Plasma membrane recruitment of dephosphorylated β-catenin upon activation of the Wnt pathway

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Summary

The standard model of Wnt signaling specifies that after receipt of a Wnt ligand at the membranous receptor complex, downstream mediators inhibit a cytoplasmic destruction complex, allowing β-catenin to accumulate in the cytosol and nucleus and co-activate Wnt target genes. Unexpectedly, shortly after Wnt treatment, we detected the dephosphorylated form of β-catenin at the plasma membrane, where it displayed a discontinuous punctate labeling. This pool of β-catenin could only be detected in E-cadherin$^{-/-}$ cells, because in E-cadherin$^{+/+}$ cells Wnt-induced, membranous β-catenin was concealed by a constitutive junctional pool. Wnt-signaling-dependent dephosphorylated β-catenin colocalized at the plasma membrane with two members of the destruction complex – APC and axin – and the activated Wnt co-receptor LRP6. β-catenin induced through the Wnt receptor complex was significantly more competent transcriptionally than overexpressed β-catenin, both in cultured cells and in early *Xenopus* embryos. Our data reveal a new step in the processing of the Wnt signal and suggest regulation of signaling output beyond the level of protein accumulation.

Supplementary material available online at
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Introduction

The Wnt pathway is a critical determinant of cell proliferation during development and regenerative processes, such as stem cell proliferation in the adult (Clevers, 2006). Aberrant activation of the pathway has been linked to oncogenesis in multiple systems. A central player in the Wnt pathway is β-catenin (official symbol CTNB1). Most of the cellular pool of β-catenin is tethered to E-cadherin (also known as CDH1 and encoded by *Cdh1* in mouse) as an adherens junction component mediating cell-cell adhesion (McCrea et al., 1991; Peifer et al., 1994a). A less abundant pool of β-catenin, often referred to as the 'free' pool, functions in complex with T-cell factor–lymphoid enhancer factor (TCF-LEF) transcription factors as a transcriptional coactivator of Wnt signaling in the nucleus (Cadigan and Nusse, 1997). In the absence of a Wnt signal, the free pool of β-catenin is tightly regulated through phosphorylation at specific N-terminal residues by a so-called 'destruction complex' consisting of the serine kinases casein kinase 1 α (CK1α) and glycogen synthase kinase 3 (GSK3) and the tumor suppressors adenomatous polyposis coli (APC) and axin (Logan and Nusse, 2004). Phosphorylated β-catenin is marked for rapid ubiquitylation and degradation by the proteasome. Receipt of a Wnt ligand at the membranous receptor complex results in inhibition of β-catenin breakdown, allowing β-catenin to accumulate, enter the nucleus and activate a Wnt target gene program (Logan and Nusse, 2004).

However, our current understanding of Wnt signal transduction and β-catenin processing suffers from significant gaps. In particular, the make-up and subcellular localization of the mature destruction complex is unclear at present. For this reason, the mechanism through which the destruction complex senses ligand engagement at the Frizzled-LRP (lipoprotein receptor-related protein) receptor complex remains unidentified. Recently, increasing evidence suggests that important regulatory steps in the turnover of the destruction complex may take place at the plasma membrane (for a review, see Cadigan and Liu, 2006). Engagement of the Frizzled (Fz) and LRP5/6 co-receptors on the cell surface by Wnt ligands, results in the phosphorylation of the intracellular domain of LRP5/6 by CK1γ and/or GSK3β. Phosphorylated LRP5/6 presents a docking site for axin that is recruited to the plasma membrane in response to Wnt stimulation (Cliffe et al., 2003; Davidson et al., 2005; Zeng et al., 2005) along with other canonical Wnt pathway components including axin, GSK3β and Fz8 (Bilic et al., 2007). The scaffold protein Dishevelled (Dvl) appears to be required for this translocation (Schwarz-Romond et al., 2007). Although it remains unproven, it has been hypothesized that cytoplasmic destruction of β-catenin is halted as a result of axin relocation, allowing β-catenin to redistribute to the nucleus.

Nuclear localization of β-catenin is considered to be a hallmark of Wnt activation, yet in many systems it is only incidentally detected in the nucleus (Anderson et al., 2002; Kobayashi et al., 2000). The nuclear level of the N-terminally dephosphorylated (or 'dephospho') form of β-catenin has been shown to correlate better with Wnt activity (Staal et al., 2002). Dephospho-β-catenin has been suggested to reflect the de novo translated form of β-catenin, which

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is involved in signal transduction (Willert et al., 2002). We set out to optimize experimental conditions for the detection of dephospho-β-catenin in cultured mammalian cell lines. In a series of colon carcinoma cell lines, dephospho-β-catenin often localizes to the plasma membrane. Although we find no correlation with either APC or β-catenin mutation status, the plasma membrane localization of dephospho-β-catenin does correlate with E-cadherin expression. Surprisingly, stimulation of E-cadherin$^{-/-}$ cells with Wnt3A resulted in the appearance of dephospho-β-catenin at the plasma membrane, where it colocalizes with the activated form of LRP6, APC and axin. By unmasking the transcriptionally competent pool of β-catenin, we provide evidence for a key step in β-catenin processing and Wnt signal transduction at the plasma membrane.

Results

Dephospho-β-catenin is present in cadherin complexes

Because N-terminally dephosphorylated β-catenin represents a better marker for Wnt signaling activity than total β-catenin, we optimized experimental conditions, allowing detection of dephospho-β-catenin by the anti-ABC (8E7) antibody (van Noort et al., 2002) in cultured mammalian cell lines. This antibody specifically reacts with an N-terminally unphosphorylated peptide (residues 36-44), which contains the GSK3β target residues S37 and T41 (van Noort et al., 2007). Reproducible detection of dephospho-β-catenin with the ABC antibody was highly dependent on fixation and blocking conditions, and further improved after antigen retrieval (see Materials and Methods). Screening of a set of colon carcinoma cell lines using these conditions showed both nuclear and plasma membrane localization of dephospho-β-catenin, depending on the cell line (supplementary material Fig. S1). Although we found no correlation with either APC or β-catenin mutation status, the plasma membrane localization of dephospho-β-catenin correlated with E-cadherin expression (supplementary material Fig. S1).

Cadherin-independent plasma membrane localization of dephospho-β-catenin upon Wnt3A stimulation

To eliminate E-cadherin expression as a confounding factor in our interpretation of endogenous Wnt-induced dephospho-β-catenin accumulation, we used the murine mammary epithelial cell line Kep1, which does not express E-cadherin because of Cre-mediated recombination of both E-cadherin alleles, and compared it with its E-cadherin$^{+/+}$ isogenic control counterpart Kp6 (Derksen et al., 2006). In the absence of Wnt stimulation, these cell lines line did not activate a Wnt-responsive luciferase reporter gene (Fig. 1A), indicating that these cell lines do not carry Wnt pathway activating mutations. Earlier studies have demonstrated that in the presence of an intact destruction complex, the loss of E-cadherin is neutral with respect to Wnt stimulation (van de Wetering et al., 2001). Very little β-catenin could be detected in unstimulated Kep1 cells, but after 2.5 hours stimulation with Wnt3A protein, a clear accumulation of the dephospho-β-catenin form was seen (Fig. 1B). By contrast, in the E-cadherin$^{+/+}$ Kp6 cells, dephospho-β-catenin was clearly detected before Wnt stimulation (Fig. 1B).

