Nkd1 Functions as a Passive Antagonist of Wnt Signaling

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Abstract

Wnt signaling is involved in many aspects of development and in the homeostasis of stem cells. Its importance is underscored by the fact that misregulation of Wnt signaling has been implicated in numerous diseases, especially colorectal cancer. However, how Wnt signaling regulates itself is not well understood. There are several Wnt negative feedback regulators, which are active antagonists of Wnt signaling, but one feedback regulator, Nkd1, has reduced activity compared to other antagonists, yet is still a negative feedback regulator. Here we describe our efforts to understand the role of Nkd1 using Wnt signaling compromised zebrafish mutant lines. In several of these lines, Nkd1 function was not any more active than it was in wild type embryos. However, we found that Nkd1’s ability to antagonize canonical Wnt/β-catenin signaling was enhanced in the Wnt/Planar Cell Polarity mutants silberblick (slb/wnt11) and trilobite (tri/vangl2). While slb and tri mutants do not display alterations in canonical Wnt signaling, we found that they are hypersensitive to it. Overexpression of the canonical Wnt/β-catenin ligand Wnt8a in slb or tri mutants resulted in dorsalized embryos, with tri mutants being more sensitive to Wnt8a than slb mutants. Furthermore, the hyperdorsalization caused by Wnt8a in tri could be rescued by Nkd1. These results suggest that Nkd1 functions as a passive antagonist of Wnt signaling, functioning only when homeostatic levels of Wnt signaling have been breached or when Wnt signaling becomes destabilized.

Introduction

Patterning of the vertebrate embryo involves the coordinated efforts of multiple signaling pathways. The regulation of these pathways must be tightly controlled in order for normal development to proceed. Regulation can occur at several different levels, but one important mechanism is the ability of a signaling pathway to invoke a negative feedback loop. The Wnt signaling pathway is a major player in development and in homeostasis of stem cells [1–4] and several negative feedback regulators have been identified including Dickopff (Dkk), Wingful/Notum, Naked (1/2), Nemo, Axin2 and β-TCRP [5–14]. For the most part, these antagonists are very efficient at blocking both ectopic and endogenous canonical Wnt/β-catenin signaling, with the exception of Nkd1. We and others have previously reported that Nkd1 is an obligate target of Wnt signaling during vertebrate development and can inhibit both canonical and non-canonical Wnt signaling [12,14–16]. Subsequently, we found that Nkd1 inhibits canonical Wnt signaling by preventing the nuclear accumulation of β-catenin [13]. As β-catenin is restricted to the canonical Wnt pathway, it is unclear how Nkd1 antagonizes non-canonical or Wnt/PCP signaling, but likely involves Dvl [17]. In our investigations, we found Nkd1 to be very efficient at reducing ectopic Wnt signaling. For example, in the boozozok mutant, there is excess Wnt signaling from the ventro-lateral mesendoderm that reduces the size of the dorsal domain, which is manifested by a reduced or absent notochord [18–20]. Overexpression of Nkd1 rescues this phenotype, along with the eyeless phenotype induced by excess Wnt8a [12]. However, in the absence of excess Wnt signaling Nkd1 activity is less obvious.

The passive effect of Nkd1 may be a universal phenomenon. In flies, absence of Nkd results in a naked cuticle phenotype (hence its name) at larval stages, but even though nkd is expressed at multiple other stages of development in domains of active Wnt signaling, its loss of function does not appreciably affect these other Wnt signaling events [21]. Double knockout of nkd1 and nkd2 in mouse results in subtle alterations in cranial bone morphology, but are otherwise normal and fertile [22] and ubiquitous overexpression of Nkd1 in the mouse or in the fly embryo does not have any obvious consequences [15,17,21]. These results are consistent with our analysis of Nkd1 function in zebrafish. While careful examination reveals a role for Nkd1 in restricting Wnt-mediated D–V patterning, there is no obvious consequence to overexpression of Nkd1. However, in contrast to wild type embryos, severe loss-of-Wg
signaling phenotypes can be induced in the embryo when Nkd is overproduced in Wg-compromised genetic backgrounds [17,21]. This suggests that Nkd activity is dependent on Wnt signaling itself and the use of compromised Wnt signaling mutants may be one avenue to further understand Nkd1 function.

