Dynamic recruitment of axin by Dishevelled protein assemblies

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Summary

Dishevelled (Dvl) proteins are cytoplasmic components of the Wnt signalling pathway, which controls numerous cell fate decisions during animal development. During Wnt signalling, Dvl binds to the intracellular domain of the frizzled transmembrane receptors, and also to axin to block its activity, which results in the activation of β-catenin and, consequently, in a transcriptional switch. We have previously reported that the DIX domain of mammalian Dvl2 allows it to form dynamic protein assemblies. Here, we show that these Dvl2 assemblies recruit axin, and also casein kinase Iϵ. Using photobleaching experiments of GFP-tagged Dvl2 and axin to study the dynamics of their interaction, we found that the recruitment of axin-GFP by Dvl2 assemblies is accompanied by a striking acceleration of the dynamic properties of axin-GFP. We also show that the interaction between Dvl2 and axin remains highly dynamic even after Wnt-induced relocation to the plasma membrane. We discuss how the recruitment of casein kinase Iϵ by Dvl2 assemblies might impact on the recruitment of axin to the plasma membrane during Wnt signalling.

Key words: Axin, DIX domain, Disheveled, Wnt signalling, Casein kinase I epsilon

Journal of Cell Science

Introduction

Wnt signalling controls numerous cell fate decisions during animal development, and is also critical for the homeostasis of adult tissues (Logan and Nusse, 2004). A key effector of the canonical Wnt pathway is β-catenin whose phosphorylation and stability are tightly regulated by a cytoplasmic protein complex formed by the axin scaffolding protein. The axin complex also contains glycogen synthase kinase 3β (GSK3β) and the adenomatous polyposis coli (APC) tumour suppressor, and loss of function of these three components causes activation of β-catenin. Inappropriate activation of β-catenin leads to cancer in many human tissues, most notably colorectal cancer (Bienz and Clevers, 2000; Polakis, 2000). The key read-out of activated β-catenin relevant for both normal and malignant development is a change in the transcriptional programme mediated by the TCF/LEF family of transcription factors to which β-catenin binds as a cofactor (Arce et al., 2006).

The axin complex is constitutively active in the absence of Wnt stimulation, phosphorylating β-catenin and thus promoting its proteasomal degradation (Kimelman and Xu, 2006). Axin itself is thought to be rate-limiting in this process as its cellular concentration is exceedingly low (Lee et al., 2003). When Wnt ligands bind to their frizzled (Fz) transmembrane receptors, the activity of the axin complex is inhibited by a mechanism that is poorly understood. This inhibition critically depends on an interaction between axin itself and the dishevelled (Dvl) proteins (Cliffe et al., 2003; Kishida et al., 1999; Li et al., 1999; Penton et al., 2002; Smalley et al., 1999) whose founding member (Dishevelled; Dsh) was discovered in *Drosophila* as a positive Wnt signalling effector (Klingensmith et al., 1994; Theisen et al., 1994). Dvl proteins are cytoplasmic proteins that can be recruited to the plasma membrane by their Fz receptors (Axelrod et al., 1998; Cong et al., 2004b; Miller et al., 1999; Rothbächer et al., 2000; Umbhauer et al., 2000; Yanagawa et al., 1995; Yang-Snyder et al., 1996) by direct binding (Wong et al., 2003). How Dvl proteins transduce the Wnt signal is not entirely clear, though various possible mechanisms have been discussed (Kimelman and Xu, 2006; Malbon and Wang, 2006; Wharton, Jr, 2003). In *Drosophila*, Dsh is required for recruitment of axin to the plasma membrane during Wingless signalling (Cliffe et al., 2003), but whether the mammalian Dvl proteins also function in this process has not yet been determined.

Dvl proteins contain a highly conserved N-terminal domain, the DIX domain (Fig. 1, top), which is essential for its signalling activity (Axelrod et al., 1998; Boutros et al., 1998; Capelluto et al., 2002; Julius et al., 2000; Kishida et al., 1999; Moriguchi et al., 1999; Park et al., 2005; Penton et al., 2002; Rothbächer et al., 2000; Schwarz-Romond et al., 2007; Smalley et al., 1999; Yanagawa et al., 1995). Remarkably, only two other proteins contain a close DIX domain relative, namely axin, in its C terminus (Fig. 1, bottom; for clarity and simplicity, we shall refer to the DIX domain of axin as the DAX domain), and also a less well-known protein called Ccd1 which is found exclusively in vertebrates and whose function, at least in zebrafish, appears to be related to that of Dvl in the transduction of the Wnt signal (Shiomi et al., 2003). Both DIX and DAX domains are known to mediate self-association in vitro and in vivo (Hsu et al., 1999; Kishida et al., 1999; Luo et al., 2004; Rothbächer et al., 2000; Sakanaka and Williams, 1999). Indeed, the recent determination of the structure of the DAX domain by X-ray crystallography revealed that this domain can form β-strand-mediated head-to-tail filaments (Schwarz-Romond et al.,

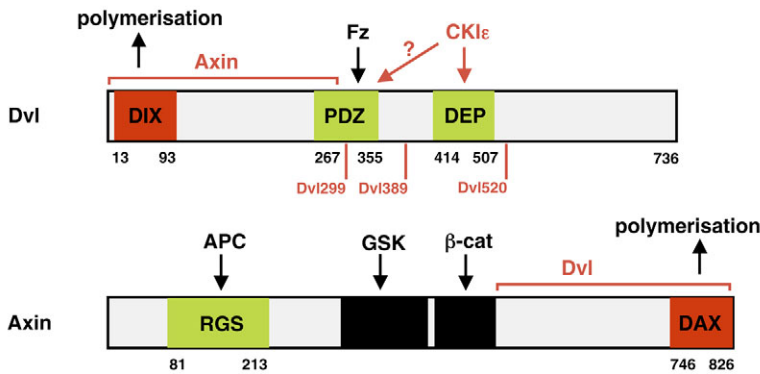

Fig. 1. The domain structures of Dvl and axin proteins. Schematic representations of Dvl2 and axin, with their ligands binding to their known structural domains PDZ (Wong et al., 2003), DEP (Wong et al., 2000) and RGS (Spink et al., 2000); the DIX and DAX domains mediate dynamic polymerisation (Schwarz-Romond et al., 2007); the sequence stretches in axin that bind to β-catenin and GSK3β are also indicated (in black); residue numbers underneath domains denote the domain boundaries. Note that the main binding domain for CKIε appears to be the DEP domain (Kishida et al., 2001) (see below, Fig. 6), but this kinase could also bind to the PDZ domain (Peters et al., 1999).

2007). Although the DIX domain appears to be necessary for the interaction between Dvl and axin (Julius et al., 2000; Kishida et al., 1999; Penton et al., 2002; Smalley et al., 1999), its contribution towards this interaction is unclear. Likewise, it has not been determined whether the DAX domain participates directly in this important interaction.

In order to understand the molecular mechanism underlying the Wnt-induced inhibition of the axin complex, we decided to focus on the rather ill-defined interaction between Dvl2 (one of the three mammalian Dvl proteins) and axin, and on the role of the DIX and DAX domains in this interaction. We also asked whether the interaction between Dvl2 and axin was sufficient for signalling, or whether additional domains of Dvl2 and their ligands may be required for the signalling activity of Dvl2. Most importantly, we used photobleaching experiments to study the dynamic aspects of the in vivo interaction between Dvl2 protein assemblies (Schwarz-Romond et al., 2005) and axin, and to see whether Wnt signalling may affect the dynamics of their interaction.

Results

As mentioned in the Introduction, in a wide variety of cells, overexpressed Dvl proteins trigger canonical Wnt pathway activity, a process that is critically dependent on their DIX domain. Although this signalling activity of Dvl bypasses the requirement for Wnt ligand, it is likely to reflect some of the same molecular properties of Dvl as its Wnt-dependent signalling activity. In particular, both result in the stabilisation of β-catenin and in the stimulation of TCF-mediated transcription (see below), so the primary effector events of Wnt-dependent and Wnt-independent Dvl activities are the same. Indeed, a key molecular effector of both activities is axin with which Dvl proteins need to interact in order to signal (Cliffe et al., 2003; Kishida et al., 1999; Penton et al., 2002; Smalley et al., 1999). We decided to focus on the Wnt-independent signalling activity of Dvl2 since this is easily accessible to study in mammalian cells, to gain insight into how this protein might interact with and inactivate axin during normal Wnt signalling. Note that Dvl proteins also function in non-canonical Wnt signalling pathways, most notably in the planar polarity pathway (Malbon and Wang, 2006; Wharton, Jr, 2003), but we shall use the term ‘signalling’ below exclusively to refer to canonical signalling mediated by axin, β-catenin and TCF/LEF transcription factors.

Wnt-dependent recruitment of Dvl2 puncta to the plasma membrane

We have previously shown that Dvl2, overexpressed in transfected COS-7 cells, forms regularly shaped cytoplasmic puncta that correspond to highly dynamic protein assemblies (Schwarz-Romond et al., 2005). Their formation is critical for both Wnt-independent and Wnt-dependent Dvl2 signalling, and they reflect a remarkable property of the DIX domain to self-associate in a reversible and concentration-dependent fashion (Schwarz-Romond et al., 2007). Indeed, similar puncta of overexpressed Dvl2 tagged with green fluorescent protein (Dvl2-GFP) were also observed in transfected 293T cells (Fig. 2A). Furthermore, exposure of these cells to Wnt3a causes recruitment of the Dvl2-GFP puncta to the plasma membrane (Fig. 2B; this recruitment also requires co-expression of other Wnt signalling components) (Bilic et al., 2007).

