Wnt ligand–dependent activation of the negative feedback regulator Nkd1

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ABSTRACT Misregulation of Wnt signaling is at the root of many diseases, most notably colorectal cancer, and although we understand the activation of the pathway, we have a very poor understanding of the circumstances under which Wnt signaling turns itself off. There are numerous negative feedback regulators of Wnt signaling, but two stand out as constitutive and obligate Wnt-induced regulators: Axin2 and Nkd1. Whereas Axin2 behaves similarly to Axin in the destruction complex, Nkd1 is more enigmatic. Here we use zebrafish blastula cells that are responsive Wnt signaling to demonstrate that Nkd1 activity is specifically dependent on Wnt ligand activation of the receptor. Furthermore, our results support the hypothesis that Nkd1 is recruited to the Wnt signalosome with Dvl2, where it becomes activated to move into the cytoplasm to interact with β-catenin, inhibiting its nuclear accumulation. Comparison of these results with Nkd function in *Drosophila* generates a unified and conserved model for the role of this negative feedback regulator in the modulation of Wnt signaling.

INTRODUCTION

Deregulation of Wnt signaling is at the core of many diseases, propelling this pathway into the spotlight as an important therapeutic target (Clevers and Nusse, 2012; Robertson *et al.*, 2014). In the Wnt signaling pathway (Wg in *Drosophila*), the scaffolding protein Dishevelled (Dsh in *Drosophila*; Dvl in vertebrates) is the only known protein whose activity is entirely Wnt ligand dependent. In canonical Wnt/Wg signaling, activated Dvl/Dsh inhibits the destruction complex, leading to transcription of numerous target genes, including negative feedback regulators (Jho *et al.*, 2002; Niida *et al.*, 2004; Zeng and Verheyen, 2004; Chamorro *et al.*, 2005; Van Raay *et al.*, 2007; Schneider *et al.*, 2010). Naked Cuticle (Nkd in *Drosophila*; Nkd1 in vertebrates) is an obligate and universal Wnt/Wg-induced negative feedback regulator that interacts with Dsh/Dvl (Rousset *et al.*, 2001; Wharton *et al.*, 2001; Yan *et al.*, 2001; Van Raay *et al.*,

2007; Schneider *et al.*, 2010). Whereas Nkd/Nkd1 is both necessary and sufficient to antagonize Wnt signaling, it functions in a pathway parallel to other negative regulators, such as Axin and Axin2 (Kishida *et al.*, 1998; Liu *et al.*, 2000; Zeng *et al.*, 2000; van de Water *et al.*, 2001; Leung *et al.*, 2002; Van Raay *et al.*, 2007; Angonin and Van Raay, 2013). This is underscored by the moderate to weak phenotypes in Nkd/Nkd1/2-knockout or -knockdown models and the lack of phenotypes when overexpressed (Zeng *et al.*, 2000; Van Raay *et al.*, 2007; Zhang *et al.*, 2007; Angonin and Van Raay, 2013). This novel aspect of Nkd1 suggests that its function is regulated or triggered only under specific circumstances, such as when a threshold of Wnt signaling has been breached (Zeng *et al.*, 2000; Van Raay *et al.*, 2007; Angonin and Van Raay, 2013). Therefore understanding the nature of Nkd/Nkd1 activity will lead to a better understanding of Wnt regulation and how to control it in disease.

Previously we demonstrated that Nkd1 interacts with β-catenin, a transcriptional coactivator that accumulates in the nucleus upon Wnt activation. Furthermore, this interaction requires Nkd1 membrane localization via its myristoylation sequence, but, curiously, Nkd1 functions in the cytoplasm to inhibit the nuclear accumulation of β-catenin (Van Raay *et al.*, 2011). The significance of the membrane localization of Nkd1 is unclear. In *Drosophila*, Nkd is localized to the membrane, the cytoplasm, and the nucleus, but how Nkd inhibits Wg signaling is also unclear. Whereas Nkd/Nkd1 interacts with Dsh/Dvl proteins in both vertebrates and invertebrates, work in *Drosophila* has demonstrated that this interaction is not required for

FIGURE 1: Cellular distribution and size of Nkd1 puncta correspond to regions of active Wnt signaling. (A–D) Injection of Nkd1^{GFP} mRNA into one of four blastomeres results in its mosaic expression in the animal pole and the V-L domain of the zebrafish blastula at 50% epiboly (A). The size of Nkd1 puncta and their distribution are different between the lateral edges and central part (animal pole) of the blastula (inset). (B) At this stage, there is active Wnt signaling along

Nkd function. The latest model for *Drosophila* suggests that the function of Dsh is to keep Nkd in the cytoplasm, where Nkd likely functions by controlling nucleocytoplasmic transport of critical signaling components such as Armadillo (Arm, β-catenin orthologue in *Drosophila*; Chan *et al.*, 2008). One of the earliest observations in *Drosophila* is that Nkd functions during active Wg signaling (Zeng *et al.*, 2000); however, the dependence of Nkd activity on Wnt ligand–mediated signaling has not been tested in this model. Here we add to the *Drosophila* model by demonstrating that in a vertebrate model, Nkd1 distribution, activity, and interaction with β-catenin are dependent on Wnt ligand–mediated signaling. We conclude that the mechanism of Nkd/Nkd1 function is evolutionarily conserved and may represent a novel target for disabling aberrant Wnt signaling in disease.

RESULTS

We first identified a novel distribution of ectopic, C-terminal green fluorescent protein (GFP)-tagged Nkd1 (Nkd1^{GFP}) upon overexpression in late zebrafish blastula (Figure 1, A–D). In the late blastula, there is an active Wnt signaling domain around the ventrolateral (V-L) perimeter of the embryo that is induced by Wnt8 (Figure 1B; Kelly *et al.*, 1995; Erter *et al.*, 2001; Lekven *et al.*, 2001; Brunet *et al.*, 2013). In contrast, the animal pole is Wnt signaling quiescent but is still responsive to ectopic Wnt signaling (Van Raay *et al.*, 2011). Injection of *nkd1^{GFP}* mRNA into one cell of a four-cell-stage embryo results in mosaic distribution of Nkd1^{GFP}. In the V-L domain, which is engaged in active Wnt signaling (Figure 1B, inset), Nkd1^{GFP} is observed on the membrane and in numerous puncta of various sizes located throughout the cytoplasm (Figure 1A, inset). By comparison, in the animal pole region, which is devoid of Wnt signaling (i.e., no nuclear β-catenin), Nkd1^{GFP} puncta appear much larger and not as uniformly distributed (Figure 1, A, inset, and D). This change in the size and number of Nkd1^{GFP} puncta in the presence of Wnt8 is emphasized in the projection image of Nkd1^{GFP} (Figure 1, F and G) and in live-cell imaging of Nkd1^{GFP} (Supplemental Movies S1 and S2). In the presence of Wnt8, the Nkd1^{GFP} puncta are smaller and more dispersed throughout the cytoplasm and appear more dynamic (Supplemental Movie S1). In contrast, without ectopic Wnt8, Nkd1^{GFP} puncta are large and more closely associated with the membrane (Supplemental Movie S2). Taken together, these observations suggest a strong correlation between the cellular distribution of Nkd1 and active Wnt signaling.

