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## Wnt3a Stimulation Elicits G Protein-Coupled Receptor Properties of Mammalian Frizzled Proteins

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**Receptors of the Frizzled (Fz) family initiate Wnt ligand-dependent signaling controlling multiple steps in organism development and carcinogenesis. Fz proteins possess seven transmembrane domains and their signaling depends on heterotrimeric G proteins in various organisms. However, Fzs constitute a distinct group within the G protein-coupled receptors (GPCR) superfamily, and Fz signaling can be G protein-independent in some experimental setups, leading to concerns about the GPCR nature of these proteins. Here we demonstrate that mammalian Fzs act as GPCRs on heterotrimeric Go/i proteins. Addition of the Wnt3a ligand to rat brain membranes or cultured cells elicits Fz-dependent guanine nucleotide exchange on Go/i. These responses were sensitive to a Wnt antagonist and to pertussis toxin, which decouples the Go/i proteins from their receptors through covalent modification. Our findings provide the long-awaited biochemical proof of the GPCR nature of Fz receptors.**

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## INTRODUCTION

Frizzled (Fz) proteins are a family of seven transmembrane helix (7-TM) receptors for secreted Wnt glycolipoproteins [1]. Initially discovered as a wing development factor in *Drosophila* and a tumorigenic gene product in mice [2, 3], Wnts further emerged as the trigger of several intricate signaling pathways [1], which are highly conserved among animal classes [4]. Wnt-Fz signal transduction plays crucial roles in organism development and homeostasis, whereas its misactivation was shown to cause numerous diseases, from degeneration to carcinogenesis [4-6].

Fz proteins regulate these diverse events through three well-recognized pathways: the canonical pathway regulating  $\beta$ -catenin-dependent transcription [7]; the planar cell polarity pathway polarizing the cytoskeleton within the epithelial plane [8]; and the calcium pathway [9]. The 19 Wnt and 10 Fz family members found in the human genome demonstrate profound differences in their specificities to each other and to the intracellular events they trigger [4, 10]. Molecular determinants which act as the immediate transducers of the signal from receptors still remain controversial [10]. One of the possible clues in this riddle is that 7-TM Fz proteins possess the same topology as the classical GPCRs [11, 12], although they are confined to a distinct group within this superfamily [13]. Fz pathways indeed were thought to be G-protein independent, until recent data showed necessity of those for Wnt signaling both in mammalian cells [14, 15] and in *Drosophila* [16-18].

As the evidence for roles of G proteins in all branches of Wnt signaling continues to grow [10, 19], the question concerning the genuine GPCR nature of Fzs remains open. Do they physically bind heterotrimeric G proteins and act as the guanine nucleotide exchange factors (GEF) protein for them? We have previously shown that this is indeed the case in a reconstituted system: bacterially expressed human Fz receptors Fz1, Fz6, and Fz7, upon activation by a panel of Wnt ligands (e.g. Wnt3a and Wnt5a), can catalyze guanine nucleotide exchange on the heterotrimeric Go protein; a physical interaction between Fz1 and Go has also been demonstrated [20]. However, despite the dependence of the physiological Fz signaling on G proteins [14-17], the GPCR properties of Fz proteins in a more physiologic cellular environment have been missing. Here we demonstrate, using mammalian brain preparations and cell cultures, that Wnt3a is capable of activating GPCR activities on several Fz receptors, coupled to the Go/i subclass of heterotrimeric G proteins.

## MATERIALS AND METHODS

**Plasmids.** Constructs encoding human *Gao*, *Gai1*, *Ga2* and *Gas* in pcDNA3.1 were from Missouri S&T cDNA Resource Center (cat. # GNA00A0000, GNAI100000, GNAI200000 and GNA0SL0000 respectively). *Ga12*, *Gaq* and *Gaz* coding sequences were obtained from ImaGenes GmbH (MGC # IRATp970H0199D, IRATp970H1164D and IRATp970B09107D, respectively) and were subcloned in the pIRES-EGFP vector (Clontech) using respective pairs of restriction sites: SacII and BamHI, PstI and BamHI, EcoRI and Sall. Sequences of human Frizzled receptors [20] were obtained from Invitrogen and subcloned in the pIRES-DsRed-Express vector.