In view of the transcriptional activation, we anticipated that in Wnt-stimulated Kep1 cells, dephospho-β-catenin would be mainly

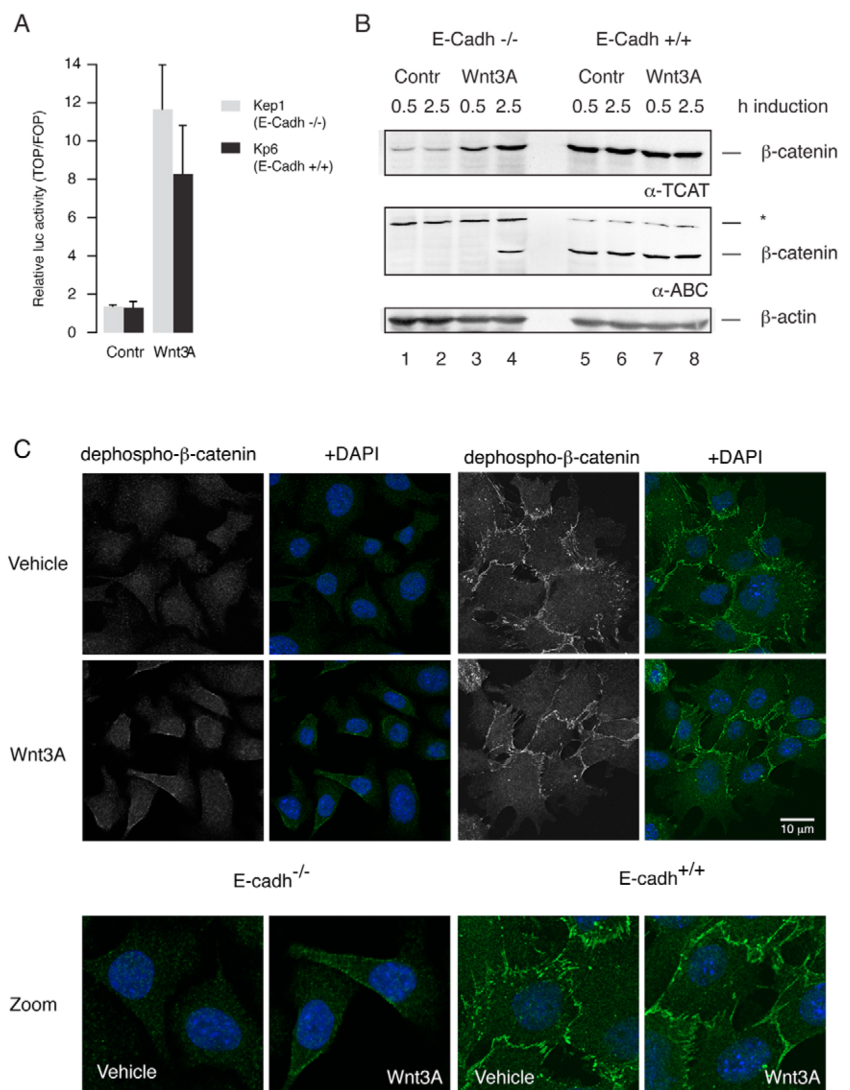

Fig. 1. Cadherin-independent plasma membrane localization of dephospho-β-catenin upon Wnt3A stimulation. (A) E-cadherin-negative cells respond normally to Wnt3a. Luciferase reporter assay in Kep1 (E-cadherin$^{-/-}$) and Kp6 (E-cadherin$^{+/+}$) cells using the TCF reporter TOP-TK and the control FOP-TK, normalized for transfection efficiency using pRL-CMV-Renilla. 24 hours after transfection, cells were stimulated overnight with Wnt3a-conditioned or control medium and luciferase activity was measured. (B) (Dephospho) β-catenin levels in Kep1 or Kp6 cells. Cells were induced with Wnt3A protein or control and analyzed 0.5 or 2.5 hours after induction by western blotting using an antibody recognizing all forms of β-catenin (TCAT) or an antibody specific for the N-terminal dephospho form (ABC). Asterisk indicates a crossreacting protein. (C) Subcellular localization of dephospho-β-catenin in E-cadherin positive or negative cells upon Wnt stimulation. Kep1 (E-cadherin$^{-/-}$) or Kp6 (E-cadherin$^{+/+}$) cells were induced with Wnt3A protein or control and analyzed 2.5 hours after induction by immunolocalization with an antibody specific for the N-terminal dephospho form (ABC). DAPI was used as a nuclear marker. Note that dephospho-β-catenin levels in E-cadherin$^{-/-}$ cells are much lower than in E-cadherin$^{+/+}$ cells, requiring unequal confocal settings to be used.

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nuclear. Surprisingly, stimulation of E-cadherin$^{-/-}$ Kep1 cells with Wnt3A resulted in the appearance of dephospho-β-catenin at the plasma membrane (Fig. 1C, left). Nuclear staining of the ABC antibody was also observed. Note that this was partly aspecific, because some nuclear staining was also observed in unstimulated Kep1 cells and NCI-H28 β-catenin knockout cells (data not shown), which is probably caused by a crossreacting protein (in Fig. 1B marked by an asterisk). As expected, a similar plasma membrane accumulation in response to Wnt stimulation in E-cadherin$^{-/-}$ Kep1 cells was confirmed with antibodies to total β-catenin (see Fig. 3C). The discontinuous punctate plasma membrane labeling of dephospho-β-catenin is strikingly similar to the plasma-membrane-associated puncta described for LRP6-axin (Bilic et al., 2007) and dishevelled (Dvl) (Schwarz-Romond et al., 2007) appearing upon Wnt treatment (see below).

In unstimulated isogenic E-cadherin$^{+/+}$ Kp6 cells, dephospho-β-catenin was prominent at the plasma membrane (Fig. 1C, right panel). This pool reflects transcriptionally inactive β-catenin, as no reporter activity was detected (Fig. 1A). Wnt3A induced reporter activity in Kp6 cells (Fig. 1A), and a minor increase in signal was indeed detected on western blot (Fig. 1B), but this increase did not translate into any noticeable increase in dephospho-β-catenin staining in situ (Fig. 1C, right panel). This indicates that the signaling-competent dephospho-β-catenin induced in response to Wnt stimulation is relatively minor in comparison to the steady-state junctional pool of dephospho-β-catenin.

The absence of classical cadherins from Kep1 cells was confirmed using a 'pan-cadherin' antibody, which recognizes E, N and P cadherin. This antibody failed to show any membrane staining in Kep1 cells, either before or after Wnt3A stimulation (supplementary material Fig. S2A). We wanted to further rule out the possibility that the recruitment of dephospho-β-catenin to the plasma membrane could be the result of a Wnt-induced upregulation of a cadherin or another membrane protein acting as a cryptic docking site. Therefore, we studied the localization of dephospho-β-catenin in Kep1 cells stimulated with Wnt3A protein in the presence or absence of the transcription inhibitor actinomycin D. Under these conditions, β-catenin was still stabilized and recruited to the plasma membrane (supplementary material Fig. S2B), indicating that this process is independent of the induction of Wnt target genes.