We analyzed in detail the effect of Wnt8 on punctum size and distribution. We coinjected *nkd1^{GFP}* mRNA with or without *wnt8* mRNA and quantified Nkd1^{GFP} punctum size and distribution in the animal pole (Figure 2). In the presence of Wnt8, Nkd1^{GFP} puncta are significantly smaller than with Nkd1^{GFP} alone (Figure 2I), and there is a trend for Nkd1^{GFP} + Wnt8–positive cells to have more cytoplasmic puncta (Figure 2, C, G, J, and K). Furthermore, ectopic Wnt8 results in decreased membrane localization of Nkd1^{GFP} (Figure 2, D, H, and L). Of importance, Wnt8 does not affect the levels of Nkd1^{GFP} (Figure 2M). Therefore we conclude that in the presence of Wnt8, Nkd1^{GFP} is released from the

membrane, forming smaller puncta that are concentrated in the cytoplasm.

The nature of these puncta or aggregates is unknown, but they are also observed with both endogenous and exogenous Nkd in *Drosophila* (Waldrop *et al.*, 2006). Nkd1 puncta are also reminiscent of oligomeric Dvl and Axin (Schwarz-Romond *et al.*, 2005, 2007). We and others have demonstrated that both endogenous and exogenous Dsh/Dvl bind exogenous Nkd/Nkd1 (Zeng *et al.*, 2000; Rousset *et al.*, 2001, 2002; Wharton *et al.*, 2001; Yan *et al.*, 2001; Miller *et al.*, 2009; Van Raay *et al.*, 2011), and these proteins colocalize in puncta when overexpressed (Waldrop *et al.*, 2006; Van Raay *et al.*, 2011). Despite these results, we do not observe strong colocalization between Nkd1^{myc} and endogenous Dvl2 by immunohistochemistry (IHC) (Figure 3, A–D). However, in the presence of ectopic Wnt8, there are obvious plasma membrane–bound Dvl2 puncta that colocalize with Nkd1^{myc} (Figure 3, E–H). Dvl2 was recently found to localize to discrete plasma membrane domains in the presence of Wnt8, indicative of a Wnt signalosome (Bilic *et al.*, 2007; Hagemann *et al.*, 2014). Taken together, these results suggest that the Wnt8-induced Dvl2-Nkd1^{myc} plasma membrane puncta are active sites of Wnt signaling. This supports our hypothesis that the distribution of Nkd1 is altered by Wnt ligands, potentially via recruitment to the Wnt signalosome by Dvl2.

Thus far, our results suggest that the Wnt ligand is responsible for the cellular distribution of Nkd1. We next hypothesized that the Wnt ligand activates Nkd1 antagonism. An alternative hypothesis is that simply increasing the concentration of cytoplasmic β-catenin, independent of the ligand, may be sufficient to activate Nkd1, as we previously demonstrated an interaction between these two proteins (Van Raay *et al.*, 2011). Therefore we set out to distinguish between Wnt ligand–dependent activation of Nkd1 and ligand-independent activation of Nkd1. To determine whether Nkd1 function was dependent on increased levels of cytoplasmic β-catenin or Wnt ligand–mediated signaling specifically, we tested the ability of Nkd1 to antagonize Wnt signaling induced downstream of the ligand–receptor complex, using constitutively active LRP6 (LRP6$^{\Delta N}$), which stabilizes cytoplasmic β-catenin independent of Wnt ligands and Frizzled receptors (Brennan *et al.*, 2004). Overexpression of LRP6$^{\Delta N}$ resulted in ectopic expression of the Wnt target genes *dkk1*, *chd*, and *gsc* in the late-blastula zebrafish embryo (Figure 4, A and B, and Supplemental Figure S1). Whereas Nkd1 is very efficient at blocking ectopic Wnt ligand–mediated signaling (Zeng *et al.*, 2000; Rousset *et al.*, 2001; Wharton *et al.*, 2001; Yan *et al.*, 2001; Van Raay *et al.*, 2007, 2011; Chan *et al.*, 2008), Nkd1 was not able to rescue the effect of LRP6$^{\Delta N}$ (Figure 4, A and B, and Supplemental Figures S1 and S2). We next tested our hypothesis that the addition of Wnt8 would activate Nkd1 to inhibit LRP6$^{\Delta N}$-induced ectopic *dkk1*, *chd*, and *gsc*. Indeed, the combination Wnt8 + LRP6$^{\Delta N}$ + Nkd1 resulted in dramatically reduced gene expression for all three probes (Figure 4, A and B, and Supplemental Figure S1). These results strongly suggest that Nkd1 requires the presence of a Wnt ligand to antagonize Wnt signaling induced by LRP6$^{\Delta N}$. In addition to Wnt signaling, *chd* and *gsc* are also transcriptionally regulated by Nodal signaling

