

ORIGINAL ARTICLE

Nuclear GSK-3 β inhibits the canonical Wnt signalling pathway in a β -catenin phosphorylation-independent manner

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β -Catenin is the central signalling molecule of the canonical Wnt pathway, where it activates target genes in a complex with lymphoid enhancer factor/T-cell factor transcription factors in the nucleus. The regulation of β -catenin activity is thought to occur via a cytoplasmic multiprotein complex that includes the serine/threonine kinase glycogen synthase kinase-3 β (GSK-3 β) that phosphorylates β -catenin, marking it for degradation by the proteasome. Here, we provide evidence showing that GSK-3 β has a nuclear function in downregulating the activity of β -catenin. Using colorectal cell lines that express a mutant form of β -catenin, which cannot be phosphorylated by GSK-3 β and ectopically expressed mutant β -catenin protein, we show that nuclear GSK-3 β functions in a mechanism that does not involve β -catenin phosphorylation to reduce the levels of Wnt signalling. We show that GSK-3 β enters the nucleus, forms a complex with β -catenin and lowers the levels of β -catenin/TCF-dependent transcription in a mechanism that involves GSK-3 β -Axin binding.

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Introduction

Wnt signalling is one of the key signalling pathways that controls cell proliferation, differentiation and morphogenesis during development and in adulthood (Giles *et al.*, 2003; Kikuchi *et al.*, 2006). Misregulation of the Wnt signalling pathway is a hallmark of many human cancers, including colon carcinoma and melanoma (Moon *et al.*, 2004). Wnt signalling regulates the stability of the intracellular protein β -catenin. In the absence of Wnt signalling, β -catenin resides in a complex with various

protein components, which include the scaffold protein Axin, the adenomatous polyposis coli (APC) tumour suppressor protein and the kinases, glycogen synthase kinase (GSK)-3 β and CKI (casein kinase I), which together comprise the destruction complex for β -catenin turnover. When this complex is assembled GSK-3 β phosphorylates β -catenin. Phosphorylated β -catenin is ubiquitinated, which results in its degradation by the proteasome (Giles *et al.*, 2003; Kikuchi *et al.*, 2006). Wnt signalling is initiated by the binding of Wnt family members to a receptor complex consisting of the Frizzled (Fz) family of transmembrane receptors, together with the co-receptors LRP (low-density lipoprotein receptor) 5/6. Wnt binding to its receptors leads to the rapid inactivation of the β -catenin degradation complex. As a result, β -catenin is no longer degraded, but accumulates and translocates into the nucleus. In the nucleus, β -catenin functions as a transcription co-factor of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family and leads to the activation of Wnt target genes. Aberrant activation of Wnt target genes is believed to be the basis for tumorigenesis (Giles *et al.*, 2003; Kikuchi *et al.*, 2006). The serine/threonine kinase GSK-3 β has been shown to be involved in several signalling pathways and to regulate several physiological responses, including protein synthesis, gene expression, subcellular localization of proteins and protein degradation (Cohen and Frame, 2001; Doble and Woodgett, 2003). As mentioned above, GSK-3 β plays an essential role in the canonical Wnt signal-transduction pathway, initiating proteasomal degradation of β -catenin by phosphorylating β -catenin on key residues.

Here, we show that GSK-3 β has an additional subcellular-dependent role in controlling the Wnt signalling levels. Our findings demonstrate that nuclear GSK-3 β controls β -catenin in a mechanism that does not involve β -catenin phosphorylation. Thus, although GSK-3 β serves as part of the cytoplasmic β -catenin degradation complex, our data suggest that GSK-3 β has an additional function in the nucleus to reduce the Wnt signalling levels.

Results

GSK-3 β represses the canonical Wnt signalling pathway in a β -catenin phosphorylation-independent manner
Inhibition of GSK-3 β , which occurs upon Wnt signalling, can be mimicked by treating cells with LiCl, an

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inhibitor of GSK-3 β (Stambolic *et al.*, 1996; Hedgepeth *et al.*, 1997), or by using other specific GSK-3 β inhibitors (Meijer *et al.*, 2004). Treating mammalian cells with LiCl leads to accumulation of non-phosphorylated β -catenin and as a result to increased levels of TCF-mediated transcription. However, this increased TCF-mediated transcription, induced by LiCl, is far greater than that produced by treatment with Wnt1, Wnt-3A or by ectopic expression of a constitutively active form of β -catenin (Staal *et al.*, 2002; Zilberberg *et al.*, 2004). This raises the possibility that GSK-3 β has an additional, yet unknown negative role in the Wnt signalling cascade.

To investigate this role of GSK-3 β , the human colorectal cancer (CRC) cell lines DLD-1, CX-1 and LS174T were used. In these cell lines, β -catenin cannot be phosphorylated by GSK-3 β due to an APC mutation (DLD-1 and CX-1) (Rowan *et al.*, 2000) or an β -catenin-stabilizing mutation (LS174T) (Yang *et al.*, 2005). These cell lines can be easily transfected and are commonly used for reporter assays. First, to confirm the notion that GSK-3 β does not phosphorylate β -catenin in these cell lines, an antibody that detects phosphorylated β -catenin was used. The results presented in Figure 1a show that these CRC cell lines do not express phosphorylated β -catenin, and moreover, ectopically expressing GSK-3 β does not lead to the expression of phosphorylated β -catenin in these cells. In comparison, our control cells (HEK293T) express phosphorylated β -catenin, which increases after the expression of GSK-3 β . Next, these CRC cell lines were transfected with pTOPFLASH—a reporter plasmid widely used for measuring transcriptional activity of TCF and β -catenin (Korinek *et al.*, 1997) and treated with LiCl to inhibit the activity of GSK-3 β . Results (Figure 1b) show that the LiCl treatment increased the levels of TCF-mediated transcription in CX-1, DLD-1 and LS174T cells as compared to untreated cells. To confirm the specificity of the TCF/ β -catenin activity, the cells were also transfected with pFOPFLASH (whose TCF-binding sites are mutated). All FOPFLASH values were low, demonstrating the specificity of the TCF/ β -catenin input (Figure 1b). Similarly, DLD-1 and LS174T cells were transfected with pTOPFLASH and treated with a

