Frizzled10 Mediates Wnt1 and Wnt3a Signaling in the Dorsal Spinal Cord of the Developing Chick Embryo

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Background: WNT1 and WNT3A drive a dorsal to ventral gradient of β-catenin-dependent Wnt signaling in the developing spinal cord. However, the identity of the receptors mediating downstream functions remains poorly understood. Results: In this report, we show that the spatiotemporal expression patterns of FZD10 and WNT1/WNT3A are highly correlated. We further show that in the presence of LRP6, FZD10 promotes WNT1 and WNT3A signaling using an 8xSuperTopFlash reporter assay. Conclusions: Together, our results identify FZD10 as a receptor for WNT1 and WNT3A in the developing chick spinal cord.

Key words: chick; FZD10; WNT1; WNT3A; LRP6; palmitate

Introduction

The proper development of the spinal cord is required for the transmission of motor and sensory information to and from the brain and for the initiation of reflexes. Wnt signals have vital roles in multiple phases of spinal cord development, including (1) the closure of the neural plate to form the neural tube and (2) the production of the correct types, number, and distribution of neuronal cells in the developing neural tube. WNT1 and WNT3A are expressed in the dorsal most spinal cord and activate a gradient of Wnt signaling that extends midway down the dorso-ventral (D/V) axis (Galli et al., 2007; Megason and McMahon, 2002). Wnts are capable of utilizing at least three distinct, but inter-related pathways: one β-catenin-dependent pathway and two β-catenin-independent pathways including the planar cell polarity (PCP) and Ca²⁺ pathways (Axelrod, 2009; Barrow, 2006; Clevers, 2006; Coombs et al., 2008; Jenny and Mlodzik, 2006; Jones and Chen, 2007; Klein and Mlodzik, 2005; Kohn and Moon, 2005; Kuhl et al., 2000; Logan and Nusse, 2004; MacDonald et al., 2009; Moon et al., 2004; Nusse, 2005; van Amerongen and Nusse, 2009; Veeman et al., 2003a; Vladar et al., 2009; Wang and Nathans, 2007). The activation of particular Wnt signaling pathways is dictated by the complement of receptors and co-receptors on the cell surface as well as their relative affinities.

LRP co-receptors often act in concert with Frizzled receptors (Brown et al., 1998; Hey et al., 1998; Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). LRP5 and LRP6 are ubiquitously expressed in multiple stages of mouse development (Diez-Roux et al., 2011; Pinson et al., 2000; Stenman et al., 2008) and in early stages of frog development (Houston and Wylie, 2002). In later stages of development in the frog, LRP6 is enriched in the spinal cord and the brain (Houston and Wylie, 2002). Consistent with its expression in the spinal cord, mutations in LRP6 are associated with neural tube defects (Andersson et al., 2010; Bryja et al., 2009; Carter et al., 2005; Joiner et al., 2013; Kubota et al., 2008; Zhou et al., 2010).

In this study, we introduce the proximity ligation assay (PLA) as a useful tool for the evaluation of ligand:receptor pairs. Using this tool in combination with other assays, we identify FZD10 as a receptor and LRP6 as a co-receptor for WNT1 and WNT3A in the developing chick spinal cord. First, we show specific spatiotemporal correlation between the expression of WNT1 and WNT3A with FZD10 in the developing spinal cord. Next, we show that FZD10 expression closely overlaps with the domain of WNT1 and WNT3A signaling via the β-catenin-dependent pathway. We further show that FZD10 and LRP6 synergize with WNT1 and WNT3A to activate the SuperTopFlash reporter in HEK293T cells. A PLA was used to confirm that WNT1 and WNT3A interact with FZD10 in the presence or absence of overexpressed LRP6. Consistent with a role for FZD10 in mediating WNT1 and WNT3A activity in the dorsal spinal cord, we show that FZD10 is required for proliferation in the dorsal most quadrant of the chick spinal cord.