We also observed puncta of endogenous Dvl2 in the cytoplasm of untransfected 293T cells (Fig. 2C). These endogenous puncta are much smaller than the Dvl2-GFP puncta, and they were only detectable by one of several tested Dvl2 antibodies (see Materials and Methods), namely an affinity-purified Dvl2 antibody purified from a high-titre rabbit antiserum that is highly specific for Dvl2 as judged by western blotting (Semenov and Snyder, 1997). However, the punctate signal clearly reflects endogenous Dvl2 since it was essentially eliminated by RNAi depletion of Dvl2 (Fig. 2D), or by prior depletion of the serum with Dvl2 antigen (Fig. 2E). Like the Dvl2-GFP puncta, these endogenous Dvl2 puncta can be recruited to the plasma membrane on Wnt3a stimulation (Fig. 2F, arrows). These findings corroborate previous observations of endogenous Dvl puncta in a number of cell types in different species (Itoh et al., 2005; Miller et al., 1999; Semenov and Snyder, 1997; Torres and Nelson, 2000; Walston et al., 2004; Yanagawa et al., 1995) that are recruited to the plasma membrane during Wnt signalling (Chang et al., 2005; Hawkins et al., 2005). As an aside, 293T cells show little if any nuclear Dvl2, either before or after Wnt3a stimulation (Fig. 2C,F), which contrasts with earlier results, using the same antiserum, of nuclear Dvl2 in 293 cells (Itoh et al., 2005).

Axin puncta formation depends on its DAX domain

Overexpressed axin-GFP also forms puncta in transfected 293T (not shown) and in COS-7 cells (Fig. 3A), as previously shown (Schwarz-Romond et al., 2005), though we found these puncta to be less dynamic, and less abundant as well as smaller,

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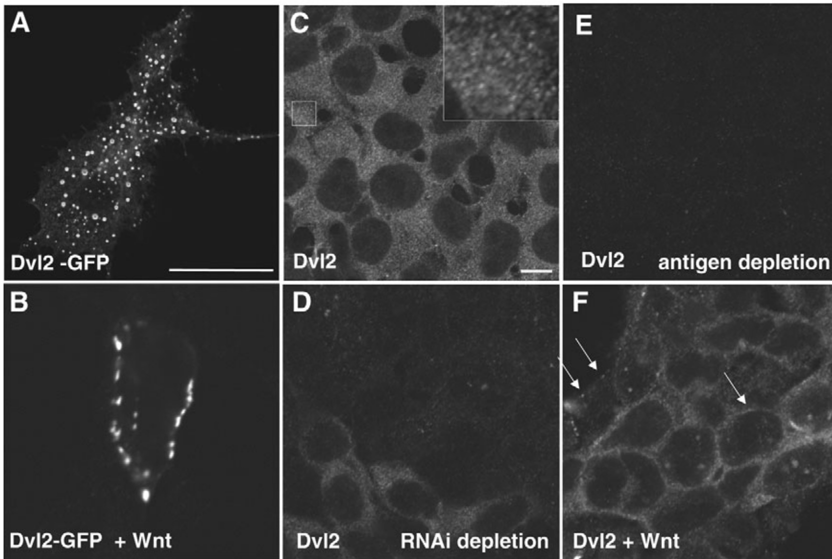

Fig. 2. Wnt-dependent relocation of Dvl2-GFP and endogenous Dvl2 puncta to the plasma membrane. (A,B) Fixed 293T cells, after transfection with Dvl2-GFP (50 ng), Fz8 (100 ng), LRP6 (100 ng), GSK3β (100 ng) and HA-axin (100 ng), (A) before or (B) after 2 hours of exposure to recombinant Wnt3a, showing Wnt-dependent recruitment of Dvl2-GFP to the plasma membrane. (C-F) 293T cells, (C-E) before or (F) after exposure to Wnt3a as in B, fixed and stained with affinity-purified anti-Dvl2 antiserum, revealing endogenous cytoplasmic Dvl2 puncta (C) that are recruited to the plasma membrane (F, arrows) in a Wnt-dependent way (insert in C, high-magnification view of cytoplasmic puncta of endogenous Dvl2); the specificity of the signals is indicated by the near-complete elimination of the staining after (D) siRNA treatment against Dvl2, or (E) prior depletion of the antiserum with 100× molar excess of recombinant GST-DIX. Scale bar, 15 μm.

than the Dvl2-GFP puncta (see below). We also noticed that the regular punctate pattern of axin-GFP (Fig. 3A) depends critically on a normal untagged N terminus (the GFP tag being at the C terminus) (Schwarz-Romond et al., 2005): N-terminally GFP-tagged axin constructs, or axin with a long artificial N-terminal extension as that found in the original mouse axin plasmid (Zeng et al., 1997), tend to produce patches and irregular speckles rather than regular puncta (e.g. Fagotto et al., 1999; Smalley et al., 1999) (not shown).

The ability of axin to form puncta depends largely on its DAX domain since ΔDAX-GFP produces a far less punctate, more diffuse expression pattern (Fig. 3B). The same is true for an M12 mutant version of axin (Fig. 3C) whose DAX domain contains three amino acid substitutions (F774S V800A F801A; Fig. 1) which, if introduced into the DIX domain, abolish self-association in vitro and puncta formation in vivo (Schwarz-Romond et al., 2007). Therefore, the DAX domain contributes significantly to the ability of axin to form puncta. However, its role in this appears to be less critical than that of the DIX domain in the formation of Dvl2 puncta, possibly because axin contains other self-associating domains (Luo et al., 2004).

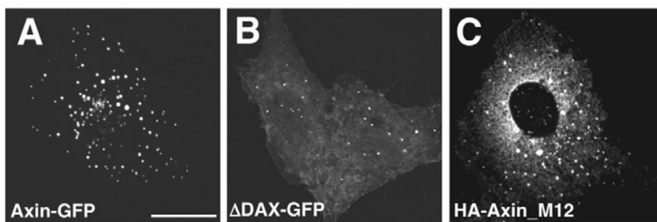

Fig. 3. The DAX domain is required for efficient puncta formation. (A-C) COS-7 cells expressing wt or mutant axin, as indicated were fixed, and (C) stained with antibody against HA (the punctate pattern of wt HA-axin is essentially the same as in A; not shown). Note that ΔDAX and HA-axin_M12 (a mutant version of axin, the self-association of which is expected to be blocked; see text) produce a less punctate, more diffuse pattern, indicating that the ability of axin to form puncta depends at least partly on its DAX domain. Scale bar, 15 μm.

Colocalisation of axin and Dvl2 in puncta depends on the DIX but not on the DAX domain

To examine the interaction between Dvl2 and axin, we co-expressed these two proteins in COS-7 cells. Partial colocalisation of co-expressed Dvl2 and axin has been reported previously (Fagotto et al., 1999; Julius et al., 2000; Kishida et al., 1999; Smalley et al., 1999). However, using axin-GFP with its normal N terminus, we found that the two proteins colocalise perfectly (Fig. 4A), in regularly shaped cytoplasmic puncta whose size and abundance resembled the puncta formed by Dvl2-GFP alone (Schwarz-Romond et al., 2005) (see below; note that the punctate pattern of axin was the same for the differently tagged axin constructs, i.e. axin-GFP and HA-axin). It thus appears that the puncta-forming ability of axin is modified as a result of its interaction with Dvl2. This implies that Dvl2 recruits axin, rather than the converse, which we confirmed by photobleaching experiments (see below).

Next, we examined the requirement for the DIX and DAX domains in the colocalisation of axin and Dvl2. We found that the DAX domain is not required for this since ΔDAX-GFP colocalises as efficiently with Dvl2 as full-length axin-GFP (Fig. 4B), confirming earlier observations (Smalley et al., 1999). By contrast, the DIX domain of Dvl2 is critical for its interaction with axin since we did not observe any significant colocalisation between axin-GFP puncta and Dvl2 without its DIX domain (Fig. 4C). The same was true when we co-expressed axin-GFP with DIX domain mutants of Dvl2 (M1 and M2) that block self-association in vitro and puncta formation in vivo (Schwarz-Romond et al., 2007) (Fig. 4D). Thus, the DIX but not the DAX domain is required for the colocalisation of co-expressed Dvl2 and axin in puncta.

