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Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p

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***MTR10*, previously shown to be involved in mRNA export, was found in a synthetic lethal relationship with nucleoporin *NUP85*. Green fluorescent protein (GFP)-tagged Mtr10p localizes preferentially inside the nucleus, but a nuclear pore and cytoplasmic distribution is also evident. Purified Mtr10p forms a complex with Npl3p, an RNA-binding protein that shuttles in and out of the nucleus. In *mtr10* mutants, nuclear uptake of Npl3p is strongly impaired at the restrictive temperature, while import of a classic nuclear localization signal (NLS)-containing protein is not. Accordingly, the NLS within Npl3p is extended and consists of the RGG box plus a short and non-repetitive C-terminal tail. Mtr10p interacts *in vitro* with Gsp1p-GTP, but with low affinity. Interestingly, Npl3p dissociates from Mtr10p only by incubation with Ran-GTP plus RNA. This suggests that Npl3p follows a distinct nuclear import pathway and that intranuclear release from its specific import receptor Mtr10p requires the cooperative action of both Ran-GTP and newly synthesized mRNA.**

Keywords: importin β-homologue/Mtr10p/nuclear import/nuclear pore complex/Ran mRNA export

Introduction

In eukaryotic cells, the nuclear interior is separated from the cytoplasm by the double nuclear membrane, and all transport between these two compartments occurs through the nuclear pore complexes (NPCs), large macromolecular assemblies embedded in the nuclear envelope (reviewed in Doye and Hurt, 1997). A number of recent discoveries have led to the development of a model for active nuclear protein import, the principles of which may also apply to active nuclear export of proteins and RNA (reviewed in Görlich and Mattaj, 1996; Corbett and Silver, 1997; Goldfarb, 1997; Nakielny *et al.*, 1997; Nigg, 1997). According to this model, import of proteins into the nucleus is mediated by soluble and mobile receptors which bind to their import substrates through recognition of sequences that function as nuclear localization signals (NLS). These receptors are responsible for targeting of the import complex to the NPC and its subsequent translocation into the nucleoplasm where the complex is dissociated, the import cargo released and the receptor recycled back into the cytoplasm.

In the case of the basic-type (classic) NLS which is found in a variety of different nuclear proteins, the receptor is a heterodimer consisting of importin α, which binds the NLS, and importin β, which mediates the interaction with the NPC through its affinity for repeat-containing nucleoporins. Importin β is the founding member of a large protein family which is characterized, despite the overall limited sequence homology, by the presence of an N-terminally located Ran-GTP-binding domain (Fornerod *et al.*, 1997b; Görlich *et al.*, 1997). Other members of this family which have been shown to function as nuclear import receptors include transportin and karyopherin β3. Transportin binds directly to the M9 domain of the hnRNP A1 protein and mediates its nuclear uptake (Pollard *et al.*, 1996; Fridell *et al.*, 1997), while it is also involved in the nuclear import of hnRNP F which lacks a similar domain (Siomi *et al.*, 1997). The yeast homologue of transportin, Kap104p, has also been shown to be required for the nuclear import of mRNA-binding proteins such as Nab2p and Nab4p (Aitchison *et al.*, 1996). Karyopherin β3 binds directly to a subset of ribosomal proteins (Yaseen and Blobel, 1997), and its yeast homologues Kap123p or Yrb4p have indeed been shown to be required for efficient nuclear import of ribosomal proteins (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997). Importin β has also been implicated in the nuclear import of U snRNPs, a process that does not, however, require importin α (Palacios *et al.*, 1997). These results demonstrate the existence of distinct nuclear import pathways, as defined by different types of NLSs and their cognate transport receptors of the importin β family.

However, other members of the importin β family recently have been implicated in nuclear export processes and therefore termed exportins (reviewed in Ullman *et al.*, 1997). CAS mediates the export of importin α from the nucleus (Kutay *et al.*, 1997), while CRM1 functions as an export receptor for the leucine-rich nuclear export signals (NES) (Fornerod *et al.*, 1997a; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). This type of NES mediates nuclear export of proteins as well as RNA–protein complexes, as is the case for the human immunodeficency virus (HIV) protein Rev which binds to unspliced or partially spliced viral transcripts (for review, see Gerace, 1995). Nuclear export of cellular RNA may proceed by a similar mechanism, as NES-containing RNA-binding proteins could facilitate nuclear export of the bound RNA. In fact, export of U snRNAs, which requires the cap-binding protein complex (CBC) (Izaurralde *et al.*, 1995), has been suggested to follow the same export route as Rev (Fischer *et al.*, 1995). Similar NESs have also

been found in other putative transport factors such as Kap95p (Iovine and Wente, 1997), RanBP1 (Richards *et al.*, 1996), Gle1p (Murphy and Wente, 1996) and Mex67p (Segref *et al.*, 1997). Another type of NES is represented by the M9 domain of hnRNP A1 (Michael *et al.*, 1995). This protein and other hnRNP proteins shuttle between the nucleus and the cytoplasm and are suggested to play a role in mRNA export from the nucleus (Izaurralde *et al.*, 1997a). It should be borne in mind, however, that a clear division of the members of the importin β family into importins and exportins may not be straightforward as, for example, the yeast proteins Kap123p and Pse1p have been implicated in both nuclear uptake of ribosomal proteins and nuclear export of mRNA (Seedorf and Silver, 1997).

Apart from importins and exportins, a central role in nucleocytoplasmic transport is played by the small GTPase Ran and its effectors (reviewed in Moore and Blobel, 1994; Koepp and Silver, 1996; Goldfarb, 1997). Hydrolysis of GTP by Ran is probably used as an energy source for the translocation of import complexes into the nucleus through the NPCs. However, recent data suggest that nuclear export of various substrates does not require Ran-dependent GTP hydrolysis, but rather the presence of nuclear Ran-GTP (Izaurralde *et al.*, 1997b; Richards *et al.*, 1997). Indeed, Ran-GTP can trigger the dissociation of the importin–import substrate complex (Rexach and Blobel, 1995; Görlich *et al.*, 1996; Izaurralde *et al.*, 1997b), while it promotes the association of an exportin with the corresponding export cargo (Fornerod *et al.*, 1997a; Kutay *et al.*, 1997). The presence of Ran-GTP in the nucleoplasm is ensured by the nuclear localization of the Ran nucleotide exchange factor RCC1 and the exclusion from the nucleus of the GTPase-activating protein RanGAP1. Ran-GDP generated in the cytoplasm or in the vicinity of the NPC by the concerted action of RanGAP1 and RanBP1/RanBP2 is required both for nuclear import and for the last step of an export reaction, the release of the export substrate (Bischoff and Görlich, 1997). Therefore, the asymmetric distribution of the components of the Ran system may determine the directionality of the nuclear transport processes. However, Ran or energy may not be required for the nuclear uptake of importins in the absence of an import substrate (Kose *et al.*, 1997).

Genetic screens in yeast for mutants defective in poly(A)$^+$ RNA export (Amberg *et al.*, 1992; Kadowaki *et al.*, 1992) and synthetic lethal screens starting with nucleoporin mutants (Doye and Hurt, 1997) have led to the identification of many factors required for poly(A)$^+$ RNA nuclear export, which are neither exportins (i.e. not belonging to the importin β-like family) nor components of the Ran system. Among these, Nup159p (Gorsch *et al.*, 1995), Mtr2p (Kadowaki *et al.*, 1994b), Gle1p (Murphy and Wente, 1996) and Mex67p (Segref *et al.*, 1997) could play a direct role in the mRNA export process, because conditionally lethal mutants exhibit a fast and strong onset of the mRNA export defect. Furthermore, Npl3p, a yeast hnRNP protein, which shuttles between the nucleus and cytoplasm, was also suggested, in analogy to hnRNP A1, to be involved in mRNA export (Lee *et al.*, 1996). These proteins could function as adaptors between exportins and RNA molecules, as elements of the NPC required for RNP docking and translocation, or they may define RNA export pathways which are not exportin-dependent. Finally, as shuttling proteins have to be re-imported into the nucleus, they should also associate with import receptors.

Yeast Nup84p was identified through its genetic interaction with the essential nucleoporin Nsp1p. It subsequently was shown that Nup84p forms a complex with five additional proteins, Nup120p, Nup85p, Sec13p, Seh1p and the C-terminal domain of Nup145p (Siniossoglou *et al.*, 1996; Teixeira *et al.*, 1997). The Nup84p complex is required for both nuclear pore distribution within the nuclear membrane and poly(A)$^+$ RNA export. We report here the identification of *MTR10* as a component that genetically interacts with *NUP85*. Mutations in the *MTR10* gene previously have been reported to cause accumulation of poly(A)$^+$ RNA in the nucleus (Kadowaki *et al.*, 1994a). Mtr10p associates with the NPC and mediates the nuclear import of Npl3p with which it also physically interacts. Taking into account the homology of Mtr10p to members of the importin β family, we suggest that Mtr10p is a nuclear import receptor specialized in the transport of Npl3p and possibly other RNA-binding proteins.

