The EMBO Journal (2012) 31, 2336–2349 | © 2012 The EMBO Journal | Some Rights Reserved 0261-4189/12
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THE EMBO JOURNAL

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Disabled-2 (Dab2) inhibits Wnt/β-catenin signalling by binding LRP6 and promoting its internalization through clathrin

Yong Jiang[1,2], Xi He[3] and Philip H Howe[1,2,*]

[1]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC, USA, [2]Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA and [3]FM Kirby Neurobiology Center, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA

Canonical Wnt signalling requires caveolin-dependent internalization of low-density lipoprotein receptor-related protein 6 (LRP6). Here we report that the tumour suppressor and endocytic adaptor disabled-2 (Dab2), previously described as an inhibitor of Wnt/β-catenin signalling, selectively recruits LRP6 to the clathrin-dependent endocytic route, thereby sequestering it from caveolin-mediated endocytosis. Wnt stimulation induces the casein kinase 2 (CK2)-dependent phosphorylation of LRP6 at S1579, promoting its binding to Dab2 and internalization with clathrin. LRP6 receptor mutant (S1579A), deficient in CK2-mediated phosphorylation and Dab2 binding, fails to associate with clathrin, and thus escapes the inhibitory effects of Dab2 on Wnt/β-catenin signalling. Our data suggest that the S1579 site of LRP6 is a negative regulatory point during LRP6-mediated dorsoventral patterning in zebrafish and in allograft mouse tumour models. We conclude that the tumour suppressor functions of Dab2 involve modulation of canonical Wnt signalling by regulating the endocytic fate of the LRP6 receptor.

The EMBO Journal (2012) **31,** 2336–2349. doi:10.1038/emboj.2012.83; Published online 10 April 2012
Subject Categories: chromatin & transcription; RNA
Keywords: CK2 kinase; disabled-2; LRP6; tumourigenesis; Wnt signalling

Introduction

Wnt signalling is critical during various developmental and physiological processes, and is implicated in human disease, including cancer (Polakis, 2000; Clevers, 2006). Stabilization and translocation of intracellular β-catenin into the nucleus are essential in canonical Wnt signalling. In the absence of Wnt, β-catenin is assembled in a multimeric β-catenin destruction complex that contains Axin, adenomatous polyposis coli (APC), casein kinase I (CKI) and glycogen synthase kinase 3 (GSK3). In this complex, β-catenin is sequentially phosphorylated by CKI and GSK3 and targeted for degradation through the $SCF^{\beta\text{-}TrCP}$-mediated ubiquitin-proteasome pathway. In the presence of Wnt, activation of the Frizzled (Fz) and low-density-lipoprotein receptor-related protein co-receptors (LRP5/6/arrow) leads to destabilization of the β-catenin destruction complex, inactivation of GSK3 and inhibition of β-catenin phosphorylation and degradation (Fuerer *et al*, 2008). Stabilized β-catenin then enters the nucleus and interacts with transcriptional regulators, including leukocyte enhancer factor-1 and T cell factor, and leads to Wnt responsive gene expression (MacDonald *et al*, 2009).

Mechanistic details of how Wnt stimulation leads to β-catenin stabilization and GSK3 inactivation are emerging, and it appears that receptor phosphorylation and internalization play critical roles. Wnt induces clustering of Fz and lipoprotein receptor-related protein 6 (LRP6) receptors on aggregates of the scaffolding protein Dvl to form endocytic 'LRP6 signalosomes' (Bilic *et al*, 2007), leading to the sequential phosphorylation of LRP6 co-receptor by a 'dual kinase' mechanism involving GSK3 and CK1 at multiple PPPSP sites. This phosphorylation of LRP6 promotes the recruitment and engagement of LRP6 with Axin and the β-catenin destruction complex (Davidson *et al*, 2005; Zeng *et al*, 2005), ultimately leading to GSK3 inactivation and accumulation of β-catenin. Wnt also stimulates the formation of phosphatidylinositol 4, 5-bisphosphate, through Fz and Dvl, to promote LRP6 aggregation and phosphorylation, as well as Axin translocation (Pan *et al*, 2008). In addition, these phospho-LRP6 signalosomes contain caveolin (Yamamoto *et al*, 2006), a marker of caveolae (cholesterol-rich invaginations of the plasma membrane), which colocalizes with LRP6 and is required for Wnt-mediated LRP6 endocytosis and β-catenin accumulation. Subsequent studies (Yamamoto *et al*, 2008) have shown that LRP6 internalization can occur through both caveolin- and clathrin-dependent pathways in order to activate and inhibit β-catenin signalling, respectively. Wnt3A stimulation induces caveolin-dependent internalization of LRP6, as well as its phosphorylation and recruitment of Axin to the plasma membrane, ultimately leading to β-catenin accumulation. In contrast, Dickkopf 1 (DKK1), which inhibits Wnt3A-dependent stabilization of β-catenin, induced the internalization of LRP6 with clathrin and failed to induce LRP6 phosphorylation and β-catenin stabilization (Yamamoto *et al*, 2008). In addition, noncanonical Wnt5a signalling requires Fz2-induced internalization through clathrin to activate Rac and inhibit β-catenin accumulation (Sato *et al*, 2010). More recent studies not only support the role of endocytosis in Wnt-mediated signalling but also mechanistically link receptor internalization to inhibition of GSK3. Wnt signalling is shown to trigger the sequestration of GSK3 from the cytosol into multivesicular bodies (MVBs) so that the enzyme becomes separated from its cytosolic

*Corresponding author. Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 86 Jonathan Lucas Street, MUSC955, Charleston, SC 29425, USA. Tel.: +1 843 792 4687; Fax: +1 843 792 3200; E-mail: howep@musc.edu

Received: 26 October 2011; accepted: 14 March 2012; published online: 10 April 2012

substrates. In such a model, the LRP6/GSK3-containing signalosomes are internalized, presumably in caveolin-containing vesicles, and recruited to the ESCRT (endosomal sorting complex required for transport) complex and sorted to vesicles destined for formation of MVBs, wherein GSK3 is sequestered from cytosolic β-catenin, which accumulates, enters the nucleus and engages in transcriptional activation (Taelman *et al*, 2010).

Disabled-2 (Dab2) is a widely expressed endocytic adaptor protein shown to be involved in several receptor-mediated signalling pathways (Xu *et al*, 1995; Hocevar *et al*, 2001; Prunier *et al*, 2004). Its expression levels have a significant impact on cancer development and cellular differentiation (Morris *et al*, 2002; Jiang *et al*, 2008). Aberrantly low levels of Dab2 protein have been found in many tumour-derived cell lines (Sheng *et al*, 2000) and its ectopic expression in these cancer cell lines results in cell cycle arrest and a decreased rate of cell proliferation (Zhoul *et al*, 2005). As such, Dab2 has been assigned the label of a tumour suppressor gene (Tong *et al*, 2010). In addition, Dab2 plays a critical role in cellular differentiation and lineage commitment. Its expression is upregulated and essential during megakaryocytic differentiation (Tseng *et al*, 2001) and during retinoic acid (RA)-induced differentiation of F9 teratocarcinoma cells into visceral endoderm-like cells (Smith *et al*, 2001).

In the current study we confirm the concept of 'two endocytic routes-two outcomes' proposed for Wnt signalling (Yamamoto *et al*, 2008) and demonstrate that Dab2 expression levels regulate whether LRP6 is internalized through the caveolin or clathrin endocytic pathways. We show that LRP6 binds directly to Dab2 in a Wnt- and casein kinase 2 (CK2)-dependent manner. Dab2 has no effect on the rate of LRP6 internalization but rather regulates its interaction with caveolin or clathrin. In the absence of Dab2 LRP6 interacts with caveolin in a Wnt-dependent manner, and in its presence Wnt stimulation leads to sequestration of LRP6 with clathrin. Collectively, the data suggest that the tumour suppressor functions of Dab2 involve modulation of Wnt/β-catenin signalling by regulating the endocytic fate of the LRP6 receptor.

Results

Caveolin-mediated endocytosis is essential for Wnt/β-catenin signalling

To evaluate the role of endocytosis in Wnt/β-catenin signalling, we employed a dominant-negative dynamin (DN) K44A that acts as an inhibitor of both clathrin and caveolin-dependent receptor endocytosis (Doherty and McMahon, 2009). Co-transfection of mouse F9 cells with the Wnt/β-catenin TOPFlash reporter and increasing concentrations of DN K44A results in dose-dependent inhibition of Wnt3A-mediated transactivation (Figure 1A). To characterize the endocytic pathway mediating Wnt3A-induced TOPFlash transactivation, we treated cells with monodansyl-cadaverine (MDC), known to block clathrin-mediated endocytosis, or nystatin, which disrupts lipid rafts and inhibits caveolin-dependent endocytosis (Yamamoto *et al*, 2008), prior to stimulation with Wnt3A. The results (Figure 1B) demonstrate that in F9 cells nystatin, and not MDC, inhibits Wnt3A-induced TOPFlash transactivation. In addition, in F9 cells ectopically expressing Dab2 (F9-Dab2 cells), TOPFlash transactivation was significantly abolished regardless of nystatin or MDC treatment (Figure 1B). Further, F9 cells are demonstrated to be Wnt signalling competent as shown by nuclear β-catenin accumulation and cyclin D1 induction (Figure 1C), whereas in F9 cells ectopically expressing Dab2 (F9-Dab2 cells) Wnt/β-catenin signalling is attenuated (Figure 1C). Confirming the TOPFlash transactivation shown above, nystatin, and not MDC, treatment of F9 cells blocks Wnt3A-induced nuclear β-catenin accumulation and cyclin D1 expression (Figure 1D), but in F9-Dab2 cells Dab2 inhibits these Wnt3A-induced responses irrespective of whether the caveolin (nystatin) or clathrin (MDC) endocytic pathway is modulated (Figure 1E). To test this further, we silenced both caveolin and clathrin in F9 and F9-Dab2 cells by siRNA. The results demonstrate that caveolin knockdown blocks Wnt/β-catenin signalling in F9 cells, whereas clathrin knockdown attenuates the inhibitory effect of Dab2 on Wnt/β-catenin signalling in F9-Dab2 cells (Supplementary Figure S1). These results suggest that Dab2 sequesters LRP6 towards clathrin, away from its interaction with caveolin, and that its inhibitory effect does not require LRP6 endocytosis.

