Intersection of the Kap123p-Mediated Nuclear Import and Ribosome Export Pathways

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Kap123p is a yeast β-karyopherin that imports ribosomal proteins into the nucleus prior to their assembly into preribosomal particles. Surprisingly, Kap123p is not essential for growth, under normal conditions. To further explore the role of Kap123p in nucleocytoplasmic transport and ribosome biogenesis, we performed a synthetic fitness screen designed to identify genes that interact with KAP123. Through this analysis we have identified three other karyopherins, Pse1p/Kap121p, Sxm1p/Kap108p, and Nmd3p/Kap119p. We propose that, in the absence of Kap123p, these karyopherins are able to supplant Kap123p’s role in import. In addition to the karyopherins, we identified Rai1p, a protein previously implicated in rRNA processing. Rai1p is also not essential, but deletion of the RAI1 gene is deleterious to cell growth and causes defects in rRNA processing, which leads to an imbalance of the 60S/40S ratio and the accumulation of halfmers, 40S subunits assembled on polysomes that are unable to form functional ribosomes. Rai1p localizes predominantly to the nucleus, where it physically interacts with Rat1p and pre-60S ribosomal subunits. Analysis of the rai1/kap123 double mutant strain suggests that the observed genetic interaction results from an inability to efficiently export pre-60S subunits from the nucleus, which arises from a combination of compromised Kap123p-mediated nuclear import of the essential 60S ribosomal subunit export factor, Nmd3p, and a ΔRAI1-induced decrease in the overall biogenesis efficiency.

In eukaryotic cells, macromolecules are actively and selectively transported between the nucleus and cytoplasm by the concerted action of soluble transport factors and the nuclear pore complex (NPC). Proteins destined for the nucleus carry a nuclear localization signal (NLS) (13), while substrates to be exported from the nucleus harbor nuclear export signals (NESs) (18, 21). The signals are recognized by a structurally related family of proteins, termed karyopherins (abbreviated as Kaps, but also known as importins or exportins) (22, 41, 57), that interact with the NPC and the small GTPase Ran to mediate translocation. The NPC is a large, octagonally symmetric structure, and its overall architecture is highly conserved from yeast to metazoans (reviewed in references 5, 44, 53, and 56). The yeast NPC contains multiple copies of ∼30 protein components, termed nucleoporins or Nups. Twelve of these Nups contain characteristic degenerate repeated peptide motifs (GLFG, FXFG, PSFG, or FG) and are thus collectively termed FG-Nups. These nucleoporins provide multiple docking sites for cargo-bearing transporters and are present throughout the NPC, extending from the cytoplasmic filaments to the nuclear basket (45). It remains a mystery how the interactions between karyopherins and the NPC mediate vectorial transport; however, karyopherins appear to derive directional cues from both specific nucleoporins and Ran (reviewed in references 10, 40, 44, and 56).

In a rapidly growing cell, the complex process of ribosome biogenesis accounts for a major proportion of nucleocytoplasmonic transport. Like all messenger transcripts, mRNAs encoding ribosomal proteins are exported to the cytoplasm, where translation occurs. Once synthesized, ribosomal proteins are imported into the nucleus and coordinateably coupled with nascent rRNA to yield immature ribosomal subunits. These subunits are then exported to the cytoplasm where they undergo the final stages of maturation and assembly onto mRNA to carry out translation. Yeast cells that divide every 1.5 h must accordingly double their ribosomal content in this time frame, necessitating the import of ∼150,000 ribosomal proteins per min (∼1,000/NPC), while simultaneously exporting ∼4,000 assembled subunits per min (55).

In Saccharomyces cerevisiae, it appears that the import leg of this biogenesis program is accomplished largely by the karyopherin Kap123p. Kap123p binds to many different ribosomal proteins including rpL25, rpS1a, rpL8a/b, rpL18a/b, rpL12a/b, rpL32, rpL11a/b, and rpL42a/b, and at least rpL25 requires Kap123p to be efficiently imported into the nucleus (46, 48). Interestingly, deletion of KAP123 from the yeast genome does not dramatically affect cell growth; thus, it is apparent that there are other ribosomal protein importers in yeast. One such candidate is Kap121p, which, among the karyopherin family members, is most similar to Kap123p. Kap121p also binds to several ribosomal proteins in the absence of Kap123p and, when overexpressed, suppresses the rpL25 import defect observed in ∆kap123 strains (46).