Results

Identification of MTR10 in a synthetic lethal screen with the nup85Δ mutant

Several members of the Nup84p nucleoporin complex including Nup85p are involved in mRNA export (Siniossoglou *et al.*, 1996). To find components which functionally interact with Nup85p and thus could belong to the mRNA transport machinery, we isolated synthetic lethal (sl) mutants of the *nup85*Δ allele (see Materials and methods). An uncharacterized sl mutant (sl125) from this screen was found to be complemented by the *MTR10* gene (DDBJ/EMBL/GenBank accession number Q99189). *MTR10* initially was isolated in another genetic screen for poly(A)$^+$ RNA export mutants (Kadowaki *et al.*, 1994a) and encodes a 972 amino acid protein with a predicted mol. wt of 110 kDa. It has been reported recently that Mtr10p belongs to a protein family characterized by a sequence motif related to the Ran-binding site of importin β (Fornerod *et al.*, 1997b; Görlich *et al.*, 1997). Members of this family have been shown to function either as nuclear import receptors (importins) or as nuclear export receptors (exportins) (reviewed in Ullman *et al.*, 1997). To study further the *in vivo* role of Mtr10p in nucleocytoplasmic transport, we generated *mtr10* mutants. Deletion of the *MTR10* gene causes a strong growth defect of cells at physiological temperatures (e.g. 30°C) and a complete growth arrest at the non-permissive temperature (37°C) (Figure 1A and B). By *in vitro* random mutagenesis of the isolated *MTR10* gene, we could isolate several thermosensitive (ts) mutants, which revealed slightly reduced growth rates at 30°C, but still arrested at 37°C. One of these ts mutants, *Ømtr10-7*, was used for further analysis (Figure 1B).

GFP-tagged Mtr10p exhibits a nuclear pore, intranuclear and cytoplasmic location

To determine the subcellular location of Mtr10p, a green fluorescent protein (GFP)-tagged version of it (GFP-Mtr10p) was expressed in the *mtr10::HIS3* disruption mutant. GFP-Mtr10p was functional and complemented

the thermosensitive growth defect of the *mtr10* mutant (Figure 1B). When living cells were analysed by fluorescence microscopy, GFP-Mtr10p accumulates inside the nucleus, but a nuclear envelope and distinct cytoplasmic staining was also noticed (Figure 2A). By digital confocal imaging which uses mathematical algorithms to deconvolve the digital image (see Materials and methods), the nuclear envelope location of GFP-Mtr10p becomes more evident (Figure 2B). To determine whether the nuclear envelope location reflects an association with the NPCs, GFP-Mtr10p location was analysed in nup133^{-} cells, in which NPCs are clustered in one or few foci. Indeed, GFP-Mtr10p was apparently localized in these clusters (Figure 2C). This shows that a pool of Mtr10p physically associates with the NPCs under steady-state conditions. This association may be dynamic as Mtr10p is present in both the cytoplasm and the nucleoplasm, and therefore may shuttle between these two compartments (see also Discussion).

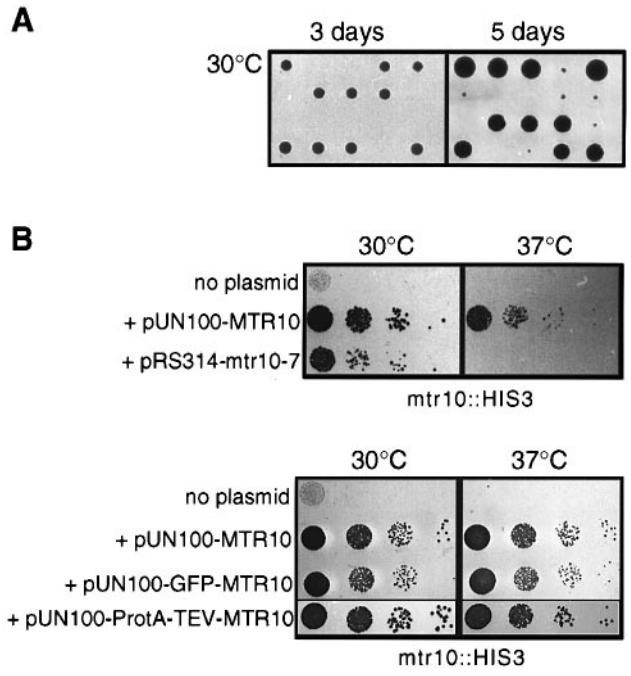

Fig. 1. Generation and characterization of *mtr10* mutants. (**A**) Tetrad analysis of a sporulated yeast diploid strain RS453 (Table I) disrupted for the *MTR10* gene. Haploid progeny carrying the *mtr10::HIS3* gene disruption exhibit a very slow growing phenotype at 30°C, so that colonies only become visible after 5 days of incubation. The fast growing colonies are *MTR10*$^{+}$ progeny. (**B**) Growth dot-spot analysis of the *mtr10::HIS3* null strain, ts *mtr10-7* cells and *mtr10::HIS3* cells complemented by ProtA–Mtr10p and GFP-Mtr10p. Pre-cultures were diluted in growth medium and equivalent amounts of cells (diluted in 10^{-1} steps) were spotted onto YPD plates. Plates were incubated for 3 days at the indicated temperatures.

Purification of Mtr10p reveals association with Npl3p and another putative RNA-binding protein

For its biochemical purification, Mtr10p was tagged at its N-terminal end with two IgG-binding domains derived from *Staphylococcus aureus* protein A. Furthermore, a cleavage site for the TEV protease comprising seven amino acids was inserted between the ProtA tag and Mtr10p (see Materials and methods). This ProtA–TEV–Mtr10p fusion protein was functional since it could complement the ts growth defect of *mtr10::HIS3* cells (Figure 1B). ProtA–TEV–Mtr10p was affinity-purified from this strain under non-denaturing conditions by IgG–Sepharose chromatography (Figure 3A). The Mtr10p protein was released from the column upon incubation with recombinant TEV protease. SDS–PAGE analysis of the released proteins revealed the presence of Mtr10p (migrating at ~100 kDa), a prominent band of 58 kDa and a weaker band of 75 kDa (Figure 3A, lane 1). Whereas the presence of the 75 kDa band varied from preparation to preparation, the 58 kDa band was always present in a similar stoichiometric ratio. Both bands were analysed by mass spectrometry in order to identify the proteins that co-purify with Mtr10p (see Materials and methods). The 75 kDa protein corresponds to Hsp70 (Ssa1p). The prominent 58 kDa band is composed of two proteins that co-migrate, Npl3p (DDBJ/EMBL/GenBank accession number Q01560) and Hrb1p (DDBJ/EMBL/GenBank accession number P38922). Npl3p is an RNA-binding protein that shuttles between the nucleus and the cytoplasm and is required for nuclear export of mRNA (Lee *et al.*, 1996). Hrb1p is an uncharacterized protein, but it appears to belong to the family of RNA-binding proteins as it contains three RNP motifs and a domain in the N-terminal part

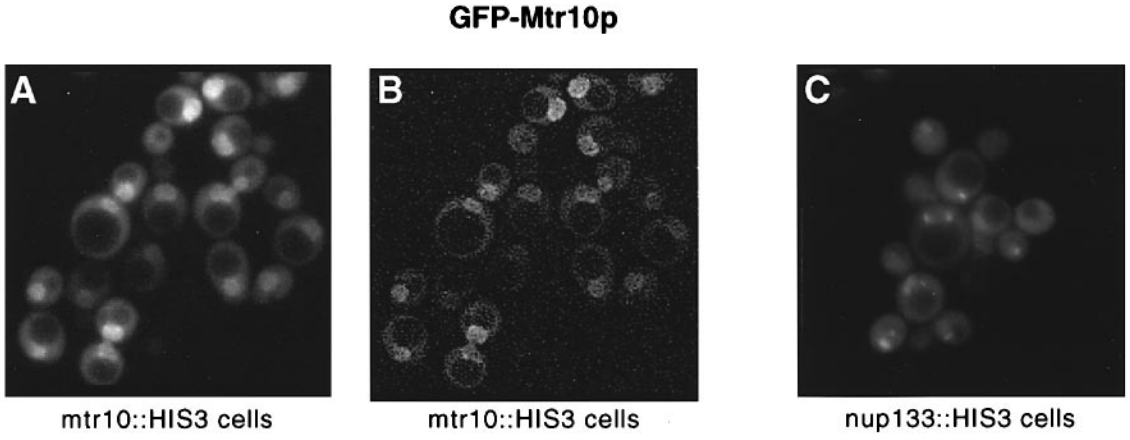

Fig. 2. Localization of GFP-tagged Mtr10p in living cells. (**A**) Fluorescence microscopy of *mtr10::HIS3* cells expressing GFP-Mtr10p. GFP-Mtr10p is located predominantly inside the nucleus, with the tendency to be more concentrated around the nuclear envelope. Less GFP-Mtr10p signal with vacuolar exclusion is seen in the cytoplasm. (**B**) Same as (A), but the digital picture was processed further by digital confocal imaging (see Materials and methods). (**C**) Fluorescence microscopy of GFP-Mtr10p in nup133^{-} cells. GFP-Mtr10p clusters together with NPCs in one or a few spots.

