

The Wnt signalling effector Dishevelled forms dynamic protein assemblies rather than stable associations with cytoplasmic vesicles

Thomas Schwarz-Romond, Christien Merrifield, Benjamin J. Nichols and Mariann Bienz*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK

*Author for correspondence (e-mail: mb2@mrc-lmb.cam.ac.uk)

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Summary

Dishevelled is a crucial effector upstream in the Wnt signalling pathway, but the molecular mechanism by which it transduces the Wnt signal remains elusive. Dishevelled is a cytoplasmic protein with a strong tendency to form puncta, which correlates with its potent activity in stimulating Wnt signal transduction when overexpressed. These puncta are thought to reflect cytoplasmic vesicles. However, we show here that the mammalian Dishevelled protein Dvl2 does not colocalise with known vesicle markers for clathrin-mediated or clathrin-independent endocytic pathways. Furthermore, Dvl2 puncta do not stain with lipid dyes, indicating that these puncta do not contain membranes. Instead, our evidence from live imaging by TIRF microscopy of Dvl2 tagged with green fluorescent protein (GFP-Dvl2) revealed that these puncta move in and out of the evanescent field near the plasma

membrane in an undirected fashion, and that they can grow by collision and fusion. Furthermore, high-resolution confocal microscopy and photobleaching experiments indicate that the GFP-Dvl2 puncta are protein assemblies; there is a constant exchange of GFP-Dvl2 between puncta and a diffuse cytoplasmic pool, which, therefore, are in a dynamic equilibrium with each other. The same is true for the DIX domain of Dvl2 itself and also for Axin-GFP, which equilibrates between the punctate and cytosolic pools. Our evidence indicates that Dvl2 and Axin puncta are dynamic protein assemblies rather than cytoplasmic vesicles.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/118/22/5269/DC1>

Key words: Dishevelled, Wnt signalling, Axin, DIX domain

Introduction

The Wnt signalling pathway is highly conserved between animals and humans (Logan and Nusse, 2004). It controls numerous steps in normal development and has also been linked to disease, in particular to cancer. Many of its components, and their assembly into a signalling pathway, were discovered through *Drosophila* genetics (Perrimon, 1994). It is now well established that there are different Wnt pathway branches that signal through distinct components to nuclear or cytoplasmic targets. Of these, the canonical Wnt pathway is perhaps the best understood; this pathway signals to the nucleus through the β -catenin effector, to stimulate the transcription of Wnt target genes. By contrast, the noncanonical Wnt pathways – including planar cell polarity (PCP) signalling – are less well defined in terms of their components and ultimate targets (Logan and Nusse, 2004).

One of the oldest but least understood Wnt signalling components are the Dvl proteins. Their founder member Dishevelled was discovered in flies (Klingensmith et al., 1994; Theisen et al., 1994), but functional relatives of Dishevelled (Dvl proteins) were subsequently found in all animals ranging from hydra to mammals (Wharton, 2003), the latter comprising three family members (Dvl1-3). Dvl is a particularly interesting Wnt signalling effector because both canonical and PCP signalling depend on its function. Furthermore, Dvl

appears to act at the point of divergence between these pathways and may thus discriminate between them (Boutros et al., 1998). Dvl acts upstream in the Wnt pathway and can be recruited to the plasma membrane by Frizzled (Fz), the transmembrane receptor for Wnt ligands (Axelrod et al., 1998; Chen et al., 2003; Cong et al., 2004; Miller et al., 1999; Rothbacher et al., 2000; Umbhauer et al., 2000; Yang-Snyder et al., 1996), apparently by direct binding through its conserved PDZ domain (Wong et al., 2003). Furthermore, Dishevelled interacts with Axin and can associate with this protein (Kishida et al., 1999; Li et al., 1999; Penton et al., 2002; Smalley et al., 1999), its key cytoplasmic target during canonical Wnt signalling. Dishevelled is required for the signalling-induced recruitment of Axin to the plasma membrane (Cliffe et al., 2003) and appears to have a role in mediating endocytosis of Fz receptors from the plasma membrane on its stimulation by Wnt ligand (Chen et al., 2003). Nevertheless, it is unclear how Dvl proteins function molecularly to transduce the Wnt signal from the Fz receptors to their cytoplasmic targets.

A striking property of Dvl proteins is their ability to form cytoplasmic puncta when overexpressed in cells (Axelrod et al., 1998; Kishida et al., 1999; Rothbacher et al., 2000; Smalley et al., 1999; Yang-Snyder et al., 1996). Importantly, puncta of endogenous Dvl have also been observed in *Drosophila*, *Caenorhabditis elegans*, *Xenopus* and mouse embryos (Chang

et al., 2005; Miller et al., 1999; Torres and Nelson, 2000; Walston et al., 2004; Yanagawa et al., 1995). Dvl puncta can be recruited to the plasma membrane by Fz receptors (see above), and their formation correlates with the ability of overexpressed Dvl to induce Wnt pathway activity (Capelluto et al., 2002). It has been suggested that these puncta represent associations of Dvl with cytoplasmic vesicles (Axelrod et al., 1998; Krylova et al., 2000; Miller et al., 1999; Yang-Snyder et al., 1996). Indeed, it was reported that the DIX domain of Dvl2 binds to the micelle-forming detergent dodecylphosphocholine (DPC), a phospholipid mimic, and it was proposed that this apparent phospholipid-binding property of the DIX domain results in the targeting of Dvl to cytoplasmic vesicles (Capelluto et al., 2002). This finding has influenced discussions of how Dvl may function in Wnt signal transduction (e.g. Cliffe et al., 2003; Cong et al., 2004; Moon, 2005).

In an attempt to identify these putative vesicles, we used immunofluorescence to examine the colocalisation between Dvl2 puncta and a panel of markers for endocytic vesicles in transfected mammalian cells. We report here our complete failure to discover any evidence for an association of Dvl2 with cytoplasmic vesicles or membranes. Instead, our analysis of the behaviour of GFP-Dvl2 puncta in living cells by total internal reflection fluorescence (TIRF) microscopy and of their morphology at high magnification by confocal microscopy suggest that, these puncta represent dynamic protein assemblies, which can grow by collision and fusion. Finally, we use photobleaching to show that the puncta of DIX-domain-containing proteins, such as Dvl2 and Axin, are in a dynamic equilibrium with diffuse cytosolic protein.

