Intracellular routing of β-catenin

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CHAPTER 3

Wnt stimulation-independent plasma membrane localization of dephospho-ß-catenin

Manuscript in preparation

Wnt stimulation-independent plasma membrane localization of dephospho-ß-catenin

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ß-Catenin is the nuclear effector of the Wnt signaling pathway. Recently, a small pool of N-terminally dephosphorylated ß-catenin was shown to transduce transcriptional activation of Wnt target genes. We show that in a panel of colon carcinoma cell lines, dephospho-ß-catenin localizes to the plasma membrane and/or nucleoplasm. Plasma membrane localization of dephospho-ß-catenin correlates with expression of E-cadherin. Dephospho-ß-catenin localizes specifically to adherens junctions while total ß-catenin staining labels along the baso-lateral membrane. Upon cellular polarization, dephospho-ß-catenin is recruited to the apical actin-based adherens junctions and colocalizes with the adenomatous polyposis coli protein. Immunohistochemistry on tissue sections shows that dephospho-ß-catenin is also enriched at apico-lateral cell-cell borders in the intestinal crypt. In fractionation experiments, neither E-cadherin-bound nor free dephospho-ß-catenin is predictive of Wnt signaling output in our panel of colon carcinoma cell lines. Our data suggest multiple levels of regulation of signaling output and emphasize the need for an E-cadherin negative background in studying the Wnt-responsive dephosphorylated pool of ß-catenin.

Wnts are secreted signaling molecules that regulate embryonic development and adult tissue homeostasis. Deregulation of the Wnt signaling pathway is implicated in tumourigenesis (Nusse, 2005). Compared to other signaling pathways, the Wnt cascade is complex as it contains numerous players (for a complete overview of the pathway see the Wnt homepage on http://www.stanford.edu/~rnusse/wntwindow.html). The output of the cascade is determined by nuclear ß-catenin levels, which regulate transcription of target genes in complex with TCF/Lef transcription factors (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). To further increase our knowledge of the Wnt signaling pathway, it is important to understand the regulatory mechanisms that control the levels and activity of nuclear ß-catenin.

The current canonical model of ß-catenin-dependent Wnt signaling holds that an important regulatory step in the pathway is the constant and rapid degradation of free ß-catenin in the cytoplasm. This mechanism is active in the absence of Wnt signaling and ensures that free ß-catenin molecules are bound and phosphorylated by a complex containing APC/Axin/GSK3/CK1 (Hart et al., 1998; Liu et al., 2002; Amit et al., 2002; Yanagawa et al., 2002). N-terminal phosphorylation marks ß-catenin for degradation by the proteasome (Hart et al., 1999; Aberle et al., 1997). Due to this constant degradation of ß-catenin in the cytoplasm, ß-catenin localization is restricted to the plasma membrane in non-stimulated epithelial cells. This pool of ß-catenin at the plasma membrane functions in cell-cell adhesion as a structural component of Ca^{2+}-dependent adherens junctions.

Another mechanism to control ß-catenin activity is retention mediated by ß-catenin binding proteins. Due to overlapping binding proteins, there is competition between binding of ß-catenin at the plasma membrane by E-cadherin, Axin, APC and ICAT in the cytoplasm/nucleus, and by TCF in the nucleus. In addition, Gottardi and Gumbiner (2004) have suggested that there are molecular forms of ß-catenin that show differential binding to E-cadherin and TCF. They showed that Wnt signaling generates a monomeric form that preferentially binds TCF over E-cadherin. This could be accomplished by a fold-back mechanism in which the C-terminus of ß-catenin binds to its final armadillo repeats, masking part of the E-cadherin binding domain (Gottardi and Gumbiner, 2004).

Staal et al. (2002) were able to show that immunoreactivity for an antibody recognizing N-terminally non-phosphorylated ß-catenin (ABC) correlates much better with Wnt activity than im-

munoreactivity for total ß-catenin. This form of ß-catenin accumulates in Wnt-activated cells and is localized only in the nucleus (Staal et al., 2002). In earlier studies, we confirmed these findings by Staal et al. and identified RanBP3 as a specific nuclear export factor for dephospho-ß-catenin (Hendriksen et al., 2005).

