# Nucleo-cytoplasmic distribution of β-catenin is regulated by retention

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## Summary

**β-catenin is the central signalling molecule of the canonical Wnt pathway, where it activates target genes in a complex with LEF/TCF transcription factors in the nucleus. The regulation of β-catenin activity is thought to occur mainly on the level of protein degradation, but it has been suggested that β-catenin nuclear localization and hence its transcriptional activity may additionally be regulated via nuclear import by TCF4 and BCL9 and via nuclear export by APC and axin. Using live-cell microscopy and fluorescence recovery after photobleaching (FRAP), we have directly analysed the impact of these factors on the subcellular localization of β-catenin, its nucleo-cytoplasmic shuttling and its mobility within the nucleus and the cytoplasm. We show that TCF4 and BCL9/Pygopus recruit β-catenin to the nucleus, and APC, axin and axin2 enrich β-catenin in the cytoplasm. Importantly, however, none of these factors accelerates the nucleo-cytoplasmic shuttling of β-catenin, i.e. increases the rate of β-catenin nuclear import or export. Moreover, the cytoplasmic enrichment of β-catenin by APC and axin is not abolished by inhibition of CRM-1-dependent nuclear export. TCF4, APC, axin and axin2 move more slowly than β-catenin in their respective compartment, and concomitantly decrease β-catenin mobility. Together, these data indicate that β-catenin interaction partners mainly regulate β-catenin subcellular localization by retaining it in the compartment in which they are localized, rather than by active transport into or out of the nucleus.**

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## Introduction

The canonical Wnt signalling pathway is highly conserved throughout the animal kingdom and regulates crucial steps in cell fate decisions, tissue development and tissue maintenance. Its deregulation has been implicated in the formation of a variety of human cancers, most prominently of colorectal and hepatocellular carcinoma (Behrens and Lustig, 2004; Morin et al., 1997). When the pathway is active, the transcriptional co-activator β-catenin can enter the nucleus and form complexes with transcription factors of the LEF/TCF family (Behrens et al., 1996; Molenaar et al., 1996), thereby activating target genes implicated in cell cycle control, cell migration and differentiation.

The principal regulatory mechanism that controls β-catenin nuclear accumulation is its proteasomal degradation in the cytoplasm (Aberle et al., 1997). In unstimulated cells, β-catenin is targeted for ubiquitination and subsequent degradation by phosphorylation by glycogen synthase kinase 3β and casein kinase 1ε (Liu et al., 2002; Rubinfeld et al., 1996) in a multiprotein complex where it is bound by the scaffold proteins axin or axin2/conductin (Behrens et al., 1998; Hart et al., 1998; Kishida et al., 1998) and the tumour suppressor adenomatous polyposis coli (APC) (Rubinfeld et al., 1993; Su et al., 1993). Upon binding of Wnt ligands to their cognate receptors Frizzled (Bhanot et al., 1996) and LRP5/6 (Pinson et al., 2000; Tamai et al., 2000), this destruction complex is inactivated, which leads to accumulation of β-catenin in the cytoplasm and its entry into the nucleus.

β-catenin has a molecular mass of 90 kDa, which should prevent its passive diffusion through the nuclear pores. Moreover, it does not contain nuclear localization signal (NLS) or nuclear export signal (NES) sequences which are required for nucleo-cytoplasmic transport by the importin/exportin system. Nevertheless, it can efficiently enter and exit the nucleus (Eleftheriou et al., 2001; Wiechens and Fagotto, 2001; Yokoya et al., 1999). On the basis of structural similarities of its armadillo repeats to the importin-β HEAT repeats, it has been suggested that β-catenin can directly interact with nuclear pore proteins (Fagotto et al., 1998), although a subsequent study has challenged this view (Suh and Gumbiner, 2003). During recent years, evidence has emerged that the transcriptional activity of β-catenin is modulated by direct regulation of its subcellular localization by a variety of interaction partners. LEF/TCF transcription factors can enrich β-catenin in the nucleus (Behrens et al., 1996; Huber et al., 1996), and a complex of B-cell lymphoma 9 (BCL9) (or its *Drosophila* homologue Legless) and its nuclear interactor Pygopus can also strongly recruit β-catenin to that compartment (Kramps et al., 2002; Townsley et al., 2004). Conversely, APC, axin and axin2/conductin, besides their role in the destruction complex, shift β-catenin to the cytoplasm (Barth et al., 1997; Tolwinski and Wieschaus, 2001). As TCF4,

BCL9, APC and axin have been shown to shuttle between nucleus and cytoplasm (Cong and Varmus, 2004; Henderson, 2000; Neufeld et al., 2000a; Neufeld et al., 2000b; Prieve et al., 1998; Townsley et al., 2004; Wiechens et al., 2004), it has been speculated that TCFs and BCL9 may act as nuclear import factors for β-catenin, whereas APC and axin may actively export β-catenin into the cytoplasm. So far, however, it has not been shown directly that these factors regulate β-catenin subcellular localization by active transport of β-catenin across the nuclear envelope.

Fluorescence recovery after photobleaching (FRAP) in living cells allowed us to analyse the kinetics of fluorescently tagged proteins moving between the nucleus and the cytoplasm. In this study, we have evaluated the shuttling of yellow fluorescent protein (YFP)-tagged β-catenin between nucleus and cytoplasm by selectively bleaching the fluorescence in one compartment with high laser power and monitoring the fluorescence recovery. Under basal conditions, β-catenin is expected to localize at a stable steady-state distribution between nucleus and cytoplasm at which equal β-catenin amounts per time unit enter and exit the nucleus. This equilibrium can be altered both by a selective increase of β-catenin nuclear import or export, and by β-catenin retention in either the nucleus or the cytoplasm. For example, a transport activity such as β-catenin co-export from the nucleus by APC would result in the accumulation of β-catenin in the cytoplasm. Unless the nuclear entry mechanism was already at its maximum, this increased cytoplasmic concentration would lead to an increased β-catenin influx back into the nucleus, resulting in a new equilibrium with increased export as well as increased nuclear entry and thus an overall acceleration of β-catenin nucleo-cytoplasmic shuttling. This faster shuttling is detectable in FRAP assays as accelerated fluorescence recovery in the bleached compartment. Furthermore, the localization shift induced by the export activity will be sensitive to a block of nuclear export, e.g. by leptomycin B (LMB). Alternatively, β-catenin binding partners could retain β-catenin in one compartment by recruiting it to immobile structures or by sterically inhibiting its passage through the nuclear pores and thus by transiently withdrawing the bound β-catenin from the shuttling pool. The remaining free β-catenin would shuttle between both compartments with its normal kinetics, so that in this case the fluorescence recovery in compartment bleach assays would remain unaffected. Sterical impairment of the passage of β-catenin through the nuclear pores could even decelerate this fluorescence recovery. The binding of β-catenin to fixed structures within either the nucleus or the cytoplasm was also tested by FRAP. To this end, we applied localized, small point bleaches in either compartment and monitored the fluorescence recovery in that region. Slower recovery in these assays would indicate association of β-catenin with an immobile interaction partner and would thus point to retention within the analysed compartment.

In summary, we have examined the nucleo-cytoplasmic distribution and shuttling of β-catenin in the absence and presence of TCF4, BCL9, Pygopus, APC, axin and axin2/conductin by live-cell microscopy and FRAP, and we have characterized the mechanism through which these factors regulate the subcellular localization of β-catenin.

