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A Novel Set of Wnt-Frizzled Fusion Proteins Identifies Receptor Components That Activate β-Catenin-dependent Signaling*

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Wnt proteins initiate the canonical (β-catenin-regulated) signaling cascade by binding to seven-transmembrane spanning receptors of the Frizzled (Fz) family together with the coreceptors LRP5 and -6, members of the low density lipoprotein receptor-related protein family (LRP). Several reports have shown physical and functional associations between various Wnt, LRP, and Frizzled molecules; however, the underlying mechanisms for selectivity remain poorly understood. We present data on a novel set of Wnt-Fz fusion constructs that are useful for elucidating mechanisms of Wnt signal transduction specificity in both *Xenopus* embryos and 293T cells. In 293T cells, coexpression of several Wnt-Fz fusion proteins with LRP6, but not LRP5, significantly activated a Wnt-responsive promoter, Optimized TOPFlash. Interestingly, Wnt proteins from both the Wnt1 and Wnt5A classes, when fused to the same Frizzled, can synergize with LRP6 to activate signaling and induce secondary axes in *Xenopus* embryos. However, when several Wnt-Fz constructs containing different Frizzled molecules were tested, it was found that all Frizzled molecules are not equivalent in their ability to activate the canonical Wnt pathway in this context. The data suggest that the distinction between the two Wnt classes lies not in intrinsic differences in the molecules but via the Frizzled molecules with which they interact.

Wnt signaling plays key roles in the development of multicellular animals ranging from Hydra to humans (1). In addition, alterations in the Wnt signaling pathway are among the most common events associated with human carcinogenesis (2–4). Wnts are a family of secreted proteins encoded by 18 separate genes in humans. Wnt proteins activate several signal transduction cascades, including one that results in the stabilization of cytosolic β-catenin through a process that involves several molecules, including heterotrimeric G proteins, dishevelled, and glycogen synthase kinase-3 (1, 5). This pathway is often referred to as the canonical Wnt signaling pathway (6). In human tumors, cytosolic β-catenin protein levels are often increased by genetic alterations in components of the Wnt signaling pathway (for reviews see Refs. 7 and 8). Wnt proteins can also initiate β-catenin-independent signaling cascades that activate protein kinase C (9, 10) or the small GTPase Rho (11–13).

Wnt proteins initiate the canonical signaling cascade by binding to a cell surface receptor complex that includes two proteins: a member of the Frizzled family of seven transmembrane-spanning receptors (encoded by nine separate genes in humans) and either low density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6)[1] (14–16). The requirement for this heterotrimeric complex in signaling is elegantly illustrated by the observation that the Dickkopf-1 (Dkk-1) protein, which binds directly to LRP6, blocks activation of the pathway, presumably by interfering with the ability of Wnt to bind LRP6 (17–19). Although our understanding of Wnt signal transduction has increased dramatically in the last few years, several important questions remain unanswered. For instance, it is unclear how specificity is generated among the 18 different Wnts, 9 Frizzleds, and 2 LRPs. Several reports have shown physical and functional associations between specific Wnt and Frizzled molecules (10, 20–23); however, the mechanisms underlying this selectivity are relatively poorly understood.

We present data based on the analysis of a novel set of Wnt-Fz fusion proteins illustrating specificity at several levels of the pathway, including differences between LRP5 and LRP6 and of the ability of different Frizzled molecules to activate the pathway in the context of these fusion proteins. In addition, members of both the Wnt1 and Wnt5A classes, when fused to Frizzled molecules, are able to interact with LRP6 to synergistically activate a Wnt-responsive reporter gene in 293T cells and induce secondary axes in *Xenopus* embryos.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—All Wnt-Fz fusions were built in the pCS2+ vector (24). A 24-amino acid linker $(GGGSGGGT)_3$ was included between any N-terminal Wnt fragments and the C-terminal Frizzled gene that had its signal sequence removed. For tagged fusions, a copy of the myc tag and 6 histidine residues were inserted between the linker and the *Frizzled* fragment. When the N-terminal *Wnt* fragment did not carry its own signal sequence, the signal sequence of human *frizzled 5* was used. A more detailed description of the cloning process and construct maps are available upon request.

Secreted versions of the XWnt8-Fz5 fusions were generated by PCR amplifying the XWnt8-Fz5 expression plasmid from the start codon to the last amino acid before the transmembrane domain in *Frizzled*. The forward primer contained a *Hin*dIII restriction site and the reverse primer contained an *Eco*RI restriction site. The PCR product was cloned into the TOPO Blunt vector (Invitrogen, Carlsbad, CA) to generate TOPO-XWnt8-Fz5s. This vector was sequenced to verify the amplified product. TOPO-XWnt8-Fz5s was digested with *Eco*RI and cloned into

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[1] The abbreviations used are: LRP, lipoprotein receptor-related protein family; OT, optimized version of the TOPFlash reporter plasmid; HRP, horseradish peroxidase; DN, dominant negative; EGF, epidermal growth factor; EGFR, EGF receptor; FRP1, Frizzled related protein 1; CRD, cysteine-rich domain; TCF, T cell factor.

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the pCDNA6-V5-HisB expression plasmid (Invitrogen, Carlsbad, CA) to generate XWnt8-Fz5sV5. The orientation of the insert was determined by *Nco*I digest. The DNXWnt8-Fz5sV5 was generated by digesting the full-length DN-XWnt8-Fz5 plasmid with *Bam*HI and *Kpn*I and inserting the fragment into the same sites in XWnt8-Fz5sV5.

To generate a C-terminal V5-tagged version of human *LRP6*, the 3′-end of the gene (bp 3163–4919) was PCR-amplified from the cDNA. The reverse primer eliminated the stop codon and inserted an *Xba*I restriction site. The PCR product was cloned into the TOPO Blunt vector (Invitrogen, Carlsbad, CA) to generate TOPO-hLRP6-IC. This vector was sequenced to verify the amplified product. The remaining 5′-end of the human *LRP6* gene was subcloned into TOPO-hLRP6-IC using the restriction sites *Bam*HI and *Afl*II to generate TOPO-hLRP6-FL. The TOPO-hLRP6-FL vector was digested with *Bam*HI and *Xba*I and the *hLRP6* fragment was cloned into the pCDNA6-V5-HisA expression plasmid (Invitrogen, Carlsbad, CA) to generate hLRP6-V5.

To generate a C-terminal V5-tagged version of human *LRP5*, the 3′-end of the gene (bp 4339–4948) was PCR-amplified from the cDNA. The forward primer contained a *Bam*HI restriction site, and the reverse primer eliminated the stop codon and inserted an *Xba*I restriction site. The PCR product was digested with *Bam*HI and *Xba*I and cloned into the pCDNA6-V5-HisA expression plasmid (Invitrogen, Carlsbad, CA) to generate hLRP5-IC-V5. This vector was sequenced to verify the amplified product. The remaining 5′-end of the human *LRP5* gene was subcloned into hLRP5-IC-V5 using the restriction sites *Hin*dIII and *Afe*I to generate hLRP5-V5.