Results and Discussion

Whole Mount In Situ Hybridization Analysis of FZD10 Expression in the Developing Chick Embryo

Previous reports demonstrate that FZD10 is expressed in the developing tail bud, limb buds, and central nervous system in a number of model systems (Borello et al., 1999; Chesnut et al., 2004; Dear-dorff et al., 2001; Endo et al., 2008; Garcia-Morales et al., 2009; Kawakami et al., 2000a; McCabe et al., 2007; Nunnally and Parr, 2004; Paxton et al., 2010; Stenman et al., 2008; Summerhurst et al., 2008; Yan et al., 2009). We were particularly interested in the expression of FZD10 in the dorsal spinal cord, which suggested a possible role in mediating WNT1 and WNT3A signaling. As such, we sought to compare the spatiotemporal expression patterns of FZD10, WNT1, and WNT3A in the spinal cord of Hamburger and Hamilton (HH) stage-6–21 chick embryos (Hamburger and Hamilton, 1951). Prior to neural tube closure, FZD10, WNT1, and WNT3A are expressed in the neural folds (Fig. 1A–D). Following neural tube closure, FZD10, WNT1, and WNT3A are all found in the spinal cord at HH10 (Fig. 1E), HH14–16 (Fig. 1F), HH18–21 (Fig. 1G) embryos. Consistent with previous reports, we also observe that FZD10 and WNT3A have overlapping and/or adjacent expression in the tail bud and the limbs (Fig. 1A–G) (Jin et al., 2001; Kawakami et al., 2000; Kengaku et al., 1998).

Section In Situ Hybridization Analysis of FZD10 Expression in the Developing Chick Spinal Cord

WNT1 and WNT3A signal via a β-catenin dependent pathway in the spinal cord (Alvarez-Medina et al., 2008; Galli et al., 2007; Megason and McMahon, 2002). Thus, we sought to refine the relationship between FZD10, WNT1, and WNT3A expression and the β-catenin activity gradient. We measured the WNT1/3A activity gradient by introducing a β-catenin-dependent reporter construct (BAT-Gal) into the right side of the spinal cord (Maretto et al., 2002). As a control, embryos were simultaneously electroporated with a GFP-expressing construct to indicate the extent of integration of our constructs in the dorsal–ventral axis of the neural tube. Whereas the FZD10 expression pattern (Fig. 2J) extends more ventrally than the region in which WNT1 and WNT3A transcripts are found (Fig. 2B, F), FZD10 gene expression correlates closely with the β-catenin activity gradient observed in BAT-Gal electroporated embryos (Fig. 2A, I).

We then detected the β-catenin activity gradient by immunostaining for nuclear β-catenin, another hallmark of β-catenin dependent signaling (Funayama et al., 1995; Huber et al., 1996; Schneider et al., 1996) (Fig. 2K). To enhance the detection of nuclear β-catenin, we used a modification of the antigen retrieval protocol reported by Borello and colleagues (Borello et al., 2006; Brabietz et al., 2000, 2001). To ensure that our method effectively distinguished nuclear β-catenin, we overexpressed constitutively activated β-catenin (Tetsu and McCormick, 1999) along with nuclear GFP in the spinal cord and then immunostained for β-catenin using conventional and antigen retrieval protocols. Sections stained with the conventional protocol reveal staining that is primarily localized to the plasma membrane (Fig. 2M, O). More intense staining is observed in cells that were successfully electroporated with the activated β-catenin (Fig. 2O). In a serial section immunostained using the antigen retrieval protocol, cells transfected with activated β-catenin exhibit very bright nuclear staining, as was expected (Fig. 2P). Importantly, nuclear staining of endogenous β-catenin (in non-transfected cells) was also enhanced using this method.

At HH stage 22, WNT1 and WNT3A expression persists exclusively in the roof plate (Fig. 2C, G), while only minimal WNT1 is observed in the floor plate. FZD10 continues to be expressed throughout the dorsal half of the spinal cord in sections through the hindlimb (Fig. 2L), mid-trunk (Fig. 2H,) and the forelimb levels (Fig. 2D). Thus, the region in which nuclear β-catenin is brightest (Fig. 2K) correlates well with FZD10 expression (Fig. 2D, H, I).

In summary, these data are consistent with a model in which FZD10 mediates the biological activity of WNT1 and/or WNT3A in the dorsal neural tube. The expression of FZD10 correlates well with β-catenin-dependent signaling activity readouts, with reduced signaling observed dorsally and a gradient that extends slightly beyond the dorsal half of the spinal cord.