## Results

### β-catenin shuttles efficiently between nucleus and cytoplasm

To analyse the kinetics of β-catenin nucleo-cytoplasmic shuttling, we performed FRAP experiments with mYFP-tagged β-catenin. We expressed mYFP-β-catenin in HEK293T cells, selectively bleached either the entire nucleus or the entire cytoplasm (‘compartment bleach’) and monitored the fluorescence recovery in this compartment (Fig. 1A). The fluorescence in the bleached compartment dropped to about 40% immediately after bleaching, and recovered to close to 100% within about 8 minutes. The recovery kinetics were identical between cells where the nucleus was bleached and cells where the cytoplasm was bleached (Fig. 1B), indicating that β-catenin shuttles at a steady-state rate between both compartments. Furthermore, there were no differences in the fluorescence recovery between cells expressing moderate amounts (used for all further experiments) or five times higher levels of mYFP-β-catenin, demonstrating that the absolute number of β-catenin molecules entering and exiting the nucleus per time unit increases proportionally when the β-catenin concentration is increased in either compartment (Fig. 1C), i.e. that the β-catenin shuttling mechanisms in both directions are not saturated in our experiments. To assess the efficiency of β-catenin shuttling between the nucleus and the cytoplasm, we compared it with that of mYFP alone and that of a YFP-CFP fusion (Mayr et al., 2001), two proteins that diffuse passively through the nuclear pores. Whereas the 30 kDa protein mYFP shuttled between both compartments with a full recovery after about 6 minutes, the fluorescence of the 60 kDa YFP-CFP fusion recovered only to 80% after 15

**Fig. 1.** β-catenin shuttles efficiently between nucleus and cytoplasm and is mobile in both compartments. (A) Time-lapse microscopy of HEK293T cells transfected with mYFP-β-catenin during compartment bleach experiments. After one initial image, the entire cytoplasm (top) or the entire nucleus (bottom) was bleached and fluorescence recovery was monitored. Bars, 10 μm. (B) Mean recovery curves from mYFP-β-catenin-expressing cells bleached in the nucleus or in the cytoplasm. The ratio of the fluorescence in the bleached compartment to that of the whole cell prior to photobleaching was set at 100%. The identical recovery kinetics in the nucleus and cytoplasm indicate that β-catenin is shuttling at an equilibrium. The cartoons in B-E designate compartment bleaches. (C) Compartment bleach analysis of cells expressing moderate amounts of mYFP-β-catenin as used for all further analyses or five times higher levels. Inset: average total cell fluorescence in both groups. (D) Compartment bleach analysis comparing the shuttling kinetics of mYFP-β-catenin or mYFP-β-cateninS45A (~120 kDa) and passively diffusing controls mYFP (~30kD) and a YFP-CFP fusion protein (~60 kDa). (E) Compartment bleach analysis of β-catenin deletion mutants and β-catenin fusion proteins with an IκBα NES alone or a MAPKK consensus NES in combination with the SV40 NLS that are actively shuttled by the exportin and the importin/exportin system, respectively. (F,G) Point bleach analysis of mYFP-β-catenin mobility in the cytoplasm (F) or the nucleus (G). A small area within one compartment (indicated by a circle) was bleached and the recovery relative to the whole compartment was monitored. Cartoons in F and G designate cytoplasmic or nuclear point bleaches. Total monitoring time was 45 seconds compared to 15 minutes in the compartment bleach analyses, due to much higher mobility of β-catenin within than between the compartments.

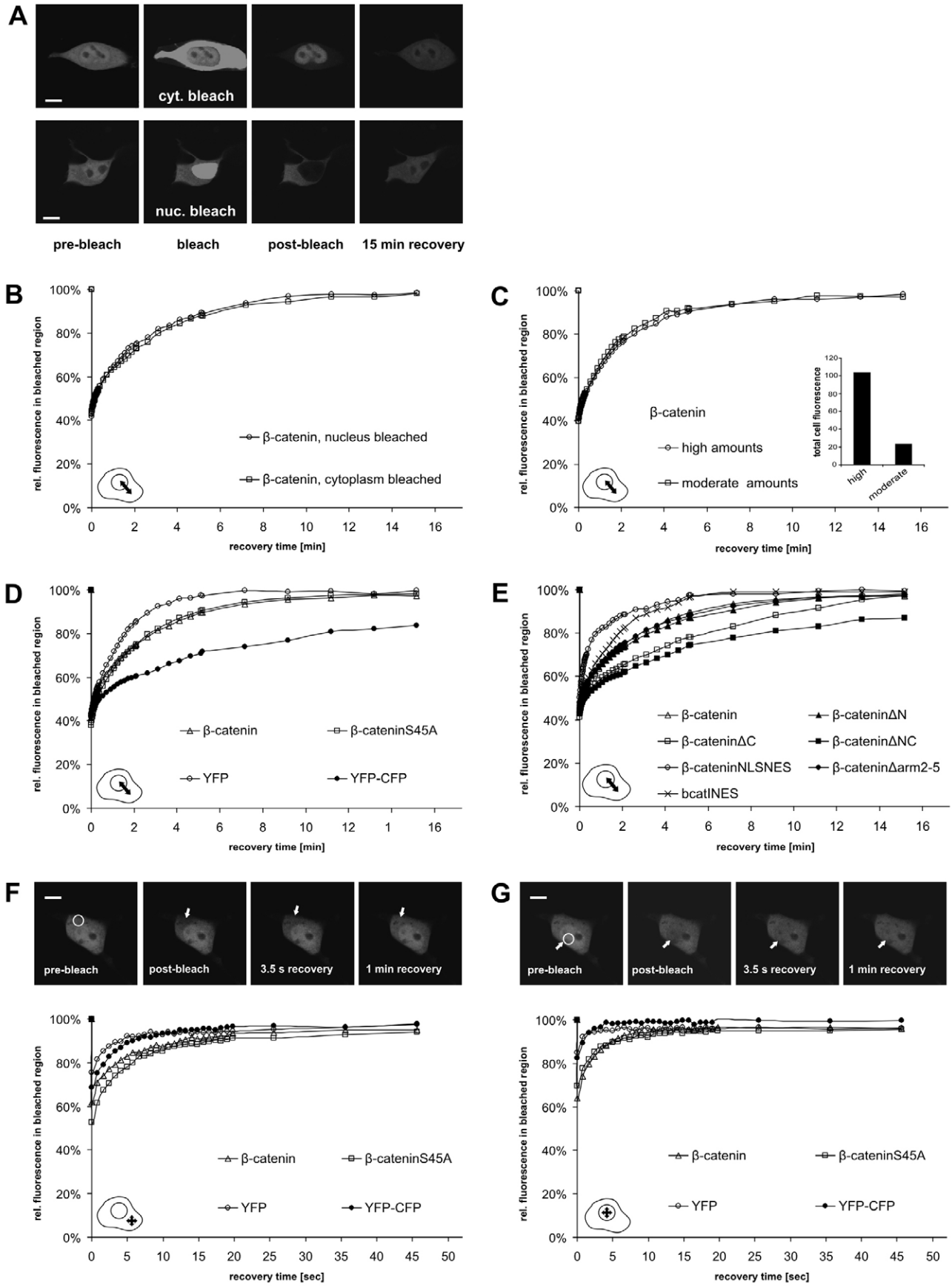


**Fig. 1.** See previous page for legend.

minutes (Fig. 1D), indicating that this protein is hardly able to diffuse through the nuclear pores because of its increased size. mYFP-β-catenin, despite its size of about 120 kDa, moved almost as fast as mYFP between both compartments. This confirms that β-catenin moves through the nuclear pores by more efficient mechanisms than mere passive diffusion. A S45A point mutant of β-catenin that is not degraded via the destruction complex shuttled indistinguishably from the wild type (Fig. 1D).

As β-catenin shuttles very efficiently between nucleus and cytoplasm by itself, we investigated whether β-catenin nucleo-cytoplasmic shuttling could be further increased by active transport, to an extent that could be detected by FRAP analysis. A β-catenin protein with the comparatively weak NES sequence of IκBα (Henderson and Eleftheriou, 2000) (β-cateninINES), which was enriched in the cytoplasm but left enough fluorescence in the nucleus to permit FRAP analyses, displayed robustly accelerated nucleo-cytoplasmic shuttling. In compartment FRAP assays, its fluorescence recovered to almost 100% after 5 to 6 minutes (Fig. 1E). This demonstrates that additional, unidirectional transport by the endogenous CRM-1 export receptor can accelerate the overall shuttling of β-catenin protein, and that we can detect this acceleration in FRAP experiments. Moreover, we used a β-catenin with both a strong MAPKK consensus NES and a SV40 NLS fused to its C terminus (β-cateninNESNLS). This protein was mainly cytoplasmic (not shown), indicating that the NES sequence was dominant, but the β-catenin amounts in the nucleus were also sufficient for FRAP analyses. β-cateninNESNLS shuttled even more efficiently between nucleus and cytoplasm than β-cateninINES, with a recovery to almost 100% after 4 minutes (Fig. 1E). This shows that β-catenin shuttling can be even further accelerated by linking it to importin-dependent transport in addition to the CRM-1 export system.

