

Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors

Sundeep Shah and Douglass J. Forbes

Background: Proteins generally enter or exit the nucleus as cargo of one of a small family of import and export receptors. These receptors bear distant homology to importin β , a subunit of the receptor for proteins with classical nuclear localisation sequences (NLSs). To understand the mechanism of nuclear transport, the next question involves identifying the nuclear pore proteins that interact with the different transport receptors as they dock at the pore and translocate through it.

Results: Two pathways of nuclear import were found to intersect at a single nucleoporin, Nup153, localized on the intranuclear side of the nuclear pore. Nup153 contains separate binding sites for importin α/β , which mediates classical NLS import, and for transportin, which mediates import of different nuclear proteins. Strikingly, a Nup153 fragment containing the importin β binding site acted as a dominant-negative inhibitor of NLS import, with no effect on transportin-mediated import. Conversely, a Nup153 fragment containing the transportin binding site acted as a strong dominant-negative inhibitor of transportin import, with no effect on classical NLS import. The interaction of transportin with Nup153 could be disrupted by a non-hydrolyzable form of GTP or by a GTPase-deficient mutant of Ran, and was not observed if transportin carried cargo. Neither Nup153 fragment affected binding of the export receptor Crm1 at the nuclear rim.

Conclusions: Two nuclear import pathways, mediated by importin β and transportin, converge on a single nucleoporin, Nup153. Dominant-negative fragments of Nup153 can now be used to distinguish different nuclear import pathways and, potentially, to dissect nuclear export.

Background

The transport of proteins through the nuclear pore is mediated by import receptors that recognize specific nuclear localization sequences (NLSs) on proteins that are targeted for import. The classical NLS, exemplified by that of SV40 large T antigen, is comprised of a short stretch of basic amino acids. A related bipartite NLS contains two clusters of basic amino acids separated by a 10 amino acid spacer [1]. Several other NLS sequences have been identified that are quite different from classical NLSs; for example, the M9 sequence of the heterogenous nuclear ribonucleoprotein hnRNPA1 is glycine-rich, whereas certain NLSs of ribosomal proteins are not only diverse in sequence but are larger than the classical NLSs [2–4]. Nuclear export sequences (NESs) have also been identified. These sequences, first observed in the protein kinase A inhibitor protein (PKI) and the HIV Rev protein [5–7], consist of a short stretch of hydrophobic leucine-rich amino acids.

Recent work has identified a family of nuclear import and export receptors that have limited homology. Individual

Address: Department of Biology 0347, University of California at San Diego, La Jolla, California 92093-0347, USA.

Correspondence: Douglass J. Forbes
E-mail: dforbes@ucsd.edu

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receptors recognize specific classes of NLSs or NESs [2,8–13]. Among these, the classical NLS receptor consists of two subunits, importin α and β . Importin α recognizes and binds to the SV40-type and bipartite-type NLSs. Importin β binds to the NLS–importin α cargo complex and allows docking of the complex on the cytoplasmic fibrils of the nuclear pore [14–20]. The exact mechanism of import is poorly understood, but it requires at least two additional soluble transport factors, the GTPase Ran and nuclear transport factor 2 (NTF2) [14,21–24]. Once translocation has occurred, the import process is believed to be terminated on the nuclear pore basket by the binding of Ran–GTP to importin β [14] which releases both importin α and the NLS cargo.

A second importin- β -like transport receptor, termed transportin, ferries cargo of a different sort into the nucleus. Transportin directly binds the M9 NLS of hnRNPA1 and mediates hnRNPA1 nuclear import [2]. Transportin also ferries certain ribosomal proteins into the nucleus [4]; in both cases, it does so without the aid of an importin- α -like subunit [2,4]. Like importin- α/β -mediated import, the

binding of Ran–GTP to transportin is believed to terminate the import process and allow release of transportin’s cargo into the nucleus [4,25]. Other importin- β -like import and export receptors have been identified. These include the export receptor CAS, responsible for recycling importin α to the cytoplasm, and the NES export receptor, Crm1 (also known as exportin 1) [8–13,26–28]. All have been shown to bind Ran–GTP and to interact with the nuclear pore.

The basic architecture of the 1.2 megadalton nuclear pore consists of three large rings: a cytoplasmic ring, a central ring of eight spokes, and a nuclear ring with additional structural elements [29]. Eight filaments extend from the cytoplasmic ring and have been implicated in the initial docking of NLS–receptor complexes at the pore [30]. These filaments contain the nucleoporins Nup358 and Nup214. A central transporter structure lies at the hub of the spokes; Nup62 and its associated proteins map in or near this central region. A structure termed the nuclear basket extends inward from the nuclear ring; it consists of eight filaments joined to a small fourth ring and is thought to play a role in nuclear export as well as import. Nucleoporins Nup98, Nup93 and Nup205 reside in or near the basket, whereas Nup153 is localized specifically to the distal ring of the basket [31]. Filaments of a different type extend from the basket several thousand angstroms into the nucleus and contain the pore-associated protein Tpr and possibly Nup153 ([31–36] and references therein).

Interest has now focused on the identity of the nucleoporins that interact with the distinct transport receptors as they traverse the nuclear pore [28–29]. Nup153 has been implicated recently in both nuclear import and export; importin β can form a strong complex with Nup153 and can do so while complexed to importin α and NLS cargo under physiological conditions [37]. Expression of a fragment of Nup153 in transfected cells leads to accumulation of polyA⁺ RNA in the nucleus, implying a possible role for Nup153 in export [38]. Other complexes between receptors and nucleoporins have also been observed in cell extracts, including interactions between Nup358 and importin β and between Nup214 and Crm1 [39–41]. Because all these nucleoporins contain various forms of phenylalanine–glycine (FG) repeats, the FG domains have been viewed as possible common binding sites for the many transport receptors [42,43]. In the case of importin β , however, this hypothesis does not seem to hold true. Although importin β can bind strongly to the FG repeat domains of Nup153 and Nup358, no interaction of importin β is seen with the FG-containing nucleoporins Nup214, Nup98 or Nup62 in *Xenopus* egg extracts or in extracts of assembled rat liver nuclear pores [37]. Similarly, in yeast, importin β interacts with certain FG nucleoporins, but not with others ([44] and references therein). In a case where recombinant Nup62 is seen to interact with importin β in

