Inhibition of microRNA suppression of *Dishevelled* results in Wnt pathway associated developmental defects

Nina Faye Sampilo[1], Nadezda A. Stepicheva[1,3], Syed Aun Murtaza Zaidi[1], Lingyu Wang[2], Wei Wu[2], Athula Wikramanayake[2], Jia L. Song[1*]

[1]Department of Biological Sciences, University of Delaware, Newark, DE 19716

[2]Department of Biology, University of Miami, Coral Gables, FL 33124

[3]Department of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

[*]Corresponding author:

jsong@udel.edu

323 Wolf Hall

Newark, DE 19716, USA

Phone: +1 (302) 831-2794

Fax: +1 (302) 831-2281

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Abbreviations:

Dvl: dishevelled; PMCs: primary mesenchyme cells; miRNATP: microRNA target protector morpholino

Summary statement: This study demonstrated that microRNAs suppress *Dishevelled* isoforms in the sea urchin embryo. Blockage of microRNA suppression of *Dishevelled* isoforms induced defects in the gut, spiculogenesis, and ciliogenesis during development.

ABSTRACT

MicroRNAs (miRNAs) are highly conserved, small non-coding RNAs that regulate gene expressions by binding to the 3'untranslated region (UTR) of target mRNAs and silence translation. Some miRNAs are key regulators of the Wnt signaling pathways which impact developmental processes. This study investigates miRNA regulation of different isoforms of *Dishevelled (Dvl/Dsh)*, a key component in the Wnt signaling pathway. The sea urchin *Dvl* mRNA isoforms have similar spatial distribution in early development, but one isoform is distinctively expressed in the larval ciliary band. We demonstrated that *Dvl* isoforms are directly suppressed by miRNAs. By blocking miRNA suppression of *Dvl* isoforms, we observed dose-dependent defects of spicule length, patterning of the primary mesenchyme cells (PMCs), gut morphology, and cilia. These defects likely result from increased Dvl protein levels, leading to perturbation of Wnt dependent signaling pathways and additional Dvl-mediated processes. We further demonstrated that overexpression of *Dvl* isoforms recapitulated some of the *Dvl* miRNATP-induced phenotypes. Overall, our results indicate that miRNA suppression of *Dvl* isoforms plays an important role in ensuring proper development and function of the PMCs and cilia.

INTRODUCTION

Dishevelled is a key regulator in the highly conserved Wnt signaling pathway and plays the paramount role of directing signaling towards the different branches of Wnt signaling cascades (Gao and Chen, 2010). Components of the Wnt signaling pathways are expressed during early stages of embryonic development to specify cell fates, direct morphogenic movements, and maintain neuronal stem cells along with many other important functions (Gao and Chen, 2010; De, 2011). Upon Wnt ligand binding to the Frizzled (Fz) receptor, Dvl is recruited to the plasma membrane as it binds to the Fz. Depending on which proteins interact with Dvl, it can activate either the canonical Wnt/β-catenin (cWnt) signaling cascades important for cell specification, the non-canonical Wnt planar cell polarity (ncWnt/PCP) or the non-canonical Wnt Calcium pathway (ncWnt/Ca^{2+}) to control cellular morphogenesis (Gao and Chen, 2010; Song et al., 2015).

The cWnt signaling pathway is a highly conserved mechanism for germ layer specification in all metazoans (Moon, 2005; Komiya and Habas, 2008). 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 the starlet sea anemone in the Cnidaria phylum (Lee et al., 2007; Kumburegama et al., 2011). Previously, it has been shown that both Dvl (Weitzel et al., 2004; Peng and Wikramanayake, 2013) and β-catenin (Logan *et al.*, 1999) localize to the vegetal pole of the sea urchin embryo, resulting in activation of endodermal and mesodermal cell specification.

The ncWnt pathways are β-catenin independent and consist of the ncWnt/PCP and the ncWnt/Ca^{2+} pathways. The ncWnt/PCP pathway regulates polarization of epithelial cells, enabling cell morphogenesis and tissue patterning. Mutation of ncWnt/PCP genes in vertebrates results in a broader and shorter body axis and neural tube closure defects due to blockage of cell movements (Simons and Mlodzik, 2008; Yin et al., 2009; Hayes et al., 2013). In *Drosophila*, PCP signaling is necessary for orientation of epithelial structures in the cuticle, wing, and eye (Gubb and Garcia-Bellido, 1982; Adler, 2002; Sepich et al., 2011). The PCP pathway also consists of multiple genes required for ciliogenesis (Dale et al., 2009).

The ncWnt/Ca^{2+} pathway, mediated by G-protein signaling, stimulates releases of intracellular Ca^{2+} and activation of protein kinase C to control actin polymerization (Gao and Chen, 2010). In *Xenopus,* embryos were found to have elevated Ca^{2+}/CaMKII levels in the ventral side of the embryo (Kuhl et al., 2000). In zebrafish, complete blockage of ncWnt/Ca^{2+} leads to a variety

of developmental defects, such as gut abnormalities and tumor formation (Yoshida et al., 2004; De, 2011).

Dvl protein has three highly conserved domains, an N-terminal DIX (**Di**shevelled, A**x**in) domain, a central PDZ (**P**ostsynaptic density 95, **D**iscs Large, **Z**onula occludens-1) domain, and a C-terminal DEP (**D**vl, **E**gl-10, **P**leckstrin) domain (Gao and Chen, 2010). In general, the DIX domain of Dvl is important for Wnt/β-catenin signaling, and the PDZ and DEP domains are both required for ncWnt/PCP and ncWnt/Ca^{2+} pathways (Axelrod et al., 1998; Tada and Smith, 2000). In addition, Dvl-DEP and C terminal region (DEP-C) interact with three discontinuous regions of Fz. The DEP-C binding of Fz stabilizes the binding of Dvl-DEP domain to Fz, which is required to drive cWnt/β-catenin activation *Xenopus* and in HEK293T cells (Tauriello et al., 2012). Overexpression of Dvl-DIX in the sea urchin resulted in a lack of nuclear localization of β-catenin (Weitzel et al., 2004), in *Drosophila* resulted in a lack of β-catenin/Wnt activity (Axelrod et al., 1998), and in *Nematostella* resulted in a lack of archenteron and endodermal epithelium (Kumburegama et al., 2011). In *Xenopus*, overexpression of truncated Dvl possessing only PDZ and DEP domains relayed Wnt signaling predominantly via ncWnt/Ca^{2+} cascade, 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). Overexpression of Dvl-DEP in sea urchin, which blocks the ncWnt pathway through a dominant negative mechanism, resulted in failed archenteron invagination (Byrum et al., 2009). Thus, depending on its interacting proteins, Dvl can direct various branches of the Wnt signling pathways.

Relatively little is known of the impact of post-transcriptional regulation of *Dvl* on Wnt signaling pathways (He et al., 2015; Huang et al., 2018). MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression by binding to the 3'UTR of target mRNAs to repress their translation and/or induce mRNA degradation (Bartel, 2009). Similar to vertebrates, each sea urchin miRNA has many predicted targets (Song et al., 2012; Stepicheva et al., 2015; Stepicheva and Song, 2015). Previous studies indicated that the sea urchin embryo contains approximately 50 miRNAs, of which *Sp*miR-31 has been identified to target multiple genes important for primary mesenchyme cell (PMC) development and function (Song et al., 2012; Stepicheva and Song, 2015). Additionally, *Sp*miRDeep2-30364, and *Sp*miR2007 have been shown to suppress *β-catenin* (Stepicheva et al., 2015). The overarching hypothesis of the current study is that miRNAs modulate components of the Wnt signaling pathways to provide critical regulation

that impacts cell functions and embryonic structures. Specifically, this study examines miRNA regulation of *Dvl*. We found that the sea urchin embryo harbors four isoforms of *Dvl*, three of which have unique 3'UTRs. We demonstrated that miRNAs directly suppress *Dvl5a* and *Dvl4a*. By removal of miRNA suppression of these *Dvl* isoforms, we observed dose-dependent defects of gut morphology, skeletal length, and PMC patterning. In addition, we observed profound ciliary defects in the *Dvl* miRNATP-injected larvae that resulted in swimming defects. Importantly, overexpression of *Dvl* isoforms recapitulated the *Dvl* miRNATP-induced phenotypes. These results indicate that miRNAs modulate the expression of *Dvl* isoforms that provided an additional regulation to ensure proper embryonic development.

RESULTS

Sea urchin embryo has four *Dishevelled* isoforms

Four different isoforms of *Dvl* were identified from a previous RNA-Seq experiment in an effort to identify vegetal cortex-enriched mRNAs (Wang, Wu, and Wikramanayake, unpublished). All isoforms of *Stronylocentrotus purpuratu*s Dvl (*Sp*Dvl5a, 1, 4a, and 4b) share the exact same protein sequence except for the last exon. Only Dvl5a contains the highly conserved DEP-C domain ('AMGNPSEFFVDVM') that is critical for interacting with Fz and its own Dvl-DEP domain to drive the cWnt/β-catenin pathway (Fig. 1A) (Tauriello et al., 2012). *Dvl4a* differs from *Dvl4b* only in their 3'UTRs, where miRNA regulation typically occurs. Dvl from another sea urchin species, *Lytechinus variegatus*, *Lv*Dvl, and *Sp*Dvl4a/4b have C-terminal amino acid residues unique to the sea urchin species ('YFDDSSVTLL') (Fig. 1A) (Weitzel et al., 2004). Dvl1 lacks the last exon and contains unique three amino acids in the most C-terminal region ('NPS'). Despite near identify of their protein sequences, these *SpDvl* isoforms have three unique 3'UTRs (Fig. 1B). *Dvl4a* differs from *Dvl4b* only in their 3'UTRs, where miRNA regulation typically occurs (Selbach et al., 2008). *Dvl1* has the same 3'UTR as *Dvl4a*. Potential mRNA regulatory sites were bioinformatically identified by searching for inverse complementary miRNA seed sequences within the *Dvl* 3'UTRs (Song et al., 2012; Stepicheva et al., 2015; Stepicheva and Song, 2015). The precise regulation and function of each of these Dvl isoforms are not known.

Dishevelled isoforms are differentially expressed

We tested the expression of *Dvl5a*, *4a/1*, and *4b* isoforms, using primers designed against their unique 3'UTRs. It was not possible to design *Dvl1*-specific primers due to its high similarity with *Dvl4a* (Fig. S1). The expression of these *Dvl* isoforms were assayed in various developmental stages (0, 6, 24, 30, and 72 hours post fertilization; hpf), using real time, quantitative PCR (QPCR). We observed that all *Dvl* transcripts were expressed throughout development and increased steadily from the egg stage up until the gastrula stage. In the larval stage, the expression of all *Dvl* isoforms decreased when normalized to the egg (Fig. 2A).

To examine the temporal and spatial expression of *Dvl* isoforms in early development, we constructed RNA *in situ* probes complementary to distinct 3'UTRs from *Dvl5a, Dvl4a/Dvl1, and Dvl4b* isoforms. Of note is that the *in situ* probe against *Dvl4a* will also recognize *Dvl1* (Fig. S1). We observed that all *Dvl* isoforms were ubiquitously expressed during early stages of development. Interestingly, *Dvl4a/Dvl1* is enriched in the ciliary band of the larval stage (Fig. 2B).

We also examined the localization of Dvl protein in blastula, gastrula and plutei stages, using a pan-Dvl antibody (Peng and Wikramanayake, 2013). In the blastula and gastrula stages, Dvl is ubiquitously expressed and enriched in the posterior of the embryo. Dvl is also enriched in the gut and PMCs of gastrulae. In the larval stage, Dvl protein is enriched in the gut, pyloric sphincter, PMCs, and the ciliary band (Fig. 2C).

Dishevelled isoforms are directly suppressed by miRNAs

We bioinformatically identified 100% seed match of annotated sea urchin miRNAs within the 3'UTRs of all *Dvl* isoforms (Fig. 1B,3A). To test the direct regulation of *Dvl5a, Dvl4a,* and *Dvl4b* by these miRNAs, we cloned *Dvl* 3'UTRs downstream of *Renilla* luciferase (Rluc). Site-directed mutagenesis was used to alter the third and fifth base pairs of the seed sequences of *Sp*miRDeep2-30364 and *Sp*miR153* in *Dvl5a*; *Sp*miRDeep2-30364, *Sp*miR2007 and *Sp*miR2002 in *Dvl4a*; and *Sp*miR200 in *Dvl4b* (Fig. 3A). Mutated seed sites would abolish endogenous miRNA binding to the target sites in the 3'UTR of these reporter constructs (Staton and Giraldez, 2011; Stepicheva et al., 2015). *In vitro* transcribed mRNAs of *Rluc* fused to the wild type or mutated *Dvl* 3'UTRs and the *Firefly* reporter construct (used as a normalization control for *Rluc* luciferase) were co-injected into newly fertilized eggs (Stepicheva et al., 2015; Stepicheva and Song, 2015).

We collected injected mesenchyme blastulae and used dual luciferase assays to test direct miRNA suppression of these *Dvl* isoforms (Fig. 3B). The *Rluc* with mutated *Sp*miRDeep2-30364 and *Sp*miR153* seed sequences resulted in a significant increase of normalized luciferase signals compared to the *Rluc* with wild type seed sequences, indicating that at least of these miRNAs directly suppress *Dvl5a* (Fig. 3B). For *Dvl4a*, *Rluc* fused with the 3'UTR with mutated seed sites for *Sp*miRDeep2-30364, *Sp*miR2007, and *Sp*miR2002 had significantly more luciferase signals compared to the Rluc fused with the wild type 3'UTR, indicating that *Dvl4a* is directly suppressed by at least one of these miRNAs. We observed increased but not statistically significant Rluc signals from *Dvl4b* construct with mutated *Sp*miR200 seed sequence compared to the *Dvl4b* construct with wild type 3'UTR, indicating that this miRNA may not be *bona fide* or plays a weak regulatory role. The overall relatively small increase in luciferase reading caused by the removal of miRNA suppression is consistent with the previous studies (Selbach et al., 2008; Nicolas, 2011; Stepicheva et al., 2015). These results indicate that *Dvl5a* and *Dvl4a* are directly suppressed by miRNAs.

Dvl miRNA target protector morpholinos (miRNATPs) modulate *Dvl* mRNA and protein levels

To test the impact of miRNA suppression of *Dvl* isoforms, we designed *Dvl* miRNATPs complementary to the miRNA regulatory binding sites of *Dvl5a* (*Sp*miR153* at +230bp), *Dvl4a* (*Sp*miRDeep2-30364 and *Sp*miR2002) and *Dvl4b* (*Sp*miR200), as identified by bioinformatics analyses and luciferase assays (Fig. 1B,3A). Zygotes were injected with a cocktail of three *Dvl* miRNATPs, each at 300μM, or Control TP at 900μM. We then assayed for changes in the *Dvl* transcript and Dvl protein levels. Results indicated that *Dvl* miRNATP-injected embryos have similar transcript levels for *Dvl* isoforms and other components of the Wnt signaling pathway (Fig. 4A). However, the transcript level of *Rac1*, which encodes a small GTPase that is downstream of the ncWnt/PCP pathway (Lindqvist et al., 2010), was significantly increased (Fig. 4A).

To test if blocking miRNA suppression of *Dvl* translation resulted in a change in Dvl, we immunolabeled control TP and *Dvl* miRNATP-injected embryos at the 32-cell stage, where *Dvl* protein is enriched at the vegetal cortex (Peng and Wikramanayake, 2013). We took confocal Z-stack images of these embryos and measured the total level of fluorescence pixels from Dvl immunolabeling. Results indicated that the *Dvl* miRNATP-injected embryos displayed an overall

1.5-fold increase of Dvl compared to the control TP-injected embryos (Fig. 4B,C). Interestingly, removal of specific miRNA suppression of *Dvl* isoforms resulted in an increase of ubiquitous Dvl protein throughout the embryo, as well as increased Dvl in the vegetal cortex.

Treatment of *Dvl* miRNATPs or *Dvl* mRNA results in dose-dependent aberrant gut morphology

Since the cWnt is involved in endomesoderm specification, we examined the effect of blocking miRNA suppression of *Dvl* on the embryonic gut (Fig. 5). The sea urchin embryonic gut consists of a muscular esophagus (foregut), a large stomach (midgut), and a tubular intestine (hindgut) (Burke, 1981; Burke and Alvarez, 1988). Newly fertilized eggs were microinjected with *Dvl* miRNATPs against all three *Dvl* isoforms at various concentrations. We used the Endo1 antibody, which recognizes antigens of the mid and hindgut (Wessel and McClay, 1985), to examine the epithelial cell lining of the gut. Results indicated that 58% of *Dvl* miRNATP-injected embryos expressed less Endo1 in the gut epithelium (the less severe phenotype) and 16% of the embryo failed to gastrulate (the more severe phenotype) (Fig. 5A).

Because blocking miRNA suppression of *Dvl* isoforms resulted in increased Dvl and various aberrant phenotypes, we overexpressed sea urchin *Dvl* isoforms to identify which phenotypes attributed to which *Dvl* isoform. We utilized the previously studied *LvDvl* that has the sea urchin-specific C-terminus residues (YFDDSVSVTLL) to examine the effect of *SpDvl4a/4b* overexpression. The *LvDvl* was modified to contain the conserved DEP-C residues that are shared by Dvl of other species which we denoted as *LvDvl**, to resemble the *SpDvl5a* form (CEFFVDVM). These *Dvl* mRNAs were injected into zygotes. We found that overexpression of each isoform alone or in combination resulted in less Endo1 expression, in comparison to *mCherry* mRNA-injected control embryos (Fig. 5B), similar to the less severe gut phenotype of the *Dvl* miRNATP-injected embryos (Fig. 5A).

To evaluate gut morphology of the less severe gut phenotype of the *Dvl* miRNATP-injected embryos, we measured the width of the midgut and blastopore and calculated their ratios. Results indicated that in the highest dose of 300µM, the width of the midgut of *Dvl* miRNATP-injected embryos was significantly narrower and the blastopore was wider than the embryos injected with the control TP (Fig. 5C). Results indicated that overexpression with *LvDvl/SpDvl4a/4b*, *LvDvl*/SpDvl5a*, or *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*-injected embryos led to significantly

wider blastopore width and narrower midgut, similar to the *Dvl* miRNATP-injected embryos (Fig. 5D).

Since we observed gut morphological differences in *Dvl* miRNATP-treated embryos, we tested if endodermal differentiation was affected by assaying for alkaline phosphatase (AP), which is an enzyme in the gut that is used as a differentiated endodermal marker (Fig. 5E) (Kumano and Nishida, 1998; Drawbridge, 2003). A significant number of *Dvl* miRNATP-treated larvae have little or no alkaline phosphatase activity present in the intestine (hindgut) as compared to the control (Fig. 5E). These results suggest that removing suppression of *Dvl* miRNAs resulted in Dvl protein increase that may negatively affect the function of the larval gut.

We investigated what might be the underlying molecular cause of these phenotypes by examining the spatial and level of expression of key transcription factors involved in endodermal specification. *Krl* and *FoxA* are expressed in the Veg2 cell lineage important for specifying the fore/midgut, and *Eve* and *Bra* are expressed in the Veg1 cell lineage important for hindgut specification (Peter and Davidson, 2011). The overall transcript levels of *Krl*, *FoxA*, *Eve*, and *Bra* did not change in *Dvl* miRNATP or combined *Dvl* mRNA (*LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*)-injected embryos compared to the control (Fig. 6A). However, the spatial expression domain of *Krl* was significantly decreased in *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to the controls (Fig. 6C). In addition, *Eve* has a significant expansion of its spatial expression domain in *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to the controls (Fig. 6D). The spatial expression changes of key transcription factors involved in endodermal specification may result in changes in the gut morphology and potentially function of the gut.

Blockage of miRNA regulation of *Dvl* isoforms or *Dvl* mRNA overexpression results in dose-dependent defects of skeletal spicules and PMC patterning

To test if the removal of miRNA suppression of *Dvl* had an impact in skeletogenesis, newly fertilized eggs were microinjected with *Dvl* miRNATPs against all three *Dvl* isoforms at various concentrations. We observed a dose-dependent decrease of the dorsoventral connecting rods (DVCs) at gastrula stage (Fig. 7A). Injection of the *Dvl* miRNATP at 300μM resulted in the most significant decrease of the DVCs. In addition, results indicated that overexpression of

LvDvl/*SpDvl4a/4b*, *LvDvl**/*SpDvl5a*, or in combination, resulted in a significant decrease in the DVCs compared to the control, similar to *Dvl* miRNATP-injected embryos (Fig. 7B).