### The β-catenin C terminus is crucial for efficient β-catenin shuttling

Previous studies have attributed shuttling activities to the N- and C-terminal domains and the arm repeats 1-8 of β-catenin (Koike et al., 2004; Wiechens and Fagotto, 2001). When we deleted the N-terminal 89 amino acids of β-catenin (β-cateninΔN), this resulted in only a slight deceleration of β-catenin nucleo-cytoplasmic shuttling, whereas deletion of the C terminus after amino acid 708 (β-cateninΔC) had a much stronger effect, and a combination of both deletions (β-cateninΔNC) further decelerated the shuttling of the truncated protein (Fig. 1E), which is in line with the results obtained with other methods in the earlier studies (Koike et al., 2004; Wiechens and Fagotto, 2001). Conversely, a β-catenin protein harbouring a deletion from the second to the fifth arm repeat (β-cateninΔarm2-5), which lacks the interaction domains for APC, axin, TCFs and BCL9 (Hoffmans and Basler, 2004; von Kries et al., 2000), shuttled with the same kinetics as the wild type.

### β-catenin is mobile in both the nucleus and the cytoplasm

β-catenin is bound by various interaction partners in the nucleus and the cytoplasm, which may reduce its mobility within those compartments and influence its subcellular distribution and/or nucleo-cytoplasmic shuttling. We therefore evaluated the mobility of mYFP-β-catenin within the nucleus or the cytoplasm by applying small localized 'point bleaches' in either compartment (Fig. 1F,G, top panels). After the bleach, the fluorescence in the bleached region dropped to about 60%, and almost completely recovered within 15 seconds in the cytoplasm and within 10 seconds in the nucleus (Fig. 1F,G), indicating that there is hardly any immobile β-catenin in either compartment. When compared to mYFP or the YFP-CFP fusion, β-catenin moved only slightly more slowly in both compartments. Given that β-catenin fluorescence recovers within about 15 seconds in both compartments, whereas the recovery between compartments takes about 10 minutes, β-catenin mobility within the nucleus and the cytoplasm very probably has no major influence on its nucleo-cytoplasmic shuttling under these conditions.

### TCF4 slows down β-catenin nucleo-cytoplasmic shuttling and nuclear mobility

TCF transcription factors can enter the nucleus by virtue of their NLS (Prieve et al., 1998) and might take β-catenin into the nucleus by a 'piggy-back' mechanism (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). As expected, TCF4 shifted β-catenin to the nucleus (Fig. 2A), whereas β-cateninΔarm2-5, which lacks the TCF interaction domain, was not affected (not shown). TCF4 slowed down rather than accelerated β-catenin nucleo-cytoplasmic shuttling (Fig. 2B), arguing against nuclear import of β-catenin by TCF factors. Moreover, TCF4 moved more slowly than β-catenin in the nucleus and also reduced β-catenin nuclear mobility to a recovery of about 80% after 10 seconds, when β-catenin alone had already fully recovered (Fig. 2C). These data suggest that β-catenin is bound by TCF4 and thereby enriched in the nucleus by retention.

### BCL9 slows down β-catenin nucleo-cytoplasmic shuttling

BCL9 may actively import β-catenin into the nucleus. Indeed, BCL9v, an N-terminal BCL9 fragment that contains all three domains conserved in *Drosophila* Legless and is sufficient to co-stimulate the activity of a TCF/β-catenin-dependent reporter gene (not shown) enriched β-catenin in the nucleus when BCL9v was itself recruited into that compartment by its nuclear binding partner Pygopus2 (Fig. 3A). Pygopus2 alone had no influence on the subcellular distribution of β-catenin (not shown). Moreover, β-cateninΔarm2-5 was not recruited into the nucleus by BCL9v and Pygopus (not shown). BCL9v itself shuttled much more slowly than β-catenin, with a fluorescence recovery to 70% after 15 minutes, and its co-expression consistently slowed down β-catenin nucleo-cytoplasmic shuttling, rather than accelerating it (Fig. 3B). The shuttling of β-cateninΔarm2-5 was not affected (see Fig. S1B in supplementary material). Additional expression of Pygopus2 had no further effect on β-catenin shuttling, which indicates that β-catenin nuclear enrichment by BCL9 together with Pygopus is independent of its decelerated shuttling by BCL9. Furthermore, BCL9v and/or Pygopus2, expressed alone or in combination, were as mobile as β-catenin within the nucleus, and consequently did not affect β-catenin mobility in that compartment (Fig. 3C). Full-size BCL9 had essentially the same effects as BCL9v on β-catenin recruitment and shuttling, although the effects were less

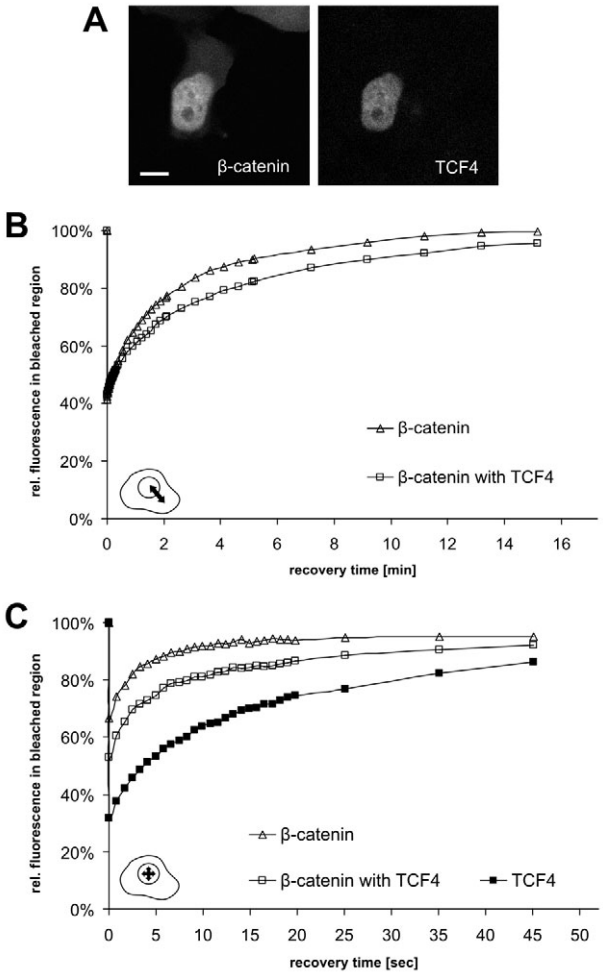


**Fig. 2.** TCF4 slows down β-catenin in the nucleus and decelerates its nucleo-cytoplasmic shuttling. (A) Live cell images of mYFP-β-cateninS45A and co-expressed TCF4-mCFP in HEK293T cells. Bar, 10 μm. (B) Compartment bleach analysis of β-catenin shuttling in the absence or presence of co-expressed TCF4-mCFP. (C) Nuclear point bleach analysis of mYFP-β-catenin mobility in the absence or presence of TCF4-mCFP. TCF4-mYFP mobility was determined in a separate experiment. The cartoons in B and C designate compartment bleaches and point bleaches, respectively.