The constant region of the mouse IgG heavy chain was PCR-amplified from an existing expression plasmid. The forward primer contained an *Xho*I restriction site, and the reverse primer contained an *Xba*I restriction site. The PCR product was digested with *Xho*I and *Xba*I, cloned into the pCDNA3 expression plasmid (Invitrogen, Carlsbad, CA) to generate mIgG-pCDNA3, and the sequence was verified. The portion of the human *LRP6* gene that encodes the N-terminal extracellular domain was cut with *Bam*HI and *Eco*RI and subcloned into mIgG-pCDNA3 to generate N-LRP6-mIgG.

The ΔN-TCF4 expression plasmid was created by PCR amplification of a full-length *TCF4* plasmid (generously provided by Hans Clevers) with primers designed to create a Kozak recognition sequence (GCCACC) immediately upstream of a translation start codon preceding the 54th amino acid of human TCF4 (threonine 54). This amino terminally truncated construct was then introduced into the pCDNA6 expression vector (Invitrogen, Carlsbad, CA) for transient transfection.

Cell Culture, Transient Transfections, and Reporter Studies—For each experiment, six-well plates were seeded with equal amounts of human 293T cells the night prior to transfection. Cells were transfected by calcium phosphate precipitation as per the manufacturer's specifications (Stratagene, La Jolla, CA). Cells were ~30% confluent at the time of transfection. To measure the ability of various constructs to activate a TCF-dependent reporter construct, 293T cells were cotransfected with 1.4 μg of the plasmid to be tested, 0.7 μg of an optimized version of the TOPFlash reporter plasmid (OT) (supplied by Bert Vogelstein, Johns Hopkins University, Baltimore, MD) (25), and 0.7 μg of the pCDNA3.1-lacZ plasmid. Empty vector DNA was added to normalize the total amount of DNA in each transfection. Transfections were performed in duplicate. Cells were harvested 36 h after transfection by lysing in 120 μl of 1× reporter lysis buffer (Promega, Madison, WI). Lysates were pelleted, and 10 μl of each supernatant was added to 50 μl of luciferase assay substrate (Promega). Luciferase activity was measured in duplicate using a Fluoroskan Ascent FL luminometer (Thermo Labsystems, Beverly, MA). Readings were normalized for transfection efficiency by measuring β-galactosidase activity (26). To monitor the protein levels from the various expression constructs, the pellets were resuspended in 100 μl of 1× SDS sample buffer, boiled for 10 min, passed through a 26-gauge needle five times, and analyzed by SDS-PAGE followed by Western blot (see below).

Examination of Cytoplasmic β-Catenin Levels— To examine the effects of the different expression constructs on cytosolic β-catenin levels, samples were prepared as described by Shimizu *et al.* (27). For each experiment, 6-cm plates were seeded with equal amounts of human 293T cells the night prior to transfection. Cells were transfected by calcium phosphate precipitation as described above except 2 μg of each DNA was used. Thirty-six hours after transfection, cells were harvested in 1 ml of isotonic lysis buffer (10 mM Tris, pH 7.4, 140 mM NaCl, and 2 mM dithiothreitol supplemented with protease inhibitors). The samples were scraped from the plates, lysed in a Dounce homogenizer, and centrifuged for 90 min at 100,000 × *g* at 4 °C. Supernatants were removed and diluted 1:1 in 2× Laemmli sample buffer. The diluted sample was then separated on a 10% Tris-glycine polyacrylamide gel, transferred to nitrocellulose, and analyzed for β-catenin expression by Western blot (see below).

Immunoprecipitation and Western Blots—After collecting the media, transfected 293T cells were washed with cold phosphate-buffered saline, and collected in 1.0 ml of phosphate-buffered saline 48 h post-transfection. The cells were pelleted at 2000 rpm for 5 min and resuspended in 500 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, with protease inhibitors) using a 26-gauge needle. Lysates were incubated for 30 min at 4 °C with constant shaking and then microcentrifuged at 5000 rpm for 5 min at 4 °C to pellet cellular debris. Immunoprecipitations were performed from both lysates and media from transfected cells. Immunoprecipitation from lysates were done in a 1.0-ml total volume with 500 μl of lysate, 450 μl of lysis buffer, and 50 μl of anti-mouse IgG-agarose beads (Sigma, St. Louis, MO) for 2 h at 4 °C with constant shaking. The beads were washed twice with 1 ml of lysis buffer, twice with 1 ml of high salt solution (1 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.5% Triton X-100), and twice with 1 ml of KSCN wash buffer (0.75 M KSCN, 10 mM Tris-HCl, pH 7.5, and 1% Triton X-100). Immunoprecipitations from the media of transfected cells were done using 1.0 ml of media and 50 μl of anti-mouse IgG-agarose beads (Sigma, St. Louis, MO) overnight at 4 °C with constant shaking. The beads were washed three times with 1 ml of 0.1% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40). Bound proteins were eluted by boiling for 5 min in SDS sample buffer. The proteins were separated on 10% Tris-glycine polyacrylamide gels, transferred to nitrocellulose, and probed with a V5 antibody by Western blot (see below).

Western blots were incubated for 1 h at room temperature in blocking solution (0.05% Tween 20 in Tris-buffered saline and 5% nonfat dry milk). Blots were immunostained for β-catenin, α-tubulin, Frizzled 5 (Fz5), V5, and myc using the following antibodies: anti-β-catenin 1:1000 (Transduction Laboratories, Lexington, KY); anti-α-tubulin (AB-1) 1:1000 (Oncogene, San Diego, CA); anti-Fz5 1:500 (Upstate Biotechnology, Lake Placid, NY); anti-V5 and anti-V5-HRP 1:2500 (Invitrogen, Carlsbad, CA); anti-myc-HRP 1:1000 (Invitrogen, Carlsbad, CA). All antibodies were diluted in blocking buffer. Western blots were incubated in the primary antibody for 1 h at room temperature with constant shaking and then washed three times in TBS-T wash buffer (0.05% Tween 20 in Tris-buffered saline). If necessary, the blots were then incubated in either anti-mouse IgG-HRP or anti-rabbit IgG-HRP secondary antibodies diluted 1:3000 (Sigma, St. Louis, MO) for 1 h at room temperature. The blots were washed three times in TBS-T wash buffer, incubated with ECL solutions per the manufacturer's specifications (Amersham Biosciences, Piscataway, NJ), and exposed to film.

Xenopus Embryo Injections—Capped mRNAs were synthesized *in vitro* using SP6 RNA polymerase from linearized pCS2+ templates. 0.1–1 ng of a given fusion mRNA was injected into one ventral blastomere of four cell-stage *Xenopus* embryos as described previously (28). Embryos were scored at stages 18–24 for the presence of a duplicated axis.

RESULTS

We have examined the ability of a number of Wnt-Frizzled fusion proteins to activate the canonical (β-catenin-regulated) signaling pathway. We have assessed this in three ways. First, we have quantitated the ability of these fusions to activate a β-catenin/TCF-responsive reporter gene, optimized TOPFlash (OT), when transiently transfected into human embryonic kidney 293T cells. Second, we have examined their ability to increase soluble levels of β-catenin when transfected into 293T cells. Finally, we have determined their ability to induce ectopic axis formation in *Xenopus* embryos.