We wished to assess the timing of plasma membrane accumulation of dephospho-β-catenin in comparison to its accumulation in the nucleus. Unfortunately, the 8E7 anti-dephospho-β-catenin antibody crossreacted with a nuclear antigen (see Fig. 1B and Fig. 4E), which precludes assessment of nuclear dephospho-β-catenin at low levels. We therefore used a polyclonal antibody (Upstate 06-734) directed against the consensus GSK3 phosphorylation sites of human β-catenin (residues 29-49), which mainly recognizes the unphosphorylated form of β-catenin (supplementary material Fig. S5). As shown in Fig. 2, Kep1 cells exhibited a significant increase in plasma membrane β-catenin labeling 30 minutes after stimulation with Wnt3A, the shortest time point after which we detected accumulation of β-catenin. Nuclear levels at this early time point showed a slight, but statistically significant, increase over the background signal. We conclude that plasma membrane localization of dephospho-β-catenin is a very early response to Wnt signaling.

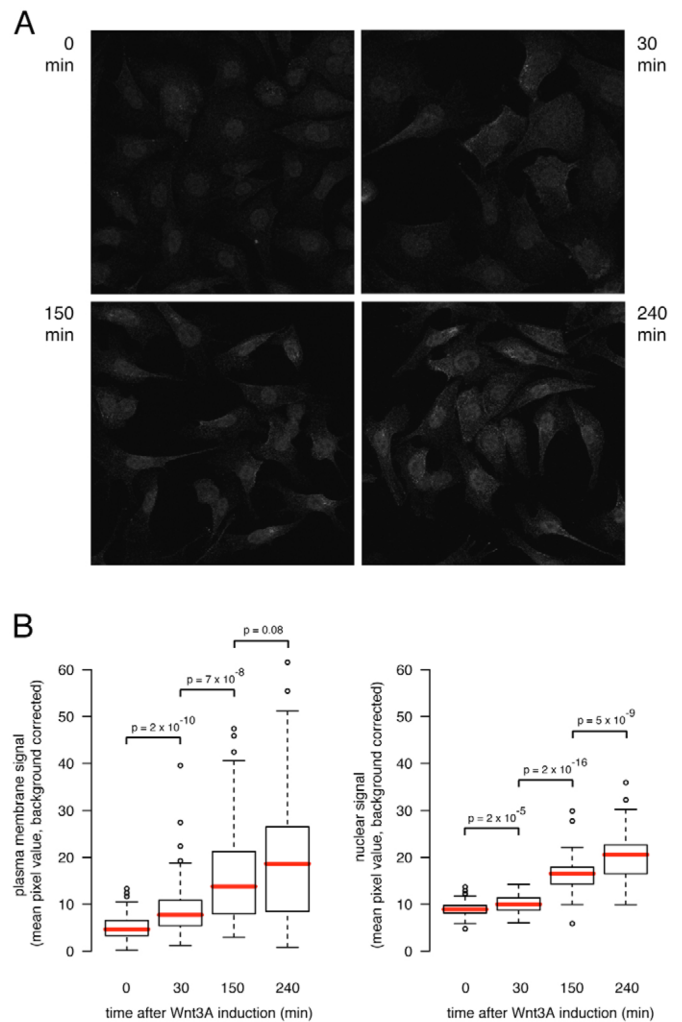

Fig. 2. Membrane recruitment of dephospho-β-catenin is an early event in the Wnt response. Kep1 cells were induced with Wnt3A for 30-240 minutes and nuclear and (dephospho-)β-catenin plasma membrane accumulation was recorded in 100-130 cells (see Materials and Methods for details on image acquisition and analysis). (A) Representative fields of cells. (B) Box plots representing quantification of plasma membrane and nuclear accumulation. Medians are indicated in red. *P* values indicated are the results of Mann-Whitney tests.

Dephospho-β-catenin colocalizes with APC, axin and LRP6 at the plasma membrane after Wnt3A stimulation

Earlier studies have suggested translocation of members of the destruction complex to the LRP6 co-receptor upon Wnt signaling (Cliffe et al., 2003; Tolwinski et al., 2003). Phosphorylation on Thr1479 of LRP6 is required for the recruitment of axin and is carried out by CK1γ in response to Wnt signaling (Davidson et al., 2005). We therefore used a phospho-specific antibody recognizing this residue of LRP6 in immunofluorescence in Kep1 cells. We found that after stimulation with Wnt3A, phospho-LRP6 colocalized with dephospho-β-catenin on the plasma membrane (Fig. 3A). Likewise, we found that axin colocalized with dephospho-β-catenin (Fig. 3B). These results suggest that the N-terminally dephosphorylated or signaling-competent form of β-catenin is translocated to the receptor complex in a Wnt-signaling-associated complex. Another component of the destruction complex, APC, has been reported to localize to the plasma membrane in different epithelial cell lines including colon carcinoma cell lines (Miyashiro et al., 1995). Since many available antibodies against APC are not reliable for immunofluorescence (Brocardo et al., 2005), we developed a monoclonal rat antibody, 3E7, which detects

