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Characterization of Karyopherin Cargoes Reveals Unique Mechanisms of Kap121p-Mediated Nuclear Import

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In yeast there are at least 14 members of the β-karyopherin protein family that govern the movement of a diverse set of cargoes between the nucleus and cytoplasm. Knowledge of the cargoes carried by each karyopherin and insight into the mechanisms of transport are fundamental to understanding constitutive and regulated transport and elucidating how they impact normal cellular functions. Here, we have focused on the identification of nuclear import cargoes for the essential yeast β-karyopherin, Kap121p. Using an overlay blot assay and coimmunopurification studies, we have identified ~30 putative Kap121p cargoes. Among these were Nop1p and Sof1p, two essential *trans*-acting protein factors required at the early stages of ribosome biogenesis. Characterization of the Kap121p-Nop1p and Kap121p-Sof1p interactions demonstrated that, in addition to lysine-rich nuclear localization signals (NLSs), Kap121p recognizes a unique class of signals distinguished by the abundance of arginine and glycine residues and consequently termed rg-NLSs. Kap104p is also known to recognize rg-NLSs, and here we show that it compensates for the loss of Kap121p function. Sof1p is also transported by Kap121p; however, its import can be mediated by a piggyback mechanism with Nop1p bridging the interaction between Sof1p and Kap121p. Together, our data elucidate additional levels of complexity in these nuclear transport pathways.

The lipid bilayers of the nuclear envelope physically separate nuclear DNA from the cytoplasm. This permits the strict coordination of key cellular processes, such as cell cycle progression, cellular differentiation, and gene expression. To maintain precise control of these processes, a vast number of macromolecules must be actively transported across the nuclear envelope. This movement occurs solely through nuclear pore complexes (NPCs) embedded in circular pores spanning the double membranes of the nuclear envelope. NPCs are comprised of ~30 distinct nucleoporins (nups), which form a highly conserved large (~45- to 60-MDa) (14, 44) octagonally symmetric structure (reviewed in references 18, 42, 55, and 59). Approximately one-third of all nups contain FG dipeptide repeat motifs (14, 44). These FG repeats have been shown to provide interaction sites for a class of soluble transport factors, termed karyopherins (kaps; also called transportins, importins, or exportins) (3, 7, 8; reviewed in references 9, 43, 51, 52, and 55).

Eukaryotic cells contain two structurally related families of kaps: the β-kaps and the α-kaps (reviewed in references 11, 19, 38, 52, and 61). There are 14 β-kaps in yeast and >20 β-kaps in higher eukaryotes. In general, each β-kap mediates macromolecular transport by interacting with the NPC and binding nuclear localization signals (NLSs) or nuclear export signals present on cargo molecules (reviewed in references 19 and 33). Members of the α-kap family (Kap60p/Srp1p in yeast and as many as six members in metazoan species [https://www.incyte.com/proteome/Retriever/index.html]) interact with classical NLS (cNLS)-containing proteins but do not interact with FG repeats. Instead, they bind to a β-kap (Kap95p in yeast or Kap β1 in mammals), which in turn interacts with the FG-nups mediating import into the nucleus (reviewed in references 9, 43, 51, 52, and 55).

β-kaps also interact with the small GTPase Ran during the transport cycle (reviewed in references 19, 26, 34, 43, and 58). Ran cycles between GTP- and GDP-bound states, and the spatial separation of these two states is believed to confer directionality to the transport cycle. Ran's guanine nucleotide exchange factor (RanGEF) is predominantly located in the nucleus, tethered to DNA, whereas the Ran GTPase-activating protein (RanGAP) is primarily localized to the cytoplasm. The compartmentalization of these two Ran regulators ensures that nuclear Ran is in the GTP-bound state, whereas cytoplasmic Ran is in its GDP-bound state. Thus, import complexes form in the cytoplasm where the concentration of RanGTP is low. They then traverse the NPC and encounter a RanGTP-rich environment on the nuclear side of the nuclear envelope. Here, RanGTP binds to the β-kap, stimulating kap/cargo complex dissociation. Conversely, export kaps bind their cargoes cooperatively with RanGTP in the nucleus. These complexes are disrupted once they reach the cytoplasm, when the RanGAP induces GTP hydrolysis (reviewed in references 19, 34, 42, and 58).

Kaps can be divided into three categories depending on their direction of transport. In yeast, the import kaps (or importins) are Srp1p/Kap60p, Kap95p, Kap104p, Sxm1p/Kap108p, Mtr10p/Kap111p, Kap114p, Nmd5p/Kap119p, Pse1p/Kap121p, Pdr6p/Kap122p, and Kap123p. The export kaps (or exportins) are

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Crm1p/Xpo1p/Kap124p, Cse1p/Kap109p, and Los1p. There are no known cargoes for Kap120p, and Msn5p/Kap142p is capable of moving cargoes in both directions (reviewed in references 19, 33, 38, 52, and 61). Although only a limited number of substrates have been defined for each kap, more than 1,500 different yeast proteins, and multiple RNA species, must be translocated across the NPC in one or both directions during their life cycle (https://www.incyte.com/proteome/Retriever/index.html). Despite this huge transport burden, only four β-kaps are essential in yeast. This suggests tremendous levels of redundancy and complexity in the specificity between kap-cargo recognition, which in turn may enable the cell to globally regulate specific transport pathways.

One of the significant metabolic burdens of a rapidly growing yeast cell is that imposed by ribosomal biosynthesis. It is estimated that under such conditions yeast allocate ~60% of the total transcription, ~50% of RNA polymerase II transcription, and ~90% of mRNA splicing activities to ribosome synthesis (57). Moreover, components must be correctly sorted and assembled. Accordingly, ~150,000 ribosomal proteins must be imported into the nucleus per min, where they are assembled by more than 60 different *trans*-acting nuclear factors into ~4,000 ribosomal subunits, which must then be exported to the cytoplasm (57).

The bulk of ribosomal protein import in yeast appears to be mediated by Kap123p (46, 47, 53). However, surprisingly, Kap123p is not essential in yeast (46). It is proposed that in the absence of Kap123p, the structurally related kap Kap121p can import ribosomal proteins (46). The ability of kaps to act redundantly appears to be a general phenomenon; indeed, many kap genes can be deleted in combination (53). We and others have sought to understand the functional relationship between kaps by focusing on the identification of kap cargoes and studying their import mechanisms (reviewed in references 19, 33, 38, 52, and 61). In focusing on Kap121p, these approaches have established that, in addition to ribosomal proteins, Kap121p is responsible for importing Pho4p (23), Spo12p (10), Ste12p (28), Pdr1p (16), Yap1p (20), and Rcs1p/Aft1p (54) into the nucleus and assembling Nup53p into the NPC (30). In each of these cases, Kap121p binds to NLSs rich in lysine residues (see also reference 30). Here, using an overlay blot assay, we identified 27 Kap121p-interacting proteins present in yeast nucleoplasm, most of which are ribosome biogenesis *trans*- and *cis*-acting factors, including two essential snoRNA-associated proteins required for rRNA processing, Nop1p and Sof1p. Characterization of the Kap121p-cargo interactions revealed a novel Kap121p-interacting rg-NLS within the N-terminal GAR domain of Nop1p, demonstrating that this β-kap can recognize more than one type of NLS. Nop1p can also be imported by Kap104p, indicating that these kaps are functionally redundant. Interestingly, we also provide evidence for a role of Kap121p in a piggyback import mechanism in which Sof1p is imported indirectly by Kap121p (or Kap104p) through its interactions with Nop1p.

MATERIALS AND METHODS

Overlay blot assay. Protein A chimeras of Kap123p (Kap123p-pA) and Kap121p (Kap121p-pA) (46) were used in an overlay assay as previously described (28). Yeast nuclear proteins were fractionated by hydroxyapatite (HA) high-pressure liquid chromatography (HPLC) and pooled column fractions were further separated by standard formic acid HPLC separation (45, 60). The proteins in each of the resulting column fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked and probed with cytosolic fractions isolated from yeast cells expressing Kap123p-pA and Kap121p-pA as previously described (1, 46). Bands of interest were identified in replicate Novex SDS-polyacrylamide gels and excised for identification by mass spectrometry (MS).

Mass spectrometric protein identification. The method used for in-gel tryptic digestion of proteins separated by SDS-PAGE was as described previously (62). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS was carried out by using a commercial instrument (Perseptive Biosystems STR, Framington, Mass.) operated in the delayed-extraction reflector mode (full-width half-maximum resolution, ~5,000). Protein identification was carried out by using the protein search engine ProFound, which uses a Bayesian algorithm to identify proteins from protein databases using mass spectrometric peptide mapping data (62). Proteins are ranked in order of their probabilities. When the probability for the first-ranked protein is close to unity and there is a large probability transition ($\geq 10^3$) from the first to second candidate, the identification of the top-ranked protein is considered to be highly confident (62).

Plasmids and strains. Yeast strains were derived from DF5 (*MAT***a**/*MAT*α *ura3-52*/*ura3-52 his3*Δ*200*/*his3*Δ*200 trp1-1*/*trp1-1 leu2-3,112*/*leu2-3,112 lys2-801*/*lys2-801*). *kap121* temperature-sensitive strains are derivatives of DF5 cells and have been described (28). The galactose-responsive *kap121-34* and *kap104-16* strains were produced by crossing *kap121-34* or *kap104-16* haploids (1) with W303 haploids (*MAT***a**/MATα *ade2-1*/*ade2-1 ura3-1*/*ura3-1 his3-11,15*/*his3-11,15 trp1-1*/*trp1-1 leu2-3,112*/*leu2-3,112 can1-100*/*can1-100*) (2). The resulting diploids were sporulated, tetrads dissected and *kap121-34* and *kap104-16* haploid strains were selected and tested for their ability to grow on galactose. These galactose-responsive strains were then tested for temperature sensitivity at 37°C. Strains that grew well on galactose at 23°C but not at 37°C were selected for further experiments.

The *NOP1-A* and *SOF1-A* strains were produced by homologous recombination (2). Briefly, the respective genes were tagged with the immunoglobulin G (IgG)-binding domains of *Staphylococcus aureus* protein A (pA) in DF5 *MAT***a** haploid cells by using the following regions for homologous recombination: *NOP1*, positions 922 to 981 and 1051 to 1110; and *SOF1*, positions 1408 to 1467 and 1531 to 1590. Viable strains expressing appropriately sized pA fusion proteins were selected and designated *NOP1-A* or *SOF1-A*.

Chromosomal *NOP1* and *SOF1* were tagged with *Aequoria victoria* green fluorescent protein (GFP) in *kap121-34*/p*KAP121-URA3* haploid cells via homologous recombination as described above and previously (17). The *NOP1 GFP*; *kap121-34*/p*KAP121-URA3* and *SOF1 GFP*; *kap121-34*/p*KAP121-URA3* strains were crossed to *kap104-16* (1), and the resulting diploid strains were sporulated to generate *NOP1 GFP*; *kap121-34*/p*KAP121-URA3*; *kap104-16* and *SOF1 GFP*; *kap121-34*/p*KAP121-URA3*; kap104-16 haploid strains. These strains, and the *NOP1 GFP*; *kap121-34*/p*KAP121-URA3* and *SOF1 GFP*; *kap121-34*/p*KAP121-URA3* strains, were plated on 5-fluoroorotic acid-containing medium, incubated at 23°C to select cells lacking p*KAP121-URA3* and designated *NOP1 GFP*; *kap121-34*; *kap104-16* and *SOF1 GFP*; *kap121-34*; *kap104-16*. Strains used to analyze genetic interactions between *KAP121* and *KAP104* were derived by crossing *kap121-34* (28) to *kap104*::*ura3*::*HIS3*/p*KAP104-URA3* (1) and isolating double mutant spores (*kap121-34*; *kap104*::*ura3*::*HIS3*/p*KAP104-URA3*). As a control, *kap121-34*; *kap108*::*ura3*::*his5*::*LEU2*/p*KAP108-URA3* double mutant spores were isolated from diploids derived from crosses of *kap121-34* with *kap108*::*ura3*::*his5*::*LEU2*/p*KAP108-URA3*.

To construct p*NOP1GFP* and p*SOF1GFP*, the open reading frames (ORFs) of *NOP1* and *SOF1* were amplified from yeast genomic DNA by PCR. The resulting *NOP1* and *SOF1* PCR products were cloned into the EcoRI and the EcoRI/BamHI restriction sites, respectively, of pYX242 (41). To place these GFP chimeras under the control of a galactose promoter (*GAL1*), each fusion was excised from pYX242 by using the 5′ EcoRI and the 3′ XhoI restriction sites and then subcloned into pYES2 (Invitrogen, Carlsbad, Calif.). The resulting plasmids were termed p*GAL1-NOP1GFP* and p*GAL1-SOF1GFP*.

p*GAL1-cNLSGFP* was made by subcloning a HindIII/XhoI *cNLS-2xGFP* fragment from p12-GFP2-NLS (50) into the HindIII/XhoI restriction sites of pYES2.