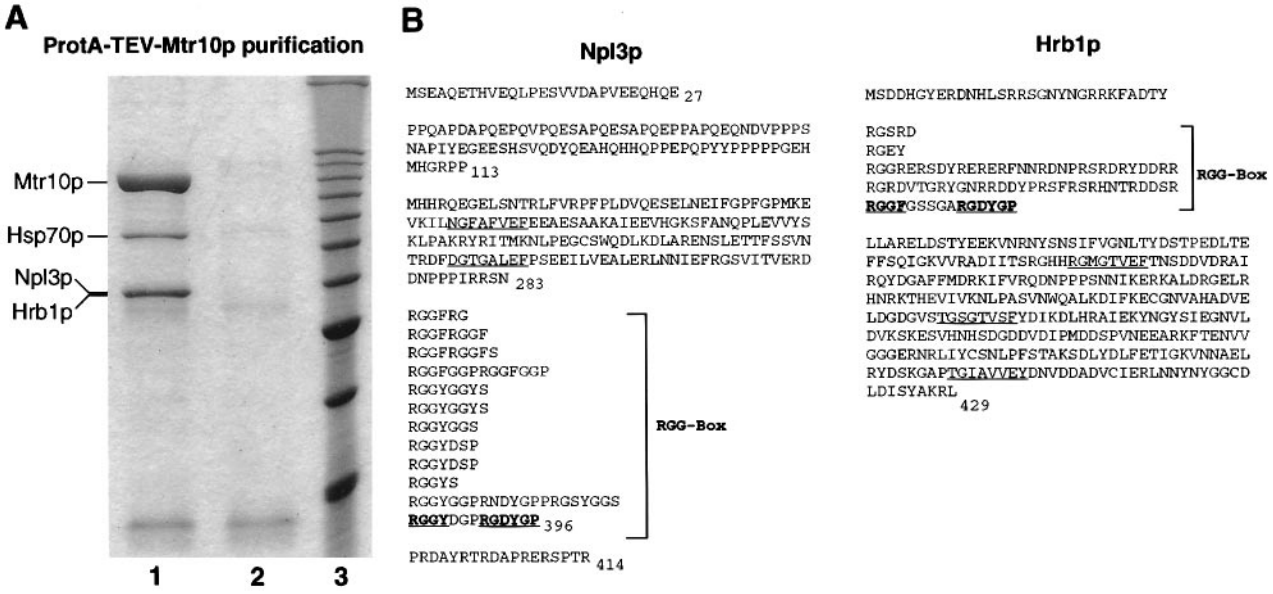

Fig. 3. Affinity purification of ProtA–TEV–Mtr10p reveals interaction with Npl3p and Hrb1p. (**A**) Affinity purification of ProtA–TEV–Mtr10p by IgG–Sepharose chromatography and release of the Mtr10p by TEV-mediated proteolytic cleavage was performed as described in Materials and methods. Shown is a Coomassie-stained SDS–polyacrylamide gel which contains: lane 1, the purified Mtr10p preparation (consisting of Mtr10p plus a prominent co-purifying 58 kDa band composed of Npl3p and Hrb1p, and a weaker staining 75 kDa band which corresponds to Ssa1p); lane 2, mock control eluate derived from a strain not expressing ProtA–TEV–Mtr10p; lane 3, protein standard with marker proteins that have a stepwise 10 kDa increase in molecular weight (the strongly stained band corresponds to 50 kDa). (**B**) Comparison of the domain organization of Npl3p and Hrb1p. The amino acid sequences of Npl3p and the other Mtr10p-interacting protein Hrb1p are shown. For Npl3p, the proline-rich domain, RNA-binding domain and the RGG box are drawn. The C-terminally located RGG domain within Npl3p is compared with a related domain found in the N-terminal part of Hrb1p. Underlined are the RNP-I motifs within Npl3p and Hrb1p. In bold and underlined is a short sequence at the end of the RGG box of Npl3p which also occurs in the corresponding RGG box of Hrb1p.

which resembles the RGG box of Npl3p (Figure 3B). Npl3p seems to be the predominant protein within the 58 kDa band, because the mass spectrometric data always gave higher intensity peptide peaks corresponding to Npl3p than for the Hrb1p peptides. The presence of Npl3p in the Mtr10p preparation was verified independently by Western blot analysis using anti-Npl3p antibodies (data not shown). We conclude that Npl3p and an uncharacterized putative RNA-binding protein are physically associated with Mtr10p.

Nuclear import of GFP-tagged Npl3p is inhibited in the thermosensitive mtr10-7 mutant

Since Npl3p binds to Mtr10p, we tested whether *mtr10* mutants are impaired in either nuclear import or export of Npl3p. Therefore, a GFP-tagged Npl3p construct under the control of an inducible GAL promoter was expressed in the ts *mtr10-7* and *mtr10* null strains. Since the *mtr10-7* mutant is already impaired in cell growth at 30°C (semi-permissive temperature; see also Figure 1B), cells were incubated at 18°C. GFP-Npl3p expression was then induced by shifting the cells from raffinose- to galactose-containing medium for 2 h. After this induction period, GFP-Npl3p expression was repressed by growing the cells in glucose-containing medium for a further 2 h. The culture was finally split and one half was left at the permissive temperature (18°C), whereas the other half was shifted for 2 h to the non-permissive temperature (37°C). The intracellular location of GFP-Npl3p was analysed by fluorescence microscopy (Figure 4). Wild-type *MTR10*$^+$ cells exibit an exclusive intranuclear location of GFP-Npl3p, both at 18 and 37°C (Figure 4A). Similarily, *mtr10-7* cells accumulate GFP-Npl3p inside the nucleus at the permissive temperature (Figure 4C). However, shifting *mtr10-7* cells to 37°C causes a strong cytoplasmic mislocation of GFP-Npl3p (Figure 4D). Strikingly, the *mtr10::HIS3* null mutant which grows very slowly at 18°C (data not shown), completely mislocalizes GFP-Npl3p to the cytoplasm under these conditions (Figure 4B). These results show that nuclear import of Npl3p is inhibited in *mtr10* mutants. To test whether this reflects a general nuclear import defect in the *mtr10* mutants, we analysed the localization of a classic NLS-containing reporter protein (NLS-GFP-lacZ). However, no inhibition of nuclear protein import of this protein was observed in the *mtr10* null mutant at the permissive or restrictive temperature (Figure 4F). The mislocalization of Npl3p in the cytoplasm may be a pleiotropic effect of impaired cell growth and inhibition of mRNA export. To exclude this possibility, the GFP-Npl3p reporter was also introduced into the *mex67-5* ts mutant, which is strongly impaired in mRNA export at the restrictive temperature (Segref *et al.*, 1997). In contrast to the *mtr10* mutants, nuclear accumulation of GFP-Npl3p was normal in the ts *mex67-5* cells at the restrictive temperature (Figure 4E). We conclude, therefore, that Mtr10p specifically mediates the nuclear import of Npl3p.

To identify the sequence within Npl3p which mediates nuclear localization, various domains of Npl3p were fused to GFP, and the *in vivo* location of the corresponding fusion proteins was determined by fluorescence microscopy. Since it has already been shown that the RGG box domain of Npl3p (Figure 3B) is necessary for nuclear location (Flach *et al.*, 1994; Lee *et al.*, 1996), we tested whether this domain alone constitutes the NLS. We could show that the RGG box (residues 283–396) plus the last

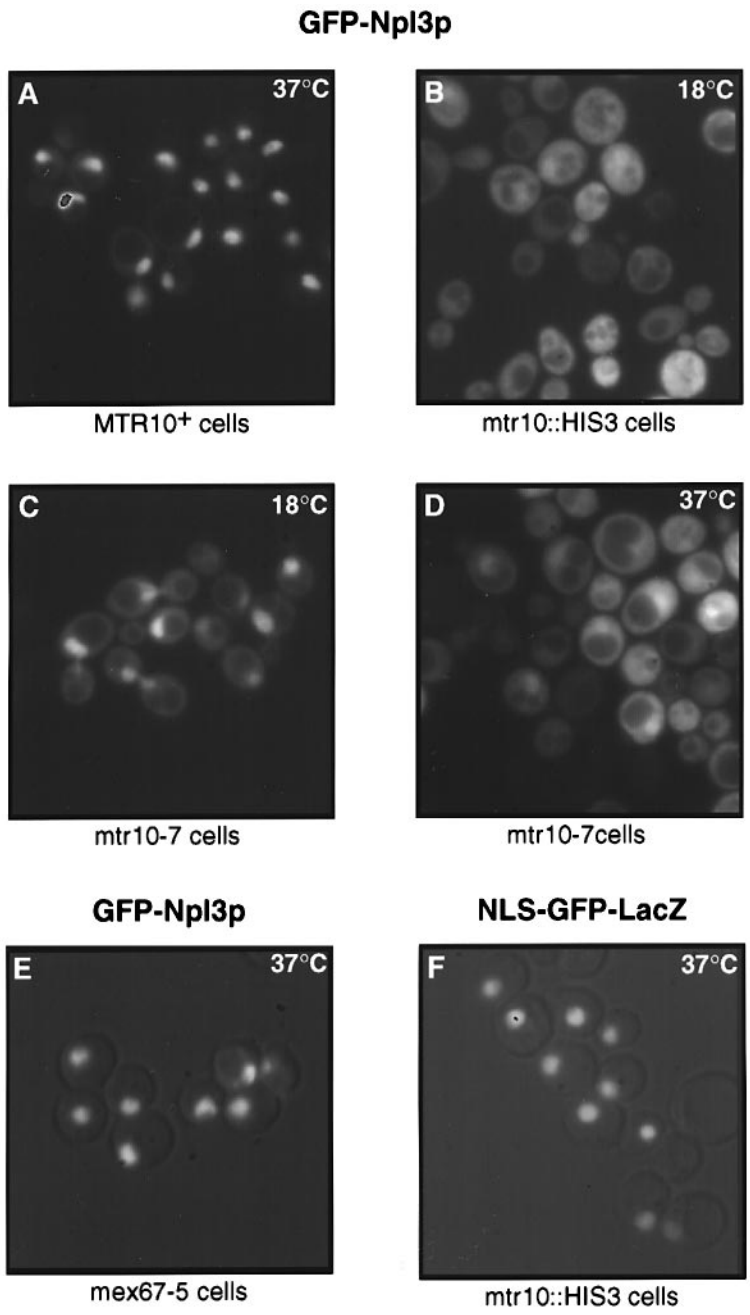

Fig. 4. Analysis of nuclear accumulation of GFP-Npl3 in *mtr10* mutants. *MTR10*$^+$ cells (**A**), *mtr10::HIS3* cells (**B**), *mtr10-7* cells (**C** and **D**) and *mex67-5* cells (**E**) were transformed with plasmid pPS811 containing the GAL::GFP-NPL3 reporter construct; *mtr10::HIS3* cells were also transformed with a plasmid containing the NLS-GFP-lacZ reporter gene (**F**). Cells expressing GFP-Npl3p or NLS-GFP-lacZ were grown at the indicated temperatures and then analysed in the fluorescence microscope for the GFP fluorescence signal as described in Lee *et al.* (1996).

