MICRORNA MODULATION OF THE NON-CANONICAL WNT SIGNALING PATHWAY IS ESSENTIAL FOR CELLULAR MORPHOGENESIS

by

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ABSTRACT

The Wnt signaling pathways are highly evolutionarily conserved in regulating cell specification, cell polarity and morphogenesis in development. The non-canonical Wnt pathways (ncWnt) consist of the Wnt/Planar Cell Polarity (Wnt/PCP) and the Wnt/calcium (Wnt/Ca^{2+}) pathways. In all Wnt signaling pathways, *Dvl* transduces Wnt ligand activation. We hypothesize that perturbation of the Wnt/Ca^{2+} pathway with drugs will impact the morphogenic movements of primary mesenchyme cells (PMCs), which give rise to the skeleton spicules that facilitate larval swimming and feeding in the sea urchin embryo. To investigate specifically the regulation of ncWnt/Ca^{2+} pathway on the directed migration of PMCs, we treated sea urchin embryos with inhibitor Cyclosporin A (CsA), activator Phorbol 12-myristate 13-acetate (PMA) and inhibitor KN-93 that disrupt the ncWnt/Ca^{2+} pathway. All drug treatments led to significantly shorter skeleton spicules compared to untreated embryos. PMA and KN-93 treated embryos also resulted in PMC patterning defects while CsA did not. These results indicate that Wnt/Ca^{2+} pathway is important for the development of PMCs. To further examine the regulation of Wnt pathways, we tested the post-transcriptional regulation of *Dvl* by microRNAs (miRNAs). miRNAs are non-coding RNA molecules that mediate post-transcriptional regulation by binding to the 3'untranslated regions (3'UTR) of target transcripts. Using luciferase constructs and site-directed mutagenesis, we identified Dvl to be directly regulated by at least one miRNA. To examine the *in vivo* impact of miRNA regulation on *Dvl*, we treated newly fertilized sea urchin eggs with Dvl miRNA Target Protector (*Dvl* miRNA TP) designed to specifically block miRNA binding to the *Dvl* mRNA. Our results indicate that the removal of miRNA regulation of *Dvl* led to defects in skeleton spicule formation. While the directed migration of PMCs were unaffected by *Dvl* miRNA TP treatment,

PMCs lack membranous connections that allow them to form a syncytium. In summary, this study reveals the important role of Wnt/Ca^{2+} in regulating PMC morphogenesis and demonstrated that at least one miRNA directly regulates *Dvl*. These results contribute to a deeper understanding of the Wnt signaling pathway in regulating PMC development and function. This in turn may help us understand underlying causes of developmental defects resulting from failed control of cell migration.

Chapter 1

Introduction

1.1 Wnt Signaling in development

The Wnt signaling regulates a variety of biological processes within the developing embryo, including cellular morphogenesis, cell fate determination (Logan and Nusse, 2004), cell proliferation, and axis formation of an embryo (Croce and McClay, 2004). This is achieved in part by activating transcription factors and mediating actin polymerization (Teo and Kahn, 2010). Aberrant Wnt signaling has been implicated in many diseases, such as colorectal cancer (Giles *et al*., 2003), leukemia, parathyroid, and breast cancer (Polakis, 2012). Thus, Wnt signaling is important for development during embryogenesis and maintenance of homeostasis in adult tissues.

There are three major branches of the Wnt signaling pathway: 1) canonical Wnt pathway (cWnt), known as the Wnt/β-catenin pathway, 2) the non-canonical Wnt/Planar Cell Polarity (ncWnt/PCP) and 3) the non-canonical Wnt/calcium (ncWnt/Ca^{2+}) pathway (Lerner and Ohlsson, 2015). The Wnt signaling pathway is initiated when the Wnt ligand bind with a Frizzled receptor (Fzd) to activate various biological processes. The Wnt ligand is a glycoprotein which typically has a highly conserved region of approximately 23 cysteine residues which are found on the carboxyl terminus of the protein (Nusse and Varmus,1992). Wnt ligands are produced within the rough endoplasmic reticulum and undergo multiple chemical modifications, including N-glycosylation and palmitoylation (Banziger *et al*., 2006). These

modifications of the Wnt ligand make it hydrophobic as it gets escorted through the secretory pathway by Wntless or Wls (Banziger *et al*., 2006). After transport from the ER through COPII vesicles, the Golgi apparatus then packages the Wnt ligand into secretory vesicles. The Wnt ligands are then transported out of the cell, allowing them to interact with their cognate Fzd (Banziger *et al*., 2006). The Fzd consists of seven transmembrane domains and has a signaling domain near its amino terminal. It is through its ligand binding domain, which also consists of multiple conserved cysteines, that the Wnt ligand binds and initiates multiple signaling cascades (Nusse and Varmus, 1992).

A transmembrane co-receptor, known as LRP 5/6, also assists in the Wnt ligand-Fzd receptor association for canonical signaling. ncWnt/PCP signaling utilizes Ror1, Ror2 and Ryk (Minami *et al*., 2010; Yoshikawa *et al*., 2014; Green *et al*., 2014). In all of the Wnt signaling pathways, this binding interaction of the Wnt ligand with the Fzd generates a signal which recruits a pivotal phosphoprotein known as Dishevelled (Dvl) towards the surface of the plasma membrane. Dvl is able to associate with a variety of different proteins and is activated through kinase phosphorylation (Bryja *et al*., 2007). One major scaffolding protein, known as β-arrestin, has been seen to associate with Dvl. Because of its role in dampening G-protein coupled receptor signaling, β-arrestin acts as an important regulator of Wnt signaling along with Dvl (Bryja *et al*., 2007).

1.2 The Canonical Wnt Signaling Pathway is Important for Development

The canonical Wnt signaling controls anterior-posterior axis formation, endoderm specification, cellular proliferation, apoptosis, and differentiation (Teo and Kahn, 2010). Prior to Wnt ligand binding (Figure 1), β-catenin, a protein used as a

transcriptional co-activator of Wnt target genes, is usually degraded by a cytoplasmic destruction complex. The destruction complex consists of the scaffolding protein axin, adenomatosis polyposis coli (APC), casin kinase 1α (CK1α), protein phosphatase 2A (PP2A) and glycogen synthase kinase 3 (GSK3) (Komiya and Habas, 2008). Upon Wnt ligand binding to LRP5/6 and Fzd, Dvl is recruited to the plasma membrane. The β-catenin destruction complex is disassembled and can no longer lead to ubiquitination of β-catenin and proteolytic destruction. Once accumulating in the cytoplasm, the β-catenin translocates to the nucleus, where it acts as a transcriptional co-activator in conjunction with the T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factor family (Grantzel *et al*.,2014). This activation enables the activation of endodermal and mesodermal genes integral for development.

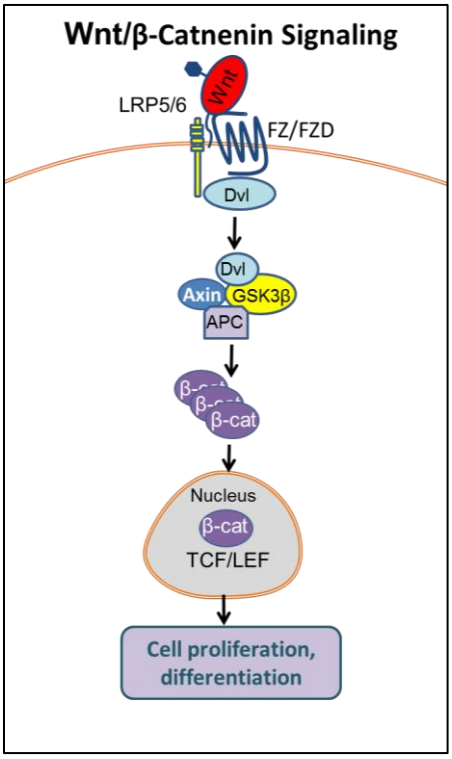


Figure 1 **Canonical Wnt signaling.** A) When there is no Wnt ligand binding, β-catenin is degraded through the proteasome destruction pathway. B) With the binding of the Wnt ligand, Dvl prevents the degradation of β-catenin, which is then allowed to accumulate and transported into the nucleus to activate genes important for cellular proliferation, apoptosis, and differentiation (Croce and McClay, 2006).

1.3 The Non-Canonical Wnt (ncWnt) Signaling Pathways are Important for Cellular Morphogenesis and Cellular Movement

The ncWnt signaling pathways, ncWnt/Ca^{2+} and ncWnt/PCP pathways, are important in morphogenic cell motility and establishment of cell polarity (Armerogen, Mikels and Nusse, 2008). The initial signaling activation in ncWnt signaling is similar as cWnt signaling in that a Wnt ligand binds to a cognate Fzd and recruits Dvl to the plasma membrane. Through the use of a guanine exchange factor, Dvl transduces signal down the Wnt/PCP pathway (Figure 2) with the help of Dvl-associated activator

of morphogenesis 1 (DAMM1), which activates RhoA small GTPase (Lerner and Ohlsson, 2015). DAMM1 also activates another protein known as Profilin, which is an actin binding protein important in actin polymerization (Lerner and Ohlsson, 2015).While RhoA is an intermediary component in Wnt/PCP signaling, it has been shown to play a role in cell cycle regulation, cytokinesis and invagination during sea urchin gastrulation (Beane et al., 2006). Since RhoA needs to have bound GTP in order to be activated, signaling down this pathway can be regulated through the use of different nucleotide exchange factors. While the guanine exchange factor (GEF) promotes the active state of RhoA, GTPase-activating proteins (GAPs) lead to RhoA inactivation (Beane et al., 2006). Another GTPase Rac1 could also be activated at this time along with the RhoA. This coactivation of these GTPases leads to the activation of downstream Rho-associated protein kinase (ROCK) and c-jun N-terminal kinase (JNK). When ROCK and myosin are activated by RhoA, they proceed to catalyze actin and cytoskeleton rearrangement and modification (Komiya and Habas, 2008). The Wnt/PCP pathway activates Rac1, leading to activation of c-Jun and ATF2, which leads to transcriptional activation of downstream genes (Lerner and Ohlsson, 2015). Through the activation of the ncWnt/PCP pathway, signals are transduced in order to control cellular motility and morphogenesis of a developing organism through convergent extension (Jenny, 2010).

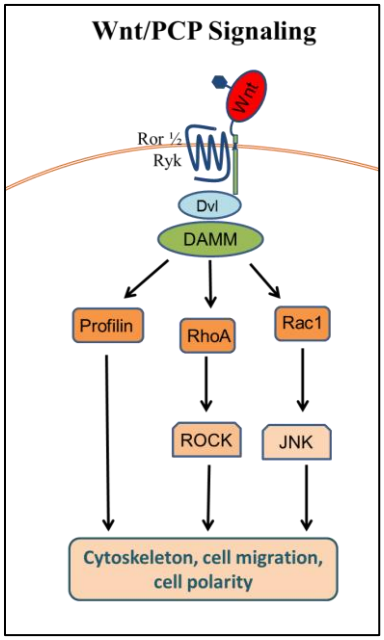


Figure 2 **The ncWnt/PCP pathway**. This signaling pathway is initiated with the binding of Wnt ligand to Fzd. Dvl (also known as Dsh) is recruited to the plasma membrane. Damm1 then associates with Dvl and causing it to transduce signals down either the Rho and ROCK pathway, the Rac and JNK pathway, or a completely different pathway associated with actin polymerization involving Profilin.

The ncWnt/Ca^{2+}utilizes intercellular calcium release from the endoplasmic reticulum (ER) in order to transduce its signaling cascades. Effects of calcium release due to Wnt/Ca^{2+} signaling have been seen in regions within developing *Danio rerio* (zebrafish) and *Xenopus* (frog) embryos that are critical during early development (Komiya and Habas, 2008). In the ncWnt/Ca^{2+} pathway, signals are carried through Dvl to a set of heterotrimeric G-proteins (Figure 3). These G-proteins lead to activation of phospholipase C (PLC). PLC is then able to cleave PIP_2 into inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG associates with protein kinase C (PKC) on the plasma membrane while IP_3 can then bind to a calcium channel on the

ER which triggers a release of calcium into the cytoplasm. This influx of released Ca^{2+} can activate several protein kinases such PKC and calcium/calmodulin kinase II (CamKII). The influx of calcium ions also activates Calcineurin (Cn). Once activated, CamKII is then able to switch on a transcription factor called NFAT which has a role in establishing ventral cell fates (Komiya and Habas, 2008). When CamKII is activated on the dorsal side of a *Xenopus* embryo, dorsal cell fates are inhibited, but when CamKII activity is reduced on the ventral side of the embryo, dorsal cell fates are promoted. This indicates that CamKII associates with promotion of ventral cell fate (Kuhl et.al, 1999). CamKII can also create an antagonistic signaling cascade to Wnt/β-catenin signaling through its activation of TGFβ activated kinase (TAK1) and Nemo-like kinase (NLK) (Komiya and Habas, 2008). When PKC is turned on, it can activate Cdc42 small GTPase. Both PKC and Cdc42 have been implicated in cardiac differentiation as well as cellular movement during gastrulation (Rao and Kühl, 2010; Komiya and Habas, 2008). Cn also interacts with NFAT and its activation triggers downstream gene expression (Rao and Kühl, 2010). It is through regulation of intercellular calcium levels that the ncWnt/Ca^{2+} pathway is able to control cytoskeleton formation and cell adhesion (Kuhl *et al*.,2000). The ncWnt/Ca^{2+} pathway has been implicated in *Xenopus* gastrulation and promoting the convergent extension of various cells from a dorsal to a ventral cell fate (Kuhl *et.al*, 1999). Activation of the Wnt/Ca^{2+} pathway is also integral for further cell morphogenesis and motility (Geng *et al*., 2016).

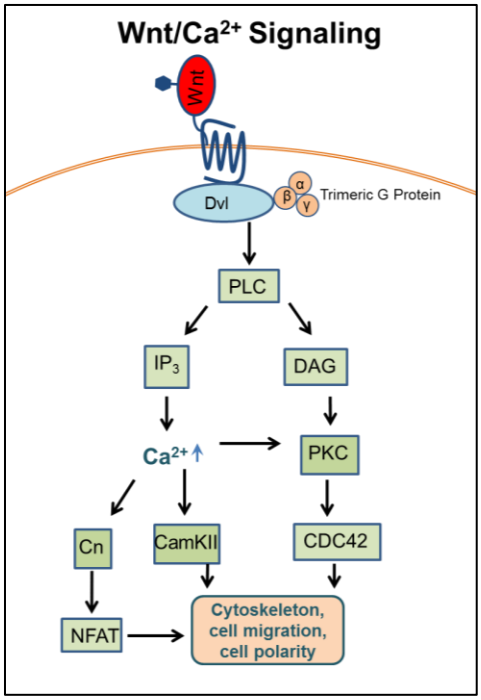


Figure 3 **The non-canonical Wnt/Calcium signaling pathway**. PLC is activated after interaction of Dvl with a G-protein. PLC leads to an activation of IP_3 which triggers a release of calcium from the ER. This calcium leads to activation of several protein kinases including PKC and CamKII. Downstream effects of ncWnt signaling are NFAT and Cdc42 activation, which leads to expression of genes involved in morphogenesis.