NOP1 fragment constructs were produced by PCR with oligonucleotides designed to amplify segments containing nucleotides (nt) 1 to 270, nt 211 to 981, or nt 658 to 981 of *NOP1* with 5′ EcoRI and HindIII and 3′ EcoRI restriction sites. HindIII- and EcoRI-digested PCR products were first cloned into p12-GFP2-NLS (pKW431) as previously described (28). The resulting GFP fusions were then excised from pKW431 and subcloned into the HindIII and XhoI restriction sites of pYES2 and termed p*GAL1*-Nop1p(aa1-90)-GFP, p*GAL1*-Nop1p(aa91-327)-GFP, and p*GAL1*-Nop1p(aa210-327)-GFP. EcoRI-digested *NOP1*, *NOP1*

(nt 1 to 270), *NOP1* (nt 271 to 981), and *NOP1* (nt 658 to 981) PCR fragments were also cloned into the EcoRI site of pGEX-4T-3 (Amersham Pharmacia Biotech), and the resulting plasmids were termed pGST-Nop1p, pGST-Nop1p(aa1-90), pGST-Nop1p(aa90-327), and pGST-Nop1p(aa210-327), respectively.

SOF1 fragment constructs were produced by PCR with oligonucleotides designed to amplify segments containing nt 1 to 390, 391 to 1140, 1141 to 1467, or 1231 to 1350 of *SOF1* with 5′ EcoRI and 3′ BamHI and SalI restriction sites. EcoRI and BamHI-digested PCR products were first cloned into pYX242. The resulting GFP fusions were then excised from pYX242 and subcloned into the EcoRI and XhoI restriction sites of pYES2 and termed p*GAL1*-Sof1p(aa1-130)-GFP, p*GAL1*-Sof1p(aa131-380)-GFP, p*GAL1*-Sof1p(aa381-489)-GFP, and p*GAL1*-Sof1p(aa411-450)-GFP. EcoRI- and SalI-digested *SOF1* (nt 1 to 390), *SOF1* (nt 391 to 1140), *SOF1* (nt 1141 to 1467), and *SOF1* (nt 1231 to 1350) PCR fragments were also cloned into the EcoRI and SalI sites of pGEX-4T-3 (Amersham Pharmacia Biotech), and the resulting plasmids were termed pGST-Sof1p(aa1-130), pGST-Sof1p(aa131-380), pGST-Sof1p(aa381-489), and pGST-Sof1p(aa411-450), respectively.

The NLS of *PHO4* (23) was amplified from yeast genomic DNA by PCR and subcloned into the BamHI and EcoRI restriction sites of pGEX-4T-3; the resulting plasmid was termed pGST-Pho4p(aa140-166).

The ORF of *KAP104* was amplified from pRS317-*KAP104* (1) by PCR, the PCR product was inserted into the SacI restriction site of pYES2, and the resulting plasmid was termed p*GAL1-KAP104*. For selection purposes, nt 1 to 599 of the *URA3* gene in pYES2 were replaced with a NdeI/ApaI-digested PCR fragment containing the *Saccharomyces cerevisiae LEU2* gene plus 200 bp upstream of the start codon. The resulting construct was termed p*GAL1-KAP104-LEU2*. The ORF of *KAP108* was amplified from *S. cerevisiae* genomic DNA by PCR. The PCR product was inserted into the SacI and XbaI restriction sites of pYES2-LEU2 and termed p*GAL1-KAP108-LEU2*.

The *NOP1cNLS* fusion was produced by PCR amplification of *NOP1*, including 700 nt upstream of the ORF, and an in-frame fusion of the simian virus 40 large T antigen NLS coding sequence (24) at its 3′ end. This PCR fragment was cloned into the HindIII site of pRS315 and termed pRS315-*NOP1cNLS. NOP1*, including 700 nt upstream of the ORF, was amplified by PCR, cloned into the HindIII site of pRS315, and termed pRS315-*NOP1*.

In vitro binding assays. (i) GST-Nop1p protein complex formation. Glutathione *S*-transferase (GST) fusion proteins were synthesized in *Escherichia coli* [BL21(DE3)/pLYS-S; Novagen, Madison, Wis.] and purified from cell lysates as previously described (28). GST fusions containing full-length Nop1p, the deletion fragments of Nop1p or GST alone were incubated with purified Kap121p, Kap104p, or Kap108p in transport buffer (TB; 20 mM HEPES-KOH [pH 7.5], 110 mM potassium acetate, 2 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 μM $CaCl_2$, 1 mM dithiothreitol, 0.1% Tween 20) plus solution P (2 μg of pepstatin A and 90 μg of phenylmethylsulfonyl fluoride/ml) (1) for 3 h at 4°C. Samples were centrifuged, the unbound protein fractions removed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to a final concentration of 1×, and the samples were heat denatured at 65°C for 15 min. The beads were washed three times with 1 ml of TB and loaded into a microcentrifuge chromatography column (Bio-Rad). 1× SDS-PAGE sample buffer was passed over the beads twice to release the bead-bound proteins, and these fractions were heat denatured as described above. SDS-PAGE and Coomassie blue staining were used to analyze the unbound and bound fractions. For elution of GST fusions, each fusion protein was purified as described above and eluted from the glutathione resin with glutathione elution buffer (20 mM reduced glutathione, 100 mM Tris-HCl [pH 8.0], 120 mM NaCl).

(ii) Sof1p-pA protein complex formation. Sof1p-pA was purified from yeast cell lysates. *SOF1-A* cells were grown to an optical density at 600 nm of 1.0 in 2 liters of yeast extract-peptone-dextrose (YPD) at 30°C, harvested, washed, and resuspended in 25 ml of ice-cold TB plus solution P. The cell suspension was passed through a microfluidizer (model M-110S; Microfluidics International Corp., Newton, Mass.) at least six times with the coil immersed in ice water. Lysis was monitored by microscopy. An equal volume of TB plus 2% Triton X-100 was added, and the lysate was clarified by centrifugation at 4,000 × *g* in a Beckman JS 4.2 rotor for 30 min. The resulting supernatant was incubated with 100 μl of a 2.0×10^6-beads/μl slurry of IgG-coupled magnetic beads (Dynal Biotech; preequilibrated in TB and blocked with TB containing 10% bovine serum albumin [Sigma] and 0.2% Casamino Acids [Difco] at room temperature for 30 min) at 4°C for 2 to 10 h. The beads were collected with a magnet, the lysate was aspirated slowly, and the beads were washed three times with 5 ml of TB. The Sof1p-interacting proteins were then removed by three washes with 5 ml of elution buffer (20 mM HEPES-KOH [pH 7.5], 0.05% Tween 20) containing 1 M $MgCl_2$. The beads were then divided into four equal fractions, and ~400 to 800 ng of the purified recombinant proteins in TB2 (TB plus 0.2% Casamino Acids and ~40 ng of bovine serum albumin/μl) was added (as indicated) to individual fractions of the immobilized Sof1p-pA, followed by incubation at 4°C for 2 h. The beads were collected by centrifugation, and the unbound protein fractions were prepared for SDS-PAGE. The bead-bound fractions were washed three times with 1 ml of TB and prepared for SDS-PAGE.

For Sof1p-pA-GST-Nop1p and Sof1p-pA-Kap121p complex challenging assays, the complexes were assembled as described above and divided in half. The bead-bound proteins from one of these fractions were released from the beads with 1× SDS-PAGE sample buffer and prepared for SDS-PAGE as described above. The remaining fraction of beads was incubated with either recombinant Kap121p (in TB2), GST-Nop1p (in TB2), or TB2 alone, as indicated, for 2 h at 4°C. The beads were collected by centrifugation, and the unbound protein fractions were prepared for SDS-PAGE. The beads were washed two times with 1 ml of TB and prepared for SDS-PAGE. SDS-PAGE and immunoblotting with α-Kap121p rabbit polyclonal antibodies (which recognizes GST, protein A, and Kap121p) (32) were then used to analyze the unbound and bound fractions.

(iii) Competition assays. Nop1p-pA was purified from *NOP1-A* cell lysates as described above. This immobilized fusion was incubated with ~400 ng of purified Kap121p (in TB2) at 4°C for 3 h, and the beads were washed three times with 1 ml of TB containing 500 mM NaCl. These Nop1p-Kap121p transport complexes were then challenged with the indicated GST-NLS fusion (in TB2 plus 500 mM NaCl) or buffer alone for 3 h at 4°C. All unbound and bead-bound fractions were analyzed as described above.

Immunopurification and immunoblotting. Lysates were prepared from *NOP1-A* and *SOF1-A* yeast cells by using a microfluidizer as described above. These lysates were incubated overnight on a rotator at 4°C with 75 μl of a slurry containing 2.0×10^6 of IgG-coupled magnetic beads (Dynal Biotech)/μl (preequilibrated with TB). The beads were collected with a magnet and washed extensively with TB, and bound proteins were eluted with increasing concentrations (50 mM, 100 mM, 250 mM, 500 mM, 1 M, 2 M, and 4 M) of $MgCl_2$ in elution buffer (20 mM HEPES-KOH [pH 7.5], 0.05% Tween 20). Proteins in each eluate fraction were precipitated with trichloroacetic acid and prepared for SDS-PAGE. As necessary, immunoblotting was performed with rabbit polyclonal α-Kap121p (32), rabbit polyclonal α-Kap108p (elicited against GST-purified Kap108p as previously described [32]), mouse polyclonal α-Kap104p (1), and mouse polyclonal α-Kap123p (46).

GFP analysis. *kap121-34*, *kap121-34*+p*KAP121*, *kap104-16*, and *kap104-16*+p*KAP104* haploid strains containing *GAL1*-inducible GFP fusion proteins [p*GAL1-NOP1GFP*, p*GAL1-SOF1GFP*, p*GAL1-cNLSGFP*, p*GAL1*-Nop1p(aa1-90)-GFP, p*GAL1*-Sof1p(aa381-489)-GFP, or p*GAL1*-Sof1p(aa411-450)-GFP] were grown overnight at 23°C in selection medium containing glucose. The cultures were divided in half and incubated at 23 or 37°C for 2 h. The cells were harvested, washed with sterile water, and resuspended in selection medium containing galactose. The cultures were incubated at 23 or 37°C for an additional 2 to 3 h, and the distributions of GFP chimeras were visualized directly by fluorescence microscopy. To identify the regions of Nop1p and Sof1p containing functional NLSs, galactose-inducible GFP chimeras containing the fragments of Nop1p or Sof1p were visualized directly by fluorescence microscopy in *kap121-34* cells grown to mid-logarithmic phase at 23°C in galactose-containing medium. For *KAP104*- and *KAP108*-regulated expression, *kap121-34*/p*GAL1-NOP1GFP* and *kap121-34*/p*GAL1-SOF1GFP* strains were transformed with p*GAL1-KAP104-LEU2* or p*GAL1-KAP108-LEU2*, and these GFP chimeras were induced and analyzed as described above. pRS315-*NOP1* and pRS315-*NOP1cNLS* were expressed in *kap121-34*/p*GAL1-SOF1GFP* cells, and the localization of Sof1p-GFP was analyzed after induction as described above. Genomically tagged Nop1p-GFP and Sof1p-GFP chimeras were grown to mid-logarithmic phase at 23°C. These cultures were then shifted to 37°C, and GFP chimeras were monitored by fluorescence microscopy at the indicated time intervals.