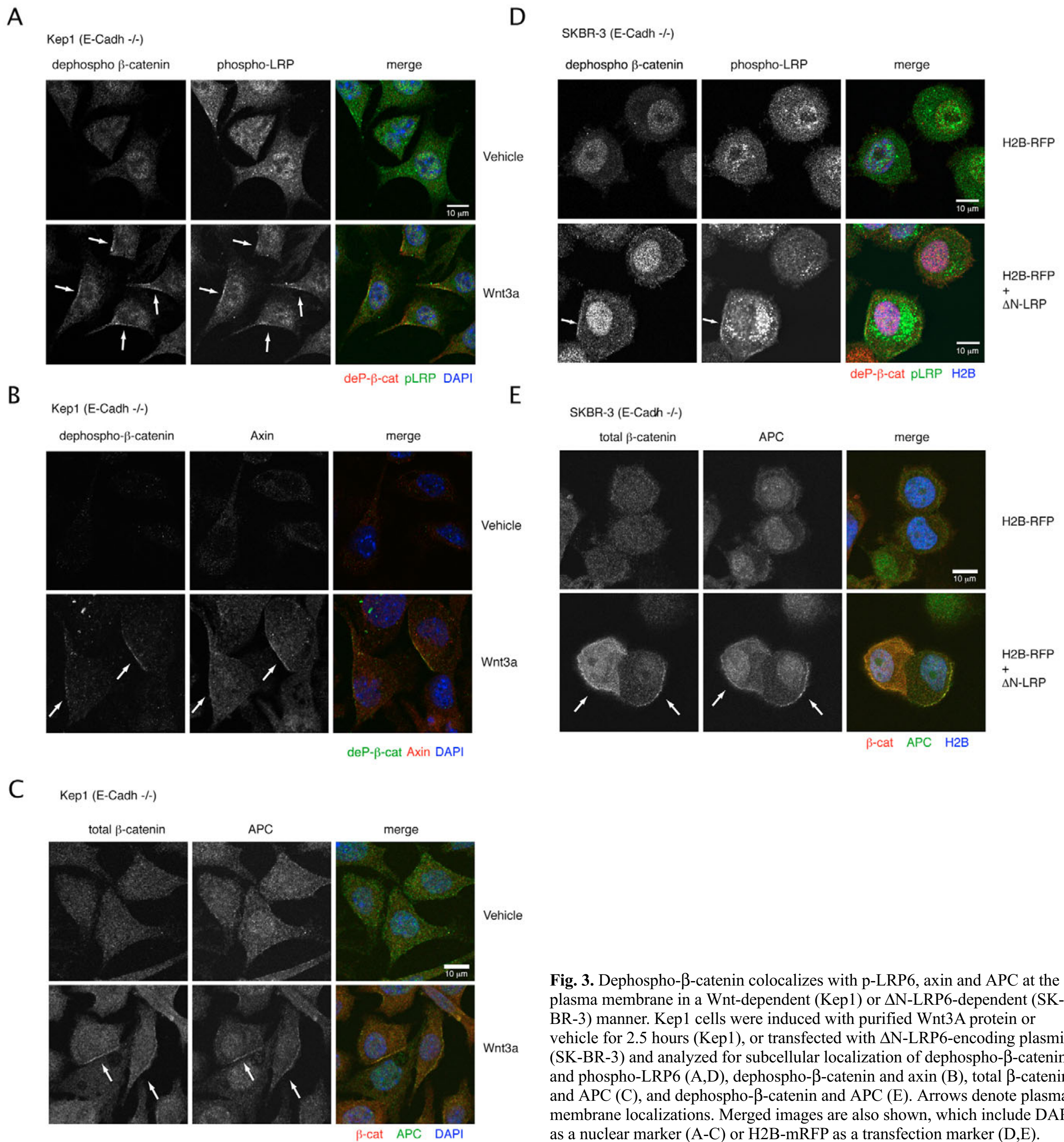

Fig. 3. Dephospho-β-catenin colocalizes with p-LRP6, axin and APC at the plasma membrane in a Wnt-dependent (Kep1) or ΔN-LRP6-dependent (SK-BR-3) manner. Kep1 cells were induced with purified Wnt3A protein or vehicle for 2.5 hours (Kep1), or transfected with ΔN-LRP6-encoding plasmid (SK-BR-3) and analyzed for subcellular localization of dephospho-β-catenin and phospho-LRP6 (A,D), dephospho-β-catenin and axin (B), total β-catenin and APC (C), and dephospho-β-catenin and APC (E). Arrows denote plasma membrane localizations. Merged images are also shown, which include DAPI as a nuclear marker (A-C) or H2B-mRFP as a transfection marker (D,E).

endogenous APC in immunofluorescence studies and on western blot (supplementary material Fig. S3). APC detected with this antibody also clearly colocalized with β-catenin on the membrane of Wnt3A-stimulated Kep1 cells (Fig. 3C).

To further characterize the involvement of the Wnt receptor complex in recruitment of β-catenin, we expressed ΔN-LRP6, a dominant active LRP6 receptor that mimics Wnt ligand engagement at the receptor complex. We could express this protein in SK-BR-3 cells, a breast cancer cell line with a homozygous deletion of E-cadherin (van de Wetering et al., 2001), but not in Kep1 cells, because it was not properly presented at the plasma membrane in these cells (data not shown). As shown in Fig. 3D, expression of ΔN-LRP6 resulted in a prominent plasma membrane localization of dephospho-β-catenin, resembling its appearance in Kep1 cells at the plasma membrane after Wnt stimulation (Fig. 3A,B). This indicates that activation of LRP6 is involved in the E-cadherin-independent plasma membrane recruitment of dephospho-β-catenin in response to Wnt signaling. Expression of ΔN-LRP6 in SK-BR-3 cells also resulted in colocalization of dephospho-β-catenin with APC (Fig. 3E). We were unable to detect axin in SK-BR-3 cells, possibly because of very low expression levels (data not shown). SK-BR-3 cells were found to be unresponsive to Wnt3A stimulation

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(data not shown) possibly because these cells lack the appropriate Frizzled receptor for this ligand. We conclude that activation of the Wnt pathway by either Wnt3A or dominant active LRP6 leads to recruitment of axin and/or APC and signaling competent β-catenin to the plasma membrane.

LRP6-initiated dephospho-β-catenin is transcriptionally significantly more competent than downstream-initiated dephospho-β-catenin

Our data so far are consistent with a model of Wnt signal transduction where, upon Wnt stimulation, de novo synthesized β-catenin is attracted to the Wnt receptor complex together with members of the destruction complex. As Wnt activation results in co-activation of genes by β-catenin in the nucleus, β-catenin is probably released from the membrane complex and routed to the nucleus. In order to test the relevance of membrane association of β-catenin, we compared the activity of β-catenin, either routed or not routed through the Wnt receptor complex. To mimic β-catenin accumulation due to receptor activation, we expressed ΔN-LRP6 in SK-BR-3 cells. To produce β-catenin accumulation without receptor activation we overexpressed wild-type β-catenin. If the transactivating potential is similar regardless of the source of dephospho-β-catenin, the amount of luciferase output is expected to closely parallel the amount of dephospho-β-catenin generated. As shown in Fig. 4, expression of ΔN-LRP6 resulted in significant upregulation of a luciferase reporter gene (Fig. 4C). A similar degree of TCF-reporter activation could be produced by transfection of wild-type β-catenin, but this was accompanied by much higher cellular levels of dephospho-β-catenin (Fig. 4D,E). Also, there was no enrichment of dephospho-β-catenin on the plasma membrane under these conditions (Fig. 4B), whereas a prominent plasma membrane localization of the comparatively minor pool of dephospho-β-catenin was induced by ΔN-LRP6 (Fig. 4A). Thus, in spite of significantly lower cellular levels of dephospho-β-catenin, a similar degree of TCF reporter output can be achieved through LRP6 co-receptor activation.