Co-Expression of FZD10 and LRP6 Promotes β-Catenin-Dependent Signaling

LRP5 and LRP6 are required for β-catenin dependent Wnt signaling in many contexts (Joiner et al., 2013; Wehrli et al., 2000). Therefore, we evaluated whether the co-expression of LRP5 or LRP6 along with FZD10 might influence the activation of β-catenin-dependent signaling as measured by the 8xSuperTopFlash reporter assay in HEK293T cells (Veeman et al., 2003). Our data show that co-expression of LRP5 and FZD10 significantly promotes β-catenin-dependent signaling as compared to expression of FZD10 or LRP6 alone (Fig. 3A, P < 0.0005). Whereas co-expression of LRP5 with FZD10 has minimal effect, it appears to be inhibitory when co-expressed with LRP6 and FZD10 (Fig. 3A).
We do not know the mechanism by which this occurs. As no Wnts were overexpressed for this experiment, we attribute the activity observed to expression of endogenous Wnts. However, we cannot rule out the unlikely possibility that FZD10 and LRP6 activate signaling in a ligand-independent manner.

**FZD10 and LRP6 Together Promote WNT1 and WNT3A Signaling in a SuperTopFlash Reporter Assay**

We then tested the capacity of FZD10 to mediate WNT1 and WNT3A activity using transient transfection of FZD10 into HEK293T cells using the 8xSuperTopFlash reporter assay (Fig. 3B). Co-expression of FZD10 along with WNT1 or WNT3A inhibited Wnt signaling as compared to WNT1 or WNT3A alone (P < 0.0005 and P < 0.05, respectively). These data suggested the possibility that (1) co-expression of FZD10 serves to uncouple WNT1:FZD10 or WNT3A:FZD10 complexes from endogenous proteins, such as LRP6, required for Wnt signal transduction, (2) overexpressed FZD10 binds to WNT1/3A, but does not signal via the β-catenin pathway (thus rendering WNT1/3A unavailable to other endogenous receptors) or (3) FZD10 is not a receptor for WNT1 and WNT3A. To distinguish between these possibilities, we next tested whether the co-expression of LRP6 along with FZD10 could promote WNT1 and WNT3A signaling activity. Consistent with the first model, co-expression of LRP6 along with FZD10 caused a significant increase in WNT1 and WNT3A signaling as compared to cells transfected with FZD10 and WNT1 or WNT3A (P < 0.0005 for both) and cells transfected with LRP6 and WNT1 or WNT3A (P < 0.0005 and P < 0.05, respectively). As a negative control, we show that co-transfection of FZD10 and LRP6 along with WNT4 has no appreciable effect on signaling (Fig. 3B).
FZD10 Interacts With WNT1 and WNT3A

To test whether FZD10 interacts with WNT1 and WNT3A, we used an in situ PLA that detects protein–protein interactions within 40 nm distance (Soderberg et al., 2006). This assay required the use of primary antibodies against WNT1/3A and FZD10 that were generated in different organisms. We previously generated anti-WNT1 and WNT3A monoclonal antibodies in the mouse (Galli et al., 2007) and identified a commercially available FZD10 polyclonal antibody generated in rabbits. For this

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**Fig. 2.**
experiment, we co-transfected COS7 cells with FZD10, WNT1, or WNT3A, and/or LRP6. Species-specific secondary antibodies, called PLA probes, were then bound to the primary antibodies. Each PLA probe is linked with a unique oligonucleotide. When the PLA probes are localized to within 40 nm of one another, the complexes of interest. Following the proximity ligation assay, we visualized specific interacting proteins in ligand-receptor complexes of interest. Following the proximity ligation assay, we used conventional fluorescence immunolabeling to identify cells co-transfected with FZD10 and WNT1 or WNT3A (Fig. 4). All antibodies yielded staining characteristic of proteins in the secretory pathway, although minor non-specific nuclear staining was observed with the FZD10 antibody.