18 C-terminal amino acids (residues 397–414) of Npl3p [called RGG(283–414)-GFP] are indeed necessary and sufficient to mediate nuclear accumulation of attached GFP (Figure 5A and B) and to bind efficiently to Mtr10p (Figure 5M, lane 3). In contrast, the N-terminal and middle domains of Npl3p, which contain the proline-rich sequence and the RNA-binding motifs, respectively, cannot mediate nuclear accumulation (Figure 5G–I) and association with Mtr10p (Figure 5M, lane 2). As anticipated, nuclear import of RGG(283–414)-GFP is inhibited in the *mtr10-7* mutant at the non-permissive temperature (Figure 5B and C). However, deletion of the last 18 C-terminal residues from the RGG box domain (Figure 5J–L) or expressing only the C-terminal tail (Figure 5D–F) renders the NLS function very inefficient and no, or only very little, nuclear accumulation takes place. Furthermore, the RGG box alone lacking the C-terminal tail no longer stably interacts with Mtr10p (data not shown). Thus, the RGG box domain requires the presence of the short non-repetitive C-terminal tail to mediate nuclear accumulation of Npl3p and to interact with its specific import receptor Mtr10p (see Discussion).

Interaction of recombinant Mtr10p with Ran-GTP

Since the N-terminal part of Mtr10p has similarity to the Ran-binding motif found in importin β-related proteins

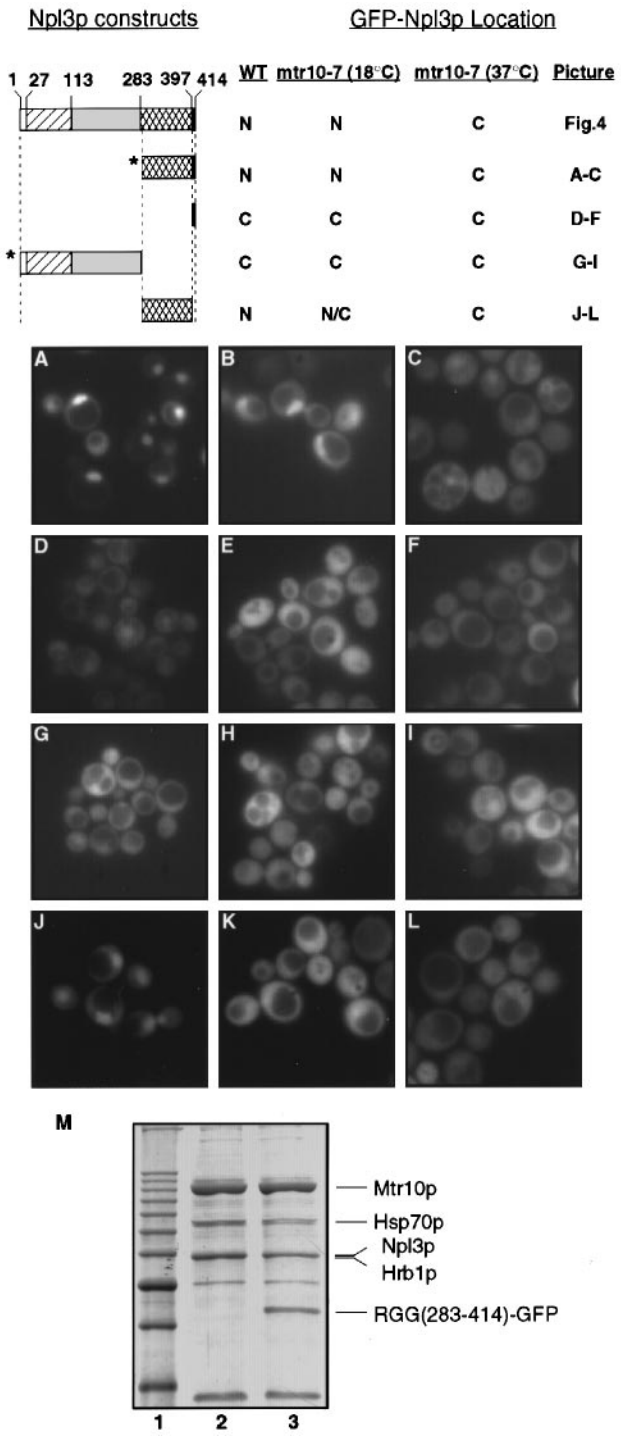

Fig. 5. The RGG domain plus the short non-repetitive C-terminal tail constitute the NLS within Npl3p. In the upper part, a schematic drawing of the Npl3p deletion constructs with the corresponding amino acid boundaries (see also Figure 3B) is shown. The constructs were fused N-terminally to GFP, expressed in NPL3$^+$ cells and their subcellular location was analysed by fluorescence microscopy (**A–L**). The asterisks indicate which GFP fusion proteins were used for ProtA–TEV–Mtr10p purification and SDS–PAGE (M). WT, wild-type; N, nuclear; C, cytoplasmic; N/C, nuclear/cytoplasmic. (**M**) SDS–PAGE analysis of a ProtA–TEV–Mtr10p purification in a NPL3$^+$ strain which co-expresses GFP-tagged Npl3p truncation proteins. Lane 1, protein standard with marker proteins that have a stepwise 10 kDa increase in molecular weight (the strongly stained band corresponds to 50 kDa); lane 2, fusion protein consisting of GFP and Npl3p(1–283); lane 3, fusion protein consisting of GFP and Npl3p(284–414).

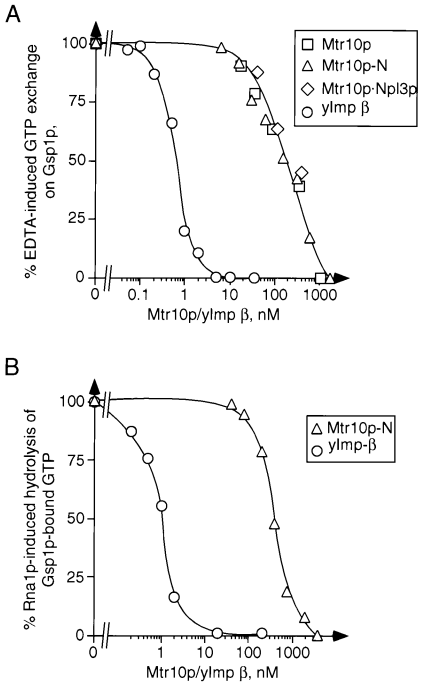

Fig. 6. Interaction of recombinant Mtr10p with Ran-GTP. Purification of recombinant full-length and the N-terminal domain of Mtr10p, importin β and Rna1p from *E.coli*, and the Mtr10p–Npl3p–Hrb1p complex from yeast is described in Materials and methods. (**A**) Inhibition of EDTA-induced GTP exchange on Gsp1p. Gsp1p-[γ-^{32}P]GTP (50 pM) was pre-incubated with either bacterially expressed Mtr10p, Mtr10p-N (residue 1–420) and yeast importin β (yImp β), or the purified yeast Mtr10p–Npl3p–Hrb1p complex, at the final concentrations indicated or with incubation buffer. Then, 40 mM EDTA and 200 nM GDP were added for another 15 min. Gsp1p-bound radioactivity finally was determined by the filter-binding assay. (**B**) Inhibition of Rna1p-induced GTP hydrolysis on Gsp1p. Gsp1p-[γ-^{32}P]GTP (50 pM) was incubated for 15 min with Mtr10p-N and yeast importin β (yImp β) at the final concentrations indicated or with incubation buffer. Rna1p (20 nM) was added and the reaction allowed to proceed for 2 min. Hydrolysis of Gsp1p-bound GTP was determined as released [^{32}P]phosphate.

(Fornerod *et al.*, 1997b; Görlich *et al.*, 1997), we tested for the ability of Mtr10p, expressed and purified from *Escherichia coli*, to interact with Gsp1p (the yeast homologue of Ran). We first tested for inhibition of nucleotide exchange on Gsp1p by Mtr10p as an assay for a physical interaction. GTP exchange on Gsp1p was induced by addition of the chelating agent EDTA which removes the magnesium ions required for tight binding of the nucleotide to Gsp1p. Employing such an *in vitro* assay, the dissociation constant for the Mtr10p–Gsp1p-GTP complex was determined to be ~200 nM (Figure 6A, Mtr10p). This would indicate that Gsp1p-GTP binds Mtr10p with an ~200-fold lower affinity than yeast importin β (Figure 6A, yImp β). Deletion of the C-terminal domain from Mtr10p (amino acids 421–972) does not change the affinity for Gsp1p-GTP (Figure 6A, Mtr10p-N), indicating that the first 420 amino acid residues of Mtr10p are sufficient for Gsp1p binding. Similar results were obtained when Mtr10p binding was monitored by inhibition of GTPase activation of Gsp1p by Rna1p (Figure 6B). Since the affinity of Mtr10p for Gsp1p is relatively low compared with other nuclear import factors of the importin β type, we tested whether binding of Npl3p to Mtr10p increases the affinity for Gsp1p-GTP. Such a mode of cooperative binding of

