# Intracellular routing of β-catenin

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## Chapter 5

# Rapid nuclear export of GFP-β-catenin via a facilitated mechanism independent of CRM1

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# Rapid nuclear export of GFP-ß-catenin via a facilitated mechanism independent of CRM1

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**Stimulation of cells with Wnt results in nuclear translocation of ß-catenin and transcriptional activation of target genes. Export of ß-catenin out of the nucleus could be an important mechanism to end transcriptional activation. Currently, there are two models for ß-catenin nuclear export. The first suggests that nuclear export of ß-catenin is mediated by co-transport of APC (or Axin) via the CRM1 nuclear export pathway. The second model predicts that ß-catenin mediates its own nucleocytoplasmic transport. We analyzed the in vivo nuclear export kinetics of GFP-ß-catenin in Hek293 cells using Fluorescence Loss In Photo bleaching (FLIP). We show that GFP-ß-catenin nuclear export is very fast and exceeds the nuclear exit by diffusion of the small GFP molecule, suggesting that GFP-ß-catenin uses a fascilitated nuclear export mechanism. Furthermore, we find that nuclear export of GFP-ß-catenin is not influenced by leptomycin B (LMB) treatment that inhibits CRM1-mediated export. We find that ß-catenin interacts with FG repeat nucleoporins Nup62, Nup153, Nup214 and Nup358. We conclude that ß-catenin mediates its own nuclear export, supporting the idea that its localisation is regulated by retention.**

The Wnt family of secreted signaling molecules regulate numerous processes during animal development and tissue homeostasis. Deregulation of the Wnt pathway is linked to many diseases including cancer (Nusse, 2005; Clevers, 2006). In canonical Wnt signaling, Wnt signals via ß-catenin to transduce the signal from the plasma membrane to the nucleus. In the nucleus, ß-catenin interacts with transcription factors of the TCF/Lef family and the co-activators Pygopus and Legless to regulate the expression of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997; Takemaru and Moon, 2000; Townsley et al., 2004).

Nuclear import and export may control the availability and thereby the activity of ß-catenin. ß-Catenin has a molecular weight of 92 kDa and is therefore expected to depend on the importin/exportin system for nucleo-cytoplasmic transport. However, ß-catenin contains no recognizable nuclear localization signal (NLS), or nuclear export signal (NES), which are required for receptor-mediated nucleo-cytoplasmic transport (Mattaj and Englmeier, 1998; Gorlich and Kutay). ß-Catenin can shuttle in and out of the nucleus (Fagotto et al., 1998; Yokoya et al., 1999; Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001; Suh and Gumbiner, 2003; Koike et al., 2004). Micro-injection and semi-permeabilized cell assays have shown that ß-catenin does not need Importin-ß, CRM1 or RanGTP to exit the nucleus (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). Structural similarities between the ß-catenin armadillo repeats and the HEAT repeats of importins, have led to the hypothesis that ß-catenin mediates its own transport by interacting directly to the NPC proteins (Cingolani et al., 1999; Fagotto et al., 1998; Yokoya et al., 1999; Wiechens and Fagotto, 2001). Nucleocytoplasmic transport of ß-catenin is, however, still under debate. An alternative model specifies, that the APC tumour suppressor exports ß-catenin out of the nucleus, resulting in the degradation of ß-catenin in the cytoplasm. This model is based upon the observation that APC shuttles between the nucleus and cytoplasm, and that ß-catenin mimics the localization of APC containing either active or inactive NESs (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). Similar to APC, Axin and LZTS2 shuttle in and out of the nucleus, and a role for these proteins in ß-catenin nuclear transport has been suggested (Cong and Varmus, 2004; Thyssen et al., 2006).