Dab2 has no effect on the internalization rate of LRP6

To address the effects of Dab2 on LRP6 endocytosis more directly, we labelled cell surface proteins with a reversible biotinylating agent, Sulfo-NHS-SS-Biotin, prior to Wnt3A treatment for various lengths of time to allow internalization of cell surface proteins including LRP6. After treatment, biotin was cleaved (reduced) or not (non-reduced) from all remaining cell surface proteins using glutathione (Semënov *et al*, 2008). Internalized cell surface proteins, including LRP6, are protected from the biotin removal procedure. We observed that total biotin-labelled LRP6 was not affected by either Wnt3A treatment or Dab2 expression (Figure 1F; 'non-reduced' lanes). We also observed a time-dependent internalization of LRP6 following Wnt3A treatment, which was not effected by the expression of Dab2. In both F9 and F9-Dab2 cells, LRP6 is rapidly internalized following Wnt3A treatment, with maximal internalization observed at 1–2 h, followed by its reappearance on the cell surface at 4 h (Figure 1F; 'reduced' lanes). α-EGFR receptor immunoblotting was used as a control, demonstrating its lack of internalization in response to Wnt3A stimulation. The results also demonstrate that nystatin, and not MDC, blocks LRP6 internalization in F9 cells, whereas in F9-Dab2 cells MDC, and not nystatin, blocks LRP6 internalization (Supplementary Figure S2). Thus, Dab2 does not affect LRP6 internalization but rather sequesters the receptor towards an interaction with clathrin and not caveolin.

Alternatively, we also compared the amount of cell surface LRP6 available for biotin labelling following Wnt3A-induced internalization. The results (Figure 1G) demonstrate that in the absence (F9 cells) or presence of Dab2 (F9-Dab2 cells) LRP6 is rapidly internalized in response to Wnt3A (maximally between 1–2 h) and recycled to the cell surface for labelling at 4 h. These Wnt3A-induced rates of LRP6 internalization are consistent with those presented above and, importantly, demonstrate that Dab2 expression does not affect the internalization rate of LRP6.

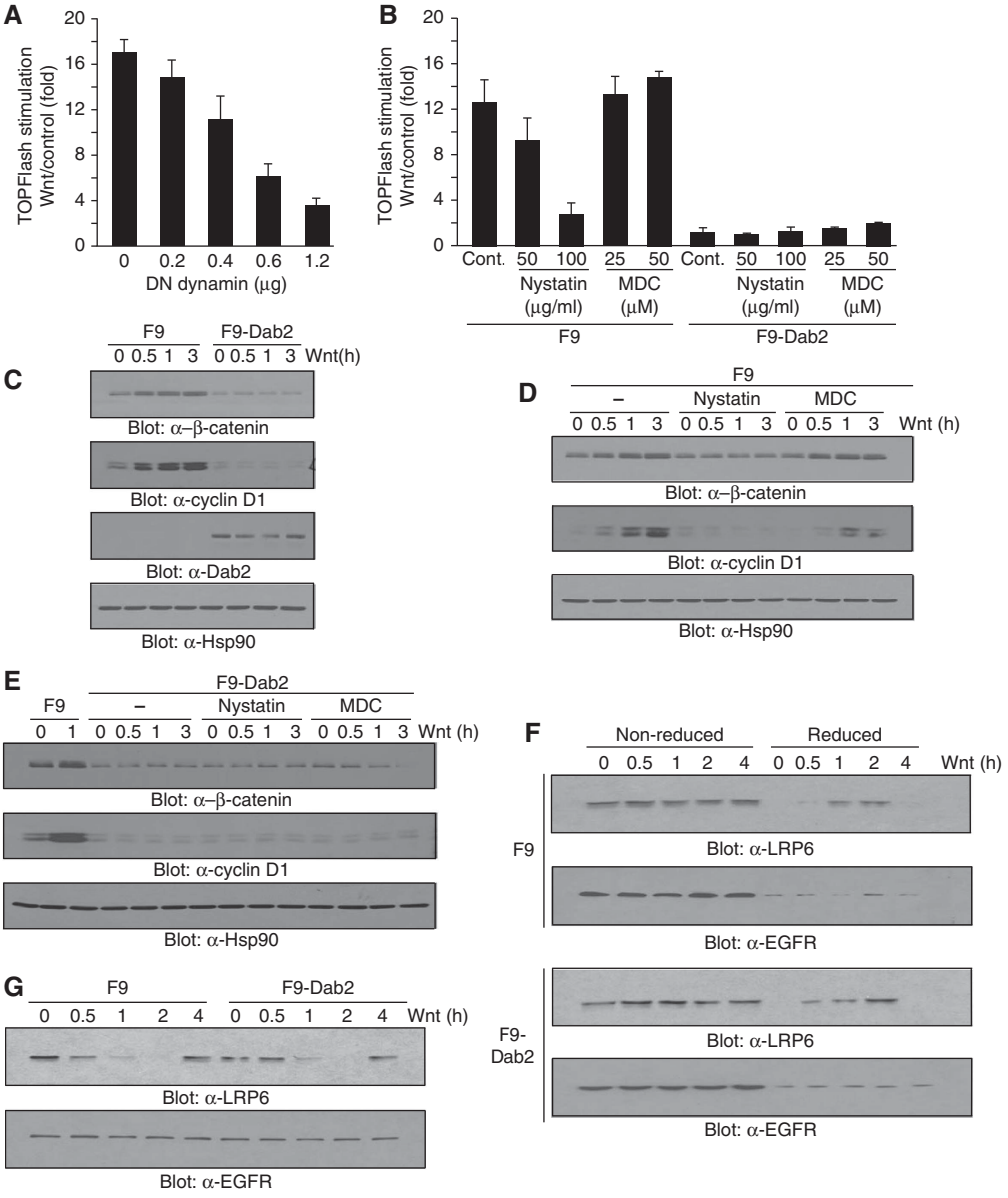

Figure 1 Caveolin-mediated endocytosis is essential for Wnt/β-catenin signalling and Dab2 has no effect on the internalization rate of LRP6. (**A**) Increasing concentrations of DN K44A were co-transfected into F9 cells with 0.5 μg of TOP/FOPFlash luciferase reporter construct and luciferase activity assays were performed and quantitated. (**B**) F9 and F9-Dab2 cells were transfected with 0.5 μg of TOP/FOPFlash luciferase reporter constructs. Cells were pre-treated for 1 h with the indicated concentration of nystatin or MDC prior to Wnt3A stimulation and luciferase activity determination. (**C**) F9 and F9-Dab2 cells were treated with Wnt3A conditioned media for the indicated times, and whole-cell lysates (WCL) were immunoblotted with α–β-catenin, α-cyclin D1 and α-Dab2 antibodies. α-Hsp90 immunoblot is used as a loading control. (**D**) F9 and (**E**) F9-Dab2 cells were pre-treated with nystatin (100 μg/ml) or MDC (50 μM) for 1 h prior to Wnt3A stimulation for the indicated times. WCL were immunoblotted with α–β-catenin and α-cyclin D1 antibodies. (**F**) F9 and F9-Dab2 cell surface proteins were biotinylated using a reversible biotinylation agent (cleavable Sulfo-NHS-SS-Biotin). Following labelling, cells were stimulated with Wnt3A for the indicated times at 37°C and cells were split into two groups. WCL from one group (non-reduced) were precipitated with avidin-agarose beads followed by immunoblot analysis with α-LRP6 antibody. The other group of cells (reduced) was treated with a glutathione-containing solution to strip away any biotinylated proteins remaining on the cell surface. WCL were prepared and internalized cell surface proteins protected from biotin stripping were precipitated with avidin-agarose beads followed by immunoblotting with α-LRP6 antibody. EGF receptor endocytosis was used as control (immunoblotting with α-EGF receptor antibody). (**G**) F9 and F9-Dab2 cells were stimulated with Wnt3A for the times indicated and placed at 4°C for a 1 h labelling with a biotinylation agent (non-cleavable Sulfo-NHS-LC-Biotin). WCL were prepared and biotin-labelled cell surface proteins were precipitated with avidin-agarose beads and analysed by immunoblotting with α-LRP6 antibody. Figure source data can be found with the Supplementary data.

Dab2 preferentially shunts LRP6 toward clathrin-dependent endocytosis

Since Dab2 had no effect on the endocytic rate of LRP6, we next examined, by co-immunoprecipitation analyses, whether it might regulate LRP6's internalization route. The results (Figures 2A and B) demonstrate that in the absence of Dab2 (F9 cells) LRP6 co-immunoprecipitates caveolin in a Wnt-dependent manner, whereas in the presence of Dab2 (F9-Dab2 cells) LRP6 co-immunoprecipitates clathrin also in a Wnt-dependent manner. Similar results were observed in