the V-L domain, as demonstrated by increased levels of cytoplasmic and nuclear β-catenin. Inset in gray scale is magnified view of the boxed region, demonstrating differences in β-catenin immunostaining between animal pole and V-L domain. The white line in A–C delineates the boundary between active Wnt-signaling and -quiescent cells based on nuclear β-catenin. (C) The distribution and punctum size of Nkd1^{GFP} are altered in the V-L domain compared with the animal pole. (E) The boxed region magnified for clarity . Visualization of the Nkd1^{GFP} particle size also demonstrates a distinct difference between these two domains (D). (F, G) Projection images of Nkd1^{GFP} (F) and Nkd1^{GFP} + Wnt8 (G) injected into one of four blastomeres and harvested for confocal microscopy at 30% epiboly. Scale bar, 50 μm (A–C), 20 μm (E, insets in A, B), 10 μm (F, G).
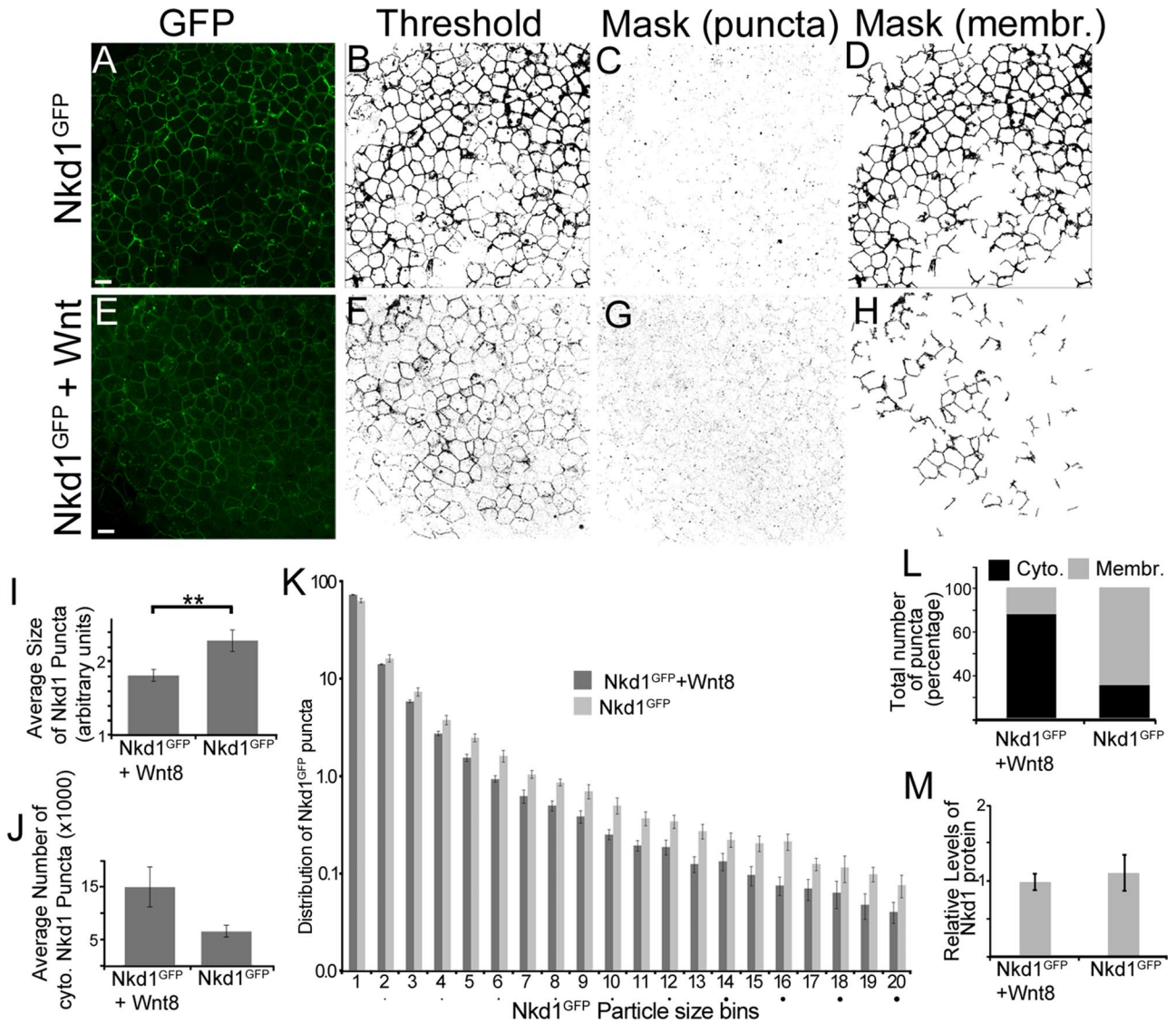
FIGURE 2: Wnt signaling converts membrane Nkd1 and large cytoplasmic Nkd1^{GFP} puncta into smaller, more-cytoplasmic puncta. (A–H) *nkd1^{gfp}* mRNA was injected at the one-cell stage, with half of the embryos also being injected with *wnt8* mRNA (E–H; views are of the animal pole). Embryos were harvested at 30–40% epiboly and GFP imaged by confocal microscopy in the animal pole of the embryo. All unmodified images were analyzed using ImageJ software for particle size (threshold, 26–255; particle analysis, 0–∞; circularity, 0.5–1). In the absence of Wnt8, Nkd1^{GFP} shows robust expression at the plasma membrane (D) and the presence of large puncta (C) compared with cells receiving ectopic Wnt8 (G, H). The average size of the puncta is significantly smaller in the presence of Wnt8 (I; Student's *t* test, *p* = 0.005, *n* = 6 for each). Analysis of just the circular puncta represented in C and G (circularity, 0.5–1.0) demonstrates that there are more cytoplasmic puncta in the presence of Wnt8, but this was not statistically significant (J; *n* = 6 for each; Student's *t* test, *p* = 0.06). The large error bars reflect the wide range in the number of puncta counted for each embryo (Nkd1^{GFP} + Wnt range: 3392–29,373 puncta, *n* = 6; Nkd1^{GFP}-alone range: 3369–11,162 puncta, *n* = 6). To determine whether there is a bias in the analysis, we performed two addition analyses. First, we compared puncta of different sizes (K; *n* = 6 for each) and found that there were more of the smallest puncta in the Nkd1^{GFP} + Wnt8–treated embryos (bin 1). We also found that as the size of the puncta increased, so did their proportion in cells without ectopic Wnt8 compared to with Wnt8 treatment. We also combined all membrane-masked Nkd1^{GFP} with all puncta-masked Nkd1^{GFP} and found overall that Nkd1^{GFP} + Wnt8 has more cytoplasmic puncta and less membrane Nkd1^{GFP} than with Nkd1^{GFP} alone (L; *n* = 6). (M) Total protein levels of exogenous Nkd1 did not change in response to Wnt8, as determined by quantification by Western analysis (*n* = 6). Scale bar, 20 µm (A, E). Data are represented as mean ± SE.

(Erter *et al.*, 1998; Shimizu *et al.*, 2000). Furthermore, the size of the *gsc* and *chd* expression domains is the result of two counteracting Wnt signaling events (Kelly *et al.*, 1995; Ramel and Lekven, 2004; Van Raay *et al.*, 2007; Angonin and Van Raay, 2013). To restrict our analysis specifically to Wnt signaling and further quantify the differ-

ence between Wnt8 and LRP6^{ΔN}, we performed quantitative reversed transcribed-PCR (qRT-PCR) to determine changes in endogenous *nkd1* expression, as *nkd1* is an obligate and universal target of Wnt signaling (Wharton *et al.*, 2001; Van Raay *et al.*, 2007; Chang *et al.*, 2008). Similar to the WMISH analysis, LRP6^{ΔN} induced the

