Identification of the Wnt signaling activator leucine-rich repeat in Flightless interaction protein 2 by a genome-wide functional analysis

Jun Liu*, Anne G. Bang†, Chris Kintner†, Anthony P. Orth†, Sumit K. Chanda‡, Sheng Ding*, and Peter G. Schultz*‡§

*Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; †The Salk Institute for Biological Studies, P.O. Box 85800, La Jolla, CA 92186; and ‡Genomics Institute of The Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, CA 92121

Contributed by Peter G. Schultz, December 17, 2004

The Wnt signaling pathway acts ubiquitously in metazoans to control various aspects of embryonic development. Wnt ligands bind their receptors Frizzled and low-density lipoprotein receptor-related protein 5/6 and function through Disheveled (Dvl), Axin, adenomatous polyposis coli, glycogen synthase kinase 3β, and casein kinase (CK) 1 to stabilize β-catenin and induce lymphocyte enhancer-binding factor (LEF)/T cell factor (TCF)-dependent transcriptional activities. To identify previously unrecognized Wnt signaling modulators, a genome-wide functional screen was performed using large-scale arrayed cDNA collections. From this screen, both known components and previously uncharacterized regulators of this pathway were identified, including β-catenin, Dvl1, Dvl3, Fbxw-1, Cullf, CK1ε, CK1δ, and γ-catenin. In particular, a previously unrecognized activator, LRRFIP2 (leucine-rich repeat in Flightless interaction protein 2), was found that interacts with Dvl to increase the cellular levels of β-catenin and activate β-catenin/LEF/TCF-dependent transcriptional activity. The function of LRRFIP2 is blocked when a dominant negative Dvl (Xd1) is coexpressed. Expression of LRRFIP2 in Xenopus embryos induced double axis formation and Wnt target gene expression; a dominant negative form of LRRFIP2 suppresses ectopic Wnt signaling in Xenopus embryos and partially inhibits endogenous dorsal axis formation. These data suggest that LRRFIP2 plays an important role in transducing Wnt signals.

Materials and Methods

High-Throughput Transfection and Reporter Assay. Human embryonic kidney (HEK) 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). High-throughput reverse transfection and screening was carried out by using an arrayed library collection containing ~20,000 cDNAs from the Origene collection and Mammalian Genome Collection described in refs. 9, 10, and 12. Briefly, each individual cDNA plasmid (62.5 ng) in the collection was spotted into a single well of 384-well cell culture plates. Serum-free DMEM (20 μl)

Abbreviations: LRRFIP2, leucine-rich repeat in Flightless interaction protein 2; GSK-3β, glycogen synthase kinase 3β; CK, casein kinase; Dvl, Disheveled; LEF, lymphocyte enhancer-binding factor; TCF, T cell factor; HEK, human embryonic kidney.

© 2005 by The National Academy of Sciences of the USA
containing a β-catenin/TCF/LEF-responsive TOPflash reporter (Upstate Biotechnology, Lake Placid, NY) and Flu-GEN6 (Roche Diagnostics) was allocated into each well before transfection. After incubation at room temperature for 30 min, 20% FBS DMEM (20 μl) containing 104 HEK293T cells was plated into each well. Cells were then cultured in a humidified incubator at 37°C in 5% CO2. After 48 h, BrightGlo (Promega) reagent (40 μl) was added to each well, and luciferase luminescence was measured with an Acquest plate reader (LJL Biosystems, Sunnyvale, CA).

** Constructs. ** Human Dvl3 was cloned by RT-PCR from human embryonic kidney 293T cells and inserted into a Myc-tagged mammalian expression vector, pMyc-cyto (Invitrogen). The Dvl mutants were generated by PCR amplification and inserted into the same vector. The mutant constructs of human LRRFIP2 were generated with PCRs and cloned into the pcS2-Flag or pMyc-cyto vectors. Xenopus LRRFIP2 was cloned by RT-PCR from the stage-20 embryos and inserted into the pcS2-Flag vector. The sequences of the primer sets used in this report are available upon request.

