Activation appears to be a major cause of cancer (3).

The pathway is also crucial in adult tissues, as its uncontrolled activation leads to cell transformation and is believed to mediate re-export of Axin from the nucleus (24, 25). However, APC is not required for export of Axin to the Axin-based complex (7–9, 12, 17). Axin can also interact with positive regulators of the pathway, which inhibit or antagonize β-catenin phosphorylation, such as Dsh (12, 18–20), GBP/Frat (21, 22), and protein phosphatase 2A (23).

Recent data have suggested nucleo-cytoplasmic transport to be an important parameter in regulation of the pathway. Both β-catenin and APC have been shown to shuttle between the cytoplasm and the nucleus (reviewed in Refs. 24 and 25). The presence of β-catenin in the nucleus is quite obviously related to its function as a transcriptional coactivator. Its re-export might be crucial to terminate its signaling activity (26). The role of nuclear APC in β-catenin regulation is still controversially discussed (25); nuclear export of APC, which follows the classical CRM1-mediated pathway (27–29), has been proposed to mediate re-export of β-catenin by a piggy-back mechanism (24, 25). However, APC is not required for export of β-catenin, which can freely translocate through the nuclear pores on its own (26). The current models of the Wnt pathway have relied on the assumption that Axin is cytoplasmic. Ectopically expressed Axin displays a typical punctate pattern in the cytoplasm and at or near the cell membrane, both in culture cell lines and in early Xenopus embryos (12), and manual isolation of nuclei from Xenopus oocytes has confirmed that, at least in this cell type, endogenous Axin is absent from the nucleus, consistent with β-catenin degradation occurring primarily in the cytoplasm (26). Here, however, we show that Axin in fact also shuttles in and out of the nucleus.

**MATERIALS AND METHODS**

*Plasmids and Protein Expression—*Myc-tagged Axin constructs in pCS2+MT used for transfection have been described previously (12), except Axin(μNES1+2), where double point mutations were introduced in each of the potential nuclear export sequence (NES) sequences to substitute valine 544, methionine 546, leucine 649, and leucine 651 with alanine residues (Fig. 2C).

**Plasmids**

[35S]Methionine-labeled proteins were produced with the rabbit reticulocyte lysate-coupled transcription/translation system (TNT kit, Promega). NPC-M9-NES, NPC-M9-M10 (30), zz-CRM1 (31), RanG12L (32), and Glutathione S-transferase.

The abbreviations used are: APC, Adenomatous polyposis coli; NES, nuclear export sequence; LMB, leptomycin B; aa, amino acid(s); GST, glutathione S-transferase.
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6×His-Axin (96–353), Axin (419–600), and Axin (419–672) (12) were expressed and purified as described previously.

**Cell Culture and Immunofluorescence**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells at ~70% density seeded on coverslips were transfected with DNA in serum-free medium (Opti-MEM I, Invitrogen) using LipofectAMINE reagent (Invitrogen). After 8–9 h at 37 °C, the medium was replaced by Dulbecco’s modified Eagle’s medium, 10% fetal calf serum with or without 5 nM leptomycin B (LMB), kindly provided by M. Yoshida, and the cells were grown for further 0.5, 4, or 13 h. Cells were fixed with 4% paraformaldehyde, 0.5% Triton X-100 in phosphate-buffered saline for 10 min, followed by blocking in 0.2% gelatin, 0.5% bovine serum albumin in phosphate-buffered saline for 30 min and incubation for 2 h with mouse anti-Myc antibody (9E10) or affinity purified rabbit anti-Axin (raised against the N terminus, aa 96–353) in 0.2% gelatin, 0.5% bovine serum albumin in phosphate-buffered saline. Secondary antibodies were Alexa488 (Molecular Probes). The cells were observed under a Zeiss Axioplan fluorescence microscope using a 63× oil immersion objective.

The distribution of endogenous Axin in control and LMB-treated cells was quantified as follows. Cells were divided in three categories: cytoplasmic but no significant nuclear staining (C), cytoplasmic and nuclear staining of roughly equal intensity (CN), and nuclear staining stronger than cytoplasmic (N). For each condition, ~100 cells were counted in each experiment. The data represent the average of three independent experiments.