In this study, we examined the nuclear export kinetics of GFP-ß-catenin in living cells, by performing *in vivo* nuclear transport assays in

Hek293 cells. We expressed GFP-tagged ß-catenin and performed Fluorescence Loss In Photo bleaching experiments (FLIP). We found that nuclear export of GFP-ß-catenin is fast, as full recovery of steady state levels is observed within 3 minutes. Furthermore, inhibition of the CRM1 pathway does not affect GFP-ß-catenin nuclear export. Furthermore, we show that ß-catenin interacts with FG repeat nucleoporins and that coupling of GFP to ß-catenin increases the nuclear export rate of GFP. We conclude that ß-catenin exits the nucleus on its own by interacting with FG repeat nucleoporins, and may act as a transport receptor itself.

## Results and discussion

**GFP-ß-catenin is active in Wnt signaling**

To analyze the kinetics of ß-catenin in living cells, we expressed a carboxy-terminal fusion of GFP to ß-catenin in Hek293 cells. In a previous study, this GFP-ß-catenin fusion protein formed rod-like aggregates in the nuclei of COS and MDCK cells (Giannini et al., 2000). We observed these aggregates as well in the nuclei of Hek293 cells but only when high levels of the plasmid were transfected and/or after long expression times (data not shown). It is conceivable that these ß-catenin aggregates are formed to protect the cell from toxic levels of GFP-ß-catenin (Giannini et al., 2000). To prevent the formation of these aggregates in the nucleus, we expressed GFP-ß-catenin at very low levels for a short period (i.e. 100 ng per $3.10^5$ cells for 20 hours). Under these conditions, the intracellular distribution of GFP-ß-catenin is uniformly distributed between the nucleus and cytoplasm, yet the nucleoli were excluded (Fig 2A left image and data not shown).

To determine the expression of our GFP-ß-catenin fusion protein, we transfected Hek293 cells and analyzed cell lysates 24 hours after transfection by western blot. An anti-GFP monoclonal antibody detected only full length GFP-ß-catenin (~115 kDa) and no free GFP (~25 kDa), showing proper expression of the GFP-ß-catenin fusion protein (Fig 1A). We next tested whether the GFP-ß-catenin fusion protein is functional in Wnt signaling by performing luciferase assays using the TCF-responsive TOP reporter (Korinek et al., 1997). In the ß-catenin deficient cell line NCI-H28, GFP-ß-catenin activates the TOP reporter but not the control FOP (Fig 1B). These results show that the GFP-ß-catenin fusion protein is properly expressed and active in transactivation.

**ß-Catenin exists in pools of different mobility**

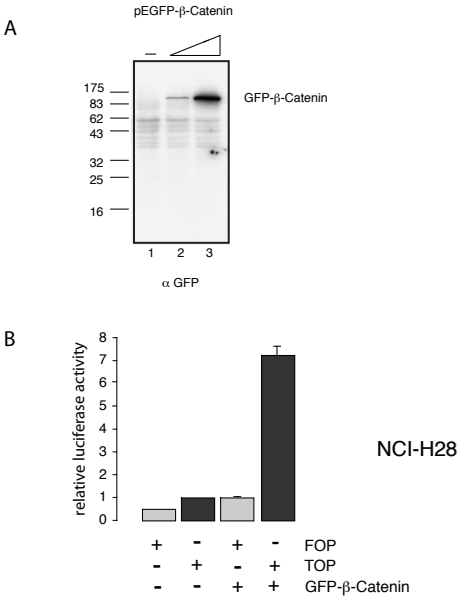


**Figure 1. GFP-ß-catenin is functional.** A. Hek293 cells were transfected with GFP-ß-catenin in increasing amounts. After 24 hours, cell lysates were analyzed by western blot using a monoclonal antibody recognizing GFP. B. NCI-H28 cells were transfected with the TOP (dark bars) or the control FOP (light bars) luciferase reporter together with pRL-CMV, in presence or absence of GFP-ß-catenin. Cell lysates were analyzed 24 hours after transfection. Shown are normalized relative luciferase values corrected with the pRL-CMV Renilla luciferase reporter. Bars represent SEMs of independent experiments.