1.4 Dvl is at the Hub of Wnt Signaling

Upon Wnt ligand binding, Dvl transduces signals downstream of every branch of the Wnt signaling pathways. Dvl consists of a central PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain, the amino terminal DIX domain (Dishevelled, Axin), and the carboxyl terminal DEP domain (Dvl, Egl-10, Pleckstrin) (Gao and Chen, 2010) (Figure 4). Each of these domains plays a different role in Wnt signaling. While PDZ seems to be needed for all the branches of Wnt signaling, the DEP seems to be required in ncWnt signaling, while the DIX domain is only required in cWnt signaling (Tada and Smith, 2000). The Dvl-β-catenin interaction is thought to

be mediated through the association of Axin with the DIX domain of Dvl (Byrum *et al*., 2009). The PDZ domain is thought to bind directly to Fzd, specifically to its C-terminal region, which makes it critical for both canonical and non-canonical Wnt signaling (Wong *et al*., 2003). The DEP domain is thought to be essential in binding to Dvl to the plasma membrane and is believed to be directly associated with both the ncWnt/PCP and ncWnt/Ca^{2+} signaling pathways (Gao and Chen, 2010). A Dvl-DEP construct overexpressed in a developing sea urchin embryo led to developmental defects of the gut and skeleton (Figure 5) (Byrum *et al*., 2009). This experiment highlights the importance of the DEP and PDZ domains of Dvl within the ncWnt signaling pathways.

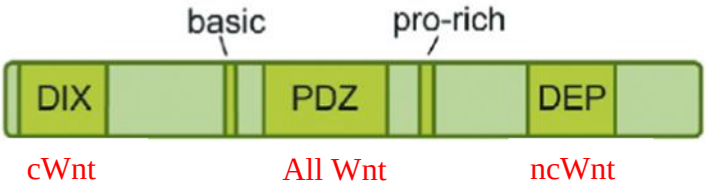


Figure 4 **The protein domains of Dvl.** There are three distinct domains of Dvl: the DIX, PDZ and DEP domains. There is also a proline rich region and a basic region where phosphorylation events take place (Gao and Chen, 2010).

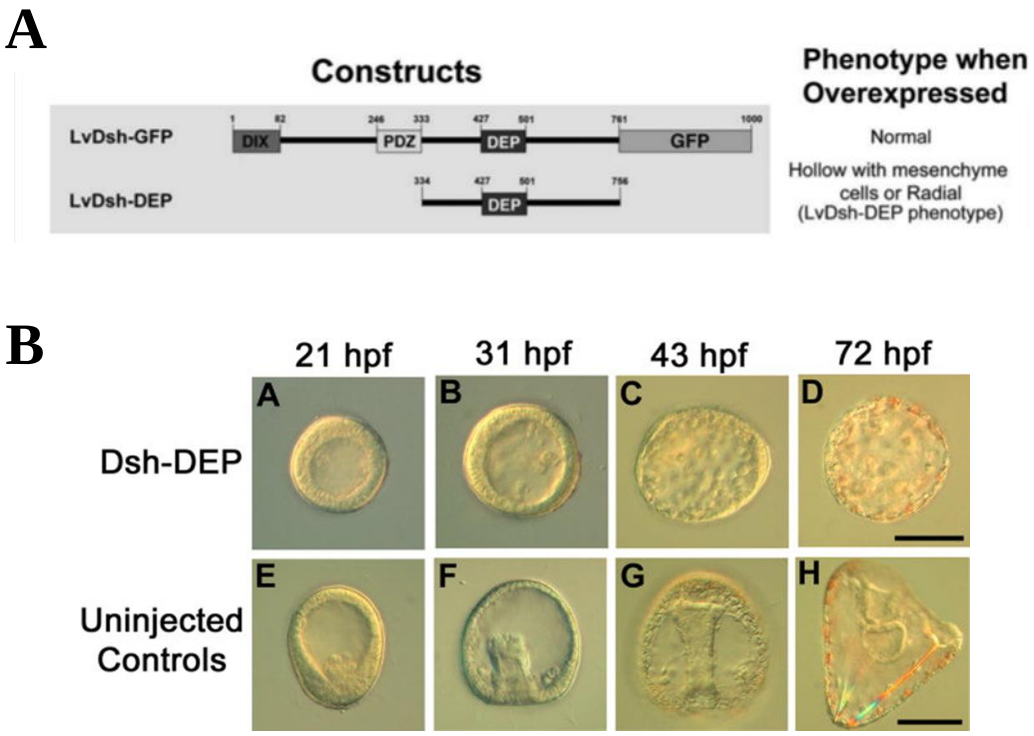


Figure 5 **Removal of DIX and PDZ domains within Dvl results in non-canonical Wnt signaling impairment.** A) A deletion construct of Dvl that was missing the DIX and PDZ domains was made and injected into newly fertilized eggs. B) Without the DIX and PDZ domains, ncWnt signaling is unable to function properly, resulting in developmental defects (Byrum *et al*., 2009).

1.5 The Purple Sea Urchin as an Experimental Model

Wnt signaling is a highly conserved signaling pathway that is utilized by all animals. A useful model for studying development and Wnt signaling is the purple sea urchin *Strongylocentrotus purpuratus*. *S. purpuratus* is a member of the Echinodermata phylum and is a close relative of chordates. *S. purpuratus* has a total of 23,000 genes and includes representatives of almost all the major gene families that are found in humans (Sea Urchin Genome Sequencing Consortium, 2006).

The advantages of using the purple sea urchin as a developmental model are that they produce a large amount of gametes, undergo external fertilization, have

transparent embryos, undergo synchronous growth, develop relatively fast, and can withstand experimental manipulations (McClay, 2011). The genome of the purple sea urchin has also been sequenced, facilitating gene function studies and understanding of development at a systems level.

1.6 The Life Cycle of the Purple Sea Urchin

After fertilization of an embryo, the zygote begins to undergo radial divisions (Figure 6). A further specification is made. The first unequal division occurs after the 4th and 5th cleavages, giving rise to micromeres, macromeres and mesomeres. These groups of cells give rise to the eventual subset of mesoderm (from micromeres), endoderm (from macromeres) and ectoderm (from mesomeres) as well as the animal and vegetal axis of the embryo (McClay, 2011). At 24 hours post fertilization (hpf), the inside of the embryo then becomes hollow, forming the blastocoel; at this stage, the embryo is referred as a blastula. After this, the skeletogenic cells from the vegetal side of the embryo, known as the PMCs, begin to ingress into the embryo (McClay, 2011). After a short period, gastrulation begins to take place as non-skelotogenic cells of the vegetal pole invaginate into the embryo. At 48 hpf, the embryo is in the gastrula stage, where gastrulation occurs by convergent extension with the gut fully developed into the foregut, midgut, and hind gut and elongates (Davidson *et al.,* 1998). At this time the PMCs undergo direction migration to form a specific pattern and form the larval skeleton. The embryo develops into the larval stage at 72 hpf when they begin to feed on its own (McClay, 2011). Depending on the species of the sea urchin, it takes the larvae 1-2 years to develop into a juvenile sea urchin.

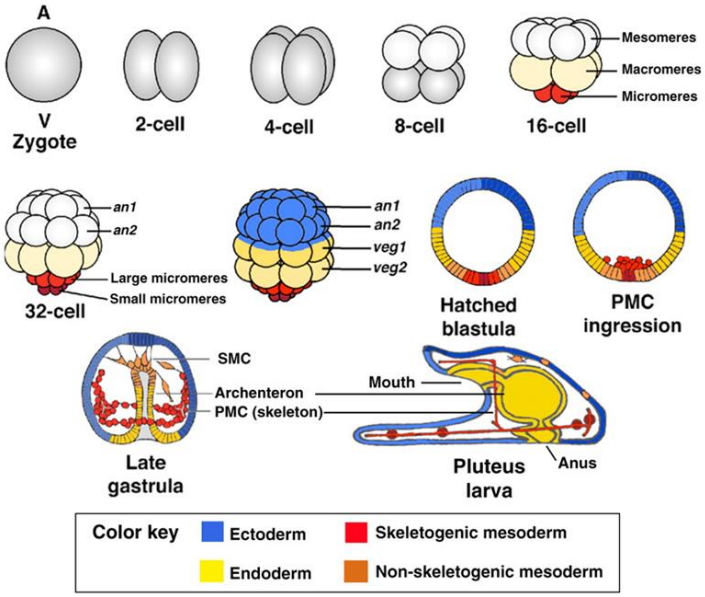


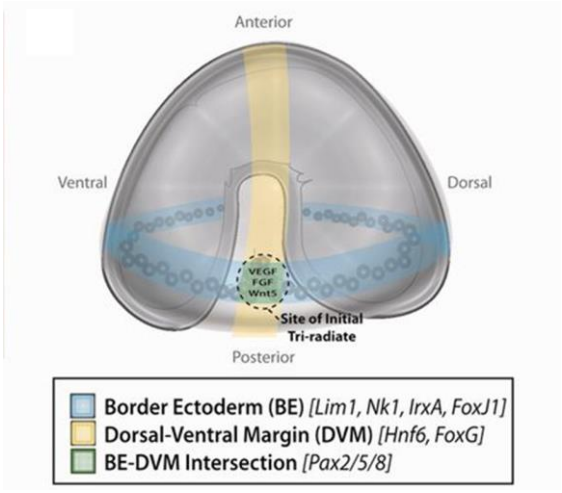
Figure 6 **Embryonic life cycle of a purple sea urchin.** The sea urchin embryo develops relatively fast into a feeding larva (three days). The 16 cell stage embryo consists of mesomeres, macromeres and micromeres. The 32 cell stage establishes the distinction of the animal and vegetal poles of the embryo. At 24 hpf the blastocoel is formed and PMCs have begun to ingress into the blastocoel. At 48 hpf the gut is fully elongated and segmented. 72 hpf marks the larval stage and when an embryo is able to feed on its own and the skeleton is fully established. (McClay 2011).

1.6 PMC: Their Development and Their Importance in Establishing Sea Urchin Skeletal Backbone

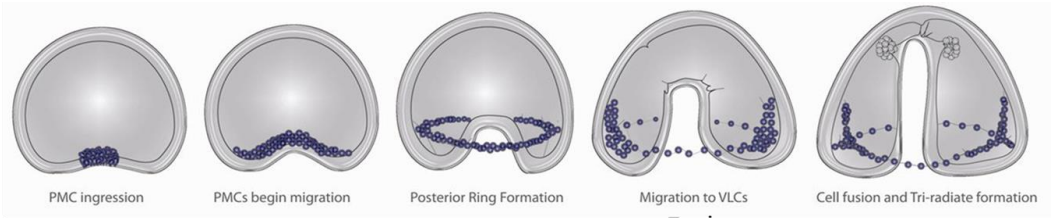
Within the purple sea urchin model, a useful cell type for studying ncWNT signaling is the PMCs, because it is specified downstream of the β-catenin/Wnt pathway and undergo dramatic directed cell migration during its development. PMCs originated from the large micromeres that arise from a 16-cell stage embryo (Wessel, Etkin and Benson, 1991).

PMCs synthesize the calcium endoskeleton found within purple sea urchins (Lyons *et al*., 2014). In order for the PMCs to form the calcium endoskeleton, they undergo epithelial to mesenchymal transition (EMT) and must first ingress into an embryo. The PMCs detach from the blastula epithelium after losing adherens junctions as they ingress into the blastocoel of the embryo at the blastula stage (Wu and McClay, 2007). The PMCs utilize both filopodia and lamellapodia to move throughout the blastocoel (Lyons *et al.*, 2014) The PMCs respond to various ectodermal growth factors from the border ectoderm (BE) and the dorsal-ventral margin (DVM) which direct them towards the bilateral side of the blastocoel (Figure 7A). The PMCs form an equatorial ring around the invaginating gut of the embryo. Slightly after the formation of the equatorial ring, many PMCs begin to cluster and fuse into a syncytium where the BE meets the DVM. These formations are called the ventral lateral clusters (VLC) and once formed, they then begin to deposit calcium carbonate which crystalizes into calcite granules that form the basis of the larval skeleton (Lyons *et al.*, 2014). Between the late blastula stage embryo (28 hpf) and the gastrula stage embryo (48 hpf), PMCs in the VLC begin to migrate anteriorly along the DVM axis, after receiving signal from several growth factors (Figure 7B) (Duoloquin *et al*., 2007). The PMCs that express vascular endothelial growth factor receptors (VegfR) receive guidance cues from Vegf deposited in domains of the ectoderm. The deposits of calcite continue to form during this time and the PMCs continue to migrate anteriorly and form different structures well into the larval stage (Figure 7C) (Lyons *et al.*, 2014).

A



B



Early Blastula
19 hpf

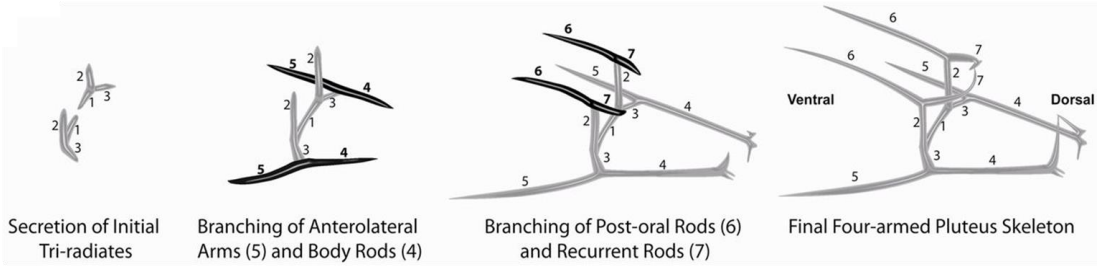
Mesenchyme Blastula
24 hpf

Early Gastrula
30 hpf

Early Gastrula
30-48 hpf

Gastrula
48 hpf

C



Secretion of Initial Tri-radiates
48 hpf

Branching of Anterolateral Arms (5) and Body Rods (4)
>48 hpf

Branching of Post-oral Rods (6) and Recurrent Rods (7)
<72 hpf

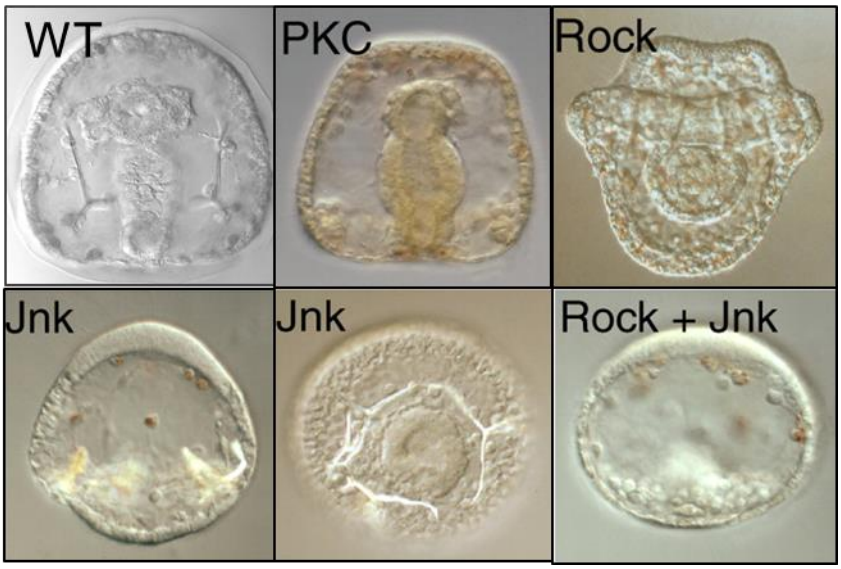
Final Four-armed Pluteus Skeleton
72- hpf

Figure 7 **PMCs migrate during development and form the skeletal backbone of the sea urchin embryo.** A) PMCs, after ingression into the blastocoel, receive ectodermal signaling from the BM and DVM though various ectodermal growth factors. They migrate toward these signals. B and C) PMCs undergo distinct patterning throughout a 72 h span, and eventually develop a full larval skeleton **(**Modified from Lyons *et al.*, 2014).

