CRM1- and Ran-independent nuclear export of β-catenin

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Background: Activation of the Wnt pathway induces β-catenin to localize inside the nucleus, where it interacts with transcription factors such as TCF/LEF-1. Regulation of the pathway occurs through a β-catenin–degrading complex based on Axin and the tumor suppressor APC. We have previously found that β-catenin import occurs independently of nuclear import factors but is similar to the import of the transport factors themselves do. APC, which can shuttle in and out of the nucleus, has been proposed to be responsible for reexport of β-catenin in a CRM1-dependent manner.

Results: We have studied β-catenin export in vivo and in semipermeabilized cells. β-catenin contains three export sequences. Export is insensitive to leptomycin B, a specific inhibitor of the CRM1-mediated pathway. It does not require nuclear RanGTP, and it can be reconstituted in the absence of additional soluble factors; this is consistent with nondirectional translocation of β-catenin. Further observations suggest that β-catenin subcellular distribution in vivo may depend primarily on retention through interaction with other cellular components. Finally, we show evidence that reexport is required for degradation of nuclear β-catenin and that nuclei lack Axin, an essential component of the degradation machinery.

Conclusions: β-catenin is exported independently of the CRM1 pathway. We propose a model of free, nondirectional nuclear translocation for β-catenin, its localization being regulated by retention in the nucleus and degradation in the cytoplasm.

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Received: **25 September 2000**
Revised: **10 November 2000**
Accepted: **17 November 2000**

Published: **9 January 2001**

Current Biology 2001, 11:18–27

0960-9822/01/$ – see front matter

Background

A crucial step in the transduction of the wingless/Wnt signal is the localization of β-catenin to the nucleus. In unstimulated cells, β-catenin is mostly confined to the plasma membrane, where it functions in cell-cell adhesion through its association with cadherins [1, 2]. Levels of free cytosolic β-catenin are kept very low by an efficient phosphorylation/ubiquitination machinery, which involves a protein complex composed of Axin, glycogen-synthase kinase-3 (GSK-3) and adenomatous polyposis coli (APC) protein (reviewed in [3, 4]). Upon activation of the Wnt pathway, degradation of β-catenin is inhibited, and free β-catenin accumulates and enters the nucleus, where it interacts with the transcription factors TCF/Lef-1 (T cell factor/lymphocyte-enhancer factor-1) and modulates their activities [5]. Nuclear localization of β-catenin is transient [6–8], which implies that it is either degraded in the nucleus or reexported.

The mechanisms of protein transport into and out of the nucleus have recently received intense scrutiny, and unifying features have emerged; both import and export pathways use specific signal sequences on the substrate, and these sequences are recognized by soluble receptors. These receptors, generically called "importins/exportins" or "karyopherins," interact directly with the nuclear pore complex and shuttle between the cytoplasm and the nucleus (for reviews see [9–12]). The "classical" import pathway involves binding of a basic amino acid–rich nuclear localization signal (NLS) to a heterodimeric receptor complex composed of importin α (karyopherin α) and importin β (karyopherin β or p97). Other nonconventional NLS (such as M9 in hnRNPA1) bind to specific receptors (transportin for M9) that are related to importin β. After translocation, the import receptors are recycled, importin α via its export factor CAS, importin β and transportin on their own. Many export substrates bear a leucine-rich nuclear export signal (NES) that binds the export receptor CRM1 (exportin 1/Xpo1). Other exportins that have been recently identified are specific for particular substrates. Exportin-t, for example, is responsible for tRNA export.

Importins and exportins constitute a superfamily of related molecules that bear a conserved Ran binding site. Ran is a small GTPase, the activity of which is crucial for import and export [13, 14]. The concentration of Ran in its GTP bound form is high in the nucleus and very low in the cytoplasm. This gradient across the nuclear pore complex (NPC) is maintained by the differential localization of the nucleotide exchange factor RCC1 in the nucleus and by that of the GTPase-activating protein RanGAP1 in the cytoplasm [12]. Hence, the concentration of RanGTP regulates the compartment-specific binding of the receptor to its cargo; the importin α/β complex binds

its substrate in the cytoplasm in the absence of RanGTP. Upon translocation, nuclear RanGTP binds importin β, which binding leads to dissociation of the importin α/β complex [11] and recycling of importin β to the cytosol. In contrast, the export factors CRM1 and CAS bind tightly to their cargo only through cooperative association with RanGTP in the nucleus [11]. Although nuclear transport is globally energy-dependent, recent results suggest that ATP/GTP hydrolysis may not be required for translocation through the NPC per se but only for the alternate binding and release of transport complexes through the Ran cycle [15–17].

β-catenin enters the nucleus by using a rather unconventional mechanism [18–19]. Unlike NLS-mediated import, β-catenin import appears to be independent of the importin α/β receptor. Instead, β-catenin can interact directly with the NPC and can translocate on its own. Furthermore, β-catenin import can be competed by importin β, and this indicates that both import pathways intersect. β-catenin and importins are structurally related because they contain similar periodic 42 amino acid repeats [20], called arm/HEAT repeats. The arm repeats are necessary and sufficient for β-catenin nuclear localization [21]. We have thus hypothesized that arm repeat–containing molecules might have related mechanisms of nuclear transport [18].

Export of β-catenin has also been observed [19, 22], but the mechanism remains unclear. Two recent papers have implicated APC in this process [23, 24]; APC can enter the nucleus (by an uncharacterized mechanism), and it is rapidly reexported. APC export is sensitive to leptomycin B, a specific inhibitor of the CRM1-dependent pathway [23–25]. Because distribution of β-catenin seemed to match APC localization under various conditions, it has been proposed that β-catenin is exported along with APC [23, 24]. However, these conclusions were based on the analysis of steady-state distribution of β-catenin, while export of β-catenin had not been directly examined. We present here the analysis of β-catenin export and show that it occurs by a leptomycin B–insensitve, RanGTP-independent mechanism.