We also tested the PMC positioning in control TP and *Dvl* miRNATP-injected embryos. We observed that 300µM of *Dvl* miRNATP-injected embryos have the most severe PMC defects with clustered PMCs at the sub-equatorial ring and a complete lack of anterior migration (Fig. 7C). The lower concentrations of *Dvl* miRNATP also resulted in some embryos with clustered PMCs at the sub-equatorial ring, but PMCs were able to partially migrate anteriorly. Similarly, *LvDvl*/*SpDvl4a/4b* or *LvDvl*/*SpDvl4a/4b* plus *LvDvl**/*SpDvl5a* co-injection resulted in scattered and clustered PMCs (Fig. 7D). No significant PMC patterning defects were observed in embryos injected with the *LvDvl**/*SpDvl5a* or in the *mCherry-LifeAct* mRNA-injected embryos, indicating that the *LvDvl*/*SpDvl4a/4b* isoform overexpression resulted in PMC patterning defects (Fig. 7D).

Importantly, the PMC patterning defects persisted into the larval stage 5 days post fertilization (dpf), where the *Dvl* miRNATP-injected and *Dvl* mRNA (*LvDvl*/*SpDvl4a/4b* plus *LvDvl**/*SpDvl5a*)-injected larvae have clustered PMCs and branched filopodia (Fig. 7E). Interestingly, we found that *Dvl* is expressed in the PMCs, suggesting that PMCs themselves may be responsive to Wnt signaling activation (Fig. 7F).

To further investigate if the failure of PMCs to migrate anteriorly might be due to defective motility, we co-injected *mCherry-LifeAct* mRNA with control TPs or *Dvl* miRNATPs into newly fertilized eggs to examine actin distribution. We observed that presumptive PMCs around the tri-radiate spicules of *Dvl* miRNATP-injected embryos have punctate actin localization, whereas the presumptive PMCs along the long skeletal spicules in the control TP-injected embryos have more even actin distribution within these cells (Fig. 7G). These results suggest that potentially actin remodeling defects may hinder proper PMC migration.

Removal of miRNA suppression of *Dvl* or *Dvl* mRNA overexpression results in decreased transcript levels of *Vegf3*

To identify the underlying molecular mechanism that led to *Dvl* miRNATP and *Dvl* mRNA-induced skeletal defects, we examined expressions of the following gene categories: 1) *Alx1* critical for PMC specification (Ettensohn et al., 2003), 2) biomineralization genes *SM29*, *SM49*, *SM50* (Adomako-Ankomah and Ettensohn, 2013), 3) *Hnf6* that regulates *SM50* (Otim et al., 2004; Otim, 2017), 4) factors that have been shown to be important for PMC positioning,

including *Nodal*, *BMP2/4* (Yaguchi et al., 2008; Duboc et al., 2010), *Pax2/5/8* (Rottinger et al., 2008), *Vegf3* (Duloquin et al., 2007; Adomako-Ankomah and Ettensohn, 2013), *Alk2/4/7*, *Slc26a5*, and *TGF-βrtII* (Piacentino et al., 2015; Piacentino et al., 2016; Sun and Ettensohn, 2017), and 5) *Kirrel* involved in PMC fusion (Ettensohn and Dey, 2017) (Fig. 8A). Results indicated no significant expression difference was observed for most genes tested, except for *Vegf3*, which had 2-fold less transcripts in both *Dvl* miRNATP and *Dvl* mRNA-injected embryos compared to the control embryos. Previous literature indicated that the Vegf3 signaling pathway provides guidance and differentiation cues to PMCs, as well as being critical for forming skeletal elements (Duloquin et al., 2007; Adomako-Ankomah and Ettensohn, 2013).

We also examined the spatial expression of *Wnt5* and *Vegf3*. Wnt5 acts as a short-range signal from the endoderm that activates the border ectoderm specification, which provides positioning cues for the PMCs (McIntyre et al., 2013). Results indicated that *Dvl* miRNATP-injected embryos did not have a significant change in the *Wnt5* expression domain, compared to the control TP-injected embryos. The spatial expression domain of *Vegf3* is significantly decreased in the *Dvl* miRNATP-injected or *Dvl* mRNA-injected embryos compared to the controls (Fig. 8C). The expression domain of *VegfR10*, which encodes the cognate receptor for Vegf3, was unchanged and expressed in all PMCs in the *Dvl* miRNATP-injected, *Dvl* mRNA-injected, and control embryos (Fig. 8D).

Blockage of miRNA suppression of *Dvl* isoforms or *Dvl* mRNA overexpression results in ciliary defects

We noticed that *Dvl* miRNATP and *Dvl* mRNA-injected larvae had swimming defects compared to the controls. The ciliary band arises after gastrulation and the ncWnt/PCP pathway has been implicated in regulating sea urchin ciliogenesis. Cilia are made up of microtubules consisting of tubulin subunits that enable the embryo to swim and feed (Yaguchi et al., 2010; Burke et al., 2014). To further examine this swimming defect, we immunolabeled larvae with β-tubulin antibody to visualize cilia. The control TP-injected embryos have long, well projected cilia, whereas the *Dvl* miRNATP-injected larvae displayed aberrant apical and body cilia (Fig. 9A-C). The body cilia of *Dvl* miRNATP-injected embryos have kinks, while control TP-injected embryos cilia are long and curved (Fig. 9B). The apical cilia of the ciliary band in the *Dvl* miRNATP-injected larvae tend to be short, bent, sparse, and disorganized compared to the controls (Fig.

9A,C). To ensure that the aberrant morphology of the cilia in *Dvl* miRNATP-injected embryos is not due to fixation artifacts, we also examined cilia in living larvae. We observed that both apical and body cilia in *Dvl* mRNATP-injected embryos are bent with irregular beating compared to the control TP-injected embryos that have long cilia with synchronous beating (movies 1-4).

Similar to the *Dvl* miRNATP-injected embryos, the overexpression of *LvDvl/SpDvl4a/4b*, *LvDvl*/SpDvl5a*, and combination of both resulted in bent ciliary defects compared to the long, protruding cilia of *mCherry* mRNA-injected control larvae (Fig. 9D and Movies 5-8).

DISCUSSION

Dishevelled is an important protein in the Wnt signaling pathway that plays a significant role in relaying cellular information to various developmental pathways (Gao and Chen, 2010). We identified various miRNAs that suppress different isoforms of *Dvl* and revealed that post-transcriptional regulation of *Dvl* is critical for proper development. Further, blockage of miRNA suppression of *Dvl* isoforms induced defects in the gut, spiculogenesis, and ciliogenesis. These developmental defects of *Dvl* miRNATP-injected embryos are mimicked by the overexpression of *Dvl* isoforms. Developmental defects in *Dvl* miRNATP and *Dvl* mRNA-injected embryos are likely due to perturbation of Wnt signaling pathways, as well as other Dvl functions in the embryo.

Dvl is ubiquitously expressed and highly enriched at the vegetal pole of early embryos (Peng and Wikramanayake, 2013). By blocking miRNA suppression of *Dvl* isoforms, we observed a 1.5-fold increase of the Dvl protein (Fig. 4B,C). This may be an underestimate, since we did not block all potential miRNA regulatory sites. Of note is that each *Dvl* miRNATP is complementary to the specific miRNA's seed sequence (6 bp) and the unique *Dvl* 3' UTR sequences flanking the miRNA seed sequence (additional 19 bp). Thus, each *Dvl* miRNATP is uniquely targeting that specific miRNA seed site within the particular *Dvl* isoform. We found that Dvl is increased in the entire *Dvl* miRNATP-injected 32-cell embryos with enhanced enrichment in the vegetal pole of the embryo compared to the control TP-injected embryos. This suggests that potentially the miRNAs we identified play a role in suppressing *Dvl* throughout the embryo. Further, the increase in Dvl protein was dose-dependent of *Dvl* miRNATP, indicating specificity of the miRNATPs (Fig. 4B,C and Fig. S2A).

Interestingly, miRDeep2-30364, one of the miRNAs that may suppress sea urchin *Dvl5a* and *Dvl4a*, also suppresses the sea urchin *β-catenin* (Stepicheva et al., 2015). Previously we have shown that miRDeep2-30364 and miR2007 directly suppressed *β-catenin* and resulted in increased gene expressions of downstream cWnt-responsive transcription factors, including *Eve*, *Bra*, *Krl*, and *FoxA* (Stepicheva et al., 2015). This is typical of miRNA function in which a miRNA targets multiple genes in the same developmental pathway (Stepicheva and Song, 2015).

Since we introduced *Dvl* miRNATP against at least one miRNA in all *Dvl* isoforms, we expect that elevation of Dvl protein would lead to the disassembly of the destruction complex and increase levels of β-catenin, which is a key effector protein of the cWnt pathway. β-catenin enters the nuclei of vegetal blastomeres where it regulates endomesoderm specification (Weitzel et al., 2004). Our laboratory previously found that blocking miRNA suppression of *β-catenin* resulted in increased β-catenin and induced aberrant gut morphology (Stepicheva et al., 2015). Similarly, here we observed that *Dvl* miRNATP or *Dvl* mRNA-injected embryos had significantly narrower midguts compared to control embryos (Fig. 5A,B). This is consistent with our observation that Dvl is localized to the gut and in the pyloric sphincter of the larvae (Figs. 2,9).

While cWnt is important for gut development, ncWnt may contribute to the wide blastopore phenotype in the *Dvl* miRNATP or *Dvl* mRNA-injected embryos. In *Xenopus*, manipulations of *Dvl* through injections of dominant-negative mutant Xdsh (Xdd1), with an internal deletion of the conserved PDZ domain, led to disruptions of ncWnt/PCP genes and resulted in failure of blastopore to close (Sokol, 1996; Ewald et al., 2004). Also in *Xenopus,* overexpression of a core PCP gene, *Strabismus* (*Stbm*), or deletion of a critical regulator of ncWnt/PCP, NEDD4L, both resulted in delayed blastopore and neural tube closing (Darken et al., 2002; Zhang et al., 2014).

Another indication that *Dvl* miRNATP-injected embryos have potential gut function defects is that they have much less alkaline phosphatase (AP) activity in the hindgut (Fig. 5E). In sea urchin larvae, AP is expressed only in differentiated endoderm (Whittaker, 1990; Kumano and Nishida, 1998; Drawbridge, 2003). These results indicate that both the morphology and potentially the function of the gut are negatively impacted by the increased Dvl, induced by blocking miRNA post-transcriptional suppression of *Dvl*.

To reveal the molecular mechanism of *Dvl* miRNATP or *Dvl* mRNA-induced gut phenotypes, we assayed for changes in transcription factors important for endodermal specification (Fig. 6). Canonical Wnt/β-catenin signaling activates endodermal regulatory genes such as *Krl* and *Eve* to give rise to the foregut, midgut, and hindgut (Howard et al., 2001; Peter and Davidson, 2010; Peter and Davidson, 2011). We observed a significant decrease in spatial expression domain of *Krl* and a significant increase in the expression domain of *Eve* in *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to the controls (Fig. 6C-F). These results provided independent evidence that increased Dvl led to expression changes in *Krl* and *Eve* (Fig. 6). *Krl* knockdown in the sea urchin blocks endoderm differentiation and results in failed gastrulation (Howard et al., 2001; Yamazaki et al., 2008). Thus, a moderate decrease in *Krl* expression domain may impact endoderm differentiation and gastrulation. *Eve* is directly activated by Wnt/β-catenin, Hox11/13b, and Eve itself (Peter and Davidson, 2011). In the intermediate germ cricket, *Gryllus bimaculatus, Krl* RNAi resulted in elimination of some Eve-regulated segmental stripes, suggesting that Krl is involved in the formation of those stripes by regulating *Eve* (Mito et al., 2006). However, *Krl* morpholino knockdown in the sea urchin indicated no effect on *Eve* expression in the early blastula stage (18 hpf). Conversely, *Eve* morpholino knockdown had insignificant effect on *Krl* expression, indicating that they are not likely to regulate each other (Peter and Davidson, 2010). Nonetheless, the decreased expression domain of *Krl* and the increased *Eve* expression domain induced by *Dvl* miRNATPs or *Dvl* mRNA overexpression may contribute to the gut morphology and developmental defects we observed.

Blockage of miRNA suppression of *Dvl* isoforms resulted in dose-dependent defects of skeletal spicules and PMC patterning (Fig. 7). We also observed that *Vegf3* expression is decreased 2-fold and its spatial expression domain is decreased in the *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to the control (Fig. 8A,C). *Dvl* mRNA-induced gene expression changes and PMC defects are likely to be specific, since a lower dosage of *Dvl* mRNA-injected embryos did not result in these changes (Fig. S2B). However, we cannot distinguish direct versus indirect effects of *Dvl* mRNA overexpression-induced phenotypes. PMCs undergo a series of directed movements where their patterning is in part in response to the Vegf signaling pathway (Duloquin et al., 2007; Adomako-Ankomah and Ettensohn, 2013). PMCs express VegfR10 receptor that receives its ligand Vegf3 expressed in the ectoderm that guides the PMCs to migrate anteriorly, as well as providing differentiation cues to the PMCs. Vegf3 also regulates the expression of many

biomineralization genes in the PMC gene regulatory network (Adomako-Ankomah and Ettensohn, 2013). A loss of VegfR10 receptor or Vegf3 result in PMCs that failed to pattern and did not secrete a skeleton. We did not detect expression changes of *VegfR10* in *Dvl* miRNATP or *Dvl* mRNA-injected embryos (Fig. 8D). The decrease in *Vegf3* level and expression domain may in part explain the *Dvl* miRNATP and *Dvl* mRNA-induced skeletal and patterning defects of the PMCs.

Another potential explanation of the PMC patterning defect in *Dvl* miRNATP-injected embryos may be due to defects in PMC motility. Since ncWnt mediates cell polarity and actin polymerization, increased Dvl may misregulate cell motility mediators, resulting in defective PMC motility. We found that Dvl protein is expressed in the PMCs, suggesting that Dvl may activate the ncWnt pathways or Wnt-independent functions in mediating cell motility of PMCs (Fig. 7G). To further investigate this, we tested the transcript levels of *RhoA*, *Rac1*, and *Cdc42* downstream of the ncWnt/PCP pathways in *Dvl* miRNATP-injected and control embryos. We found that *Rac1* transcript levels was elevated in *Dvl* miRNATP-injected embryos by almost a four-fold (Fig. 4A). Since Rac1 regulates actin polymerization (Chung et al., 2000), we used *mCherry-LifeAct* reporter, which encodes a 17 amino acid peptide that binds to filamentous actin (Riedl et al., 2008), to examine the actin dynamics in the early embryo. Results indicated that more punctate actin structures are present in the *Dvl* miRNATP-injected presumptive PMCs compared to the control (Fig. 7H). In chicken embryo fibroblasts, overexpression of Rac proteins induced disassembly in existing F-actin-containing stress fibers, consisting of long, highly branched bundled actin-rich protrusions that resulted in dramatic changes in cell morphology (Albertinazzi et al., 1999). The punctate actin structures in *Dvl* miRNATP-injected embryos may indicate defective actin polymerization, contributing to PMCs' inability to migrate properly.

These PMC patterning defects are not likely to be acting on the cWnt signaling, since our previous study indicated that removing miRNA suppression of *β-catenin* had no impact on PMC patterning (Stepicheva et al., 2015). In addition, the overexpression of the *LvDvl/SpDvl4a/4b* resulted in significantly shorter DVCs and PMC patterning defects (Fig. 7B,D). These results indicated that the skeletal and PMC defects observed in the *Dvl* miRNATP-injected embryos are likely due to the increased *SpDvl4a/4b* isoform. The molecular mechanism of how Dvl4a/4b regulates skeletogenesis remains unclear.

An unexpected and interesting finding we observed is that *Dvl* miRNATP, *LvDvl/SpDvl4a/4*b, or *LvDvl*/SpDvl5a*-injected embryos all had swimming and ciliary defects (Fig. 9 and Movies 1-8). Sea urchins have motile monocilia on their ectodermal cells that allow the embryos and larvae to swim with coordinated ciliary beating and feed (Kinukawa and Vacquier, 2007; Mizuno et al., 2017). A previous study indicated that Dvl-DEP overexpression resulted in embryos that failed to swim (Byrum et al., 2009). The swimming defect observed in *Dvl* miRNATP or *Dvl* mRNA-injected embryos may result from improper ciliary coordination, where the direction of ciliary movement depends on the orientation of the basal body, which is primarily determined by the PCP pathway (Marshall and Kintner, 2008; Kunimoto et al., 2012; Mizuno et al., 2017). Dvl in *Xenopus* is found at the base of the cilia and is essential for apical positioning of the basal bodies and positioning of cilia (Park et al., 2008; Mlodzik, 2016). Knockdown of *Dvl1* and *Dvl3* in *Xenopus* and knockdown of *Stbm* in *Clytia* resulted in phenotypes of fewer and shorter cilia (Park et al., 2008; Momose et al., 2012), indicating that Dvl plays an evolutionarily conserved role in proper cilia formation. PCP signaling plays a critical role in ciliogenesis; however, the Wnt/β-catenin also regulates ciliogenesis via *foxj1a* expression in zebrafish Kupffer's vesicle. Downregulation of cWnt/β-catenin leads to depletion of Lef1 and Tcf7, inhibiting *foxj1a* transcription. This results in shorter and fewer cilia with loss of cilia motility that can be rescued with overexpression *foxj1a* (Lin and Xu, 2009; Caron et al., 2012; Zhu et al., 2015). Dvl has also been shown to interact with DCDC2, which binds to tubulin to enhance microtubule polymerization and localizes to the ciliary axoneme (Schueler et al., 2015). Wnt inhibitor treatment rescued the effects of *dcdc2* knockdown induced ciliopathy in renal spheroid cells and zebrafish embryos, suggesting cWnt signaling in the process of ciliation (Schueler et al., 2015). Thus, the ciliary defect observed in *Dvl* miRNATP-injected embryos may result from both ncWnt/PCP and cWnt pathway perturbations.

Conclusions

This study demonstrated that miRNAs suppress various *Dvl* isoforms in the sea urchin embryo. We have shown that the Wnt signaling pathway components are controlled post-transcriptionally by miRNAs (Fig. 10). Importantly, the level of increased Dvl protein, induced by blocking miRNA suppression of *Dvl* isoforms, is sufficient to induce various defects in the gut, spiculogenesis, and ciliogenesis during development.

MATERIALS AND METHODS

Animals

Adult *Strongylocentrotus purpuratus* were obtained from Point Loma Marine Invertebrate Lab, Lakeside, California. Adult males and females were given 0.5 M KCl intracoelomic injections for obtaining sperm and eggs. Filtered natural sea water (collected from Indian River Inlet; University of Delaware) or artificial seawater was used for embryo cultures incubated at 15°C.

Whole mount *in situ* hybridization

Krl, *Eve*, *Wnt5* and *Vegf3* were cloned into Blunt-TOPO vector (Thermo Fisher Scientific, Grand Island, NY) (Stepicheva et al., 2015; Stepicheva and Song, 2015). *Krl*, *Eve*, *Wnt5*, and *Vegf3* were linearized with restriction enzymes *EcoR*I, *EcoR*I, *Not*I, and *BamH*I, respectively. All were *in vitro* transcribed with Sp6 RNA polymerase, except for *Vegf3* which was *in vitro* transcribed with T7 RNA polymerase using the DIG RNA Labeling Kit (Sigma Aldrich, St. Louis, MO). *Dvl*5a and *Dvl4b* 3'UTRs were synthesized as gBlock DNA fragments from IDTdna.com (Integrated DNA Technologies, Inc., Coralville, Iowa) and cloned into Blunt-TOPO vectors. *Dvl4a* 3'UTR was PCR amplified with Dvl4a For 5' GGCCTCGAGAGTGCGGAAATT TTGAAATCAT 3' and Dvl4a Rev 5' GGCGCGGCCGCCAATAATGACCGCTCAATTTTT 3' and cloned into Blunt-TOPO vectors. *Xho*I and *Not*I cut sites are underlined. All *Dvl* probes are linearized with *Not*I and *in vitro* transcribed with Sp6 RNA polymerase. All *Dvl in situs* were examined in between 180 to 300 embryos for each stage in 3-8 replicates.

Cloning of luciferase reporter constructs

For generating *Dvl* 3'UTR luciferase reporter constructs, *Dvl5a* and *Dvl4b* 3'UTR was synthesized as a gBlock DNA fragment (Integrated DNA Technologies, Inc., Coralville, Iowa). *Dvl4a* 3'UTR in Blunt-TOPO was subcloned into the *Renilla* luciferase construct. All seed sequences were mutated at positions 3 and 5 within the miRNA seed sequences (Gregory et al., 2008; Stepicheva et al., 2015). *Sp*miRDeep2-30364 was modified from 5' GUGCAAU 3' to 5' GUACGAU 3'; *spu*-miR-153* was modified from 5' AAAAAT3' to 5' AAGAGT 3'; *Sp*miR2002 was modified from 5' CTGAAAT 3' to 5' CTTACAT 3'; *Sp*miR2007 was modified from 5' CTGAAAT 3' to 5' CTTACAT 3'; and *Sp*miR200 was modified from 5' GTATGAT 3' to 5'

GTGTAA 3'. Mutated nucleotides are underlined. All positive clones were identified by DNA sequencing (Genewiz, Inc., South Plainfield, NJ). Firefly luciferase was used as loading control as previously described (Stepicheva et al., 2015). Luciferase constructs containing the *Dvl* 3'UTR were linearized with *EcoR*I and *in vitro* transcribed using mMessage machine kit with either T7 (for *Dvl* mRNAs) or Sp6 (for firefly luciferase mRNAs) RNA polymerases (Ambion, Carlsbad, CA) according to the manufacturer's instructions. mRNAs were purified by using Macherey-Nagel Nucleospin® RNA Clean-up kit (Macherey-Nagel, Bethlehem, PA) according to manufacturer's instructions. *In vitro* transcribed mRNAs were loaded onto the Millipore spin columns (Millipore, Billerica, MA) to further clean the mRNAs prior to injections.