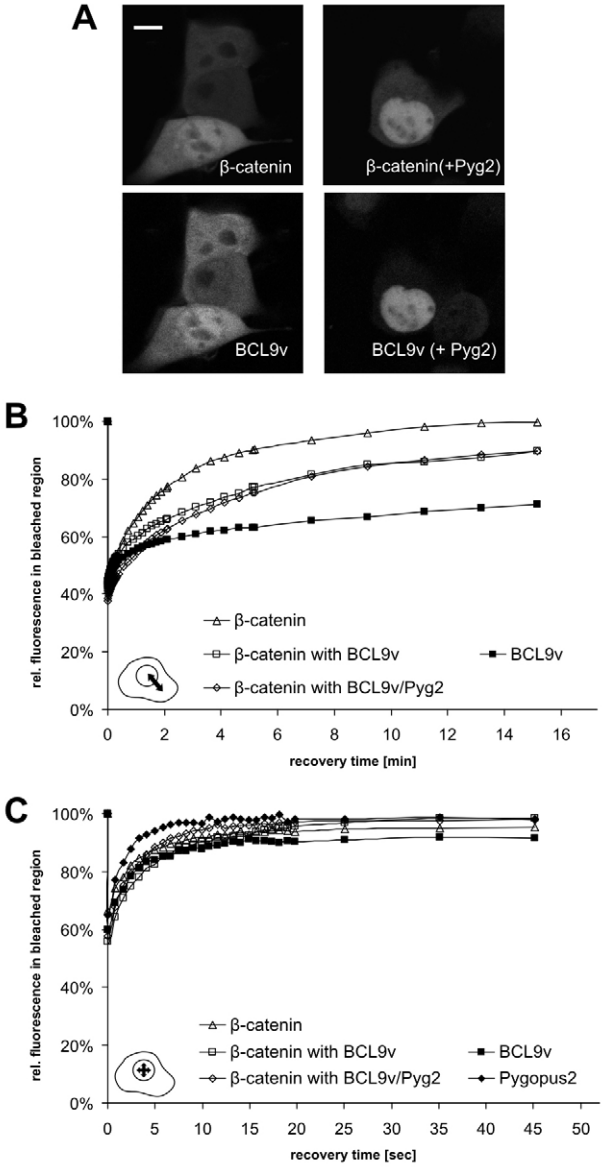


**Fig. 3.** BCL9 slows down β-catenin nucleo-cytoplasmic shuttling. (A) Live cell images of mYFP-β-cateninS45A and co-transfected mCFP-BCL9v in the absence (left panels) or presence (right panels) of mRFP-Pygopus2. Bar, 10 μm. (B) Compartment bleach analysis of mYFP-β-catenin shuttling in the absence or presence of mCFP-BCL9v and mRFP-Pygopus2. mYFP-BCL9v shuttling was analysed in a separate experiment. (C) Nuclear point bleach analysis of mYFP-β-catenin in the absence or presence of mCFP-BCL9v and mRFP-Pygopus2. The mobilities of mYFP-BCL9v and mYFP-Pygopus were determined in separate experiments. The cartoons in B and C designate compartment bleaches and point bleaches, respectively.

pronounced because of its lower expression levels (data not shown).

## APC slows down β-catenin nucleo-cytoplasmic shuttling and cytoplasmic mobility

The APC tumour suppressor is almost exclusively cytoplasmic and recruits β-catenin into that compartment (Fig. 4A). APC can shuttle between nucleus and cytoplasm (Brocardo et al., 2005; Henderson, 2000; Neufeld et al., 2000a; Neufeld et al., 2000b; Rosin-Arbesfeld et al., 2000), raising the possibility that it actively exports β-catenin from the nucleus. APC co-expression, however, did not increase, but rather mildly decreased β-catenin nucleo-cytoplasmic shuttling (Fig. 4B, compare with Fig. 1E). Furthermore, APC moved slowly within the cytoplasm, with a fluorescence recovery of only about 85% after 15 seconds in the cell body, and it slowed down β-catenin to a similar extent in that compartment. At the tips of cellular protrusions, where APC accumulates (Nathke et al., 1996), both APC and β-catenin were even slower, with recoveries of only 20% and 30%, respectively, rather than 100% after 15 seconds (Fig. 4A,C). Again, the APC binding mutant β-cateninΔarm2-5 was not affected (see Fig. S1C in supplementary material).

### Axin and axin2/conductin slow down β-catenin cytoplasmic mobility

Similarly to APC, axin has been shown to be a nucleo-cytoplasmic shuttling protein (Cong and Varmus, 2004; Wiechens et al., 2004), which may therefore co-export β-catenin from the nucleus. Axin2/conductin is a functional homologue of axin with respect to its participation in the β-catenin degradation complex (Behrens et al., 1998), and can enrich β-catenin in the cytoplasm to a similar extent as axin (Fig. 5A). In the presence of either factor, β-catenin nucleo-cytoplasmic shuttling was not affected (Fig. 5B). However, both axin and axin2 moved slowly in the cytoplasm and could also slow down β-catenin, which recovered to about 70% rather than 100% after 15 seconds (Fig. 5C), but they did not slow down β-cateninΔarm2-5 (see Fig. S7C in supplementary material). As it was reported that deletion of the axin Dix domain greatly enhances the ability of the truncated protein to shuttle between the nucleus and the cytoplasm (Cong and

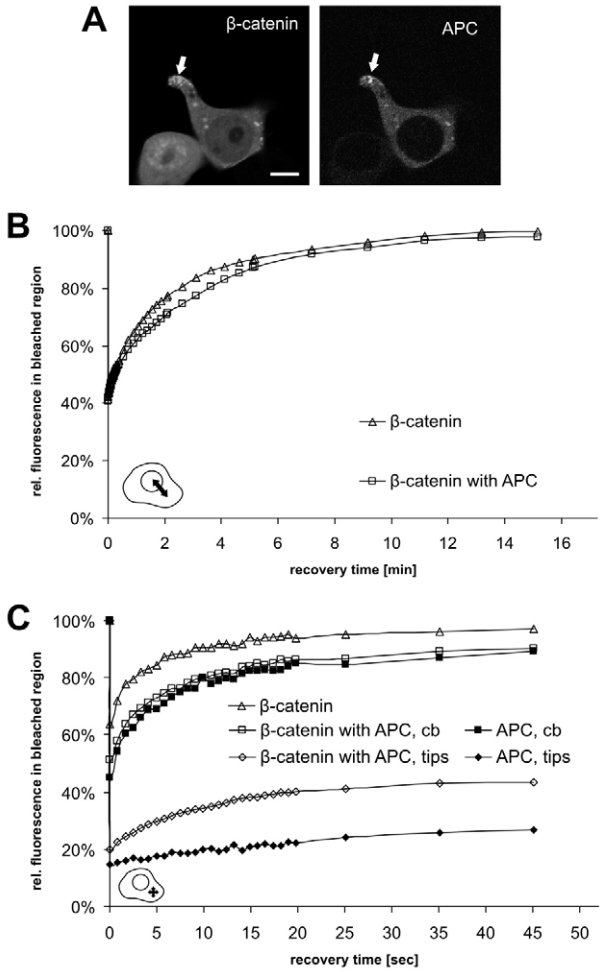


**Fig. 4.** APC slows down β-catenin in the cytoplasm and slightly decelerates β-catenin nucleo-cytoplasmic shuttling. (A) Live cell images of mYFP-β-cateninS45A and co-expressed mCFP-APC. The tip of a protrusion where APC and β-catenin co-accumulate is indicated by an arrow. Bar, 10 μm. (B) Compartment bleach analysis of mYFP-β-cateninS45A shuttling in the absence or presence of mCFP-APC. (C) Cytoplasmic point bleach analysis of mYFP-β-cateninS45A in the absence or presence of mCFP-APC. mYFP-APC mobility was determined in a separate experiment. Note the low mobility of APC and β-cateninS45A at the tips of cellular protrusions (tips) as compared to the cell body (cb). The cartoons in B and C designate compartment bleaches and point bleaches, respectively.

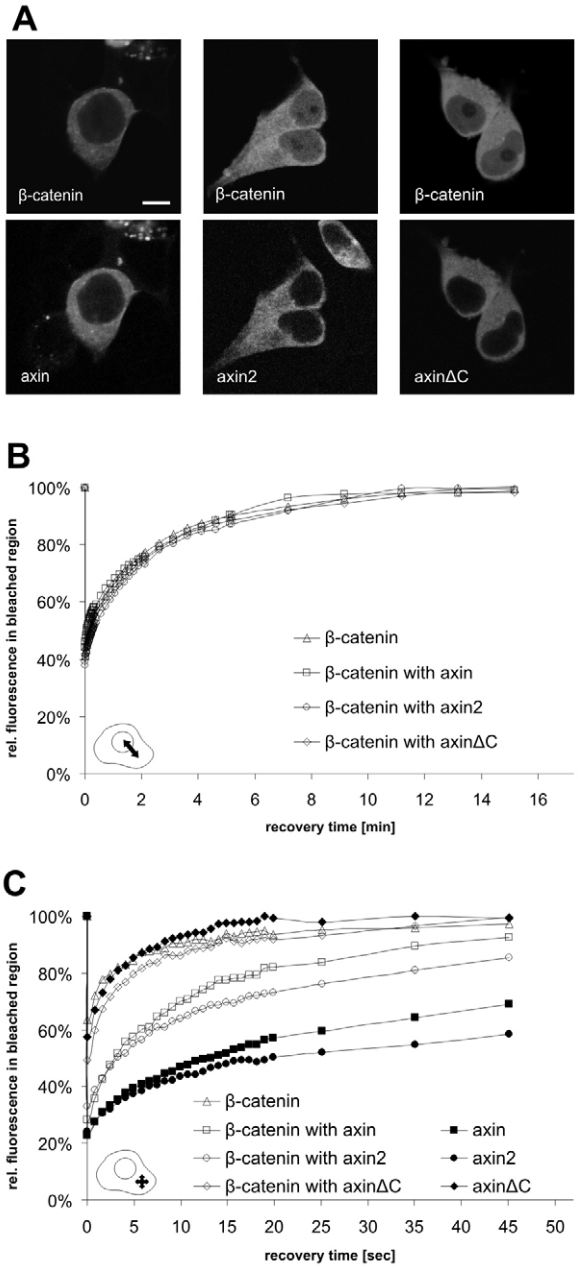


**Fig. 5.** Axin and axin2 slow down β-catenin in the cytoplasm and do not accelerate β-catenin nucleo-cytoplasmic shuttling. (A) Live cell images of mYFP-β-cateninS45A (upper panels) and co-expressed mCFP-tagged axin, axin2 or axinΔC (lower panels). Bar, 10 μm. (B) Compartment bleach analysis of mYFP-β-catenin in the absence or presence of axin, axin2 or axinΔC. (C) Cytoplasmic point bleach analysis of mYFP-β-catenin in the absence or presence of axin, axin2 or axinΔC. The mobilities of mYFP-tagged axin, axin2 and axinΔC were determined in separate experiments. The cartoons in B and C designate compartment bleaches and point bleaches, respectively.