Materials and Methods

Plasmids and antibodies

The following plasmids were used: HA-Dvl2 (Schwarz-Romond et al., 2002); GFP-Dvl2 (Smalley et al., 1999); clathrin-light-chain-DsRed (Merrifield et al., 2002); Caveolin1-GFP, AP180C-GFP (Nichols, 2002); Arf6-HA (Naslavsky et al., 2004); Dynamin2-K44A-GFP (Henley et al., 1998); Rab7-GFP (kindly provided by Sean Munro, MRC LMB, Cambridge, UK). HA- Δ DIX is equivalent to HA-Dvl2 without its N-terminus (amino acids 1-240 deleted). The Dvl2 mutant M68/69 (Capelluto et al., 2002) was generated using Quickchange mutagenesis (Stratagene). To generate C-terminally tagged GFP-DIX, the DIX domain of Dvl2 (amino acids 1-114) was subcloned as an *EcoRI*/*Bam*HI fragment into pEGFP-N1 (Clontech). Axin was tagged with GFP at its C-terminus by subcloning a *Hind*III/*Eco*RI fragment [spanning the 862 amino acids of the full-length isoform a of human Axin and a short N-terminal extension of 38 amino acids (Kusano and Raab-Traub, 2002)] into pEGFP-N2. We noticed that the puncta of various Axin proteins are highly sensitive to their N-termini: N-terminal tagging of human Axin with red fluorescent protein compromised its puncta-forming ability, as did long N-terminal extensions, like those found in certain mouse Axin plasmids (e.g. Fagotto et al., 1999; Smalley et al., 1999; our own unpublished observations). Likewise, Axin puncta were detectable in *Drosophila* embryos only with C-terminally, but not with N-terminally tagged *Drosophila* Axin (Cliffe et al., 2003) (A. Cliffe and M.B., unpublished observations).

HA-Dvl2 was detected with antibody against the HA tag (clone 3F10; Roche, UK). The same punctate pattern was seen when an antibody against Dvl2 (clone 10B5, Santa Cruz, CA) was used, or when untagged Dvl2 was expressed and detected with the latter antibody. The following antibodies were also used: α -EEA1, α -GBF1, α -Golgin-245 (called p230) (BD Biosciences); α -AP-2 (kindly

provided by Harvey McMahon, MRC LMB, Cambridge, UK). Secondary Alexa antibodies and Fluoro-Ruby 10k dextran were from Molecular Probes (Eugene, OR). Cy3 fluorophore for conjugation to cholera toxin B (CTxB; see below) was from AP Biotech (Little Chalfont, UK).

Cell transfection, uptake experiments and fixation

COS-7 and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) and 1% penicillin-streptomycin, using standard techniques. Transfection of cells with 1-3 μ g plasmid DNA was carried out by the calcium phosphate-DNA precipitation methods as described (Schwarz-Romond et al., 2002).

For uptake experiments, cells were washed once in phosphate-buffered saline (PBS) and serum-starved for 1 hour prior to the uptake. They were then incubated in DMEM without serum at 37°C, typically for 45 minutes, with the following fluorescently-labelled conjugates: FITC-conjugated ConA (1 mg/ml; Sigma), Cy3-conjugated CTxB (0.5 mg/ml), rhodamine-labelled 10k dextran (5 mg/ml), FITC-conjugated human transferrin (10 mg/ml; Sigma), Nile Red (10 μ g/ml), FM4-64 (500 nm final concentration). Incubations for the lipid dyes were also carried out for 30, 60 and 90 minutes. After incubation, cells were washed three times in PBS before fixation in 4% paraformaldehyde. For antibody staining, cells were permeabilised with 0.5% Triton X-100 in PBS for 5 minutes.

Luciferase assays

pTOPFLASH was used for transcriptional read-out of nuclear β -catenin in 293T cells (Korinek et al., 1997). pRL-SV40 (Promega) served as internal control, and luciferase assays were performed with the Dual Luciferase Reporter Assay System (Promega). Relative luciferase activities were obtained by dividing pTOPFLASH values by pRL-SV40 values. Experiments were performed as triplicates, and error bars represent standard deviations (s.d.).

Confocal image analysis

Images were obtained using BioRad 1024 (Hemel Hempstead, UK) and Zeiss LSM510 (Jena, Germany) confocal microscopes. Double-label images are of Cy5-Cy3 or Cy5-GFP-FITC conjugates, detected with standard filter-sets and laser lines. In the case of GFP fusion proteins, cells with low fluorescence levels were selected for imaging. Soluble, purified GFP for calibration was a gift from John Kilmartin, MRC LMB, Cambridge, UK.

Images of orthogonal sections through z-stacks (composed of images obtained at 0.2 μ m intervals) were calculated with Zeiss LSM 3.2 software. Two-dimensional deconvolution of these sections was carried out with a pre-determined point-spread function within the same software package.

Photobleaching experiments

FRAP (fluorescence-recovery after photobleaching) and FLIP (fluorescence-loss in photobleaching) experiments were carried out essentially as described (Nichols, 2002). For FRAP measurements, single average-sized GFP-Dvl2 puncta were bleached with five maximum-intensity scans with the 488 and 514 nm lines of a 40 mW argon laser (Zeiss LSM510 confocal). Recovery was monitored by acquisition of images for the following 3 minutes, at 5-second intervals. The fluorescence intensity of individual neighbouring, unbleached puncta was also monitored. All fluorescence intensities (Fig. 9D,H,L) were normalised to the mean fluorescence intensity of the whole cell (as a control for nonspecific photobleaching during imaging).