In vertebrates it has been well established that the Wnt/PCP pathway can antagonize canonical Wnt/β-catenin signaling, but at what level in the pathway remains controversial [23–27]. Nonetheless, Wnt/PCP mutants may provide the necessary sensitivity to understand Nkd1 function. Along these lines, we evaluate how Nkd1 functions in several zebrafish Wnt mutant lines (canonical and non-canonical) and find that Nkd1 has the greatest activity in silberblick (slb/wnt11) and trilobite (tri/vangl2) mutants, both of which are specific for the Wnt/Planar Cell Polarity pathway. These mutant lines are sensitive to canonical Wnt signaling, but Nkd1 can rescue this sensitivity.

**Results**

We have previously determined that Nkd1 is both necessary and sufficient to antagonize both canonical and non-canonical Wnt signaling [12]. However, in comparison to other Wnt antagonists such as Axin2 and Dkk1, the effect of Nkd1 is subtle [28–30]. As shown in Figure 1, overexpression or knockdown of Nkd1 does not have an overt phenotype during somitogenesis (Figure 1A–F) or at 1 dpf (G–I). If knockdown of Nkd1 resulted in dorsalization of the embryo, we would have observed a characteristic elongated and ovoid morphology during early somitogenesis,

![Figure 1. Nkd1 does not influence normal development and patterning of the early embryo.](image)

**Figure 1.** Nkd1 does not influence normal development and patterning of the early embryo. Injection of nkd1 mRNA or morpholino (MO) does not have an obvious affect during early somitogenesis (A–F) or at 1 dpf (G–I). The number and width of somites in injected individuals is indistinguishable from uninjected embryos. At 1 dpf, injection of nkd1 MO results in neural necrosis, which ranges from moderate (H) to more severe (I). doi: 10.1371/journal.pone.0074666.g001

![Figure 2. Nkd1 is sufficient to antagonist ectopic Wnt8a.](image)

**Figure 2.** Nkd1 is sufficient to antagonize ectopic Wnt8a. Overexpression of Wnt8a (25pg) results in an eyeless phenotype that can be rescued by co-injection of nkd1 (A, B) which is quantified in (C). Numbers above each column represent n values. Overexpression of high Wnt8a (200pg) results in ectopic gsc expression along the ventral-lateral domain at 50% epiboly (E). Co-injection of high wnt8a with nkd1 mRNAs dramatically reduces the ectopic gsc expression, but leaves the putative endogenous gsc domain intact (F).

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Similarly, Wnt8 can induce ectopic distribution of phenotypes in significant differences between Nkd1 overexpression, Nkd1 spectrum of dorsal-ventral (D–V) phenotypes ranging from a doi: 10.1371/journal.pone.0074666.g003
is quantified in (D), with numbers above each column representing n values. Consistent with the 1 dpf phenotype, varying degrees dependent on if it is endogenous or extra-D-V, are toxic beginning at late epiboly, which is also effectively reduced by the addition of Nkd1 (Figure 3E–G; Exp +Nkd n=34). It is unknown if this N-terminal region of β-catenin is required for interaction with Nkd1.
To further explore the function of Nkd1, we tested whether Nkd1 was sufficient to inhibit ectopic Wnt signaling in the headless (hdltcf7l1a (tcf3)) mutant, which has a mutation in the tcf7l1a transcription factor gene. In the absence of β-catenin, tcf7l1a is a transcriptional repressor and only in the presence of activated Wnt signaling and stabilized β-catenin is the inhibition released and transcription initiated. However, a frameshift mutation in the hdl mutant results in a truncated protein that can still bind β-catenin but cannot bind DNA [33]. As such the hdl mutant mimics the Wnt8a overexpression phenotype in that both lack eyes (Figure 2A c/w Figure 4D). Knockdown of Nkd1 or overexpression of Nkd1 has no effect on the overall development of the early hdl mutant embryo (Figure 4A–C). At 1 dpf, ectopic Nkd1 results in a kinked axis phenotype, but no rescue of the eyeless phenotype (Figure 4E). Knockdown of Nkd1 has no effect other than slight necrosis at 1 dpf (Figure 4F). This data is consistent with the role of Nkd1 acting between the ligand-receptor complex and the nuclear accumulation of β-catenin [13].

