Negative feedback regulation of Wnt signaling by Gβγ-mediated reduction of Dishevelled

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Abbreviations: APC, adenomatous polyposis coli; GPCR, G protein coupled receptors; GSK3β, glycogen synthase kinase 3β; $G\beta_2$, guanine nucleotide binding protein β_2; NSCLC, non small cell lung cancer; PP2A, protein phosphatase type 2A; Iso, isoproterenol; β_2AR, β_2 adrenergic receptor

Abstract

Wnt signaling is known to be important for diverse embryonic and post-natal cellular events and be regulated by the proteins Dishevelled and Axin. Although Dishevelled is activated by Wnt and involved in signal transduction, it is not clear how Dishevelled-mediated signaling is turned off. We report that guanine nucleotide binding protein beta 2 (Gnb2; $G\beta_2$) bound to Axin and $G\beta_2$ inhibited Wnt mediated reporter activity. The inhibition involved reduction of the level of Dishevelled, and the $G\beta_2\gamma_2$ mediated reduction of Dishevelled was countered by increased expression of Axin. Consistent with these effects in HEK293T cells, injection of $G\beta_2\gamma_2$ into *Xenopus* embryos inhibited the formation of secondary axes induced either by XWnt8 or Dishevelled, but not by β-catenin. The DEP domain of Dishevelled is necessary for both interaction with $G\beta_2\gamma_2$ and subsequent degradation of Dishevelled via the lysosomal pathway. Signaling induced by $G\beta_2\gamma_2$ is required because a mutant of $G\beta_2$, $G\beta_2$ (W332A) with lower signaling activity, had reduced ability to downregulate the level of Dishevelled. Activation of Wnt signaling by either of two methods, increased Frizzled signaling or transient transfection of Wnt, also led to increased degradation of Dishevelled and the induced Dishevelled loss is dependent on $G\beta_1$ and $G\beta_2$. Other studies with agents that interfere with PLC action and calcium signaling suggested that loss of Dishevelled is mediated through the following pathway: Wnt/Frizzled → Gβγ → PLC → Ca^{+2}/PKC signaling. Together the evidence suggests a novel negative feedback mechanism in which $G\beta_2\gamma_2$ inhibits Wnt signaling by degradation of Dishevelled.

Keywords: dishevelled proteins; feedback, biochemical; Frizzled receptors; heterotrimeric GTP-binding proteins; type C phospholipases; Wnt proteins

Introduction

Wnt signaling plays a pivotal role in diverse biological processes in embryonic development and in adult organisms (Clevers, 2006; Logan and Nusse, 2004; White *et al*., 2007). Mammals have 19 different Wnt ligands, 10 Frizzled receptors which are similar to serpentine G protein coupled receptors (GPCR) (Schulte and Bryja, 2007), plus other co-receptors such as LRP5/6 (He, 2003; Logan and Nusse, 2004). Binding of Wnt to its receptors activates downstream signaling events, such as the "canonical" pathway, mainly mediated by controlling the level of β-catenin via the ubiquitination/proteasome degradation pathway, or the "non-canonical" pathway which is controlled either via Rac/Rho or Ca^{+2} signaling (Kohn and Moon, 2005; Veeman *et al*., 2003). In some specific combinations of Wnt/Frizzled signaling the intracellular protein Dishevelled determines whether signaling proceeds by β-catenin-dependent or independent pathways. It is obvious that tight regulation of the signaling is critical since mis-regulation of Wnt signaling during embryonic development or post-natal life leads to different types of developmental defects, or diseases such as cancer, Alzheimer's disease or osteoporosis and others (http://www.stanford.edu/~rnusse/wntwindow.html).

Axin is a scaffold protein that interacts with a number of proteins to regulate Wnt/β-catenin signaling (Lee *et al*., 2003; Tolwinski and Wieschaus, 2004; Kikuchi *et al*., 2006). In the absence of Wnt,

Axin is part of a complex with adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β) whose action is to phosphorylate cytoplasmic β-catenin and thus target it for ubiquitin- and proteasome-mediated degradation. When, however, Wnt binds to Frizzled and Dishevelled is activated then β-catenin degradation is inhibited, and after accumulating in the cytoplasm translocates to the nucleus where it binds to and activates TCF/LEF transcription factor (Logan and Nusse, 2004; Kikuchi *et al.*, 2006). Although several mechanisms have been proposed how Dishevelled is activated in the presence of Wnt (Li *et al.*, 1999; Chen *et al.*, 2001), it is not clear how the activated Dishevelled signaling is later turned off. One way to turn off activated Dishevelled signaling is by activating the expression of its antagonist *naked cuticle* which causes down-regulation of Dishevelled (Zeng *et al.*, 2000; Creyghton *et al.*, 2005).

The involvement of trimeric G protein signaling in the regulation of Wnt signaling was suggested by the finding that Frizzled interacts with the Gαsubunit of trimeric G proteins in mammalian cells and that G protein signaling directly transduces Frizzled signaling in *Drosophila* (Katanaev *et al.*, 2005; Liu *et al.*, 2005; Wang *et al.*, 2006). Gα-protein signaling synergistically activates the Wnt/β-catenin pathway in colon cancer cells (Castellone *et al.*, 2005; Yang *et al.*, 2005), and bone formation is promoted by non-canonical Wnt-mediated G-protein signaling (Tu *et al.*, 2007). We have found a role of Gβγ in Wnt signaling through the discovery that in yeast two-hybrid screening Axin binds to Guanine nucleotide binding protein, beta 2 (Gnb2; $G\beta_2$). Here, we provide evidence for a novel negative feedback loop for Wnt signaling in which activated Gβγ signaling can turn off Wnt signaling via degradation of Dishevelled.

Results

Gβ interacts with Axin

To confirm that the interaction between Axin and $G\beta_2$ occurs in mammalian cells, Myc-tagged Axin