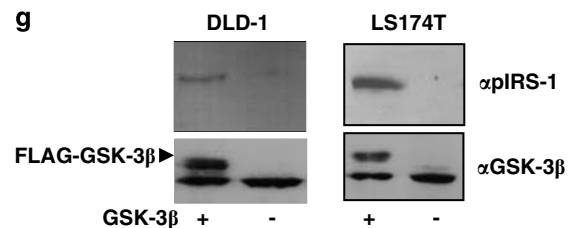
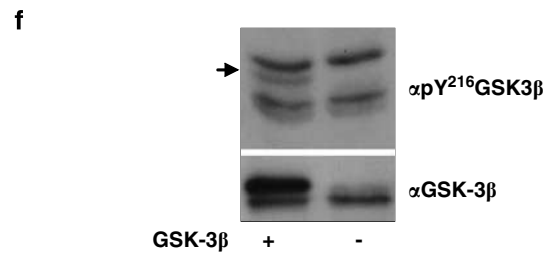
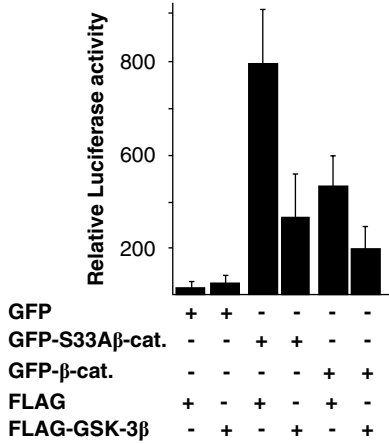
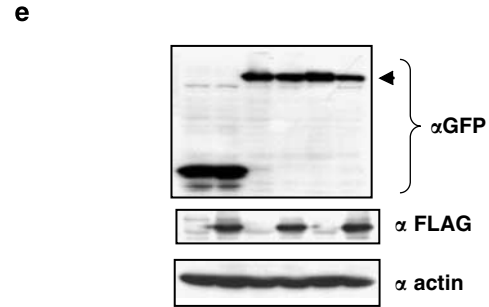
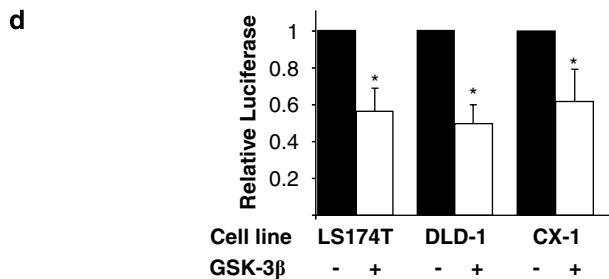
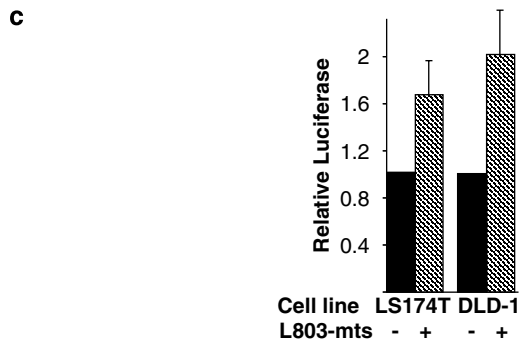
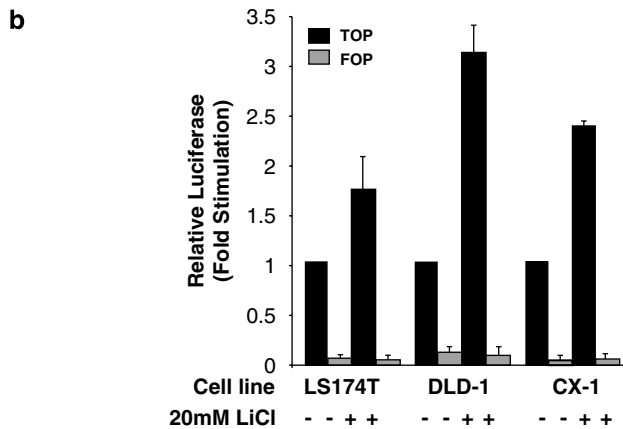
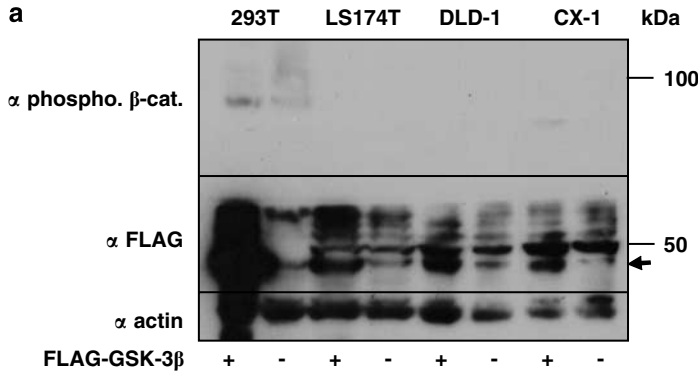
specific GSK-3 β inhibitor—L803-mts (Plotkin *et al.*, 2003). As with LiCl, treatment with L803-mts led to an increased TCF/ β -catenin activity (Figure 1c). As GSK-3 β cannot phosphorylate β -catenin in these cell lines, these results suggest that GSK-3 β affects Wnt signalling independently of its ability to phosphorylate β -catenin. To further address this point, GSK-3 β was over-expressed in the CRC cell lines, and the extent of TCF-mediated transcription was measured using the TOPFLASH assay. As can be seen from Figure 1d, ectopically expressed GSK-3 β led to a decrease in the levels of TCF-mediated transcription in the CRC cell lines. Similarly, as shown in Figure 1e, GSK-3 β can reduce the transcription activity of a stable ectopically expressed β -catenin protein containing a missense mutation of alanin for serine at codon 33 (S33A), which is a critical GSK-3 β phosphorylation site. This effect of GSK-3 β does not result from a decrease in the protein levels of stabilized β -catenin as in the case of wild-type β -catenin (arrowhead, Figure 1e). In our experiments, the activity of LiCl was more robust than that of L803-mts. This may result from the fact that although L803-mts is a substrate-specific, inhibitor of GSK-3 β , shown to selectively block GSK-3 β activity without affecting the activity of other kinases (Plotkin *et al.*, 2003), LiCl inhibits GSK-3 β , GSK-3 α (Jean-Martin *et al.*, 2004) and other kinases. To confirm that GSK-3 β is functional in CRC cells, we have used the anti-phosph-GSK (Tyr²¹⁶), which labels active GSK-3 β (Hughes *et al.*, 1993; Cole *et al.*, 2004) (Figure 1f). We further examined the phosphorylation of IRS-1 (insulin receptor substrate-1), which is a GSK-3 β target (Lieberman and Eldar-Finkelman, 2005). Enhanced IRS-1 phosphorylation at serine 332 confirmed that the ectopic GSK-3 β was active (Figure 1g). Recently it has been shown that there is a functional redundancy of GSK-3 α and GSK-3 β in Wnt/ β -catenin signalling (Doble *et al.*, 2007). Thus, it is conceivable that the effect of LiCl seen in our experiments is an accumulative effect of LiCl on GSK-3 β , GSK-3 α and other kinases. Taken together, these results indicate that in addition to regulating the levels of β -catenin by phosphorylation, GSK-3 β can regulate the levels of signalling active β -catenin by a β -catenin phosphorylation-independent mechanism.

