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Wnt3a stimulation elicits G-protein-coupled receptor properties of mammalian Frizzled proteins

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

Key words: Frizzled (Fz), G-protein-coupled receptor (GPCR), guanine-nucleotide-exchange factor (GEF), Wnt3a.

INTRODUCTION

Fz (Frizzled) proteins are a family of 7-TM (seven transmembrane helix) 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 signalling pathways [1] which are highly conserved among animal classes [4]. Wnt–Fz signal transduction plays crucial roles in organism development and homoeostasis, whereas its misactivation has been shown to cause numerous diseases, from degeneration to carcinogenesis [4–6].

Fz proteins regulate these diverse events through three well-recognized pathways: (i) the canonical pathway regulating β-catenin-dependent transcription [7]; (ii) the planar cell-polarity pathway polarizing the cytoskeleton within the epithelial plane [8]; and (iii) the calcium pathway [9]. The 19 Wnt and ten 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 (G-protein-coupled receptors) [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 data showed the necessity of those for Wnt signalling both in mammalian cells [14,15] and in *Drosophila* [16–18].

As the evidence for roles of G-proteins in all branches of Wnt signalling continues to grow [10,19], the question concerning the genuine GPCR nature of Fz proteins remains open. Do they physically bind heterotrimeric G-proteins and act as the GEFs (guanine-nucleotide-exchange factors) for them? We have shown previously 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 catalyse guanine-nucleotide exchange on the heterotrimeric G_o protein, a physical interaction between Fz1 and G_o has also been demonstrated [20]. However, despite the dependence of the physiological Fz signalling on G-proteins [14–17], the GPCR properties of Fz proteins in a more physiological cellular environment have been missing. In the present study we demonstrate, using mammalian brain preparations and cell cultures, that Wnt3a is capable of activating GPCR activities on several Fz receptors coupled to the $G_{o/i}$ subclass of heterotrimeric G-proteins.

MATERIALS AND METHODS

Plasmids

Constructs encoding human $G_{\alpha o}$, $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha s}$ in pcDNA3.1 were from the Missouri S&T cDNA Resource Center (catalogue numbers GNA0OA0000, GNAI100000, GNAI200000 and GNA0SL0000 respectively). $G_{\alpha 12}$, $G_{\alpha q}$ and $G_{\alpha z}$ coding sequences were obtained from ImaGenes (MGC numbers IRATp970H0199D, IRATp970H1164D and IRATp970B09107D respectively) and were subcloned into the pIRES-EGFP vector (Clontech) using respective pairs of restriction sites: SacII and BamHI, PstI and BamHI, and EcoRI and SalI. Sequences of human Fz receptors [20] were obtained from Invitrogen and were subcloned into the pIRES-DsRed-Express vector.

Isolation of rat brain membranes

Wistar rat pups (5-day postnatal) were decapitated and the cerebellum was dissected on ice. The resting parts of the brain from five animals were immediately transferred into 5 vol. of ice-cold lysis buffer [50 mM Hepes/NaOH (pH 7.4), 1 mM EGTA

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Fz, Frizzled; GEF, guanine-nucleotide-exchange factor; GPCR, G-protein-coupled receptor; Ptx, Pertussis toxin; sFRP, secreted Fz-related protein; 7-TM, seven transmembrane helix.

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and Complete™ protease inhibitor cocktail (Roche)]. Tissue was gently homogenized in a Dounce homogenizer and the nuclear fraction was precipitated by centrifugation at 2000 ***g*** for 10 min at 4 °C. The membrane fraction was isolated by additional ultracentrifugation at 51 000 rev./min for 10 min using a Beckman Coulter Type 70.1 Ti rotor, and then the pellet was resuspended in the same amount of lysis buffer and re-centrifuged. After the second identical wash, membranes were resuspended at a concentration of 3 mg/ml total protein (as determined using the Bradford assay) in 50 mM Hepes/NaOH (pH 7.4), 1 mM EGTA, 1 mM $MgCl_2$, 50 mM NaCl and Complete™ protease inhibitor cocktail, and was stored frozen at − 80 °C. Animal experiments were approved by the review board of Regieungspräsidum Freiburg.