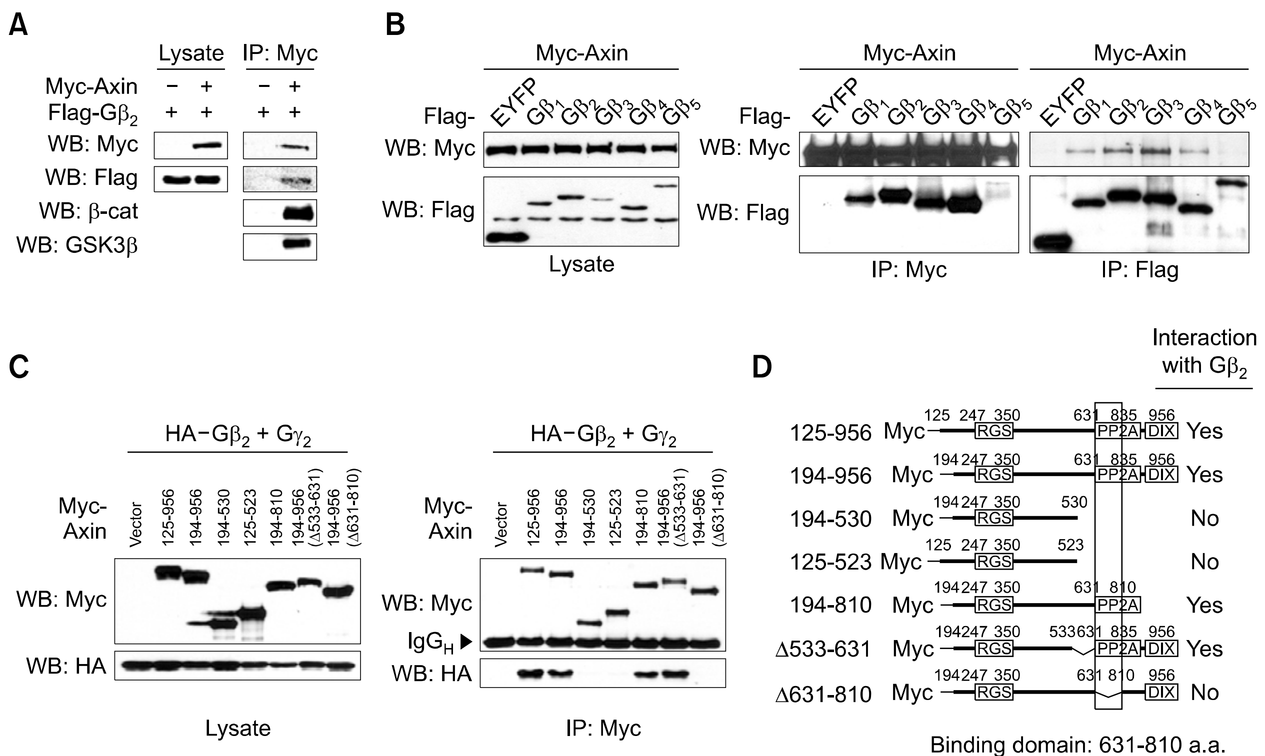

Figure 1. Gβ interacts with Axin. (A) Co-immunoprecipitation of myc-Axin and Flag-$G\beta_2$. Myc-tagged Axin and FLAG-tagged $G\beta_2$ were transfected into HEK293T cells. The lysates were first subjected to immunoprecipitation (IP) using anti-Myc antibody, followed by western blotting (WB) using the antibodies indicated on the left side. β-catenin and GSK3β were used for positive controls. (B) Myc-tagged Axin with FLAG-tagged isoforms of Gβ or EYFP were transfected into HEK293T cells. Immunoprecipitation followed by western blotting was performed to test the specificity of interaction between Axin and isoforms of Gβ. (C) Deletion constructs of Axin with HA-tagged $G\beta_2$ and $G\gamma_2$ were transfected into HEK293T cells and the expression was confirmed by western blot (left panel). Immunoprecipitation with anti-Myc antibody followed by western blotting was performed to examine specific interaction (right panel). (D) Schematic diagram of deletion constructs and summary of interaction between Axin and $G\beta_2$ was depicted.

and FLAG-tagged $G\beta_2$ were co-transfected in HEK293T cells followed by co-immunoprecipitation (co-IP) experiments (Figure 1A). With the Axin to β-catenin/GSK3β interaction serving as a positive control we found that Axin also interacts with $G\beta_2$. All five isoforms of the Gβ family of heterotrimeric G-protein subunits were tested and found to interact strongly with Axin (Figure 1B) except for $G\beta_5$ which has the least sequence similarity to the other four isoforms $G\beta_{1\text{-}4}$. Because Gβ forms a dimer with Gγ to make a functional unit (Birnbaumer, 2007), we tested whether the dimerization affects binding to Axin. When $G\gamma_2$, known to form dimers with $G\beta_1$ or $G\beta_2$ is co-expressed the interaction between $G\beta_2$ and Axin was unaffected (Supplemental Data Figure S1A). Additional co-IP experiments using deletion constructs of Axin determined that amino acids 631-810 of Axin, a sequence which had previously been shown to interact with protein phosphatase 2A (Hsu *et al.*, 1999), were required for interacting with $G\beta_2$ (Figure 1C, 1D and Supplemental Data Figure S1B).

$G\beta_2\gamma_2$ inhibits Wnt/β-cat signaling by reducing the level of Dishevelled

We next examined the functional significance of the Gβγ to Axin interaction through the effect of transient transfection of Gβγ on Wnt mediated TOP-FLASH reporter activity. While it is known that ectopic-expression of Gα activates canonical Wnt signaling (Castellone *et al.*, 2005), we found that co-transfection of $G\beta_2\gamma_2$ inhibited signaling (Supplemental Data Figure 2A). Results from co-transfection experiments designed to individually express either Wnt1, Dishevelled, or β-catenin showed that Gβγ inhibits canonical Wnt signaling at a level

