Supplemental Data

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

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Results

Gβ interacts with Axin

To confirm that the interaction between Axin and Gβ2 occurs in mammalian cells, Myc-tagged Axin and FLAG-tagged Gβ2 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 Gβ2. 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 Gβ5 which has the least sequence similarity to the other four isoforms Gβ1-4. Because Gβ forms a dimer with Gγ to make a functional unit (Birnbaumer, 2007), we tested whether the dimerization affects binding to Axin. When Gγ2, known to form dimers with Gβ1 or Gβ2 is co-expressed the interaction between Gβ2 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 Gβ2 (Figure 1C, 1D and Supplemental Data Figure S1B).

Gβ2γ2 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 Gβ2γ2 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 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 Gβ2γ2 (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 Gγ2, alone caused a significant reduction, co-transfection of Gβ2γ2 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 Gβ2γ2 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 Gβ2γ2 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 Gβ2 and Gβ2γ2, but not Gγ2 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 Gβ2 mRNA alone (but not by Gγ2 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 Gβ2γ2 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 Gβ2, Gγ2 and Gβ2γ2 (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 Gβ3 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 Gβ2γ2. Only the DEP domain including C-terminal domain was found to be necessary for Gβ2γ2-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 Gβ2γ2 (Figure 3C). We reasoned that ΔDEP-Dishevelled could not be down-regulated by Gβ2γ2 since it could not bind to Gβ2. To test this hypothesis we performed co-IP experiments and found that as expected ΔDEP-Dishevelled does not bind to Gβ2 (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 Gβ2γ2 (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 Gβ2γ2 assessed. Although the myr-ΔDEP-Dishevelled protein localized to the membrane, its level was not reduced by Gβ2γ2 (Figure 3E). Additionally, the robust luciferase-reporter activity induced by myr-γDEP-Dishevelled was not blocked by Gβ2γ2 (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 Gβ2γ2, 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 tion of PKCα from the cytoplasm to the plasma membrane (Sheldahl *et al*., 1999) (Supplemental Data Figure S4A) we used a mutant form Gβ2 (W332A) which is able to bind to Gγ but has reduced signaling activity (Ford *et al*., 1998) in order to determine whether signaling activity of Gβγ is needed. Gβ2 (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 Gβγ plays an important role in the downregulation of Dishevelled. Gβ2 (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 Gβ2 in co-IP experiments (Supplemental Data Figure S4D).

Having shown that activation of downstream signaling by Gβγ 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 Gβγ-mediated loss of Dishevelled (Figure 4C). Staurosporine, a PKC inhibitor, blocked whereas PMA, a PKC activator, increased the loss of Dishevelled by Gβγ (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 Gβγmediated loss of Dishevelled.

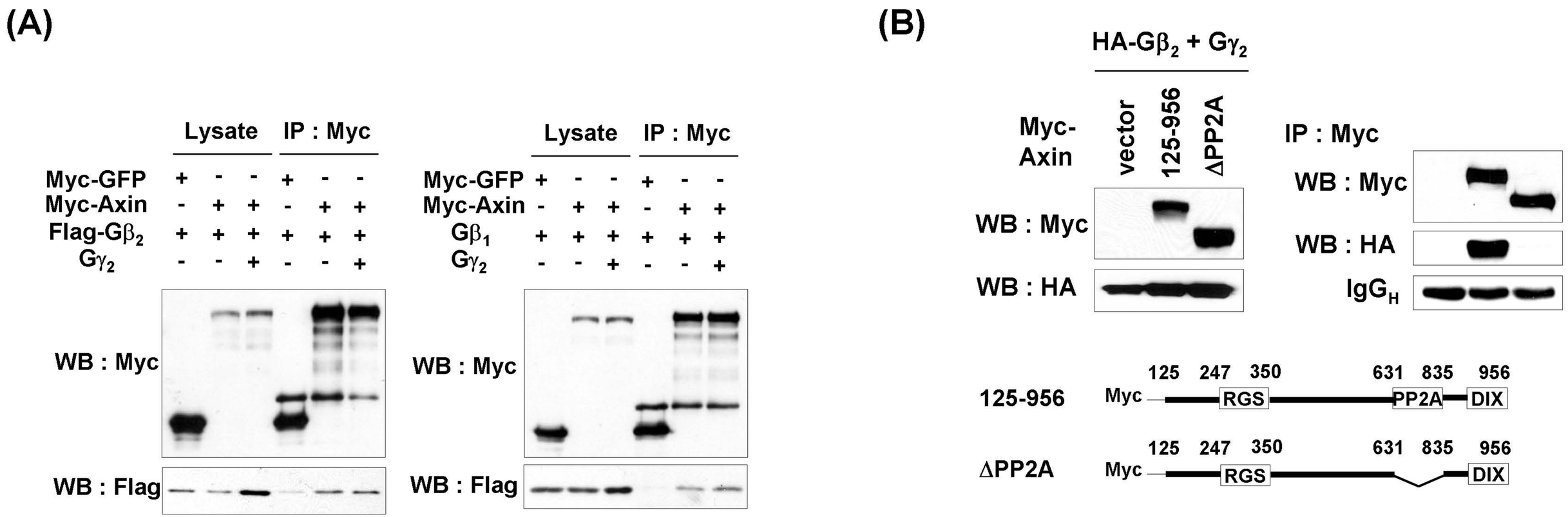
Axin blocks Gβγ-mediated loss of Dishevelled