**Nuclear Transport Assay in Xenopus Oocytes**—The assay was performed as described previously in detail (26). Briefly, 35S-labeled in vitro translated proteins were injected directly into the oocyte nucleus. At various time points, the cytoplasm and the nucleus were separated by dissection of the oocytes into nuclei and cytoplasm (examples of dissection and manual staining of roughly equal intensity (CN), and nuclear staining stronger than cytoplasmic (N)). For each condition, ~100 cells were counted in each experiment. The data represent the average of three independent experiments.

**Results and Discussion**

Axin Shuttles in and out of the Nucleus—Exogenous, epitope-tagged full-length Axin (aa 12–956) shows a punctate pattern in the cytoplasm and at the cell periphery, both in mammalian cell lines and in *Xenopus* embryos (Ref. 12 and Fig. 1, A and C). When determining the subcellular distribution of various deletion mutants transfected in HEK293 cells, we observed that some of them had a nuclear localization (Fig. 1E). This observation suggested that Axin might be capable of shuttling in and out of the nucleus and that the deletion variants showing nuclear localization were missing the export signal. Nuclear export is generally mediated by a leucine-rich NES, which binds in a Ran-GTP-dependent manner to the export receptor CRM1 (exportin 1/Xpo 1; Ref. 33). The interaction NES-CRM1 can be blocked specifically by an antibiotic, LMB. We tested whether LMB influenced the distribution of Axin. We expressed Myc-tagged full-length Axin in HEK293 cells and analyzed its distribution by immunofluorescence. We found that LMB treatment caused indeed a significant amount of Axin to accumulate in the nucleus of most cells (Fig. 1B and D). We conclude that Axin indeed shuttles through the nuclear pore and that its export is CRM1-dependent.

Nuclear enrichment after LMB treatment is good evidence, but not proof, that the protein under investigation can be re-exported from the nucleus, as other explanations cannot be excluded (Axin could for example become more stable in the nucleus). To obtain direct evidence for Axin nuclear export, we used the *Xenopus laevis* oocyte assay (32); *in vitro* translated, 35S-labeled Axin variants were microinjected in the oocyte nucleus, and their final localization was determined by manual dissection of the oocytes into nuclei and cytoplasm (examples shown in Fig. 3 and data summarized in Fig. 2B). As full-length Axin was not well translated, we used our longest C-terminal deleted construct Ax-(12–810) to confirm that Axin is indeed exported (Fig. 3B). Note that some variable degradation of the Axin fragment was observed, in agreement with the known instability of Axin (34, 35). Note also that the kinetics of export of Ax-(12–810) varied between experiments (compare Fig. 3, B and C), which is likely to be due to variations in amounts of Axin-interacting proteins between oocyte batches, which may affect not only stability, but also for instance nuclear retention.

Export of proteins containing a classical NES is mediated by an interaction with CRM1 that requires cooperation of the small GTPase Ran in its GTP form (33). As shown in Fig. 3, B and C, export of Ax-(12–810) is inhibited by incubation with LMB or by coinjection of a recombinant RanGAP (Rnap1; Ref. 36), which rapidly depletes RanGTP from the nucleus (32). Thus, we conclude that Axin is export via the “canonical” CRM1- and RanGTP-dependent pathway.

We also wanted to determine whether endogenous Axin shuttles in and out of the nucleus. For this purpose, we needed first to establish the localization of endogenous Axin in untreated HEK293 cells. We used an affinity-purified polyclonal antibody raised against the N terminus of Axin. This antibody recognizes a single band around 90kD in HEK293 cell extracts, which corresponds to the N-terminal half of Axin, localized constitutively to the nucleus (arrows). G and H, Ax-(531–956), corresponding to the C-terminal half of Axin, had a diffuse cytoplasmic distribution in control cells and accumulated in the nuclei of LMB-treated cells.
fractionation experiments using HEK293 cells. We have also observed a very dense Axin pool, but we think that it corresponds to large complexes/organelles, possibly associated with cytoskeletal elements, rather than to a genuine nuclear localization.

In cells treated with LMB, however, Axin disappeared from the pericytoplasmic spots and accumulated in the nucleus (Fig. 4, B and C). The nuclear signal was abolished by preincubation of the antibody with the corresponding antigen (Fig. 4, E). Note that in these controls a strong cytoplasmic staining was observed. This is most likely due to binding of the recombinant Axin fragment to cellular structures. Apparently, sequestration of endogenous Axin in the nucleus upon LMB treatment creates free Axin binding sites in the cytoplasm.

