The EMBO Journal (2011) 30, 1433–1443 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

THE EMBO JOURNAL

Amer1/WTX couples Wnt-induced formation of PtdIns(4,5)P_2 to LRP6 phosphorylation

Kristina Tanneberger[1], Astrid S Pfister[1], Katharina Brauburger[1], Jean Schneikert[1], Michel V Hadjihannas[1], Vitezslav Kriz[2,3], Gunnar Schulte[4], Vitezslav Bryja[2,3] and Jürgen Behrens[1,*]

[1]Nikolaus-Fiebiger-Center, University Erlangen-Nürnberg, Erlangen, Germany, [2]Faculty of Science, Institute of Experimental Biology, Masaryk University, Brno, Czech Republic, [3]Department of Cytokinetics, Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic and [4]Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

Phosphorylation of the Wnt receptor low-density lipoprotein receptor-related protein 6 (LRP6) by glycogen synthase kinase 3β (GSK3β) and casein kinase 1γ (CK1γ) is a key step in Wnt/β-catenin signalling, which requires Wnt-induced formation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Here, we show that adenomatous polyposis coli membrane recruitment 1 (Amer1) (also called WTX), a membrane associated PtdIns(4,5)P_2-binding protein, is essential for the activation of Wnt signalling at the LRP6 receptor level. Knockdown of Amer1 reduces Wnt-induced LRP6 phosphorylation, Axin translocation to the plasma membrane and formation of LRP6 signalosomes. Overexpression of Amer1 promotes LRP6 phosphorylation, which requires interaction of Amer1 with PtdIns(4,5)P_2. Amer1 translocates to the plasma membrane in a PtdIns(4,5)P_2-dependent manner after Wnt treatment and is required for LRP6 phosphorylation stimulated by application of PtdIns(4,5)P_2. Amer1 binds CK1γ, recruits Axin and GSK3β to the plasma membrane and promotes complex formation between Axin and LRP6. Fusion of Amer1 to the cytoplasmic domain of LRP6 induces LRP6 phosphorylation and stimulates robust Wnt/β-catenin signalling. We propose a mechanism for Wnt receptor activation by which generation of PtdIns(4,5)P_2 leads to recruitment of Amer1 to the plasma membrane, which acts as a scaffold protein to stimulate phosphorylation of LRP6.

The EMBO Journal (2011) **30,** 1433–1443. doi:10.1038/emboj.2011.28; Published online 8 February 2011
Subject Categories: signal transduction
Keywords: Amer1; LRP6; PtdIns(4,5)P_2; Wnt; WTX

*Corresponding author. Nikolaus-Fiebiger-Center, University Erlangen-Nürnberg, Glückstr. 6, 91054 Erlangen, Germany.
Tel.: +49 9131 8529109; Fax: +49 9131 8529111;
E-mail: jbehrens@molmed.uni-erlangen.de

Received: 9 August 2010; accepted: 18 January 2011; published online: 8 February 2011

Introduction

The Wnt/β-catenin signalling pathway regulates cell proliferation, differentiation and apoptosis, and has an important role during embryonic development, adult tissue homoeostasis and various diseases including cancer (Lustig and Behrens, 2003; Clevers, 2006). In the absence of extracellular Wnt ligands the levels of cytoplasmic β-catenin are kept low by the action of a multiprotein destruction complex that targets β-catenin for proteasomal degradation. The core components of this complex are the scaffold proteins Axin and its homologue Conductin (Axin2), the tumour suppressor adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β), which phosphorylates β-catenin and thereby earmarks it for ubiquitin-mediated degradation in the proteasome (MacDonald *et al*, 2009). The binding of Wnt ligands to the transmembrane receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein 6 (LRP6) initiates a signalling cascade that results in the inhibition of β-catenin phosphorylation and degradation leading to the activation of β-catenin-dependent transcription (Huang and He, 2008; Angers and Moon, 2009; MacDonald *et al*, 2009).

A key step after Wnt stimulation is the phosphorylation of the intracellular domain (ICD) of LRP6 at five reiterated PPPSPxS motifs and adjacent Ser/Thr clusters (Tamai *et al*, 2004; Davidson *et al*, 2005; Zeng *et al*, 2005; MacDonald *et al*, 2008; Supplementary Figure S7A). Phosphorylation at the PPPSPxS motifs (e.g., at Ser1490) is mediated by GSK3β, whereas the Ser/Thr clusters (e.g., at Thr1479) are phosphorylated by casein kinase 1γ (CK1γ) (Davidson *et al*, 2005; Zeng *et al*, 2005). The phosphorylated PPPSPxS motifs provide docking sites for Axin (Mao *et al*, 2001; Tamai *et al*, 2004; Davidson *et al*, 2005) and can directly inhibit the activity of GSK3β (Cselenyi *et al*, 2008; Piao *et al*, 2008; Wu *et al*, 2009).

Phosphorylation of LRP6 by GSK3β requires binding of Dishevelled (Dvl) to Fz, which in turn leads to recruitment of the Axin/GSK3β complex (Zeng *et al*, 2008). In contrast, CK1γ is constitutively localized to the plasma membrane (Davidson *et al*, 2005). It was recently suggested that co-clustering of Fz-LRP6 receptors together with Axin and Dvl in so-called LRP6 signalosomes is involved in LRP6 phosphorylation (Bilic *et al*, 2007). Signalosome formation depends on the ability of Dvl to dynamically polymerize, which might provide a high density of phosphorylation sites for GSK3β and CK1γ (Bilic *et al*, 2007; Schwarz-Romond *et al*, 2007a, b). Recent evidence indicates that the Wnt-induced generation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the plasma membrane is required for LRP6 phosphorylation by GSK3β as well as CK1γ and for signalosome formation (Pan *et al*, 2008). This process is mediated by Dvl, which binds and activates phosphatidylinositol 4-kinase type IIα (PI4KIIα) and phosphatidylinositol-4-phosphate 5-kinase type Iβ (PIP5KIβ) sequentially acting to produce PtdIns(4,5)P_2 (Pan *et al*, 2008; Qin *et al*, 2009).

Amer1 (APC membrane recruitment 1) was initially described by our group as an APC-binding protein, which can associate with the plasma membrane via two N-terminal PtdIns(4,5)P_2-binding domains (Grohmann *et al*, 2007). Amer1 is identical to the tumour suppressor WTX (Wilms Tumour gene on the X chromosome) mutated in a significant fraction of Wilms tumours (Rivera *et al*, 2007) and in the inherited disease OSCS (osteopathia striata congenita with cranial sclerosis) (Jenkins *et al*, 2009). Amer1 is found in complexes with β-catenin and components of the β-catenin destruction machinery such as APC, Axin and β-TrCP, and can block canonical Wnt signalling by inducing proteasomal degradation of β-catenin (Major *et al*, 2007). Studies in Zebrafish and Xenopus also suggest a negative role of Amer1 in Wnt signalling (Major *et al*, 2007).

Several key players involved in LRP6 phosphorylation have been identified (Fz, Dvl, Axin, GSK3β, CK1γ and PtdIns(4,5)P_2), but a coherent picture of their interactions and the sequence of events are still missing. In particular, the mechanism by which Wnt-induced PtdIns(4,5)P_2 formation results in LRP6 phosphorylation has remained elusive. In the present study, we identify an unexpected role of Amer1 as a positive regulator of Wnt signalling acting at the LRP6 receptor level by showing that Amer1 links Wnt-induced formation of PtdIns(4,5)P_2 to LRP6 phosphorylation.

Results

Amer1 is required for Wnt-induced LRP6 phosphorylation

To investigate whether Amer1 has a function in Wnt signalling at the receptor level, we knocked down its expression using two different siRNAs and analysed Wnt-induced phosphorylation of LRP6 in plasma membrane fractions by western blotting. We found that Amer1 knockdown prevented LRP6 phosphorylation at Ser1490 and Thr1479 in Wnt3A-treated HEK293T cells (Figure 1A and B; Supplementary Figure S1A). Amer1 knockdown also prevented LRP6 phosphorylation in SW480 colon carcinoma cells (Supplementary Figure S1B). Reciprocally, overexpression