The results from the FRAP experiments (Fig. 9) were confirmed by FLIP experiments, in which half of the cytoplasm of a given cell was

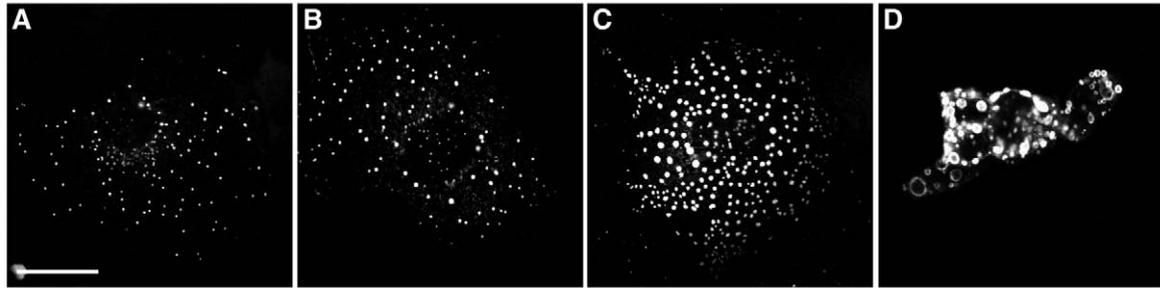


Fig. 1. Dvl2 puncta in transfected mammalian cells. COS-7 cells expressing HA-Dvl2 were fixed and stained with anti-HA antibody at the following time-points after transfection: (A) 18 hours; (B) 24 hours; (C) 36 hours; (D) 48 hours. Bar, 15 μm .

bleached and fluorescence loss was monitored from the puncta in the unbleached territory. Rapid loss of fluorescence of unbleached puncta was observed, with the puncta adjacent to the bleached area losing their fluorescence first.

TIRF microscopy

TIRF imaging was done as previously described (Merrifield et al., 2002). The acquisition of images was done at 1 frame/2 seconds; the speed of playback is 6 frames/second.

Results and Discussion

The formation of Dvl2 puncta correlates with Wnt signalling activity

Confirming previous reports (see Introduction), we found that Dvl2 forms striking puncta when overexpressed in transfected simian COS-7 cells (Fig. 1A-D). Very similar puncta are also observed in transfected human 293T or SW480 colorectal cancer cells (data not shown), and they are seen regardless of the protein tag (see Materials and Methods). Notably, similar

puncta can be observed in live cells after tagging Dvl2 with GFP (Miller et al., 1999; Smalley et al., 1999) (see below). The Dvl2 puncta become first detectable as minute pin-prick-like dots approximately 16 hours after transfection, but they have a tendency to grow with time (Fig. 1A-C) and can become very large indeed (Fig. 1D). We estimate that their diameters typically range from <200 nm to ~ 2 μm , in rough agreement with previous measurements of Dvl puncta in *Xenopus* embryos (Miller et al., 1999). Within any given transfected cell, the majority of the Dvl2 puncta tend to be of a relatively uniform size (Fig. 1A-C). Their growth appears to be not only a function of time, but also of increasing Dvl2 protein levels (data not shown).

As already mentioned, the ability of Dvl2 to form puncta correlates with its signalling activity, indicating that this property of Dvl2 might be functionally significant (Capelluto et al., 2002). This conclusion was largely based on a mutant Dvl2 with a substitution of two neighbouring amino acids in its DIX domain. We confirmed that this mutant (called M68/69) cannot form puncta (though very rarely, puncta can be observed in cells that express very high levels of Dvl2), but instead produces a diffuse cytoplasmic staining (Fig. 2B, compare with A). Diffuse cytoplasmic staining is also seen with a Dvl2 construct that lacks its DIX domain (ΔDIX) (Fig. 2C), whereas the DIX domain by itself is capable of forming puncta (see below), confirming that the DIX domain is necessary and sufficient for puncta formation (Capelluto et al., 2002). Importantly, as previously shown (Capelluto et al., 2002), the ability of M68/69 to induce Wnt signalling activity as measured by the transcription of the

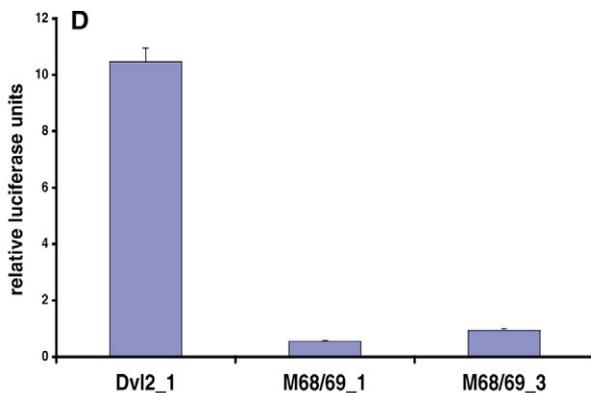
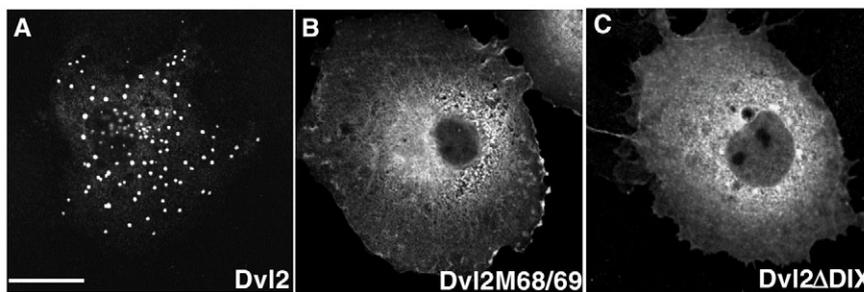


Fig. 2. Correlation between Dvl2 puncta formation and signalling activity. COS-7 cells expressing (A) wild-type, (B) M68/69 or (C) ΔDIX mutant HA-Dvl2, were fixed and stained as in Fig. 1. Notice that neither mutant is able to form puncta (see also Capelluto et al., 2002). (D) Wnt signalling activity of wild-type and M68/69 mutant HA-Dvl2 in transfected 293T cells, as measured by TOPFLASH transcription assays (see Materials and Methods). Numbers underneath bars represent μg of DNA used for transfection (1, 1 and 3 μg). Protein expression levels were confirmed by western blotting (not shown). Bar, 15 μm .

Wnt-specific reporter TOPFLASH (Korinek et al., 1997) is reduced to <10% of wild-type Dvl2 (Fig. 2D). Since the two crucial DIX-domain residues M68 and M69 were implicated in the direct binding to the phospholipid mimic DPC (Capelluto et al., 2002), the interpretation was that these residues mediated the targeting of Dvl2 to cytoplasmic vesicles. The latter was corroborated by the observation that the Dvl2 puncta overlapped with concanavalin A (conA) staining (Capelluto et al., 2002), a lectin that detects sugar-modified proteins in the plasma membrane and in cytoplasmic vesicles. This is the only known bona-fide vesicle marker for which a colocalisation with Dvl puncta has been reported.