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. Cells from the Wnt3a-expressing immortalized murine fibroblast line (Wnt-3A L-cells, A.T.C.C. catalogue number CRL-2647) were passaged 1:10 into a Nunclon 500 cm^2 TripleFlask with 200 ml of DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS (fetal bovine serum; HyClone) and cultivated for 4 days. The conditioned medium was harvested, centrifuged at 3200 ***g*** for 10 min and stored at 4 °C while cells were incubated for a further 4 days with 200 ml of fresh medium. After identical harvesting, both batches of Wnt3a-conditioned medium were combined and filtered through a 1-μm glass fibre filter (Pall). Filtrate was then adjusted to 50 mM Hepes/NaOH (pH 7.5) and 1 % Triton X-100, re-filtered through 0.22-μm nitrocellulose filters (Millipore) and applied to a 30 ml Blue−Sepharose column equilibrated with 50 mM Hepes/NaOH (pH 7.5), 1 % Triton X-100 and 150 mM NaCl. The column was washed with 2 column vol. of the equilibration buffer and then with 2 vol. of 50 mM Hepes/NaOH (pH 7.5), 1 % CHAPS and 150 mM NaCl. Wnt3a was eluted in one step with buffer containing 50 mM Hepes/NaOH (pH 7.5), 1 % CHAPS and 1 M NaCl, and the effluent was collected in fractions of 10 ml. These were analysed by Western blotting with a monoclonal rat antibody against mouse Wnt3a (R&D Systems, catalogue number MAB1324, at a 1:1000 dilution) and those with a higher content of Wnt3a protein were collected, concentrated in Amicon Ultra-15 centrifugal filter units with a 10 000 Da cut-off to a final volume of 500 μl. This sample was separated on a Superose 12 size-exclusion chromatography column (Pharmacia) pre-equilibrated with 50 mM Hepes/NaOH (pH 7.5), 1 % CHAPS and 150 mM NaCl. Fractions of 1 ml were collected and analysed as described above, and those with the higher content of Wnt3a were applied to the 5 ml HiTrap Heparin HP column equilibrated with 50 mM Hepes/NaOH (pH 7.5), 1 % CHAPS and 150 mM NaCl, and eluted with the same buffer containing 1 M NaCl in 1 ml fractions. The total protein concentration was measured using the Bradford assay, and the purity was estimated by SDS/PAGE followed by Coomassie Blue R-250 staining.

GTP-binding assay in rat brain membranes

The GTP-binding activity in membranes was assayed using the DELFIA GTP-binding kit (PerkinElmer) according to the manufacturer's protocol, with modifications as described previously [23]. The assay was performed in 96-well AcroWell filter plates in a total volume of 100 μl per well with a final concentration of 50 μg/ml membrane protein per well, 50 mM Hepes/NaOH (pH 7.4), 1 mM $MgCl_2$, 65 mM NaCl, 0.2 % CHAPS, 0.05 % saponin (Sigma) and the 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 above) from the final step of purification to ensure a uniform CHAPS concentration. After addition of all components the reaction mixtures were additionally incubated for 10 min on a shaker and the assay was initiated by addition of GTP-Eu to a concentration of 5 nM. The reaction proceeded for 45 min and was then stopped by filtration followed by an immediate wash with 150 μl of ice-cold buffer containing 25 mM Tris/HCl (pH 8.0) and 0.1 mM $MgCl_2$. Fluorescence was measured within 30 min after washing on the Victor3 Multilabel counter (PerkinElmer) according to the manufacturer's protocol.

Ptx (pertussis toxin) treatment

Brain membranes were pre-treated with the A protomer of the toxin from *Bordetella pertussis* (Merck, catalogue number 516854) as described previously [24]. The membrane suspension was adjusted to a protein concentration of 1 mg/ml in 20 mM DTT (dithiothreitol), 0.2 mM ATP (New England Biolabs), 50 mM NaCl, 0.05 % saponin and 50 μM NAD^+ (New England Biolabs), with or without (control) 1 μg/ml Ptx A protomer. Following a 2 h 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, A.T.C.C. catalogue number CRL-2648) were grown in 96-well plates (TPP) until 60−70 % confluent. They were transfected with 6 μg/ml total plasmid DNA in serum-free Opti-MEM medium (Invitrogen) using the Lipofectamine™ 2000 (Invitrogen) reagent according to the manufacturer's protocol. After transfection, the medium was replaced with DMEM supplemented with 10 % FBS and cells were grown for an additional 36−48 h. If specified, holotoxin from *B. pertussis* (Sigma) was added to a final concentration of 2 μg/ml in the medium 12−16 h prior to the experiment. The medium was then removed, the cell layer was quickly rinsed twice with 100 μl of 50 mM Hepes/NaOH (pH 7.5), 150 mM NaCl and 1 mM $MgCl_2$ per well, and then the cells were permeabilized with the same buffer containing 0.05 % saponin for 10 min with shaking. The permeabilizing buffer was then replaced with 40 μl of DMEM supplemented with 10 % FBS and 0.05 % saponin. The Wnt3a ligand was supplied in 2 μl of elution buffer bringing the final concentration of NaCl in the medium to 160 mM, and that of CHAPS to 0.05 %. Following a 10 min incubation, the reaction was started by the addition of 10 μl of 50 nM GTP-Eu, and was allowed to proceed for 45 min. Cells were then scraped, the suspension was transferred to 96-well AcroWell filter plates and immediately filtered. Washing and measuring steps were performed as described above 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 G_o in Wnt signalling pathways. G_o and G_i are the most abundant heterotrimeric G-proteins in the mammalian nervous system [27]. Furthermore, several Fz proteins, including Fz1, Fz3, Fz6, Fz7 and Fz9, are strongly expressed in this tissue [28]. To test the capacity of

