Article

A divergent canonical WNT-signaling pathway regulates microtubule dynamics: Dishevelled signals locally to stabilize microtubules

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Dishevelled (DVL) is associated with axonal microtubules and regulates microtubule stability through the inhibition of the serine/threonine kinase, glycogen synthase kinase 3β (GSK-3β). In the canonical WNT pathway, the negative regulator Axin forms a complex with β-catenin and GSK-3β, resulting in β-catenin degradation. Inhibition of GSK-3β by DVL increases β-catenin stability and TCF transcriptional activation. Here, we show that Axin associates with microtubules and unexpectedly stabilizes microtubules through DVL. In turn, DVL stabilizes microtubules by inhibiting GSK-3β through a transcription- and β-catenin–independent pathway. More importantly, axonal microtubules are stabilized after DVL localizes to axons. Increased microtubule stability is correlated with a decrease in GSK-3β–mediated phosphorylation of MAP-1B. We propose a model in which Axin, through DVL, stabilizes microtubules by inhibiting a pool of GSK-3β, resulting in local changes in the phosphorylation of cellular targets. Our data indicate a bifurcation in the so-called canonical WNT-signaling pathway to regulate microtubule stability.

Introduction

Microtubules are essential cytoskeleton components that regulate vesicle transport, directed cell migration, cell division, and cell polarity. Neurons are highly polarized cells in which microtubules are required for the formation and stability of axons and dendrites (Baas, 1999). Microtubules are highly dynamic polymers with a short half-life of minutes to seconds (for review see Desai and Mitchison, 1997). The dynamic instability of microtubules allows cells to adapt to rapid changes in the environment. Extensive analyses have led to the understanding that microtubule dynamics are regulated by intracellular components that directly interact with microtubules. However, relatively little is known about how extracellular stimuli regulate the microtubule cytoskeleton.

WNT factors are secreted proteins that regulate cell fate decisions, cell polarity, cell migration, axonal morphology, and synaptic differentiation (Lucas and Salinas, 1997; Wodarz and Nusse, 1998; Hall et al., 2000; Patapoutian and Reichardt, 2000; Huelsken and Birchmeier, 2001; Peifer and McEwen, 2002). Analyses in various developmental systems have shown that WNT proteins can signal through three pathways. In the canonical pathway, the binding of WNTs to their receptor Frizzled leads to activation of Dishevelled (DVL) and inhibition of the serine/threonine kinase, glycogen synthase kinase 3β (GSK-3β). As a consequence, β-catenin accumulates and translocates to the nucleus, where it activates TCF-mediated transcription (Arias et al., 1999; Huelsken and Birchmeier, 2001). In the planar polarity pathway, activation of DVL signals to the Jun-kinase pathway rather than to the GSK-3β pathway (Boutros et al., 1998). In the WNT-Ca^{2+} pathway, binding of WNTs to Frizzled receptors increases intracellular Ca^{2+} and activates PKC in a DVL-dependent manner (Sheldahl et al., 2003). More recently, DVL, PKC, and FAK have been found downstream of the DWNT-4/dFZ2 signaling pathway to regulate

Abbreviations used in this paper: ΔPDZ-DVL, mouse DVL in which the PDZ site has been deleted; DVL, Dishevelled; DVL-ER, mouse DVL fused with the estrogen receptor; GSK-3β, glycogen synthase kinase 3β; NB2a, neuroblastoma 2a.

cell motility (Cohen et al., 2002). Thus, WNTs can activate at least three different pathways through DVL, a cytoplasmic protein. DVL contains four domains: DIX, PDZ, PR (proline-rich), and DEP, which are required for signaling through different pathways (Penton et al., 2002). The DEP domain is required for planar polarity (Boutros et al., 1998). The PDZ and DEP domains are involved in the activation of Rac and Rho GTPases, respectively (Habas et al., 2003), whereas all domains are required for the canonical pathway (Yanagawa et al., 1995; Penton et al., 2002).

Several reports suggest that DVL functions as a scaffolding protein by bringing different intracellular components together to regulate intracellular signaling (Boutros and Mlodzik, 1999). In the absence of WNTs, a complex between Axin, APC, GSK-3β, and β-catenin causes the phosphorylation of β-catenin by GSK-3β and targets it for degradation (Behrens et al., 1998). Upon activation of the WNT pathway, DVL recruits FRAT-1 (a positive regulator of the pathway) to the Axin/APC/GSK-3β/β-catenin complex, causing its disassembly and therefore resulting in the accumulation and translocation of β-catenin to the nucleus (Li et al., 1999; Salic et al., 2000). Thus, DVL regulates nuclear events through inhibition of GSK-3β and changes in β-catenin levels.

Recent reports show that interactions between DVL and GSK-3β regulate the cytoskeleton. DVL is associated with the most stable pool of microtubules in neurons (Krylova et al., 2000). More importantly, DVL increases microtubule stability through inhibition of GSK-3β (Krylova et al., 2000). Here, we examine the mechanism by which DVL regulates microtubule dynamics. Unexpectedly, we found that endogenous Axin associates with microtubules and increases microtubule stability through DVL. In turn, DVL stabilizes microtubules through GSK-3β, but in a transcriptional-independent manner. Using a post-translational inducible system, we show that DVL functions locally to stabilize microtubules. Inhibition of GSK-3β by DVL or Axin results in changes in the phosphorylation of MAP-1B, a protein that regulates microtubule dynamics. Our findings that DVL acts locally by regulating signaling complexes within a cellular compartment have important implications for the role of DVL in cell polarity.

Results

DVL induces axonal remodeling and stabilizes microtubules in developing neurons

Previously, we have shown that WNTs induce axonal remodeling in developing neurons, a process characterized by increased growth cone size, increased axon diameter, and decreased axonal outgrowth (Lucas and Salinas, 1997; Hall et al., 2000). These changes in axonal morphology are associated with an increased number of stable microtubules at the axon shaft and an increased number of looped stable microtubules at enlarged growth cones (Hall et al., 2000). The axonal remodeling activity of WNTs is mediated through the inhibition of GSK-3β (Lucas and Salinas, 1997; Hall et al., 2000, 2002). Here, we examined whether DVL induces similar changes to those observed with WNTs. Indeed, we found that expression of DVL-1 in dif-
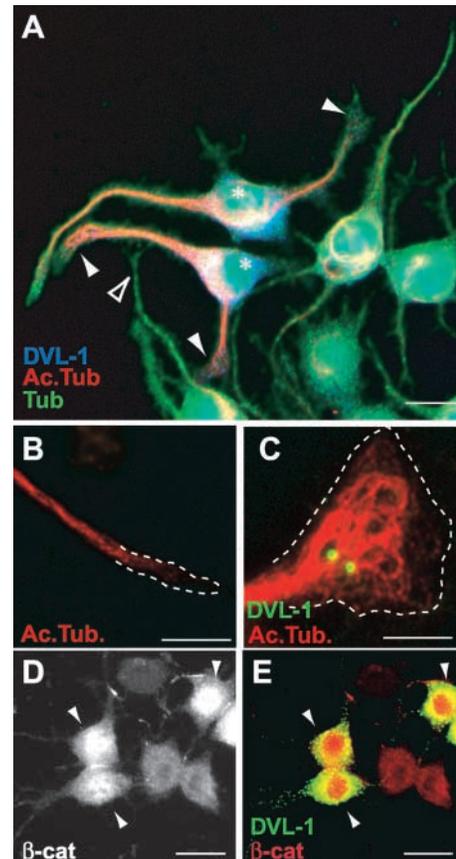
Figure 1. **DVL-1 mimics the axonal remodeling activity of WNTs.** (A) DVL-1 expression (asterisk) induces growth cone enlargement and increased axonal diameter, and decreases axon length (filled arrowheads) compared with untransfected differentiated neurons from NB2a cells (empty arrowhead). (B) Control neurons have thin axons with relatively small growth cones and few stable microtubules. (C) Expression of DVL-1 increases the number of stable microtubules in axons and induces looping and unbundling of stable microtubules at enlarged growth cones. (D and E) Expression of DVL-1 induces the translocation of endogenous β-catenin into the nucleus (arrowheads). Dashed lines show the shape of the growth cone. Bars: (A, D, and E) 15 μm, (B and C) 2 μm.