The Dvl2 puncta do not colocalise with known vesicles markers

To examine this colocalisation between Dvl2 puncta and conA-positive vesicles, we repeated these experiments, based on uptake of fluorescently-labelled conA (see Materials and Methods). However, we were unable to detect any significant overlap between conA and Dvl2 puncta (Fig. 3A-C). We noticed that the conA fluorescence was partly punctate, presumably reflecting cytoplasmic vesicles, but in addition also produced relatively high levels of disperse fluorescence throughout the cytoplasm (Fig. 3B) (Capelluto et al., 2002). As a result, a small fraction of Dvl2 puncta appeared to coincide with conA, as indicated by occasional yellow signals in some of the merged images (Fig. 3C, arrows). However, these yellow signals were rare and were not seen in every cell. They thus do not appear to be significant.

We therefore looked for other, more specific, markers for cytoplasmic vesicles to corroborate the putative association of Dvl2 with membranous organelles. We co-stained cells transfected with Dvl2 with antibodies against its protein tag and against endogenous markers for clathrin-coated pits and early endosomes, such as endogenous early endosomal antigen 1 (EEA1) and AP-2. Both EEA1 and AP-2 stainings are clearly punctate, marking clathrin-coated pits and early endosomes as expected (Robinson, 2004; Stenmark et al., 1996), but there is no significant colocalisation with the Dvl2 puncta (Fig. 4A,B).

Dvl2 puncta also fail to colocalise with staining against endogenous Golgi markers, such as GBF1 (Fig. 4C) or Golgin-245 (data not shown). Furthermore, when we coexpressed GFP-tagged caveolin to visualise clathrin-independent endocytic vesicles (Nichols, 2003; Pelkmans et al., 2001), we also failed to detect a significant colocalisation between this marker and Dvl2 (Fig. 4D). Two further markers for endocytic vesicles were tested after coexpression (HA-Rab7 and Arf6-HA), which label late endosomes and an Arf6-positive endocytic compartment, respectively (Naslavsky et al., 2004), with the same negative results (Fig. 4E,F). Finally, we co-transfected COS-7 cells with dominant-negative constructs of AP180 or dynamin that interfere with endocytosis (Henley et al., 1998; Nichols, 2002) to see whether this affected the numbers or distribution of the Dvl2 puncta. However, neither of these constructs colocalise with the Dvl2 puncta, nor do they affect their abundance in any detectable way (Fig. 4G,H). Therefore, we failed to obtain any evidence that the Dvl2 puncta associated stably with any of these known internal membranous compartments.

Next, we carried out further uptake experiments to assay for endocytic vesicles in a way that does not rely on staining for known vesicle markers. We thus incubated serum-starved cells with fluorescently-labelled 10k dextran (as a marker for bulk fluid-uptake), with transferrin, which is internalised through classical clathrin-mediated endocytosis, or with fluorescently-labelled CTxB subunit, which is internalised through coated pits and through less well-characterised clathrin-independent endocytic pathways (Kirkham et al., 2005; Nichols, 2002). Cells were fixed after various periods of incubation time (see Materials and Methods), and internalisation of the fluorescently-labelled markers was confirmed. However, none of these experiments showed any significant colocalisation of the fluorescent label and the Dvl2 puncta (Fig. 5A-C). Again, these results argue against a stable association of Dvl2 with endocytic vesicles.

Dvl2 puncta do not stain with lipid dyes

It remained possible that Dvl2 associates with a novel type of cytoplasmic vesicle that is neither positive for known vesicle markers nor takes up any of the above compounds. We thus stained cells expressing HA-Dvl2 with Nile Red, a fluorescent dye specific for intracellular lipid droplets (Greenspan et al., 1985), and also with the lipid dye FM4-64, which allows the detection of cytoplasmic organelles with a significant lipid content, including endocytic vesicles in mammalian cells

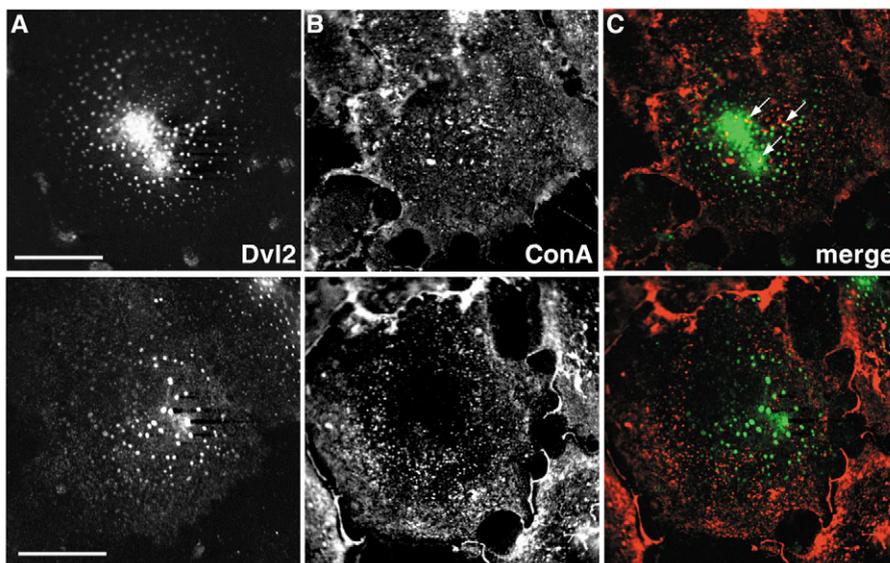


Fig. 3. Dvl2 puncta do not overlap significantly with conA fluorescence. COS-7 cells expressing HA-Dvl2 were fixed and stained as in Fig. 1 after incubation with fluorescently-labelled conA for 45 minutes; two representative cells are shown. Arrows indicate rare yellow signals that reflect coincidental green (Dvl2) and red (conA) fluorescence. Bars, 15 μ m.

