Journal of Cell Science (2014) 127, 2967 2982 doi:10.1242/jcs.150888

trigger canonical Wnt signalling in a ligand-independent manner. Following treatment with SB415286, SaOS-2 cells showed activation and translocation of b-catenin to the nucleus, with some active b-catenin also present at intercellular contacts. By contrast, NHU cells, which already exhibited a high level of nuclear active b-catenin in control cultures, displayed only a modest increase in nuclear labelling after treatment with SB415286 (Fig. 1A). These findings indicate that proliferative NHU cells have an activated Wnt–b-catenin pathway, with a pool of active b-catenin present in the nucleus.

TCF promoter activity was assessed using the TOPFLASH/FOPFLASH luciferase reporter assay. SaOS-2 cells showed a significant increase in luciferase expression when treated with SB415286 (Fig. 1B); normalised TOPFLASH reporter activity after SB415286 treatment was 7.5-fold higher ($P<0.001$) than that of a vehicle-control-treated sample (Fig. 1B), confirming the functionality of the pharmacological activator and its ability to mimic active Wnt signalling. By contrast, there was no significant change ($P>0.05$) in TOPFLASH luciferase activity observed in NHU cells after SB415286 treatment (Fig. 1B).

The FOPFLASH control reporter provided a normalisation control, as it showed constant activity in NHU cells under different culture conditions, although it tended to give higher readouts than were achieved with TOPFLASH; similar observations were also made with untreated SaOS-2 cells (Fig. 1B) and have been reported in other epithelial cells (Papkoff and Aikawa, 1998). TOPFLASH and FOPFLASH are engineered from the same vector backbone and the only difference is the presence of a series of tandem LEF1/TCF-binding sites within TOPFLASH that are mutated in FOPFLASH. However, also extant within the promoter inserts of both vectors are a plethora of other transcription-factor-binding sites, including those of nuclear receptor sub-family 2, GATA and PPARc, which account for the high basal activity seen.

Blockade of EGFR ERK and PI3K AKT pathways reveals positive feedback between EGFR ERK and β-catenin signalling

In NHU cell cultures grown under standard culture conditions either with or without exogenous EGF, the expression of active b-catenin fluctuated over time. At a seeding density of 2.5×10^4 cells/cm^2, nuclear labelling for active b-catenin was most intense at 48 hours post-seeding, after which it was seen to decrease as cells reached confluence (Fig. 2A, Control; supplementary material Fig. S2). Western blotting results from parallel cultures corroborated the immunofluorescence microscopy findings, with active b-catenin expression peaking at 48 hours of culture (Fig. 2B). In parallel, the expression of phosphorylated ERK1/2 (phospho-ERK) also peaked at the 48-hour time-point, indicating a reciprocal pattern of expression for b-catenin and phospho-ERK. To determine whether b-catenin activation was due to a change in the activity of the destruction complex, we probed for the inactive form of GSK3b, which is phosphorylated at Ser9 (da Costa et al., 1999). Our results revealed that the increase in active b-catenin expression seen at 48 hours post-seeding was accompanied by an increase in the amount of inactive phospho-GSK3b (Fig. 2B).

Functional inactivation of EGFR by the pharmacological inhibitor PD153035 blocked the expression of nuclear active b-catenin (Fig. 2A,B). This was accompanied by loss of induction of phospho-GSK3b (Fig. 2B). We have previously shown that EGFR signalling in proliferative NHU cells is predominantly relayed across the MEK–ERK intracellular signalling axis (MacLaine et al., 2008; Varley et al., 2005). Functional ERK blockade by the MEK inhibitor U0126 caused marked attenuation of active b-catenin expression and inhibited the induction of phospho-GSK3b (Fig. 2B). By contrast, functional blockade of PI3K–AKT signalling with LY294002 caused a marked increase in the amount of nuclear active b-catenin at all time-points (Fig. 2A). In this latter state, some cells showed membrane-localised b-catenin, which was not evident in the control cultures (Fig. 2A). Notably, phospho-GSK3b expression was dramatically induced in PI3K–AKT-blocked cells at both the 24- and 48-hour time-points, yet it was diminished by 72 hours (Fig. 2B). Moreover, PI3K–AKT inhibition resulted in a substantial elevation in the levels of phospho-ERK.

To confirm the functionality of b-catenin activation and the downstream effects of RTK signalling blockade, we assessed the expression of Axin2 as a direct target of b-catenin–TCF transcription. The expression of Axin2 was significantly downregulated (by greater than twofold) after treatment with the EGFR inhibitor PD153035 for 24, 48 and 72 hours in culture (Fig. 2C), implying that inhibition of EGFR reduces TCF-mediated transcription. Treatment with the ERK inhibitor (U0126) also reduced the expression of Axin2 at the 24- and 48-hour time-points and, to a lesser extent, at the 72-hour time-point (Fig. 2C). By contrast, PI3K–AKT inhibition (with LY294002) did not alter Axin2 transcription (Fig. 2C). Similar observations were made for another b-catenin–TCF transcriptional target, c-Myc (not shown). We also examined the expression of E-cadherin (CDH1) mRNA, a repressed indirect target of b-catenin–TCF signalling (due to repression by the Wnt target Twist). In contrast to Axin2, CDH1 mRNA expression was significantly higher in EGFR-blocked cultures (Fig. 2C), an observation that mirrored our findings at the level of E-cadherin protein expression (Fig. 2B). Inhibition of neither ERK nor PI3K–AKT affected the expression of the CDH1 transcript (Fig. 2C).

Blockade of EGFR reveals the capacity for proliferative Wnt signalling in NHU cells

The observation that an EGFR–ERK-driven b-catenin–TCF pathway dominated in proliferative NHU cells (Fig. 2), combined with our finding that Wnt activation did not promote TCF activity over the high baseline (Fig. 1), led us to hypothesise that a blockade of EGFR would permit the influence of endogenous/exogenous mediators of b-catenin signalling to be observed. In EGFR-blocked NHU cell cultures, pharmacological activation of Wnt signalling using the GSK3b antagonist SB415286 resulted in intense punctate active b-catenin localisation in the nucleus (Fig. 3A). A comparison of mean nuclear fluorescence intensities showed that induction of Wnt signalling in EGFR-blocked cells restored nuclear active b-catenin levels to those observed in control EGFR-responsive NHU cells (Fig. 3A, right panel). The activation of Wnt signalling in NHU cells treated with the ERK inhibitor U0126 did not result in any profound changes in the expression or localisation of active b-catenin, and b-catenin localisation was more diffuse across both nuclear and cytoplasmic compartments (Fig. 3A). Moreover, in a large proportion of cells, active b-catenin labelling was also evident at the cell membrane (Fig. 3A). Inhibition of PI3K–AKT signalling resulted in a major increase in nuclear active b-catenin expression in comparison to controls (in agreement with Fig. 2), and activation of Wnt signalling by