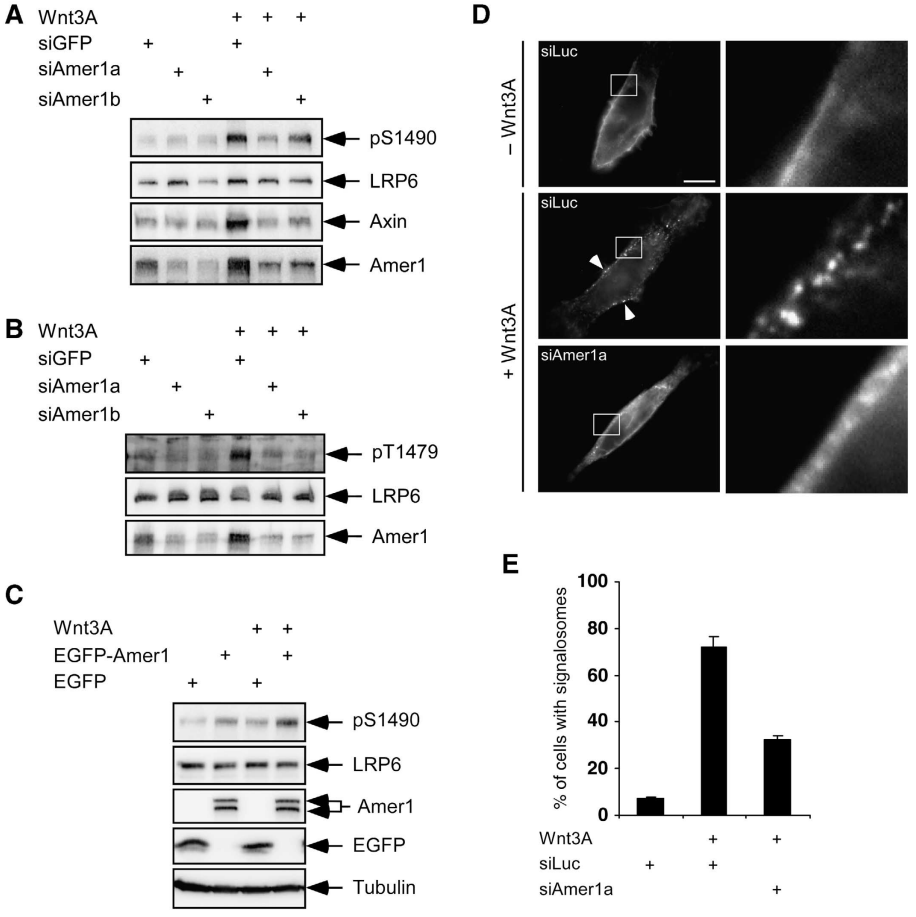

Figure 1 Amer1 is required for Wnt-induced LRP6 phosphorylation and signalosome formation. (**A**, **B**) Amer1 is required for LRP6 phosphorylation at Ser1490 (**A**) and Thr1479 (**B**). HEK293T cells stably expressing LRP6-EGFP were transfected with the indicated siRNAs and incubated with Wnt3A for 1 h. Membrane fractions were analysed by western blotting. **(C)** Overexpression of Amer1 promotes LRP6 phosphorylation at Ser1490. HEK293T cells stably expressing VSVG-LRP6 were transiently transfected with EGFP-Amer1 and treated with Wnt3A for 20 min. Note that Amer1 is expressed in two splice variants represented by the two bands on the anti-GFP western blot (cf. Supplementary Figure S3A). In panels (**A**) and (**B**), the anti-Amer1 antibody detects only the larger splice variant of endogenous Amer1. (**D**, **E**) Amer1 is required for Wnt-induced signalosome formation. (**D**) Signalosome formation in HeLa cells co-expressing LRP6-EYFP and Fz8-EYFP transfected with the indicated siRNAs. Images show EYFP fluorescence with and without Wnt3A treatment for 1 h. Right-hand panels represent higher magnifications of the boxed regions from the left. Arrowheads point to signalosomes. Scale bar is 10 μm. **(E)** Quantification of signalosome formation in cells from (**D**). Error bars indicate s.e.m. from three independent experiments.

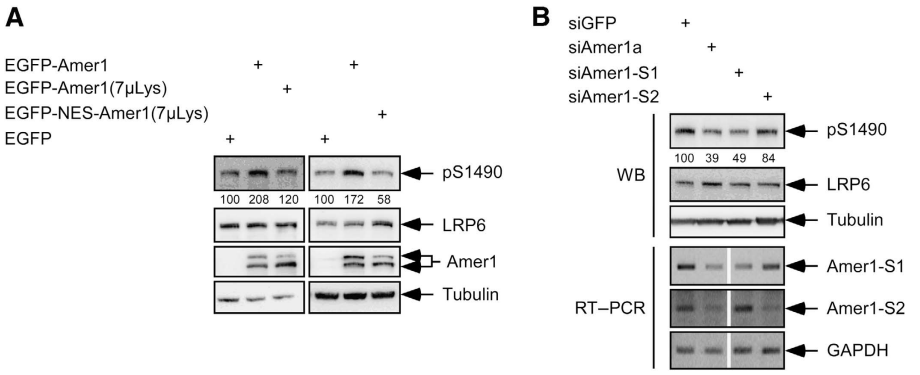

Figure 2 Membrane localization of Amer1 through PtdIns(4,5)P_2 binding is required for its effect on LRP6 phosphorylation. (**A**) N-terminal lysine mutants of Amer1 (EGFP-Amer1(7μLys), EGFP-NES-Amer1(7μLys)) lacking PtdIns(4,5)P_2 binding and membrane association are deficient for LRP6 phosphorylation. HEK293T cells stably expressing VSVG-LRP6 were transiently transfected with EGFP-tagged Amer1 constructs as indicated and treated with Wnt3A for 20 min. For details on the constructs, see Supplementary Figure S2A–C. (**B**) Effect of specific knockdown of Amer1 splice variants Amer1-S1 and Amer1-S2 on LRP6 phosphorylation. HeLa cells transfected with the indicated Amer1 siRNAs were incubated with Wnt3A for 1 h and endogenous LRP6 was examined by western blotting (WB). Specific RT–PCR for expression of the splice variants is shown below. See also Supplementary Figure S3A–D. In (**A**) and (**B**) numbers below the lanes reflect relative levels of phosphorylated LRP6 (pS1490) normalized to LRP6 as determined by densitometry. Experiments were repeated at least three times.

of Amer1 stimulated LRP6 phosphorylation at Ser1490 (Figure 1C; Supplementary Figure S1C). Notably, the previously described recruitment of Axin to the plasma membrane after Wnt stimulation was also abolished after knockdown of Amer1 (Figure 1A; Mao *et al*, 2001). These data show that Amer1 is essential for Wnt-induced LRP6 phosphorylation and Axin translocation to the plasma membrane and point to a positive role of Amer1 in the activation of Wnt signalling at the receptor level.

Amer1 is required for Wnt-induced signalosome formation

Given the role of Amer1 in Wnt receptor activation we analysed whether it is involved in signalosome formation. Signalosomes were detected by monitoring the aggregation of YFP-tagged LRP6 and Fz8 after Wnt3A stimulation. Indeed knockdown of Amer1 efficiently reduced formation of LRP6 signalosomes (Figure 1D and E). These results show that Amer1 is required for Wnt-induced signalosome formation and corroborate a role for Amer1 in the activation of the Wnt pathway at the receptor level.

Membrane localization of Amer1 through PtdIns(4,5)P_2 binding is required for its effect on LRP6 phosphorylation

We next analysed whether LRP6 activation depends on plasma membrane localization of Amer1, which is mediated by two short domains in its N-terminus. These domains bind to PtdIns(4,5)P_2 and are characterized by a high proportion of highly conserved lysine residues known for their capacity to interact with PtdIns(4,5)P_2 (Kagan and Medzhitov, 2006; Grohmann *et al*, 2007; Supplementary Figure S2A). Mutation of seven of these lysine residues to alanine (Amer1(7μLys)) abolished interaction with PtdIns(4,5)P_2 as well as membrane association of Amer1 (Supplementary Figure S2B and C). Importantly, this mutant was defective in stimulating LRP6 phosphorylation in HEK293T cells (Figure 2A, left panels). Because the Amer1(7μLys) mutant is enriched in the nucleus, this experiment does not rule out a role of Amer1 in the cytoplasm. Fusion of the Amer1(7μLys) mutant to a nuclear export sequence (NES-Amer1(7μLys)) led to cytoplasmic localization of Amer1, but did not restore its ability to stimulate LRP6 phosphorylation (Figure 2A, right panels; Supplementary Figure S2C). Together, these results show that PtdIns(4,5)P_2-mediated membrane association of Amer1 is required for its effect on LRP6 phosphorylation and that cytoplasmic localization alone is not sufficient.

Amer1 is expressed in two splice isoforms termed Amer1-S1 or Amer1-S2, which differ by the presence or absence of amino acids 50–326, comprising a large part of the membrane association/PtdIns(4,5)P_2-binding domain (Supplementary Figure S3A; Jenkins *et al*, 2009). While Amer1-S1, which binds to the plasma membrane, stimulated LRP6 phosphorylation, Amer1-S2 lacking the membrane association failed to do so (Supplementary Figure S3B and C). Importantly, specific knockdown of the Amer1-S1 isoform reduced Wnt-induced LRP6 phosphorylation, whereas knockdown of Amer1-S2 had no effect (Figure 2B; Supplementary Figure S3A and D). These data further support the importance of membrane localization of Amer1 for its effect on LRP6 phosphorylation.