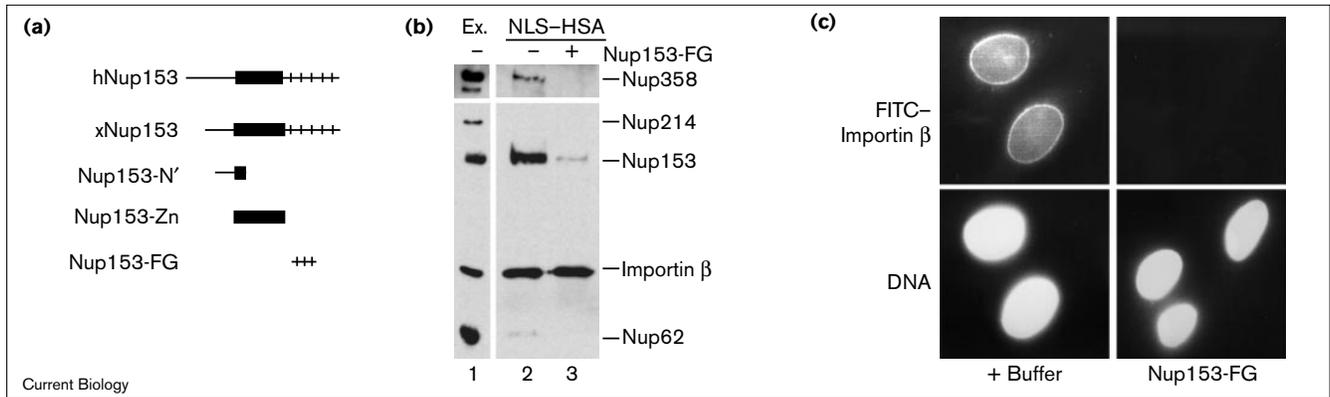
solution, it does so through the coiled-coil domain of Nup62, not the FG domain [45]. Lastly, importin β has been found in cells in a strong complex with Tpr, which lacks FG repeats altogether [37]. It is clear from these results that importin β does not interact with all FG-repeat-containing nucleoporins and does interact with other nucleoporins that do not contain repeats. It appears that import receptors will have multiple specific targets among the nucleoporins with which they interact: some of these nucleoporins will contain FG repeats, whereas some will not. Importin β may bind first to the FG region of Nup358 on the cytoplasmic filaments, pass perhaps transiently to Nup62, bind in a strong complex to the FG region of Nup153 on the basket, while still carrying importin α and NLS cargo, and is then disassembled from Nup153 by the binding of Ran–GTP to importin β . Importin β also binds to Tpr when it is free of NLS cargo; interaction of importin β with Tpr might occur either because this is a more downstream site in the importin β import pathway or because the Tpr filaments might represent a parallel pathway that importin β takes when it carries cargo lacking a classical NLS.

Do all transport receptors follow an identical pathway to that of importin β or do they use distinct pathways? In this study we have analyzed two receptors, transportin and Crm1, asking first whether they bind to the importin β binding site present on Nup153. We found that an FG repeat fragment of Nup153 (termed Nup153-FG), when added in excess, prevented the interaction of importin β with the nuclear pore and prevented NLS-mediated import. Nup153-FG had no effect, however, on the activity of transportin or on the pore binding of Crm1. Thus, Nup153-FG does not comprise a common binding site for all importin- β -related transport factors. Indeed, we found that a separate domain within Nup153 interacted with transportin and, when a fragment containing this more amino-terminal domain was added in excess, it blocked the import activity of transportin but not that of importin α/β . The interaction of this separate Nup153 domain with transportin was disrupted by a non-hydrolyzable form of GTP, GMP-PNP, as well as by a Ran mutant unable to hydrolyze GTP (Ran^{G19V}). We conclude that Nup153 contains distinct binding sites for interaction with two different import pathways.

Results

A Nup153 FG repeat fragment inhibits NLS-directed nuclear import but not transportin-mediated import

Sequence analysis has identified a family of related import and export receptors, including importin β , transportin, Crm1, CAS, exportin-tRNA, RanBP5 and RanBP7 [2,8–15,26]. We wished to determine whether the FG-repeat-containing region of Nup153, which has strong affinity for importin β [37], is also a major binding site for other transport receptors. If this Nup153 domain is acting

Figure 1

The Nup153-FG fragment blocks the binding of importin β to the nuclear pore. **(a)** The structure of full-length human Nup153 protein (hNup153), and the protein encoded by the partial *Xenopus* Nup153 cDNA (xNup153) from which Nup153-N', Nup153-Zn and Nup153-FG were derived. Boxes represent the putative zinc finger repeat region; human Nup153 has four repeats [46], whereas *Xenopus* Nup153 has five [37]. Vertical lines represent the FG repeat region. Nup153-N' contains the equivalent of amino acids 431–723 of human Nup153 (amino acids 53–334 of the *Xenopus* partial sequence; [37]), and consists of unique amino-terminal amino acids and the first *Xenopus* zinc finger. Nup153-Zn contains amino acids 272–544 of the *Xenopus* partial sequence and represents the entire putative zinc finger domain of Nup153. Nup153-FG contains the equivalent of amino acids 976–1198 of human Nup153 (amino acids 618–828 of the *Xenopus* partial sequence; [37]) and has nine FG repeats. **(b)** Nup153-FG prevents the interaction of importin β with Nup153 and Nup358. *Xenopus* egg extract with 1.9 μ g NLS-HSA (see later) was diluted 100-fold in ELBS [37] with or without 4 μ g Nup153-FG, followed by the addition of

anti-HSA antibody and protein-A-Sepharose. After 2 h, the immunoprecipitated proteins were transferred to a PVDF membrane and probed with anti-importin β and mAb414 (which recognizes the repeat-containing nucleoporins Nup358, Nup214, Nup153 and Nup62). Without Nup153-FG, this results in the coimmunoprecipitation of a complex containing NLS-HSA, importin α , importin β and either Nup153 or Nup358 (lane 2; [37]). Addition of Nup153-FG resulted in a loss of endogenous Nup153 and Nup358 from the immunoprecipitates (lane 3). Lane 1 contains 0.2 μ l *Xenopus* egg extract (Ex.) probed with the antibodies. **(c)** Nup153-FG blocks binding of FITC-importin β to assembled nuclear pores. Permeabilized HeLa cells were incubated with *Xenopus* egg extract, energy mix, FITC-importin β , and either buffer or Nup153-FG (8 μ M). In buffer, the FITC-importin β bound to the rim of permeabilized cell nuclei. With the addition of Nup153-FG, this nuclear rim binding was efficiently blocked. (The removal of FITC-importin β was proportional to the amount of Nup153-FG fragment added; data not shown). The lower panels of the figure show overexposed DNA stains of the cells in the upper panels visualized with Hoechst 33258 dye.

as a universal binding site for nuclear transport receptors, then addition of an FG fragment containing the importin β binding site to a permeabilized cell assay might block the ability of other transport receptors to bind and advance through the pore. A soluble fragment of the *Xenopus* Nup153 FG domain, homologous to amino acids 976–1198 of human Nup153 (Figure 1a; [46,47]) and consisting of about half of the FG domain from the *Xenopus* protein, was expressed and purified. Normally, Nup153 and Nup358 are found in complexes with importin α/β in *Xenopus* egg extracts. Added Nup153-FG fragment strongly competed Nup153 and Nup358 away from these importin α/β complexes (Figure 1b).