By comparison to the import leg, ribosomal subunit export is less straightforward, as assembly and export appear to be temporally and physically coupled. Initially, the 35S rRNA is transcribed by RNA polymerase I and the 5S rRNA is separately transcribed by RNA polymerase III. Over 60 trans-acting fac-

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they undergo a receptor-mediated, energy-dependent process (7), where independently exported through the NPC to the cytoplasm by several nucleoporins and karyopherins are required for this yeast mutants, it has been established that the Ran cycle and sfA7 we employed a genetic interaction screen to identify compo-

dents in the ribosome biogenesis pathway that interact with the major ribosomal protein nuclear import factor, Kap123p. In addition, we identified a mutant allele of the gene encoding Rai1p, a protein previously shown to be involved in 60S subunit biogenesis (58). Interestingly, cells lacking Rai1p and Kap123p, but not other karyopherins assayed, were defective in 60S ribosomal subunit export. Here, we report on the character-

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**MATERIALS AND METHODS**

Yeast strains and microbiological techniques. The *S. cerevisiae* strains used in the study were derivatives of the diploid DF5 strain, unless specified otherwise, and are listed in Table 1. All yeast genetic manipulations were performed according to established procedures (23). Gene knockout marker modules were switched as required by using "marker swap" plasmids, as described previously (11).

Deletion of the *RAI1* open reading frame (ORF) was accomplished by integrative transformation of DF5 cells with a PCR-synthesized *HIS3* marker containing short flanking homology to the upstream and downstream regions of the *RAI1* ORF, generated using the following oligonucleotides: RA11-5’-CCG GAA TTC AAG CTT ATG GGT GTT AGT GCA AAT TGC-3’ and RA11 3’-CCG GAA TTC TTA ACC AGA AGT TTT CCA CTC-3’. Transformants were selected on synthetic complete (SC) plates lacking histidine (SC-His plates).
used as secondary antibodies, and immunoreactive bands were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce).

**Isolation of Rai1p-pA interacting proteins.** Immunoprecipitations from yeast whole-cell lysates were performed as previously described (12, 38). After immunoprecipitation in coupled extracts, IgG-Sepharose beads were washed 10 times with 1 ml of wash buffer containing 20 mM Na$_2$HPO$_4$ (pH 7.5), 150 mM NaCl, 0.1 mM MgCl$_2$, 0.1% Tween 20, 0.4 µg of pepstatin A/ml, and 180 µg of phenylmethylsulfonyl fluoride/ml. Bound proteins were eluted in wash buffer containing either 0.2 or 1 mM magnesium chloride and trichloroacetic acid precipitated. Copurified proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue or silver staining (50). Bands of interest were then excised from the gel, subjected to in-gel digestion, and identified by mass spectrometry (25).

**Northern blot analysis.** RNA was isolated by the hot-phenol technique (6). The analysis of rRNA by Northern hybridization was performed as described previously (28). The following primers were used: A3-B1L (5'-5' CAG TTA AAC ATT GTC GAC GGT GAA GCT CAA AAA CTT AAT-3'), A2-A3 (5'-TGT TAC CTC TGG GCC C-3'), E-C2 (5'-GCG CAA TAT CTT CAA GTA-3'), 255 (5'-CTC CGC TTA TTG ATA TGC-3'), D-A2 (5'-GCT ATC TGG CTA CCA-3'), and 185 (5'-CAT GCC TTA ATT GAG AC-3').

**Polysome analysis.** For polysome preparations, yeast cultures were grown to an optical density at 600 nm (OD$_{600}$) of 0.6, treated with cycloheximide (100 µg per ml of culture), harvested, and disrupted by glass bead lysis as described previously (19, 33). For each sample, 200 µg units of cell lysate supernatants were collected after centrifugation at 14,000 × g for 6 h at 141,000 × g in a Beckman SW28 rotor at 4°C. Sucrose gradient fractions were analyzed by continuous monitoring at 254 nm with a UV monitor (UV-M II; Pharmacia LKB).