**Isolation of rat brain membranes.** 5-day postnatal Wistar rat pups were decapitated and cerebellum was dissected on ice. The resting parts of the brain from 5 animals were immediately transferred into the 5 volumes of the ice-cold lysis buffer (50mM HEPES-NaOH pH 7.4, 1mM EGTA and Complete protease inhibitor cocktail (Roche)). Tissue was gently homogenized in Dounce homogenizer and nuclear fraction was precipitated by centrifugation for 10 min at 2000g, 4°C. The membrane fraction was isolated by additional ultracentrifugation at 180000g for 10 min, then the pellet was resuspended in the same amount of the lysis buffer and re-centrifuged. After the second identical wash membranes were resuspended to the concentration of 3 mg/ml of total protein (as determined by the Bradford assay) in the 50mM HEPES-NaOH pH 7.4, 1mM EGTA, 1mM MgCl<sub>2</sub>, 50 mM NaCl, 1x Complete protease inhibitor cocktail and stored frozen at -80°C.

**Purification of the Wnt3a protein.** Wnt3a was purified in three steps from conditioned medium essentially as described previously [21] with modifications [22], which yielded a final stock concentration of 300-500 nM. Wnt3a-expressing immortalized murine fibroblasts line (L Wnt-3A cells, ATCC #CRL-2647) were passaged 1:10 into Nunclon 500cm<sup>2</sup> TripleFlask with 200 ml of DMEM supplemented with 10% FCS (HyClone) and cultivated for 4 days. The conditioned medium was harvested, centrifuged at 3200g for 10 min and stored at 4°C while cells were incubated for 4 more days with 200 ml of fresh medium. After identical harvesting, both batches of Wnt3a-conditioned medium were combined and filtered through a 1- $\mu$ m glass fiber filter (Pall). Then filtrate was adjusted to 50mM HEPES-NaOH (pH 7.5) and 1% Triton X-100, re-filtered through 0.22- $\mu$ m nitrocellulose filters (Millipore) and applied to a 30-ml Blue Sepharose column equilibrated with 50mM HEPES-NaOH (pH 7.5), 1% Triton X-100, and 150mM NaCl. The column was washed with 2 column volumes of the equilibration buffer and then with 2 volumes of 50mM HEPES-NaOH (pH 7.5), 1% CHAPS, and 150mM NaCl. Wnt3a was eluted in one step with buffer containing 50mM HEPES-NaOH (pH 7.5), 1% CHAPS, and 1M NaCl and the eluate was collected in fractions of 10 ml. They were analyzed by Western blotting with monoclonal rat antibody against mouse Wnt3a (R&D Systems, cat. # MAB1324, at a 1:1000 dilution) and those with higher contents of Wnt3a protein were collected, concentrated in Amicon Ultra-15 centrifugal filter units with 10,000 Da cut-off to the final volume of 500 $\mu$ l. This sample was separated on the Superose 12 size-exclusion chromatography column (Pharmacia), pre-equilibrated with the 50mM HEPES-NaOH (pH 7.5), 1% CHAPS, and 150mM NaCl buffer. Fractions of 1ml were collected and analyzed as above and those with the higher content of Wnt3a were applied to the 5ml HiTrap Heparin HP column equilibrated with the 50mM HEPES-NaOH (pH 7.5), 1% CHAPS, and 150mM NaCl and eluted with the same buffer containing 1M NaCl in 1 ml fractions. The total protein concentration was measured by the Bradford assay, and the purity was estimated with the SDS-PAGE followed by Coomassie Blue R-250 staining.