Journal of Cell Science (2014) 127, 2967 2982 doi:10.1242/jcs.150888

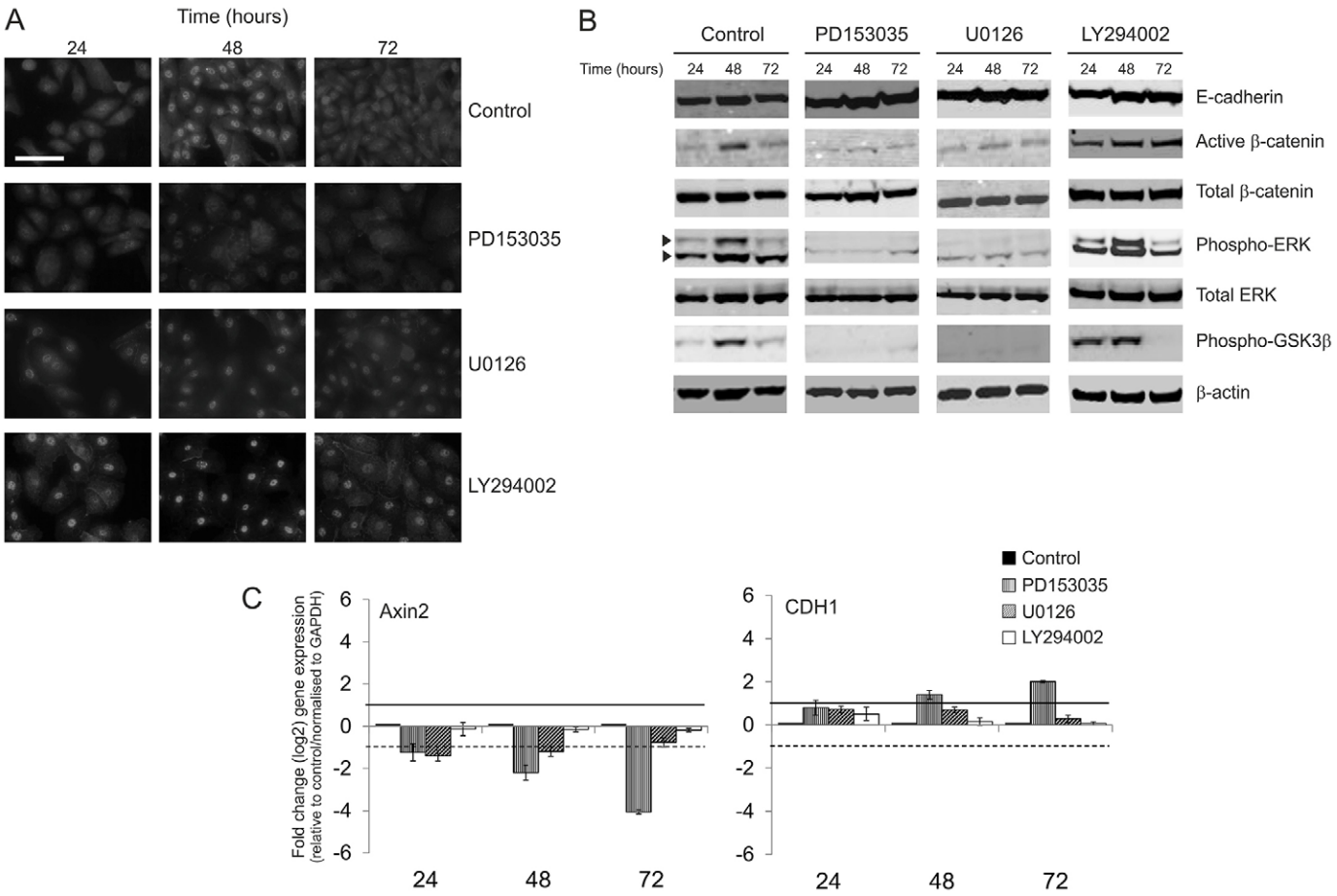


Fig. 2. Effect of inhibition of EGFR, MEK ERK and PI3K AKT on the Wnt β-catenin signalling pathway in NHU cells. (A) NHU cells were cultured for 24, 48 and 72 hours post-seeding in standard culture medium (Control) or medium containing 1μM EGFR inhibitor (PD153035), 5 μM MEK ERK inhibitor (U0126) or 5 μM PI3K AKT inhibitor (LY294002). These parallel cultures were then immunolabelled to detect the expression of active b-catenin. Results are representative of at least three independent experiments. Scale bar: 50μM. (B) NHU cells were cultured as described for A, before whole-cell lysates were prepared and the expression of E-cadherin, b-catenin (active and total), ERK (phospho- and total) and phospho-GSK3b (serine 9) was assessed by western blotting. For phospho-ERK, detection of both ERK1 (upper band) and ERK2 (lower band) isoforms of molecular mass 44 and 42 kDa, respectively, is indicated by arrowheads. Detection of b-actin served as a loading control. Results are representative of experiments with two NHU lines. (C) NHU cells were cultured as described for A, total RNA was isolated, cDNA was prepared and quantitative real-time RT-PCR was performed for the direct downstream b-catenin TCF target Axin2 (left). E-cadherin (CDH1) mRNA expression was also quantified (right). Data were initially normalised to GAPDH house-keeping controls and then expressed as fold change relative to solvent-balanced controls. Data represent the log2 mean expression (±s.d.) of three technical replicates. Solid and dotted lines represent twofold upregulation and twofold downregulation, respectively.

SB415286 had little effect on the overall intensity of active b-catenin expression, although the localisation appeared more diffuse (Fig. 3A).

The blockade of EGFR in NHU cells by PD153035 reduced basal TCF promoter activity, as was reflected by low levels of luciferase reporter activity in the TOPFLASH assays. This allowed for the first time the detection of significant induction of TCF promoter activity and, upon Wnt activation with SB415286, there was an approximately threefold increase in TOPFLASH (but not FOPFLASH) reporter activity (Fig. 3B), signifying substantial TCF promoter activation. Wnt-b-catenin activation in NHU cells treated with the MEK-ERK inhibitor U0126 had little effect on reporter activity (Fig. 3B). By contrast, reporter assays in PI3K–AKT-blocked NHU cells showed the previously observed (Fig. 1B) high basal TCF reporter activity, and GSK3b inhibition showed a modest yet non-significant increase in promoter activity (Fig. 3B)

The functional blockade of EGFR severely diminished NHU cell proliferation, but this could in part be overcome by pharmacological activation of Wnt signalling (Fig. 3C). Wnt stimulation in MEK–ERK-blocked NHU cells caused a small yet consistent increase in cell growth that was, however, not statistically significant (Fig. 3C). Inhibition of the PI3K–AKT pathway resulted in an initial retardation of NHU cell proliferation, consistent with our previous findings (MacLaine et al., 2008). However, Wnt activation had little effect on proliferation in PI3K–AKT-blocked NHU cell cultures (Fig. 3C).

β-catenin modulates ERK and AKT signalling as part of a bi-directional positive-feedback signalling loop

To investigate the role of b-catenin itself, we used a retroviral shRNA approach to generate NHU sub-lines carrying a stable b-catenin knockdown (NHU-b-cat-KD cells). Immunoblotting confirmed a substantial decrease in the expression of both