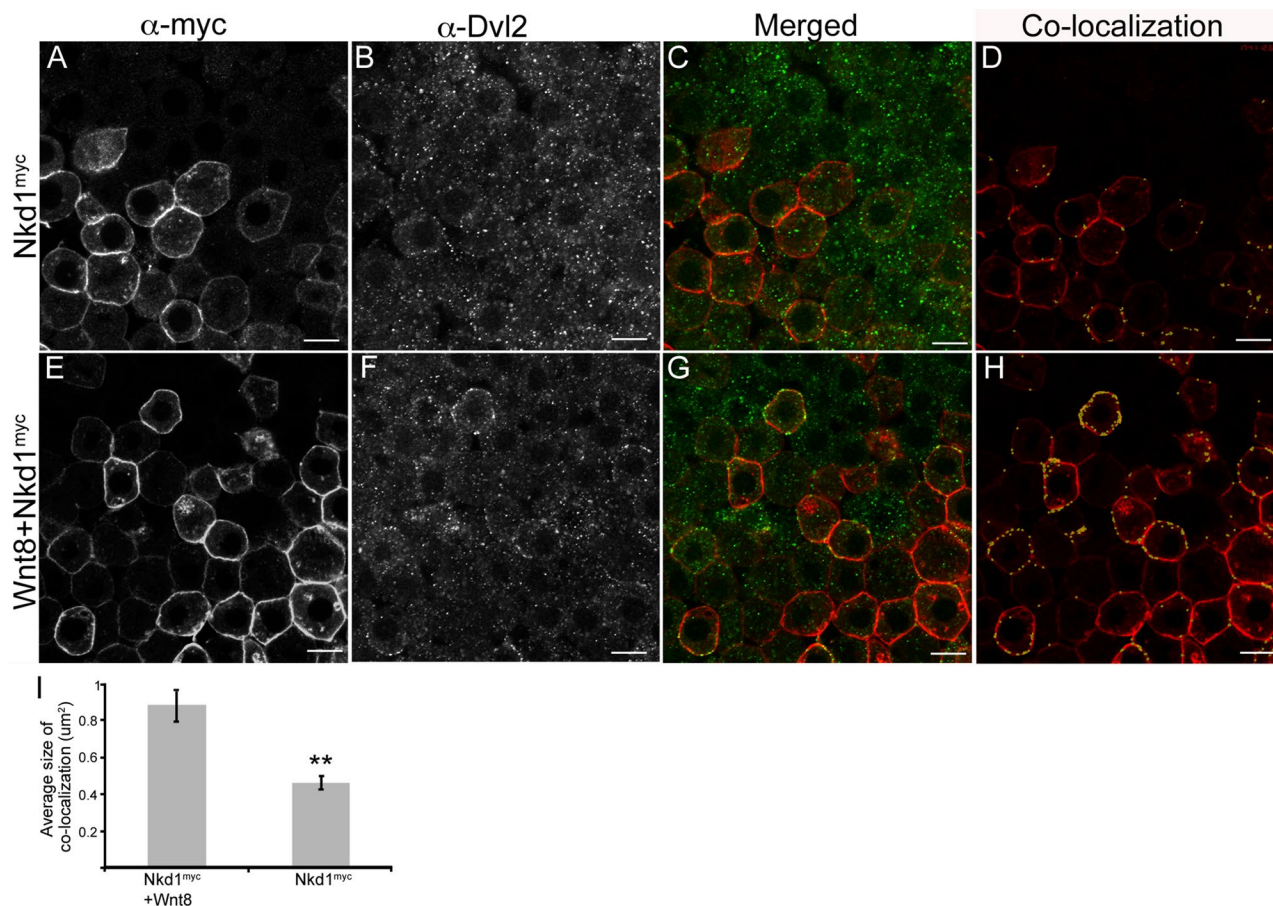|	α-myc	α-Dvl2	Merged	Co-localization

FIGURE 3: Wnt signaling induces Nkd1-Dvl2 signalosomes. (A–C) Mosaic expression of Nkd1^myc in the animal pole of 30% blastula-stage zebrafish embryo shows characteristic membrane-localized and cytoplasmic Nkd1 puncta (A). Costaining with endogenous Dvl2 antibodies (B) shows characteristic Dvl2 puncta but little colocalization with Nkd1^myc at the membrane or in the cytoplasm (C, D). Coexpression of Nkd1^myc with Wnt8 results in discreet domains of membrane-enriched localized Dvl2 puncta (G, H) that colocalize with Nkd1^myc (G). Yellow puncta in D and H represent sites of colocalization that were used to calculate the average size of colocalization puncta (I). Double asterisks denote significance by Student's *t* test (*p* < 0.005). Note that the majority of colocalization occurs on the membrane in both Wnt8- positive and -negative injections. Scale bar, 20 μm. Data are represented as mean ± SE.

expression of *nkd1*, which could not be reduced by ectopic Nkd1. Also consistent with the WMISH data, Nkd1 activity required the presence of Wnt8 to inhibit the expression of endogenous *nkd1* induced by LRP6^ΔN (Figure 4C). This suggests that Nkd1 is functional only in the presence of a Wnt ligand. In contrast, Axin2 is sufficient to inhibit LRP6^ΔN without the need of a Wnt ligand (Figure 4D).

Given that the distribution and activity of Nkd1 are dependent on Wnt ligand–mediated signaling, we predicted that Nkd1 would not be able to inhibit the nuclear accumulation of β-catenin induced by LRP6^ΔN. As we previously showed, mosaic expression of Nkd1^GFP plus Wnt8 results in nuclear accumulation of β-catenin in cells next to Nkd1^GFP-expressing cells but reduced nuclear β-catenin accumulation in GFP-positive cells (Figure 5, A–C; Van Raay et al., 2011). The ability of Wnt8 to signal in both GFP-positive and -negative cells is due to the non–cell-autonomous nature of the Wnt ligand. In contrast, LRP6^ΔN acts cell autonomously, similarly to Nkd1^GFP, but Nkd1^GFP was unable to prevent the nuclear accumulation of β-catenin induced by LRP6^ΔN (Figure 5, D–G). We did observe a difference in the cellular distribution of Nkd1^GFP in these cells: whereas the puncta appear smaller compared with Nkd1^GFP alone, LRP6^ΔN appears to increase the proportion of these puncta,

possibly stabilizing Nkd1^GFP. This observation supports our finding that the ability of Nkd1 to antagonize Wnt signaling is Wnt ligand dependent.