Our results imply that the DIX and DAX domains do not interact directly in vivo, consistent with earlier observations (Julius et al., 2000; Kishida et al., 1999; Smalley et al., 1999). In support of this, we observed two sets of distinct puncta when we co-expressed HA-axin with the DIX domain of Dvl2 (Fig. 4E). Evidently, Dvl2 depends on its DIX domain as well as on sequences outside this domain for its colocalisation with axin. Consistent with these in vivo findings, we confirmed that the

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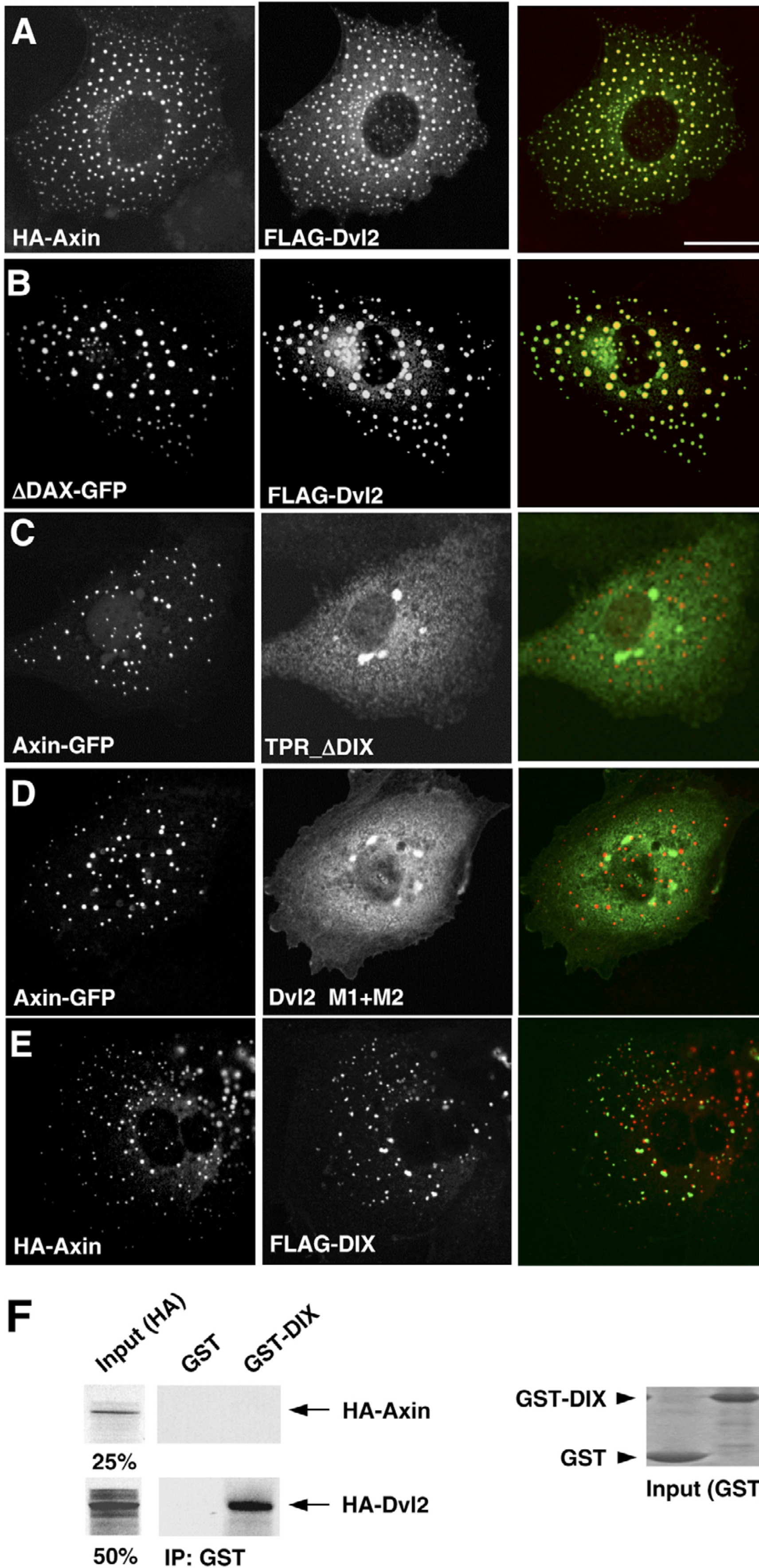

Fig. 4. The recruitment of axin into Dvl2 puncta depends on the DIX but not the DAX domain. (A-E) COS-7 cells co-expressing wt and mutant axin (left-hand panels, and red) and Dvl2 (middle panels, and green) as indicated (merges in right-hand panels), fixed and stained with antibodies against Dvl2 and (A,E) HA. Axin colocalises precisely with Dvl2 puncta (A), regardless of its DAX domain (B), but dependent on a functional DIX domain of Dvl2 (C,D; the ΔDIX construct used here also contains the TPR dimerisation domain, but this domain does not detectably change its behaviour or subcellular distribution; not shown). Note that two sets of puncta are observed if the DIX domain is co-expressed with axin, indicating that the DIX and DAX domains do not interact directly. (F) Pull-down assays between bacterially expressed GST-tagged DIX domain and in vitro translated HA-tagged axin or Dvl2, as indicated (GST input proteins are shown on the right). The DIX domain binds efficiently to itself (within full-length HA-Dvl2, arrow), but not to the DAX domain (within full-length HA-axin, arrow). Scale bar, 15 μm.

isolated DIX and DAX domains do not bind to each other directly, either in vitro (Kishida et al., 1999) (Fig. 4F) or in yeast two-hybrid assays (T.S.-R., unpublished data). Indeed, it has been shown that the minimal fragments that mediate direct binding between Dvl1 and axin in vitro include not only the DIX and DAX domains, respectively, but also extensive flanking sequences (Kishida et al., 1999) (Fig. 1).

Recruitment of axin into Dvl2 puncta is not sufficient for signalling

Based on previous functional analyses of other Dvl proteins (Axelrod et al., 1998; Kishida et al., 2001; Rothbächer et al., 2000; Yanagawa et al., 1995), we expected that the DIX domain of Dvl2 on its own would not exhibit signalling activity. We confirmed this to be the case, by measuring TCF-dependent transcription in 293T cells transfected with a TCF-specific luciferase reporter (Korinek et al., 1997) (not shown). However, we reckoned that the putative minimal axin-binding sequence of Dvl2 (Kishida et al., 1999) might be sufficient to recruit axin into puncta and to signal. We thus tested an N-terminal fragment of Dvl2 that spans this sequence (Dvl299; Fig. 1), but this did not show any signalling activity either (Fig. 5A), despite being expressed at levels comparable to full-length Dvl2 (Fig. 5B). We thus tested the ability of Dvl299 to recruit HA-axin into puncta in cotransfected HeLa cells (which are equally suitable as

COS-7 cells for these recruitment assays, but they are also responsive to Wnt ligands; see below). However, whereas we observed complete co-localisation between FLAG-Dvl2 and HA-axin puncta in these cells (Fig. 5C), we observed two non-overlapping sets of puncta when we co-expressed Dvl299 with HA-axin (Fig. 5D). Thus, either the minimal axin-binding sequences of Dvl2 are more extensive than those of Dvl1 (Kishida et al., 1999) or, more likely given the sequence conservation between the two proteins, the minimal in vitro binding sequences are not sufficient for efficient interaction of the two proteins in vivo.

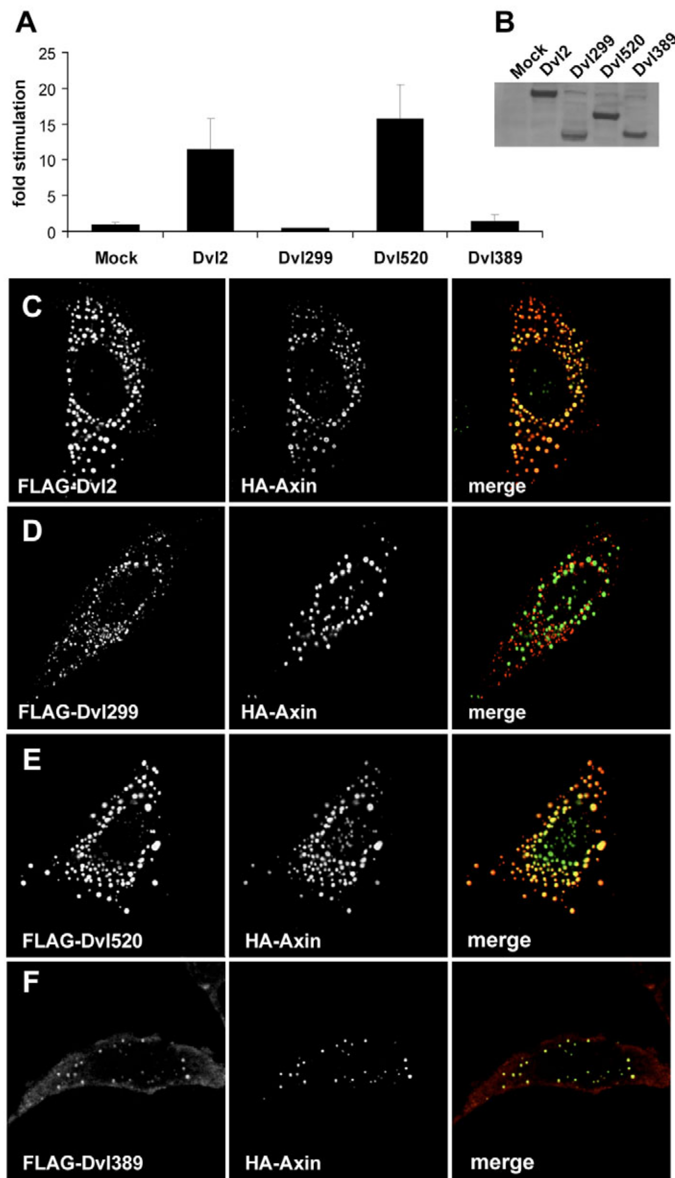

Fig. 5. The recruitment of axin into Dvl2 puncta requires extensive DIX-domain flanking sequences. (A) TCF transcription assays in 293 cells, transfected with a TCF-dependent firefly luciferase reporter (SuperTOP; 50 ng) and an internal control (CMV-renilla; 25 ng), and with full-length Dvl2 (200 ng) or Dvl2 truncations as indicated (500 ng); bars indicate standard deviations. (B) Western blot, probed with anti-FLAG antibody, showing expression levels of Flag-Dvl2 constructs, as indicated. (C-F) HeLa cells, cotransfected with HA-axin (green) and full-length FLAG-Dvl2 or truncations (red), as indicated in panels, fixed and stained with antibodies against their tags; note the two sets of cytoplasmic puncta in D (red or green), but the co-localisation of Dvl and axin puncta in F (yellow), indicating that the minimal axin-recruiting sequences of Dvl2 are contained within amino acids 1-389. Scale bar, 15 μm.