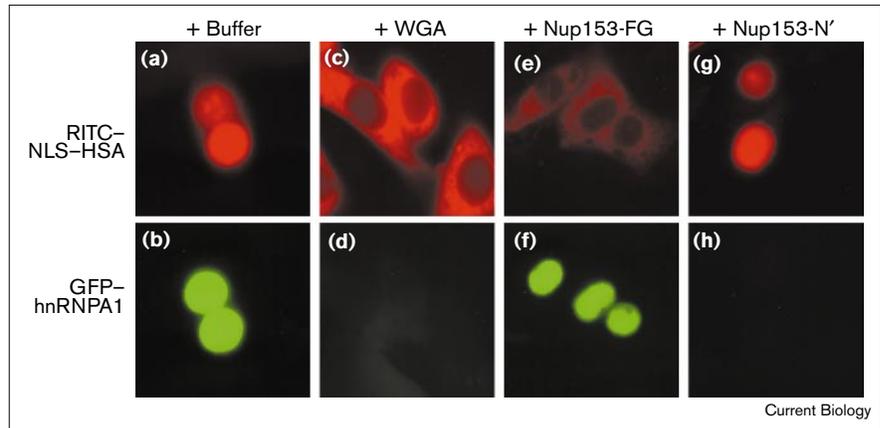
To ask whether the Nup153-FG fragment could block the binding of importin β to assembled nuclear pores, a permeabilized cell assay was employed [48]. HeLa cells grown on coverslips were permeabilized with digitonin, washed, then inverted onto a reaction mix containing fluorescein isothiocyanate (FITC)-labeled importin β , *Xenopus* egg cytosol, an energy mix (see Materials and methods), and either buffer or Nup153-FG. In the presence of cytosol plus buffer, the FITC-importin β showed efficient

binding to the nuclear rims of permeabilized cells (Figure 1c; top left). Upon addition of Nup153-FG, however, we observed a block in the ability of FITC-importin β to bind to the nuclear rim (Figure 1c; top right). This indicates that in the presence of excess Nup153-FG, importin β is not able to interact with assembled nuclear pore complexes.

We next asked whether classical NLS-directed import is inhibited by the Nup153-FG fragment; one would predict that this import would be completely blocked because importin β cannot bind to the nuclear pore in the presence of excess Nup153-FG. Permeabilized cells were incubated with reticulocyte lysate, energy mix, and two different import substrates. These were rhodamine B isothiocyanate-labeled human serum antigen fused to multiple SV40-type NLSs (RITC-NLS-HSA), and a green fluorescent protein fused to hnRNPA1 (GFP-hnRNPA1). To this, either buffer, the Nup153-FG fragment, or the transport inhibitor wheat germ agglutinin (WGA) [49] was added. NLS import was monitored by the nuclear accumulation of rhodamine-labeled NLS transport substrate (Figure 2, top panels). We found that the import

Figure 2

The Nup153-FG fragment blocks importin- β -mediated import, and the Nup153-N' fragment inhibits transportin-mediated import. Permeabilized HeLa cells were incubated with reticulocyte lysate, energy mix, RITC-NLS-HSA, GFP-hnRNPA1, plus one of the following: (a,b) buffer, (c,d) 1 mg/ml wheat germ agglutinin (WGA), (e,f) 25 μ M Nup153-FG, or (g,h) 8 μ M Nup153-N'. (a,c,e,g) Analysis of RITC-NLS-HSA import. (b,d,f,h) GFP-hnRNPA1 import in the same cells. (d,h) When GFP-hnRNPA1 was excluded it showed no affinity for cytoskeletal elements and the cells appeared blank, as described previously [2]. (c,e) Excluded RITC-NLS-HSA did show affinity for cytoplasmic elements, as described [2].



of the RITC-labeled NLS-HSA was completely blocked by the addition of Nup153-FG (Figure 2a,e), indicating that the fragment is indeed a dominant-negative inhibitor for classical NLS-mediated import.

The import receptor transportin, which shares limited homology to importin β , mediates the nuclear import of hnRNPA1 and certain ribosomal proteins [2,4]. If the Nup153-FG region is also a binding site for transportin, one would expect that hnRNPA1 import would be blocked by the fragment. Surprisingly, the import of GFP-hnRNPA1 was completely unaffected by the Nup153-FG fragment (Figure 2b,f). This indicates that transportin has a much lower, or possibly negligible, affinity for Nup153-FG compared with importin β , and thus transportin and importin β receptors do not have identical binding sites on the nuclear pore.

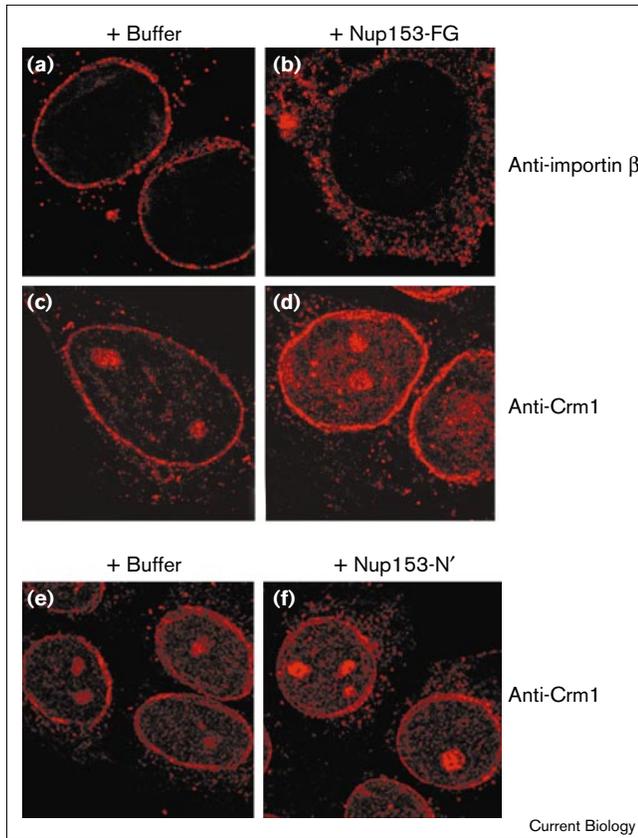
Nuclear rim binding of the NES receptor Crm1 is unaffected by the Nup153-FG fragment

We next asked whether the Nup153-FG fragment would alter the ability of the nuclear export receptor Crm1 to bind to the pore. Coverslips containing permeabilized HeLa cells were added to a reaction mix containing *Xenopus* egg cytosol, energy mix, and either buffer or Nup153-FG. After a short incubation (20 minutes), cells were fixed and immunostained for importin β or Crm1. Inclusion of Nup153-FG in the reaction caused loss of endogenous importin β from the nuclear rim (Figure 3a,b). Thus, Nup153-FG not only prevents FITC-labeled importin β from binding to the nuclear pores of permeabilized cells (Figure 1c), but also removes endogenous importin β from the pores (Figure 3b), perhaps by trapping endogenous importin β as it cycles off the pore. In contrast, Crm1 binding to the nuclear rim was unaffected or perhaps slightly increased by addition of Nup153-FG to permeabilized cells (Figure 3c,d). Crm1 staining remained rim-like with

some staining on the nucleoli, a previously reported pattern [39]. These results indicate that Crm1 does not appear to have an affinity for the FG fragment of Nup153 used here, a fragment which binds strongly to importin β and has a dominant-negative effect on importin- β -mediated import.