Journal of Cell Science (2014) 127, 2967 2982 doi:10.1242/jcs.150888

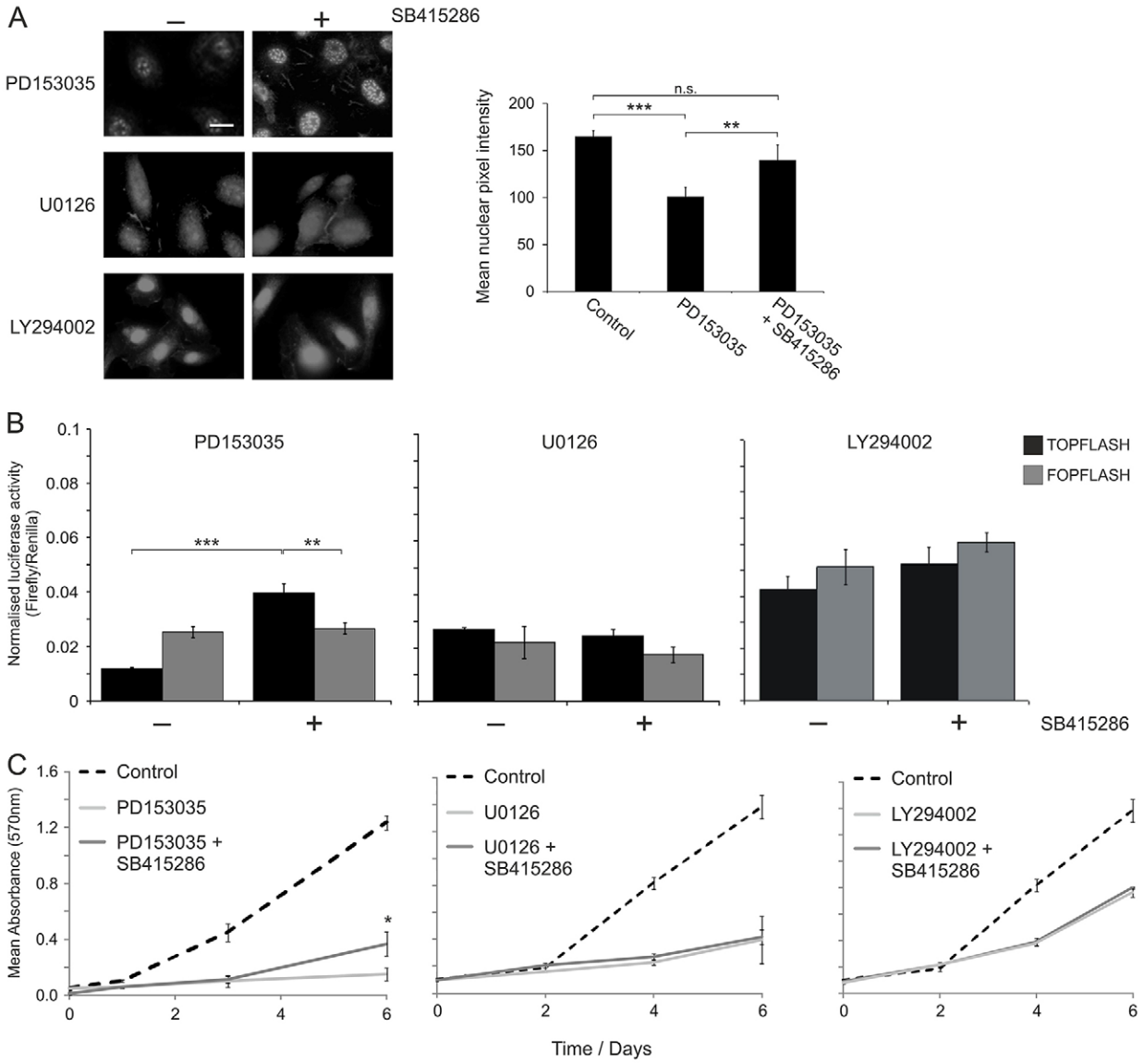


Fig. 3. Pharmacological activation of Wnt β-catenin signalling in NHU cells treated with EGFR, ERK and AKT inhibitors: effects on β-catenin localisation, TCF transcriptional activity and cell growth. (A) Left, NHU cells were pre-treated for 24 hours with medium supplemented with 1 μM EGFR inhibitor (PD153035), 5 μM MEK ERK inhibitor (U0126) or 5 μM PI3K AKT inhibitor (LY294002). Thereafter, the culture medium was replenished and supplemented with 10 μM GSK3b inhibitor (SB515286) or solvent [0.1% (v/v) DMSO] alone (Control) in the continued presence of signalling inhibitors for a further 24 hours. Cells were then immunolabelled to detect expression of active b-catenin. Results are representative of at least three independent experiments. Scale bar: 20 μM. Right, nuclear translocation of active b-catenin in control cells (micrographs not shown) and PD153035-treated NHU cells in the absence (PD153035) or presence (PD153035 + SB415286) of 10 μM GSK3b inhibitor was also quantified (as described in Fig. 1). Data represent the mean nuclear pixel intensity (±s.d.) from independent randomly selected b-catenin-positive cells (n=20). (B) NHU cells were transfected with either TOPFLASH or FOPFLASH plasmid along with pRL-CMV vector as a transfection control. At 16 hours post-transfection, cells were pre-treated with 1 μM EGFR inhibitor (PD153035), 5 μM MEK ERK inhibitor (U0126) or 5 μM PI3K AKT inhibitor (LY294002) for 24 hours. Cells were then incubated for a further 24 hours with 10 μM GSK3b inhibitor (SB415286) in the continued presence of signalling inhibitors. Subsequently, dual luciferase reporter assays were performed. Data show the mean (±s.d.) firefly luciferase activity [three technical replicates following normalisation to the transfection control (Renilla), at least three independent experiments]. **P<0.01; *** P<0.001; n.s., non-significant. (C) NHU cells were seeded and allowed to attach overnight. On day 0, cells were cultured in medium supplemented with 1 μM EGFR inhibitor (PD153035), 5 μM MEK ERK inhibitor (U0126), 5 μM PI3K AKT inhibitor (LY294002) or solvent [0.1% (v/v) DMSO] alone (Control). On day 1, the pathway inhibitor (or solvent) was replenished and, for some cultures, the medium was also supplemented with 10 μM GSK3b inhibitor (SB415286). Cell proliferation was determined using the MTT assay on days 0, 1, 4 and 6, with the respective culture medium in each case replenished on day 3. Data show the mean (±s.d.) absorbance at 570 nm (six technical replicates, three independent experiments); *P<0.05 when comparing the growth of cells treated with GSK3b inhibitor (SB415286) in the presence of EGFR inhibitor (PD153035) versus that of cells treated with PD153035 alone.

active and total b-catenin in NHU-b-cat-KD cells in comparison with NHU-Con control-shRNA-expressing isogenic counterparts (Fig. 4A). Immunofluorescence microscopy confirmed these findings and showed that both nuclear active and total b-catenin expression were reduced in NHU-b-cat-KD cultures in comparison with controls (Fig. 4B).

Journal of Cell Science (2014) 127, 2967 2982 doi:10.1242/jcs.150888

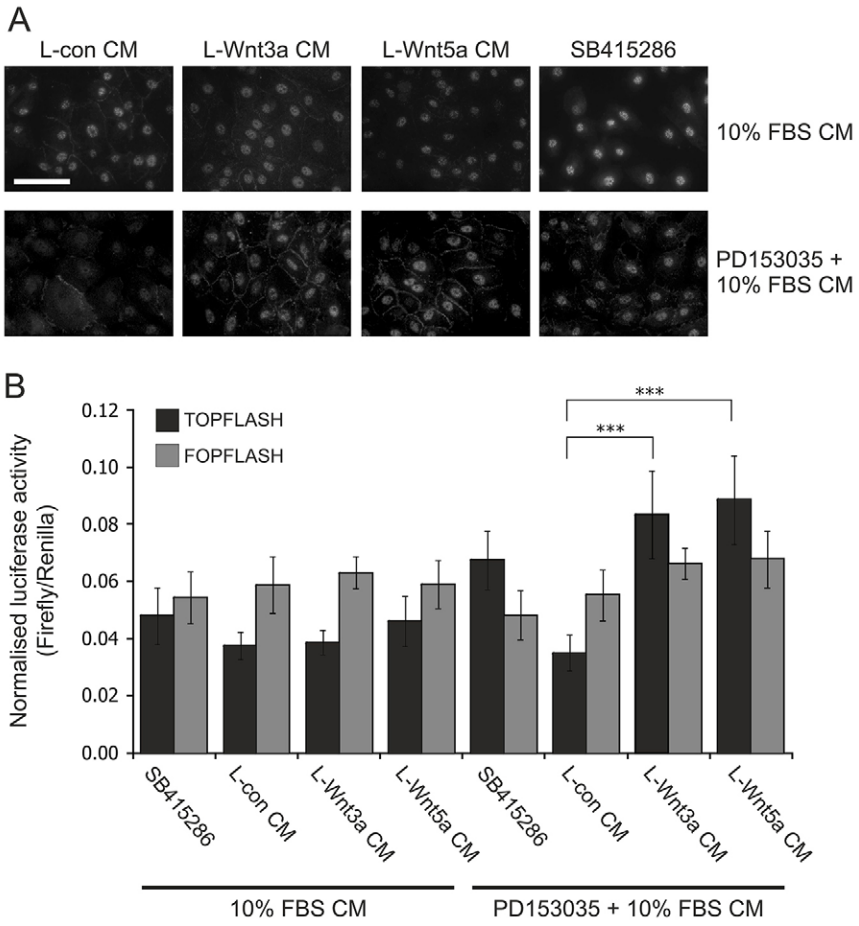


Fig. 7. Paracrine Wnt β-catenin signalling activation in NHU cells: treatment with exogenous Wnt3a and Wnt5a ligands in EGF-responsive and EGFR-blocked cells. (A) To detect active b-catenin protein expression by indirect immunofluorescence microscopy, NHU cells were pre-treated for 24 hours in the presence or absence of 1 μM EGFR inhibitor (PD153035). This was followed by a 24-hour treatment with conditioned medium (CM) from control (L-con), L-Wnt3a and L-Wnt5a cells (L-Con CM, L-Wnt3a CM and L-Wnt5a CM, respectively) obtained from L cells cultured in medium supplemented with 10% (v/v) serum. These treatments were carried out in the absence (10% FBS CM) or presence (PD153035+10% FBS CM) of EGFR inhibitor. Parallel cultures additionally treated with 10 μM GSK3b inhibitor (SB415286) were used as positive controls for b-catenin nuclear translocation. Results are representative of three experiments. Scale bar: 50 μm. (B) NHU cells were transfected with either TOPFLASH or FOPFLASH plasmid along with transfection control pRL-CMV vector. Starting at 16 hours post-transfection, cells were pre-treated with or without 1 μM EGFR inhibitor (PD153035) for 24 hours. Following this, cells were cultured in L-Con CM, L-Wnt3a CM or L-Wnt5a CM obtained from L cells cultured in medium supplemented with 10% (v/v) serum. These treatments were performed in the absence or presence of EGFR inhibitor. At 24 hours post-treatment, dual luciferase assays were performed. Data show the mean (±s.d.) firefly luciferase activity (three or four technical replicates following normalisation to transfection control, three independent experiments). ***$P<0.001$.

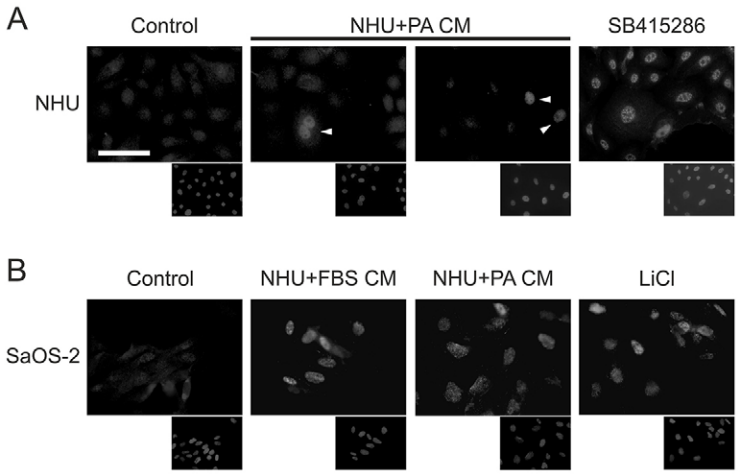


Fig. 8. Autocrine Wnt β-catenin signalling in NHU cells. (A) NHU cells were pre-treated for 24 hours with standard medium (KSFMc) supplemented with 1 μM EGFR inhibitor (PD153035), before treatment for 24 hours with conditioned medium (CM) from NHU cells cultured in KSFMc supplemented with 80 μM palmitic acid (NHU+PA CM). Expression of active b-catenin was detected by immunofluorescence microscopy. Parallel cultures treated with 10 μM GSK3b inhibitor (SB415286) were used as positive controls for b-catenin nuclear translocation. Negative controls included treatment with conditioned medium from NHU cells cultured in KSFMc containing 0.1% (v/v) ethanol alone (Control), as well as non-treated cells (not shown). Two representative images from one to three independent experiments are shown for NHU cells treated with conditioned medium (NHU+PA CM). White arrowheads, strong nuclear labelling in a subset of treated NHU cells. (B) SaOS-2 cells were treated for 24 hours with conditioned medium from NHU cells cultured in standard medium (KSFMc) in the presence of 10% (v/v) serum (NHU+FBS CM) or conditioned medium from NHU cells cultured in standard medium supplemented with 80 μM palmitic acid. Expression of active b-catenin was detected by immunofluorescence microscopy. Treatment with 20 mM LiCl served as a positive control for b-catenin activation. Negative controls were as described in A. For both A and B, lower panels show Hoechst 33258 labelling of nuclei. Scale bar: 50 μm.