Based on our work examining the transcriptionally active pool of ß-catenin, we determined the intracellular localization of dephospho-ß-catenin in a panel of colon carcinoma cell lines. Surprisingly, nuclear dephospho-ß-catenin is observed in only 3 out of 8 cell lines, whereas the majority of cell lines in our panel show plasma membrane localization. Plasma membrane localization of dephosphorylated ß-catenin correlates with E-cadherin expression. Upon close inspection of HCT15 cells, we find that total ß-catenin antibodies label along the baso-lateral membrane, while the ABC antibody specifically labels the apical region of the baso-lateral membrane. We confirmed the apical localisation of dephospho-ß-catenin using single polarized cells. In this system, dephospho-ß-catenin localizes to the apical actin cap along with APC. Next, we determined the localization of dephospho-ß-catenin in normal adult small intestine and found that in the crypt area where Wnt signaling is active, dephospho-ß-catenin was enriched at the apico-lateral cell border. Total ß-catenin did not show a preferential accumulation. Finally, by focusing on the free pool of dephosphorylated ß-catenin we demonstrate that the correlation between this pool and Wnt signaling activity in colon carcinoma cell lines is poor, which suggests multiple levels of regulation of signaling output. We stress that an E-cadherin null background is required in studying the dephosphorylated pool of ß-catenin in Wnt signal transduction.

Results and Discussion

Nuclear localization of ß-catenin has long been a surrogate marker for Wnt signaling activity, even though it correlates poorly with TCF reporter activity in *in vitro* assays. The ABC antibody, which specifically recognizes N-terminally unphosphorylated ß-catenin, was shown to correlate much better with Wnt signaling activity when compared to antibodies recognizing total ß-catenin (Staal et al., 2002). To gain more insight into the behaviour of unphosphorylated (and therefore possibly signaling competent) ß-catenin, we have investigated the intracellular localization of the dephosphorylated form of ß-catenin in various colon cancer cell lines. In these Wnt-activated cells, we detected dephospho-ß-catenin in the nucleus of SW480, LS174T and Colo320 cells (Fig 1A) as was suggested by the work of (Staal et al., 2002). Interestingly, several cell lines showed prominent dephospho-ß-catenin staining at the plasma membrane, including HCT15, Colo205, SW48, DLD1 and Caco2. Low amounts of dephospho-ß-catenin were detected at the cell-cell contacts of Lovo cells, whereas dephospho-ß-catenin was not detected in HT29 cells (Fig 1A, data not shown for DLD1 and Caco2). Plasma membrane localization of dephospho-ß-catenin in these cell lines correlates with total ß-catenin staining. This localization of the dephosphorylated form of ß-catenin is somewhat surprising. A previous study has claimed that N-terminally dephosphorylated ß-catenin can localize to the plasma membrane in epithelial cells (Gottardi and Gumbiner, 2004). However, the anti-dephospho-ß-catenin antibody used in the this study has recently been shown to be aspecific (van Noort et al., 2007). To our knowledge, we are the first to describe that a large pool of dephospho-ß-catenin resides at the plasma membrane.

As the dephosphorylated pool of ß-catenin has been equated with the signaling competent pool of ß-catenin (Staal et al., 2002), we focused on this membrane-associated pool more closely. Close inspection of our colon carcinoma cell lines expressing plasma membrane dephospho-ß-catenin revealed that localization patterns of dephospho-ß-catenin and total ß-catenin do not overlap. Confocal scanning showed that, whereas total ß-catenin labels along the lateral plasma membrane, dephospho-ß-catenin accumulates at the apico-lateral cell-cell border (Fig 1B and C). The observed difference in localization is most apparent in cell lines, such as HCT15 cells, that retain the ability to grow in monolayer and therefore show proper polarization. We sought to investigate differences in plasma membrane accumulation of dephospho-ß-catenin between colon cancer cell lines in further detail. The known mutation status of ß-catenin and APC did not reveal any associations with nuclear or plasma membrane dephospho-ß-catenin levels (Table 1). The localization of dephospho-ß-catenin was investigated in relation to the expression of E-cadherin in our panel of cell lines. Using quantitative western blot analysis, we find a relationship between plasma membrane localization of dephospho-ß-catenin and E-cadherin protein levels. Cell lines with little membrane-associated dephospho-ß-catenin express low (SW480) to undetectable (Colo320, LS174T) levels of E-cadherin (Figure 1A and D), whereas cell lines showing prominent plasma membrane staining of de-

