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Gβγ Activates GSK3 to Promote LRP6-Mediated β-Catenin Transcriptional Activity

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Abstract

Evidence from *Drosophila* and cultured cell studies support a role for heterotrimeric G proteins in Wnt signaling. Wnt inhibits the degradation of the transcriptional regulator β-catenin. We screened the α subunits of major families of recombinant G protein subunits and Gβγ subunits in a *Xenopus* egg extract system that reconstitutes β-catenin degradation. We found that Gα_o, Gα_q, Gα_{i2}, and Gβγ inhibited β-catenin degradation. Gβ₁γ₂ promoted phosphorylation and activation of the Wnt co-receptor low density lipoprotein receptor-related protein 6 (LRP6) by recruiting synapse kinase 3 (GSK3) to the membrane and enhancing its kinase activity. In both a reporter gene assay and an in vivo assay, c-βARK, an inhibitor of Gβγ, blocked LRP6 activity. Several components of the Wnt/β-catenin pathway formed a complex: Gβ₁γ₂, LRP6, GSK3, axin, and dishevelled. We propose that heterotrimeric G protein activation results in formation of free Gβγ and Gα, which act cooperatively to inhibit β-catenin degradation and activate β-catenin-mediated transcription.

INTRODUCTION

Heterotrimeric G proteins, which consist of a Gα subunit and an associated Gβγ dimer, mediate a multitude of physiological responses from a host of diverse ligands. Activated G protein-coupled receptors (GPCRs) catalyze the exchange of GDP for GTP on Gα, resulting

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in subunit dissociation and G protein activation. The signal is terminated upon hydrolysis of GTP by $G\alpha$ by the intrinsic guanosine triphosphatase (GTPase) activity of the $G\alpha$ subunit and its subsequent reassociation with $G\beta\gamma$. Both $G\alpha$ and $G\beta\gamma$ activate many downstream effectors.

The pathway from the ligand Wnt to the transcriptional regulator β -catenin (Wnt/ β -catenin pathway) is conserved throughout metazoa and required for coordination of diverse developmental programs, stem cell maintenance, and cell growth and proliferation. In the absence of the Wnt ligand, the cytoplasmic β -catenin concentrations are kept low by the destruction complex, which is composed of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3 (GSK3). GSK3 phosphorylates β -catenin, thereby marking it for ubiquitination and proteasome-mediated degradation. Binding of Wnt to the co-receptors Frizzled and low density lipoprotein receptor-related Protein 6 (LRP6), a single transmembrane domain protein, inhibits β -catenin destruction, allowing cytoplasmic β -catenin concentrations to increase. β -catenin enters the nucleus to initiate a transcriptional program with T cell factor and lymphoid enhancer factor (TCF/LEF) family members [1]. The initial discovery that the Wnt receptor, Frizzled, contains seven predicted transmembrane domains, a topology characteristic of GPCRs, suggested that Frizzled signals through heterotrimeric G proteins, although direct evidence for coupling between Frizzled and G proteins is lacking [1]. Activation of the transcriptional activity of β -catenin (the β -catenin/TCF pathway), however, has been reported to occur through GPCRs independent of Wnt and Frizzled. Signaling through the gonadotrophin-releasing hormone receptor [2], prostaglandin receptor [3], or parathyroid receptor [4] activates the transcriptional activity of β -catenin.

During Wnt signaling, activation of LRP6 depends on phosphorylation of conserved motifs within its intracellular domain [5, 6]. A pool of axin-bound GSK3 that translocates to the membrane through a process involving dishevelled (Dsh) may contribute to the initial phosphorylation of LRP6 at the plasma membrane [6, 7]. Phosphorylated LRP6 provides additional docking sites for cytoplasmic axin-GSK3 complexes to promote further phosphorylation of LRP6. Binding of the axin-GSK3 complex to phosphorylated LRP6 inhibits GSK3, leading to a subsequent decrease in β -catenin phosphorylation [8-10]. Thus, GSK3 in the destruction complex promotes β -catenin degradation, whereas GSK3-mediated phosphorylation of LRP6 at the plasma membrane contributes to stabilization of β -catenin; thus both GSK3 activity and inhibition of its activity are required for LRP6-mediated β -catenin stabilization. Additionally, LRP6 is also a substrate for and an inhibitor of GSK3. We provide evidence that $G\beta\gamma$ recruits GSK3 to the plasma membrane, thereby promoting its activity toward LRP6, which places $G\beta\gamma$ in a pivotal role in the initiation of LRP6 activation.

RESULTS

Biochemical screen identifies G proteins that regulate β -catenin turnover

The *Xenopus* egg extract system recapitulates numerous complex GTP-dependent phenomena, such as microtubule dynamics, translation, DNA replication, nuclear envelope reformation, and mitotic spindle assembly [11-13]. It contains cytosol and other cellular components, including plasma membrane, organelles, amino acids, and nucleotides at or near physiological levels (Fig. S1) [14]. Furthermore, this system contains the components of the Wnt pathway responsible for the regulation and degradation of β -catenin and thus can be used to recapitulate β -catenin regulation by monitoring the abundance of exogenous [35 S] β -catenin or endogenous β -catenin phosphorylation in the presence of additional factors [15]. To assess whether *Xenopus* egg extract could be used to test the role of G proteins in regulating β -catenin stability, we used purified G protein heterotrimers (primarily G_o) from

brain for reconstitution [16] (Fig. 1A). We detected modest inhibition of [^{35}S] β -catenin degradation upon addition of the G protein heterotrimer (Fig. 1B), with greater inhibition upon G protein activation (and disassociation into the active subunits α and $\beta\gamma$) by AlF_4^- . Partial activity of the heterotrimer in the absence of AlF_4^- may have resulted from partial activation of the heterotrimer due to nucleotide exchange (GDP for GTP) of G_o within the crude egg extract, which contains high concentrations of GTP [12, 14].