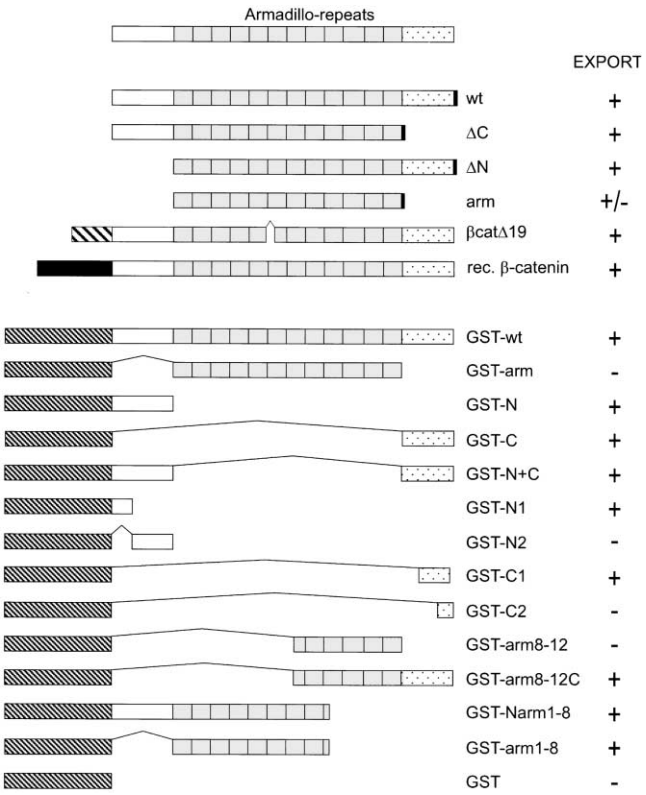
Results

Nuclear export of β-catenin occurs via the N-terminal and C-terminal domains

We have studied nuclear export of β-catenin in *Xenopus* oocytes, a well-established assay for protein and RNA export [26]. ^{35}S-labeled β-catenin protein was microinjected into the oocyte nucleus. At various time points, we manually dissected the oocytes to separate the nuclei from the rest of the oocyte. The nuclear and the cytoplasmic fractions thus obtained were analyzed by SDS-PAGE and fluorography. Because some degradation of β-catenin occurs in the cytoplasm, total extracts from undissected oocytes were also loaded for comparison. Note that the loading of the gels (1 nucleus: 1 cytoplasm) allows comparison of amounts (nuclear + cytoplasmic = total) but does not reflect the relative concentrations in the two compartments as the volume of the nucleus is obviously much smaller. We observed that β-catenin (wild type) was efficiently exported, as seen by the shift from the nuclear to the cytoplasmic fraction (Figure 2a). Fifty percent of β-catenin was exported within 1 1/2 hr. Hemoglobin, abundant in the reticulocyte lysate, was completely retained within the nucleus (not shown), and this demonstrated the integrity of the nuclei injected during our experiments.

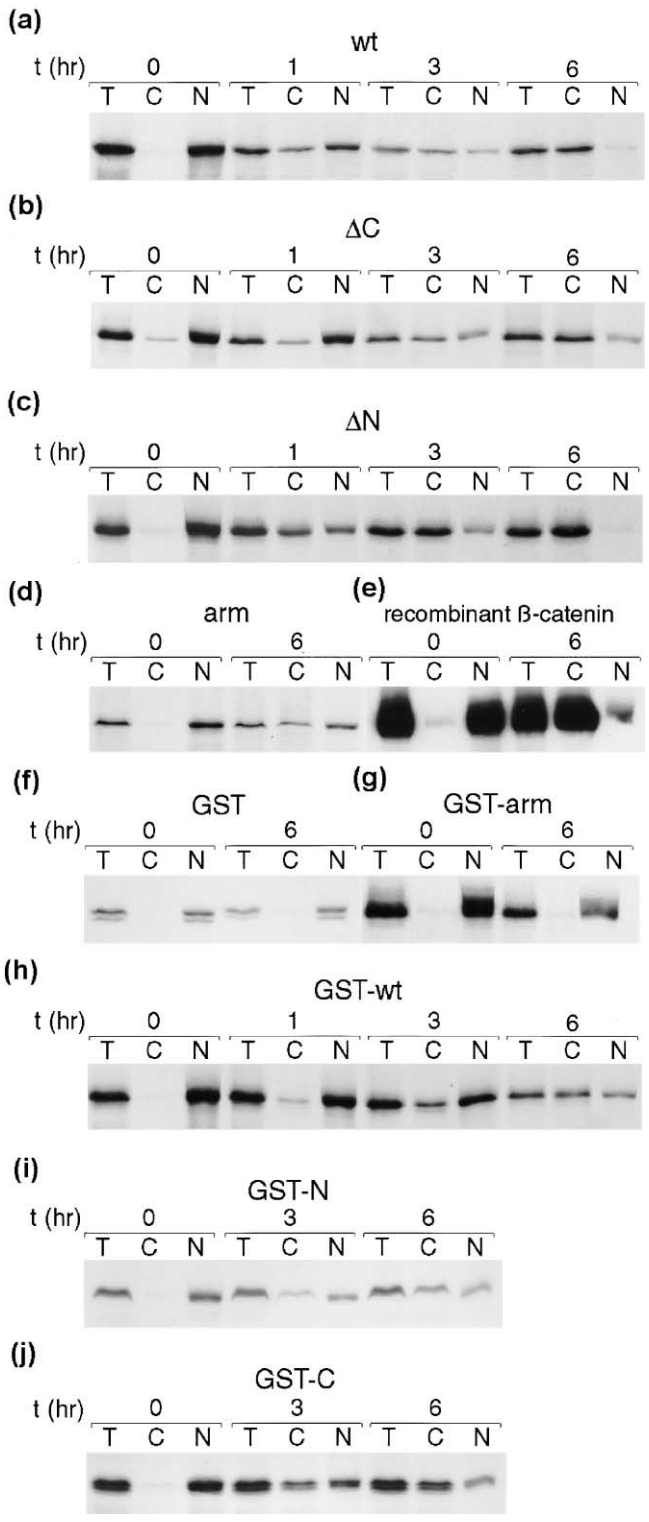
We determined the sequence requirements for β-catenin export by testing a series of deletion mutants (Figures 1 and 2). β-catenin can be subdivided into three major domains, as follows: the N-terminal domain, the central 12 arm repeats, and the C-terminal domain (Figure 1). Variants lacking the C terminus (ΔC) or the N terminus (ΔN) (Figure 2b,c) were still efficiently exported; ΔN had