Figure 1 GSK-3 β inhibits the canonical Wnt signalling pathway in CRC cell lines. (a) HEK293T, LS174T, DLD-1 and CX-1 cells were transfected with pCMV-FLAG-GSK-3 β or pCMV-FLAG as a control. Forty-eight hours later, the cells were harvested and analysed by western blotting using anti-phospho- β -catenin, anti-FLAG and anti-actin antibodies. The specific FLAG band is indicated by an arrow. Note that the FLAG antibody detects some unspecific proteins, especially in the CRC cells. (b) The pTOPFLASH or pFOPFLASH reporter plasmids were co-transfected into LS174T, DLD-1 and CX-1 cells with pRL-CMV (0.1 μ g) that served to normalize for transfection efficiency. Twenty-four hours later, the cells were treated with 20 mM LiCl over night (ON) as indicated. The cells were lysed and luciferase levels were measured. Data are presented as mean values and s.d. for at least three independent experiments performed in duplicate. (c) LS174T and DLD-1 were transfected as described in (a). Twenty-four hours post-transfection, cells were treated ON with 40 μ M L803-mts. Luciferase levels were measured as described above. (d) LS174T, DLD-1 and CX-1 cells were transfected as described in (a) together with a control or a GSK-3 β -expressing plasmid. Luciferase levels were measured as described above. (e) HEK293T cells were co-transfected with pTOPFLASH, pRL-CMV as described above and pCMV-FLAG-GSK-3 β or pEGFP- β -catenin or pEGFP-S33A β -catenin. Forty-eight hours later, cells were lysed and subjected to western blot analysis (upper panel) using anti-GFP, anti-FLAG and anti-actin antibodies or were measured for the levels of luciferase and β -Gal activities as described. (f) COLO320 cells were transfected with FLAG-GSK-3 β . Forty-eight hours later, cells were lysed and subjected to western blot analysis using anti-phosph-GSK3 (Tyr²¹⁶) or anti-GSK-3 β as indicated. Arrowhead points to phosphorylated GSK-3 β . (g) DLD-1 and LS174T cells were transfected with FLAG-GSK-3 β . Forty-eight hours later, cells were lysed and subjected to western blot analysis using anti-phosph-IRS-1 or anti-GSK-3 β antibodies as indicated. * P <0.05 treated vs control. CRC, colorectal cancer; GFP, green fluorescent protein; GSK-3 β , glycogen synthase kinase-3 β .

Inhibition of GSK-3 β does not decrease the levels of E-cadherin in CRC cells

Recently, it has been shown that inhibition of GSK-3 β altered the assembly of adherens junctions by upregulating the transcriptional repressor Snail, which decreases E-cadherin expression (Zhou *et al.*, 2004; Yook *et al.*, 2005). This process was suggested to facilitate the

intracellular transfer of E-cadherin-bound β -catenin to a pool of free cytosolic/nuclear β -catenin, which is able to activate Wnt signalling (Bachelder *et al.*, 2005). Thus, the inhibition of GSK-3 β may increase the levels of β -catenin/TCF-mediated transcription in a β -catenin phosphorylation-independent mechanism via alterations of the E-cadherin levels. To explore this possibility,



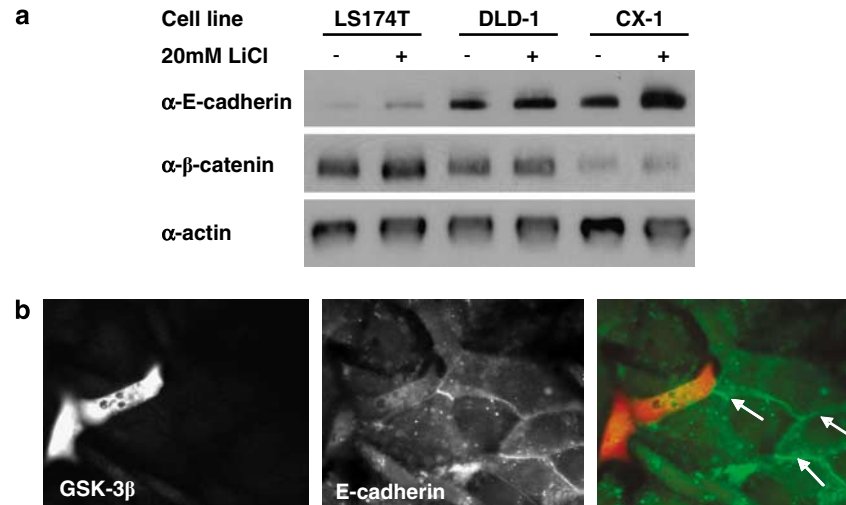


Figure 2 GSK-3 β expression does not change the levels of E-cadherin in CRC cells. (a) LS174T, DLD-1 and CX-1 cells were treated ON (overnight) with 20mM LiCl as indicated. Cell lysates (100 μ g) were analysed by western blotting for the level of expression of E-cadherin, β -catenin or actin using specific antibodies. (b) The levels of E-cadherin (indicated by the arrows) were detected by immunofluorescence in semiconfluent (1×10^4 cells cm^{-2}) SW480 cells overexpressing GSK-3 β . CRC, colorectal cancer; GSK-3 β , glycogen synthase kinase-3 β .