We thus designed two larger Dvl2 truncations, one that spanned the DIX and PDZ domains (Dvl389), and one that spans all three domains (Dvl520; Fig. 1). Dvl520 behaved essentially the same as full-length Dvl2: it was fully active in stimulating the transcriptional activity of the TCF-specific SuperTOP reporter (DasGupta et al., 2005) in transfected 293 cells (Fig. 5A), and it also colocalised completely with HA-axin puncta in transfected HeLa cells (Fig. 5E). By contrast, Dvl389 appeared somewhat dysfunctional in these assays: it showed diffuse cytoplasmic staining and its ability to form puncta was reduced, however the rudimentary puncta did contain HA-axin (Fig. 5F), indicating that Dvl389 was able to recruit axin. The same was observed in cotransfected COS-7 cells (not shown). This indicates that the minimal sequences mediating in vivo interaction with axin, and recruitment into Dvl2 puncta, are contained within amino acids 1-389 of Dvl2.

Somewhat to our surprise, Dvl389 barely showed any signalling activity in transfected 293 cells (Fig. 5A), suggesting that the ability of Dvl2 to recruit axin may not be sufficient for signalling. And although our result with Dvl389 contrasts with those from comparable truncations of *Drosophila* and *Xenopus* Dvl proteins (spanning both DIX and PDZ domains) that appeared to be active in signalling through Armadillo or β-catenin (Axelrod et al., 1998; Rothbächer et al., 2000; Yanagawa et al., 1995), our results are in close agreement with those of Kishida et al. (Kishida et al., 2001) who found that a similar (if slightly longer) Dvl1 fragment was only partially active in mammalian cells, whereas a longer, DEP-domain-containing truncation was fully active. It thus appears that the mammalian Dvl proteins and/or cell assays are somewhat different from the *Xenopus* and *Drosophila* ones, though we cannot rule out that the apparent differences are due to the levels of Dvl overexpression, or even to the precise details of the Dvl constructs used.

We conclude that the DEP domain and/or its N-terminal flanking sequences are critical for the ability of Dvl2 to signal in mammalian cells. This is consistent with earlier conclusions that the DEP domain is critical for efficient canonical signalling activity of other Dvl proteins (Kishida et al., 2001; Penton et al., 2002), whereas the PDZ domain is not always essential (Julius et al., 2000; Rothbächer et al., 2000). Perhaps, a DEP domain-interacting protein assists Dvl2 in signalling, as previously proposed for Dvl1 (Kishida et al., 2001).

Recruitment of casein kinase Iϵ into Dvl2 puncta

An obvious candidate to assist Dvl2 in signalling is casein kinase Iϵ (CKIϵ), which has been shown to bind to, and act through, the DEP domain to synergise with Dvl1 (Kishida et al., 2001). It is well-established that CKIϵ phosphorylates Dvl proteins and enhances their canonical signalling activities (Cong et al., 2004a; Gao et al., 2002; Hino et al., 2003; Kishida et al., 2001; Klein et al., 2006; Klimowski et al., 2006; McKay et al., 2001; Peters et al., 1999; Sakanaka et al., 1999) (reviewed by Price, 2006), but there is some uncertainty as to whether the DEP domain is the only CKIϵ-binding domain,

Journal of Cell Science

given that the PDZ domain of *Xenopus* Dsh has also been shown to bind to CKIϵ (Peters et al., 1999) (Fig. 1).

We first confirmed that the signalling activity of Dvl2 in transfected 293 cells was much enhanced by co-expressed CKIϵ (Fig. 6A), in agreement with previous findings (Cong et al., 2004a; Hino et al., 2003; Kishida et al., 2001; Klimowski et al., 2006). Furthermore, we found that CKIϵ also strongly synergised with Dvl520, but only moderately with Dvl389, and barely at all with Dvl299 (Fig. 6A). Thus, the DEP domain is the primary Dvl2 domain mediating a functional interaction with CKIϵ, in agreement with previous conclusions regarding Dvl1 (Kishida et al., 2001).

We also investigated whether Dvl2 might recruit CKIϵ into puncta, although we were aware that a previous study had found that co-expression of CKIϵ with GFP-tagged Dvl1 in 293 cells caused the Dvl1 puncta to dissolve (Cong et al., 2004a). However, if we co-expressed limiting amounts of HA-CKIϵ with FLAG-Dvl2 in HeLa cells, we found that CKIϵ was recruited efficiently into the Dvl2 puncta, leaving very little diffuse HA-CKIϵ staining (Fig. 6B; expressed on its own, HA-CKIϵ was distributed diffusely throughout the cytoplasm; not shown) (see also Kishida et al., 2001). The same was true in similarly cotransfected COS-7 cells (not shown). Significantly, ΔDIX did not affect the diffuse cytoplasmic staining of HA-CKIϵ in any way (not shown), so the DIX domain of Dvl2, and its ability to form puncta, is critical for recruitment not only of axin, but also of CKIϵ. We note that this could explain why overexpressed wild-type (wt) Dvl2 was phosphorylated in vivo, but DIX domain mutants that could not form puncta were not (Capelluto et al., 2002).

Next, we tested the various Dvl2 truncations, to identify the domain(s) required for recruitment of CKIϵ into Dvl2 puncta. We found that Dvl520 recruited CKIϵ into the Dvl2 puncta efficiently (Fig. 6C), whereas Dvl299 did not affect the diffuse cytoplasmic CKIϵ staining at all (Fig. 6D). Likewise, CKIϵ was not recruited into the rudimentary puncta formed by Dvl389 (Fig. 6E). However, we did find some recruitment of CKIϵ into puncta in COS-7 cells transfected with Dvl389 (not shown), perhaps reflecting the previously observed ability of the PDZ domain to bind to CKIϵ (Peters et al., 1999). Nevertheless, we conclude that the DEP domain and/or its N-terminal flanking sequences are critical for robust recruitment of CKIϵ into Dvl2 puncta. Taken together with our results on axin recruitment by Dvl389 (Fig. 6E), our findings suggest that the recruitment of CKIϵ into Dvl2 puncta may assist, but is not essential for, the recruitment of axin.

Previous loss-of-function experiments showed that CKIϵ is required for a full Wnt response in *Xenopus*, *Drosophila* and mammalian cells (Cong et al., 2004a; Hino et al., 2003; Klein et al., 2006; Peters et al., 1999; Sakanaka et al., 1999), but the role of CKIϵ with regard to Dvl2 signalling was not entirely