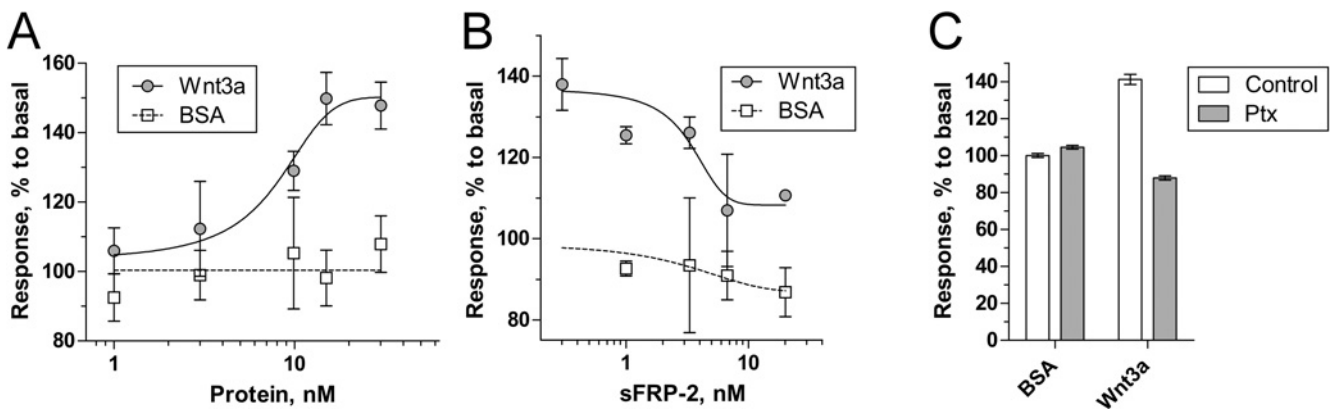

Figure 1 Wnt3a-dependent specific activation of G-proteins in rat postnatal brain membranes

(**A**) Wnt3a, but not BSA (control), in a dose-dependent manner enhanced binding of GTP-Eu by endogenous G-proteins to the rat brain membranes. (**B**) Activity was sensitive to increasing concentrations of sFRP-2, a Wnt antagonist. (**C**) Activation was abolished by pre-treatment of membranes by the Ptx A protomer, known to deactivate GPCR signalling by ADP-ribosylating G-proteins of the $G_{o/i}$ family.

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 observed is similar to the stimulation achieved by other activated GPCRs in cellular membranes or in reconstituted systems [23,29−31]. To further prove the specificity of this process, we tested the sensitivity of Wnt-dependent G-protein activation to the natural antagonist of the Wnt pathway, sFRP-2 (secreted Fz-related protein 2). The general mechanism of the action of sFRPs has been 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 $G_{o/i}$ proteins can be decoupled from their cognate GPCRs by Ptx, which ADP-ribosylates the conserved cysteine residue in the C-terminus of G_{α} subunits, blocking this important site of interaction with receptors [33,34]. We found that Ptx pre-treatment of rat brain membranes completely abolished G-protein activation in response to Wnt3a (Figure 1C). Cumulatively, these findings 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 sFRP-2 and Ptx inhibition. Thus endogenous brain receptors for the Wnt3a ligand act as typical $G_{o/i}$-coupled GPCRs.