CRC cells were treated with LiCl and the levels of endogenous E-cadherin and β -catenin were determined. The data presented in Figure 2a show that the LiCl treatment did not decrease the levels of E-cadherin or β -catenin in the CRC cell lines tested (LS174T, DLD-1 and CX-1) and unexpectedly led to a slight increase in E-cadherin levels. As E-cadherin has a potentially long half-life, cells were treated with LiCl for 72–96 h however the results did not show any differences in E-cadherin levels.

In the next step, immunofluorescence microscopy was used to examine possible changes in the levels of E-cadherin in the human colon carcinoma cell line SW480, which similarly to CX-1 and DLD-1 cells express a mutant APC protein. SW480 cells grown in sparse culture are characterized by weak staining of E-cadherin. In contrast, cells grown as dense cultures displayed a more intense E-cadherin staining confined to cell–cell contacts (Conacci-Sorrell *et al.*, 2003). FLAG-tagged GSK-3 β was expressed in semiconfluent SW480 cells and its impact on E-cadherin levels was examined by indirect immunofluorescence. Overexpression of GSK-3 β did not lead to increased levels of E-cadherin in adjacent SW480 cells (Figure 2b). Arrows in Figure 2b point to high levels of junctional E-cadherin in cells not overexpressing GSK-3 β . Together, the results suggest that changes in the levels of GSK-3 β expression do not alter the levels of E-cadherin in the CRC tested cells, implying that inhibition of Wnt signalling in CRC cells by GSK-3 β is not mediated through changes in E-cadherin levels.

GSK-3 β and β -catenin form a nuclear complex that does not lead to β -catenin degradation

Recent studies have indicated that the localization of GSK-3 β is dynamically regulated. Although GSK-3 β is

found predominantly in the cytoplasm of most cells, it has also been found in the nucleus where it regulates the subcellular distribution and function of a number of proteins. As β -catenin functions in the nucleus, we examined whether GSK-3 β and β -catenin interact in the nucleus. In these experiments, the COLO320 CRC cell line was used. Unlike SW480, COLO320 cells do not express E-cadherin (Langford *et al.*, 2006) and have high levels of β -catenin. Our results using nuclear and cytoplasmic extracts of COLO320 cells show that endogenous β -catenin can co-immunoprecipitate with endogenous GSK-3 β in both the cytoplasmic and the nuclear fractions (Figure 3a). As COLO320 cells lack a functional APC protein, the β -catenin–GSK-3 β interaction does not lead to cytoplasmic degradation of β -catenin (Figure 3b). Similarly, in the nuclear fraction, there was no detectable degradation of β -catenin (Figure 3b). To further confirm the nuclear interaction between GSK-3 β and β -catenin, and to examine whether the nuclear GSK-3 β – β -catenin complex exists also in cells where the Wnt signal is switched off, nuclear fractions from HEK293T cells transfected with FLAG-GSK-3 β (Figure 3c) or with FLAG- Δ 45- β -catenin (Figure 3d) were immunoprecipitated with an anti-FLAG antibody and blotted with anti- β -catenin (Figure 3c) or anti-GSK-3 β (Figure 3d) antibodies. As can be seen in Figures 3c and d, FLAG-GSK-3 β or FLAG- β -catenin co-immunoprecipitated with endogenous β -catenin or with endogenous GSK-3 β , respectively. These results indicate that GSK-3 β can associate with β -catenin in the nucleus. As HEK293T cells contain an intact APC protein and a functional β -catenin degradation complex, the cytoplasmic association of FLAG-GSK-3 β and endogenous β -catenin resulted in β -catenin degradation (Figure 3e). Interestingly, the nuclear GSK-3 β – β -catenin interaction did not lead to β -catenin degradation (Figure 3e).