Co-expression of Wnt1 or Wnt3A with LRP6 or LRP5 Synergistically Transactivates the OT Reporter—Transient transfection of LRP6 into 293T cells results in the transactivation of the Wnt-responsive luciferase reporter OT, inducing the reporter between 5- and 10-fold (Fig. 1*A*). Consistent with other reported data (17), cotransfection of either mouse Wnt1 or Wnt3A with LRP6 results in both the transactivation of the Wnt-responsive luciferase reporter OT, synergistically inducing the reporter 30- to 40-fold over baseline levels, and stabilization of cytosolic levels of β-catenin (Fig. 1*B*). We found that *Xenopus* Wnt8 (XWnt8) and LRP6 synergized in a similar manner (data

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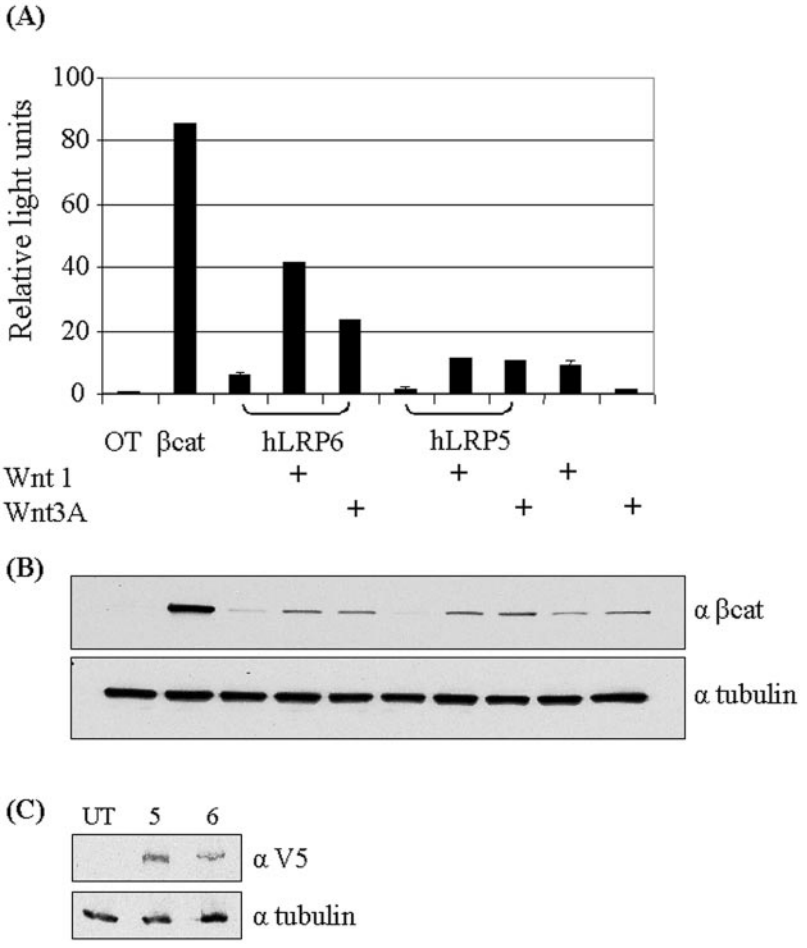

FIG. 1. **Coexpression of Wnt1 or Wnt3A with LRP5 or LRP6 synergistically transactivates the OT reporter.** *A*, relative transactivation of the OT reporter upon transfection of the indicated plasmids. All values are expressed relative to the levels of luciferase activity seen in cells transfected with empty vectors and are normalized for transfection efficiency by determining levels of β-galactosidase activity expressed from the pCMV-βgal plasmid equally transfected in each experiment. Values represent the average of duplicate experiments, and *bars* representing the standard error are shown. *βcat* is S37A β-catenin. *B*, immunoblot for β-catenin protein (*top*) and α-tubulin (*bottom*) in cytosolic fractions from cell lysates transfected with the indicated plasmids. The anti-tubulin blot is included to confirm equivalent protein loading in each lane. *C*, immunoblot for the V5 epitope (*top*) on lysates from untransfected (*UT*) 293T cells and cells transfected with expression vectors encoding versions of LRP5 (*5*) and LRP6 (*6*) tagged on the carboxyl terminus with the V5 epitope. An anti-tubulin blot (*bottom*) is again shown to confirm equivalent loading.

not shown). Although a lower level of synergy was seen between Wnts of this class and LRP5 (Fig. 1*A*), the differences observed between LRP5 and LRP6 are not due to differences in expression levels, because carboxyl-terminally, epitope-tagged versions acted similarly to the wild type, untagged proteins in these assays and were expressed at similar levels (Fig. 1*C*). Also, the addition of increasing amounts of LRP5 with Wnt1 or Wnt3A did not increase the activation of OT (data not shown).

We extended our analysis by testing various Frizzled molecules with LRP5 or -6 and Wnts for activation. However, we found that several Frizzled expression constructs, especially human Frizzled 5 (hFz5), diminished transfection efficiencies (as measured by the levels of β-galactosidase used to monitor transfection efficiency; see "Experimental Procedures") (data not shown). This observation is consistent with other reports suggesting that Frizzled molecules can induce apoptosis in some contexts (29, 30).

We set out to generate a constitutively active Frizzled receptor that would be capable of initiating canonical Wnt signaling in a ligand-independent manner. In the absence of known activating mutations in Frizzled genes, we reasoned that a covalent link between Wnt and Frizzled should generate such a constitutively active Frizzled construct (Fig. 2). Transfection of these plasmids into 293T cells was not associated with reductions in transfection efficiencies, unlike the native forms of several Frizzled molecules (data not shown). Although this difference between the fusion proteins and the parental Frizzled construct is of great interest, for the purposes of this report we have focused on using these constructs to address issues of specificity in the Wnt receptor complex. In addition to the ability to express these fusions in cells, there are other advantages to the use of these constructs. We speculated that, even beyond the effects on cell viability, they should more strongly induce signaling in cells, because only two, instead of three, molecules need to associate to form a functional receptor complex. Also, it is likely that, by fusing the Wnt and Frizzled proteins together, the ability of each fusion partner to interact with other Wnts and Frizzleds is restricted. This reduces the complexity of analysis, because the pool of endogenous Wnt and Frizzled molecules will be less likely to interact with the exogenous Wnt and Frizzled proteins in the fusion.

Both XWnt8-Fz5 and DNWnt8-Fz5 Induce Secondary Axis in Xenopus Embryos—Prior to the linkage of LRP5 and LRP6 to the Wnt signaling pathway, we had completed preliminary analyses in *Xenopus* embryos of a fusion protein joining XWnt8 with human Frizzled 5 (XWnt8-Fz5). In *Xenopus*, components of the canonical Wnt pathway have previously been found to be essential for body axis formation (31). The earliest sign of axis formation is β-catenin accumulation on the dorso-anterior side of the embryo, and overexpression of β-catenin induces formation of a secondary embryonic axis (32). We found that injection of mRNA-encoding XWnt8-Fz5 induced secondary axes in *Xenopus* embryos. Although human Frizzled 5 on its own is unable to induce secondary axes in *Xenopus*, XWnt8 can do so. To exploit our observation that the fusion protein retained biological activity, we created a fusion protein between a truncated form of XWnt8 and human Frizzled 5. This truncation (DNWnt8) not only is unable to induce secondary axes by itself but has properties of a dominant negative (DN) molecule, because it blocks the signaling activities of the full-length molecule when they are coexpressed (33). Interestingly, we observed that a fusion protein between this dominant negative form of