Figure 1



Summary diagram of the β-catenin constructs used in this study. The three domains of β-catenin are indicated as follows: N terminus (white), 12 arm repeats (grey), and C terminus (dotted). The wild type, ΔC, ΔN, and arm contained a C-terminal hemagglutinin epitope tag sequence (black). βcatΔ19 was N terminally Myc-tagged (boldly crosshatched). Recombinant β-catenin was N terminally His-tagged (black). The other variants were fused to glutathione-S-transferase (GST, crosshatched). GST alone was used as a control. The results of the nuclear export assay are summarized by plus or minus signs.

Figure 2

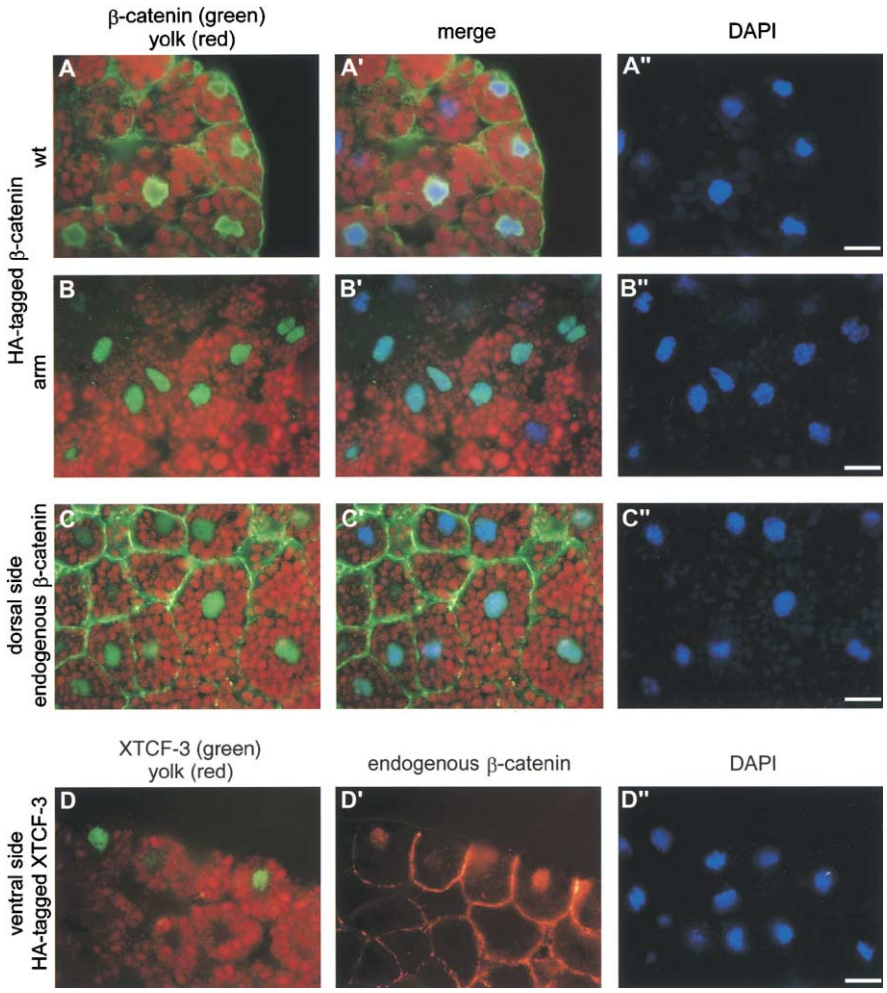


Identification of the domains responsible for nuclear export of β-catenin**. (a)** ^{35}S-labeled, in vitro–translated full-length β-catenin, **(b–d, g–j)** β-catenin variants, and **(e)** bacterial recombinant β-catenin were injected into *Xenopus* oocyte nuclei. Extracts from total oocytes (T) or cytoplasmic (C) or nuclear (N) fractions were prepared immediately after injection (0 hr) or after 1, 3, or 6 hr incubation and analyzed by SDS-PAGE and fluorography (western blot for [e] recombinant β-catenin). (a) Full-length β-catenin (wt) and (b, c) mutants lacking the C terminus (ΔC) or the N terminus (ΔN) were rapidly exported, but (d) the arm repeats alone showed only a very slow export. (e) Export of recombinant β-catenin (3 mg/ml) was complete within 6 hr. **(f, g)** Neither GST alone nor GST fused to the arm repeats (GST-arm) showed any significant export. (h) GST fused to full-length β-catenin (GST-wt) was also exported, although more slowly than β-catenin alone. (i, j) Fusion of the N terminus (GST-N) or the C terminus (GST-C) was sufficient to cause export of GST.