Wnt induces plasma membrane translocation of Amer1, which requires the formation of PtdIns(4,5)P_2

Next, we studied whether activation of Wnt signalling alters the association of Amer1 with the plasma membrane and whether PtdIns(4,5)P_2 is involved. Wnt3A treatment induced a rapid increase of endogenous Amer1 in the plasma membrane fraction of HEK293T cells, whereas total amounts of Amer1 in whole cell lysates were not altered (Figure 3A). To determine whether Wnt-induced association of Amer1 with the plasma membrane depends on the formation of PtdIns(4,5)P_2 we knocked down PI4KIIα, which was shown to be essential for PtdIns(4,5)P_2 synthesis after Wnt stimulation (Pan *et al*, 2008). Wnt-induced plasma membrane recruitment of Amer1 was strongly reduced when PI4KIIα was knocked down by two different siRNAs (Figure 3B; Supplementary Figure S4). Neomycin binds PtdIns(4,5)P_2 and can block its interaction with proteins (Gabev *et al*, 1989; Pilot *et al*, 2006). Pre-treatment of cells with neomycin abolished Wnt-induced membrane association of Amer1 and at the same time strongly reduced LRP6 phosphorylation and

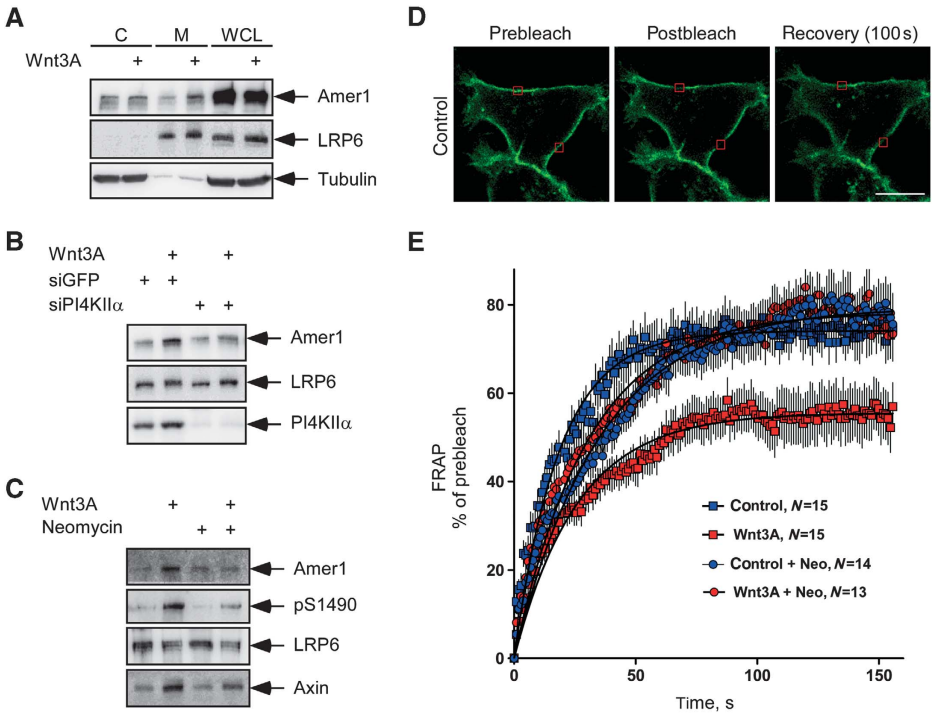

Figure 3 Wnt induces plasma membrane translocation of Amer1, which requires the formation of PtdIns(4,5)P_2. (**A**) Wnt3A induces plasma membrane translocation of Amer1. HEK293T cells stably expressing LRP6-EGFP were incubated with Wnt3A for 1 h and then subjected to subcellular fractionation. Cytoplasmic fractions (C), membrane fractions (M) and whole cell lysates (WCL) were analysed by western blotting. α-Tubulin and LRP6 were used to mark cytoplasmic and membrane fractions, respectively. (**B**) Knockdown of PI4KIIα prevents Wnt-induced plasma membrane recruitment of Amer1. siRNA transfected HeLa cells were incubated with Wnt3A for 1 h and membrane fractions were analysed by western blotting. (**C**) Neomycin abolishes Wnt-induced membrane translocation of Amer1. HEK293T cells stably expressing VSVG-LRP6 were treated with 10 mM neomycin for 30 min before incubation with Wnt3A plus neomycin for 1 h and membrane fractions were analysed by western blotting. (**D**, **E**) Wnt decreases membrane dynamics of Amer1. HEK293 cells transiently transfected with EGFP-Amer1 were subjected to FRAP analysis. (**D**) Typical example of EGFP-Amer1 distribution and changes in EGFP fluorescence before and after bleaching. Scale bar is 10 μm. (**E**) Statistical analysis of FRAP experiments using cells pre-stimulated with PBS (control) or Wnt3A with or without 10 mM neomycin as indicated (*N*, number of cells analysed per condition). The graphs show mean values and s.e.m., and the best fitting curve model, which was used for calculation of the mobile pool of EGFP-Amer1 (% of fluorescence recovered) and of the recovery halftime ($T_{1/2}$).

Wnt-induced recruitment of Axin to the plasma membrane (Figure 3C). Together, these data demonstrate that Amer1 is translocated to the plasma membrane in a PtdIns(4,5)P_2-dependent manner after Wnt stimulation.

Wnt3A decreases membrane dynamics of Amer1

In order to test for effects of Wnt stimulation on the membrane dynamics of Amer1, we employed fluorescence recovery after photobleaching (FRAP) methodology. Selected membrane regions of EGFP-Amer1-expressing cells were bleached by a laser pulse and the recovery of EGFP fluorescence was analysed for 150 s (see Figure 3D for a typical experiment). Pre-incubation with Wnt3A decreased the mobile pool of Amer1 from ~74% (95% confidence interval: 73.1–75.1%) to 55% (95% confidence interval: 54.4–56.7%) and at the same time increased the halftime required for recovery of Amer1 from 13 to 18 s (Figure 3E; Supplementary Table). This indicates that Wnt signalling generates an immobile pool of Amer1 at the plasma membrane that does not readily exchange with neighbouring or cytoplasmic Amer1. Interestingly, pre-treatment of cells with neomycin restored recovery in the presence of Wnt3A suggesting that Wnt-induced changes in the mobility of membrane Amer1 are dependent on PtdIns(4,5)P_2 (Figure 3E).

Amer1 is required for PtdIns(4,5)P_2-induced LRP6 phosphorylation

We previously found that treatment of cells with ionomycin which activates phospholipase C and thereby leads to breakdown of PtdIns(4,5)P_2 resulted in release of Amer1 from the plasma membrane (Varnai and Balla, 1998; Grohmann *et al*, 2007). Interestingly, ionomycin treatment abolished LRP6 phosphorylation after Wnt treatment (Figure 4A). In line, inhibition of PtdIns(4,5)P_2 formation by knockdown of PI4KIIα prevents LRP6 phosphorylation after Wnt stimulation (Pan *et al*, 2008; Figure 4B). To analyse whether this is due to loss of Amer1 from the plasma membrane we asked whether tethering of Amer1 to the membrane independently of PtdIns(4,5)P_2 would be able to restore LRP6 phosphorylation. Indeed, reduced LRP6 phosphorylation after knockdown of PI4KIIα was prevented by Amer1 when fused to the transmembrane domain from the low-density lipoprotein (LDL) receptor (Figure 4B; Zeng *et al*, 2005). Recent data demonstrate that carrier-mediated transfer of exogenous PtdIns(4,5)P_2 lipids into cells enhances Wnt-induced LRP6 phosphorylation (Pan *et al*, 2008). We found that knockdown of Amer1 abolished the stimulation of LRP6 phosphorylation by PtdIns(4,5)P_2 (Figure 4C). These data demonstrate that Amer1 mediates the effect of PtdIns(4,5)P_2 on LRP6 phosphorylation.