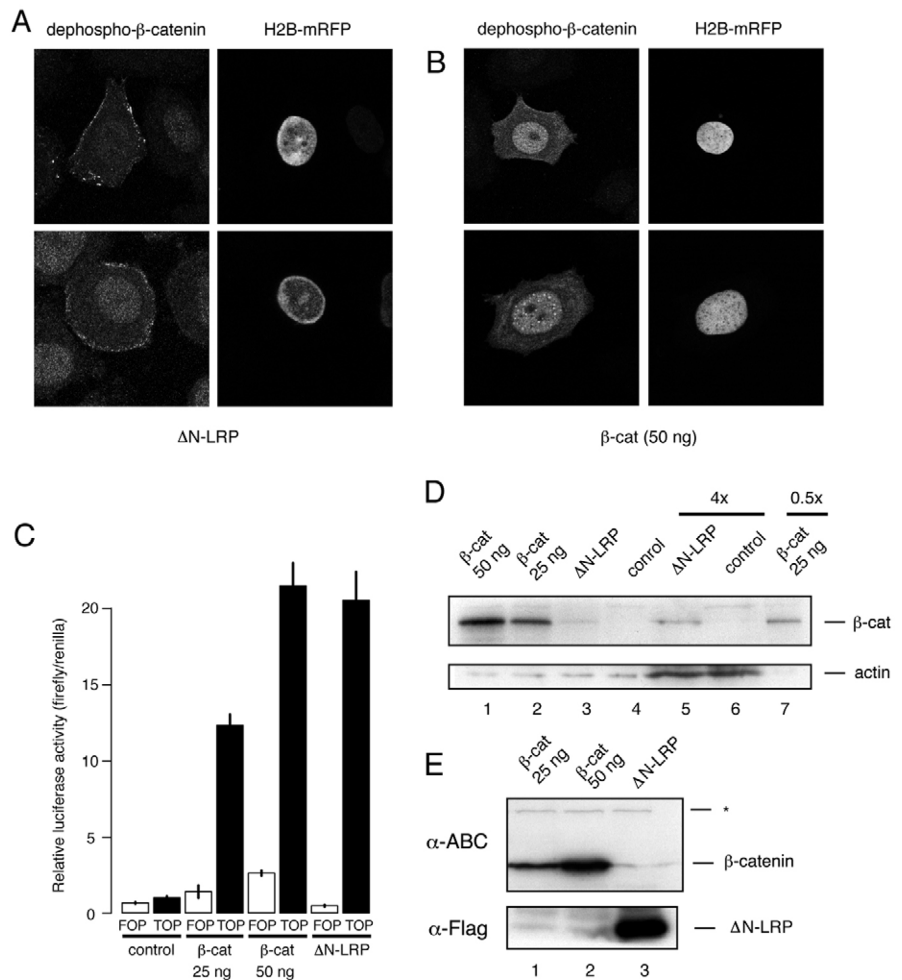

Fig. 4. LRP6-initiated dephospho-β-catenin is transcriptionally more active than downstream-initiated dephospho-β-catenin. Cadherin-deficient SK-BR-3 breast carcinoma cells were transiently transfected with 25 or 50 ng plasmid encoding wild-type β-catenin or 160 ng of a plasmid encoding ΔN-LRP6 and analyzed by immunolocalization (A,B), TCF transcriptional activity (C) and western blotting (D,E). (A,B) Immunolocalization of dephospho-β-catenin in cells exogenously expressing β-catenin or ΔN-LRP6, identified by coexpression of mRFP-tagged histone H2B. (C) TCF-dependent transcriptional activity in SK-BR3 cells transfected with indicated plasmids 24 hours after transfection. TOP, TCF-reporter luciferase activity; FOP, mutated TCF-reporter activity. Values were normalized to a transfection control (constitutive Renilla luciferase reporter). (D,E) Western blot analysis of cells shown in A-C, detecting total (D) or dephospho-β-catenin (E) levels. 4×, fourfold amount loaded; 0.5×, one half amount loaded. Asterisk indicates a cross-reacting protein.

Supraphysiological levels of exogenous β-catenin are required to mimic Wnt activity in *Xenopus* embryos

We sought to support these observations in a second model. Wnt signaling activity in the early *Xenopus* embryo can be readily monitored by the formation of an ectopic body axis. We thus compared the levels of exogenously expressed β-catenin required to induce secondary axes to the levels produced by the endogenous dorsalizing center or by a much stronger activation of the pathway by ectopic Wnt expression (Fig. 5G).

The endogenous Wnt pathway is active in the blastula (stage 8.5-9.5) and can be detected as nuclear accumulation of β-catenin strongest in the dorsal side, but also spread throughout the prospective mesoderm, whereas the signal remains lower in the ectoderm (Schohl and Fagotto, 2002; Schohl and Fagotto, 2003). With 50 pg Wnt8a mRNA, which is in excess of the amount required to induce complete dorsalization, β-catenin nuclear levels were found to be only slightly higher than levels induced by the endogenous pathway (Fig. 5). However, the levels of exogenous β-catenin corresponding to induction of a secondary axis were well beyond physiological levels, both in the cytoplasm and in the nucleus. These observations indicate that exogenous β-catenin is less effective at activating the pathway than endogenous β-catenin regulated by Wnt signals, consistent with the hypothesis that Wnt-induced β-catenin is qualitatively different.

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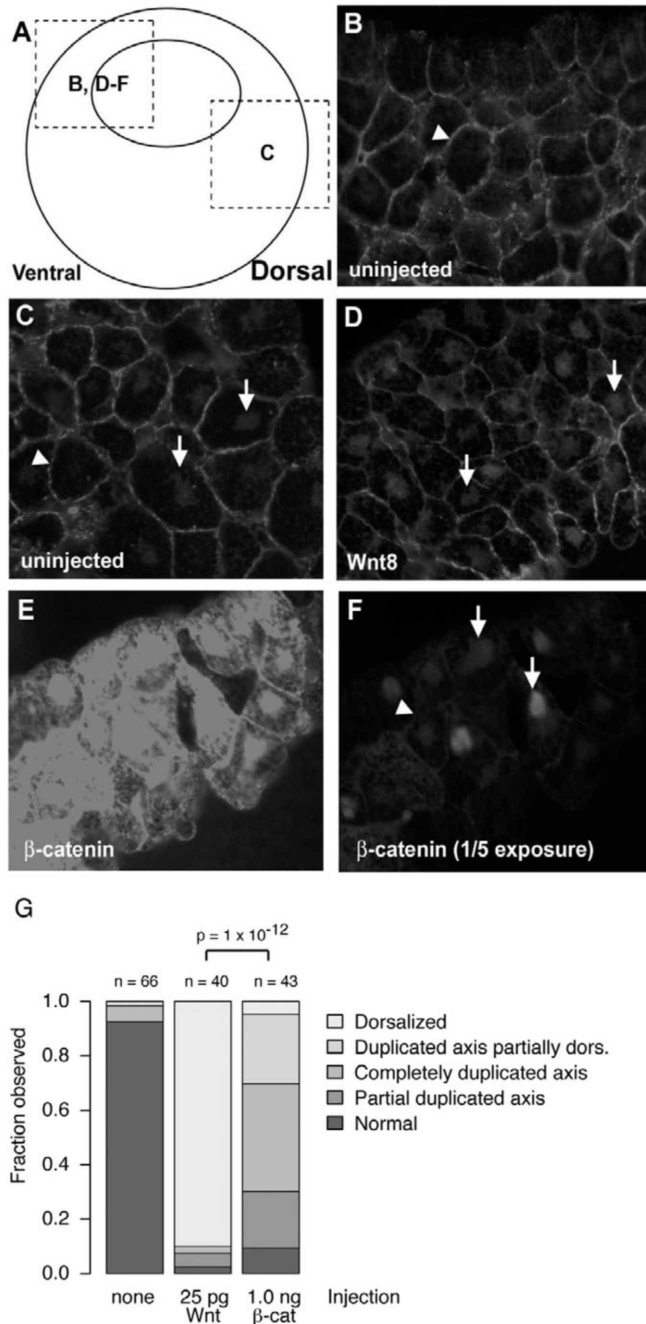

Fig. 5. Supraphysiological levels of exogenous β-catenin are required to mimic Wnt activity in *Xenopus* embryos. β-catenin (1.0 ng) or Wnt8 (50 pg) mRNA was injected into the ventral side of four-cell-stage embryos and total β-catenin levels achieved at blastula stage were estimated by immunofluorescence on cryosections. (A) Diagram of cross-section of a blastula embryo indicating the areas used to compare β-catenin staining. (B,C) Ventral and dorsal regions of uninjected embryos. Arrows indicate nuclear β-catenin and arrowheads, the plasma membranes. (D) Ventral region from Wnt8-injected embryo. Note the increased nuclear signal. (E) Ventral region from β-catenin-injected embryo. Note the very strong signal throughout the cells. (F) The same field recorded with a five times shorter exposure time. (G) Comparison of dorsalizing activity of Wnt and β-catenin in early *Xenopus* embryos. The degree of dorsalization obtained was scored at the tailbud stage. The phenotypes were classified into five categories of increasing dorsalization: normal; partial duplicated axis; complete duplicated axis; complete duplicated axis with shortened axis indicating partial global dorsalization; and completely dorsalized, i.e. global dorsalization with reduced or no axis and radial head structures. *P* value according to χ^2 test.