**GTP-binding assay in rat brain membranes.** GTP-binding activity in membranes was assayed according to the DELFIA GTP-binding kit (PerkinElmer) manual, with modifications as described [23]. The assay was performed in 96-well AcroWell filter plates in the total volume of 100 $\mu$ l per well with

the final concentration of 50 µg/ml of membrane protein per well, 50mM HEPES-NaOH pH 7.4, 1mM MgCl<sub>2</sub>, 65 mM NaCl, 0.2% CHAPS, 0.05% Saponin (Sigma) and indicated amounts of Wnt3a or BSA. In all experiments dilution of the samples, as well as solutions of the BSA control, were prepared in the Wnt3a column elution buffer (see below) from the final step of purification, to ensure uniform CHAPS concentrations. After addition of all components the reaction mixtures were additionally incubated for 10 minutes on a shaker and the assay was initiated by addition of GTP-Eu to 5nM. The reaction proceeded for 45 minutes and then was stopped by filtration followed by immediate wash with the 150µl ice-cold buffer containing 25mM Tris-HCl pH 8.0, 0.1 mM MgCl<sub>2</sub>. Fluorescence was measured within 30 min after washing on the Victor3 Multilabel counter (PerkinElmer) according to the manufacturer's protocol.

**Pertussis toxin treatment.** Brain membranes were pretreated with the A protomer of the toxin from *Bordetella pertussis* (Merck, cat. # 516854) as described [24]. Membrane suspension was adjusted to the protein concentration of 1mg/ml, 20mM DTT, 0.2mM ATP (New England Biolabs), 50mM NaCl, 0.05% Saponin, 50µM NAD<sup>+</sup> (New England Biolabs), with or without (control) 1µg/ml of Pertussis toxin A protomer. Following 2h incubation at 35°C membranes were used in the GTP-binding assay as described above.

**GTP-binding assay in intact mouse fibroblasts (L cells).** Murine fibroblasts (L cells, ATCC cat.# CRL-2648) were grown in the 96-well plates (TPP) until 60-70% confluent monolayer. They were transfected with the 6µg/ml of total plasmid DNA in serum-free Opti-MEM medium (Invitrogen) using the Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's recommendations. After transfection the medium was replaced with DMEM supplemented with 10% FCS and cells were grown for additional 36-48 hours. If specified, holotoxin from *Bordetella pertussis* (Sigma) was added to the final concentration of 2µg/ml in the medium 12-16h prior to experiment. Then the medium was removed, cell layer was quickly rinsed 2 times with 100µl per well 50mM HEPES-NaOH, 150mM NaCl, 1mM MgCl<sub>2</sub> and then permeabilized with the same buffer containing 0.05% saponin for 10min with shaking. The permeabilizing buffer was then replaced with 40µl of DMEM supplemented with 10% FCS and 0.05% saponin. The Wnt3a ligand was supplied in 2µl elution buffer bringing the final concentration of NaCl in the medium to 160mM, and that of CHAPS to 0.05%. Following 10min incubation, reaction was started by addition of 10µl 50nM GTP-Eu, and was allowed to proceed for 45 minutes. Cells were then scraped, the suspension was transferred to the 96-well AcroWell filter plates and immediately filtered. Washing and measuring steps were performed as it described before for the membrane-based GTP-binding assay.

## RESULTS AND DISCUSSION

Multiple studies performed with cell lines [14, 25, 26], as well as *Drosophila* epistasis experiments [16, 17] suggest an important function of the heterotrimeric protein Go in Wnt signaling pathways. Go and Gi are the most abundant heterotrimeric G proteins in the mammalian nervous system [27]. Further, several Fz proteins, including Fz1, Fz3, Fz6, Fz7, and Fz9, are strongly expressed in this tissue [28]. To test the capacity of endogenous Fz proteins to activate endogenous G proteins, we treated membrane isolations from rat brains with purified Wnt3a.