Transportin interacts with Nup153 at a site distinct from the importin β binding site

It was possible that the transportin-mediated import pathway might have unique and specific interactions with Nup153. To test this, two other regions of *Xenopus* Nup153, referred to as Nup153-N', which contains a more amino-terminal region of unique sequence along with one zinc finger repeat, and Nup153-Zn, which contains the entire zinc finger domain of *Xenopus* Nup153 (Figure 1a), were assessed for transportin binding. Recombinant Nup153-N', Nup153-Zn and Nup153-FG were added separately to rabbit reticulocyte lysate, which contains both endogenous transportin and importin β . GFP-hnRNPA1 protein was added to an aliquot of lysate as a control. Immunoprecipitation was performed using an antibody against the T7 tag that was present on the recombinant proteins. The immunoprecipitates were immunoblotted with anti-transportin and anti-importin β antibodies. Importin β was detected only in the Nup153-FG immunoprecipitate (Figure 4a, lane 5; see also [37]). Transportin was not detected in the Nup153-FG or Nup153-Zn immunoprecipitates (Figure 4a, lanes 4,5). It was detected in the control GFP-hnRNPA1 immunoprecipitate, as expected because hnRNPA1 is a normal cargo for transportin (Figure 4a, lane 3). Surprisingly, transportin was strongly detected in Nup153-N' immunoprecipitates (Figure 4a, lanes 1,2). The presence of transportin in the Nup153-N' immunoprecipitate implicates this region of Nup153 as a potential transportin binding site. The Nup153-N' region does not show significant homology to hnRNPA1. Although it contains a single FG motif, it does

Figure 3

Neither the Nup153-FG fragment nor the Nup153-N' fragment alter the binding of Crm1 at the nuclear rim. **(a–d)** Permeabilized HeLa cells were incubated with *Xenopus* egg extract, energy mix, and either buffer or Nup153-FG (35 μ M), then immunostained with (a,b) anti-importin β or (c,d) anti-Crm1 antisera, and RITC-labeled secondary antibody. Cells were examined with confocal microscopy (at 3600 \times magnification). **(e,f)** A similar experiment using either buffer or Nup153-N' (20 μ M) captured at 2400 \times magnification.

not resemble the Nup153-FG fragment which contains nine canonical FXFG repeats.

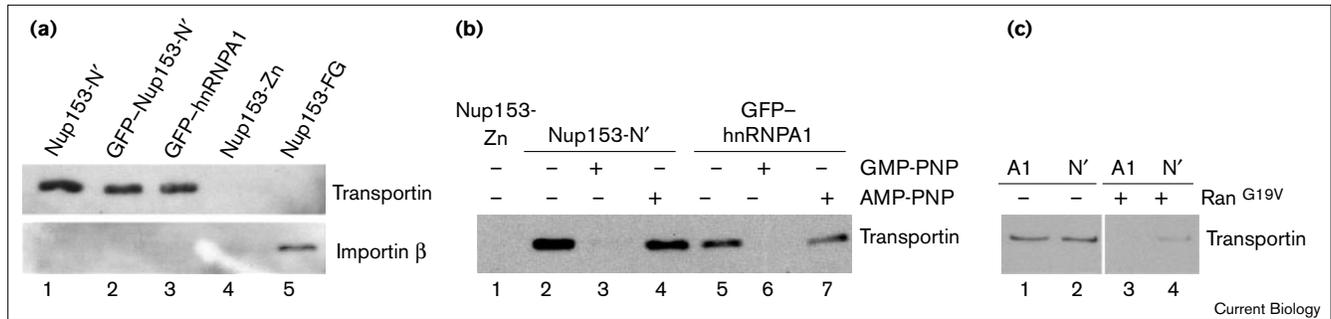
To further examine the hypothesis that the Nup153-N' fragment is a transportin binding region, we tested it for an effect on hnRNPA1 import. Indeed, Nup153-N' completely blocked the import of GFP-hnRNPA1 in permeabilized cells (Figure 2b,h). It had no effect on the import of RITC-NLS-HSA in the same cells (Figure 2g), nor did it inhibit the binding of Crm1 to the nuclear envelope in permeabilized cells (Figure 3e,f). These results confirm that, of the two import pathways, Nup153-N' specifically affects transportin-mediated import. Further, because Nup153-N' did not alter Crm1 localization at the nuclear periphery, this fragment does not have Crm1 binding activity, at least in the context of permeabilized cells — a situation presumed to be similar to that found *in vivo*.

Interaction of Nup 153-N' and transportin is sensitive to GMP-PNP and Ran^{G19V}

In many cases, Ran has proved an important switch for determining whether a transport receptor binds to its cargo and similarly whether it binds to the nuclear pore. Ran-GTP dissociates importin α/β from NLS cargo *in vitro* [50,51] and dissociates importin β from Nup153 and Tpr [37]. Ran-GTP also dissociates transportin from both hnRNPA1 and the ribosomal protein rpL23a [4,25]. We asked whether GMP-PNP prevented transportin from co-immunoprecipitating with the Nup153-N' fragment. Reticulocyte lysate, which contains endogenous transportin and Ran, was mixed with T7-tagged Nup153-N' or T7-tagged GFP-hnRNPA1, in the presence or absence of non-hydrolyzable GMP-PNP or AMP-PNP. The immunoprecipitates were then probed for transportin (Figure 4b). As expected, incubation with GMP-PNP led to a loss of transportin from the control GFP-hnRNPA1 immunoprecipitate (Figure 4b, lane 6), whereas incubation with AMP-PNP did not (lane 7). Strikingly, GMP-PNP also caused a loss of transportin from the Nup153-N' immunoprecipitate (Figure 4b, lane 3), whereas AMP-PNP did not (lane 4). No transportin was seen co-immunoprecipitating with the negative control Nup153-Zn fragment (Figure 4b, lane 1). These results indicate that the interaction of transportin with Nup153-N' is disrupted by GMP-PNP, presumably because Ran in a GMP-PNP-bound form acts to disassemble the Nup153-N'-transportin complex. In support of this conclusion, endogenous transportin immobilized on a column of GTP-bound Ran^{Q69L} (a mutant deficient in GTPase activity) did not bind Nup153-N' (data not shown). Moreover, when T7-tagged Nup153-N' was added to reticulocyte lysate and a Nup153-N'-transportin complex isolated (Figure 4c, lanes 2,4), the addition of Ran^{G19V} dissociated transportin from Nup153-N'.