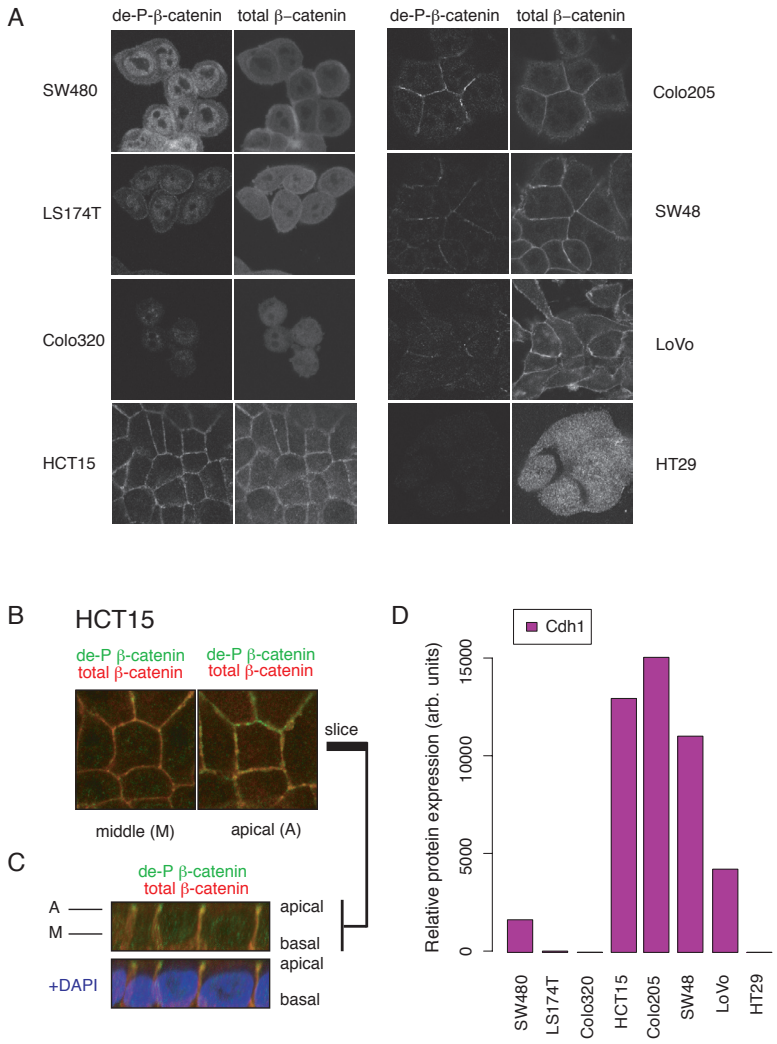

Figure 1. Plasma membrane localization of dephospho-ß-catenin correlates with E-cadherin expression. A. Subcellular localization of total and dephospho-ß-catenin in colon carcinoma cell lines. B. Dephospho-ß-catenin is concentrated at adherens junctions. Confocal sections taken from a z-series through the mid (left) and apical (right) planes of confluent HCT15 cells stained for total (red) or dephospho-ß-catenin (green). C. Orthogonal slice of z-series, labelled as in B. The lower panel includes the DAPI channel to visualize the positions of the nuclei (blue). D. Relative Cdh1 protein levels in cell lines shown in Figure 1A. 20 µg of total cellular protein was separated on SDS-PAGE, blotted and probed with an anti-Cdh1 antibody. Western blot signals were quantified using a luminoscan analyzer. Equal loading was confirmed using ß-actin detection, levels of which varied less than 25%.

phospho-ß-catenin (HCT15, Colo205, SW48 and Lovo) all express high levels of E-cadherin (Figure 1A and D). Low E-cadherin levels in SW480 and LS174T have been described in earlier reports (Gottardi et al., 2001; Elefstathiou et al., 1999; Muller et al., 2002). This shows that the amount of membrane-associated dephospho-ß-catenin correlates with E-cadherin expression, and suggests that the pool of dephospho-ß-catenin likely resides in a junctional complex. These results are consistent with previous findings showing that exogenously expressed N-terminal truncation mutants of ß-catenin colocalize with E-cadherin at cell-cell contacts in MDCK epithelial cells

mutation status (grey is LOH)