** Immunoprecipitation and Western Blot Analysis. ** Coimmunoprecipitation and Western blot analysis were performed as described in ref. 15. Briefly, whole-cell lysates were pretreated with mouse IgG and protein G agarose. Flag-tagged proteins were immunoprecipitated from supernatants by mouse anti-Flag monoclonal antibody (M2, Sigma) conjugated to agarose (Sigma). For immunoblotting, immunoprecipitants or cellular proteins were mixed with Laemmli SDS sample buffer. Total cellular proteins (40 μg per lane) were separated by 4–20% gradient Tris-glycine SDS/PAGE (Invitrogen) and transferred to a nitrocellulose membrane. Proteins were detected with primary antibodies and horseradish peroxidase-conjugated secondary antibodies by using an enhanced chemiluminescence kit (Amersham Biosciences). Primary antibodies used were mouse anti-ε-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology), mouse anti-Flag monoclonal antibody (M2, Sigma), mouse anti-γ-tubulin monoclonal antibody (Sigma), and mouse anti-β-catenin monoclonal antibody (C-19220, Transduction Laboratories, Lexington, KY).

** Xenopus Microinjection. ** Xenopus eggs were in vitro fertilized following established methods (16). Embryos were raised in 0.1× Marc’s modified Ringer’s solution and staged according to the methods of Nieuwkoop and Faber (17). Capped RNAs were transcribed in vitro by using the mMessage-mMachine kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Synthetic RNAs were microinjected into the two- or four-cell-stage embryos. After 48 h, the axis duplication phenotype was scored. For the animal cap experiments, Xenopus RNAs were microinjected into the animal pole of two-cell stage embryos. At stage 8.5, animal caps were dissected and allowed to develop to stage 10.5. Explants were harvested, and RNAs were extracted by using TRIzol (Invitrogen). The first-strand cDNAs were synthesized by using the SuperScript III First Strand System (Invitrogen) according to the manufacturer’s instructions. One to two microliters of the cDNA-synthesizing reaction was used for PCRs. The sequences of the primers used for RT-PCR are available upon request. Histology section and staining were performed as described in ref. 16.

** Results and Discussion. ** Identification of LRRFIP2 as a Wnt Signaling Modulator. To identify additional modulators of Wnt signaling, a genome-wide functional screen was performed with a large-scale cDNA collection containing ~20,000 cDNAs (described in detail in Materials and Methods). Briefly, cDNAs were prespotted on 384-well cell culture plates as individual clones and then assayed by transfection into HEK293T cells along with a TOPflash reporter. The level of TOPflash reporter activity was used as an indicator of TCF/LEF-dependent transcriptional activity and, indirectly, β-catenin levels (18). Each well that scored positive from a luminescence readout of reporter luciferase activity was restested on both TOPFlash and on a negative control FOPflash construct carrying mutations in the β-catenin/TCF-responsive elements (18).

A variety of known components/modulators of the Wnt pathway were identified in the screen (Table 1, which is published as supporting information on the PNAS web site), including β-catenin, Dvl1, Dvl3, Fbxw-1, CK1ε, CK1δ, and γ-catenin (1, 2). Culll and the ubiquitin-conjugating enzyme E2, which are components of the Skp1–Cull1–Fbox complex that regulates the ubiquitination of β-catenin (19), also were identified. In addition, the screen identified previously unrecognized proteins that have not been associated with β-catenin regulation and/or Wnt signaling, including KIAA1190, ectoptic viral insertion site 3 (Evi3), Ring finger gene 4 (Rnf4), and LRRFIP2 (Table 1). KIAA1190 is an uncharacterized protein containing eight putative zinc fingers. Evi3, which contains 30 zinc fingers, is frequently up-regulated in mouse B cell lymphomas and functions in B cell development and oncogenesis (20). Rnf4 is a Ring finger-containing factor that is involved in transcription factor activation, nuclear trafficking, and ubiquitin conjugation (21–23). These proteins may play a role in β-catenin nuclear translocation and formation of active TCF/LEF transcription complexes.