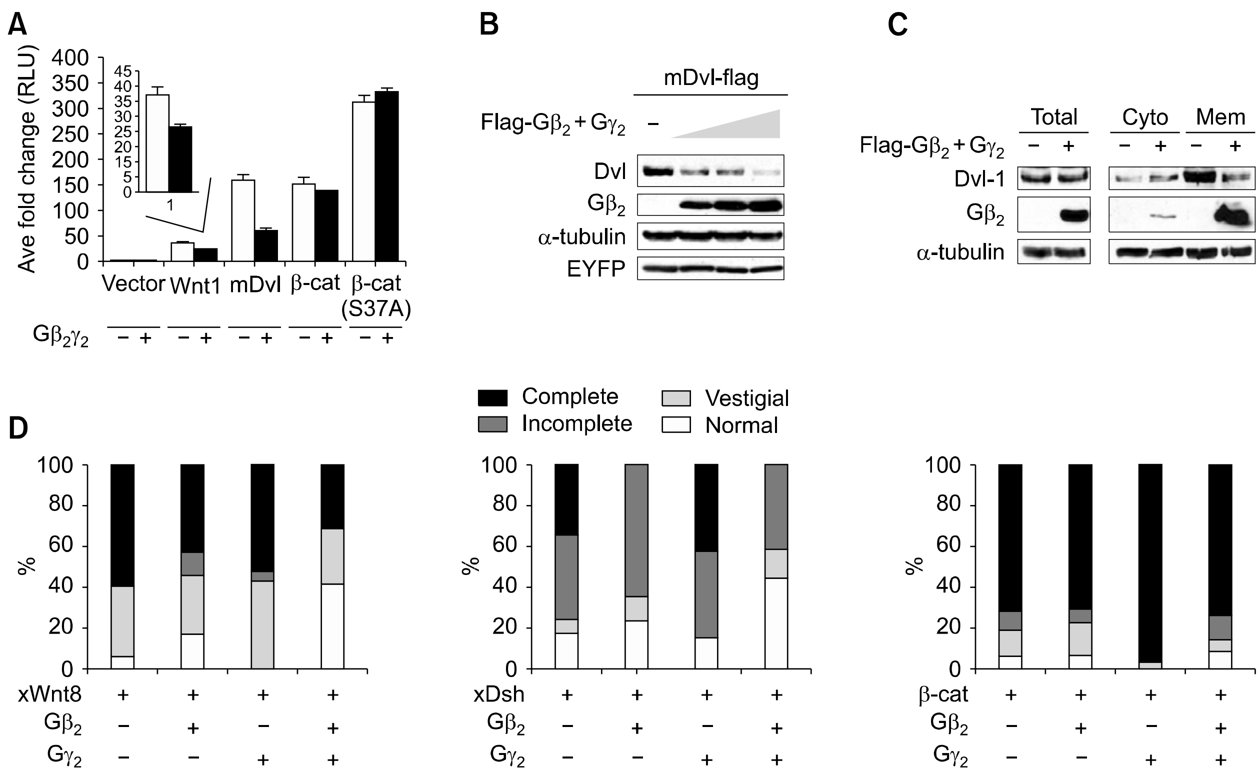

Figure 2 $G\beta_2\gamma_2$ inhibits Wnt signaling by reducing the level of Dishevelled (Dvl). (A) Epistasis experiments show Gβγ acts downstream of or in parallel to Dishevelled and upstream of β-catenin. SuperTOPFlash plasmid with empty-vector, Wnt1, mDvl-1, β-catenin or β-catenin S37A was transfected along with either empty-vector or $G\beta_2\gamma_2$, respectively. After transfection (36 h), luciferase assay was performed. The reporter activity induced by transfection of Wnt1 or mDvl-1 was significantly inhibited by co-transfection of $G\beta_2\gamma_2$ (P value < 0.01, $n = 3$). (B) FLAG-tagged mDvl-1 was transfected alone or with 0.5, 1 and 2 μg of FLAG-tagged $G\beta_2$ and $G\gamma_2$ each into HEK293T cells and western blotting was performed to examine the level of mDvl-1. EYFP was used for equal transfection control. (C) To measure the effect of $G\beta_2\gamma_2$ on the level of endogenous Dishevelled-1, HEK293T cells were transfected with Flag tagged $G\beta_2$ and $G\gamma_2$ plasmids. After transfection (36 h), cells were lysed and the lysates were fractionated into cytosolic and membrane fractions. The endogenous level of Dishevelled-1 was examined by western blotting. (D) XWnt8 mRNA (10 pg, A, $n = 127$ embryos), XDsh (500 pg, B, $n = 139$) or Xβcat (1 ng, C, $n = 130$) were injected alone or with 1 ng of $G\beta_2$, $G\gamma_2$ or both $G\beta_2$ and $G\gamma_2$ mRNAs into ventral vegetal blastomeres of 8 cell stage embryos. Axis duplication was scored at tailbud stage (stage35). The percentages of embryos that show varying degrees of axis duplication are presented as bar graphs.

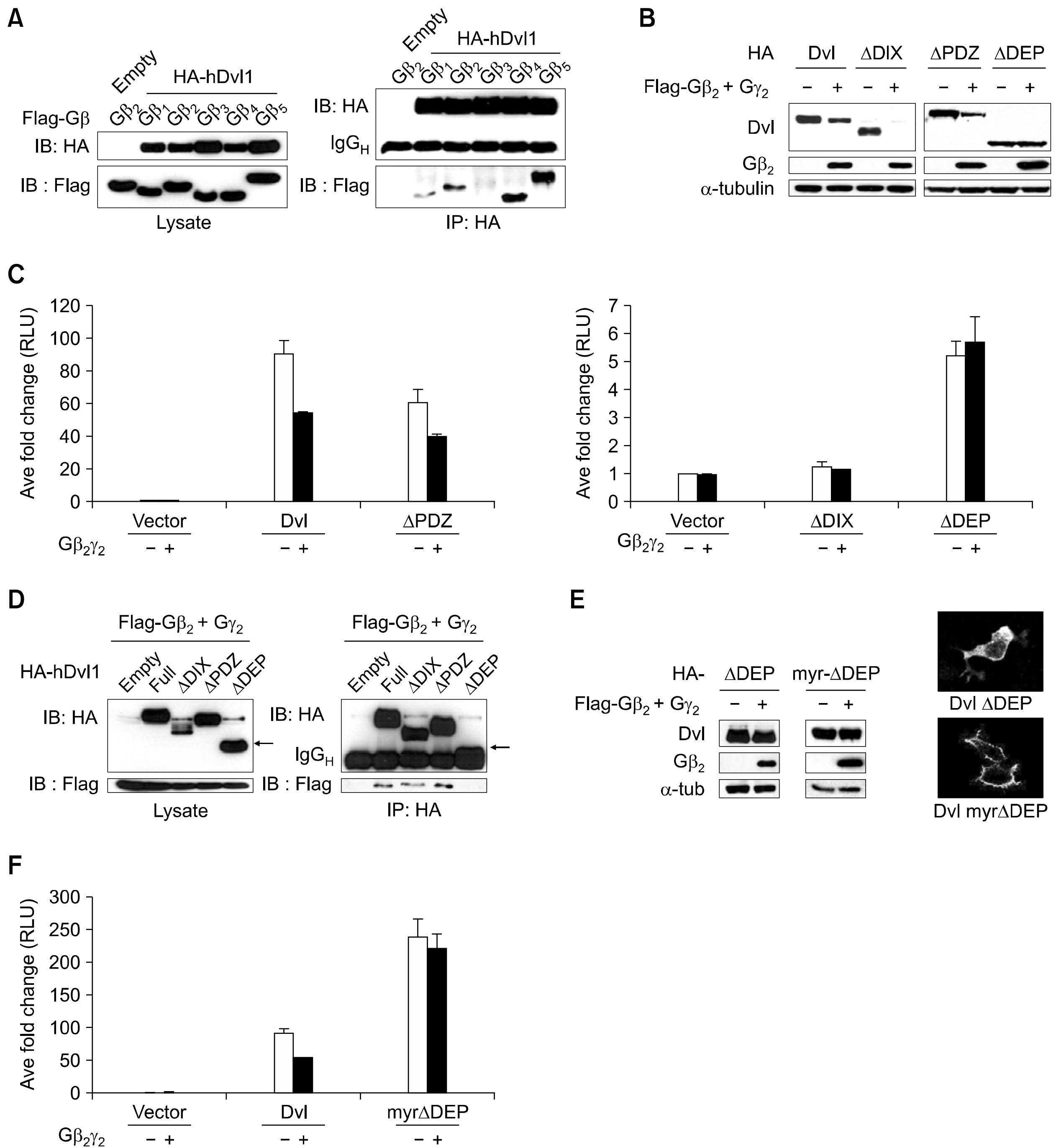

Figure 3. DEP domain of Dishevelled (Dvl) is required for degradation of Dishevelled by Gβγ. (A) Dishevelled interacts with all isoforms of Gβ except for $G\beta_3$. HA-tagged human Dv-1 was co-transfected with all isoforms of Gβ into HEK293T cells. Immunoprecipitation followed by western blotting was performed. (B) The level of DEP domain deleted Dishevelled was not reduced by $G\beta_2\gamma_2$. Various deletion constructs of Dishevelled were co-transfected with $G\beta_2\gamma_2$ into HEK293T cells and western blotting was performed. (C) The data from luciferase assay confirm the result obtained in (B). Luciferase assay was performed using superTOPFlash and pTK-*renilla* luciferase after co-transfection of various deletion constructs of Dishevelled and $G\beta_2\gamma_2$. (D) DEP domain of Dishevelled is required for interaction with $G\beta_2\gamma_2$. Various deletion constructs of Dishevelled with $G\beta_2\gamma_2$ were co-transfected into HEK293T cells and immunoprecipitation followed by western blotting was performed. Arrow indicates ΔDEP-Dishevelled. (E) Membrane localization of ΔDEP-Dishevelled via myristoylation/ palmitoylation signal is not sufficient for the down-regulation of Dishevelled by $G\beta_2\gamma_2$. ΔDEP-Dishevelled and myr-ΔDEP, which has a myristoylation/ palmitoylation signal sequence to the N-terminal of ΔDEP-Dishevelled, were transfected alone or with $G\beta_2\gamma_2$ into HEK293T cells. After fractionation, western blotting was performed using the membrane fraction (left panel). Indirect immunofluorescence analysis with anti-HA antibody shows myr-ΔDEP-Dishevelled is highly localized in plasma membrane (right panel). (F) Luciferase assay using superTOPFlash was conducted to confirm the result obtained in (E).

upstream of β-catenin and downstream of, or in parallel to, the level of Dishevelled (Figure 2A). We focused on Dishevelled as the target of the inhibition of this since Gβ is known to interact with Dishevelled (Angers *et al.*, 2006). We found that as reporter activity induced by Dishevelled was decreased in a dose-dependent manner by increasing $G\beta_2\gamma_2$ (Supplemental Data Figure S2B) the level of Dishevelled was dramatically reduced; the level of a control protein EYFP was unaffected (Figure 2B). Furthermore although Gβ2 itself (it may form a dimer with endogenous Gγ, but not $G\gamma_2$, alone caused a significant reduction, co-transfection of $G\beta_2\gamma_2$ resulted in a greater decrease in the level of Dishevelled than either subunit alone (Supplemental Data Figure S2C).