Cloning of *LvDvl*

To identify the contribution of *Dvl* isoforms to *Dvl* miRNATP phenotypes, we injected mRNA coding for two *Dvl* isoforms into zygotes. LvDvl protein is 93% identical to SpDvl4a/4b. The sea urchin species *L. variegatus* diverged from *S. purpuratus* about 50 million years ago. These two sister species of sea urchin have many similarities at the genomic as well as at the gene regulatory levels (Crain and Bushman, 1983; Ettensohn et al., 2004). Because of the relatively close evolutionary distance of *L. variegatus* to *S. purpuratus* and their high protein identify especially at the C-terminus of the protein, we used the available LvDvl clone (Weitzel et al., 2004) to represent the SpDvl4a/4b protein (Fig. 1). Because of the difficulty of cloning the *SpDvl5a* isoform, we used PCR to replace the sequence coding for the amino acids "YFDDSVSVTLL" in LvDvl to a sequence coding for the highly conserved "CEFFVDVM" amino acid sequence. Since the goal was to test if the overexpression of SpDvl isoforms would induce phenotypes similar to Dvl miRNATPs-injected embryos, we did not add the endogenous 3'UTRs to these protein coding sequences. Thus, LvDvl was used to mimic SpDvl4a/4b (denoted as LvDvl/SpDvl4a/4b) and LvDvl+ CEFFVDVM was used to mimic SpDvl5a (denoted as LvDvl*/SpDvl5a). To obtain*LvDvl**, the *LvDvl:Flag* construct was modified using Q5® Site-Directed Mutagenesis Kit (NEB, Ipswich, MA). Primers used to change the amino acids "YFDDSVSVTLL" on the C-terminal of LvDvl:Flag to "CEFFVDVM" are: For 5' GTCGATGTCATGTGAATTCAAGGCCTCTCG 3' and Rev 5' AAAGAACTCACAAGGGTTTCCCATAGCCAT3'. The Flag tag region was deleted after mutation. Plasmids were sequenced to verify that correct mutations were introduced. These

plasmids were *in vitro* transcribed and mRNA coding for the two Dvl isoforms were injected into zygotes.

Microinjections

Microinjections were performed as previously described with modifications (Cheers and Ettensohn, 2004; Stepicheva and Song, 2014). 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. The stock injection solution contained 150, 250 or 300µM of each *Dvl* miRNA TP or 450, 750 or 900 µM of control TP 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, 2015). For *Dvl* miRNATPs, we blasted each of the *Dvl* miRNATPs against the annotated sea urchin genome and identified them to be complementary to only the *Dvl* genes. Each *Dvl* miRNATP is complementary to the miR's seed sequence (6 bp) and the unique *Dvl* 3' UTR sequences flanking the miR seed sequence. Thus, each *Dvl* miRNATP is uniquely targeting that specific miR seed site within the particular *Dvl* isoform. We microinjected zygotes a cocktail of *Dvl* miRNATPs corresponding to miR153* at +230 position of *Dvl5a* (5' CTAGCATTTTTTTTTTGAAAGCTGT 3'), to miR-2002 and miR-Deep2-35240 at positions +1198 to +1224 position of the *Dvl4a* (5' TTGCACTTCATGATGCATAGAATAC 3') and to miR-200 at position +219 position of the *Dvl4b* (5' ATTAATATCATACCCAAAACATATT 3') at 300µM each or 900µM of the negative control (5' CCTCTTACCTCAGTTACAATTTATA 3') that corrects a splicing error of the human *β-globin* pre-mRNA (Kang et al., 1998).

To examine the actin dynamics in control TP and *Dvl* miRNATP, we microinjected the newly fertilized eggs with 500 ng of mCherry-LifeAct reporter mRNA, 0.5 µl 100% glycerol, and 0.5 µl of Texas Red in a 2.5 µl injection solution (Riedl et al., 2008; Stepicheva et al., 2017).

To identify the impact of individual Dvl isoforms, *LvDvl/SpDvl4a/4b* mRNA, encoding the sea urchin-specific form of Dvl ending with 'YFDDSSVTLL', and *LvDvl*/SpDvl5a* mRNA modified to contain the conserved DEP-C domain ('CEFFVDVM), were injected into newly fertilized eggs. Stock injection solutions of 2.5 µl consisted of 0.5 µl of 100% glycerol, 2.5 µg of *LvDvl/SpDvl4a/4b* and *LvDvl*/SpDvl5a* or combinations of both (each with 1.25 µg of mRNA)

with 500 ng of mCherry mRNA. The control solution contained 500 ng of mCherry without *Dvl* mRNA.

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 blastula stage were collected at 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 (FF) signal and the *Renilla* luciferase (Rluc) 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. The values of Rluc containing mutated seed sites were normalized to the corresponding Rluc containing wild type miRNA target sites.

Real time, quantitative PCR (QPCR)

100 *Dvl* miRNATP or control TP-injected blastulae were collected. Total RNA was extracted by using the Macherey-Nagel Nucleospin® RNA Clean-up XS kit (Macherey-Nagel, Bethlehem, PA) according to manufacturer's instructions. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). QPCR was performed using 2.5 or 7.5 embryo equivalent for each reaction with the Fast SYBER or PowerUp Green PCR Master Mix (Thermo Fisher Scientific, Grand Island, NY) in the QuantStudio 6 Real-Time PCR cycler system (Thermo Fisher Scientific, Grand Island, NY). Results were normalized to the mRNA expression of the housekeeping gene ubiquitin and shown as fold changes compared to control embryos that were injected with the control morpholino using the ΔΔCt method as previously described (Stepicheva et al., 2015). Primer sequences were designed using the Primer 3 Program (Rozen and Skaletsky, 2000) and are listed in Table S1. 3-6 biological replicates were conducted. Statistical significance was calculated using the 2 tailed unpaired Student T-test (Goni *et al.*, 2009).

Detection of endogenous alkaline phosphatase activity

The endogenous alkaline phosphatase was used to assay for differentiated endoderm of the larvae as previously described (Hinman and Degnan, 1998; Drawbridge, 2003; Annunziata et al., 2013; Stepicheva et al., 2015). Since we observed a significant difference in the width of the midgut of control TP and *Dvl* miRNATP-injected embryos, we used this assay to examine the differentiated endoderm. Five day old larvae were fixed in MOPS-paraformaldehyde based 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 with alkaline phosphatase buffer three times (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20), followed by treatment with the staining solution (0.1 M Tris pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 1 mM Levamisole, 10% Dimethylformamide, 45 µl of 75 mg/mL NBT and 35 µl of 50 mg/mL BCIP per 10 ml of solution). Once the color development was observed, the staining was terminated 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 connected to a Zeiss Observer Z1 microscope.

Immunofluorescence

Gastrula (48 hpf) and larval stage (120 hpf) embryos were fixed in 4% paraformaldehyde (20% stock; EMS, Hatfield, PA) in artificial sea water for overnight at 4°C. 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 or ID5 antibody (McClay et al., 1983) at 1:50 overnight at 4°C. Embryos were washed three times for 15 minutes each with PBS-Tween followed by goat anti-mouse Alexa 488 (Thermo Fisher Scientific, Grand Island, NY) conjugated secondary antibody at 1:300. After three more PBS-Tween washes, embryos were incubated with Hoechst dye at 1:1000 for 5 minutes, followed by two more PBS-Tween washes.

To examine the level of Dvl protein changes in *Dvl* miRNATP-injected embryos, we injected the control TP and the *Dvl* miRNATP with Texas Red dextran. These 32-cell stage embryos (4-5 hpf) were fixed in 4% paraformaldehyde (20% stock; EMS, Hatfield, PA) in phosphate buffered saline (PBS) for 20 minutes, post fixed with 100% ice cold methanol for 10 minutes, and washed with several washes of 1x PBS (Peng and Wikramanayake, 2013). Injected embryos were then incubated with primary rabbit anti-SUDdsh-C antibody (1:400 overnight at 4°C). Following this

incubation, the embryos were washed with PBS-Tween with 0.01% BSA three times and then incubated with goat anti-rabbit Cy3 (Thermo Fisher Scientific, Grand Island, NY) conjugated secondary antibody at 1:300. After three more PBS-Tween washes, embryos were incubated with Hoechst dye at 1:1000 for 2 minutes, followed by three more PBS-Tween washes.

Larval stage (120 hpf) embryos were fixed in 4% paraformaldehyde (20% stock; EMS, Hatfield, PA) in artificial sea water for overnight at 4°C. Four 10 minute PBS-Tween washes were performed, followed by 1 hour of blocking with 4% sheep serum (Sigma Aldrich, St. Louis, MO). Double immunofluorescence was performed with primary antibodies overnight at 4°C, using the following the dilutions: anti-SUDdsh-C antibody at 1:400 and β-tubulin (Developmental Studies Hybridoma Bank, catalog # E7) at 1:10,000. Embryos were washed three times for 15 minutes each with PBS-Tween followed by 1 hour room temperature incubation with goat anti-mouse Alexa 647 (Thermo Fisher Scientific, Grand Island, NY) and goat anti-rabbit Cy3 at 1:300, sequentially. After three more PBS-Tween washes, embryos were incubated with Hoechst dye at 1:1000 for 2 minutes, followed by two more PBS-Tween washes. These immunolabled embryos in PBS-Tween were mounted onto protamine sulfate-coated slides and imaged using a LSM 780 scanning confocal miscroscope (Zeiss Incorporation, Thorwood, NY) and data analysis was performed using the Zen software (Zeiss Incorporation, Thorwood, NY).

Dvl protein quantitation

To measure semi-quantitatively the amount of Dvl in control TP and *Dvl* miRNATP-treated embryos, confocal Z-stack images of 32-cell embryos were collected. The maximum projections of Z-stacks of each embryo were collected. The amount of fluorescence pixels were quantitated with Metamorph (Molecular Devices, LLC, San Jose, CA). The level of Dvl of *Dvl* miRNATP-injected embryos was normalized to the control TP-injected embryos.

Phenotyping

To assay for the DVC length, we took a Z-stack of DIC and 1D5 immunolabeled images with Zeiss Observer Z1 microscope. Normal PMC phenotypes have these criteria: 1) sub-equatorial ring formation of PMCs at the vegetal pole of the embryo, 2) anterior migration of the PMCs, and 3) syncytial cables formed among the PMCs. Normal gut phenotypes fulfill these criteria: 1) fully extended gut tube at gastrula stage and 2) Endo1 antibody stain for mid- and hindgut epithelia. To

assay for ciliary defects in live embryos, we acquired images using the time-lapse function at the LSM 780 scanning confocal miscroscope (Zeiss Incorporation, Thorwood, NY).

Statistical analysis

For spicule length, gut/blastopore widths, and expression domains, Student T-test was used to assess the statistical significance between control and experimental groups. For patterning of PMCs, the Cochran-Mantel Haenszel test was used to assess the statistical significance of the percent of normal embryos of control TP-injected embryos compared to the *Dvl* miRNATP-injected embryos. In all graphs, standard error bars were graphed.

Competing interests

The authors declare no competing financial interests.

Author contributions

NFS, NAS, and JLS conducted all the experiments. SAMZ cloned the *Dvl* 3'UTRs into luciferase constructs. LW, WW, and AW identified the four isoforms of *Dvl* and supplied the Dvl antibody. NFS and JLS wrote the manuscript.

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Figures

Figure 1. Four isoforms of *Dvl* with shared protein domains and three unique 3'UTRs. (A) All *SpDvl* isoforms contain the conserved DIX, PDZ, and DEP domains. Partial multiple alignment of Dvl C-terminus protein sequences from various species indicated that *SpDvl4a, SpDvl4b, SpDvl1* and *SpDvl5a* have different amino acids in the last exon. Only *Sp*Dvl5a contains the conserved DEP-C domain highlighted in yellow. (B) *SpDvl1* isoform lacks one exon at the end of its coding region and has identical 3'UTR as *Dvl4a*. *SpDvl5a*, *SpDvl4a/Dvl1*, and *SpDvl4b* have unique 3'UTRs. The potential miRNA regulatory sites within *SpDvl* 3'UTRs were identified bioinformatically, based on the inverse complementary seed sequences that have a perfect match to the corresponding miRNA. *Dvl* miRNATPs used to block miRNA binding of the *SpDvl* transcripts are shown in blue.+1 is the first basepair of the 3'UTR.
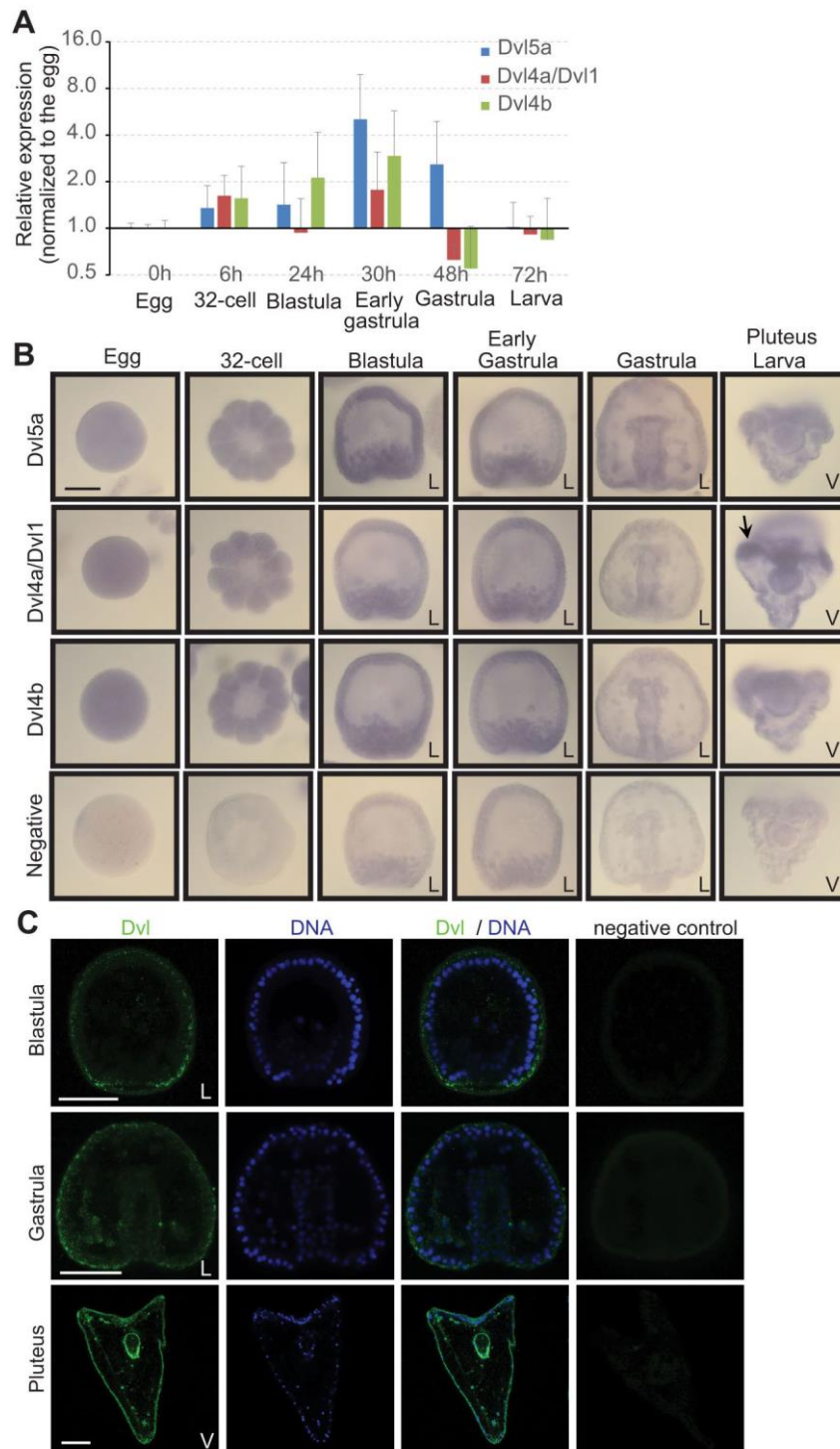
Figure 2. *Dvl* isoforms are expressed during early embryogenesis. (A) 200 eggs or embryos were collected and subjected to QPCR analysis. Genes in each of the developmental stage were not significantly different than the egg stage (p>0.1; Student T-test). (B) Embryos were hybridized