To identify G proteins involved in regulating β -catenin stability, we tested the four major classes of $\text{G}\alpha$ subunits for their capacity to inhibit degradation of [^{35}S] β -catenin in the *Xenopus* egg extract (Fig. 1A,C). The specificity of each G protein in inhibiting β -catenin degradation was also assessed by immunoblotting for endogenous β -catenin phosphorylation by GSK3 (Fig. 1C; Fig. S2B). Both inactive (GDP-bound) and active forms were tested. For activation, proteins were bound to a $\text{GTP}\gamma\text{S}$ analog or, for $\text{G}\alpha_i$ and $\text{G}\alpha_q$, which exhibit slow nucleotide exchange in the absence of receptor activation, were treated with GDP-AlF_4^- . We found that, in addition to the previously implicated $\text{G}\alpha_o$ [17, 18] and $\text{G}\alpha_q$ [19, 20], $\text{G}\alpha_{i2}$ also inhibited exogenous β -catenin degradation and phosphorylation of endogenous β -catenin by GSK3. β -catenin was stabilized in egg extract when the GDP-bound forms of $\text{G}\alpha_o$ and $\text{G}\alpha_{i2}$, but that of not $\text{G}\alpha_q$, were added to the extract. This is likely due to differences in affinity for GTP and the rates of nucleotide exchange between $\text{G}\alpha$ subunits (GDP dissociation rate of $\text{G}\alpha_o = 0.19 \text{ min}^{-1}$ at 30°C ; GDP dissociation rate of $\text{G}\alpha_{i2} = 0.07 \text{ min}^{-1}$ at 30°C ; $\text{G}\alpha_q$ has very low affinity for GTP in the absence of receptor) [21, 22]. $\text{G}\beta\gamma$ protein purified from brain, as well as recombinant $\text{G}\beta_1\gamma_2$, inhibited β -catenin phosphorylation and degradation (Fig. 1D,E; Fig. S3). Additionally, $\text{G}\beta\gamma$ synergized with $\text{G}\alpha_o$ to inhibit β -catenin degradation, suggesting that $\text{G}\alpha_o$ and $\text{G}\beta\gamma$ act cooperatively (Fig. 1F).

The effective concentrations of $\text{G}\alpha$ and $\text{G}\beta\gamma$ that we used in the *Xenopus* egg extract system are in the low micromolar range, well below the predicted concentrations of heterotrimeric G proteins at the plasma membrane ($\sim 1 \text{ mM}$) [23]. Furthermore, because membrane localization of G proteins is not homogeneous, local concentrations of G proteins within the plasma membrane may actually exceed 1 mM [23]. The micromolar concentrations of $\text{G}\alpha$ and $\text{G}\beta\gamma$ required to inhibit β -catenin degradation in the *Xenopus* egg extract system may also reflect the presence of competing binding proteins for $\text{G}\alpha$ and $\text{G}\beta\gamma$. For example, the concentration of tubulin in the *Xenopus* egg extract has been estimated as $\sim 24 \mu\text{M}$ (4.1% of total protein), and the *Xenopus* egg extract contains an abundance of microtubules [24]. Microtubules bind $\text{G}\beta\gamma$ [25] and thus, are likely to compete with β -catenin regulatory components for $\text{G}\beta\gamma$ binding.

$\text{G}\beta\gamma$ binds and recruits GSK3 to the membrane

We hypothesized that the mechanism by which $\text{G}\beta\gamma$ inhibits β -catenin degradation in the egg extract involves sequestration of GSK3 at membranes, thereby preventing phosphorylation of cytoplasmic β -catenin. Isoprenylation of $\text{G}\gamma$ targets $\text{G}\beta\gamma$ subunits to the plasma membrane. Thus, $\text{G}\beta\gamma$ added to the *Xenopus* egg extract, which contains membranous organelles, is likely incorporated into membranes. Furthermore, when $\text{G}\beta\gamma$ interacts with any of three of its kinase effectors [G protein coupled-receptor kinase 2 (GRK2), Bruton's tyrosine kinase (BTK), and phosphoinositide 3 kinase γ (PI3K γ)] these become translocated to the membrane [26]. In support of the hypothesis that $\text{G}\beta\gamma$ interacts with GSK3, addition of GSK3 (but not casein kinase I) to the extract reversed the inhibitory effects of $\text{G}\beta\gamma$ on β -catenin degradation (Fig. 2A). Interaction with free $\text{G}\beta\gamma$ subunit likely sequesters cytoplasmic GSK3 to the membrane pool in the *Xenopus* egg extract because AlF_4^- -activated (but not the unactivated) brain G protein shifted the endogenous GSK3 pool to the membrane fraction (Fig. S4). To confirm an interaction between GSK3 and $\text{G}\beta\gamma$, we showed that endogenous GSK3 co-immunoprecipitated with endogenous $\text{G}\beta\gamma$ from the extract (Fig. 2B). When co-expressed in cultured mammalian cells, Myc-tagged GSK3 similarly co-

immunoprecipitated with hemagglutinin (HA)-tagged $G\beta_1\gamma_2$ (Fig. 2C). In contrast, we were unable to demonstrate co-immunoprecipitation of GSK3 and $G\alpha_o$ when both were co-expressed in cultured mammalian cells (Fig. S5). We also observed an interaction between purified $G\beta\gamma$ and GSK3 (Fig. 2D), suggesting that GSK3 and $G\beta\gamma$ directly interact.

To test whether the interaction between $G\beta\gamma$ and GSK3 promoted membrane localization of GSK3, we expressed Myc-GSK3 with or without HA- $G\beta_1\gamma_2$ in HeLa cells and isolated membrane-associated proteins. An increased fraction of GSK3 was membrane-associated when $G\beta_1\gamma_2$ was co-expressed (Fig. 2E). Because cellular knockdown of $G\beta\gamma$ is complicated by subsequent loss of $G\alpha$ signaling [27, 28], we used the C-terminal domain of β -adrenergic receptor kinase (c- β ARK) to bind and inhibit free $G\beta\gamma$ [29]. Inhibition of both HA- $G\beta_1\gamma_2$ and endogenous $G\beta\gamma$ by c- β ARK reduced the membrane localization of Myc-GSK3 (Fig. 2E).