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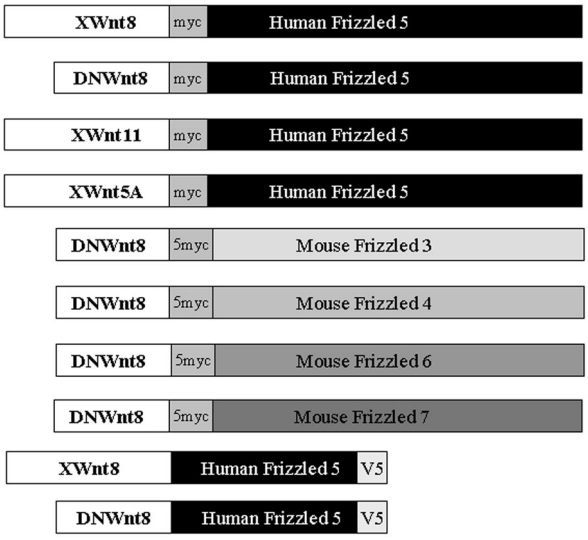

FIG. 2. **Schematic diagram of constructs.** The constructs used in this work are linearly represented. *Xenopus* Wnt8 (*XWnt8*) was fused to human Frizzled 5 via a linker containing a single myc tag and a glycine-serine rich region to provide steric flexibility. A dominant negative (*DN*) form (missing the last 98 amino acids) of XWnt8, as well as full-length XWnt11 and XWnt5A were also fused to hFz5 and produced in a similar manner. Constructs containing DNWnt8 fused to mouse Fz3, -4, -6, and -7 were also produced that contained a glycine-serine rich linker region and also contained five copies of the myc epitope. Finally, fusion proteins containing either full-length XWnt8 or DNWnt8 fused to a form of hFz5 truncated prior to the first transmembrane domain and epitope tagged with the V5 epitope were also created.

TABLE I
Analysis of axis duplication in injected Xenopus embryos

Embryos were scored at stages 18–24 for the presence of a duplicated axis. mRNA was injected into one ventral blastomere of four cell-stage Xenopus embryos as described (28). None of the Frizzled proteins induce an axis on their own (20).

Ventrally injected RNA	Number	Axis duplication	No axis duplication[a]
		%	%
Uninjected	30	0	100
hFz5	18	0	100
XWnt11	15	0	80
XWnt11-Fz5	21	67	33
XWnt5A-Fz5	20	40	60
DNWnt8	20	0	90
DNWnt8-Fz3	21	14	86
DNWnt8-Fz4	21	19	81
DNWnt8-Fz5	22	64	36
DNWnt8-Fz6	19	16	84
DNWnt8-Fz7	25	48	52

[a] Includes normal embryos and embryos with a shortened body axis.

XWnt8 and human Frizzled 5 (DNWnt8-Fz5) was also able to induce secondary axes in *Xenopus* (Table I).

Both XWnt8-Fz5 and DNWnt8-Fz5 Synergize with LRP6 to Strongly Transactivate the OT Reporter—We next tested the ability of the XWnt8-Fz5 and DNWnt8-Fz5 fusion proteins to transactivate the OT reporter upon transient transfection of 293T cells. Although we found that neither the XWnt8-Fz5 nor the DNWnt8-Fz5 fusion significantly transactivated the OT reporter (Fig. 3*A*), both fusion proteins were found to be expressed as assayed by immunoblots of cell lysates (Fig. 3*C*). Based on the observed synergy between Wnt and LRP5 and LRP6 (Fig. 1*A*), we examined the ability of the XWnt8-Fz5 fusion to transactivate the OT reporter when cotransfected with LRP5 or LRP6. We found that cotransfection of either XWnt8-Fz5 or DNWnt8-Fz5 with LRP6 transactivated the reporter 300- to 400-fold over baseline levels, whereas transfection of either fusion alone or LRP6 alone induced less than 10-fold (Fig. 3*A*). The Wnt-Fz fusion transactivated the OT reporter in the presence of LRP5 as well but to a much lesser extent (~30-fold) than LRP6. The difference between LRP5- and LRP6-mediated transactivation is not due to differences in expression levels, because epitope-tagged versions of the proteins are expressed at similar levels and are identical to native forms of each protein in their abilities to synergistically activate the OT reporter with the fusion proteins (Fig. 1*C*). Furthermore, the addition of increasing amounts of the LRP5 expression plasmid did not cause further increases in transactivation.

A conserved motif (KT*XXX*W), located two amino acids after the seventh transmembrane domain in the Frizzled receptor, has been demonstrated to be required for the activation of the canonical Wnt pathway (34). To demonstrate that the XWnt8-Fz5 fusion functions like the native un-fused proteins, we generated a point mutation (W530G) within this motif in Frizzled 5 and measured the ability of the mutant to activate the OT reporter when cotransfected with LRP5/6 into 293T cells. As expected, the XWnt8-Fz5W530G mutant was unable to activate the OT reporter even though the mutant protein was expressed at levels similar to the wild-type protein, as detected by Western blot analysis (data not shown). This demonstrates that the biological function of Frizzled is required for the activity of the Wnt-Fz fusion.

Both XWnt8-Fz5 and DNWnt8-Fz5 Stabilize Cytosolic β-Catenin—To further characterize the effects of these fusion proteins on Wnt signaling, we assayed their effects on the levels of cytosolic β-catenin upon transfection into 293T cells. We found that expression of the Wnt-Fz fusions effectively increased the levels of cytosolic β-catenin over baseline levels even when transfected alone (Fig. 3*B*). In fact, there appeared to be little difference in cytosolic β-catenin levels between these lysates and lysates in which transactivation of the OT reporter was 40-fold higher (compare Xwnt8-Fz5 alone and XWnt8-Fz5 cotransfected with LRP6), suggesting that other aspects of signaling, aside from stabilization of cytosolic levels of β-catenin, are involved in establishing this high level of transactivation. Although the focus of this report is on using these fusion constructs to answer questions about signaling specificity and mechanisms at the level of the receptor complex, we recognize that the mechanisms underlying the potency of this signaling may also help reveal other signals important for this pathway.

Both XWnt8-Fz5 and DNWnt8-Fz5 Physically Interact with LRP6—It has been shown that LRP6 binds to Wnt and forms a physical complex with Frizzled in a Wnt-dependent manner (15). If the Wnt-Fz fusion proteins we have created function in a similar manner to the native un-fused proteins, then the fusion proteins we have described should bind to LRP6. To test this, we utilized secreted forms of our XWnt8-Fz5 fusions in which the proteins were truncated just prior to the transmembrane domain and fused to the V5 epitope tag (XWnt8-Fz5-V5 and DNWnt8-Fz5-V5). We also utilized a secreted form of LRP6, which consisted of the first two epidermal growth factor (EGF)-like repeats of LRP6 fused to a mouse immunoglobulin Fc tag (N-LRP6-IgG). 293T cells were transfected with either the secreted Wnt-Fz fusion construct alone or the secreted Wnt-Fz fusion construct with N-LRP6-IgG. As expected, N-LRP6-IgG coprecipitated both XWnt8-Fz5 and DNWnt8-Fz5 from cell lysates and culture media, demonstrating that the fusion proteins are secreted and physically interact with LRP6 in solution (Fig. 3*D*).