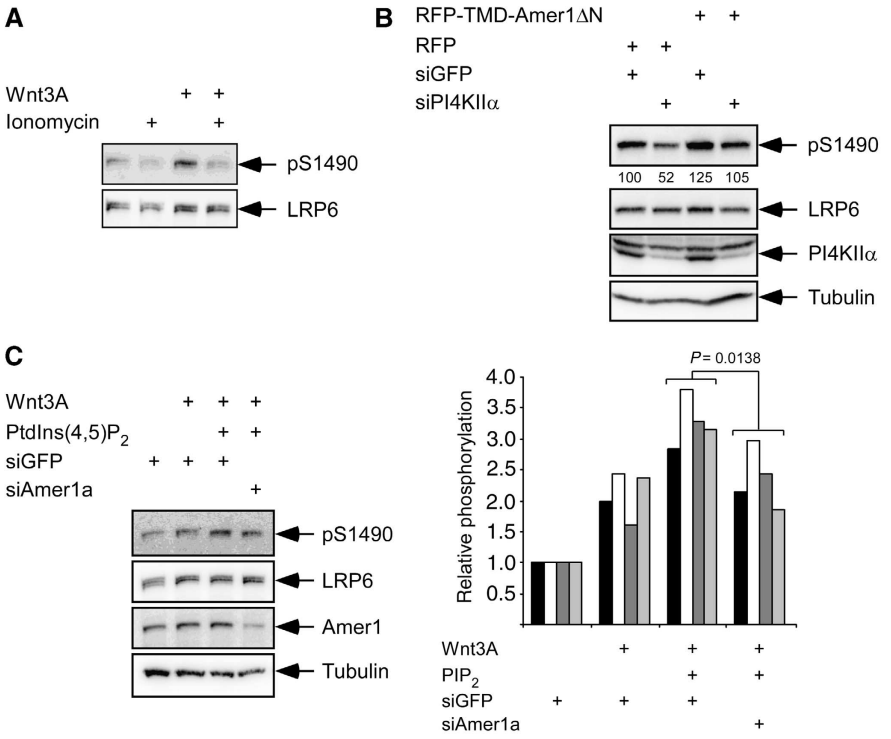

Figure 4 Amer1 is required for PtdIns(4,5)P_2-induced LRP6 phosphorylation. (**A**) Ionomycin treatment prevents Wnt-induced LRP6 phosphorylation. HEK293T cells stably expressing VSVG-LRP6 were treated with Wnt3A in the presence or absence of 10 μM ionomycin for 30 min. (**B**) Amer1ΔN (amino acids 207–1135) linked to the transmembrane domain of the LDL receptor (RFP-TMD-Amer1ΔN) rescues phosphorylation of endogenous LRP6 after knockdown of PI4KIIα. HeLa cells stably expressing RFP or RFP-TMD-Amer1ΔN were transfected with the indicated siRNAs and incubated with Wnt3A for 1 h. The numbers below the lanes reflect relative levels of phosphorylated LRP6 (pS1490) normalized to LRP6 as determined by densitometry. Data are representative of four independent experiments. (**C**) Amer1 is required for LRP6 phosphorylation stimulated by PtdIns(4,5)P_2. HEK293T cells stably expressing VSVG-LRP6 and transfected with the indicated siRNAs were treated with PtdIns(4,5)P_2 for 10 min before Wnt3A conditioned medium was added for another 20 min (left). Quantification of LRP6 phosphorylation from four independent experiments (right). Statistical analysis was done using an unpaired Student's *t*-test. The *P*-value reflects statistically significant differences. The black bars represent the experiment shown on the left.

Amer1 recruits Axin and GSK3β to the plasma membrane and promotes complex formation between Axin and LRP6

Our data show that Amer1 is required for Wnt-induced Axin translocation to the plasma membrane (see Figure 1A). Therefore, Amer1 might stimulate LRP6 phosphorylation through recruitment of Axin (Zeng *et al*, 2005, 2008). It is possible, however, that membrane association of Axin is a consequence rather than a cause of increased LRP6 phosphorylation induced by Amer1 because the phosphorylated PPPSPxS motifs in the cytoplasmic domain of LRP6 can serve as docking sites for Axin (Mao *et al*, 2001; Tamai *et al*, 2004; Davidson *et al*, 2005). To rule out this possibility, we treated cells with LiCl in order to inhibit GSK3β-mediated LRP6 phosphorylation and monitored Wnt-induced Axin translocation to the plasma membrane. LiCl treatment efficiently inhibited LRP6 phosphorylation but had no effect on Axin recruitment (Figure 5A). This shows that phosphorylation of LRP6 is not required for the association of Axin with the plasma membrane after Wnt treatment, suggesting that Amer1 promotes Axin translocation independently of prior LRP6 phosphorylation. We therefore analysed whether Amer1 can directly recruit Axin and the associated GSK3β. Amer1 formed endogenous complexes with Axin as shown by immunoprecipitation (Supplementary Figure S5A). In agreement with previous reports, Axin was diffusely distributed in the cytoplasm in a dotty pattern when exogenously expressed in MCF-7 cells (e.g., Schwarz-Romond *et al*, 2005; Figure 5B). In contrast, Axin was localized to the plasma membrane when Amer1 was expressed (Figure 5B; Supplementary Figure S5B). Similarly, endogenous Conductin was redistributed by Amer1 to the plasma membrane in SW480 colon carcinoma cells (Supplementary Figure S5C). Axin and Conductin were not redirected to the plasma membrane by Amer1(7μLys) mutants, indicating that membrane association of Amer1 is required (Supplementary Figure S5B and C). Importantly, in the presence but not in the absence of Axin Amer1 was also able to recruit GSK3β to the plasma membrane (Figure 5B). In line, GSK3β co-immunoprecipitated with Amer1 in the presence of wild-type Axin but not in the presence of a mutant that lacks GSK3β binding (AxinL396Q; Zeng *et al*, 2008), indicating that Axin proteins link GSK3β to Amer1 (Figure 5C). Together, these data suggest that Amer1 stimulates LRP6 phosphorylation by recruiting the Axin/GSK3β complex. In support of this, a C-terminal deletion mutant of Amer1 that retains Axin/Conductin binding (Amer1(2–601)) stimulated LRP6 phosphorylation whereas a mutant lacking the Axin/Conductin-binding region (Amer1(2–530)) failed to do so (Figure 5D; Supplementary Figure S6A–C).

Next, we analysed whether Amer1 binds to LRP6 by co-immunoprecipitation experiments. We found that Amer1 and