with *Dvl* mRNA *in situ* probes. At the larval stage, distinct ciliary band staining (arrow) is observed in *Dvl4a/Dvl1* labeled embryos. Scale bar is 40 µm. (C) Dvl proteins are expressed throughout development. From the blastula to gastrula stages, Dvl is enriched in the posterior end of the embryos. Dvl is also expressed in the gut and PMCs of gastrulae. In the larval stage, Dvl protein is enriched in the PMCs, endoderm, pyloric sphincter and ciliary band. Negative controls are embryos incubated with only the secondary antibody. L=lateral view. V=ventral view with ventral toward the top. Scale bar is 50 µm.
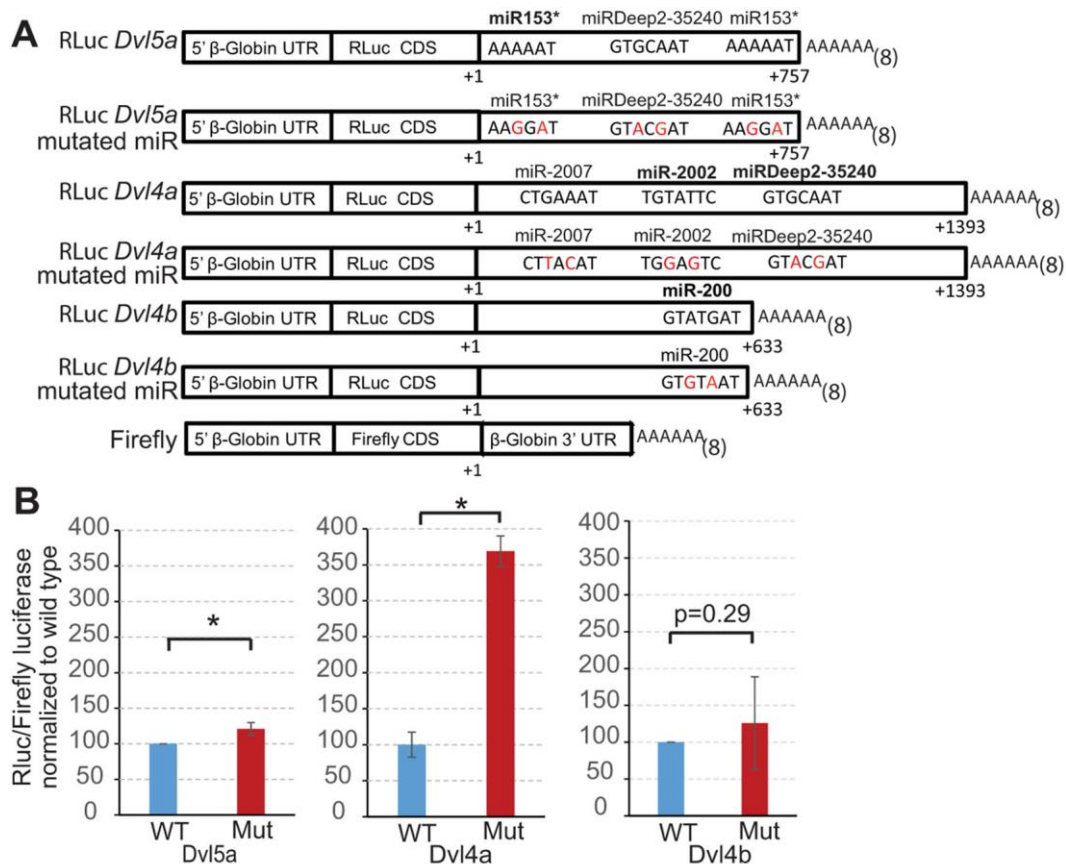
Figure 3. miRNAs directly suppress *Dvl5a* and *Dvl4a*. (A) Unique 3'UTRs of various *Dvl* isoforms were cloned downstream of *Renilla* luciferase (Rluc). Site-directed mutagenesis was used to mutate select seed sequences of predicted miRNAs regulatory binding sites. (B) *Firefly* (control) and *Rluc* mRNAs were injected into newly fertilized eggs. Dual luciferase assays were conducted with mesenchyme blastulae at 24hpf. *Dvl5a* and *Dvl4a* are directly suppressed by miRNAs. *Dvl* miRNATPs were designed against specific miRNA binding sites shown in bold (miR153* of *Dvl5a*, miR2002 and miRDeep2-30364 of *Dvl4a*, and miR200 of *Dvl4b*). *p<0.05.
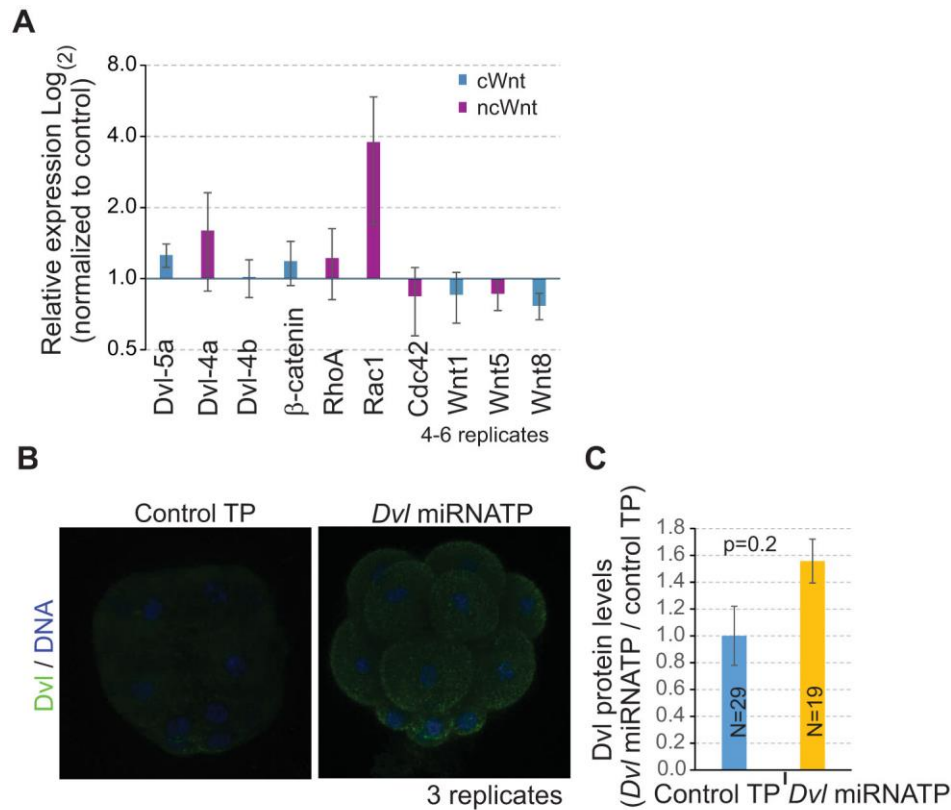
Figure 4. Removing miRNA suppression of *Dvl* leads to increased *Rac1* expression and Dvl protein. (A) QPCR was used to measure transcriptional changes of genes in Wnt signaling pathways in control TP and *Dvl* miRNATP-injected blastulae (300µM of each TP) at 24hpf. *Rac1* increased by almost four-fold. Designation of cWnt or ncWnt components are based on previous studies and this study (Range et al., 2013; Cui et al., 2014; Minegishi et al., 2017) (B) 32-cell stage embryos were immunolabeled with Pan-Dvl antibody. (C) *Dvl* miRNATP-injected embryos had an overall increase of 1.5-fold of Dvl compared to control TP at 32-cell stage. Confocal Z-stack images were taken with scanning confocal microscopy. Fluorescence signals of maximum projections were quantified with Metamorph.
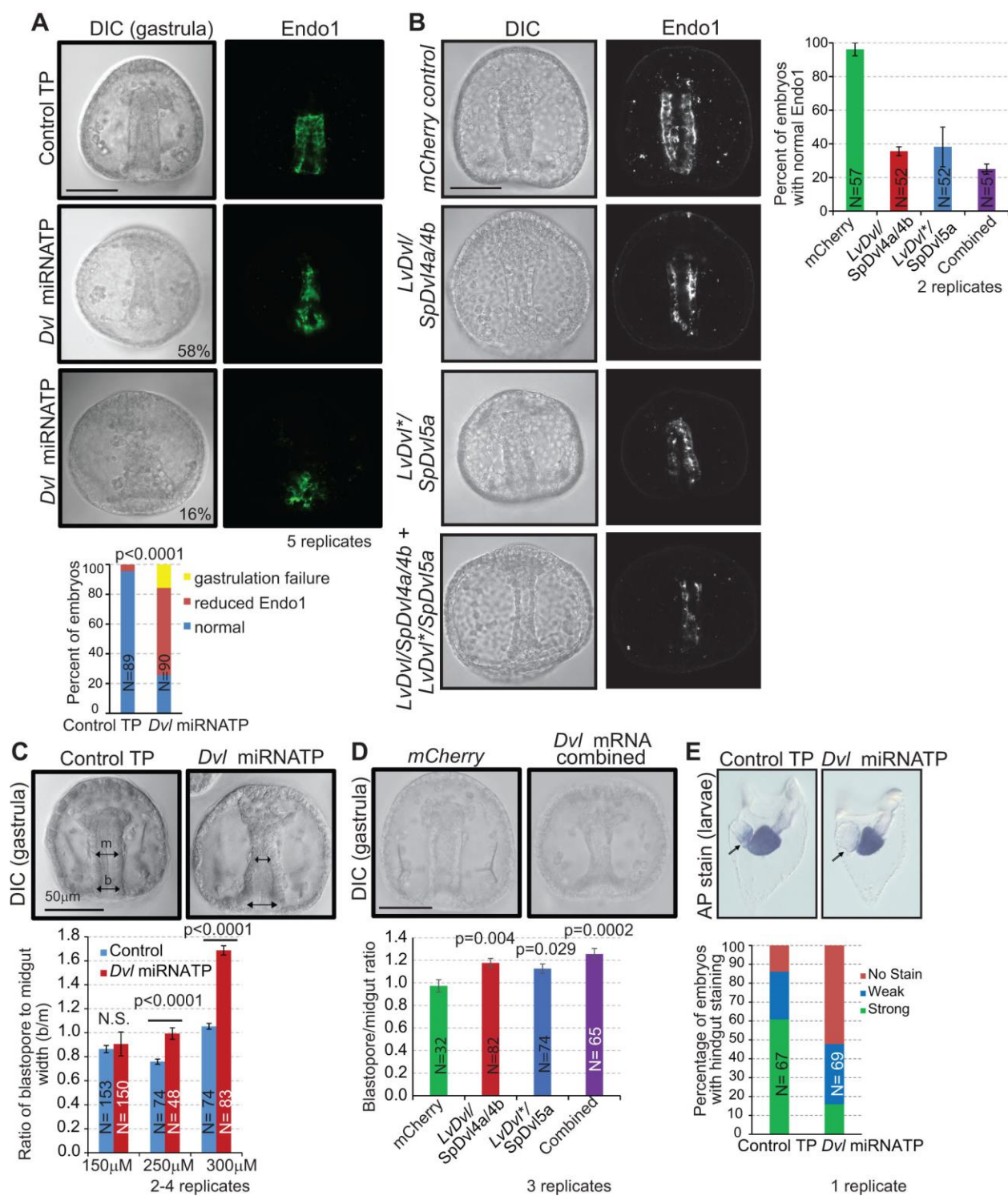
Figure 5. Removal of miRNA suppression of all *Dvl* isoforms or *Dvl* isoform overexpression results in aberrant gut morphology. (A-B) Gastrulae were immunolabeled with Endo1. *Dvl* miRNATP-injected embryos have either less Endo1 expression or gastrulation failure. *LvDvl/SpDvl4a/4b*, *LvDvl*/SpDvl5a*, and *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*-injected embryos are either normal or exhibited less Endo1 expression. (C-D) In the embryos that had properly gastrulated, the ratio of the width of the blastopore (b) to the midgut (m) were significantly higher in *Dvl* miRNATP or *Dvl* mRNA (*LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*)-injected gastrulae compared to the controls. (E) Control TP-injected embryos have more intense alkaline phosphate (AP) activity in the hindgut compared to the *Dvl* miRNATP-treated embryos (arrows).
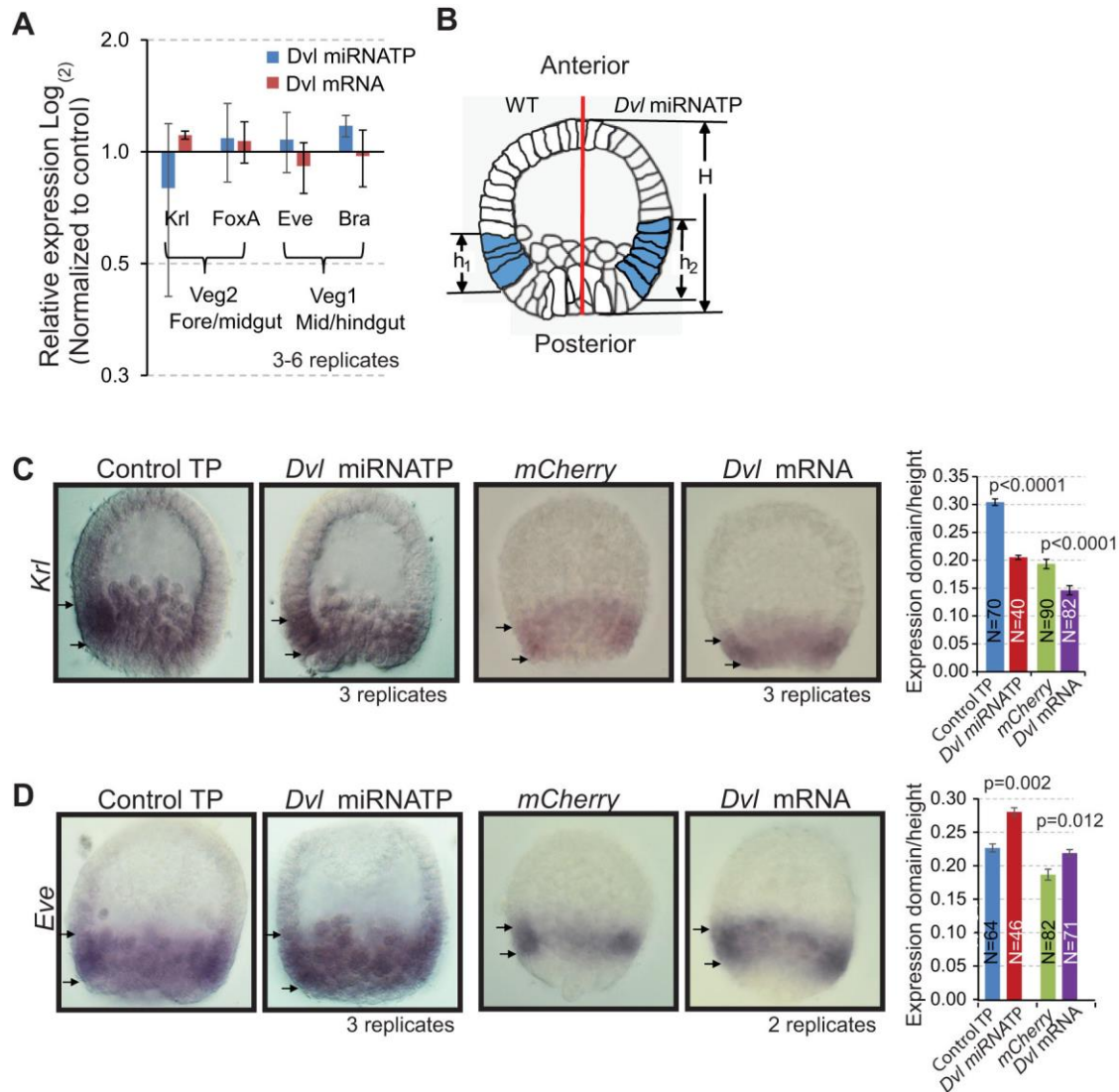
Figure 6. Removal of miRNA suppression of *Dvl* isoforms or *Dvl* mRNA overexpression results in decreased *Krl* spatial expression and expanded *Eve* spatial expression. (A) QPCR was used to measure transcriptional changes of Wnt-responsive endodermal genes in control TP and *Dvl* miRNATP-injected embryos at 24hpf. Overall transcript levels of *Eve*, *Bra*, *Krl* and *FoxA* did not change in *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to the control embryos. (B) The expression domain is measured by taking the ratio of h_1 or h_2/H. (C) The expression domain of *Krl* is significantly decreased in *Dvl* miRNATP or *Dvl* mRNA-injected embryos. (D) The spatial expression domain of *Eve* is significantly expanded in *Dvl* miRNATP or *Dvl* mRNA-injected embryos. L=lateral view.
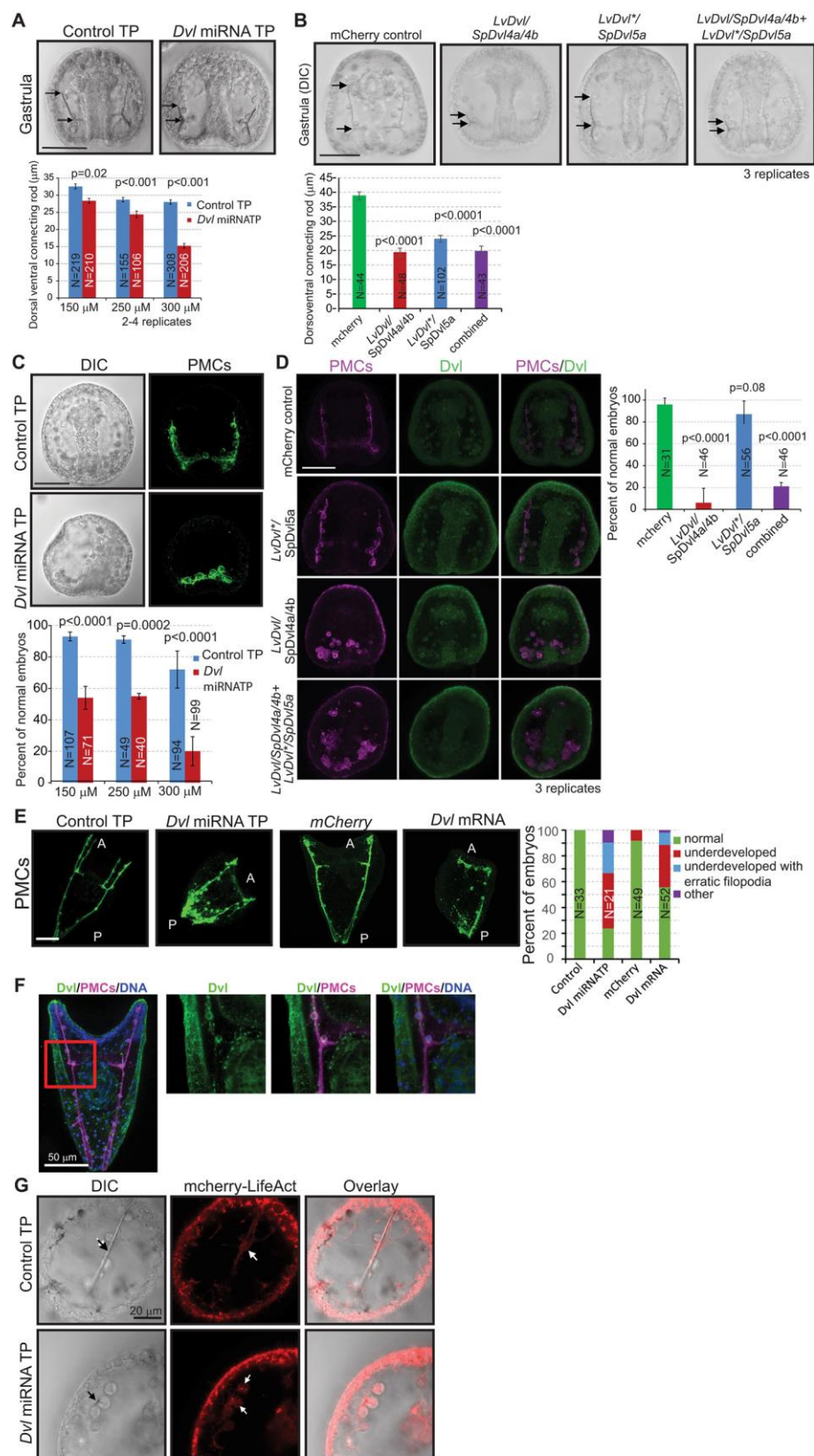
Figure 7. Blockage of miRNA suppression of *Dvl* isoforms or *Dvl* mRNA overexpression results in dose-dependent defects of spicule length and patterning of the PMCs. (A) *Dvl* miRNATP-injected embryos displayed dose-dependent, shortened dorsoventral connecting rods (DVCs) in comparison to the control embryos. (B) *LvDvl/SpDvl4a/4b*, *LvDvl*/SpDvl5a* and *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*-injected embryos had shorter DVCs in comparison to *mCherry*-injected controls. (C) PMCs were immunostained with PMC-specific marker 1D5. *Dvl* miRNATP-injected embryos displayed PMC clustering and lack of anterior migration in comparison to the control. (D) *LvDvl/SpDvl4a/4b* and *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*-injected embryos exhibited scattered and severe clustering of PMCs. (E) PMC defects persist into larval stage (5 days post fertilization; dpf), displaying PMC clustering and branched filopodia for both *Dvl* miRNATP and *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*-injected embryos. A=Anterior, P=Posterior. Scale is 50 µm. (F) *Dvl* is expressed in PMCs and PMC connections. Embryos were collected 5 dpf and immunolabeled with Pan-Dvl antibody (green), 1D5 against PMCs (magenta), and Hoechst dye against DNA (blue). Inset boxed in red is enlarged to show Dvl in PMCs. (G) Embryos were co-injected with *mCherry-LifeAct* to assay for actin in live gastrulae using scanning confocal microscopy. *Dvl* miRNATP-injected embryos have punctate actin localization (white arrows) in presumptive PMCs clustered at the tri-radiate spicules (black arrow), whereas the presumptive PMCs along the long skeletal spicule in the control TP-injected embryos have more even actin distribution within these cells.
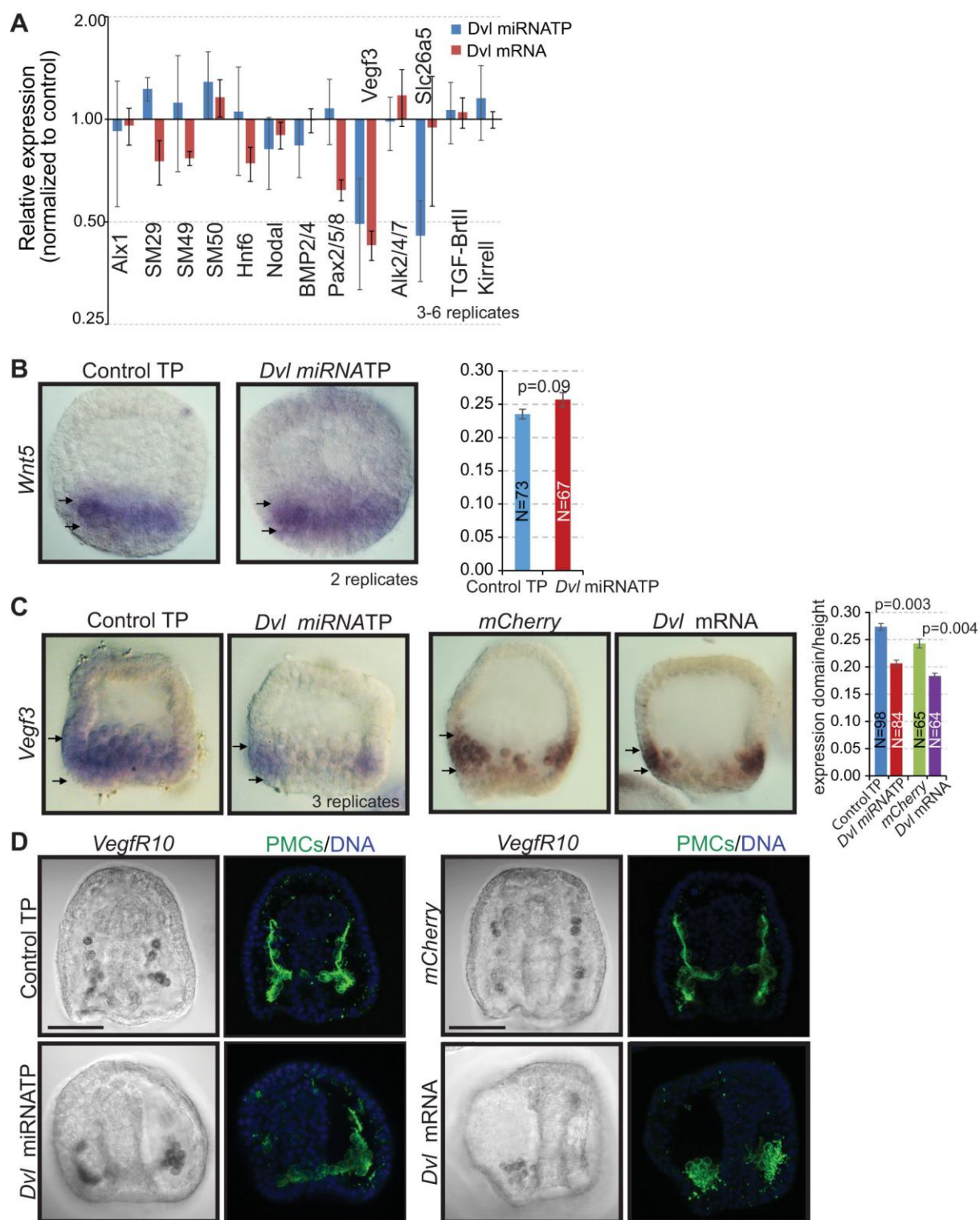
Figure 8. Removal of miRNA suppression of *Dvl* or *Dvl* overexpression results in decreased *Vegf3*. (A) QPCR was used to measure transcriptional changes of genes involved in PMC development and function in *Dvl* miRNATP, *Dvl* mRNA (*LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a*)-injected embryos and controls at 24hpf. Results indicate that *Vegf3* have 2-fold less transcripts in *Dvl* miRNATP or *Dvl* mRNA-injected embryo compared to the controls. (B) *Wnt5* has a slightly expanded spatial expression domain compared to the controls at 15hpf. (C) *Vegf3* spatial expression domain is significantly decreased in the *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to their corresponding controls. (D) *VegfR10* expression is not altered in the *Dvl* miRNATP or *Dvl* mRNA-injected embryos compared to controls. 21-32 embryos in 1 replicate.