RESULTS

Identification of novel Kap121p-interacting nucleoplasmic proteins. Yeast nuclear proteins separated in three dimensions (HA HPLC, reversed-phase HPLC, and SDS-PAGE) were probed by an overlay assay using protein A-tagged Kap121p chimera (Kap121p-pA) to detect previously unidentified interactions between Kap121p and nuclear proteins. The relevant sections of the blot are shown in Fig. 1 (see also reference 28). Because Kap121p and Kap123p are functionally similar and it

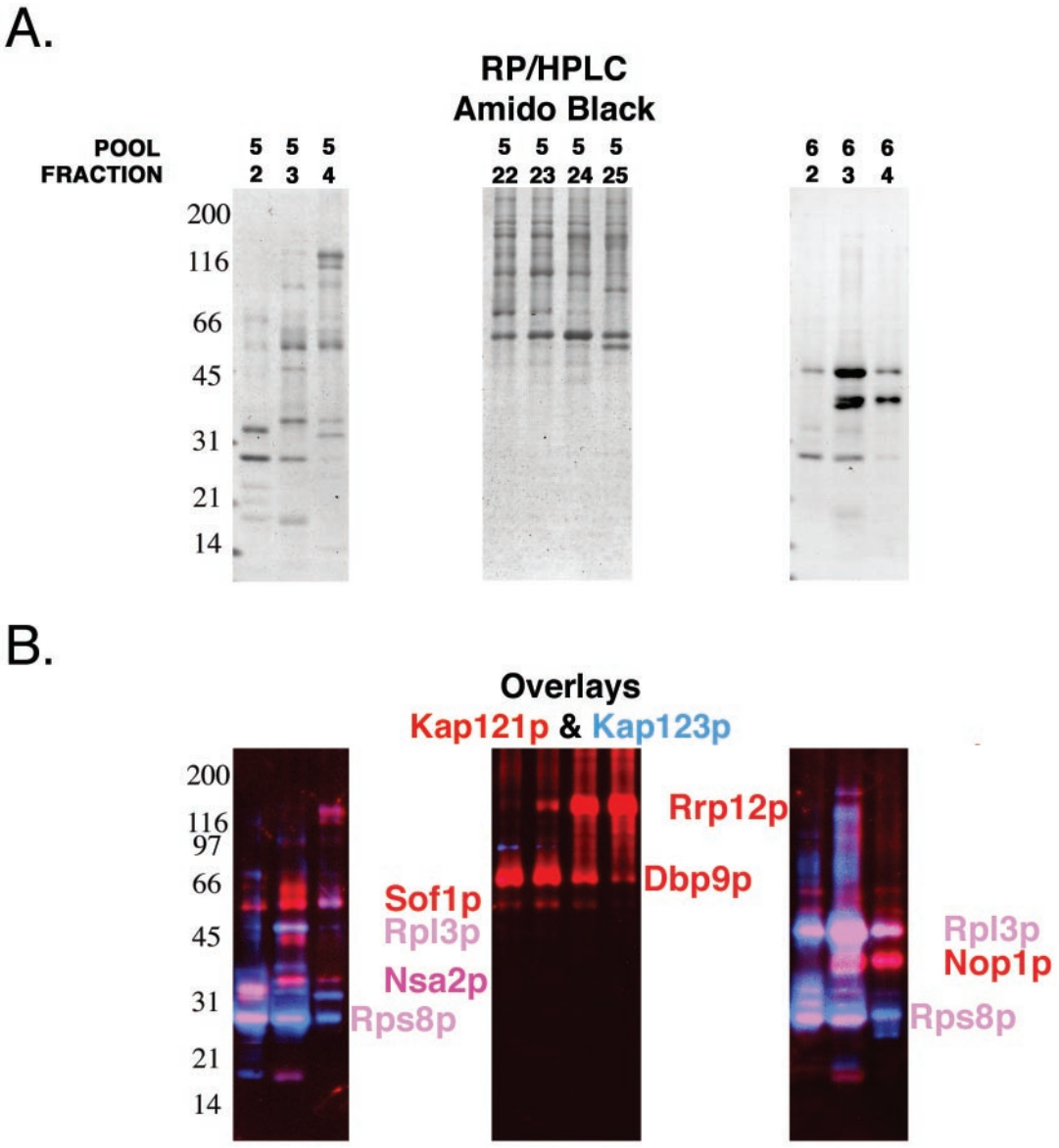

FIG. 1. Kap121p interacts with numerous nuclear proteins. (A) Yeast nuclear proteins were purified, HA HPLC fractionated, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with cytosols from cells synthesizing either Kap121p-pA or Kap123p-pA (see also reference 28). Fractions enriched with Kap121p-pA-interacting proteins were pooled, and proteins were further fractionated by reversed-phase HPLC (RP/HPLC), separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by amido black staining. (B) Membranes were separately probed with cytosols from cells producing Kap121p-pA or Kap123p-pA, and bound chimeras were detected with horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence. Overlaid, colorized images of each blot (red, Kap121p-pA; blue, Kap123p-pA) are shown. Karyopherin-interacting proteins were identified by MS of protein bands in an identical gel.

is well established that they bind to many of the same cargoes (46), the overlay blot assay was repeated with protein A-tagged Kap123p (Kap123p-pA) as a control. The Kap121p-pA and Kap123p-pA profiles were compared, and all Kap121p-interacting proteins were identified by MALDI-TOF MS. Despite the fact that the probe is part of a total yeast lysate, we have previously used this procedure to identify direct interactions between cargoes and kaps. This includes Ste12p, an authentic import cargo of Kap121p, and numerous ribosomal cargoes of Kap123p (28, 46). Here, we have identified 27 Kap121p-interacting proteins. In agreement with previous studies, many of these proteins, including ribosomal proteins, also interacted with Kap123p; however, 11 proteins favored Kap121p over Kap123p under these conditions (http://systemsbiology.org/Default.aspx?pagename=data).

The NLS regions of previously characterized Kap121p cargoes [Rpl25p (amino acids [aa] 1 to 62) (46); Pho4p (aa 140 to 166) (23); Spo12p (aa 76 to 143) (10); histone H2A (aa 1 to 46) (37); histone H2B (aa 1 to 52) (37) Yap1p (aa 5 to 59) (16), Ste12p (aa 494 to 688) (28) and Rcs1p/Aft1p (aa 198 to 225 and 332 to 365) (54)] are rich in lysine residues and in this sense resemble the cNLSs or NLSs of ribosomal proteins.

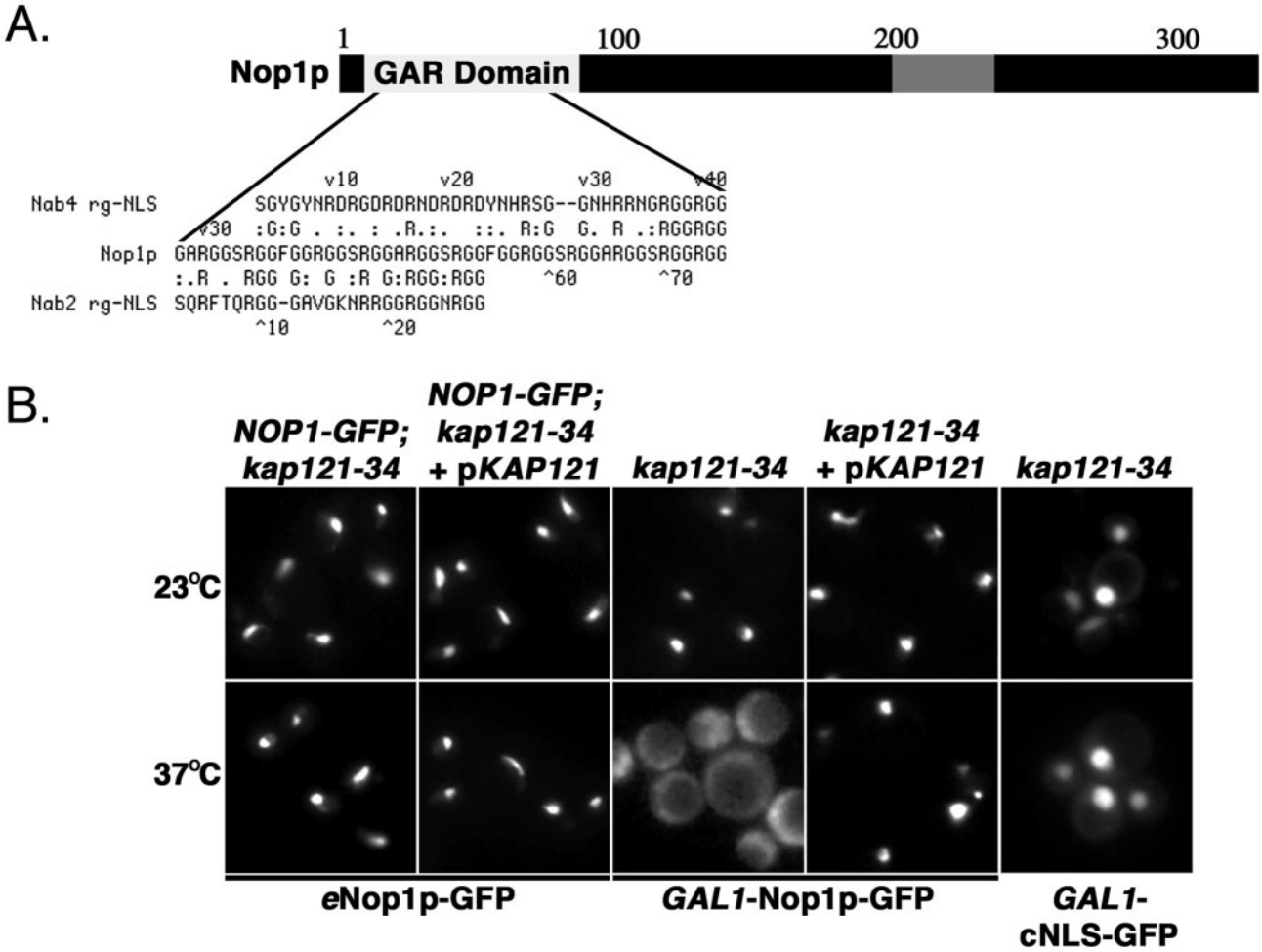

FIG. 2. Nop1p mislocalizes in *kap121-34* cells. (A) Schematic diagram of Nop1p. The white segment highlights the RG-rich GAR domain of Nop1p. The gray segment represents a basic stretch of amino acids (199 to 219; SHRPGRELISMAKKRPNIIP) that is most similar to the previously identified Kap121p NLS sequences. The NLS sequences of Nab2p and Nab4p (27) were compared to full-length Nop1p by using MegAlign (Lipman-Pearson: ktuple, 2; gap penalty, 4; gap length penalty, 12). (B) The distribution of endogenously tagged *NOP1* (*e*Nop1p-GFP) and galactose-inducible *NOP1GFP* (*GAL1*-Nop1p-GFP) were monitored by direct fluorescence microscopy in *kap121-34* cells. *NOP1 GFP*; *kap121-34* cells were visualized after growth to mid-logarithmic phase at the permissive temperature (23°C) and after 3 h of growth at the restrictive temperature (37°C). Although the galactose-inducible *NOP1GFP* chimera was expressed in *kap121-34* cells at 23 or 37°C, *e*Nop1p-GFP remains nucleolar in *hap121-34* cells after 3h of growth at 37°C. However, *GAL1*-Nop1p-GFP accumulates in the cytoplasm of *kap121-34* cells when expressed at the nonpermissive temperature. This contrasts with control *kap121-34* cells containing a wild-type copy of *KAP121* (*kap121-34*+p*KAP121*) or *kap121-34* cells expressing a *GAL1*-cNLS-GFP chimera.

Indeed, they share limited primary sequence similarities and a bipartite consensus Kap121p NLS has been proposed (30). Similarly, all of the potential Kap121p cargoes identified in the overlay assays also contain clusters of lysine-rich regions that we suspected represent functional Kap121p NLS sequences (data not shown). This was also true for Nop1p; a potential lysine-rich Kap121p NLS is located between aa 199 and 219 (Fig. 2A). However, Nop1p also contains an N-terminal GAR (glycine/arginine-rich) domain. This domain is very similar to the arginine-glycine rich RNA-binding domains of Nab2p and Nab4p/Hrp1p (Fig. 2A) which, in *S. cerevisiae*, are also nuclear import signals (rg-NLSs) recognized by Kap104p (27). Moreover, localization studies of Nop1p homologues identified the evolutionarily conserved N-terminal domain as possessing nuclear localization activity. In the case of human Nop1p (fibrillarin), the domain was required, but not sufficient, for nuclear import (49), whereas in *Arabidopsis* the GAR domain of AtFbr1 was both necessary and sufficient to target this protein to nucleoli (40). These data suggested that if Kap121p indeed mediates the import of Nop1p, it may do so through a kind of NLS that is different from those previously characterized. We therefore sought to focus our analysis on the nature of the Kap121p-Nop1p interaction.

Kap121p imports Nop1p into the nucleus. To determine whether Nop1p is an authentic Kap121p cargo, it was C terminally tagged with GFP, and the cellular localization of the chimera was examined in *kap121-18*, *kap121-34*, and *kap121-41* strains (28) by direct fluorescence microscopy at permissive (23°C) and nonpermissive (37°C) temperatures. Under similar nonpermissive (and semipermissive) conditions, previously

characterized Kap121p cargoes redistribute from the nucleus to the cytoplasm (10, 16, 23, 28, 37, 46). Surprisingly, Nop1p fused to GFP under the control of its endogenous promoter (*e*Nop1p-GFP) remained exclusively nucleolar at 23, 34, and 37°C (Fig. 2B and data not shown). However, inhibition of nuclear transport induced by azide and deoxyglucose also did not result in the equilibration of *e*Nop1p-GFP (data not shown). Together, these findings suggest that Nop1p is not a soluble nucleoplasmic protein and that mechanisms such as binding and retention within the nucleolus maintains the nuclear concentration of Nop1p during Kap121p inactivation. We therefore imagined that if Nop1p is a bona fide Kap121p cargo, inducing its expression after inactivation of Kap121p would reveal its import pathway. *NOP1 GFP* was placed under the control of a galactose-inducible promoter (*GAL1*), and then expression was induced after temperature inactivation of kap121-34p and the cellular distribution of Nop1p-GFP was examined by fluorescence microscopy. After 3 h of growth under inducing conditions at 37°C, Nop1p-GFP accumulated in the cytoplasm of *kap121-34* cells but remained nuclear at 23°C or when a wild-type copy of *KAP121* was present (Fig. 2B). By comparison, when synthesized under the same conditions, under the control of the *GAL1* promoter, cNLS-GFP remained nuclear. These data indicated that the efficient nuclear import of Nop1p was specifically dependent on the Kap121p transport pathway.