XWnt11-Fz5 and XWnt5A-Fz5 Induce Secondary Axes in Xenopus Embryos and Synergize with LRP6 to Strongly Transactivate the OT Reporter in 293T Cells—We next tested the ability of different Wnt molecules to cooperate with LRP5 and

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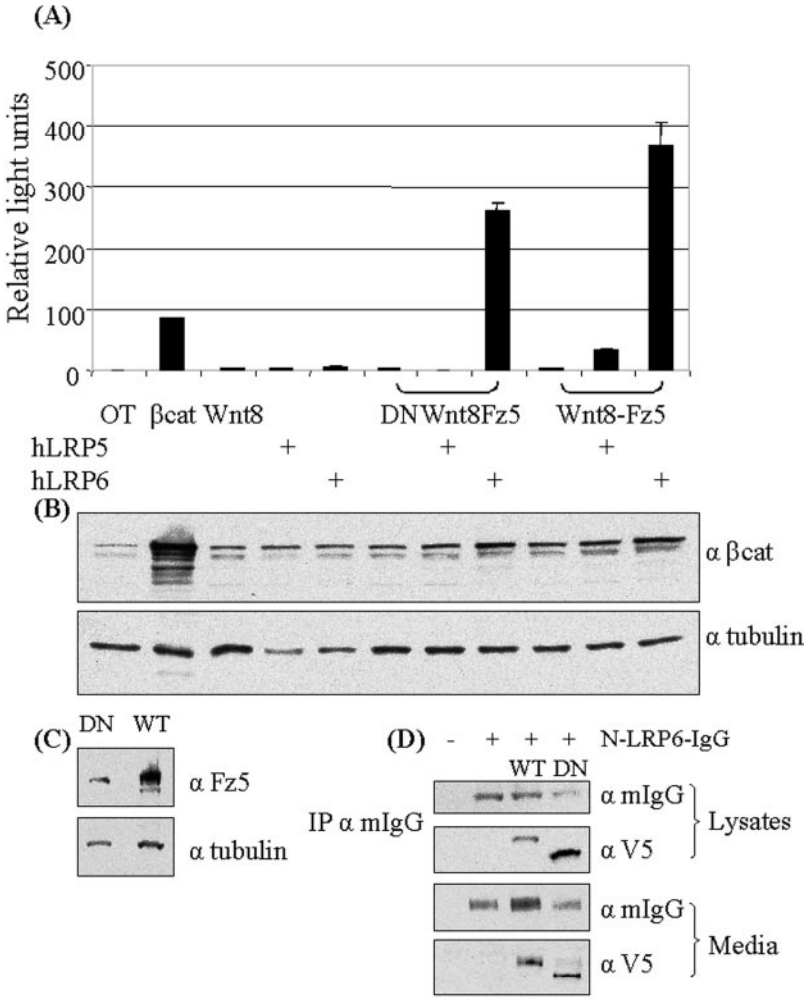

FIG. 3. **Both an XWnt8-Fz5 Fusion and a DNWnt8-Fz5 fusion protein synergize with LRP6 to strongly transactivate the OT reporter and stabilize β-catenin.** *A*, optimized TOPFlash luciferase reporter gene assay. Experiments were performed as described in Fig. 1*A*. Values represent the average of duplicate experiments, and *bars* representing the standard error are shown. Note the difference in the scale of the *y* axis between Fig. 1 and this and the following figures. *B*, Western blot of cytoplasmic β-catenin (*top*) and α-tubulin (*bottom*) on 293T cell lysates transfected with the expression plasmids indicated in *A*. *C*, immunoblot for Frizzled 5 protein in whole cell lysates from cells transfected with full-length XWnt8-Fz5 fusion (*WT*) and the DNWnt8-Fz5 (*DN*) fusions. An α-tubulin immunoblot is also shown. *D*, immunoprecipitation analysis performed on cell lysates and media from 293T cells transfected with different combinations of the following expression plasmids: an amino-terminally truncated form of LRP6 fused to the constant region of mouse IgG (N-LRP6-IgG, see text) and either full-length XWnt8 (*WT*) or a dominant negative form of XWnt8 (*DN*) fused to hFz5 truncated prior to the first transmembrane domain and tagged with the V5 epitope. Proteins were immunoprecipitated with anti-mouse IgG, and the immunoprecipitate was immunoblotted for the V5 epitope.

LRP6 to activate the Wnt pathway when fused to Fz5 (Fig. 2). Wnt11 and Wnt5A were chosen because they belong to the class of Wnt molecules that does not typically signal through the canonical (β-catenin-dependent) pathway (35). It was previously shown that, although neither XWnt5A, XWnt11, nor hFz5 alone was able to cause axis duplication in *Xenopus*, coinjection of XWnt5A with hFz5 resulted in secondary axis formation (20). Here we show that XWnt11-Fz5 and XWnt5A-Fz5 fusions caused axis duplication when injected as mRNA into one ventral blastomere at the four-cell stage, suggesting they behaved as expected for a constitutively active Frizzled, which signals through the canonical Wnt pathway (Table I and Fig. 4). Both XWnt11-Fz5 and XWnt5A-Fz5 fusion proteins synergized with LRP6 to potently transactivate the OT reporter (Fig. 5*A*) and increased levels of soluble β-catenin (Fig. 5*B*). Western blot analysis of cells transfected with these constructs indicated that similar amounts of each polypeptide were produced (Fig. 5*C*).

Fusion of Different Frizzled Genes to DNWnt8 Reveals Differences in Their Ability to Activate the Canonical Pathway—To examine the role of Frizzled specificity in this fusion, we created several constructs in which different Frizzled molecules were fused to DNWnt8 and found that some Frizzled molecules conferred upon the fusion construct the ability to synergize strongly with LRP6, whereas others did not (Fig. 6*A*). For instance, DNWnt8-Fz4 and -5 synergized with LRP6 to potently transactivate the OT reporter (~240- and 260-fold, respectively), whereas DNWnt8-Fz3 and -6 did so to a much lesser extent (~40- and 18-fold, respectively). Furthermore,

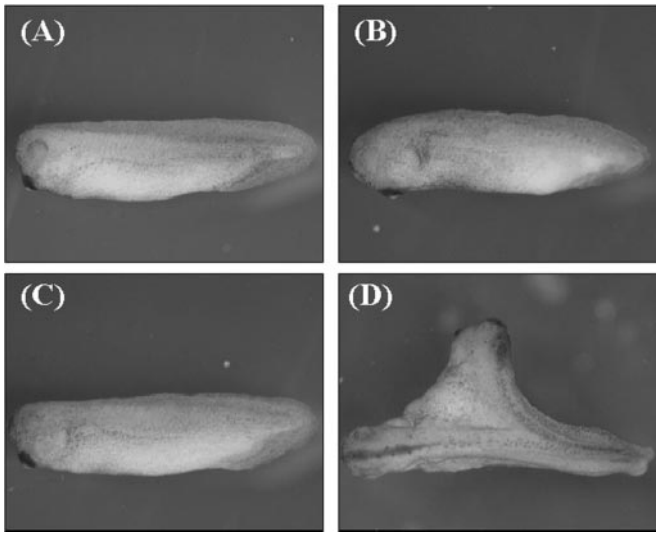

FIG. 4. **XWnt11-Fz5 induced ectopic secondary axis in *Xenopus* embryos.** 0.1–1 ng of a given fusion mRNA was injected into one ventral blastomere of four cell-stage *Xenopus* embryos. Embryos were scored at stages 18–24 for the presence of a duplicated axis. *A*, uninjected; *B*, XWnt11 injected; *C*, hFz5 injected; *D*, XWnt11-Fz5 injected.