Adult mammalian brain expresses several Fz proteins [28]. To identify which Fz protein could respond to $G_{o/i}$ 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 Wnt signalling and possesses endogenous Wnt pathway components, as it responds to Wnt ligands by the characteristic β-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 analysed the ability of different human Fz proteins to enhance this activation. Sequences encoding human Fz1, Fz6, Fz9 and Fz10 were cloned into the pIRES2-DsRed-Express vector for mammalian expression and transfected into cells prior to Wnt stimulation. All Fz proteins tested, 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 described previously [36]. To confirm the specificity of the GPCR responses described above, we repeated the experiments with Fz1- and control-transfected cells, stimulated by Wnt3a in the presence of an 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 additional proof for the $G_{o/i}$-coupling of Fz1 in L-cells, we treated cells with Ptx prior to stimulation. As expected, cells expressing Fz1 alone or together with $G_{\alpha o}$ 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 statistical significance.

To investigate systematically which G-proteins were coupled to the Wnt3a−Fz1 pair, we co-transfected different G_{α} 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 G-proteins with weak intrinsic activity could be reliably investigated [37]. Of the G_{α} subunits we analysed, members of the $G_{o/i}$ subtype ($G_{\alpha o}$, $G_{\alpha i1}$ and $G_{\alpha i2}$) were responsive to Wnt3a−Fz1 activation, whereas $G_{\alpha z}$, $G_{\alpha s}$, $G_{\alpha q}$ 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, whereas transfection with $G_{\alpha 12}$ suppressed this response (Figure 2E). These results may suggest that the endogenous Wnt3a-binding Fz protein(s) is G_s-coupled; the suppression of this endogenous response by $G_{\alpha 12}$ will require additional investigation.

Mammalian Fz proteins investigated in the present study fall into different groups divided by the type of signalling pathway they initiate. Our results demonstrate that Fz receptors transducing the canonical β-catenin-dependent pathway (Fz1 and Fz10) [36,38], as well as the planar cell-polarity pathway (Fz6) [39], all behave as $G_{o/i}$-coupled GPCRs. In the present study, we find