1.7 ncWnt Signaling Pathway regulates PMCs

Previous studies have shown that perturbation of various branches of the ncWnt signaling pathways resulted in PMC and/or gastrulation defects (Figure 8). Two specific inhibitors had been used to completely block the two branches of Wnt/PCP signaling, ROCK and JNK. When only ROCK was inhibited, skeletogenesis was prevented and no spicules were formed. When JNK was inhibited, invagination and gastrulation was prevented. A combined inhibition of both ROCK and JNK resulted in defects where skeleton and blastopore failed to form (Croce *et al*., 2006). In addition, treatment of the sea urchin pre-blastula embryos with Bisindolylmaleimide I, a specific inhibitor of PKC within the ncWnt/Ca^{2+} pathway, inhibited skeleton spicule formation (Croce *et al*., 2006). As mentioned previously, the ncWnt/Ca^{2+} pathway has three branches (PKC, Cn, and CamKII; Figure 3), of which the effect of disrupting Cn or CamKII on PMC development has not been examined.

A


WT
PKC
Rock
Jnk
Jnk
Rock + Jnk

B

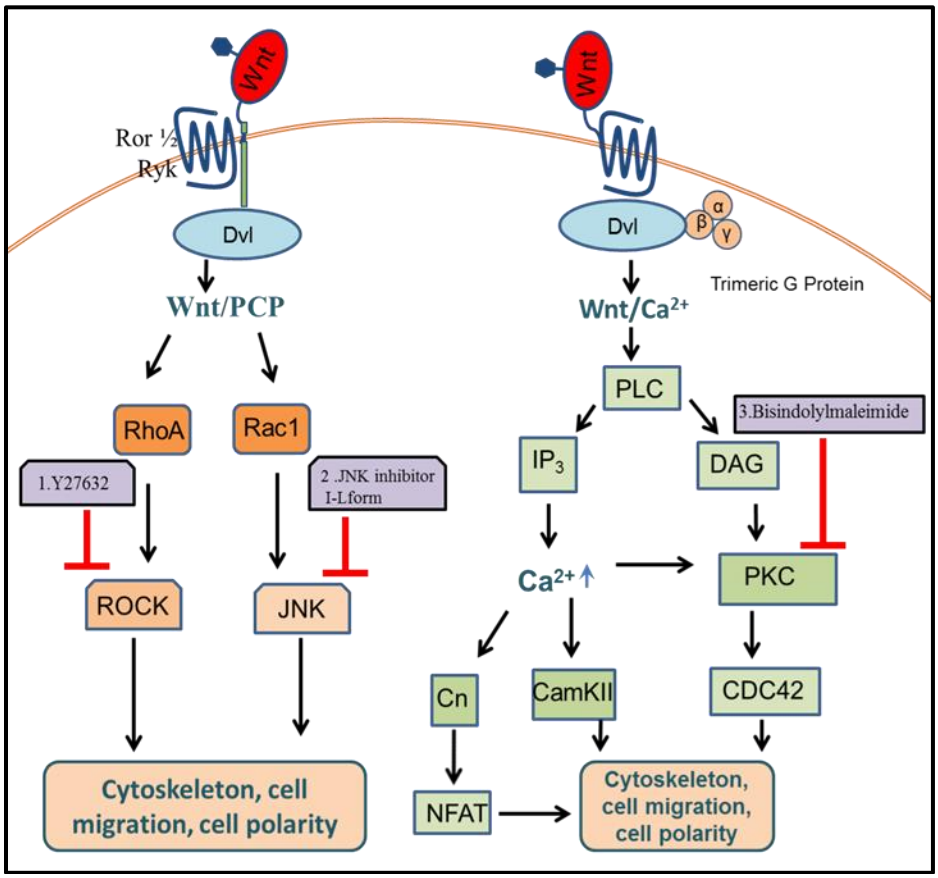

Wnt
Ror ½
Ryk
Dvl
Wnt/PCP
RhoA
Rac1
1.Y27632
2 .JNK inhibitor I-Lform
ROCK
JNK
Cytoskeleton, cell migration, cell polarity
Wnt
Dvl
α
β
γ
Trimeric G Protein
Wnt/Ca2+
PLC
3.Bisindolylmaleimide
IP3
DAG
Ca2+
PKC
Cn
CamKII
CDC42
NFAT
Cytoskeleton, cell migration, cell polarity

Figure 8 **Drug inhibition of ncWnt signaling results in distinct developmental phenotypes.** This figure shows the phenotypes that result from inhibiting PKC, ROCK and JNK as well double inhibition of ROCK and JNK (Modified from Croce *et al.* 2006). B) A Schematic of where the drug inhibited PKC, ROCK and JNK on the ncWnt signaling pathway (Modified from Song *et al*., 2015)

1.8 microRNA biogenesis and regulatory mechanisms

Another important way that the Wnt signaling pathway is regulated is through post-transcriptional regulation by miRNAs (Stepicheva *et al*., 2015; Song *et al*., 2015). miRNAs are small 19-23 nucleotide noncoding RNAs encoded in the genomes. They can be located within the introns of protein coding genes, or within the introns of noncoding RNAs (Kim and Nam, 2005). Primary miRNA transcripts, or pre-miRNAs, are produced by transcription of DNA through RNA Polymerase II/III (Figure 9). After production of the pre-miRNA, the transcript may be edited by adenosine deaminases (ADARs). ADARS facilitate the change of adenosines into inosines and this action can manipulate the binding and structure of the miRNA transcript (Winter *et al*., 2009). The primary miRNA is then cleaved by an enzyme complex known as Drosha-DGCR8 which consists of the RNase III enzyme Drosha, and the DiGeorge critical region 8 protein. This complex facilitates the cleavage of the miRNA, creating a stem loop structure consisting of around 70 nucleotides (Shukla *et al*., 2011) After Drosha processing, there is additional RNA splicing which takes place. Following the spicing, the miRNA transcript is transported out of the nucleus through the use of an Exportin complex. Once in the cytoplasm, another RNase III enzyme known as Dicer further processes the miRNA. Dicer along with RNA binding domain proteins, PACT and TRBP, and the central domain protein called Argonaute-2 (Ago2) form the RISC loading complex, or RLC. This RLC facilitates the removal of the hairpin turn, leaving

around a 22 nucleotide segment of double- stranded RNA. After cleavage due to the RLC, the miRNA-Induced Silencing Complex can bind one strand of miRNA, (called the guide stand) while the other strand is sent for destruction.

The RISC complex directs the miRNA strand towards its mRNA target (Winter *et al*., 2009; Shukla *et al*., 201l). miRNAs mediate post transcriptional regulation by binding to 3' untranslated regions of target mRNAs (Shukla *et al*., 201l). The miRNA does not need to have complete complementarity to the target mRNA; however, miRNAs have a distinct sequence of 6-8 nucleotides known as the "seed sequence" that is critical for target mRNA recognition (Shukla *et al*., 201l). Other regions within the miRNA, such as the "anchor" region is also implicated in the miRNA to target interaction (Lewis *et al*., 2005). Despite having seed sequence complementarity for a given miRNA regulation element (MRE) sequence, miRNAs are often able to bind more than one target. The mRNAs themselves can be regulated by more than one miRNA. miRNAs repress of translation and/or induce degradation of target mRNAs. miRNA induced target mRNA degradation can be achieved through chemical modification, such as deadenylation of nucleotides within mRNA, or removal of the 5' cap on the mRNA (Shukla *et al*., 201l). These modifications would destabilize the mRNA structure and lead to its subsequent destruction.

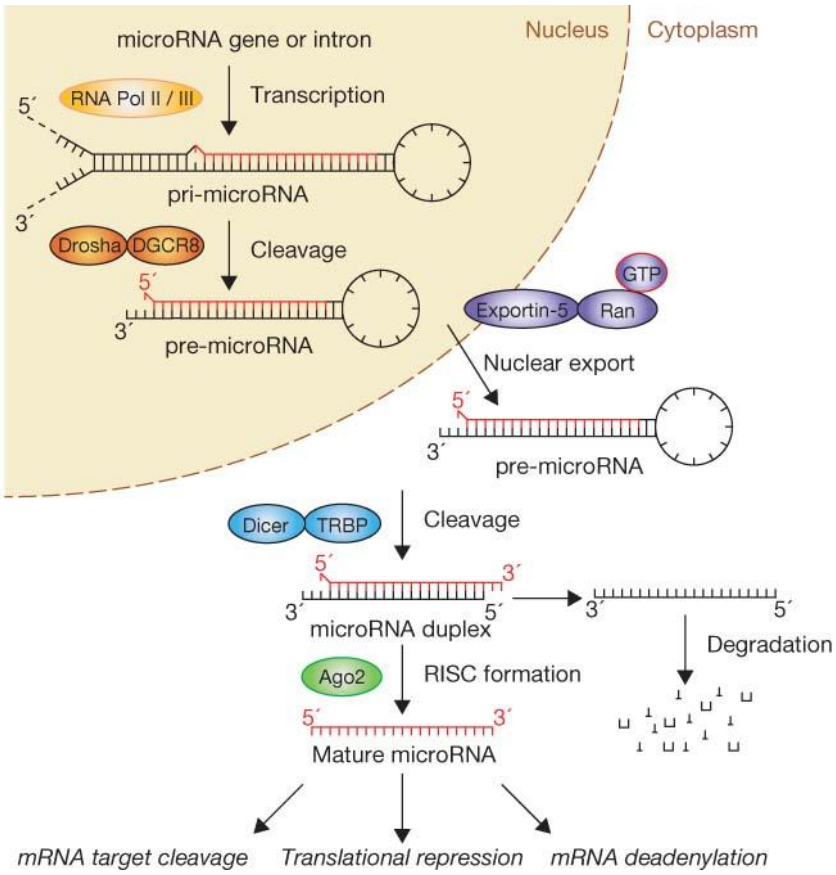


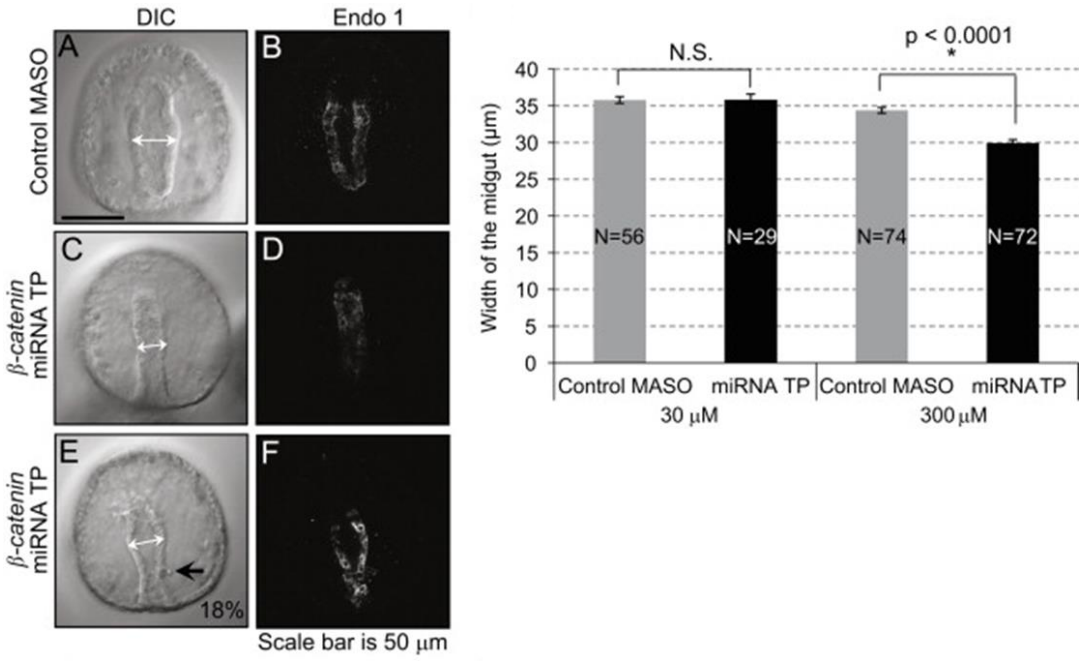
Figure 9 **The biogenesis pathway of microRNAs**. Pri-miRNAs are produced through RNA Polymerase transcription of intron regions of protein coding genes and noncoding RNAs. Pre-miRNAs are produced after Drosha/DGCR8 cleavage and then exported out of the nucleus by an Exportin complex. RLC cleaves the miRNA into a duplex structure and RISC facilitates the miRNA interaction with mRNA (Winter *et al.*, 2009).

1.9 miRNAs Regulate Components of the Wnt Signaling Pathway

miRNA have been shown to regulate cellular differentiation and developmental events (He *et al.*, 2004). miRNAs have been found to regulate multiple components of the Wnt signaling pathways (Stepicheva *et al.*, 2015; Song *et al.*, 2015).

Previous work in our laboratory indicated that knockdowns of Dicer and/or Drosha resulted in sea urchin embryos that lack endodermally and mesodermally derived structures such as the gut and PMCs (Song *et al*., 2012). Since the Wnt signaling pathway is critical for endoderm specification and axis formation, we hypothesized that the phenotypes we observed with Dicer and/or Drosha knockdowns may be due to miRNA regulation of the Wnt signaling pathways. We further demonstrated that *β-catenin* of the cWnt pathway is directly regulated by at least two miRNAs (Stepicheva *et al*., 2015). Blocking miRNA regulation of β -catenin *in vivo* led to morphological defect of the gut (Figure 10A), as well as alteration of mRNA levels of transcription factors known to be important for endoderm specification (Figure 10B).