$G\beta\gamma$ is required for maximal LRP6 stimulation of β -catenin transcriptional activity

By promoting membrane localization of GSK3, $G\beta_1\gamma_2$ could enhance the activity of GSK3 towards membrane-localized substrates, such as LRP6. To monitor LRP6-mediated signaling, we analyzed HEK 293 Super TOPflash (STF) cells (HEK 293 cells stably transformed with the Wnt reporter construct TOPflash) expressing LRP6 fused to the vesicular stomatitis virus G protein (VSVG-LRP6), which results in constitutive activation of β -catenin/TCF-mediated transcription [30], quantified as activity of the TOPflash reporter. HA- $G\beta_1\gamma_2$ enhanced VSVG-LRP6-stimulated TOPflash activity and this was blocked by co-expression of c- β ARK (Fig. 3A, B). Consistent with the importance of $G\beta_1\gamma_2$ membrane localization for its enhancement of LRP6 activity, overexpression of a $G\gamma_2$ CAAX mutant lacking the isoprenylation modification (required for $G\gamma$ membrane localization) failed to enhance VSVG-LRP6-stimulated TOPflash activity (Fig. 3C). We also noted that c- β ARK alone decreased TOPflash activation by VSVG-LRP6 (Fig. 3A,B), suggesting that endogenous $G\beta\gamma$ contributes to LRP6 signaling. To confirm that c- β ARK was not affecting other components of the β -catenin pathway, we showed that c- β ARK did not coimmunoprecipitate with axin, GSK3, or Dsh (Fig. S6).

To provide in vivo evidence that the interaction between $G\beta\gamma$ and LRP6 occurs, we investigated their interactions in *Xenopus laevis* embryos and ectodermal explants. Ectopic activation of the β -catenin/TCF pathway in ventral blastomeres (for example by injection of LRP6-encoding mRNA) induces formation of a partial ectopic axis in *Xenopus* embryos [31]. We found that coinjection of mRNA for LRP6 with mRNA encoding c- β ARK inhibited secondary axis formation (Fig. 4A). In contrast, coinjection of mRNAs for LRP6 and $G\beta_1\gamma_2$ promoted axis duplication in *Xenopus* embryos (Fig. 4B) and expression of the Wnt target genes, *Siamois* and *Xnr3*, in *Xenopus* ectodermal explant (Fig. 4C).

Residues clustered on blades 1, 3, and 4 of $G\beta_1$ are involved in GSK3 binding and stimulation of LRP6 activity

Previous studies identified critical residues on $G\beta_1$ that mediate its interaction with specific effectors [32, 33]. To determine whether these sites also mediate GSK3 binding, LRP6 activation, or both, we generated a series of single alanine mutants of $G\beta_1$, of which a subset exhibited a decreased capacity to activate LRP6, reflected as decreased stimulation of the TOPflash reporter in VSVG-LRP-expressing cells (Fig. 5A). $G\beta$ is a WD40-repeat protein that forms a seven-bladed β -propeller structure [26]. Based on previous X-ray crystal structures of $G\beta$, residues altered in this subset of mutants are clustered within two regions of $G\beta_1$, predominantly within blades 1, 3, and 4 (Fig. 5C). For the subset of $G\beta_1$ mutants with impaired LRP6 activation, we performed co-immunoprecipitation experiments to test the capacity of each mutant to bind GSK3. Several of these $G\beta_1$ mutants bound GSK3 more

avidly than did wild-type $G\beta_1$ (Fig. 5B). These results support a model in which a dynamic interaction between $G\beta\gamma$ and GSK3 is required to promote LRP6 activation.

$G\beta\gamma$ and Dsh are both needed for maximal stimulation of β -catenin transcriptional activity

To investigate what effect $G\beta_1\gamma_2$ had in Wnt-mediated activation of β -catenin signaling, we analyzed the effects of overexpression of HA- $G\beta_1\gamma_2$ or inhibition of $G\beta_1\gamma_2$ with c- β ARK in cultured cells exposed to Wnt3a. HA- $G\beta_1\gamma_2$ did not affect Wnt3a-stimulated TOPflash activity in cultured HEK 293 STF cells, which contain the TOPflash reporter and endogenously express Frizzled and LRP6 receptors (Fig. S7A). The presence of c- β ARK also had no effect on Wnt3a-stimulated activity (Fig. S7B). This may suggest that, similar to CK1 γ , $G\beta_1\gamma_2$ becomes limiting only when LRP6 is present in excess [5]. Alternatively, $G\beta\gamma$ may be activated through Wnt-independent pathways to signal through a distinct mechanism upstream of LRP6.

We investigated at what point $G\beta\gamma$ functioned in the β -catenin pathway. Dsh acts downstream of Frizzled and upstream or at the level of the β -catenin degradation complex in the Wnt pathway and is required for LRP6 phosphorylation [34]. Similar to LRP6, excess Dsh activates β -catenin/TCF signaling in the absence of other stimuli [35]. In HEK 293 STF cells that are overexpressing Dsh, β -catenin is stabilized in the absence of ligand and the TOPflash reporter is expressed. Cells coexpressing c- β ARK and Dsh exhibited less TOPflash activity than did cells only expressing Dsh (Fig. 6A), indicating a requirement of $G\beta\gamma$ for Dsh to activate β -catenin signaling. Introduction of a dominant-negative mutant of Dsh (Xdd) [36] with VSVG-LRP6 and $G\beta_1\gamma_2$ into the HEK 293 STF cells prevented $G\beta_1\gamma_2$ from enhancing the activity of the TOPflash reporter (Fig. 6B). Xdd also inhibited activation of TOPflash by LRP6 alone (Fig. 6B), consistent with a role for Dsh in mediating LRP6 activation [7, 34]. These studies suggest that both $G\beta\gamma$ and Dsh contribute to LRP6 activation. There was no detectable effect of HA- $G\beta_1\gamma_2$ or c- β ARK when the pathway was stimulated by LiCl, a GSK3 inhibitor, confirming that $G\beta\gamma$ acts upstream of the β -catenin destruction complex (Fig. S7C,D). These data suggest that $G\beta\gamma$ functions upstream of the destruction complex at the level of LRP6 and Dsh.