Cells co-transfected with FZD10 and WNT1 or WNT3A showed positive PLA punctae in the presence or absence of over-expressed LRP6 (Fig. 4A–D). Quantitation of these data showed a similar number of PLA punctae when WNT1 and FZD10 are co-expressed in the presence or absence of exogenous LRP6. However, there seemed to be a slight reduction in the number of punctae corresponding to a WNT3A:FZD10 interaction when these proteins were expressed without exogenous LRP6 (P < 0.05, Fig. 5). Thus, our data indicate that WNT1/WNT3A and FZD10 are able to interact in the absence of exogenous LRP6. Upon omission of the WNT1 or WNT3A cDNA, the numbers of punctae were reduced to background levels (Figs. 4E, G, 5). Likewise, omission of the anti-WNT1 (Figs. 4F, 5) or the anti-FZD10 antibody (Figs. 4H, I, 5) resulted in minimal punctae being observed in the presence of only one of the two required antibodies (Figs. 4F, H, I, 5).

It has been recently reported that the palmitate moiety on WNT3a is important, but not essential, for binding to FZD8 (Janda et al., 2012). To test whether the palmitoylation of WNT3A is required for binding to FZD10, we performed the PLA using a WNT3A construct in which the palmitoylated serine residue (S209) was substituted with an alanine residue. Consistent with the results of Janda and colleagues, WNT3A S209A continues to associate with FZD10 in the PLA, indicating that palmitoylation is not absolutely required for binding in this context (Figs. 4J, 5). However, it is important to note that it is not yet possible to assess the affinity of the WNT/FZD interaction using the PLA.

**FZD10 Knockdown Causes a Reduction in Proliferation in the Dorsal Quadrant of the Spinal Cord**

WNT1 and WNT3A drive a dorsal to ventral proliferation gradient in the developing spinal cord (Dickinson et al., 1994; Ikeya et al., 1997; Megason and McMahon, 2002). To test the requirement of FZD10 for proliferation, we introduced FZD10 morpholino into...
the chick spinal cord via electroporation. As the FZD10 antibody used in the PLA studies above does not detect endogenous FZD10 protein (data not shown), we needed to develop a method to ensure that the FZD10 morpholino inhibited the translation of FZD10 transcripts. To do this, we first generated a pCIG construct engineered to overexpress a bi-cistronic transcript encoding a truncated FZD10-myc variant (containing the target sequences) and a nuclear GFP variant (Megason and McMahon, 2002). We then electroporated this construct into the spinal cord along with control or FZD10 morpholinos. Using a protocol developed in our lab, we were able to deliver the expression construct to both sides of the spinal cord while delivering the morpholinos to only the

Fig. 4. FZD10 protein interacts with WNT1 and WNT3A in COS7 cells. COS7 cells were co-transfected as indicated with a combination of FZD10 and WNT1, WNT3A, WNT3A S209A, and/or LRP6, WNT1 (A, B, H), WNT3A (C, D, I), WNT3A S209A (J), and FZD10 (A–G, J) positive cells are detected as expected. The proximity ligation assay was carried out using polyclonal antibodies against FZD10 and monoclonal antibodies against WNT1 or WNT3A (A–J). PLA “PLUS” and “MINUS” secondary antibodies were added to all samples. After the PLA, cells were also immunolabeled for WNT1 or WNT3A (green) and FZD10 (red) using conventional secondary antibodies and DAPI was used to visualize the nuclei. PLA-positive punctae are shown in red (A–D, J). Images were collected via confocal microscopy. Representative images from 10 different fields are shown.
left side. GFP expression was used to assess the efficiency of transfection on both sides of the spinal cord while FZD10-myc expression was used to evaluate the ability of morpholinos to inhibit translation of FZD10. Thus, by comparing the expression of FZD10-myc expression on the right (no morpholino) and left sides (with morpholino) in sections with roughly equal GFP expression on both sides, we were able to assess the efficiency of our morpholinos.

Using this approach, we show that the presence of control morpholino on the left side has little or no effect on the levels of FZD10-myc (Fig. 6A–C). By contrast, co-electroporation of FZD10-myc with FZD10 morpholinos shows a substantial reduction in the levels of FZD10-myc protein on the left side of the spinal cord as compared to the right (Fig. 6D–F). Though we do not observe complete inhibition of translation, the inhibition is notable in light of the fact that the FZD10-myc protein is undoubtedly expressed at much higher levels than endogenous FZD10.