Journal of Cell Science (2014) 127, 2967 2982 doi:10.1242/jcs.150888

phosphorylation of b-catenin at Tyr654 by Src or EGFR (disrupting the cadherin–b-catenin complex) and Tyr142 phosphorylation by Fer or Fyn (abrogating the interaction of b-catenin with a-catenin) (Kinch et al., 1995; Müller et al., 1999; Piedra et al., 2001; Piedra et al., 2003; Roura et al., 1999; Takeichi, 1995). By contrast, confluent cultures mainly express non-Tyr-phosphorylated, Ser/Thr-phosphorylated b-catenin, which localises at the membrane as an intrinsic component of the adherens junction (Müller et al., 1999; Shibamoto et al., 1994; Takeichi, 1995). Also, long-term EGF exposure can enhance E-cadherin repression by b-catenin–TCF-mediated expression of the transcriptional repressors Snail and Twist, suggesting a positive-feedback mechanism between the two pathways (Lickert et al., 2000), which is in agreement with our observation made here of Twist expression in proliferative NHU cells where b-catenin is actively signalling.

Seeding NHU cells at low density resulted in prolonged nuclear expression of b-catenin, whereas high seeding densities resulted in weak nuclear expression. The most obvious explanation for this apparent confluence-dependent effect on b-catenin activity is provided by our previously published findings showing EGFR downregulation in confluent NHU cell cultures (Varley et al., 2005). The present work corroborates these published findings, as a reduction in the expression of phospho-ERK was observed as NHU cultures became more densely populated. The ability of adherens junction formation to modulate the availability of b-catenin for nuclear translocation by sequestering the protein at the cell membrane could potentially be an important link in modulating the proliferation-quiescence switch in NHU cells. In low-Ca^{2+} growth medium, such as KSFMc, Ca^{2+}-dependent cell–cell engagement is weak and b-catenin expression at the cell membrane is low. However, stimulating adherens junction engagement by increasing the concentration of extracellular Ca^{2+} did not reduce the levels of nuclear b-catenin or the activity of TCF. These results suggest that, once released from the destruction complex, b-catenin preferentially translocates to the nucleus. How this is regulated remains unclear, but evidence suggests that activated EGFR can phosphorylate b-catenin at Tyr654, negating its ability to interact with E-cadherin at the adherens junction (Kinch et al., 1995; Piedra et al., 2003). Our findings support this scenario, as blockade of EGFR not only reduced the level of nuclear b-catenin but also increased the amount of b-catenin at the membrane. Therefore, our observations demonstrate that the quality of cell–cell contacts, although important in increasing the level of active b-catenin sequestered at sites of contact, does not alter the ability of b-catenin to translocate to the nucleus, nor does it interfere with its function in epithelial growth. Instead, b-catenin activation is predominantly dictated by proliferative signalling cues, and its gradual downregulation in cultured cells coincides with progression from cell proliferation to quiescence. Collectively, our study suggests that b-catenin might be crucial in the proliferation-quiescence switch seen during tissue regeneration. Overall, there appears to be a complex communication network between EGFR signalling, Wnt–b-catenin signalling and cell–cell contact, as summarised in Fig. 9. We suggest that b-catenin plays a central role in the regulation of this network, which strengthens the already prominent role of b-catenin in the maintenance of normal epithelial tissue homeostasis and renders it a potential target for deregulation in the transition from normal to malignant cell growth.

In addition to pharmacological activation of Wnt signalling, we examined whether biologically active Wnt ligand family members could trigger canonical Wnt signalling in NHU cells. For this purpose, we utilised L-cell derivatives producing Wnt ligands in conditioned medium. Previous reports on the production of bioactive Wnt ligand have recommended the inclusion of 10% (v/v) serum when harvesting conditioned medium, as serum contains albumin-bound palmitic acid that is required for Wnt post-translational palmitoylation (Povelones and Nusse, 2005). However, this imposed a practical difficulty for studying exogenous Wnt signalling in NHU cell cultures and for assessing whether NHU cells were capable of producing autocrine Wnt ligand, as serum is known to induce NHU differentiation (Cross et al., 2005). Wnt ligands are hydrophobic in nature owing to the many essential lipid modifications that occur during the maturation process. Post-translational modification of Wnt occurs in the ER and begins with the addition of a hydrophobic palmitate moiety, a process known as palmitoylation, which occurs on the first, absolutely conserved cysteine residue (Cys77 in Wnt 3a and Cys104 in Wnt 5a) (Reichsman et al., 1996; Willert et al., 2003) and which is essential for activity but not for secretion itself (Komekado et al., 2007; Willert et al., 2003). In our study, conditioned medium from serum-reduced Wnt-secreting L cell lines did not produce significant amounts of bioactive Wnt ligand and thus supported the results presented in previous publications (Willert et al., 2003; Willert, 2008). To overcome this, palmitic acid was added to the growth medium of L Wnt-3a cells to replace serum. Because of issues with the solubility and precipitation of palmitic acid in aqueous medium (not shown) a maximum concentration of 80 μM palmitic acid was obtained (compared with 110 μM in medium containing 10% FBS, as measured by gas-liquid chromatography; not shown). This was adequate to restore Wnt ligand secretion and activity to ~50% of that observed with serum-supplemented medium, thus confirming that palmitic acid was required for the production of bioactive Wnt ligand. This observation provides the first conclusive evidence that serum can be reduced when harvesting bioactive Wnt ligand, but only if the growth medium is supplemented with palmitic acid. More importantly, by exploiting the successful palmitic-acid-based approach to produce active Wnt ligands, we were able to demonstrate for the first time that biologically active Wnt ligands can induce canonical Wnt–b-catenin signalling in NHU cells.

Our studies also provided some intriguing observations when we examined the ability of NHU cells to carry out autocrine/paracrine Wnt signalling by endogenously produced Wnt ligands. We demonstrated that EGFR-blocked NHU cells that had been incubated with conditioned medium from isogenic palmitic-acid-treated NHU cell cultures showed limited overall Wnt pathway activation, which was restricted to a small proportion of NHU cells that displayed high levels of active nuclear b-catenin after treatment. At present, the basis for this heterogeneity and its implications are unclear; however, the data imply that there might be a small subset of cells capable of driving self-renewal in response to autocrine/paracrine Wnt signalling. It is tempting to speculate that this might represent a subpopulation of cells with the ability to initiate their own programme of self-renewal, such as a stem cell population.

The presence of resident adult stem cell populations has been described for many organs, including brain, lung and heart, as well as many epithelial tissues, including liver, colon and skin (Mimeault and Batra, 2008). Although research into urothelial stem cells is ongoing, no unequivocal resident stem cell population has been identified in human urothelium, although

Journal of Cell Science (2014) 127, 2967 2982 doi:10.1242/jcs.150888

Fig. 9. Model of Wnt β-catenin signalling crosstalk with EGFR ERK and cell cell-contact-mediated β-catenin regulation and their roles in NHU cell proliferation. (A) At low density, EGFR present on the NHU cell surface is activated by EGF ligand. EGFR activation leads to phosphorylation of ERK (P-ERK), subsequent translocation of phospho-ERK to the nucleus and induction of cell proliferation. Phospho-ERK inactivates the destruction complex, allowing b-catenin to accumulate and enter the nucleus. (B) As culture density increases, the intense signalling crosstalk between b-catenin and EGFR ERK results in a positive-feedback loop that accelerates growth and supports a highly proliferative (regenerative) phenotype, thus leading to rapid increases in cell numbers. Interestingly, the activities of b-catenin and AKT appear to be mutually exclusive. (C) Confluence-induced reduction in the expression of EGFR leads to a decline in EGFR signalling and reduction in phospho-inhibition of GSK3b. As a consequence, the activity of the destruction complex is reinstated. Moreover, cytosolic b-catenin is sequestered to the cytoplasmic tail of E-cadherin and, as a result, extensive adherens junctions form. Once a critical number of cell cell contacts have been established, excess b-catenin is targeted for degradation by the destruction complex. Phospho-ERK and active b-catenin levels are low, cells enter G1 growth arrest and cells exit the regenerative response.

in situ observations have been used to infer a basal progenitor (Gaisa et al., 2011). In the rat, a subpopulation of highly clonogenic BrdU-label-retaining (i.e. long lived) basal cells have been identified and have been shown to express markers consistent with stem cells in other tissues (including Bcl-2, p63, KRT14 and b1 integrin) (Kurzrock et al., 2008). In the mouse, a subset of $KRT5^{+}$ basal urothelial cells have been shown to express Sonic hedgehog (Shh), a ligand that is important during embryonic development. The relevance of these studies to human urothelium remains unclear, as there appear to be fundamental differences in the regulation of urothelial regeneration between human and rodent urothelium in vivo (Chopra et al., 2008), and the plasticity to revert from a suprabasal to a basal phenotype is a feature of human urothelial cells, at least in vitro (Wezel et al., 2013). The role of Wnt–b-catenin signalling has been more widely studied in rodent urothelium than in human, and the pathway has been found to play an important role in tissue homeostasis. In the mouse, proliferation in response to bacterially or chemically induced injury is regulated by signal feedback between the basal urothelial cells and the underlying stromal cells. After injury, basal urothelial cells were seen to secrete Shh, evoking the expression of Wnt ligands from the underlying stroma. Both stromal and urothelial cells proliferated in response to Wnt ligand, restoring urothelial integrity (Shin et al., 2011). Our observation of autocrine/paracrine Wnt–b-catenin activation in a subset of NHU cells might represent an important step towards identifying self-renewal mechanisms in human urothelium.