$G\beta\gamma$ enhances LRP6 phosphorylation by GSK3

To further the mechanism by which $G\beta_1\gamma_2$ enhances LRP6 signaling, we transfected cells with $G\beta_1\gamma_2$ and GSK3, isolated membrane-associated proteins, and immunoblotted with an antibody that recognizes LRP6 phosphorylated at sites that could be phosphorylated by GSK3. When GSK3 was co-expressed with $G\beta_1\gamma_2$, there was an increase in phosphorylated LRP6 above that observed when GSK3 was expressed alone (Fig. 6C). $G\beta_1\gamma_2$ coimmunoprecipitated with several proteins involved in β -catenin regulation: Dsh (Fig. 6D), LRP6 (Fig. 6E), axin (Fig. 6F), and GSK3 (Fig. 2C). These results are consistent with previous reports of coimmunoprecipitation of $G\beta_1\gamma_2$ with axin [37] and with Dsh [37-39]. Thus, $G\beta\gamma$ appears to act in a complex with Dsh, axin, and GSK3 to promote phosphorylation of LRP6.

In vitro kinase assays showed that $G\beta\gamma$ enhanced GSK3 phosphorylation of Tau, a GSK3 substrate, whereas addition of either heterotrimeric Go or bovine serum albumin (BSA) did not enhance GSK3 activity towards Tau (Fig. 6G; Fig. S8A-C). $G\beta\gamma$ also enhanced GSK3 phosphorylation of the intracellular domain of LRP6 (LRP6ICD) on the PPPSP motif, which is required for LRP6 activity and provides a docking site for axin (Fig. 6H; Fig. S8D). Higher concentrations of $G\beta\gamma$ were required to stimulate GSK3 phosphorylation of LRP6ICD relative to required to stimulate Tau phosphorylation (Fig. 6G, H). This may reflect differences in the affinity of GSK3 for Tau versus LRP6ICD and a previously

reported feedback mechanism by which phosphorylated LRP6 inhibits GSK3 activity [8-10]. Alternatively, LRP6 could simply compete with $G\beta\gamma$ for binding to GSK3. This latter model is consistent with our observation that $G\beta_1$ mutants with increased affinity for GSK3 failed to activate β -catenin/TCF signaling.

DISCUSSION

With this report, GSK3 becomes the fourth identified $G\beta\gamma$ kinase effector (in addition to GRK2, BTK, and PI3K γ) [26]. In each case, the mechanism of action of $G\beta\gamma$ appears to involve membrane translocation and, in the cases of PI3K γ , BTK, and GSK3, stimulation of kinase activity. During zebrafish gastrulation, regulation of GSK3 by $G\beta\gamma$ plays a critical role in controlling the stability of the GSK3 substrate Snail [40]. Thus, $G\beta\gamma$ may control diverse signaling pathways through regulation of GSK3.

$G\beta\gamma$ has been reported to both negatively and positively influence Wnt signaling. $G\beta_{2\gamma 2}$ has been reported to negatively regulate Wnt1 signaling by a feedback mechanism that stimulates the degradation of Dsh in HEK 293T cells [37]; whereas a study in *Drosophila* indicated that $G\beta\gamma$ enhances β -catenin/TCF signaling [39, 41]. Knockdown of $G\beta$ by RNAi during *Drosophila* wing development results in malformation of wing structures consistent with downregulation of Wg, the *Drosophila* ortholog of Wnt, signaling, and downregulation of $G\beta$ reduced expression of Wg target genes in wing imaginal discs [39, 41]. In accord with our study, the authors did not observe ectopic activation of the Wg pathway when $G\beta\gamma$ was overexpressed. Together, our experiments with *Xenopus* embryos (Fig. 4) and the previous study with *Drosophila* [39, 41] provides evidence that $G\beta\gamma$ plays a positive role in β -catenin/TCF signaling in vivo.

The simplest model to explain the activation of β -catenin/TCF signaling by $G\beta\gamma$ would invoke a receptor-mediated mechanism. In contrast to its effects on Dsh and LRP6, however, c- β ARK did not inhibit Wnt3a activation of β -catenin/TCF signaling. One possibility is that receptor-mediated activation of the heterotrimer and subsequent association of $G\beta\gamma$ and GSK3 is a rapid and tightly coupled process. Indeed, there is evidence that certain GPCRs and their G proteins remain tightly associated upon activation by ligand [41]. Thus, overexpression of c- β ARK, which binds free $G\beta\gamma$, may not be sufficient to disrupt Frizzled receptor-associated $G\beta\gamma$. Alternatively, signaling through $G\beta_{1\gamma 2}$ may be independent of Wnt activation of Frizzled. Various GPCRs have been reported to activate β -catenin/TCF signaling. For example, the parathyroid receptor I activates LRP6 to stimulate β -catenin/TCF-mediated transcription, and the gonadotrophin-releasing hormone receptor inhibits GSK3 activity and stimulates β -catenin/TCF-mediated transcription [2, 4]. It remains to be determined whether $G\beta\gamma$ mediates β -catenin signaling through Frizzled or another GPCR.