Wnt/PCP mutants are sensitive to Nkd1
Previously we determined that Nkd1 can inhibit non-canonical Wnt/PCP signaling independent of Nkd1’s role in canonical Wnt/β-catenin signaling [12]. Furthermore, it is well established that the Wnt/PCP pathway can antagonize canonical Wnt/β-catenin signaling [23–27]. Dependent on where in the signaling cascade this antagonism occurs, we would predict that some Wnt/PCP mutants would be more sensitive than others to alterations in canonical Wnt signaling. Coincident with this, homeostatic levels of canonical Wnt signaling may fluctuate more in Wnt/PCP mutants than in wild type embryos, which in turn may uncover a more active role for Nkd1. There are several lines of evidence supporting the sensitivity of Wnt/PCP mutants to canonical Wnt signaling [24,34–36]. For example in Drosophila, the vacuolar (V)-ATPase proton pump (VhaPRR) is required in the Wnt/PCP

Figure 3. Nkd1 is insufficient to antagonize constitutively active β-catenin. Overexpression of ∆N-β-catenin results in a spectrum of dorsal-ventral (D–V) phenotypes ranging from a severe phenotype (A), which shows dramatic reduction in both dorsal and ventral structures to a moderate phenotype (B), which have reduced dorsal and ventral structures. The addition of Nkd1 does not ameliorate the effect of ∆N-β-catenin. The distribution of phenotypes in ∆N-β-catenin and nkd1 injections is quantified in (D), with numbers above each column representing n values. Consistent with the 1 dpf phenotype, ∆N-β-catenin overexpression results in expansion of gsc expression (E, F), which is not reduced by the addition of Nkd1 (G) (unij n=40; ∆N-β-catenin n=32; ∆N-β-catenin+nkd1 n=34).

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characteristic of increased canonical Wnt signaling [31,32]. Unfortunately, morpholinos targeting nkd1, either at the start ATG or at a splice site, are toxic beginning at late somitogenesis, which is obvious by the neural necrosis at 1 dpf (Figure 1I). Despite this, there do not appear to be any significant differences between Nkd1 overexpression, Nkd1 knockdown and uninjected embryos at 1 dpf.

While Nkd1 has little apparent affect on overall development, it is still sufficient to antagonize Wnt signaling, although to varying degrees dependent on if it is endogenous or exogenous Wnt signaling (Figure 2). Overexpression of Wnt8 in zebrafish embryos results in an eyeless phenotype at 1 dpf, which is effectively rescued by Nkd1 and overall development appears normal, albeit with a smaller eye (Figure 2A–C). Similarly, Wnt8 can induce ectopic gsc expression at 50% epiboly, which is also effectively reduced by the addition of Nkd1 (Figure 2D–F). Note however, that there is also a subtle effect on endogenous Wnt signaling: expansion of dorsal gsc
pathway, but was also found to restrict the expansion of Wg morphogen [34]. In zebrafish, the maternal-zygotic wnt5a mutant, pipetail (ppt), has a highly dorsalized phenotype [24] and loss of vangl2 (strabismus or tri mutants) results in embryos highly sensitive to canonical Wnt signaling [35,36]. Importantly, in these zebrafish mutants, activation of, or sensitivity to, canonical Wnt signaling (or dorsalization) occurs before the onset of gastrulation, strongly suggesting that Wnt/PCP specific components regulate canonical Wnt signaling prior to activation of the Wnt/PCP pathway.

To determine if Nkd1 has a more active role in Wnt/PCP mutants, we overexpressed and knocked down Nkd1 function in several Wnt/PCP mutants. Knockdown of Nkd1 in embryos from wnt11-/- (silberblick, slb) homozygous parents did not have a dorsalizing effect on somitogenesis stage embryos (Figure 5A, B). At later stages, the necrotic effect of the nkd1 MO prevented accurate analysis (not shown). However, by measuring the width of gsc expression at 30% epiboly, Nkd1 MO resulted in a slightly expanded organizer (Figure 5G, H), consistent with our previous results in wild type embryos [12].