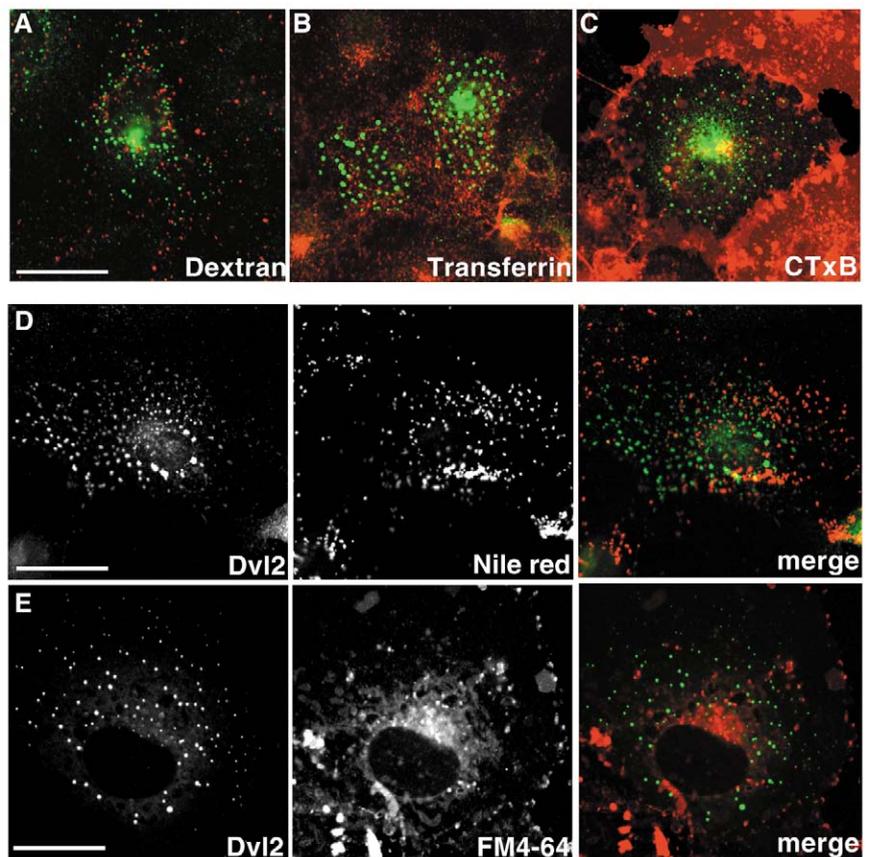
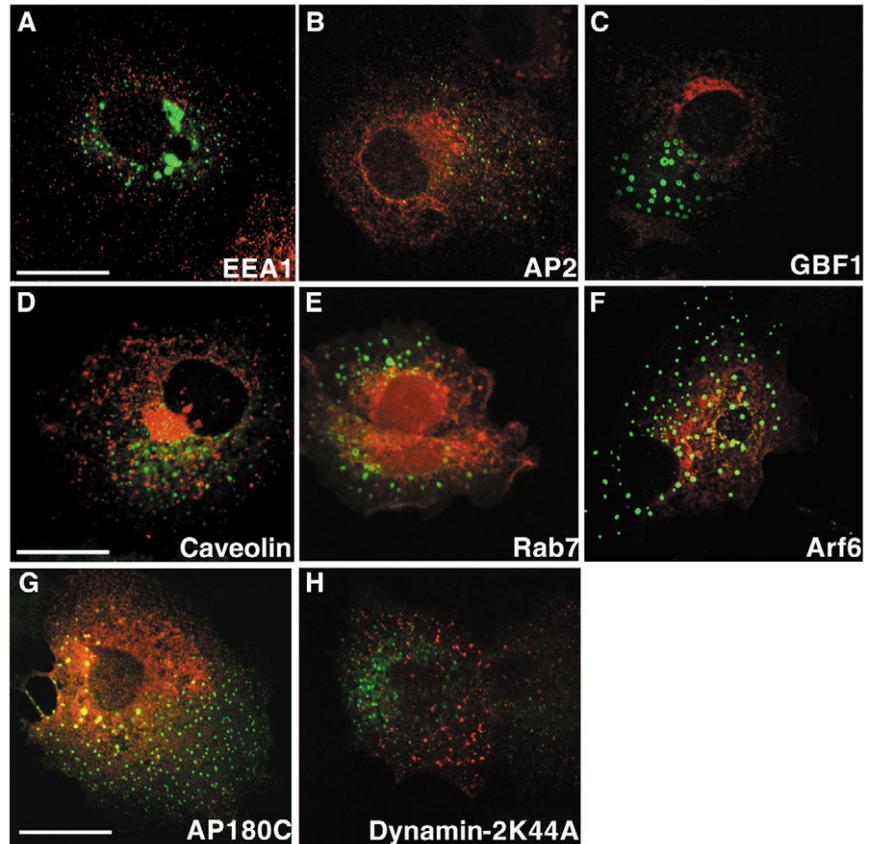
Fig. 4. Lack of colocalisation between Dvl2 puncta and endocytic markers. COS-7 cells expressing HA-Dvl2, were fixed and double-stained with antibodies against HA (green), and various endocytic markers (red) as indicated. (A-C) Endogenous endocytic and Golgi proteins; (D-F) exogenous endocytic proteins. (G,H) Overexpression of dominant-negative mutants that block endocytosis, which neither affected the abundance nor the distribution of the Dvl2 puncta. Bars, 15 μ m.

(e.g. Taraska and Almers, 2004). Once again, we failed to observe any significant colocalisation of these lipid stains with the Dvl2 puncta (Fig. 5D,E). We conclude that the Dvl2 puncta are highly unlikely to reflect stable associations with cytoplasmic organelles that are enclosed by membranes. This is supported by a biochemical analysis of endogenous Dvl in two types of human cells, which revealed that the small subcellular fraction of Dvl, pelleted with high-speed centrifugation (i.e. the P100 fraction) sedimented with the dense fractions during subsequent density-equilibrium-flotation on sucrose gradients, rather than floating with the membrane fractions (Reinacher-Schick and Gumbiner, 2001).