The RG-rich N-terminal GAR domain of Nop1p is required for Kap121p interaction and contains a functional NLS. To identify the functional Kap121p NLS within Nop1p, three fragments encoding aa 1 to 90, 91 to 327, and 210 to 327 of Nop1p were fused to the 5′ end of two tandem repeats of the ORF encoding GFP (Fig. 3A). The expression of these *NOP1GFP*(x2) chimeras was regulated by using the *GAL1* promoter in *kap121-34* cells as described above, and their cellular localizations were determined by fluorescence microscopy at 23°C (Fig. 3B). Only Nop1p(aa1-90)-GFP, the RG-rich GAR domain-containing fragment, was targeted to the nucleus, whereas Nop1p(aa91-327)-GFP and Nop1p(aa210-327)-GFP were diffusely distributed throughout the cell. Notably, Nop1p(aa1-90)-GFP lacks the aforementioned lysine-rich region (aa 199 to 219) most closely related to other proposed Kap121p NLSs (30). Moreover, the import of Nop1p(aa1-90)-GFP was dependent on Kap121p function. When *kap121-34* cells expressing Nop1p(aa1-90)-GFP were shifted from 23 to 37°C for 3 h, the chimera mislocalized from the nucleus to the cytoplasm (Fig. 3C). Introduction of a wild-type copy of *KAP121* rescued this nuclear import defect.

To address whether Kap121p interacts directly with Nop1p, GST fusion proteins containing either full-length Nop1p or the Nop1p deletion fragments were constructed, synthesized in *E. coli*, immobilized on glutathione-Sepharose, and incubated with recombinant Kap121p. In agreement with the in vivo localization studies, Kap121p bound directly to GST-Nop1p(aa1-90) and full-length GST-Nop1p but failed to interact to any appreciable extent with either GST-Nop1p(aa91-327), GST-Nop1p(aa210-327), or GST alone (Fig. 3D and data not shown). The weak binding to GST-Nop1p(aa91-327) may reflect the presence of a basic region (aa 199 to 219) similar to other NLSs but which, as shown above, is inactive as an NLS in vivo (Fig. 3B). Previous studies have shown that the binding of RanGTP to import-bound Kap121p contributes to the displacement of import substrates (23, 28). To test whether RanGTP displaced Kap121p from GST-Nop1p(aa1-90), GST-Nop1p(aa1-90)-Kap121p complexes were immobilized on glutathione-Sepharose and then incubated with either RanGTP, RanGDP, or GTP-loading buffer. RanGTP did not release Kap121p from the rg-NLS of Nop1p (data not shown). This is similar to previous studies demonstrating that a combination of RanGTP and nucleic acids are required to release many nucleic acid-binding proteins from their kaps (27, 39, 48). Taken together, these data show that Kap121p interacts directly with an rg-NLS sequence present in the N-terminal GAR domain of Nop1p, which is both necessary and sufficient for its Kap121p-mediated import.

Kap104p mediates Nop1p nuclear import in the absence of Kap121p function. The similarity between the rg-NLS sequences of Nop1p and those of the Kap104p cargoes Nab2p and Nab4p suggests possible overlap between the cargoes recognized by Kap121p and Kap104p. We therefore used a synthetic lethal genetic assay to test for a functional interaction between the two pathways. A conditional *kap121*::*LEU2*; *kap104*::*ura3*::*HIS3* double-deletion haploid strain expressing a temperature-sensitive allele of *KAP121* (*kap121-34*) and a *URA3* selectable wild-type allele of *KAP104* was isolated (*kap121-34*; Δ*kap104*/p*KAP104-URA3*). As a control, a *kap121-34;* Δ*kap108*/p*KAP108-URA3* strain was also isolated. An equal number of cells from each culture were spotted in serial dilutions on YPD and 5-FOA-containing solid medium, followed by incubation at 23°C. The double-deletion strains grew equally well on rich medium; however, when cells were grown on 5-FOA-containing medium (to select for cells lacking p*KAP104-URA3* or p*KAP108-URA3*) the *kap121-34*; Δ*kap104*/p*KAP104-URA3* strains failed to grow, whereas the *kap121-34*; Δ*kap108*/p*KAP108-URA3* grew as well as the parental strains (Fig. 4A). Thus, a wild-type copy of *KAP104*, but not *KAP108*, is required for growth of cells compromised in Kap121p function, and this suggests that under such conditions Kap104p imports essential Kap121p cargoes. In contrast, we observed no synthetic lethal interaction between *kap121-34* and Δ*kap108*, suggesting a specific relationship between the Kap121p and the Kap104p import pathways.

To directly test whether Kap104p could supplant Kap121p activity, *KAP104*, *KAP108* and *KAP123* were overexpressed in *kap121-34*/p*GAL1-NOP1GFP* cells. Cultures were prepared as previously described, the localization of *GAL1*-Nop1p-GFP was determined by fluorescence microscopy, and *KAP104* and *KAP108* overexpression were confirmed by Western blotting (Fig. 4B and C). Cells overexpressing *KAP104* showed nucleolar localization of Nop1p in *kap121-34* cells at both the permissive and nonpermissive temperatures, whereas *GAL1*-Nop1p-GFP accumulated in the cytoplasm of *kap121-34* cells overexpressing *KAP108* or *KAP123* (Fig. 4B and data not shown). These findings suggest that Kap104p is also capable of importing Nop1p into the nucleus. Kap104p thus probably contributes to the nuclear localization of Nop1p upon inactivation of Kap121p, and this at least partially explains why it was necessary to regulate Nop1p-GFP expression to reveal its cytoplasmic accumulation in *kap121* mutant cells (Fig. 2B).

These findings prompted us to investigate whether Kap104p, in addition to Kap121p, could interact directly with Nop1p. To

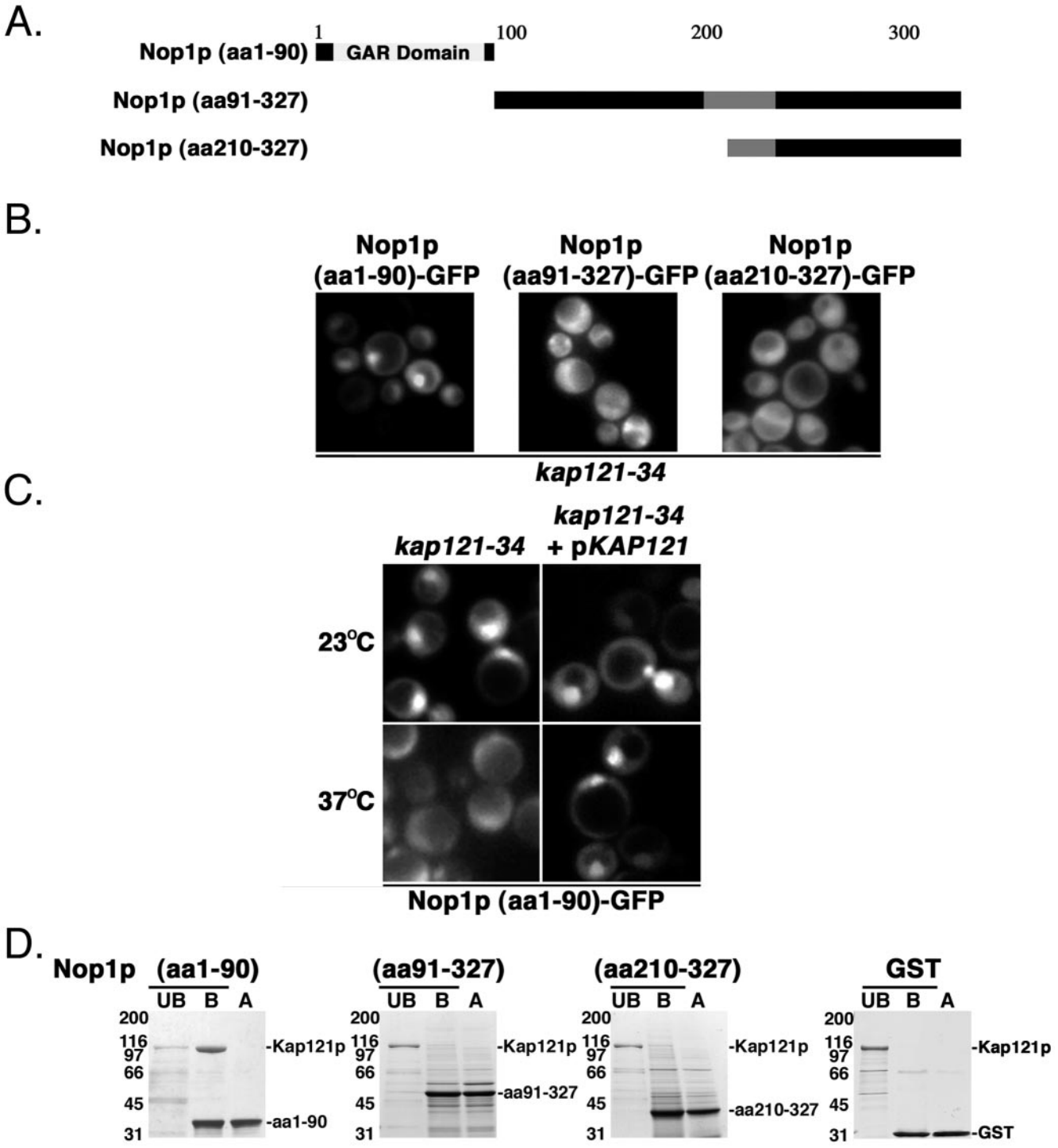

FIG. 3. The GAR domain of Nop1p interacts directly with Kap121p and contains a functional NLS. (A) A schematic diagram representing the fragments of Nop1p expressed as GST and GFP fusion proteins. The GAR domain and a putative lysine-rich NLS (aa 199 to 219 [shaded gray]) are indicated. (B) Galactose-inducible GFP fusion proteins containing the three fragments of Nop1p were synthesized at 23°C in *kap121-34* cells and analyzed by direct fluorescence microscopy. Note the nuclear localization of Nop1p(aa1-90)-GFP. (C) The cellular localization of Nop1p(aa1-90)-GFP was determined at both 23 and 37°C in *kap121-34* and *kap121-34*+p*KAP121* cells. Note that Nop1p(aa1-90)-GFP mislocalized to the cytoplasm of in *kap121-34* cells at 37°C. (D) GST fusions containing fragments of Nop1p (~400 ng) or GST alone were immobilized on glutathione-Sepharose 4B beads and incubated with recombinant Kap121p (~200 ng). Equal amounts of the unbound fractions (UB), bound fractions (B), and the GST fusions alone (A) were analyzed by SDS-PAGE and Coomassie blue staining. Note recombinant Kap121p interacts directly with GST-Nop1p(aa1-90).