ferentiated neuroblastoma 2a (NB2a) neurons induces axonal remodeling (Fig. 1 A). Staining for total tubulin (β-tubulin) shows that expression of DVL-1 increases axon diameter and growth cone size when compared with control untransfected cells (Fig. 1 A). Staining for acetylated tubulin reveals the presence of very few stable microtubules in control growth cones (Fig. 1, A and B). In contrast, DVL-1 increases the number of stable microtubules along the axon and induces the formation of looped microtubules at the growth cone (Fig. 1, A and C). In addition, DVL-1 increases the level of endogenous β-catenin and induces its translocation to the nucleus (Fig. 1, D and E). Thus, expression of DVL-1 in NB2a neurons mimics the effect of WNTs in developing axons. These results suggest that the axonal remodeling activity of WNTs is accomplished through activation of the WNT canonical pathway. However, it remains to be established whether DVL stabilizes microtubules through β-catenin and nuclear events or through direct signaling to microtubules.
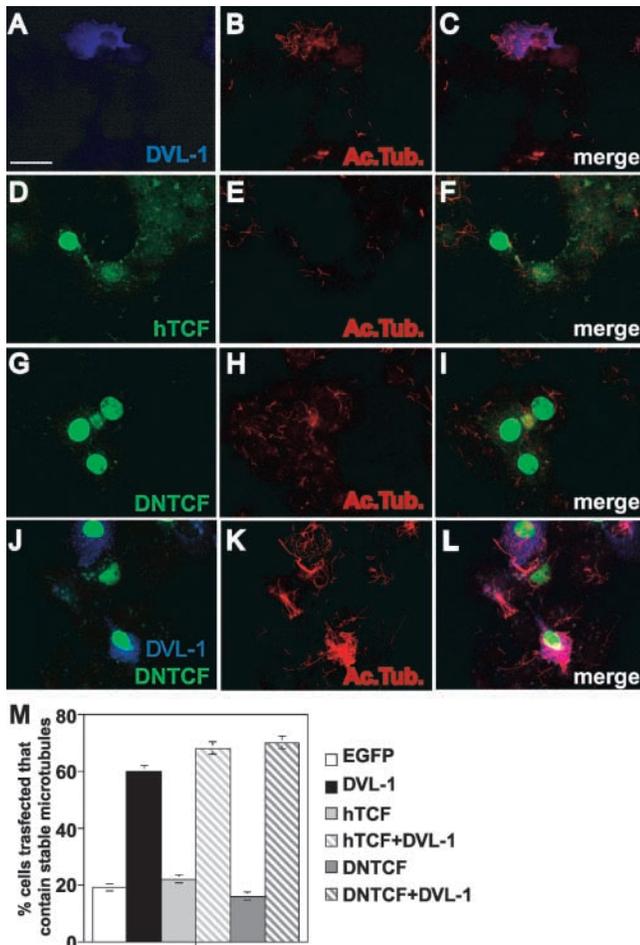
Figure 2. **TCF is not required for DVL microtubule-stabilizing function.** (A–C) Expression of DVL-1 protects microtubule against nocodazole. (D–I) Neither hTCF nor ΔNTCF protect microtubules against nocodazole. (J and L) Expression of ΔNTCF in DVL-expressing cells does not affect the ability of DVL to stabilize microtubules against nocodazole. Bar, 50 μm. (M) Graph shows the percentage of cells containing microtubules after nocodazole treatment. Values are mean ± SEM from three different experiments (at least 30 neurons were counted in each experiment).

TCF factors are not required for DVL microtubule stabilizing function

In the canonical WNT pathway, inhibition of GSK-3β by DVL results in the elevation of β-catenin and subsequent transcriptional activation through β-catenin binding to LEF/TCF transcription factors (Wodarz and Nusse, 1998). To establish whether the microtubule-stabilizing function of DVL is accomplished through this pathway, we examined the effect of TCF-4 on microtubule stability. To assess microtubule stability, we treated COS-7 cells with nocodazole, a microtubule-depolymerizing drug (Hoebeke et al., 1976) that has been used to determine the function of microtubule-stabilizing proteins (Bosc et al., 1996; Hergovich et al., 2003). As we previously observed, DVL-1 is able to stabilize microtubules against nocodazole in COS cells (Krylova et al., 2000; Fig. 2, A–C). In contrast, expression of full-length hTCF does not protect microtubules against nocodazole (Fig. 2, D–F). Next, we tested the effect of a dominant-negative hTCF-4 (ΔNTCF-4), carrying a deletion of the

β-catenin binding site that has been shown to block transcriptional activation by several TCF factors (Roose et al., 1999). Expression of ΔNTCF-4 does not protect microtubules against nocodazole (Fig. 2, G–I). More importantly, cells expressing both ΔNTCF-4 and DVL-1 contain a large number of stable microtubules after nocodazole, similar to cells expressing DVL-1 only (Fig. 2, J–L). Quantification indicates that ΔNTCF-4 does not block DVL-1 function on microtubules (Fig. 2 M). These results show that DVL-1 regulates microtubules through a TCF-independent mechanism.

An immediate effect of the activation of DVL by WNTs is the elevation of β-catenin and its translocation to the nucleus (Cadigan and Nusse, 1997). Indeed, expression of DVL-1 in differentiating neurons increases endogenous β-catenin levels and its translocation to the nucleus (Fig. 1, D and E). Interestingly, β-catenin has been implicated in the regulation of the actin cytoskeleton (Fukata and Kaibuchi, 2001; Gottardi and Gumbiner, 2001) and microtubule organization through interactions with dynein or APC (Ligon et al., 2001; McCartney et al., 2001). Thus, it is possible that the microtubule-stabilizing function of DVL could be mediated through β-catenin. To test this notion, we examined the effect of β-catenin on microtubule stability in COS cells. Cells expressing β-catenin lose their microtubules after nocodazole treatment (Fig. 3, A–C), even though expression of β-catenin increases TCF-mediated transcription as determine by luciferase assays (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200309096/DC1). In addition, expression of a constitutive active form of β-catenin (CA β-catenin) does not protect the cells against nocodazole (unpublished data). Next, we examined the effect of a dominant-negative β-catenin mutant (ARM β-catenin), carrying a deletion of the transactivation domain required for binding to TCF/LEF factors (Zhurinsky et al., 2000). We found that ARM β-catenin does not affect the ability of DVL-1 to stabilize microtubules (Fig. 3, D–G). Quantification of these experiments shows that β-catenin does not stabilize microtubules and that neither β-catenin nor ARM β-catenin affect DVL microtubule-stabilizing function (Fig. 1 H). These findings demonstrate that β-catenin is not required for DVL function on microtubule stability.