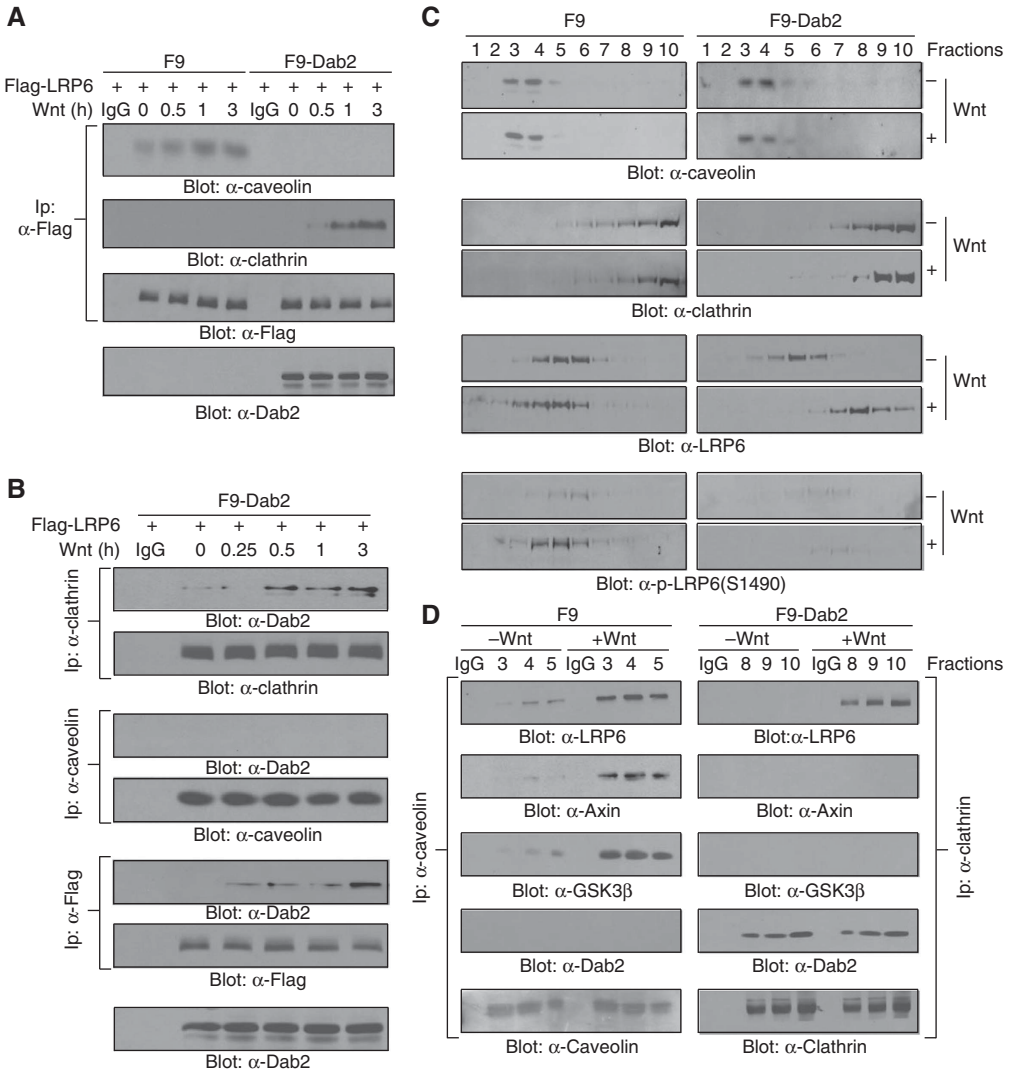

Figure 2 Dab2 shunts LRP6 towards clathrin-dependent endocytosis. (**A**) Flag-tagged LRP6 (10 μg) was transfected into F9 and F9-Dab2 cells. Following transfection, cells were treated with Wnt3A for the times indicated and WCL were immunoprecipitated with control α-IgG or α-Flag antibody. Immunocomplexes were subjected to immunoblot analysis with α-caveolin, α-clathrin and α-Flag antibodies. WCL were also immunoblotted with α-Dab2 antibody to demonstrate relative Dab2 levels. (**B**) Flag-tagged LRP6 (10 μg) was transfected into F9 Dab2 cells and, following transfection, stimulated with Wnt3A for the indicated times. WCL were subjected to immunoprecipitation with α-clathrin, α-caveolin and α-Flag antibodies. Immunocomplexes were immunoblotted with α-Dab2 antibody to detect clathrin/Dab2, caveolin/Dab2 and Flag-LRP6/Dab2 interactions. WCL were also immunoblotted with α-Dab2 antibody for demonstrating relative Dab2 levels. IgG antisera was used as negative control for the various immunoprecipitations. (**C**) WCL from F9 and F9-Dab2 cells treated with or without Wnt3A for 1 h were fractionated by sucrose density gradient centrifugation. Fractions were collected and aliquots of each were analysed by immunoblotting with α-caveolin, α-clathrin, α-LRP6 and α-p-LRP6 (S1490). (**D**) Pooled sucrose gradient fractions (light fractions no. 3–5 and heavy fractions no. 8–10) were immunoprecipitated with α-caveolin (left panels) and α-clathrin (right panels) antibodies. Immunocomplexes were immunoblotted with α-LRP6, α-Axin, α-GSK3β, α-Dab2, and α-caveolin or α-clathrin. IgG antisera was used as negative control for the various immunoprecipitations. Figure source data can be found with the Supplementary data.

two different stable Dab2 F9 clones (Supplementary Figure S3). To further examine this redistribution of LRP6 towards clathrin by Dab2, we fractionated the lipid raft (caveolin-containing) and nonlipid raft (clathrin-containing) fractions by sucrose density gradient centrifugation. The detergent-resistant membranes including caveolin-1 (Figure 2C, fractions 3, 4 and 5) were present in the lighter-density fractions, whereas the soluble membranous and non-membranous materials including clathrin (fractions 7–10) were present in the higher-density fractions. As depicted in Figure 2C, Wnt3A stimulation had little effect on the relative distribution of either caveolin or clathrin, either in the absence (F9 cells) or in the presence of Dab2 (F9-Dab2 cells). LRP6 and phospho-LRP6 (S1490) distributions, on the other hand, were significantly impacted by the presence of Dab2. In the absence of Dab2, in F9 cells, LRP6 is shown to co-sediment with the lighter caveolin-containing fractions and Wnt3A stimulation appears to promote this distribution. In the presence of Dab2, in F9-Dab2 cells, LRP6 similarly co-sediments with the caveolin-containing fractions; however, following Wnt3A stimulation, LRP6 redistributes with the higher-density clathrin-containing fractions (Figure 2C). Furthermore, phospho-LRP6 (S1490, indicative of activated Wnt signalling), which co-sediments with caveolin-containing fractions in

Wnt3A-stimulated F9 cells, is not present in F9-Dab2 cells following Wnt3A stimulation. To further probe the activation of Wnt/β-catenin signalling in the various gradient fractions, we pooled the lower (fractions 3–5) and higher (fractions 8–10) density fractions and determined the relative associations and distributions of other β-catenin modulators (Figure 2D). As shown in the figure, in F9 cells, α-caveolin Ips co-immunoprecipitate LRP6, Axin and GSK3β in a Wnt-dependent manner, whereas in F9-Dab2 cells, α-clathrin Ips co-immunoprecipitate only LRP6 in a Wnt3A-dependent manner, and not Axin or GSK3β. Collectively, these results suggest that Dab2 regulates the localization of the LRP6 receptor following Wnt3A stimulation. In the presence of Dab2, Wnt3A promotes LRP6 association with clathrin, resulting in its failure to interact with and be phosphorylated by the β-catenin destruction complex.

To directly observe the effects of Dab2 on the endocytic fate of the LRP6 receptor, we carried out immunofluorescence analyses of LRP6 colocalization with caveolin or clathrin in the absence (F9 cells) or presence (F9-Dab2 cells) of Dab2. The results show that LRP6 is primarily localized peripherally along the cell surface and that caveolin is also membrane associated, as well as found in other regions of the cell. Wnt stimulation, in the absence of Dab2, promotes internalization of LRP6 and its co-localization with caveolin (Figure 3A). Quantitatively, ~41% of the LRP6 puncta were co-localized with caveolin and not clathrin (Supplementary Figure S4A). In the presence of Dab2, although LRP6 is

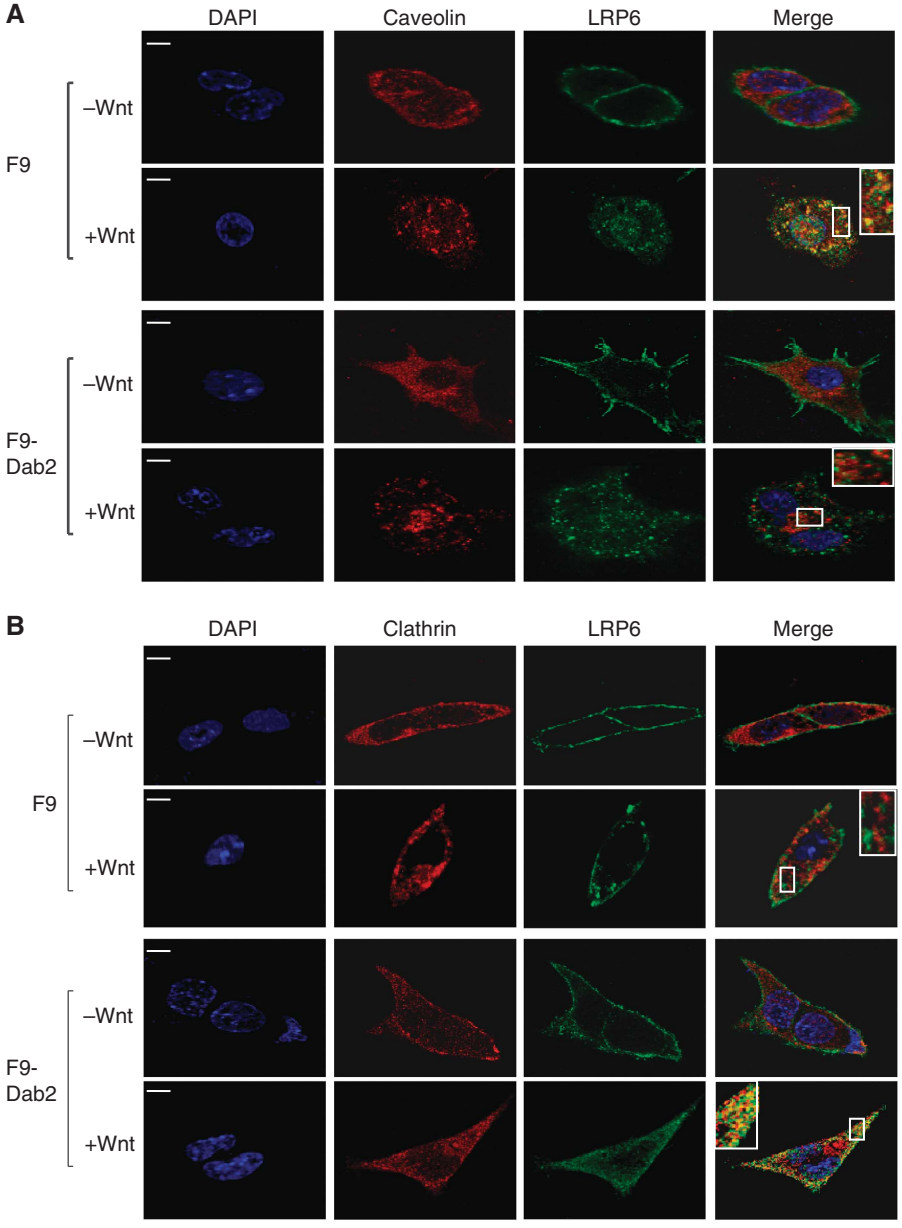

Figure 3 Subcellular distribution and co-localization of LRP6/caveolin and LRP6/clathrin in the absence and presence of Dab2. Immunofluorescence of caveolin and LRP6 (**A**) and clathrin and LRP6 (**B**) in F9 and F9-Dab2 cells treated with or without Wnt3A for 1 h. After treatment all cells were fixed and stained with α-LRP6, α-caveolin or α-clathrin antibodies. LRP6 is shown in green, and caveolin and clathrin are shown in red. Co-localization of LRP6 with caveolin or clathrin is depicted by the yellow in the panels labelled 'merge'. DAPI was used to stain the nuclei. Scale bars, 5 μm.

similarly internalized following Wnt3A stimulation, it fails to co-localize with caveolin (Figure 3A). Clathrin staining in F9 cells is primarily restricted to the cell surface (Figure 3B), and following Wnt3A stimulation it fails to co-localize with the LRP6 receptor. In contrast, in F9-Dab2 cells, Wnt3A treatment promotes LRP6/clathrin co-localization in cytoplasmic puncta (Figure 3B). Quantitatively, ~44% of the LRP6 puncta co-localize with clathrin and not caveolin (Supplementary Figure S4B). Meanwhile, in F9 cells ~57% of internalized LRP6 co-localizes with Axin and ~39% with GSK3β, confirming Dab2's inhibitory effect on Wnt/β-catenin signalling (Supplementary Figure S5). These immunocytochemical analyses confirm the biochemical assays and demonstrate that Dab2 regulates the internalization of LRP6 and dictates whether it is endocytosed through the caveolin or clathrin pathways.