We found that addition of purified Wnt3a, but not equivalent amounts of a control protein (BSA), to the rat brain membrane preparations indeed induced the nucleotide exchange on endogenous G proteins in a concentration-dependent manner (Figure 1A). The 50-100% stimulation of the G proteins we describe is similar to the stimulation achieved by other activated GPCRs in cellular membranes or in reconstituted systems [23, 29-31]. To further prove specificity of this process, we tested the sensitivity of the Wnt-dependent G protein activation to the natural antagonist of the Wnt pathway – sFRP-2. The general mechanism of sFRPs' action was proposed to be the competitive binding for the Wnt ligands [32], thus preventing activation of Fz receptors. Indeed, increasing concentrations of sFRP-2 diminished Wnt3a-dependent G protein activation in rat brain membranes (Figure 1B).

The heterotrimeric Go/i proteins can be decoupled from their cognate GPCRs by pertussis toxin (Ptx), which ADP-ribosylates the conserved cysteine residue in the C-terminus of G $\alpha$ -subunits, blocking this important site of interaction with receptors [33, 34]. We found that Ptx pretreatment of rat brain membranes completely abolished G protein activation in response to Wnt3a (Figure 1C). Cumulatively, these data demonstrate that in the endogenous system from rat brains, a Wnt ligand is able to induce a characteristic activation of heterotrimeric G proteins, which is sensitive to both sFRP2- and Ptx-inhibition. Thus, endogenous brain receptors for the Wnt3a ligand act as typical Go/i-coupled GPCRs.

Adult mammalian brain expresses several Fz proteins [28]. To identify which Fz could respond to Go/i activation upon stimulation with the Wnt3a ligand, we transiently transfected L-cells (murine fibroblasts) with different Fz-expressing plasmids. This cell line has been extensively used in the analysis of the Wnt signaling and possesses the endogenous Wnt pathway components, as it responds to Wnt ligands by the characteristic  $\beta$ -catenin-dependent transcription [21].

First, we evaluated the capacity of non-transfected L-cells to activate intrinsic G proteins upon stimulation of the pathway. As expected, addition of increasing concentrations of Wnt3a revealed small although significant activation of endogenous G proteins (Figures 2A and 2B). Next, we analyzed the ability of different human Fz proteins to enhance this activation. Sequences encoding human Fz1, Fz6, Fz9, and Fz10 were cloned into pIRES2-DsRed-Express vector for mammalian expressions and transfected into cells prior to Wnt stimulation. All tested Fzs, with the exception of Fz9, substantially increased G-protein activation upon addition of Wnt3a (Figures 2A and 2B). The most likely explanation for the inability of Fz9 to respond in our assay is that it does not efficiently bind Wnt3a [35].

The Wnt3a-Fz1 interaction has been previously documented [36]. To confirm specificity of the GPCR responses described above, we repeated the experiments with the Fz1- and control-transfected cells, stimulated by Wnt3a in the presence of increasing concentration of sFRP-2. In both experimental setups, sFRP-2 inhibited Wnt3a-induced G protein activation (Figure 2D), proving that the effect was indeed Wnt3a-dependent. To obtain the additional proof for the Go/i-coupling of Fz1 in L-cells, we treated the cells with Ptx prior to stimulation. As expected, cells expressing Fz1 alone or together with Gao completely lost their responsiveness to Wnt3a after Ptx treatment (Figure 2C). In contrast, a certain degree of G protein activation of non-transfected cells appeared to persist, although the low level of the remaining response was below the statistical significance.