Our initial finding that Axin binds Gβ2 (Figure 1) led to the hypothesis that interaction with Gβγ might control the loss of Dishevelled but left the role of 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).

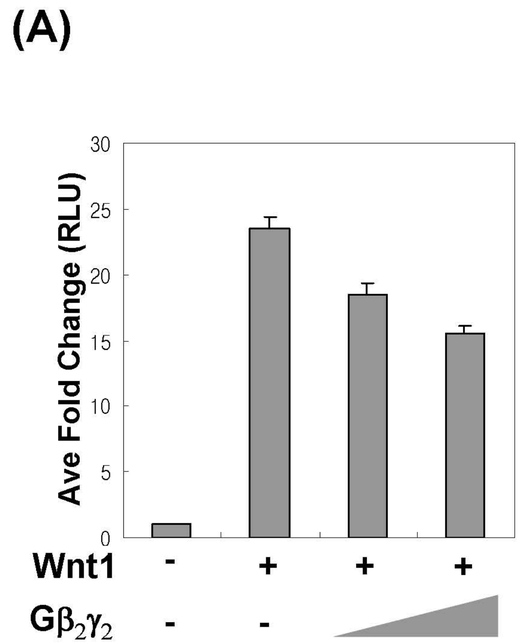
Activation of Frizzled signaling induces loss of Dishevelled

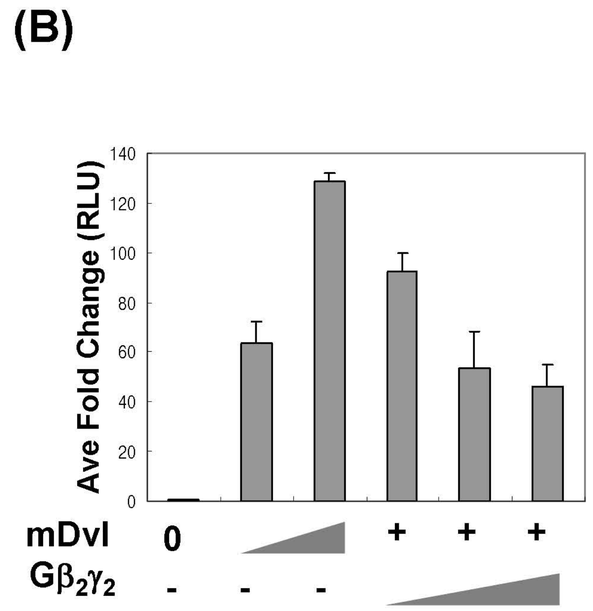
Because Frizzled is known to be a G-protein coupled receptor (Schulte and Bryja, 2007) and would be expected to increase the level of dissociated Gβγ from Gα in a more physiological manner, we examined whether activation of Frizzled signaling could induce loss of Dishevelled. To activate Frizzled signaling HEK293T cells were transfected with β2AR/RFz1 and β2AR/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 β2AR/RFz1 and β2AR/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 β2AR 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 β2AR/RFz1 and β2AR/RFz2, but not β2AR, 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, β2AR/RFz1 and β2AR/RFz2 can induce translocation of Dishevelled to the plasma membrane, but β2AR 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 β2AR/RFz1 and β2AR/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 β2AR/RFz1 and β2AR/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 β2AR/RFz1 or β2AR/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.

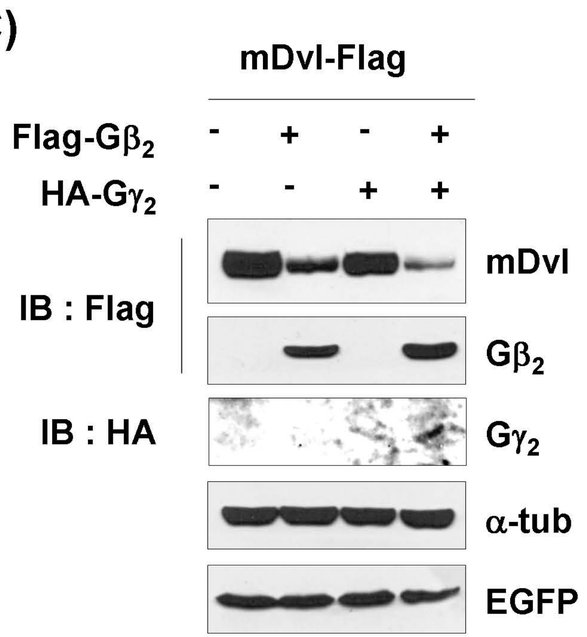
Supplementary Fig. 1.