In summary, our study provides evidence for a bi-directional signalling loop between Wnt–b-catenin and RTK-driven MAPK signalling pathways that serves to drive proliferation in a normal epithelial cell population. This has important implications for normal epithelial physiology, where the crosstalk could represent an extremely efficient mechanism to rapidly initiate, accelerate and sustain cell growth during tissue regeneration, for instance, following tissue damage. Upon completion of tissue regeneration and establishment of contact inhibition, rapid cell-contact-mediated downregulation of RTK signalling (combined with the induction of inhibitory Wnt components) would attenuate b-catenin signalling, thus switching off the signalling feedback loop and, subsequently, cell proliferation. Moreover, this efficient mechanism would represent a molecular target in carcinogenesis, as its dysregulation (constitutive activation) would provide a strong growth advantage during malignant transformation (Ahmad et al., 2011a; Ahmad et al., 2011b).

MATERIALS AND METHODS

Reagents and antibodies

Pharmacological inhibitors PD153035, U0126 and LY294002 were purchased from VWR (Merck). GSK3b inhibitors SB415286 and LiCl were from Sigma Aldrich. The antibodies used were against; active b-catenin dephosphorylated on Ser37 and Thr41 (8E7; a kind gift from Hans Clevers, Utrecht University), total b-catenin (C2206; Sigma Aldrich), b-actin (AC-15; Sigma), E-cadherin (HECD-1; Abcam), total ERK (16; Transduction Laboratories), phospho-42/44 MAPK (D13.14.4E; Cell Signalling Technology), AKT (7; BD Biosciences),

Funding
The study was part funded by the Engineering and Physical Sciences Research Council (EPSRC) [grant number GR/S62338/01], on which N.T.G. was employed; L.A.K. was supported by a postgraduate studentship from York Against Cancer, and J.S. holds a research chair supported by York Against Cancer. Deposited in PMC for immediate release.

Supplementary material
Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.150888/-/DC1

References
Ahmad, I., Morton, J. P., Singh, L. B., Radulescu, S. M., Ridgway, R. A., Patel, S., Woodgett, J., Winton, D. J., Taketo, M. M., Wu, X. R. et al. (2011a). b-Catenin activation synergizes with PTEN loss to cause bladder cancer formation. Oncogene **30**, 178-189.
Ahmad, I., Patel, R., Liu, Y., Singh, L. B., Taketo, M. M., Wu, X. R., Leung, H. Y. and Sansom, O. J. (2011b). Ras mutation cooperates with b-catenin activation to drive bladder tumourigenesis. Cell Death Dis. **2**, e124.
Bikkavilli, R. K. and Malbon, C. C. (2009). Mitogen-activated protein kinases and Wnt/beta-catenin signaling: Molecular conversations among signaling pathways. Comm. Integr. Biol. **2**, 46-49.
Bock, M., Hinley, J., Schmitt, C., Wahlicht, T., Kramer, S. and Southgate, J. (2014). Identification of ELF3 as an early transcriptional regulator of human urothelium. Dev. Biol. **386**, 321-330.
Carmon, K. S. and Loose, D. S. (2008). Wnt7a interaction with Fzd5 and detection of signaling activation using a split eGFP. Biochem. Biophys. Res. Commun. **368**, 285-291.
Cavallaro, U. and Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat. Rev. Cancer **4**, 118-132.
Chopra, B., Hinley, J., Oleksiewicz, M. B. and Southgate, J. (2008). Trans-species comparison of PPAR and RXR expression by rat and human urothelial tissues. Toxicol. Pathol. **36**, 485-495.
Clevers, H. and Nusse, R. (2012). Wnt/b-catenin signaling and disease. Cell **149**, 1192-1205.
Cross, W. R., Eardley, I., Leese, H. J. and Southgate, J. (2005). A biomimetic tissue from cultured normal human urothelial cells: analysis of physiological function. Am. J. Physiol. **289**, F459-F468.
da Costa, L. T., He, T. C., Yu, J., Sparks, A. B., Morin, P. J., Polyak, K., Laken, S., Vogelstein, B. and Kinzler, K. W. (1999). CDX2 is mutated in a colorectal cancer with normal APC/beta-catenin signaling. Oncogene **18**, 5010-5014.
Ding, Q., Xia, W., Liu, J. C., Yang, J. Y., Lee, D. F., Xia, J., Bartholomeusz, G., Li, Y., Pan, Y., Li, Z. et al. (2005). Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. Mol. Cell **19**, 159-170.
Fleming, J. M., Shabir, S., Varley, C. L., Kirkwood, L. A., White, A., Holder, J., Trejdosiewicz, L. K. and Southgate, J. (2012). Differentiation-associated reprogramming of the transforming growth factorb receptor pathway establishes the circuitry for epithelial autocrine/paracrine repair. PLoS ONE **7**, e51404.
Gaisa, N. T., Graham, T. A., McDonald, S. A., Canadillas-Lopez, S., Poulsom, R., Heidenreich, A., Jakse, G., Tadrous, P. J., Knuechel, R. and Wright, N. A. (2011). The human urothelium consists of multiple clonal units, each maintained by a stem cell. J. Pathol. **225**, 163-171.
Georgopoulos, N. T., Kirkwood, L. A., Walker, D. C. and Southgate, J. (2010). Differential regulation of growth-promoting signalling pathways by E-cadherin. PLoS ONE **5**, e13621.
Georgopoulos, N. T., Kirkwood, L. A., Varley, C. L., MacLaine, N. J., Aziz, N. and Southgate, J. (2011). Immortalisation of normal human urothelial cells compromises differentiation capacity. Eur. Urol. **60**, 141-149.
Guturi, K. K., Mandal, T., Chatterjee, A., Sarkar, M., Bhattacharya, S., Chatterjee, U. and Ghosh, M. K. (2012). Mechanism of b-catenin-mediated transcriptional regulation of epidermal growth factor receptor expression in glycogen synthase kinase 3 b-inactivated prostate cancer cells. J. Biol. Chem. **287**, 18287-18296.
Howe, L. R., Watanabe, O., Leonard, J. and Brown, A. M. (2003). Twist is up-regulated in response to Wnt1 and inhibits mouse mammary cell differentiation. Cancer Res. **63**, 1906-1913.
Hubbell, E., Liu, W. M. and Mei, R. (2002). Robust estimators for expression analysis. Bioinformatics **18**, 1585-1592.
Hughes, T. A. and Brady, H. J. (2005). Cross-talk between pRb/E2F and Wnt/beta-catenin pathways: E2F1 induces axin2 leading to repression of Wnt signalling and to increased cell death. Exp. Cell Res. **303**, 32-46.
Jho, E. H., Zhang, T., Domon, C., Joo, C. K., Freund, J. N. and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell. Biol. **22**, 1172-1183.
Kinch, M. S., Clark, G. J., Der, C. J. and Burridge, K. (1995). Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J. Cell Biol. **130**, 461-471.
Komekado, H., Yamamoto, H., Chiba, T. and Kikuchi, A. (2007). Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. Genes Cells **12**, 521-534.
Krejci, P., Aklian, A., Kaucka, M., Sevcikova, E., Prochazkova, J., Masek, J. K., Mikolka, P., Pospisilova, T., Spoustova, T., Weis, M. et al. (2012). Receptor tyrosine kinases activate canonical WNT/b-catenin signaling via MAP kinase/LRP6 pathway and direct b-catenin phosphorylation. PLoS ONE **7**, e35826.
Kurzrock, E. A., Lieu, D. K., Degraffenried, L. A., Chan, C. W. and Isseroff, R. R. (2008). Label-retaining cells of the bladder: candidate urothelial stem cells. Am. J. Physiol. **294**, F1415-F1421.
Latasa, M. U., Salis, F., Urtasun, R., Garcia-Irigoyen, O., Elizalde, M., Uriarte, I., Santamaria, M., Feo, F., Pascale, R. M., Prieto, J. et al. (2012). Regulation of amphiregulin gene expression by b-catenin signaling in human hepatocellular carcinoma cells: a novel crosstalk between FGF19 and the EGFR system. PLoS ONE **7**, e52711.
Li, J., Ying, J., Fan, Y., Wu, L., Ying, Y., Chan, A. T., Srivastava, G. and Tao, Q. (2010). WNT5A antagonizes WNT/b-catenin signaling and is frequently silenced by promoter CpG methylation in esophageal squamous cell carcinoma. Cancer Biol. Ther. **10**, 617-624.
Lickert, H., Bauer, A., Kemler, R. and Stappert, J. (2000). Casein kinase II phosphorylation of E-cadherin increases E-cadherin/beta-catenin interaction and strengthens cell-cell adhesion. J. Biol. Chem. **275**, 5090-5095.
Lobban, E. D., Smith, B. A., Hall, G. D., Harnden, P., Roberts, P., Selby, P. J., Trejdosiewicz, L. K. and Southgate, J. (1998). Uroplakin gene expression by normal and neoplastic human urothelium. Am. J. Pathol. **153**, 1957-1967.
Lu, Z., Ghosh, S., Wang, Z. and Hunter, T. (2003). Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell **4**, 499-515.
Ma, L., Zhang, G., Miao, X. B., Deng, X. B., Wu, Y., Liu, Y., Jin, Z. R., Li, X. Q., Liu, Q. Z., Sun, D. X. et al. (2013). Cancer stem-like cell properties are regulated by EGFR/AKT/b-catenin signaling and preferentially inhibited by gefitinib in nasopharyngeal carcinoma. FEBS J. **280**, 2027-2041.
MacLaine, N. J., Wood, M. D., Holder, J. C., Rees, R. W. and Southgate, J. (2008). Sensitivity of normal, paramalignant, and malignant human urothelial cells to inhibitors of the epidermal growth factor receptor signaling pathway. Mol. Cancer Res. **6**, 53-63.
Meijer, L., Flajolet, M. and Greengard, P. (2004). Pharmacological inhibitors of glycogen synthase kinase 3. Trends Pharmacol. Sci. **25**, 471-480.
Mimeault, M. and Batra, S. K. (2008). Recent progress on tissue-resident adult stem cell biology and their therapeutic implications. Stem Cell Rev. **4**, 27-49.
Morali, O. G., Delmas, V., Moore, R., Jeanney, C., Thiery, J. P. and Larue, L. (2001). IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition. Oncogene **20**, 4942-4950.
Muller, T., Choidas, A., Reichmann, E. and Ullrich, A. (1999). Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J. Biol. Chem. **274**, 10173-10183.
Muller, T., Bain, G., Wang, X. and Papkoff, J. (2002). Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Exp. Cell Res. **280**, 119-133.
Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. Science **303**, 1483-1487.
Papkoff, J. and Aikawa, M. (1998). WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. Biochem. Biophys. Res. Commun. **247**, 851-858.
Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M. and de Herreros, A. G. (2001). Regulation of beta-catenin structure and activity by tyrosine phosphorylation. J. Biol. Chem. **276**, 20436-20443.
Piedra, J., Miravet, S., Castano, J., Palmer, H. G., Heisterkamp, N., Garc a de Herreros, A. and Dunach, M. (2003). p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. Mol. Cell. Biol. **23**, 2287-2297.
Povelones, M. and Nusse, R. (2005). The role of the cysteine-rich domain of Frizzled in Wingless-Armadillo signaling. EMBO J. **24**, 3493-3503.
Qiang, Y. W., Hu, B., Chen, Y., Zhong, Y., Shi, B., Barlogie, B. and Shaughnessy, J. D., Jr (2009). Bortezomib induces osteoblast differentiation via Wnt-independent activation of beta-catenin/TCF signaling. Blood **113**, 4319-4330.
Reichsman, F., Smith, L. and Cumberledge, S. (1996). Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. J. Cell Biol. **135**, 819-827.
Roura, S., Miravet, S., Piedra, J., Garc a de Herreros, A. and Dunach, M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. J. Biol. Chem. **274**, 36734-36740.
Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. and Ito, F. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell Adhes. Commun. **1**, 295-305.
Shin, K., Lee, J., Guo, N., Kim, J., Lim, A., Qu, L., Mysorekar, I. U. and Beachy, P. A. (2011). Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. Nature **472**, 110-114.
Southgate, J., Hutton, K. A., Thomas, D. F. and Trejdosiewicz, L. K. (1994). Normal human urothelial cells in vitro: proliferation and induction of stratification. Lab. Invest. **71**, 583-594.
Southgate, J., Masters, J. R. W. and Trejdosiewicz, L. K. (2002). Culture of human urothelium. In Culture of Epithelial Cells (ed. R. I. Freshney and M. G. Freshney), pp. 381-399. New York, NY: John Wiley and Sons., Inc.
Tago, K., Nakamura, T., Nishita, M., Hyodo, J., Nagai, S., Murata, Y., Adachi, S., Ohwada, S., Morishita, Y., Shibuya, H. et al. (2000). Inhibition of Wnt