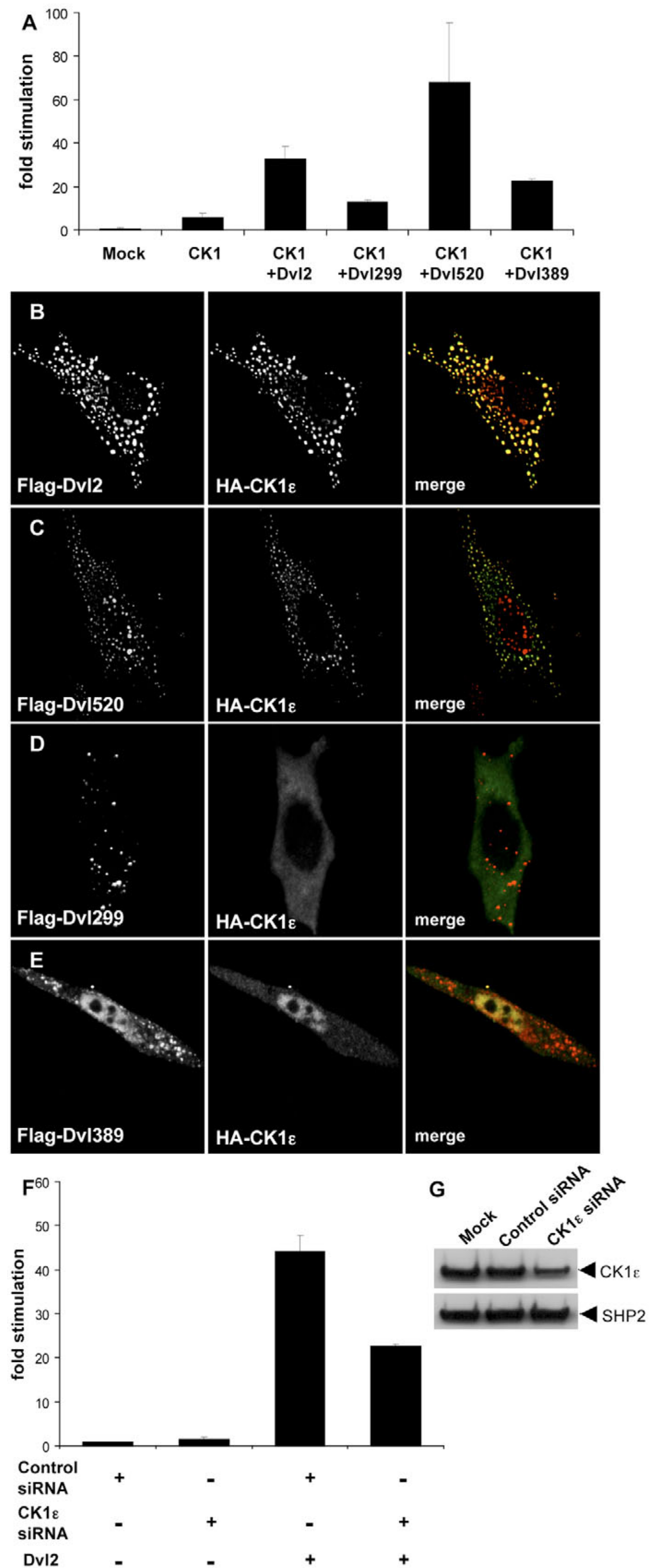

Fig. 6. Recruitment of CKIϵ into Dvl2 puncta. (A) SuperTOP assays in transfected 293 cells as in Fig. 5A; limiting amounts of HA-CKIϵ (100 ng) were co-expressed with full-length FLAG-Dvl2 (200 ng) or truncations (500 ng), as indicated. (B-E) HeLa cells, cotransfected with HA-CKIϵ (green) and FLAG-Dvl2 or truncations (red) as indicated in panels, fixed and stained as in Fig. 5. Note that Dvl299 does not affect the HA-CKIϵ staining, which remains diffuse cytoplasmic (D), indistinguishable from the staining of HA-CKIϵ expressed by itself, or co-expressed with ΔDIX (not shown).
(F) SuperTOP assays in 293T cells transfected with FLAG-Dvl2, after RNAi-mediated depletion of endogenous CKIϵ (to <50% of normal levels, as show by western blotting, G), revealing that the signalling activity of Dvl2 depends on CKIϵ. Scale bar, 15 μm.

clear: inhibitor experiments suggested that CKIϵ was required for the signalling activity of overexpressed Dvl in *Xenopus* (Peters et al., 1999), but in *Drosophila* S2 cells, CKIϵ depletion did not affect the signalling activity of overexpressed Dsh (Cong et al., 2004a). We thus depleted endogenous CKIϵ by RNAi in 293T cells transfected with full-length Dvl2, to test the functional relevance of CKIϵ recruitment for the signalling activity of Dvl2. We found that CKIϵ reduced the signalling activity of Dvl2 significantly (Fig. 6F; western blot analysis confirmed that CKIϵ was reduced to <50% of its normal level, Fig. 6G). Thus, our results provide evidence that Dvl2 recruits CKIϵ into puncta in order to signal efficiently.

Recruitment of axin into Dvl2 puncta changes the dynamics of axin self-assembly

To study the dynamic aspects of the interaction between Dvl2 and axin, and also to ask whether Dvl2 recruits axin into puncta, or vice versa, we performed photobleaching experiments with GFP-tagged proteins expressed in COS-7 cells in which we measured the kinetics of exchange between punctate and diffuse cytoplasmic protein (Schwarz-Romond et al., 2005). In these experiments, rates of fluorescence recovery are determined after photobleaching individual puncta (see Materials and Methods). We previously found that the recovery of axin-GFP puncta was much slower, and much less complete, than that of Dvl2-GFP puncta (Schwarz-Romond et al., 2005): typically, axin-GFP puncta recovered with a $t_{1/2}$ value of >1 minute, and to only ~20-30% of the initial fluorescence level (Fig. 7A), whereas Dvl2-GFP recovered with a $t_{1/2}$ value of 10-20 seconds, and to ~80% of the initial fluorescence level (Fig. 7B). Very similar kinetic values were measured in HeLa cells transfected with Dvl2-GFP, or axin-GFP (not shown). Thus, axin puncta are far less dynamic than Dvl2 puncta in both cell types.

Interestingly, however, the recovery of axin-GFP fluorescence was much accelerated, and increased, by co-expressed Dvl2 (Fig. 7C). Accelerated recovery was also observed if ΔDAX-GFP instead of full-length axin was co-expressed with Dvl2 (Fig. 7D); indeed, ΔDAX-GFP seemed to be even more efficiently recruited into Dvl2 puncta than full-length axin-GFP (Fig. 7, compare D with C), perhaps because this truncation interacts less efficiently with other axin ligands. Conversely, neither the DIX domain of Dvl2 itself, nor Dvl2 without its DIX domain, accelerated the recovery of axin-GFP fluorescence (Fig. 7E; not shown), consistent with their failure to recruit axin into puncta (Fig. 4). Taken together, these results confirm that, at least under overexpression conditions, Dvl2 recruits axin rather than the converse. Furthermore, they imply that the recruitment of axin by Dvl2 triggers a change in axin that alters its ability to self-associate and/or to associate with other binding partners (see Discussion).

We previously found that the signalling by Dvl2 depended on its ability to polymerise rather than dimerise (Schwarz-Romond et al., 2007). This was based on studies of two sets of Dvl2 mutants, namely, (i) co-expression of M1 and M2 mutants of Dvl2, neither of which can polymerise, although they can heterodimerise, and (ii) a ΔDIX construct whose dimerisation was restored by linkage to a heterologous dimerisation domain called TPR (TPR_ΔDIX; TPR is derived from an oncogenic Met receptor and mediates dimerisation through a leucine zipper motif) (Rodrigues and Park, 1993).

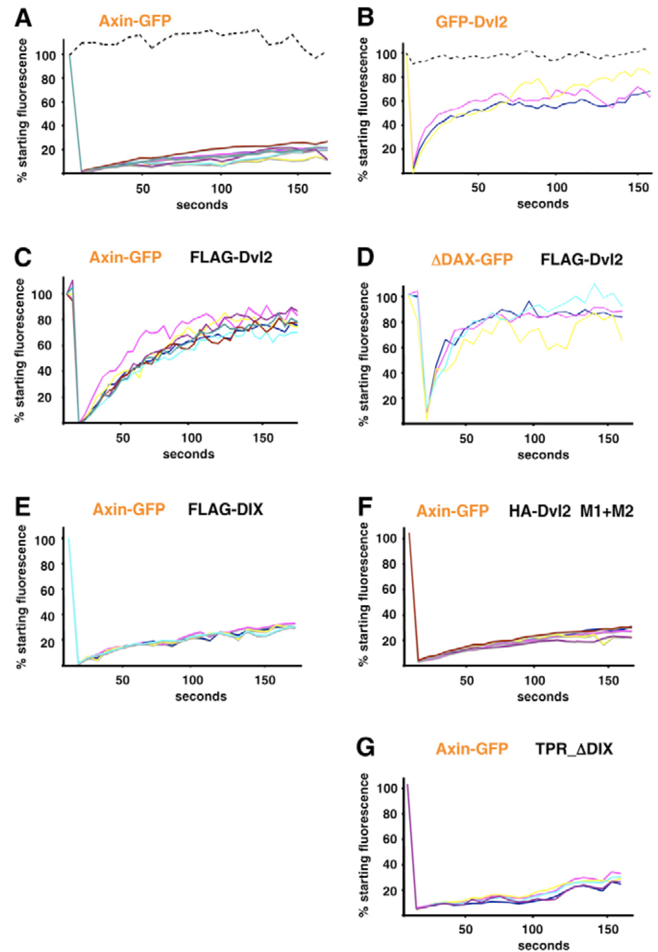

Fig. 7. Dvl2 accelerates the recruitment of axin into dynamic protein assemblies. (A,B) Graphs of FRAP experiments conducted in COS-7 cells, in which individual (A) axin-GFP or (B) Dvl2-GFP puncta were bleached, and fluorescence recovery within the bleached boxes was monitored every 5 seconds (coloured lines represent different puncta; values were normalised with respect to unbleached control puncta, shown by dotted line). (C-G) Graphs of FRAP experiments of axin-GFP as in A, but in the presence of co-expressed wt or mutant Dvl2, as indicated. Note that wt but not mutant Dvl2 accelerates the recovery rates of axin-GFP.

Significantly, neither co-expressed M1 and M2 mutants nor TPR_ΔDIX affected the recovery rates of co-expressed axin-GFP fluorescence into puncta (Fig. 7F,G), reflecting their inability to form puncta (Schwarz-Romond et al., 2007). Thus, Dvl2 polymers rather than dimers are required for the efficient recruitment of axin.

We also attempted to examine biochemically the in vivo interaction between Dvl2 and axin. It has been reported that these proteins can apparently be co-immunoprecipitated after co-expression in mammalian cells (Julius et al., 2000; Kishida et al., 1999; Li et al., 1999; Smalley et al., 1999), though at least in one report, the specificity of this biochemical interaction depended on the precise buffer conditions (Smalley et al., 1999). However, despite testing various buffer conditions (including the previously reported ones), we were unable to get robust and specific co-immunoprecipitation signals that reflected the abilities of wt versus mutant Dvl2 to colocalise

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with axin. This might be expected, given the highly dynamic and reversible nature of this interaction (Fig. 7C,D).

Wnt stimulation does not affect the dynamic nature of the Dvl2-axin interaction

As shown above (Fig. 2B), Wnt3a stimulation of transfected 293T cells triggered a relocation of most of the cytoplasmic Dvl2 puncta to the plasma membrane (as mentioned, this relocation also required co-expression with other signalling components such as Fz, low density lipoprotein receptor-related protein (LRP) and GSK3β) (Bilic et al., 2007). The same was observed in Wnt3a-stimulated HeLa cells, cotransfected with Dvl2 and axin-GFP (Fig. 8A,B), or with Dvl2-GFP and axin (Fig. 8C,D). We thus conducted FRAP experiments on the membrane-associated GFP puncta in these cells, to determine whether the dynamic association between Dvl2 and axin changed after Wnt3a stimulation.