Dvl2 puncta are mobile and can grow by collision and fusion

We asked whether the Dvl2 puncta can associate transiently with the plasma membrane in live cells. We thus used TIRF microscopy on live COS-7 cells transfected with GFP-Dvl2, to focus at their cortical region immediately underneath the plasma membrane facing the glass cover slip (Steyer and Almers, 2001). This revealed that many of the smaller GFP-Dvl2 puncta are fairly mobile, moving in and out of the evanescent field, i.e. the \sim 100 nm cortical zone underneath the plasma membrane, within which fluorescence is detectable by this method (Steyer and Almers, 2001); the larger puncta tend to be less mobile and simply seem to tumble (supplementary material, Movie 1). We cannot detect any obvious directionality of the movements of the GFP-Dvl2 puncta. Notably, we occasionally observed collisions of two

Fig. 5. Dvl2 puncta neither coincide with endocytic vesicles nor stain with lipid dyes. COS-7 cells expressing HA-Dvl2 were fixed and stained with anti-HA antibody (green) after loading of cells with (A-C) fluorescently-labelled conjugates or (D, E) lipid dyes as indicated (red). Bars, 15 μ m.



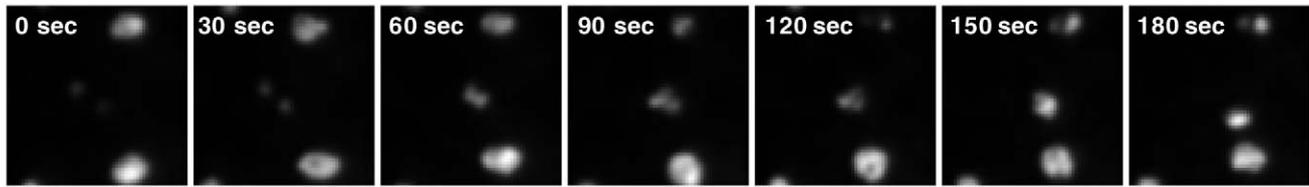


Fig. 6. GFP-Dvl2 puncta can grow by collision and fusion. Series of individual frames (30 seconds apart) taken from supplementary material Movie 2, depicting the fusion of two GFP-Dvl2 puncta in transfected COS-7 cells.

puncta, which appeared to result in a fused particle with a larger diameter than the two original puncta (Fig. 6; supplementary material, Movie 2). Some of these collision events are followed by fragmentation (supplementary material, Movie 1), indicating that the fusion of the two original puncta was not successful. Similar undirected movements of Dvl2 puncta, and collisions between them, have previously been observed in transfected mammalian cells (Smalley et al., 1999). These observations suggest that the growth of Dvl2 puncta over time (Fig. 1) is, at least partly, due to collision and fusion.

We also used two-colour TIRF microscopy to compare the GFP-Dvl2 puncta with clathrin-DsRed puncta that label coated pits and vesicles that bud off the plasma membrane into the underlying cortical cytoplasm (Merrifield et al., 2002). In this case, we focussed on cells with smaller GFP-Dvl2 puncta whose fluorescence was comparable to that of clathrin-DsRed. This revealed that the two types of puncta are distinct in almost every aspect, in particular in their localisation and mobility (Fig. 7A-C; supplementary material, Movie 3). This confirms our earlier conclusion that Dvl2 puncta do not reflect known sites of endocytosis nor incipient endocytic vesicles. Again, we were able to detect collision and fusion events of Dvl2 puncta (supplementary material, Movie 3).

Dvl2 puncta appear to contain solid protein

As mentioned above, the Dvl2 puncta, as detected by GFP fluorescence, look similar to those detected by antibody staining (Fig. 1A-C). However, in contrast to the latter, which occasionally seem hollow, especially when they are large (Fig. 1D, Fig. 4C,F), the GFP-Dvl2 puncta appear consistently more solid (Fig. 8A,B). This suggests that the apparent hollowness

of the puncta that are detected by immunofluorescence is likely to reflect an artefact due to lack of antibody accessibility to their centres. This can be demonstrated directly by antibody staining of GFP-Dvl2 puncta (Fig. 8C), which appear as green (GFP signal) and red-rimmed (antibody signal) at high magnification (Fig. 8C, merged image).

We further examined the GFP-Dvl2 puncta at high magnification by confocal microscopy (after fixation, because this improved the resolution), to see whether their morphology is consistent with that of a vesicle. We focussed on relatively large puncta with an average size of 1-2 μm (Fig. 8A, boxed). Single confocal sections through individual puncta revealed rings of strong fluorescence surrounding a centre of lower fluorescence (Fig. 8B,D-F). Some of these 'puncta' turned out to be multi-centred (Fig. 8B), somewhat reminiscent of aggregated soap bubbles. The multi-centred particles probably reflect the products of the observed fusions (Fig. 6; supplementary material, Movies 1-3). Reconstruction of orthogonal sections from z -stacks revealed a thinning of their fluorescence at opposite poles of individual puncta (Fig. 8D-F). This is particularly visible after 2D deconvolution (Fig. 8D,E). These reconstructions suggest that the three-dimensional shapes of the Dvl2 puncta resemble that of a doughnut, although we cannot rule out that the apparent asymmetry is an artefact of the reconstruction and they are, in fact, spherical. Often, the large puncta also showed signs of a grainy substructure (Fig. 8D). The morphology of these puncta and their apparent substructure are somewhat reminiscent of the previously observed nuclear inclusion bodies of aggregating proteins such as RED and Ataxin-1 (Rich et al., 1999).

Quantification of the fluorescence intensity in sections across the doughnut-like GFP-Dvl2 structures indicates a

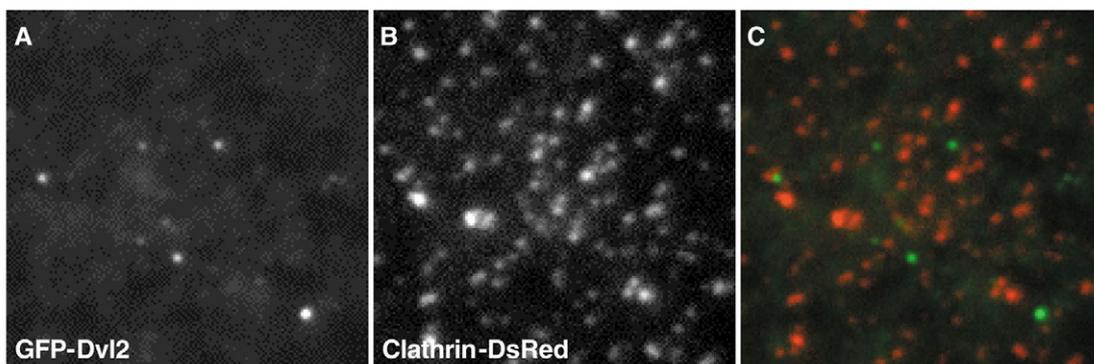


Fig. 7. GFP-Dvl2 puncta do not colocalise with clathrin-DsRed in live COS-7 cells. An individual frame, taken from supplementary material Movie 3 (frame 15), shows distinct (A) GFP-Dvl2 puncta [green in merged image (C)] and (B) clathrin-DsRed puncta in live transfected COS-7 cells.