To analyze the kinetics of ß-catenin nuclear export in living cells, we expressed GFP-ß-catenin at low levels in Hek293 cells and performed Fluorescence Loss In Photo bleaching (FLIP) experiments. In these experiments, the entire nucleus or cytosol is bleached after which time laps images are taken to monitor the recovery rate. To control the precision of the laser beam, we performed similar bleach experiments on formalin fixed cells expressing GFP-ß-catenin. A well-defined spot was permanently bleached showing that there was no leakage of the laser beam to regions outside our indicated region of interest (data not shown). Many nuclear proteins, including transcription factors are highly mobile as measured by FRAP analysis (Fluorescence Recovery After Photo bleaching). Although these proteins move rapidly, their movements are not as fast as a free diffusing molecule such as GFP (Misteli, 2001).

We noticed that the mobility of GFP-ß-catenin in both the nucleus and cytoplasm was very high, as we could bleach either compartment effectively and uniformly by using 4 point bleaches of 3 seconds, suggesting that during this short bleaching time, all GFP-ß-catenin molecules in the bleached compartment had passed the laser beam.
Typically, after bleaching, fluorescence dropped in the bleached compartment to ~80%, whereas the compartment that was not bleached lost only ~20% (data not shown). To measure nuclear export, we bleached the whole cytoplasm and used time-lapse microscopy to monitor the recovery. The subsequent increase in cytoplasmic fluorescence of GFP-ß-catenin was equal to the decrease in nuclear fluorescence, suggesting that the cytoplasmic increase was the result of nuclear transport events. From the recovery curves, we calculated the tau value, i.e. the time needed for the fluorescence to recover to 63% between its level after bleach and the plateau

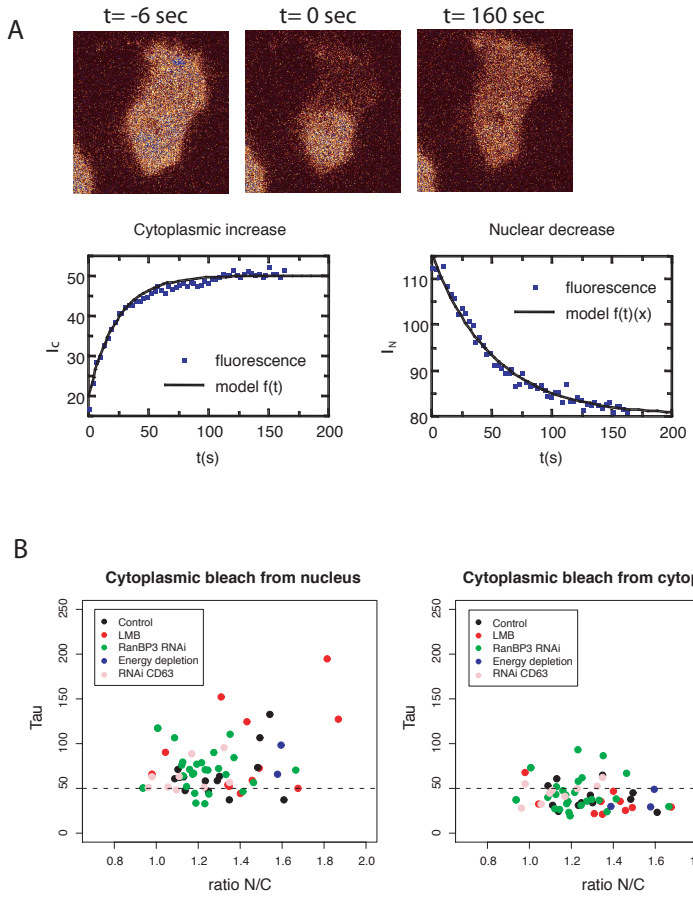


**Figure 2. Time-lapse confocal images and recovery curves of a representative FLIP experiment.** A. Hek293 cells were transfected with GFP-ß-catenin for 24 hours, after which FLIP experiments were carried out in imaging medium at 37°C. Two initial images (T = -6 sec) were taken after which the cytoplasm was bleached using 4 x 3 sec point bleaches. T = 0 is the first image of the time lapse that was used to monitor the fluorescent recovery. Steady state levels were reached within 160 seconds. Recovery curves were corrected for bleach depth, normalized and fitted to a single exponential function. B. Time constants (tau) of multiple experiments and experimental conditions were plotted against the nuclear/cytoplasmic (N/C) ratio of GFP-ß-catenin before bleaching. Observed from the cytoplasm, taus from a cytoplasmic bleach are close to 40 seconds independent of the N/C ratio (right). From a nuclear view, taus are higher and increase as the N/C ratio rises (left).