DNWnt8-Fz7 transactivated the OT reporter when cotransfected with LRP6 but to a lesser extent than Fz4 and -5 (~70-fold). Cytosolic β-catenin levels were also found to be less in cells cotransfected with DNWnt8-Fz3 and LRP5/6 or DNWnt8-

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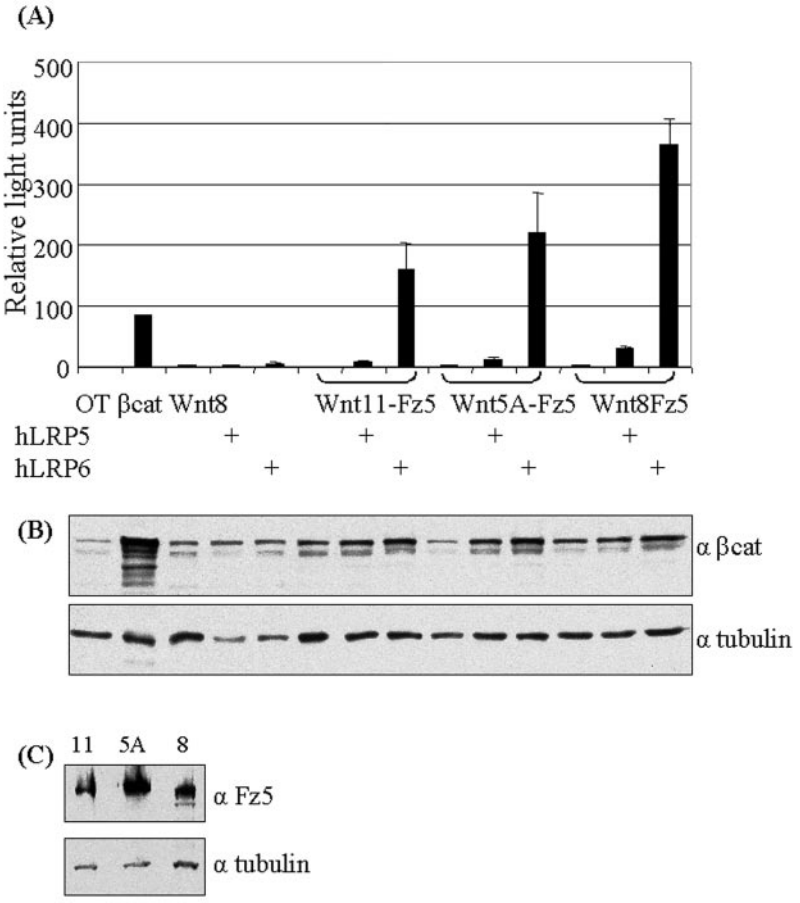

FIG. 5. **Fusion proteins containing either class of Wnt molecules fused to Frizzled 5 synergize with LRP6 to activate the OT reporter in 293T cells.** *A*, OT reporter assays performed on cell lysates transfected with the indicated expression vectors. Values are determined and represented as described in Fig. 1. *B*, β-catenin and tubulin immunoblots performed on cell lysates transfected with the expression plasmids indicated in *A*. *C*, immunoblot with anti-hFz5 antibody of lysates from cells transfected with Xwnt11-Fz5 (*11*), XWnt5A-Fz5 (*5A*), or XWnt8-Fz5 (*8*) fusion protein expression vectors.

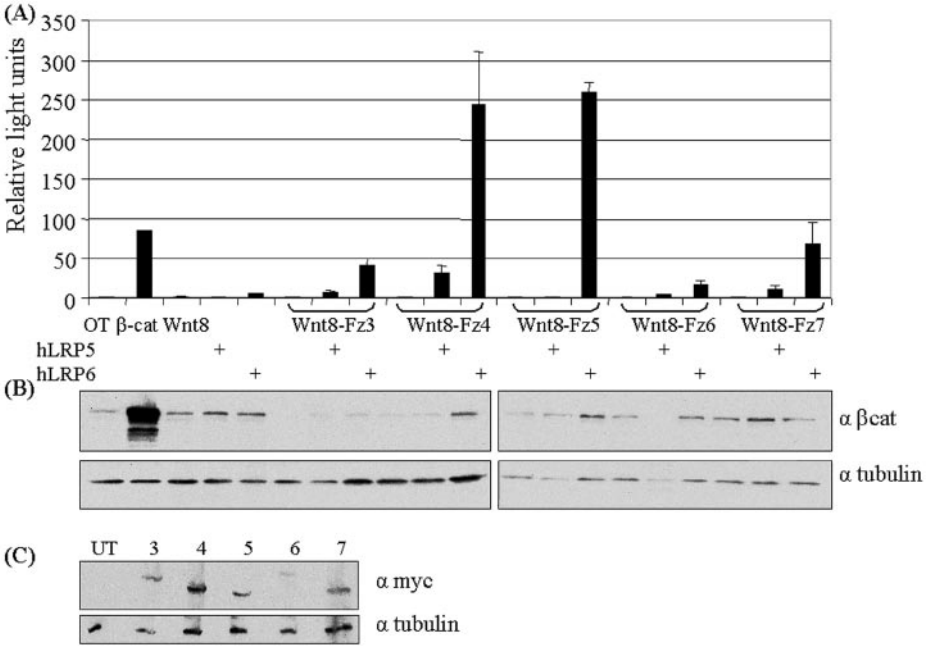

FIG. 6. **Fusion of different Frizzled genes to DNWnt8 reveals differences in their ability to activate the canonical pathway.** *A*, OT reporter gene assay on lysates from 293T cells transfected with the indicated expression plasmids. Assays are represented as described in Fig. 1. *B*, Western blot of cytoplasmic β-catenin and α-tubulin levels in cells transfected with the plasmids indicated in *A*. *C*, immunoblot with antibody to the myc epitope on cell lysates from 293T cells either untransfected (*UT*) or transfected with expression vectors encoding fusions of DNWnt8 to mouse Frizzled 3 (*3*), mouse Frizzled 4 (*4*), human Frizzled 5 (*5*), mouse Frizzled 6 (*6*), or mouse Frizzled (*7*). Note that five copies of the myc epitope have been inserted between the Wnt and Frizzled portions of the fusion protein (see Fig. 2).