	SW480	LS174T	Colo320	HCT15	Colo205	SW48	LoVo	HT29	HCT116	CaCo2
β-cat	wt	S45F	wt	wt	N287S	S33Y	wt	wt	Δ45	G245A
APC	1338	wt	811	1416	1554	wt	1114	853 / 1555	wt	1367

Table 1. ß-Catenin and/or APC mutation status of colon carcinoma cell lines used in this study.

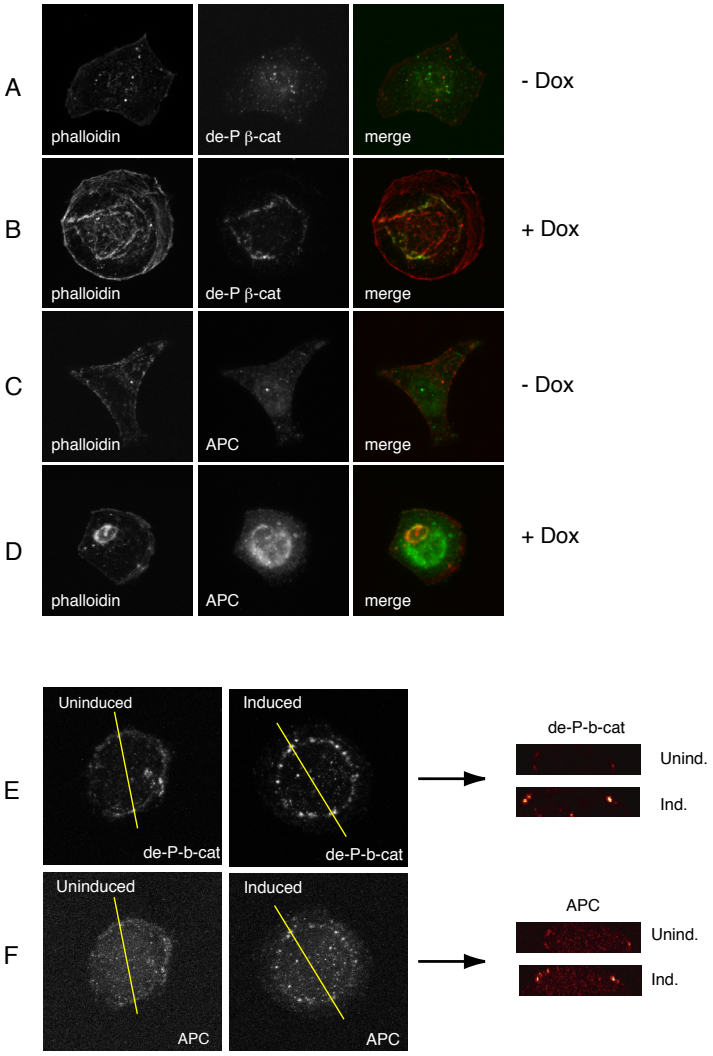

Figure 2. Dephospho-ß-catenin and APC localize to the apical membrane in polarized cells A-D. Immunofluorescence images of DLD1-W5 cells before and after polarization induced with doxicycline, stained with indicated antibodies. Dephospho-ß-catenin and APC colocalize with actin in the apical brush border after polarization. E and F. Z-stack projection of confocal images of DLD1-W5 cells before and after induction with doxicycline, showing apical localization of dephopho-ß-catenin (E) and APC (F) after polarization.