fluorescence (Fig 2A).
To measure export, we bleached GFP in the cytoplasm and measured the recovery time in both the nucleus and cytoplasm. The recovery curves were different depending on whether measurements were taken from the nucleus or the cytoplasm. Plotting the tau values of the recovery curves from the nucleus against the nuclear/cytoplasmic ratio, reveals that there are two pools of GFP-ß-catenin; a slow and a fast pool. The slow pool dominates export measured from a nuclear view and likely represents retention of GFP-ß-catenin in the nucleus, whereas the fast pool represents free GFP-ß-catenin. Increasing nuclear/cytoplasmic ratio's correlate with increasing tau values, suggesting that the slow pool of GFP-ß-catenin that is seen from the nucleus, is dependent on expression levels. However, when export was measured from the cytoplasm, we mostly detected the fast pool with no effects on expression levels (Fig 2B). We therefore studied GFP-ß-catenin nuclear export by measuring the fluorescent recovery in the cytoplasm.
The slow pool of GFP-ß-catenin that we observe in the nucleus may reflect nuclear retention by the formation of rod-like aggregate formation as described in Gianni et al. (Giannini et al., 2000). Nuclear levels of GFP-ß-catenin indeed rise upon higher expression levels and increased expression time. Alternatively, the slow and fast pool of GFP-ß-catenin in the nucleus may represent different molecular forms of ß-catenin that have been described earlier (Gottardi and Gumbiner, 2004). Higher levels of GFP-ß-catenin may shift the equilibrium to a molecular form of ß-catenin that is more likely to interact with nuclear proteins, such as TCF/Lef and BCL9 resulting in increased nuclear retention. Although retention is a likely mechanism to regulate ß-catenin, the different molecular conformations of ß-catenin remain speculative as no evidence for this theory was found in thermodynamic experiments on ß-catenin and its interacting partners (Choi et al., 2006).

**Nuclear export of GFP-ß-catenin is fast and mediated by a facilitated transport pathway**
To measure the export rate of GFP-ß-catenin in living cells, we bleached the cytoplasm of low expressing Hek293 cells and monitored the recovery time. Full recovery of fluorescence was observed within 160 seconds (2 min 40 sec) after bleaching, after which steady state levels were reached. We measured an averaged tau of 45 seconds, indicating that nuclear export of GFP-ß-catenin in Hek cells is fast (Fig 3). The nuclear export rate that we measure for GFP-ß-catenin in Hek293 cells is more than twice as fast as described for YFP-ß-catenin in COS cells (Townsley et al., 2004). Differences in expression levels or cell types may account for this discrepancy. To compare the nuclear export kinetics of GFP-ß-catenin to those of other export substrates, we compared export of GFP-ß-catenin to the nuclear exit of free GFP, that can freely pass through the NPC by diffusion. Interestingly, the nuclear exit of the small GFP molecule was 1.5 times slower than that of GFP-ß-catenin, indicating that GFP-ß-catenin export is very efficient and mediated by a facilitated process. To confirm these findings in another cell line, we used the human colon cancer cell line SW480, which shows constitutively active Wnt signaling due to a truncation in APC. Nuclear export of GFP-ß-catenin in SW480 was as fast as in Hek293 cells and, likewise, the nuclear exit of free GFP was much slower than that of GFP-ß-catenin (Fig 3). These results indicate that differences in tissue origin and Wnt activation between these two cell types do not result in different nucleocytoplasmic transport kinetics of GFP-ß-catenin. Furthermore, the nuclear export rate of GFP-ß-catenin is faster than the nuclear exit of free GFP, indicating that ß-catenin export is mediated by an active transport mechanism (Ben-Efraim and Gerace, 2001).