Dab2 interactions with LRP6

To further characterize Dab2/LRP6 interactions, we performed co-immunoprecipitation analyses in F9-Dab2 cells with LRP6 membrane-tethered intracellular domain constructs (Figure 4A). The results demonstrate that the full-length intracellular domain of LRP6 (ΔN) containing the 5 PPP(S/T)P motifs is capable of immunoprecipitating Dab2 (Figure 4A). The ΔNG construct that contains the C-terminal 3 PPP(S/T)P motifs is also capable of interacting with Dab2. Deletion and/or mutation of the N-terminal 2 PPP(S/T)P motifs has no effect on Dab2 interaction. But for interaction with Dab2, the C-terminal domain-containing 3 PPP(S/T)P motifs are required (Figure 4A). Importantly, the data also demonstrate that overexpression of these constructs, without Wnt3A stimulation, is sufficient to induce interaction with Dab2, supporting previous studies demonstrating that mere overexpression of these motifs activates Wnt/β-catenin signalling (Tamai *et al*, 2004). Reciprocally, the N-terminal 247 amino acids of Dab2, including the phosphotyrosine-binding domain (PTB), are required for interacting with endogenous LRP6 (Figure 4B), and this interaction requires Wnt stimulation, in that in the absence of Wnt3A treatment full-length Dab2 could not co-immunoprecipitate LRP6 (Figure 4B; FL-). Direct interaction of Dab2 with LRP6 is shown in Figure 4C, demonstrating that a GST fusion of the PTB domain of Dab2 (GST-PTB) is capable of precipitating overexpressed intracellular LRP6 (ΔN) and the C-terminal 3 PPP(S/T)P (ΔNG) LRP6 constructs ectopically expressed in cells. The C-terminal phosphorylation motifs are required in that the ΔN1 construct, lacking these, fails to bind the Dab2 PTB domain. Thus, the PTB domain of Dab2 is required for binding directly to the intracellular domain of LRP6.

Wnt-induced phosphorylation of LRP6 by GSK3 promotes the engagement of LRP6 with the scaffolding protein Axin (Zeng *et al*, 2005). To determine whether phosphorylation of LRP6 is required for Dab2 interactions, we performed *in situ* mutagenesis of all possible phosphorylation sites within the ΔNG construct region of Flag-tagged full-length LRP6. We mutated Ser, Thr and Tyr residues to Ala (Figure 4D and Supplementary Figure S6A) in the context of the full-length Flag-tagged LRP6 construct and one of the mutant constructs containing Ala substitutions in all three of the C-terminal PPP(S/T)P motifs (nos. 1572, 1590 and 1607). The data demonstrate that of all the possible phosphorylation sites within this region, the only mutant that completely inhibited Wnt3A-induced Dab2/LRP6 interaction was the phospho-mutant at Ser1579 (Figure 4D). When transfected in F9-Dab2 cells with the TOP/FOPFlash reporter, this mutant (S1579A) also failed to attenuate Wnt-induced transactivation of this promoter as efficiently as the other phospho-mutant LRP6 constructs (Supplementary Figure S6B). Analysis of this site (SAEE) suggests that it is conserved in LRP5/6 and *Arrow*, and that it conforms to the CK2 phosphorylation consensus SxEE/D (Pinna, 2002).

Further support for CK2-mediated phosphorylation of LRP6 at S1579 is provided by the *in vitro* kinase assay using *in vitro* transcribed and translated recombinant WT (wild type)-ΔNG LRP6 or phospho-mutant S1579A ΔNG LRP6 as substrate and either purified recombinant CK2 kinase or α-CK2 immunoprecipitates as the source of kinase. As shown in Supplementary Figure S7A (upper panel), when [^{35}S]-methionine-labelled substrate is employed, purified CK2 and Ips from Wnt3A-stimulated cells promote the upward mobility shift of the WT-ΔNG LRP6 and not the phospho-mutant S1579A ΔNG LRP6. In the absence of purified CK2 or with α-CK2 Ips from control, non-stimulated cells, neither the band corresponding to WT-ΔNG LRP6 nor the phospho-mutant S1579A ΔNG LRP6 is upwardly shifted. When the *in vitro* assay is performed with [^{32}P]-γ-ATP, again only the WT-ΔNG LRP6 and not the phospho-mutant S1579A ΔNG LRP6 is directly phosphorylated by either purified CK2 or α-CK2 Ips from Wnt3A-stimulated cells (Supplementary Figure S7A, lower panel). Further, employing an *in vitro* kinase assay with specific CK2 peptide substrate, the data (Supplementary Figure S7B) demonstrate that Wnt3A stimulation of either F9 or F9-Dab2 cells results in a time-dependent induction of CK2 activity.

To test whether CK2-mediated phosphorylation of this site modulated LRP6/Dab2 interactions, we transfected F9-Dab2 cells with Flag-tagged WT or S1579A phospho-mutant LRP6 constructs and treated the cells with apigenin, a selective inhibitor of CK2, prior to stimulation with Wnt3A (Figure 4E). WT-LRP6, and not the phospho-mutant S1579A construct, is shown to co-immunoprecipitate Dab2 and clathrin following Wnt3A stimulation (Figure 4E). Co-immunoprecipitation of LRP6 with Dab2 and clathrin does not occur in the absence of Wnt3A stimulation. The phospho-mutant S1579A construct is still capable of co-immunoprecipitating caveolin in Wnt3A-stimulated cells, suggesting that the S1579 site is specific for LRP6 association with Dab2 and clathrin and not caveolin. Further, apigenin inhibits, in a dose-dependent manner, Wnt3A-induced interaction of WT-LRP6 with Dab2 and clathrin. These data are supported by the TOP/FOPFlash luciferase data (Supplementary Figure S8) demonstrating that, in F9-Dab2 cells, Dab2 inhibits reporter transactivation induced by the WT-LRP6 receptor but not by the S1579A phospho-mutant LRP6 receptor. Further, Dab2 inhibition of WT-LRP6-mediated reporter transactivation is sensitive to apigenin whereas the S1579A phospho-mutant LRP6 is not. In addition, sucrose sedimentation analysis (Supplementary Figure S9) demonstrates that in the presence of Dab2 (F9-Dab2), the phosphomutant S1579A LRP6 receptor fails to shift towards the heavier clathrin fractions upon Wnt3A stimulation as does the WT-LRP6 (compare with Figure 2C). The distribution of the S1579A LRP6 receptor is similar ± Wnt3A stimulation in the absence (F9 cells) or presence (F9-Dab2) of Dab2. This indicates that the mutant can interact with caveolin and shift distribution towards the lighter