Having established that Gβγ signaling inhibits Wnt signaling via reduction of the level of Dishevelled, we next determined if membrane-associated or cytoplasmic, or both, forms of Dishevelled are targeted. To do this cell lysates were separated into cytoplasm and membrane fractions and analyzed for Dishevelled. After ectopic expression of $G\beta_2\gamma_2$ both endogenous and ectopically expressed Dishevelled present in the membrane fractions were clearly reduced, while the level of Dishevelled in the cytoplasmic fraction was not (Figure 2C and Supplemental Data Figure S2D). Therefore the evidence suggests that $G\beta_2\gamma_2$ inhibits Wnt/β-cat signaling primarily by reducing the level of membrane-associated Dishevelled.

To confirm that Gβγ can regulate Wnt signaling in an *in vivo* system, we used *Xenopus* embryos because Wnt, Dishevelled and β-catenin induce a secondary embryonic axis if they are injected into the vegetal side of early blastomeres. We expected that Gβγ would inhibit the formation of secondary axes induced by the injection of XWnt8, and XDsh but not β-catenin mRNA. The results show that $G\beta_2$ and $G\beta_2\gamma_2$, but not $G\gamma_2$ alone, significantly reduced secondary axis formation after injection of XWnt8; the percentage of normal embryos was increased from 6 to 41 (Figure 2D, left panel). Similarly, secondary axes induced by the injection of XDsh mRNA were considerably reduced by the co-injection of $G\beta_2$ mRNA alone (but not by $G\gamma_2$ mRNA), and reduced even more when Gβ2 and Gγ2 mRNAs were co-injected; the percentage of normal embryos increased from 17 to 44 (Figure 2D, middle panel). In addition, the percentage of embryos that showed complete axis duplication was reduced from 34 to 0 when $G\beta_2\gamma_2$ mRNA was co-injected with XDsh mRNA. As expected from the lack of an effect on β-catenin-mediated signaling in cell culture the increased secondary axis formation induced by the injection of β-catenin mRNA was unaffected by the co-injection of $G\beta_2$, $G\gamma_2$ and $G\beta_2\gamma_2$ (Figure 2D). Taken together these results from cell culture and *Xenopus* development suggest that Gβγ inhibits the Wnt/β-cat pathway *in vivo* by creating a signal for the loss of Dishevelled.

Interaction between Gβ and the DEP domain and membrane localization of Dishevelled is necessary for down-regulation of Dishevelled

We determined if Dishevelled can interact with Gβ family members by co-IP experiments and found that all Gβ proteins except for $G\beta_3$ interact with Dishevelled (Figure 3A). Dishevelled has three conserved domains; DIX, PDZ and DEP (Wallingford and Habas, 2005). To identify which domain of Dishevelled is involved in the degradation induced by Gβγ, we used deletion constructs (Supplemental Data Figure S3) which were co-transfected with $G\beta_2\gamma_2$. Only the DEP domain including C-terminal domain was found to be necessary for $G\beta_2\gamma_2$-mediated degradation of Dishevelled (Figure 3B). It is known that DIX domain is necessary for the induction of the level of β-catenin (Kishida *et al.*, 1999). Consistent with this published result ectopic expression of DIX domain-deleted Dishevelled (ΔDIX-Dishevelled) neither induce β-catenin/Tcf mediated reporter activity nor respond to Gβγ (Figure 3C), while the level of ΔDIX-Dishevelled was reduced by Gβγ (Figure 3B). As expected luciferase-reporter activity induced by DEP domain-deleted Dishevelled (ΔDEP-Dishevelled) was not inhibited by the co-expression of $G\beta_2\gamma_2$ (Figure 3C). We reasoned that ΔDEP-Dishevelled could not be down-regulated by $G\beta_2\gamma_2$ since it could not bind to $G\beta_2$. To test this hypothesis we performed co-IP experiments and found that as expected ΔDEP-Dishevelled does not bind to $G\beta_2$ (Figure 3D). These results suggest that interaction of Gβ with the DEP domain of Dishevelled is necessary for down-regulation of Dishevelled by Gβγ. Since it is known that the DEP domain is necessary for the membrane localization of Dishevelled when Frizzled is over-expressed (Axelrod *et al.*, 1998), and the membrane fraction of Dishevelled is targeted for loss mediated by $G\beta_2\gamma_2$ (Figure 2C and Supplemental Data Figure 2D), the ΔDEP-Dishevelled protein may be resistant to down-regulation partly due to its lack of membrane localization. To test this hypothesis, a myristoylation/palmitoylation modification sequence (Simons *et al.*, 2005) was added to ΔDEP-Dishevelled (myr-ΔDEP-Dishevelled) to target localization to the membrane and the effect of $G\beta_2\gamma_2$ assessed. Although the myr-ΔDEP-Dishevelled protein localized to the membrane, its level was not reduced by $G\beta_2\gamma_2$ (Figure 3E). Additionally,

the robust luciferase-reporter activity induced by myr-γDEP-Dishevelled was not blocked by $G\beta_2\gamma_2$ (Figure 3F). These data suggest that membrane localization of Dishevelled alone may not be enough to induce degradation, although it may aid degradation of Dishevelled (See Figure 5B). It is of course also possible that the forced localization of Dishevelled in the membrane does not sufficiently mimic the position in the membrane or interaction with membrane proteins after normal Wnt/Frizzled signaling. The results of these experiments suggest that the interaction between Gβ and the DEP domain and membrane localization of Dishevelled is necessary for down-regulation of Dishevelled.

Dishevelled degradation by Gβ2γ2 is mediated via the lysosomal degradation pathway and Ca^{+2}/PKC signaling is involved in that process

Dishevelled has been shown to be degraded using either the proteasomal or lysosomal pathway (Miyazaki *et al.*, 2004; Creyghton *et al.*, 2005; Simons *et al.*, 2005; Angers *et al.*, 2006; Zhang *et al.*, 2006). Which pathway is involved in the Gβγ-mediated reduction of Dishevelled was tested using pathway-specific inhibitors. Treatment with MG132, a proteasome-pathway inhibitor, did not block Gβγ mediated reduction of Dishevelled (Figure 4A) nor were any cleavage products of N-terminal and C-terminal epitope-tagged Dishevelled observed (Figure 4A). Additional proteasome pathway calpain inhibitors ALLN or ALLM also did not block the degradation of Dishevelled (data not shown). Dishevelled degradation mediated by $G\beta_2\gamma_2$, however, was blocked by the lysosomal-pathway degradation inhibitor chloroquine (Figure 4B) suggesting that Gβγ-mediated reduction of Dishevelled occurs in lysosomes.