Previously we demonstrated that Dvl and β-catenin compete for binding to Nkd1 and that myristoylation of Nkd1 is important for the Nkd1–β-catenin interaction but not for the Nkd1-Dvl interaction (Van Raay et al., 2011). On the basis of the evidence thus far, we hypothesize that Wnt ligand–mediated activation of Nkd1 at the plasma membrane is necessary for it to interact with β-catenin. To test this, we first reevaluated the distribution of the Nkd1^GFP puncta with and without ectopic Wnt8. Without Wnt8, the puncta are large and localized near the plasma membrane. However, with the addition of Wnt8, the Nkd1^GFP not only are puncta smaller, but there is also a tendency to observe these puncta juxtaposed to the nuclear membrane (Figure 6, C and D), which was not observed without ectopic Wnt8 (Figure 6, A and B). This suggests that upon activation, Nkd1 not only becomes more cytoplasmic, but also is enriched in the perinuclear region. To evaluate this further, we looked for colocalization of cytoplasmic β-catenin and Nkd1^GFP. As a control, we used an N-terminal–bound GFP Nkd1 (Nkd1^N-GFP), which abolishes the myristoylation sequence and its function (Van Raay et al., 2011;
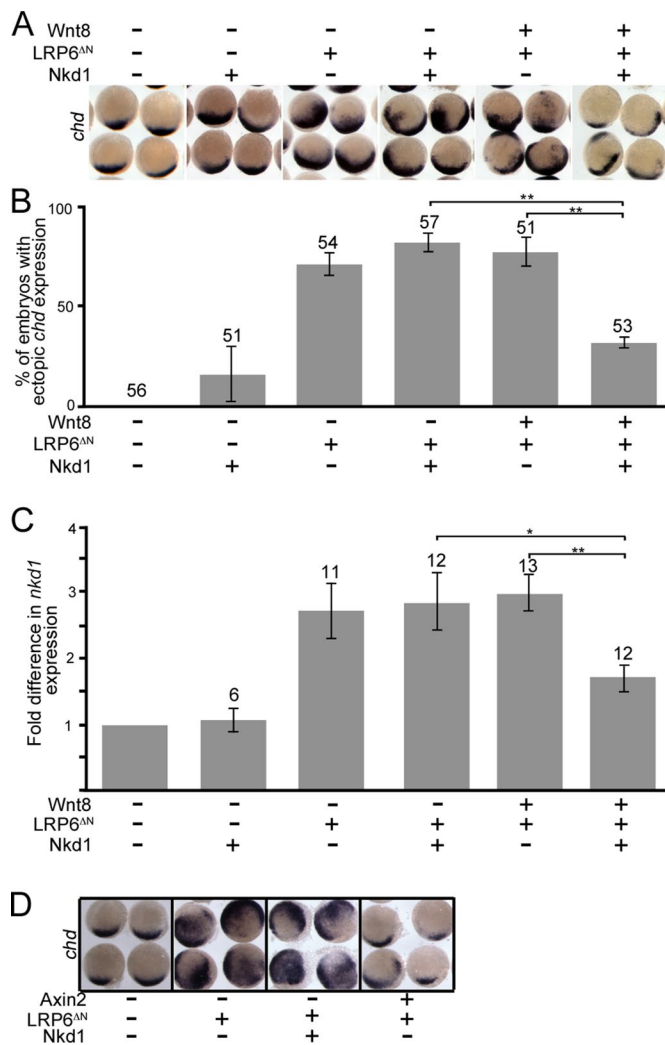
FIGURE 4: Nkd1 activity is dependent on Wnt ligand. (A) Whole-mount in situ hybridization with *chd* probe at 30–40% epiboly. (B) The changes in gene expression are quantified at the bottom, representing the percentage of embryos with ectopic *chd* expression. Numbers above the columns represent the total number of embryos analyzed from three independent experiments. Double asterisks denote significance by Student's *t*-test ($p < 0.005$). (C) Quantitative RT-PCR on embryos at 50% epiboly analyzed for endogenous *nkd1* expression (using the 5′ untranslated region). Numbers above the columns represent the number of experiments (each performed in triplicate) used in the analysis. Asterisks denote significance by Student's *t* test (*$p = 0.026$; **$p = 0.001$). (D) In contrast to Nkd1, Axin2 does not require Wnt ligand–mediated signaling, as it is sufficient to reduce the ectopic expression of *chd* induced by LRP6$^{\Delta N}$ alone. Data are represented as mean ± SE. The complete data set is shown in Supplemental Figure S1.

unpublished data). In the majority of cells, it is difficult to detect cytoplasmic puncta of β-catenin, but in the rare cells in which we can detect it, there are selected puncta that colocalize with Nkd1^{GFP} (Figure 6, E–G) but not with Nkd1^{N-GFP} (Figure 6, H–J). Similar to the above, any colocalization could simply be due to increased levels of cytoplasmic β-catenin due to activation of the pathway. To distinguish between these two possibilities, we coinjected Nkd1^{flag} with Wnt8 or with LRP6$^{\Delta N}$ and immunoprecipitated endogenous β-catenin at 30% epiboly. Without exogenous Wnt, Nkd1 associates with a basal level of β-catenin, likely due to the endogenous Wnt

signaling occurring at this stage of development (Figure 1B). This interaction is increased in the presence of ectopic Wnt8 but not in the presence of LRP6$^{\Delta N}$, even though they stabilize equivalent levels of cytoplasmic β-catenin (Figure 7, A and B). Thus we conclude that the interaction between Nkd1 and β-catenin is dependent on the presence of a Wnt ligand.

DISCUSSION

Interaction of the Wnt ligand with its receptors sets off a signaling cascade that controls many biological processes. One common event is initiation of the expression of feedback regulators to control the intensity and/or duration of the signal. Here our findings strongly suggest that Nkd1 is a negative feedback regulator that requires activation by the Wnt ligand. Using zebrafish blastula cells that can actively respond to Wnt signaling (Van Raay *et al.*, 2011; Hagemann *et al.*, 2014), we found that in Wnt-quiescent cells, the cellular distribution of Nkd1 is primarily at the plasma membrane or in large puncta adjacent to the membrane. On stimulation of the pathway specifically at the level of the ligand–receptor interaction, Nkd1 colocalizes with Dvl2 at the plasma membrane in putative Wnt signalosomes, and the large puncta decrease in size, becoming enriched in the cytoplasm, specifically around the nuclear membrane. A Wnt ligand is also required for Nkd1 to interact with β-catenin to restrict its nuclear accumulation, thereby reducing the active Wnt signaling program.

Thus far, Dvl is the only other protein whose activation is entirely dependent on Wnt ligand activation of the pathway (Gonzalez-Sancho *et al.*, 2004, 2013). Surprisingly, we know very little about how Dvl becomes activated by canonical Wnt/β-catenin signaling (Malbon and Wang, 2006; Gao and Chen, 2010; Gonzalez-Sancho *et al.*, 2013). Aside from Dvl, no other proteins have been identified whose function is entirely dependent on the Wnt ligand–receptor interaction. Although there are several kinases that function during Wnt signaling, their activation is unknown, and these kinases may be constitutively active or regulated independently of Wnt ligand–mediated activation of the receptor (Cruciat *et al.*, 2013; Yim and Virshup, 2013). One model that has been suggested is that Nkd/Nkd1 simply sequesters Dsh/Dvl, allowing reactivation of the Arm/β-catenin destruction complex (Rousset *et al.*, 2001; Wharton *et al.*, 2001). More recent evidence presented here and elsewhere, including studies in *Drosophila*, no longer supports this model (Waldrop *et al.*, 2006; Chan *et al.*, 2008; Van Raay *et al.*, 2011). First and foremost, the Nkd-Dsh interaction is dispensable for Nkd activity (Waldrop *et al.*, 2006). Second, we have demonstrated that a myristoylation-deficient form of Nkd1 (Nkd1^{G2A}) binds as efficiently to Dvl as does wild-type Nkd1 but is not able to antagonize Wnt signaling (Van Raay *et al.*, 2011). Third, Nkd1 interacts with β-catenin in a manner dependent on the Nkd1 myristoylation sequence (Van Raay *et al.*, 2011). Fourth, membrane localization is required for both vertebrate and invertebrate Nkd/Nkd1 function (Chan *et al.*, 2007; Van Raay *et al.*, 2011). Finally, here we demonstrate that the Nkd1–β-catenin interaction is also dependent on Wnt ligand activation of the pathway. Therefore the Dsh/Dvl sequestration model is insufficient to fully describe the function of Nkd/Nkd1.