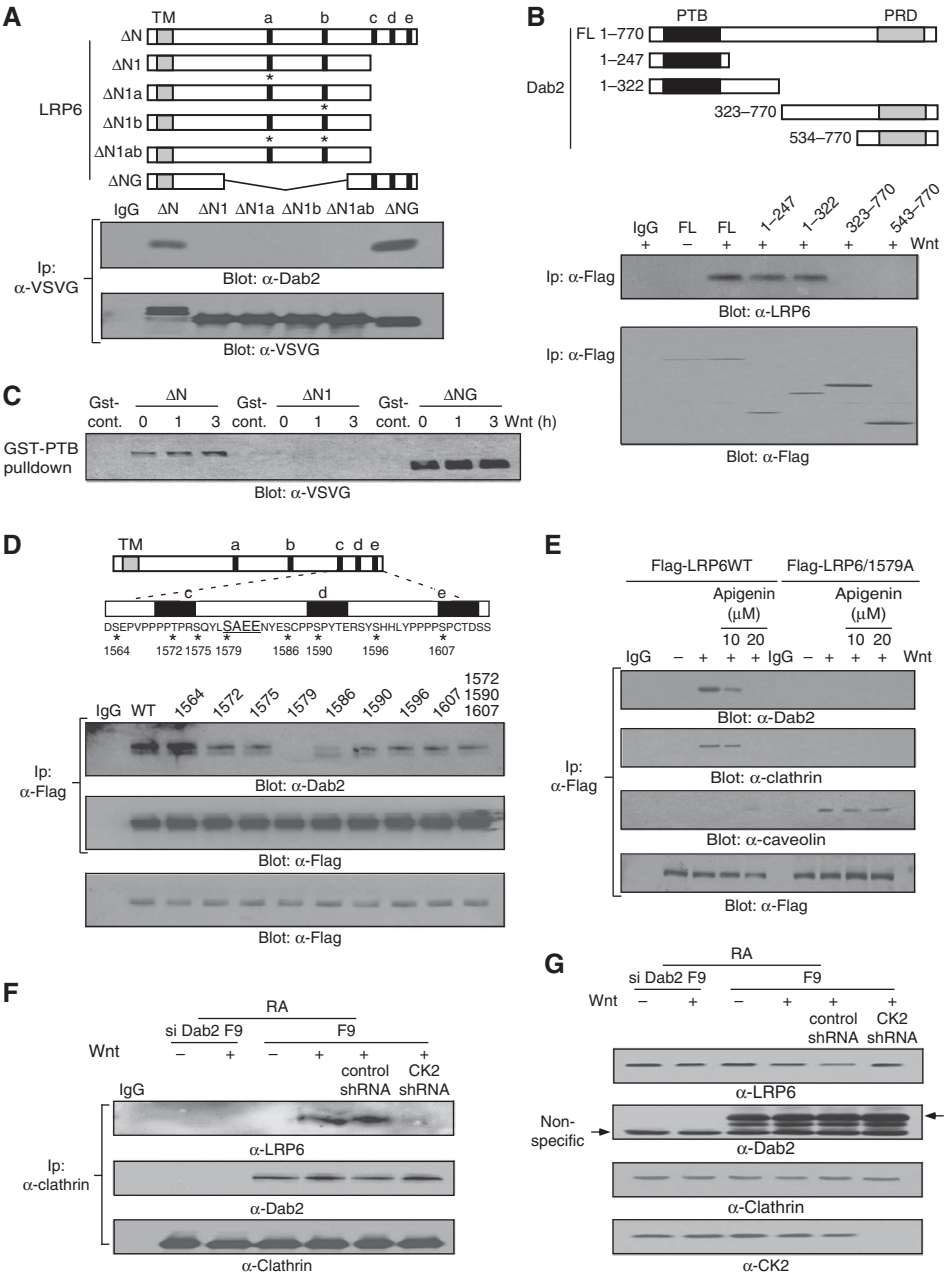

Figure 4 Dab2 interacts with LRP6. (**A**) Schematic of the VSVG-tagged LRP6 intracellular constructs utilized. The five reiterated PPPS/TP motifs are designated as a, b, c, d and e, and the asterisks (*) indicate an S or T to A substitution. 'TM' indicates the position of the transmembrane region of LRP6. Lower panels: all constructs were transfected into F9-Dab2 cells and WCL were prepared and subjected to immunoprecipitation with either α-VSVG antibody or α-IgG antisera (as negative control). Immunocomplexes were analysed by immunoblotting with α-Dab2 antibody to detect LRP6/Dab2 interactions and with α-VSVG to detect the expression of the various constructs. (**B**) Schematic of various Flag-tagged Dab2 constructs utilized. Lower panels: Dab2 constructs were transfected into F9 cells and, following transfection, treated with Wnt3A for 2 h. WCL were subjected to immunoprecipitation analysis with α-Flag antibody and α-IgG antisera (as negative control). Immunocomplexes were analysed by immunoblotting with α-LRP6 to detect Dab2/LRP6 interactions and with α-Flag to detect the expression of the various Dab2 constructs. (**C**) VSVG-tagged LRP6 intracellular mutant constructs ΔN, ΔN1 and ΔNG were transfected into F9 cells and treated with Wnt3A for the times indicated. WCL were subjected to GST-PTB pulldown assays (GST fusion protein with the N-terminal PTB domain of Dab2). The precipitated complexes were analysed by immunoblotting with α-VSVG to detect PTB/LRP6 interactions. (**D**) Schematic of the intracellular domain of LRP6 and of the various phospho-mutant sites generated. These phospho-mutants were generated in the context of full-length LRP6 and cloned in Flag-pCS2 expression vector. Also depicted is the consensus CK2 phosphorylation site 'SAEE' in LRP6. Constructs were transfected into F9 Dab2 cells and following transfection treated with Wnt3A for 2 h. WCL were immnunoprecipitated with α-Flag antibody and α-IgG antisera (as negative control). Immunocomplexes were analysed by immunoblotting with α-Dab2 to detect Dab2/LRP6 interactions and with α-Flag to detect the expression of the various Dab2 constructs. (**E**) Flag-tagged WT and phospho-mutant S1579A LRP6 constructs were transfected into F9 Dab2 cells. Following transfection, cells were pre-treated with apigenin (10 and 20 μM) for 30 min prior to a 2 h Wnt3A stimulation. WCL were immunoprecipitated with α-Flag antibody followed by immunoblotting with α-Dab2, α-clathrin and α-caveolin. (**F**) siDab2 F9 cells, WT F9 cells, control shRNA F9 cells and CK2 shRNA F9 cells were treated with 100 nM RA for 3 days. Following treatment, cells were stimulated ± Wnt3A for 2 h and WCL were immunoprecipitated with α-clathrin. The precipitated complexes were analysed by immunoblotting with α-Dab2 and α-LRP6 to detect clathrin/LRP6 and clathrin/Dab2 interactions. (**G**) Relative protein expression in the cells in (**F**) was detected by immunoblotting with α-LRP6, α-Dab2, α-clathrin and α-CK2 antibodies. Figure source data can be found with the Supplementary data.

caveolin fraction upon Wnt3A stimulation, whereas it fails to interact with clathrin and fails to redistribute with the heavier clathrin fractions upon Wnt3A stimulation. These data suggest that Wnt3A-mediated phosphorylation of S1579 of LRP6 by CK2 is required for its association with Dab2 and clathrin.

To determine whether endogenous Dab2 can mediate LRP6/clathrin interactions, we treated F9 and siDab2-F9 cells with RA for 3 days to induce endogenous Dab2 expression (Jiang *et al*, 2008). In siDab2 F9 cells, without Dab2, there is no clathrin/LRP6 interaction even in the presence of Wnt3A, while RA-induced endogenous Dab2 in parental F9 cells mediates clathrin/LRP6 interactions in the presence of Wnt3A and this interaction is abrogated by CK2 knockdown (Figure 4F). The relative LRP6, Dab2, clathrin and CK2 expression levels in siDab2 F9 cell and F9 cells are shown in Figure 4G). Confirming that endogenous Dab2 inhibits Wnt/β-catenin signalling, Wnt3A-induced phospho-LRP6, Axin2 and β-catenin accumulation are shown to be attenuated in RA-treated F9 cells but not in RA-treated siDab2 F9 cells (Supplementary Figure S10). These data confirm that endogenous Dab2 can mediate clathrin/LRP6 interactions in the presence of Wnt and that these interactions are CK2-dependent.

LRP6 S1579A mutant neutralizes Dab2 inhibition of Wnt3A-mediated dorsoventral patterning in zebrafish

Previous studies have shown that ectopic expression of Dab2 phenocopies Axin overexpression, resulting in inhibition of Wnt/β-catenin signalling and altered dorsoventral patterning (Rui *et al*, 2007; Jiang *et al*, 2009). To determine whether modulation of LRP6/Dab2 interactions, through the S1579 site of LRP6, mediates any effects on Wnt-mediated dorsoventral patterning, we suppressed endogenous zebrafish LRP6 expression, by antisense morpholinos, and rescued LRP6 expression using human WT or S1579A mutant LRP6, not targeted by the zebrafish-specific LRP6 anti-sense morpholinos, in the absence or presence of Dab2. Injection of antisense morpholinos specific for zebrafish LRP6 (MO zLRP6), and not control morpholinos (control MO), into fertilized one-cell-stage embryos resulted in two different populations of abnormal embryos 24 h post fertilization (Figure 5A). The ventralized phenotype is caused by injection and accumulation of nucleotides in the future dorsal side, whereas the dorsalized phenotype results from microinjection and nucleotide accumulation in the future ventral side (Rui *et al*, 2007). Altered phenotypes of the zLRP6 knockdown (MO zLRP6) can be rescued by co-injection with either the human WT LRP6 (WT hLRP6) or the S1579A mutant LRP6 (hLRP6 S1579A), demonstrating that human LRP6 can complement zLRP6 in mediating dorsoventral patterning (Figure 5A). Rescue of the zLRP6 knockdown altered phenotype by WT hLRP6 is not observed if microinjection is performed in the presence of Dab2 mRNA (MO zLRP6 + WT hLRP6 + Dab2). Injection of Dab2 mRNA inhibits the ability of the WT hLRP6 receptor to rescue the zLRP6 knockdown phenotype. In contrast, co-microinjection of Dab2 mRNA with the S1579A LRP6 mutant has no inhibitory effect on the ability of the hLRP6 S1579A receptor to rescue the zLRP6 knockdown phenotype (MO zLRP6 + hLRP6 1579A + Dab2). These representative morphological views are expanded and confirmed by the data shown in Figure 5B, where dose-dependent effects of the various morpholinos and microinjected mRNAs are shown demonstrating that human WT and S1579A mutant LRP6 expression can rescue the zLRP6 knockdown, and that only the human WT LRP6 receptor, and not the S1579A mutant, is susceptible to attenuation by Dab2. Appropriate expression of the injected hLRP6 receptors and Dab2 mRNAs in oocyte extracts is shown in Figure 5C, and the levels of WT hLRP6, hLRP6 S1579A mutant and Dab2 are quantitated and presented (Supplementary Figure S11). Collectively, the data demonstrate that mutation of the phospho-Ser1579 site has no effect on Wnt-mediated signalling, but that this site is essential for LRP6/Dab2 interactions and for Dab2 to mediate its inhibitory activity on Wnt-mediated dorsoventral patterning.

LRP6 S1579A mutant neutralizes Dab2 inhibition of Wnt/β-catenin-mediated tumourigenesis

To determine whether LRP6/Dab2 interactions, and specifically interactions through S1579, might be involved in the putative tumour suppressor functions of Dab2, we performed orthotopic allograft tumour studies in athymic nude mice. Initially, we established stable cell lines in the mouse F9 background ectopically expressing Dab2, the ΔNG LRP6 receptor, ΔNG LRP6 receptor + Dab2, ΔNG S1579A mutant LRP6 receptor and ΔNG S1579A mutant LRP6 receptor + Dab2. Ectopic expression of the LRP6 receptor constructs in the presence or absence of Dab2 is shown in Figure 6A. Confirming that expression of the intracellular portion of the LRP6 receptor is sufficient to mediate Wnt/β-catenin signalling (Tamai *et al*, 2004), stable expression of either ΔNG LRP6 or ΔNG S1579A mutant LRP6 receptor results in increased TOPFlash activity (Figure 6B) and cyclin D1 accumulation (Figure 6C). Stable co-expression of Dab2 with the ΔNG constructs demonstrates that Dab2 inhibits ΔNG receptor-mediated signalling but not that of the ΔNG S1579A mutant LRP6 receptor (Figures 6B and C).

Next, the various cell lines were injected into the left-side flanks of athymic nude mice and as a common control F9-Dab2 cells were injected into the right-side flanks, 10 mice for each cell line. At 45 days post inoculation, tumour growth was monitored photographically (Figure 6D) or by tumour weight (Figure 6E). The data show (left-side flanks of the mice in panel D) that parental F9 cells form small tumours that reached a mean mass of ∼1 g. Injection of F9-Dab2 cells (right-side flanks of the mice) failed to induce tumour growth in either of the mice, indicating that Dab2 inhibits F9 tumour formation. F9 cells expressing either the ΔNG or the mutant ΔNG S1579A LRP6 receptors induce tumourigenesis, with tumours reaching a mean mass of ∼3–4 g. Growth of tumours induced by expression of the ΔNG LRP6 receptor can be inhibited by co-expression of Dab2 (F9ΔNG-Dab2), whereas tumours induced by the mutant ΔNG S1579A LRP6 receptor cannot be abrogated by co-expression of Dab2 (Figures 6D and E). Cells isolated from the primary tumours and expanded in culture (Figure 6F, 'cultured cells'), as well as extracts prepared directly from excised tumours (Figure 6F, 'primary tumour'), maintain stable ectopic expression of the LRP6 constructs (α-VSVG blot) and of Dab2 (α-Dab2 blot). Further, haematoxylin and eosin (H&E) staining (Figure 7A; H/E) and immunohistochemical (IHC) staining of tumour sections revealed an increased expression of cyclin D1 and β-catenin (Figure 7B