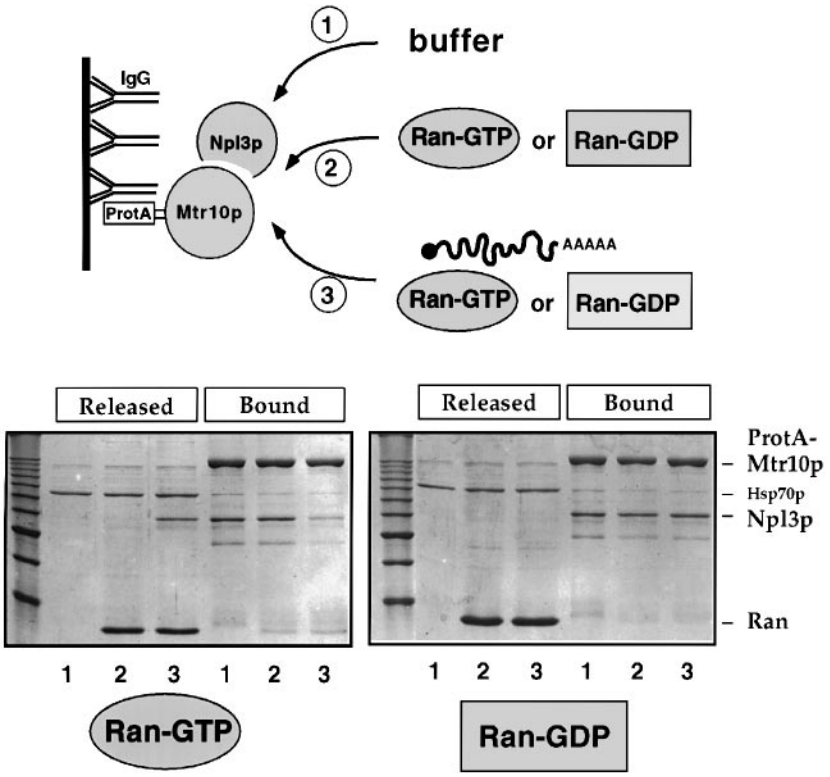

Fig. 7. Release of Npl3p from Mtr10p is mediated in a cooperative fashion by Ran-GTP and RNA. Purification of Mtr10p complex is performed as described in Materials and methods. The upper panel represents a schematic view of the experiment performed. Immobilized Mtr10p complex is eluted with buffer (1), RanGDP (2), RanGTP (2), RanGDP and RNA (3), RanGTP and RNA (3). The lower panel shows the SDS–PAGE analysis of the eluates. The numbering of the lanes is in agreement with the numbering used in the upper panel. The acetic acid elution is reported as 'Bound' because it remains bound to the beads. The type of Ran used (GTP or GDP) is also indicated.

Ran-GTP to a transport receptor in the presence of the transport substrate was seen in the case of human CAS which functions as a nuclear export receptor for importin α (Kutay *et al.*, 1997). However, the affinity of Gsp1p-GTP for Mtr10p was not changed when the purified yeast Mtr10p–Npl3p–Hrb1p complex was tested in the *in vitro* assay (Figure 6A), suggesting that Npl3p is an import rather than an export substrate for Mtr10p (see also Discussion).

Release of Npl3p from Mtr10p is mediated by the cooperative action of Ran-GTP and RNA

Since Mtr10p exhibits a low affinity for Ran-GTP, which is not stimulated by Npl3p, we tested whether RNA as the nuclear target of Npl3p increases this affinity. Therefore, ProtA–TEV–Mtr10p with bound Npl3p was first immobilized on IgG–Sepharose beads as described before (see also Figure 3A). However, it was not eluted by TEV protease, but incubated with buffer, human Ran-GDP or Ran-GTP, and Ran-GDP or Ran-GTP plus yeast total RNA, respectively. Release of Npl3p was analysed finally by SDS–PAGE and Coomassie staining (Figure 7). Buffer, Ran-GDP alone, Ran-GTP alone and Ran-GDP plus RNA were not capable of dissociating Npl3p from Mtr10p. Interestingly, the combination of Ran-GTP plus RNA caused release of Npl3p from Mtr10p (Figure 7). The released band was also identified as Npl3p by Western blotting (data not shown). When the bound material which remained on the column was analysed, the Npl3p band was significantly diminished only in the presence of Ran-GTP plus RNA. Under these conditions, Ran-GTP was clearly bound to the immobilized ProtA–TEV–Mtr10p (Figure 7, bound). This shows that the combination of Ran-GTP plus RNA causes a release of Npl3p from immobilized Mtr10p. Since no cooperative binding of total RNA and Gsp1p-GTP to monomeric Mtr10p could be detected (data not shown), this could indicate that it is Npl3p that binds to the RNA (see also Discussion).

Discussion

We have shown that Mtr10p is required for the nuclear import of Npl3p, a protein shuttling between the nucleus and the cytoplasm and implicated in nuclear mRNA export (Lee *et al.*, 1996). Mtr10p was first identified in a genetic screen for mRNA export mutants (Kadowaki *et al.*, 1994a) and is shown here to functionally interact with the nucleoporin *NUP85*, which is also required for efficient mRNA export. Recently, Mtr10p was grouped by sequence homology into the family of importin β-like transport factors, which bind to Ran-GTP (Görlich *et al.*, 1997). For some members of this family, a role not only in nuclear protein import, but also in nuclear protein export was demonstrated recently (reviewed in Ullman *et al.*, 1997). However, since the sequence identity is generally low between the various members of this protein family, only a functional analysis can prove that a putative member is indeed a transport receptor. Our data now demonstrate that Mtr10p functions as a nuclear import receptor for Npl3p. Mtr10p is likely to be evolutionarily conserved because distinct homo-

logues of yeast Mtr10p were found in the data libraries, including uncharacterized open reading frames (ORFs) from *Drosophila* and *Caenorhabditis elegans* and several expressed sequence tags from human (E.Hurt, unpublished data).

During the course of this work, Mtr10p was identified independently as the import receptor for Npl3p (Pemberton *et al.*, 1997). However, this group reported that Mtr10p is located mainly in the cytoplasm. In constrast, we see Mtr10p accumulating more inside the nucleus. This apparent discrepancy is not completely clear, but could be due to different tagging strategies (GFP tagging versus ProtA tagging; N tagging versus C tagging) and the type of location studies (*in vivo* analysis versus fixation and indirect immunofluorescence).

Although *MTR10* is not essential in yeast, cells lacking its gene grow extremely slowly at physiological temperatures and stop cell growth at 37°C. The direct cause of this growth inhibition could be that the essential Npl3p protein is not or only inefficiently imported into the nucleus, and, accordingly, maturation and/or export of mRNA which require nuclear Npl3p would be impaired. Indeed, the Mtr10p function correlates with a nuclear location of Npl3p, as Npl3p strongly mislocalizes to the cytoplasm in the *mtr10* null mutant at all temperatures and in the *mtr10-7* ts mutant at the restrictive temperature. Interestingly, both the *mtr10-7* and *mtr10* null mutant exhibit only a weak mRNA export defect under conditions where nuclear import of Npl3p is severely inhibited (B.Senger, unpublished results). This suggests that there might be a residual nuclear import of Npl3p that would allow mRNA export to continue in *mtr10* mutant cells at semi-permissive temperatures. It is likely that other importin β-like transport factors overlap with the Mtr10p function and partially compensate for the Npl3p import defect in the *mtr10* null mutant. Nevertheless, Mtr10p appears to be the main import receptor for Npl3p. We cannot, however, exclude that Mtr10p also plays a role as an import receptor for other proteins which bind with lower affinity and whose association with Mtr10p is therefore lost during purification.

Mtr10p and Npl3p (as well as Hrb1p) form a complex which is stable during biochemical purification. Nuclear import receptors (importins) in general can bind to their transport substrates in the absence of Ran-GTP, whereas Ran-GTP triggers the dissociation of the transport substrate–importin complex (Görlich *et al.*, 1996). Conversely, export receptors (exportins) such as CAS and CRM1 require both Ran-GTP plus the specific transport substrate for cooperative complex formation (Fornerod *et al.*, 1997a; Kutay *et al.*, 1997). Therefore, the firm physical interaction between Mtr10p, as a transport receptor, and Npl3p, as its cargo, in the absence of Ran-GTP implies that Mtr10p functions as an importin. Interesting in this context is the observation that the stable association between Mtr10p and Npl3p was not affected by overexpressing, in the same cells, the dominant-negative Ran mutant Gsp1p (G21V), which is blocked in the Gsp1p-GTP form (B.Senger, unpublished data). This suggests that the Mtr10p–Npl3p–Hrb1p complex exhibits low affinity for the GTP-bound form of Gsp1 and, therefore, is not easily dissociated. A similar conclusion can be drawn from the *in vitro* binding assay. Recombinant Mtr10p or yeast purified Mtr10p complexed to Npl3p–Hrb1p exhibit a much lower affinity for Gsp1p-GTP as compared with other importins such as importin β or Yrb4p/Kap123p.

Since the affinity of Mtr10p for Ran-GTP is low, its import substrate Npl3p may not be released efficiently from Mtr10p by nuclear Ran-GTP alone. We therefore tested whether the nuclear target of Npl3p, which is RNA (Lee *et al*., 1996), mediates release of Npl3p from Mtr10p in the presence of Ran-GTP. Indeed, dissociation of Npl3p from immobilized Mtr10p was induced *in vitro* by the action of both Ran-GTP and total yeast RNA, but not by Ran-GDP plus RNA. We therefore suggest that Mtr10p first transports Npl3p into the nucleus, but Ran-GTP-induced dissociation of this transport complex only occurs when Npl3p meets its nuclear substrate, which is nascent mRNA (Figure 8). Npl3p finally returns together with mRNA into the cytoplasm using an as yet unidentified export mechanism. It has been suggested previously that Npl3p exits the nucleus only in association with mRNA (Lee *et al.*, 1996). Alternatively, since the low affinity for Ran-GTP turns out to be a property of export receptors like CAS and CRM1, it is possible that Mtr10p might also be involved in the nuclear export of Npl3p. In this case, the release of the import cargo (Npl3p) from Mtr10p and the cooperative binding of Mtr10p to Ran-GTP plus the export cargo (Npl3p–RNA complex) could be one and the same event.