The clone encoding LRRFIP2 was one of the strongest positives identified in the screen, inducing the TOPflash reporter activity 20- to 30-fold over background and, therefore, was characterized more fully. Human LRRFIP2 was originally identified in a two-hybrid screen using human Flightless-I (24), an actin-binding protein (25), as a bait. However, the significance of this potential interaction and the genetic and biochemical function of LRRFIP2 are unknown. LRRFIP2 was shown to be expressed as a long and short form, representing alternatively spliced variants (24). Although the screen only identified the long form, subsequent analysis showed that the short form has similar albeit weaker activity in the TOPflash reporter assay. The obvious structural features of LRRFIP2 are a predicted tailed-coiled-coil domain at its carboxyl terminus (24) and a Ser-rich region at the amino terminus, a potential site for posttranslational modification. A search of public databases did not reveal homologous orthologs of LRRFIP2 in either Caenorhabditis elegans or Drosophila, although the genes CG8578-PA (accession no. AAF48525) in Drosophila and F57B9.7 (accession no. AAM48538) in C. elegans are partially homologous to the carboxyl terminus of LRRFIP2. However, we were able to isolate a cDNA encoding the long form of Xenopus LRRFIP2 from an embryonic cDNA library by PCR. Alignment of the frog and human proteins indicates a high level of overall amino acid conservation (75%), with particularly high levels in the amino terminus (92%) as well as in the predicted coiled-coiled domain (94%) (Fig. 6, which is published as supporting information on the PNAS web site), suggesting that these regions encode conserved essential functions.

Overexpression of LRRFIP2 in HEK293T cells robustly activates the TOPflash reporter activity in a dose-dependent manner (Fig. 14). The specificity of this activation was confirmed by using the negative control FOPflash reporter as well as other reporter constructs, such as a p53-responsive reporter and a sonic hedgehog-responsive reporter (data not shown). No activity was detected with LRRFIP2 using either of these reporters. To test whether LRRFIP2 activates the canonical Wnt signaling pathway by modulating the level of free β-catenin (1, 2), LRRFIP2 was transfected into HEK293T cells and the levels of cytoplasmic β-catenin were measured by Western blot anal-
Analysis. As shown in Fig. 1D, expression of LRRFIP2 in HEK293T cells results in a significant increase in the cytoplasmic level of β-catenin, suggesting that the induction of the TOPflash reporter activity by LRRFIP2 is mediated through β-catenin.

Injection of LRRFIP2 in Xenopus Embryos Induces Double Axis Formation and Wnt Target Gene Expression. Dorsal axis formation in Xenopus embryos is a well established model for assaying activators and inhibitors of the canonical Wnt signaling pathway (1, 2). Ectopic activation of this pathway in early embryos leads to double axis formation (26). To examine the activity of LRRFIP2 in Xenopus, capped synthetic LRRFIP2 RNAs were injected at the ventral marginal zone into two-cell-stage embryos. As shown in Fig. 1B, LRRFIP2 induces axis duplication with high penetrance (80%, n = 110/139), whereas embryos injected with a GFP RNA control developed normally (100%, n = 50). Histological staining (Fig. 1C) revealed that the LRRFIP2-induced second axis contained a secondary notochord and neural tube morphologically indistinguishable from those in the primary axis. Thus, ectopic expression of LRRFIP2 in embryos produces a phenotype consistent with Wnt activation.

Activation of canonical Wnt signaling in early embryos produces a secondary dorsal axis by inducing the expression of Spemann organizer genes, including Siamois and Xnr3 (1, 2). To test whether LRRFIP2 also promotes dorsal axis formation by inducing Wnt target gene expression, the induction of Siamois and Xnr3 RNA by LRRFIP2 was measured in animal cap assays. Animal caps were isolated at stage 8.5 from embryos injected at the two-cell stage with LRRFIP2 or GFP RNA, cultured to stage 10.5, and then assayed by using RT-PCR for Siamois and Xnr3 RNA. As shown in Fig. 1E, LRRFIP2 strongly induces the expression of Siamois and Xnr3 to levels comparable to those induced by β-catenin; injection of a GFP RNA control had no effect. These data suggest that LRRFIP2 can indeed function as a Wnt signaling activator in Xenopus.