DVL stabilizes microtubules through a transcription-independent mechanism

Our results described above suggest that DVL stabilizes microtubules through a β-catenin– and TCF-independent mechanism. However, DVL could still signal to the nucleus through a novel pathway. To test this possibility, we used a post-translational inducible system to express DVL in cells after blocking all transcriptional activity with actinomycin D. DVL-2 was fused to the estrogen receptor ligand-binding site (DVL-ER) such that the fused protein becomes active upon β-estradiol treatment. DVL-ER protein is translated, but is not functional in the absence of β-estradiol as assayed by β-catenin stabilization. In the presence of β-estradiol, DVL-ER activates TCF-mediated transcription like full-length DVL (unpublished data). Using this inducible system, we tested in COS-7 cells whether transcriptional activity is required for DVL to stabilize microtubules. After 1 h actinomycin D treatment, cells were then treated with control or β-estradiol

Figure 3.   **β-catenin is dispensable for DVL-stabilizing function.** (A–C) Expression of wild-type β-catenin does not stabilize microtubules against nocodazole. (D–G) Mutant β-catenin (ARM β-cat) lacking the transactivation domain does not interfere with the ability of DVL to stabilize microtubules. Bar, 50 μm. (H) Graph shows the percentage of cells containing microtubules after nocodazole treatment. In the first three columns, the error bars are too small to be seen. Values are mean ± SEM from three different experiments (at least 70 neurons were counted in each experiment).
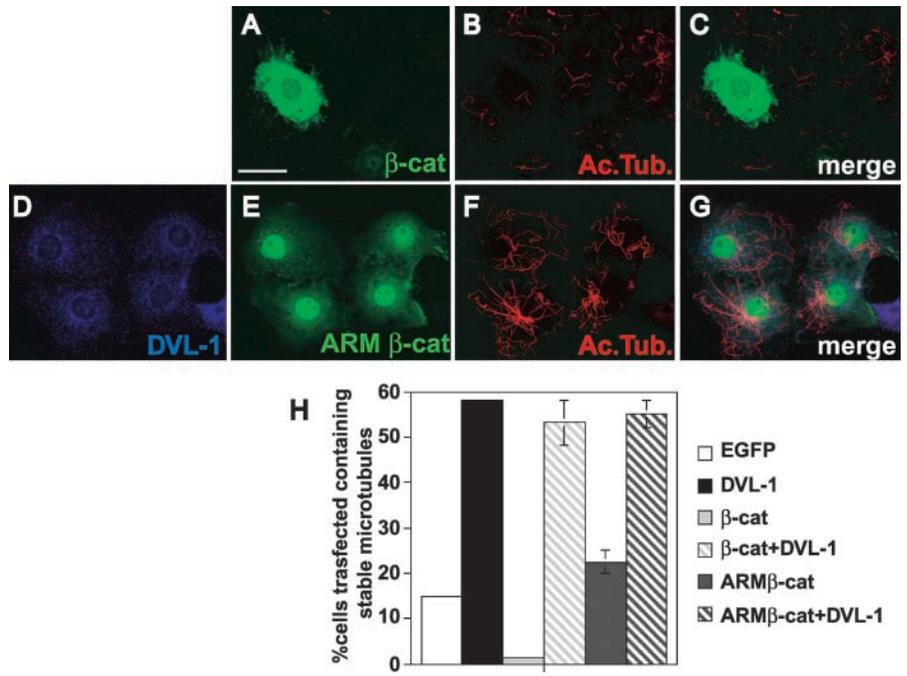
containing media in the continued presence of actinomycin D for a further 3 h. Under these conditions, expression of EGFP, used as control, is completely abolished (unpublished data). To determine the effect of DVL on microtubules, nocodazole was added in the last hour of the culture period (Fig. 4 A). In the absence of β-estradiol, the DVL-ER protein is uniformly distributed in the cytoplasm, and microtubules are not present after depolymerization with nocodazole (Fig. 4, B–D). Upon induction with β-estradiol, DVL adopts a punctate distribution in the cytoplasm that coincides with its ability to signal to the nucleus as assayed by β-catenin stabilization (unpublished data). More impor-

tantly, microtubules are preserved after nocodazole treatment in DVL-ER–expressing cells even in the presence of actinomycin D (Fig. 4, E–G). These results are consistent with our findings that TCF factors do not affect DVL function on microtubules, and demonstrate that transcription is not required for DVL function on microtubule dynamics.

Localization of DVL to axons coincides with its ability to stabilize axonal microtubules

The finding that DVL stabilizes microtubules in a transcriptional-independent manner, as well the observation of its association with microtubules, suggests the possibility that DVL

Figure 4.   **The microtubule-stabilizing function of DVL does not require transcriptional activity.** (A) Schematic representation of the experimental design. (B–D) Cells expressing DVL-ER in the absence of β-estradiol do not have stable microtubules when treated with nocodazole and actinomycin D. (E–G) After β-estradiol, DVL-ER–expressing cells contain nocodazole-resistant microtubules, even in the presence of actinomycin D. Bar, 50 μm.

Figure 5. **Local effect of DVL microtubule-stabilizing function.** (A) In the absence of nocodazole and β-estradiol, DVL-ER is evenly distributed in NB2a differentiated neurons. (B) A significant amount of DVL-ER has a punctate distribution in the cell body after 1 h β-estradiol induction. (C) DVL-ER is present at significant levels along the axon after 5 h induction with β-estradiol. (D and D′) In the presence of nocodazole but in the absence of β-estradiol, neurons lose their microtubules. (E and E′) After 1 h of β-estradiol, microtubules are stabilized mainly in the cell body after nocodazole. (F and F′) After 5 h of β-estradiol induction, a significant number of neurons contain axonal microtubules after nocodazole treatment, coinciding with the presence of DVL along the axon (arrowheads) Bar, 15 μm. (G) Quantification of the percentage of DVL-expressing cells that have stable microtubules in the cell body and axon under different periods of β-estradiol treatment. (H) Graph shows the correlation between the localization of DVL-ER in axons and the presence of stable microtubule along the axons. Values are mean ± SEM from three experiments (at least 70 neurons were counted in each experiment).

could locally signal to microtubules. To address this issue, we took advantage of the polarity of neurons. We used differentiated NB2a neurons expressing DVL-ER where its relocalization and ability to stabilize axonal microtubules can be examined over different time periods. DVL-ER is evenly distributed along the cell body and axons in the absence of β-estradiol (Fig. 5 A). After 15 min of β-estradiol induction, DVL-ER adopts a punctate distribution (unpublished data) similar to that observed with endogenous DVL-1 (Krylova et al., 2000). After 1 h of β-estradiol treatment, a significant increase in punctate DVL-ER is observed in the cell body of most cells (Fig. 5 B), although DVL-ER is also found in some axons (unpublished data). After 5 h of β-estradiol treatment, a significant increase in DVL-ER levels is observed along the axon of all neurons (Fig. 5 C). Next, we examined the ability of DVL to stabilize axonal microtubules in the presence of nocodazole. In the absence of β-estradiol, DVL-ER does not stabilize microtubules compared with control cells (Fig. 5, D and D′). After 1 h of β-estradiol treatment, stable microtubules are mainly found in the cell body of DVL-ER–expressing cells (Fig. 5, E and E′). In contrast, stable microtubules are present along the axon in the majority of the DVL-ER–expressing cells after 5 h of β-estradiol treatment (Fig. 5, F and F′). Next, we measured the percentage of DVL-ER–expressing cells that contain stable microtubules in the cell body and along the axon. In the absence of β-estradiol, 26% of cells have stable microtubules in the cell body, whereas 12% have

them along the axon (Fig. 5 H). These results are similar to control untransfected cells (unpublished data). After 1 or 5 h β-estradiol, the number of neurons with stable microtubules in the cell body increases to 67 and 88%, respectively (Fig. 5 G). In contrast, the number of neurons with stable microtubules in axons increases to 41 and 70%, respectively (Fig. 5 G). These findings show that DVL microtubule-stabilizing function is proportional to the levels of functional DVL. How many cells containing stable microtubules along the axon also have DVL in the axon? At both time points, a 100% correlation is observed between DVL localization in axons and its ability to stabilize axonal microtubules (Fig. 5 H). These results strongly suggest that stabilization of microtubules occurs in the cellular compartment in which DVL is localized.