width of the annulus of ~500-700 nm, which is wider than the limit of resolution of light microscopy (~400 nm). Moreover, the fluorescence profile across the annulus (e.g. along the horizontal green line in Fig. 8E) cannot be fitted to a simple Gaussian distribution (with a significant deviation from this model, with $P < 0.0001$, in a Runs test). It is thus unlikely that this profile is generated by the membrane of a vesicle-like structure, and argues against the possibility that GFP-Dvl2 particles are generated by assembly of a layer of GFP-Dvl2 on a membrane surface.

Calibration of the confocal microscope with a known concentration of purified, soluble GFP allowed us to estimate the concentration of GFP molecules within GFP-Dvl2 puncta (e.g. those in Fig. 8B). These are 100-500 \times brighter than 5 mg/ml GFP imaged under identical conditions, implying a concentration of GFP molecules within these puncta of >500-2500 mg/ml. This translates to ~0.1 M GFP or approximately one GFP molecule per 1-10 nm³. GFP itself, even without the larger Dvl2 protein, occupies a volume of ~5 nm³ (Ormo et al., 1996), so the observed fluorescence intensity of GFP-Dvl2-positive puncta is consistent with these structures being composed predominantly or entirely of this protein.

Dynamic equilibrium between punctate and cytosolic pools of DIX-domain proteins

We used selective photobleaching to address whether GFP-Dvl2 is stably associated with puncta or, alternatively, constantly exchanges into and out of these structures. After bleaching individual puncta to <10% of starting fluorescence, fluorescence intensity of the puncta recovered to typically 70% of the pre-bleach intensity with a half-time in the range of 10-40 seconds (Fig. 9A-D). This demonstrates that the diffuse cytosolic GFP-Dvl2 is rapidly and continuously recruited into Dvl2 puncta. Given that the intensity of neighbouring unbleached puncta did not increase within the time frame of these photobleaching experiments (Fig. 9D, dotted line), it can be argued that this process is fully reversible, in other words, that GFP-Dvl2 dissociates from the puncta as rapidly as it is recruited into them. This is supported by the results from FLIP experiments, in which half of a given cell was bleached and the fluorescence loss from unbleached puncta was monitored: although the rates of fluorescence loss depended on the position of unbleached puncta with regard to the bleached area (see Materials and Methods), we estimate that the average fluorescence loss from these puncta occurs at rates similar to the recovery rates measured in the FRAP experiments (Fig. 9D). Taken together, these photobleaching experiments demonstrate that the steady-state distribution of GFP-Dvl2 in protein assemblies or puncta results from a constant dynamic exchange between punctate and cytosolic pools.

As mentioned above, a minimal DIX-domain-containing

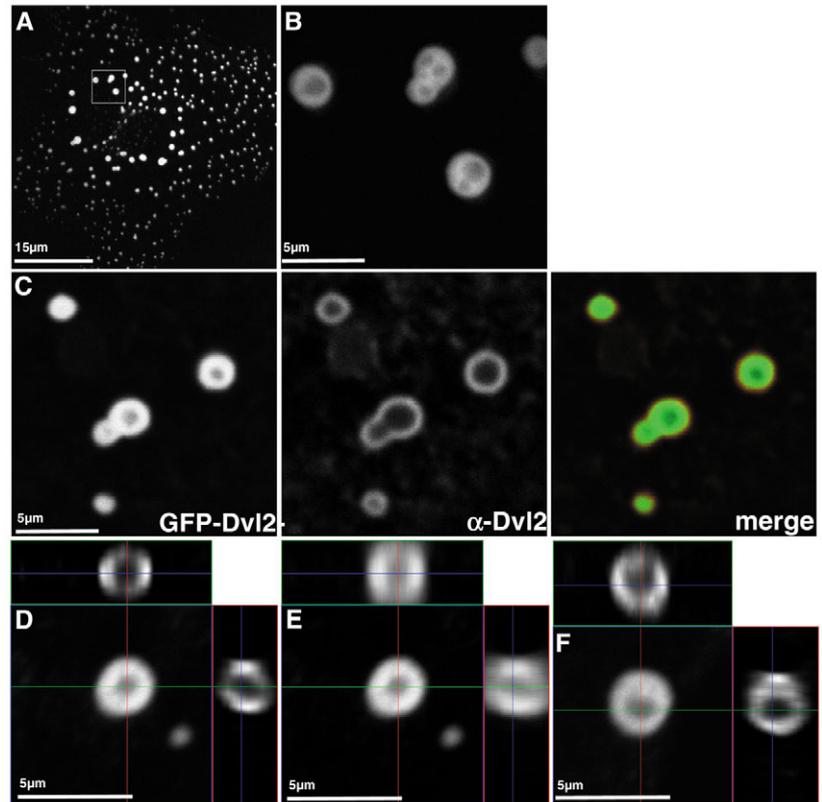


Fig. 8. Morphology of GFP-Dvl2 puncta at high magnification. (A,B) Single confocal sections of COS-7 cells expressing GFP-Dvl2; (C) GFP-Dvl2 puncta (green in merged image) were also stained with anti-Dvl2 antibody (red in merge); high magnification view in (B) corresponds to boxed section in (A). Notice the multi-centred 'puncta' in (B). (D-F) Single confocal sections through individual GFP-Dvl2 puncta (bottom left) and orthogonal reconstructions along the two coloured axes as indicated (above and to the right, respectively) from image stacks. (D,E) deconvoluted images; (F) original image corresponding to (E). All cells were fixed before imaging.

construct (GFP-DIX) forms similar puncta as full-length GFP-Dvl2 (Fig. 9E). Furthermore, the kinetics of fluorescence recovery after photobleaching are comparable to those of GFP-Dvl2, although GFP-DIX recovers slightly faster and to virtually 100% of the pre-bleaching fluorescence intensity (Fig. 9E-H). We conclude that, the puncta-forming properties of Dvl2 are conferred by its DIX domain, which mediates rapid assembly into, and disassembly from, puncta. The slightly slower recovery rate of GFP-Dvl2 compared to GFP-DIX suggests that, the exchange of the full-length protein between punctate and cytosolic pool is attenuated by its interactions with other cytosolic partners, which are mediated by its additional protein-interaction domains (see Introduction).