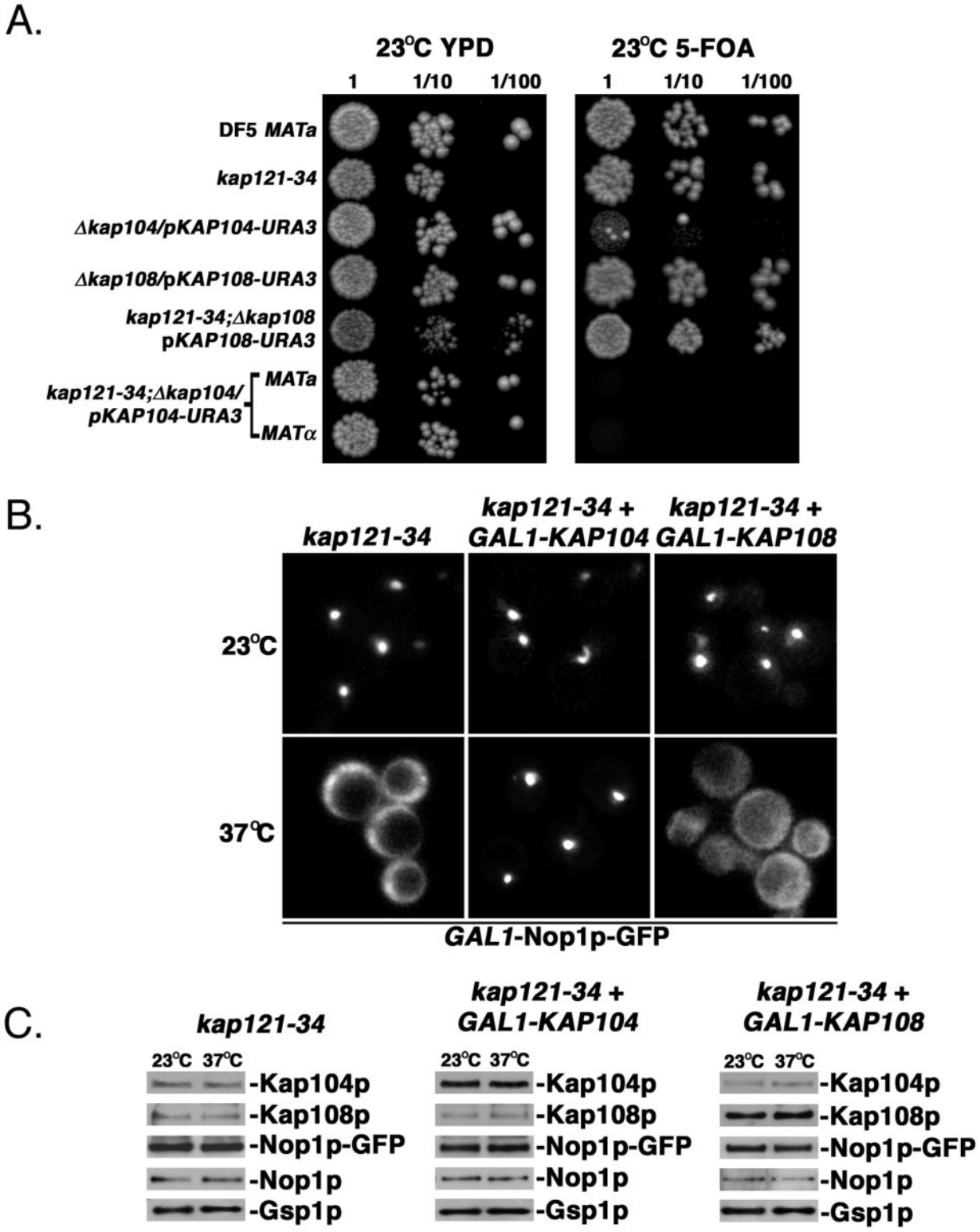

FIG. 4. Kap104p can supplant the function of Kap121p in Nop1p import. (A) *kap121-34* and *kap104*::*ura3*::*HIS5* are synthetically lethal in combination. The mutant strains shown were tested for their ability to withstand the loss of a wild-type copy of *KAP104* or *KAP108* at an otherwise permissive temperature. The indicated dilutions of cultures containing each strain were spotted onto YPD or 5-FOA-containing medium (to select against cells containing p*KAP104-URA3* or p*KAP108-URA3*) and incubated at 23°C for 4 days. (B) Overexpression of *KAP104* rescues the mislocalization of *GAL1*-Nop1p-GFP. Kap104p (p*GAL1-KAP104*) and Kap108p (p*GAL1-KAP108*) were induced in *kap121-34* cells containing *GAL1*-Nop1p-GFP (p*GAL1-NOP1GFP*) by a shift to galactose-containing medium for 3 h at restrictive (37°C) or permissive (23°C) temperatures. Under these conditions, in the absence of additional Kap104p, Nop1p-GFP accumulates in the cytoplasm but is nuclear upon coinduction of Kap104p. (C) *kap121-34*, *kap121-34*+p*GAL1-KAP104*, and *kap121-34*+p*GAL1-KAP108* whole-cell lysates from the same cultures as those used for microscopy were probed with rabbit polyclonal α-Kap104p, α-Kap108p, and α-Gsp1p and mouse monoclonal α-Nop1p (MAb D77 [5]) antibodies. The Western blots indicate the relative levels of Kap104p, Kap108p, Nop1p-GFP, endogenous Nop1p, and Gsp1p (loading control).

this end, Nop1p-pA protein complexes were purified from a *NOP1-A* yeast whole-cell lysate by using IgG-Sepharose. Bound proteins were eluted from the beads with increasing concentrations of $MgCl_2$, and the fractions were immunoblotted with four kap-specific polyclonal antibodies directed against Kap121p, Kap104p, Kap123p, and Kap108p. Although inherent variations of antigen recognition for each kap-specific antibody precluded quantitative comparisons between these immunoblots, qualitative examination of these data suggested that of the β-kaps tested, only Kap121p and Kap104p interacted significantly with Nop1p-pA (Fig. 5A). In parallel, GST-Nop1p(aa1-90) was immobilized on glutathione-Sepharose incubated with lysates derived from yeast strains expressing pA-tagged copies of Kap121p, Kap104p, Kap123p, and Kap108p. Proteins that bound to each chimera were analyzed by immunoblotting. In agreement with the Nop1p-pA immunopurification studies, Kap121p-pA and Kap104p-pA both enriched with GST-Nop1p(aa1-90), with Kap121p-pA appearing to bind to Nop1p with a higher affinity (Fig. 5B). In contrast, neither Kap123p nor Kap108p enriched with GST-Nop1p(aa1-90).

Considering that previous studies demonstrated that Kap104p interacts directly with the rg-NLS sequences of Nab2p and Nab4p (27), we reasoned that Kap104p would also bind directly to the N-terminal rg-NLS of Nop1p. To address this, GST-Nop1p, GST-Nop1p(aa1-90), GST-Nop1p(aa91-327), and GST alone were immobilized on glutathione-Sepharose and incubated with recombinant Kap104p or Kap108p. In agreement with the immunopurification studies, Kap108p did not interact with these GST fusions (Fig. 5C and data not shown). However, like Kap121p, Kap104p interacted directly with both full-length Nop1p and the rg-NLS-containing GAR domain of Nop1p (Fig. 5C) but not with GST-Nop1p(aa91-327) OR GST alone.

Collectively, these data suggest that the Kap104p and Kap121p transport pathways overlap in their cargo specificities. It is thus likely that Kap104p can supplant the activity of Kap121p upon its inactivation or disruption of its import activity (and vice versa). Nevertheless, as has been observed for other transport pathways, each kap appears to prefer its own cargo. Indeed, a significant change in the localization of Nop1p-GFP was not observed when its expression was induced in *kap104-16* cells (1) at 37°C (Fig. 5D) and the rg-NLS of Nab2p was not mislocalized in *kap121-34* cells at nonpermissive temperatures (data not shown).

Kap121p is required for the nuclear import of Sof1p. Numerous nuclear transport studies have demonstrated that a given kap tends to transport cargoes involved in similar cellular processes, and this may serve as a means to coordinate various cellular functions (27, 35–37, 46). In support of this observation, our overlay assays also identified Sof1p, which interacts physically and functionally with Nop1p. Sof1p and Nop1p are essential members of the U3 (box C+D) snoRNP complex required for site-specific 2′ O methylation of pre-rRNA in the nucleolus (reviewed in references 6, 25, and 56). However, unlike Nop1p, Sof1p does not contain an RG-rich NLS. Indeed, amino acid sequence alignments of Sof1p with other Kap121p NLSs identified a potential lysine-rich NLS (aa 413 to 446) that is more typical of other Kap121p cargoes (Fig. 6A). Presented with the opportunity to study the import of two functionally related proteins carrying distinct NLSs, we investigated the mechanism of Sof1p import.

To this end, we first sought to establish whether Sof1p is a Kap121p cargo. Endogenous Sof1p was C terminally tagged with GFP, and its localization in *kap121-34* (28) was examined at permissive (23°C) and nonpermissive (37°C) temperatures. Like Nop1p, *e*Sof1p-GFP was retained in the nucleolus under these conditions (Fig. 6B); but when its expression was regulated by using the *GAL1* promoter, *GAL1*-Sof1p-GFP accumulated in the cytoplasm of *kap121-34* cells at 37°C (Fig. 6B). Furthermore, this chimera remained nuclear at 23°C or when a wild-type copy of *KAP121* was present. These findings indicate that Kap121p function is required for the efficient nuclear import of Sof1p.

To investigate whether Sof1p bound to Kap121p, we immunopurified Sof1p-pA from *SOF1-A* yeast whole-cell lysates and followed the copurification of interacting proteins by immunoblotting. Both Kap121p and Nop1p coenriched with Sof1p-pA, with the majority of Kap121p eluting in the 100 mM $MgCl_2$ fraction and Nop1p eluting in the 250 mM $MgCl_2$ fraction (Fig. 6C). To further examine this interaction, we attempted to reconstitute the import complex by using recombinant proteins and solution binding experiments. Since we could not synthesize full-length GST-Sof1p in sufficient quantities with an *E. coli* expression system, Sof1p-pA was isolated from yeast lysates, and any copurifying yeast proteins were removed by excessive washes with 1 M $MgCl_2$ (see Fig. 6B). The resulting immobilized Sof1p-pA fusion was incubated with recombinant Kap121p or GST-Nop1p (Fig. 6D). Both proteins interacted directly with Sof1p. In addition to confirming the previously detected interaction between Sof1p and Nop1p (21), these data also suggested that Sof1p can bind directly to Kap121p.

Sof1p is imported by Kap121p through a lysine-rich NLS. As mentioned above, amino acid alignments identified aa 413 to 446 of Sof1p as a potential archetypal lysine-rich Kap121p NLS (Fig. 6A). To address the nature of the Kap121p-Sof1p protein-protein interaction, GST fusion proteins containing aa 1 to 130, 131 to 380, 381 to 489, and 411 to 450 of Sof1p were constructed and synthesized in *E. coli*. These GST chimeras were immobilized on glutathione-Sepharose and then incubated with recombinant Kap121p. Kap121p interacted directly with the GST-Sof1p(aa381-489) and GST-Sof1p(aa411-450) but failed to bind to either the N-terminal (aa 1 to 130) or central (aa 131 to 380) portion of the protein (Fig. 7A). Kap104p did not interact with any of these GST fusion proteins, suggesting that the observed interactions are specific to Kap121p. Extending the Kap121p interacting fragment of Sof1p from aa 411 to 450 to aa 381 to 489 significantly improved the observed protein-protein interaction, indicating that flanking sequences also contribute to the NLS.

To evaluate the import potential of each fragment in vivo, the Sof1p protein fragments were C terminally fused to GFP. The Sof1p-GFP chimeras were expressed under the control of the *GAL1* promoter in *kap121-34* cells, and their cellular localizations were monitored by fluorescence microscopy at 23°C (Fig. 7B). In agreement with the in vitro binding experiments, the Kap121p-interacting fragments [Sof1p(aa381-489)-GFP and Sof1p(aa411-450)-GFP] were targeted to the nucleus, whereas Sof1p(aa1-130)-GFP and Sof1p(aa131-380)-GFP were diffusely distributed throughout the cell. Furthermore,