Indeed, the dynamics of axin-GFP recovery in Wnt-induced membrane-associated Dvl2-containing puncta were essentially the same as those observed for cytoplasmic Dvl2-containing puncta (Schwarz-Romond et al., 2005) (Fig. 7C; not shown), with average $t_{1/2}$ values of about 10-30 seconds and recovery levels of ~80% (Fig. 8E). The same $t_{1/2}$ values and recovery levels were obtained if the fluorescence recovery of membrane-associated Dvl2-GFP puncta were monitored (Fig. 8F). Therefore, neither dynamics were altered significantly by Wnt-induced recruitment of the puncta to the plasma membrane.

Discussion

Our work was focussed on the in vivo interaction between Dvl2 and axin, and the relevance of this interaction for the signalling activity of Dvl2. Our main conclusion is that the function of Dvl2 to signal depends on its ability to form a dynamic platform to recruit axin, and additional binding partners such as CKIϵ. As discussed below, we think it likely that this property of Dvl2 to act as a dynamic recruitment platform is also relevant for its activity to transduce the Wnt signal.

The roles of the DIX and DAX domains in the Dvl2-axin interaction

We have shown that the DIX domain of Dvl2 is critical for its ability to recruit axin, and also CKIϵ. Both these ligands appear to bind to Dvl1 with relatively low affinity (Kishida et al., 2001; Kishida et al., 1999). We have proposed previously that the DIX-dependent dynamic self-association of Dvl2 results in a high local concentration of ligand binding sites (e.g. within the PDZ and DEP domains) that allows Dvl2 to recruit low-affinity binding partners (Schwarz-Romond et al., 2007).

By contrast, the DAX domain of axin was not required for its recruitment into Dvl2 puncta. This was somewhat unexpected, given that the DAX domain has similar molecular properties to the DIX domain, mediating self-association in vitro (Hsu et al., 1999; Kishida et al., 1999; Luo et al., 2004; Sakanaka and Williams, 1999) and dynamic self-assembly in vivo (Fig. 7). Furthermore, purified DAX domain can form filaments in vitro by head-to-tail interactions that are mediated by β-strands (Schwarz-Romond et al., 2007). Although we cannot rule out that endogenous axin may depend on DAX-dependent self-association for its interaction with Dvl2 during Wnt signalling, our evidence nevertheless suggests that the DAX domain may function in a process other than responding to Dvl2. Given that the DAX domain contributes to the signalling function of axin (Kishida et al., 1999; Sakanaka and Williams, 1999), one possibility is that this domain may mediate the efficient assembly of the destruction complex. As in the case of the DIX domain, the underlying principle might be the creation of a high local concentration of binding sites for ligands to accelerate their efficient recruitment. This could be a critical functional property, given that the physiological intracellular concentration of axin appears to be exceedingly low (Lee et al., 2003).

How does recruitment of axin by Dvl2 lead to Wnt signalling activity?

It was previously shown in cell-free systems that recombinant Dvl can inhibit the activity of axin to promote GSK3β phosphorylation of β-catenin (Kishida et al., 1999) and to destabilise it (Lee et al., 2003). Thus, the interaction between Dvl and axin could trigger a direct conformational change of axin that blocks its stimulatory effect on GSK3β (Kishida et al., 1999; Lee et al., 2003). Alternatively, this block could involve a third component, for example CKIϵ: as we have shown, CKIϵ can also be recruited into Dvl protein assemblies, and can synergise with Dvl2 to mediate efficient signalling.

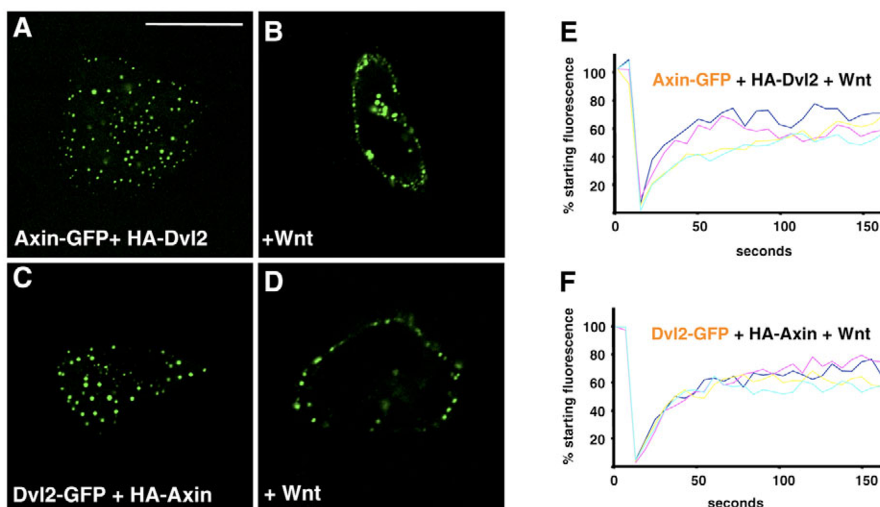

Fig. 8. Dvl2-axin puncta remain dynamic after Wnt-induced recruitment to the plasma membrane. (A-D) Fixed HeLa cells, co-expressing Fz8, LRP6 and GSK3β (as in Fig. 1B) in addition to (A,B) HA-Dvl2 plus axin-GFP or (C,D) Dvl2-GFP plus HA-axin, (A,C) before or (B,D) after 2 hours of exposure to recombinant Wnt3a. (E,F) Graphs of FRAP experiments conducted in HeLa cells, transfected and Wnt-stimulated as in B,D; individual membrane-associated puncta were bleached, and fluorescence recovery of (E) axin-GFP or (F) Dvl2-GFP was monitored, as in Fig. 7. Note that the recovery rates of these puncta are similar to those of cytoplasmic Dvl2-GFP puncta, and of axin-GFP recruited into cytoplasmic Dvl2 puncta, in HeLa cells (not shown) and in COS-7 cells (Fig. 7B-D).

Importantly, we have demonstrated that the interaction between Dvl2 and axin is highly dynamic and reversible (Fig. 7), even after recruitment to the plasma membrane after Wnt stimulation (Fig. 8). This implies that Dvl2 does not simply sequester axin, and prevent it from assembling the β-catenin destruction complex. Indeed, in *Drosophila*, Dsh promotes the recruitment not only of axin-GFP, but also of endogenous APC, to the plasma membrane during Wingless signalling (Cliffe et al., 2003), implying that the recruitment step per se does not block the binding between axin and its other interaction partners.

However, we found that the interaction between Dvl2 and axin alters the *dynamic* behaviour of axin: Dvl2 increases the on- and off-rates of axin into puncta (Fig. 7), indicating that Dvl2 accelerates the mobility of axin. This implies that the affinity between axin and some of its ligands is decreased as a result of its interaction with Dvl2, possibly reflecting a conformational change of axin (see above), its phosphorylation by CKIϵ, or modification by another co-recruited enzyme. Quite possibly, these axin ligands are the components of the β-catenin destruction complex, notably APC and GSK3β. We should emphasize that this change in the dynamic behaviour of axin (and/or its ligands) could not have been detected by steady-state analysis approaches, such as colocalisation studies by immunofluorescence (e.g. Cliffe et al., 2003; Fagotto et al., 1999; Kishida et al., 1999; Smalley et al., 1999), since these cannot distinguish between stable or even irreversible interactions (largely determined by low off-rates), and highly transient mobile interactions that are based on high on- and off-rates.

Transduction of the Wnt signal by Dvl2

We have focussed on the Wnt-independent signalling activity of Dvl2, and its interaction with axin and CKIϵ. These proteins are clearly functionally relevant, not only for the Wnt-independent activity of Dvl proteins, but also during normal transduction of the Wnt signal (Cliffe et al., 2003; Cong et al., 2004a; Hino et al., 2003; Kishida et al., 2001; Kishida et al., 1999; Penton et al., 2002; Peters et al., 1999; Price, 2006; Smalley et al., 1999). We note that Dvl proteins are also substrates of CKIϵ (Gao et al., 2002; Hino et al., 2003; Klein et al., 2006; Klimowski et al., 2006; McKay et al., 2001; Peters et al., 1999), so phosphorylation of Dvl protein might change their activity (Klein et al., 2006; Klimowski et al., 2006), but this phosphorylation may also simply reflect a 'side-effect' of the recruitment (consistent with findings in *Drosophila* that at least some of the CKIϵ phosphorylation sites of Dsh are not required for its rescue activity) (Strutt et al., 2006).

In *Drosophila*, Dsh is required for, and promotes, the recruitment of axin to the plasma membrane during Wnt signalling (Cliffe et al., 2003). Furthermore, Wnt stimulation of cells results in the phosphorylation of LRP6 by CKIγ (Davidson et al., 2005; Zeng et al., 2005), which in turn results in the recruitment of axin to the plasma membrane (Tamai et al., 2004). Indeed, recent results have shown that Dvl proteins are required for the phosphorylation of LRP6 by CKIγ (Bilic et al., 2007). Note also that Dvl protein, by virtue of its binding to the receptor Fz (Wong et al., 2003), could be brought into proximity with LRP coreceptors during Wnt stimulation, since the action of Wnt ligand clusters Fz and LRP (Carron et al., 2003; Cong et al., 2004b).