In contrast, overexpression of Nkd1 resulted in a reduction in expression of both gsc and chd genes at 50% epiboly, more so than observed in wild type embryos (Figure 5C–F) [12]. As we have previously shown, overexpression of Nkd1 in slb mutants resulted in increased cyclopia at 1 dpf [12] (and not shown), consistent with its role in Wnt/PCP signaling. Thus, the reduced gsc and chd expression in these mutants in the presence of excess Nkd1 suggest it may have a more active role in regulating canonical Wnt signaling in the dorsal organizer in this Wnt/PCP mutant compared to wild type embryos. This is because in wild type embryos we mainly observe decreased gsc/chd expression only when Nkd1 is injected early, prior to fertilization, affecting maternal Wnt/β-catenin signaling. In more standard 1-2 cell stage injections, overexpression of Nkd1 results in expanded gsc/chd expression due to its ability to antagonize zygotic, ventral-lateral Wnt8 signaling [12]. Interestingly, even though Nkd1 was sufficient to reduce the expression of gsc/chd in slb mutants, both markers of the dorsal organizer, we did not observe ventralized embryos at 1 dpf. This is likely due to the short half-life of Nkd1, which would

Figure 4. Nkd1 does not rescue the hdl/tcf71a mutant. Homozygous deletion of tcf71a results in an eyeless phenotype due to activated canonical Wnt signaling (D). Overexpression of Nkd1 in embryos from a homozygous hdl-/- parental cross (B, E) does not rescue the eyeless phenotype (E) and does not affect development of the early embryo (B), although at 1 dpf, nkd1 injected embryos typically have a kinked axis. Injection of nkd1 MO into embryos from a hdl +/- X hdl +/- parental cross has no affect on early (C) or 1 dpf (F) development (Uninj hdl +/- n= 14, hdl +/- n= 16; Nkd1 MO injected hdl +/- n= 15, hdl +/- n=22). Experiments in embryos from homozygous hdl-/- parental crosses had similar results (not shown).

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Figure 5. The silberblick (Wnt11) mutant is sensitive to Nkd1. The slb mutant undergoes normal convergence and extension (A), which is not affected by knockdown of Nkd1 with morpholinos (B). Overexpression of Nkd1 in slb-/- reduces gsc (D) and chd (F) expression (arrows), relative to controls (C, E) at 50% epiboly (Nkd1 injected: 47% of embryos with reduced chd expression (n=81); 50% of embryos with reduced gsc expression (n=46)). In contrast, knockdown of Nkd1 results in a slight expansion of gsc expression at 30% epiboly (G: ave gsc width=0.35 mm; n=13, H: ave gsc width=0.38 mm; n=22). All embryos are homozygous slb, derived from homozygous slb parents.

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allow gsc and chd expression to recover later in development [37] (data not shown).

We also tested the sensitivity of this mutant to canonical Wnt signaling. Overexpression of Wnt8a resulted in a slight increase in organizer gene expression but had other curious effects at 1 dpf. In multiple experiments, 50% of the embryos died by 1 dpf and of the 50% that remained, 37% had no eye whereas wild type embryo controls had 75% with no eye (slb n=69; wt n=92). The surviving embryos also had varying degrees of dorsalization and slight rescue of cyclopia (not shown). Taken together, this suggests that slb mutant embryos are more sensitive to canonical Wnt signaling and that this sensitivity increases the antagonistic activity of Nkd1. Furthermore, Wnt8 may also affect the Wnt/PCP pathway, which is currently being investigated.

We next tested the glypican mutant, knypek/glypican 4 (kny/gpc4). Overexpression or knockdown of Nkd1 did not have any effect on the development or morphology of somitogenesis stage or 1 dpf embryos (aside from MO toxicity) (Figure 6). In addition, overexpression of wnt8a resulted in an eyeless phenotype similar to injections into wild type embryos but otherwise Wnt8a had no effect on the kny phenotype and thus we concluded that the kny mutant is not sensitive to canonical Wnt signaling. Due to this lack of phenotype we did not determine if Nkd1 or Wnt8a affected organizer gene expression.

Lastly we determined the effect of Nkd1 in the trilobite/vang-like 2 tri/vangl2 (also known as strabismus/stbm) mutant, which has a mutation in the transmembrane vangl2 gene [36,38]. Vangl2 is specific for Wnt/PCP and is involved in cell polarity and directed cell migration [38–41]. Knockdown of Nkd1 or overexpression of Nkd1 does not appear to affect development of the early somitogenesis stage embryo (Figure 7 A–J). However, overexpression and knockdown of Nkd1 results in increased cyclopia (Figure 7 K,L,O). Cyclopia results from decreased extension of the axial mesoderm and is a classical Wnt/PCP phenotype (eg. Slb/Wnt11) [42,43]. It is also well established that inhibiting or overactivation of Wnt/PCP gives similar phenotypes, so observing similar effects with knockdown and overexpression of Nkd1 in tri mutants is consistent with Nkd1 affecting Wnt/PCP signaling [42,44,45]. Thus, similar to slb mutants, Nkd1 affects the expressivity of the Wnt/PCP mutant phenotypes, but in the case of kny, Nkd1 has no effect at all.