A

Dvl | Tubulin/DNA | Dvl/Tubulin/DNA

Control TP

Anterior — CB — Posterior

50 µm

Dvl miRNA TP

Anterior — CB — Posterior

B

Control TP | *Dvl* miRNATP

Anterior | Anterior

β-tubulin

CB | CB

50 µm

Posterior | Posterior

3 replicates

C

Dvl/Tubulin/DNA

Control TP — Anterior — CB

Dvl miRNATP — Anterior — CB

Control TP

Dvl miRNATP

Percent of embryos with normal cilia

N=17 | N=18

Control TP | *Dvl* miRNATP

D

mCherry | *LvDvl/SpDvl4a/4b* | *LvDvl*/SpDvl5a* | *LvDvl/SpDvl4a/4b+ LvDvl*/SpDvl5a*

DIC

Anterior | Anterior | Anterior | Anterior

20 µm | N=8 | N=10 | N=9 | N=9

Figure 9. Removal of miRNA suppression of *Dvl* isoforms or *Dvl* overexpression results in shorter and aberrantly structured cilia. (A-C) Embryos were collected 5 dpf and immunolabeled with Pan-Dvl antibody (green), β-tubulin antibody (magenta), and Hoechst dye (blue) against DNA. (B) The epithelial cilia in the anterior larval body were imaged. *Dvl* miRNATP-injected embryos have kinks in body cilia compared to control embryos. (C) *Dvl* miRNATP-injected embryos had shorter cilia in bent orientation in the ciliary band compared to controls. CB=ciliary band. (D) Live imaging of *Dvl* mRNA-injected larvae indicated similar ciliary defects as *Dvl* miRNATP-injected larvae.
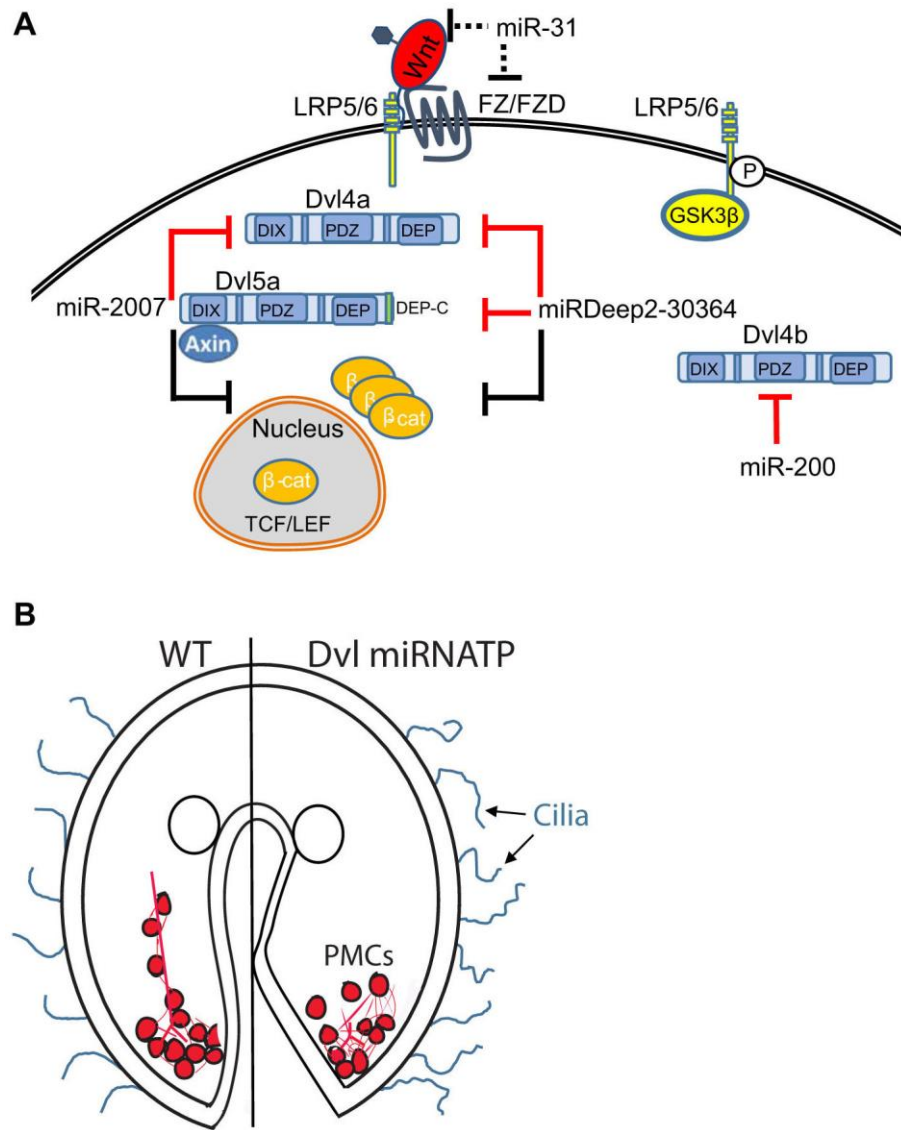
Figure 10. Post-transcriptional regulation of the Wnt signaling components by miRNAs. (A) Current knowledge of sea urchin miRNAs that regulate the Wnt signaling components (Stepicheva et al., 2015; Stepicheva and Song, 2015). (B) Removal of miRNA suppression of *Dvl* isoforms induces gut, PMC, and ciliary defects.

A.

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Dvl-5a      ATGGAGGAGACTAAGATTATATATCATATAGATGATGAAGACACTCCATATCTCGTAAAA
Dvl-1       ATGGAGGAGACTAAGATTATATATCATATAGATGATGAAGACACTCCATATCTCGTAAAA
Dvl4a-4b    ATGGAGGAGACTAAGATTATATATCATATAGATGATGAAGACACTCCATATCTCGTAAAA
            ************************************************************

Dvl-5a      TTACCAATTCCTGCCGCCGATGTAACTCTTGGAGATTTCAAGAATGTCCTCAATCGGCCG
Dvl-1       TTACCAATTCCTGCCGCCGATGTAACTCTTGGAGATTTCAAGAATGTCCTCAATCGGCCG
Dvl4a-4b    TTACCAATTCCTGCCGCCGATGTAACTCTTGGAGATTTCAAGAATGTCCTCAATCGGCCG
            ************************************************************

Dvl-5a      AACTACAAATTCTTCTTCAAATCGATGGACGATGATTTTGGAGTTGTAAAAGAAGAAATA
Dvl-1       AACTACAAATTCTTCTTCAAATCGATGGACGATGATTTTGGAGTTGTAAAAGAAGAAATA
Dvl4a-4b    AACTACAAATTCTTCTTCAAATCGATGGACGATGATTTTGGAGTTGTAAAAGAAGAAATA
            ************************************************************

Dvl-5a      GTTGATGATGACACCAAATTACCTTGTTTTAATGGCCGTGTTGTGTCATGGCTGGTGCCA
Dvl-1       GTTGATGATGACACCAAATTACCTTGTTTTAATGGCCGTGTTGTGTCATGGCTGGTGCCA
Dvl4a-4b    GTTGATGATGACACCAAATTACCTTGTTTTAATGGCCGTGTTGTGTCATGGCTGGTGCCA
            ************************************************************

Dvl-5a      GCAGAGGGCAGCACCAACGGGTCAGACACAGCGTCGGTCACGACAGATACGAGAGGAGAC
Dvl-1       GCAGAGGGCAGCACCAACGGGTCAGACACAGCGTCGGTCACGACAGATACGAGAGGAGAC
Dvl4a-4b    GCAGAGGGCAGCACCAACGGGTCAGACACAGCGTCGGTCACGACAGATACGAGAGGAGAC
            ************************************************************

Dvl-5a      TCGCAACTTCCGCCCGAGAGGACAGGTGGTATAGGAGACTCTAGACCACCTTCATTTCAT
Dvl-1       TCGCAACTTCCGCCCGAGAGGACAGGTGGTATAGGAGACTCTAGACCACCTTCATTTCAT
Dvl4a-4b    TCGCAACTTCCGCCCGAGAGGACAGGTGGTATAGGAGACTCTAGACCACCTTCATTTCAT
            ************************************************************

Dvl-5a      TCGAGTGGAGGAGGGGTCCAAGATAAATTCGACGACACAGACACATGTACAGAATCAGAG
Dvl-1       TCGAGTGGAGGAGGGGTCCAAGATAAATTCGACGACACAGACACATGTACAGAATCAGAG
Dvl4a-4b    TCGAGTGGAGGAGGGGTCCAAGATAAATTCGACGACACAGACACATGTACAGAATCAGAG
            ************************************************************

Dvl-5a      CGAAGCTCAAGAAGAGGTCACCATCGCATGAGGAGAGACAAGTACAATAACTTTTCAAGA
Dvl-1       CGAAGCTCAAGAAGAGGTCACCATCGCATGAGGAGAGACAAGTACAATAACTTTTCAAGA
Dvl4a-4b    CGAAGCTCAAGAAGAGGTCACCATCGCATGAGGAGAGACAAGTACAATAACTTTTCAAGA
            ************************************************************

Dvl-5a      AGCAAGAGGGACCATCACCATCGAAGCGGGCATGGATACGAGAGTTCGTCAACGTTGATG
Dvl-1       AGCAAGAGGGACCATCACCATCGAAGCGGGCATGGATACGAGAGTTCGTCAACGTTGATG
Dvl4a-4b    AGCAAGAGGGACCATCACCATCGAAGCGGGCATGGATACGAGAGTTCGTCAACGTTGATG
            ************************************************************

Dvl-5a      AGCAGCGACATTGACTCCACAAGCTGCTTTGAATCAACAGACGACGACAGCAGTAGATTC
Dvl-1       AGCAGCGACATTGACTCCACAAGCTGCTTTGAATCAACAGACGACGACAGCAGTAGATTC
Dvl4a-4b    AGCAGCGACATTGACTCCACAAGCTGCTTTGAATCAACAGACGACGACAGCAGTAGATTC
            ************************************************************

Dvl-5a      AGTAGTGCCACAGAAAGGAGTAGCTTAGCCAGGCCGATGAGGGGACCACCAAAGAACCGC
Dvl-1       AGTAGTGCCACAGAAAGGAGTAGCTTAGCCAGGCCGATGAGGGGACCACCAAAGAACCGC
Dvl4a-4b    AGTAGTGCCACAGAAAGGAGTAGCTTAGCCAGGCCGATGAGGGGACCACCAAAGAACCGC
            ************************************************************

Dvl-5a      AAGAAGAAGAGGAGATCTAAGATGCCACCAGTACACAGAGCATCATCTTTCAGTAGTATA
Dvl-1       AAGAAGAAGAGGAGATCTAAGATGCCACCAGTACACAGAGCATCATCTTTCAGTAGTATA
Dvl4a-4b    AAGAAGAAGAGGAGATCTAAGATGCCACCAGTACACAGAGCATCATCTTTCAGTAGTATA
            ************************************************************

Dvl-5a      ACAGACTCAACAATGTCCCTCAACATCATTAATGTCACATTAAACTTAGATAAAATTAAT
Dvl-1       ACAGACTCAACAATGTCCCTCAACATCATTAATGTCACATTAAACTTAGATAAAATTAAT
Dvl4a-4b    ACAGACTCAACAATGTCCCTCAACATCATTAATGTCACATTAAACTTAGATAAAATTAAT
            ************************************************************

Dvl-5a      TTTCTTGGCATCAGTATTGTTGGTCAGAGTAATAAAGGAGGAGACGGTGGCATTTATGTG
Dvl-1       TTTCTTGGCATCAGTATTGTTGGTCAGAGTAATAAAGGAGGAGACGGTGGCATTTATGTG
Dvl4a-4b    TTTCTTGGCATCAGTATTGTTGGTCAGAGTAATAAAGGAGGAGACGGTGGCATTTATGTG
            ************************************************************
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Dvl-5a     GGCTCCATAATGAAAGGGGGCGCAGTAGCAGCAGATGGGAGGATAGAACCTGGAGATATG
Dvl-1      GGCTCCATAATGAAAGGGGGCGCAGTAGCAGCAGATGGGAGGATAGAACCTGGAGATATG
Dvl4a-4b   GGCTCCATAATGAAAGGGGGCGCAGTAGCAGCAGATGGGAGGATAGAACCTGGAGATATG
           ************************************************************

Dvl-5a     ATCTTACAGGTCAACGAGGTCAGCTTTGAGAACATGAGTAACGATGATGCGGTCAGAGTA
Dvl-1      ATCTTACAGGTCAACGAGGTCAGCTTTGAGAACATGAGTAACGATGATGCGGTCAGAGTA
Dvl4a-4b   ATCTTACAGGTCAACGAGGTCAGCTTTGAGAACATGAGTAACGATGATGCGGTCAGAGTA
           ************************************************************

Dvl-5a     TTAAGAGAAGCAGTACATCAACCAGGTCCTATCAAATTAGTGGTAGCTAAATGCTGGGAC
Dvl-1      TTAAGAGAAGCAGTACATCAACCAGGTCCTATCAAATTAGTGGTAGCTAAATGCTGGGAC
Dvl4a-4b   TTAAGAGAAGCAGTACATCAACCAGGTCCTATCAAATTAGTGGTAGCTAAATGCTGGGAC
           ************************************************************

Dvl-5a     CCCTCACCGAAAGGATACTTCACCATTCCAAGAAGTGAGCCGGTAAGACCCATCGACCCA
Dvl-1      CCCTCACCGAAAGGATACTTCACCATTCCAAGAAGTGAGCCGGTAAGACCCATCGACCCA
Dvl4a-4b   CCCTCACCGAAAGGATACTTCACCATTCCAAGAAGTGAGCCGGTAAGACCCATCGACCCA
           ************************************************************

Dvl-5a     GGTGCATGGGTAGCACACACAAATGCCATGAAAGTTGCTGCAGAGTATCAAGGAAGGGCG
Dvl-1      GGTGCATGGGTAGCACACACAAATGCCATGAAAGTTGCTGCAGAGTATCAAGGAAGGGCG
Dvl4a-4b   GGTGCATGGGTAGCACACACAAATGCCATGAAAGTTGCTGCAGAGTATCAAGGAAGGGCG
           ************************************************************

Dvl-5a     GGGCCTATGAGTCCGTCAATGACCTCTATGACCTCTACAAGCTCTTCCATCACCAGCTCA
Dvl-1      GGGCCTATGAGTCCGTCAATGACCTCTATGACCTCTACAAGCTCTTCCATCACCAGCTCA
Dvl4a-4b   GGGCCTATGAGTCCGTCAATGACCTCTATGACCTCTACAAGCTCTTCCATCACCAGCTCA
           ************************************************************

Dvl-5a     CTACCAGAGTCAGAGAGGTTAGAAGACTTTGGACGCCTCACCCTCAACACGGACATGACG
Dvl-1      CTACCAGAGTCAGAGAGGTTAGAAGACTTTGGACGCCTCACCCTCAACACGGACATGACG
Dvl4a-4b   CTACCAGAGTCAGAGAGGTTAGAAGACTTTGGACGCCTCACCCTCAACACGGACATGACG
           ************************************************************

Dvl-5a     ACCATCGCTAGGGCCATGGCAGCTCCTGATTCCGGCCTCGACATTAGAGACAGAATGTGG
Dvl-1      ACCATCGCTAGGGCCATGGCAGCTCCTGATTCCGGCCTCGACATTAGAGACAGAATGTGG
Dvl4a-4b   ACCATCGCTAGGGCCATGGCAGCTCCTGATTCCGGCCTCGACATTAGAGACAGAATGTGG
           ************************************************************

Dvl-5a     CTCAAAATCACCATCTCAAGCGCTTTCATAGGTTCTGATGTAGTGGAATGGTTGTTCACG
Dvl-1      CTCAAAATCACCATCTCAAGCGCTTTCATAGGTTCTGATGTAGTGGAATGGTTGTTCACG
Dvl4a-4b   CTCAAAATCACCATCTCAAGCGCTTTCATAGGTTCTGATGTAGTGGAATGGTTGTTCACG
           ************************************************************

Dvl-5a     CACGTAGAAGGGTTCCAGGACAGGAGAGAAGCGAGGAAGTACGCCTGCAATCTCTTAAAA
Dvl-1      CACGTAGAAGGGTTCCAGGACAGGAGAGAAGCGAGGAAGTACGCCTGCAATCTCTTAAAA
Dvl4a-4b   CACGTAGAAGGGTTCCAGGACAGGAGAGAAGCGAGGAAGTACGCCTGCAATCTCTTAAAA
           ************************************************************

Dvl-5a     GCCGGCTTCATCAGACACACCGTCAACAAGATCACATTCTCCGAGCAGTGCTACTATGTC
Dvl-1      GCCGGCTTCATCAGACACACCGTCAACAAGATCACATTCTCCGAGCAGTGCTACTATGTC
Dvl4a-4b   GCCGGCTTCATCAGACACACCGTCAACAAGATCACATTCTCCGAGCAGTGCTACTATGTC
           ************************************************************

Dvl-5a     TTTGGTGACCTCTGCGGAAACATGGCTTCTCTGTCCTTAGGAGACGAAGCATCCGAGGCG
Dvl-1      TTTGGTGACCTCTGCGGAAACATGGCTTCTCTGTCCTTAGGAGACGAAGCATCCGAGGCG
Dvl4a-4b   TTTGGTGACCTCTGCGGAAACATGGCTTCTCTGTCCTTAGGAGACGAAGCATCCGAGGCG
           ************************************************************

Dvl-5a     GACAGGGACACGTTAGCACCCCTACCCCAGCAGGGCCATTGGATGCCCCCGCCCCTCCCC
Dvl-1      GACAGGGACACGTTAGCACCCCTACCCCAGCAGGGCCATTGGATGCCCCCGCCCCTCCCC
Dvl4a-4b   GACAGGGACACGTTAGCACCCCTACCCCAGCAGGGCCATTGGATGCCCCCGCCCCTCCCC
           ************************************************************

Dvl-5a     ACTGCACCCCCAATGCCTTATCAGATGCCCCCAGGGGTGCCGGGCTACACCAGCTTTGAC
Dvl-1      ACTGCACCCCCAATGCCTTATCAGATGCCCCCAGGGGTGCCGGGCTACACCAGCTTTGAC
Dvl4a-4b   ACTGCACCCCCAATGCCTTATCAGATGCCCCCAGGGGTGCCGGGCTACACCAGCTTTGAC
           ************************************************************
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Dvl-5a      ACTGCTAGCTATACAAGCTTCGGTGCCACCAGCATCGGTAGTGGAAGCGGAGGAAGCAGT
Dvl-1       ACTGCTAGCTATACAAGCTTCGGTGCCACCAGCATCGGTAGTGGAAGCGGAGGAAGCAGT
Dvl4a-4b    ACTGCTAGCTATACAAGCTTCGGTGCCACCAGCATCGGTAGTGGAAGCGGAGGAAGCAGT
            ************************************************************

Dvl-5a      GATTCTGGTCACAGCCAAGCCAAAGCCATGGCGGCCAAAGGAGGGTCAGGAAGCAAGGGC
Dvl-1       GATTCTGGTCACAGCCAAGCCAAAGCCATGGCGGCCAAAGGAGGGTCAGGAAGCAAGGGC
Dvl4a-4b    GATTCTGGTCACAGCCAAGCCAAAGCCATGGCGGCCAAAGGAGGGTCAGGAAGCAAGGGC
            ************************************************************