The Amino Terminus of LRRFIP2 Functions as a Dominant Negative Form. To begin to analyze the molecular function of LRRFIP2, a series of deletion mutants was constructed as shown in Fig. 2 and then tested for their ability to activate the TOPflash reporter when expressed in HEK293T cells. The ability of LRRFIP2 to activate the TOPflash reporter was severely reduced after truncation of either the carboxyl terminus or the amino terminus of LRRFIP2 in Xenopus, capped synthetic LRRFIP2 RNAs were injected at the ventral marginal zone into two-cell-stage embryos. As shown in Fig. 1B, LRRFIP2 induces axis duplication with high penetrance (80%, n = 110/139), whereas embryos injected with a GFP RNA control developed normally (100%, n = 50). Histological staining (Fig. 1C) revealed that the LRRFIP2-induced second axis contained a secondary notochord and neural tube morphologically indistinguishable from those in the primary axis. Thus, ectopic expression of LRRFIP2 in embryos produces a phenotype consistent with Wnt activation.

Activation of canonical Wnt signaling in early embryos produces a secondary dorsal axis by inducing the expression of Spemann organizer genes, including Siamois and Xnr3 (1, 2). To test whether LRRFIP2 also promotes dorsal axis formation by inducing Wnt target gene expression, the induction of Siamois and Xnr3 RNA by LRRFIP2 was measured in animal cap assays. Animal caps were isolated at stage 8.5 from embryos injected at the two-cell stage with LRRFIP2 or GFP RNA, cultured to stage 10.5, and then assayed by using RT-PCR for Siamois and Xnr3 RNA. As shown in Fig. 1E, LRRFIP2 strongly induces the expression of Siamois and Xnr3 to levels comparable to those induced by β-catenin; injection of a GFP RNA control had no effect. These data suggest that LRRFIP2 can indeed function as a Wnt signaling activator in Xenopus.

The Amino Terminus of LRRFIP2 Functions as a Dominant Negative Form. To begin to analyze the molecular function of LRRFIP2, a series of deletion mutants was constructed as shown in Fig. 2 and then tested for their ability to activate the TOPflash reporter when expressed in HEK293T cells. The ability of LRRFIP2 to activate the TOPflash reporter was severely reduced after truncation of either the carboxyl terminus or the amino termi-
panels. Myc- or Flag-tagged proteins in cell lysates are shown in bottom blot panels. Bands with the expected sizes are indicated with asterisks.

When cell lysates were immunoprecipitated with an anti-Flag antibody and associated Flag-LRRFIP2 protein was detected by immunoblotting using an anti-Flag antibody. The Flag-LRRFIP2 and Myc-Dvl3 in cell lysates and immunoprecipitates were detected by Western blot analysis. (B) Immunoprecipitation analyses to examine the interaction between the truncation mutants of LRRFIP2 and Dvl3. HEK293T cells were transfected with the indicated plasmids. The cell lysates were immunoprecipitated by using an anti-Myc antibody, and associated Flag-LRRFIP2 protein was detected by immunoblotting with an anti-Flag antibody. The Flag-LRRFIP2 and Myc-Dvl3 in cell lysates and immunoprecipitates were detected by Western blot analysis. (B and C) Immunoprecipitation analyses to examine the interaction between the truncation mutants of LRRFIP2 and Dvl3. HEK293T cells were transfected with the indicated plasmids. The cell lysates were immunoprecipitated by using an anti-Flag antibody (B) or an anti-Myc antibody (C), and associated protein was detected by immunoblotting with an anti-Myc antibody (B) or an anti-Flag antibody (C) (top blot panels). Immunoprecipitated tagged proteins are shown in middle blot panels. Myc- or Flag-tagged proteins in cell lysates are shown in bottom blot panels. Bands with the expected sizes are indicated with asterisks.