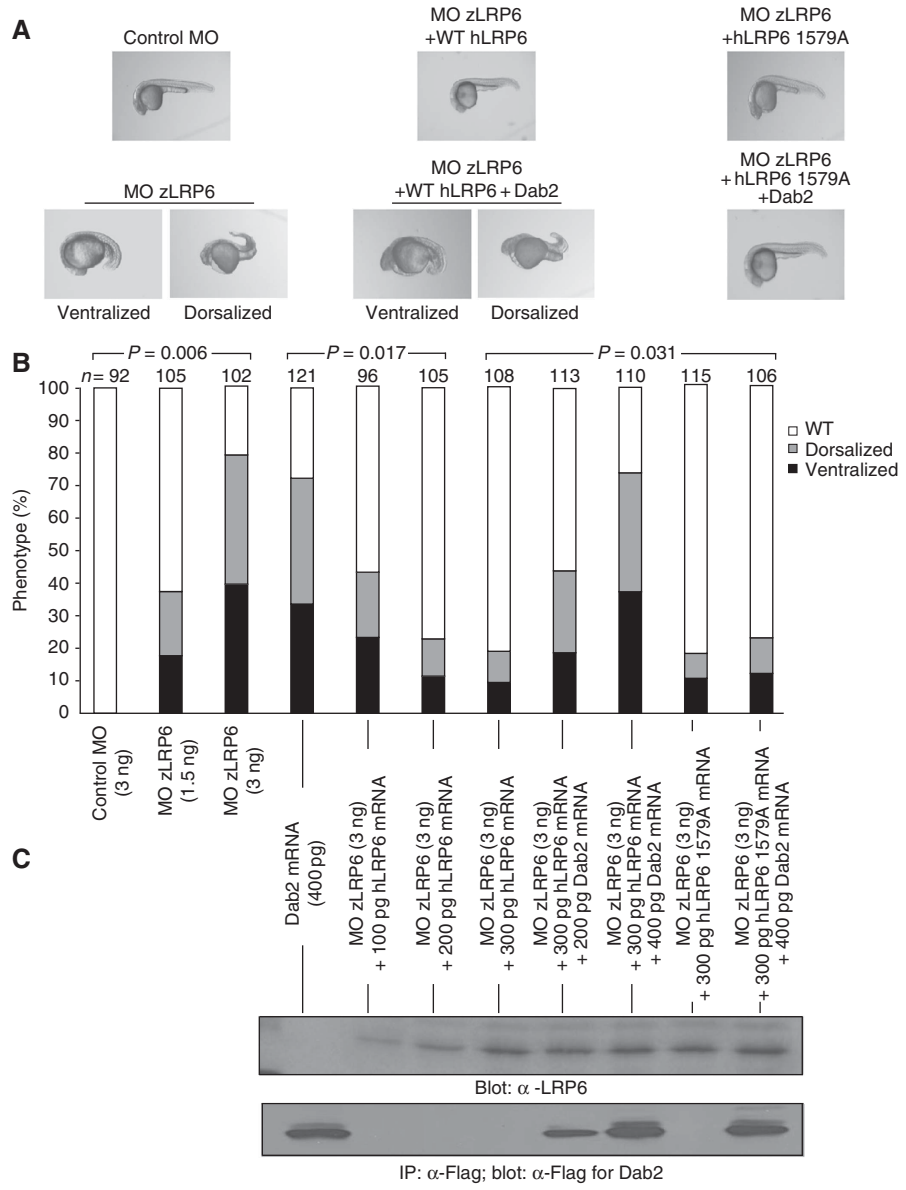

Figure 5 Dab2 fails to attenuate phospho-mutant S1579A LRP6-mediated dorsoventral patterning during zebrafish embryonic development. (**A**) Left panels: Control (MO) and zebrafish LRP6 anti-sense (zLRP6) morpholinos (3 ng) were injected into one-cell-stage fertilized zebrafish embryos and 24 h post fertilization (hpf) photos were taken using a Leica MZ95 dissection microscope. Injection of zLRP6 MO yielded the dorsoventralized phenotypes. Middle panels: Injection of zLRP6 MO (3 ng) together with human LRP6 mRNA (hLRP6; 300 pg) into one-cell-stage zebrafish embryos rescued the dorsoventralized phenotypes (MO zLRP6 + WT hLRP6). Injection of zLRP6 MO (3 ng) and hLRP6 mRNA (300 pg) and Dab2 mRNA (400 pg) resulted in the dorsoventral phenotypes. Right panels: Co-injection of zLRP6 MO (3 ng) with the hLRP6 phospho-mutant S1579A (hLRP6 1579A; 300 pg) rescued the dorsoventralized phenotypes, and injection of these together with Dab2 (zLRP6 MO + hLRP6 1579A + Dab2) failed to cause the dorsoventralized phenotypes. (**B**) Statistical analysis of the results from the injection assay of panel A. (**C**) A set of injected embryos described in (**A**) were collected and proteins were extracted, followed by immunoblot and immunoprecipitation analysis to detect expression levels of LRP6 and Dab2 using α-LRP6 and α-Flag antibodies. Figure source data can be found with the Supplementary data.

and C), supporting our orthotopic allograft tumour studies. These tumour studies confirm the cellular analyses and demonstrate that Wnt/LRP6-mediated tumourigenesis is inhibited by Dab2 expression and that this inhibition requires LRP6/Dab2 interactions through the S1579 site of LRP6.

Knockdown of CK2 levels inhibits the tumour suppressor functions of Dab2

To confirm the role of CK2 in Dab2's ability to modulate LRP6-mediated tumourigenesis, we shRNA-silenced CK2 expression in F9-Dab2 cells and examined its effects on Wnt/β-catenin signalling. Successful shRNA-mediated silencing of CK2 was achieved (Figure 8A) and co-immunoprecipitation analysis (Figure 8A, middle panel) revealed that Dab2 and LRP6 fail to interact in the CK2-attenuated cells (F9-Dab2 CK2 KD) compared to the control shRNA cells (F9-Dab2 cont.). We also compared Wnt/β-catenin signalling in F9 and F9 CK2 knockdown cells, and no significant differences were observed (Supplementary Figure S12). Further, compared with F9-Dab2 cells, in the CK2 knockdown (F9-Dab2 CK2 KD) Dab2 fails to attenuate transactivation of TOPFlash (Figure 8A, right panels).

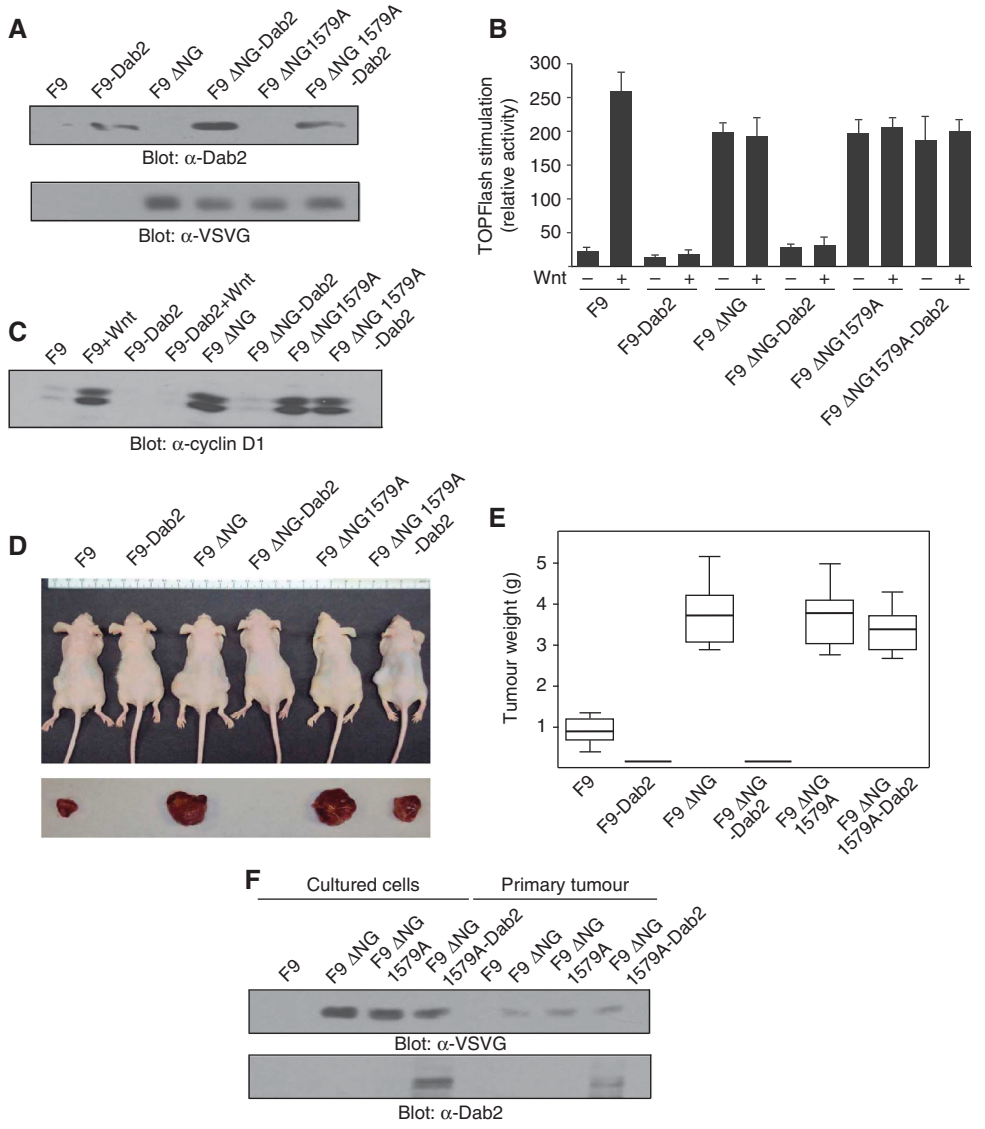

Figure 6 Dab2 fails to attenuate phospho-mutant S1579A LRP6-mediated tumourigenesis. (**A**) WCL from the indicated stably transfected cell lines were analysed by immunoblot analysis using α-Dab2 and α-VSVG antibodies to detect the expression levels of Dab2 and the intracellular domain of LRP6, respectively. (**B**) The relative TOP/FOPFlash luciferase activities and (**C**) expression levels of cyclin D1 in the stably transfected cell lines stimulated with or without Wnt3A were determined. Data are presented as means ± s.d. for three independent experiments ($n = 3$), each experiment being performed in triplicate. Cyclin D1 expression levels were determined by immunoblot analysis using α-cyclin D1 antibody. (**D**) Subcutaneous inoculation of 5×10^5 cells of the indicated stably transfected cell lines in the left hind flank of 6-week-old BalbC athymic nude mice (*nu/nu*) was performed. Each animal was also inoculated with 5×10^5 cells of the F9-Dab2 cell line in the right hind flank serving as a control. Images were taken 45 days post injection. (**E**) Tumours were excised and tumour weight was evaluated as a box-and-whisker plot to analyse differences between mean tumour weights among the various cells used. Data are presented as means ± s.e.m. for $n = 10$ samples per group. (**F**) Protein extracts from cultured cells established from the excised tumours (cultured cells) or the primary tumours themselves (primary tumours) were analysed by immunoblot analysis using α-VSVG and α-Dab2 to detect relative expression levels of Dab2 and VSVG-tagged ΔNG and ΔNG S1579A. Figure source data can be found with the Supplementary data.