A


DIC
Endo 1
Control MASO
β-catenin miRNA TP
β-catenin miRNA TP
A
B
C
D
E
F
18%
Scale bar is 50 μm
N.S.
p < 0.0001
*
Width of the midgut (μm)
40
35
30
25
20
15
10
5
0
N=56
N=29
N=74
N=72
Control MASO
miRNA TP
Control MASO
miRNA TP
30 μM
300 μM

B

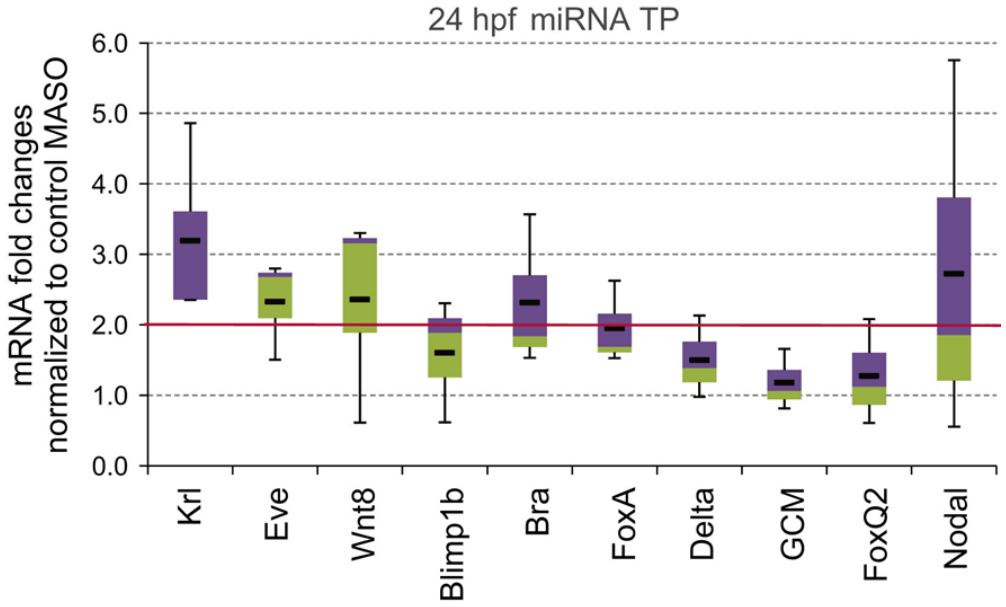

24 hpf miRNA TP
mRNA fold changes normalized to control MASO
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Krl
Eve
Wnt8
Blimp1b
Bra
FoxA
Delta
GCM
FoxQ2
Nodal

Figure 10 **β-catenin is directly suppressed by at least 2 miRNAs .** A) Blocking miRNA binding sites on *β-catenin* with a miRNA TP led to significantly thinner guts. 18 % of treated embryos also had aberrant hindgut morphology. Gut widths were measured for embryos treated with 30μM and 300μM of the miRNA TP, but only the 300 μM embryos were found to have a significant difference in gut width. Scale bar is 50 μm B) This QPCR data shows relative transcript levels that are normalized to a standard. The levels on the y-axis indicate fold change levels of transcripts in miRNA TP injected embryos normalized to the control embryos. The transcripts tested are involved in specification of the endoderm, mesoderm and ectoderm. The majority of transcripts have a 2-fold increase, indicating that miRNA regulation is important in controlling cWnt signaling driven specification (Stepicheva *et al*., 2015).

Using bioinformatics approach, our lab identified miRNAs to target several components of the Wnt signaling pathway. Work from graduate students Priya Nigam and Aun Zaidi showed that Dvl is directly regulated by one of the previously identified miRNA that also regulates *β-catenin*.

1.11 Goals of the Research Study

The goal of my study is to examine how ncWnt/Ca^{2+} regulates PMC morphogenesis and to test the impact of miRNA regulation of Dvl.

Specific Aim 1: The first aim is to bridge the current knowledge gap of how ncWnt/Ca^{2+} regulates PMC morphogenesis. I will use drugs to inhibit the ncWnt Ca^{2+} Cn, and CamKII branches, and PMA to activate PKC. I will observe the development and function of PMCs. This will be mainly assayed by measuring the length of the dorsoventral connecting rods and also phenotyping the positioning of PMCs using a PMC-specific antibody.

Specific Aim 2: My second aim is to test the hypothesis that miRNA regulation of Dvl impacts early development. Our lab has previously identified that Dvl is directly regulated by at least one miRNA. My project is to test the impact of blocking miRNA regulation of Dvl on early development. I will acquire images of Differential Interference Contrast (DIC) and PMC immunofluorescent images to examine skeleton spicules and PMC positioning, respectively.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

The *Strongylocentrotus purpuratus* used in this study were collected in the Pacific Ocean by the Point Loma Marine Company in California. These animals are shed for their sperm and eggs with intracoelomic injections using 0.5 M KCl. Sperm and eggs were combined in glass culture dishes filled with filtered natural sea water taken from the University of Delaware Lewes campus or artificial sea water. The fertilized embryos were then cultured at 15 °C.

2.2 Drug Studies Targeting ncWNT Signaling

Various drugs known to have an effect on different components of ncWNT were tested to examine how they affect PMC development. Drugs tested include 1) phorbol 12-mysristate 13-acetate (PMA), which is a known PKC activator, 2) KN-93, which is a known CamKII inhibitor, and 3) Cyclosporine A (CsA), which is a known inhibitor of Cn that prevents NFAT dephosphorylation and subsequent trans location into the nucleus (Ferrari *et al.,* 2003; Cheng *et al.,* 2008; Kohn and Moon, 2005). These drugs (Enzo Life Sciences, Inc., Farmingdale, NY) were a gift from Dr. Randall Duncan's lab. Each of these drugs were taken from a 10 mM stock solutions in DMSO and diluted with filtered natural sea water to empirically identify non-lethal concentrations. The concentrations tested for PMA were 15, 25 and 35 nM. The concentrations tested for KN-93 were 1, 1.5 and 3 μM. The concentrations tested for

CsA were 0.1, 0.5 and 1 μM. DMSO used to dissolve these drugs was used as the negative control. Living sea urchin embryos taken at 19 hpf were spun down using a hand centrifuge. The sea water was aspirated out and the remaining embryos were added to the various drug dilutions. These embryos were treated with their respective drugs for 3 hours while incubating at 15 °C. This time period was chosen because this is the developmental time point when PMCs undergo EMT (McClay, 2011). After 3 hours of incubation, the drugs were washed out by centrifuging the embryos at 9000 RPM for 1 minute, followed by two sea water washes. These embryos were then incubated at 15 °C until the gastrula stage (48 hpf). At 48 hpf these embryos were fixed with 4 % paraformaldehyde and stored at 4 °C until they were imaged or immunolabeled.

2.3 Imaging and Image Analysis

Embryos were examined under light microscopes and imaged using both a Zeiss Axio Observer Z1 microscope for DIC and fluorescence microscopy and a Zeiss LSM 780 confocal microscope. Images were analyzed using the Axiovision program (Zeiss, Thornwood, NY).

2.4 Immunofluorescence

When utilizing immunofluorescence, embryos were fixed with 4 % paraformaldehyde and were allowed to sit for at least 10 minutes at room temperature. After fixation, the embryos were washed at least four times using a 1% PBST solution. After washing, the embryos were treated with a 4% sheep serum (Sigma Aldrich, St. Louis, MO) blocking solution diluted with 1X PBST (1:10 dilution) for 1 hour. After blocking, the embryos were treated with a primary antibody, either Endo1 (1:200

dilution in 4% sheep serum) for gut immunolabeling or 1d5 (1:50 dilution in 4% sheep serum) for PMC immunolabeling overnight in humid chamber at 4 °C. After overnight incubation, the embryos were again washed at least 3 times with 1X PBST. After these washes, the embryos were treated with the secondary goat anti-mouse antibody Alexa 488 (Invitrogen, Carlsbad, CA) at a 1:300 dilution in PBST. After a 40 min incubation time, the embryos were washed at least 3 more times with 1X PBST. These embryos were then imaged with the Zeiss Axio Observer Z1 microscope and in some instances, the Zeiss LSM 780 confocal microscope.

2.5 Real time, Quantitative PCR (QPCR)

Drug treated embryos were collected at 24, 30 and 48 hpf for QPCR. Total RNA was extracted using the Nucleospin XS kit (Macherey-Nagel, Bethlehem, PA). cDNA was generated with the iScript (BioRad, Hercules, CA) according to the manufacturer's instructions. QPCR was performed using 1.25 embryos equivalent for each reaction with the FAST SYBER Green PCR Master Mix (Thermo Fisher Scientific, Grand Island, NY) using the QuantStudio 6 Real-Time PCR cycler system (Thermo Fisher Scientific, Grand Island, NY). Results were normalized to the mRNA expression of housekeeping gene ubiquitin and shown as fold changes.

2.6 Cloning Luciferase Constructs

We bioinformatically searched the 3'UTR of Dvl for 100% seed match of annotated sea urchin miRNAs and identified two putative miRNA binding sites. Since *spu*-miR-153* had no normalized sequence reads from our previous study, we focused on *spu*-miRDeep2-30364 which have over 500 normalized sequence reads in all developmental stages (Song *et al*., 2012). In order to test the direct regulation of spu-

miRDeep2-30364 of Dvl, we cloned the Dvl its 3'UTR downstream of *Renilla* luciferase (Rluc). We cloned the 3'UTR of Dvl with mutated spu-miRDeep2-30364 seed sequence (at third and fifth base pairs) downstream of Rluc to abolish endogenous miRNA binding to the target sites in the 3'UTR of reporter constructs. Firefly luciferase reporter construct was used as a control for Rluc luciferase normalization as previously described (Stepicheva *et al*., 2015; Stepicheva and Song, 2015).

In vitro transcribed mRNAs of *Renilla* reporter constructs fused to the Dvl 3'UTR and the Firefly reporter construct were co-injected into newly fertilized eggs. We collected mesenchyme blastula stage embryos to measure miRNA regulation on Dvl using the dual luciferase assay.

2.7 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/FF ratio. Finally, the values of mutants were normalized to that of wild type.

2.8 Microinjections

In order to perform a luciferase assay, microinjection is necessary to get the various mutant constructs into an embryo. When injecting for luciferase assays, other lab members, (Jia Song, Priya Nigam and Nadia Stepicheva) would create two 2.5 µl injecting solutions which contained 100 ng of *Renilla* luciferase and 60 ng of firefly luciferase respectively along with 0.5 µl of 100% glycerol and Texas Red dextran. The stock injection solution contains 15 µM of the *Dvl* miRNA TP or 45 µM of control MASO in 20% sterile glycerol, 2 mg/ml 10,000 MW Texas Red lysine charged dextran (Molecular Probes, Carlsbad, CA as previously described (Stepicheva *et al*., 2015; Stepicheva and Song, 2014). The *Dvl* miRNA TP morpholino sequence designed against *spu*-miRDeep2-30364-35240 is at position +253 5' CTAGCATTTTTTTTTGAAAGCTGT 3'. Position +1 is defined as the first nucleotide after the stop codon. The sea urchin eggs that were utilized for these experiments needed to be dejellied to make them more permeable to the injection needle used. Dejellying was achieved by placing the eggs into acidic sea water with a pH of 5.15 for a 10 minute period. These dejellied eggs were then stuck onto protamine sulfate coated petri dishes which prevented them from moving. The eggs on the petri dishes were then fertilized with sperm treated with 1mM 3-amino-triazol The Femto Jet injection system (Eppendorf) was used to inject the zygotes while the vertical needle puller PL-10 was used to remove the injection needle after injection.

Chapter 3

RESULTS

3.1 PMA, KN-93 and CsA Caused Skeletal Defects.

While previous studies have shown the importance of ncWnt signaling in developmental morphogenic events such as spiculogenesis and gastrulation (Croce *et al*., 2006), the effect of disrupting all branches of the ncWnt on cell morphogenesis has not been systematically examined. In this study, I tested both inhibition and activation of other ncWnt signaling components. PMA was used to activate PKC, while KN-93 and CsA were used to inhibit CamKII and Cn, respectively (Figure 11). A wide range of drug concentrations was used to identify the optimal concentrations which would not result in embryonic lethality but has an impact on cellular morphogenesis.

Results indicated that the dorsoventral connecting (DVC) rods were decreased in a dose-dependent manner in the presence of all the drugs tested (Figures 12-14). Digital slices of DIC images were acquired to measure the length of the DVC, starting at the tri-radiate junction to the most anterior part of the embryo.

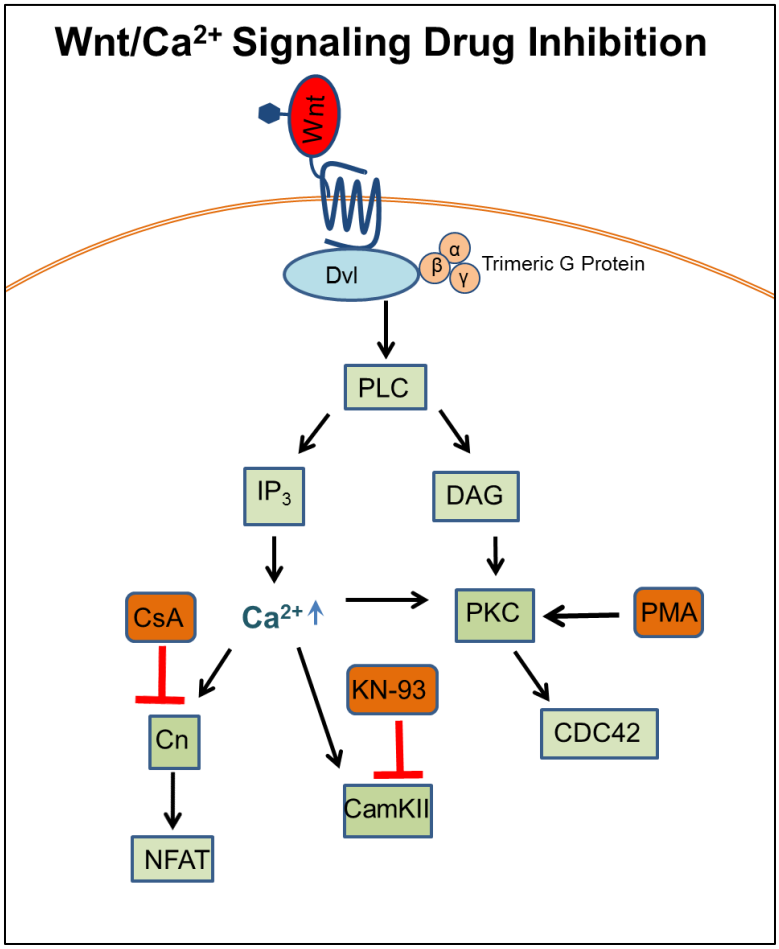
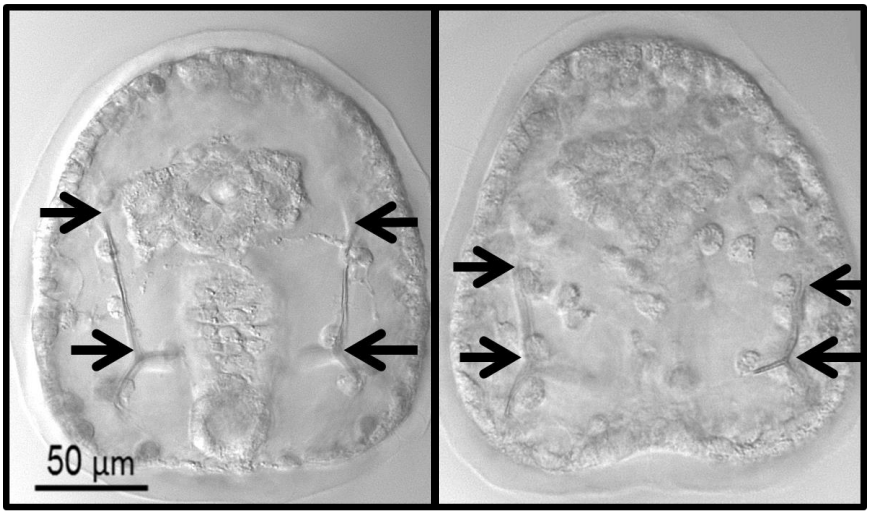


Figure 11 **Drug inhibition of the ncWnt/ Ca^{2+} Signaling Pathway.** Several drugs were utilized to target different parts of ncWnt/Ca^{2+} signaling. Two inhibitors, CsA and KN-93, block Calcineurin and CamKII, respectively, while the PMA activates PKC.