DVL stabilizes microtubules through inhibition of GSK-3 in a FRAT-independent manner

Next, we examined the role of GSK-3β in DVL function in neurons. We found that treatment of differentiated NB2a neurons with lithium, a direct GSK-3 inhibitor, mimics the effect of DVL on microtubule stability. In the presence of 20 mM lithium, a significant number of nocodazole-treated neurons contain stable microtubules in the cell body and axons compared with control NaCl-treated neurons (Fig. 6, A–C). These results, together with our previous work (Krylova et al., 2000), strongly suggest that DVL-1 stabilizes axonal microtubules through inhibition of GSK-3. This inhibition could be

Figure 6. **DVL stabilizes microtubule via inhibition of GSK-3β without the requirement of FRAT.** (A and B) NB2a neurons treated with lithium show a significant increase in the number of stable microtubules after nocodazole treatment when compared with control NaCl-treated neurons. (C) Graph shows the percentage of neurons with stable microtubules after nocodazole treatment. Note the proportional increase in microtubule stability with increased concentrations of lithium. (D–F) Full-length FRAT does not protect microtubules against nocodazole. (G–J) Expression of ΔCFRAT, lacking the GSK-3β binding domain, does not affect the ability of DVL-1 to protect microtubules against nocodazole (arrows). Bars, 15 μm.
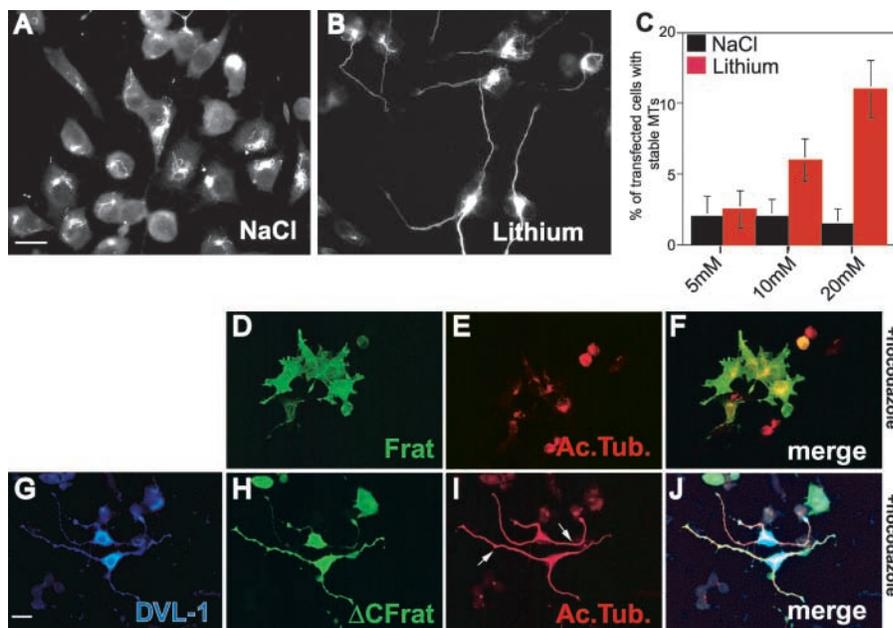
accomplished by increasing serine-9 phosphorylation in GSK-3, as has been demonstrated in the insulin pathway (Cohen and Frame, 2001). However, we found that expression of DVL-1 in NB2a neurons does not affect the levels of serine-9 phosphorylation (unpublished data). These findings are in agreement with previous reports on the effect of WNTs on GSK-3β phosphorylation (Andersen and Bi, 2000).

In the canonical WNT-signaling pathway, the interaction of DVL with GSK-3β is mediated by FRAT/GBP, a positive regulator (Yost et al., 1998; Li et al., 1999). To begin to address the mechanism by which DVL interacts with GSK-3β to regulate microtubule stability, we tested whether DVL requires FRAT to inhibit GSK-3β. FRAT-expressing neurons lose their microtubules in the presence of nocodazole like untransfected cells (Fig. 6, D–F), even though FRAT increases TCF-mediated transcription in these neurons and COS-7 cells as determined by TOP-FLASH luciferase assay (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200309096/DC1). In addition, FRAT increases the level of β-catenin in NB2a cells (Fig. S1 B). Next, we examined whether expression of ΔCFRAT, carrying a deletion of the GSK-3β binding site (Franca-Koh et al., 2002), could interfere with the interaction of DVL with GSK-3β. We found that ΔCFRAT does not block the ability of DVL to stabilize microtubules (Fig. 6, G–J). We also found that ΔNFRAT, a mutant FRAT carrying a deletion of the DVL binding site, does not affect the microtubule-stabilizing function of DVL (unpublished data). Quantification of these experiments shows that 60% of cells expressing both DVL and ΔNFRAT or ΔCFRAT maintain their microtubules after nocodazole treatment (unpublished data). These findings suggest that FRAT does not mediate the interaction between DVL and GSK-3β to regulate microtubule stability.

Axin binds microtubules and regulates microtubule stability through DVL

Axin is a negative regulator of the canonical WNT-signaling pathway (Zeng et al., 1997). It is believed that Axin is a scaf-fold protein that brings together GSK-3β with APC and β-catenin to form a "destruction complex" that leads to the phosphorylation of β-catenin and its subsequent degradation (Huelsken and Birchmeier, 2001). We wish to test whether Axin is also a negative regulator for DVL microtubule-stabilizing function. First, we examined whether Axin is associated with microtubules. Using specific antibodies against Axin, we found that endogenous Axin is associated with microtubules isolated from rat brain (Fig. 7 A). Brain microtubules were obtained after one and two cycles of depolymerization and repolymerization (P1 and P2). Endogenous Axin was found in both microtubule pellets (Fig. 7 A). Moreover, endogenous Axin colocalizes with microtubules along the axon and at the growth cone in cerebellar granule neurons in which only proteins associated with the cytoskeleton have preserved (Fig. 7 B). These findings demonstrate that endogenous Axin is tightly associated with microtubules.

As DVL is tightly associated with microtubules, its ability to stabilize microtubules could be accomplished by regulating the level of GSK-3β and/or Axin on microtubules. For these experiments, we used microtubules from COS cells transfected with DVL. We found that expression of DVL in COS cells does not affect the endogenous level of Axin or GSK-3β associated with microtubules (Fig. 7 C).