The identification of the signal pathway downstream of Gβγ which leads to lysosomal degradation of Dishevelled is also important. After confirming that activation of Gβγ signaling causes transloca-

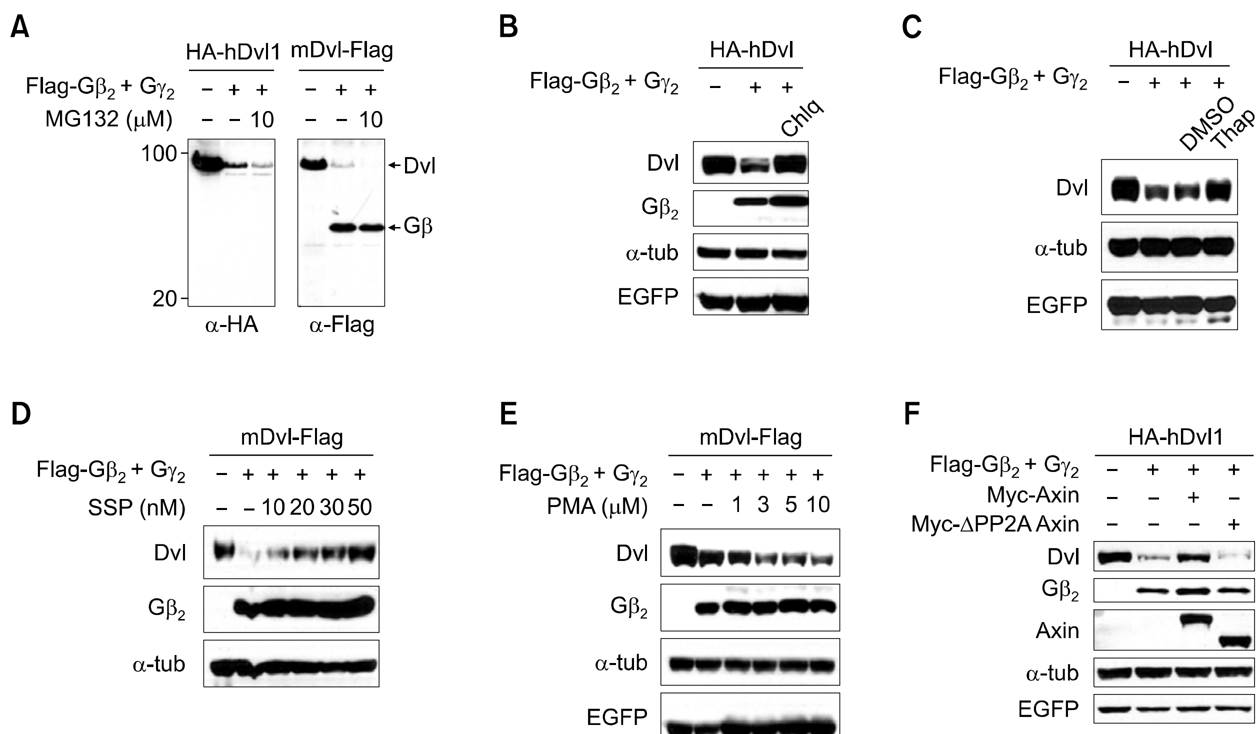

Figure 4. Dishevelled (Dvl) degradation by $G\beta_2\gamma_2$ is mediated via the lysosomal degradation pathway and Ca^{+2}/PKC signaling is involved in that process. (A-C) HA-tagged human Dishevelled-1 or Flag tagged mouse Dishevelled-1 plasmid was transfected alone or with $G\beta_2\gamma_2$ into HEK293T cells and the cells were treated with MG132 (10 μM for 4 h before harvest, (A)), chloroquine (50 μM for 16 h before harvest, (B)) and thapsigargin (5 μM for 1h and cells were incubated for 24 h more without drug before harvest, (C)) and the level of Dishevelled was measured by western analysis. (D and E) Flag tagged mouse Dishevelled-1 plasmid was co-transfected with $G\beta_2\gamma_2$ and EGFP into HEK293T cells and the cells were treated with different concentrations of PKC antagonist (16h before harvest, (D)) or agonist (16 h before harvest, (E)). Western blot analysis was then performed with antibodies indicated in the figure. (F) Axin blocks degradation of Dishevelled by $G\beta_2\gamma_2$. Dishevelled was transfected alone (lane 1) or with $G\beta_2\gamma_2$ (lanes 2-4) into HEK293T cells along with Myc-tagged Axin (lane 3) or ΔPP2A Axin (lane 4) and western blotting was performed with antibodies indicated in the figure. EGFP was used for equal transfection control.

tion of PKCα from the cytoplasm to the plasma membrane (Sheldahl *et al.*, 1999) (Supplemental Data Figure S4A) we used a mutant form $G\beta_2$ (W332A) which is able to bind to $G\gamma$ but has reduced signaling activity (Ford *et al.*, 1998) in order to determine whether signaling activity of $G\beta\gamma$ is needed. $G\beta_2$ (W332A) had a much lower ability than the wild type to reduce Dishevelled abundance (Supplemental Data Figure S4B) and it had reduced ability to inhibit Wnt-mediated luciferase-reporter activity (Supplemental data Figure S4C). These data suggest that activation of downstream signaling by $G\beta\gamma$ plays an important role in the downregulation of Dishevelled. $G\beta_2$ (W332A)'s lower ability (Supplemental Data Figure S4B and S4C) was not a result of reduced interaction with Dishevelled because it can interact with Dishevelled as well or even better than wild type $G\beta_2$ in co-IP experiments (Supplemental Data Figure S4D).

Having shown that activation of downstream signaling by $G\beta\gamma$ is required for the degradation of Dishevelled we examined the role of Ca^{+2}/PKC signaling (Sheldahl *et al.*, 1999) by using inhibitors or activators of this pathway. Thapsigargin, which inhibits cytoplasmic calcium signaling by diverting Ca^{+2} released from the ER from the cytoplasm to outside of the cell (Westfall *et al.*, 2003) clearly blocked $G\beta\gamma$-mediated loss of Dishevelled (Figure 4C). Staurosporine, a PKC inhibitor, blocked whereas PMA, a PKC activator, increased the loss of Dishevelled by $G\beta\gamma$ (Figure 4D and 4E). Although the many isoforms of PKC precluded easy identification of the specific isoforms responsible for the effect, these preliminary data suggest that Ca^{+2}/PKC signaling is involved in the $G\beta\gamma$mediated loss of Dishevelled.