Our observations here provide evidence that the Wnt negative feedback regulator Nkd1, like Dvl, is dependent on Wnt interacting with its receptor. Nkd1 interacts with Dvl in the same domain in which Dvl is phosphorylated by Wnt signaling (Rousset *et al.*, 2001; Wharton *et al.*, 2001; Gao and Chen, 2010). Thus it is possible that Wnt-induced phosphorylation of Dvl may disrupt the interaction between Dvl and Nkd1, allowing Nkd1 to then interact with β-catenin. In support of this model, we observed colocalization of Nkd1^{myc} and
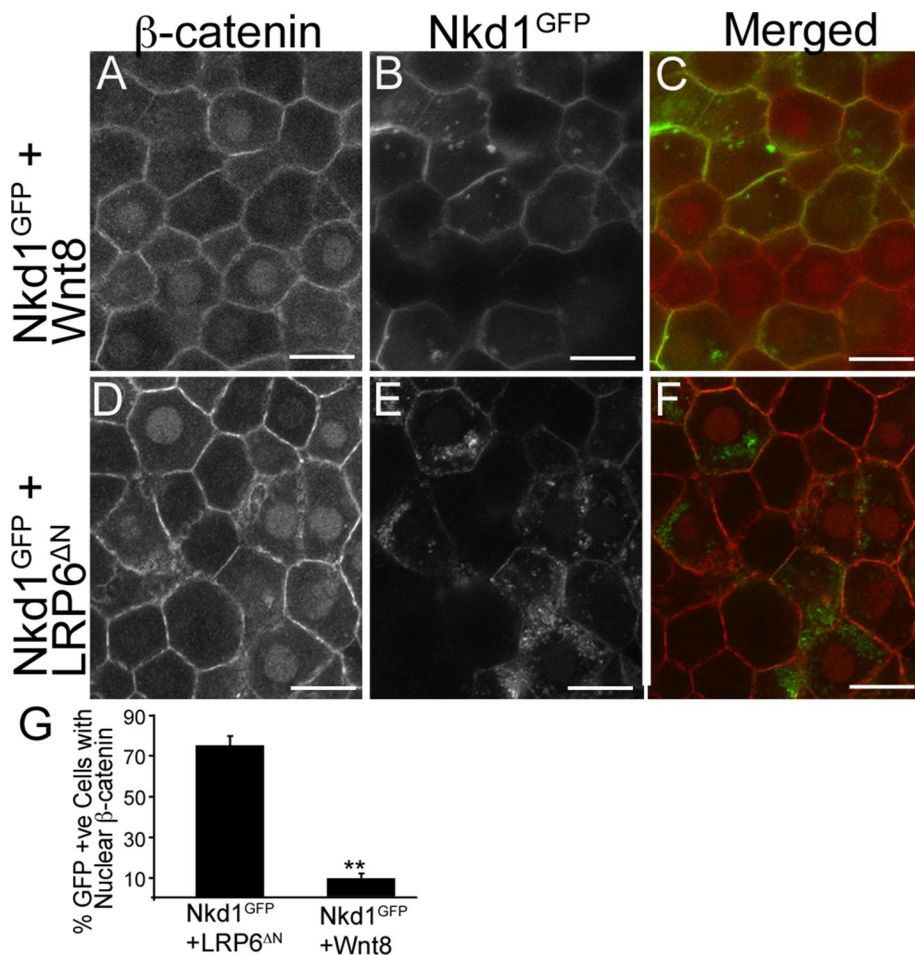
FIGURE 5: Nkd1 does not inhibit LRP6$^{\Delta N}$-induced nuclear accumulation of β-catenin. Nkd1^{GFP} is coinjected with either Wnt8 (A–C) or LRP6$^{\Delta N}$ (D–F) into one cell at the four-cell stage and harvested at 50% epiboly. Embryos were incubated with anti–β-catenin (A, C, D, F) and processed for IHC. Images are from animal pole cells. Nkd1^{GFP} + Wnt8–positive cells display nuclear β-catenin in cells juxtaposed to the GFP-positive cells but have low levels of nuclear β-catenin in Nkd1^{GFP}-positive cells (A–C). In contrast, Nkd1^{GFP} is not sufficient to inhibit nuclear accumulation of β-catenin induced by LRP6$^{\Delta N}$, as Nkd1^{GFP}-positive cells are also nuclear β-catenin positive (D–F). (G) Quantification of GFP-positive, nuclear β-catenin–positive cells from 10 embryos from three independent experiments. Double asterisks denote significance by Student's t test ($p < 0.001$). Data are represented as mean ± SE.

Nkd1 and Dvl2 to putative Wnt signalosomes suggests that the Dvl-Nkd1 interaction is required for activation of Nkd1 but that Nkd1 functions downstream of Dvl to inhibit the nuclear accumulation of β-catenin.

The interaction between Nkd1 and β-catenin is an important part of our model. Previously we demonstrated that the Nkd1–β-catenin interaction is dependent on the myristoylation sequence in Nkd1 and that Dvl can compete out this interaction (Van Raay et al., 2011). Here we demonstrate that the interaction is Wnt ligand dependent and not simply due to the increase in cytoplasmic levels of β-catenin. It is important to note that no interaction was found between Drosophila Nkd and Arm when tested using a yeast two-hybrid assay (Rousset et al., 2001). This result is entirely in line with our present findings, as yeast does not contain a Wnt/Wg signaling program to activate Nkd. Therefore Nkd should not interact with Arm in a yeast two-hybrid assay. Furthermore, it has been demonstrated that Nkd requires active Wg signaling in Drosophila, but the requirement for Nkd activity induced by Wg has not been tested, or at least reported (Zeng et al., 2000; Rousset et al., 2001). Finally, fly Nkd was found to interact with Importin α3, which promoted the nuclear localization of Nkd (Chan et al., 2008). Importin α3 contains 10 ARM repeats, which forms a concave groove homologous to the Arm/β-catenin protein for which the ARM repeats are named (Sharma et al., 2012; reviewed in Fagotto, 2013). It is proposed that this groove interacts with the basic residues of nuclear localization sequences found in Drosophila Nkd (Chan et al., 2008). Although this sequence is not conserved in vertebrate Nkd1, there is a conserved region of several basic residues that could perform a similar function (unpublished data). Although we have observed colocalization between Nkd1 and cytoplasmic β-catenin by IHC, these are rare events, as the vast majority of cytoplasmic β-catenin is clearly nonpunctate in Wnt8-expressing cells (Figures 1 and 5; Brunet et al., 2013). This may suggest that nonoligomeric Nkd1 interacts with β-catenin in the presence of a Wnt ligand. This is supported by the significant decrease in the size of cytoplasmic Nkd1 puncta upon Wnt ligand–mediated signaling. Taken together with our results, this suggests that the ability of Nkd/Nkd1 to interact with ARM containing proteins is conserved. A simple model would be that the interaction stoichiometrically prevents β-catenin from passing through the nuclear pore complex. However, it remains to be determined where Nkd1 and β-catenin interact and if Nkd1 directly interacts with β-catenin or potentially other ARM-containing proteins, which would influence the nucleocytoplasmic shuttling of β-catenin.