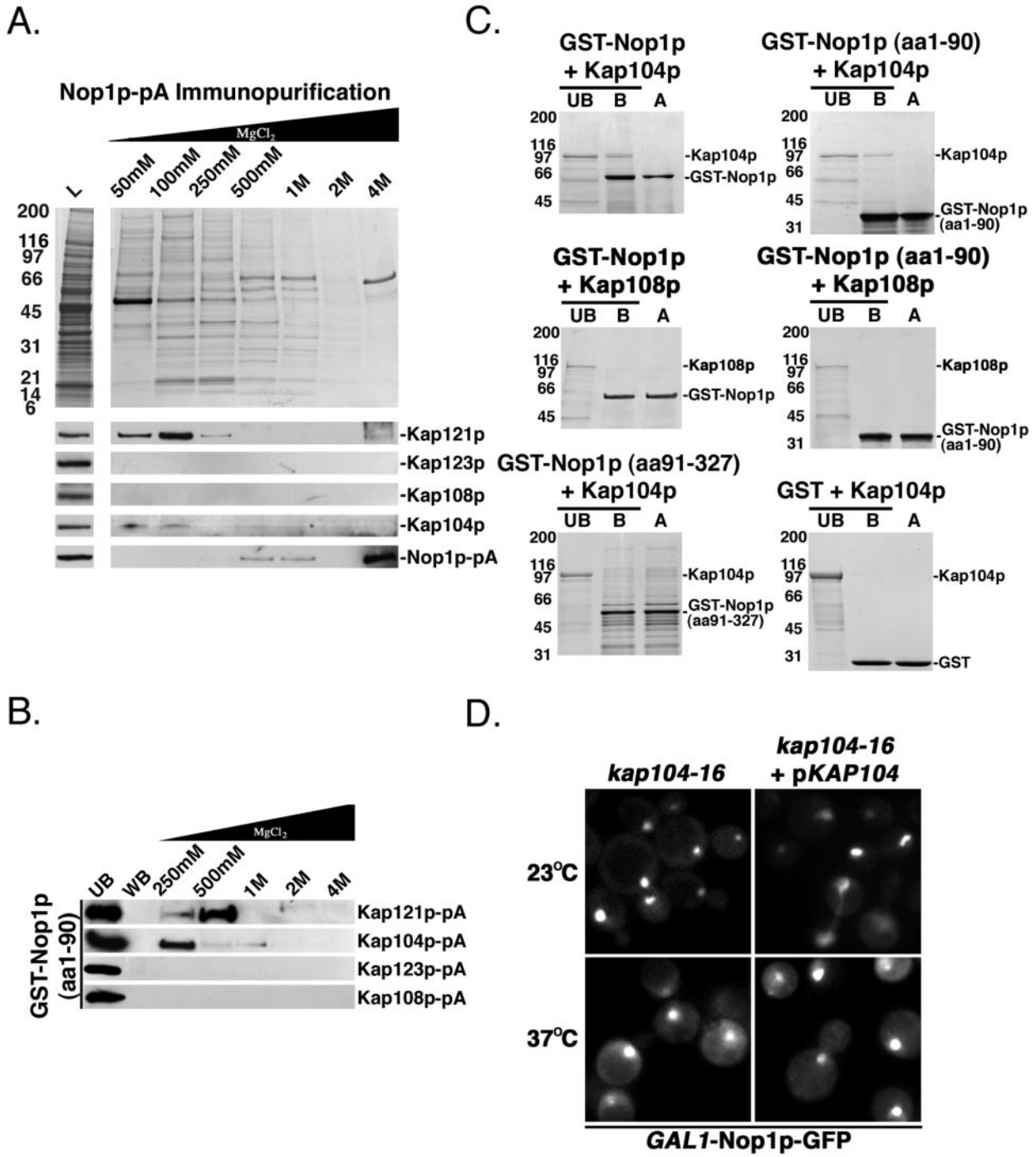

FIG. 5. Kap104p interacts directly with Nop1p. (A) Nop1p-pA was immunopurified from *NOP1-A* cells, and copurifying proteins were eluted with a step gradient of $MgCl_2$ as indicated. Equal volumes of the lysate (L) and elution fractions were separated by SDS-PAGE and either stained with Coomassie brilliant blue (top panel) or transferred to nitrocellulose for immunoblot analysis (bottom panel) with kap-specific polyclonal antibodies as indicated. Nop1p-pA was also detected. The membranes were probed with kap-specific polyclonal antibodies as shown. (B) Recombinant GST-Nop1p(aa1-90) was immobilized on glutathione-Sepharose and incubated with yeast whole-cell lysates (1 mg/ml) from strains expressing Kap121p-pA, Kap104p-pA, Kap123p-pA, and Kap108p-pA. Bound protein complexes were washed and eluted with a step gradient of $MgCl_2$ as indicated. Proteins from each eluate fraction, the unbound fraction (lane UB), and the wash buffer (lane WB) fraction were separated by SDS-PAGE and transferred to nitrocellulose membranes. Protein A chimeras were detected by immunoblotting with horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence. Note that Kap121p preferentially copurified with both Nop1p-pA and GST-Nop1p(aa1-90) and that minimal amounts of Kap104p also copurified with these Nop1p fusion proteins. (C) GST-Nop1p (~400 ng), GST-Nop1p(aa1-90) (~400 ng), GST-Nop1(aa91-327) (~400 ng), and GST alone (~400 ng) were immobilized on glutathione-Sepharose 4B beads and incubated with recombinant Kap104p (~200 ng) or Kap108p (~200 ng) as indicated. Equal amounts of unbound fractions (lanes UB), bound fractions (lanes B), and the GST fusions alone (lanes A) were analyzed by SDS-PAGE and Coomassie blue staining. Kap104p interacted directly with both Nop1p and the N-terminal rg-NLS-containing GAR domain of Nop1p. (D) Nop1p is correctly localized to the nucleolus in *kap104* cells. The galactose-inducible *NOP1GFP* chimera (*GAL1*-Nop1p-GFP) was expressed in *kap104-16* cells (in the presence or absence of a wild-type copy of *KAP104*) at permissive (23°C) or restrictive (37°C) temperatures and visualized by direct fluorescence microscopy. Note that there was no significant change in the localization of Nop1p in these strains at the nonpermissive temperature.

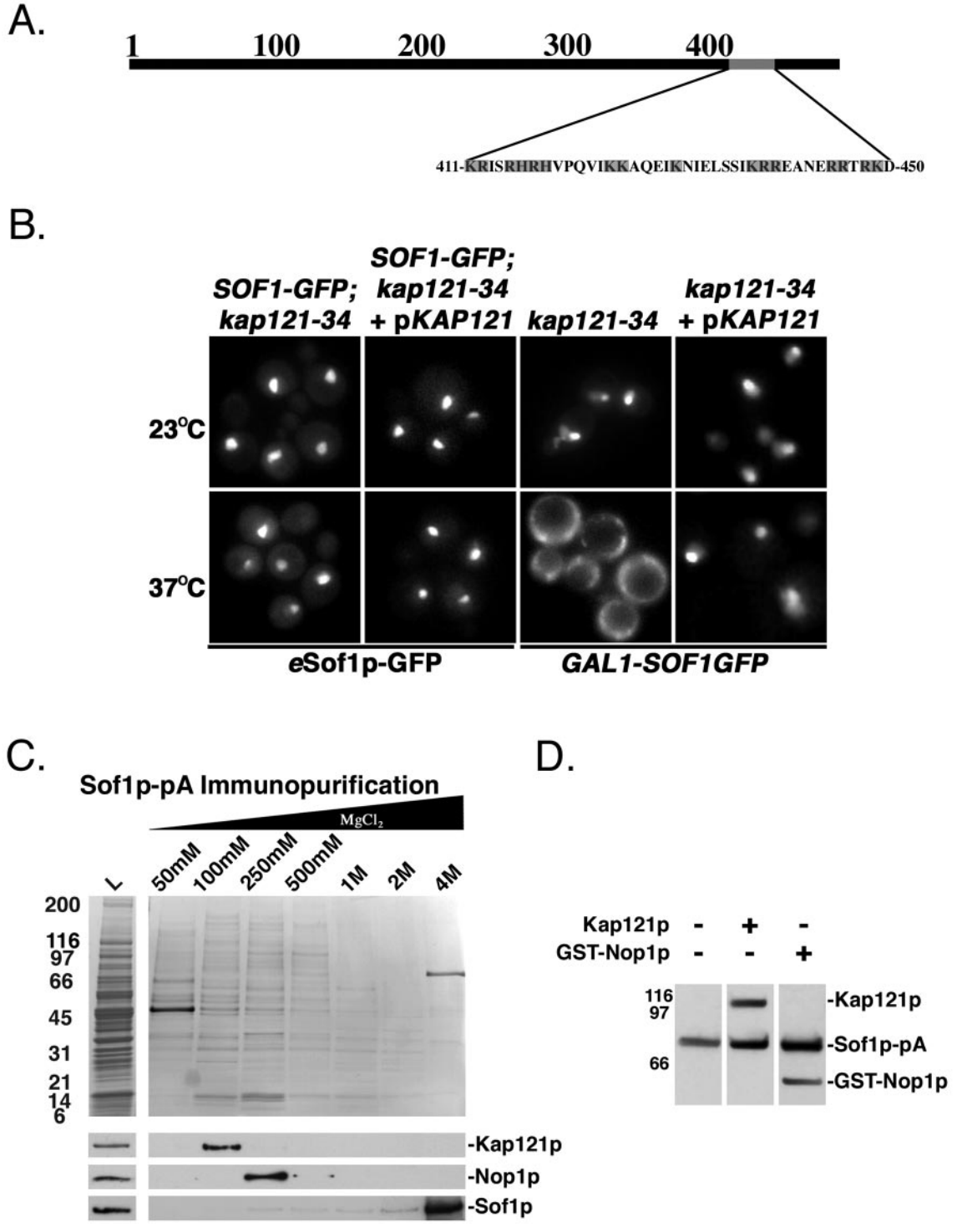

FIG. 6. Kap121p imports Sof1p. (A) Schematic diagram of Sof1p. The gray segment represents a basic stretch of amino acids that is most similar to the previously identified Kap121p NLS sequences. (B) Endogenously tagged *SOF1 GFP* (*e*Sof1p-GFP) and galactose-inducible *SOF1GFP* (*GAL1*-Sof1p-GFP) were monitored by direct fluorescence microscopy in *kap121-34* cells at permissive (23°C) and nonpermissive (37°C) temperatures as described previously. Note the cytoplasmic mislocalization of *GAL1*-Sof1p-GFP at 37°C. (C) Sof1p-pA protein complexes were purified from *SOF1-A* whole-cell lysates. These immobilized complexes were washed extensively, and the bound proteins were eluted with a step gradient of $MgCl_2$ as indicated. Equal volumes of the lysate (lane L) and eluted fractions were separated by SDS-PAGE and either stained with Coomassie brilliant blue (top panel) or transferred to nitrocellulose and analyzed by immunoblotting with rabbit α-Kap121p polyclonal and α-Nop1p monoclonal (MAb D77 [5]) antibodies (bottom panel). Note that both Kap121p and Nop1p copurified with Sof1p-pA. (D) Sof1p-pA was purified from *SOF1-A* whole-cell lysates, and the copurified proteins were removed by a wash with 1 M $MgCl_2$ (left panel). The beads were incubated with GST-Nop1p or recombinant Kap121p (middle and right panels, respectively) and equal fractions of the bead-bound proteins were analyzed by immunoblotting with α-Kap121p antibodies. Both Nop1p and Kap121p interacted directly with Sof1p.

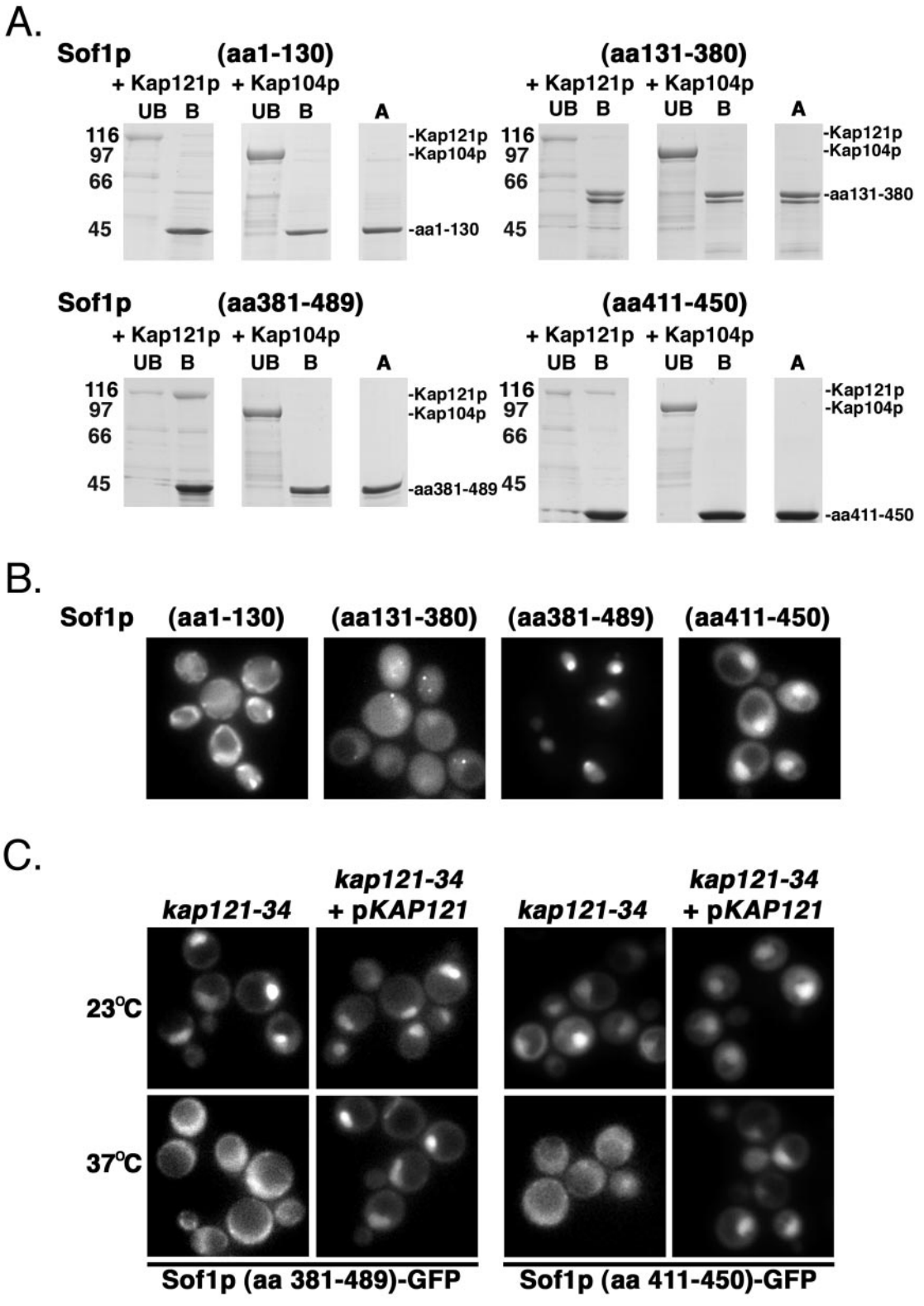

FIG. 7. aa 381 to 489 of Sof1p contains a functional Kap121p NLS. (A) GST fusions containing fragments of Sof1p (~400 ng) or GST alone were immobilized on glutathione-Sepharose 4B beads and incubated with recombinant Kap121p (~200 ng) or Kap104p (~300 ng). Equal amounts of the unbound fractions (lanes UB), bound fractions (lanes B), and GST fusions alone (lanes A) were analyzed by SDS-PAGE and Coomassie blue staining. Note that recombinant Kap121p, but not Kap108p, interacts strongly with GST-Sof1p(aa381-498) and weakly with GST-Sof1p(aa411-450). (B) Galactose-inducible GFP fusion proteins containing the four fragments of Sof1p were synthesized at 23°C in *kap121-34* cells and analyzed by direct fluorescence microscopy. Note the nucleolar localization of Sof1p(aa381-489)-GFP and the nuclear accumulation of Sof1p(aa411-450)-GFP. (C) The cellular localizations of Sof1p(aa381-489)-GFP and Sof1p(aa411-450)-GFP were determined at both 23 and 37°C in *kap121-34* and *kap121-34*+p*KAP121* cells. Note that the nuclear import of these Sof1p fragments is dependent on Kap121p function.