**Nuclear export of ß-catenin does not dependent of the CRM1 export pathway**
Proteins of up to 30 kDa can diffuse through the NPC, yet larger proteins generally pass by a facilitated transport process that requires Ran and recognition by transport receptors (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999; Frey and Gorlich, 2007). The CRM1 nuclear export receptor is responsible for a considerable fraction of protein export out of the nucleus and recognizes its cargo by the NES. CRM1 binds directly to the NES in a RanGTP-dependent manner (Fornerod et al., 1997; Kudo et al., 1997; Stade et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997).
To test the involvement of the CRM1 pathway in ß-catenin nuclear export, we measured the nuclear export of GFP-ß-catenin in living cells in the presence and absence of the CRM1 inhibitor LMB. The earlier described shuttling substrate NLS-Rev-NES-GFP was used to control for the activity of LMB (Henderson and Eleftheriou, 2000). This substrate localizes to the cytosol in the absence of LMB due to its NES, but it accumulates in nucleoli when LMB is added. Hek293 cells were transfected with GFP-ß-catenin or

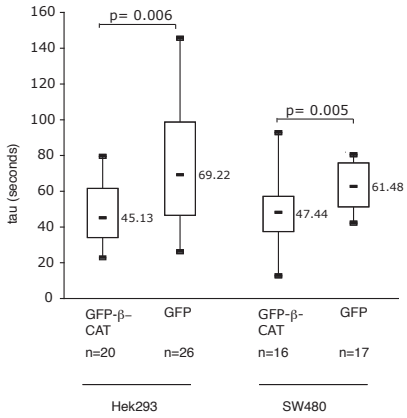


**Figure 3. Nuclear export of GFP-ß-catenin is faster than that of GFP alone.** Hek293 (left) and SW480 (right) cells were transfected with either GFP alone or with GFP-ß-catenin. After 24 hours, FLIP experiments were carried out to measure nuclear export using cytoplasmic bleaching. Kinetics of the cytoplasmic fluorescence recovery were analyzed by timelapse microscopy, corrected for bleach depth, normalized and fitted to a single exponential function. Taus are represented in a box plot. Indicated values are medians. P values are according to Mann-Whitney tests.

NLS-Rev-NES-GFP and either mock treated or incubated in imaging medium containing 50 nM LMB. Within 30 min, the NLS-Rev-NES-GFP reporter shifted from the cytoplasm to the nucleus, indicating that LMB was fully active (Fig 4A). We performed FLIP experiments on Hek293 cells expressing GFP-ß-catenin, and cultured the cells for at least 30 minutes to a maximum of 1.5 hours under LMB conditions. As shown in Figure 4B, the median tau value for GFP-ß-catenin decreased from 45 seconds without LMB to 34 seconds with LMB, but this drop was not significant (Fig 4B). This indicates that blocking the CRM1 pathway does not affect the nuclear export rate of GFP-ß-catenin. The steady state localization of GFP-ß-catenin was neither affected by LMB treatment, not even after treatment with LMB for more than 3 hours (data not shown). Our results show that in living cells, GFP-ß-catenin can exit the nucleus independently of the CRM1 nuclear export pathway. These results are in line with previous studies (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001) and are not consistent with a role of APC, Axin or LTZS2 in ß-catenin nuclear export as these proteins all rely on the CRM1 nuclear export receptor to exit the nucleus.

**ß-Catenin associates with FG repeat nucleoporins**

As GFP-ß-catenin can exit the nucleus independently of the CRM1 export pathway, we sought for evidence that ß-catenin can translocate by itself through the nuclear pore. We therefore tested whether ß-catenin can interact with FG repeat nucleoporins using immobilized GST-tagged *X. laevis* ß-catenin to pull down interacting proteins from *X. leavis* egg extracts, which are highly concentrated in nucleoporins. We analyzed interacting proteins by western blot using monoclonal antibody 414 that recognizes a subset of FG repeat containing nucleoporins. Importantly, both full length and the armadillo repeat region of ß-catenin specifically interacted with FG repeat nucleoporins Nup62, Nup153, Nup214 and Nup358. Furthermore, a small amount of Nup214 also bound full length GST-ß-catenin (Figure 5). The interaction was not stimulated or weakened by non-hydrolysable forms of RanGTP (data not shown), indicating that the ß-catenin interaction was not mediated by nuclear trans-