signaling by ICAT, a novel beta-catenin-interacting protein. Genes Dev. **14**, 1741-1749.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. Curr. Opin. Cell Biol. **7**, 619-627.

Tang, Y., Simoneau, A. R., Liao, W. X., Yi, G., Hope, C., Liu, F., Li, S., Xie, J., Holcombe, R. F., Jurnak, F. A. et al. (2009). WIF1, a Wnt pathway inhibitor, regulates SKP2 and c-myc expression leading to G1 arrest and growth inhibition of human invasive urinary bladder cancer cells. Mol. Cancer Ther. **8**, 458-468.

Ueda, M., Ouhtit, A., Bito, T., Nakazawa, K., Lubbe, J., Ichihashi, M., Yamasaki, H. and Nakazawa, H. (1997). Evidence for UV-associated activation of telomerase in human skin. Cancer Res. **57**, 370-374.

Urakami, S., Shiina, H., Enokida, H., Kawakami, T., Tokizane, T., Ogishima, T., Tanaka, Y., Li, L. C., Ribeiro-Filho, L. A., Terashima, M. et al. (2006). Epigenetic inactivation of Wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical Wnt/beta-catenin signaling pathway. Clin. Cancer Res. **12**, 383-391.

Varley, C., Hill, G., Pellegrin, S., Shaw, N. J., Selby, P. J., Trejdosiewicz, L. K. and Southgate, J. (2005). Autocrine regulation of human urothelial cell proliferation and migration during regenerative responses in vitro. Exp. Cell Res. **306**, 216-229.

Wezel, F., Pearson, J. and Southgate, J. (2013). Plasticity of in vitro-generated urothelial cells for functional tissue formation. Tissue Eng. Part A. **20**, 1358-1368.

Willert, K. H. (2008). Isolation and application of bioactive Wnt proteins. Methods Mol. Biol. **468**, 17-29.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., III and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature **423**, 448-452.

Yun, M. S., Kim, S. E., Jeon, S. H., Lee, J. S. and Choi, K. Y. (2005). Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation. J. Cell Sci. **118**, 313-322.

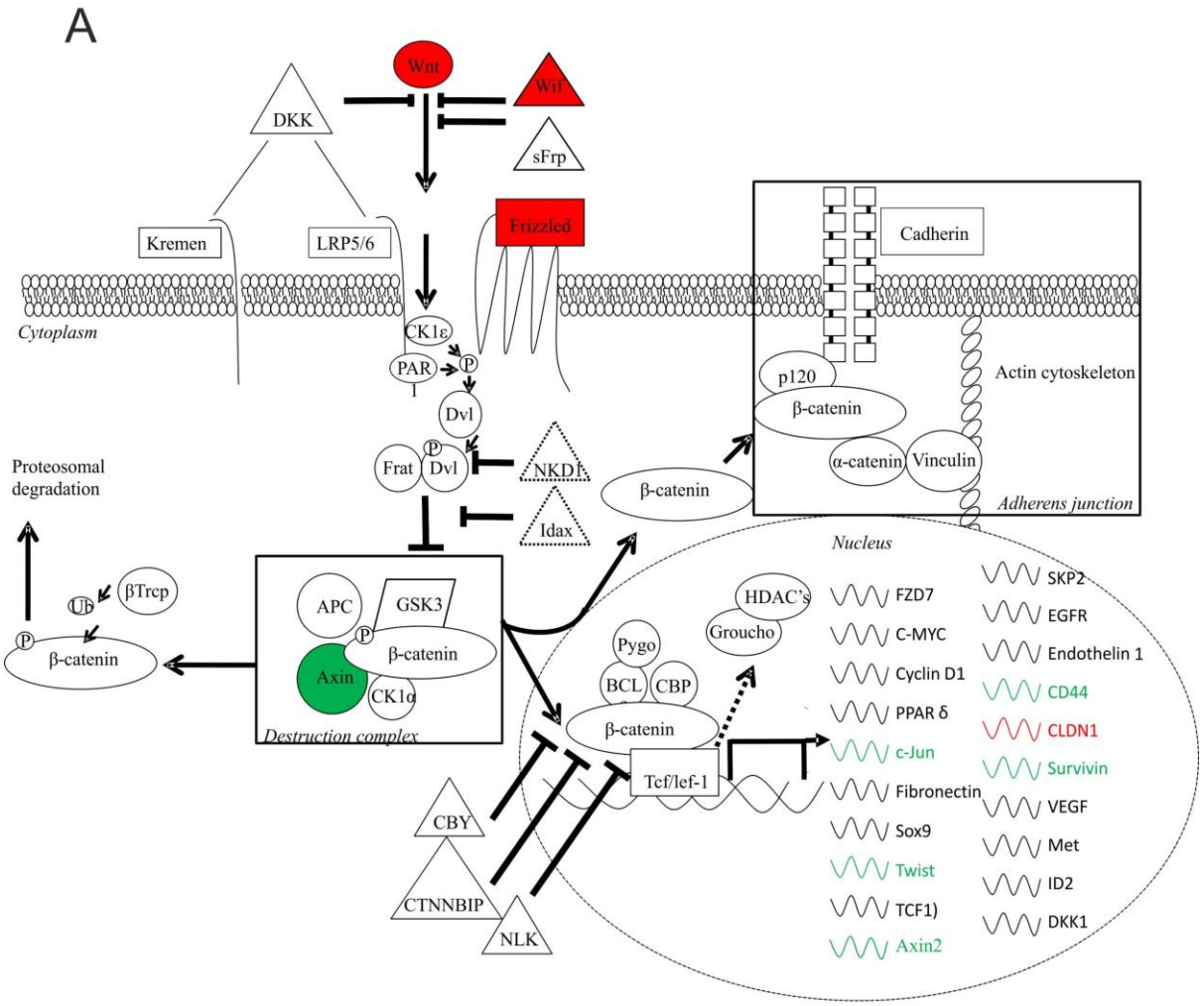
Zhang, T., Otevrel, T., Gao, Z., Gao, Z., Ehrlich, S. M., Fields, J. Z. and Boman, B. M. (2001). Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res. **61**, 8664-8667.