We then assessed whether FZD10 is required for proliferation in the spinal cord by electroporating the FZD10 morpholino into the left side of the spinal cord (Fig. 6G–I). Consistent with a reduction in proliferation, embryos electroporated with FZD10 morpholino consistently showed a reduction in the overall size of the left side of the spinal cord. To detect differences in proliferation on either side of the midline, we counted the number of proliferative cells marked by anti-phospho histone H3 (Hendzel et al., 1997; Wei et al., 1999), in four equally spaced quadrants along the dorsal to ventral axis (Fig. 6J). To normalize the number of mitotic cells back to the total number of cells, we divided the number of mitotic cells in a quadrant by the area in that quadrant to generate a “proliferative index” (Fig. 6J).

This method of normalization is valid because we have previously determined that cell size in the spinal cord is uniform at this stage of development (Galli et al., 2006). These data show that FZD10 is required for proliferation in the dorsal-most quadrant of the developing spinal cord and are consistent with a role for FZD10 in mediating the biological activity of WNT1 and WNT3A in vivo.

In summary, our results demonstrate that FZD10 expression overlaps with that of WNT1 and WNT3A in the developing chick spinal cord and that FZD10 is capable of mediating WNT1 and WNT3A signaling. We further show that although LRP6 is not needed for binding of WNT1 or WNT3A to FZD10, it is required for efficient β-catenin dependent signaling via this complex. Given that LRP6 is ubiquitously expressed at multiple stages of mouse development (Diez-Roux et al., 2011), we think it highly likely that it is also present in the chick spinal cord. These data suggest possible roles for FZD10 in the development of the dorsal spinal cord. However, as Fzd10 knockout mice have no overt phenotype (Mouse Genome Database: MGI: 2136761), it seems likely that at least one other Frizzled family member can compensate for the loss of FZD10 (Borello et al., 1999; Bourhis et al., 2010; Dear-dorf et al., 2001; Endo et al., 2008; Holliday et al., 1995; Janda et al., 2012; Kawakami et al., 2000a; Nunnally and Parr, 2004; Parr et al., 1993, Summerhurst et al., 2008; Tanai et al., 2000; Wang et al., 2006; Yan et al., 2009). Though FZD4 and 9 are the most closely related to FZD10, neither has been linked to dorsal spinal cord development. Of the other Frizzled family members implicated in the development of the dorsal spinal cord, the expression of FZD8 (in the mouse) is most closely aligned with what we observed for FZD10 (Borello et al., 1999; Summerhurst et al., 2008). We suspect that similar compensation would have been observed in our FZD10 knockdown experiment if we had extended it over a longer time frame. Our results also highlight the utility of PLA to predict specific Wnt/Frizzled interactions. The value of this simple approach is tremendous as it overcomes technical issues relating to Wnt production, solubility, and activity that have historically hindered classic binding studies.

**Experimental Procedures**

We thank Randy Moon for supplying 8xSuperTopflash and 8xSuperFopflash (negative control) (Veeman et al., 2003). We thank Elena Frolova for partial chick WNT1 cDNA (Fokina and Frolova, 2006), Frank McCormick for the constitutively activated form of β-catenin (Tetsu and McCormick, 1999), Tsutomu Nohno for partial chick WNT3A cDNA (Kawakami et al., 2000a), Xi He for human LRP5 and LRP6 (Tanai et al., 2000), and Michael Stark for FZD10 (Stark et al., 2000).

**Materials**

Materials used were Fugene HD Transfection Reagent, Dual Luciferase Reporter Assay System (Promega, Madison, WI); Lipofectamine 2000 (Invitrogen, Carlsbad, CA); Duolink PLA In Situ Kit (Sigma, St. Louis, MO); COS7 and HEK293T cells (ATCC); fertile eggs (Petaluma Farms, Petaluma, CA).

**Antibodies**

Mouse anti-WNT1 5F1-G11-D1 and mouse anti-WNT3A 3E9-1B11-H3 were made in the Burrus lab and used at a 1:10 dilution (Galli et al., 2007). The FZD10 polyclonal antibody was acquired from Aviva (ARP41263) and was used at a 1:333 dilution. The β-galactosidase antibody (JIE7) was from the Developmental Studies
Hybridoma Bank (Indianapolis, IN) and was used at a 1:100 dilution. The β-catenin antibody (used at 1:1,000 dilution) was from Sigma (Billerica, MA) and used at 1:1,000 dilution. Cy2-anti-mouse, Cy3-anti-mouse, and Cy3-anti-rabbit secondary antibodies were from Jackson Immunoresearch Labs (West Grove, PA).