Dvl-5a      AGTGGTAGTGAGTCTTCTGACCAAGCCTCGACCGTGGCCGGGGACATCCCTCCTGCCCTC
Dvl-1       AGTGGTAGTGAGTCTTCTGACCAAGCCTCGACCGTGGCCGGGGACATCCCTCCTGCCCTC
Dvl4a-4b    AGTGGTAGTGAGTCTTCTGACCAAGCCTCGACCGTGGCCGGGGACATCCCTCCTGCCCTC
            ************************************************************

Dvl-5a      ATGGGCAGCATGCAGGGCATCGGTCCCCCCTCCGTCACCAACACTGGCATGATGGTCCCC
Dvl-1       ATGGGCAGCATGCAGGGCATCGGTCCCCCCTCCGTCACCAACACTGGCATGATGGTCCCC
Dvl4a-4b    ATGGGCAGCATGCAGGGCATCGGTCCCCCCTCCGTCACCAACACTGGCATGATGGTCCCC
            ************************************************************

Dvl-5a      GGCGGCGCACCCTGCGGTAGCCTTGGTAGCCTCGGTAGCCACGGTGCCCTGTCCAACCAC
Dvl-1       GGCGGCGCACCCTGCGGTAGCCTTGGTAGCCTCGGTAGCCACGGTGCCCTGTCCAACCAC
Dvl4a-4b    GGCGGCGCACCCTGCGGTAGCCTTGGTAGCCTCGGTAGCCACGGTGCCCTGTCCAACCAC
            ************************************************************

Dvl-5a      GGCATCGGTCCCCCGTCCATGGGCGGGGTCCAGCACCAAGTCATCGGCTTTGGTCCCCCT
Dvl-1       GGCATCGGTCCCCCGTCCATGGGCGGGGTCCAGCACCAAGTCATCGGCTTTGGTCCCCCT
Dvl4a-4b    GGCATCGGTCCCCCGTCCATGGGCGGGGTCCAGCACCAAGTCATCGGCTTTGGTCCCCCT
            ************************************************************

Dvl-5a      CCCGGTGTCCCCCACCAGCTGCAGCACGTGATCGGGCCCCCACCGCACCAGACCCAGGGG
Dvl-1       CCCGGTGTCCCCCACCAGCTGCAGCACGTGATCGGGCCCCCACCGCACCAGACCCAGGGG
Dvl4a-4b    CCCGGTGTCCCCCACCAGCTGCAGCACGTGATCGGGCCCCCACCGCACCAGACCCAGGGG
            ************************************************************

Dvl-5a      ATCGGCCCCGCCCAGGGCATCGGTCCCCCGAGCCAGGGGGTGCCCCAGATGATGGTACCA
Dvl-1       ATCGGCCCCGCCCAGGGCATCGGTCCCCCGAGCCAGGGGGTGCCCCAGATGATGGTACCA
Dvl4a-4b    ATCGGCCCCGCCCAGGGCATCGGTCCCCCGAGCCAGGGGGTGCCCCAGATGATGGTACCA
            ************************************************************

Dvl-5a      ATGATGCCTCGACAACTCGGTAGCGTGCCTGAGGATCTCTCCGGCAGCAGACAGTCATTC
Dvl-1       ATGATGCCTCGACAACTCGGTAGCGTGCCTGAGGATCTCTCCGGCAGCAGACAGTCATTC
Dvl4a-4b    ATGATGCCTCGACAACTCGGTAGCGTGCCTGAGGATCTCTCCGGCAGCAGACAGTCATTC
            ************************************************************

Dvl-5a      CGCATGGCGATGGGAAACCCTTGTGAGTTCTTTGTCGATGTCATGTAA---------
Dvl-1       CGCATGGCCATGGGAAACCCTTCCTAG-----------------------------
Dvl4a-4b    CGCATGGCCATGGGAAACCCTTATTTTGATGATTCAGTCAGCGTCACACTTTTGTGA
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B.

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Dvl5a_3UTR    --------------------------------------------------ACAAAAATAT 10
Dvl4b_3UTR    AGTGCGGAAATTTTGAAATCATTTTGATTAGGTACAGTAGTGTTTGATTGAAGATAAATA 60
Dvl4a_3UTR    AGTGCGGAAATTTTGAAATCATTTTGATTAGCCTAGGACGCTAAAGTCTTGCGAATTTAA 60
Dvl1_3UTR     ----------------------------------GACGCTAAAGTCTTGCGAATTTAA 24
                                                                        *

Dvl5a_3UTR    AT-CCAAACCGACAACCAACCCCAATCCAGCTAGACTAAATCCTCTTTT------ATATT 63
Dvl4b_3UTR    -----TTGCACAATGTT-------AAAGAGCTAAAATAACTT---TGGGAGGTCAAATTG 105
Dvl4a_3UTR    AGAAGCTGCCCCCGCCGGCGCCGCCGCGAGCAAGCCGGAATCATGGTGCACCTCAAGCTC 120
Dvl1_3UTR     AGAAGCTGCCCCCGCCGGCGCCGCCGCGAGCAAGCCGGAATCATGGTGCACCTCAAGCTC 84
                       *               *** *     * *               *  *

Dvl5a_3UTR    CTTTCTCCCACCTAGCATCAGTTACCCATGACACCTCTCGCTTGTATCATATAGAAATAT 123
Dvl4b_3UTR    GAATGACCTTGTCAAGGCTG--GG--GTTGGAAATTTGAACTTGATGCTGACAAAATTCA 161
Dvl4a_3UTR    ATCGCTCCCTCCCCGCCTTC--GGCCCCCCGCAACTCTCCCACGAAGCACACGGGTCTGT 178
Dvl1_3UTR     ATCGCTCCCTCCCCGCCTTC--GGCCCCCCGCAACTCTCCCACGAAGCACACGGGTCTGT 142
                   **                  *  *     *  *    *  *       *

Dvl5a_3UTR    ATAT-------------------------------------------------------- 127
Dvl4b_3UTR    TTTTAA-CAATCTAAAATAGATTCTGGGGACCATGATTTG--ACTAGAGTACAAAGAATA 218
Dvl4a_3UTR    AAAGCACAACTGTCAAAATGTTGAAAGGGAGAAAAAGTCGAGAGAGGAATGCTTTCTGCT 238
Dvl1_3UTR     AAAGCACAACTGTCAAAATGTTGAAAGGGAGAAAAAGTCGAGAGAGGAATGCTTTCTGCT 202

Dvl5a_3UTR    -------TATCTACCTGTGTGTTTTGTTTGAGTTGAAATA------------------- 160
Dvl4b_3UTR    TTAATATGTTTTGGGTA-TGATATTAATATGGTACAAATTTTCCATTCTAATATTATTTG 277
Dvl4a_3UTR    CGAACATGATTTTGTTGTTGTTTCTGACTTGATTGAAATTAGCTGATTTTGAGTT----- 293
Dvl1_3UTR     CGAACATGATTTTGTTGTTGTTTCTGACTTGATTGAAATTAGCTGATTTTGAGTT----- 257
                      *  *    *    *  *       *  ****

Dvl5a_3UTR    ----------------------------------------GCATGCATCTATTATACTTTA 181
Dvl4b_3UTR    TGTTTTATTTTATTGTTTTCTTTTGTTTGATCATGAGTTGGATCGTATAGGGTCAGTTTC 337
Dvl4a_3UTR    GGTTCAATGTTGGACTTTTGTATT---AGATGTCAACC-CGCTGCTTTTGGGGCAGATTG 349
Dvl1_3UTR     GGTTCAATGTTGGACTTTTGTATT---AGATGTCAACC-CGCTGCTTTTGGGGCAGATTG 313
                                                         *           *  **

Dvl5a_3UTR    TATTTGTCTGTGTGTTGCCGATAGCT---GTGGT-AGGAAGT----------------AC 221
Dvl4b_3UTR    AA---ATCTAGGATTTAATATCAAGCCGGGCCTTAA------------TGCATAAAGGT 381
Dvl4a_3UTR    TTTTTGTTTTTGTTTTAATTGAAAGAGTTTCGTTATGCAAGCATGGCTTTGCCATGTGCT 409
Dvl1_3UTR     TTTTTGTTTTTGTTTTAATTGAAAGAGTTTCGTTATGCAAGCATGGCTTTGCCATGTGCT 373
                  *   *    *  **       *             *

Dvl5a_3UTR    AGCTTTCAAAAAAAAAATGCTAGATGATGTAACATAGAGCTTCTT--------------- 266
Dvl4b_3UTR    TGCTATCGAATACAAATG-------AAAATCATTAACAAATTTGTAACGTTAG---CCCT 431
Dvl4a_3UTR    TGGTTTCACCAGGCCATTGTTATATTTTGTGATTCACAACTATTTTTCCTCTGTTTTTCC 469
Dvl1_3UTR     TGGTTTCACCAGGCCATTGTTATATTTTGTGATTCACAACTATTTTTCCTCTGTTTTTCC 433
                  * * **        *          * *   * *  *     *

Dvl5a_3UTR    ----------------------------------------------------------- 266
Dvl4b_3UTR    TCAGGATCGATAGGAT---------ACATGTAGCTGTACTATTG---------AGTATAG 473
Dvl4a_3UTR    TCTGTTTTTATTCAACTCATTGTTATCACTTATGTGCTGTATTTTGGATGTTTTTCATTG 529
Dvl1_3UTR     TCTGTTTTTATTCAACTCATTGTTATCACTTATGTGCTGTATTTTGGATGTTTTTCATTG 493

Dvl5a_3UTR    ----------------------------------------------------------- 266
Dvl4b_3UTR    GTCCAGATTGACATATAAAGGAGAATTATGCTTGATTGTAACTGCGTGATTCCAAAAATG 533
Dvl4a_3UTR    TTCCAACCTTAGCTTTAT-TTTCAGTTATTGTTCTCTCTATCTCTCTCTTTCTCTCTGTC 588
Dvl1_3UTR     TTCCAACCTTAGCTTTAT-TTTCAGTTATTGTTCTCTCTATCTCTCTCTTTCTCTCTGTC 552

Dvl5a_3UTR    ----------------------------------------------------------- 266
Dvl4b_3UTR    CAACAGGCCT--------------------TGGGTCAAAATTCACTTTCAGATTCGTTT 572
Dvl4a_3UTR    TCCCTCCCTCTCTCTCTTTCTCTTTTTTTCTCCCCCCCCCTCTCTCTCTCCCTATCCTTC 648
Dvl1_3UTR     TCCCTCCCTCTCTCTCTTTCTCTTTTTTTCTCCCCCCCCCTCTCTCTCTCCCTATCCTTC 612

Dvl5a_3UTR    ----------------------------------------------------------- 266
Dvl4b_3UTR    GCAATTGTTGTATTGATTACTT------------------------------------- 594
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Dvl4a_3UTR   CCACTTCATCTCTTTCTACATTATTATTCATTTTTGTATTTCACTATTTTTGTTCATGTT 708
Dvl1_3UTR    CCACTTCATCTCTTTCTACATTATTATTCATTTTTGTATTTCACTATTTTTGTTCATGTT 672


Dvl5a_3UTR   ------------------------------------------------------------ 266
Dvl4b_3UTR   ------------------------------------------------------------ 594
Dvl4a_3UTR   TGTCCAATCTTTTCATCCTTCTCCTCATTTTAATTTTCTGTTTTAACCTGTTTTTCTGTG 768
Dvl1_3UTR    TGTCCAATCTTTTCATCCTTCTCCTCATTTTAATTTTCTGTTTTAACCTGTTTTTCTGTG 732


Dvl5a_3UTR   ------------------------------------------------------------ 266
Dvl4b_3UTR   ---------------------------------------------------------GTTAA 599
Dvl4a_3UTR   CCGACCATTTCTATCACCCTACATGTATGTCTCCACATTATATCTGTCATTTCATTTTTA 828
Dvl1_3UTR    CCGACCATTTCTATCACCCTACATGTATGTCTCCACATTATATCTGTCATTTCATTTTTA 792


Dvl5a_3UTR   -------------------------------------GTGCAA------TGTTGAT 279
Dvl4b_3UTR   CGAATATATGTTGT-------------------------------GTCCTCATTAAA 625
Dvl4a_3UTR   AAGAAAGATACTGTGCCCATCAAAATGAAGTTCATACATATGTGCACTATTCTTGAAAAT 888
Dvl1_3UTR    AAGAAAGATACTGTGCCCATCAAAATGAAGTTCATACATATGTGCACTATTCTTGAAAAT 852
                                                                         *

Dvl5a_3UTR   TGATCTGGAGAGAGAAAGGTCAGTCGTGTAATG---------CTTATTTTTTTGT----T 326
Dvl4b_3UTR   TCATCATA---------------------------------------------------- 633
Dvl4a_3UTR   TCAACAAACCCGAGAATGCTCAATGCTTTCCATCTCTTACTCCATCTCTTTCCA------ 942
Dvl1_3UTR    TCAACAAACCCGAGAATGCTCAATGCTTTCCATCTCTTACTCCATCTCTTTCCATCTCTT 912
             * * *

Dvl5a_3UTR   GCGATTAGTAGTAT--TCTGGCAAGAATCATAGTAGATTCTTT-GTTCAATGTGTGATTT 383
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   ----TCTCTCTCCTGCTCTCTCTCCTGCTCTCTCTCACTCTCTCTTACTCTCTCTCACTT 998
Dvl1_3UTR    TCCATCTCTCTCCTGCTCTCTCTCCTGCTCTCTCTCACTCTCTCTTACTCTCTCTCACTT 972


Dvl5a_3UTR   TTAAAAACCTTTTGTCTAAATT--ATCCTTGACTGAATTTTTAAGGACATTAATTCAGAT 441
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   TAACAATCTCAATCACTACCCTTTATTTCTTTCTTCCCCTCTCGCGACATTTTATTTTTT 1058
Dvl1_3UTR    TAACAATCTCAATCACTACCCTTTATTTCTTTCTTCCCCTCTCGCGACATTTTATTTTTT 1032



Dvl5a_3UTR   GCTTTTGAGGACATTAATTATACTGCCATGATACAACTATGCGTGTATCTGATGAATGAG 501
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   TCT-CCCATTTCTCTTTTTATCTTTCCCTGCTCTCTATATGTCTCCCTGAAATCCTAAAG 1117
Dvl1_3UTR    TCT-CCCATTTCTCTTTTTATCTTTCCCTGCTCTCTATATGTCTCCCTGAAATCCTAAAG 1091


Dvl5a_3UTR   ACA------------------GGATTACAAAAATTATGCTTATGGACAATATCAAAACG 542
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   TCTATTTGCTGCCATGCTTAACTATTTTCACTCCTAAATGATCTTGTGTCTACCGAGGCA 1177
Dvl1_3UTR    TCTATTTGCTGCCATGCTTAACTATTTTCACTCCTAAATGATCTTGTGTCTACCGAGGCA 1151


Dvl5a_3UTR   AGTATAGGAAATTGCCTTTGAAAAATCTTAATTTCAAGATATATACTTGGGAAGGGTGGA 602
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   AGCAAAGGCAGACTCCACTTTGTATTCTAT--------------------GCATCATGAA 1217
Dvl1_3UTR    AGCAAAGGCAGACTCCACTTTGTATTCTAT--------------------GCATCATGAA 1191


Dvl5a_3UTR   GAGGGGTATGGCTTACACAAAAATATGTGAAATACCTTGCATGATAT----------CAA 652
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   GTGCAATTTGGCCTCTACATCTCTCCCTTTGATAATTTACATCATTGAGAATAAAAGAAA 1277
Dvl1_3UTR    GTGCAATTTGGCCTCTACATCTCTCCCTTTGATAATTTACATCATTGAGAATAAAAGAAA 1251


Dvl5a_3UTR   AAAGCTATACTAGTTACT--------AATT--------GTAGAGAATGCTTTTGACT--- 693
Dvl4b_3UTR   ------------------------------------------------------------ 633
Dvl4a_3UTR   AAAAATATGCTTGTACAATCATCCACAATCATGCACAGTGATGGCATCCTGTTGGCACTC 1337
Dvl1_3UTR    AAAAATATGCTTGTACAATCATCCACAATCATGCACAGTGATGGCATCCTGTTGGCACTC 1311
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Dvl5a_3UTR    ------------ATGACTTTGTTTTCTGTGAT-GAAAAATGCAGTGTTTAGGAGCAAGTA 740
Dvl4b_3UTR    ----------------------------------------------------------- 633
Dvl4a_3UTR    AAATTCCAAATTATCCCTTATTCTTCATTCATTCAAAAATTGAGCGGTCATT------- 1389
Dvl1_3UTR     AAATTCCAAATTATCCCTTATTCTTCATTCATTCAAAAATTGAGCGGTCATT------- 1363


Dvl5a_3UTR    ACCTAACATATTGT          754
Dvl4b_3UTR    --------------          633
Dvl4a_3UTR    ---------ATTG-          1393
Dvl1_3UTR     ---------ATTG-          1367
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Figure S1. Sequence analysis of *Dvl* isoforms. (A) Multiple sequence alignment of the coding region of *SpDvl* isoforms using CLUSTAL Omega (1.2.4). (B) Multiple sequence alignment of the 3'UTR of *SpDvl* isoforms using CLUSTAL Omega (1.2.4). QPCR primers are underlined.

Figure S2. Dose response of *Dvl* mRNATP and *Dvl* mRNA overexpression effects. (A) Zygotes were injected with either control TP with fluorescein dextran or *Dvl* miRNATP with Texas Red dextran. All injected embryos were mixed and incubated in one well with primary rabbit anti-SUDdsh-C antibody, followed with goat anti-rabbit Alexa Fluor 647 secondary staining. The level of Dvl protein increase is dependent on the dosage of injected *Dvl* miRNATP. (B) Lower dose of *Dvl* mRNA overexpression did not induce significant PMC or gut developmental defects. Zygotes were injected with either 0.25µg/µl of *mCherry* or combinations of *LvDvl/SpDvl4a/4b* and *LvDvl*/SpDvl5a* mRNA. Gastrulae were fixed and immunolabeled with antibodies against the PMCs (1D5) or mid- and hindgut (Endo1).

Figure S3. miRNA sequence reads throughout development. Sequence reads of miRNAs examine in this study are expressed throughout early development (Song et al., 2012).

Table S1. Real time, quantitative PCR primers.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
Ubq	CACAGGCAAGACCATCACAC	GAGAGAGTGCGACCATCCTC
Dvl 5a	GCATCAGTTACCCATGACACC	TCGGCAACACACAGACAAAT
Dvl 4a	TGTGCCGACCATTTCTATCA	ATTGAGCATTCTCGGGTTTG
Dvl 4b	AGCCCTTCAGGATCGATAGG	ATTTTGACCCAAGGCCTGTT
β-catenin	GACATCAACGTGGTGACCTG	GCTGGCTCTGTGATTTCCTC
RhoA	AGATGAAGCAGGAACCGGTG	CGTCTCAAACACTTCCCGGA
Rac1	CGGAGCTGTGGGAAAGACAT	ATCCTAAGTTCACTGGGCGG
Cdc42	ACCAGAGATCACCCACCACT	TTTGGCCATCCTCTCACCAG
Wnt 1	TGCGATCTTATGTGCTGCTC	GAAACGACGTGCACTCTTCA
Wnt5	TGCTGTGGAAGAGGCTACAA	TTCTGCACTTCCGACACTTG
Wnt8	TGGGTGAAGCAGAGCTGTAG	ACCTGACAACACCAAACGAA
SM29	GCTGATGGAACCGAATTCTT	GAGTTATCCGGTCGGTTGTT
SM49	CCGCTGAACCATTTTACAGA	CATGAACTCAATCGCTTGCT
SM50	GGTGCTCTGGCTTCAGTTTC	GTGCCATCTTCCCAAAAGAA
Hnf6	TGCAGCTTCTCTGCATACCA	ACTCCAACATGCCTCCAAAC
Nodal	GACAACCCAAGCAACCACG	CGCACTCCTGTACGATCATG
BMP2/4	GACACACGGTTAGTCGACGT	GATGGTCTGCCCCTTGAGTC
Pax2/5/8	CCAAAGGTGGTGTCGAAGAT	ATCGAGCTGACACTGGGAAC
Vegf3	TGCAAATGTTCCCATTACGA	GTTCGTTTGGTTATGCGTCA
Alk4-5-7	CATAGGCACAAAGCGCTACA	CCAATCTCCCAGAGAACCAA
SLC26a5	TGATATCTTCCTGGGCCTTG	TCGAGGGTTCAAAACCAATC
TGFbrtII	CGAGCTCATTGGAAAAGGAC	GCTCTTTCTCGACGTTCCAC
KirrelL	TCGCATCCCATTCTCTCAGC	GACCCAGCAGCCCATGATAA
Eve	TTCAACCAGCAGTTCATCCA	ATGTCGGGATTGGTGGTG
Bra	ACACATCGACCCATCATCAA	CATGGTGTCGTATCTTGGAAAG
Krl	TCTTCACTCCTGTCCCTTGG	GTGATCATTTCCAGCTGTCG
FoxA	CCAACCGACTCCGTATCATC	CGTAGCTGCTCATGCTGTGT

Movie 1. Control TP-injected embryos have long apical cilia with synchronous beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 2. *Dvl* miRNATP-injected embryos have bent apical cilia with irregular beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 3. Control TP-injected larvae have long body cilia with synchronous beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 4. *Dvl* miRNATP-injected larvae have bent body cilia with irregular beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 5. Embryos injected with *mCherry* have normal apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 6. Embryos injected with *LvDvl/SpDvl4a/4*b mRNA have defective apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 7. Embryos injected with *LvDvl*/SpDvl5a* mRNA have defective apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 8. Embryos injected with *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a* mRNA have defective apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

A.