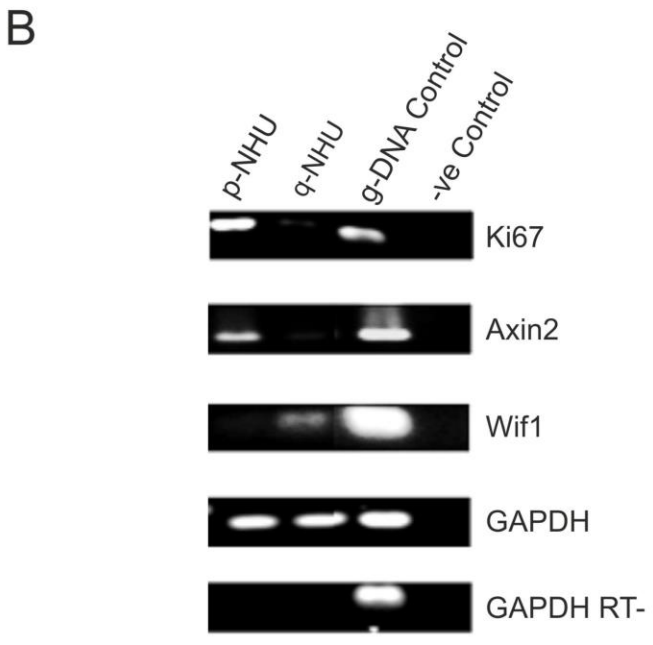
Supplementary data: A novel bidirectional positive feedback loop between Wnt/β-catenin and EGFR/ERK: role of context-specific signalling crosstalk in modulating epithelial tissue regeneration

Supplementary Table 1

DNA sequences of forward (FWD) and reverse (REV) oligonucleotide primers used in this study.

Gene target	FWD 5′ → 3′	REV 5′ → 3′
Axin2	CAAGGGCCAGGTCACCAA	CCCCCAACCCATCTTCGT
CDH1	AATCTGAAAGCGGCTGATACTGA	CGGAACCGCTTCCTTCATAG
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
Ki67	CAAGAGCATCAGAACGTTTAAGGA	TTCTTGGCCACTTCTTCATTCC
Wif1	GGCACCTTTTACACATGATTTCAG	TGGAATGGATATTGACAGGAATAGC
Wnt3a (mouse)	CTGGCAGCTGTGAAGTGAAG	TGGGTGAGGCCTCGTAGTAG
Wnt5a (mouse)	CTGGCTCCTGTAGCCTCAAG	AATCTCCGTGCACTTCTTGC


A
Wnt
Wif
DKK
sFrp
Kremen
LRP5/6
Frizzled
Cadherin
Cytoplasm
CK1ε
PAR
P
Dvl
Frat
NKD1
Idax
Actin cytoskeleton
p120
β-catenin
α-catenin
Vinculin
Adherens junction
Proteosomal degradation
Ub
βTrcp
APC
GSK3
Axin
CK1α
Destruction complex
Nucleus
HDAC's
Groucho
Pygo
BCL
CBP
Tcf/lef-1
CBY
CTNNBIP
NLK
FZD7
C-MYC
Cyclin D1
PPAR δ
c-Jun
Fibronectin
Sox9
Twist
TCF1)
Axin2
SKP2
EGFR
Endothelin 1
CD44
CLDN1
Survivin
VEGF
Met
ID2
DKK1

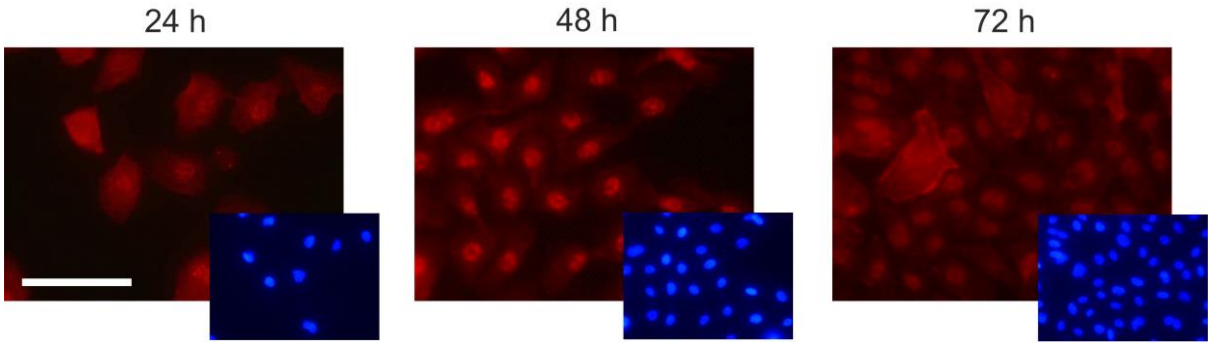

B
p-NHU
q-NHU
g-DNA Control
-ve Control
Ki67
Axin2
Wif1
GAPDH
GAPDH RT-

Supplementary Figure 1

Expression of Wnt/β-catenin pathway components in NHU cells assessed by expression arrays and RT-PCR

A) Schematic representation of the components of the canonical Wnt cascade present in NHU cells and gene changes relating to the pathway in quiescent cells. Wnt pathway components in the diagram (white or in colour) were classified as 'present' from MAS5 normalised Affymetrix™ microarray data. Genes with at least a 2-fold change in expression in quiescent NHU cultures compared to proliferative cultures are labelled as red (up-regulated) or green (down-regulated) and fold-change gene expression data are provided in appropriate tables. Components in white with dashed borders were classed as absent in the proliferating NHU culture.