Because Vangl2 has been implicated in regulating canonical Wnt signaling we looked at the ability of ectopic Nkd1 to reduce the expression of dorsal organizer markers. Consistent with our results using the slb mutant, we observed a significant decrease in gsc gene expression in more than 50% of the embryos obtained from tri heterozygote crosses injected with nkd1 mRNA (Figure 7 M, N). Considering we crossed heterozygote tri parents, we expected 25% of the embryos (tri-/-) to have a phenotype, the remaining 75% (tri+/-; +/+) to be similar to nkd1 injections into wild type embryos and have slightly expanded gsc expression [12]. These results suggest that heterozygous tri embryos are haploinsufficient for the tri gene with respect to its ability to regulate canonical Wnt signaling, but the genotype of the individual embryos has not been determined. The effect of Nkd1 on dorsal gene expression in tri mutants is similar to, but greater than, that observed in slb mutants from homozygous mutant parental crosses. We conclude that Nkd1 function in canonical Wnt signaling is more active in vangl2 mutants compared to slb mutants and wild type embryos, potentially as a result of

**Figure 6. The knypek (glypican 6) mutant is not sensitive to Nkd1.** Knockdown of Nkd1 in kny -/- (C, D) or in kny +/-; +/- (G, H) does not have any affect on the kny mutant phenotype during somitogenesis (A–H) or at 1 dpf (I; n=19 kny +/-). Note the high levels of neural necrosis in nkd1 MO injected embryos at 1 dpf (I). Consistent with the lack of sensitivity, overexpression of Nkd1 has no obvious effects during early somitogenesis (J–M) or at 1 dpf (N; n=31 kny -/-). There is no change in the ratio of wild-type: mutant phenotypes.

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reduced Wnt/PCP control over canonical Wnt/β-catenin signaling. Curiously, and as mentioned above for the slb mutants, the majority of the embryos with reduced or absent gsc expression must recover later in development, because we did not observe significant D–V patterning defects at 1 dpf (Figure 7L).

To explore the function of Nkd1 and the sensitivity of vangl2 mutants further, we overexpressed the canonical Wnt ligand Wnt8a. A dose of wnt8a that causes an eyeless phenotype in 75% of wild type embryos, but no dorsalization (Figure 2 A,C) was lethal to the majority of embryos from a tri +/- X tri+/+ parental cross are sensitive to Nkd1 overexpression, demonstrated by a reduction or absence of gsc expression (M; n=32, N; 56% of embryos with reduced expression, n=25). (O) The cyclopic index was calculated using criteria established in Marlow et al., 1998 [43]. n values reflect the number of tri/- embryos. Error bars represent standard error.

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Figure 7. Trilobite (vangl2) mutants are sensitive to Nkd1. Knockdown of Nkd1 with morpholinos (D, E) or overexpression of Nkd1 (I, J) does not have an obvious effect during early somitogenesis. However, at 1 dpf, knockdown or overexpression of Nkd1 in tri mutants results in an increase in cyclopia (K, L, O). Before the onset of gastrulation, at 50% epiboly, embryos generated from a tri +/- X tri+/+ parental cross are sensitive to Nkd1 overexpression, demonstrated by a reduction or absence of gsc expression (M; n=32, N; 56% of embryos with reduced expression, n=25). (O) The cyclopic index was calculated using criteria established in Marlow et al., 1998 [43]. n values reflect the number of tri/- embryos. Error bars represent standard error.