kinetics similar to those of full-length β-catenin, and ΔC export was slightly slower. Export of the arm repeats alone (arm, Figure 2d) was very slow. Because constructs smaller than 60 kDa may in principle freely diffuse through the nuclear pore, we fused all our small fragments, including arm (approximately 55 kDa), to glutathione-S-transferase (GST). Despite its small size (27 kDa), GST does not diffuse through the nuclear pore (Figure 2f). We verified that full-length β-catenin fused to GST (GST–wild type) was still exported (Figure 2h), although more slowly than the wild type alone (50% export in about 4 hr). GST fused to the central arm repeats (GST-arm, Figure 2g) did not show any significant export. On the contrary, the N terminus (GST-N) and the C terminus (GST-C) were both able to promote export of GST (Figure 2i,j). We further localized the N-terminal and C-terminal export activities, respectively, within the first 48 amino acids (GST-N1) and between amino acids 701 and 770 (GST-C1) (see Supplementary material). Note that neither fusion of GST C-terminal to the β-catenin N or C terminus (not shown), addition of a "spacer" such as in the GST-arm8–12C construct, nor juxtaposition of the C terminus to the N terminus (see Supplementary material) altered the export of the termini. Rapid export was also observed for mutant Δ19 (see Supplementary material), a β-catenin variant with a deletion in the arm repeats that ablates its binding to APC [22]. We conclude that export of β-catenin relies on two redundant signals located in the N and C termini. The C-terminal signal appears to be the most efficient one and can largely account on its own for the full export (compare ΔN with the wild type and GST-C with GST–wild type).

When analyzing the localization of overexpressed β-catenin in *Xenopus* embryos, we observed that full-length β-catenin was enriched along the nuclear rim but, in most cells, did not accumulate significantly inside the nucleus (Figure 3a). In contrast, the arm repeat domain alone (arm) always accumulated strongly in the nucleoplasm and was not detected at the nuclear membrane (Figure 3b). These data suggest that in embryos, as well, full-length β-catenin can shuttle in and out of the nucleus while the central region alone remains trapped inside the nucleus.

Figure 3



The N and C termini influence steady-state distribution of β-catenin. Full-length β-catenin (wt), the arm repeat domain (arm), or XTCF-3 were expressed in early *Xenopus* embryos. Exogenous proteins (all were HA tagged) and endogenous β-catenin were detected by indirect immunofluorescence. Chromatin was counterstained with DAPI, and the yolk was counterstained with Eriochrome Black. **(a)** The wild-type β-catenin localized throughout the cell, but was mainly enriched at the plasma membrane and at the nuclear rim. Nucleoplasmic levels were low in most cells. **(b)** Arm mainly accumulated in the nucleus. Cytoplasmic levels were low, and no signal was observed at the nuclear membrane. **(c)** In the dorsal side of the early *Xenopus* embryo, where the Wnt pathway is activated, endogenous β-catenin accumulates inside the nucleus in addition to the constitutive localization at the plasma membrane. **(d)** XTCF-3 overexpression induces nuclear accumulation of endogenous β-catenin even in the ventral side, where the Wnt pathway is not active. The scale bar represents 20 μm.

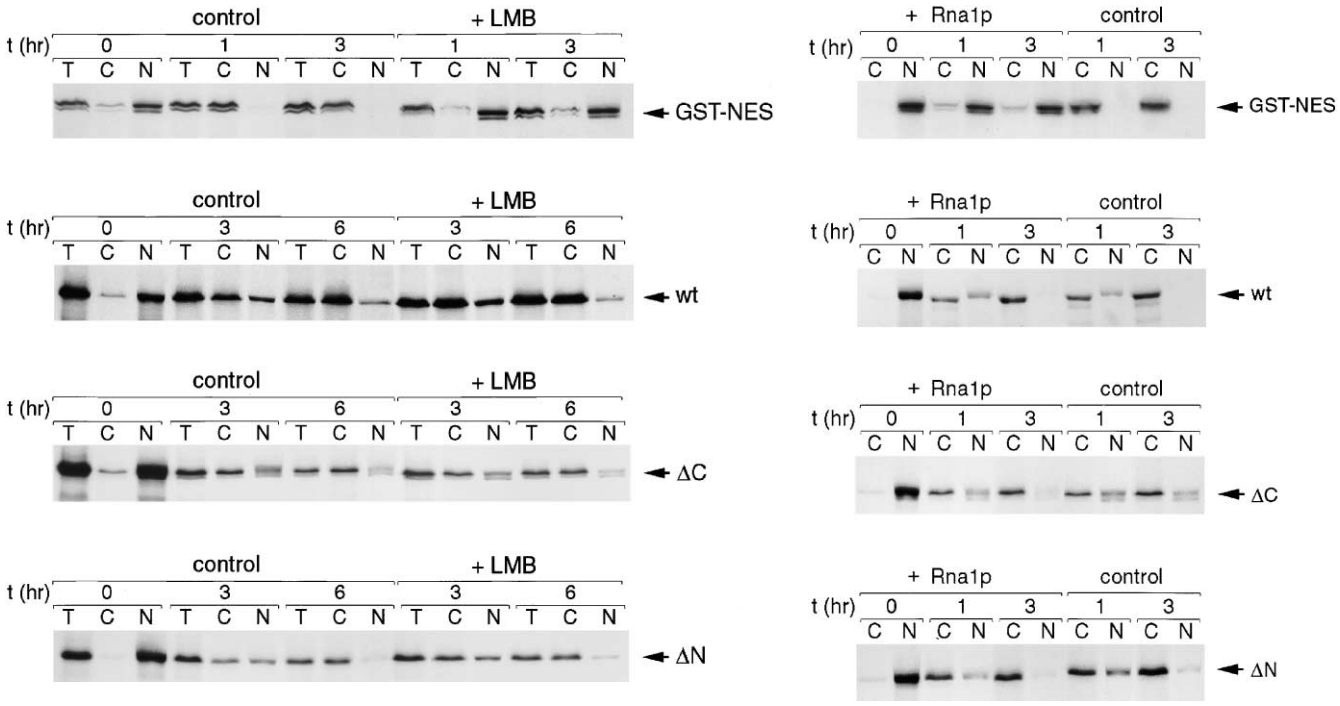
β-catenin export is saturable but independent of CRM1 and Ran

Export of wild-type β-catenin and of the individual termini GST-N and GST-C was strongly inhibited by coinjection of excess unlabeled recombinant β-catenin (see Supplementary material), and this indicates that β-catenin export involves specific interactions with the export machinery. In the "classical" export pathway, the substrate interacts through a leucine-rich NES with CRM1. No obvious classical NES motif can be detected in the β-catenin sequence, but it is conceivable that β-catenin could contain a noncanonical NES [27–29] or be piggy-backed by another NES-containing protein, such as APC. We found that an excess of NES substrate did not lessen β-catenin export (see Supplementary material). We also tested the effect of leptomycin B (Figure 4a), a specific inhibitor of CRM1-mediated export [30]. Leptomycin B, which strongly inhibited ^{35}S-labeled GST-NES, had no effect on wild-type β-catenin export.