Fz6 and LRP5/6 (Fig. 6*B*). The differences in transactivation and β-catenin stabilization were not due to a lack of protein expression, because all the fusion proteins were expressed, as detected on immunoblots of lysates with an antibody to the common myc epitope tag (Fig. 6*C*). DNWnt8-Fz6 was found to be expressed at slightly lower levels than the other Wnt-Fz fusions. All of the DNWnt8-Fz fusions were capable of inducing secondary axes in *Xenopus* embryos but to varying degrees. DNWnt8-Fz5 was the strongest inducer of a secondary axis in the embryos (64%), whereas DNWnt8-Fz3 and DNWnt8-Fz6 were the weakest inducers of a secondary axis (14 and 16%, respectively). The fact that all of the DNWnt8-Fz fusions were able to induce axis duplication, even weakly, suggests that this assay in *Xenopus* is potentially more promiscuous than the 293T system in its ability to utilize Frizzled molecules in signaling. We note that other reports have indicated clear differences between the *Xenopus* and the 293T transfection system we describe here (36). Together these data reinforce the idea

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that activation of different Frizzled molecules is a point where specificity is generated in the pathway. Finally, the data consistently show that LRP6 is much more potent in synergizing with all the fusion constructs than LRP5, pointing to another clear area where specificity in signaling can be generated.

Comparison of the primary amino acid sequences of the Frizzled proteins used in this study revealed that Frizzled proteins 4, 5, and 7 contain a strong consensus binding site for some PDZ domains (37), *i.e.* (S/T)*X*(V/I), located in the final 3 residues of the proteins, whereas Frizzled proteins 3 and 6 do not contain this consensus sequence at their C termini. Although previous studies showed that this region is not required for signaling via the canonical pathway (34, 38), we speculated it might play a role in the intensity of the signal. To test this, we eliminated the last 3 residues of DNWnt8-Fz5 (DNWnt8-Fz5ΔSHV) and compared its activity to that of the full-length molecule in 293T cells. No significant difference in signaling was observed between the two constructs, further reinforcing the idea that this motif is not required for activation of the canonical pathway (data not shown).

Transactivation of the OT Reporter Induced by the XWnt8-Fz5 Expression Vector Is Inhibited by Dominant Negative Forms of LRP6 and TCF4 in a Dose-dependent Manner—To further characterize the interaction between XWnt8-Fz5 and LRP6, we utilized known inhibitors to block the activation of the OT reporter. It has previously been shown that the N terminus of LRP6, lacking the transmembrane and cytoplasmic domains, acts in a dominant negative fashion, presumably by its ability to bind Wnt but its inability to signal due to the lack of the cytoplasmic domain (15). We titrated increasing amounts of N-LRP6-IgG DNA with XWnt8-Fz5 and LRP6 DNA and cotransfected them into 293T cells with the OT reporter. As expected, N-LRP6-IgG blocked the ability of XWnt8-Fz5 to activate the Wnt signaling pathway in a dose-dependent manner (Fig. 7*A*). We also tested a dominant negative TCF4 construct (ΔN-TCF4) as a downstream inhibitor of the pathway. ΔN-TCF4 is thought to act as a dominant negative, because it lacks the N terminus, containing the β-catenin binding site, but still associates with other TCF/lymphoid enhancer factor family members (39). We titrated increasing amounts of ΔN-TCF4 DNA with XWnt8-Fz5 and LRP6 DNA and cotransfected them into 293T cells with the OT reporter. As expected, ΔN-TCF4 blocked the ability of both constructs to activate the Wnt signaling pathway in a dose-dependent manner (Fig. 7*A*). Western blot analysis of extracts from cells transfected with these constructs indicated that the decreased activation observed in the presence of the inhibitors was not due to decreased levels of LRP6 or XWnt8-Fz5 (Fig. 7, *B* and *C*, respectively). Additionally, we tested the Wnt inhibitor Frizzled related protein 1 (FRP1) (40) by titrating increasing amounts of FRP1 DNA with XWnt8-Fz5, XWnt5A-Fz5, or XWnt11-Fz5 and LRP6 DNA in 293T cells. FRP1 blocked the ability of all of these fusions to activate the Wnt signaling pathway in a dose-dependent manner (data not shown).

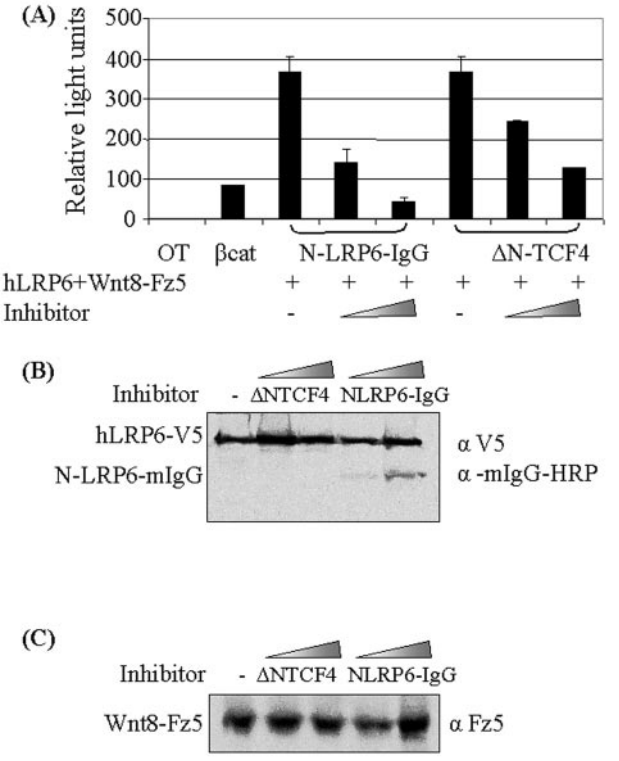

FIG. 7. **Transactivation of the OT reporter induced by the XWnt8-Fz5 expression vector is inhibited by dominant negative forms of LRP6 and TCF4 in a dose-dependent manner.** *A*, OT reporter gene assay on cells transfected with the indicated expression plasmids. Note that the *shaded triangle* represents increasing amounts of N-LRP6-IgG fusion or ΔN-TCF4 protein transfected into the cells. *B*, Western blot of LRP6-V5 and N-LRP6-mIgG expression in the absence or presence of increasing amounts of the inhibitors. *C*, Western blot of XWnt8-Fz5 expression in the absence or presence of increasing amounts of the inhibitors.

DISCUSSION

We report here the creation and analysis of various fusion constructs to assess mechanisms underlying Wnt signal transduction. We show that, in a 293T cell transient transfection system, an XWnt8-Fz5 fusion protein synergizes with LRP6 to potently transactivate the Wnt-responsive reporter OT and show that the specificity of this interaction is generated at several levels. Despite being expressed at similar levels, LRP6 is much more potent in this interaction than LRP5. Similarly, all Frizzled molecules are not equivalent in their ability to activate the canonical Wnt pathway in this context. Finally, Wnt molecules from both functionally defined classes can activate the β-catenin-dependent pathway in this system.

The difference between LRP5 and LRP6 is consistent with previous observations showing LRP6 to be a more potent inducer of ectopic embryonic axis in *Xenopus* embryos than LRP5 (15). Even though LRP5 is much less active in both the *Xenopus* model and data presented here, the physiological importance of LRP5 in Wnt signaling is underscored by the recent report that loss of function mutations in LRP5 are the cause of osteoporosis pseudoglioma and that the normal role of LRP5 in osteoblasts is to transduce signals through β-catenin (41). Future work using the observed differences in signaling activity may identify specific motifs that mediate these effects.