To further explore the sensitivity of tri to canonical Wnt signaling, we used antisense oligonucleotide morpholino’s to knockdown Vangl2 protein levels, which effectively recapitulates the tri phenotype [35,38] and data not shown. Co-injection of vangl2 MO with wnt8a results in significant lethality by 1 dpf (not shown), similar to the effect of Wnt8a in tri mutants. We also determined the effect on dorsal-ventral patterning by assaying the expression of gsc. Injection of vangl2 MO or wnt8a individually did not have a significant affect on the size of the gsc expression domain (Figure 8J, K, M), which is not surprising considering we did not observe wnt8a, approximately 15% of the embryos had a strong dorsalization phenotype which phenocopies the maternal wnt5a mutant, ppt (Figure 8C, I) [24]. Therefore, we hypothesized that there would be a significant increase in dorsal gene expression in wnt8a overexpressing embryos, but the levels of gsc and boz, a direct target of maternal Wnt signaling [18], were relatively normal at 40% epiboly (Figure 8E–H).
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changes in gsc or boz expression in the tri mutants. However, co-injection of vangl2 MO with wnt8a would occasionally result in ectopic gsc (2-5%), but the endogenous domain was not significantly perturbed (Figure 8L). This inconsistency between highly dorsalized 1 dpf embryos and a lack of increase in dorsal gene expression, while surprising, is consistent with previous reports. Knockdown of Rack1, which is required for Vangl2 membrane localization and inhibition of canonical Wnt signaling, also had low percentages of ectopic chordin (dorsal gene expression, while surprising, is consistent with previous reports. Knockdown of Rack1, which is required for Vangl2 membrane localization and inhibition of canonical Wnt signaling, also had low percentages of ectopic chordin (dorsal gene expression, while surprising, is consistent with previous reports. 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advantage of the Wnt/PCP mutant sensitivity to canonical Wnt signaling to determine exactly how Nkd1 functions to antagonize Wnt signaling and how the Wnt/PCP pathway restricts/reduces or inhibits Wnt/β-catenin signaling. In conclusion, our results support a model whereby Nkd1 acts as a passive Wnt antagonist. Under homeostatic levels of Wnt signaling, Nkd1 becomes an active and potent inhibitor of Wnt signaling.

Methods

Ethics Statement

All animal work has been done in accordance with national and international guidelines and has been approved by the University of Guelph Animal Care Committee AUP #1295.

Fish maintenance

Adult zebrafish and embryos were raised at 28.5°C and were staged by anatomical criteria or hours or days post fertilization (hpf and dpf, respectively) according to Kimmel et al [49]. Maternal zygotic or zygotic hdl, sib2[51], knyL199, tri2[207] or tri1747, knyN[119] homozygous or heterozygous embryos (all in AB* backgrounds) were collected from pairwise matings of homozygous or heterozygous adults, respectively.

Whole-mount in situ hybridization

Staged embryos were fixed overnight at 4°C in phosphate-buffered saline solution containing 4% paraformaldehyde. In situ hybridization was carried out as described by Thissee et al. (1999). The following antisense digoxigenin-labeled probes were used: chordin [50], goosecoid [51,52] and boozok [18]. Probes were synthesized using T7, T3, or SP6 RNA polymerases (Ambion) and precipitated with LiCl and EtOH and resuspended in RNase free. by spectrophotometry and quality was assayed using gel electrophoresis.

mRNA and morpholino oligonucleotide injections

Capped mRNA was synthesized using Ambion’s mMessage mMachine kit. Following transcription, the RNA was purified over a G-50 sephadex column (Roche) and diluted in RNase free water, and its quantity and quality was analyzed as described above. The following mRNA concentrations were used: wnt8a [32,53]; Figure 2E, 200 pg; Figure 2A–C, 25 pg; Figure 8L, M, N, 25 pg; 25 pg for kny and sib mutant injections (not shown); Figure 8A–L, 5 pg; ∆N-β-catenin 10pg; nkd1 mRNA: 800 pg, except for Figure 3 G: 1600pg; nkd1 ATG MO 4 ng (5’-GAAGTTTACCCATTTCTCTGCATCG-3’); Vangl2 MO has been previously described [36]. 4 ng of Vangl2 MO was used. MO’s and mRNA’s were pressure injected into the yolk cell of one- to two-cell-stage embryos. Antisense morpholino oligonucleotides (MOs) were dissolved and diluted in water containing 1% phenol red. At all but the 0.5 ng dose tested for the Nkd1 MO, there was non-specific necrosis by mid-segmentation stages. All experiments were repeated at least once.

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Author Contributions

Conceived and designed the experiments: TVR. Performed the experiments: DA TVR. Analyzed the data: DA TVR. Contributed reagents/materials/analysis tools: TVR. Wrote the manuscript: TVR.

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