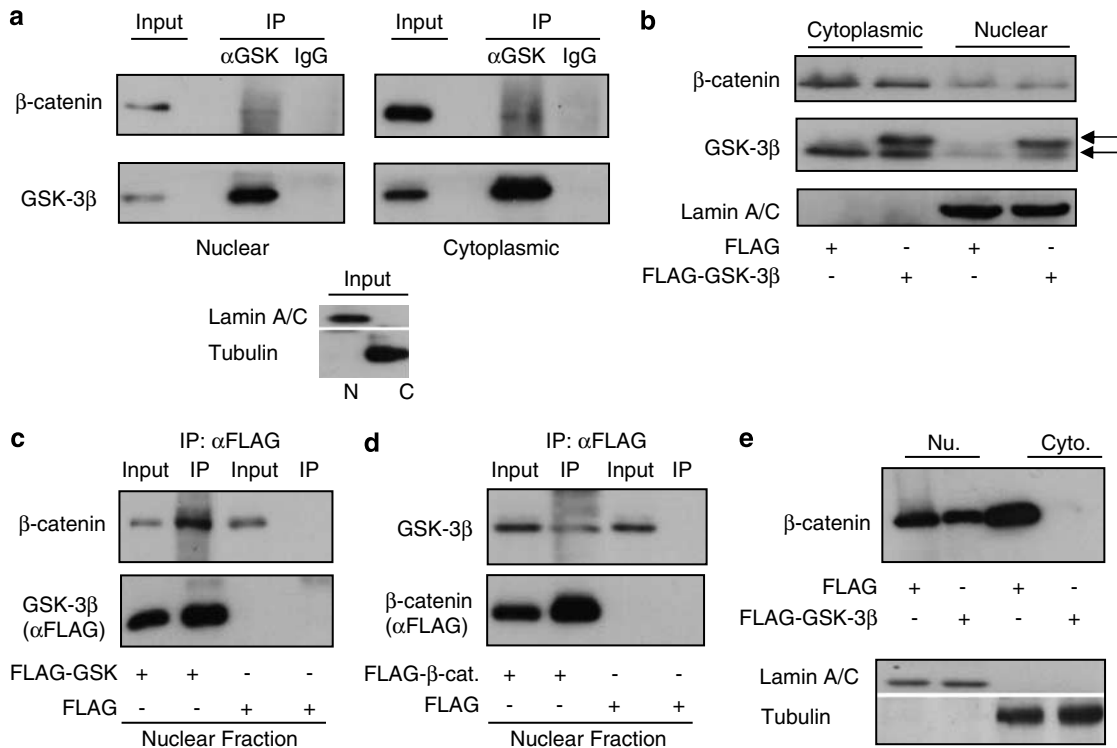


Figure 3 GSK-3 β and β -catenin form a complex in the nucleus. **(a)** Nuclear and cytoplasmic fraction of COLO320 cells were immunoprecipitated with anti-GSK-3 β or with normal rabbit serum (IgG) as a control. Western blot was performed using anti- β -catenin and anti-GSK-3 β -specific antibodies. Lamin A/C and tubulin served as markers for nuclear (N) and cytoplasmic (C) fractions, respectively. **(b)** COLO320 cells were transfected with FLAG-GSK-3 β or the control vector and were fractionated into nuclear and cytoplasmic fractions 48 h later. The levels of endogenous β -catenin were detected by western blot with anti- β -catenin-specific antibodies. Ectopically expressed GSK-3 β was detected using an anti-GSK-3 β antibody. Endogenous GSK-3 β is shown by an arrow and exogenous by an arrowhead. **(c and d)** HEK293T cells were transfected with FLAG-GSK-3 β **(c)**, FLAG- Δ 45- β -catenin **(d)** or empty FLAG vector **(c and d)**. Nuclear fractions were prepared 48 h post-transfection. Immunoprecipitation was performed using anti-FLAG agarose beads. Western blot of lysates containing FLAG-GSK-3 β or FLAG vector was performed with anti- β -catenin and anti-FLAG-specific antibodies. Lysates containing FLAG- Δ 45- β -catenin or FLAG vector were blotted with anti-GSK-3 β and anti-FLAG antibodies **(d)**. **(e)** Nuclear and cytoplasmic fractions of HEK293T cells transfected with FLAG-GSK-3 β were prepared as described in **(c)**. Western blot was performed using anti- β -catenin antibodies. CRC, colorectal cancer; GSK-3 β , glycogen synthase kinase-3 β ; IP, immunoprecipitation.

Nuclear GSK-3 β reduces the levels of β -catenin/ TCF-mediated transcription

To further examine the finding that GSK-3 β associates with β -catenin in the nucleus, GSK-3 β was targeted or excluded from the nucleus by linking it to a synthetic NLS (nuclear localization sequence) or NES (nuclear export sequence), respectively. HEK293T cells were co-transfected with pTOPFLASH, GSK-3 β , NLS-GSK-3 β , NES-GSK-3 β and β -catenin as indicated (Figure 4). Results show that expression of GSK-3 β or NES-GSK-3 β reduces both the protein levels of β -catenin and the TCF-mediated transcription (Figure 4a, lanes 4 and 8). In contrast, although nuclear targeting of GSK-3 β (NLS-GSK-3 β) resulted in only a moderate decrease in the β -catenin protein levels (lane 6), it led to a similar decrease in the levels of TCF-induced transcription. These results suggest that nuclear GSK-3 β can reduce the levels of TCF-mediated transcription without leading to degradation of β -catenin as in the case of cytoplasmic GSK-3 β .

Nuclear GSK-3 β does not alter the subcellular distribution of β -catenin

Our results raised the possibility that nuclear GSK-3 β may affect the subcellular distribution of β -catenin and subsequently the levels of Wnt signalling. This possibility was examined by using leptomycin B (LMB), a highly specific inhibitor of the protein nuclear export receptor CRM1 (Fukuda *et al.*, 1997). For these experiments, DLD-1 cells, which are relatively large, have a flat morphology and express high levels of overexpressed proteins, were used. DLD-1 cells ectopically expressing FLAG-GSK-3 β were treated with LMB, and the subcellular distribution of GSK-3 β and β -catenin was detected by indirect immunofluorescence. As expected from previous studies showing that GSK-3 β can enter and exit the nucleus, the LMB treatment led to nuclear accumulation of GSK-3 β . Importantly, this nuclear accumulation of GSK-3 β did not alter the subcellular distribution of β -catenin (Figure 5a). Next, the levels of TCF-mediated transcription were measured