**Morpholinos**

The GenBank accession number for the sequence used to design the chick FZD10 morpholino is AF224320. FZD10 (5’CAGGTCCCCTGGCCTGCCCATG 3’) and control (5’CCCTTACCTCACTAAATTTATA 3’) morpholinos were purchased from Gene Tools (Philomath, OR).

**Constructs**

The myc-tagged soluble version of chick FZD10 was created by ligating the cysteine-rich domain of chick FZD10 into pcDNA3.1 resulting in a fusion protein containing the amino acids 1–215 of chick FZD10 along with myc and his6 epitope tags. This cDNA was then subcloned into pCIG to generate FZD10-myc. A constitutively activated form of β-catenin was also subcloned into pCIG for use in electroporations (Tetsu and McCormick, 1999).

**In Situ Hybridizations**

Whole mount and section in situ hybridizations were carried out as previously described (Baranski et al., 2000; Chapman et al., 2002; Galli et al., 2007; Terry et al., 2000).

**In Ovo Electroporations**

For the BAT-Gal studies (Fig. 2), the spinal cords of HH12–13 chick embryos were electroporated with psiSTRIKE (Promega) containing an inert (scrambled) insert and BAT-Gal (Maretto et al., 2003) constructs as previously described (Galli et al., 2007). psiSTRIKE expresses hmgF under the control of a CMV promoter while BAT-Gal expresses β-galactosidase under the control of a promoter with multiple TCF sites. β-galactosidase expression was visualized by immunostaining. Images were collected via confocal microscopy.

The pCIG construct encoding activated β-catenin and nuclear GFP was electroporated as previously described (Galli et al., 2007).

For the knockdown experiments, we electroporated the spinal cord of HH stage 11–14 embryos with FZD10 or control morpholinos (final concentration = 0.5–1.0 mM) and/or pCIG.FZD10CRD-myc/his (final concentration = 3.75 mg/ml DNA). Using platinum electrodes, 3 x 50 ms pulses were delivered at 30V with the positive

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**Fig. 6.** FZD10 is required for proliferation in the dorsalmost quadrant of the spinal cord. To demonstrate that the FZD10 morpholino is able to inhibit the translation of FZD10, we engineered a bi-cistronic construct designed to simultaneously express a truncated form of FZD10 tagged with a myc epitope (FZD10-myc) and nuclear GFP. GFP fluorescence was used to assess transfection level, whereas expression of the myc tag was used to assess inhibition of translation via FZD10 morpholino. Co-electroporation of the FZD10-myc construct and control morpholino result in the expression of GFP (green) on both sides of the spinal cord, showing that both sides have been successfully transfected with the FZD10-myc construct (A, B). The morpholino (also in green) is also observed on the left side of the spinal cord, but is not detected with the exposures shown here. Immunostaining with the anti-myc antibody (red) shows that the levels of FZD10-myc are very similar on both sides of the spinal cord for control morpholino (C). By contrast, co-electroporation of FZD10 morpholino along with the FZD10-myc construct significantly reduced the levels of FZD10-myc on the left side of the spinal cord (D–F). DAPI (blue) was used to visualize nuclei (A and D). Embryos electroporated with FZD10 morpholino were sectioned and immunostained with anti-phosphohistone H3 (white) and β-catenin (red) (G–I). β-catenin immunostaining was carried out using a conventional protocol, which primarily detects β-catenin localized to the plasma membrane. The spinal cord was divided into 4 quadrants along the dorsal-ventral axis for counting (I). J: Quantitation of the proliferation data are shown. The proliferation index represents the number of proliferative cells divided by the area of the quadrant. These data represent 39 sections taken from 6 different embryos. Error bars show ± standard error. A Student’s t-test was performed to assess statistical significance. The asterisk indicates that P < 0.05 for quadrant 1.
electrode on the right side and the negative electrode on the left side of the embryo. For experiments using morpholinos in combination with pCIG.FZD10-myc, the electrodes were then reversed for an additional 3 pulses. Embryos were incubated overnight at 39 °C and processed as previously described. Embryos for control and FZD10 morpholinos were electroporated on the same day.