Another key principle of "classical" export is Ran requirement. Both CRM1 and CAS bind tightly to their substrates only through cooperative binding with RanGTP. After export of the complex, cytoplasmic RanGAP1 stimulates GTP hydrolysis, and the complex dissociates [12]. The experimental injection of Rna1p (the homolog of RanGAP1 in yeast [31]) causes rapid depletion of RanGTP and inhibits NES export [26]. We found that coinjection of Rna1p, which blocked export of GST-NES, had no effect on export of wild-type β-catenin (Figure 4b). Similarly, ΔC and ΔN were both insensitive to leptomycin B and to Rna1p (Figures 4a,b). We conclude that β-catenin export, whether mediated by its N or C terminus, is independent of the classical NES- and CRM1-mediated pathway and does not require RanGTP.

We also tested export of β-catenin in digitonin-permeabilized cells. This assay was designed to identify cytosolic components required for nuclear transport [32]. In particular, Ran is depleted during permeabilization and must be replenished to reconstitute classical nuclear transport [33, 34]. Nuclei were loaded by incubation with recombinant β-catenin, then the cells were washed, and export (disap-

Figure 4



β-catenin export is leptomycin B insensitive and RanGTP independent. **(a)** Export of ^{35}S-labeled GST-NES, full-length β-catenin (wild type), and deletion variants ΔC and ΔN was tested in the presence or in the absence of 1 mM leptomycin B (LMB). Export of GST-NES was strongly inhibited by LMB, but export of the wild-type β-catenin, ΔC, and ΔN was not affected. **(b)** ^{35}S-labeled GST-NES, the wild-type β-catenin, ΔC, and ΔN were injected with or without 20 μM (1.3 mg/ml) recombinant Rna1p. GST-NES export was blocked by coinjection of Rna1p, but export of the wild-type β-catenin, ΔC, and ΔN was indistinguishable in the presence or in the absence of Rna1p.

pearance from the nucleus) was measured. We found that β-catenin could enter and subsequently exit the nucleus rapidly in the absence of cytosolic factors (Figure 5). These findings further support the conclusion that nuclear translocation of β-catenin is Ran independent.

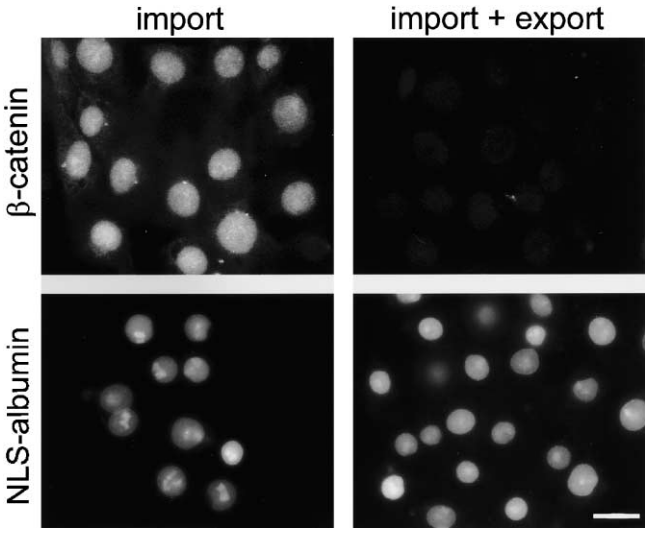
Deletion mutants reveal that the arm repeats have latent export capacities and that export is required for degradation

Although the central arm repeat region had shown no significant export abilities, we wanted to test whether smaller repeat fragments may reveal some role in export, either by themselves or in combination with the adjacent N- or C-terminal signals. The last five repeats did not show significant export and did not influence export by the C terminus (see Supplementary material). Constructs that contained the first eight arm repeats, either alone (GST-arm1–8) or with the N terminus (GST-Narm1–8) were rapidly degraded (Figure 6a) and their ability to be exported could not be readily detected.

To determine if degradation of these two constructs occurred in the nucleus or in the cytoplasm, we coinjected wheat germ agglutinin (WGA), which binds to O-glycosylated nucleoporins and blocks nuclear transport [35]. In nuclear coinjections, WGA efficiently retained wild-type β-catenin in the nucleus. WGA also caused stabilization of GST-Narm1–8 and GST-arm1–8, which then appeared in the nuclear fraction (Figure 6a). WGA had no effect on degradation of GST-Narm1–8 and GST-arm1–8 when it was coinjected in the cytoplasm (Figure 6a). These results show that nuclear transport is required for degradation and thus imply that the first eight repeats are capable of export and that degradation occurs in the cytoplasm. The dramatic difference in stability of GST-Narm1–8 and GST-arm1–8 compared to wild-type β-catenin is probably due to the fact that these mutants cannot associate with cadherins (see Discussion). Export mediated by the arm repeats also appeared to be independent of RanGTP because coinjection of Rna1p had no effect on degradation of GST-arm1–8 (Figure 6b).