Varmus, 2004), we also tested C-terminally truncated axin (axinΔC), a protein that was highly mobile in the cytoplasm (Fig. 5C). Co-transfection of axinΔC substantially enriched β-catenin in the cytoplasm (Fig. 5A), but did not accelerate β-catenin nucleo-cytoplasmic shuttling (Fig. 5B), arguing against active nuclear export of β-catenin by axin.

### The β-catenin enrichment in the cytoplasm by APC or axin is largely CRM-independent

Since both APC and axin contain NES sequences (Cong and Varmus, 2004; Henderson, 2000; Neufeld et al., 2000b; Wiechens et al., 2004) and thus might co-export β-catenin via the export receptor CRM-1, we investigated the influence of the CRM-1 inhibitor leptomycin B on the ability of APC and axin to enrich β-catenin in the cytoplasm. Neither endogenous β-catenin nor exogenously expressed mYFP-β-catenin shifted to the nuclei of HEK293T cells after 1 hour of LMB treatment (Fig. 6A). In the presence of either APC or axin, β-catenin was strongly enriched in the cytoplasm, but treatment of the cells with LMB for 1 hour did not significantly change its subcellular distribution. In line with recent publications (Brocardo et al., 2005; Wiechens et al., 2004), APC and axin themselves only marginally accumulated in the nucleus during that time (data not shown). By contrast, β-catenin with a MAPKK consensus NES fused to its C terminus (β-cateninNES) was almost exclusively cytoplasmic, but re-localized to the nucleus within about 20 minutes upon LMB addition, and was indistinguishable in its distribution from wild-type β-catenin after 1 hour of treatment. In contrast to full-length axin, axinΔC strongly accumulated in the nucleus within 1 hour in response to LMB treatment (not shown) and recruited β-catenin into the nucleus upon LMB treatment (Fig. 6A), demonstrating that axinΔC can enrich β-catenin independently of its ability to shuttle between nucleus and cytoplasm. The nucleo-cytoplasmic shuttling of mYFP-β-catenin in the presence or absence of APC and axin was unaltered by LMB (Fig. 6A and data not shown). Together with our FRAP analyses, the LMB data suggest that β-catenin nucleo-cytoplasmic distribution is determined mainly by retention in both compartments rather than co-transport across the nuclear envelope.

### Wnt signalling does not affect β-catenin nucleo-cytoplasmic shuttling

Activation of the Wnt pathway was suggested to shift β-catenin to the nucleus (Staal et al., 2002). We therefore activated the Wnt pathway by either co-expression of Wnt3a or dominant active LRP6 (LRP6da). Both activators led to stabilization of endogenous β-catenin (Fig. 7A,B), and particularly LRP6da could also stabilize mYFP-β-catenin. Both activators also enhanced the activation of a TCF/β-catenin-dependent reporter (TOPglow) by mYFP-β-catenin (Fig. 7C). In the presence of either Wnt3a or LRP6da, however, mYFP-β-catenin did not shift into the nucleus, and β-catenin nucleo-cytoplasmic shuttling was altered marginally, at best (Fig. 7D). The intracompartmental mobility of β-catenin was also not

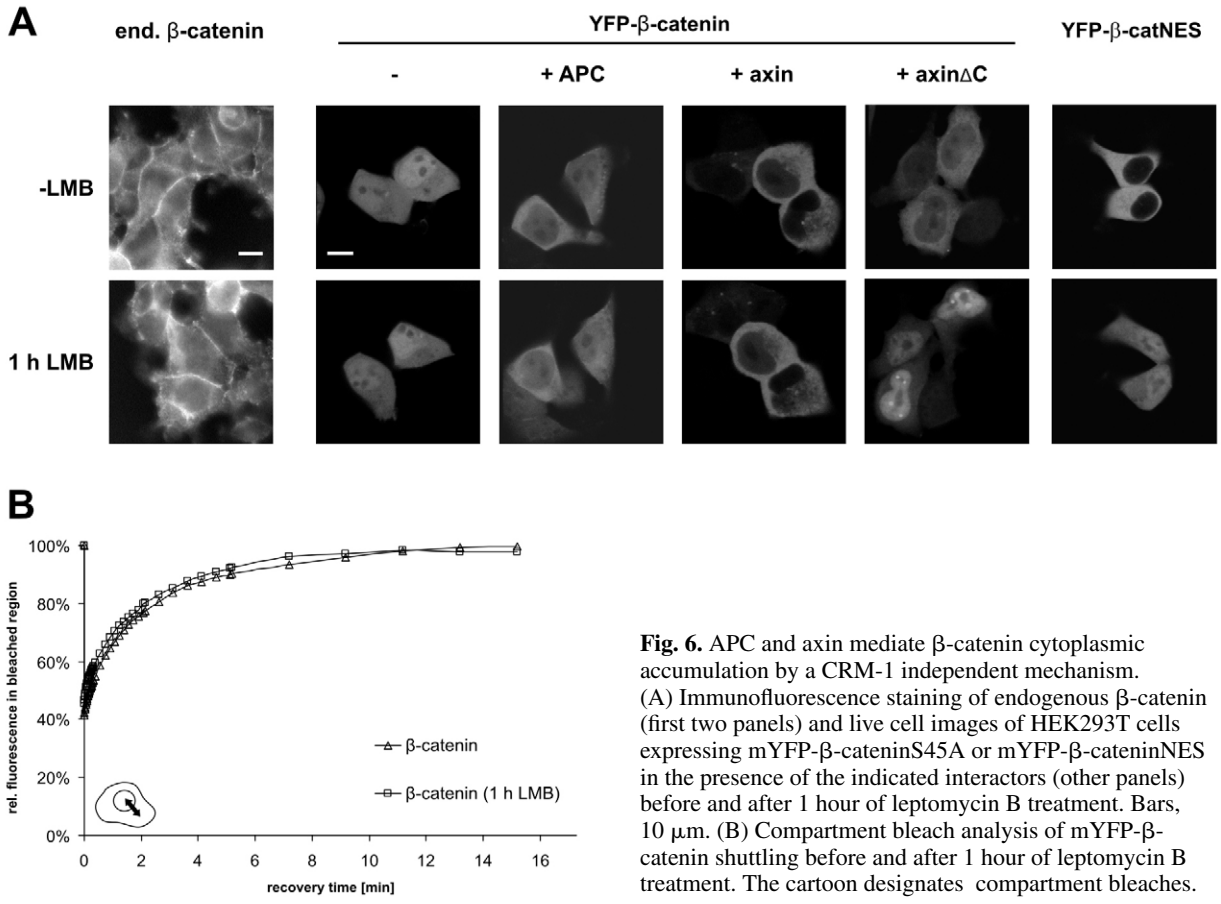


**Fig. 6.** APC and axin mediate β-catenin cytoplasmic accumulation by a CRM-1 independent mechanism. (A) Immunofluorescence staining of endogenous β-catenin (first two panels) and live cell images of HEK293T cells expressing mYFP-β-cateninS45A or mYFP-β-cateninNES in the presence of the indicated interactors (other panels) before and after 1 hour of leptomycin B treatment. Bars, 10 μm. (B) Compartment bleach analysis of mYFP-β-catenin shuttling before and after 1 hour of leptomycin B treatment. The cartoon designates compartment bleaches.