To search for candidate dominant negative forms, the deletion constructs were cotransfected along with full-length LRRFIP2 into HEK293T cells. The LRRFIP2 mutant containing amino acids 1–400 weakly stimulates reporter activity, precluding its use as a dominant negative. The LRRFIP2 mutant containing the carboxyl-terminal 250 amino acids, although inactive in reporter assay, was unable to inhibit the activation of TOPflash by full-length LRRFIP2. However, mutants M4 and M5, consisting of amino-terminal fragments containing amino acids 1–200 and 1–370, respectively, strongly inhibit the activity of LRRFIP2 and function as dominant negative mutants. Taken together, the above results indicate that protein interactions at both the amino and carboxyl termini are required for LRRFIP2 to activate Wnt signaling.

LRRFIP2 Interacts with Dvl3. To explore the mechanism by which LRRFIP2 activates Wnt signaling, the binding of LRRFIP2 to components in the Wnt pathway was assayed by coimmunoprecipitation. A Flag-tagged form of LRRFIP2 was introduced into HEK293T cells, along with Myc-tagged forms of Dvl3, Axin, or GSK-3β by cotransfection. After 48 h, Myc-tagged and associated proteins were recovered from cell lysates by immunoprecipitation and then probed by Western blots for Flag-LRRFIP2 with an anti-Flag antibody (Fig. 3A). The results show that LRRFIP2 can be coimmunoprecipitated in a complex with Dvl3 but not detectably with Axin or GSK-3β (data not shown).

The region of LRRFIP2 that binds to Dvl3 was determined from binding assays of Dvl3 with the LRRFIP2 deletion constructs described in Fig. 2. Extracts were prepared from HEK293T cells that were cotransfected with a Myc-tagged Dvl3 and the various Flag-tagged deletion mutants of LRRFIP2. Binding interactions were scored by immunoprecipitating Myc-Dvl and probing with a Flag-tag antibody on Western blots or vice versa. As shown in Fig. 3B, Myc-Dvl3 was detected in the immunoprecipitants of Flag-LRRFIP2-M4, Flag-LRRFIP2-M5, and Flag-LRRFIP2-M6. When cell lysates were immunoprecipitated with an anti-Myc antibody, Flag-LRRFIP2-M5 and Flag-LRRFIP2-M6 were readily detected in the immunoprecipitants as shown in Fig. 3C (Flag-LRRFIP2-M4 migrates at the same position as the light chain of IgG, making it undetectable in this assay). Together, these data indicate that LRRFIP2 binds to Dvl3 through its amino terminus. Consistent with this interpretation, a LRRFIP2 mutant containing only the region required for binding to Dvl3 functions as a dominant negative mutant.

The carboxyl terminus of LRRFIP2, which does not detectably interact with Dvl3, also seems to be required for its function (Fig. 2), presumably through interactions with other components of the Wnt pathway (or yet unknown factors), which function synergistically with LRRFIP2 to activate Dvl. It is worth noting that Western blot analysis of the LRRFIP2 truncated mutant afforded high molecular weight bands at trace levels, raising the possibility that LRRFIP2 is posttranslationally modified and that this modification could modulate its ability to interact with other proteins.

Dvl3 mediates Wnt signaling through three key domains: the DIX domain at the amino terminus, the PDZ domain in the central region, and the DEP domain near the carboxyl terminus (1, 2). Each of these domains likely mediates protein–protein interactions that allow the Dvl proteins to act as scaffolding proteins during Wnt signaling (27–36). To determine which of these domains binds LRRFIP2, a series of truncation mutants of Dvl3 (Fig. 7A, which is published as supporting information on the PNAS web site) was assayed with the coimmunoprecipitation protocol described above. Extracts were prepared from HEK293T cells coexpressing Flag-tagged LRRFIP2-M5 and Myc-tagged deletions of Dvl3, subjected to immunoprecipitation with an anti-Flag antibody, and analyzed by Western blots to detect the bound Myc-tagged Dvl3 proteins. As shown in Fig. 7B, the region containing both the PDZ and DEP domains is responsible for the interaction with LRRFIP2-M5. Each domain alone cannot bind LRRFIP2-M5 efficiently. Similar results were obtained when the Dvl3 mutant constructs were cotransfected with the full-length Flag-tagged LRRFIP2 (data not shown).