(Barth et al., 1997). Their data suggest that cellular dephospho-ß-catenin levels per se are not to be equated with ongoing Wnt signaling activity.
We continued to study the localization of dephospho-ß-catenin in a model of cellular polarization. For this, we used the human colon cancer cell line DLD1-W5 that can be induced to polarize at the single cell level (Baas et al., 2004). Upon doxicycline-induced expression of STRAD, isolated DLD-1-W5 cells show several hallmarks of polarization, such as organization of the actin cytoskeleton including a prominent apical ring-like actin cap (Fig. 2B and D). While there was no co-localization between actin (phalloidin) and dephospho-ß-catenin before polarization, in the polarized HCT15 monolayer, dephospho-ß-catenin localized to this apical actin structure (Fig. 2A and B). Dephospho-ß-catenin co-localized specifically with apical actin, whereas it did not co-localize with a well-known tight-junction marker ZO-1 (data not shown). Data from *in vivo* systems both in the *D. melanogaster* embryonic epidermis (Yu et al., 1999; McCartney et al., 1999; Cliffe et al., 2004) and in the human adult gastrointestinal epithelium (Anderson et al., 2002) have revealed that APC localizes to adherens junctions along with ß-catenin. However, the exact location of APC in cultured mammalian cell lines has remained unclear (Brocardo et al., 2005). We therefore stained polarized and unpolarized DLD-1-W5 cells with the N-APC monoclonal antibody (Midgley et al., 1997) that is a specific probe for endogenous APC in cultured cells (Kita et al., 2006). Like dephospho-ß-catenin, APC localizes to the ring-like apical actin cap in polarized epithelial cells (Fig. 2D), whereas no co-localization is apparent before polarization (Fig. 2C). We conclude that upon cellular polarization both dephospho-ß-catenin and APC are recruited to the presumptive apical adherens junction in this model system. Our results confirm studies in *D. melanogaster* showing that E-APC localizes to adherens junctions, where it co-localizes with ß-catenin and E-cadherin (Yu et al., 1999).
In order to determine the localization of dephospho-ß-catenin in the adult human system *in vivo*, we stained paraffin-embedded consecutive sections of normal adult small intestinal epithelium with antibodies recognizing either the total or dephosphorylated pool of ß-catenin. We find that both antibodies reveal an increased labelling on the plasma membrane at the level of the

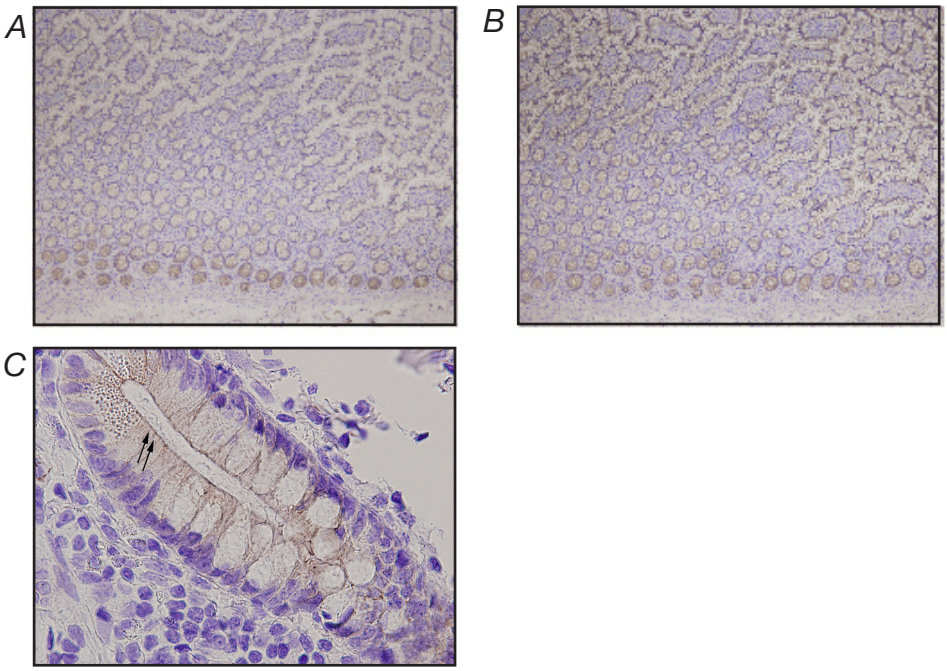

Figure 3. Immunohistochemistry of total (A) and dephospho-ß-catenin (B) in normal human small intestine. Both antibodies reveal increased membrane labeling at the level of the intestinal crypt. C. Zoom-in Fig 3B. Dephospho-ß-catenin shows a punctuate staining at the apico-lateral cell-cell border.

crypt over the villous epithelial cells (Fig 3A and B). By performing a dilution series we find that at limiting dilution, dephospho-ß-catenin accumulates on the apico-lateral membrane at the presumptive adherens junctions (Fig 3C), whereas dilution series for total ß-catenin did not reveal a similar preferential accumulation, in line with our data obtained in the polarized cell lines HCT15 and DLD-1-W5. Importantly, this apico-lateral accumulation is specific for crypt compared to villous epithelial cells, suggesting that the apical accumulation of dephospho-ß-catenin might be linked to active Wnt signal transduction.