A


DMSO
PMA
50 µm

B

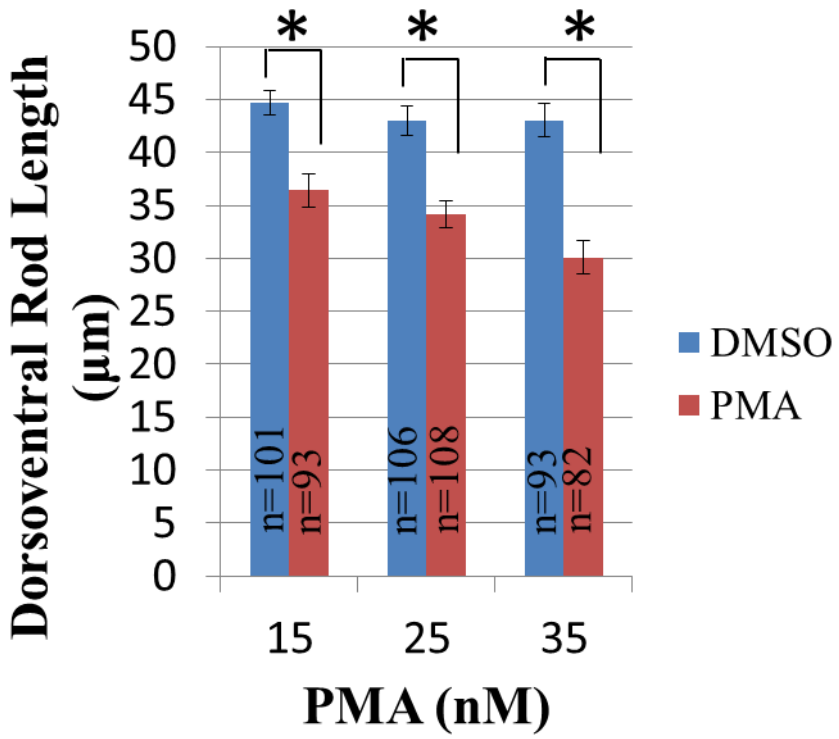

Dorsoventral Rod Length (µm)
50
45
40
35
30
25
20
15
10
5
0
*
*
*
n=101
n=93
n=106
n=108
n=93
n=82
15
25
35
PMA (nM)
DMSO
PMA

Figure 12 **PMA treatment results in a significant decrease of the dorsoventral connecting rod (DVC) length in a dose-dependent manner.** A) DIC images of embryos treated with or without PMA. Measurement of DVC are from the tri-radiate to the most anterior part of the embryo as indicated by the arrows. B) PMA induced a dose-dependent decrease in the length of the DVC (n is the number of embryos. 3 biological replicates, Student T-test with $p < 0.0001$).

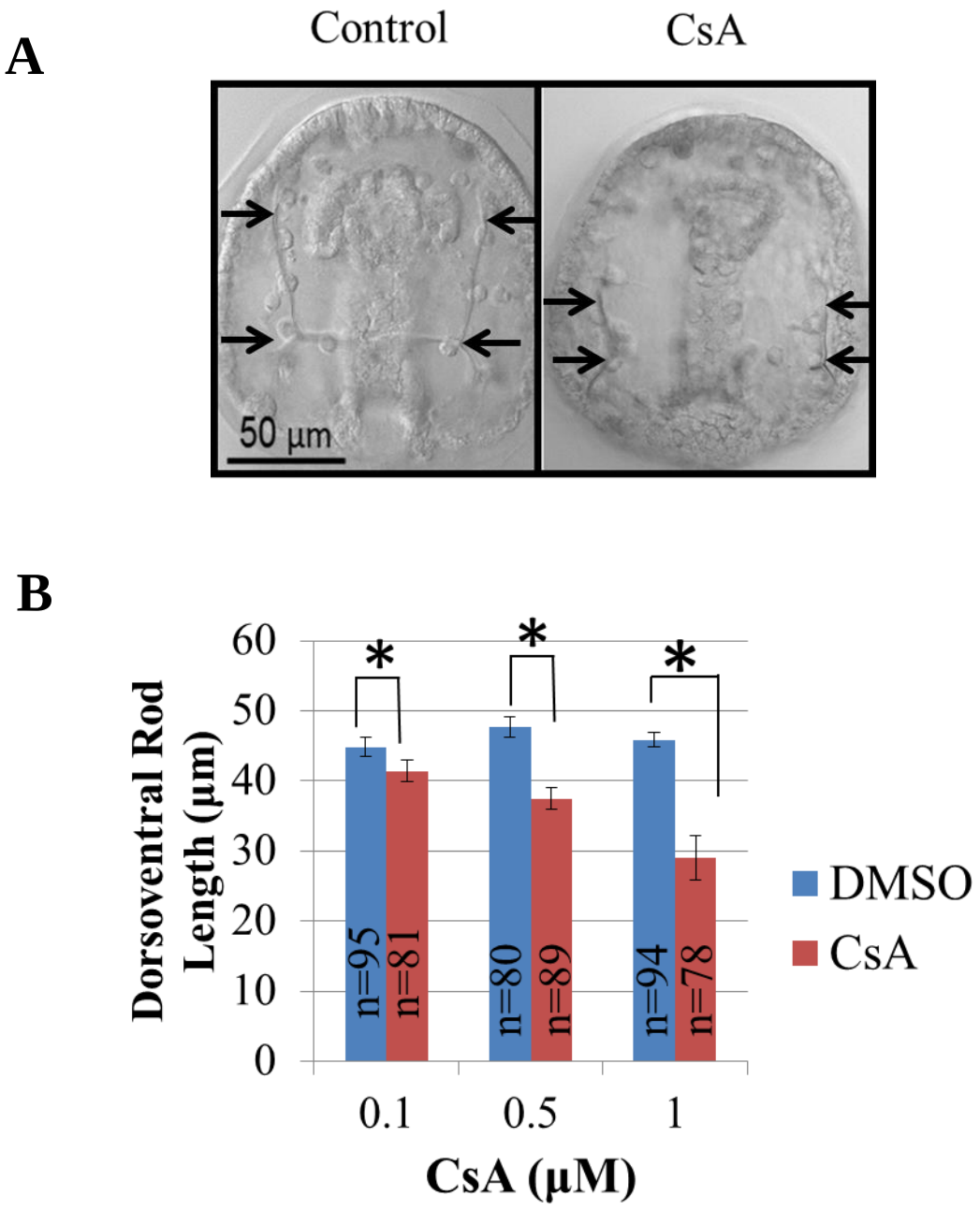


Figure 13 **CsA caused a shorter dorsoventral connecting rod in a dose dependent manner**. A) DIC images of embryos treated with or without CsA. Similar to PMA, when CsA is added to 19 hpf embryos for 3 hours, the DVC rod is significantly decreased. Arrows indicate the measured DVC length. B) CsA induced defects are dose-dependent (n is the number of embryos. 3 biological replicates; Student T-test, p <.009).

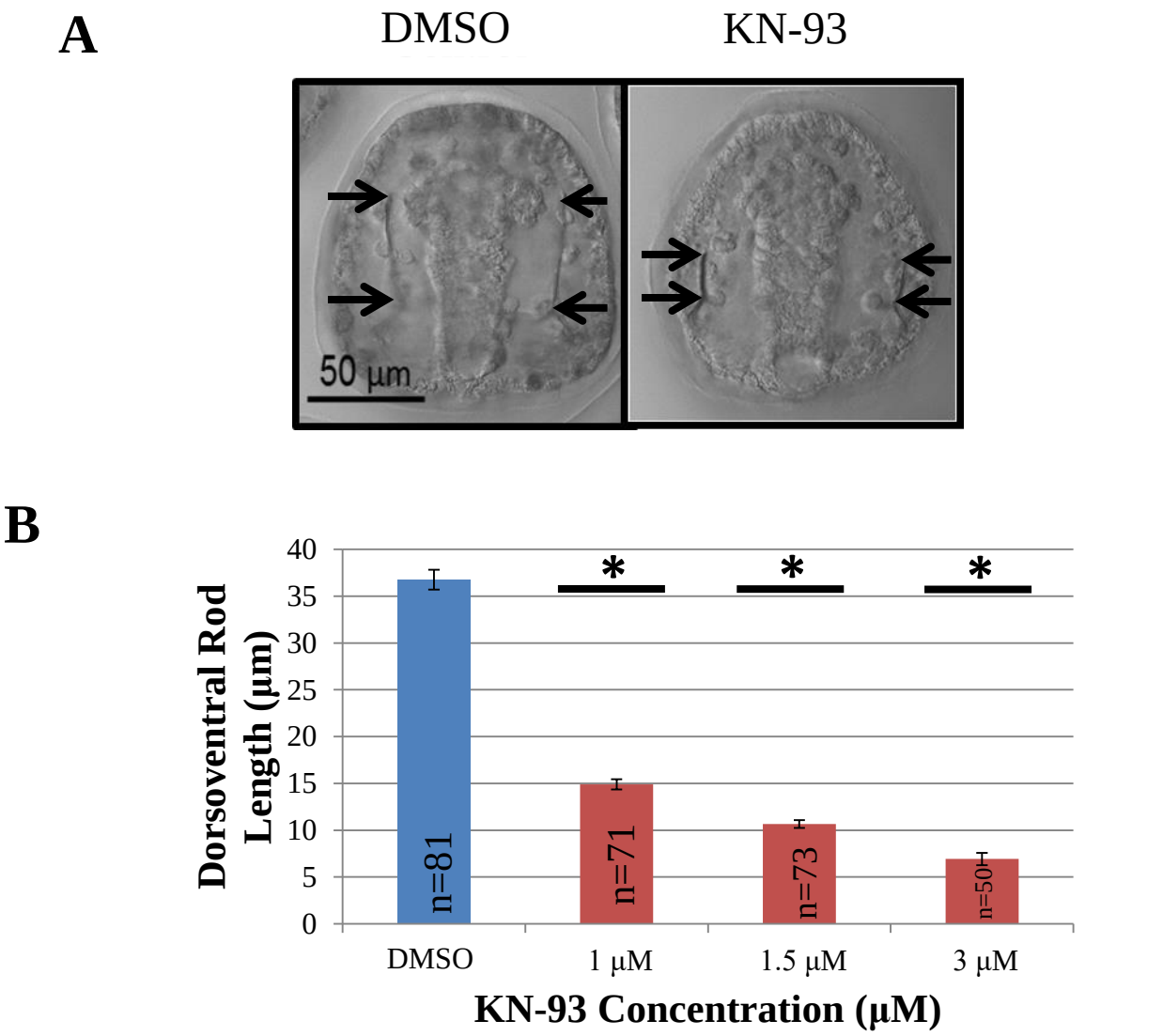
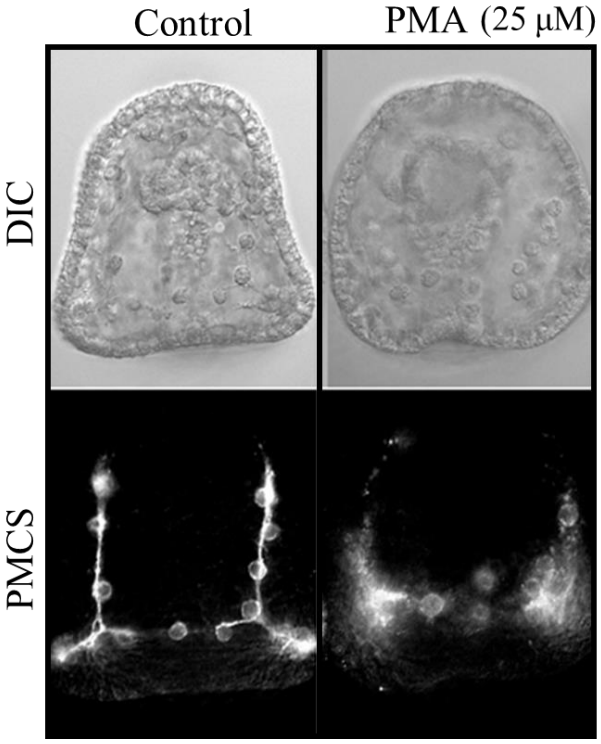


Figure 14 **KN-93 caused a decrease in DVC connecting rod length in a dose dependent manner**. A) DIC images of embryos with or without KN-93Different concentrations of KN-93 was added to embryos at 19 hpf for 3 hours. Arrows indicate the measured DVC. B) KN-93 caused a dose-dependent decrease in the length of the dorsoventral rod. (n is the number of embryos examined. 2 biological replicates; Student T-test with p <0.00001)

3.2 KN-93 and PMA caused PMC Patterning

In order to test the effect of perturbation of the ncWnt/Ca^{2+} pathway on PMC morphogenesis, we used various drugs. The PMCs of the control and drug-treated embryos were immunolabeled with an antibody 1D5 to assess the positioning of the PMCs (McClay *et al.,* 1983). When compared with a DMSO control (1:1000 dilution in natural sea water), the positioning of PMCs in KN-93 and PMA-treated embryos had clustered PMCs that failed to migrate anteriorly. On the other hand, PMC patterning is normal in CsA treated embryos (Figure 15-17).