Combining the most recent in vivo evidence from Drosophila with our in vivo data, there is now convincing evidence that Nkd1 is activated by a Wnt ligand to antagonize Wnt signaling. In our

endogenous Dvl2 in putative Wnt signalosomes but not in Dvl2 or Nkd1^{myc} puncta in the cytoplasm. This is in contrast to our previous results showing robust colocalization between exogenous Dvl2^{HA} and Nkd1^{myc} at the membrane and in cytoplasmic puncta by IHC (Van Raay et al., 2011). Nonetheless, the interaction between Nkd1^{myc} and endogenous Dvl2 by immunoprecipitation (Van Raay et al., 2011) and by IHC shown here suggests that this interaction may represent a critical step in the activation of Nkd1, such as the recruitment of Nkd1 to the Wnt signalosome for activation. This is supported by recent evidence with Dvl2-Cherry in zebrafish blastula cells. Coexpression of Dvl2-Cherry with Wnt8-GFP resulted in few membrane-localized puncta where these two proteins colocalized, representing a Wnt signalosome. However, the size and frequency of these membrane puncta dramatically increased upon coexpression of the transmembrane receptor Frizzled, further supporting the Wnt signalosome theory (Hagemann et al., 2014). Therefore the redistribution of Nkd1 upon Wnt ligand activation, the dependence of Nkd1 activity on Wnt ligand activation, and the clustering of
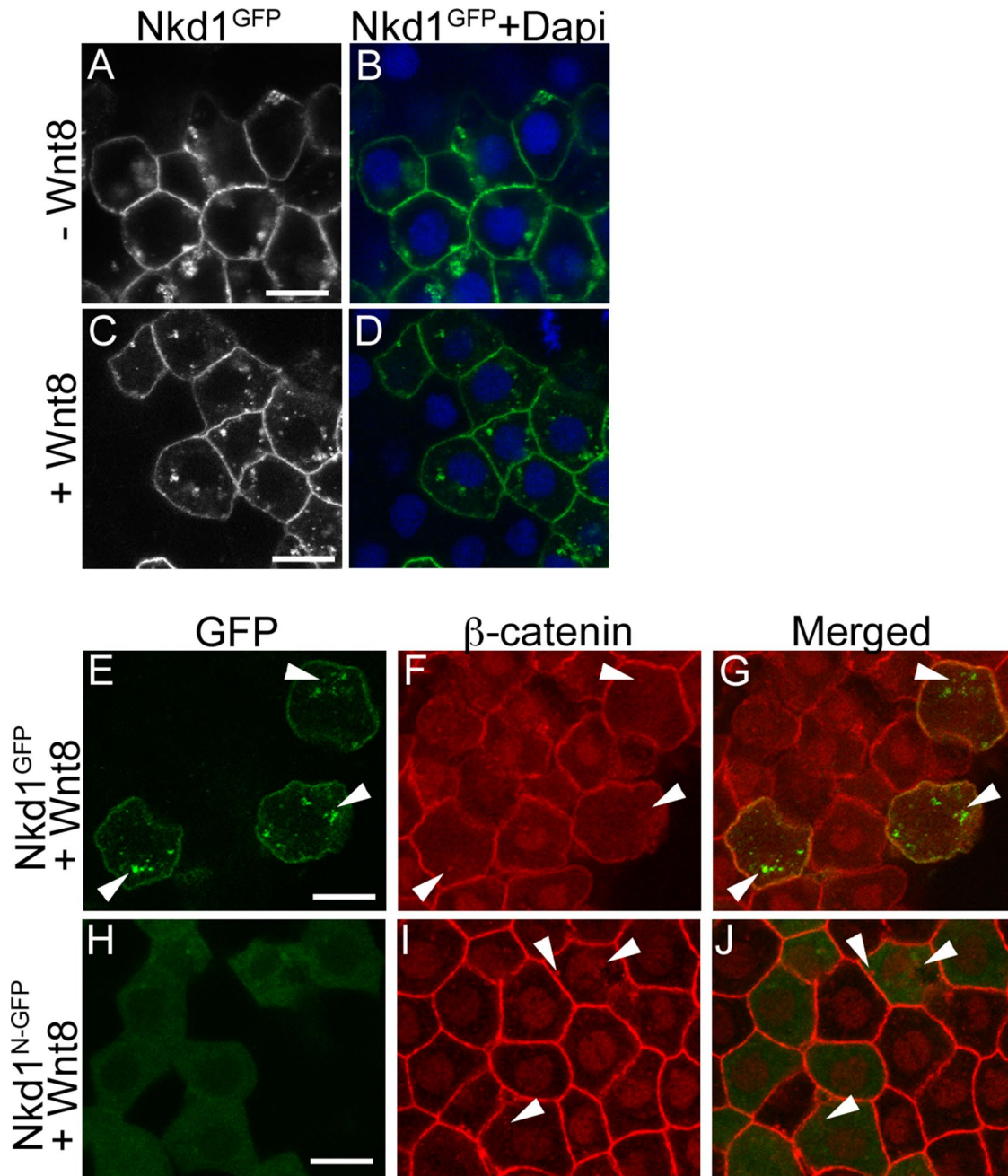
FIGURE 6: Wnt signaling enriches for perinuclear Nkd1[GFP] and colocalization with β-catenin. (A, B) In the absence of Wnt signaling, Nkd1[GFP] form large puncta enriched adjacent to the plasma membrane. (C, D) Coexpression of Nkd1[GFP] plus Wnt8 results in smaller puncta that tend to become enriched around the nucleus. 4′,6-Diamidino-2-phenylindole staining identifies the nucleus. (E–G) Ectopic Wnt8 stabilizes cytoplasmic β-catenin and in rare cases forms small puncta, some of which colocalize with Nkd1[GFP] (arrowheads). (H–J) In the absence of a myristoylation sequence (Nkd1[N-GFP]), we do not observe any colocalization between the rare β-catenin puncta and the evenly distributed Nkd1[N-GFP]. Arrowheads in I and J identify β-catenin puncta. Scale bar, 20 μm.

vertebrate model Wnt signaling induces the expression of Nkd1, which becomes cotranslationally modified with myrsitate, interacts with Dvl, and colocalizes with Dvl at the Wnt signalosome. At the Wnt signalosome, Nkd1 becomes activated by the proximal events occurring between the Wnt ligand interacting and its receptors. We speculate that activated Nkd1 is released from Dvl and the membrane and/or acquires a higher affinity for cytoplasmic β-catenin, ultimately preventing nuclear accumulation of β-catenin and attenuating Wnt signaling.