Sof1p(aa381-489)-GFP was imported more efficiently than Sof1p(aa411-450)-GFP. Amino acids 381 to 489 localized the GFP reporter exclusively to the nucleolus. Although the smaller fragment (aa 411 to 450) also concentrated the reporter in the nucleus, in agreement with the in vitro binding data, its import was less efficient. In addition, Kap121p function was required for the import of Sof1p(aa381-489)-GFP and Sof1p(aa411-450)-GFP since inactivation of Kap121p resulted in the mislocalization of these reporters (Fig. 7C). Our findings demonstrated that Kap121p interacts directly with Sof1p through a C-terminal, lysine-rich NLS sequence that is both necessary and sufficient for its Kap121p-mediated import.

Sof1p can be imported by a piggyback mechanism. The data presented here and previously (10, 16, 20, 23, 28, 37, 46) suggest that Kap121p can interact with two different types of NLSs. This raises the possibility that Kap121p might be capable of interacting with two distinct NLSs simultaneously. Therefore, in vitro competition assays were performed to determine whether Kap121p could interact with a lysine-rich NLS and the rg-NLS of Nop1p at the same time (Fig. 8A). Nop1p-pA was prepared from *NOP1-A* whole-cell lysates, and interacting yeast proteins were removed by extensive washing with 1 M $MgCl_2$. The immobilized Nop1p-pA chimera was incubated with recombinant Kap121p to assemble Nop1pA-Kap121p import complexes. These heterodimers were then challenged with GST alone; GST fusion proteins containing the Kap121p-specific NLS sequences of Nop1p [GST-Nop1p(aa1-90)], Ste12p [GST-Ste12p(aa494-688)] (28), or Pho4p [GST-Pho4p(aa140-166)] (23); or buffer alone. Under these conditions, challenging Nop1p-Kap121p protein complexes with an rg-NLS [GST-Nop1p(aa1-90)] or lysine-rich NLS sequences [GST-Ste12p(aa494-688) or GST-Pho4p (aa140-166)] displaced Kap121p from Nop1pA (Fig. 8A). These data suggest that, at least for the different NLSs tested here, Kap121p binds directly to only one cargo at a time. Thus, either Kap121p contains only one NLS binding pocket or, if it contains two binding pockets, then occupation at one sterically prevents the simultaneous occupation at the other. More precise experiments are required to distinguish these possibilities.

The Sof1p-pA immunopurification studies suggest that the Sof1p-Nop1p protein-protein interaction was stronger than the Sof1p-Kap121p interaction, since Nop1p eluted in the 250 mM $MgCl_2$ fraction, whereas Kap121p eluted in the 100 mM $MgCl_2$. We therefore reasoned that if Nop1p and Sof1p interact in the cytoplasm, then the cytoplasmic pool of Nop1p could inhibit the formation of a Kap121p-Sof1p import complex. In vitro binding assays were used to address this point. In agreement with this idea, we found that when a Sof1p-Kap121p complex was preformed and Nop1p was added, then Kap121p was displaced and Nop1p in turn bound to Sof1p. These findings further suggest that, like the other NLSs examined, Sof1p NLS and Nop1p NLS cannot interact with Kap121p simultaneously. However, when a complex between Nop1p and Sof1p was preformed and Kap121p was added subsequently, a tertiary complex was formed without displacing either component (Fig. 8B). We interpreted these data to suggest that a complex of Nop1p-Sof1p was capable of interacting with Kap121p and considered the possibility that this complex could be imported into the nucleus by Kap121p. Because Kap121p was displaced from Sof1p by the addition of Nop1p, we suggest that in such a scenario the Nop1p rg-NLS would be engaged and Sof1p, in turn, would interact with Nop1p and be carried piggyback into the nucleus.

To investigate this possibility, we appended a cNLS (recognized by Kap95p/Kap60p) to Nop1p, bypassing the requirement of Kap121p for Nop1p nuclear import. If Nop1p and Sof1p can be imported as a single complex, this chimera should restore the nuclear import defect of Sof1p in the *kap121-34* cells. *kap121-34*/p*GAL1-SOF1GFP* cells were transformed with a single-copy plasmid containing either *NOP1* or a *NOP1* fusion containing the cNLS from simian virus 40 large T antigen (24) at its 3′ end (*NOP1cNLS*), thereby allowing Nop1p to be imported by the Kap95p/Kap60p transport pathway. Remarkably, the nuclear import of Sof1p-GFP was restored when *NOP1cNLS* was expressed in *kap121-34* cells but remained mislocalized in the presence of *NOP1* alone (Fig. 9A). Taken together, it appears that Sof1p can be imported as a complex with Nop1p, which in turn is bound to a kap. If correct, we predicted that, since overexpression *KAP104* rescued the mislocalization of Nop1p-GFP in *kap121-34* cells (Fig. 4B), its overexpression would also restore the mislocalization of Sof1p-GFP. Indeed, upon *KAP104* overexpression in *kap121-34*/p*GAL1-SOF1GFP* cells the nuclear localization of Sof1p-GFP was restored (Fig. 9A). This was not observed upon overexpression of the control kap, Kap108p, although Western blotting of whole-cell lysates from these cells confirmed that *KAP104* and *KAP108* (Fig. 9B) were both induced to similar extents. Together, these data demonstrate that Nop1p and Sof1p can be simultaneously imported by Kap104p or, if provided with a cNLS, by the Kap95p/Kap60p complex. These findings, together with the in vitro binding data, demonstrate that Sof1p can be imported as a piggyback complex with Nop1p and Kap121p.

DISCUSSION

Karyopherin escort a variety of macromolecular cargoes between the nucleus and cytoplasm through the nuclear pore. Comprehensive molecular and structural analyses of the NPC, its components, kaps, and other soluble transport factors have yielded tremendous insights into the molecular mechanisms of directional, selective carrier-cargo transport. Importantly, however, our knowledge of which cargoes are transported by each kap remains inadequate. Indeed, nearly one-third of the proteins in the yeast proteome must be translocated into the nucleus at some stage of their life cycle (13, 15). These include factors involved in regulated gene expression, macromolecular assembly, and nuclear architecture. Understanding regulated transport and the phenotypic consequences of altering transport pathways demands a comprehensive knowledge of the various transport pathways, their cargoes, and their functional redundancy.

Here, we have initiated the large-scale identification of Kap121p cargoes. Overlay blot assays probing an entire nuclear extract identified 27 Kap121p-interacting proteins. Some of these proteins also interacted with Kap123p, supporting the previously reported redundancy between these two kaps (20, 37, 46). However, 11 proteins preferentially interacted with Kap121p (http://systemsbiology.org/Default.asp?pagename=data). Given that approximately one-third of all

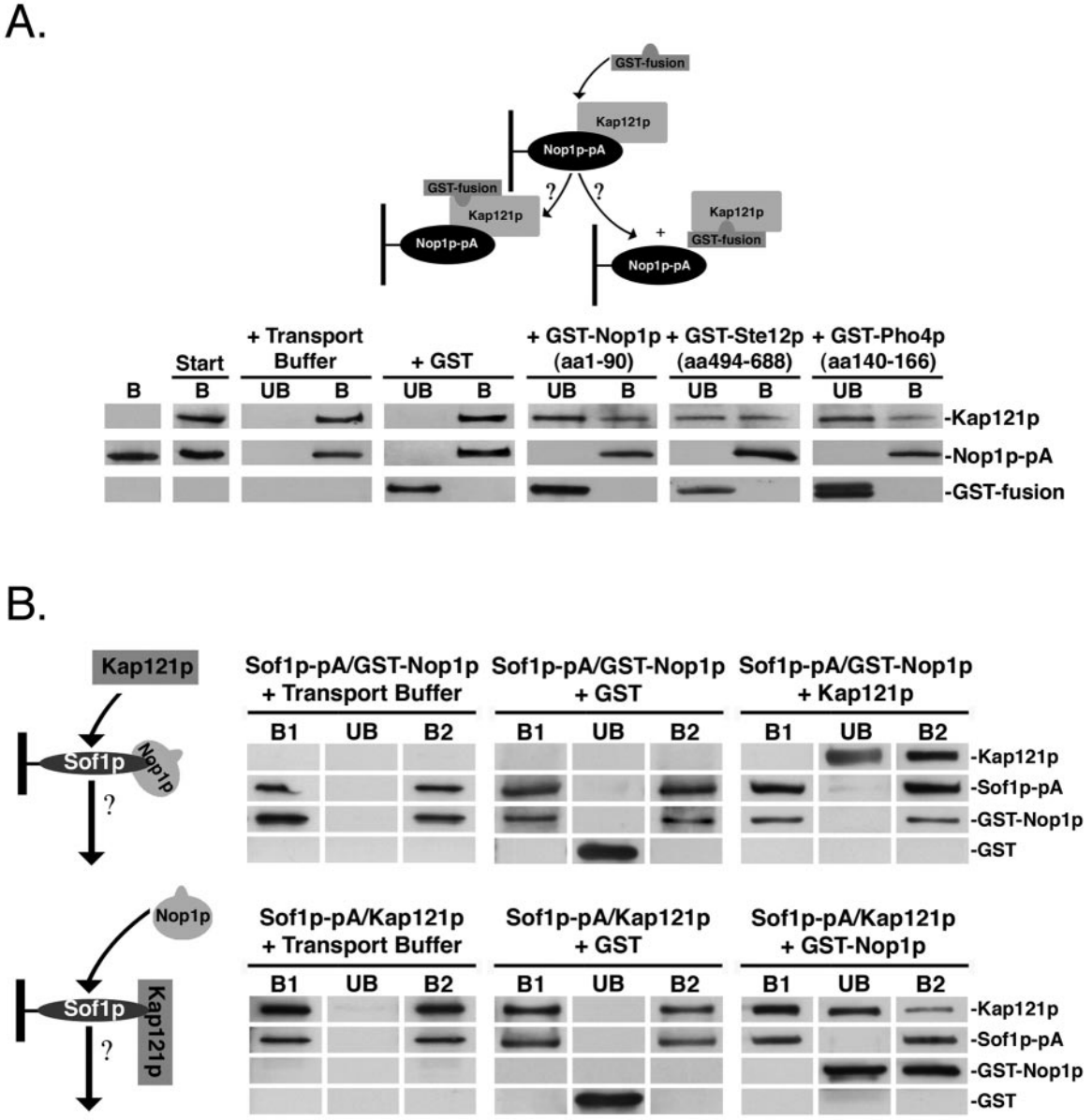

FIG. 8. Heterotrimeric protein complexes containing Sof1p, Kap121p, and Nop1p assemble in vitro. (A) Nop1p-pA was purified from *NOP1-A* whole-cell lysates, and the copurified proteins were removed by washing them with 1 M $MgCl_2$ (far left panel). The immobilized pA fusion was then incubated with recombinant Kap121p (~400 ng), forming Nop1p-pA-Kap121p protein complexes (Start). These import complexes were then incubated with transport buffer, GST alone, or GST fusion proteins (~600 to 800 ng) containing the Kap121p-specific NLS sequences of Nop1p [GST-Nop1p(aa1-90)], Ste12p [GST-Ste12p(aa494-688)], or Pho4p [GST-Pho4p(aa140-166)]. After a washing step, equal volumes of the unbound (lanes UB) and bound (lanes B) fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with α-GST-Kap121p antibodies (which react with GST, protein A, and Kap121p). Note that the addition of the NLS sequences of Nop1p, Ste12p, or Pho4p disrupted the Nop1p-pA-Kap121p import complexes. (B) Sof1p-Nop1p (Sof1p-pA/GST-Nop1p) or Sof1p-Kap121p (Sof1p-pA/Kap121p) complexes were assembled as previously described (Fig. 6D) and incubated with recombinant Kap121p, GST-Nop1p, GST, or transport buffer as indicated. After an extensive washing, the initial complex (lanes B1), unbound (lanes UB), and bound (lanes B2) fractions were prepared for SDS-PAGE, separated, transferred to nitrocellulose, and probed with α-GST-Kap121p antibodies (which react with GST, protein A, and Kap121p). Note that Nop1p dissociates Kap121p from Sof1p, whereas Kap121p interacts with the Sof1p-Nop1p protein complex without displacing Nop1p.