Axin blocks $G\beta\gamma$-mediated loss of Dishevelled

Our initial finding that Axin binds $G\beta_2$ (Figure 1) led to the hypothesis that interaction with $G\beta\gamma$ might control the loss of Dishevelled but left the role of

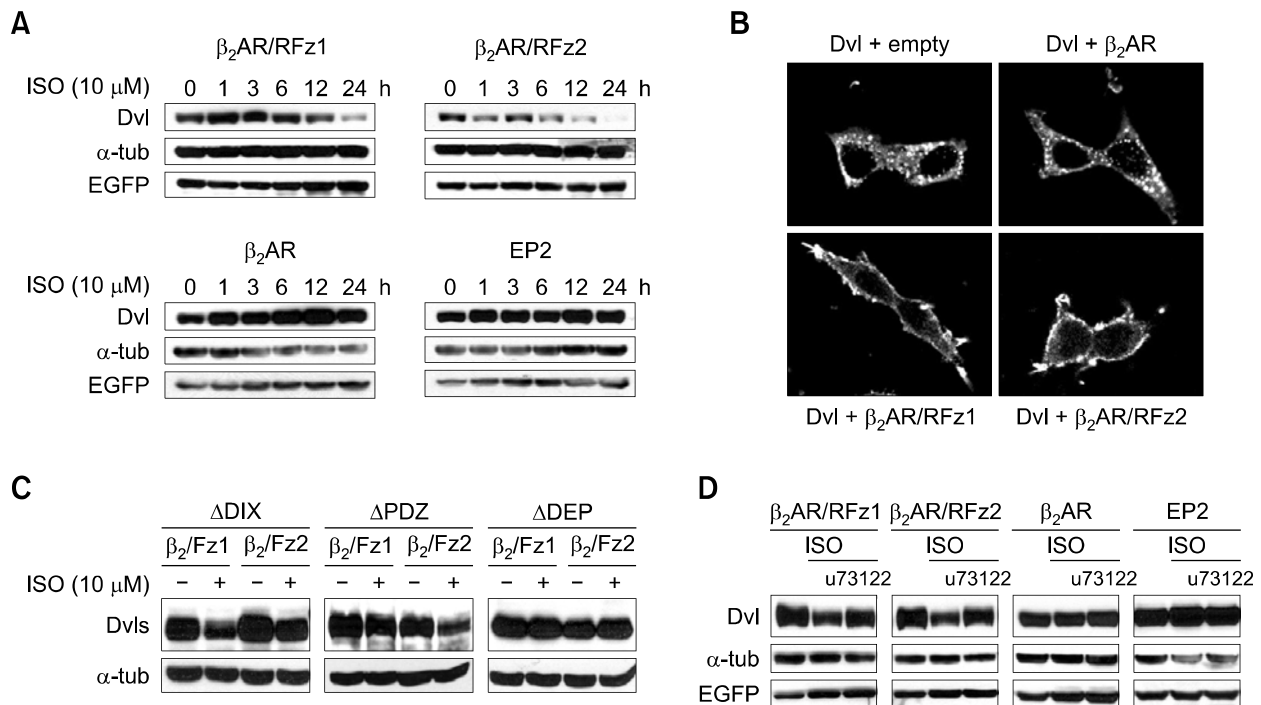

Figure 5. Activation of Fz leads to down-regulation of Dishevelled (Dvl) via PLC signaling. (A) Activation of Frizzled leads to down-regulation of Dishevelled. Dishevelled was co-transfected with β_2-adrenergic/rat Fz1 (β_2AR/RFz1), β_2-adrenergic/rat Fz2 chimeric receptor (β_2AR/RFz2), Gs-coupled receptor β_2-adrenergic receptor (β_2AR) or EP2 into HEK293T cells. Isoproterenol was added at 10 μM at the times indicated in the figure. The level of Dishevelled was measured by western blot analysis. EGFP was used for equal transfection control. (B) Activation of chimeric Frizzled recruits Dishevelled to plasma membrane. Dishevelled was co-transfected with receptors described in figure into HEK293T cells and the cellular localization of Dishevelled was detected via indirect immunofluorescence. (C) DEP domain is necessary for the downregulation of Dishevelled via activation of β_2AR/RFz1 and β_2AR/RFz2. β_2AR/RFz1 and β_2AR/RFz2 plasmids were co-transfected with Dishevelled constructs (Supplemental Data Figure S3) as indicated in figure and the cells were treated with isoproterenol (10 μM for 24 h before harvest) followed by western blot analysis. (D) Down-regulation of Dishevelled by Frizzled activation is blocked by treatment with a PLC inhibitor. Dishevelled was co-transfected with each receptor described in figure into HEK293T cells, which were treated with isoproterenol at 10 μM for 24 h before harvest (36 h). U73122, a PLC inhibitor, was added at 10 μM for 24 h before harvest. Western analysis was performed with antibodies indicated in figure.

Axin unexplained. As previously mentioned co-transfection of Axin blocked Gβγ-mediated loss of Dishevelled, but an ΔPP2A-Axin construct which lacks the interaction domain with protein phosphatase 2A (PP2A) and does not interact with Gβγ (Supplemental Data Figure S1B), did not inhibit loss of Dishevelled by Gβγ (Figure 4F). It is conceivable that Axin sequesters Gβγ from interaction with Dishevelled thereby blocking loss of Dishevelled mediated by Gβγ. The possibility that Axin blocks downregulation of Dishevelled by Gβγ through competitive inhibition of interaction between Gβγ and Dishevelled or interference of Gβγ signaling is discussed later in a summary model (Figure 7).

Activation of Frizzled signaling induces loss of Dishevelled

Because Frizzled is known to be a G-protein coupled receptor (Schulte and Bryja, 2007) and would be expected to increase the level of dissociated Gβγ from Gα in a more physiological manner, we examined whether activation of Frizzled signaling could induce loss of Dishevelled. To activate Frizzled signaling HEK293T cells were transfected with β_2AR/RFz1 and β_2AR/RFz2, which are chimeric receptors that are used to activate the canonical and non-canonical Wnt/Frizzled signaling pathways, respectively, when isoproterenol (Iso) is added (Liu *et al.*, 2001; Ahumada *et al.*, 2002). As expected after cells with β_2AR/RFz1 and β_2AR/RFz2 were treated with isoproterenol (Iso) Dishevelled levels were reduced (Figure 5A) similarly to that found when Gβγ was increased by transfection. Control GPCRs, which are not related with Wnt/Frizzled signaling, like β_2AR that can, or EP2 that cannot be activated by the treatment of Iso were both found to be unable to cause loss of Dishevelled (Figure 5A). Why the chimeric receptors β_2AR/RFz1 and β_2AR/RFz2, but not β_2AR, leads to loss of Dishevelled while both types can activate a Gβγ signal upon Iso treatment could be a result of a difference in ability to induce membrane translocation of Dishevelled. We therefore examined Dishevelled localization after co-expressing these same chimeric or non-chimeric receptors. Consistent with this hypothesis, β_2AR/RFz1 and β_2AR/RFz2 can induce translocation of Dishevelled to the plasma membrane, but β_2AR could not (Figure 5B). Thus the activation of Gβ signaling alone from a receptor is normally insufficient to allow Dishevelled level to be reduced and other signaling events initiated by β_2AR/RFz1 and β_2AR/RFz2 are necessary. Although ectopic expression of Gβγ alone led to the downregulation of Dishevelled (Figure 2 and Supplemental Data Figure S2), it is possible that overexpression bypasses the requirement for Frizzled for membrane localization of Dishevelled in that situation. Which domain of Dishevelled is necessary for the β_2AR/RFz1 and β_2AR/RFz2 mediated loss of Dishevelled was examined next. Of the Dishevelled deletion constructs used (Supplemental Data Figure S3) only the ΔDEP-Dishevelled was not degraded after the activation of β_2AR/RFz1 or β_2AR/RFz2 (Figure. 5C). This result is consistent with the previous results (Figure 3) that showed that the DEP domain plays a critical role in the Gβγ mediated degradation of Dishevelled.