Silencing of CK2 also inhibits the ability of Dab2 to inhibit tumourigenesis (Figure 8B). Compared with F9-Dab2 cells that fail to form tumours following inoculation (injected in the left flanks), F9-Dab2 cells in which CK2 has been attenuated form tumours with a mean weight of ~1 g (right flanks). These data (Figures 8A and B) demonstrate that CK2-mediated phosphorylation of LRP6 is required for Dab2/LRP6 interactions and for Dab2's ability to inhibit Wnt3A-mediated signalling.

As depicted in our model (Figure 8C), the LRP6 receptor has two endocytic fates following Wnt stimulation. In the absence of Dab2, LRP6 is internalized in caveolin-containing vesicles that presumably couple with and allow activation of the β-catenin destruction complex. In cells that express Dab2, Wnt3A-mediated activation of CK2 leads to LRP6 phosphorylation at S1579, which promotes Dab2/LRP6 interactions and shunting of the receptor to the clathrin endocytic pathway. Wnt-mediated phosphorylation of LRP6 phosphorylation at S1490, postulated to be required for interaction with and modulation of the β-catenin destruction complex, is not observed when the receptor is internalized in clathrin-containing vesicles. Thus, Dab2-mediated internalization of

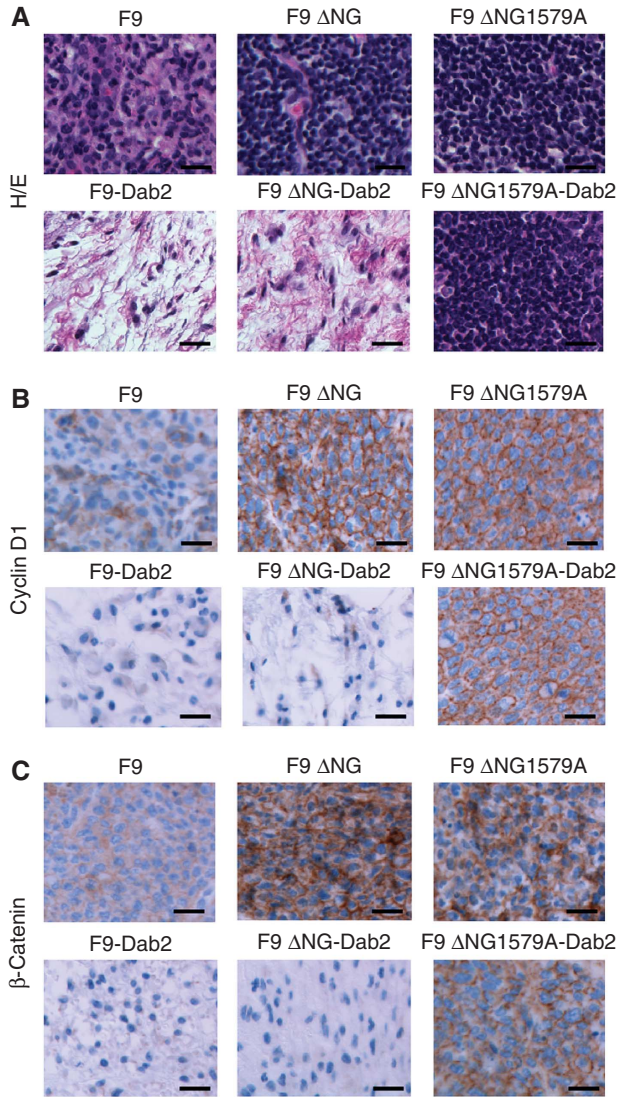

Figure 7 IHC analysis of tumour tissues. (**A**) H&E staining of excised tumours from mice inoculated with the indicated cell lines. IHC analysis of paraffin sections from the excised tumours was performed with α-cyclin D1 (**B**) and α–β-catenin (**C**). Scale bars, 50 μm.

LRP6 through the clathrin pathway fails to couple with the destruction complex.

Discussion

We provide evidence that the tumour suppressor Dab2 mediates antagonistic effects on Wnt/β-catenin signalling through its effects on LRP6 internalization. In the absence of Dab2, Wnt induces the internalization of LRP6 through caveolin-mediated endocytosis, resulting in Wnt/β-catenin signalling, whereas in its presence Dab2 binds LRP6, in a Wnt and CK2-dependent manner, and shunts LRP6 towards clathrin-mediated endocytosis and suppression of β-catenin signalling (Figure 8C). We postulate, therefore, that cellular Dab2 levels modulate Wnt/β-catenin signalling by regulating the endocytic fate of the LRP6 receptor.

Dab2 is an adaptor involved in endocytosis and protein trafficking (Bonifacino and Traub, 2003). Dab2 binds to clathrin and the clathrin adaptor AP-2 and displays a punctate 'dot-like' staining pattern in cells suggestive of its association with intracellular vesicles (Morris and Cooper, 2001). On the basis of these data, and recent reports demonstrating that internalization of Wnt receptor complexes, through either caveolin or clathrin-mediated routes, is critical for regulating Wnt signalling (Yamamoto *et al*, 2008), we did observe an interaction between Dab2 and LRP6. This interaction requires activation of Wnt signalling or mere overexpression of the intracellular domain of LRP6. Dab2 interaction with LRP6 does not involve the five reiterated 'PPPS/TP' sites required for LRP6 to interact with Axin (Tamai *et al*, 2004), but rather requires the highly conserved Ser site at 1579. The data demonstrate that phosphorylation of this site by CK2, in response to Wnt, promotes binding of Dab2 and LRP6. Dab2/LRP6 interactions do not affect the endocytic rate of LRP6, in that in the presence or absence of Dab2 LRP6 receptors are internalized with similar kinetics. Rather, Dab2's binding to LRP6 shunts the receptor away from its interaction with and endocytosis by caveolin, and promotes its binding to and endocytosis by clathrin. Mutation of S1579 does not abrogate LRP6/Wnt-mediated signalling but prevents LRP6's ability to be phosphorylated by CK2, its interaction with Dab2 and its shunting toward the clathrin endocytic pathway, and thus inhibits Dab2's antagonistic effect on Wnt/β-catenin signalling. Significantly, in our allograftic tumour studies attenuation of Wnt signalling and tumour growth by Dab2 is observed only with the WT LRP6 ΔNG and not with the phospho-S1579A LRP6 mutant ΔNG that fails to interact with Dab2. Thus, S1579 of LRP6 represents a significant negative regulatory point of Wnt/β-catenin signalling and suggests that mutation of this site may be targeted in tumourigenesis.

Our findings also corroborate previous studies by Yamamoto *et al*, demonstrating that S1490 phosphorylation is independent of LRP6 internalization but is essential for β-catenin accumulation. Indeed, we did not observe Axin and GSK3β in the LRP6 complex in the presence of Dab2, suggesting that LRP6's association with components of the β-catenin complex, and ultimately β-catenin accumulation, occurs downstream of LRP6 internalization in caveolin-containing vesicles. In the presence of Dab2 and CK2 phosphorylation of LRP6, the co-receptor is internalized through clathrin-containing vesicles and fails to couple and modulate the β-catenin destruction complex. Thus, although LRP6 is internalized, it fails to mediate accumulation of β-catenin in the presence of Dab2.

DKK1, similarly to Dab2, inhibits Wnt3A-dependent stabilization of β-catenin. However, while both DKK1 and Dab2 inhibit Wnt/β-catenin signalling by promoting LRP6 internalization through the clathrin pathway, their inhibitory actions differ in several important aspects: (1) whereas Dab2 interacts with LRP6's intracellular domain, DKK1 interacts with the extracellular domain of LRP6; (2) mere expression of DKK1 in cells induces internalization of LRP6 (Yamamoto *et al*, 2008), whereas Dab2 expression has no effect on basal or Wnt3A-induced LRP6 internalization. DKK1 does not suppress Wnt3A-induced accumulation of β-catenin when clathrin-mediated endocytosis is disrupted using MDC or in clathrin knockdown cells (Yamamoto *et al*, 2008), suggesting that DKK1-dependent internalization of LRP6 through clathrin is required for its inhibitory effects on β-catenin signalling. In contrast, Dab2's inhibitory effects on