We propose a model in which both $G\alpha$ and $G\beta\gamma$ regulate β -catenin/TCF signaling (Fig. 7). Analysis of purified recombinant proteins in the *Xenopus* egg extract system demonstrated a role for $G\alpha_o$ and $G\alpha_q$, as well as $G\alpha_{i2}$, in the inhibition of β -catenin degradation (Fig. 1). Additionally, our biochemical studies, in vivo assays, and experiments with cultured mammalian cells support a positive role for $G\beta\gamma$ in β -catenin/TCF signaling. Artificially tethering GSK3 to the plasma membrane is sufficient to stimulate Wnt signaling in cultured mammalian cells and axis duplication in *Xenopus laevis* embryos; thus, the β -catenin/TCF pathway appears to be very sensitive to GSK3 membrane localization [6]. Our data support a model in which $G\beta\gamma$ acts in concert with Dsh to recruit and activate GSK3 (either free or possibly in association with axin) at the plasma membrane; membrane-associated GSK3 phosphorylates LRP6, which then inhibits the β -catenin degradation complex to stabilize β -catenin, allowing β -catenin to translocate to the nucleus and mediate transcription.

MATERIALS AND METHODS

Constructs and purified proteins

Human G β_1 was subcloned into pCS2-HA vector. hG β_1 alanine mutations were constructed with the QuikChange site mutagenesis kit (Stratagene) following the manufacturer's protocol (Primer sequences: T47A F 5'-agaatccaaatgcgcgcgaggaggacactgc, R 5'-gcagtgtcctcctcgcgcgcatttgattct; L55A F 5'-cactgcggggcacgcggccaagatctacg, R 5'-cgtagatcttgccgcgtgccccgcagtg; K57A F 5'-gggggcacctggccgcgactctacgccatgc, R 5'-gcatggcgtagatcgcggccagtgcccc; Y59A F 5'-cacctggccaagatcgccgccatgcactgggg, R 5'-ccccagtgcatggcgcgcatcttgccagtg; K78A F 5'-gtcagtgccctgcgaggatggtgcacttatcatctgggac, R 5'-gtcccagatgataagtgcacatcctgcgaggcactgac; M80A F 5'-tgccctgcaggtgtaaaacttgccatctgggacagcta, R 5'-tgccctgcaggtgtaaaacttgccatctgggacagcta; K89A F 5'-agctacaccaccaacgcggtccacgccatccc, R 5'-gggatggcgtggaccgcgttggtgtagct; W99A F 5'-ctctgcgtcctccgcggtcatgacctgtg, R 5'-cacaggtcatgaccgcggaggagcgcagag; M101A F 5'-cgctcctcctgggtgcgacctgtgcatatgc, R 5'-gcatatgcacaggtcgcgaccagaggagcgc; L117AF 5'-gtggcctcgcgttgccgcggataacatttctcc, R 5'-ggagcaaatgtatccgcgccaccgcagggccac; T143A F 5'-agctggcagacacgcaggttacctgtcc, R 5'-ggacaggtaacctgcgtgtcctgccagct; Y145A F 5'-ggcaggacacacaggtgcctgtcctgtcc, R 5'-ggcagcaggacaggcacctgtgtcctgcc; D186A F 5'-ccggacacactggagctgtcatgagccttc, R 5'-gaaaggctcatgacagctccagtggtccgg; M188A F 5'-ggacacactggagatgtcgcgagcctttcttctcc, R 5'-ggagcaagagaaaggctcgcgacatctccagtggtcc; N230A F 5'-ggccacagtgctgacatcgtgccatttctcttcc, R 5'-ggaaagaagcaaatggcagcgtatgcagactcgtggcc; W332A F 5'-gtggcgacagggtccgcggatagcttctccaa, R 5'-ttgaggaagctatccgcggaccctgtgccac. Sequence encoding amino acids 493-689 of mouse adrenergic receptor kinase β_1 was subcloned into pCS2 vector (Primer sequences: F 5'-gcaaggccggccaatgggaatcaagtactggac, R 5'-gcaaggcgcgcggaggccgttgccactgcc). G γ 2CAAX pCS2 was generated by PCR-based site-directed mutagenesis (F5'-gcaaggccggccaatggccagcaacaacaccgcc R5'-gcaaggcgcgccttaaaggatggcagagaaaaaac). All other constructs used have been described previously. G α_s and myristoylated G α_{0A} , G α_{0B} , G α_{i2} , and G α_{i3} were expressed and purified from bacteria, and G $\beta_1\gamma_2$ and G α_q were purified from *Sy9* cells as previously described [42-46]. Heterotrimeric G proteins were purified from porcine brain (Wampler's Sausage Factory, TN) and G α_t was purified from bovine rod outer segments following previously described protocols [16, 47]. G $\beta\gamma$ was concentrated to 1 mg/mL in 50 mM HEPES pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM MgSO₄, and 40 mM β -octyl glucoside. GSK3 and CKI proteins were purchased from New England Biolabs.

G protein activation

To activate G α_{0A} , G α_{0B} , G α_{i2} , G α_{i3} , and G α_s , proteins were diluted 1:20 (v/v) with 50 mM HEPES pH 8, 1mM EDTA, 1 mM DTT, 10 mM MgSO₄, and 10 μ M GTP γ S and incubated at 30°C for 30 min. Proteins were concentrated to their original volumes using Centricon-10 (Millipore). To activate G α_{i2} , protein was diluted 1:1 (v/v) with 80 mM HEPES pH 8, 1 mM EDTA, 1 mM DTT, 22 mM MgSO₄, 0.05% Lubrol, and 20 μ M GTP γ S and incubated at 30°C for 90 min. G α_{i3} was activated by diluting 1:1 (v/v) with 80 mM HEPES pH 8, 1mM EDTA, 1 mM DTT, 3 mM MgSO₄, 0.05% Lubrol, and 20 μ M GTP γ S and incubated at 30°C for 90min. Excess GTP γ S was removed using Zeba Desalt Spin Column (Pierce). To activate purified brain heterotrimer, G α_t and G α_q , proteins were incubated with 10 mM NaF, 30 mM AlCl₃, and 10 mM MgCl₂ for 45min on ice.