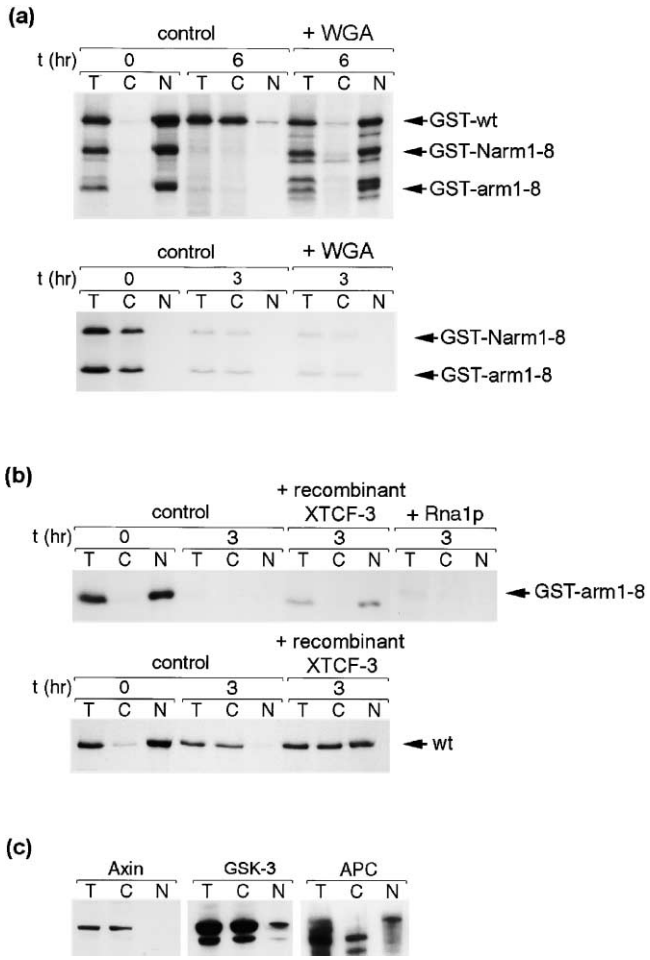
β-catenin degradation is mediated by a multimeric complex containing Axin, APC, and GSK-3. When nuclear extracts were analyzed for the presence of these three components (Figure 6c), they were found to contain APC and GSK-3 but no Axin. The absence of Axin provides an obvious explanation for the stability of β-catenin in the nucleus.

Figure 5



β-catenin is imported and exported in the absence of cytosolic factors. Digitonin-permeabilized BRL cells, thus depleted of cytosolic factors including Ran, were incubated with 9 μM Oregon Green–labeled recombinant β-catenin for 20 min. The cells were then fixed either immediately (import) or after they were washed with import buffer for 20 min (export). As a control, 3 μM rhodamine-labeled NLS-albumin was imported in the presence of cytosol and ATP (import) and then washed with buffer (export). β-catenin was rapidly reexported, while control NLS-albumin remained in the nuclei. The scale bar represents 20 μm.

Figure 6



(a) Arm repeats 1–8 show export-dependent degradation. ^{35}S-labeled GST-arm1–8, GST-Narm1–8, and GST–wild type were injected in the nucleus with or without 50 mg/ml WGA (upper panel). Export of GST–wild type was blocked by WGA. GST-arm1–8 and GST-Narm1–8 were completely degraded after 6 hr in the absence of WGA, but they were retained in the nucleus and stabilized by WGA. WGA had no effect on degradation of GST-arm1–8 and GST-Narm1–8 when it was coinjected in the cytoplasm (lower panel). **(b)** XTCF-3 inhibits export of β-catenin. ^{35}S-labeled GST-arm1–8 and full-length β-catenin (wild type) were injected with or without 0.5 mg/ml recombinant XTCF-3. Export of full-length β-catenin (wild type) was reduced by XTCF-3, and GST-arm1–8 was stabilized inside the nucleus. Coinjection of 20 μM (1.3 mg/ml) recombinant Rna1p did not stabilize GST-arm1–8, and this finding suggests that the export of the arm repeats is RanGTP independent. **(c)** Western blot analysis of cytoplasmic and nuclear fractions for endogenous Axin, GSK-3, and APC. GSK-3 and APC, but not Axin, are present inside the nucleus.

XTCF-3 inhibits β-catenin export

The observations that β-catenin export is Ran independent in the oocyte assay and that it occurs in the absence of additional factors in semipermeabilized cells suggest that β-catenin may be able to shuttle freely in and out of the nucleus. Enrichment of overexpressed β-catenin at the nuclear rim of embryonic cells (Figure 3a) is consistent with this hypothesis. However, endogenous β-catenin can accumulate in the nucleus upon activation of the Wnt pathway, for example in the dorsal side of early *Xenopus* embryos (Figure 3c). Nuclear accumulation could also be induced by XTCF-3 (Figure 3d, see also [36, 37]), which would suggest a retention mechanism. We thus tested the effect of XTCF-3 on β-catenin export. As shown in Figure 6b, coinjection of recombinant XTCF-3 significantly inhibited export of β-catenin. GST-arm1–8, which can still bind XTCF-3 in vitro (see Supplementary material), was similarly stabilized in the nucleus (Figure 6b).

Discussion

Recent studies on APC export have suggested that β-catenin is exported by APC in a CRM1-dependent fashion [23, 24]. However, this conclusion was based on indirect observation of steady-state distribution, which can be influenced by many factors. Our results on export in oocytes and in semipermeabilized cells show that β-catenin is efficiently exported out of the nucleus independently of the classical NES pathway and of RanGTP.

β-catenin export was found to rely on two regions, located in the N terminus and C terminus, respectively. Either of these two terminal domains could mediate efficient export of the central arm repeat region, and both could

induce export of GST. The C-terminal domain appeared to be the most active one and can account by itself for the export of full-length β-catenin. The two export sequences correspond to regions with other attributed functions; the N-terminal sequence contains the GSK-3 consensus site involved in regulation of β-catenin stability [3], and the C-terminal domain has been characterized for its transactivation activity [38, 39]. However, so far these regions have not been implicated in transport or localization of β-catenin. They have no homology with known export signals or molecules involved in nuclear transport. Export via these terminal domains does not appear to follow any conventional pathway; the export processes they mediated were not negatively affected by excess NES, were insensitive to leptomycin B, and did not require RanGTP. Both pathways, however, were saturable. This indicates that they rely on specific interactions, which remain to be characterized. The terminal domains may be transported by specific exportins with the use of an unconventional RanGTP-independent mechanism. Alternatively, they may be capable of direct translocation through the nuclear pore. Other molecules, such as RCC1 and NTF2, which show no sequence similarity to the repeats of the importin/exportin family, can indeed cross the nuclear pore independently of other transporters [11, 12, 40, 41].