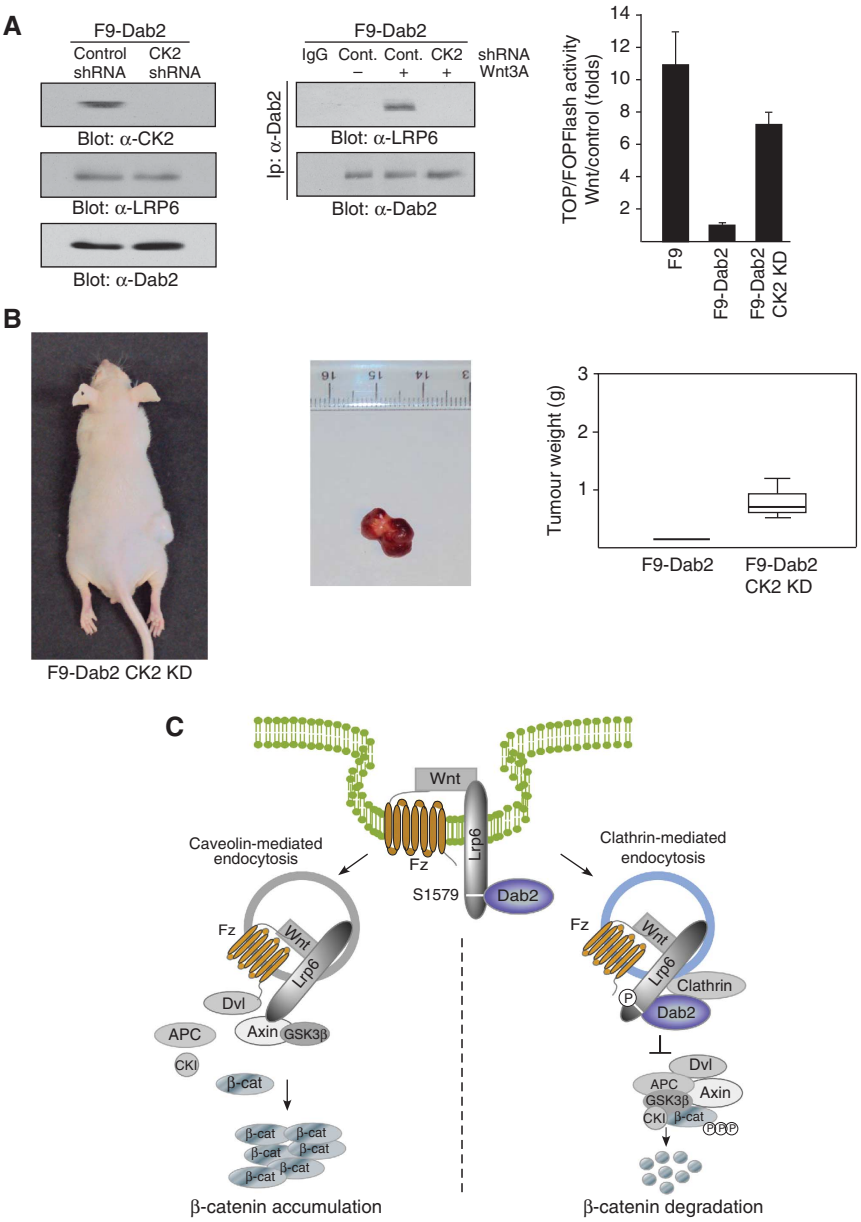

Figure 8 Knockdown of CK2 inhibits the tumour suppressor functions of Dab2. (**A**) Left panels: F9-Dab2 stably expressing control shRNA or CK2 shRNA were generated as described in Materials and methods and analysed by immunoblot analysis for expression levels of CK2, LRP6 and Dab2. Middle panels: Dab2 immunoprecipitates from F9-Dab2 stably expressing control shRNA or CK2 shRNA treated ± Wnt3A were also analysed for co-immunoprecipitation of LRP6. Right panel: Wnt3A-stimulated TOP/FOPFlash luciferase activity was determined in parental F9 cells, F9-Dab2-expressing cells with control shRNA and F9-Dab2-expressing cells in which CK2 is stably attenuated (F9-Dab2 CK2 KD). (**B**) Subcutaneous inoculation of F9-Dab2 shCK2 cells (5×10^5) in the right hind flank of 6-week-old BalbC athymic nude mice (*nu/nu*) was performed. Each animal was also inoculated with 5×10^5 F9-Dab2 cells with control shRNA in the left hind flank to serve as a control. Images were taken 45 days post injection. Tumours were excised (middle panel) and tumour weight was evaluated as a box-and-whisker plot (right panel) to analyse the differences between mean tumour weights. Data are presented as means ± s.e.m. for $n = 10$ samples per group. (**C**) Model depicting the shunting of LRP6 towards the clathrin endocytic pathway by Dab2. In the absence of Dab2, LRP6 is internalized through the caveolin pathway, resulting in β-catenin accumulation, whereas in its presence LRP6 is internalized through the clathrin pathway and fails to inhibit the β-catenin destruction complex. Figure source data can be found with the Supplementary data.

β-catenin accumulation do not require LRP6 internalization in that the MDC inhibitor does not attenuate Dab2's inhibitory effect; only clathrin knockdown inhibits Dab2's attenuation of Wnt signalling. Presumably, in the absence of clathrin, Dab2 sequestration of LRP6 does not occur and the receptor is free to internalize and signal through the caveolin pathway upon Wnt3A stimulation. This indicates that Dab2's inhibitory effect requires interaction with clathrin and LRP6 resulting in sequestration of the receptor from the caveolin pathway, and not LRP6 internalization.

There is now accumulating evidence that Wnt-induced endocytosis is not only an obligatory step for β-catenin signalling (Blitzer *et al*, 2006; Taelman *et al*, 2010) but is also necessary for PCP signalling (Gagliardi *et al*, 2008; Ohkawara *et al*, 2011). A 'two endocytic routes–two outcomes' model for canonical Wnt/β-catenin signalling has

been proposed whereby LRP6 internalization can occur through caveolin- or clathrin-dependent pathways resulting in activation or inhibition of β-catenin, respectively (Yamamoto *et al*, 2008). Our results confirm this concept and show that the endocytic adaptor Dab2 regulates the sequestration of LRP6 towards clathrin-mediated endocytosis. Thus, Dab2, presumably through its binding to clathrin and the AP2 adaptor (Morris and Cooper, 2001), links LRP6 to the endocytic machinery, while a similar adaptor molecule linking LRP6 with the caveolin endocytic machinery has yet to be identified. It appears, from our data, that although LRP6 is internalized with similar kinetics in the presence or absence of Dab2, once internalized the endocytic fate of the receptor is distinct. In Dab2/clathrin-containing endosomes, Wnt3A-mediated phosphorylation of LRP6 at S1490 does not occur, suggesting that after being internalized these Dab2 vesicles fail to engage the β-catenin destruction complex and its associated CKI and GSK3β kinases to phosphorylate LRP6. Presumably this endocytic pathway is involved in receptor recycling, but whether it mediates other Wnt signalling remains to be determined. On the other hand, caveolin-containing endosomes, once internalized, appear to couple with the β-catenin destruction complex in that LRP6 phosphorylation at S1490 is observed. Collectively, these data not only highlight the significance of LRP6 internalization in Wnt/β-catenin signalling but also support the so-called 'signalling endosome' model whereby endosomal vesicles serve as intracellular platforms for the assembly of regulatory molecules and effectors that confer signalling specificity and diversity. Perhaps the composition of endocytic adaptor molecules such as Dab2 within these endocytic platforms regulates signalosome formation and thus signalling outcomes.

Materials and methods

Preparation of cell lysates, immunoblot analysis and immunoprecipitation

For immunoprecipitation and immunoblot analysis, cells were lysed in buffer D and immunoprecipitation was performed as described previously (Jiang *et al*, 2008).

Biotin labelling of cell surface proteins and internalization assays

For cell surface protein labelling, cells were treated in the presence or absence of Wnt3A conditioned media at 37°C for the times indicated and washed three times with ice-cold phosphate-buffered saline (PBS; pH 8.0) to remove any contaminating proteins. Cells (2.5×10^7 cells/ml) were resuspended in PBS and 50 μl of 20 mM Sulfo-NHS-SS-Biotin per millilitre of reaction volume was added. Following a 1-h incubation on ice, cells were washed with Sulfo-NHS-SS-Biotin blocking reagent (50 mM NH_4Cl in PBS containing 1 mM $MgCl_2$; 0.1 mM $CaCl_2$) to quench free Sulfo-NHS-SS-Biotin, followed by several ice-cold PBS washes. Cell lysates were prepared in buffer D followed by NeutrAvidin agrose beads (Pierce) precipitation. Precipitates were washed three times with buffer D and analysed by SDS–PAGE and immunoblotting with α-LRP6 and α-EGFR antibodies. For internalization assays, cell surface proteins were biotin-labelled as described above at room temperature for 1 h, followed by treatment with or without Wnt3A for the indicated times at 37°C. Following stimulation, cells were incubated with ice-cold glutathione solution (60 mM glutathione, 0.83 M NaCl, with 0.83 M NaOH and 1% bovine serum albumin (BSA) added before use) for two 30-min incubations, followed by ice-cold PBS washes four times. Cells were collected and lysed with buffer D and biotinylated proteins precipitated with immobilized NeutrAvidin agarose beads followed by SDS–PAGE and immunoblot analysis with α-LRP6 and α-EGFR antibodies.

Immunofluorescence and imaging

F9 and F9-Dab2 cells were treated with E1 A control media or Wnt3A conditioned media for 1 h. Following treatment, cells were fixed for 20 min in PBS buffer containing 4% (w/v) paraformaldehyde, followed by permeabilization with PBS containing 0.2% (w/v) Triton X-100 and 2% BSA for 20 min. Cells were incubated with primary antibodies α-LRP6/α-caveolin or α-LRP6/α-clathrin for 1 h in permeabilization buffer followed by three washes with PBS. Cells were incubated in secondary antibodies Alexa fluor 488 (green) goat α-mouse and Alexa fluor 568 (red) goat α-rabbit at room temperature for 1 h followed by three washes with PBS before being observed with confocal laser scanning on a Leica TCS SP2 confocal microscope (Leica Microsystems, Germany).

In vivo tumourigenecity studies

Tumourigenesis was performed using subcutaneous injection of F9, F9-Dab2, F9-ΔNG, F9-ΔNG-Dab2, F9-ΔNG S1579A or F9-ΔNG S1579A-Dab2 cells (5×10^5) into the hind left flank of 6-week-old BalbC athymic nude mice (*nu/nu*), and F9-Dab2 cells were injected into the hind right side as standard control. Ten animals were used for each cell type. Post injection (45 days), all mice were killed and images were taken. Tumours in mice were excised for weight measurement and images taken. All experiments were performed according to approved protocols of the Institutional Animal Care and Use Committee (IACUC), Medical University of South Carolina.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections as described (Hussey *et al*, 2011). Briefly, tissue sections were incubated with primary antibody α–β-catenin (Cell Signalling Technology, 1:100) or α-cyclin D1 (Cell Signalling Technology, 1:100) for 2 h at 25°C. The sections were washed with PBS before being incubated with the biotinylated secondary antibody for 30 min at 25°C. The stain was developed with diaminobenzidine tetrahydrochloride (DAB) chromogen. Haematoxylin/eosin staining was carried out with Ehrlich haematoxylin/eosin solution (Sigma-Aldrich). All slides were observed and photos taken under a Leica DM 2000 microscope.

Zebrafish embryo microinjection

The WT AB zebrafish strain was maintained and bred under standard conditions and embryo microinjection was performed following the standard protocol (Westerfield, 2007). For more detailed information, see Supplementary data.

Statistical analysis

All experiments were carried out at least three times. Data were represented as mean ± s.e.m. Significance of differences between two groups was tested by Student's *t* test or ANOVA. A *P* value less than 0.05 was regarded as significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank the members of our laboratory for reagents and DNA constructs preparation, zebrafish embryo injection, helpful discussions and critical insights. We are grateful to Yvonne Parker for her invaluable help with the animal studies. We thank Drs Leof and Niehrs for the dominant-negative DN K44A and pCS2-Flag-tagged LRP6 constructs, respectively. This work was supported by grants CA55536 and CA154663 from the National Cancer Institute to PHH.

Author contributions: YJ performed the experiments. YJ, XH and PHH suggested and designed the experiments. YJ and PHH analysed the experimental results and wrote the article.

Conflict of interest

The authors declare that they have no conflict of interest.

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