A shift from cytoplasmic to nuclear could be already detected in some cells after 30 min of LMB treatment (Fig. 4G). After 4 h, Axin was found in most nuclei and further accumulated during overnight treatment (Fig. 4G). Thus, Axin accumulated in LMB-treated cells with kinetics comparable with what has been observed for APC (27) but much slower than expected for a freely shuttling protein. This is consistent with the fact that

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2 F. Schwaak and F. Fagotto, unpublished results.

3 K. Heinle and F. Fagotto, unpublished results.
most Axin is associated with large protein complexes. The extent of shuttling may be then dictated by the rates of association/dissociation of these complexes.

Axin Nuclear Import—The sequence requirements for nuclear import appear to be complex and will need further characterization. All fragments examined could localize to the nucleus, either constitutively or upon LMB treatment, indicating the presence of multiple NLSs (the large size of the fragments examined (>50 kDa) precludes simple diffusion through the nucleopores). The primary sequence shows two potential classical NLSs. The first sequence is positioned at aa 59, and the second bipartite NLS consensus is at aa 849 (6). However, a fragment lacking both of these sequences (aa 194–672) was still very efficiently retained in the nucleus upon LMB treatment of the nuclei with 4',6-diamidino-2-phenylindole. Axin localized to the nucleus in LMB-treated HEK293 cells. Nuclei showed no significant staining. B and C, Axin localized to the nucleus in LMB-treated cells. Large arrows and arrowheads point to nuclei with respectively high and low levels of Axin. D and E, specificity control: untreated (D) and LMB-treated (E) cells stained with anti-Axin antibody. G, quantitation of cytoplasmic/nuclear Axin signal after LMB treatment. C, mostly cytoplasmic; CN, cytoplasmic and nuclear; N, enriched in nuclei. Examples for all three categories are presented in A–C: small arrowheads = C, arrowheads = CN, large arrows = N.

Fig. 4. Endogenous Axin distribution depends on CRM1 export. A, endogenous Axin localized in spots at or near the plasma membrane (small arrows) in untreated HEK293 cells. Nuclei showed no significant staining. B and C, Axin localized to the nucleus in LMB-treated cells. Large arrows and arrowheads point to nuclei with respectively high and low levels of Axin. D and E, specificity control: untreated (D) and LMB-treated (E) cells stained with anti-Axin antibody. G, quantitation of cytoplasmic/nuclear Axin signal after LMB treatment. C, mostly cytoplasmic; CN, cytoplasmic and nuclear; N, enriched in nuclei. Examples for all three categories are presented in A–C: small arrowheads = C, arrowheads = CN, large arrows = N.

4 F. Schwaak and F. Fagotto, unpublished results.

Fig. 5. Direct binding of the central region of Axin to CRM1. Recombinant Axin fragments were incubated with recombinant zCRM1 in the presence or absence of RanQ69L (CTP-bound). zCRM1 was pulled down using IgG-agarose beads. Two small recombinant proteins containing a classical NES (NPC-M9-NES) or a mutated NES unable to bind CRM1 (NPC-M9-M10) were used, respectively, as positive and negative controls. Filled arrowheads point to the recombinant fragments, V-shaped arrowheads point to RanQ69L, and asterisks indicate IgG heavy chains leaking from the beads. Both Ax-(419–672) and Ax-(419–600) bound to CRM1 in a RanGTP-dependent manner, while Ax-(96–353), a more N-terminal fragment, did not.

Characterization of the Axin Nuclear Export Domains—Axin has no obvious classical consensus export sequence (LxRxLxLxL, Ref. 38). To determine the sequence requirements for Axin export, we first examined the localization of a series of Axin variants, in the presence or absence of LMB (examples in Fig. 1, E–H, see summary in Fig. 2A). The patterns observed for each variant were very reproducible, with virtually all cells showing a similar distribution. Mutants with C-terminal deletions up to aa 600 were exclusively cytoplasmic in untreated cells and redistributed to the nucleus in the presence of LMB. Further deletion to aa 531, however, led to constitutive localization to the nucleus. The same deletion also blocked export in the oocyte assay (Fig. 3A). Thus, the region responsible for export appears to be located between aa 531 and 600. On the other hand, N-terminal deletions and internal deletions showed that fragments lacking the first 600 aa were still shuttling in a LMB-sensitive manner, while further deletion to aa 631 caused constitutive nuclear localization, indicating the presence of a second export sequence between aa 600 and 631.