Our data show that total cellular levels of dephospho-ß-catenin are not predictive of Wnt signaling activity due to cadherin-mediated dephospho-ß-catenin membrane sequestration. However, we were interested to analyze whether subpools of dephospho-ß-catenin might correlate better with Wnt signaling activity. The lectin protein Concavalin A (Con A) binds with high affinity to glycosylated proteins and has been used by several laboratories to distinguish between E-cadherin-bound and free ß-catenin (Aghib and McCrea, 1995; Funayama et al., 1995). We used Con A to precipitate E-cadherin and associated proteins, including pools of ß-catenin from cell lysates, and analyzed fractions on semi-quantitative western blot. In our panel of colon cancer cell lines, 4 cell lines showed a dispersed growth pattern, absence of ß-catenin in cell-cell contacts and low (SW480) to undetectable E-cadherin levels (HT29, LS174T, Colo320) (Fig 1A and D). Analysis of total ß-catenin protein levels before and after Con A binding did not reveal a correlation between E-cadherin expression and Con A-bound ß-catenin (Fig 4A). A possible explanation could be expression of other cadherins in these cell lines. We next determined dephospho-ß-catenin levels and found that 3 out of 4 cell lines with low E-cadherin levels show high levels of non-Con A-bound or free dephospho-ß-catenin (SW480, Colo320 and LS174T, Fig 4B). Out of these 3 cell lines, only SW480 and Colo320 show high Wnt signaling activity in the TOP/FOP assay as a readout for TCF-dependent transcriptional activation (Fig 4C). We conclude that high levels of free dephospho-ß-catenin still correlate poorly with Wnt signaling activity as only two out of three cell lines match high levels of free dephospho-ß-catenin to robust TCF reporter output.

It remains to be established whether quantitative analyses of the pool of dephospho-ß-catenin at the plasma membrane correlates with Wnt signaling activity. Our analyses in an E-cadherin null background provide evidence for plasma membrane recruitment of dephospho-ß-catenin upon Wnt treatment (Chapter 4). From the results of the Con A-bound pool of dephospho-ß-catenin in our panel of cell lines, it is clear that there is no strict correlation between this pool and Wnt signaling activity, much like the situation for free dephospho-ß-catenin. Therefore, our data of Con A-bound versus free dephospho-ß-catenin provide no evidence for a correlation between either of these pools and Wnt signaling activity. This underscores the importance of an E-cadherin null background in studying signaling competent dephospho-ß-catenin. Moreover, levels of free dephospho-ß-catenin still correlate poorly with Wnt signaling output, even if cell lines expressing low levels of E-cadherin are scored separately, which suggests multiple levels of regulation of signaling output. This is in accordance with data obtained in an E-cadherin null background (Chapter 4). To further investigate the impact of E-cadherin expression on dephospho-ß-catenin localization, we compared dephospho-ß-catenin localization in HCT15 cells grown at different densities. Nuclear levels of dephospho-ß-catenin were found to be higher in HCT15 cells grown in low density compared to confluent cells (Fig 4D). This suggests that increased cell-cell contacts can downregulate nuclear dephospho-ß-catenin levels. Indeed, overexpression of E-cadherin in these cells reduced TCF-dependent transcription (Fig 4E). Earlier studies have also shown that modulation of E-cadherin levels can affect Wnt signaling output. In particular, overexpression of E-cadherin antagonizes Wnt signaling by sequestering ß-catenin at the plasma membrane (Heasman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996; Orsulic et al., 1999). Likewise, reduction in E-cadherin increased armadillo signaling in Drosophila (Cox et al., 1996). However, E-cadherin does not appear to regulate the Wnt pathway *in vivo* as loss of cadherin function did not enhance Wnt signaling in either human tumors or murine cancer models (Caca et al., 1999; Smits et al., 2000; Vasioukhin et al., 2001; van de Wetering et al., 2001; Derksen et al., 2006).