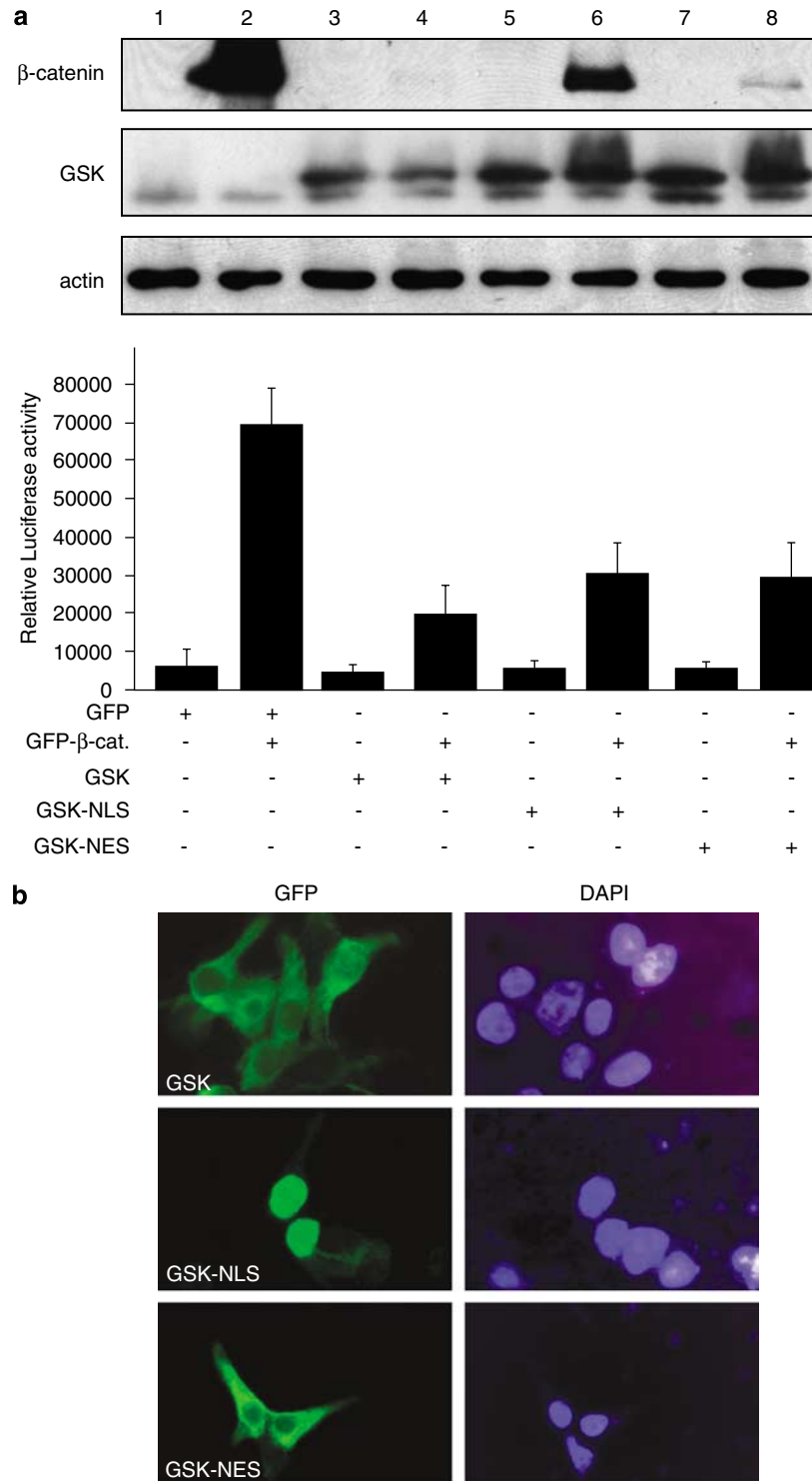


Figure 4 The effect of nuclear GSK3 on β -catenin. HEK293T cells were co-transfected with pEGFP-GSK-3 β , pEGFP-NLS-GSK-3 β , pEGFP-NES-GSK-3 β or pEGFP- β -catenin as indicated. **(a)** Forty-eight hours later, the cells were harvested and subjected to western blot analysis using anti-GFP, anti-GSK-3 β and anti-actin antibodies. For luciferase assay, the pTOPFLASH reporter and β -Gal plasmids were added to the transfection. After 48 h, the cells were lysed and luciferase levels were measured as described. **(b)** Forty-eight hours post-transfection, the cells were fixed and stained with DAPI. DAPI, 4'-6-diamidino-2-phenylindole; GFP, green fluorescent protein; GSK-3 β , glycogen synthase kinase-3 β .

in DLD-1 cells overexpressing GSK-3 β that were treated with LMB. Results show that the LMB treatment did not change the levels of TCF-mediated transcription

in control cells, but did lead to reduced levels of TCF-mediated transcription in GSK-3 β -expressing cell (Figure 5b, lanes 3 and 4). These results suggest that

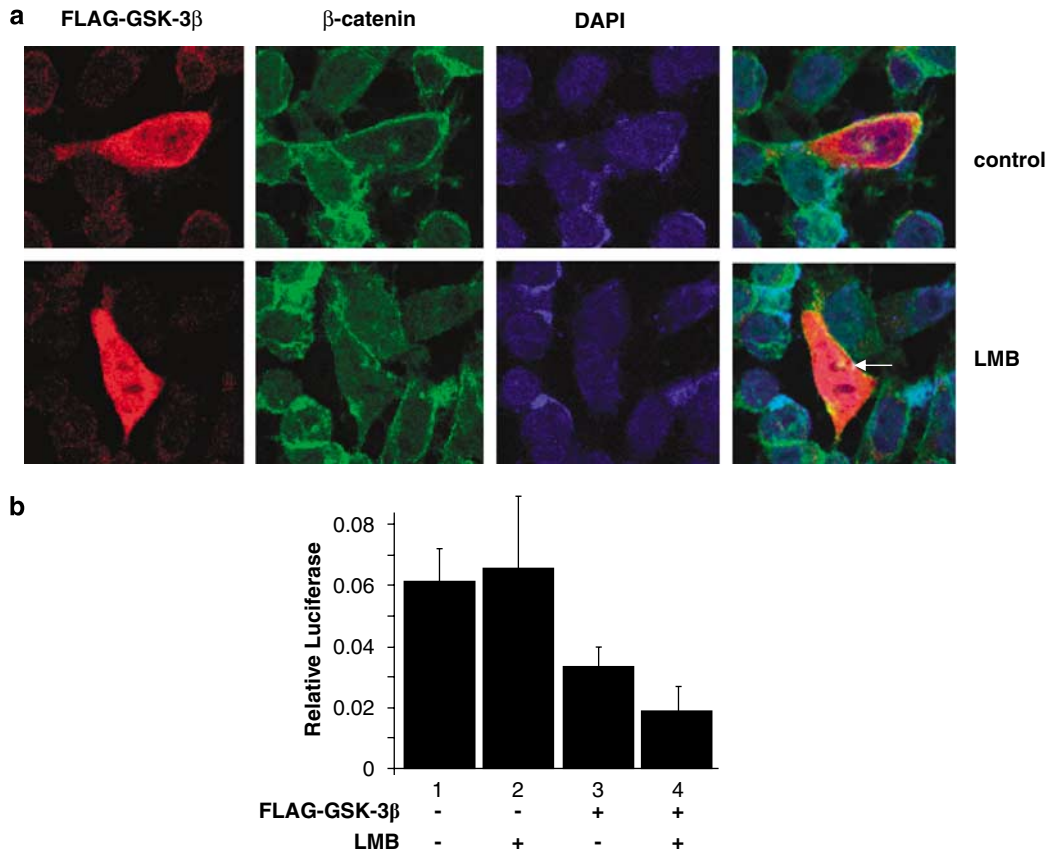


Figure 5 Nuclear GSK-3 β and β -catenin. (a) FLAG-GSK-3 β was transfected into DLD-1 cells. Forty-eight hours post transfection, the cells were mock-treated or treated with 5 ng LMB for 5 h. The subcellular distribution of exogenous GSK-3 β and endogenous β -catenin were detected using an anti-FLAG or anti- β -catenin antibody, respectively. DAPI was used to mark the nucleus. (b) DLD-1 cells were transfected with the pTOPFLASH reporter plasmid as described in 1a. The cells were co-transfected with a control or a GSK-3 β -expressing plasmid and treated with LMB as indicated. DAPI, 4',6'-diamidino-2-phenylindole; GSK-3 β , glycogen synthase kinase-3 β ; LMB, leptomycin B.

nuclear GSK-3 β can affect the β -catenin/TCF-mediated transcription levels without altering the subcellular distribution of β -catenin.