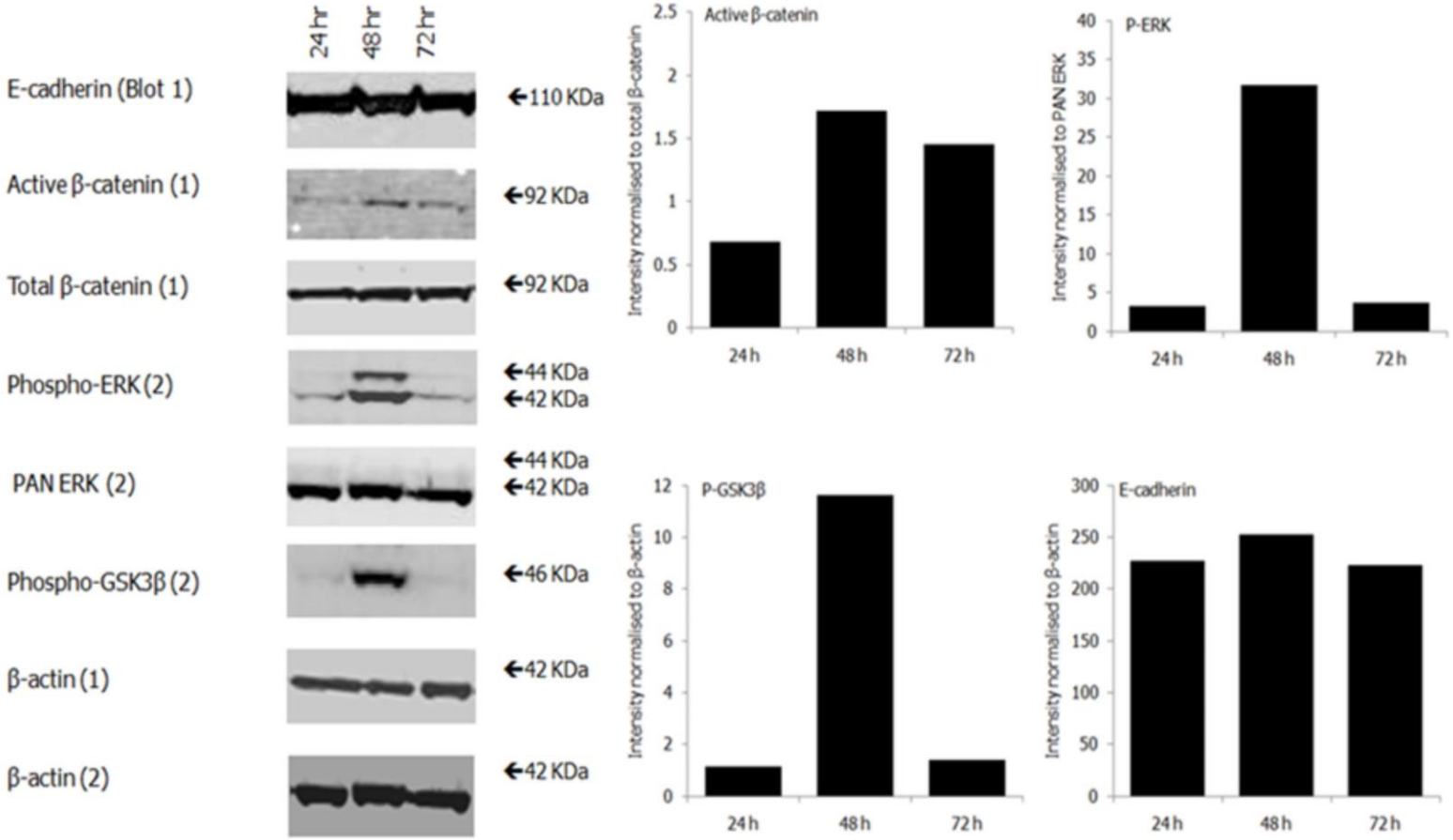
B) Expression of Wnt components in NHU cells grown in culture as a proliferative monolayer (p-NHU) and in fully-confluent, quiescent cultures (q-NHU) was assessed by RT-PCR (using primers the sequences of which are provided in Supplementary Table 1). Human genomic DNA (g-DNA) was used as a template control, whilst water only was used as a no-template negative control (-ve Control). GAPDH was used as a housekeeping control to verify intact cDNA. Reverse transcriptase (RT) negative samples (GAPDH RT-) were included to verify lack of genomic DNA contamination in the cDNA template.



Supplementary Figure 2

Expression and localisation of active β-catenin in NHU cells cultured without exogenous recombinant human EGF (rhEGF)

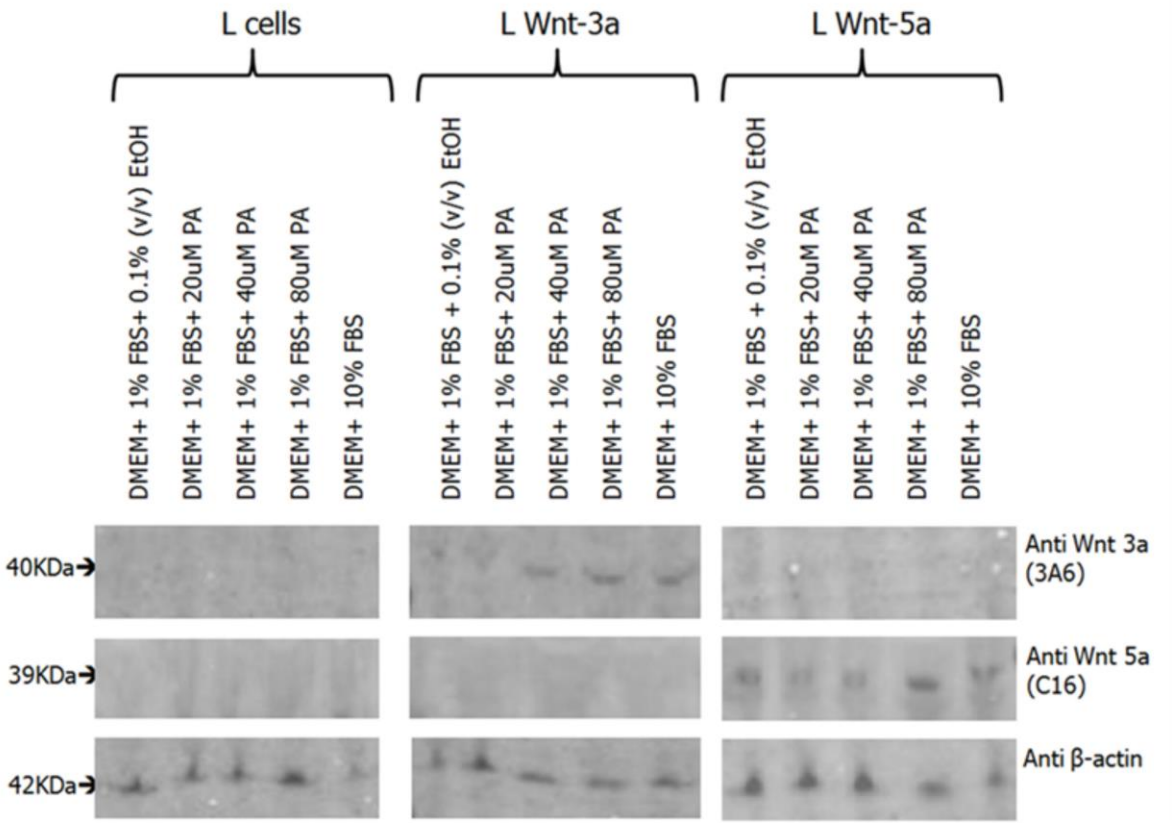
NHU cells were cultured for 24, 48 and 72 hours in KSFM medium supplemented with BPE but in the absence of rhEGF ligand (-Exogenous EGF). Cells were formalin-fixed and immunolabelled with active β-catenin antibody (8E7) and rabbit anti-mouse IgG Alexa 594 secondary antibody. Cells were also stained with Hoechst 33258 to delineate the nucleus. Labelling was visualised under epifluorescence illumination. Scale bar: 50μm.


24 hr
48 hr
72 hr
E-cadherin (Blot 1)
←110 KDa
Active β-catenin (1)
←92 KDa
Total β-catenin (1)
←92 KDa
Phospho-ERK (2)
←44 KDa
←42 KDa
PAN ERK (2)
←44 KDa
←42 KDa
Phospho-GSK3β (2)
←46 KDa
β-actin (1)
←42 KDa
β-actin (2)
←42 KDa
Active β-catenin
Intensity normalised to total β-catenin
2.5
2
1.5
1
0.5
0
24 h
48 h
72 h
P-ERK
Intensity normalised to PAN ERK
40
35
30
25
20
15
10
5
0
24 h
48 h
72 h
P-GSK3β
Intensity normalised to β-actin
12
10
8
6
4
2
0
24 h
48 h
72 h
E-cadherin
Intensity normalised to β-actin
300
250
200
150
100
50
0
24 h
48 h
72 h

Supplementary Figure 3

Expression of Wnt/β-catenin pathway components in NHU cells over a 72 hour time-course in physiological calcium conditions

NHU cells were cultured as described in Figure 5B, before whole cell lysates were prepared and expression of E-cadherin, β-catenin (Active and Total), ERK (Phospho- and Total), and Phospho-GSK3β (Serine 9) was assessed by Western blotting. Detection of β-actin served as a loading control. Results (blots shown on the left) are representative of experiments with two NHU lines. For the blots shown, densitometric analysis was also carried out to quantify β-catenin expression. Bars (on the right) represent relative band intensity of active β-catenin, phospho-ERK, phospho-GSK3β and E-cadherin after background subtraction and following normalisation to respective β-actin band intensity. The appropriate blot number ("1" or "2") and its corresponding β-actin expression are indicated.



Supplementary Figure 4

Assessing the effect of palmitic acid (PA) on Wnt ligand production

Whole cell lysates were prepared from L-Wnt3a, L-Wnt5a and L-Con cells cultured for 72 hours in medium (DMEM) containing 1% FBS (v/v) and supplemented with 20-80μM palmitic acid (PA). Positive and negative controls were 10% (v/v) FBS and 0.1%(v/v) EtOH (solvent only), respectively. 20μg of each protein lysate was subjected to SDS-PAGE within a 4-12% (w/v) Bis-Tris gel under denaturing conditions and immunoblotted onto PVDF membrane. Membranes were probed with anti-Wnt 3a (mouse 3A6; Santa Cruz), anti-Wnt 5a (goat C16; Santa Cruz) and β-actin (AC-15; Sigma) antibodies followed by goat anti-mouse IgG Alexa 680 for Wnt 3a and β-actin or donkey anti-goat IgG Alexa 680 for Wnt-5a. Antibody binding was visualised as described in the Methods section.