The result that export of β-catenin requires its terminal domains but not the arm repeat region was surprising given the structural and functional analogies between β-catenin and the importin/exportin family. Importins and exportins consist largely of HEAT repeats, which are related to the arm repeats of β-catenin [20], and translocate directly through the nuclear pore. Similarly, import of β-catenin relies on the arm repeats [21, 22] and seems to occur by direct interaction with the nuclear pores [18, 19]. In fact, our present data indicate that the arm repeats do have the capacity for export but that this property is inhibited in vivo. Indeed, although we could not formally demonstrate this third export pathway, the clear stabilization of the "vanishing" arm1–8 mutants when nuclear pores are blocked by WGA argues for export and subsequent degradation in the cytoplasm. Stabilization of arm1–8 appears to be Rna1p-insensitive, and this observation is incompatible with a CRM1-mediated export. The relative contribution of the three pathways may depend on the cell type and on physiological conditions; although export by the arm repeats seems to be repressed in the oocyte and in the embryo, it may become prominent in other circumstances.

The Ran cycle ensures directionality and seems to account for the energy dependence of nuclear transport. Unlike NES substrates, importin α, or RNAs [26], however, β-catenin export does not require RanGTP, as none of the three pathways described here is affected by Rna1p. RanGTP-independent export has been reported only for cargo-free nuclear transport receptors, namely importin β [42] and transportin [43, 44]. Import of β-catenin, studied in semipermeabilized cells, does not require Ran [18, 19] (see also Supplementary material for further discussion). We have now confirmed in semipermeabilized cells that export of β-catenin can occur in the absence of additional factors. Thus, β-catenin may well cross the nuclear pore freely in either direction.

It is commonly assumed that raising the levels of soluble β-catenin, either through Wnt signaling or artificially by overexpression, automatically leads to nuclear accumulation. Instead, β-catenin appears to shuttle between the cytoplasm and the nucleus. We were surprised by the fact that sheer overexpression of full-length β-catenin in *Xenopus* embryos only leads to rather mediocre nuclear levels (Figure 3). Enrichment at the nuclear rim is strikingly reminiscent of importin β localization and suggests that in vivo β-catenin may show a short life span in the nucleoplasm and constant recycling.

Several mechanisms can nevertheless produce an asymmetrical distribution of β-catenin even in the absence of Ran-dependent directional transport. Sequestration by cadherins at the plasma membrane and degradation of soluble β-catenin in the cytoplasm tend to deplete the nuclear pool of β-catenin. Nuclear retention, on the other hand, can shift the steady-state distribution of β-catenin toward the nucleus (see Supplementary material for further discussion). Nuclear accumulation of endogenous β-catenin can be induced experimentally, for instance by overexpression of TCF/Lef-1 in culture cells [36, 37] and in *Xenopus* embryos (Figure 3d). XTCF-3 inhibits export of β-catenin (Figure 6b), possibly by binding to chromatin. We propose that such a retention mechanism may also account for the nuclear accumulation of β-catenin observed under physiological conditions upon the stimulation of Wnt signaling (see, for example, [7, 8] and our data). However, additional nuclear factors must be involved because β-catenin mutants defective in TCF binding still accumulate in the nucleus [6, 22].

Stabilization of our two "vanishing" mutants by WGA, which blocks nuclear transport, strongly suggests that β-catenin degradation does not occur in the nucleus but only in the cytoplasm. This conclusion is confirmed by the fact that Axin, which is required for β-catenin degradation, is excluded from the oocyte nucleus. Overexpressed Axin also appearedto be exclusively cytoplasmic in *Xenopus* embryos and in mammalian culture cells [45]. A glance at the various mutants used in this study shows that their stability depends on the ability to bind to Axin; mutants that lack the first arm repeats, responsible for Axin binding (not shown), are stable. In addition, mutants that include the full arm repeat domain, and which thus can bind Axin, can nevertheless be "protected" from degradation if they

are sequestered in an appropriate subcellular compartment. They can either bind to cadherins after export (e.g., wild type; see Materials and methods) and thus be stabilized [3], or they can be retained in the nucleus (e.g., arm). The two quickly disappearing mutants can bind Axin (not shown) but not cadherins [46], and they are not retained in the nucleus. Note that the N terminus is not required for rapid degradation of the "vanishing" mutants (see GST-arm1–8). This is consistent with the observation that some truncated forms of β-catenin are highly unstable independently of GSK activity [47]. In any case, degradation of β-catenin, either full-length or mutant, requires association with Axin [4], which is absent from the nucleus.

The fact that the Axin-based complex is localized in the cytoplasm may at least partly account for the sensitivity of steady-state distribution of β-catenin to leptomycin B [23, 24]; leptomycin B–induced relocalization of APC in the nucleus will impair the function of the degradation complex and lead indirectly to stabilization and nuclear accumulation of β-catenin, as observed when degradation is blocked directly with proteasome inhibitors. Overexpression of APC or APC mutants will further influence β-catenin stability and, consequently, its localization. Overexpressed APC forms will also provide excess binding sites for β-catenin in a particular cellular compartment (nucleus or cytoplasm). The combination of these effects is expected to shift steady-state distribution of β-catenin and produce the impression that β-catenin "shuttles" with APC. APC may certainly be able to carry out some β-catenin. However, to what extent this pathway plays a role in β-catenin export awaits the availability of cell lines null for APC and the direct measurement of export in these cells. Considering that β-catenin can shuttle rapidly in the absence of additional factors, we expect from our data that this contribution will be minor under physiological conditions.