In this study, we have shown that a pool of dephospho-ß-catenin resides at the apico-lateral cell-cell border of the plasma membrane. Plasma membrane localization of dephospho-ß-catenin correlates with E-cadherin expression, which suggests that at least part of this pool is involved in cell-cell adhesion. Therefore, the mere presence of dephospho-ß-catenin is not predictive of Wnt signaling activity. However, since Wnt treatment induces plasma membrane recruitment of

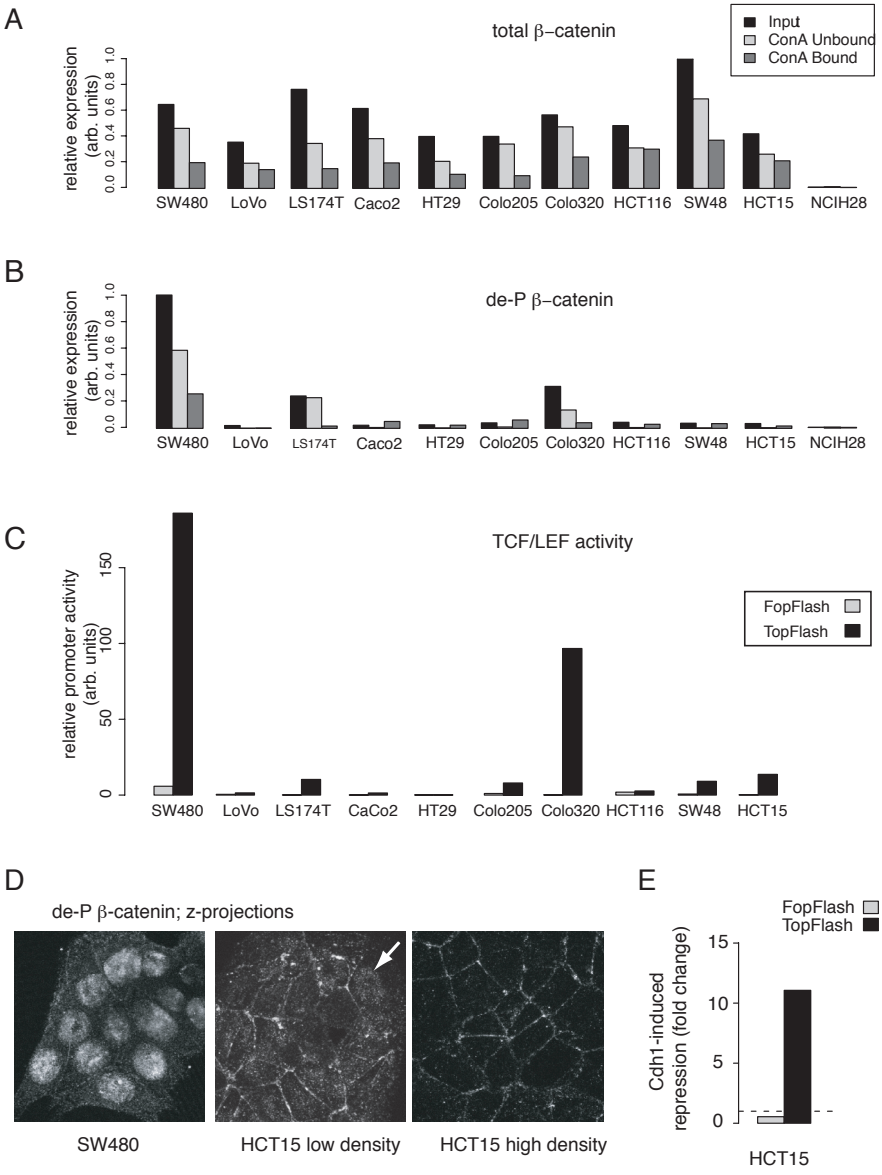

Figure 4. Relationship between ß-catenin levels, E-cadherin binding and Wnt signalling activity. A and B. Cell lysates from indicated colon carcinoma cell lines were subjected to binding to Con A to pull down E-cadherin-binding proteins. Input, Con A-bound (E-cadherin-bound) and Con A-unbound fractions were analyzed by semi-quantitative western blot and analyzed with an antibody recognizing total (A) or dephospho-ß-catenin (B). C. ß-Catenin/TCF-mediated transcriptional activity in colon carcinoma cell lines. Cells were transfected with TOP or the control FOP luciferase reporter to measure Wnt signalling activity 24 hours after transfection. Co-transfection of the pRL-CMV Renilla construct was used to correct for transfection efficiency. D. Detection of dephospho-ß-catenin in low density (middle) and high density (right) HCT15 cells. SW480 cells stained in parallel and imaged with the same settings are shown for comparison (left). Images represent projections of top-to-bottom confocal z-series. An arrow marks nuclear staining in low-density HCT15 cells. E. HCT15 cells were assayed for TCF/LEF-dependent transcriptional activity using TOP and FOP-TK-luciferase reporters in the presence or absence of Cdh1 expression. Ratios with/without Cdh1 are plotted as fold change. Dashed line: fold change equals 1.

dephospho-ß-catenin (Chapter 4), a fraction of this membrane-associated pool might in fact be involved in Wnt signal transduction. The mechanistic details of dephospho-ß-catenin routing in response to Wnt stimulation are unclear. Therefore, it is impossible to discern a resident junctional pool from a recruited signaling competent pool of dephospho-ß-catenin residing at the plasma membrane. It is imperative that future work addresses these issues to develop adequate immunological tools. In this respect, it is interesting to note that dephospho-ß-catenin accumulates at the apico-lateral cell-cell border to a greater degree in crypt epithelial cells, which are