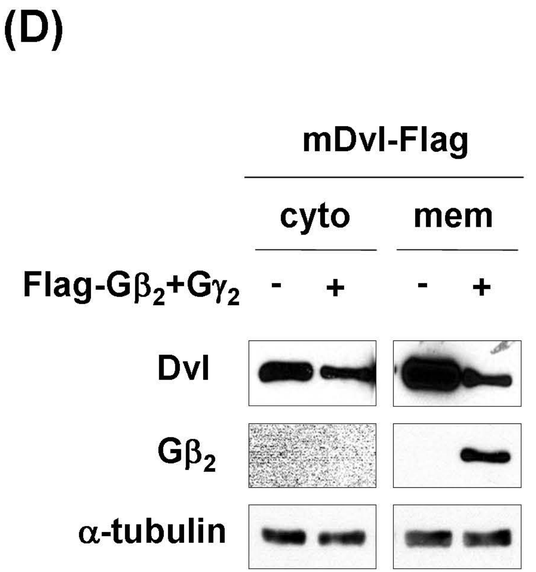


Supplementary Fig. 2.









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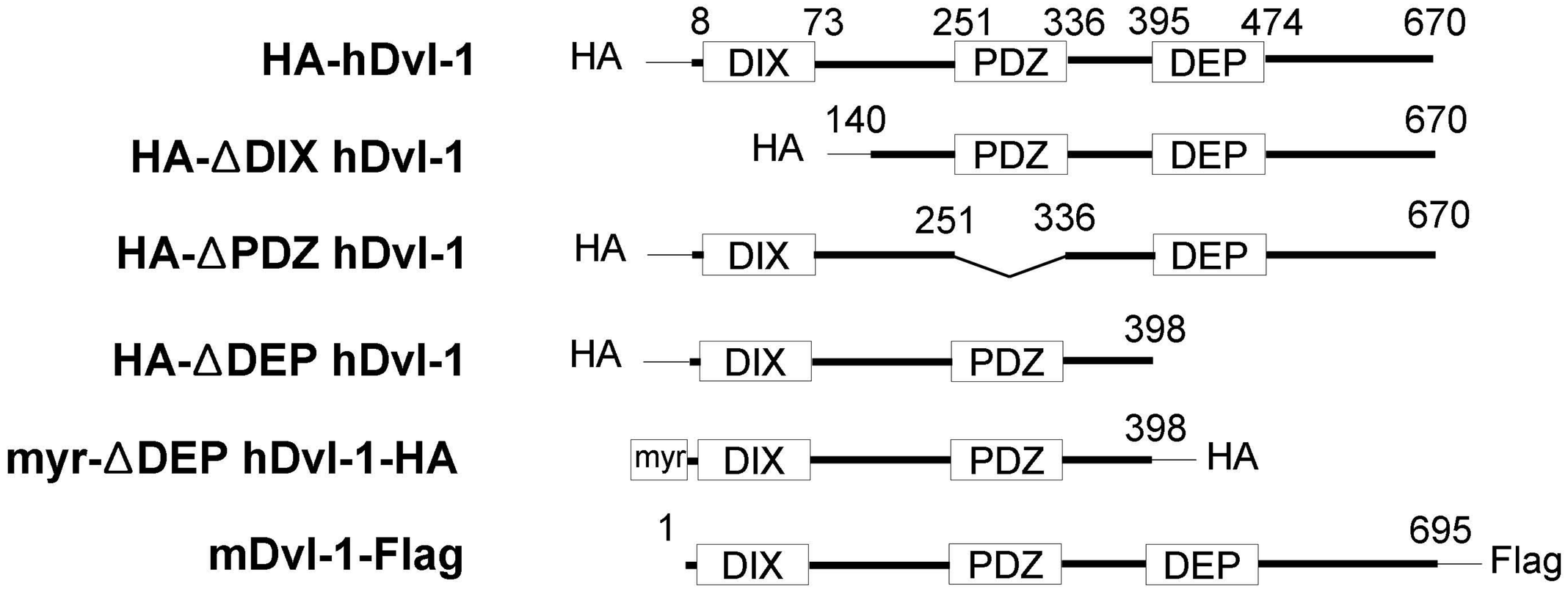
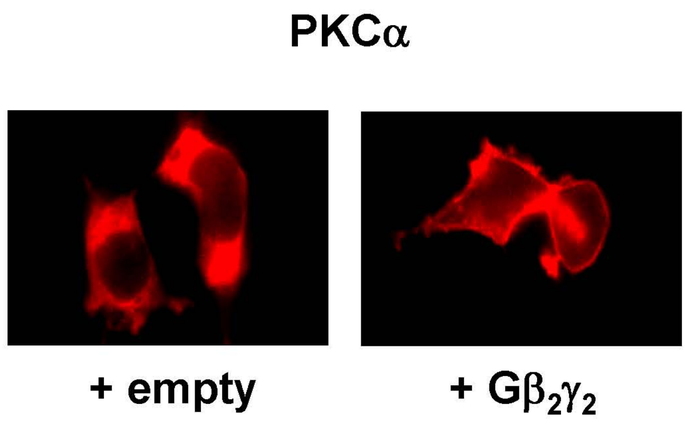
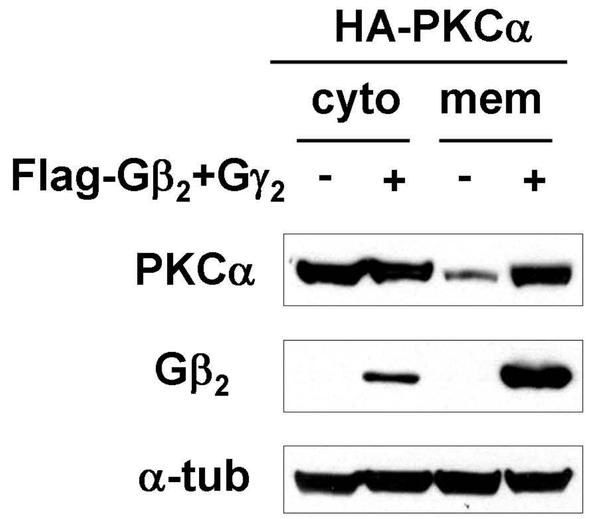