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Dvl-5a      ATGGAGGAGACTAAGATTATATATCATATAGATGATGAAGACACTCCATATCTCGTAAAA
Dvl-1       ATGGAGGAGACTAAGATTATATATCATATAGATGATGAAGACACTCCATATCTCGTAAAA
Dvl4a-4b    ATGGAGGAGACTAAGATTATATATCATATAGATGATGAAGACACTCCATATCTCGTAAAA
            ************************************************************

Dvl-5a      TTACCAATTCCTGCCGCCGATGTAACTCTTGGAGATTTCAAGAATGTCCTCAATCGGCCG
Dvl-1       TTACCAATTCCTGCCGCCGATGTAACTCTTGGAGATTTCAAGAATGTCCTCAATCGGCCG
Dvl4a-4b    TTACCAATTCCTGCCGCCGATGTAACTCTTGGAGATTTCAAGAATGTCCTCAATCGGCCG
            ************************************************************

Dvl-5a      AACTACAAATTCTTCTTCAAATCGATGGACGATGATTTTGGAGTTGTAAAAGAAGAAATA
Dvl-1       AACTACAAATTCTTCTTCAAATCGATGGACGATGATTTTGGAGTTGTAAAAGAAGAAATA
Dvl4a-4b    AACTACAAATTCTTCTTCAAATCGATGGACGATGATTTTGGAGTTGTAAAAGAAGAAATA
            ************************************************************

Dvl-5a      GTTGATGATGACACCAAATTACCTTGTTTTAATGGCCGTGTTGTGTCATGGCTGGTGCCA
Dvl-1       GTTGATGATGACACCAAATTACCTTGTTTTAATGGCCGTGTTGTGTCATGGCTGGTGCCA
Dvl4a-4b    GTTGATGATGACACCAAATTACCTTGTTTTAATGGCCGTGTTGTGTCATGGCTGGTGCCA
            ************************************************************

Dvl-5a      GCAGAGGGCAGCACCAACGGGTCAGACACAGCGTCGGTCACGACAGATACGAGAGGAGAC
Dvl-1       GCAGAGGGCAGCACCAACGGGTCAGACACAGCGTCGGTCACGACAGATACGAGAGGAGAC
Dvl4a-4b    GCAGAGGGCAGCACCAACGGGTCAGACACAGCGTCGGTCACGACAGATACGAGAGGAGAC
            ************************************************************

Dvl-5a      TCGCAACTTCCGCCCGAGAGGACAGGTGGTATAGGAGACTCTAGACCACCTTCATTTCAT
Dvl-1       TCGCAACTTCCGCCCGAGAGGACAGGTGGTATAGGAGACTCTAGACCACCTTCATTTCAT
Dvl4a-4b    TCGCAACTTCCGCCCGAGAGGACAGGTGGTATAGGAGACTCTAGACCACCTTCATTTCAT
            ************************************************************

Dvl-5a      TCGAGTGGAGGAGGGGTCCAAGATAAATTCGACGACACAGACACATGTACAGAATCAGAG
Dvl-1       TCGAGTGGAGGAGGGGTCCAAGATAAATTCGACGACACAGACACATGTACAGAATCAGAG
Dvl4a-4b    TCGAGTGGAGGAGGGGTCCAAGATAAATTCGACGACACAGACACATGTACAGAATCAGAG
            ************************************************************

Dvl-5a      CGAAGCTCAAGAAGAGGTCACCATCGCATGAGGAGAGACAAGTACAATAACTTTTCAAGA
Dvl-1       CGAAGCTCAAGAAGAGGTCACCATCGCATGAGGAGAGACAAGTACAATAACTTTTCAAGA
Dvl4a-4b    CGAAGCTCAAGAAGAGGTCACCATCGCATGAGGAGAGACAAGTACAATAACTTTTCAAGA
            ************************************************************

Dvl-5a      AGCAAGAGGGACCATCACCATCGAAGCGGGCATGGATACGAGAGTTCGTCAACGTTGATG
Dvl-1       AGCAAGAGGGACCATCACCATCGAAGCGGGCATGGATACGAGAGTTCGTCAACGTTGATG
Dvl4a-4b    AGCAAGAGGGACCATCACCATCGAAGCGGGCATGGATACGAGAGTTCGTCAACGTTGATG
            ************************************************************

Dvl-5a      AGCAGCGACATTGACTCCACAAGCTGCTTTGAATCAACAGACGACGACAGCAGTAGATTC
Dvl-1       AGCAGCGACATTGACTCCACAAGCTGCTTTGAATCAACAGACGACGACAGCAGTAGATTC
Dvl4a-4b    AGCAGCGACATTGACTCCACAAGCTGCTTTGAATCAACAGACGACGACAGCAGTAGATTC
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Dvl-5a      AGTAGTGCCACAGAAAGGAGTAGCTTAGCCAGGCCGATGAGGGGACCACCAAAGAACCGC
Dvl-1       AGTAGTGCCACAGAAAGGAGTAGCTTAGCCAGGCCGATGAGGGGACCACCAAAGAACCGC
Dvl4a-4b    AGTAGTGCCACAGAAAGGAGTAGCTTAGCCAGGCCGATGAGGGGACCACCAAAGAACCGC
            ************************************************************

Dvl-5a      AAGAAGAAGAGGAGATCTAAGATGCCACCAGTACACAGAGCATCATCTTTCAGTAGTATA
Dvl-1       AAGAAGAAGAGGAGATCTAAGATGCCACCAGTACACAGAGCATCATCTTTCAGTAGTATA
Dvl4a-4b    AAGAAGAAGAGGAGATCTAAGATGCCACCAGTACACAGAGCATCATCTTTCAGTAGTATA
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Dvl-5a      ACAGACTCAACAATGTCCCTCAACATCATTAATGTCACATTAAACTTAGATAAAATTAAT
Dvl-1       ACAGACTCAACAATGTCCCTCAACATCATTAATGTCACATTAAACTTAGATAAAATTAAT
Dvl4a-4b    ACAGACTCAACAATGTCCCTCAACATCATTAATGTCACATTAAACTTAGATAAAATTAAT
            ************************************************************

Dvl-5a      TTTCTTGGCATCAGTATTGTTGGTCAGAGTAATAAAGGAGGAGACGGTGGCATTTATGTG
Dvl-1       TTTCTTGGCATCAGTATTGTTGGTCAGAGTAATAAAGGAGGAGACGGTGGCATTTATGTG
Dvl4a-4b    TTTCTTGGCATCAGTATTGTTGGTCAGAGTAATAAAGGAGGAGACGGTGGCATTTATGTG
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Dvl-5a       GGCTCCATAATGAAAGGGGGCGCAGTAGCAGCAGATGGGAGGATAGAACCTGGAGATATG
Dvl-1        GGCTCCATAATGAAAGGGGGCGCAGTAGCAGCAGATGGGAGGATAGAACCTGGAGATATG
Dvl4a-4b     GGCTCCATAATGAAAGGGGGCGCAGTAGCAGCAGATGGGAGGATAGAACCTGGAGATATG
             ************************************************************

Dvl-5a       ATCTTACAGGTCAACGAGGTCAGCTTTGAGAACATGAGTAACGATGATGCGGTCAGAGTA
Dvl-1        ATCTTACAGGTCAACGAGGTCAGCTTTGAGAACATGAGTAACGATGATGCGGTCAGAGTA
Dvl4a-4b     ATCTTACAGGTCAACGAGGTCAGCTTTGAGAACATGAGTAACGATGATGCGGTCAGAGTA
             ************************************************************

Dvl-5a       TTAAGAGAAGCAGTACATCAACCAGGTCCTATCAAATTAGTGGTAGCTAAATGCTGGGAC
Dvl-1        TTAAGAGAAGCAGTACATCAACCAGGTCCTATCAAATTAGTGGTAGCTAAATGCTGGGAC
Dvl4a-4b     TTAAGAGAAGCAGTACATCAACCAGGTCCTATCAAATTAGTGGTAGCTAAATGCTGGGAC
             ************************************************************

Dvl-5a       CCCTCACCGAAAGGATACTTCACCATTCCAAGAAGTGAGCCGGTAAGACCCATCGACCCA
Dvl-1        CCCTCACCGAAAGGATACTTCACCATTCCAAGAAGTGAGCCGGTAAGACCCATCGACCCA
Dvl4a-4b     CCCTCACCGAAAGGATACTTCACCATTCCAAGAAGTGAGCCGGTAAGACCCATCGACCCA
             ************************************************************

Dvl-5a       GGTGCATGGGTAGCACACACAAATGCCATGAAAGTTGCTGCAGAGTATCAAGGAAGGGCG
Dvl-1        GGTGCATGGGTAGCACACACAAATGCCATGAAAGTTGCTGCAGAGTATCAAGGAAGGGCG
Dvl4a-4b     GGTGCATGGGTAGCACACACAAATGCCATGAAAGTTGCTGCAGAGTATCAAGGAAGGGCG
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Dvl-5a       GGGCCTATGAGTCCGTCAATGACCTCTATGACCTCTACAAGCTCTTCCATCACCAGCTCA
Dvl-1        GGGCCTATGAGTCCGTCAATGACCTCTATGACCTCTACAAGCTCTTCCATCACCAGCTCA
Dvl4a-4b     GGGCCTATGAGTCCGTCAATGACCTCTATGACCTCTACAAGCTCTTCCATCACCAGCTCA
             ************************************************************

Dvl-5a       CTACCAGAGTCAGAGAGGTTAGAAGACTTTGGACGCCTCACCCTCAACACGGACATGACG
Dvl-1        CTACCAGAGTCAGAGAGGTTAGAAGACTTTGGACGCCTCACCCTCAACACGGACATGACG
Dvl4a-4b     CTACCAGAGTCAGAGAGGTTAGAAGACTTTGGACGCCTCACCCTCAACACGGACATGACG
             ************************************************************

Dvl-5a       ACCATCGCTAGGGCCATGGCAGCTCCTGATTCCGGCCTCGACATTAGAGACAGAATGTGG
Dvl-1        ACCATCGCTAGGGCCATGGCAGCTCCTGATTCCGGCCTCGACATTAGAGACAGAATGTGG
Dvl4a-4b     ACCATCGCTAGGGCCATGGCAGCTCCTGATTCCGGCCTCGACATTAGAGACAGAATGTGG
             ************************************************************

Dvl-5a       CTCAAAATCACCATCTCAAGCGCTTTCATAGGTTCTGATGTAGTGGAATGGTTGTTCACG
Dvl-1        CTCAAAATCACCATCTCAAGCGCTTTCATAGGTTCTGATGTAGTGGAATGGTTGTTCACG
Dvl4a-4b     CTCAAAATCACCATCTCAAGCGCTTTCATAGGTTCTGATGTAGTGGAATGGTTGTTCACG
             ************************************************************

Dvl-5a       CACGTAGAAGGGTTCCAGGACAGGAGAGAAGCGAGGAAGTACGCCTGCAATCTCTTAAAA
Dvl-1        CACGTAGAAGGGTTCCAGGACAGGAGAGAAGCGAGGAAGTACGCCTGCAATCTCTTAAAA
Dvl4a-4b     CACGTAGAAGGGTTCCAGGACAGGAGAGAAGCGAGGAAGTACGCCTGCAATCTCTTAAAA
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Dvl-5a       GCCGGCTTCATCAGACACACCGTCAACAAGATCACATTCTCCGAGCAGTGCTACTATGTC
Dvl-1        GCCGGCTTCATCAGACACACCGTCAACAAGATCACATTCTCCGAGCAGTGCTACTATGTC
Dvl4a-4b     GCCGGCTTCATCAGACACACCGTCAACAAGATCACATTCTCCGAGCAGTGCTACTATGTC
             ************************************************************

Dvl-5a       TTTGGTGACCTCTGCGGAAACATGGCTTCTCTGTCCTTAGGAGACGAAGCATCCGAGGCG
Dvl-1        TTTGGTGACCTCTGCGGAAACATGGCTTCTCTGTCCTTAGGAGACGAAGCATCCGAGGCG
Dvl4a-4b     TTTGGTGACCTCTGCGGAAACATGGCTTCTCTGTCCTTAGGAGACGAAGCATCCGAGGCG
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Dvl-5a       GACAGGGACACGTTAGCACCCCTACCCCAGCAGGGCCATTGGATGCCCCCGCCCCTCCCC
Dvl-1        GACAGGGACACGTTAGCACCCCTACCCCAGCAGGGCCATTGGATGCCCCCGCCCCTCCCC
Dvl4a-4b     GACAGGGACACGTTAGCACCCCTACCCCAGCAGGGCCATTGGATGCCCCCGCCCCTCCCC
             ************************************************************

Dvl-5a       ACTGCACCCCCAATGCCTTATCAGATGCCCCCAGGGGTGCCGGGCTACACCAGCTTTGAC
Dvl-1        ACTGCACCCCCAATGCCTTATCAGATGCCCCCAGGGGTGCCGGGCTACACCAGCTTTGAC
Dvl4a-4b     ACTGCACCCCCAATGCCTTATCAGATGCCCCCAGGGGTGCCGGGCTACACCAGCTTTGAC
             ************************************************************
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Dvl-5a      ACTGCTAGCTATACAAGCTTCGGTGCCACCAGCATCGGTAGTGGAAGCGGAGGAAGCAGT
Dvl-1       ACTGCTAGCTATACAAGCTTCGGTGCCACCAGCATCGGTAGTGGAAGCGGAGGAAGCAGT
Dvl4a-4b    ACTGCTAGCTATACAAGCTTCGGTGCCACCAGCATCGGTAGTGGAAGCGGAGGAAGCAGT
            ************************************************************

Dvl-5a      GATTCTGGTCACAGCCAAGCCAAAGCCATGGCGGCCAAAGGAGGGTCAGGAAGCAAGGGC
Dvl-1       GATTCTGGTCACAGCCAAGCCAAAGCCATGGCGGCCAAAGGAGGGTCAGGAAGCAAGGGC
Dvl4a-4b    GATTCTGGTCACAGCCAAGCCAAAGCCATGGCGGCCAAAGGAGGGTCAGGAAGCAAGGGC
            ************************************************************

Dvl-5a      AGTGGTAGTGAGTCTTCTGACCAAGCCTCGACCGTGGCCGGGGACATCCCTCCTGCCCTC
Dvl-1       AGTGGTAGTGAGTCTTCTGACCAAGCCTCGACCGTGGCCGGGGACATCCCTCCTGCCCTC
Dvl4a-4b    AGTGGTAGTGAGTCTTCTGACCAAGCCTCGACCGTGGCCGGGGACATCCCTCCTGCCCTC
            ************************************************************

Dvl-5a      ATGGGCAGCATGCAGGGCATCGGTCCCCCCTCCGTCACCAACACTGGCATGATGGTCCCC
Dvl-1       ATGGGCAGCATGCAGGGCATCGGTCCCCCCTCCGTCACCAACACTGGCATGATGGTCCCC
Dvl4a-4b    ATGGGCAGCATGCAGGGCATCGGTCCCCCCTCCGTCACCAACACTGGCATGATGGTCCCC
            ************************************************************

Dvl-5a      GGCGGCGCACCCTGCGGTAGCCTTGGTAGCCTCGGTAGCCACGGTGCCCTGTCCAACCAC
Dvl-1       GGCGGCGCACCCTGCGGTAGCCTTGGTAGCCTCGGTAGCCACGGTGCCCTGTCCAACCAC
Dvl4a-4b    GGCGGCGCACCCTGCGGTAGCCTTGGTAGCCTCGGTAGCCACGGTGCCCTGTCCAACCAC
            ************************************************************

Dvl-5a      GGCATCGGTCCCCCGTCCATGGGCGGGGTCCAGCACCAAGTCATCGGCTTTGGTCCCCCT
Dvl-1       GGCATCGGTCCCCCGTCCATGGGCGGGGTCCAGCACCAAGTCATCGGCTTTGGTCCCCCT
Dvl4a-4b    GGCATCGGTCCCCCGTCCATGGGCGGGGTCCAGCACCAAGTCATCGGCTTTGGTCCCCCT
            ************************************************************

Dvl-5a      CCCGGTGTCCCCCACCAGCTGCAGCACGTGATCGGGCCCCCACCGCACCAGACCCAGGGG
Dvl-1       CCCGGTGTCCCCCACCAGCTGCAGCACGTGATCGGGCCCCCACCGCACCAGACCCAGGGG
Dvl4a-4b    CCCGGTGTCCCCCACCAGCTGCAGCACGTGATCGGGCCCCCACCGCACCAGACCCAGGGG
            ************************************************************

Dvl-5a      ATCGGCCCCGCCCAGGGCATCGGTCCCCCGAGCCAGGGGGTGCCCCAGATGATGGTACCA
Dvl-1       ATCGGCCCCGCCCAGGGCATCGGTCCCCCGAGCCAGGGGGTGCCCCAGATGATGGTACCA
Dvl4a-4b    ATCGGCCCCGCCCAGGGCATCGGTCCCCCGAGCCAGGGGGTGCCCCAGATGATGGTACCA
            ************************************************************

Dvl-5a      ATGATGCCTCGACAACTCGGTAGCGTGCCTGAGGATCTCTCCGGCAGCAGACAGTCATTC
Dvl-1       ATGATGCCTCGACAACTCGGTAGCGTGCCTGAGGATCTCTCCGGCAGCAGACAGTCATTC
Dvl4a-4b    ATGATGCCTCGACAACTCGGTAGCGTGCCTGAGGATCTCTCCGGCAGCAGACAGTCATTC
            ************************************************************

Dvl-5a      CGCATGGCGATGGGGAAACCCTTGTGAGTTCTTTGTCGATGTCATGTAA---------
Dvl-1       CGCATGGCCATGGGAAACCCTTCCTAG-----------------------------
Dvl4a-4b    CGCATGGCCATGGGAAACCCTTATTTTGATGATTCAGTCAGCGTCACACTTTTGTGA
            ********  *************
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B.