Nup153 exists in an endogenous complex with transportin in *Xenopus* egg extract

The experiments described above identify a domain of Nup153 that both interacts with transportin and blocks transportin-mediated import when added in excess to permeabilized cells. To determine whether an interaction between endogenous full-length Nup153 and transportin occurs, *Xenopus* egg extract which contains the disassembled components of millions of nuclear pores in assembly-competent subcomplexes was analyzed [52]. Nup153 was immunoprecipitated from egg extract and the immunoprecipitate probed for the presence of transportin. Transportin could be clearly detected in the Nup153 immunoprecipitate, but not in a control immunoglobulin G (IgG) immunoprecipitate (Figure 5a, lanes 2,4). This indicates that not only Nup153-N', but also endogenous full-length Nup153 in *Xenopus* egg extracts, exists in a stable complex with transportin. Theoretically, transportin could bind to Nup153 either directly or through the bridging effect of another protein, such as hnRNPA1 or Ran. To test whether

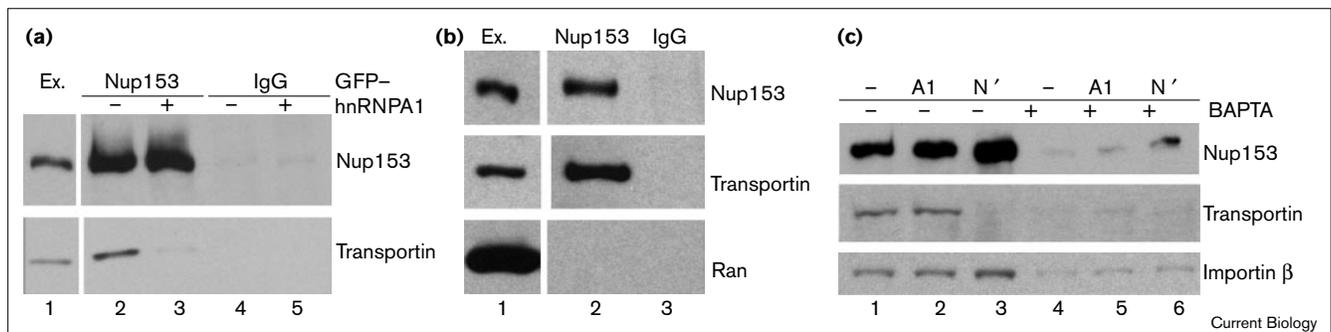
Figure 4


The Nup153-N' fragment binds transportin specifically and this complex is sensitive to GMP-PNP and Ran^{G19V}. **(a)** To determine whether any of the Nup153 fragments contain a transportin binding site, the recombinant proteins (750 ng) described in Figure 1a were added to 5 μ l rabbit reticulocyte lysate together with an antibody to the T7 tag (2 μ g). GFP-hnRNPA1 was used as a positive control for transportin binding. After dilution with IP buffer (see Materials and methods), protein-A-Sepharose beads were added. The immunoprecipitates were analyzed by immunoblotting with anti-transportin and anti-importin β antibodies. **(b)** Sensitivity of the transportin-Nup153-N' complex to a non-hydrolyzable GTP analog. Reticulocyte lysate was mixed with either buffer, GMP-PNP or AMP-PNP and diluted 25-fold in IP buffer (final nucleotide concentration 2 mM). Recombinant T7-tagged Nup153-Zn, Nup153-N'

or GFP-hnRNPA1 was then added together with anti-T7 antibody and protein-A-Sepharose beads. Immunoprecipitates were analyzed by immunoblotting with anti-transportin antisera. Immunoblotting with anti-T7 antibody indicated that all the recombinant proteins in (a,b) were successfully immunoprecipitated (data not shown). **(c)** The Nup153-N'-transportin complex is disassembled by Ran^{G19V}. T7-tagged GFP-hnRNPA1 (A1) or Nup153-N' (N') were added to reticulocyte lysate, incubated for 2 h, then immunoprecipitated with anti-T7 antibody and protein-A-Sepharose beads. The immunoprecipitates were washed, and split into two aliquots to which either GMP-PNP (1 mM) or GMP-PNP (1 mM) plus Ran^{G19V} (5 μ M) were added in IP buffer. After a further fivefold dilution in IP buffer, the beads were incubated for 1 h at 4°C. Bound proteins were eluted and analyzed for transportin by immunoblotting.

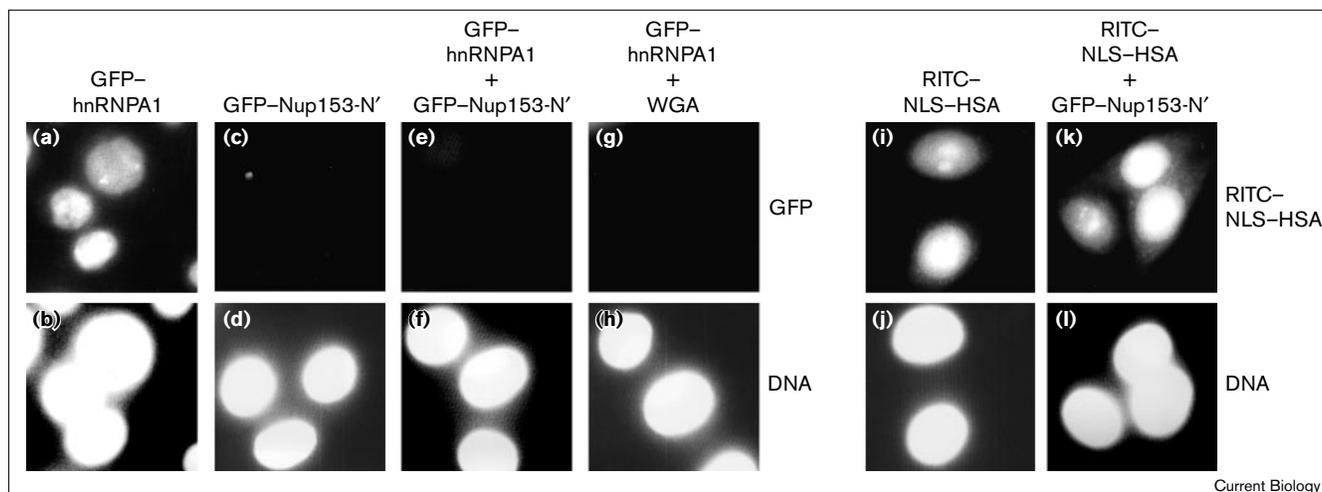
hnRNPA1 serves as a bridge, T7-tagged hnRNPA1 was added to *Xenopus* egg cytosol, immunoprecipitated, and probed for transportin and Nup153. Transportin, but not Nup153, was present in the hnRNPA1 immunoprecipitate (data not shown). To test for a potential Ran bridging effect, endogenous Nup153 was immunoprecipitated from