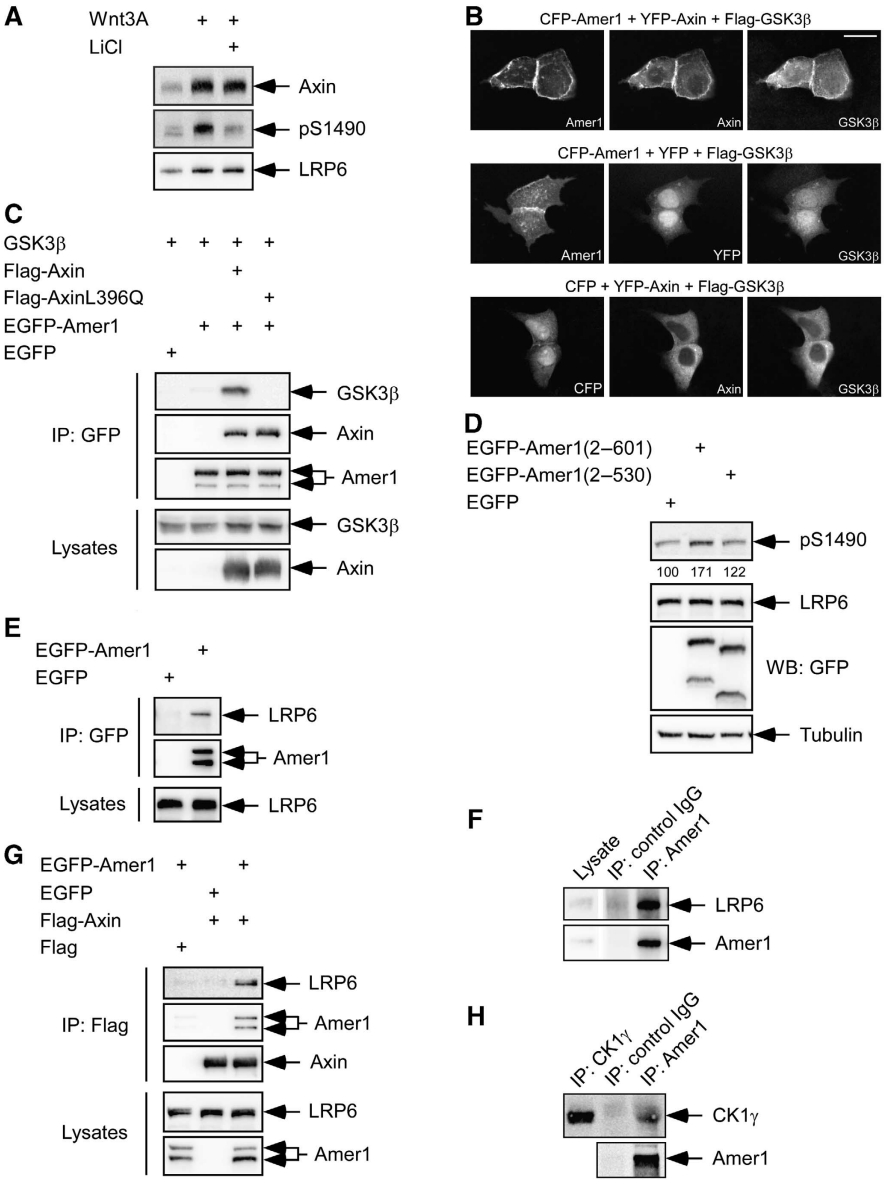

Figure 5 Amer1 recruits Axin and GSK3β to the plasma membrane and promotes complex formation between Axin and LRP6. (**A**) Inhibition of LRP6 phosphorylation by LiCl does not prevent Wnt-induced Axin translocation to the plasma membrane. HEK293T cells stably expressing EGFP-LRP6 were incubated with 50 mM LiCl for 30 min before Wnt3A treatment for 1 h and membrane fractions were analysed by western blotting. (**B**, **C**) Amer1 associates with GSK3β and recruits it to the plasma membrane via the interaction with Axin. (**B**) MCF-7 cells were co-transfected as indicated above the panels. Expressed proteins were detected by CFP and YFP fluorescence and anti-Flag immunofluorescence. Scale bar is 20 μm. (**C**) GSK3β co-immunoprecipitates with EGFP-Amer1 in the presence of Flag-Axin but not Flag-AxinL396Q, which is defective in GSK3β binding. (**D**) The binding of Amer1 to Axin/Conductin is required for its effect on LRP6 phosphorylation. HEK293T cells stably expressing VSVG-LRP6 were transiently transfected with EGFP or EGFP-tagged Amer1 mutants as detailed in Supplementary Figure S6A–C. The numbers below the lanes reflect relative levels of phosphorylated LRP6 (pS1490) normalized to LRP6 as determined by densitometry. Data are representative of four independent experiments. (**E**, **F**) Amer1 interacts with LRP6. **(E)** Co-immunoprecipitation of LRP6 with EGFP-Amer1 upon transient transfection of HEK293T cells stably expressing VSVG-LRP6. **(F)** Co-immunoprecipitation of endogenous Amer1 and LRP6 from lysates of HEK293T cells. Immunoprecipitations were performed with mouse anti-Amer1 or control IgG antibodies. (**G**) Amer1 links Axin to LRP6. VSVG-LRP6 stably expressed in HEK293T cells co-immunoprecipitated with Flag-Axin in the presence but not in the absence of EGFP-Amer1. **(H)** Amer1 interacts with CK1γ. Co-immunoprecipitation of endogenous CK1γ and Amer1 from lysates of SW480 cells. Immunoprecipitations were performed with mouse anti-Amer1 or control IgG antibodies. As CK1γ levels in lysates were very low, immunoprecipitation with anti-CK1γ antibodies is shown.

LRP6 form complexes after overexpression and at the endogenous level (Figure 5E and F). Amer1 also co-immunoprecipitated with LRP6 lacking the extracellular domain (LRP6ΔE(1–4); Supplementary Figure S7A and B). Immunoprecipitation with serial LRP6 C-terminal deletion mutants (Davidson *et al*, 2005) showed that Amer1 interacts with a fragment retaining the membrane proximal PPPSPxS motif and flanking Ser/Thr clusters (LRP6ΔE(1–4)Δ87; Supplementary Figure S7A and B). Deletion of the PPPSPxS motif abolished the interaction (LRP6ΔE(1–4)Δ127). Amer1 did not interact with a fragment consisting only of the PPPSPxS motif and Ser/Thr clusters, indicating that these motifs are not sufficient for Amer1 binding (LDLRΔN-miniC; Supplementary Figure S7A and B). Moreover, alanine substitutions

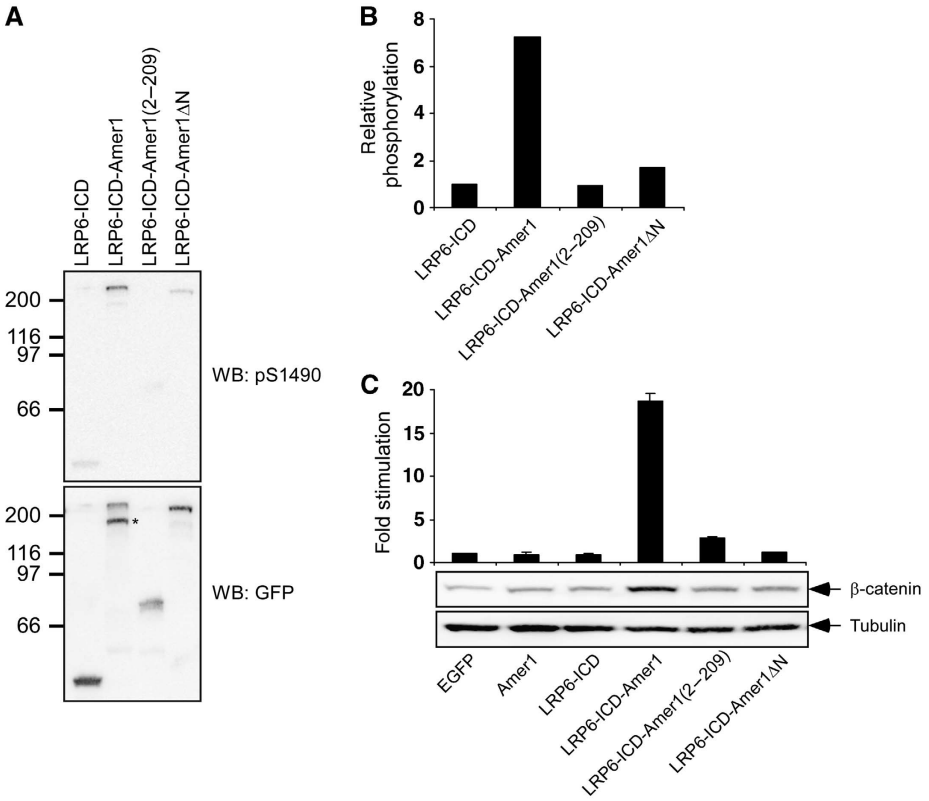

Figure 6 Fusion of Amer1 to the cytoplasmic domain of LRP6 induces LRP6 phosphorylation and stimulates downstream Wnt/β-catenin signalling. **(A, B)** Amer1 fused to the LRP6 intracellular domain (ICD) suffices to induce LRP6 phosphorylation. (**A**) Phosphorylation of the EGFP-LRP6-ICD–Amer1 fusion constructs (Supplementary Figure S9) as determined by anti-pS1490 western blotting. The band indicated by the asterisk most likely represents the Amer1-S2 splice variant generated from the LRP6-ICD–Amer1 fusion, which does not stimulate phosphorylation of the LRP6-ICD. **(B)** Quantification of the experiment from (**A**) as determined by densitometry. The intensity of the pS1490 bands was normalized to the intensity of the GFP bands with the LRP6-ICD set to 1. **(C)** The LRP6-ICD–Amer1 fusion protein activates a β-catenin-dependent luciferase reporter stably expressed in HEK293T cells (Major *et al*, 2007) and induces stabilization of cytoplasmic β-catenin. Fold changes of luciferase activity were determined by normalization to the EGFP control. Error bars indicate s.d.

of serines and threonines in the PPPSPxS motifs (LRP6m10; Zeng *et al*, 2005) did not affect Amer1 binding to LRP6 (data not shown). Together, these data demonstrate that Amer1 binds close to the signalling motifs phosphorylated by GSK3β and CK1γ, but that phosphorylation of these motifs is not required for Amer1 binding. Deletion analysis of Amer1 demonstrated that both central and C-terminal parts interact with LRP6, suggesting that there are multiple LRP6 interaction sites in Amer1 (data not shown).

Axin is suggested to form a complex with LRP6 (Mao *et al*, 2001; Tamai *et al*, 2004; Davidson *et al*, 2005). Because Amer1 binds to both Axin and LRP6, it might promote complex formation between the two proteins. Indeed, while LRP6 was only poorly co-immunoprecipitated with Axin in the absence of Amer1, it was strongly immunoprecipitated when Amer1 was present (Figure 5G). Conversely, Axin might link LRP6 to Amer1. However, this is unlikely, because Axin did not increase the amounts of LRP6 co-immunoprecipitated with Amer1 (Supplementary Figure S7C).

In co-immunoprecipitation experiments endogenous complexes of Amer1 and CK1γ were found (Figure 5H). Amer1 did not interact with a CK1γ mutant lacking the membrane association domain (Supplementary Figure S8A and B). These results are consistent with the finding that Amer1 is necessary for LRP6 phosphorylation at the CK1γ site Thr1479 (cf. Figure 1B; Supplementary Figure S1A).