thought to be Wnt responsive. Whether this reflects ongoing Wnt signal transduction requires further study. Lastly, we show by fractionation experiments that neither the Con A-bound pool of dephospho-ß-catenin nor the free fraction of dephospho-ß-catenin correlates with Wnt signaling output. In addition to underscoring the importance of an E-cadherin null background, our results suggest multiple levels of regulation of Wnt signaling output.

Materials and methods

Cell culture and luciferase reporter assay
All cell lines 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. For reporter assays, cells were cultured in 12-wells plates and transfected with 200 ng TOP-Tk-lluc or the control FOP-Tk-luc together with 1 ng pRL-CMV Renilla to control for transfection efficiency. Cells were lysed after 48 hours and luciferase activity was measured using the Dual-luciferase reporter assay system (Promega).

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% skim milk (Oxio, Hampshire, England) at room temperature for one hour. Note that detection of dephospho-ß-catenin with the ABC antibody was inhibited by certain brands/lots of skim milk. Primary antibodies were incubated in 1% skim milk for 2 hours at room temperature in the following dilutions: E-cadherin 1:1500; ß-catenin mAb C19220 1:5000, ABC 1:500; actin 1:5000. 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 min and permeabilized for 5 min in 0.2% Triton/PBS. Primary antibodies were incubated for 2 hours in 1% purified BSA/PBS using the following dilutions; ABC 1:200; total ß-catenin C19220 1:250. Cells were washed shortly in PBS and incubated in conjugated fluorescent secondary antibodies (Molecular Probes) and DAPI in 1% BSA/PBS for 30 min, washed shortly in PBS and mounted in Mowiol. Images were recorded using a Leica NT, SP2 or SP2 AOBS confocal microscope. Antibodies used were against ß-catenin (C19220) (Transduction Labs), active ß-catenin (ABC 8E7), E-cadherin (C20820, Transduction Labs).

Concavalin A purification
For Concavalin A (Con A) purification, cells were lysed in 0.1% NP-40, 20 mM HEPES-KOH (pH 7.9), 200 mM NaCl 1 mM 2-mercaptoethanol and protease inhibitors (Complete-EDTA; 0.5 tablet per 10 ml), cleared by centrifugation and bound to 10 microliter Con A Sepharose 4B (Pharmacia) for 2.5 h at 4ºC. Beads were washed 3 times in lysis buffer and eluted using SDS-PAGE sample buffer.

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

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