Conclusions

The cell has devised a panel of control mechanisms to prevent deregulation of β-catenin signaling, which can lead to severe consequences, including cancer [4]. If β-catenin degradation is restricted to the cytoplasm, nuclear export is likely to be an important additional mechanism for regulation of Wnt signaling by ensuring rapid termination of the signal as well as retrieval and degradation of any "escaped" molecule in nonstimulated cells. The occurrence of three independent export domains may further ensure a tight control on nuclear β-catenin. Note that two of these domains, i.e., the arm repeats and the C terminus, overlap with interaction domains for XTCF-3 and for components of the transcription machinery, respectively [38, 39], while the N terminus includes the GSK-3 phosphorylation site and could be influenced by Wnt signaling. β-catenin distribution and activity may thus not only depend on β-catenin levels but also on regulation of multiple interactions and/or modifications of these domains.

Materials and methods

Nuclear export assay in Xenopus *oocytes*

The assay was performed as in the study by Kambach and Mattaj [48], with slight modifications; in vitro–translated and recombinant proteins (see Supplementary material for a precise description of the constructs) were equilibrated in PBS/8.7% glycerol. Protein (30 nl) was injected into the nucleus of stage V–VI *Xenopus* oocytes, which were incubated at 20°C. At various time points, the cytoplasm and the nuclei were separated by dissection in 10 mM HEPES (pH 7.6), 0.1 mM EDTA, 7 mM $MgCl_2$, 70 mM NH_4Cl, and 10% glycerol. The red color of the nuclei, due to the hemoglobin present in the injected reticulocyte lysate, was used to control the accuracy of nuclear injections and the integrity of the nuclei. Dissected nuclei were placed directly in SDS-PAGE loading buffer (15 mM Tris [pH 6.8], 10% glycerol, 100 mM DTT, and 0.3% bromophenol blue). The cytoplasm was transferred into cold NP40 Buffer (10 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% Nonidet NP40, and 0.1% NaN_3) and homogenized. NP40-insoluble components (mainly yolk and pigment granules) were removed by centrifugation, and the supernatant was acetone precipitated and redissolved in SDS-PAGE loading buffer. For each sample, six oocytes were pooled, and the estimated extract from 2.1 oocytes was loaded onto SDS polyacrylamide gels, which were analyzed by fluorography (Amplify, Amersham Pharmacia Biotech). Recombinant β-catenin (Figure 2e) was detected with a polyclonal rabbit anti–β-catenin antibody [7]. Note that in the original protocol, the cytoplasmic fraction was homogenized in the absence of detergents. However, we found that cytoplasmic β-catenin rapidly entered a sedimentable fraction but could be recovered after extraction with NP40. Concanavalin A precipitation confirmed association of this pool with cadherins (not shown). For leptomycin B inhibition experiments, oocytes were preincubated in 1 mM leptomycin B for 2 hr prior to injection and then further incubated in the presence of 1 mM leptomycin B (gift of M. Yoshida) [49].

Nuclear import/export in permeabilized cells

The import assay using BRL cells permeabilized with digitonin has been previously described [18, 50]. Recombinant His-tagged β-catenin was labeled with Oregon Green (Molecular Probes) (approximately 0.6 molecules dye per molecule protein). Import was achieved by the incubation of permeabilized cells with approximately 9 μM β-catenin diluted in import buffer (100 mM potassium acetate, 20 mM HEPES-KOH [pH 7.4], 2 mM magnesium acetate, 2 mM DTT, and 1 mM EGTA). In controls, permeabilized cells were incubated with 3 μM rhodamine-labeled, NLS coupled–human serum albumin, 30 mg/ml cytosol, 1 mM ATP, and an ATP-regenerating system as previously described [50]. After 20 min, cells were either briefly rinsed and immediately fixed with 4% paraformaldehyde (import), or further incubated for 20 min with import buffer alone before fixation (export).

Western blot analysis of nuclear and cytoplasmic fractions

Uninjected oocytes were dissected, six oocytes were pooled, and for each fraction an equivalent of 1.4 oocytes was loaded onto 4%–12% Bis-Tris polyacrylamide gels (Novex). Western blots were stained with a polyclonal-rabbit anti-Axin (raised against the mouse Axin fragment consisting of amino acids 419–600), a polyclonal-rabbit anti-APC (C-20, Santa Cruz Biotechnology) or a monoclonal-mouse anti–GSK-3 (clone 4G-1E, Upstate Biotechnology) antibody.

Indirect immunofluorescence

HA-tagged β-catenin (wild type), arm, and HA-tagged XTCF-3 mRNAs (1–5 ng) were injected in early cleaving embryos, which were fixed at early gastrula stage and processed for cryosectioning and immunofluorescence as described [51]. Primary antibodies used were rabbit anti-HA (Santa Cruz Biotechnology), mouse anti-HA 12CA5, and rabbit anti–β-catenin. Secondary antibodies were Alexa488 goat anti-rabbit and anti-mouse (Molecular Probes) and Cy3 goat anti-rabbit (DAPKO).

Supplementary material
Additional "Results and discussion" and "Materials and methods" sections, as well as four additional figures, are available with the electronic version of this article at http:\\current-biology.supmat.com.

Acknowledgements

We thank Anne Schohl for technical assistance. We are grateful to Drs. Iain Mattaj, Ludwig Englmeier, Scott Kuersten (EMBL), Doris Wedlich (Ulm), Tobias Pukrop (Ulm), Marian Waterman (California), and Minoru Yoshida (Tokyo) for kindly providing many reagents. We thank Dr. Mattaj, Dr. Mutsuhito Ohno, and Alexandra Segref for help in setting the export assay. We thank Dr. David Edwards for critical reading of the manuscript.

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