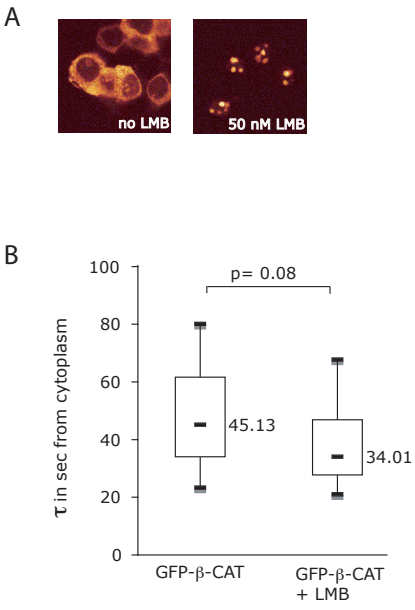


**Figure 4.** A. The CRM1 nuclear export pathway is efficiently blocked by addition of 50 nM LMB for 30 minutes. Hek293 cells were transfected with the shuttling substrate NLS-Rev-NES-GFP (Henderson and Eleftheriou, 2000). At 24 hours after transfection, cells were exposed to either normal imaging medium or imaging medium containing 50 nM LMB for 30 min. Thereafter, the cells were imaged to record the localization of the reporter. B. Blockade of the CRM1 pathway does not influence the nuclear export kinetics of GFP-ß-catenin. Hek293 cells were transfected with GFP-ß-catenin and export kinetics were determined as described in Figure 3.

port receptors. These FG repeat nucleoporins are commonly known to bind nuclear transport receptors including those of the Importin-ß and NTF2 family (Moroianu et al., 1995; Bayliss et al., 2000; Fribourg et al., 2001; Conti and Izaurralde, 2001; Vasu and Forbes, 2001; Bayliss et al., 2002). As ß-catenin shares structural homology with Importin-ß, it is interesting that it interacts with these common nucleoporins. Our results are in line with a previous study in which recombinant *X. laevis* ß-catenin was shown to bind to the yeast Nup1 (Fagotto et al., 1998). Our data contradict a study (Stuh and Gumbiner, 2003) in which ß-catenin was tested for its ability to bind to the FG nups recognized by mAb 414, i.e. the same nups as tested here. In that study, the relative binding of ß-catenin to FG repeat nups was compared to that of Importin-ß, but no interaction could be detected (Suh and Gumbiner, 2003). The affinity of Importin-ß for FG repeat nups is very high compared to other transport receptors (Ben-Efraim and Gerace, 2001). Therefore, it is imaginable that the relative weak interaction of ß-catenin with FG nups is below detection limits

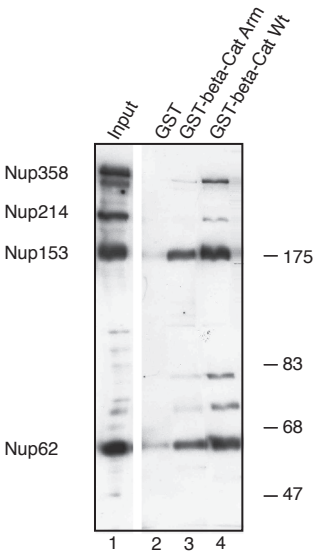


**Figure 5. ß-Catenin interacts with FG- rich nucleoporins Nup62, Nup153, Nup214 and Nup 358** *in vitro***.** GST (lane 2), GST-ß-catenin Arm (lane 3) and GST-ß-catenin (lane 4) were used to pull down interacting nucleoporins from *X. laevis* egg extracts in the presence of 2 μM RanQ69L. Bound proteins were analyzed by western blot using monoclonal antibody 414, recognizing FG repeat nucleoporins.