How does Dvl promote the recruitment of axin to the plasma membrane during Wnt signalling? One possible scenario is that Dvl primarily controls the phosphorylation of LRP by relocating Dvl-associated CKIϵ from the cytoplasm to the Fz-LRP receptor complex, thus promoting LRP phosphorylation directly by CKIϵ, or indirectly by CKIϵ, through CKIγ. In this scenario, the recruitment of axin to the plasma membrane would be a secondary consequence of a primary Dvl-mediated phosphorylation event. Alternatively, Dvl could function primarily to relocate axin from the cytoplasm to the membrane during Wnt signalling. Since this relocation includes APC (at least in *Drosophila*) (Cliffe et al., 2003), and most likely other axin-associated proteins such as GSK3β, these could prime the CKI-mediated phosphorylation of LRP (Zeng et al., 2005). The latter would then increase the binding between axin and LRP, resulting in the retention of axin at the plasma membrane.

Possibly, both recruitment activities of Dvl contribute to its normal function in transducing the Wnt signal. Indeed, they are expected to reinforce each other: Dvl-mediated recruitment of CKIϵ to LRP could promote LRP phosphorylation, and thus axin recruitment, whereas Dvl-mediated recruitment of axin to LRP could also promote LRP phosphorylation, albeit indirectly, and thus axin retention. Importantly, our evidence indicates that the effects of Dvl on its targets during Wnt signalling are highly transient (Fig. 8). Thus, Dvl appears to be an exquisitely dynamic adaptor, or clustering factor, mediating rapid and flexible interactions between its transient binding partners.

Materials and Methods

Plasmids

The following previously described plasmids were used: HA-Dvl2, HA-CKIϵ (Schwarz-Romond et al., 2002); M1 and M2 mutant derivatives of HA-Dvl2, TPR_ΔDIX, and GST-DIX (Schwarz-Romond et al., 2007); Dvl2-GFP (Smalley et al., 1999); HA-ΔDIX, GFP-DIX, axin-GFP (Schwarz-Romond et al., 2005); HA-axin (Kusano and Raab-Traub, 2002); *Xenopus* Fz8 and GSK3β (He et al., 1997; He et al., 1995); human LRP6 (Zeng et al., 2005).

For FLAG-Dvl2, full-length Dvl2 was subcloned as a *Bam*HI/*Eco*RI fragment into pCDNA FLAG (Invitrogen); Dvl299, Dvl389 and Dvl520 (spanning amino acids 1-299, 1-389 and 1-520 of Dvl2, respectively; Fig. 1) were inserted into the same vector. For FLAG-DIX, residues 1-114 of mouse Dvl2 and flanking linker sequences (N′, LKRASET; C′, LSGRIVTD) (see also Schwarz-Romond et al., 2007) were subcloned into the *Not*I site of pCDNA FLAG. ΔDAX was generated by deleting the DAX domain from axin-GFP (Schwarz-Romond et al., 2005). Site directed mutagenesis was used to produce the M12 mutant version (F774S V800A F801A in human axin) of HA-tagged axin (Kusano and Raab-Traub, 2002). All constructs were confirmed by sequencing.

Cell transfection, immunofluorescence and luciferase assays

Simian COS-7, and human HeLa, HEK 293 and HEK 293T cells were grown in 10% CO_2 and DMEM containing 10% foetal calf serum (FCS) and 1% penicillin-streptomycin, using standard techniques. Transfection of cells with a total of 1-3 μg plasmid DNA per well (in 6-well plates) was carried out by the calcium phosphate-DNA precipitation method, for immunofluorescence analysis and FRAP (fluorescence recovery after photobleaching), as described (Schwarz-Romond et al., 2002), or by FuGENE 6 (Roche) according to the manufacturers' instructions (for Figs 5 and 6). Endogenous Dvl2 or CKIϵ in HEK 293T cells were depleted by siRNA against Dvl2 or CKIϵ (100 nM Smartpool, Dharmacon Inc.) using Lipofectamine 2000 (Invitrogen).

Fixation of cells and immunofluorescence were carried out as described previously (Schwarz-Romond et al., 2005); image analysis was done typically 24 hours, or 30-36 hours, post-transfection for Dvl2 or axin puncta, respectively. A range of antibodies against Dvl2 was tested (α-Dvl2 #3216, Cell Signaling Technology; E-15, H-75, C-19, G-19, Santa Cruz Biotechnology), but none of these was useable as a tool to detect endogenous Dvl2 (either by immunofluorescence, or by western blotting without prior immunoprecipitation). Indeed, the only antibody that produced a specific signal by immunofluorescence was an affinity-purified anti-Dvl2 rabbit antiserum [previously characterised (Semenov and Snyder, 1997) and kindly provided by M. Semenov]. Overexpressed Dvl2 (Fig. 4) was detected with a mouse monoclonal antibody against mouse Dvl2 (clone 10B5, Santa

Cruz Biotechnology). The following mouse monoclonal antibodies were also used: α-HA (clone 3F10, Roche, UK); α-FLAG M2 (F1804, Sigma); α-CKIϵ (BD Transduction Laboratories).

To measure the signalling activity of Dvl2 in transfected HEK 293T or 293 cells, we used a quantitative reporter assay as a highly specific read-out for transcription mediated by TCF/LEF transcription factors based on TOPFLASH (Korinek et al., 1997) or SuperTOP (DasGupta et al., 2005) containing tandem binding sites for TCF transcription factors. Luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega), essentially as described previously (Rosin-Arbesfeld et al., 2000): relative luciferase values were obtained from duplicate or triplicate samples (from two to four independent experiments), by dividing firefly luciferase values from SuperTOP by renilla luciferase values from an internal control (CMV-renilla), to control for transfection efficiency, and the degree of Dvl2-mediated stimulation ('fold stimulation') was determined relative to transfected control cells without Dvl2 overexpression; standard deviations were calculated.

To stimulate the Wnt pathway, recombinant Wnt3a (R&D Systems) was added to the culture medium according to the manufacturer's instructions (200 ng/ml of serum-free medium).

Confocal image analysis and photobleaching experiments

Images were obtained using Bio-Rad 1024 (Hemel Hempstead, UK) and Zeiss LSM510 (Jena, Germany) confocal microscopes (Schwarz-Romond et al., 2005). Double-label images are of Cy5/Cy3 or Cy5/GFP-FITC conjugates, detected with standard filter-sets and laser lines. FRAP (fluorescence recovery after photobleaching) experiments in COS-7 or HeLa cells were carried out as described previously (Nichols, 2002; Schwarz-Romond et al., 2005).

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References

Arce, L., Yokoyama, N. N. and Waterman, M. L. (2006). Diversity of LEF/TCF action in development and disease. *Oncogene* **25**, 7492-7504.

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* **12**, 2610-2622.

Bienz, M. and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311-320.

Bilic, J., Huang, Y.-L., Davidson, G., Zimmermann, T., Cruciat, C.-M., Bienz, M. and Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes Dishevelled-dependent LRP6 phosphorylation. *Science* **316**, 1619-1622.

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

Capelluto, D. G., Kutateladze, T. G., Habas, R., Finkielstein, C. V., He, X. and Overduin, M. (2002). The DIX domain targets dishevelled to actin stress fibres and vesicular membranes. *Nature* **419**, 726-729.

Carron, C., Pascal, A., Djiane, A., Boucaut, J. C., Shi, D. L. and Umbhauer, M. (2003). Frizzled receptor dimerization is sufficient to activate the Wnt/β-catenin pathway. *J. Cell Sci.* **116**, 2541-2550.

Chang, W., Lloyd, C. E. and Zarkower, D. (2005). DSH-2 regulates asymmetric cell division in the early C. elegans somatic gonad. *Mech. Dev.* **122**, 781-789.

Cliffe, A., Hamada, F. and Bienz, M. (2003). A Role of Dishevelled in relocating Axin to the plasma membrane during Wingless signaling. *Curr. Biol.* **13**, 960-966.

Cong, F., Schweizer, L. and Varmus, H. (2004a). Casein kinase Iϵ modulates the signaling specificities of dishevelled. *Mol. Cell. Biol.* **24**, 2000-2011.

Cong, F., Schweizer, L. and Varmus, H. (2004b). Wnt signals across the plasma membrane to activate the β-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. *Development* **131**, 5103-5115.

DasGupta, R., Kaykas, A., Moon, R. T. and Perrimon, N. (2005). Functional genomic analysis of the Wnt-wingless signaling pathway. *Science* **308**, 826-833.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A. and Niehrs, C. (2005). Casein kinase 1γ couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* **438**, 867-872.

Fagotto, F., Jho, E., Zeng, L., Kurth, T., Joos, T., Kaufmann, C. and Costantini, F. (1999). Domains of axin involved in protein-protein interactions, wnt pathway inhibition, and intracellular localization. *J. Cell Biol.* **145**, 741-756.

Gao, Z. H., Seeling, J. M., Hill, V., Yochum, A. and Virshup, D. M. (2002). Casein kinase I phosphorylates and destabilizes the β-catenin degradation complex. *Proc. Natl. Acad. Sci. USA* **99**, 1182-1187.

Hawkins, N. C., Ellis, G. C., Bowerman, B. and Garriga, G. (2005). MOM-5 Frizzled regulates the distribution of DSH-2 to control C. elegans asymmetric neuroblast divisions. *Dev. Biol.* **284**, 246-259.

He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. and Dawid, I. B. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in Xenopus embryos. *Nature* **374**, 617-622.

He, X., Saint-Jeannet, J. P., Wang, Y., Nathans, J., Dawid, I. and Varmus, H. (1997). A member of the Frizzled protein family mediating axis induction by Wnt-5A. *Science* **275**, 1652-1654.