GSK-3 β does not affect β -catenin/TCF-4 complex formation

Transcription of Wnt target genes is dependent on β -catenin/TCF interaction. To investigate whether nuclear GSK-3 β interferes with the ability of β -catenin to interact with TCF-4, HEK293T cells were co-transfected with a FLAG- Δ 45- β -catenin, HA-TCF4 and either HA-GSK-3 β or empty vector. Data showed that the same amounts of HA-TCF4 co-immunoprecipitated with FLAG- β -catenin in the presence or absence of GSK-3 β (Figure 6). Hence, GSK-3 β does not interfere with TCF- β -catenin complex formation. Next, we investigated whether GSK-3 β would affect the binding of TCF- β -catenin complex to target DNA. Nuclear extracts were prepared from HEK293T cells, co-transfected with a FLAG- Δ 45- β -catenin, HA-TCF4 and either HA-GSK-3 β or empty vector. EMSA (electrophoretic mobility shift assays) experiments showed that the ectopically expressed GSK-3 β did not reduce the formation of TCF 4- β -catenin-containing

complexes at the TCF DNA-binding sites (data not shown). These data show that the GSK-3 β -induced repression is not due to an effect on TCF- β -catenin complex formation or from an inhibitory effect of GSK-3 β on TCF DNA-binding activity.

The function of GSK-3 β in reducing Wnt signalling levels in CRC cells is Axin-binding dependent

Axin, a member of the β -catenin degradation complex, is phosphorylated by GSK-3 β (Yamamoto *et al.*, 1999). Axin shuttles in and out of the nucleus (Cong and Varmus, 2004; Wiechens *et al.*, 2004), a function that may regulate the nuclear export of β -catenin. Recently, it has been shown that Axin2 acts as a nucleocytoplasmic chaperone for GSK-3 β (Yook *et al.*, 2006). To test whether the GSK-3 β -Axin interaction is important for the ability of GSK-3 β to reduce the levels of TCF-mediated transcription activity in a β -catenin degradation-independent mechanism, the GSK-3 β K85R and GSK-3GR (a generous gift from Dr TC Dale) mutants were used. The GSK-3 β K85R is a kinase dead form of GSK-3 β that can bind Axin, whereas GSK-3GR is kinase active form but cannot bind Axin (Franca-Koh *et al.*, 2002). LS174T cells were transfected with

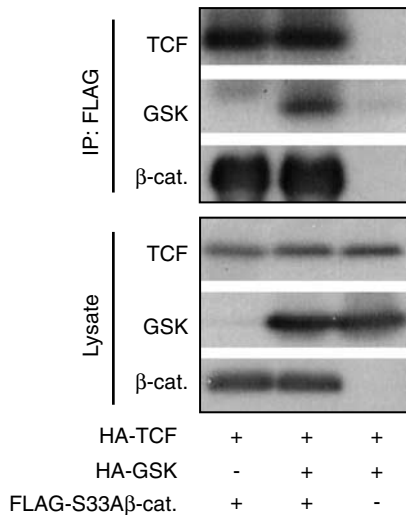


Figure 6 GSK-3 β does not interfere with the β -catenin–TCF complex. HEK293T cells were co-transfected with pCDNA3-HA-TCF4, FLAG- Δ 45- β -catenin and pCDNA-HA-GSK-3 β . The cells were harvested 48 h post-transfection and immunoprecipitation was performed using anti-FLAG agarose beads. Extracts and IP complexes were analysed by western blot analysis using anti-HA (hemagglutinin) and anti- β -catenin antibodies. GSK-3 β , glycogen synthase kinase-3 β ; TCF, T-cell factor.

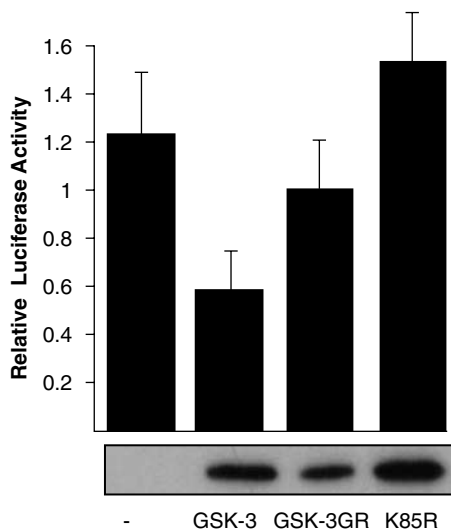


Figure 7 Axin binding and kinase activity are both required for the function of GSK-3 β in CRC cells. LS174T cells were co-transfected with pCDNA-HA plasmids containing WT GSK-3 β , mutated GSK-3 β -GR or K85R and pTOPFLASH reporter plasmid (pRL-CMV was used to normalize transfection efficiency). Forty-eight hours later, the cells were lysed and luciferase levels were measured as described. Bottom panel shows protein levels of the expressed proteins. GSK-3 β , glycogen synthase kinase-3 β ; TCF, T-cell factor.

HA-GSK-3 β , HA-GSK-3 β K85R and HA-GSK-3GR, and the levels of TCF-mediated transcription were measured. Results show that the inhibitory function of GSK-3 β is impaired when it can no longer bind Axin or when its phosphorylation activity is abolished (Figure 7). These results indicate that the ability of GSK-3 β to reduce the TCF-induced transcription levels without leading to

β -catenin degradation depends both on the kinase activity of GSK-3 β and the ability of GSK-3 β to bind Axin.

Discussion

GSK-3 β is a multifunctional serine/threonine kinase, being an important regulator of various signal-transduction pathways including the Wnt pathway (Patel *et al.*, 2004). As a central negative regulator of the Wnt pathway, GSK-3 β phosphorylates β -catenin on key residues marking it for ubiquitination and subsequent proteasomal degradation (Orford *et al.*, 1997). Here, we provide evidence showing that in addition to this established role, GSK-3 β can inhibit the Wnt pathway in a mechanism that does not involve β -catenin phosphorylation and degradation. We show that treatment with LiCl, a well-established inhibitor of GSK-3 β , increases the rate of Wnt signalling without affecting the phosphorylation levels of β -catenin. Our results demonstrate that GSK-3 β can inhibit the levels of TCF-mediated transcription in both CRC cell lines that lack an intact β -catenin degradation complex and in CRC cell lines that express a β -catenin protein that cannot be phosphorylated. These results suggest that GSK-3 β can inhibit Wnt signalling independently to its activity on β -catenin phosphorylation. It has been shown that expression of GSK-3 β can change the levels of E-cadherin, which may result in decreasing the pool of free β -catenin that is able to activate Wnt signalling. Thus, the possibility that GSK-3 β alters the levels of E-cadherin was investigated. As both inhibition and overexpression of GSK-3 β did not lead to a decrease in E-cadherin levels, this possibility was ruled out. Interestingly, the LiCl treatment led to a slight increase in the E-cadherin levels. This may result from the activity of lithium on other pathways, such as, triggering Akt inactivation which regulates E-cadherin expression.