[³⁵S]-Catenin degradation and phospho- β -catenin assays

Xenopus egg extract preparation and [³⁵S] β -catenin degradation assays were performed as previously described [15]. Samples were removed at 0 and 3hr for SDS-PAGE and autoradiography. To detect β -catenin phosphorylated by GSK3 in extract, anti- β -catenin-

phospho-Ser33/Ser37/Thre41 antibody (Cell Signaling) was used for immunoblotting. Tubulin detected with anti- α -tubulin (DM1 α , Sigma) served as the loading control.

TOPflash assay

HEK-293 Super TOPflash (STF) cells were cultured in DMEM media supplemented with 100 units/mL penicillin, 100 unit/mL streptomycin, 10% FBS. Lipofectamine 2000 transfection reagent (Invitrogen) was used for all transfections. Cells were lysed 48 hr post-transfection with 1X Passive Lysis Buffer (Promega) and luciferase activity was measured with Steady Glo (Promega). Luciferase activities were normalized to cell number calculated with Cell Titre Glo (Promega).

Embryo studies: Axis duplication assay and β -catenin target gene expression analysis

Capped RNA for embryo injection was synthesized from linearized plasmid DNA templates by using mMessage mMachine (Ambion). *Xenopus* embryos were fertilized in vitro, dejellied, and injected and cultured as previously described [48]. 2-4 cell *Xenopus* embryos were injected with 10nl mRNAs using Narishige IM 300 microinjector according to published methods [48]. Axis duplication was scored when embryos reached stage 21 (staging of embryos performed as previously described [49]). Animal caps were cut from stage 9 embryos and cultured in 75% Marc's modified Ringers (MMR) [0.1 M NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM Hepes (pH 7.4)] until stage 11. RT-PCR for *Siamois*, *Xnr3*, and *ODC* were performed by using primers and conditions previously described (www.hhmi.ucla.edu/derobertis/index.html).

Co-immunoprecipitation studies

For co-immunoprecipitation, cells were washed with cold PBS and then lysed on ice for 30 min with nondenaturing lysis buffer (NDLB): 50 mM Tris pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, and protease inhibitors (1mg/ml leupeptin, pepstatin, and chymostatin). Lysates were diluted to 1 mg/ml with NDLB and incubated with antibody beads for 3 hr with rotation at 4°C. Beads were then washed 3X with NDLB and 1X with PBS. Proteins were eluted from beads with sample buffer and processed for SDS-PAGE followed by immunoblotting. To prepare antibody beads, mouse anti-HA (12CA5), rat anti-HA (Roche), or rabbit anti-G β (1:300, Sigma) antibodies were cross-linked to Protein G or Protein A magnetic beads (New England Biolabs) following the manufacturer's protocols. The following antibodies were used for immunoblotting: rabbit anti-G β (Sigma), mouse anti-HA (12CA5), mouse anti-Myc (9E10), and rabbit anti-VSVG (Bethyl Laboratories). Tubulin was detected with anti- α -tubulin (DM1 α , Sigma) and served as the loading control. For co-immunoprecipitation from *Xenopus* egg extracts, extracts were diluted 10X with PBS, incubated with anti-G β beads, and beads were processed as above. Eluted samples were immunoblotted using anti-G β and anti-GSK3 (BD Transduction).

Membrane protein isolation and detection of LRP6

Hela cells were cultured in DMEM media supplemented with 100 units/mL penicillin, 100 unit/mL streptomycin, 10% FBS. Lipofectamine 2000 transfection reagent (Invitrogen) was used for all transfections. For membrane preparations, Hela cells were incubated in media containing 0.6mM sodium vanadate in DMEM (without serum) for 3hr. Cells were then lysed and membrane-associated proteins isolated using ProteoExtract Native Membrane Protein Extraction kit (Calbiochem). To detect endogenous LRP6, endogenous phospho-LRP6, and transfected VSVG-LRP6, the following antibodies were used: rabbit anti-LRP6 (Cell Signaling), rabbit anti-phospho-LRP6 (Ser1490) (Cell Signaling), and rabbit anti-VSVG (Bethyl Laboratories), respectively.

In vitro protein binding assay

For in vitro binding of $G\beta_1\gamma_2$ and GSK3, recombinant $G\beta_1\gamma_2$ was incubated with Maltose binding protein (MBP) or MBP-GSK3 protein in binding buffer (10 mM HEPES pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, and 0.5 mg/mL BSA) at 4°C for 45 min. Protein G magnetic beads (New England Biolabs) crosslinked to anti-MBP antibody (Sigma) were added and the reaction was incubated at 4°C for 45 min. Beads were washed 4X with binding buffer, 2X times with PBS, and samples were eluted and analyzed by immunoblotting.

In vitro kinase assay

Tau (200 nM; r-Peptide, Tau-441) or LRP6ICD (100 nM) were pre-incubated with 200 nM or 400 nM GSK3 (New England Biolabs), respectively, 200 μ M ATP, 20 μ Ci [γ - ^{32}P]ATP, GSK3 kinase buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 2 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, 0.2% Tween-20) and increasing concentrations of recombinant $G\beta_1\gamma_2$, G_o , or BSA in a total reaction volume of 20 μ l. Samples were incubated at room temperature and aliquots removed at 30 min (Tau) or 15 min (LRP6ICD) for analysis by SDS-PAGE and autoradiography.

Molecular modeling

The software program MacPyMol was used to generate a model of the $G\beta_1\gamma_2$ dimer (Protein Databank entry 1XHM)[50].

Quantification and statistical analyses

Quantitation of β -catenin abundance and immunoblots was performed by scanning images with Adobe Photoshop CS4 (Adobe Systems, Mountain View, CA, USA) and intensity of the bands was quantified with NIH ImageJ with correction for background. Statistical significance was determined by t-test, with $P < 0.005$ considered significant.