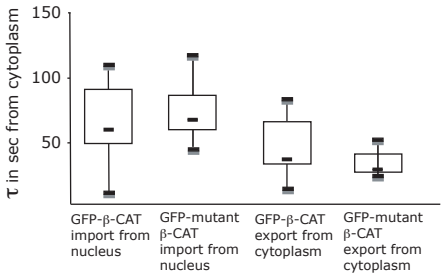


**Figure 6. Nuclear import and export kinetics of GFP-tagged ΔGSK3 mutant ß-catenin are not different from wild type GFP-ß-catenin.** Hek293 cells were transfected with either GFP-ß-catenin or GFP-ΔGSK3 ß-catenin. After 24 hours, import and export kinetics were measured. To measure import, the nucleus was bleached followed by monitoring the recovery of fluorescence in the nucleus. Transport kinetics were measured, analyzed and represented as in Figure 3.

when compared to the strong affinity of Importin-ß for FG nups. Moreover, weak interactions of transport receptors with FG repeats have been suggested to play an important role for efficient translocation through the inner channel of the NPC (Frey et al., 2006; Frey and Gorlich, 2007). In line with our observation that ß-catenin can exit the nucleus independent of CRM1, we find that ß-catenin interacts with FG repeat nups, suggesting that ß-catenin mediates its own translocation through the NPC.

**ß-Catenin nuclear export is insensitive to GSK3ß phosphorylation**

Our data so far have shown that GFP-ß-catenin can exit the nucleus by itself, most likely by mediating its own nuclear export by interacting with the FG repeat nucleoporins. In recent years, it has been shown that not all ß-catenin molecules are equally active in transcriptional activation. Inhibition of ß-catenin degradation by abolishing proteosomal degradation results in increased ß-catenin levels, but not in increased transcription. However, blocking the phosphorylation of ß-catenin on its N-terminal GSK3/CK1 phosphorylation sites does increase transcription. The use of the ABC antibody, which specifically recognizes ß-catenin that is not phosphorylated on its N-terminus, has been shown to report Wnt signaling activity more faithfully than antibodies directed against an epitope elsewhere in the protein (Staal et al., 2002; Chan et al., 2002; Hendriksen et al., 2005).

To test whether N-terminally dephosphorylated

ß-catenin exhibits distinct nucleocytoplasmic shuttling behavior, we mimicked dephospho-ß-catenin by using the ΔGSK3 mutant ß-catenin in which all 4 GSK3 phosphorylation sites on its N-terminus are mutated to alanine. We tagged this protein to GFP and performed FLIP experiments to study its nuclear transport kinetics. In addition to measuring export, we also measured nuclear import of this substrate and compared it to the import of wild type GFP-ß-catenin. To measure import, we bleached the nucleus and analyzed the subsequent nuclear increase in fluorescence. We did not measure any significant differences in tau values of both the nuclear import and export between ΔGSK3 mutant and wild type GFP-ß-catenin (Fig 6). These results suggest that our ΔGSK3 mutant GFP-ß-catenin, which is more active in Wnt signaling, enters the nucleus as fast as GFP-ß-catenin. Furthermore, the results imply that N-terminal phosphorylations on positions 33, 37, 41 and 45 do not affect the ability of ß-catenin to interact with FG repeats to mediate its nuclear transport. However, as alanine mutations are not the same as natural non-phosphorylated residues, it is not clear whether this mutant is a good representative of active or dephospho-ß-catenin. As long as the exact nature of transcriptionally active or dephospho-ß-catenin remains elusive we cannot be totally sure whether the nuclear import of this protein is enhanced or its export decreased. Preliminary experiments using LiCl to block ß-catenin phosphorylation, however, did not show any differences in nuclear export kinetics either (data not shown).