To test a possible function of Axin on microtubules, we expressed Axin in neurons from differentiated NB2a cells. After nocodazole treatment, a significant number of Axin-expressing cells retain their microtubules when compared with control EGFP-transfected neurons (Fig. 7, D and E). Expression of Axin protects microtubules to the same extent as DVL (Fig. 7, F and I). These unexpected results indicate that Axin, like DVL, stabilizes microtubules. Next, we examined whether Axin function depends on DVL. To test this notion, we expressed mouse DVL in which the PDZ site has been deleted (ΔPDZ-DVL), which has a very low level of microtubule-stabilizing function (Krylova et al., 2000). We found that most neurons lose stable microtubules after nocodazole when both Axin and ΔPDZ-DVL were expressed (Fig. 7 G).
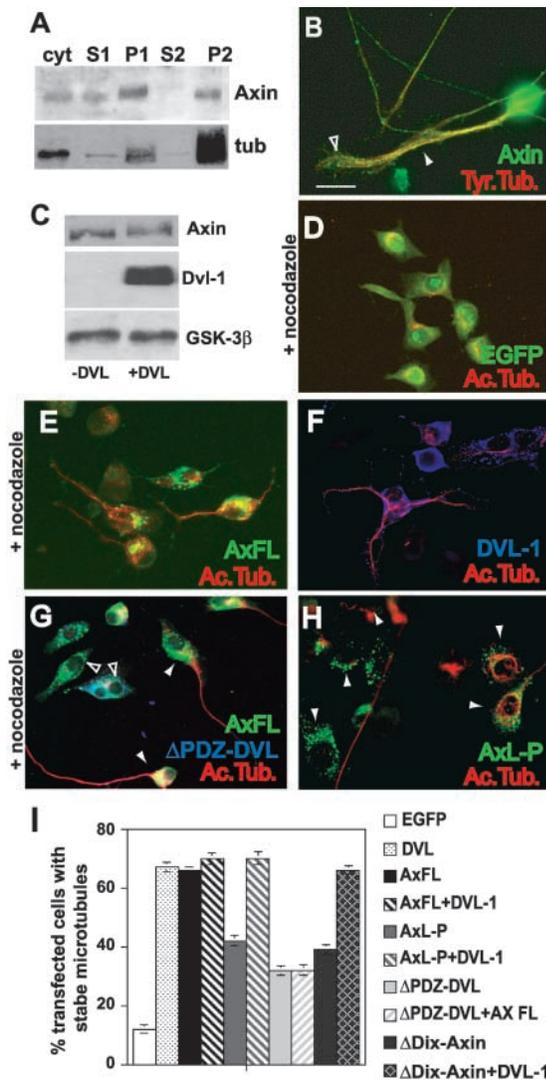
Figure 7. **Axin associates with microtubules and stabilizes microtubules through DVL.** (A) Endogenous Axin comes down with brain microtubule pellets obtained through one and two rounds of polymerization and repolymerization. (B) Endogenous Axin is associated with microtubules along the axon (filled arrowhead) and at growth cones (empty arrowhead) of cerebellar granule cell neurons. (C) Expression of DVL in COS7 cells does not change the level of endogenous Axin or GSK-3β associated with microtubules. (D–F) Expression of DVL or Axin in NB2a neurons protects microtubules from nocodazole. (G) Neurons expressing both Axin and ΔPDZ-DVL lose their microtubules after nocodazole treatment (open arrowheads), whereas cells expressing Axin alone (filled arrowheads) retain their microtubules. (H) Fewer cells expressing AxinL-P carrying a mutation in the GSK-3β binding site exhibit microtubule stability (arrowheads). Bar, 10 μm. (I) Graph shows that DVL and Axin have similar levels of microtubule stabilizing function. Expression of both DVL and Axin do not have an additive effect. AxL-P and ΔDIX-Axin exhibit a lower microtubule-stabilizing function when compared with wild-type Axin. Full-length DVL rescues both mutant forms of Axin. In contrast, ΔPDZ-DVL has a low level of microtubule stabilizing function when compared with full length DVL. Expression of Axin was unable to rescue microtubule stability in cells expressing ΔPDZ-DVL. Values are mean ± SEM.

Quantification reveals that the percentage of neurons with stable microtubules is similar to those obtained with ΔPDZ-DVL alone, indicating that ΔPDZ-DVL blocks Axin func-

tion (Fig. 7, G and I). We also tested the effect of Axin mutant lacking the DIX domain required for binding to DVL and to Axin itself. We found that ΔDIX-Axin (mouse Axin in which the DIX domain has been deleted) confers low level of microtubule stability and this effect is rescued by expression of full length DVL (Fig. 7 I). These results suggest that Axin requires DVL to stabilize microtubules.

Axin directly interacts with GSK-3β (Ikeda et al., 1998; Sakanaka et al., 1998; Ferkey and Kimelman, 2002). Therefore, Axin could regulate microtubule stability through GSK-3β. To test this, we expressed an allele of Axin carrying a mutation in the GSK-3β–binding domain (AxL-P) in NB2a neurons. AxL-P confers low levels of microtubule stability when compared with full-length DVL or Axin (Fig. 7, H and I). Interestingly, DVL fully restores microtubule stability in cells expressing AxL-P (Fig. 7 H). Together with the results obtained with ΔPDZ-DVL, these findings strongly suggest that Axin requires DVL to stabilize microtubules.

Axin and DVL-1 decrease the phosphorylation of MAP-1B, a microtubule-associated protein that controls microtubule dynamics

Next, we examined whether DVL was able to stabilize microtubules in primary neurons. Cerebellar granule cell neurons transfected with EGFP do not contain stable microtubules after treatment with nocodazole, although some neurons have some stable microtubules in the cell body and proximal regions of the axon (Fig. 8, A–C). In contrast, granule cell neurons expressing DVL-1 maintain their stable microtubules in the cell body and along the entire axon after nocodazole treatment. (Fig. 8, D–F). Together with our experiments in COS and NB2a cells, these results demonstrate that DVL stabilizes microtubules in dividing cells and in primary neurons.

DVL could stabilize microtubules by regulating the phosphorylation of microtubule-binding proteins. GSK-3β, a serine/threonine kinase, directly phosphorylates proteins such as MAP-1B, Tau, and kinesin, which regulate microtubule function (Hanger et al., 1992; Lucas et al., 1998; Morfini et al., 2002). Interestingly, recent works have shown that phosphorylation of MAP-1B by GSK-3β is required to maintain microtubules in a more dynamic state (Goold et al., 1999). Therefore, we examined the effect of DVL on MAP-1B phosphorylation as a mechanism to regulate microtubule stability and also as a read-out for GSK-3β activity. We found that in control untransfected or EGFP-expressing neurons, phosphorylated MAP-1B is found along axons and at growth cones (Fig. 8, G–I). In contrast, expression of DVL-1 decreases the level of phosphorylated MAP-1B in the axon (Fig. 8, J–L). Expression of Axin in cerebellar granule neurons also decreases the level of MAP-1B phosphorylation as observed with DVL-1 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200309096/DC1). These results suggest that DVL-1 and Axin increase microtubule stability, at least in part, by modulating MAP-1B phosphorylation.

Discussion

DVL has been proposed to function as a scaffold protein to bring signaling components together. After activation of