For TOPFlash assays, analysis of variance (ANOVA) was used to test the global hypothesis of equal mean intensities for all treatment conditions. The global hypothesis of all ANOVA analyses was rejected if $P < 0.05$. All possible pairwise contrasts were evaluated using Tukey's HSD procedure, which corrects for multiple comparisons within each ANOVA experiment [51]. As distributional assumptions are difficult to assess due to small sample size, a non-parametric Kruskal-Wallis test was conducted for each experiment. All experiments had significant Kruskal-Wallis p-values, indicating rejection of the null hypothesis of equal population medians among all treatment groups. All luciferase assays were repeated at least three times in triplicate. Values are presented as mean $\pm$ standard deviation.

For *Xenopus* embryo injection data, statistical significance was determined by Fisher's exact test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

G proteins inhibit β-catenin degradation in a *Xenopus* egg extract system. (A) Schematic of assay used to assess the effects of G protein subunits on in vitro translated (IVT) [³⁵S] β-catenin degradation in *Xenopus* egg extract. (B) Addition of purified brain G protein (5 μM) inhibits degradation of radiolabeled (IVT) [³⁵S] β-catenin in *Xenopus* egg extract and inhibition is potentiated by the G protein activator AIF₄⁻. (C) In the *Xenopus* egg extract system, the activities of the major classes of G proteins subunits on IVT [³⁵S]β-catenin turnover and phosphorylation of endogenous β-catenin at GSK3 consensus sites vary (see Fig. S2 for details). (D) Both recombinant Gβ₁γ₂ (recom., 5 μM) and brain Gβγ (5 μM) inhibit β-catenin degradation. (E) Addition of brain Gβγ (5 μM) to *Xenopus* egg extract inhibits phosphorylation of endogenous β-catenin at GSK3 consensus sites (Ser33/Ser37/Thr41). Extract was analyzed after 2 hr incubation. Tubulin is loading control. (F) Gβ₁γ₂ synergizes with Gα_{oA} to inhibit β-catenin degradation. [³⁵S]β-catenin was added to *Xenopus* egg extract and incubated with buffer, Gβ₁γ₂ (0.5 μM), Gα_{oA}-GTPγS (0.25 μM), or Gβ₁γ₂ (0.5 μM) plus Gα_{oA}-GTPγS (0.25 μM). Gβ₁γ₂ and Gα_{oA}-GTPγS were added at sub-threshold concentrations such that they did not inhibit β-catenin degradation on their own. Figures are representative of experiments performed three times.

Fig. 2.

G β ₁ γ ₂ binds and promotes GSK3 membrane localization. **(A)** Addition of GSK3 blocked the ability of G β ₁ γ ₂ to inhibit β -catenin degradation in *Xenopus* egg extract. [³⁵S] β -catenin was added to extract and incubated with G β ₁ γ ₂ (5 μ M), G β ₁ γ ₂ plus GSK3 (10 units/ μ l), or G β ₁ γ ₂ plus CKI (100 units/ μ l). **(B)** GSK3 co-immunoprecipitates with G β from *Xenopus* egg extract. Beads coupled to anti-G β antibody were incubated with extract, washed, eluted with sample buffer, and immunoblotted for G β and GSK3. Representative blot is shown on left; quantitation on right (**P*<0.005, *n* $\geq$ 3, t-test). **(C)** GSK3 co-immunoprecipitates with G β ₁ γ ₂ from cultured cells. HEK-293 cells were transfected with Myc-GSK3 and HA-G β ₁ γ ₂, lysed, immunoprecipitated with an antibody against HA, and Myc-GSK3 was detected by immunoblotting for Myc. **(D)** Purified GSK3 binds recombinant G β ₁ γ ₂ in vitro. GSK3 fused to (MBP-GSK3, 0.66 μ M) or MBP (0.66 μ M) was incubated with G β ₁ γ ₂ (0.6 μ M) and proteins were immunoprecipitated with anti-MBP antibody. Samples were washed, eluted, and immunoblotted for G β and MBP. Representative blot is shown on left; quantitation on right (**P*<0.005, *n* $\geq$ 3, t-test). **(E)** G β ₁ γ ₂ enhances association of GSK3 with membrane fractions, and this effect is reversed by c- β ARK. HeLa cells were transfected with c- β ARK, Myc-GSK3, and HA-G β ₁ γ ₂. Cell membranes were isolated and immunoblotted for Myc and HA. Representative blot is shown on left; quantitation on right (*, **, *** *P*<0.005, *n* $\geq$ 3, t-test)

Fig. 3. LRP6 activation of β -catenin/TCF signaling is regulated by $G\beta_1\gamma_2$. **(A)** $G\beta_1\gamma_2$ enhances LRP6 stimulation of TOPflash, and c- β ARK inhibits the effects of $G\beta_1\gamma_2$ on LRP6 activity. HEK-293 STF cells were transfected with various combinations of HA- $G\beta_1\gamma_2$, VSVG-LRP6, and c- β ARK. Lysates were assayed by TOPflash and immunoblotting and quantified [ANOVA (family-wise), $*P=8.38\times 10^{-13}$, $**P=3.35\times 10^{-12}$, $***P=1.09\times 10^{-6}$]. Tubulin (tub) served as the loading control **(B)** VSVG-LRP6 activation was inhibited by c- β ARK. HEK-293 STF cells were transfected with VSVG-LRP6 plus increasing amounts of plasmid encoding c- β ARK. Lysates were assayed by TOPflash and immunoblotting and quantified [ANOVA (family-wise), $*P=0.001$, $**P=9.94\times 10^{-6}$, $***P=3.41\times 10^{-7}$]. **(C)** Membrane localization of $G\beta_1\gamma_2$ is required for it to enhance LRP6 activity. In contrast to wild-type $G\gamma_2$, expression of a $G\gamma_2$ CAAX mutant (lacking a signal for isoprenylation) did not enhance LRP6 activity as assessed by TOPflash [ANOVA, (family-wise), $*P=4.12\times 10^{-7}$, $**P=6.81\times 10^{-7}$]. The amount of plasmid transfected is indicated. Luciferase activity is fold-induction compared to VSVG-LRP6 alone (mean $\pm$ s.d.). Experiments were performed in triplicate.