A


Control
PMA (25 µM)
DIC
PMCS

B

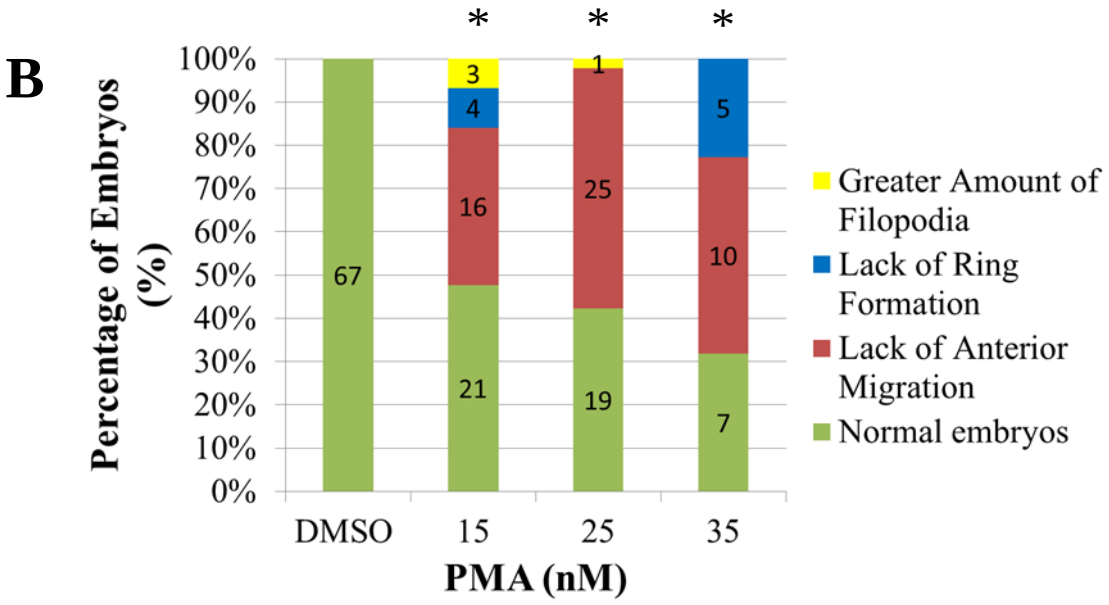
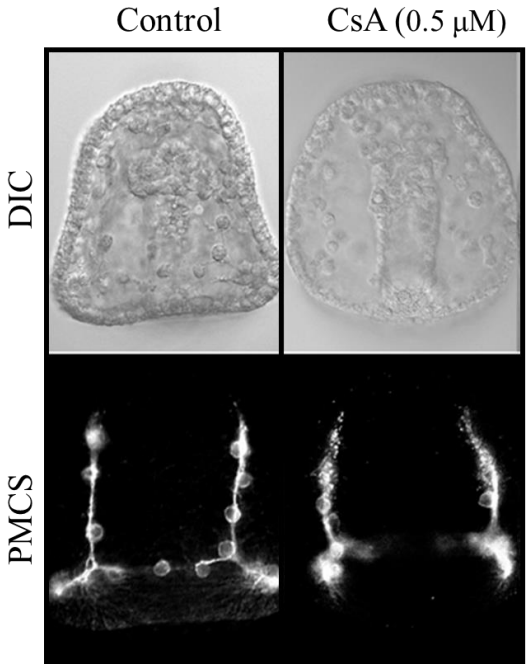

*
*
*
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Embryos (%)
67
3
4
16
21
1
25
19
5
10
7
DMSO
15
25
35
PMA (nM)
Greater Amount of Filopodia
Lack of Ring Formation
Lack of Anterior Migration
Normal embryos

Figure 15 **PMA treatment resulted in PMC patterning defects.** A) The panel displays representative images of the PMC phenotypes in embryos treated with or without PMA. PMA treated embryos had defects in PMC migration, compared to DMSO treated (negative control) embryos. B) PMA drug treatment resulted in PMC defects, including lack of anterior PMC migration, ring formation and extensive filopodia compared to control. (n is the number of embryos examined. 1 biological replicate for PMA. The asterisks represent statistical significance using Fisher's exact test when comparing the amount of normal/abnormal embryos in the control to the amount of normal/abnormal embryos in the drug-treated embryos. All $p<.00001$)

A


Control
CsA (0.5 µM)
DIC
PMCS

B

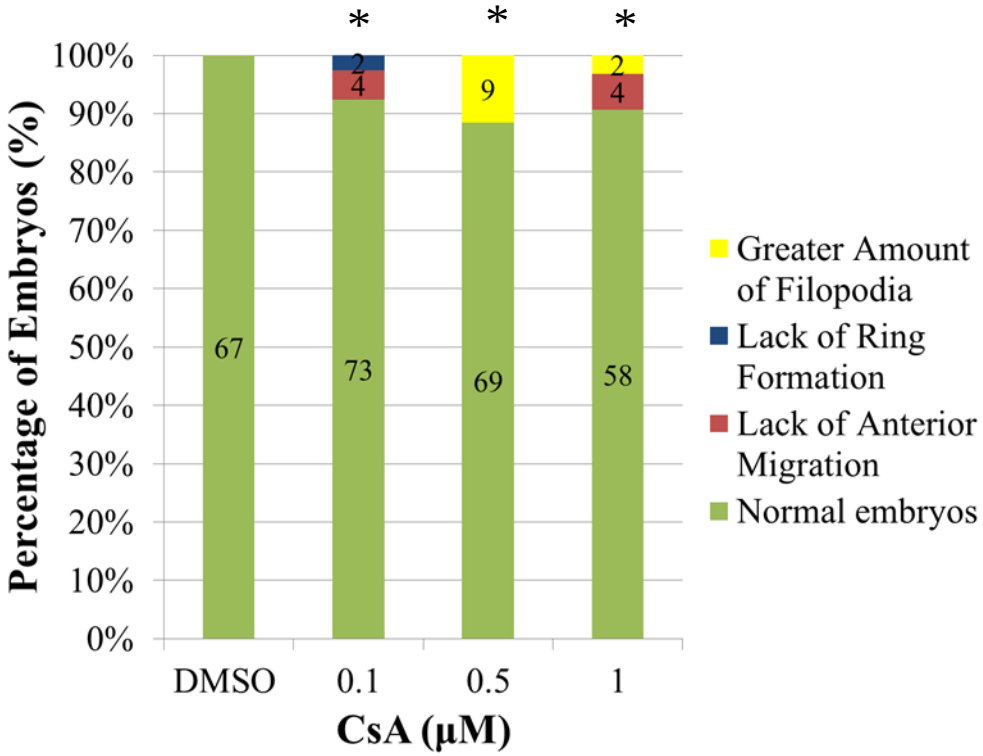
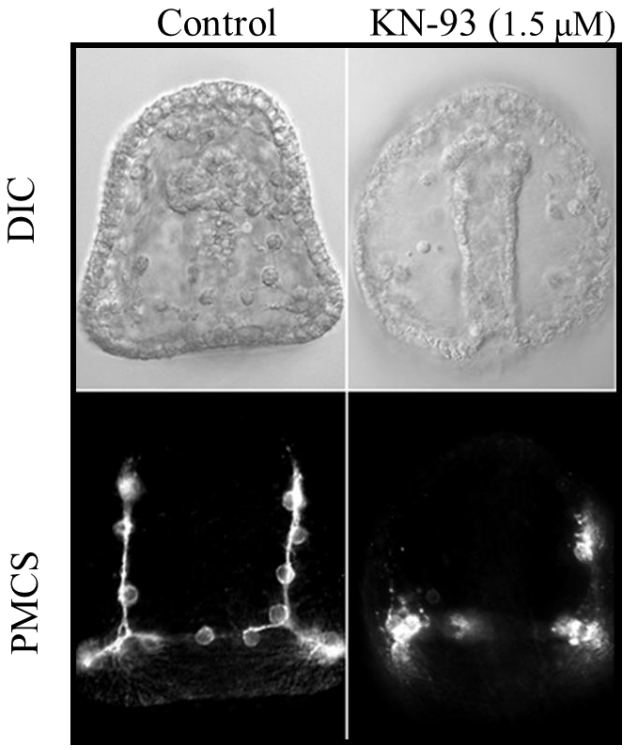

*
*
*
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Embryos (%)
67
2
4
73
9
69
2
4
58
DMSO
0.1
0.5
1
CsA (µM)
Greater Amount of Filopodia
Lack of Ring Formation
Lack of Anterior Migration
Normal embryos

Figure 16 **CsA treatment did not result in PMC patterning defects**. A) The panel displays representative images of the PMC phenotypes in embryos treated with or without CsA. CsA treated embryos did not exhibit significant defects in PMC migration, compared to DMSO treated (negative control) embryos. B) CsA drug treatment did not result in PMC defects. (n is the number of embryos examined. 2 biological replicate for CsA There was no statistical significance using the Cochran-Mantel-Haenszel test between the number of normal /abnormal embryos in control embryos compared to the number of normal/abnormal embryos in drug-treated embryos. $p<0.005$

A


Control
KN-93 (1.5 µM)
DIC
PMCS

B

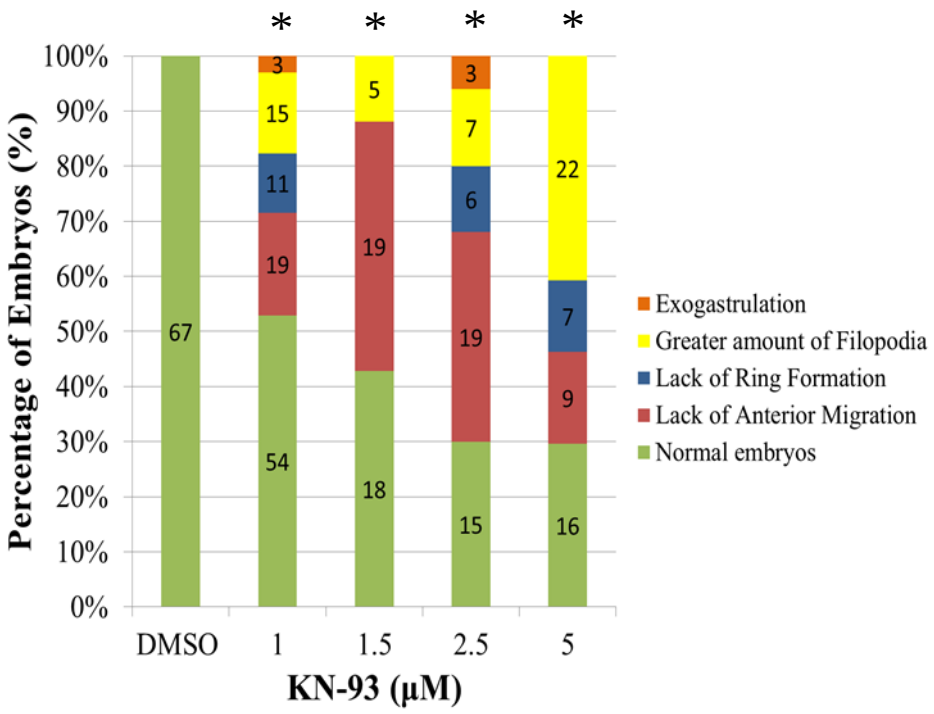

*
*
*
*
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of Embryos (%)
67
3
15
11
19
54
5
19
18
3
7
6
19
15
22
7
9
16
DMSO
1
1.5
2.5
5
KN-93 (µM)
Exogastrulation
Greater amount of Filopodia
Lack of Ring Formation
Lack of Anterior Migration
Normal embryos

Figure 17 **KN-93 treatment resulted in PMC positioning defect.** A) The panel displays representative images of the KN-93 phenotypes in embryos treated with or without KN-93. KN-93 treated embryos had defects in PMC migration compared to DMSO treated (negative control) embryos. B) KN-93 drug treatment resulted in PMC defects, including lack of anterior PMC migration, ring formation and extensive filopodia compared to control. (n is the number of embryos examined, 2 biological replicates for KN-93 The asterisks represent statistical significance using the Cochran-Mantel-Haenszel test when comparing the number of normal/abnormal embryos in the control to the number of normal/abnormal embryos in the drug-treated. $p<0.00001$.

3.3 KN-93 Treated Embryos Have Decreased Expression in PMC Patterning and Biomineralization Genes.

In order to understand the molecular mechanism of these drugs on PMC development, we assayed for transcript level changes of genes known to be important for PMC positioning (*Vegf3* and *VegfR10)* and genes encode for biominearalization genes (*p16, p19, SM50, and Clectin*) (Duloquin *et al*., 2007; Matranga *et al.,* 2013). We observed that in embryos treated with KN-93 (1.5 µM), *Vegf3* almost had a 2-fold decrease in transcript levels compared to the control (Figure 18). A majority of the biomineralization genes tested (*p19, sm50 and clectin*) also had slightly decreased levels in KN-93 drug treated embryos at 24 hpf compared to the control. This data also supports the lack of fully formed DVCs phenotypes observed in KN-93 treated embryos. It is necessary to repeat the QPCR experiments for further time points.

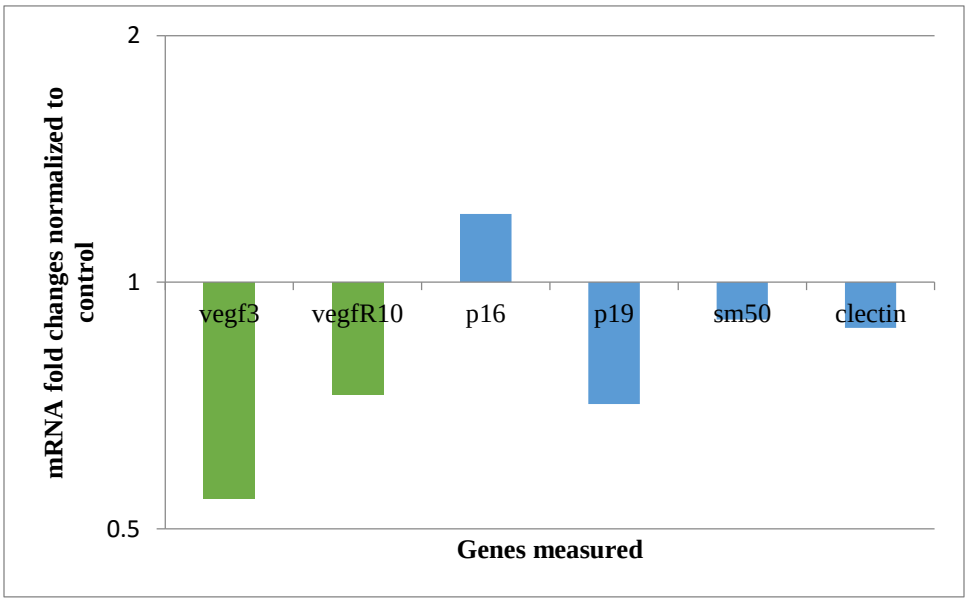


Figure 18 **KN-93 treated embryos have decreased expression of PMC patterning and biomineralization genes**. 100 Embryos were collected for QPCR at the 24 hpf. Several genes which had a potential role in the KN-93 treated embryo phenotypes were tested. PMC patterning genes (green) *Vegf3* and *VegfR10* had decreased transcript levels. Biomineralization genes (blue) *p19*, *SM50*, and *clectin* also had decreased transcript levels.

3.3 Dvl is directly regulated by miRNAs.

The PMA, CsA and the KN-93 drug study data suggest that the ncWnt signaling pathways have a significant effect on skeletal spicule formation and PMC migration within a developing embryo. In order to further examine the regulation of ncWnt signaling, we tested the post-transcriptional regulation of ncWnt pathway by miRNAs. Previous work in our lab demonstrated that Dvl is directly regulated by miRNA (Priya Nigam, Aun Zaidi, and Nadezda Stepicheva). It was bioinformatically determined that there were two putative miRNA binding sites within the 3'UTR of Dvl (miR-153* and miRDeep2-35240). We did not test the regulation of miR153* because previous sequence reads indicated that this miRNA is at undetectable levels in

all developmental stages (Song et al., 2012). Thus, our focus is on testing the direct regulation of miRDeep2-35240 on Dvl.

The 3'UTR of Dvl was cloned downstream of *Renilla* luciferase (Rluc) (Aun Zaidi, unpublished). The third and fifth base pairs of the spu-miRDeep2-30364 seed sequence was mutated to prevent endogenous miRNA binding to the 3'UTR target site in the reporter constructs. *In vitro* transcribed mRNAs of *Renilla* reporter constructs fused to the Dvl 3'UTR and the Firefly reporter construct were co-injected into newly fertilized eggs. Mesenchyme blastula stage embryos were collected to measure miRNA regulation on Dvl using the dual luciferase assay (Fig. 19a). The *Renilla* luciferase construct with mutated spu-miRDeep2-30364 seed sequence resulted in a significant increase compared to the *Renilla* luciferase construct with wild type spu-miRDeep2-30364 seed, indicating that miRDeep-2-35240 directly suppresses Dvl (Figure 19b) The relatively small increase in luciferase reading caused by the removal of miRNA regulation is consistent with the previous studies (Nicolas, 2011; Selbach *et al*., 2008; Stepicheva *et al*., 2015).