Xenopus egg extract and probed for the presence of transportin and Ran; only transportin was present in the Nup153 immunoprecipitate to any significant extent (Figure 5b, lane 2). These results indicate that the interaction between Nup153 and transportin is not bridged by hnRNPA1 cargo or Ran, consistent with a direct interaction occurring

Figure 5


Nup153 is in a complex with transportin in *Xenopus* egg extracts that is competed by excess GFP-hnRNPA1. **(a)** *Xenopus* egg extract (5 μ l), diluted 100-fold, was mixed with either anti-Nup153 antiserum or total rabbit IgG. Buffer or GFP-hnRNPA1 (30 μ g) was subsequently added to each reaction. Immunoprecipitates were electrophoresed and immunoblotted with anti-Nup153 and anti-transportin antisera. **(b)** Ran is not present in the Nup153-transportin complex. Nup153 was immunoprecipitated from *Xenopus* egg extract and probed with antibodies specific for Nup153, transportin and Ran. A control IgG immunoprecipitate was also examined. In (a,b), lane 1

contains 0.2 μ l *Xenopus* egg extract (Ex.) analyzed on the same blot with the same exposure time. **(c)** Nup153-N' inhibits the association of transportin with nuclear pores assembled *in vitro*. Annulate lamellae were formed from *Xenopus* egg extracts and membranes in the presence of buffer (lanes 1,4), 12.5 μ M GFP-hnRNPA1 (A1; lanes 2,5), or 8 μ M Nup153-N' (N'; lanes 3,6). BAPTA, which blocks pore assembly but not membrane fusion, was added as indicated. Annulate lamellae were then isolated by centrifugation through a sucrose cushion and analyzed by immunoblotting for Nup153, transportin or importin β .

Figure 6

The Nup153-N' fragment does not localize to nuclei. **(a)** Permeabilized cells were mixed with reticulocyte lysate, energy mix, and one of the following: **(a,b)** GFP-hnRNPA1, **(c,d)** GFP-Nup153-N', **(e,f)** GFP-hnRNPA1 and GFP-Nup153-N', or **(g,h)** GFP-hnRNPA1 and 1 mg/ml of the transport inhibitor WGA. Native protein gels confirmed that GFP-Nup153-N' and GFP-hnRNPA1 had equivalent

fluorescence per microgram of protein (data not shown).

(i-l) Importin- β -mediated import of RITC-NLS-HSA was not blocked by the addition of GFP-Nup153-N'. The lower panels of the figure show overexposed DNA stains of the cells in the upper panels visualized with Hoechst 33258 dye.

between transportin and Nup153, although it is still possible that an unknown factor could be present in the Nup153-transportin complex.

The interaction between Nup153 and importin β occurs even when importin β is bound to importin α and NLS cargo [37]. To ask whether the Nup153-transportin complex can exist when transportin is bound to hnRNPA1 cargo, excess amounts of GFP-hnRNPA1 were added to *Xenopus* egg extracts, then Nup153 was immunoprecipitated. We found that GFP-hnRNPA1 caused a loss of transportin from Nup153 immunoprecipitates (Figure 5a, lane 3; GFP-hnRNPA1 had no effect on the interaction of importin β with Nup153, data not shown). These results indicate that transportin cannot interact with Nup153 when transportin is carrying an M9 cargo such as hnRNPA1.

The Nup153-N' fragment is not imported *in vitro*

The finding that transportin cannot bind hnRNPA1 and Nup153 simultaneously was consistent with one of several possibilities. The most interesting possibility is that Nup153, located on the nuclear side of the pore, binds transportin and plays a role in the disassembly of the transportin-hnRNPA1 complex after its import through the pore. A second possibility is that a sequence contained within Nup153-N' is itself an M9-like nuclear import signal that competes with hnRNPA1 for binding to transportin. To assess this second possibility, a GFP-Nup153-N' fusion was constructed. When added to reticulocyte lysate, it retained the ability to bind transportin (Figure 4a, lane 2).

Permeabilized HeLa cells on coverslips were incubated with reticulocyte lysate, energy mix and either GFP-hnRNPA1, GFP-Nup153-N', GFP-hnRNPA1 mixed with GFP-Nup153-N', or GFP-hnRNPA1 mixed with WGA. Significantly, the GFP-Nup153-N' reporter, unlike GFP-hnRNPA1, was not significantly imported into nuclei as judged by GFP fluorescence (Figure 6c) or by immunofluorescence using anti-Nup153 antisera (data not shown). GFP-Nup153-N' once again efficiently inhibited the import of GFP-hnRNPA1, however (Figure 6e), whereas it had no effect on import of RITC-NLS-HSA (Figure 6k). We conclude that Nup153-N' does not resemble an import cargo in its interaction with transportin.

The Nup153-N' fragment prevents the interaction of transportin with assembled nuclear pores

The findings that Nup153 interacts with transportin and that Nup153-N' inhibits transportin-mediated import of hnRNPA1 strongly suggest that the Nup153-N' region is acting as a docking site for transportin during its passage through the nuclear pore. If Nup153 is indeed a docking site for a transportin complex on the nuclear pore, then the addition of excess Nup153-N' should act as a sink and prevent transportin from interacting with the nuclear pore. To test this hypothesis, the ability of transportin to interact with *in vitro* assembled nuclear pores in the presence or absence of excess Nup153-N' was analyzed. Using *Xenopus* egg extracts, stacks of double nuclear membranes replete with numerous assembled nuclear pores, termed annulate lamellae, were formed *in vitro* (Figure 5c; [53]).

Annulate lamellae containing pores were assembled in the presence of buffer, GFP-hnRNPA1 or Nup153-N', then separated from unincorporated proteins by centrifugation through a sucrose cushion. Annulate lamellae nuclear pores were then probed for Nup153, importin β and transportin. In the presence of buffer or GFP-hnRNPA1, annulate lamellae pores were seen to contain Nup153 and associated importin β and transportin, as determined by immunoblotting (Figure 5c, lanes 1,2). The addition of BAPTA, which blocks pore assembly but not membrane fusion [54], prevented the association of Nup153, importin β , and transportin with membranes lacking annulate lamellae pores, demonstrating that the association of these proteins is dependent on pore formation (Figure 5c, lanes 4–6). Excess Nup153-N' did not prevent Nup153 or importin β from copurifying with annulate lamellae pores (Figure 5c, lane 3), nor did it prevent the association of nucleoporins Nup62, Nup214 or Nup358 with annulate lamellae (data not shown). Nup153-N' completely prevented the association of transportin with annulate lamellae pores, however (Figure 5c, lane 3), indicating that excess Nup153-N' prevents transportin from interacting with nuclear pores assembled *in vitro*.