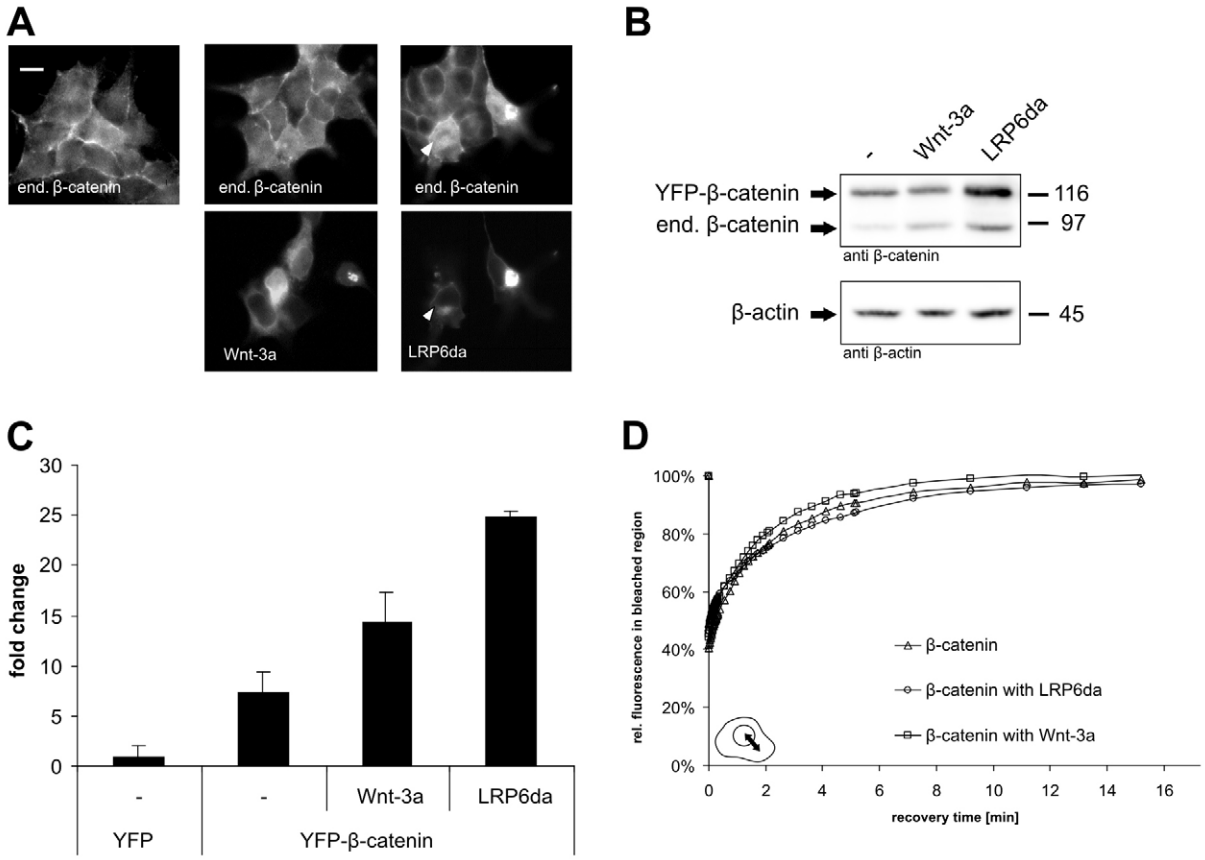


**Fig. 7.** Wnt signalling does not influence β-catenin nucleo-cytoplasmic shuttling. (A) Immunofluorescence staining of endogenous β-catenin (upper panels) in HEK293T cells in the absence or presence of mRFP-tagged Wnt-3a or dominant active LRP6 (LRP6da). Lower panels depict the fluorescence of the RFP-tagged activators in the same microscopic field as in the upper panels. Bar, 10 μm. (B) Western blot of hypotonic lysates of HEK293T cells transfected with mYFP-β-catenin and the indicated activators, stained for β-catenin (top) and β-actin (bottom). (C) TOPglow reporter assay with mYFP-β-catenin and indicated interactors. Fold changes were determined by calculating ratios of relative luciferase activities of TOP- to FOP samples and normalizing to the mYFP control. Error bars indicate standard deviations. (D) Compartment bleach analysis of mYFP-β-catenin in the absence or presence of dominant active LRP6 or Wnt3a. The cartoon designates compartment bleaches.

significantly affected by either factor (not shown). The effects of Wnt signalling on β-catenin subcellular distribution, shuttling and mobility were not analysed in the presence of interaction partners, as the repressing activity of APC and axin in TOPglow reporter assays was poorly reversible by Wnt3a or LRP6da (data not shown).

## Discussion

β-catenin can enter and exit the nucleus independently of the importin/exportin system or RanGTP (Eleftheriou et al., 2001; Wiechens and Fagotto, 2001; Yokoya et al., 1999), but the exact mechanism for its passage through the nuclear pores remains to be elucidated. We found that free β-catenin equilibrates between nucleus and cytoplasm within about 10 minutes. This time frame is comparable to the shuttling of other signal transducers such as the catalytic subunit of protein kinase A or SMAD proteins, which accumulate in the nuclei of stimulated cells within 30 minutes or 1 hour, respectively (Hagiwara et al., 1993; Nicolas et al., 2004). By contrast to these factors, which already pre-exist in the cytoplasm, β-catenin is continuously degraded in the absence of a signal and only accumulates over a period of several hours after the onset of Wnt signalling (Lustig et al., 2002). This makes it unlikely that the speed of the nucleo-cytoplasmic shuttling of β-catenin is limiting for efficient signal transduction. Nevertheless, the nucleo-cytoplasmic distribution of β-catenin and hence control of Wnt signalling might be altered by active transport or by retention of β-catenin in either compartment. In this study we have characterized the mechanism by which several interaction partners of β-catenin direct its localization in the nucleus or cytoplasm.

Both BCL9, in conjunction with its nuclear binding partner Pygopus2, and TCF4 on its own, efficiently enrich β-catenin in the nucleus. The fact that both factors reduce rather than stimulate nucleo-cytoplasmic shuttling of β-catenin argues that this enrichment is not due to active import, but rather to impairment of the exit of β-catenin from the nucleus. The mechanisms by which BCL9/Pygopus and TCF4 keep β-catenin in the nucleus appear to differ, as we found that only TCF4 is able to reduce β-catenin mobility in the nucleus. This is in line with the established role of TCF4 as a transcription factor that binds β-catenin and recruits it to chromatin, thereby effectively reducing the β-catenin population that is free to cross the nuclear pores. In contrast, BCL9 and Pygopus do not

seem to be tightly anchored in the nucleus, as judged by their high intranuclear mobility, and thus cannot sequester β-catenin away from the nuclear pores. Yet, they can obviously impair β-catenin exit from the nucleus, possibly by sterically inhibiting its passage through the nuclear pores. Taken together, our data indicate that both factors enrich β-catenin in the nucleus by retention, rather than by actively importing it.

Previous studies have suggested the possibility that nuclear export of β-catenin by APC or axin may play an important role in controlling Wnt signalling (Aberle et al., 1997; Cong and Varmus, 2004; Henderson, 2000; Neufeld et al., 2000b; Rosin-Arbesfeld et al., 2003). Our FRAP analyses show that neither APC nor axin accelerate β-catenin nucleo-cytoplasmic shuttling, arguing against efficient co-export mediated by these factors. Furthermore, the cytoplasmic enrichment of β-catenin by APC and axin persists for more that 1 hour in the presence of the CRM-1 inhibitor leptomycin B, suggesting that continuous nuclear export of these two proteins is not required for the maintenance of the β-catenin enrichment in the cytoplasm. This differs from the cytoplasmic enrichment of a β-cateninNES fusion protein, which is also linked to the CRM-1 export pathway. In this case, LMB treatment leads to rapid re-equilibration of β-cateninNES to the wild-type distribution. This strongly suggests that APC and axin mainly utilize means other than CRM-1-dependent co-export to recruit β-catenin into the cytoplasm. Remarkably, APC, axin and axin2 are much less mobile in the cytoplasm than β-catenin, probably because of their association with fixed structures such as the cytoskeleton (Ciani et al., 2004; Mogensen et al., 2002), and they substantially reduce β-catenin mobility in the cytoplasm. This effect is reminiscent of the retention of β-catenin by TCF4 in the nucleus, and argues that APC, axin and axin2 also anchor β-catenin in the compartment in which they are localized. The effects of these factors on β-catenin subcellular localization, cytoplasmic mobility and shuttling are very similar to that of E-cadherin, an integral membrane protein which retains β-catenin at the plasma membrane and at intracellular vesicular structures (see Fig. S2 in supplementary material), and differ from the effects of fusing the IκBα NES sequence to the β-catenin C terminus, which also enriched β-catenin in the cytoplasm, but robustly accelerated its nucleo-cytoplasmic shuttling (Fig. 1E).