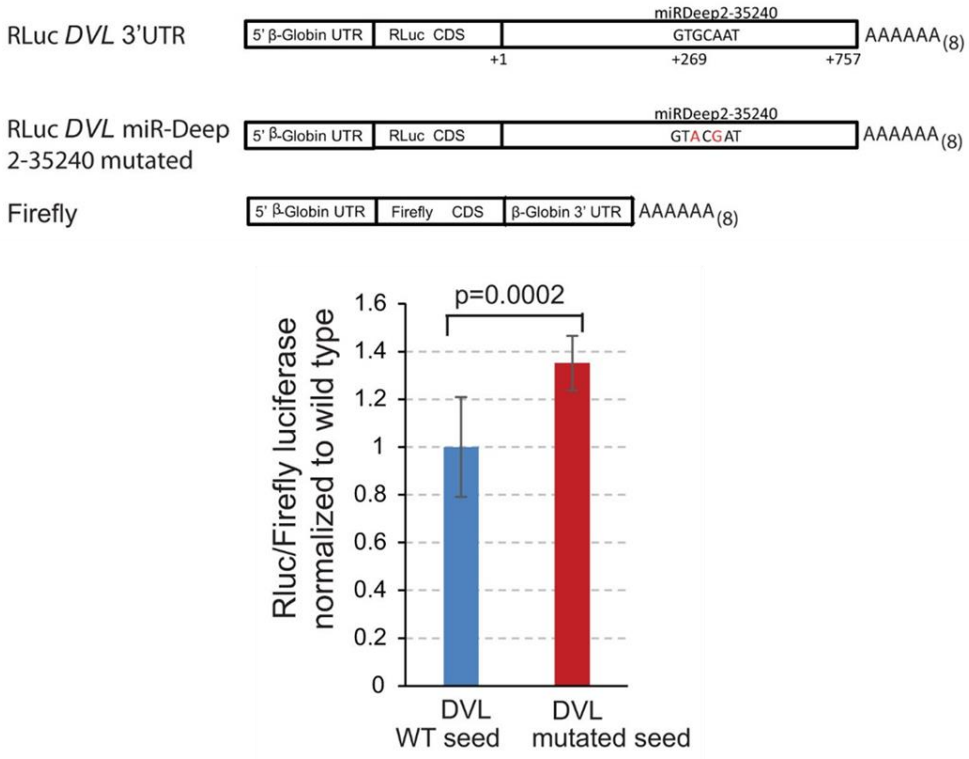


Figure 19 **Dvl is directly suppressed by *spu*-miRDeep2-30364.** The *Renilla* luciferase (Rluc) construct with mutated Dvl miRNA binding site had a significantly increased Rluc/Firefly luciferase reading when normalized to the Rluc with wild type Dvl miRNA binding site. Mutated Dvl miRNA binding site would prevent *spu*-miRDeep2-30364 from binding and suppressing its translation, leading to increased translation. This increase in Rluc with mutated Dvl miRNA site indicated that Dvl is directly regulated by miR-Deep 2-35240.

3.4 Inhibited miRNA Binding of Dvl Resulted in Minor Gut Defects

Once it was determined that Dvl was directly suppressed by miR-Deep 2.35240, miRNA target protector morpholinos (miRNA TP) were designed to specifically block the binding site of this miRNA within the 3'UTR of *Dvl* (Staton and Giraldez, 2011). The antisense morpholino sequences were specifically designed for the complementary miRNA binding site sequences identified within the 3'UTR of

Dvl. The Dvl miRNA TP was used to block miRNAs from binding, which would result in its failure to repress translation (Figure 20). A range of concentrations from 3 μM to 300 μM of Dvl miRNA TP was tested.

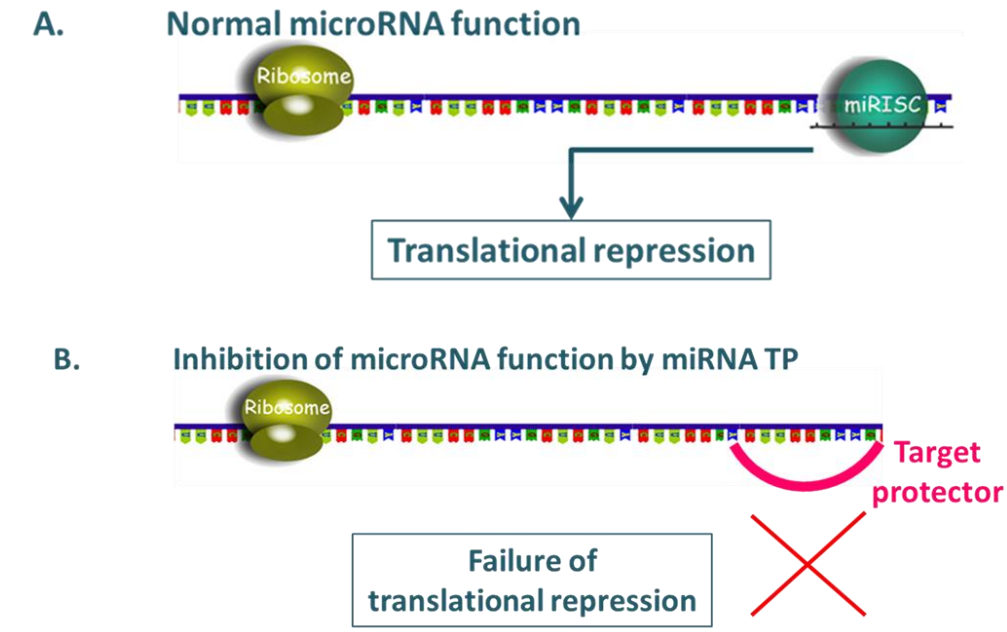


Figure 20 **miRNA TPs block miRNA regulation of its target mRNA**. A) Normal miRNA binding results in subsequent translational repression. B) By blocking miRNA binding sites with a miRNA TP, miRNA binding would be blocked, resulting in a lack of translational repression (Priya Nigam's Thesis, 2015).

Treating embryos with Dvl miRNA TPs resulted in gut defects. Newly fertilized eggs were microinjected with either control antisense morpholino oligonucleotide (MASO) or Dvl miRNA target protector (Dvl miRNA TP). The embryos were imaged using DIC at 48 hpf (Figure 21). In a normal 48 hpf embryo, the gut is fully elongated and differentiated. At this stage, it consists of a muscular

esophagus (foregut), a large stomach (midgut), and a tubular intestine (hindgut) (Burke, 1981; Burke and Alvarez, 1988)). In the Dvl miRNA TP treated embryos, their midgut width was significantly thinner (Figure 21).

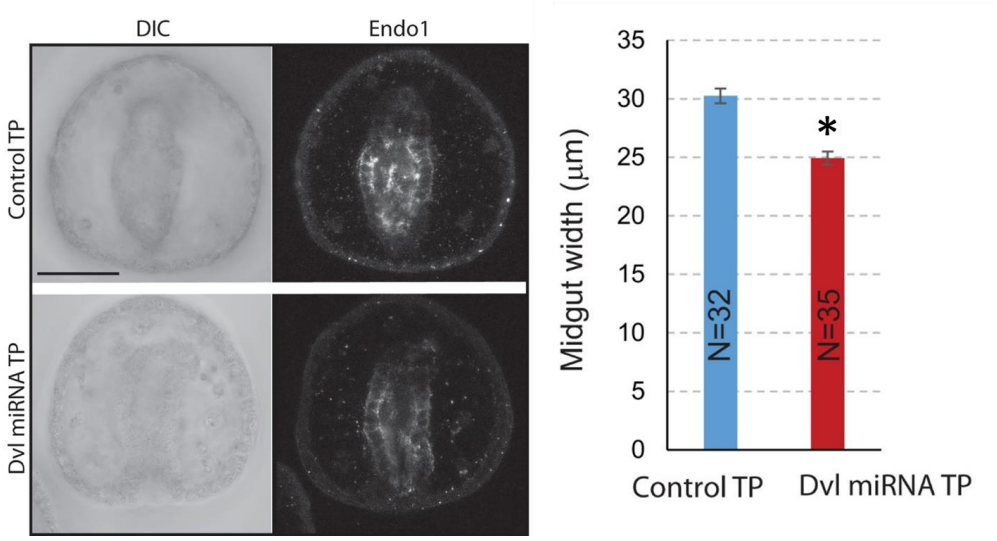


Figure 21 **miRNA TP treated embryos displayed thinner midgut width**. The midgut width (middle compartment) was measured for control and miRNA TP treated embryos. The Dvl miRNA TP treated embryos had significantly thinner mid-gut width. B) The endodermal cells of the gut were also examined using Endo1 stain and the miRNA TP treated embryos did not have any defects in morphology. * indicated statistical significance with Student T-test with $p<0.001$. Scale bar is 50 μm.

3.5 Blockage of miRNA Regulation of Dvl Resulted in PMC Defects

Dvl miRNA TP injected embryos also exhibited a defects in skeletal formation. The Dvl miRNA TP treated embryos DVC had dose-dependent decrease in

DVC rod length compared to the control (Figure 22). Results indicate that the PMCs in the majority of Dvl miRNA TP treated embryos (80%) lacked syncytial cables that normally connect the PMCs (Figure 23). A proportion of Dvl miRNA TP treated embryos (17%) have PMCs that lacked syncytial cables and have defective positioning.

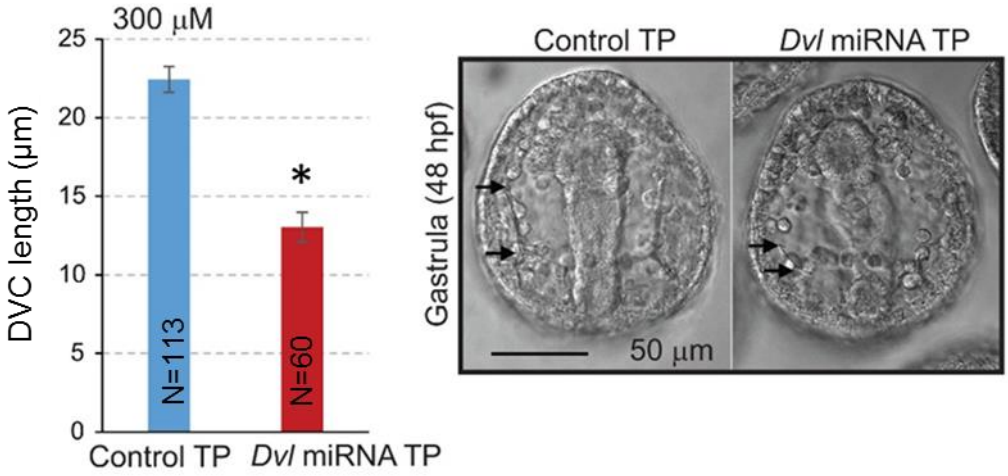
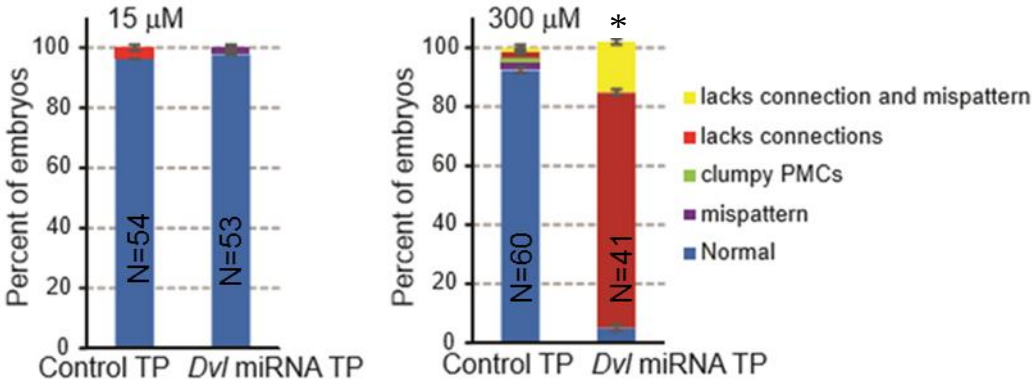


Figure 22 **Dvl miRNA TP treated embryos resulted in a decrease in DVC length**. Embryos were treated with 300 µM Dvl miRNA TP. Arrows indicate the measured DVC rod length. Two biological replicates, Student T-test $p<0.001$.


15 µM
300 µM
*
Percent of embryos
100
80
60
40
20
0
N=54
N=53
N=60
N=41
Control TP
Dvl miRNA TP
lacks connection and mispattern
lacks connections
clumpy PMCs
mispattern
Normal

B

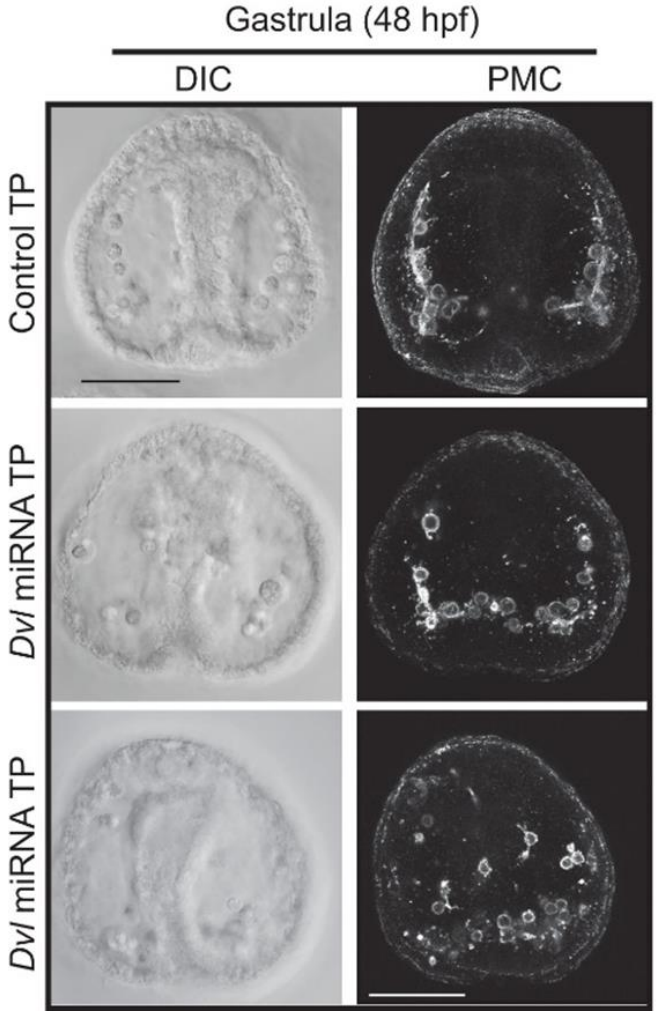
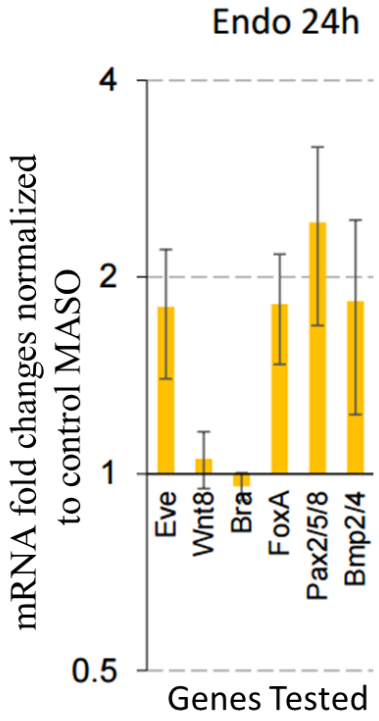

Gastrula (48 hpf)
DIC
PMC
Control TP
Dvl miRNA TP
Dvl miRNA TP

Figure 23 **Dvl miRNA TP treated embryos resulted in defects in PMC connections.** A) A dose-dependent defect in PMC connections and positioning were observed with Dvl miRNA TP injected embryos. B) Embryos were immunolabeled for PMCs using 1d5 antibody. The middle image depicted the lack of syncytial cables in 80% of Dvl miRNA TP-treated embryos. The bottom image depicted Dvl miRNA TP-treated embryo that lacks PMC syncytial cables and had defective PMC positioning in 17% of the embryos (Nadezda Stepicheva).