Quantitative differences in dephospho-β-catenin plasma membrane labeling in Wnt-responding tissues in vivo

Our data in the isogenic Kep1 and Kp6 cell lines demonstrate that dephospho-β-catenin is recruited to the plasma membrane in response to Wnt stimulation in an E-cadherin independent fashion. Such an unbiased analysis would not be feasible in other in vitro model systems such as the commonly used colon cancer cell lines where the Wnt pathway is constitutively active and E-cadherin expression varies even amongst subclones of low-expressing cell lines such as LS174T (our unpublished data). We found that in our model system, the increase in dephospho-β-catenin is subtle and not detectable in situ in an E-cadherin$^{+/+}$ background. However, based on earlier data in the *Drosophila* system from the Wieschaus lab (see below), we optimized our staining protocol for the detection of dephospho-β-catenin in other systems as well. As a first approach, we double-stained *Xenopus* embryo sections for total and dephospho-β-catenin (Fig. 6). We detected a clear signal for dephospho-β-catenin at the plasma membrane of mesodermal cells, but less in ectodermal cells (Fig. 6B). Wnt overexpression led to membrane recruitment of dephospho-β-catenin in ectodermal cells (Fig. 6A) and increased membrane staining of total β-catenin. Despite the fact that this model system lacks the advantage of an E-cadherin negative background, the observations in a physiological setting of a quantitative difference in (dephospho-) β-catenin accumulation at the plasma membrane in Wnt-responding versus Wnt-nonresponding cells, are in agreement with the observations in our initial model system. However, to unambiguously assign translocation of Wnt-induced dephospho-β-catenin as E-cadherin independent, an E-cadherin-negative background is required. Note that the results on dephospho-β-catenin plasma membrane accumulation in response to Wnt stimulation in the *Xenopus* model system parallel earlier data obtained in *Drosophila* where Wg signaling similarly increases plasma membrane levels of Armadillo (the fly β-catenin homologue) in Wg-responding stripe regions, in addition to elevating cytosolic levels of the protein (Peifer et al., 1994b). This suggests that an increase in Armadillo at the plasma membrane is also important for endogenous Wg signaling in the fruit fly.

We also tested Wnt-induced dephospho-β-catenin plasma membrane enrichment in a second in vivo model of Wnt signalling. The intestinal epithelium of the human small intestine is organized into flask-shaped invaginations called crypts and finger-like projections termed villi. Wnt signaling has been shown to be essential for maintaining stem cell turnover in the intestinal crypt (Korinek et al., 1998). We stained normal human small intestinal epithelium for dephospho-β-catenin and found that similarly to the situation in *Xenopus*, dephospho-β-catenin is enriched at the plasma membrane of Wnt-responsive crypt epithelial cells when compared with differentiated cells on the villus epithelium (Fig. 7, supplementary material Fig. S4).

We emphasize that it remains to be tested in these two in vivo examples of Wnt signaling, whether the plasma membrane localization of (dephospho-) β-catenin, which coincides with regions of known Wnt activity, represents the E-cadherin-independent signaling-competent form, because this form could still potentially be masked.

Discussion

The currently prevailing model of Wnt signal transduction specifies that upon receipt of a Wnt ligand at the membranous receptor complex, β-catenin proteolysis is prevented and the

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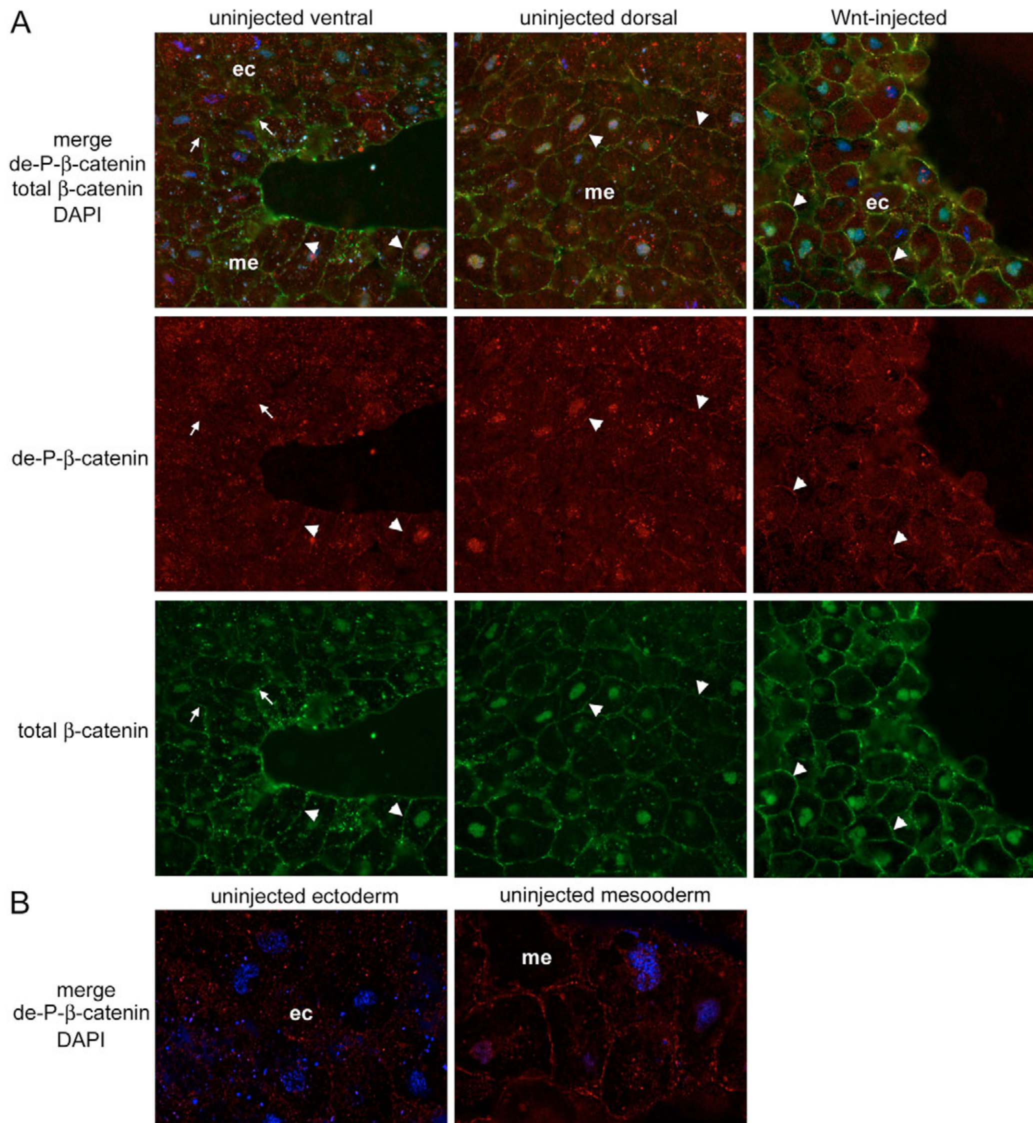

Fig. 6. Membrane localization of dephospho-β-catenin in the early *Xenopus* embryo. Cryosections of stage 9 embryos were stained for dephospho-β-catenin (ABC) and total β-catenin (H102) and nuclei were counterstained with DAPI. (A) Selected fields of the ventral presumptive ectoderm and mesoderm (uninjected ventral), dorsal mesoderm (uninjected dorsal), and ventral ectoderm of an embryo injected with 50 pg Wnt8 mRNA (Wnt-injected). In uninjected embryos, ABC stains the outlines of mesodermal cells (me) (arrowheads) but not ectodermal cells (ec) (arrows). Membrane ABC is detected in ectodermal cells of Wnt-injected embryos (arrowheads). Small bright cytoplasmic spots in all three color channels correspond to autofluorescent pigment granules. (B) Higher magnification view of ABC staining of ectodermal (ec) and mesodermal (me) cells of uninjected cells. These two images were obtained by collection of *z*-stacks followed by 2D nearest neighbors deconvolution and merging 10 images from each stack.