Previous studies have shown that β-catenin co-accumulates with APC or axin in the nuclei of cells when nuclear export is inhibited by NES mutation of these interaction partners or LMB treatment (Henderson, 2000; Rosin-Arbesfeld et al., 2003; Wiechens et al., 2004). These findings can be interpreted as evidence for nuclear export of β-catenin by APC and axin, but they can also be readily explained by retention of β-catenin by these factors in the nucleus. Nevertheless, APC or axin-mediated nuclear export of β-catenin might contribute to its cytoplasmic enrichment, as NLS-mutants of these factors, which cannot enter the nucleus, are less efficient in recruiting or degrading β-catenin (Cong and Varmus, 2004; Neufeld et al., 2000b). As we have found no evidence for active transport of β-catenin between nucleus and cytoplasm by full-length APC or axin, we propose that β-catenin subcellular localization is predominantly determined by retention in both compartments.

Nuclear and cytoplasmic retention factors could compete for binding to β-catenin as has been demonstrated in several studies (Hamada and Bienz, 2004; Henderson et al., 2002; Hulsken et al., 1994; Lee et al., 2001; Tolwinski and Wieschaus, 2001). Such competition could fine-tune the outcome of Wnt signalling at different steps along the pathway. For instance, the nuclear translocation of β-catenin by BCL9 and its binding partner Pygopus which is essential for Wnt signalling could be counteracted by cytoplasmic retention by APC and axin. Intriguingly, the axin homologue axin2/conductin is a Wnt target itself (Jho et al., 2002; Lustig et al., 2002), thus possibly constituting a negative feedback factor that could limit the extent of Wnt signalling by retaining cytoplasmic β-catenin. It has also been suggested that truncated APC in colorectal cancer, which can still bind to β-catenin but cannot promote its degradation anymore, may attenuate β-catenin signalling by recruiting it into the cytoplasm (Henderson, 2000), thereby possibly protecting tumour cells from the pro-apoptotic effects of β-catenin (Albuquerque et al., 2002). Furthermore, loss of the cytoplasmic anchor E-cadherin has been shown to augment β-catenin signalling in some mammalian systems (Orsulic et al., 1999). Wnt signalling, in turn, can also control the degree of retention by decreasing the affinity of APC, axin and E-cadherin for β-catenin (Gottardi and Gumbiner, 2004; Ha et al., 2004; Willert et al., 1999; Xing et al., 2004). β-catenin bound to these factors could be set free in response to the activation of the pathway and thus serve as a reservoir for the rapid activation of the pathway. Thus, retention of β-catenin may be an important modulator of Wnt signalling in both normal development and cancer.

## Materials and Methods

### Plasmids

All cDNAs were cloned in frame into the *Xma*I-*Bam*HI sites of mYF-C2 or mCF-C2 (Mayr et al., 2001; Mayr et al., 2005), which contain amino acids (aa) 1-227 of monomeric (A206K; m), yellow (YFP), or cyan fluorescent protein (CFP) (Zacharias et al., 2002) at the N terminus of the resulting fusion protein, or into mYF-N2 or mCF-N2, which contain C-terminal mYFP or mCFP, respectively. For red fluorescent fusion proteins, cDNAs were inserted into mRF-N2 or mRF-C2, in which the parental mYFP was replaced by aa 1-225 of monomeric red fluorescent protein 1 (mRFP1) (Campbell et al., 2002). Inserts were amplified by PCR and verified by sequencing, and internal *Bam*HI and *Xma*I sites were removed by introducing silent point mutations.

YFP-β-catenin was created by insertion of the full-length human β-catenin coding sequence (CDS) for aa 2-781 from myc-β-catenin (Hulsken et al., 1994) into mYF-C2. mYFP-β-cateninS45A was obtained by site-directed mutagenesis from mYFP-β-catenin. mYFP-β-cateninΔN, lacking aa 2-89, was created by *Xma*I/*Xho*I digest of mYFP-β-catenin, blunting and religation. mYFP-β-cateninΔC, lacking aa 709-781, was created by *Eco*RV/*Bam*HI digest, blunting and religation. mYFP-β-cateninΔNC is a combination of both deletions. mYFP-β-cateninΔarm2-5, which lacks the overlapping interaction domains for APC, axin, TCF and BCL9 from aa 184 to 323, was created by deletion of the internal 5′ *Hin*dIII fragment. mYFP-β-cateninNES was created by insertion of an oligonucleotide coding for a consensus MAPKK NES (NLVDLQKKLEELELDEQQ) (Henderson and Eleftheriou, 2000) into the *Bam*HI-*Not*I sites of a mYFP-β-catenin construct without a stop codon, and for mYFP-β-catenin NESNLS, an additional oligonucleotide coding for a SV40 NLS (PKKKRKVPP) was inserted into the BamHI site. mYFP-β-cateninINES was created by inserting an oligonucleotide for the IκBα NES (PSTRIQQQLGQ-LTLENLQ) (Henderson and Eleftheriou, 2000) into the *Bam*HI site of mYFP-β-catenin without STOP codon.

The full size human APC CDS from CMV-APC (aa 2-2845) (Kinzler et al., 1987), the CDS for aa 2-831 of the short form of mouse axin (generous gift from Hans Clevers, The Netherlands Institute for Developmental Biology, Utrecht, The Netherlands) and the human axin2 CDS (Mai et al., 1999) were inserted into both mYF-C2 and mCF-C2. mCFP-axinΔC was created from mCFP-axin by *Eco*RI/*Not*I digest, blunting and religation, resulting in a truncation of the axin protein after aa 671. The full-length TCF4 cDNA (kindly provided by Hans Clevers) from pGLOmyc-TCF4 was inserted into mYF-C2 or mCF-N2. mCFP- and mYFP-BCL9v were created by insertion of aa 2-728 of the human coding sequence from flag-BCL9 (Brembeck et al., 2004) into mCF-N2 and mYF-N2. For mCFP-BCL9,

the CDS for aa 729-1394 was inserted into the *Bam*HI and *Not*I sites of mCFP-BCL9v. The full-length CDS for human Pygopus2 (from flag-Pygopus2, kindly provided by Walter Birchmeier, Max Delbrück Centre, Berlin, Germany) was inserted into mRF-C2. For the experiments with E-cadherin, pBATEM2 (Nose et al., 1988) was used. Dominant active, mRFP1-tagged LRP6 (LRP6da) was created by PCR by inserting the CDS for the signal peptide (aa 2-25) of hLRP6 (Tamai et al., 2000) into the *Age*I site in front of the mRFP1 of mRF-C2 and insertion of the CDS for the transmembrane and cytoplasmic domains of LRP6 (aa 1362-1613) into the *Xma*I/*Bam*HI sites of the resulting plasmid. The CDS for aa 2-352 of mouse Wnt3a (Shimizu et al., 1997) was inserted into mRF-N2, yielding a C-terminally tagged Wnt3a protein.

For the mammalian-2-hybrid assay, the mYFP tags from mYFP-β-catenin and mYFP-β-cateninΔarm2-5 were replaced with the GAL4 DNA binding domain of pSG424 (Sadowski and Ptashne, 1989) and the mYFP tag in mYFP-BCL9v was replaced with the VP16 domain from pVP16 (Clontech). For control FRAP experiments with non-CFP-tagged proteins, the mYFP insert in mCFP-axin and mCFP-axin2 was removed by *Age*I/*Xma*I digest and religation and the BCL9v insert from mCFP-BCL9v was transferred to flag-C2. Integrity of expressed proteins was determined by western blot (see Fig. S3 in supplementary material).

## Cell culture and FRAP experiments

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and antibiotics. For FRAP experiments, $3\times10^5$ cells were grown on ∅ 40 mm coverslips and transfected with up to 2.5 μg plasmids per well in serum-free DMEM with 5 μl EscortIV (Sigma)/μg DNA for 4-8 hours. For co-expression experiments, cells were transfected with 600 ng of mYFP-β-catenin plasmid or derivatives and equivalent amounts of mCFP-tagged interactor, or 300 ng of mRFP-tagged activator as indicated. Key experiments were also reproduced with untagged β-catenin interactors (see Fig. S4 in supplementary material). To this end, the cells were co-transfected with the 600 ng of mYFP-β-catenin plasmid and up to 1.2 μg of interactor plasmid. In this case, cells were selected for analysis by virtue of their β-catenin enrichment in one compartment.