To investigate systematically which G proteins were coupled to the Wnt3a/Fz1 pair, we co-

transfected different  $G\alpha$ -subunits with human Fz1 prior to L-cell stimulation with Wnt3a. Overexpression of heterotrimeric G protein intensifies their signal in cellular GTP-binding assays, so that even the G proteins with the weak intrinsic activity could be reliably investigated [37]. Out of the  $G\alpha$ -subunits we analyzed, members of the Go/i subtype ( $G\alpha_o$ ,  $G\alpha_{i1}$ , and  $G\alpha_{i2}$ ) were responsive to the Wnt3a-Fz1 activation, while the  $G\alpha_z$ ,  $G\alpha_s$ ,  $G\alpha_q$ , and  $G\alpha_{12}$  subunits were not (Figure 2E). In contrast, transfection of L-cells with  $G\alpha_s$  stimulated the response of the endogenous receptor(s) to Wnt3a, while transfection with  $G\alpha_{12}$  suppressed this response (Figure 2E). These data may suggest that the endogenous Wnt3a-binding Fz protein(s) is Gs-coupled; the suppression of this endogenous response by  $G\alpha_{12}$  will require additional investigation.

Mammalian Fz proteins investigated in our work fall into different groups divided by the type of the signaling pathway they initiate. Our data demonstrate that Fz receptors transducing the canonical  $\beta$ -catenin-dependent pathway (Fz1, Fz10) [36, 38], as well as the planar cell polarity pathway (Fz6) [39], all behave as Go/i-coupled GPCRs. In the present work, we find the ability of Wnt3a to activate Fz1, Fz6, and Fz10. In general, no systematic identification of the specificities among numerous Wnt and Fz family members has been performed. Together with our previous data [20], our assay lays the ground for such systematic investigation.

Our results also enable the detailed analysis of which Wnt-Fz pairs couple to which heterotrimeric G proteins. In the results we present, all the tested Fz proteins efficiently interacted with the Go, Gi1, and Gi2 proteins, which are close to each other and form the Ptx-sensitive subgroup within the heterotrimeric G proteins [40]. Additionally, we provide evidence in favor of coupling of an unidentified Fz(s) endogenous to L-cells to Gs. Previous investigations on rat Fz1 performed in zebrafish and cultured cells showed the presence of a Ptx-sensitive component, identified in some studies as Go, in the signal transduction elicited by these receptors [14, 15, 25, 41, 42]. A recent report implicated Gi2 in Wnt signaling in *Xenopus* eggs [43]; Gi proteins are also involved in signaling by the Fz-related receptor Smoothed [44, 45]. Additionally, murine microglia cell line N13 was recently found to activate Gi-type heterotrimeric G proteins through an uncharacterized receptor(s) [46].

Despite the fact that the heterotrimeric G protein Gq has been previously implicated in Fz signaling [14, 42, 43, 47], we failed to show any ability of  $G\alpha_q$  to stimulate Wnt3a-Fz1-induced G protein activation in L-cells. It is clear that our current analysis of the G protein involvement in different Wnt-Fz pair-induced G protein activation is not exhaustive. Additional studies in this direction will be required to test which Wnt ligands can elicit coupling of which Fzs to the non-Go/i heterotrimeric G proteins.

Overall, our data provide the first firm and definitive biochemical demonstration that Fz receptors, expressed in their natural cellular environment, act as typical GPCRs serving as guanine nucleotide exchange factors on heterotrimeric G proteins upon stimulation with Wnt ligands. Together with our previous report on bacterially expressed Fz proteins [20], our results resolve a long-standing debate over the GPCR nature of Fz receptors, initiated by the original demonstration of the GPCR topology of Fz receptors [11, 12]. The GPCR activities of Fz receptors can be used as a platform to screen chemical libraries for small molecule agonists and antagonists of the Wnt-Fz interactions. Given the high-degree involvement – positive or destructive – of the Wnt/Fz signaling in such medically important processes as carcinogenesis [6], tissue regeneration [48], stem cell proliferation [49], and neurodegeneration [5], the agonists and antagonists of Wnt/Fz interactions might be of a highest potential importance. Such a platform would be directed to detect the highly desired agents acting directly at the level of the Wnt ligand-Fz receptor interaction [50].