These experiments suggest that binding of LRRFIP2 to Dvl3 involves residues from both the PDZ and DEP domains and/or the hinge region between the two. Moreover, forms of Dvl3...
lacking the DIX domain were more strongly associated with LRRFIP2, suggesting that the DIX domain may be inhibitory for this interaction.

*In vitro* GST pull-down assays also were carried out to test the direct interaction between LRRFIP2 and Dvl. GST-LRRFIP2 (1–370) indeed could pull down *in vitro* translated Dvl3 (80–510) but with much lower efficiency than immunoprecipitation in HEK293T cells (data not shown). It is possible that phosphorylation of Dvl is required for the direct binding, because Dvl is heavily phosphorylated when expressed in cells, whereas the *in vitro* translated Dvl is not.

It was next determined whether LRRFIP2 functions within the Wnt pathway at the level of Dvl. A dominant negative form of Dvl (Xdd1) that lacks the PDZ domain (37) was assayed for its ability to inhibit LRRFIP2-induced TOPflash reporter activity in cultured cells. As shown in Fig. 4A, coexpression of Xdd1 totally abolished the activity of LRRFIP2. Furthermore, we tested whether the dominant negative mutant, LRRFIP2-M5, blocks the ability of Dvl3 to activate the TOPflash reporter in cultured cells. Again, dominant negative LRRFIP2 dramatically reduced Dvl3-activated reporter activity (Fig. 4B) but not the reporter activity promoted by β-catenin (data not shown). Taken together, these data suggest that LRRFIP2 may function in the Wnt pathway in association with Dvl3, and vice versa, both acting upstream of β-catenin.

**LRRFIP2 Plays a Role in Axis Formation in Xenopus.** The results above show that LRRFIP2 is a potent activator of Wnt signaling in Xenopus embryos. To test whether LRRFIP2 is required for Wnt signaling in this context, its expression pattern during Xenopus development was examined by RT-PCR. As shown in Fig. 5A, a PCR product corresponding to the shorter form of LRRFIP2 is expressed at high levels maternally and throughout early development. In addition, a PCR band corresponding to the longer form also was detected but only after the midblastula transition at low levels, becoming readily detectable after stage 14. As described above, the short form of LRRFIP2 lacks amino acids 61–294, which are found in the longer form, but retains the conserved regions and also activates Wnt signaling, although with a lower efficiency compared with the long form of LRRFIP2. This result indicates that LRRFIP2 is expressed in early embryos, where it could function in Wnt signaling, and, moreover, raises the possibility that its functional capacity could be regulated by alternative splicing.

![Fig. 4. LRRFIP2 functions at the level of Dvl. (A) LRRFIP2 and Dvl3 function in a mutually dependent manner. HEK293T cells were transfected with a reporter construct (TOPflash or FOPflash), an internal control (Renilla luciferase), and the indicated plasmids. TOPflash activities are represented by light-colored bars; FOPflash activities are represented by dark-colored bars. The amount of DNA in each transfection was kept constant by addition of empty expression vector. Dominant negative Dvl (Xdd1) could block LRRFIP2’s activity by inducing the reporter. (B) Similarly, the function of Dvl3 is blocked by a dominant negative LRRFIP2.](image)