FRAP analysis was performed 24-hours post transfection in a FCS2 live cell chamber (Bioptechs, Butler, PA) at 37°C in either $CO_2$-independent medium (Gibco) or Hepes-buffered DMEM. For leptomycin B treatment, the chamber was perfused with 5 ng/ml LMB (Biomol, Hamburg, Germany) in imaging medium. Photobleaching was performed on a confocal laser scanning microscope (TCS SP2, Leica). Cells expressing mYFP fusion proteins in moderate amounts, i.e. sufficiently high fluorescence above background levels, were monitored using the 514 laser line of the 20 mW argon laser at 4% power and bleached at 100% laser power. For compartment bleaches, about 80% of either the nucleus or the cytoplasm were bleached with seven subsequent bleach frames; for localized point bleaches, a single strong laser pulse was applied for 0.5 seconds in either the nucleus or the cytoplasm. Subsequently, images were taken as follows: 25 frames at 0.85-second intervals, 11 frames at 10-second intervals, 7 frames at 30-second intervals and 6 frames at 2-minute intervals. Average intensities in regions of interest were measured using MetaVue (Universal Imaging Corporation). The ratio of the average fluorescence intensity in the bleached area to the average total cell fluorescence (for compartment bleaches) or to the average fluorescence intensity of the entire compartment (for point bleaches) was plotted over time. The ratio in the pre-bleach image was set to 100%, and the first post-bleach image was set as time point 0. The recovery curves shown are averages of the recovery curves of at least eight cells (or five cells for some point bleach experiments), and are representative of at least two independent experiments (see Table S1 in supplementary material for mean values and standard errors of the experiments shown). A significant contribution of new protein synthesis to the fluorescence recovery in these experiment was ruled out by analysis of total cell fluorescence during the recovery time, which only increased by less than 3% within 15 minutes (see Fig. S5 in supplementary material). To detect any deviations from a steady state equilibrium, in all compartment bleach experiments, half of the cells were bleached in the nucleus and the other half in the cytoplasm, but only the average curves are shown because both groups showed identical kinetics in all experiments. Owing to technical constraints, the first image in point bleach experiments could only be taken about 0.8 seconds after the end of the bleach pulse, which resulted in some recovery before the first image was acquired. Consequently, depending on the mobility of the protein of interest, the bleach efficiency sometimes seemed low and bleached areas were not well defined. Together with a high overall fluorescence loss from a single bleach this was interpreted as high mobility of the analysed protein.

## Reporter and mammalian-2-hybrid assays

To evaluate TCF/β-catenin-dependent gene transcription, HEK293T cells were seeded at 75,000 cells per 12-well plate and transfected 24 hours later with 25 ng RSV-β-gal, 75 ng TOP- or FOPglow reporter plasmid (van de Wetering et al., 2001) and 50 ng mYFP-β-catenin plus 25 ng of activator plasmids as indicated. All transfections were performed in duplicate, and experiments were reproduced at least once. Cells were harvested and luciferase activity was determined 24 hours post-transfection and normalized to β-gal values. For mammalian two-hybrid assays, cells were transfected with 25 ng RSV-β-gal, 75 ng pFRluc (Stratagene), 100 ng of the indicated GAL4 plasmids and 300 ng of VP16 or control plasmids and processed as above. The data shown represent changes in relative luciferase activity (for TOPglow assays, the ratios of TOP to FOP activity) compared to the empty vector control.

## Immunofluorescence staining and western blot

For immunofluorescence, $2\times10^5$ HEK293T cells were plated on coverslips in 6-well plates and transfected with 150 ng of the indicated plasmids. Cells were fixed with 3% PFA in PBS 24 hours later, permeabilized with 0.5% Triton X-100 in PBS and stained with H102 rabbit anti β-catenin (Santa Cruz Biotechnology). When indicated, cells were incubated with 5 ng/ml LMB (Biomol) for 1 hour prior to fixation. Images were acquired with an Axiophot 2 microscope (Zeiss).

For western blot analysis of the cytosolic β-catenin fraction, cells were plated without coverslips and transfected as above. Hypotonic lysates were prepared by scraping the cells into cold hypotonic buffer (25 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM DTT) and incubating for 20 minutes on ice, centrifuging for 5 minutes at top speed in a tabletop centrifuge and saving the supernatant. Equal amounts of total protein were separated on 10% polyacrylamide SDS gels, blotted onto nitrocellulose membranes and detected with mouse anti-β-catenin (BD Transduction Laboratories) or mouse anti-β-actin (Sigma) and HRP-coupled goat anti-mouse antibodies (Dianova, Hamburg, Germany) followed by chemiluminescence (Western Lightning, Perkin Elmer Life Sciences).

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# Table S1: Reproducibility of the shuttling kinetics

| transfected | co-transfected | Half Rec. Time (min) | SEM |
|---|---|---|---|
| β–catenin | - | 2:12 | 0:12 |
| β–cateninS45A | - | 2:07 | 0:09 |
| mYFP | - | 1:09 | 0:07 |
| YFP-CFP | - | 4:51 | 0:36 |
| β–cateninΔN | - | 2:52 | 0:10 |
| β–cateninΔC | - | 4:12 | 0:16 |
| β–cateninΔNC | - | 5:02 | 0:28 |
| β–cateninNESNLS | - | 0:40 | 0:06 |
| β–cateninINES | - | 1:03 | 0:08 |
| β–cateninΔarm2-5 | - | 2:09 | 0:13 |
| β–catenin | CFP-APC | 2:35 | 0:11 |
| β–catenin | CFP-axin | 2:22 | 0:16 |
| β–catenin | CFP-axin2 | 2:20 | 0:08 |
| β–catenin | CFP-axinΔC | 2:19 | 0:05 |
| β–catenin | CFP-BCL9v | 4:19 | 0:05 |
| β–catenin | CFP-BCL9fs | 3:17 | 0:21 |
| β–catenin | CFP-BCL9v/RFP-Pyg2 | 4:19 | 0:16 |
| BCL9v | - | 9:30 | 0:20 |
| β–catenin | E-cadherin | 2:19 | 0:46 |
| β–catenin | TCF-CFP | 3:17 | 0:07 |

Mean Half Recovery Times (HRTs) and standard error (SEM) (minutes) of compartment bleach experiments with 8-12 cells each; HRTs were calculated as $t_{½} = \frac{\ln(½)}{k}$ with the curves fitted to the equation $fluorescence = 1 - e^{(k \cdot time)}$.

| transfected | co-transfected | mean HRT cyto (sec) | SEM | mean HRT nuc | SEM |
|---|---|---|---|---|---|
| β–catenin | - | 3,4 | 0,3 | 2,4 | 0,2 |
| β–cateninS45A | - | 2,8 | 0,3 | 2,6 | 0,3 |
| YFP | - | 1,7 | 0,6 | 0,9 | 0,2 |
| YFP-CFP | - | 2,8 | 0,3 | 0,9 | 0,1 |
| β–catenin | CFP-APCfs | 7,8 | 1,7 | - | - |
| APCfs | - | 7,6 | 1,1 | - | - |
| β–catenin | CFP-axin | 7,2 | 1,3 | - | - |
| axin | - | 12,7 | 2,0 | - | - |
| β–catenin | CFP-axin2 | 11,1 | 2,2 | - | - |
| axin2 | - | 25,4 | 6,6 | - | - |
| β–catenin | CFP-axinΔC | 3,0 | 0,1 | - | - |
| axinΔC | - | 2,9 | 0,4 | - | - |
| β–catenin | TCF-CFP | - | - | 5,6 | 1,1 |
| TCF4 | - | - | - | 11,8 | 1,7 |
| β–catenin | CFP-BCL9v | 4,5 | 0,8 | 3,3 | 0,6 |
| BCL9v | - | 3,5 | 0,5 | 3,3 | 0,6 |
| β–catenin | CFP-BCL9v + RFP-Pyg2 | 4,6 | 0,6 | 2,6 | 0,4 |
| Pygopus2 | - | - | | 1,6 | 1,2 |
| β–catenin | E-cadherin | 8,0 | 1,2 | - | - |

Mean Half Recovery Times and standard error (seconds) of point bleach experiments in the indicated compartments with at least 5 cells; HRTs were determined directly from the individual recovery curves.