Another implication from our studies is that different mRNA-binding proteins use different nuclear import pathways. So far, transportin has been found to mediate nuclear uptake of two higher eukaryotic hnRNP proteins, hnRNP A1 and hnRNP F (Siomi *et al.*, 1997). In yeast, Kap104p was shown to bind to and import Nab2p and Nab4p, two mRNA-binding proteins of unknown function (Anderson *et al.*, 1993; Aitchison *et al.*, 1996). We show here that Npl3p, another yeast mRNA-binding protein, uses a distinct importin (Mtr10p) for its nuclear re-import. Whether the other Mtr10p-associated protein Hrb1p is also imported into the nucleus via Mtr10p remains to be shown. All this together establishes that yeast has at least two distinct import routes for returning nuclear mRNA-binding proteins to the nucleus. Either these mRNA-binding proteins are so abundant that they require different import pathways to ensure efficient transport back into the nucleus, or various mRNA-binding proteins are involved in different intranuclear functions and therefore may be targeted via specific importins to specific intranuclear locations. Our data strongly suggest a model in which Mtr10p is involved not only in nuclear import of Npl3p, but also in intranuclear transport to the sites where mRNA is transcribed and/or processed (Figure 8).

Such a mechanism of directed intranuclear transport may also apply to other import complexes, in which cargo release from the import receptor requires interaction with the nuclear target of the imported molecule at distinct intranuclear sites, away from the nuclear pores. Accordingly, nuclear Ran-GTP may not always be sufficient to release the transport substrate from its specific importin after arrival at the nuclear site of the NPC (Görlich *et al*., 1996). Cargo–importin complexes with low affinity for Ran-GTP may have to meet their intranuclear target (e.g. newly transcribed RNA, but one can envisage other targets such as chromatin, nucleolar components, etc.) before

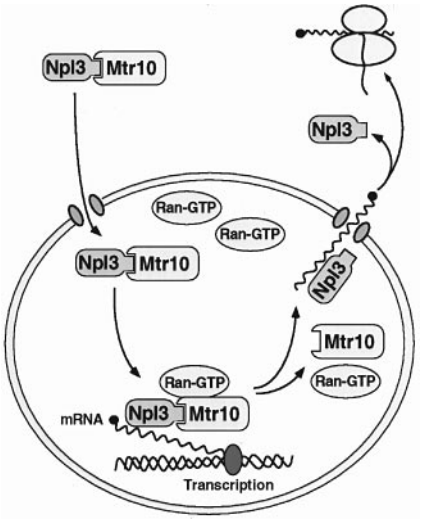

Fig. 8. Model of nuclear import and intranuclear transport of Npl3p mediated by Mtr10p.

Ran-GTP-induced disassembly of the cargo–importin complex can take place. Accordingly, the release of the import substrate from the importin is only triggered at the site of assembly. Such a mechanism would ensure directionality during intranuclear transport processes towards the sites of assembly. Thus, intranuclear Ran-GTP may play an important role not only in nuclear import and export mechanisms, but also in intranuclear targeting and assembly.

It has been shown previously that signals required for proper nuclear localization of Npl3p lie within its glycine-rich C-terminus (Lee *et al.*, 1996). This RGG box thus may represent an extended NLS, similar to the M9 NLS present in hnRNP A1. Interestingly, Hrb1p contains an RGG-like domain in its N-terminus and within a short sequence motif RGG-$(X)_{3–5}$-RGDYGP, which is also present in Npl3p (see Figure 3B). We have shown here that the RGG box (residues 283–395) plus the non-repetitive, C-terminal tail of Npl3p (residues 396–414) constitute the NLS that binds to Mtr10p and targets Npl3p into the nucleus. Thus, the RGG box domain including its short C-terminal tail is necessary and sufficient to mediate nuclear accumulation of Npl3p and to interact with its specific import receptor Mtr10p. Whether the entire RGG box is required for NLS activity remains to be determined. Our data are thus consistent with the previous finding that a mutation in the C-terminal tail of Npl3p (E409) impairs nuclear accumulation of Npl3 (Lee *et al.*, 1996).

Finally, why and how Mtr10p is functionally linked to the Nup84p complex is not clear. It is possible that the Nup84p complex, and in particular Nup85p, plays a direct role in Mtr10p-mediated nuclear import of Npl3p by providing docking sites and facilitating translocation through the NPC. Alternatively, the synergistic inhibition of cell growth is due to impaired mRNA export which is caused by both Mtr10p and Nup85p mutations. In either case, the interplay between soluble transport factors and structural components of the NPC, although evident by the genetic analysis, remains a complex and unresolved issue.

Materials and methods

Strains, plasmids and microbiological techniques

The yeast strains employed in this study are given in Table I. Growth and transformation of yeast and *E.coli*, plasmid recovery, mating, sporulation and tetrad analysis were done as described earlier (Segref *et al.*, 1997). The *MTR10* gene contained within a 4 kb *Nco*I–*Sph*I restriction fragment was blunt-ended and cloned into the centromeric ARS/CEN plasmids pUN100-LEU2, pRS316-URA3 and pRS314-TRP1. Other plasmids were pPS811 carrying the *GFP-NPL3* gene fusion under the control of the GAL promoter (Lee *et al.*, 1996) (kindly provided by P.Silver, Boston, MA) and a pRS315 derivative containing the *NOP1* promoter fused to protein A and followed by the TEV proteolytic cleavage site (kindly provided by S.Siniossoglou and K.Hellmuth).

Isolation of MTR10 in a synthetic lethal screen with the nup85Δ allele

Isolation of a collection of different synthetically lethal mutants of the *nup85*Δ allele was reported recently (Segref *et al.*, 1997). One of the remaining non-characterized sl mutants (sl125) was used to clone the wild-type gene responsible for the sl phenotype by complementation with a yeast genomic plasmid libarary. After sequence analysis of the minimal complementing DNA restriction fragment, it turned out that the *MTR10* gene complemented synthetic lethality of strain sl125.

Construction of fusion genes and gene disruption

To construct ProtA–TEV–Mtr10p, the protein A tag with two IgG-binding domains followed by a TEV proteolytic cleavage site (S.Sinniossoglou, in preparation) was fused to the 5′ end of the *MTR10* gene. In order to tag Mtr10p with GFP, the P_{NOP1}-GFP cassette (K.Hellmuth, unpublished data) was used, yielding plasmid pUN100-P_{NOP1}-GFP-MTR10. The GFP was the S65T/V163A variant which shows enhanced fluorescence properties (Kahana and Silver, 1996; Shibasaki *et al.*, 1996).

To determine the NLS within Npl3p, we cloned downstream of a P_{NOP1}-*GFP* cassette several domains of Npl3p. These domains were obtained by PCR amplification and were delimited as follows (numbering according to the amino acid sequence of Npl3p; see Figure 3B): Npl3p(284–414), Npl3p(397–414), Npl3p(1–283) and Npl3p(284–396).

For disruption of the *MTR10* gene, the entire *MTR10* ORF was excised from the gene and replaced by the *HIS3* gene. This was possible because unique DNA restriction sites previously were PCR-generated at both the ATG start and TAA stop codon of the *MTR10* gene. The *mtr10::HIS3* construct with sufficient overhanging 5′ and 3′ non-coding sequences was excised from the plasmid and used to transform the diploid strain RS453 and for selection for HIS^+ transformants. Heterozygous diploids harbouring the correct *mtr10::HIS3* disruption at the *MTR10* gene locus were verified by 'PCR Southern'. Such positive strains were sporulated and tetrad analysis was performed. After 3 days of incubation on YPD plates at 30°C, a 2:2 segregation pattern for cell growth was observed, but longer incubations revealed two additional slower growing colonies which were always His^+. To construct an *MTR10* shuffle strain, pRS316-MTR10 was transformed into the *mtr10::HIS3* disrupted haploid strain. This shuffle strain was able to grow well on YPD plates, but grows very slowly at 30°C on 5-fluoro-orotic acid (FOA)-containing plates.

Generation of ts mtr10 mutants

Mutagenesis of double-stranded plasmid DNA containing the *MTR10* gene (pRS314-MTR10) was done with hydroxylamine (Amberg *et al.*, 1993). Mutagenized plasmid DNA was transformed into the *MTR10* shuffle strain. Approximately 3000 transformants were picked and plated at 30°C (permissive temperature) on 5-FOA-containing YPD plates. After 4 days, growing colonies (lacking pURA3-MTR10 and complemented by mutagenized pRS314-MTR10) were re-streaked on YPD plates and analysed for their growth properties at 30 and 37°C. From this screen, several ts mutants were obtained. Recovery of plasmid DNA from the cells was achieved by isolating total DNA and re-transformation of competent *E.coli* MC1061 cells. The plasmid pRS314-mtr10-7 was re-transformed in the *MTR10* shuffle strain and, after 5-FOA selection, the ts phenotype of the recovered *mtr10-7* allele was reproduced.