In this study, we have confirmed that GFP-ß-catenin can exit the nucleus independently of CRM1 and hence, independently of APC and Axin (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). We suggest that ß-catenin interacts with FG repeat nucleoporins to mediate its own nuclear export, which explains why GFP-ß-catenin nuclear export is very efficient even during LMB treatment. Moreover, coupling GFP to ß-catenin increases the nuclear export rate of GFP, suggesting that ß-catenin not only exits the nucleus similar to the transport receptors, but may also carry along substrates. Indeed, it has recently been shown that Lef1 can function as a natural nuclear import substrate for ß-catenin (Asally and Yoneda, 2005). It is fascinating that not only the mobility of GFP-ß-catenin within the cytoplasm and nucleus is very high, but also that GFP-ß-catenin is capable of shuttling quickly between these two compartments. Therefore, ß-catenin seems capable of relocalizing quickly in the cell to meet its binding partners. A previously proposed model of retention seems applicable here (Tolwinski and Wieschaus, 2001). In this model, the binding partners of ß-catenin regulate its subcellular localization and therefore its activity. E-cadherin binds ß-catenin at the plasma membrane, APC, Axin and Dvl in the cytoplasm, and BCL9 and TCF in the nucleus. Therefore, Wnt signaling could regulate the availability of these pools of ß-catenin allowing the cell to respond quickly to the extracellular Wnt signal.

## Materials and Methods

**Plasmids**

GST-ß-catenin and GST-ARM ((Wiechens and Fagotto, 2001)), GFP-ß-catenin (Giannini et al., 2000), pSUPER, pSUPER-RanBP3, Top-Tk and Top-Tk, pRL-CMV Renilla plasmids were previously described (Hendriksen et al., 2005). pEGFP-N1 was obtained from Clontech.

***In vitro* binding studies**

GST-ß-catenin binding studies were performed as described before (Hendriksen et al., 2005).

**Luciferase reporter assays**

NCI-H28 cells were cultured in 12-wells plates and transfected with 200 ng Top/Fop-Tk, 1 ng pRL-CMV Renilla and 25 and 100 ng GFP-ß-catenin. Luciferase activity was measured 48 h after transfection using the Dual-luciferase reporter assay system (Promega).

**Western blotting**

To detect GFP-ß-catenin 25 µg cell lysate was analyzed by SDS-PAGE. Western blotting was performed as described before (Hendriksen et al., 2005).

**Cell culture, transfection and photo bleaching experiments**

All cell lines were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) and were transfected using Fugene-6 (Roche) or Lipfofectamine (Invitrogen) as instructed by the supplier. For FLIP experiments, 3.105 cells were grown on 40 mm coverslips and transfected with 100 ng pEGFP, GFP-ß-catenin or ΔGSK3-ß-catenin, up to 500 ng in total using pcDNA3 as stuffer DNA. FRAP experiments were performed 24 hours after transfection using a live cell chamber at 37°C in bicarbonate-buffered saline (containing: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 23 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES at pH 7.2).

Photo bleaching and imaging was done on a confocal laser scanning microscope (SP2 TCS AOBS, Leica). Cells expressing low amounts of tagged-ß-catenin were selected and imaged at 4% laser power using the 488 laser line of the 20 mW argon laser and bleached at 100% laser power. The cytoplasm was bleached uniformly by using 4 point-bleaches of 3 seconds each. Using Leica time lapse, 2 images were taken before bleach using an interval of 1.68 seconds, after bleach 50 frames were imaged using a 3 second interval. Averaged intensities of regions of interest were measured using Image J and recovery curves and taus were determined using R software. LMB was used at 50 nM for 30 min up to 1.5 hours (Wolff et al., 1997). To control for LMB activity, cells were transfected with 1 µg of NLS-Rev-NES-GFP per 40 mm coverslip (Henderson and Eleftheriou, 2000).

**Data analyis**

Statistical analysis was done using the R software package (R Development Core Team, 2005). Nuclear and cytoplasmic decay curves were fitted to a single exponential function I = I0 - A * (1 - exp(-t/tau)), using the optim() function in R. Quality of the fit was assessed by "goodness of fit" (R2) = 1- (sum of squared residuals) /(sum of squared differences from mean), where 1 equals a perfect fit and 0 no fit. A small number of fits with an R2 of < 0.8 were not used in further analysis. The low quality of these fits could be traced back to movement of cells during recording or very low signal to noise. The mean R2 of data excluding these outliers was 0.97.

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