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## REFERENCES

- 1 MacDonald, B. T., Tamai, K. and He, X. (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* **17**, 9-26
- 2 Cabrera, C. V., Alonso, M. C., Johnston, P., Phillips, R. G. and Lawrence, P. A. (1987) Phenocopies induced with antisense RNA identify the wingless gene. *Cell* **50**, 659-663
- 3 Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987) The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* **50**, 649-657
- 4 Logan, C. Y. and Nusse, R. (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**, 781-810
- 5 De Ferrari, G. V. and Moon, R. T. (2006) The ups and downs of Wnt signaling in prevalent neurological disorders. *Oncogene* **25**, 7545-7553
- 6 Polakis, P. (2007) The many ways of Wnt in cancer. *Curr Opin Genet Dev* **17**, 45-51
- 7 Cadigan, K. M. (2008) Wnt-beta-catenin signaling. *Curr Biol* **18**, R943-947
- 8 Simons, M. and Mlodzik, M. (2008) Planar cell polarity signaling: from fly development to human disease. *Annu Rev Genet* **42**, 517-540
- 9 Kuhl, M. (2004) The WNT/calcium pathway: biochemical mediators, tools and future requirements. *Front Biosci* **9**, 967-974
- 10 Angers, S. and Moon, R. T. (2009) Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol* **10**, 468-477
- 11 Vinson, C. R., Conover, S. and Adler, P. N. (1989) A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains. *Nature* **338**, 263-264
- 12 Park, W. J., Liu, J. and Adler, P. N. (1994) The *frizzled* gene of *Drosophila* encodes a membrane protein with an odd number of transmembrane domains. *Mech Dev* **45**, 127-137
- 13 Fredriksson, R., Lagerstrom, M. C., Lundin, L. G. and Schioth, H. B. (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**, 1256-1272
- 14 Liu, T., Liu, X., Wang, H., Moon, R. T. and Malbon, C. C. (1999) Activation of rat *frizzled-1* promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via pathways that require Galpha(q) and Galpha(o) function. *J Biol Chem* **274**, 33539-33544
- 15 Liu, X., Rubin, J. S. and Kimmel, A. R. (2005) Rapid, Wnt-induced changes in GSK3beta associations that regulate beta-catenin stabilization are mediated by Galpha proteins. *Curr Biol* **15**, 1989-1997
- 16 Katanaev, V. L., Ponzielli, R., Semeriva, M. and Tomlinson, A. (2005) Trimeric G protein-dependent *frizzled* signaling in *Drosophila*. *Cell* **120**, 111-122
- 17 Katanaev, V. L. and Tomlinson, A. (2006) Dual roles for the trimeric G protein Go in asymmetric cell division in *Drosophila*. *Proc Natl Acad Sci U S A* **103**, 6524-6529
- 18 Purvanov, V., Koval, A. and Katanaev, V. L. (2010) A direct and functional interaction between Go and Rab5 during G protein-coupled receptor signaling. *Sci Signal* **3**, ra65
- 19 Egger-Adam, D. and Katanaev, V. L. (2008) Trimeric G protein-dependent signaling by *Frizzled* receptors in animal development. *Front Biosci* **13**, 4740-4755
- 20 Katanaev, V. L. and Buestorf, S. (2009) *Frizzled* Proteins are bona fide G Protein-Coupled Receptors. Available from Nature Precedings <<http://hdl.handle.net/10101/npre.2009.2765.1>>
- 21 Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd and Nusse, R. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452
- 22 Kishida, S., Yamamoto, H. and Kikuchi, A. (2004) Wnt-3a and Dvl induce neurite retraction by