Fig. 4.

Gβγ enhances LRP6 activation of β-catenin signaling in vivo (**A**) Secondary axis formation by injection of LRP6 mRNA (2 ng) into one ventral blastomere of 4-cell *Xenopus* embryos is inhibited by coinjection of c-βARK RNA (1 ng). Arrow head marks secondary axis. Frequency of axis duplication is plotted (Fisher's exact test, * $P < 0.0001$). (**B**) Coinjecting Gβ₁γ₂ mRNA (0.5 ng) enhanced LRP6-stimulated (1 ng) secondary axis formation (Fisher's exact test, * $P = 0.004$). Injection of cβARK mRNA (n=31) or Gβ₁γ₂ mRNA (n=27) alone into ventral blastomere did not perturb axis formation. (**C**) Coinjection of LRP6 mRNA (0.5 ng) and Gβ₁γ₂ mRNA (0.5 ng) promotes ectopic transcription of Wnt/β-catenin targets genes, *Xnr3* and *Siamese*, in ectopic explants (animal caps) to a greater extent than injection of LRP6 alone as assayed by RT-PCR. WE, whole embryos; Caps, animal caps; WE-RT, no reverse transcriptase added; *ODC*, ornithine decarboxylase (loading control). Asterisk marks intervening lanes that were removed.

Fig. 5.

Scanning alanine mutagenesis of Gβ₁ identifies amino acids that mediate LRP6 activation and GSK3 interaction. **(A)** Mutagenesis of Gβ₁ reveals specific amino acids required for enhancement of LRP6 activity measured by TOPflash. VSVG-LRP6 (40 ng) was transfected alone or with the indicated Gβ₁ mutant (50 ng) plus Gγ₂ (50 ng) into HEK-293 STF cells. Lysates were assayed by TOPflash and immunoblotting (anti-HA for HA-Gβ₁ and anti-VSVG for VSVG-LRP6). Red and white bars represent mutants that map to two clusters on the surface of Gβ₁. For TOPflash assays, experiments were performed in triplicate, and luciferase activity is fold induction compared to LRP6 (mean ± s.d.) [ANOVA (family-wise), **P*<0.005]. **(B)** Co-immunoprecipitation of GSK3 with Gβ₁ point mutants. HEK-293 cells were transfected with Myc-GSK3, HA-Gβ₁ point mutants, and Gγ₂. Cells were lysed, HA-Gβ₁ immunoprecipitated, and Myc-GSK3 was detected by immunoblotting. Each experiment was repeated three times with representative results are shown. **(C)** The crystal coordinates of Gβ₁γ₂ (PDB 1XHM). Gβ₁ is shown in teal and Gγ₂ in gray. The seven β-propellers of Gβ are labeled B1-B7. Two clusters of amino acids required for Gβ₁-enhancement of LRP6 activity are in red and white.

Fig. 6.

Gβ₁γ₂ promotes Dsh-mediated activation of β-catenin signaling and enhances phosphorylation of LRP6 by GSK3. (A) HEK-293 STF cells were transfected with Myc-Dsh plus increasing amounts of plasmid encoding c-βARK. Transfected cells were then lysed and processed for TOPflash or immunoblotting [mean ± s.d., ANOVA (family-wise), * $P=3.85 \times 10^{-5}$]. (B) Dsh is required for LRP6-mediated TOPflash activation. Dominant-negative Dsh (Xdd; 20 ng, 50 ng, 125 ng) inhibited LRP6 (40 ng) activation of TOPflash, and Gβ₁γ₂ (50 ng each) enhanced LRP6 activation of TOPflash in HEK-293 STF cells [mean ± s.d., ANOVA (family-wise), * $P=0.0009$]. (C) Gβ₁γ₂ increases phosphorylation of endogenous LRP6 when co-transfected with GSK3. HEK-293 cells were transfected with Myc-GSK3 with or without HA-Gβ₁γ₂ and lysates immunoblotted with anti-phospho-LRP6 (Ser¹⁴⁹⁰) antibody. Representative blot shown on left, quantification on right (3 experiments, t-test, * $P<0.005$). (D) Dsh co-immunoprecipitates with Gβ₁γ₂ from cultured cells. HEK-293 cells were transfected with HA-Gβ₁ (1 μg), Gγ₂ (1 μg), and Myc-Dsh (1 μg). Cells were lysed, HA-Gβ₁ immunoprecipitated, and Myc-Dsh was detected by immunoblotting for the Myc tag. (E) LRP6 co-immunoprecipitates with Gβ₁γ₂ from cultured cells. Membrane proteins were isolated from HEK-293 cells transfected with HA-Gβ₁ (1 μg), Gγ₂ (1 μg), and VSVG-LRP6 (1 μg). HA-Gβ₁ was immunoprecipitated and VSVG-LRP6 detected by immunoblotting for the VSVG tag. (F) Axin co-immunoprecipitates with Gβ₁γ₂ from cultured cells. HEK-293 cells were transfected with Gβ₁ (1 μg), Gγ₂ (1 μg), and Myc-Axin (1 μg). Cells were lysed, Gβ₁ immunoprecipitated, and Myc-Axin was detected by immunoblotting for the Myc tag. (G,H) Gβ₁γ₂ enhances GSK3 phosphorylation of Tau (F) and LRP6ICD (G) in vitro. Each experiment was repeated three times with representative results shown.

Fig. 7.

Model for regulation of β-catenin/TCF signaling by Gα and Gβγ. Receptor activation dissociates Gα and Gβγ. Gβγ, along with Dsh, recruits and activates GSK3 (either free or possibly in association with axin) at the plasma membrane such that GSK3 can then phosphorylate and activate LRP6. Signaling downstream of the activated LRP6 receptor and Gα signaling events inhibit the β-catenin degradation complex, inhibiting β-catenin turnover. It is unclear whether the receptor is a classical GPCR or whether Frizzled is involved in transduction of the signal.