Figure 8. **DVL stabilizes microtubules in primary neurons and down-regulates the level of GSK-3β–mediated phosphorylation of MAP-1B in developing axons.** (A–F) Cerebellar granule cell neurons expressing DVL-1 contain stable microtubules (filled arrowheads), whereas EGFP-expressing control neurons lose their microtubules after nocodazole treatment (open arrowheads). (G–I) Cerebellar granule cell neurons expressing EGFP exhibit a significant level of GSK-3β–phosphorylated MAP-1B-P along the axon with similar levels to untransfected neurons (open arrowheads). (J–L) Expression of DVL-1 in cerebellar granule cell neurons decreases the level of GSK-3β–phosphorylated MAP-1B-P along the axon (filled arrowheads). Bar, 10 μm.
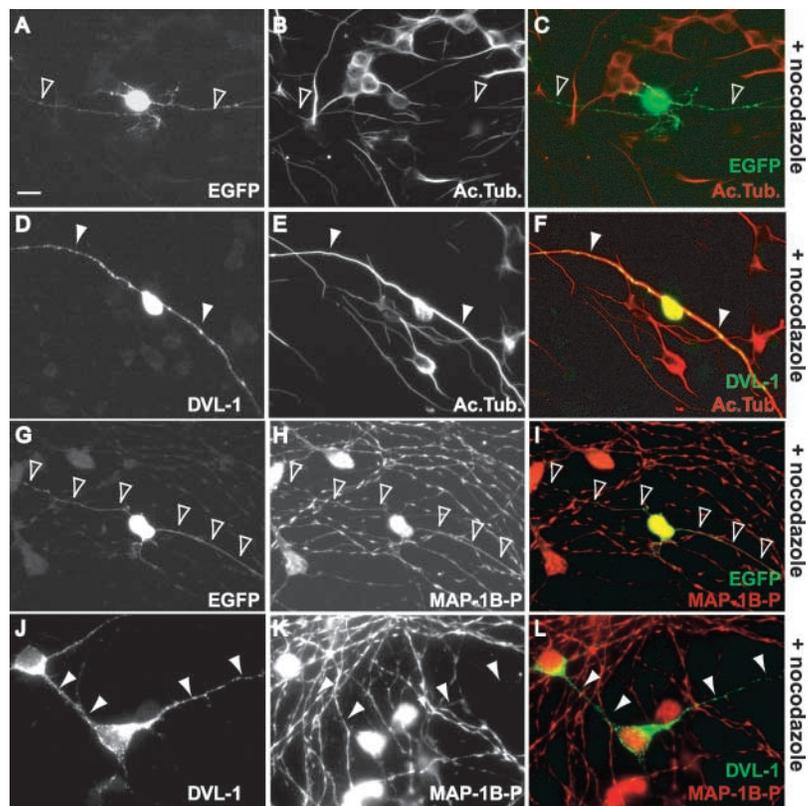
the canonical WNT pathway, DVL forms a complex with Axin, APC, GSK-3β, and β-catenin (Huelsken and Birchmeier, 2001). In this complex, DVL interacts with GSK-3β through FRAT, resulting in the inhibition of GSK-3β and in the dissociation of the complex. As a consequence, β-catenin is no longer phosphorylated by GSK-3β and accumulates in the cytoplasm. Subsequently, β-catenin translocates to the nucleus where it activates transcription by forming complexes with TCF/LEF transcription factors (Wodarz and Nusse, 1998; Huelsken and Birchmeier, 2001). Thus, the main function of DVL in the canonical WNT pathway is to increase β-catenin stability and to promote TCF-mediated transcriptional activation (Wodarz and Nusse, 1998; Huelsken and Birchmeier, 2001). However, the finding that endogenous DVL is tightly associated to microtubules and increases microtubule stability (Krylova et al., 2000) suggested that DVL-1 might not just function as a scaffold protein to regulate signaling to the nucleus.

Although DVL increases the levels of cytoplasmic and nuclear β-catenin, we showed that β-catenin is not required for DVL function on microtubule stability. First, β-catenin does not mimic the effect of DVL on microtubules. Interestingly, β-catenin decreases the percentage of cells with stable microtubules when compared with control EGFP-expressing cells, possibly by sequestering a pool of DVL from microtubules. Second, a mutant ARM β-catenin, carrying a deletion of the domain required for binding to TCF/LEF factors, does not block the ability of DVL to stabilize microtubules. These results indicate that DVL-1 stabilizes microtubules through a β-catenin–independent pathway.

DVL increases TCF-mediated transcription while increasing microtubule stability in the same cells. However, experiments using dominant-negative TCF and actinomycin D show that DVL does not require TCF function or transcriptional activity to signal to the microtubules. Interestingly, DVL signals to microtubules through the GSK-3β pathway. Three experiments support this notion. First, expression of wild-type GSK-3β blocks DVL function on microtubules (Krylova et al., 2000). Second, lithium, an inhibitor of GSK-3β, mimics the effect of DVL in neurons. Third, the PDZ domain of DVL, implicated in signaling through GSK-3β in the canonical WNT pathway, is required for microtubule stability (Krylova et al., 2000). These findings indicate that DVL stabilizes microtubules through a novel pathway that bifurcates downstream of GSK-3β.

Axin, a scaffold protein that functions as a negative regulator of the canonical WNT-signaling pathway, is involved in the formation of a destruction complex together with APC, GSK-3β, and β-catenin (Li et al., 1999; Huelsken and Birchmeier, 2001). Surprisingly, we found that endogenous Axin is associated with microtubules in cell lines and in brain. More importantly, Axin increases microtubule stability in diving cells (unpublished data) and in neurons. This novel function of Axin requires its GSK-3β–binding domain, suggesting that Axin stabilizes microtubules through the GSK-3β pathway. More importantly, epistatic analyses reveal that Axin requires DVL to stabilize microtubules. Axin mutants with a low stabilizing activity can be rescued by expression of full-length DVL. Conversely, expression of the mutant ΔPDZ-DVL blocks the effect of full-length Axin. These findings suggest that DVL functions downstream of Axin to regulate microtubule stability. It is intriguing that Axin functions as a negative regulator of β-catenin stability and TCF-mediated transcription (Zeng et al., 1997;

Sakanaka et al., 1998) while acting positively to regulate microtubule stability. A possible explanation for this apparent discrepancy is that Axin brings GSK-3β in close proximity to its targets or other signaling molecules. In the WNT/β-catenin canonical pathway, Axin brings together GSK-3β and β-catenin, therefore inducing the degradation of β-catenin. In contrast, on microtubules, Axin could increase the chance for DVL to interact with GSK-3β, resulting in GSK-3β inhibition. Future analyses may provide clues to the apparent differences in Axin function.

How does DVL inhibit GSK-3β to regulate microtubule stability? In the canonical WNT pathway, the interaction of DVL with GSK-3β is mediated by FRAT/GBP (Yost et al., 1998; Li et al., 1999). It has been proposed that DVL recruits FRAT to the complex formed between Axin, APC, GSK-3β, and β-catenin, resulting in the inhibition of GSK-3β–mediated phosphorylation of β-catenin and Axin (Thomas et al., 1999; Salic et al., 2000). In addition, recent reports show that FRAT regulates nuclear export of GSK-3β (Franca-Koh et al., 2002), suggesting that FRAT mediates nuclear events in the canonical WNT-signaling pathway. Consistent with this idea, we found that FRAT does not mediate the interaction between DVL with GSK-3β to signal to microtubules. How DVL interacts with GSK-3β to regulate microtubules remains to be established.

Localized DVL regulates microtubule stability in axons. In developing primary neurons, endogenous DVL is localized to axonal microtubules and is associated with the most stable pool of microtubules (Krylova et al., 2000). The ability of DVL to bind to microtubules suggests that DVL could locally regulate cytoskeletal changes. Using the estrogen receptor post-translational inducible system, we found that after a short period of activation with β-estradiol, DVL stabilizes microtubules within the neuronal cell body. In contrast, longer periods of activation are required to stabilize axonal microtubules. One interpretation of these results is that a higher level of active DVL is required for the stabilization of axonal microtubules. However, when DVL is only localized in the cell body, microtubules are stabilized in this cellular compartment, but not in the axon. Later, stabilized axonal microtubules are only observed in neurons that have DVL along the axons. Indeed, a perfect correlation between axonal microtubule stabilization and the presence of DVL in the axon was found. These findings strongly suggest that DVL induces signal transduction events resulting in local changes in microtubule stability.

Local inhibition of GSK-3β by DVL could result in changes in the phosphorylation of target proteins such as MAP-1B and Tau that regulate microtubule stability in axons (Hanger et al., 1992; Lucas et al., 1998). For example, phosphorylation of MAP-1B by GSK-3β has been shown to maintain microtubules in a more dynamic state (Goold et al., 1999). In contrast, inhibition of GSK-3β by lithium or WNTs decreases GSK-3β–mediated phosphorylation of MAP-1B (Lucas et al., 1998) and increases stable microtubules in axons (Hall et al., 2000). Here, we show that DVL mimics the effect of WNTs on axonal remodeling. DVL-expressing neurons exhibit thicker and shorter axons with enlarged growth cones containing stable looped microtubules, as observed in neurons exposed to WNTs (Lucas et al., 1998; Hall et al., 2000). Our data indicate that DVL could regulate microtubule dynamics by changing the activity of GSK-3β and the function of its cellular targets. Indeed, DVL decreases the level of phosphorylated MAP-1B in developing axons, a readout of GSK-3β activity. Consistent with the role of Axin in the regulation of microtubule stability, Axin also decreases MAP-1B phosphorylation by GSK-3β and suggests that Axin decreases GSK-3β activity in neurons. We propose a model in which DVL regulates signaling events within a cellular compartment through a nuclear-independent pathway. By virtue of its association with microtubules, DVL could locally regulate microtubule dynamics in processes such as cell migration, cell polarity, and axonal remodeling.