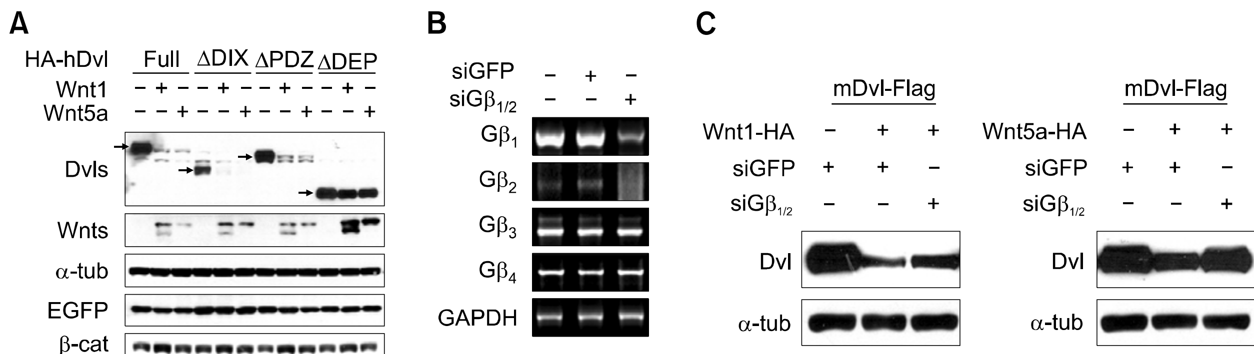

Figure 6. Expression of Wnts reduces the level of Dishevelled (Dvl) and the degradation is blocked by knock-down of $G\beta_1$ and $G\beta_2$. (A) Dishevelled is down-regulated by Wnt expression and DEP domain of Dishevelled is required for this down-regulation. Full length and deletion constructs of Dishevelled were co-transfected with mouse Wnt1 or Wnt5a into HEK293T cells and western blot analysis was performed. EGFP was used equal transfection control and the level of β-catenin was measured to show that Wnt1 signaling is properly conducted. (B) HEK293T cells were transfected with siRNA for GFP or $G\beta_1$/$G\beta_2$, and the level of isoforms of $G\beta_{1-4}$ was measured by RT-PCR analysis using specific primers targeting each Gβ isoform at 36 h after transfection. Gβ5 was not shown since its expression seems to be very low. (C) Wnt induced down-regulation of Dishevelled is blocked by knock-down of $G\beta_1$ and $G\beta_2$. Dishevelled was co-transfected with Wnt1 or Wnt5a into HEK293T cells. siRNAs for GFP or $G\beta_{1/2}$ was transfected as depicted in figure. Western analysis was performed to measure the level of Dishevelled.

We next turned our attention to the signaling events occurring after the Frizzled receptor and especially the possible role of phospolipase C (PLC) signaling, which is known to be activated by Gβγ (Ahumada *et al.*, 2002). Addition of a specific PLC inhibitor, u73122 (Lockhart and McNicol, 1999) to cells expressing β_2AR/RFz1 or β_2AR/RFz2 and induced with Iso prevented the loss of Dishevelled (Figure 5D). Because PLC involvement occurs after Gβγ and before Ca^{+2}/PKC the pathway involved in the downregulation of Dishevelled is expected to follow this order (Wnt/Frizzled→Gβγ→ PLC→Ca^{+2}/PKC signaling) (Figure 4C-E and 5D).

Gβγ signaling is necessary for the Wnt-mediated loss of Dishevelled

Interestingly, co-transfection of Wnt1 or Wnt5A with Dishevelled can reduce the level of Dishevelled (but not a control protein EGFP) just as with activation of Frizzled receptors by Iso (Figure 6A). As expected all deletion versions of Dishevelled except ΔDEP-Dishevelled were degraded by the co-expression of Wnt1 or Wnt5a. For these experiments we confirmed that the Wnt1 signaling pathway is intact and functional as indicated by an increased level of β-catenin upon co-transfection of Wnt1 (Figure 6A). To further confirm that Gβγ signaling is necessary for the Wnt-mediated loss of Dishevelled, siRNA that can knockdown both $G\beta_1$ and $G\beta_2$ (Krumins and Gilman, 2006) was added to HEK293T cells. The cells with that had been given siRNA had lowered $G\beta_1$ and $G\beta_2$, but not other control, mRNAs (Figure 6B) and had the expected diminished block in the loss of Dishevelled induced by either Wnt1 or Wnt5a (Figure 6C). The lack of complete blocking may be a result of incomplete downregulation of $G\beta_1$ and $G\beta_2$ or functional redundancies of other Gβ proteins whose mRNA levels were not reduced with these siRNAs. It is of course also possible that Wnt signaling causes downregulation of Dishevelled by pathways in addition to those involving Gβγ signaling. Nevertheless our data support the view that endogenous Gβγ signaling plays a critical role in loss of Dishevelled caused by Wnt.

Discussion

These data on the role of Gβγ signaling in Wnt mediated loss of Dishevelled can be incorporated into the following model based on work by others and our current results (Figure 7). In the presence of Wnt, Dishevelled and Axin (for canonical Wnt signaling) are translocated to membrane-bound Frizzled and LRP, respectively, and initiate downstream signaling (Mao *et al.*, 2001; Tamai *et al.*, 2004). Released Gα subunits from Gβγ may then synergistically enhance canonical Wnt signaling (Castellone *et al.*, 2005). During this time if LRP-bound Axin binds the Gβγ subunit and blocks the downstream signaling of Gβγ (as we show in Figure 1 and 4F) then Dishevelled will not be degraded and signaling will continue. Upon further Wnt signaling LRP-bound Axin is degraded (Willert *et al.*, 1999; Mao *et al.*, 2001) and now Gβγ subunits interact with Dishevelled to lead to its destruction via a signaling pathway including PLC, Ca^{+2} and PKC (Figure 3-5). The activation of the

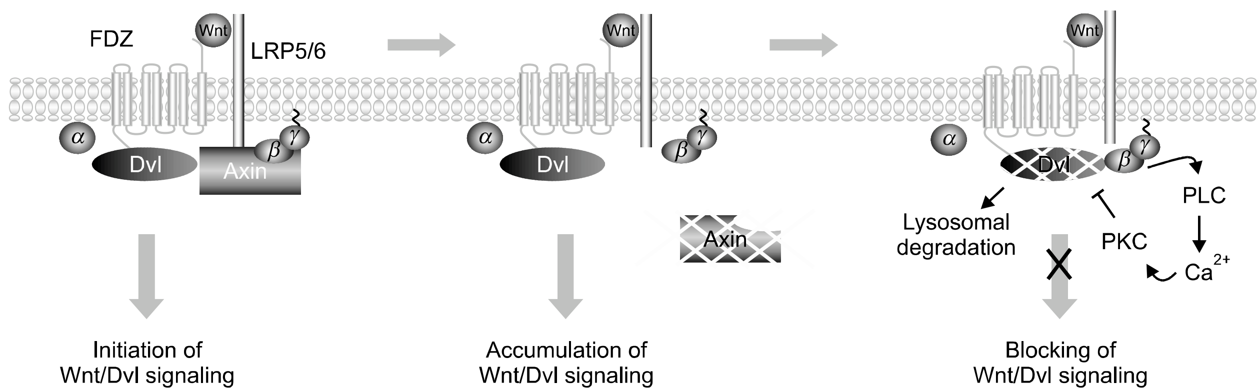

Figure 7. Model for a negative feedback mechanism of Wnt signal transduction by Gβγ signaling. Left panel: In the presence of Wnt, Dishevelled (Dvl) and Axin (for canonical Wnt signaling) are translocated to membrane-bound Frizzled and LRP, respectively, and initiate downstream signaling. Released Gα subunits from Gβγ may then synergistically enhance canonical Wnt signaling. During this time if LRP-bound Axin binds the Gβγ subunit and blocks the downstream signaling of Gβγ then Dishevelled will not be degraded and signaling will continue. Middle panel: Upon further Wnt signaling LRP-bound Axin is degraded which leads to release of Gβγ subunits from Axin. Right panel: The released Gβγ subunits interact with Dishevelled, which lead to its destruction via a signaling pathway including PLC, Ca^{+2} and PKC. The activation of the Gβγ signaling cascade leads to degradation of Dishevelled in lysosomes and thereby turns off Wnt signaling.