The expression of Nkd1 and Wnt3a is elevated in the intestinal crypt stem cells and in a significant number of colorectal cancers (Yan *et al.*, 2001; Caldwell *et al.*, 2008; Guo *et al.*, 2009; Voloshanenko *et al.*, 2013; Stancikova *et al.*, 2015). Thus one would predict that this would be sufficient to antagonize the constitutively activated Wnt signaling induced by mutations in adenomatous polyposis coli. We have started to analyze the role of Nkd1 in mammalian cells and human cancer cells in vitro but, surprisingly, have found that in most cases, Nkd1 is not localized to the membrane.
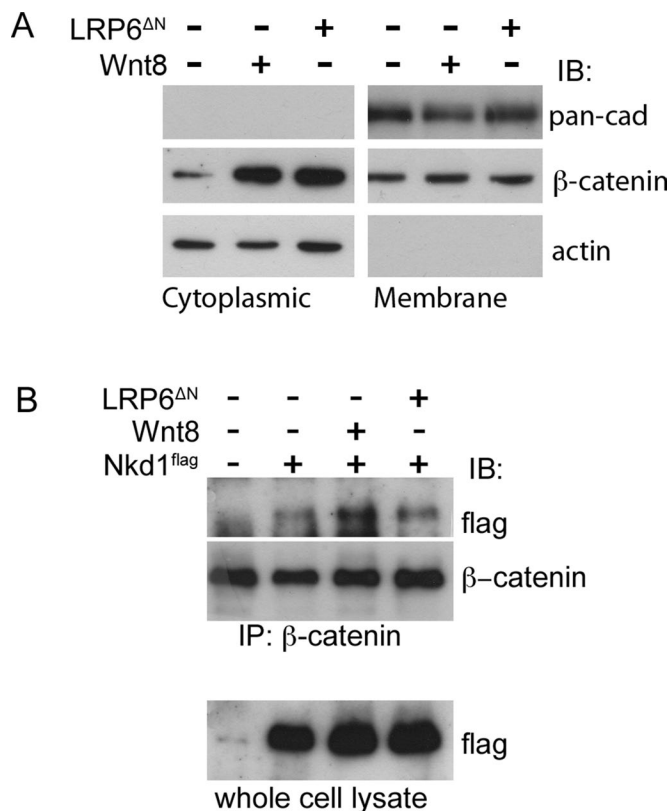
FIGURE 7: Interaction between Nkd1^{flag} and β-catenin is dependent on Wnt ligand. Nkd1^{flag} was coinjected with Wnt8 or LRP6^{ΔN} at the one-cell stage and harvested at 30–50% epiboly. (A) Activation of Wnt signaling by Wnt8 or LRP6^{ΔN} is confirmed by observing increasing levels of cytoplasmic β-catenin. Actin and pan-cadherin (pan-cad) antibodies were used as loading controls and to test for the relative purity of the cytoplasmic and membrane fractions, respectively. (B) Endogenous β-catenin was pulled down, Western blotted, and probed for the Flag epitope on Nkd1 or for β-catenin itself. An increase in the association between Nkd1^{flag} and β-catenin was observed only in the presence of Wnt8, not LRP6^{ΔN}. We attribute the low level of interaction between β-catenin and Nkd1^{flag} in the Nkd1^{flag}-alone and Nkd1^{flag} + LRP6^{ΔN} injections to endogenous Wnt signaling that is occurring in the blastula at this stage (Figure 1B). The results in B are representative of two independent experiments.

We speculate that this may be due to the lack of a three-dimensional matrix and/or cellular polarity that may be required for Nkd1 membrane localization, which is being investigated.

MATERIALS AND METHODS

Nkd1^{GFP} and Wnt8 have been described previously (Van Raay et al., 2011). LRP6^{ΔN} was constructed by removing the extracellular domain, leaving the signal sequence and the transmembrane domain intact. Nkd1^{flag} was constructed by placing two Flag epitopes between amino acids 317 and 318 of zebrafish Nkd1 using site-directed mutagenesis. The C-terminus of Nkd1 contains a highly conserved histidine-rich domain with unknown function. Nkd1 containing C-terminal-tagged GFP or Myc can antagonize Wnt signaling similar to the nontagged forms but with less efficiency. Therefore we constructed an internally tagged form of Nkd1 with two Flag tags in a region of the protein that has sequence length variability and is not well conserved. We tested this construct relative to

the other tagged and untagged forms of Nkd1, and Nkd1^{Flag} is as efficient as untagged Nkd1 in antagonizing ectopic Wnt8 in an overexpression assay (unpublished data).

For whole-mount in situ hybridization, 800 pg of Nkd1^{flag}, 25 pg of Wnt8, and 100 pg of LRP6^{ΔN} were injected at the one-cell stage and harvested at 30–50% epiboly. Probes have been described previously (Van Raay et al., 2011). For IHC, 200 pg of Nkd1^{GFP} or Nkd1^{myc}, 25 pg of Wnt8, and 25 pg of LRP6^{ΔN} were injected into one cell of a four-cell-stage embryo. Embryos were harvested at 30–50% epiboly and processed for IHC as previously described (Van Raay et al., 2011). Primary β-catenin and myc (9E10) antibodies (Sigma-Aldrich, St. Louis, MO) were used at 1:500 along with Alexa Fluor 594 goat anti-mouse secondary at 1:500. The polyclonal zebrafish Dvl2 antibody has been described (Lum et al., 2011) and was used at 1:1000 with Cy2 conjugated anti-rabbit at 1:200. Images were taken on a confocal microscope (Leica SP5). Relative levels of Nkd1^{GFP} protein were analyzed using densitometry analysis with ImageJ (National Institutes of Health, Bethesda, MD). Analysis of colocalization between Nkd1^{myc} and zDvl2 was performed using the Intersect tool in Volocity Quantitation (PerkinElmer Cetus, Waltham, MA).

Projection image was generated with Volocity software. For Western analysis, 800 pg of Nkd1^{flag}, 25 pg of Wnt8, and 100 pg of LRP6^{ΔN} were injected at the one-cell stage and harvested at 30–50% epiboly. For fractionations, 10 embryos from each experiment were mechanically deyolked and probed with antibodies as previously described (Van Raay et al., 2011). The equivalent of one embryo is run in each lane. For immunoprecipitations, 800 pg of Nkd1, 25 pg of Wnt8, and 200 pg of LRP6^{ΔN} were injected at the one-cell stage, and ~40 embryos (yolk plus chorion) were harvested at 30–40% epiboly in immunoprecipitation buffer as previously described (Van Raay et al., 2011).

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