Discussion

Nuclear transport is mediated by a set of discrete import and export receptors. These distantly related receptors might bind to similar sites on the nuclear pore or to distinct ones. Nup153, on the distal ring of the nuclear pore basket, is the most intranuclear component of the pore and is thus located in a region that is presumably of central importance to both import and export. Our previous data indicated that Nup153 contains a strong binding site for the importin α/β -NLS cargo complex and might thus bind this complex as it translocates into the nucleus [37]. In this study, we have identified a short FG repeat fragment of Nup153 that acts as a strong dominant-negative inhibitor of classical NLS-mediated import. This Nup153-FG fragment efficiently blocked the binding of importin β to the nucleoporins Nup153 and Nup358, prevented binding of FITC-importin β to the nuclear pores of permeabilized cell nuclei, and prevented rim docking of NLS substrates on nuclear pore complexes (data not shown). Disruption of the ability of importin β to interact with the pore clearly results in the dominant-negative effect of Nup153-FG on importin- α/β -mediated nuclear import.

We then used the permeabilized cell import assay to determine whether Nup153 might play a major role in other pathways of nuclear transport. Nup153-FG had no effect, however, on the ability of two other transport receptors, Crm1 or transportin, to bind to and/or traverse the nuclear pore. The fact that Nup153-FG blocks the docking and function of importin β but not that of Crm1 and transportin strengthens the conclusion that FG domains are not common and indiscriminate binding

motifs for all transport receptors. Instead, we found that transportin interacts with a more amino-terminal fragment of Nup153, Nup153-N'. This fragment, homologous to amino acids 431–723 of human Nup153, is a potent dominant-negative inhibitor of hnRNPA1 import, but had no effect on the import of RITC-NLS-HSA, an importin β cargo. Immunoprecipitates of Nup153 contained transportin but no significant amount of Ran, whereas immunoprecipitates of recombinant hnRNPA1 from egg extracts contained transportin but not Nup153 (data not shown). Together, these latter results strongly argue that the Nup153-N' region is a transportin-specific interaction site, one that requires neither Ran nor hnRNPA1 for binding transportin. Nup153-N' could either bind transportin directly or potentially bind a complex of transportin and an as yet unidentified protein.

Nup153 is present in an endogenous complex with transportin in nuclear-assembly-competent *Xenopus* egg extracts (Figure 5a), as well as in extracts of rat liver nuclei (data not shown). Moreover, we find that transportin is bound to the pores of annulate lamellae and this binding can be disrupted by the Nup153-N' fragment. This fragment shows no nuclear accumulation, arguing that it does not contain an NLS and does not bind transportin as if it were an M9 NLS. Taken together, the data are consistent with a model in which Nup153 in assembled pores acts as a structural platform involved in the transport process itself. Within the pore, the Nup153 FG region would mediate importin β binding, and the Nup153 N' region would act as a docking site for transportin complexes on the nuclear pore.

The next question to be considered concerns at what point during import the interaction of Nup153 with transportin is likely to take place. Clues can be found from characterizing the conditions under which the transportin-Nup153 complexes assemble and disassemble. Relevant to this, we found that only hnRNPA1-free transportin binds to Nup153. A second clue to when transportin and Nup153 might interact comes from the finding that the interaction of transportin with Nup153-N' is disrupted by GMP-PNP and by Ran^{G19V}, a Ran mutant unable to hydrolyze GTP. To date, almost all import receptors release their cargo in the presence of Ran-GTP. Additionally, importin β dissociates from Nup153 and Tpr in the presence of GMP-PNP [37]. These results and the finding that only transportin that is free of hnRNPA1 binds to Nup153 suggest two alternative possibilities: first, that Nup153 is encountered late in transportin's import through the pore, with Nup153 perhaps aiding transportin in releasing its hnRNPA1 cargo; or alternatively, that Nup153 on the pore interacts only with the class of transportin molecules importing ribosomal protein cargo [4]. If transportin uses two separate pathways through the nuclear pore, one for M9 cargo and one for

ribosomal protein cargo, it might be the ribosomal protein import pathway that converges on Nup153. Excess Nup153-N' would bind all the transportin, preventing it from functioning in either transportin pathway.

It should be noted that a study by Bastos *et al.* [38], using transfection of the entire human Nup153 FXFG domain into tissue culture cells, did not find an effect on NLS-directed import of the glucocorticoid receptor. In that study, however, the relative ratio of the fragment to importin β was not determined. It could well be that the FG repeat fragment was not expressed in transfected cells in high enough quantity to inhibit importin- β -mediated import in a dominant-negative manner. Interestingly, in that study transfection of the full-length FG repeat domain caused accumulation of polyA⁺ RNA in the nucleus [38]. This, in addition to evidence that Nup153 is involved in multiple RNA and protein export pathways [55], reflects an even more multi-dimensional role for Nup153 in nuclear transport. Examination of the binding of Nup153 to different export factors will be of interest. The three Nup153 domains examined here did not show obvious effects on the presence of Crm1 at the pore. It might be that other regions of Nup153 engage Crm1, a possibility that will be a subject for future studies.

Conclusions

The nuclear pore must import and export multiple types of cargo. As an increasing number of transport receptors are identified, the way in which they are individually recognized by the pore becomes a complex question. As all the known nuclear transport receptors have homology to importin β , one possible prediction has been that they would all recognize the same binding sites on the nuclear pore. In this study, we show that for import mediated by importin β and transportin this prediction is not true. Although a single nucleoporin, Nup153, interacts with the receptors for two separate import pathways, it does so using distinctly different domains. Both domains bind the different receptor complexes in a GTP-regulated manner. Significantly, these domains can be used to eliminate the individual pathways of import in a dominant-negative manner without affecting the binding of the export receptor Crm1 to the nuclear rim.

Materials and methods

Constructs and expression of protein fragments

Fragments of the gene encoding *Xenopus* Nup153 were placed in pET28 vectors for expression. Construction of Nup153-N' (amino acids 53–334 of the *Xenopus* partial Nup153 sequence) and Nup153-FG (amino acids 618–828 of the *Xenopus* partial Nup153 sequence) has been described ([37]; see constructs 3 and 8 therein). For generation of the Nup153-Zn construct, PCR was used to amplify the zinc finger domain (amino acids 272–544) encoded by the *Xenopus* Nup153 partial cDNA (Genbank accession number AF045567). This fragment was subsequently cloned into a pET28 expression vector. An expression vector encoding GFP fused to *Xenopus* hnRNPA1 was obtained from M. Michael and a construct encoding GFP–Nup153-N' was made

by subcloning the Nup153 cDNA fragment from construct 3 ([37], and see above) into the vector created by removal of hnRNPA1 from the construct encoding GFP–hnRNPA1. GST-Ran^{G19V} and ZZ-RanQ69L constructs [11] were obtained from S. Kornbluth and D. Gorlich, respectively. Expression and purification of all the recombinant fragments was as described [37].