3.6 Gene Expression associated with Wnt Signaling is Affected By *Dvl* miRNA TP

To identify the molecular mechanism behind the skeleton and gut phenotypes caused by *Dvl* miRNA TP injection, we examined the transcript levels of genes that were involved in the Wnt signaling pathway, biomineralization, fusion, and cellular movement. *VegfR10* and *Pax 2/5/8* had more than 2-fold increase in transcript levels in *Dvl* miRNA TP injected embryos compared to the control. VegfR10 is a growth factor receptor expressed by PMCs that is involved in PMC patterning (Röttinger *et al*., 2008). *Pax 2/5/8* are proteins that are expressed in distinct regions of the ectoderm that is upstream of *Vegf3* (McIntryre *et al., 2013*). *Lasp1, kirrell, hypp1164, prestin* and *slc26a5* were also increased to almost 2-fold in *Dvl* miRNA TP injected embryos compared to the control. In mammalian cells, Lasp1 has recently been shown to mediate the directional migration of leukocytes by binding to the chemotactic receptor CXCR2 (Raman *et al*., 2010). Lasp1 may be a motility-related protein in PMCs (Rafiq *et al*, 2012). Kirrell and hypp1164 have been found to regulate PMC cell fusion (Rafiq *et al*., 2014). Prestin and Slac26a5 are solute carrier proteins that recently have been found to regulate the ventral skeletal patterning (Piacentino *et al*., 2016). Other transcripts were affected but had minor changes (Figure 24).

A


Endo 24h
mRNA fold changes normalized to control MASO
4
2
1
0.5
Eve
Wnt8
Bra
FoxA
Pax2/5/8
Bmp2/4
Genes Tested

B

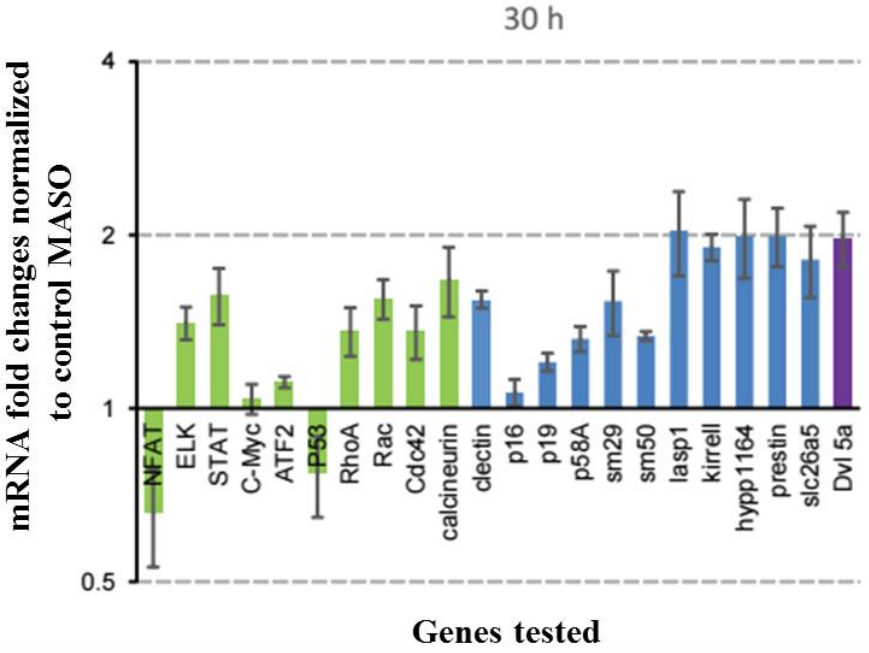

30 h
mRNA fold changes normalized to control MASO
4
2
1
0.5
NFAT
ELK
STAT
C-Myc
ATF2
P53
RhoA
Rac
Cdc42
calcineurin
dectin
p16
p19
p58A
sm29
sm50
lasp1
kirrell
hypp1164
prestin
slc26a5
Dvl 5a
Genes tested

Figure 24 ***Dvl* miRNA TP affected gene expression of several genes involved in PMC development.** *Dvl* miRNA TP injected embryos were collected at (A) 24 and (B) 30 hpf for QPCR. Several transcripts, including *Pax 2/5/8* and *VegfR10*, had a greater than 2-fold increase in *Dvl* miRNA TP treated embryos compared to the control. At 30 hpf, genes involved in biomineralization did not significantly change. However, *Lasp1*, *kirrell*, *hypp1164*, *prestin*, and *Dvl* were increased to almost 2-fold when treated with *Dvl* miRNA TPs (Nadezda Stepicheva and Jia L. Song).

Chapter 4

DISCUSSION

The results of the drug studies and the *Dvl* miRNA TP experiments indicated the importance of ncWnt signaling pathway in regulating PMC development and function. Inhibition of Calcineurin (with CsA) or CaMKII with (KN-93) or activation of PKC (with PMA) all resulted in significantly shorter DVC skeleton rod lengths compared to the control. We further examined post-transcriptional regulation of Dvl by miRNAs and found that miRNA regulation of Dvl was important for DVC skeleton rod development and the formation of filopodial connections between the PMCs.

Knowing that the ncWnt/Ca^{2+} pathway is important for cell morphogenesis and motility, we hypothesized that altering the activity of different ncWnt/Ca^{2+} components would result in morphogenic defects of PMCs (Geng *et al.,* 2016). While both PMA and KN-93 drug treatments resulted in PMC positioning defects, it is surprising that inhibiting Cn with CsA did not result in similar PMC positioning defects. One explanation for this might be that the concentrations used in the drug treatments were not high enough to elicit a phenotypic response. These results also suggest that DVC rods are more sensitive to CsA than PMC patterning.

Previous study indicated that inhibition of PKC resulted in a similar skeleton defects. Inhibition with the drug Bisindolylmaleimide I lead to a complete prevention of skeleton spicule formation (Croce *et al.,* 2006). Thus, altering PKC levels either through activation (Fig. 11) or inhibition resulted in a lack of full DVC skeleton rod formation. In the future, it will be important to monitor later time points, specifically

the 72 hpf larval stage where embryonic skeleton formation is complete, in order to determine if the DVC phenotype persists.

CsA has been shown to inhibit renal morphogenesis in mammals. In mammalian kidney development, an uteric bud receives signals from and adjacent metanephric mesenchyme which cause the bud to undergo morphogenesis and branching, to initiate nephron differentiation. In mammalian kidney organ cultures treated with CsA, a marked decreased in nephrons and uteric bud tips (renal morphogenesis) was observed, which was restored when CsA was removed (Burn *et al.,* 2011). In mammals 4 out of 5 NFAT transcription factors are regulated by Cn (Burn *et al.,* 2011). The renal morphogenesis phenotypes are the result of preventing activation of Calcineurin-dependent NFAT transcription factors. In the sea urchin genome, only one NFAT has been annotated. The decrease in DVC skeleton rod in the CsA treatments is likely to be attributed to the inhibition of Calcineurin-dependent activation of NFAT. In the future we will treat embryos and use NFAT specific inhibitor to test if NFAT inhibition would lead to similar phenotypes as the CsA treatment.

Wnt signaling has previously been linked to morphogenic processes such as neural patterning and development of neurites (Freese *et al*., 2010). Using KN-93, a previous study identified that CaMKII is involved in axon outgrowth during neuron polarization (Horigane *et al.,* 2016). KN-93 is a broad spectrum drug against all CamK family species (Horigane *et al.,* 2016). Even though CamKII isoforms may be involved in different signaling pathways such as the Ras/Raf-1/Mek/Erk pathways, they are known to regulate cellular morphogenesis (Illario *et al., 2003;* Chen *et al.,*

2011; Minichiello,*2009;* Gartner *et al.,* 2004 Horigane *et al.,* 2016). Thus, the results of my study is consistent with previous studies.

The QPCR data collected for KN-93 at 24 hpf has decreased levels of the growth factor *Vegf3* by 2-fold, suggesting that Vegf3 expression may be regulated by CamKII and potentially contribute to the PMC positioning defect. The growth factor receptor *VegfR10* and biomineralization genes *p19*, *Sm50* and *clectin* were also decreased slightly and may explain the shortened DVC phenotype upon KN-93 treatment.

Larger scale studies conducted previously on the effects of removing calcium all together using calcium chelators and thapsigargin, which eliminates intracellular calcium stores (Markova and Lenne, 2012), have indicated that coordinated movements such as convergent extension in frogs, and epiboly in zebrafish and newts were prevented (Wallingford *et al.,* 2001; Cheng, Miller and Webb, 2004; Takano *et. al* 2011). These studies support the importance of calcium in regulating cellular morphogenesis.

One of the issues with attributing the drug inhibition phenotypes strictly to the ncWnt/Ca^{2+} pathway is that many of the components involved in other pathways (Gartner *et al.,* 2004; Reyland, 2009; Rusnak and Mertz, 2000). This suggests that the drug inhibition phenotypes might not solely be attributed to ncWnt/Ca^{2+} inhibition. While PMA, CsA and KN-93 are highly specific in activating PKC isoforms and inhibiting Cn and CamKII isoforms, respectively, the phenotypes they caused could be the result of altering isoforms found outside of ncWnt/Ca^{2+} pathway.

A different approach to examine the effect of Wnt signaling on PMC morphogenesis is to test how this pathway may be regulated by miRNAs. The *Dvl*

miRNA TP treatment led to almost 2-fold increase of *Dvl* transcripts at both 24 and 30 hpf. This accumulation of *Dvl* mRNA may lead to increased Dvl protein in the embryo. It is important to note however, that miRNAs are used for fine tuning within biological systems, so when we refer to protein increases, there is likely a small change in protein levels (Selbach *et al.,* 2008; Stepicheva *et al*., 2015). Our lab previously demonstrated that treatment of *β-catenin* miRNA TP led to a 1.5-fold increase in β -catenin protein using western blot. Due to the lack of Dvl antibodies, we were not able to test Dvl protein levels. Since blocking miRNA regulation of Dvl would abolish miRNA-mediated translation and since we showed Dvl transcript level increase with *Dvl* miRNA TP treatment, these results indicate that the *Dvl* miRNA TP induced phenotypes are likely due to the increase in Dvl protein.

It is not clear if any Dvl isoform is strictly associated with any Wnt signaling pathway (Gentzel *et al.,* 2015). The isoform Dvl-*5a* we focused in this study has conservation at the protein level with the Dvl isoform that is associated with the cWnt However, depending on its interacting proteins with specific protein domains, Dvl can activate ncWnt pathway as well (Byrum *et al*., 2009). Thus, it is not clear if *Dvl* miRNA TP treatment would activate a specific branch of the Wnt pathway.

Dvl miRNA TP treatment led to shortened DVC, similar to CsA, KN-93, and PMA studies, suggesting that *Dvl 5a* may be regulating PMCs through the ncWnt pathway. In support of this possibility, our lab had previously shown that blocking miRNA regulation of β-catenin of the cWnt pathway did not impact DVC or PMC positioning (Stepicheva *et al*., 2015). It would be informative to test and compare the expression of genes of *Dvl* miRNA TP treated embryos with the drug-treated (KN-93, CsA, and PMA) embryos to identify molecular similarities. We would expect that

immunolabeling of KN-93, CsA, and PMA-treated embryos with Endo1 would give similar results as the *Dvl* miRNA TP treatment.

To elucidate how *Dvl* miRNA TP might impact downstream Wnt pathways, we assayed for transcript changes in ncWnt components. We found that the transcript levels of *NFAT* and *p53* were slightly decreased, whereas *calcineurin*, *RhoA*, *Rac*, *Cdc42*, *Elk* and *Stat* were increased in *Dvl* miRNA TP treated embryos (Figure 22). The level of transcript changes of these genes were relatively minor, and without examining their protein levels and activities, we cannot conclude that *Dvl* miRNA TP treatment impacted a particular branch of the Wnt pathways.

The QPCR results indicated that *Kirrell* and *Hypp1164* have increased transcripts in *Dvl* miRNA TP treated embryos. Previous studies indicated that these genes are closest to proteins that were important for myoblast interactions prior to fusion (Abmayr and Paylath, 2012; Rafiq *et al*., 2014). This may explain the phenotype of PMCs lacking proper connections. Other studies have found that decreased *Kirrell* levels prevented cell fusion (Durcan *et al.* 2013), whereas our results indicated that an increase in *Kirrell* transcripts correlated with a lack of PMC connections. Thus, how increased *Kirrell* and *Hypp1164* would abrogate proper cell to cell fusion or formation of filopodial structures among PMCs remains unknown.

Several of the transcripts have almost a 2-fold increase of transcripts in *Dvl* miRNA TP embryos at 24 hpf, including *VegfR10*, *Pax2/5/8, BMP2/4*, *Eve, and FoxA.* The PMCs express *VegfR10* to receive the *Vegf3* ligand, so increased *VegfR10* may affect the ability of PMCs to receive chemotactic signals. In response to short range Wnt5 signaling, *Pax2/5/8* expression is restricted to the border ectoderm (McIntryre *et al., 2013*). Wnt5a has primarily been established as ncWNt/Ca^{2+} Wnt ligand, so an

increase in *Pax2/5/8* level suggests that the increase in Dvl may lead to an increase in ncWnt/Ca^{2+} signaling. Therefore, this increase in ncWnt/Ca^{2+} could potentially be playing a role in the morphogenic phenotypes observed in the *Dvl* miRNA TP embryos. Both *BMP2/4* and *Pax 2/5/8* are important in controlling ectoderm specification and pattering in the border ectoderm (McIntryre *et al., 2013*). Overexpression of these genes might also be causing the *Dvl* miRNA TP phenotypes as well since PMC patterning was affected.

Eve and *FoxA* are key transcription factors important for gut development. *FoxA* is expressed at high levels in the foregut region of a developing embryo during gastrulation and is crucial for endodermal specification (Oliveri *et al.* 2006). Eve is also a factor involved in gastrulation and is expressed in Veg cells found on the periphery of the vegetal plate of the embryo (Peter and Davidson, 2010). Their alterations may lead to changes of the gut morphology.

Overall, results from this study indicated that disruption of Wnt/Ca^{2+} led to shortened DVC rod length and PMC mispatterning in the sea urchin embryo. We further demonstrated that Dvl of the Wnt pathway is directly regulated by miRNAs and blocking this miRNA regulation of Dvl is sufficient to negatively impact the length of DVC and the development of PMCs. Future experiments would be conducted to further elucidate the molecular mechanisms of Wnt/Ca^{2+} on cellular morphogenesis.

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