Hino, S., Michiue, T., Asashima, M. and Kikuchi, A. (2003). Casein kinase Iϵ enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of β-catenin. *J. Biol. Chem.* **278**, 14066-14073.

Hsu, W., Zeng, L. and Costantini, F. (1999). Identification of a domain of Axin that binds to the serine/threonine protein phosphatase 2A and a self-binding domain. *J. Biol. Chem.* **274**, 3439-3445.

Itoh, K., Brott, B. K., Bae, G. U., Ratcliffe, M. J. and Sokol, S. Y. (2005). Nuclear localization is required for Dishevelled function in Wnt/β-catenin signaling. *J. Biol.* **4**, 3.

Julius, M. A., Schelbert, B., Hsu, W., Fitzpatrick, E., Jho, E., Fagotto, F., Costantini, F. and Kitajewski, J. (2000). Domains of axin and disheveled required for interaction and function in wnt signaling. *Biochem. Biophys. Res. Commun.* **276**, 1162-1169.

Kimelman, D. and Xu, W. (2006). The β-catenin destruction complex: insights and questions from a structural perspective. *Oncogene* **25**, 7482-7491.

Kishida, M., Hino, S., Michiue, T., Yamamoto, H., Kishida, S., Fukui, A., Asashima, M. and Kikuchi, A. (2001). Synergistic activation of the Wnt signaling pathway by Dvl and casein kinase Iϵ. *J. Biol. Chem.* **276**, 33147-33155.

Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M. and Kikuchi, A. (1999). DIX domains of dvl and axin are necessary for protein interactions and their ability To regulate β-catenin stability. *Mol. Cell. Biol.* **19**, 4414-4422.

Klein, T. J., Jenny, A., Djiane, A. and Mlodzik, M. (2006). CKIϵ/discs overgrown promotes both Wnt-Fz/β-catenin and Fz/PCP signaling in Drosophila. *Curr. Biol.* **16**, 1337-1343.

Klimowski, L. K., Garcia, B. A., Shabanowitz, J., Hunt, D. F. and Virshup, D. M. (2006). Site-specific casein kinase 1ϵ-dependent phosphorylation of Dishevelled modulates β-catenin signaling. *FEBS J.* **273**, 4594-4602.

Klingensmith, J., Nusse, R. and Perrimon, N. (1994). The Drosophila segment polarity gene disheveled encodes a novel protein required for response to the wingless signal. *Genes Dev.* **8**, 118-130.

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

Kusano, S. and Raab-Traub, N. (2002). I-mfa domain proteins interact with Axin and affect its regulation of the Wnt and c-Jun N-terminal kinase signaling pathways. *Mol. Cell. Biol.* **22**, 6393-6405.

Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M. W. (2003). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol.* **1**, E10.

Li, L., Yuan, H., Weaver, C. D., Mao, J., Farr, G. H., 3rd, Sussman, D. J., Jonkers, J., Kimelman, D. and Wu, D. (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.

Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.

Luo, W., Zou, H., Jin, L., Lin, S., Li, Q., Ye, Z., Rui, H. and Lin, S. C. (2004). Axin contains three separable domains that confer intramolecular, homodimeric, and heterodimeric interactions involved in distinct functions. *J. Biol. Chem.* **280**, 5054-5060.

Malbon, C. C. and Wang, H. Y. (2006). Dishevelled: a mobile scaffold catalyzing development. *Curr. Top. Dev. Biol.* **72**, 153-166.

McKay, R. M., Peters, J. M. and Graff, J. M. (2001). The casein kinase I family in Wnt signaling. *Dev. Biol.* **235**, 388-396.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L. and Moon, R. T. (1999). Establishment of the dorsal-ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J. Cell Biol.* **146**, 427-437.

Moriguchi, T., Kawachi, K., Kamakura, S., Masuyama, N., Yamanaka, H., Matsumoto, K., Kikuchi, A. and Nishida, E. (1999). Distinct domains of mouse dishevelled are responsible for the c-Jun N-terminal kinase/stress-activated protein kinase activation and the axis formation in vertebrates. *J. Biol. Chem.* **274**, 30957-30962.

Nichols, B. J. (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell Biol.* **4**, 374-378.

Park, T. J., Gray, R. S., Sato, A., Habas, R. and Wallingford, J. B. (2005). Subcellular localization and signaling properties of dishevelled in developing vertebrate embryos. *Curr. Biol.* **15**, 1039-1044.

Penton, A., Wodarz, A. and Nusse, R. (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.

Peters, J. M., McKay, R. M., McKay, J. P. and Graff, J. M. (1999). Casein kinase I transduces Wnt signals. *Nature* **401**, 345-350.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev.* **14**, 1837-1851.

Price, M. A. (2006). CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev.* **20**, 399-410.

Rodrigues, G. A. and Park, M. (1993). Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. *Mol. Cell. Biol.* **13**, 6711-6722.

Rosin-Arbesfeld, R., Townsley, F. and Bienz, M. (2000). The APC tumour suppressor has a nuclear export function. *Nature* **406**, 1009-1012.

Rothbächer, U., Laurent, M. N., Deardorff, M. A., Klein, P. S., Cho, K. W. and Fraser,

S. E. (2000). Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. *EMBO J.* **19**, 1010-1022.

Sakanaka, C. and Williams, L. T. (1999). Functional domains of axin. Importance of the c terminus as an oligomerization domain. *J. Biol. Chem.* **274**, 14090-14093.

Sakanaka, C., Leong, P., Xu, L., Harrison, S. D. and Williams, L. T. (1999). Casein kinase Iϵ in the wnt pathway: regulation of β-catenin function. *Proc. Natl. Acad. Sci. USA* **96**, 12548-12552.

Schwarz-Romond, T., Asbrand, C., Bakkers, J., Kuhl, M., Schaeffer, H. J., Huelsken, J., Behrens, J., Hammerschmidt, M. and Birchmeier, W. (2002). The ankyrin repeat protein Diversin recruits Casein kinase Iϵ to the β-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling. *Genes Dev.* **16**, 2073-2084.

Schwarz-Romond, T., Merrifield, C., Nichols, B. J. and Bienz, M. (2005). The Wnt signalling effector Dishevelled forms dynamic protein assemblies rather than stable associations with cytoplasmic vesicles. *J. Cell Sci.* **118**, 5269-5277.

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P. J. G., Kikuchi, A., Yoshiki, Y. and Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nat. Struct. Mol. Biol.* **14**, 484-492.

Semenov, M. V. and Snyder, M. (1997). Human dishevelled genes constitute a DHR-containing multigene family. *Genomics* **42**, 302-310.

Shiomi, K., Uchida, H., Keino-Masu, K. and Masu, M. (2003). Ccd1, a novel protein with a DIX domain, is a positive regulator in the Wnt signaling during zebrafish neural patterning. *Curr. Biol.* **13**, 73-77.

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

Spink, K. E., Polakis, P. and Weis, W. I. (2000). Structural basis of the Axin-adenomatous polyposis coli interaction. *EMBO J.* **19**, 2270-2279.

Strutt, H., Price, M. A. and Strutt, D. (2006). Planar polarity is positively regulated by casein kinase Iϵ in Drosophila. *Curr. Biol.* **16**, 1329-1336.

Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z. and He, X. (2004). A mechanism for Wnt coreceptor activation. *Mol. Cell* **13**, 149-156.

Theisen, H., Purcell, J., Bennett, M., Kansagara, D., Syed, A. and Marsh, J. L. (1994). dishevelled is required during wingless signaling to establish both cell polarity and cell identity. *Development* **120**, 347-360.

Torres, M. A. and Nelson, W. J. (2000). Colocalization and redistribution of dishevelled and actin during Wnt-induced mesenchymal morphogenesis. *J. Cell Biol.* **149**, 1433-1442.

Umbhauer, M., Djiane, A., Goisset, C., Penzo-Mendez, A., Riou, J. F., Boucaut, J. C. and Shi, D. L. (2000). The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/β-catenin signalling. *EMBO J.* **19**, 4944-4954.

Walston, T., Tuskey, C., Edgar, L., Hawkins, N., Ellis, G., Bowerman, B., Wood, W. and Hardin, J. (2004). Multiple Wnt signaling pathways converge to orient the mitotic spindle in early C. elegans embryos. *Dev. Cell* **7**, 831-841.

Wharton, K. A., Jr (2003). Runnin' with the Dvl: proteins that associate with Dsh/Dvl and their significance to Wnt signal transduction. *Dev. Biol.* **253**, 1-17.

Wong, H. C., Mao, J., Nguyen, J. T., Srinivas, S., Zhang, W., Liu, B., Li, L., Wu, D. and Zheng, J. (2000). Structural basis of the recognition of the dishevelled DEP domain in the Wnt signaling pathway. *Nat. Struct. Biol.* **7**, 1178-1184.

Wong, H. C., Bourdelas, A., Krauss, A., Lee, H. J., Shao, Y., Wu, D., Mlodzik, M., Shi, D. L. and Zheng, J. (2003). Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell* **12**, 1251-1260.

Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J. and Nusse, R. (1995). The dishevelled protein is modified by wingless signaling in Drosophila. *Genes Dev.* **9**, 1087-1097.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J. and Moon, R. T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr. Biol.* **6**, 1302-1306.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., 3rd, Lee, J. J., Tilghman, S. M., Gumbiner, B. M. and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90**, 181-192.

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J. and He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438**, 873-877.

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