Permeabilized cell assays

Permeabilized cell assays were conducted essentially as described [48]. HeLa cells, grown on coverslips, were permeabilized with digitonin [48] and incubated with a reaction mix containing the following: 5 μ l rabbit reticulocyte lysate [48], 1 μ l energy mix (0.5 mM ATP, 10 mM creatine phosphate, 50 μ g/ml creatine phosphokinase), 0.75 μ g GFP–hnRNPA1, and/or RITC–NLS–HSA transport substrate (1 μ l; [56]). In complete cytosol, the energy mix ensured the production of both ATP and GTP through interconversion. In certain reactions, 3 μ g GFP–Nup153-N' was included. Similarly, in some reactions, 2 μ l recombinant Nup153-N' fragment (see figure legends for concentrations), Nup153-FG fragment (in PBS–0.25M sucrose), or 2 μ l transport buffer [48] was added. After incubation for 20 min at room temperature, coverslips were washed twice with PBS, fixed on a drop of 50 μ l 4% formaldehyde in PBS for 10 min at room temperature, then washed twice with PBS. Cells were mounted on a drop of 90% glycerol, 10% PBS, containing 1 μ g/ml Hoechst 33258 fluorescent DNA dye, and 1 mg/ml anti-fading agent *p*-phenylenediamine. Coverslips were viewed with a Zeiss Photo III fluorescence microscope (63 \times lens) and import was measured by the accumulation of fluorescence in cell nuclei, as captured with a COHU CCD camera. Wheat germ agglutinin (Vector Labs) was included in some reactions at 1 mg/ml final concentration as a transport inhibitor [52]. Recombinant proteins were used at the following concentrations: 8 μ M Nup153-N' and 10–25 μ M Nup153-FG. To assess the nuclear rim binding of added FITC–importin β in permeabilized cells, the permeabilized cell assay was performed as described above, but *Xenopus* egg extract cytosol was used in the place of rabbit reticulocyte lysate and FITC-labeled importin β prepared as described [8] was substituted for the transport substrates. *Xenopus* egg extract cytosol was prepared as described [37]. Nup153-FG was used at 8 μ M in this experiment.

Production and affinity purification of antibodies

Anti-Nup153 antisera to amino acids 334–828 of the *Xenopus* Nup153 partial sequence has been described [37]. Affinity purification of this antibody, anti-Nup153 (363), herein simply referred to as anti-Nup153, was also as described [37].

Immunoprecipitation and immunoblotting

Immunoprecipitation from *Xenopus* egg extract or rabbit reticulocyte lysate (Promega) was conducted as follows: 5 μ l extract or lysate was diluted 100-fold in IP buffer (PBS containing 1 mM PMSF, 1 μ g/ml aprotinin and 1 μ g/ml leupeptin) or ELBS buffer [37]; 10 μ l protein-A–Sepharose (Pharmacia Fast Flow; Pharmacia Biotech) was added along with the appropriate antibody and recombinant protein. Antibodies were used in the following amounts per immunoprecipitation: 6 μ g anti-HSA coupled to protein-A–Sepharose [37], 2 μ g anti-T7 antibody (Novagen), 1.5 μ g total rabbit IgG (Calbiochem) and 1.5 μ g anti-Nup153 antibody. For immunoprecipitation of recombinant proteins added to rabbit reticulocyte lysate (see Figure 4), 750 ng recombinant protein was used. For immunoprecipitation from *Xenopus* egg extracts in the presence of GFP–hnRNPA1, 30 μ g GFP–hnRNPA1 was added. Immunoprecipitation of the HSA–NLS conjugate from *Xenopus* egg extract was performed as described [37]. Immunoprecipitations were rotated for 2 h at 4°C. The beads were pelleted and washed four times with IP buffer as described [37]. Immunoprecipitated proteins were eluted from the protein A beads by the addition of 25 μ l 100 mM glycine (pH 2.5) or 25 μ l SDS–PAGE loading buffer [37]. Samples eluted with glycine were neutralized with 22.5 μ l sample buffer and 2.5 μ l 100 mM Tris (pH 8.0) prior to gel electrophoresis. Samples were electrophoresed and immunoblotted as described [37]. Immunoprecipitation from rabbit reticulocyte lysate in the presence of GMP–PNP or

AMP-PNP was performed as above except that the lysate was first diluted 1:1 with a 100 mM stock of nucleotide in 100 mM HEPES, pH 8.0, and incubated at room temperature for 2 min. The mix was then diluted 25-fold into IP buffer at 4°C (final nucleotide concentration was 2 mM). For immunoblotting, primary antibodies were used at the following concentrations: 1:200 anti-transportin/karyopherin beta2 antibody (Santa Cruz Biotechnology, SC-6914; 250 µg/ml), 1:1000 monoclonal antibody 414 (Babco ascites MMS-120R-500), 1:40,000 anti-human importin β antibody, 1:6000 anti-Nup153 antibody [37], 1:2000 anti-Ran antibody (Transduction Laboratories, R32620) and 1:2000 anti-T7 tag antibody (Novagen). Secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:2000 (goat anti-rabbit HRP, goat anti-mouse HRP and donkey anti-goat HRP). The components for small-scale annulate lamellae reactions were mixed as in Meier *et al.* [53], except that 20 µl GFP-hnRNP1 (1.5 mg/ml) or 20 µl Nup153-N' (0.5 mg/ml) in PBS-0.25 M sucrose were added at t=0 min to 20 µl of the standard annulate lamellae reaction mix. Reactions were incubated at room temperature for 3 h, then the annulate lamellae harvested as described [53]. Incorporated proteins were analyzed by immunoblotting as described in the main text and figure legend.

Immunofluorescence microscopy

Immunofluorescence staining of permeabilized cells was as follows: HeLa cells were grown on coverslips and permeabilized with digitonin as described [48]. Coverslips were inverted on a reaction mix containing the following: 2 µl *Xenopus* egg extract, 1 µl energy mix, 2–7 µl recombinant protein (20 µM Nup153-N' or 35 µM Nup153-FG, final concentrations), and transport buffer (added to a final volume of 10 µl). Cells were incubated for 20 min at room temperature, washed twice with PBS, fixed on a 50 µl drop of 4% formaldehyde in PBS, and washed twice with PBS. Cells were then immunostained with either anti-importin β (1:400) or anti-Crm1 (1:100; [39]) antisera as described [37]. The anti-human importin β antisera and anti-human Crm1 antisera were the kind gifts of Dirk Gorlich and Gerard Grosveld [39]. The cells were viewed with a Nikon confocal microscope (model PCM2000). Images were captured on computer and analyzed with Adobe Photoshop 5.0.

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