Materials and methods

Cell cultures and transfections
COS and NB2a cells were maintained in DME containing 10% FCS at 37°C in 5% CO₂. For immunofluorescence analyses, cells were cultured on glass coverslips and transiently transfected using LipofectAMINE™ (Invitrogen) for 3 h. NB2a cells were differentiated into neurons by treatment with 1 mM dibutyryl-cyclic-AMP overnight, and were then fixed. When the inducible system was used, differentiated neurons were treated with 1 μM β-estradiol for different periods of times and fixed with 4% PFA in PBS for 15 min. Cells were then permeabilized with 0.02% Triton X-100 in PBS for 5 min or with a decreasing concentration of ethanol. To visualize proteins bound to microtubules, cells were fixed according to Krylova et al. (2000). For microtubule-stabilization assays, COS cells and NB2a cells were treated with nocodazole at a concentration of 10 μM for 1 h and 5 μM for 30 min, respectively. To inhibit transcription, actinomycin D was used at 10 μg/ml, a concentration that inhibits all cellular RNA polymerase (Perry and Kelley, 1970). This concentration of actinomycin D completely blocks transcription, as estimated by the lack of expression of EGFP in COS cells.

Primary neuronal transfections
Cerebellar granule cells were obtained from neonatal mice as described previously (Krylova et al., 2000). After dissection, 5×10^4 cells were plated on coverslips previously treated with 500 μg/ml poly-D-lysine for 1 h at RT and 30 μg/ml laminin for 3 h at RT. After 2 d in vitro, neurons were transiently transfected using LipofectAMINE™ 2000 (Invitrogen) for 2 h and were cultured for an additional 24 h. To test microtubule stability, primary neurons were treated with 5 μM nocodazole for 6 h, and were then fixed. Cells were fixed with 4% PFA in 4% sucrose for 20 min, and were permeabilized with 0.02% Triton X-100 for 5 min.

Immunofluorescence microscopy
Fixed cultures were blocked with 5% BSA in PBS and then incubated with primary antibodies overnight at 4°C. Primary antibodies used were against DVL-1 (Krylova et al., 2000), total tubulin, and acetylated tubulin (purchased from Sigma-Aldrich); anti-hemagglutinin (HA) antibody (purchased from Boehringer); anti-Myc antibody and anti-FLAG antibody (purchased from Abcam); mAb against phosphorylated MAP-1B (SMI 31 purchased from Affinity BioReagents, Inc.); and mAb against β-catenin (from Transduction Laboratories). pAb against Axin was provided by Drs. Nusse and Willert (Howard Hughes Medical Institute, Stanford, CA). Secondary antibodies were Alexa® 350, Alexa® 488, and Alexa® 546 (purchased from Molecular Probes, Inc.). Fluorescent images were captured with a microscope (model BX60; Olympus) using a CCD camera (Orca ER; Hamamatsu Corporation), acquired with MetaMorph® software (Universal Imaging Corp.), and processed with Adobe Photoshop®.

Microtubule preparation and Western blot
Purified microtubules were prepared from P9 rat brain using two cycles of temperature-dependent polymerization and depolymerization in Pipes buffer (Vallee, 1986). When COS cells were used, microtubules were obtained using cycles of assembly and disassembly using GTP and Taxol® (Vallee, 1982). Microtubules were run on an 8% SDS-PAGE. Endogenous proteins bound to microtubules were visualized using an mAb against GSK-3β and a polyclonal anti-Axin antibody.

Plasmid constructs

The expression of EGFP and DVL-1-HA were under the control of the cytomegalovirus promoter (Krylova et al., 2000). DVL-ER was generated by fusion of HA-DVL-2 with the estrogen receptor ligand-binding domain fusion protein. Residues 282–595 (SAGD to PATV) of the human estrogen receptor containing the β-estradiol binding site were isolated from the EFG2ERP plasmid (a gift from Tariq Enver, Institute of Cancer Research, London, UK), and were fused to HA-DVL-2 (Smalley et al., 1999). This domain of the estrogen receptor contains a mutation (G440V) that reduces its affinity for β-estradiol, thus preventing its activation by β-estradiol present in serum or by phenol red present in the culture media. The DVL-ER construct activates TCF-mediated transcription in HEK293 cells. Myc-tagged hTCF-4 and ΔNTCF-4 constructs were provided by Dr. Hans Clevers (Center for Biomedical Genetics, Utrecht, Netherlands) (Roose et al., 1999). β-catenin and mutant ARM β-catenin constructs were provided by Dr. A. Ben-Ze'ev (Weizmann Institute of Science, Rehovot, Israel) (Zhurinsky et al., 2000). Constitutive active β-catenin fused to Myc (Giannini et al., 2000) was provided by Dr. Robert Kypta (University College London, London, UK). FRAT constructs were described previously (Fraser et al., 2002). Plasmids for transfection were isolated using a Maxi-Prep Endotoxin-Free kit (QIAGEN).

Online supplemental material

Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200309096/DC1. Fig. S1 (A) shows transcription activation by DVL-1, β-catenin, and FRAT measured by luciferase assay. Fig. S1 (B) shows that FRAT increases the level of endogenous β-catenin and its translocation into the nucleus. Fig. S2 shows that Axin down-regulates GSK-3β–mediated phosphorylation of MAP-1B.

References

Andersen, S.S., and G.Q. Bi. 2000. Axon formation: a molecular model for the generation of neuronal polarity. *Bioessays.* 22:172–179.

Arias, A.M., A.M. Brown, and K. Brennan. 1999. Wnt signalling: pathway or network? *Curr. Opin. Genet. Dev.* 9:447–454.

Baas, P.W. 1999. Microtubules and neuronal polarity: lessons from mitosis. *Neuron.* 22:23–31.

Behrens, J., B.A. Jerchow, M. Wurtele, J. Grimm, C. Asbrand, R. Wirtz, M. Kuhl, D. Wedlich, and W. Birchmeier. 1998. Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. *Science.* 280:596–599.

Bosc, C., J.D. Cronk, F. Pirollet, D.M. Watterson, J. Haiech, D. Job, and R.L. Margolis. 1996. Cloning, expression, and properties of the microtubule-stabilizing protein STOP. *Proc. Natl. Acad. Sci. USA.* 93:2125–2130.

Boutros, M., and M. Mlodzik. 1999. Dishevelled: at the crossroads of divergent intracellular signaling pathways. *Mech. Dev.* 83:27–37.

Boutros, M., N. Paricio, D.I. Strutt, and M. Mlodzik. 1998. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell.* 94:109–118.

Cadigan, K.M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. *Genes Dev.* 11:3286–3305.

Cohen, E.D., M.C. Mariol, R.M. Wallace, J. Weyers, Y.G. Kamberov, J. Pradel, and E.L. Wilder. 2002. DWnt4 regulates cell movement and focal adhesion kinase during *Drosophila* ovarian morphogenesis. *Dev. Cell.* 2:437–448.

Cohen, P., and S. Frame. 2001. The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* 2:769–776.

Desai, A., and T.J. Mitchison. 1997. Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* 13:83–117.