yeast proteins are imported into the nucleus, we believe that this list represents a subset of all Kap121p import cargoes. Indeed, only 2 of the 12 previously characterized Kap121p cargoes were among these 27 proteins (10, 16, 20, 23, 28, 36, 37, 46, 54). This was not surprising, since the nature of this approach, with a kap as the probe to identify cargoes, demands that the cargoes be sufficiently abundant in order to detect the interactions. In the other cases, Kap121p was identified by using the cargo as the bait (Spo12p) or by monitoring the cargos (Yap1p, Pdr1p, Pho4p, and Aft1p) in different kap mutants. The majority of the Kap-interacting proteins (23 of 27) identified here are abundant factors required for ribosome

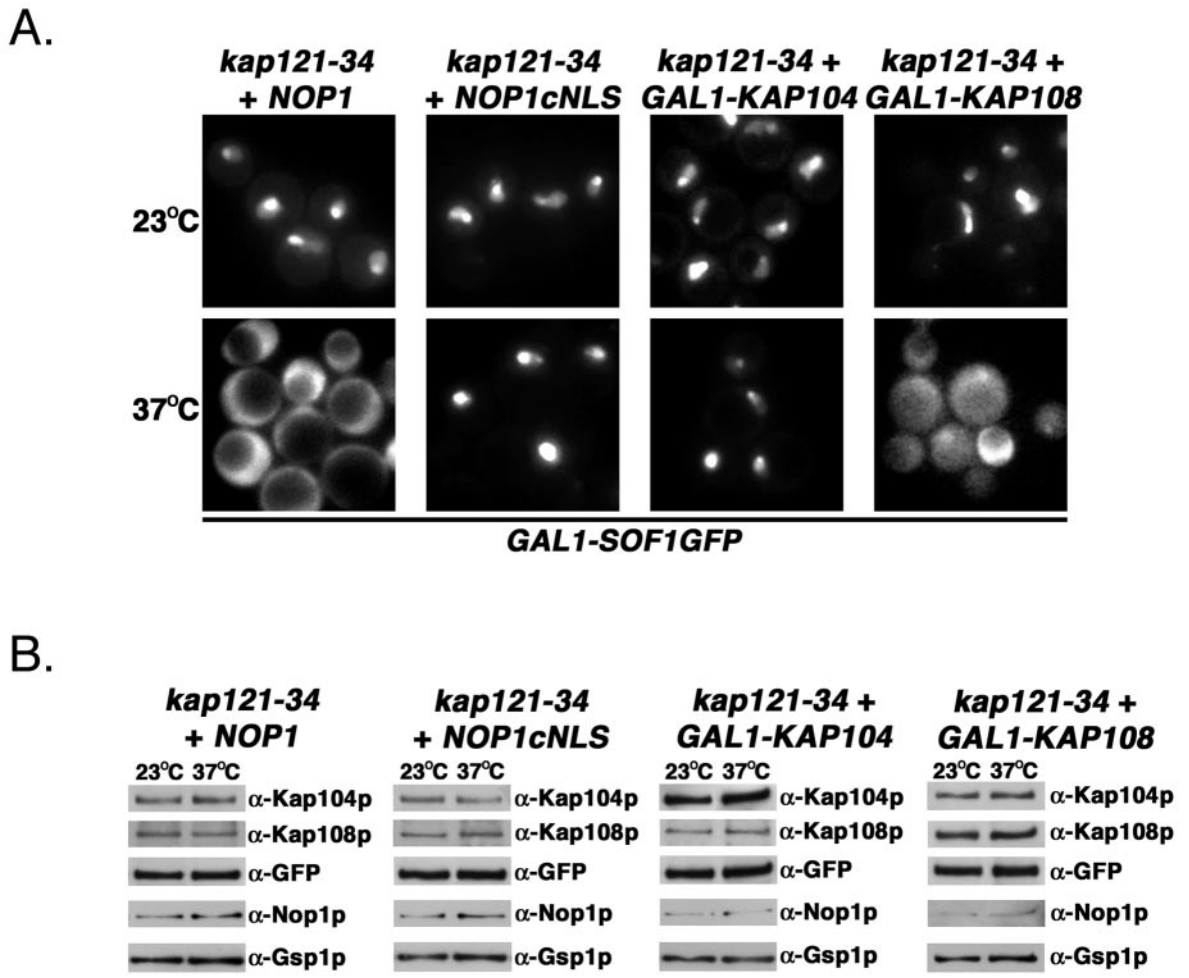

FIG. 9. Sof1p can be imported by piggyback. (A) Sof1p is imported by interacting with Nop1p bound to a kap. The galactose-inducible *SOF1GFP* (*GAL1*-Sof1p-GFP) chimera was expressed in *kap121-34* cells that contained a plasmid-linked copy of wild-type *NOP1* (*NOP1*), *NOP1* fused to a cNLS (*NOP1cNLS*), galactose-inducible *KAP104* (*GAL1-KAP104*), or galactose-inducible *KAP108* (*GAL1-KAP108*) at permissive (23°C) or restrictive (37°C) temperatures as previously described. Note that the expression of *NOP1cNLS* or overexpression of *KAP104* rescued the cytoplasmic mislocalization of Sof1p-GFP at the nonpermissive temperature (37°C). (C) Whole-cell lysates from the same cultures as those used for microscopy (as shown in panel A) were probed with rabbit polyclonal α-Kap104p, α-Kap108p, α-Gsp1p, and α-GFP and mouse monoclonal α-Nop1p (MAb D77 [5]) antibodies.

biogenesis and, as such, represent the most abundant potential Kap121p cargoes.

Amino acid sequence alignments revealed that most of these potential Kap121p import cargoes contained lysine-rich sequences typical of the previously identified NLS sequences of Kap121p cargoes (see reference 30). Interestingly, although Nop1p contains a weakly related lysine-rich region, it also contains an N-terminal GAR domain that shares significant sequence homology with NLS sequences recognized by another β-kap, Kap104p. Nop1p is an essential nucleolar protein that has remained extremely well conserved, both structurally and functionally (4, 6, 22), throughout evolution. Nop1p (fibrillarin) is also an autoantigen in humans and has been shown to be implicated in ~8% of the cases of the autoimmune disease scleroderma (5, 29). Given the unique NLS characteristics of this putative Kap121p transport substrate, the essential functions carried out by Nop1p, and the potential extrapolation to vertebrates, we sought to investigate the Kap121p-Nop1p interaction.

Characterization of the Kap121p-Nop1p interaction demonstrated that Nop1p is a bona fide import cargo for Kap121p. However, Kap121p mediates its import through a direct interaction with the N-terminal rg-NLS. This establishes Nop1p as the first member of a novel class of Kap121p cargoes and extends previous studies implicating the Nop1p GAR domain in nuclear localization (40, 49). Interestingly, this novel Kap121p-recognized rg-NLS sequence is similar to the rg-NLS sequences of the Kap104p import cargoes Nab2p and Nab4p. This high degree of sequence similarity suggested that these seemingly distinct nuclear transport pathways might converge and mediate the nuclear import of some common cargoes. Genetic studies demonstrated that mutations in both *KAP121* and *KAP104* are synthetically lethal, establishing a functional relationship between these two β-kaps and strongly suggesting

that these two transport pathways overlap. Although rg-NLS sequences were originally characterized for Kap104p, and Kap104p can interact with the rg-NLS of Nop1p in vitro, Kap121p does so with higher affinity and is primarily responsible for its import. Kap104p thus provides an alternative transport route for Nop1p in the absence of Kap121p function.

Why should an NLS be recognized by different kaps? The weaker affinities of each class of NLS for other kaps probably allow kaps to back each other up. This may allow cells to respond to particularly high transport burdens during high growth rates or in response to different environmental conditions, external cues, or developmental stages. Interestingly, recent studies have demonstrated that mutations in *KAP121* cause cell cycle defects and that Kap121p transport is specifically attenuated at certain stages of the cell cycle (31). This has the potential to alter the transport of numerous Kap121p cargoes essential for cell cycle progression. Promiscuity among kap-cargo liaisons creates a situation in which the loss of individual kap function (either temporarily through regulation, or constitutively through genetic perturbation) may only affect a small number of cargoes specific for that kap. Thus, the cell can shut down the transport of classes of molecules by modifying the kap (or a nucleoporin with which it specifically interacts) without affecting all cargoes normally transported by that kap.

Interestingly, the majority of the proteins that interacted specifically with Kap121p in the overlay assay, (Nop1p, Sof1p, Dbp9p, Imp4p, Nop12p, and Rrp12p) are *trans*-acting protein factors required for ribosome biogenesis. We followed up on Sof1p because it is an essential nucleolar protein that suppresses a *nop1* temperature-sensitive mutant and forms a dimeric complex with Nop1p (21). We demonstrated that Kap121p mediates the nuclear import of Sof1p through a C-terminal lysine-rich NLS. These studies illustrated that, whereas aa 411 to 450 were sufficient for Kap121p interaction and subsequent import, aa 381 to 489 contained all of the information required for wild-type, nucleolar localization of Sof1p. Furthermore, two nuclear import complexes containing Sof1p could be generated in vitro: Sof1p-Kap121p and Sof1p-Nop1p-Kap121p. Recent studies with mammalian Kap β1 established that this kap mediates the nuclear import of a fragment of kap α and nonclassical NLS-containing cargoes simultaneously (12), indicating that an individual β-kap can recognize, interact with, and mediate the nuclear import of cargoes containing unrelated NLS sequences. However, in vitro competition assays demonstrated that, unlike mammalian Kap β1, Kap121p does not interact with previously identified lysine-rich Kap121p NLSs and the rg-NLS of Nop1p at the same time. Our data nevertheless indicate that Nop1p and Sof1p can be imported together by Kap121p. In this case, however, Nop1p and Sof1p form a complex that can be recognized by Kap121p, and the trimeric complex traverses the NPC. Solution binding and immunopurification studies showed that under the conditions explored here Kap121p appears to have a higher affinity for the rg-NLS of Nop1p than that present in Sof1p, whereas Sof1p has a higher affinity for Nop1p than Kap121p. Furthermore, expression of *NOP1cNLS* chimera or overexpression of *KAP104* rescued the mislocalization of Sof1p-GFP in *kap121-34* cells, suggesting that Nop1p bridges the interaction between Sof1p and Kap121p.

Recent work from numerous groups has begun to reveal the complex network of interactions between import kaps and their cargoes. The simple idea of one kap for each kind of NLS was soon overturned by several studies showing that, whereas many NLSs are recognized mainly by one kind of kap, they can also be recognized to a lesser extent by other kaps, providing a high degree of redundancy to the nuclear import of many cargoes. As well as further highlighting this kind of redundancy, the present study has added another level of complexity. Here, we show that a single kap (in this case, Kap121p) can recognize both a lysine-rich NLS and an rg-NLS. We had previously assumed that these two very different NLSs would be recognized by different kaps. But it is apparent that cells can exploit surprising flexibility in kap cargo recognition, providing an extra level of redundancy to nuclear transport. Moreover, we also show that associating proteins can be imported as complexes by a single kap. Transporting cargoes in this fashion would ensure that essential, functionally dependent cellular components (such as the rRNA-processing factors transported by Kap121p and Kap104p) are properly localized, in appropriate molar ratios, to the subcellular compartments where they function. Furthermore, we envision that nucleocytoplasmic transport networks like this one would allow the cell to adapt to minor perturbations in individual nuclear transport pathways without drastically affecting other cellular processes. Exploring how these newly discovered levels of transport redundancy are regulated and interconnected is the next challenge in uncovering the network of nuclear transport.

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