We have used the oocyte assay to further dissect the regions responsible for export. For this purpose, we have tested small fragments fused to glutathione S-transferase (GST), which does not diffuse on its own through the nuclear pore (Fig. 3L). The results are summarized in Fig. 2B, and examples are shown in Fig. 3, D–K. Consistent with the results from cell transfections, we could thus identify two small fragments mediating export, aa 532–560 (Fig. 3, E and H) and aa 641–667 (Fig. 3G). The presence of a short sequence between these two regions, aa 601–640 (Fig. 3, F and K), appeared to decrease the efficiency of export via the first fragment (Fig. 3F). Possible explanations for this inhibition could be steric hindrance by an interacting partner or inappropriate conformation in the context of these small fragments. Full export was restored by addition of the second fragment (aa 532–667, Fig. 3J).

As mentioned above, the Axin sequence does not reveal any classical leucine-rich NES. However, the two identified fragments mediating Axin export have sequences remotely resembling a classical NES (LxxLxxVxxM and VxxLxxLxxL, respectively, Fig. 2C). To assess the role of these residues we introduced double point mutations in both of these sequences (Ax(mutNES1+2) in Fig. 2A). These mutations did not prevent Axin shuttling in HEK293 cells, confirming that Axin export does not rely on a classical NES. It is known that CRM1-mediated export can also be mediated by atypical NESs (39, 40), and in the case of the U snRNP receptor snuportin1, a much larger domain appears to bind to CRM1 (41). Further work will be required to determine the essential residues within the two Axin export domains.
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Direct Binding of Axin to CRM1—We next wanted to examine whether, despite the absence of a typical NES, Axin could bind directly to CRM1. To address this issue, we tested binding of Axin to CRM1 in vitro, using recombinant proteins (Fig. 5). As the specific interaction of a transport substrate with CRM1 must be RanGTP dependent, binding was assayed in the presence or absence of RanQ69L, a Ran mutant unable to hydrolyze GTP. We tested three His-tagged Axin fragments, Ax-(419–600) and Ax-(414–672), which encompass the region of the NESs, and a control fragment Ax-(96–353), which corresponds to a more N-terminal region without export capacities. We found that Ax-(419–600) and Ax-(414–672), but not Ax-(96–353), could bind CRM1 in a RanGTP-dependent manner. We conclude that Axin can interact directly with CRM1, although via non-classical NES sequences.

Axin Shuttling and the Wnt Pathway—Models about regulation of the Wnt pathway have so far assumed that β-catenin and APC could dynamically shuttle between the cytoplasm and the nucleus, while the Axin complex was stably anchored in the cytoplasm. The fact that Axin can also shuttle in and out of the nucleus prompts to a re-evaluation of our current view of the pathway. One of the models, in particular, has proposed that APC plays the role of a shuttle mediating export of β-catenin (24). This hypothesis was based mainly on two observations: 1) LMB treatment can cause accumulation of β-catenin in the nuclei of some cell lines (27), a phenotype that has been interpreted as the direct consequence of inhibition of CRM1-mediated export of APC. 2) Expression of APC mutants that failed to affect shuttling of a second β-catenin binding protein, Axin, which, in principle, could then also contribute to β-catenin export. However, there are other possible interpretations of these data. For instance, sequestration of β-catenin-binding proteins in the nucleus, either by LMB treatment or mutation of the export sequences, can certainly cause passive retention of β-catenin in this compartment. In the case of Axin, β-catenin faithfully colocalizes with various overexpressed Axin variants, whether in the cytoplasm or in the nucleus. In fact, the consequences of LMB treatment may be complex and cell type-dependent. At least in HEK293 cells, we could not observe any obvious effect of LMB treatment on β-catenin—β-catenin levels were not increased, and there was no detectable β-catenin in the nuclei. Axin may also have yet unknown functions in the nucleus or may even simply enter the nucleus fortuitously, e.g. carried along by one of its many interacting partners, and would then need to be retrieved to the cytoplasm to preserve on the long term the proper organization of the complex. While we found Axin to be largely cytoplasmic in various cell lines, a diffuse nuclear staining has been observed in epithelial cells of normal human colon tissue (37), suggesting that nuclear localization of Axin may be regulated under physiological conditions. We have not been able to test the role of Axin export by mutating the export domain, because modifying the sequence in this region will affect the overlapping binding sites for β-catenin and PP2A (see Fig. 2C). We thus need to find other approaches to unravel this challenging issue, which is a prerequisite for understanding how the Wnt pathway really works.

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