Ferkey, D.M., and D. Kimelman. 2002. Glycogen synthase kinase-3 β mutagenesis identifies a common binding domain for GBP and Axin. *J. Biol. Chem.* 277:16147–16152.

Franca-Koh, J., M. Yeo, E. Fraser, N. Young, and T.C. Dale. 2002. The regulation of glycogen synthase kinase-3 nuclear export by Frat/GBP. *J. Biol. Chem.*

277:43844–43848

Fraser, E., N. Young, R. Dajani, J. Franca-Koh, J. Ryves, R.S. Williams, M. Yeo, M.T. Webster, C. Richardson, M.J. Smalley, et al. 2002. Identification of the Axin and Frat binding region of glycogen synthase kinase-3. *J. Biol. Chem.* 277:2176–2185.

Fukata, M., and K. Kaibuchi. 2001. Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nat. Rev. Mol. Cell Biol.* 2:887–897.

Giannini, A.L., M.M. Vivanco, and R.M. Kypta. 2000. Analysis of β-catenin aggregation and localization using GFP fusion proteins: nuclear import of α-catenin by β-catenin/Tcf complex. *Exp. Cell Res.* 255:207–220.

Goold, R.G., R. Owen, and P.R. Gordon-Weeks. 1999. Glycogen synthase kinase 3β phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. *J. Cell Sci.* 112:3373–3384.

Gottardi, C.J., and B.M. Gumbiner. 2001. Adhesion signaling: how β-catenin interacts with its partners. *Curr. Biol.* 11:R792–R794.

Habas, R., I.B. Dawid, and X. He. 2003. Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev.* 17:295–309.

Hall, A.C., F.R. Lucas, and P.C. Salinas. 2000. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell.* 100:525–535.

Hall, A.C., A. Brennan, R.G. Goold, K. Cleverley, F.R. Lucas, P.R. Gordon-Weeks, and P.C. Salinas. 2002. Valproate regulates GSK-3-mediated axonal remodeling and synapsin I clustering in developing neurons. *Mol. Cell. Neurosci.* 20:257–270.

Hanger, D.P., K. Hughes, J.R. Woodgett, J.P. Brion, and B.H. Anderton. 1992. Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci. Lett.* 147:58–62.

Hergovich, A., J. Lisztwan, R. Barry, P. Ballschmieter, and W. Krek. 2003. Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. *Nat. Cell Biol.* 5:64–70.

Hoebeke, J., G. Van Nijen, and M. De Brabander. 1976. Interaction of nocodazole (R 17934), a new antitumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.* 69:319–324.

Huelsken, J., and W. Birchmeier. 2001. New aspects of Wnt signaling pathways in higher vertebrates. *Curr. Opin. Genet. Dev.* 11:547–553.

Ikeda, S., S. Kishida, H. Yamamoto, H. Murai, S. Koyama, and A. Kikuchi. 1998. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. *EMBO J.* 17:1371–1384.

Korinek, V., N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. *Science.* 275:1784–1787.

Krylova, O., M.J. Messenger, and P.C. Salinas. 2000. Dishevelled-1 regulates microtubule stability: a new function mediated by glycogen synthase kinase-3β. *J. Cell Biol.* 151:83–94.

Li, L., H. Yuan, C.D. Weaver, J. Mao, G.H. Farr, III, D.J. Sussman, J. Jonkers, D. Kimelman, and D. Wu. 1999. Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. *EMBO J.* 18:4233–4240.

Ligon, L.A., S. Karki, M. Tokito, and E.L. Holzbaur. 2001. Dynein binds to β-catenin and may tether microtubules at adherens junctions. *Nat. Cell Biol.* 3:913–917.

Lucas, F.R., and P.C. Salinas. 1997. WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons. *Dev. Biol.* 192:31–44.

Lucas, F.R., R.G. Goold, P.R. Gordon-Weeks, and P.C. Salinas. 1998. Inhibition of GSK-3β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J. Cell Sci.* 111:1351–1361.

McCartney, B.M., D.G. McEwen, E. Grevengoed, P. Maddox, A. Bejsovec, and M. Peifer. 2001. *Drosophila* APC2 and Armadillo participate in tethering mitotic spindles to cortical actin. *Nat. Cell Biol.* 3:933–938.

Morfini, G., G. Szebenyi, R. Elluru, N. Ratner, and S.T. Brady. 2002. Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. *EMBO J.* 21:281–293.

Patapoutian, A., and L.F. Reichardt. 2000. Roles of Wnt proteins in neural development and maintenance. *Curr. Opin. Neurobiol.* 10:392–399.

Peifer, M., and D.G. McEwen. 2002. The ballet of morphogenesis: unveiling the hidden choreographers. *Cell.* 109:271–274.

Penton, A., A. Wodarz, and R. Nusse. 2002. A mutational analysis of *dishevelled* in

Drosophila defines novel domains in the Dishevelled protein as well as novel suppressing alleles of *axin*. *Genetics.* 161:747–762.

Perry, R.P., and D.E. Kelley. 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose–response of different RNA species. *J. Cell. Physiol.* 76:127–139.

Roose, J., G. Huls, M. van Beest, P. Moerer, K. van der Horn, R. Goldschmeding, T. Logtenberg, and H. Clevers. 1999. Synergy between tumor suppressor APC and the β-catenin-Tcf4 target Tcf1. *Science.* 285:1923–1926.

Sakanaka, C., J.B. Weiss, and L.T. Williams. 1998. Bridging of β-catenin and glycogen synthase kinase-3β by axin and inhibition of β-catenin-mediated transcription. *Proc. Natl. Acad. Sci. USA.* 95:3020–3023.

Salic, A., E. Lee, L. Mayer, and M.W. Kirschner. 2000. Control of β-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. *Mol. Cell.* 5:523–532.

Sheldahl, L.C., D.C. Slusarski, P. Pandur, J.R. Miller, M. Kuhl, and R.T. Moon. 2003. Dishevelled activates Ca^{2+} flux, PKC, and CamKII in vertebrate embryos. *J. Cell Biol.* 161:769–777.

Smalley, M.J., E. Sara, H. Paterson, S. Naylor, D. Cook, H. Jayatilake, L.G. Fryer, L. Hutchinson, M.J. Fry, and T.C. Dale. 1999. Interaction of Axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. *EMBO J.* 18:2823–2835.

Thomas, G.M., S. Frame, M. Goedert, I. Nathke, P. Polakis, and P. Cohen. 1999. A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of axin and β-catenin. *FEBS Lett.* 458:247–251.

Vallee, R.B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J. Cell Biol.* 92:435–442.

Vallee, R.B. 1986. Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins, and MAP fragments. *Methods Enzymol.* 134:89–104.

Wodarz, A., and R. Nusse. 1998. Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* 14:59–88.

Yanagawa, S.I., F. Van Leeuwen, A. Wodarz, J. Klingensmith, and R. Nusse. 1995. The dishevelled protein is modified by wingless signaling in *Drosophila*. *Genes Dev.* 9:1087–1095.

Yost, C., G.H.r. Farr, S.B. Pierce, D.M. Ferkey, M.M. Chen, and D. Kimelman. 1998. GBP, an inhibitor of GSK-3, is implicated in *Xenopus* development and oncogenesis. *Cell.* 93:1031–1041.

Zeng, L., F. Fagotto, T. Zhang, W. Hsu, T.J. Vasicek, W.L. Perry, J.J. Lee, S.M. Tilghman, B.M. Gumbiner, and F. Costantini. 1997. The mouse fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell.* 90:181–192.

Zhurinsky, J., M. Shtutman, and A. Ben-Ze'ev. 2000. Differential mechanisms of LEF/TCF family-dependent transcriptional activation by β-catenin and plakoglobin. *Mol. Cell. Biol.* 20:4238–4252.