An Amer1–LRP6 fusion protein activates downstream Wnt/β-catenin signalling independently of Wnt

Our data indicate that Amer1 acts by recruiting the Axin/GSK3β complex to LRP6. To test whether close proximity between Amer1 and LRP6 would be sufficient to trigger LRP6 phosphorylation and, consequently, downstream β-catenin signalling, we fused Amer1 to the ICD of LRP6 (Supplementary Figure S9). After transfection in HEK293T cells phosphorylation of the LRP6-ICD was strongly stimulated by fusion to Amer1, but not by fusion to the membrane targeting domain of Amer1 (amino acids 2–209) or to Amer1ΔN lacking this domain (Figure 6A and B). Consistent with this, the LRP6-ICD–Amer1 fusion protein robustly induced stabilization of cytoplasmic β-catenin and activation of a TCF/β-catenin-dependent transcriptional reporter (Figure 6C). In contrast, the LRP6-ICD alone or its fusion to the Amer1 deletion mutants had no or only a minor stimulatory activity. Thus, Amer1 can directly induce LRP6 phosphorylation and activation of downstream Wnt/β-catenin signalling when fused to the cytoplasmic domain of LRP6. Notably, Amer1 alone did not stimulate β-catenin stabilization or reporter gene expression in spite of its ability to induce LRP6 phosphorylation (see Figure 1C; Supplementary Figure S1C). This is presumably due to its concurrent function in promoting β-catenin degradation (Major *et al*, 2007), and indicates that its negative regulatory activity is abolished in the LRP6-ICD–Amer1 fusion construct (see Discussion).

Discussion

It is meanwhile well established that LRP6 phosphorylation is a key and early event in Wnt signalling. However, although several key players are known in this process it has remained elusive how LRP6 is linked to the activating kinases GSK3β and CK1γ and how Wnt-induced formation of PtdIns(4,5)P_2 is involved. We propose that Amer1 is an essential intermediate in the process of Wnt receptor activation and that it has a specific role in connecting PtdIns(4,5)P_2 to LRP6 phosphorylation.

Mechanism of LRP6 phosphorylation induced by Amer1

Our knockdown and overexpression experiments showed that Amer1 is necessary and sufficient for LRP6 phosphorylation. We suggest that this function is based on the complex formation of Amer1 with Axin/GSK3β and CK1γ, and on the Wnt-regulated dynamic interaction of these complexes with the plasma membrane. It was previously shown that Axin and its relative Conductin are crucial for LRP6 phosphorylation through binding to GSK3β (Zeng *et al*, 2005, 2008). We found that Amer1 interacts with Axin and the associated GSK3β and can link Axin to LRP6. When directly fused to the cytoplasmic domain of LRP6 Amer1 promotes phosphorylation of LRP6 at the GSK3β phosphorylation site Ser1490, and induces downstream Wnt/β-catenin signalling. This suggests that Amer1 acts as a scaffold protein to recruit GSK3β to LRP6 via Axin, and thereby promotes LRP6 phosphorylation, very much like Axin acts as a scaffold for β-catenin phosphorylation (Behrens *et al*, 1998; Ikeda *et al*, 1998). Amer1 also interacts with CK1γ, the second kinase responsible for LRP6 phosphorylation. Unlike GSK3β, CK1γ is constitutively present at the plasma membrane (Davidson *et al*, 2005) and it is not known how it is activated by Wnt signalling. It is possible that Amer1 recruits CK1γ from the lateral plasma membrane to the vicinity of LRP6 receptors and thereby positions CK1γ for optimal phosphorylation of LRP6. In line with a scaffolding role, Amer1 binds close to the membrane proximal phosphorylation sites of GSK3β and CK1γ in LRP6 (Supplementary Figure S7A and B).

Of interest, Amer1 was initially identified as an APC-binding protein, which contains three independent APC interaction sites (Supplementary Figure S3A). We noticed that Wnt-induced LRP6 phosphorylation does not depend on APC (Supplementary Figure S10), suggesting that this interaction is not of relevance for the function of Amer1 in LRP6 phosphorylation.

From our immunoprecipitation experiments and published proteomic studies (Major *et al*, 2007), Amer1/Axin/GSK3β and Amer1/CK1γ complexes seem to form constitutively in cells. This raises the question why these complexes are inactive with respect to LRP6 phosphorylation in the absence of Wnts and how they become activated by Wnt signalling. We found that Amer1 is recruited to the plasma membrane after Wnt stimulation through formation of PtdIns(4,5)P_2, and that membrane localization of Amer1 is essential for stimulation of LRP6 phosphorylation. Moreover, Amer1 is required for plasma membrane localization of Axin after Wnt stimulation and can directly recruit Axin/GSK3β to the membrane. Altogether, this points to a crucial role of Wnt-induced membrane localization of Amer1 for activation of LRP6 phosphorylation. In the absence of Wnts, a fraction of Amer1 together with its associated proteins might be present at the plasma membrane but at too low concentrations to promote LRP6 phosphorylation, whereas after Wnt stimulation the mere increase of plasma membrane bound Amer1 leads to activation of LRP6 phosphorylation. Moreover, it could well be that PtdIns(4,5)P_2 is preferentially formed in the vicinity of the Fz/LRP6 receptor complexes due to local engagement of Dvl by Fz, which stimulates PtdIns(4,5)P_2 synthesis after Wnt stimulation (Pan *et al*, 2008). This could generate a high density of binding sites for Amer1 and its associated kinases at the receptors and thereby allow efficient phosphorylation of LRP6. In support of this, increased PtdIns(4,5)P_2 levels were found to be associated with LRP6 aggregates, that is, signalosomes, as compared with non-aggregated LRP6 in sucrose density fractions (Pan *et al*, 2008). Altogether, we suggest a model (Figure 7) in which PtdIns(4,5)P_2 molecules formed after Wnt stimulation through Dvl serve as docking sites for Amer1 at the plasma membrane and attract Amer1 to LRP6. Amer1 recruits Axin/GSK3β and CK1γ to these sites, binds to LRP6 and promotes LRP6 phosphorylation. Thus, Amer1 seems to act as a scaffold protein at the plasma membrane that connects LRP6 receptors to the activating kinases in a Wnt-inducible and PtdIns(4,5)P_2-dependent manner. This model implies that the PtdIns(4,5)P_2-dependent recruitment of Amer1 is of functional relevance for LRP6 phosphorylation and positions Amer1 within the Wnt-Dvl-PtdIns(4,5)P_2 pathway described previously (Tamai *et al*, 2004; Davidson *et al*, 2005; Zeng *et al*, 2005, 2008; Pan *et al*, 2008). In fact, our studies show that Amer1 is required for LRP6 phosphorylation stimulated by PtdIns(4,5)P_2 and that fusion of Amer1 to an unrelated membrane targeting domain rescues LRP6 phosphorylation after inhibition of PtdIns(4,5)P_2 synthesis by knockdown of PI4KIIα.

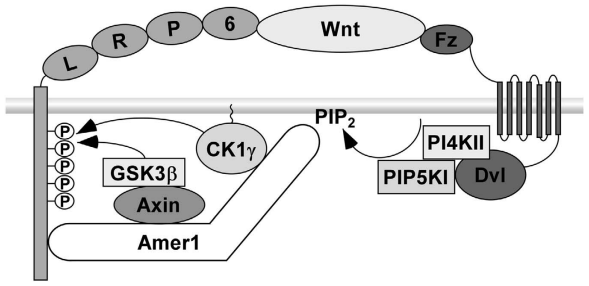

Figure 7 A model for the involvement of Amer1 in PtdIns(4,5)P_2-mediated LRP6 phosphorylation. Wnt binding to the Fz-LRP6 receptor complex leads to recruitment of Dvl, which induces the formation of PtdIns(4,5)P_2 by binding and activating the phosphatidylinositol kinases PI4KII and PIP5KI. The generation of PtdIns(4,5)P_2 in regions of receptor activity triggers the recruitment of Amer1 proteins, which in turn promote LRP6 phosphorylation by recruiting Axin/GSK3β and CK1γ to LRP6.

Integration of Amer1 into current models of LRP6 phosphorylation

According to an initiation-amplification model, LRP6 phosphorylation by GSK3β occurs in two steps. Initial recruitment of the Axin/GSK3β complex to the plasma membrane allows phosphorylation at PPPSPxS motifs such as at Ser1490 and thus creates docking sites for more Axin/GSK3β complexes, which in turn induce further phosphorylation at other

PPPSPxS sites resulting in amplification of the signal (Zeng *et al*, 2008; MacDonald *et al*, 2009). It was proposed that initiation occurs through Fz/Dvl-mediated recruitment of Axin/GSK3β complexes to LRP6 (Cliffe *et al*, 2003; Zeng *et al*, 2008). Amer1 may have a similar role as Dvl in mediating the initial phosphorylation steps because it can recruit Axin/GSK3β independently of prior phosphorylation of LRP6. In fact, Dvl and Amer1 might cooperate in the recruitment of Axin/GSK3β: Dvl might recruit Axin by direct interaction, and/or indirectly by promoting PtdIns(4,5)P_2 synthesis and, as a consequence, Amer1 translocation to the plasma membrane. Amer1 does not seem to be conserved in invertebrates such as Drosophila where phosphorylation of the LRP6 orthologue arrow is presumably also critical. This may indicate that initiation relies entirely on the Dvl-dependent Axin recruitment in these organisms and that Amer1 provides an additional level of regulation in vertebrates.