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Dvl5a_3UTR    ------------------------------------------------------ACAAAAATAT 10
Dvl4b_3UTR    AGTGCGGAAATTTTGAAATCATTTTGATTAGGTACAGTAGTGTTTGATTGAAGATAAATA 60
Dvl4a_3UTR    AGTGCGGAAATTTTGAAATCATTTTGATTAGCCTAGGACGCTAAAGTCTTGCGAATTTAA 60
Dvl1_3UTR     ----------------------------------GACGCTAAAGTCTTGCGAATTTAA 24
                                                                        *

Dvl5a_3UTR    AT-CCAAACCGACAACCAACCCCAATCCAGCTAGACTAAATCCTCTTTT------ATATT 63
Dvl4b_3UTR    -----TTGCACAATGTT-------AAAGAGCTAAAATAACTT---TGGGAGGTCAAATTG 105
Dvl4a_3UTR    AGAAGCTGCCCCCGCCGGCGCCGCCGCGAGCAAGCCGGAATCATGGTGCACCTCAAGCTC 120
Dvl1_3UTR     AGAAGCTGCCCCCGCCGGCGCCGCCGCGAGCAAGCCGGAATCATGGTGCACCTCAAGCTC 84
                       *              *** *     * *             * *

Dvl5a_3UTR    CTTTCTCCCACCTAGCATCAGTTACCCATGACACCTCTCGCTTGTATCATATAGAAATAT 123
Dvl4b_3UTR    GAATGACCTTGTCAAGGCTG--GG--GTTGGAAATTTGAACTTGATGCTGACAAAATTCA 161
Dvl4a_3UTR    ATCGCTCCCTCCCCGCCTTC--GGCCCCCCGCAACTCTCCCACGAAGCACACGGGTCTGT 178
Dvl1_3UTR     ATCGCTCCCTCCCCGCCTTC--GGCCCCCCGCAACTCTCCCACGAAGCACACGGGTCTGT 142
                    **                   *   *     *     *     *         *

Dvl5a_3UTR    ATAT--------------------------------------------------------- 127
Dvl4b_3UTR    TTTTAA-CAATCTAAAATAGATTCTGGGGACCATGATTTG--ACTAGAGTACAAAGAATA 218
Dvl4a_3UTR    AAAGCACAACTGTCAAAATGTTGAAAGGGAGAAAAAGTCGAGAGAGGAATGCTTTCTGCT 238
Dvl1_3UTR     AAAGCACAACTGTCAAAATGTTGAAAGGGAGAAAAAGTCGAGAGAGGAATGCTTTCTGCT 202

Dvl5a_3UTR    -------TATCTACCTGTGTGTTTTGTTTGAGTTGAAATA-------------------- 160
Dvl4b_3UTR    TTAATATGTTTTGGGTA-TGATATTAATATGGTACAAATTTTCCATTCTAATATTATTTG 277
Dvl4a_3UTR    CGAACATGATTTTGTTGTTGTTTCTGACTTGATTGAAATTAGCTGATTTTGAGTT----- 293
Dvl1_3UTR     CGAACATGATTTTGTTGTTGTTTCTGACTTGATTGAAATTAGCTGATTTTGAGTT----- 257
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Dvl5a_3UTR    -----------------------------------------GCATGCATCTATTATACTTTA 181
Dvl4b_3UTR    TGTTTTATTTTATTGTTTTCTTTTGTTTGATCATGAGTTGGATCGTATAGGGTCAGTTTC 337
Dvl4a_3UTR    GGTTCAATGTTGGACTTTTGTATT---AGATGTCAACC-CGCTGCTTTTGGGGCAGATTG 349
Dvl1_3UTR     GGTTCAATGTTGGACTTTTGTATT---AGATGTCAACC-CGCTGCTTTTGGGGCAGATTG 313
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Dvl5a_3UTR    TATTTGTCTGTGTGTTGCCGATAGCT---GTGGT-AGGAAGT----------------AC 221
Dvl4b_3UTR    AA---ATCTAGGATTTAATATCAAGCCGGGCCTTAA------------TGCATAAAGGT 381
Dvl4a_3UTR    TTTTTGTTTTTGTTTTAATTGAAAGAGTTTCGTTATGCAAGCATGGCTTTGCCATGTGCT 409
Dvl1_3UTR     TTTTTGTTTTTGTTTTAATTGAAAGAGTTTCGTTATGCAAGCATGGCTTTGCCATGTGCT 373
                   *  *   *  **      *          *

Dvl5a_3UTR    AGCTTTCAAAAAAAAAATGCTAGATGATGTAACATAGAGCTTCTT-------------- 266
Dvl4b_3UTR    TGCTATCGAATACAAATG-------AAAATCATTAACAAATTTGTAACGTTAG---CCCT 431
Dvl4a_3UTR    TGGTTTCACCAGGCCATTGTTATATTTTGTGATTCACAACTATTTTTCCTCTGTTTTTCC 469
Dvl1_3UTR     TGGTTTCACCAGGCCATTGTTATATTTTGTGATTCACAACTATTTTTCCTCTGTTTTTCC 433
                * * **         *              *  *    * *    *     *

Dvl5a_3UTR    ------------------------------------------------------------ 266
Dvl4b_3UTR    TCAGGATCGATAGGAT---------ACATGTAGCTGTACTATTG---------AGTATAG 473
Dvl4a_3UTR    TCTGTTTTTATTCAACTCATTGTTATCACTTATGTGCTGTATTTTGGATGTTTTTCATTG 529
Dvl1_3UTR     TCTGTTTTTATTCAACTCATTGTTATCACTTATGTGCTGTATTTTGGATGTTTTTCATTG 493

Dvl5a_3UTR    ------------------------------------------------------------ 266
Dvl4b_3UTR    GTCCAGATTGACATATAAAGGAGAATTATGCTTGATTGTAACTGCGTGATTCCAAAAATG 533
Dvl4a_3UTR    TTCCAACCTTAGCTTTAT-TTTCAGTTATTGTTCTCTCTATCTCTCTCTTTCTCTCTGTC 588
Dvl1_3UTR     TTCCAACCTTAGCTTTAT-TTTCAGTTATTGTTCTCTCTATCTCTCTCTTTCTCTCTGTC 552

Dvl5a_3UTR    ------------------------------------------------------------ 266
Dvl4b_3UTR    CAACAGGCCT--------------------TGGGTCAAAATTCACTTTCAGATTCGTTT 572
Dvl4a_3UTR    TCCCTCCCTCTCTCTCTTTCTCTTTTTTTTCTCCCCCCCCCTCTCTCTCTCCCTATCCTTC 648
Dvl1_3UTR     TCCCTCCCTCTCTCTCTTTCTCTTTTTTTTCTCCCCCCCCCTCTCTCTCTCCCTATCCTTC 612

Dvl5a_3UTR    ------------------------------------------------------------ 266
Dvl4b_3UTR    GCAATTGTTGTATTGATTACTT-------------------------------------- 594
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Dvl4a_3UTR    CCACTTCATCTCTTTCTACATTATTATTCATTTTTGTATTTCACTATTTTTGTTCATGTT 708
Dvl1_3UTR     CCACTTCATCTCTTTCTACATTATTATTCATTTTTGTATTTCACTATTTTTGTTCATGTT 672


Dvl5a_3UTR    ------------------------------------------------------------ 266
Dvl4b_3UTR    ------------------------------------------------------------ 594
Dvl4a_3UTR    TGTCCAATCTTTTCATCCTTCTCCTCATTTTAATTTTCTGTTTTAACCTGTTTTTCTGTG 768
Dvl1_3UTR     TGTCCAATCTTTTCATCCTTCTCCTCATTTTAATTTTCTGTTTTAACCTGTTTTTCTGTG 732


Dvl5a_3UTR    ------------------------------------------------------------ 266
Dvl4b_3UTR    ---------------------------------------------------------GTTAA 599
Dvl4a_3UTR    CCGACCATTTCTATCACCCTACATGTATGTCTCCACATTATATCTGTCATTTCATTTTTA 828
Dvl1_3UTR     CCGACCATTTCTATCACCCTACATGTATGTCTCCACATTATATCTGTCATTTCATTTTTA 792


Dvl5a_3UTR    ------------------------------------------GTGCAA------TGTTGAT 279
Dvl4b_3UTR    CGAATATATGTTGT-----------------------------GTCCTCATTAAA 625
Dvl4a_3UTR    AAGAAAGATACTGTGCCCATCAAAATGAAGTTCATACATATGTGCACTATTCTTGAAAAT 888
Dvl1_3UTR     AAGAAAGATACTGTGCCCATCAAAATGAAGTTCATACATATGTGCACTATTCTTGAAAAT 852
                                                                         *

Dvl5a_3UTR    TGATCTGGAGAGAGAAAGGTCAGTCGTGTAATG---------CTTATTTTTTTGT----T 326
Dvl4b_3UTR    TCATCATA---------------------------------------------------- 633
Dvl4a_3UTR    TCAACAAACCCGAGAATGCTCAATGCTTTCCATCTCTTACTCCATCTCTTTCCA------ 942
Dvl1_3UTR     TCAACAAACCCGAGAATGCTCAATGCTTTCCATCTCTTACTCCATCTCTTTCCATCTCTT 912
              * * *

Dvl5a_3UTR    GCGATTAGTAGTAT--TCTGGCAAGAATCATAGTAGATTCTTT-GTTCAATGTGTGATTT 383
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    ----TCTCTCTCCTGCTCTCTCTCCTGCTCTCTCTCACTCTCTCTTACTCTCTCTCACTT 998
Dvl1_3UTR     TCCATCTCTCTCCTGCTCTCTCTCCTGCTCTCTCTCACTCTCTCTTACTCTCTCTCACTT 972


Dvl5a_3UTR    TTAAAAACCTTTTGTCTAAATT--ATCCTTGACTGAATTTTTAAGGACATTAATTCAGAT 441
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    TAACAATCTCAATCACTACCCTTTATTTCTTTCTTCCCCTCTCGCGACATTTTATTTTTT 1058
Dvl1_3UTR     TAACAATCTCAATCACTACCCTTTATTTCTTTCTTCCCCTCTCGCGACATTTTATTTTTT 1032



Dvl5a_3UTR    GCTTTTGAGGACATTAATTATACTGCCATGATACAACTATGCGTGTATCTGATGAATGAG 501
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    TCT-CCCATTTCTCTTTTTATCTTTCCCTGCTCTCTATATGTCTCCCTGAAATCCTAAAG 1117
Dvl1_3UTR     TCT-CCCATTTCTCTTTTTATCTTTCCCTGCTCTCTATATGTCTCCCTGAAATCCTAAAG 1091


Dvl5a_3UTR    ACA-----------------GGATTACAAAAATTATGCTTATGGACAATATCAAAACG 542
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    TCTATTTGCTGCCATGCTTAACTATTTTCACTCCTAAATGATCTTGTGTCTACCGAGGCA 1177
Dvl1_3UTR     TCTATTTGCTGCCATGCTTAACTATTTTCACTCCTAAATGATCTTGTGTCTACCGAGGCA 1151


Dvl5a_3UTR    AGTATAGGAAATTGCCTTTGAAAAATCTTAATTTCAAGATATATACTTGGGAAGGGTGGA 602
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    AGCAAAGGCAGACTCCACTTTGTATTCTAT--------------------GCATCATGAA 1217
Dvl1_3UTR     AGCAAAGGCAGACTCCACTTTGTATTCTAT--------------------GCATCATGAA 1191


Dvl5a_3UTR    GAGGGGTATGGCTTACACAAAAATATGTGAAATACCTTGCATGATAT----------CAA 652
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    GTGCAATTTGGCCTCTACATCTCTCCCTTTGATAATTTACATCATTGAGAATAAAAGAAA 1277
Dvl1_3UTR     GTGCAATTTGGCCTCTACATCTCTCCCTTTGATAATTTACATCATTGAGAATAAAAGAAA 1251


Dvl5a_3UTR    AAAGCTATACTAGTTACT--------AATT--------GTAGAGAATGCTTTTGACT--- 693
Dvl4b_3UTR    ------------------------------------------------------------ 633
Dvl4a_3UTR    AAAAATATGCTTGTACAATCATCCACAATCATGCACAGTGATGGCATCCTGTTGGCACTC 1337
Dvl1_3UTR     AAAAATATGCTTGTACAATCATCCACAATCATGCACAGTGATGGCATCCTGTTGGCACTC 1311
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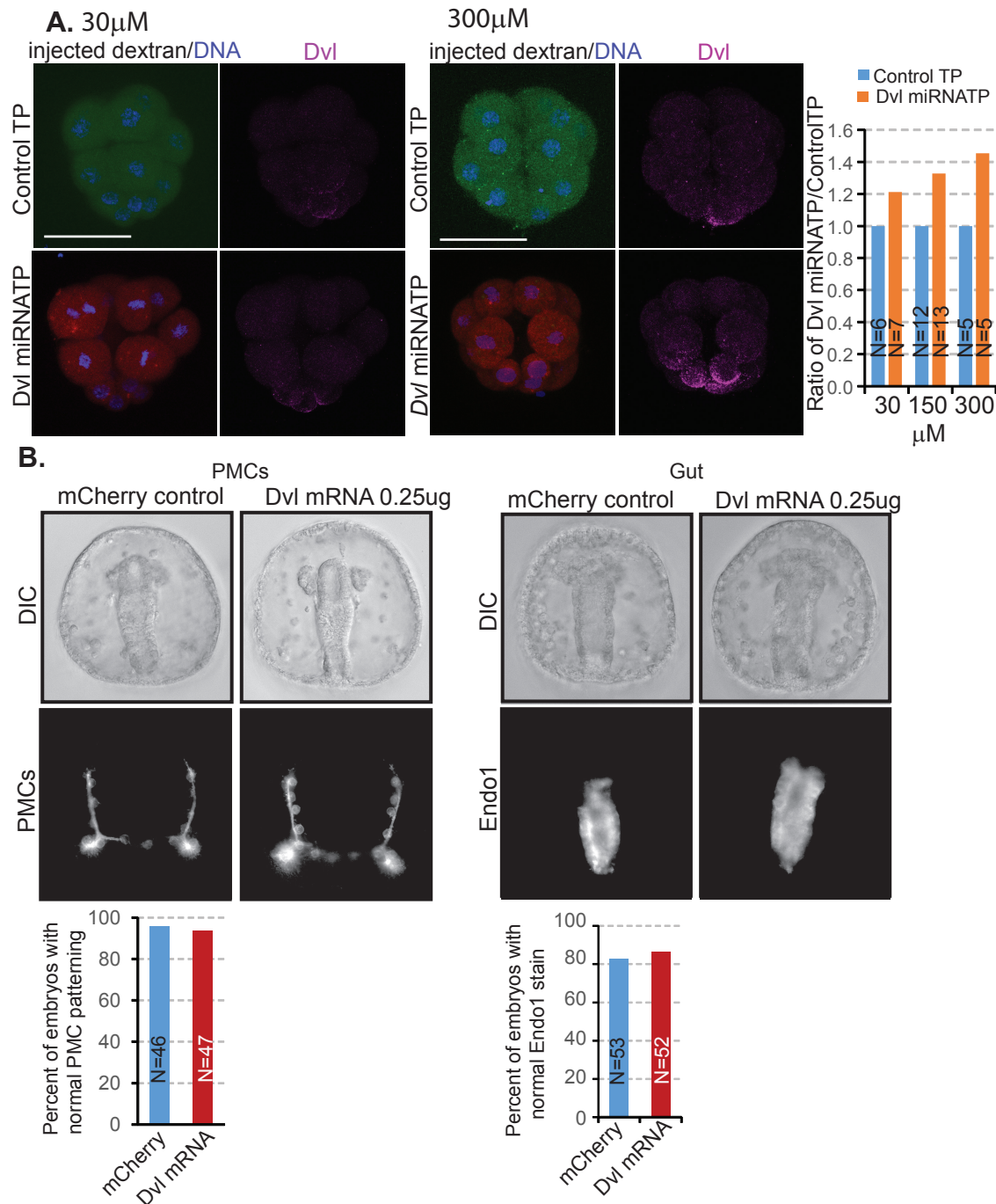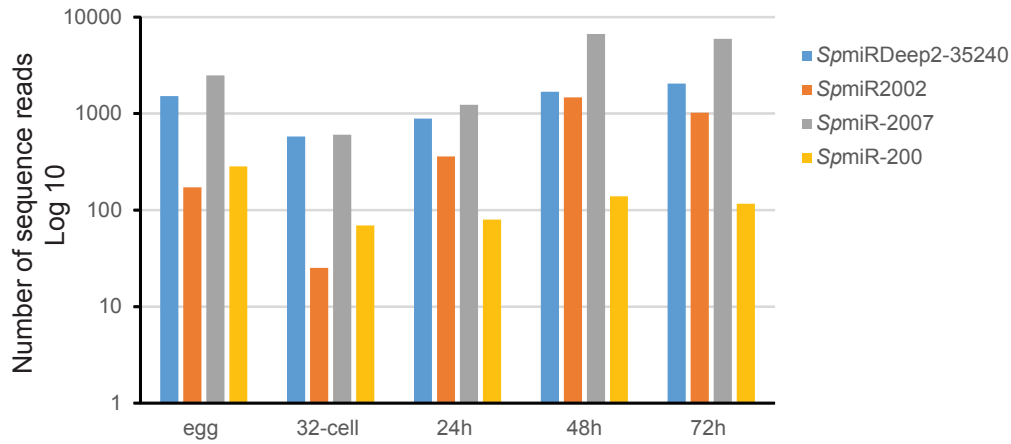
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Dvl5a_3UTR     ------------ATGACTTTGTTTTCTGTGAT-GAAAAATGCAGTGTTTAGGAGCAAGTA 740
Dvl4b_3UTR     ------------------------------------------------------------ 633
Dvl4a_3UTR     AAATTCCAAATTATCCCTTATTCTTCATTCATTCAAAAATTGAGCGGTCATT------- 1389
Dvl1_3UTR      AAATTCCAAATTATCCCTTATTCTTCATTCATTCAAAAATTGAGCGGTCATT------- 1363


Dvl5a_3UTR     ACCTAACATATTGT          754
Dvl4b_3UTR     --------------          633
Dvl4a_3UTR     --------ATTG-           1393
Dvl1_3UTR      --------ATTG-           1367
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Figure S1. Sequence analysis of *Dvl* isoforms. (A) Multiple sequence alignment of the coding region of *SpDvl* isoforms using CLUSTAL Omega (1.2.4). (B) Multiple sequence alignment of the 3'UTR of *SpDvl* isoforms using CLUSTAL Omega (1.2.4). QPCR primers are underlined.

Figure S2. Dose response of *Dvl* mRNATP and *Dvl* mRNA overexpression effects. (A) Zygotes were injected with either control TP with fluorescein dextran or *Dvl* miRNATP with Texas Red dextran. All injected embryos were mixed and incubated in one well with primary rabbit anti-SUDdsh-C antibody, followed with goat anti-rabbit Alexa Fluor 647 secondary staining. The level of Dvl protein increase is dependent on the dosage of injected *Dvl* miRNATP. (B) Lower dose of *Dvl* mRNA overexpression did not induce significant PMC or gut developmental defects. Zygotes were injected with either 0.25µg/µl of *mCherry* or combinations of *LvDvl/SpDvl4a/4b* and *LvDvl*/SpDvl5a* mRNA. Gastrulae were fixed and immunolabeled with antibodies against the PMCs (1D5) or mid- and hindgut (Endo1).

Figure S3. miRNA sequence reads throughout development. Sequence reads of miRNAs examine in this study are expressed throughout early development (Song et al., 2012).

Table S1. Real time, quantitative PCR primers.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
Ubq	CACAGGCAAGACCATCACAC	GAGAGAGTGCGACCATCCTC
Dvl 5a	GCATCAGTTACCCATGACACC	TCGGCAACACACAGACAAAT
Dvl 4a	TGTGCCGACCATTTCTATCA	ATTGAGCATTCTCGGGTTTG
Dvl 4b	AGCCCTTCAGGATCGATAGG	ATTTTGACCCAAGGCCTGTT
β-catenin	GACATCAACGTGGTGACCTG	GCTGGCTCTGTGATTTCCTC
RhoA	AGATGAAGCAGGAACCGGTG	CGTCTCAAACACTTCCCGGA
Rac1	CGGAGCTGTGGGAAAGACAT	ATCCTAAGTTCACTGGGCGG
Cdc42	ACCAGAGATCACCCACCACT	TTTGGCCATCCTCTCACCAG
Wnt 1	TGCGATCTTATGTGCTGCTC	GAAACGACGTGCACTCTTCA
Wnt5	TGCTGTGGAAGAGGCTACAA	TTCTGCACTTCCGACACTTG
Wnt8	TGGGTGAAGCAGAGCTGTAG	ACCTGACAACACCAAACGAA
SM29	GCTGATGGAACCGAATTCTT	GAGTTATCCGGTCGGTTGTT
SM49	CCGCTGAACCATTTTACAGA	CATGAACTCAATCGCTTGCT
SM50	GGTGCTCTGGCTTCAGTTTC	GTGCCATCTTCCCAAAAGAA
Hnf6	TGCAGCTTCTCTGCATACCA	ACTCCAACATGCCTCCAAAC
Nodal	GACAACCCAAGCAACCACG	CGCACTCCTGTACGATCATG
BMP2/4	GACACACGGTTAGTCGACGT	GATGGTCTGCCCCTTGAGTC
Pax2/5/8	CCAAAGGTGGTGTCGAAGAT	ATCGAGCTGACACTGGGAAC
Vegf3	TGCAAATGTTCCCATTACGA	GTTCGTTTGGTTATGCGTCA
Alk4-5-7	CATAGGCACAAAGCGCTACA	CCAATCTCCCAGAGAACCAA
SLC26a5	TGATATCTTCCTGGGCCTTG	TCGAGGGTTCAAAACCAATC
TGFbrtII	CGAGCTCATTGGAAAAGGAC	GCTCTTTCTCGACGTTCCAC
KirrelL	TCGCATCCCATTCTCTCAGC	GACCCAGCAGCCCATGATAA
Eve	TTCAACCAGCAGTTCATCCA	ATGTCGGGATTGGTGGTG
Bra	ACACATCGACCCATCATCAA	CATGGTGTCGTATCTTGGAAAG
Krl	TCTTCACTCCTGTCCCTTGG	GTGATCATTTCCAGCTGTCG
FoxA	CCAACCGACTCCGTATCATC	CGTAGCTGCTCATGCTGTGT

Movie 1. Control TP-injected embryos have long apical cilia with synchronous beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 2. *Dvl* miRNATP-injected embryos have bent apical cilia with irregular beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 3. Control TP-injected larvae have long body cilia with synchronous beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 4. *Dvl* miRNATP-injected larvae have bent body cilia with irregular beating. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 5. Embryos injected with *mCherry* have normal apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 6. Embryos injected with *LvDvl/SpDvl4a/4*b mRNA have defective apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 7. Embryos injected with *LvDvl*/SpDvl5a* mRNA have defective apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.

Movie 8. Embryos injected with *LvDvl/SpDvl4a/4b* plus *LvDvl*/SpDvl5a* mRNA have defective apical cilia. Live embryos were collected 5 dpf and mounted in filtered natural sea water on microscope slides. Images were taken with an LSM 780 scanning confocal microscope using the time lapse function. Images were compiled using Windows Movie Maker to make a movie using 0.1s between images.