Although GSK-3 β is generally considered to be a cytoplasmic protein, several lines of evidence suggest that GSK-3 β also functions in the nucleus and that the subcellular localization of GSK-3 β is a highly dynamic process: GSK-3 β was found to enter the nucleus during S phase of the cell cycle and during apoptosis (Diehl *et al.*, 1998; Bijur and Jope, 2001) and to exit the nucleus of cardiac myocytes upon isoproterenol stimulation (Morisco *et al.*, 2001). Nuclear GSK-3 β also plays a role in controlling the nuclear/cytoplasmic distribution of several proteins such as cyclin D1, STAT, GATA-4 c-myc, NRF2, Snail and p53 (Diehl *et al.*, 1998; Ginger *et al.*, 2000; Morisco *et al.*, 2001; Watcharasit *et al.*, 2002; Gregory *et al.*, 2003; Linseman *et al.*, 2004; Yook *et al.*, 2005; Salazar *et al.*, 2006). Importantly, FRAT/GBP, a positive regulator of β -catenin, was reported to actively export GSK-3 β from the nucleus (Franca-Koh *et al.*, 2002), and recent data show that Axin2 acts as a nucleocytoplasmic chaperone for GSK-3 β (Yook *et al.*, 2006), indicating a nuclear function for GSK-3 β in the regulation of Wnt signalling.

Although nuclear GSK-3 β appears to have many different functions, its effects on β -catenin and Wnt

signalling have not been investigated so far. Here, we provide evidence showing that GSK-3 β forms nuclear complexes with β -catenin. These nuclear complexes lead to reduced TCF-mediated transcription but do not result in β -catenin degradation. Although we show that GSK-3 β can partially accumulate in the nucleus, in response to LMB treatment, this does not affect the subcellular distribution of β -catenin but, rather, results in an inhibition of β -catenin-dependent Wnt target gene expression. Together these results suggest that in addition to its known cytoplasmic function in keeping the cytoplasmic levels of β -catenin low, GSK-3 β may have a nuclear function that impairs the Wnt pathway without affecting the protein levels or the subcellular distribution of β -catenin.

In the nucleus, β -catenin forms complexes with members of the TCF family of proteins to transactivate Wnt target genes, and we speculated that GSK-3 β might affect this process. However, our data rule out the possibility that GSK-3 β interferes with the nuclear β -catenin/TCF4 complex or has an effect on TCF DNA-binding activity.

In the cytoplasm, both GSK-3 β and Axin are components of the β -catenin degradation complex where GSK-3 β directly interacts and phosphorylates Axin (Yamamoto *et al.*, 1999). Furthermore, both Axin and GSK-3 β are expressed and function in the nucleus, and it is thus conceivable that they interact in the nucleus. GSK-3 β has numerous nuclear substrates and Axin may be one of them. In the nucleus, GSK-3 β may activate Axin by phosphorylation which in turn can result in reduced β -catenin activity. It is important to emphasize that this function of GSK-3 β may result from the ability of GSK-3 β to phosphorylate a yet unknown substrate that in turn inhibits the Wnt pathway. Supporting this notion is our data showing that GSK-3 β needs to possess both its Axin-binding ability and its kinase ability to reduce the levels of TCF-mediated transcription in CRC cells that express a stabilized β -catenin protein.

Although several components of the β -catenin destruction complex such as Axin, APC and Dsh have been shown to enter the nucleus and regulate the subcellular localization and function of β -catenin (Henderson, 2000; Rosin-Arbesfeld *et al.*, 2000; Cong and Varmus, 2004; Wiechens *et al.*, 2004; Itoh *et al.*, 2005), a correlation between nuclear GSK-3 β and Wnt signalling has not been demonstrated so far. The current models of Wnt pathway regulation suggest that β -catenin, APC, Axin and Dsh can dynamically shuttle between the cytoplasm and the nucleus, while the whole β -catenin degradation complex is stably anchored in the cytoplasm, and β -catenin degradation occurs in the cytoplasm. The fact that GSK-3 β can enter the nucleus

and regulate the levels of Wnt signalling prompts to re-evaluate our current view of the pathway. Here, we raise the possibility that nuclear GSK-3 β binds to nuclear β -catenin in an Axin-binding-dependent manner. This nuclear complex that exists in both wild-type and CRC cells is compromised in CRC cells due to mutations in different Wnt signalling members. This nuclear interaction could be a safeguard function of GSK-3 β , operating in parallel to its other cytoplasmic functions in phosphorylating and targeting β -catenin for degradation. Another possibility could be that GSK-3 β phosphorylates APC and Axin in the nucleus to enhance their nuclear export similar to the effect of GSK-3 β on proteins such as NRF2, STAT and Snail. Moreover, as GSK-3 β , Axin and APC all have a nuclear function in regulating the Wnt signal, it is conceivable that the β -catenin degradation complex is assembled in the nucleus. These mechanisms could minimize β -catenin signalling activity in cells that do not receive a Wnt signal or could quickly downregulate β -catenin signalling activity when the Wnt signal is withdrawn.

Materials and methods

Complete details are given in Supplementary Materials.

Plasmids and reagents

Complete details of all plasmids can be obtained in the Supplementary Materials.

Cell cultures, transfections and luciferase reporter assays

HEK293T and CRC cell lines (LS174T, DLD-1, CX-1, SW480 and COLO320) were used. To assay TCF-mediated transcription, the pFOPFLASH/pTOPFLASH reporters were used.

Nuclear extracts, immunoprecipitation, immunofluorescence and western blot analysis

Complete details are given in Supplementary Materials.

Abbreviations

CRC, colorectal cancer; GSK-3 β , glycogen synthase kinase-3 β ; TCF/LEF, T-cell factor/lymphoid enhancer factor.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).