According to the signalosome model, Fz-LRP6 receptor pairs aggregate due to polymerization of Dvl and co-cluster with Axin leading to LRP6 phosphorylation (Bilic *et al*, 2007; Schwarz-Romond *et al*, 2007a, b). We found that Amer1 is essential for signalosome formation. This role of Amer1 is probably independent of its function in LRP6 phosphorylation because signalosome formation did not require LRP6 phosphorylation by CK1γ (Bilic *et al*, 2007). Instead, Amer1 might be directly involved in receptor aggregation, for example, through its ability to connect LRP6 to Axin, which in turn can interact with Dvl. Our FRAP analysis shows that Amer1 is recruited to an immobile pool at the plasma membrane upon Wnt stimulation, which might correspond to signalosomes. So far, we have not been able to detect Amer1 in signalosomes probably owing to the suboptimal stoichiometry of the many overexpressed proteins in this assay. Of note, PtdIns(4,5)P_2 was shown to be required for signalosome formation (Pan *et al*, 2008), possibly through interaction with Amer1.

Amer1 has a dual positive and negative role in Wnt signalling

Our results show that Amer1 acts as an activator of the Wnt signalling pathway at the LRP6 receptor level, whereas loss of function and biochemical studies suggest a role of Amer1 as a negative regulator of Wnt signalling by inducing degradation of β-catenin (Major *et al*, 2007). Indeed, overexpression of Amer1 repressed the activity of a TCF/β-catenin-dependent transcriptional reporter (Supplementary Figure S11). Thus, Amer1 has a dual functional role as an activator and inhibitor of the Wnt pathway. Because of its downstream role in β-catenin degradation only the negative regulatory role becomes apparent in loss of function studies. The dual role of Amer1 is similar to that of Axin and GSK3β, which are required for activation of LRP receptors but are also essential for β-catenin degradation. In the case of GSK3β, these functions may be spatially separated between plasma membrane and cytoplasm (Zeng *et al*, 2005, 2008). In contrast, both activation and inhibition of Wnt signalling by Amer1 require its localization in the plasma membrane (Figure 2; KT and JB unpublished data) raising the question as to how these activities are regulated. It is possible that the differential localization of Amer1 between the general membrane compartment and clustered Fz/LRP6 receptors determines the balance between inhibiting and activating functions of Amer1 and provides a switch mechanism between these activities. In the simplest model, a constitutive pool of Amer1 at the plasma membrane might be involved in the steady state degradation of β-catenin. After Wnt stimulation, Amer1 is recruited close to LRP6 leading to its phosphorylation as discussed above. Thereafter, phosphorylated LRP6 might directly inhibit Amer1 function in β-catenin degradation by blocking GSK3β via its phosphorylated PPPSPxS motifs (Cselenyi *et al*, 2008; Piao *et al*, 2008; Wu *et al*, 2009) and/or by interfering with binding of Amer1 to interaction partners such as β-catenin and APC. In line, when present in a fusion protein with the ICD of LRP6 Amer1 can lead to stabilization of β-catenin and activation of Wnt/β-catenin-dependent transcription, indicating that the function of Amer1 in β-catenin degradation is blocked in the complex with LRP6 (Figure 6C). Alternatively, Amer1 might initially act as an activator of the signal and turn into an inhibitor, as part of a negative feedback regulation mediated by the TCF/β-catenin-dependent upregulation of Conductin (Lustig *et al*, 2002).

Materials and methods

Cell culture, Wnt treatment and subcellular fractionation

HEK293T cells stably expressing VSVG-LRP6 (Zeng *et al*, 2005) were kindly provided by X He and HEK293T cells stably expressing LRP6-EGFP (Kategaya *et al*, 2009) or pBAR/*Renilla* (Major *et al*, 2007) by RT Moon. HeLa cells stably expressing RFP-TMD-Amer1ΔN or RFP were selected after transfection in medium containing 1 mg/ml geneticin (G418, Invitrogen) and enriched using a MoFlo high-speed cell sorter (Dako Cytomation). Wnt3A conditioned medium was produced from mouse L cells stably expressing Wnt3A (American Type Culture Collection CRL-2647) and added 48 h after transfection for knockdown experiments, or 16 h after transfection for overexpression experiments. Ionomycin was obtained from Calbiochem. Subcellular fractionation of cells was carried out using the ProteoJET Membrane Protein Extraction Kit (Fermentas) according to the manufacturer's instructions.

Plasmids

The pEGFP-Amer1(7μLys) mutant was obtained by PCR mutagenesis. pEGFP-NES-Amer1(7μLys) was created by the insertion of an oligonucleotide coding for a consensus MAPKK-NES (NLVDLQKK LEELELDEQQ) (Henderson and Eleftheriou, 2000) between the EGFP- and Amer1-coding sequences of pEGFP-Amer1(7μLys). To obtain the pEGFP-LRP6-ICD–Amer1 fusion constructs, the coding sequence of the LRP6-ICD (residues 1394–1613) was inserted between the coding sequences of EGFP and the respective Amer1 constructs. mRFP-TMD-Amer1ΔN was generated by replacing the LRP6-coding sequence of mRFP-daLRP6 (Krieghoff *et al*, 2006) with the transmembrane domain of the LDL receptor (residues 781–849; Zeng *et al*, 2005) fused to the Amer1(207–1135)-coding sequence. To generate pEGFP-Amer1-S1, three single nucleotide changes (147 A>C, 150 T>C, 153 G>A) were introduced leading to the ablation of the internal splice donor site without changing the amino-acid sequence. pEGFP-Amer1-S2 contains an in-frame deletion of residues 50–326. Details of the plasmids are available upon request.

Lipid-binding assay and PtdIns(4,5)P_2 delivery

GST-Amer1 proteins were expressed in *Escherichia coli* BL21 and freshly purified before the experiment using Glutathione Sepharose 4B beads (GE Healthcare) as described previously (Grohmann *et al*, 2007). Membrane Lipid Strips (Echelon Biosciences Inc.) were incubated with the GST fusion proteins at a concentration of 1 μg/ml at 4°C overnight and detected by an anti-GST antibody, according to the manufacturer's instructions. For delivery of PtdIns(4,5)P_2 into cells, lipids and Carrier 3 (Echelon Biosciences Inc.) were pre-incubated at a 1:1 molar ratio (100 μM final concentration each) for 10 min at room temperature and then added to the cells at a final concentration of 10 μM. After incubation of the cells for 10 min at 37°C,

the same volume of Wnt3A conditioned medium was added for another 20 min.

Immunofluorescence microscopy

Immunofluorescence stainings were performed as described previously (Hadjihannas *et al*, 2006; Grohmann *et al*, 2007). For signalosome experiments, cells were transfected with 100 pmol siRNA together with 600 ng LRP6-EYFP, 200 ng MESD, 200 ng Fz8-EYFP, 200 ng Flag-Axin, 200 ng Flag-GSK3β and 100 ng CFP-Dvl2 using TransIT-TKO (Mirus, Madison, WI, USA). Photographs were taken with a CCD camera (Visitron, Munich, Germany) on a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany, ×63 objective or ×100 objective) and MetaMorph software (Molecular Devices).

Amer1 antibodies

The mouse monoclonal antibody against Amer1 has been described before (Grohmann *et al*, 2007). The Amer1-specific rabbit polyclonal antibody was produced by Pineda (Berlin, Germany) by immunizing rabbits with amino acids 2–285 of recombinant human Amer1 generated as a GST fusion in bacteria. The serum was affinity purified using CNBr-activated Sepharose 4B beads (GE Healthcare) coupled to GST-Amer1(2–285).

Reporter assays

β-Catenin reporter assays were carried out in HEK293T cells stably expressing a β-catenin responsive firefly luciferase reporter (pBAR) along with a *Renilla* luciferase, which serves as an internal control (Major *et al*, 2007). Cells were seeded in 12-well plates, transfected with 200 ng of the indicated constructs and harvested 24 h post-transfection. Firefly and *Renilla* luciferase activities were determined according to standard procedures, and firefly luciferase values were normalized to *Renilla* values. All experiments were performed in duplicates and reproduced at least twice.

Fluorescence recovery after photobleaching

HEK293 cells were plated on 35-mm glass bottom dish (MatTek Corp.) pre-coated with 0.1% collagen (Sigma) and transfected with the calcium phosphate method using 1.6 μg of EGFP-Amer1 plasmid/dish. FRAP analysis was carried out 24 h after transfection on a Zeiss LSM710 laser scanning microscope (488 nm laser line) with a C-Apochromat ×40 water immersion objective, NA = 1.2 (Zeiss, Jena, Germany). During live cell imaging, cells were kept at 37°C in serum-free DMEM supplemented with 20 mM HEPES and 0.1% BSA. Stimulation with 100 ng/ml purified, recombinant Wnt3A (R&D Systems) and/or 10 mM neomycin (Sigma) was performed for 30 min prior image acquisition. One or two ~5 μm^2 square regions of interest (ROI) were selected per cell. Basal signal intensity was measured in 1 s intervals for 18 s. Applied laser power was 1% to minimize photobleaching. Then, the ROI was bleached for 4 s with two scans with maximum power using the 488-nm line argon laser. Fluorescence recovery was recorded in 1 s intervals for 154 s. Image processing and data analysis were done using the Zen 2009 software (Zeiss, Jena, Germany). The raw data were normalized with the average fluorescence of 20 data points before bleaching as 100% and the first value after bleaching as 0%. Sum of all datasets with stable recovery have been statistically analysed and the quantitative information (mobile pool, recovery halftime) was obtained by the non-linear one phase association using the least squares fitting method. The normalization and statistical analysis have been performed using GraphPad Prism software (GraphPad Software Inc.).