Axin is one of only three distinct protein species known to contain a DIX domain. Like Dvl, Axin forms puncta after overexpression in *Drosophila*, *Xenopus* and mammalian cells (Cliffe et al., 2003; Fagotto et al., 1999; Smalley et al., 1999). As expected, GFP-tagged human Axin (GFP-Axin) forms puncta similar to those of GFP-Dvl2, although they tend to be somewhat smaller (Fig. 9I; see also Materials and Methods). These puncta are also dynamic structures, although the

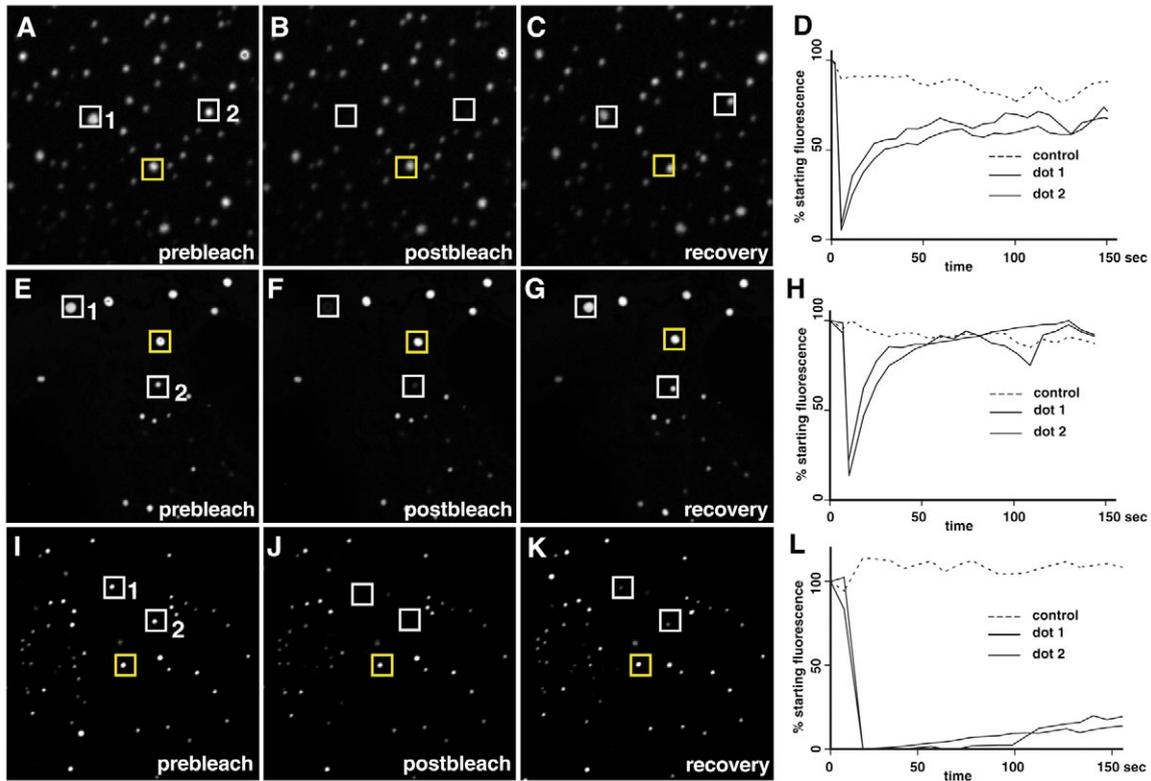


Fig. 9. Dynamic exchange of DIX-domain proteins between punctate and cytosolic pools. Average-sized puncta of (A-C) GFP-Dvl2, (E-G) GFP-DIX or (I-K) GFP-Axin were highlighted with white boxes and bleached, and fluorescence recovery was monitored for the following 3 minutes (at 5-second intervals); unbleached neighbouring puncta (yellow boxes) served as internal controls. (D,H,L) Graphs showing mean fluorescence intensities within the boxes indicated (of bleached puncta, or unbleached controls), were normalised to the mean fluorescence intensity of the whole cell.

recovery rates in the FRAP experiments are far slower than those of GFP-Dvl2, with only limited recovery (Fig. 9I-L). This suggests that the exchange of Axin between cytosolic and punctate pools is attenuated more than that of Dvl2, again probably due to interactions with other cytosolic proteins. Nevertheless, we conclude that Axin, like Dvl2, exchanges constantly between punctate and cytosolic pools.

Conclusions

Our results strongly argue against the idea, widespread in the literature, that Dvl puncta reflect stable associations of Dvl with cytoplasmic vesicles. Therefore, models that envisage a vesicle-based function of Dvl in transducing the Wnt signal (e.g. Capelluto et al., 2002; Chen et al., 2003; Cliffe et al., 2003; Moon, 2005) will need revisiting. Nevertheless, given that Dvl protein can be recruited by Fz receptors to the plasma membrane (see Introduction), it is conceivable that Dvl proteins could be transiently associated with endocytic vesicles if, and when, Fz receptors are endocytosed after Wnt ligand binding (Chen et al., 2003).

Instead, the morphology of the Dvl puncta at high resolution, their apparent growth by collision and fusion, and their constant exchange with soluble Dvl2 indicate that these puncta are in fact dynamic assemblies of Dvl2 protein. It is perhaps relevant in this context that Dvl proteins can self-associate in

vitro and in vivo (Kishida et al., 1999; Rothbacher et al., 2000). Whether this property is functionally relevant for their signalling activity remains to be seen.

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