Affinity purification of ProtA–TEV–Mtr10p

Affinity purification of the ProtA–TEV–Mtr10p by IgG–Sepharose chromatography was done as described earlier (Siniossoglou *et al.*, 1996), with modifications, and elution from the IgG–Sepharose column by recombinant TEV protease (Life Technologies, Cat. No. 10127-017) was according to S.Siniossoglou and E.Hurt (in preparation) and

Table I. Yeast strains

Strain	Genotype
RS453	*mata/α, ade2/ade2, his3/his3, leu2/leu2, trp1/trp1, ura3/ura3* (Segref *et al.*, 1997)
mtr10::HIS3	*matα, ade2, his3, leu2, trp1, ura3, mtr10::HIS3* (null mutant)
MTR10 shuffle	*matα, ade2, his3, leu2, trp1, ura3, mtr10:HIS3* (pRS316-URA3-MTR10)
mtr10-7	*matα, ade2, his3, leu2, trp1, ura3, mtr10::HIS3* (pRS314-TRP1-mtr10-7)
GFP-MTR10	*matα, ade2, his3, leu2, trp1, ura3, mtr10:HIS3* (pUN100-LEU2-GFP-*MTR10*)
ProtA–TEV–MTR10	*matα, ade2, his3, leu2, trp1,ura3, mtr10:HIS3* (pRS315-LEU2-ProtA-TEV-MTR10)
mtr10-7/GFP-NPL3	*matα, ade2, his3, leu2, trp1, ura3, mtr10:HIS3* (pRS314-TRP1-mtr10-7) (pPS811-GFP-NPL3)
mex67-5/GFP-NPL3	*mat**a**, ade2, his3, leu2, trp1, ura3, mex67:HIS3* (pRS314-mex67-5) (pPS811-GFP-NPL3)
nup133:HIS3/GFP-MTR10	*matα, ade2, his3, leu2, ura3, trp1, nup133::HIS3* (pUN100-GFP-MTR10)

K.Hellmuth and E.Hurt (in preparation). Cells disrupted for *mtr10::HIS3* and complemented with ProtA–TEV–Mtr10p were grown in SDC –leu medium to an OD_{600} of 2. Cells were harvested by centrifugation and spheroplasted. Five grams of spheroplasts were lysed in 15 ml of a lysis buffer containing 20 mH HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 0.5% Tween-20 and a cocktail of protease inhibitors (Boehringer, Mannheim). The crude cell extract was then centrifuged at 50 000 r.p.m. in a Beckmann Ti 70 rotor for 1 h at 4°C. The supernatant was mixed with 300 µl of IgG–Sepharose beads (Pharmacia, Freiburg, Germany) and incubated at 4°C for 2 h. The lysate–beads mixture was then packed onto a column and washed extensively with lysis buffer in a total volume of 30 ml. In order to perform the TEV cleavage, the beads were equilibrated with 5 ml of cleavage buffer containing 20 mM HEPES, pH 7.4, 100 mM potassium acetate, 1 mM dithiothreitol (DTT), 0.1% Tween-20 and 0.5 mM EDTA. One volume of beads was mixed with one volume of cleavage buffer, and 50 U of TEV protease was added. This was incubated for 2 h at 16°C. The elution was performed with cleavage buffer after transferring the whole mixture onto a spin column. Then 10–20 µl of the eluate were analysed by SDS–PAGE, followed by Coomassie staining. This one-step affinity purification yielded ~10–20 µg of Mtr10p starting with 5 g wet weight of cells.

In vitro release of Npl3p from ProtA–TEV–Mtr10p upon incubation with Ran-GTP and RNA

ProtA–TEV–Mtr10p was affinity-purified as described above starting from 12 g of spheroplasted yeast cells and using a 250 µl bed volume of IgG–Sepharose beads. However, a final wash of the column was performed with lysis buffer that contained no protease inhibitors and only 0.1% Tween-20. The beads were not eluted with TEV protease, but split and packed onto five spin columns. Each column, which had a 50 µl bed volume, was then resuspended in 50 µl of buffer containing or not Ran-GDP (~20 µg), Ran-GTP (~20 µg) or RNA [~10 µg; total yeast RNA including poly(A)$^+$ RNA]. The whole mixture was incubated for 1 h at 25°C on a turning wheel. The eluate plus a 50 µl wash were finally collected and pooled. The proteins which were still bound to the IgG–Sepharose beads were eluted with 300 µl of acetic acid (0.5 M, pH 3.4), lyophilized and dissolved in the same volume of SDS loading buffer as the first eluate. Twenty µl of all the eluates were analysed by SDS–PAGE and Coomassie staining.

Mass spectrometric analysis

The protein bands of interest were excised from the one-dimensional SDS–polyacrylamide gel (see Figure 3A) and processed as described (Shevchenko *et al.*, 1996). The tryptic in-gel digestion was carried out for 3 h at 37°C. The samples for mass spectrometric analysis were prepared according to Jensen *et al.* (1996) using the thin film technique (Vorm *et al.*, 1994). Tryptic peptide maps were recorded on a Bruker REFLEX matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometer equipped with delayed ion extraction. Trypsin autolysis products were used for internal calibration to give an accuracy, on average, better than 30 p.p.m. Proteins were uniquely identified using the Peptide Search software package and a non-redundant protein sequence database (maintained by C.Sander EMBL/EBI). The protein band p58 which co-purifies with Mtr10p was identified as a mixture of two yeast proteins by MALDI peptide mass mapping. Initially, database searching with a list of peptide masses retrieved Npl3p/Nop3p (DDBJ/EMBL/GenBank accession number Q01560) as the most prominent protein. Seventeen peptides detected with mass error of $<$30 p.p.m. covered 43% of the sequence. The second component of the mixture was identified as Hrb1p (DDBJ/EMBL/GenBank accession number P38922) using iterative database searching algorithm (Jensen *et al.*, 1997). Fifteen tryptic peptides matched to the Hrb1p protein (sequence coverage 31%).

Expression and localization of GFP-Mtr10p and GFP-Npl3p

To determine the *in vivo* location of Mtr10p, GFP-tagged Mtr10p was expressed in *mtr10::HIS3* disrupted strains. The GFP signal was examined in the fluorescein channel of a Zeiss Axioskop fluorescence microscope and pictures were obtained with a Xillix Microimager CCD camera. In some cases, digital pictures were processed further by digital confocal imaging applying deconvolution to images, available as a module within the software program Openlab (Improvision, Coventry, UK). Analysis of the location of GFP-Npl3p (which was induced by growing cells for 2 h in galactose-containing medium) in *mtr10-7* and *mex67-5* ts mutants was done as described (Lee *et al.*, 1996).

Purification of E.coli recombinant proteins

Full-length *MTR10* (from the ATG to the stop codon) was inserted into pET8c (pET8c-MTR10) by PCR amplification of the entire gene using *Sal*I (5′) and *Mlu*I (3′) primers and insertion of the *Sal*I–*Mlu*I fragment into the pET8c-HIS vector, previously cut with *Xho*I and *Mlu*I. Construction of the HIS-tagged N-terminal domain of Mtr10p (Mtr10p-N) ranging from residue 1 to 420 (corresponding to an internal *Pst*I site) was done in a similar way (pET8c-MTR10-N). The vectors containing the fusion genes were transformed into *E.coli* BL21 cells and 500 ml cultures were grown in minimal medium at 37°C to OD 0.7, shifted to 25°C and induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial cell pellet was lysed by sonication in 10 ml of 50 mM KPi, 200 mM NaCl, 0.05% TX-100, 10 mM β-mercaptoethanol, pH 8.0. The lysate was cleared by centrifugation and applied onto a Ni-NTA resin (Qiagen, Hilden, Germany) column. Bound proteins were eluted with 200 mM imidazole in lysis buffer, dialysed against 20 mM Tris–HCl, 50 mM NaCl, 1 mM DTT, 0.05% Triton X-100, pH 7.4, and further purified by chromatography on a Mono Q HR 5/5 column (Pharmacia) equilibrated in the same buffer and eluted with a NaCl gradient. The expression and purification of Rna1p and yeast importin β were done as described previously (Bischoff *et al.*, 1995a; Görlich *et al.*, 1996).

Enzymatic Ran-GTP binding assays

Enzymatic assays were carried out as described (Bischoff *et al.*, 1994, 1995b; Schlenstedt *et al.*, 1997). Briefly, 30 µl of Gsp1p-[γ-^{32}P]GTP were pre-incubated at 25°C with 10 µl of the corresponding Gsp1p-binding protein in incubation buffer (20 mM HEPES–NaOH, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02% sodium azide, 0.05% hydrolyzed gelatin). After 15 min, 10 µl of 200 mM EDTA, pH 7.5, 1 mM GDP were added. After 15 min, Gsp1p-bound radioactivity was determined by the filter-binding assay (Bischoff and Ponstingl, 1991). For GAP assays, 50 pM Gsp1p-[γ-^{32}P]GTP was incubated with 10 µl volumes of the corresponding Gsp1p-binding protein in incubation buffer. After a 2 min GTPase reaction at 25°C,

released [^{32}P]phosphate was determined by the charcoal assay (Bischoff *et al*., 1994).

Miscellaneous

DNA recombinant work including restriction analysis, end filling, ligation, PCR amplification and DNA sequencing were performed as described in Maniatis *et al.* (1982). SDS–PAGE and Western blotting was performed according to Siniossoglou *et al.* (1996).

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References

Aitchison,J.D., Blobel,G. and Rout,M.P. (1996) Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science*, **274**, 624–627.

Amberg,D.C., Goldstein,A.L. and Cole,C.N. (1992) Isolation and characterization of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.*, **6**, 1173–1189.

Amberg,D.C., Fleischmann,M., Stagljar,I., Cole,C.N. and Aebi,M. (1993) Nuclear PRP20 protein is required for mRNA export. *EMBO J.*, **12**, 233–241.

Anderson,J.T., Wilson,S.M., Datar,K.V. and Swanson,M.S. (1993) NAB2: a yeast nuclear polyadenylated RNA-binding protein essential for cell viability. *Mol. Cell. Biol.*, **13**, 2730–2741.

Bischoff,F.R. and Görlich,D. (1997) RanBP1 is crucial for the release of RanGTP from importin β-related nuclear transport factors. *FEBS Lett.* **419**, 249–254.

Bischoff,F.R and Ponstingl,H. (1991) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature*, **354**, 80–82.

Bischoff,F.R., Klebe,C., Kretschmer,J., Wittinghofer,A. and Ponstingl,H. (1994) RanGAP1 induces GTPase activity of nuclear Ras-related Ran. *Proc. Natl Acad. Sci. USA*, **91**, 2587–2591.

Bischoff,F.R., Krebber,H., Kempf,T., Hermes,I. and Ponstingl,H. (1995a) Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl Acad. Sci. USA*, **92**, 1749–1753.

Bischoff,F.R., Krebber,H., Smirnova,E., Dong,W. and Ponstingl,H. (1995b) Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO J.*, **14**, 705–715.