RT–PCR

RT–PCR was performed according to standard protocols (see Supplementary Experimental Procedures) using the following sense and antisense primers: Amer1-S1 (5′-GCGAATTCGGAGACCC AAAAGGATGAAGCTGCTCAG-3′, 5′-CCTTGCTCTTCCGGTGACGGC GGATACTGC-3′), Amer1-S2 (5′-GCGAATTCGGAGACCCAAAAGG ATGAAGCTGCTCAG-3′, 5′-CATCATCATCTGGCAAGGCCATCTC-3′) and GAPDH (5′-CTTCACCACCATGGAGAAGG-3′, 5′-CCTGCTTCACC ACCTTCTTG-3′). Note that PCR for Amer1-S2 leads to the co-amplification of products from Amer1-S1 and Amer1-S2 as the primers flank the alternatively spliced intron. GAPDH was used for normalization.

Supplementary data

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

Acknowledgements

We thank C Niehrs, X He, RT Moon, P De Camilli and A Kikuchi for cell lines and reagents; J Mašek for technical assistance with FRAP analysis; I Wacker for helpful discussions; A Döbler for secretarial assistance. This work was supported by the DFG research grant BE 1550/6-1 and the IZKF research grant TPD12 to Jürgen Behrens and the DFG research grant SCHN 1189/1-1 to Jean Schneikert. Vitezslav Bryja is supported by grants from the Czech Science Foundation (204/09/0498, 204/09/J030), EMBO Installation Grant, the Ministry of Education Youth and Sports of the Czech Republic (MSM 0021622430) and by Academy of Sciences of the Czech Republic (AVOZ50040507, AVOZ50040702). Gunnar Schulte is supported by Knut and Alice Wallenberg Foundation (KAW2008.0149), Swedish Research Council (K2008-68P-20810-01-4, K2008-68X-20805-01-4) and The Swedish Foundation for International Cooperation in Research and Higher Education (STINT).

Author contributions: KT planned and performed most of the experimental work. KT, ASP, KB, MVH and JS performed co-immunoprecipitation and immunofluorescence experiments. ASP generated the Amer1-specific rabbit polyclonal antibody. VK, GS and VB performed FRAP experiments. JB coordinated the project and assisted with planning the experiments and data analysis. The manuscript was written by JB and KT.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Angers S, Moon RT (2009) Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol* **10:** 468–477

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W (1998) Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* **280:** 596–599

Bilic J, Huang YL, Davidson G, Zimmermann T, Cruciat CM, Bienz M, Niehrs C (2007) Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science* **316:** 1619–1622

Clevers H (2006) Wnt/beta-catenin signalling in development and disease. *Cell* **127:** 469–480

Cliffe A, Hamada F, Bienz M (2003) A role of dishevelled in relocating axin to the plasma membrane during wingless signalling. *Curr Biol* **13:** 960–966

Cselenyi CS, Jernigan KK, Tahinci E, Thorne CA, Lee LA, Lee E (2008) LRP6 transduces a canonical Wnt signal independently of axin degradation by inhibiting GSK3's phosphorylation of beta-catenin. *Proc Natl Acad Sci USA* **105:** 8032–8037

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

Gabev E, Kasianowicz J, Abbott T, McLaughlin S (1989) Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP2). *Biochim Biophys Acta* **979:** 105–112

Grohmann A, Tanneberger K, Alzner A, Schneikert J, Behrens J (2007) AMER1 regulates the distribution of the tumor suppressor APC between microtubules and the plasma membrane. *J Cell Sci* **120:** 3738–3747

Hadjihannas MV, Bruckner M, Jerchow B, Birchmeier W, Dietmaier W, Behrens J (2006) Aberrant Wnt/beta-catenin signaling can induce chromosomal instability in colon cancer. *Proc Natl Acad Sci USA* **103:** 10747–10752

Henderson BR, Eleftheriou A (2000) A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp Cell Res* **256:** 213–224

Huang H, He X (2008) Wnt/beta-catenin signalling: new (and old) players and new insights. *Curr Opin Cell Biol* **20:** 119–125

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

Jenkins ZA, van Kogelenberg M, Morgan T, Jeffs A, Fukuzawa R, Pearl E, Thaller C, Hing AV, Porteous ME, Garcia-Minaur S, Bohring A, Lacombe D, Stewart F, Fiskerstrand T, Bindoff L, Berland S, Ades LC, Tchan M, David A, Wilson LC *et al* (2009) Germline mutations in WTX cause a sclerosing skeletal dysplasia but do not predispose to tumorigenesis. *Nat Genet* **41:** 95–100

Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signalling. *Cell* **125:** 943–955

Kategaya LS, Changkakoty B, Biechele T, Conrad WH, Kaykas A, Dasgupta R, Moon RT (2009) Bili inhibits Wnt/beta-catenin signalling by regulating the recruitment of axin to LRP6. *PLoS One* **4:** e6129

Krieghoff E, Behrens J, Mayr B (2006) Nucleo-cytoplasmic distribution of {beta}-catenin is regulated by retention. *J Cell Sci* **119:** 1453–1463

Lustig B, Behrens J (2003) The Wnt signalling pathway and its role in tumor development. *J Cancer Res Clin Oncol* **129:** 199–221

Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, Behrens J (2002) Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* **22:** 1184–1193

MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signalling: components, mechanisms, and diseases. *Dev Cell* **17:** 9–26

MacDonald BT, Yokota C, Tamai K, Zeng X, He X (2008) Wnt signal amplification via activity, cooperativity, and regulation of multiple intracellular PPPSP motifs in the Wnt co-receptor LRP6. *J Biol Chem* **283:** 16115–16123

Major MB, Camp ND, Berndt JD, Yi X, Goldenberg SJ, Hubbert C, Biechele TL, Gingras AC, Zheng N, Maccoss MJ, Angers S, Moon RT (2007) Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. *Science* **316:** 1043–1046

Mao J, Wang J, Liu B, Pan W, Farr III GH, Flynn C, Yuan H, Takada S, Kimelman D, Li L, Wu D (2001) Low-density lipoprotein receptor-related protein-5 binds to axin and regulates the canonical Wnt signaling pathway. *Mol Cell* **7:** 801–809

Pan W, Choi SC, Wang H, Qin Y, Volpicelli-Daley L, Swan L, Lucast L, Khoo C, Zhang X, Li L, Abrams CS, Sokol SY, Wu D (2008) Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. *Science* **321:** 1350–1353

Piao S, Lee SH, Kim H, Yum S, Stamos JL, Xu Y, Lee SJ, Lee J, Oh S, Han JK, Park BJ, Weis WI, Ha NC (2008) Direct inhibition of GSK3beta by the phosphorylated cytoplasmic domain of LRP6 in Wnt/beta-catenin signaling. *PLoS One* **3:** e4046

Pilot F, Philippe JM, Lemmers C, Lecuit T (2006) Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. *Nature* **442:** 580–584

Qin Y, Li L, Pan W, Wu D (2009) Regulation of phosphatidylinositol kinases and metabolism by Wnt3a and Dvl. *J Biol Chem* **284:** 22544–22548

Rivera MN, Kim WJ, Wells J, Driscoll DR, Brannigan BW, Han M, Kim JC, Feinberg AP, Gerald WL, Vargas SO, Chin L, Iafrate AJ, Bell DW, Haber DA (2007) An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. *Science* **315:** 642–645

Schwarz-Romond T, Fiedler M, Shibata N, Butler PJ, Kikuchi A, Higuchi Y, Bienz M (2007a) The DIX domain of dishevelled confers Wnt signalling by dynamic polymerization. *Nat Struct Mol Biol* **14:** 484–492

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

Schwarz-Romond T, Metcalfe C, Bienz M (2007b) Dynamic recruitment of axin by dishevelled protein assemblies. *J Cell Sci* **120:** 2402–2412

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

Varnai P, Balla T (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. *J Cell Biol* **143:** 501–510

Wu G, Huang H, Garcia Abreu J, He X (2009) Inhibition of GSK3 phosphorylation of beta-catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. *PLoS One* **4:** e4926

Zeng X, Huang H, Tamai K, Zhang X, Harada Y, Yokota C, Almeida K, Wang J, Doble B, Woodgett J, Wynshaw-Boris A, Hsieh JC, He X (2008) Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development* **135:** 367–375

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