It is not surprising that Frizzled molecules differ in their ability to synergize in signaling in this context, because many reports have shown specificity among different Frizzled molecules. For example, specificity of binding of an XWnt8 alkaline phosphatase (XWnt-8-AP) fusion protein to cells expressing different Frizzled molecules has been demonstrated (23). Interestingly, XWnt8-AP bound to Fz4 and -5 and, to a lesser extent, to Fz7, but not to Fz3 and -6. This is similar to the transactivation profile we observed with the XWnt8-Fz fusions (Fig. 6*A*). This correlation suggests that certain Frizzled molecules may not be as capable of activating the canonical pathway in 293T cells, even when they are in a complex with Wnt and LRP5 or LRP6. Alternatively, the correlation may suggest that, for the Wnt-Fz fusions to activate the canonical pathway, Wnt must bind to the cysteine-rich domain (CRD) of the Fz to which it is fused. We tested this by generating XWnt11-Fz5 constructs that lacked portions of the CRD essential for Wnt binding

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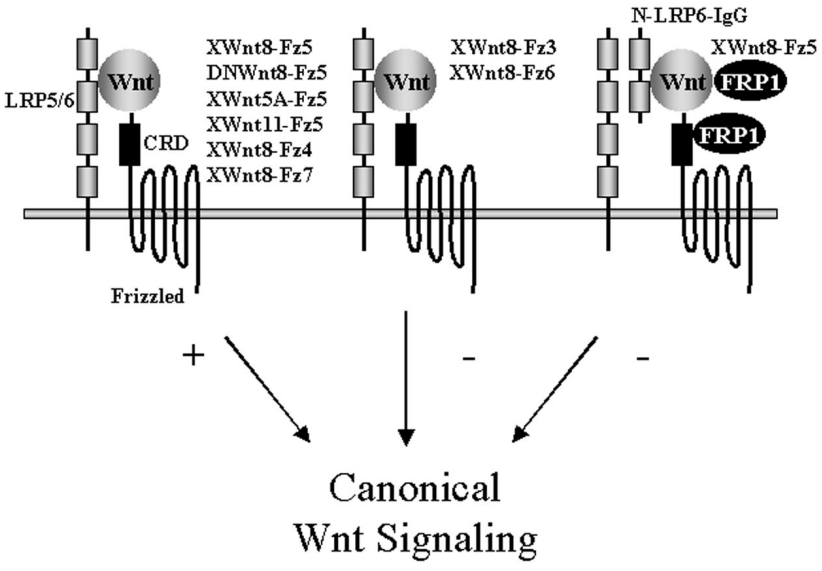

FIG. 8. **Model of Wnt-Fz fusion protein function.** The four epidermal growth factor repeat (EGFR) regions of LRP5/6 are represented as *gray boxes*, with EGFR regions 1 and 2 interacting with Wnt. The CRD of Frizzled is represented by a *black box*. Wnt is fused to the N terminus of Frizzled and functions to bring LRP5/6 into a physical complex with the Frizzled member of the fusion, which then signals to stabilize β-catenin and activate the canonical Wnt pathway. The following Wnt-Fz fusions are capable of signaling in synergy with LRP5/6 to activate the canonical Wnt pathway XWnt8-Fz5, DNWnt8-Fz5, XWnt5A-Fz5, XWnt11-Fz5, XWnt8-Fz4, and XWnt8-Fz7. XWnt8-Fz3 and XWnt8-Fz6 do not strongly activate the canonical Wnt pathway. A dominant negative form of LRP6 (N-LRP6-IgG), consisting of only the first two EGFR regions, as well as Frizzled related protein 1 (*FRP1*), block the synergistic signaling activity of XWnt8-Fz5 with LRP5/6, presumably by interfering with formation of the Wnt-Fz·LRP5/6 complex.

(XWnt11-Fz5 116–588 and XWnt11-Fz5 71–588) (42). Both of these constructs were expressed at levels similar to full-length XWnt11-Fz5 and synergized with LRP6 to transactivate the OT reporter to similar levels (data not shown), suggesting that there is not a requirement for Wnt binding to the Frizzled CRD in this system. To address this further we used the Wnt inhibitor FRP1, which can compete with Frizzleds for Wnt ligands (43–46), or block signaling by forming complexes with Frizzled receptors via their homologous CRDs (40). FRP1 blocked the ability of XWnt8-Fz5, XWnt5A-Fz5, and XWnt11-Fz5 to synergize with LRP6 to activate the Wnt signaling pathway in a dose-dependent manner (data not shown). Because FRP1 has been shown to antagonize Wnt1 and Wnt8, but not Wnt3A, -5A, or -11 (45), the effect we observed is consistent with the idea that FRP1 can bind to Frizzled CRDs and inhibit their function.

We were somewhat surprised that members of both functional Wnt classes could activate signaling in the context of being fused to Frizzled 5. Because both Wnt11 and Wnt5A are unable to induce a secondary axis when mRNA is injected into developing *Xenopus* embryos (35, 47), we anticipated that fusion of these Wnts to Frizzled 5 would not activate the canonical signaling pathway. It should be noted that in some contexts, for instance coexpression of Wnt5A and Frizzled 5 in developing *Xenopus* embryos, Wnt5A could activate the pathway (20). Because we have removed the necessity for Wnt5A and Wnt11 to bind to specific Frizzleds by fusing them to Frizzled 5, these data suggest that all Wnt molecules may be equivalent in their activities once they are bound to a Frizzled.

In summary, we propose that the Wnt-Fz fusions function to simply bring LRP5 and LRP6 into a physical complex with the Frizzled member of the fusion, which activates the canonical Wnt pathway. Given that the CRD deletion mutants of XWnt11-Fz5 can synergize with LRP6 in this system, our hypothesis is that the main function of Wnt is to simply nucleate the formation of a physical complex between LRP5 or -6 and a Frizzled molecule (Fig. 8). We are currently pursuing methods to induce the association between LRP6 and Frizzleds in a Wnt-independent manner to analyze signaling in this context.

Whereas this report has focused on using these reagents to assess specificity and mechanisms of signaling at the plasma membrane, we are pursuing further work using these constructs to address several other questions. For example, the demonstration that fusion of the dominant negative form of XWnt8 to Frizzled 5 creates a protein that has equivalent signaling activity to full-length XWnt8-Fz5 suggests that the dominant negative form of XWnt8 may function as a dominant negative, because it fails to bind to Frizzleds. By fusing the molecule directly to Frizzled we may have overcome the need to bind to Frizzled to induce signaling. Alternatively, the deleted residues may be required to properly localize the Wnt molecule to the plasma membrane, perhaps by mediating interactions with proteoglycan molecules (48–51). We are currently testing these hypotheses.

Although we have focused on the canonical Wnt signaling pathway in this report, Wnt ligands activate at least two other intracellular signaling pathways. One pathway, the planar cell polarity pathway, signals through the small GTPase Rho to organize polarity within tissues by signaling through a JNK pathway (11–13). In addition, a signaling cascade that activates isoforms of protein kinase C can also be initiated by Wnt ligands (9, 10, 52). Future work will focus on assessing the ability of these and other fusion constructs to activate these pathways and understanding what signals determine which of these Wnt-dependent pathways are activated in various contexts.

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A Novel Set of Wnt-Frizzled Fusion Proteins Identifies Receptor Components That Activate β-Catenin-dependent Signaling

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