Supplementary Fig. 4.

(A)





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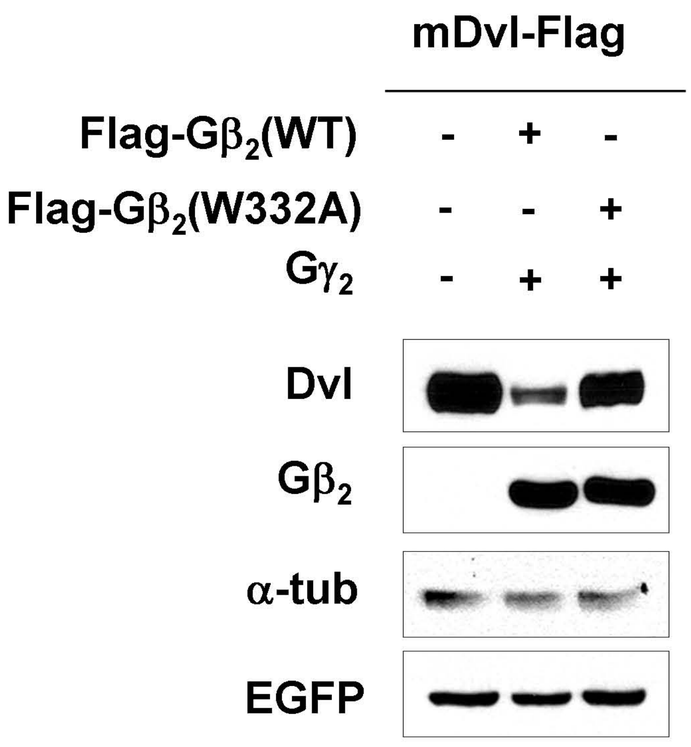

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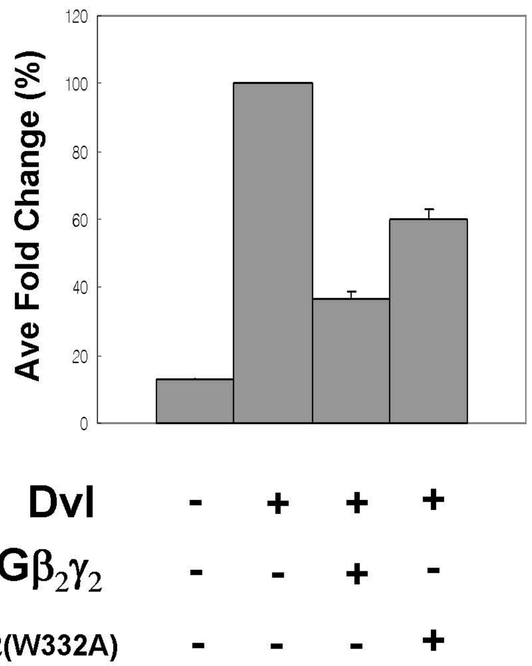

(D)