protein accumulates in the cytosol. It has remained unclear how cytosolic destruction complexes sense ligand engagement at the plasma membrane. Recent studies have shown that Wnt treatment induces the formation of LRP6 co-receptor aggregates at the plasma membrane (Bilic et al., 2007). These receptor aggregates in turn promote the recruitment of canonical Wnt pathway components including Dishevelled, axin and GSK3β (Bilic et al., 2007; Schwarz-Romond et al., 2007). These observations parallel earlier data obtained in the *Drosophila* system, where similar recruitment of pathway components to the plasma membrane has been recorded upon activation of the pathway (Cliffe et al., 2003). How the inhibition of β-catenin proteolysis links up with the formation of these LRP6 signalosomes and whether β-catenin itself may translocate to the plasma membrane along with its canonical destruction complex members is unknown. Here we show, using an in vitro model system, that endogenous dephosphorylated β-catenin appears on the plasma membrane upon Wnt3A treatment. This translocation occurs independently of E-cadherin, and dephosphorylated β-catenin colocalizes at the plasma membrane with phospho-LRP6, axin and APC. Together, our results suggests that Wnt signal transduction may be regulated at multiple levels other than, or in addition to, the inhibition of breakdown, and that routing of de novo synthesized β-catenin through the Wnt receptor complex is required for optimal transcriptional activity of the protein. This step in the processing of signaling-competent β-catenin may have remained difficult to detect so far because of plasma membrane masking by the junctional pool of β-catenin. We note that the punctate plasma membrane labeling observed in

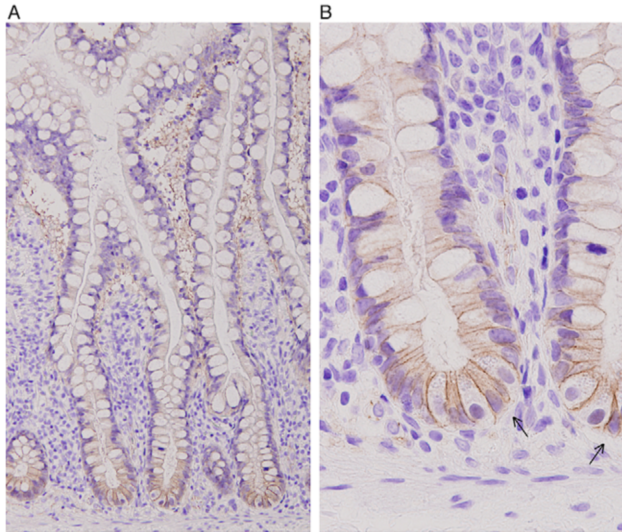

Fig. 7. Dephospho-β-catenin is enriched on the plasma membrane in human intestinal crypts. (A) Low-power photomicrograph of a normal human small intestinal crypt-villus axis. Dephospho-β-catenin is enriched on the crypt epithelial plasma membrane when compared with the plasma membrane labeling on differentiated villus cells. (B) High-power photomicrograph of crypt compartment shown in A. Crypt epithelial cells including the presumptive intestinal epithelial stem cells or crypt base columnar (CBC) cells (Barker et al., 2007) show robust plasma membrane labelling (arrows).

our model system bears a striking resemblance to the plasma membrane labeling described in earlier studies (Bilic et al., 2007; Schwarz-Romond et al., 2007). Our time-course analysis indicates that the kinetics of β-catenin accumulation on the plasma membrane in response to Wnt3A is similar to that for the formation of axin-LRP6 aggregates in response to Wnt treatment as described in the earlier studies. Indeed, we also observe colocalization of dephospho-β-catenin, phospho-LRP6 and axin at the plasma membrane upon Wnt induction. Thus, plasma membrane accumulation of dephospho-β-catenin upon Wnt treatment is an early event, occurring, at most, 30 minutes after Wnt3A treatment. At present, we cannot make a spatiotemporal discrimination between plasma membrane and nuclear accumulation, because at the earliest time point we also detected a small but statistically significant increase in nuclear β-catenin. The kinetics we observed agree with the fact that the Wnt response depends on de novo synthesized β-catenin (Willert et al., 2002), and β-catenin accumulation to detectable levels is probably slower than intracellular traffic. Novel approaches are required to trace β-catenin routing upon Wnt signaling at these low endogenous levels.

On the basis of our observations, we propose that upon Wnt stimulation, β-catenin is activated at the plasma membrane in a Wnt-receptor complex, which also contains components of the destruction complex, generating a signaling-competent form. In line with this, Gottardi and Gumbiner have shown that Wnt stimulation generates a monomeric form of β-catenin, which selectively binds TCF and not E-cadherin (Gottardi and Gumbiner, 2004). The activation step may for example constitute this transition. Plasma membrane activation is not absolutely required for signal transduction, because increasing β-catenin to supraphysiological levels by interfering with its degradation will also lead to transactivation.

Previous work in *Xenopus* has generated evidence for the idea that β-catenin stability alone may not explain Wnt signaling outcome (Guger and Gumbiner, 2000; Nelson and Gumbiner, 1999). Later studies in the fly embryo using hypomorphic Armadillo alleles show that modulation of Wg signaling can occur in the presence of uniformly high levels of Armadillo (Tolwinski et al., 2003; Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004). Moreover, studies in cultured mammalian cell lines show that receptor-mediated signal transduction events, such as Wnt stimulation or secreted Frizzled-related protein (sFRP) inhibition, can impinge on Wnt signaling output even when downstream mutations prevent β-catenin breakdown (He et al., 2005; Suzuki et al., 2004). We are currently in the process of studying potential post-translational modifications on β-catenin using the E-cadherin$^{-/-}$ Kep1 cell line.

A model of β-catenin activation at the receptor complex would allow β-catenin output to be regulated on a direct stoichiometric 'per molecule' basis, in theory allowing one Wnt molecule to liberate a predetermined quanta of signaling-competent β-catenin molecules. If correct, this regulation would be considerably more efficient than the currently proposed models in which Wnt signaling input is titrated against the activity of cytoplasmic degradation complexes to regulate gene expression in the nucleus. Regulation at multiple levels is similarly observed in the Hedgehog signal transduction pathway where stabilization of the transcriptional co-activator does not suffice for full activation (Methot and Basler, 1999). It is currently not known what mediates this activation step in Hedgehog signal transduction at the plasma membrane (Hooper and Scott, 2005). Regulation at multiple levels beyond the mere inhibition of proteolysis would allow the Wnt pathway to join other developmental pathways, such as the Hedgehog and Notch signaling pathways, in which the transcriptional co-activator at the plasma membrane receives the permission ('licence') to signal.

Materials and Methods

Data analysis

Statistical analysis was done using the R software package (R Development Core Team, 2005).