![Fig. 5. Expression of a dominant negative LRRFIP2 in Xenopus embryos causes hypodorsalization. (A) RT-PCR to examine the expression pattern of Xenopus LRRFIP2 during embryonic development. Total RNAs were isolated from embryos at the indicated developmental stages. (Top) RT-PCRs were performed to examine the expression of xLRRFIP2. The lower band represents the short form of xLRRFIP2; the upper band represents the long form. (Middle) RT-PCRs with primers specific for the long form of xLRRFIP2 were also performed. (Bottom) Histone H4 serves as a loading control. (B) A dominant negative LRRFIP2 (LRRFIP2-M5) blocks xWnt8-induced double axis formation. Coinjection of xWnt8 (50 pg) with DN-LRRFIP2 (2 ng) or β-gal (2 ng) into stage-2 embryos; 48 h later, embryos were scored for double axis formation. (C) Effects of a dominant negative LRRFIP2 (LRRFIP2-M5) on axis formation. Stage-2 embryos were injected with DN-LRRFIP2 (2 ng) at multiple positions along the dorsal ventral axis and were scored by using the dorsoanterior index (DAI) at tadpole stages (39). Thirty-one percent of the injected embryos have gastrulation defects. Among gastrulated embryos, 23% show mild ventralization phenotypes (DAI 1–3), represented by the dark-colored bar; 7% show microcephalic or acephalic phenotypes (DAI 4), represented by the light-colored bar. 7% of the embryos show microcephalic or acephalic phenotypes (DAI 1–3), represented by the dark-colored bar. The combined hypodorsalized embryos are 30%.](image)
We then tested whether expression of the dominant negative mutant, LRRFIP2-M5, would suppress double axis formation in response to expression of xWnt8. Injection of RNA encoding LRRFIP2-M5 led to a significant reduction of double axis formation induced by xWnt8 (Fig. 5B) but had little or no effect on double axis formation by β-catenin (data not shown). These data suggest that LRRFIP2 may indeed be required for Wnt signaling and operate upstream of β-catenin, as suggested by the results in tissue culture cells described above.

To determine whether LRRFIP2 is involved in endogenous axis formation, RNA encoding the dominant negative LRRFIP2 was injected at multiple positions along the dorsal ventral axis. A small but reproducible number of injected embryos showed defects in gastrulation and of its interactions with Dvl in other arms of the Wnt signaling pathway. The amino terminus of LRRFIP2 is responsible for interaction with the terminal of LRRFIP2 are required for its function. The amino termini of LRRFIP2 are responsible for interaction with the central region of Dvl, which contains the PDZ and DEP domains; a mutant of LRRFIP2 containing only the amino terminus acts as a dominant negative form. One possibility is that LRRFIP2 interacts with Dvl and acts as a scaffold protein to facilitate the activation of Dvl, leading to the stabilization of β-catenin; disruption of this interaction with a dominant negative LRRFIP2 abolishes the activities of both. LRRFIP2 promotes the formation of a second dorsal axis in Xenopus embryos and induces the expression of organizer genes in animal caps. The dominant negative LRRFIP2 mutant suppresses xWnt8 dorsalizing activity, partially inhibits dorsal axis formation, and, in some embryos, reduces the levels of embryonic myogenesis, a process that is also known to require Wnt signaling within the dorsal and ventral marginal zone after the midblastula transition. Taken together, these results provide compelling evidence that LRRFIP2 transduces Wnt signals through the canonical pathway.

The lack of homologs of LRRFIP2 in invertebrates may be an indication that the primary function of LRRFIP2 in vertebrates may not be as a core component of Wnt signaling but as a modulator that is used to expand the signaling potential of the pathway, thus adding a new level of regulation during embryonic development. In this respect, LRRFIP2 could be akin to other signal modulators, such as Dkk1, Frodo, and Dapper, that modulate Wnt signaling but are unique to vertebrates (30, 31, 38).

We gratefully thank Drs. R. White (University of California, San Francisco), S. Sokol (Harvard Medical School, Boston), H. Clevers (University of Utrecht, Utrecht, The Netherlands), and H. Susaki (Osaka University, Osaka) for providing plasmids. We also thank Dr. B. Mitchell for assistance in frog handling. We are grateful to S. White, M. Medina, and J. Pendergraft for technical assistance. This work was supported by Novartis and The Skaggs Institute for Chemical Biology. This is manuscript 17126-CH of The Scripps Research Institute.