8xSuperTopflash Assay

Transfections of HEK293T cells and luciferase measurements were performed as previously described (Galli et al., 2011). Briefly, HEK293T cells were transfected with Lipofectamine 2000 according to the manufacturer’s protocol and incubated overnight. The cells were lysed and subjected to Dual Luciferase Assay as per the manufacturer’s protocol.

Immunostaining

Total β-catenin was detected by use of a previously reported “conventional” staining protocol (Galli et al., 2007). Because β-catenin is most abundant at the plasma membrane, this protocol primarily detected β-catenin at the plasma membrane.

We used an antigen retrieval protocol to enhance the immunostaining of nuclear β-catenin (Borello et al., 2006; Brabletz et al., 2000, 2001). HH22–23 embryos were harvested, fixed, and cryosectioned. Slides were washed to remove the OCT and then immersed in 0.01 M citric acid, pH 6, in a 250-ml plastic slide holder that was then placed in a beaker with water and microwaved for 5 min on high and 30 min on low. After cooling, slides were blocked for 30 min at room temperature in Tris-buffered saline (0.05 M Tris, pH 7.4, with 0.15 M NaCl) containing 3% sheep serum. Slides were incubated for 2 hr with anti-mouse IgG IgM Cy3 (diluted 1:200 in blocking buffer) overnight at room temperature. The next day the slides were washed 3 times in Tris-buffered saline, blocked for 15 min, and then incubated for 2 hr with anti-mouse IgG IgM Cy3 (diluted 1:200 in blocking buffer) at room temperature. After washing, slides were post fixed in 4% paraformaldehyde, washed, and mounted in slowfade.

Chick embryos electroporated with pCIG.FZD10-myc, in combination with FZD10 or control morpholinos, were sectioned and immunostained with a conventional immunostaining protocol (Galli et al., 2007). DAPI was used to visualize nuclei. Images were collected on a Zeiss (Thornwood, NY) LSM 710 confocal microscope.

In Situ Proximity Ligation Assay

Transfections of COS7 cells were carried out with Fugene according to the manufacturer’s instructions. Cells were transfected with WNT1, WNT3A, FZD10, and/or LRP6. We used WNT1 or WNT3A monoclonal antibodies developed in our laboratory and a commercial FZD10 polyclonal antibody for this assay (Galli et al., 2007). The proximity ligation assay was carried out as per the manufacturer’s instructions using a kit designed to fluoresce in the far-red region of the spectrum (Olink Bioscience, Uppsala, Sweden). After the PLA, conventional secondary antibodies were added to immunolabel WNT1, WNT3A, and FZD10. Cy2 coupled anti-mouse antibody was used for WNT1 and WNT3A while Cy3 labeled anti-rabbit antibody was used for FZD10. Images were collected via confocal microscopy.

We used Adobe Photoshop CS6 in combination with NIH ImageJ to count PLA-positive punctae. After outlining WNT/FZD10-positive cells in Adobe Photoshop, the PLA channel was then copied and pasted into a new Photoshop document and saved in a PNG format. We then ran a macro on the png file in ImageJ that highlighted the area we wanted to count and saved that image as a tif file in a new folder. Using modified parameters defined in our laboratory, we used the Image-Based Tool for Counting Nuclei (ITCN) to count PLA punctae.

Proliferation Analysis

For the proliferation analysis, embryos electroporated with FZD10 morpholinos were sectioned and immunostained with anti-phospho histone H3 and anti-β-catenin using a conventional protocol (Galli et al., 2007). Whereas the phospho histone H3 antibodies marked cells in late G2 and M phase, the β-catenin antibodies primarily marked the plasma membranes. Images were either collected on a Nikon Eclipse E600 with a SPOT RSlider camera or on a Nikon C1 confocal microscope. Images were then processed in Photoshop where a quadrant grid was pasted and resliced over the spinal cord. The number of phospho histone H3-positive cells was manually counted. The gridded image was then opened in SPOT Advanced 3.5.6 to measure the area of the individual quadrants.

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