Cell culture, transfection and reporter assays

SK-BR-3, Kep1 (E-cadherin$^{-/-}$, p53$^{-/-}$), Kp6 (E-cadherin$^{+/+}$, p53$^{-/-}$), SW480, LS174T, Colo320, HCT15, Colo205 and SW48 were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) and were transfected using Fugene-6 (Roche) as instructed by the supplier. 1×10^5 cells were transfected with 300 ng TOP/FOP-TK-luc, 1.5 ng pRL-CMV, 325 ng ΔNLRP6, 50 or 100 ng β-catenin, 10 ng Wnt1 and 50 ng H2B-mRFP. Luciferase reporter activity was measured 24 hours after transfection in SK-BR-3. 24 hours after transfection with Top/Fop-TK-luc, Kep1 cells were stimulated with Wnt3A for 7 hours, after which luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

APC antibodies

Domains of mouse APC, termed APC-A (amino acids 788-1038), APC-B (amino acids 2170-2394), and APC-C (amino acids 2644-2845), were fused to GST and purified in bacteria. Rabbit polyclonal antisera were prepared as described (Hoogenraad et al., 2000). Rat monoclonal antibodies against APC were generated by Absea (China) using the same GST fusion proteins. Hybridomas were first tested for specific recognition of the respective GST fusion proteins. Positive clones (51 hybridomas for APC-A, 44 hybridomas for APC-B, and 58 hybridomas for APC-C) were subsequently tested on western blot for recognition of eCFP-tagged APC domains and by immunofluorescence for detection of endogenous APC. We screened nine rabbit polyclonals against the A, B and C domains, but none of the rabbit polyclonal antibodies were monospecific (data not shown). We subsequently screened 51 antibody-producing rat hybridomas for APC-A (amino acids 788-1038), 44 hybridomas for APC-B (amino acids 2170-2394) and 58 hybridomas for APC-C (amino acids 2644-2845). Two rat monoclonal antibodies (13F7, APC-A-derived, and 3E7, APC-B-derived) detected both overexpressed, eCFP-tagged APC (supplementary material Fig. S3C), as well as endogenous APC on western blots

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(supplementary material Fig. S3D). These antibodies also recognized GFP-tagged full-length APC in transfected COS-1 cells (data not shown) and endogenous APC in different cell lines (supplementary material Fig. S3D and data not shown). As 3E7 recognized clusters of full-length APC in MDCK cells, and not in SW480 cells that contain truncated APC (supplementary material Fig. S3E,F), we conclude that 3E7 detects endogenous APC in immunofluorescence studies.

Other antibodies and reagents

Further antibodies used were against β-catenin (C19220) (Transduction Labs) and H-102 (Santa Cruz), active β-catenin (ABC 8E7), actin (Ab-1, Oncogene), N-axin (Fagotto), E-cadherin (C20820, Transduction Labs), pan-cadherin (C3678, Sigma), Tp1479 LRP6 (Niehrs), M2 mAb Flag (Sigma). Purified recombinant Wnt3A was a kind gift from R. Nusse (Stanford, CA) or obtained from R&D Systems. 4′,6-diamidino-2-phenylindole (DAPI) and actinomycin D were obtained from Sigma.

Plasmids

Top/Fop-TK, pRL-CMV and pRK5SK-β-catenin were described before (Hendriksen et al., 2005) and ΔN-LRP6 was a kind gift from H. Clevers (Hubrecht Laboratory, Utrecht, The Netherlands).

Western blotting

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (25 μg per lane) and Western blotting using Immobilon-P transfer membrane (Millipore). Aspecific sites were blocked with 5% skimmed milk (Oxio, Hampshire, England) at room temperature for 1 hour. Note that detection of dephospho-β-catenin with the ABC antibody was inhibited by certain brands/lots of skimmed milk. Primary antibodies were incubated in 1% skimmed milk for 2 hours at room temperature in the following dilutions: E-cadherin 1:1500; β-Catenin mAb C19220 1:5000, ABC (8E7) 1:500; dephospho-β-catenin Upstate 06-734, 1:1000; Actin 1:5000; mFLAG M2 1:500. Blots were washed with phosphate-buffered saline (PBS)/0.05% Tween 20. Enhanced chemiluminescence (Amersham) was used for detection of proteins.

Immunofluorescence and confocal microscopy

For immunofluorescence, cells were grown on glass coverslips coated with fibronectin (Sigma) and fixed in 3.7% formalin in PBS for 10 minutes and permeabilized for 5 minutes in 0.2% Triton-PBS. For antigen retrieval, cells were incubated in 10 mM citric acid buffer pH 6 at 95°C for 20 minutes and blocked in 5% BSA/PBS at room temperature for 10 minutes. Primary antibodies were incubated for 2 hours in 1% purified BSA/PBS using the following dilutions; ABC (8E7) 1:200; total β-catenin C19220 1:250; total β-catenin H102 1:65; dephospho-β-catenin Upstate 06-734, 1:200; N-axin 1:50; N-APC 1:100; APC 3E7 1:100; p-LRP6 1:250; LRP6 1:300; pan-cadherin 1:5000. Cells were shortly washed in PBS and incubated in fluorescently conjugated secondary antibodies (Molecular Probes) and DAPI in 1% BSA-PBS for 30 minutes, washed shortly in PBS and mounted in Mowiol. Images were recorded using a Leica NT, SP2 or SP2 AOBS confocal microscope.

In situ quantification

Kep1 cells were grown on coverslips to 40-50% confluency and induced with Wnt3A-conditioned medium for 30-240 minutes. Cells were fixed and immunostained as described, using the antibody Upstate 06-734 directed against the consensus GSK3 phosphorylation site of human β-catenin (amino acids 29-49), which mainly recognizes the unphosphorylated form (supplementary material Fig. S5) at 5 μg/ml for 1 hour. Cells were stained with Alexa Fluor 488-linked secondary antibody and counterstained with DAPI. Images were acquired as described using a 63× oil objective at 1024×1024 pixel resolution. Random fields from duplicate experiments were selected and imaged using an eight-slice *Z*-series (pinhole 1.00 airy units) totalling 2 μm in depth. Projected *Z*-series were quantified in ImageJ. Images displayed uniform background levels (11.0±0.1). Per condition, all cells from three fields were quantified (100-130 cells). Plasma membrane signals were recorded as mean pixel intensities over 3 μm of highest staining membrane. Nuclear signals were recorded as mean pixel intensity from a 25 μm^2 nuclear area. Mean pixel values were background and base line (median of t=0) subtracted. Statistical analysis was done in R. *P* values were derived from Mann-Whitney tests.

Embryo injections and immunofluorescence

Four-cell-stage embryos were injected in one ventral blastomere with 25 or 50 pg Wnt8 mRNA or 1000 pg myc-tagged β-catenin mRNA as previously described (Fagotto et al., 1996). Stage 9 embryos were fixed in 3-4% paraformaldehyde and sections were prepared and stained as previously described (Schohl and Fagotto, 2002). Antibodies used were total anti-β-catenin H102 diluted 1:50, ABC 1:250, and secondary goat Alexa Fluor 546 or 488 anti-rabbit/anti-mouse (Molecular Probes). Images were recorded with a Leica microscope using a narrow Cy3 filter and a 20× oil-immersion objective.

Immunohistochemistry

Sections (4 μm) were deparaffinized and antigen retrieval was carried out by boiling for 10 minutes in 10 mM Tris-HCl, 1 mM EDTA (pH 9). Subsequently slides were immersed in 0.3% hydrogen peroxide in methanol for 30 minutes and nonspecific binding was blocked with 5% normal goat serum for 1 hour at room temperature. The sections were incubated for 1 hour at room temperature in primary antibodies against total β-catenin (C19220 Transduction Labs) and active β-catenin (ABC 8E7). The ultravision antipolyvalent HRP detection system (Lab Vision, Fremont, CA) was used to visualize antibody-binding sites with 3,3′-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin.

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