Corbett,A.H. and Silver,P.A. (1997) Nucleocytoplasmic transport of macromolecules. *Microbiol. Mol. Biol. Rev.*, **61**, 193–211.

Doye,V. and Hurt,E.C. (1997) From nucleoporins to nuclear pore complexes. *Curr. Opin. Cell Biol.*, **9**, 401–411.

Fischer,U., Huber,J., Boelens,W.C., Mattaj,I.W. and Lührmann,R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–483.

Flach,J., Bossie,M., Vogel,J., Corbett,A., Jinks,T., Aker Willins,D. and Silver,P.A. (1994) A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. *Mol. Cell. Biol*., **14**, 8399–8407.

Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997a) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, **90**, 1051–1060.

Fornerod,M., Vandeursen,J., Vanbaal,S., Reynolds,A., Davis,D., Gopal Murti,K., Fransen,J. and Grosveld,G. (1997b) The human homologue of yeast Crm1 is in a dynamic subcomplex with Can/Nup214 and a novel nuclear pore component Nup88. *EMBO J.*, **16**, 807–816.

Fridell,R.A., Truant,R., Thorne,L., Benson,R.E. and Cullen,B.R. (1997) Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β. *J. Cell Sci.*, **110**, 1325–1331.

Gerace,L. (1995) Nuclear export signals and the fast track to the cytoplasm. *Cell*, **82**, 341–344.

Goldfarb,D.S. (1997) Nuclear transport—whose finger is on the switch? *Science*, **276**, 1814–1816.

Gorsch,L.C., Dockendorff,T.C. and Cole,C.N. (1995) A conditional allele of the novel repeat-containing yeast nucleoporin *RAT7/NUP159* causes both rapid cessation of mRNA export and reversible clustering of nuclear pore complexes. *J. Cell Biol.*, **129**, 939–955.

Görlich,D. and Mattaj,I.W. (1996) Protein kinesis—nucleocytoplasmic transport. *Science*, **271**, 1513–1518.

Görlich,D., Panté,N., Kutay,U., Aebi,U. and Bischoff,F.R. (1996) Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.*, **15**, 5584–5594.

Görlich,D., Dabrowski,M., Bischoff,F.R., Kutay,U., Bork,P., Hartmann,E., Prehn,S. and Izaurralde,E. (1997) A novel class of RanGTP binding proteins. *J. Cell Biol.*, **138**, 65–80.

Iovine,M.K. and Wente,S.R. (1997) A nuclear export signal in Kap95p is required for both recycling the import factor and interaction with the nucleoporin GLFG repeat regions of Nup116p and Nup100p. *J. Cell Biol.*, **137**, 797–811.

Izaurralde,E., Lewis,J., Gamberi,C., Jarmolowski,A., McGuigan,C. and Mattaj,I.W. (1995) A cap-binding protein complex mediating U snRNA export. *Nature*, **376**, 709–712.

Izaurralde,E., Jarmolowski,A., Beisel,C., Mattaj,I.W., Dreyfuss,G. and Fischer,U. (1997a) A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. *J. Cell Biol.*, **137**, 27–35.

Izaurralde,E., Kutay,U., von Kobbe,C., Mattaj,I.W. and Görlich,D. (1997b) The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J.*, **16**, 6535–6547.

Jensen,O.N., Podtelejnikov,A. and Mann,M. (1996) Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. *Rapid Commun. Mass Spectrom.*, **10**, 1371 - 1378.

Jensen,O.N., Podtelejnikov,A.V. and Mann,M. (1997) Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. *Anal. Chem.*, **69,** 4741 –4750.

Kadowaki,T., Zhao,Y. and Tartakoff,A.M. (1992) A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm. *Proc. Natl Acad. Sci. USA*, **89**, 2312–2316.

Kadowaki,T. *et al.* (1994a) Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (*mtr*) mutants. *J. Cell Biol.*, **126**, 649–659.

Kadowaki,T., Hitomi,M., Chen,S. and Tartakoff,A.M. (1994b) Nuclear mRNA accumulation causes nucleolar fragmentation in yeast *mtr2* mutant. *Mol. Biol. Cell*, **5**, 1253–1263.

Kahana,J. and Silver,P. (1996) Use of the *A.victoria* green fluorescent protein to study protein dynamics *in vivo*. *Curr. Prot. Mol. Biol.*, **9**, 722–728.

Koepp,D.M. and Silver,P.A. (1996) A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? *Cell*, **87**, 1–4.

Kose,S., Imamoto,N., Tachibana,T., Shimamoto,T. and Yoneda,Y. (1997) Ran-unassisted nuclear migration of a 97 kDa component of nuclear pore-targeting complex. *J. Cell Biol.*, **139**, 841–849.

Kutay,U., Bischoff,F.R., Kostka,S., Kraft,R. and Görlich,D. (1997) Export of importin α from the nucleus is mediated by a specific nuclear transport factor. *Cell*, **90**, 1061–1071.

Lee,M.S., Henry,M. and Silver,P.A. (1996) A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. *Genes Dev.*, **10**, 1233–1246.

Maniatis,T., Fritsch,E.T. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Michael,W.M., Choi,M.Y. and Dreyfuss,G. (1995) A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell*, **83**, 415–422.

Moore,M.S. and Blobel,G. (1994) A G protein involved in nucleocytoplasmic transport: the role of Ran. *Trends Biochem. Sci.*, **19**, 211–216.

Murphy,R. and Wente,S.R. (1996) An RNA-export mediator with an essential nuclear export signal. *Nature*, **383**, 357–360.

Nakielny,S., Fischer,U., Michael,W.M. and Dreyfuss,G. (1997) RNA transport. *Annu. Rev. Neurosci.*, **20**, 269–301.

Neville,M., Stutz,F., Lee,L., Davis,L.I. and Rosbash,M. (1997) The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr. Biol.*, **7**, 767–775.

Nigg,E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature*, **386**, 779–787.
Ossareh-Nazari,B., Bachelerie,F. and Dargemont,C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science*, **278**, 141–144.
Palacios,I., Hetzer,M., Adam,S.A. and Mattaj,I.W. (1997) Nuclear import of U snRNPs requires importin β. *EMBO J.*, **16**, 6783–6792.
Pemberton,L.F., Rosenblum,J.S. and Blobel,G. (1997) A distinct and parallel pathway for nuclear import of an mRNA-binding protein. *J. Cell Biol.*, **139**, 1645–1653.
Pollard,V.W., Michael,W.M., Nakielny,S., Siomi,M.C., Wang,F. and Dreyfuss,G. (1996) A novel receptor-mediated nuclear protein import pathway. *Cell*, **86**, 985–994.
Rexach,M. and Blobel,G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell*, **83**, 683–692.
Richards,S.A., Lounsbury,K.M., Carey,K.L. and Macara,I.G. (1996) A nuclear export signal is essential for the cytosolic localization of the ran binding protein, RanBP1. *J. Cell Biol.*, **134**, 1157–1168.
Richards,S.A., Carey,K.L. and Macara,I.G. (1997) Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. *Science*, **276**, 1842–1844.
Rout,M.P., Blobel,G. and Aitchison,J.D. (1997) A distinct nuclear import pathway used by ribosomal proteins. *Cell*, **89**, 715–725.
Schlenstedt,G., Smirnova,E., Deane,R., Solsbacher,J., Kutay,U., Görlich,D., Ponstingl,H. and Bischoff,F.R. (1997) Yrb4p, a yeast Ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. *EMBO J.*, **16**, 6237–6249.
Seedorf,M. and Silver,P.A. (1997) Importin/karyopherin protein family members required for mRNA export from the nucleus. *Proc. Natl Acad. Sci. USA*, **94**, 8590–8595.
Segref,A., Sharma,K., Doye,V., Hellwig,A., Huber,J. and Hurt,E.C. (1997) Mex67p which is an essential factor for nuclear mRNA export binds to both poly(A)$^+$ RNA and nuclear pores. *EMBO J.*, **11**, 3256–3271.
Shevchenko,A. *et al.* (1996) Linking genome and proteome by mass spectrometry: large scale identification of yeast proteins from two dimensional gels. *Proc. Natl Acad. Sci. USA*, **93**, 14440–14445.
Shibasaki,F., Price,E.R., Milan,D. and McKeon,F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature*, **382**, 370–373.
Siniossoglou,S., Wimmer,C., Rieger,M., Doye,V., Tekotte,H., Weise,C., Emig,S., Segref,A. and Hurt,E.C. (1996) A novel complex of nucleoporins, which includes Sec13p and a Sec13p homolog, is essential for normal nuclear pores. *Cell*, **84**, 265–275.
Siomi,M.C., Eder,P.S., Kataoka,N., Wan,L.L., Liu,Q. and Dreyfuss,G. (1997) Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. *J. Cell Biol.*, **138**, 1181–1192.
Stade,K., Ford,C.S., Guthrie,C. and Weis,K. (1997) Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell*, **90**, 1041–1050.
Teixeira,M.T., Siniossoglou,S., Podtelejnikov,S., Bénichou,J.-C., Mann,M., Hurt,E.C. and Fabre,E. (1997) *In vivo* cleavage of Nup145p generates two functionally distincts domains. *EMBO J.*, **16**, 5086–5097.
Ullman,K.S., Powers,M.A. and Forbes,D.J. (1997) Nuclear export receptors: from importin to exportin. *Cell*, **90**, 967–970.
Vorm,O., Roepstorff,P. and Mann,M. (1994) Matrix surfaces made by fast evaporation yield improved resolution and very high sensitivity in MALDITOF. *Anal. Chem.*, **66**, 3281–3287.
Yaseen,N.R. and Blobel,G. (1997) Cloning and characterization of human karyopherin β3. *Proc. Natl Acad. Sci. USA*, **94**, 4451–4456.

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