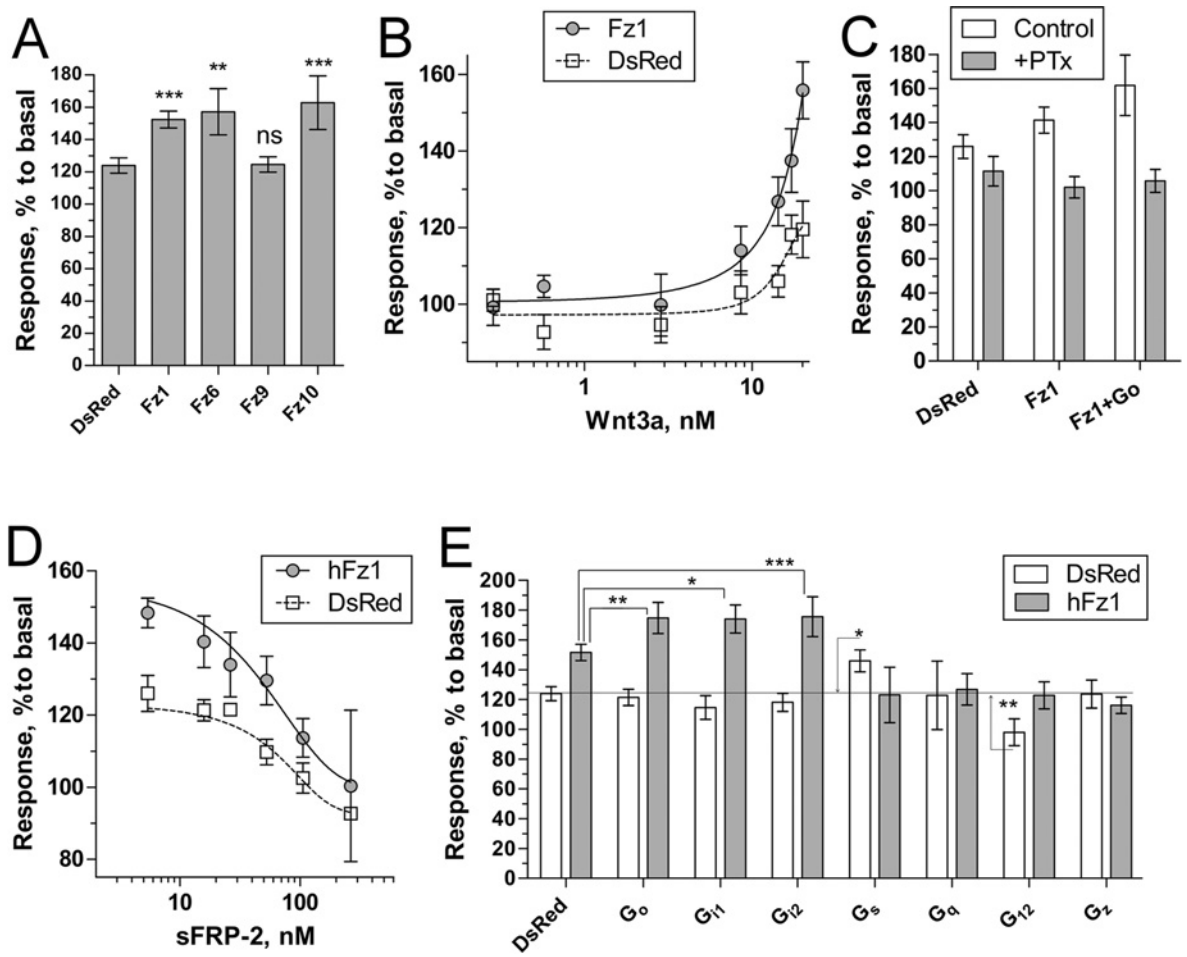

Figure 2 L-cell cultures reveal the $G_{o/i}$-coupled nature of human Fz1 protein in GTP-binding assays

(**A**) Control pIRES2-DsRed vector-transfected cells demonstrate a moderate increase in GTP-binding activity upon stimulation with Wnt3a, improved significantly by transfection of Fz1, Fz6 and Fz10, but not Fz9, constructs. (**B**) Wnt3a induces the G-protein activation response in a concentration-dependent manner both in control and Fz1-transfected cells. (**C**) Ptx-pretreated cells, transfected with pIRES-DsRed, or Fz1 alone or co-transfected with $G_{\alpha o}$ fail to show any increase in G-protein activation. (**D**) Progressive inhibition of the Wnt3a effect by sFRP-2 in Fz1- or DsRed-transfected cells. (**E**) Results of simultaneous co-transfection of control or human Fz1 plasmids with those encoding a set of G_{α} subunits reveal enhanced GTP binding for G_o, G_{i1} and G_{i2} protein in response to Wnt3a. The response of the endogenous Fz receptor(s) was stimulated by overexpression of G_s and suppressed by G_{12}. All of the results are shown as the level of G-protein activation achieved in the presence of Wnt3a, as a percentage of that produced in the presence of the equal amount of BSA (% of basal on the Figure). Statistical significance (measured using a Student's *t* test) is shown as $^{***}P < 0.0005$, $^{**}P < 0.005$ and $^{*}P < 0.05$. ns, non-significant ($P > 0.05$).

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. Taken together with our previous data [20], the assay in our present study lays the foundations for such systematic investigation.

The results of the present study also enable the detailed analysis of which Wnt–Fz pairs couple to which heterotrimeric G-proteins. All of the Fz proteins tested efficiently interacted with the G_o, G_{i1} and G_{i2} proteins, which are close to each other and form the Ptx-sensitive subgroup within the heterotrimeric G-proteins [40]. Additionally, we provide evidence in favour of coupling of an unidentified Fz(s) endogenous to L-cells to G_s. Previous investigations on rat Fz1 performed in zebrafish and cultured cells showed the presence of a Ptx-sensitive component, identified in some studies as G_o, in the signal transduction elicited by these receptors [14,15,25,41,42]. A recent report has implicated G_{i2} in Wnt signalling in *Xenopus* eggs [43]; G_i proteins are also involved in signalling by the Fz-related receptor Smoothened [44,45]. Additionally, the murine microglia cell line N13 was recently found to activate G_i-type heterotrimeric G-proteins through an uncharacterized receptor(s) upon stimulation with Wnt5a [46].

Despite the fact that the heterotrimeric G-protein G_q has been implicated previously in Fz signalling [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 Fz proteins to the non-$G_{o/i}$ heterotrimeric G-proteins.

Overall, the results of the present study provide the first firm and definitive biochemical demonstration that Fz receptors, expressed in their natural cellular environment, act as typical GPCRs serving as GEFs on heterotrimeric G-proteins upon stimulation with Wnt ligands. Taken 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 Wnt–Fz signalling in such medically important processes, such 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 high potential importance. Such a platform would be used to detect the highly desired agents acting directly at the level of the Wnt–Fz receptor interaction [50].

AUTHOR CONTRIBUTION

Alexey Koval performed the experiments, and contributed to data analysis and preparation of the paper. Vladimir Katanaev designed and analysed the experiments, and wrote the paper.

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