Gβγ signaling cascade leads to degradation of Dishevelled in lysosomes (Figure 3) and thereby turns off Wnt signaling. This model suggests that the downregulation of Dishevelled by Gβγ signaling is a novel negative feedback mechanism for regulation of Wnt signaling and provides some ideas for further investigation regarding the role of Gβγ signaling in Wnt signaling.

We showed that Axin does not interact with $G\beta_5$ (Figure 1B), whereas Dishevelled does not interact with $G\beta_3$ (Figure 3A) and the interaction between Dishevelled and $G\beta_2$ is necessary for the down-regulation of Dishevelled by $G\beta_2$. Therefore we expected that $G\beta_3$ has weaker effect while $G\beta_5$ shows stronger effect than $G\beta_2$ on the down-regulation of Dishevelled. Figure 3A and repeated experiments (data not shown) reproducibly show that $G\beta_2$ has the strongest effect while $G\beta_3$ and $G\beta_5$ exhibit very weak effect. The result with $G\beta_3$ is consistent with our proposed model, while the result with $G\beta_5$ is not easily explainable. It may be possible that the interaction between Axin and Gβ plays other unknown roles, such as recruitment of Dishevelled/Axin complex to Gβγ, in the regulation of Dishevelled.

Although the mechanism is not clear, Dishevelled can, depending on the nature of the upstream Wnt/Frizzled signaling, selectively determine whether canonical or non-canonical signaling is used (Schulte and Bryja, 2007). Here we found that β_2AR/RFz2, which is known to activate non-canonical Wnt signaling (Ahumada *et al.*, 2002), or Wnt5a led to loss of Dishevelled (Figure 5 and 6). Therefore it is conceivable that Gβγ signaling can control both canonical and non-canonical Wnt signaling by determining the amount of Dishevelled at the membrane. Although it is not known whether Axin is translocated to the membrane in non-canonical Wnt signaling, it is possible that Axin plays the same role in reduction of Dishevelled to turn off non-canonical Wnt signaling. Other unknown molecules may have similar roles to control Gβγ signaling for the downregulation of Dishevelled.

We can now add Gβγ to the list of proteins including NEDL1 (Miyazaki *et al.*, 2004), Naked cuticle (Creyghton *et al.*, 2005), inversin (Simons *et al.*, 2005), KLHL12 (Angers *et al.*, 2006), and dapper (Zhang *et al.*, 2006) that are responsible for the regulation of Dishevelled level in cells. Naked cuticle in particular is similar to Gβγ's role in that both establish a negative feedback loop to shut down Wnt signaling (Creyghton *et al.*, 2005). That there are so many proteins involved suggests that the precise regulation of Dishevelled level is crucial to many processes. Mis-regulation of Dishevelled is known to be involved in nephronophthis type II (Simons *et al.*, 2005), an autosomal cystic kidney disease, and non small cell lung cancer (NSCLC) (Uematsu *et al.*, 2003). Whether Gβγ is playing a role in these diseases will be important to determine.

Methods

Plasmids

All isoforms of Gβ cDNAs were obtained by RT-PCR using total RNA from whole mouse embryos and cloned in pCMV4-Flag and pCS2-HA backbone vectors. HA-tagged hDishevelled constructs were obtained from Dr. Kikuchi (Hiroshima University, Japan). Details about myc-tagged Axin and Flag-tagged mDishevelled constructs are described elsewhere (Fagotto *et al.*, 1999). SuperTopFlash reporter was obtained from Dr. Moon (University of Washington). β_2AR/RFz1 and β_2AR/RFz2 were obtained from Dr. Malbon (State University of New York at Stony Brook). β_2AR and EP2 were obtained from Dr. Gutkind (National Institute of Health).

Tissue culture and transfection

HEK293T cells were grown in DMEM supplemented with 10% FBS in a 37°C humidified incubator containing 5% CO_2. Transient transfections of plasmids were performed via the calcium phosphate method.

Co-immunoprecipitation and western analysis

For the co-immunoprecipitation, HEK293T cells (5×10^6) were lysed in RIPA buffer (25 mM Tris-HCl at pH 8.0, 150 mM NaCl, 10% glycerol, 1% Igepal CA-630, 0.25% deoxycholic acid, 2 mM EDTA, 1 mM NaF, 50 mM glycerophosphate). Lysates were cleared by centrifugation and immunoprecipitation was performed using monoclonal anti Myc or anti HA (sc-7392; Santa Cruz Biotechnology, Santa Cruz, CA) or anti Flag (F3165; Sigma) antibodies and protein A/G plus agarose. Western blot experiments were performed as previously described (Fagotto *et al.*, 1999).

Membrane-cytosolic fractionation

Cells were washed in PBS and scraped in lysis buffer containing 10 mM Tris-HCL (pH7.4), 140 mM NaCl, 5 mM EDTA, 2 mM DTT, 0.5 mM PMSF and 1 mg/ml leupeptin. Cells were lysed by strokes in a chilled homogenizer at 4°C. The lysates were centrifuged at $1,000 \times g$ for 10 min to remove unbroken cells and nuclei. The cleared lysates were subject to ultracentrifugation at $100,000 \times g$ for fractionation. The pellet was dissolved using RIPA buffer (containing 0.05% SDS).

Luciferase reporter assay

Cells seeded in 12-well plates were co-transfected with 500 ng superTOP-FLASH, 50 ng pTK-*Renilla* (Promega) and effector plasmids. The final amount of DNA in all transfections was brought to a total of 1,000-1,500 ng with

empty vectors. Assays were performed in accordance with the dual luciferase assay protocols (Promega) using the luminometer. The *Renilla* activity was used to normalize TOP-FLASH activity.

Axis duplication assay using Xenopus embryo

10 pg of Wnt8, Dsh and β-catenin mRNAs were co-injected with 1 ng of $G\beta_2$ and $G\gamma_2$ to ventral-vegetal one blastomere of 8 cell-stage embryos. Axis duplication was monitored at tailbud stage (stage 37), and embryos were classified as complete axis duplication, incomplete axis duplication, vestigial or normal. The phenotypes for the classification are followings: complete axis duplication, duplicated trunk and complete secondary head structures including duplicated cement gland and pair of eyes; incomplete axis duplication, duplicated trunk only or duplicated trunk and incomplete head structures; vestigial, no duplicated trunk but slight dorsalization.

Usage of siRNA

The sequences of siRNA for $G\beta_1$ and $G\beta_2$ were adapted from Krumins and Gilman (2006). The sequences of siRNAs; $siG\beta_1/G\beta_2$, 5'-UACGACGACUUCAACUGCATT-3'/5'-UGC-AGUUGAAGUCGUCGUATT-3'; siGFP, 5'-GUUCAGCGU-GUCCGGCGAGTT-3'/5'-CUCGCCGGACACGC-UGAACTT-3'. 200 nM siRNAs were transfected into HEK293T using calcium phosphate.

Supplemental data

Supplemental Data include four figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-41-10-02.pdf.

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