8

- activating Rho-associated kinase. *Mol Cell Biol* **24**, 4487-4501
- 23 Koval, A., Kopein, D., Purvanov, V. and Katanaev, V. L. (2010) Europium-labeled GTP as a general nonradioactive substitute for [(35)S]GTPgammaS in high-throughput G protein studies. *Anal Biochem* **397**, 202-207
- 24 Kurose, H. and Ui, M. (1983) Functional uncoupling of muscarinic receptors from adenylate cyclase in rat cardiac membranes by the active component of islet-activating protein, pertussis toxin. *J Cyclic Nucleotide Protein Phosphor Res* **9**, 305-318
- 25 Bikkavilli, R. K., Feigin, M. E. and Malbon, C. C. (2008) G{alpha}o mediates WNT-JNK signaling through Dishevelled 1 and 3, RhoA family members, and MEKK 1 and 4 in mammalian cells. *J Cell Sci* **121**, 234-245
- 26 Feigin, M. E. and Malbon, C. C. (2007) RGS19 regulates Wnt-beta-catenin signaling through inactivation of Galpha(o). *J Cell Sci* **120**, 3404-3414
- 27 Gierschik, P., Milligan, G., Pines, M., Goldsmith, P., Codina, J., Klee, W. and Spiegel, A. (1986) Use of specific antibodies to quantitate the guanine nucleotide-binding protein Go in brain. *Proc Natl Acad Sci U S A* **83**, 2258-2262
- 28 Regard, J. B., Sato, I. T. and Coughlin, S. R. (2008) Anatomical profiling of G protein-coupled receptor expression. *Cell* **135**, 561-571
- 29 Frang, H., Mukkala, V. M., Syysto, R., Ollikka, P., Hurskainen, P., Scheinin, M. and Hemmila, I. (2003) Nonradioactive GTP binding assay to monitor activation of g protein-coupled receptors. *Assay Drug Dev Technol* **1**, 275-280
- 30 Milligan, G. (2003) Principles: extending the utility of [35S]GTP gamma S binding assays. *Trends Pharmacol Sci* **24**, 87-90
- 31 Weiland, T. and Jakobs, K. H. (1994) Measurement of receptor-stimulated guanosine 5'-O-(gamma-thio)triphosphate binding by G proteins. *Methods Enzymol* **237**, 3-13
- 32 Wawrzak, D., Metioui, M., Willems, E., Hendrickx, M., de Genst, E. and Leys, L. (2007) Wnt3a binds to several sFRPs in the nanomolar range. *Biochem Biophys Res Commun* **357**, 1119-1123
- 33 Moss, J. and Vaughan, M. (1988) ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins. *Advances in Enzymology and Related Areas of Molecular Biology* **61**, 303-379
- 34 Oldham, W. M. and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* **9**, 60-71
- 35 Karasawa, T., Yokokura, H., Kitajewski, J. and Lombroso, P. J. (2002) Frizzled-9 is activated by Wnt-2 and functions in Wnt/beta -catenin signaling. *J Biol Chem* **277**, 37479-37486
- 36 Gazit, A., Yaniv, A., Bafico, A., Pramila, T., Igarashi, M., Kitajewski, J. and Aaronson, S. A. (1999) Human frizzled 1 interacts with transforming Wnts to transduce a TCF dependent transcriptional response. *Oncogene* **18**, 5959-5966
- 37 Harrison, C. and Traynor, J. R. (2003) The [35S]GTPgammaS binding assay: approaches and applications in pharmacology. *Life Sci* **74**, 489-508
- 38 Terasaki, H., Saitoh, T., Shiokawa, K. and Katoh, M. (2002) Frizzled-10, up-regulated in primary colorectal cancer, is a positive regulator of the WNT - beta-catenin - TCF signaling pathway. *Int J Mol Med* **9**, 107-112
- 39 Guo, N., Hawkins, C. and Nathans, J. (2004) Frizzled6 controls hair patterning in mice. *Proc Natl Acad Sci U S A* **101**, 9277-9281
- 40 Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**, 615-649
- 41 Slusarski, D. C., Corces, V. G. and Moon, R. T. (1997) Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410-413

- 42 Liu, T., DeCostanzo, A. J., Liu, X., Wang, H., Hallagan, S., Moon, R. T. and Malbon, C. C. (2001) G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. *Science* **292**, 1718-1722
- 43 Jernigan, K. K., Cselenyi, C. S., Thorne, C. A., Hanson, A. J., Tahinci, E., Hajicek, N., Oldham, W. M., Lee, L. A., Hamm, H. E., Hepler, J. R., Kozasa, T., Linder, M. E. and Lee, E. (2010) Gbetagamma activates GSK3 to promote LRP6-mediated beta-catenin transcriptional activity. *Sci Signal* **3**, ra37
- 44 Riobo, N. A., Saucy, B., Dilizio, C. and Manning, D. R. (2006) Activation of heterotrimeric G proteins by Smoothed. *Proc Natl Acad Sci U S A* **103**, 12607-12612
- 45 Ogden, S. K., Fei, D. L., Schilling, N. S., Ahmed, Y. F., Hwa, J. and Robbins, D. J. (2008) G protein Galpha(i) functions immediately downstream of Smoothed in Hedgehog signalling. *Nature*
- 46 Kilander, M., Dijksterhuis, J., Ganji, R. S., Bryja, V. and Schulte, G. WNT-5A stimulates the GDP/GTP exchange at pertussis toxin-sensitive heterotrimeric G proteins. *Cell Signal*
- 47 Salmanian, S., Najafi, S. M., Rafipour, M., Arjomand, M. R., Shahheydari, H., Ansari, S., Kashkooli, L., Rasouli, S. J., Jazi, M. S. and Minaei, T. (2010) Regulation of GSK-3beta and beta-Catenin by Galphaq in HEK293T cells. *Biochem Biophys Res Commun* **395**, 577-582
- 48 Stoick-Cooper, C. L., Moon, R. T. and Weidinger, G. (2007) Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. *Genes Dev* **21**, 1292-1315
- 49 Reya, T. and Clevers, H. (2005) Wnt signalling in stem cells and cancer. *Nature* **434**, 843-850
- 50 Barker, N. and Clevers, H. (2006) Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discov* **5**, 997-1014

**FIGURE LEGENDS****Figure 1 Wnt3a-dependent specific activation of G proteins in rat postnatal brain membranes**

(A) Wnt3a, but not BSA (control) in a dose-dependent manner enhance binding of GTP-Eu by G proteins endogenous to the rat brain membranes. (B) This activity is shown to be sensitive to increasing concentrations of sFRP-2, a Wnt antagonist. (C) Activation is abolished by pretreatment of membranes by A protomer from Bordetella pertussis toxin (Ptx), known to deactivate GPCR signaling by ADP-ribosylating G proteins of Go/i family.

**Figure 2 L-cell culture reveal Go/i-coupled nature of human Frizzled1 protein in GTP-binding assay**

(A) Control pIRES2-DsRed vector transfected cells demonstrate moderate increase in GTP-binding activity upon stimulation with Wnt3a, improved significantly by transfection of Fz1, Fz6 and Fz10 but not Fz9 constructs. (B) Progressive inhibition of the Wnt3a effect by sFRP-2 in Fz1- or DsRed-transfected cells. (C) Wnt3a induces the G protein activation response in a concentration-dependent manner both in control and Fz1-transfected cells. (D) Ptx-pretreated cells, transfected with pIRES-DsRed, or Fz1 alone or co-transfected with G $\alpha$  fail to show any increase in G protein activation. (E) Results of simultaneous co-transfection of control or human Fz1 plasmids with those encoding a set of G $\alpha$  subunits reveal enhanced GTP binding for Go, Gi1 and Gi2 protein in response to Wnt3a. The response of the endogenous Fz receptor(s) was stimulated by overexpression of Gs and suppressed by G12. All the data are shown as the level of G protein activation achieved in the presence of Wnt3a, as percent of that produced in the presence of the equal amount of BSA (“% of basal” on the Figure). Statistical significance (t-test) is shown as P<0.0005 (\*\*\*), P<0.005 (\*\*), P<0.05 (\*), and non-significant (ns, P>0.05).