**RESULTS**

**KAP123 synthetic fitness screen.** KAP123p plays an integral role in ribosome biogenesis by importing ribosomal proteins into the nucleus prior to their incorporation into assembling preribosomal particles (46, 48). To investigate how cells compensate for the loss of KAP123 and its interaction with other components of the ribosome assembly pathways, we have isolated null mutants that, in combination with kap123 null mutants, exhibit a synthetic fitness defect. The genes encoding the β-karyopherins Sxm1p/Kap108p, Nmd5p/Kap119p, and Pse1p/Kap121p complemented strains sfa7 and sfa9, sfl5, and sfl2 mutants, respectively, suggesting that the nuclear import pathways mediated by these karyopherins overlap with those mediated by Kap123p (Fig. 1). Indeed, genetic and functional
interactions have previously been reported between KAP123 and KAP121 (46, 49). Furthermore, Sxm1p/Kap108p interacts with ribosomal proteins, suggesting a role in importing this class of proteins (42). Such functional redundancy between multiple members of this family is underscored by the fact that each of these synthetic fitness mutants was viable, displaying synergistic fitness defects but not synthetic lethality at 30°C. To confirm these results, we constructed strains harboring double knockouts of genes encoding Sxm1p/Kap108p, Nmd5p/Kap119p, and Kap123p in all three combinations, along with a \( \Delta \)kap123/kap121ts double mutant strain. All of these strains were viable, but their growth rates were dramatically reduced (data not shown).

Interestingly, sf17 was not complemented by a gene encoding a karyopherin, but rather by the gene YGL246C/RAI1 (Fig. 1). Rai1p stabilizes and augments the function of Rat1p (58), a 5'-exonuclease required for the trimming of the 27A3 precursor rRNA to the 27SBs and degradation of excised pre-rRNA spacer fragments during 60S ribosomal subunit biogenesis (36, 54). In agreement with the original report on Rai1p (58), deletion of YGL246C was not lethal but compromised cell growth. Moreover, these cells showed no specific temper-

FIG. 1. KAP123 interacts genetically with several karyopherins and YGL246C/RAI1. (A) Summary of genetic interactions uncovered by the KAP123 synthetic fitness screen. (B) Top: The chromosomal region contained in the sf17 rescuing plasmid is indicated by the dashed box. Bottom: Plasmids containing candidate genes (RAI1, YGL247W, and PDE1) and genes encoding several karyopherins (SXM1/KAP108, NMD5/KAP119, KAP95, and KAP121) were analyzed using a plasmid shuffling assay in sf17 cells. Note that only cells transformed with YGL246C/RAI1 were able to grow in the absence of pJA8, as assayed by growth on 5-FOA. (C) Top: Cells lacking the RAI1 ORF were viable in a \( \Delta \)kap123 background but exhibited a synthetic fitness interaction with KAP123. Bottom: The reduced growth rate of \( \Delta \)rai1 cells was complemented by plasmids encoding either Rai1p or Rai1p-GFP. (D) Sequencing of the RAI1 ORF in the sf17 mutant revealed an A-to-T transversion at position 443, which introduced the nonsense mutation shown.
Furthermore, diploid cells resulting from a cross of ∆rail cells with sf17 cells displayed a similar growth defect as the haploid strains, suggesting that the mutation in sf17 was indeed allelic to RAIL (data not shown). This mutation was termed rail-1. To evaluate the specificity of the ribfl/kap123 genetic interaction, double mutant strains containing ∆rail/∆sxm1, ∆rail/∆nmd5, and ∆rail/kap123ts were compared to cells containing single deletions of each gene and the rail-1/kap123 and ∆rail/ ∆kap123 strains. In contrast to the dramatic growth defect associated with the ∆rail/kap123 double deletion, ∆rail/ ∆sxm1, ∆rail/∆nmd5, and ∆rail/kap121-34 cells grew only slightly slower than ∆rail cells (data not shown). Direct DNA sequencing of the PCR-amplified rail gene in sf17 cells revealed an A→T transversion at position 443, resulting in a nonsense mutation of lysine 147 that predicts an expressed product containing approximately the amino-terminal one-third of Rai1p (Fig. 1). Interestingly, this mutation seemed more detrimental to cells than did complete loss of Rai1p, as the ∆rail/kap123 strain was not as defective in growth as the rail-1/kap123 strain (data not shown).

**Rail1p is a nuclear protein.** Because of Kap123p’s well-established role in nucleocytoplasmic transport, we first investigated whether the localization of Rai1p was dependent on Kap123p. Rai1p was therefore C-terminally tagged with GFP and localized by fluorescence microscopy. In wild-type cells, Rai1p-GFP localized primarily to the nucleus but also revealed a distinct cytoplasmic pool. Surprisingly, we detected no change in the Rai1p-GFP nuclear signal in cells lacking Kap123p, Sxm1lp/Kap108p, or Nmd5p/Kap119p, or in cells carrying temperature-sensitive mutants of Kap121p (kap121-34) or Kap95p (kap95-14) (data not shown). Furthermore, despite numerous attempts, including immunopurification, overlay, and solution binding assays, we failed to detect a physical interaction between Rai1p and Kap123p. Together, these data suggest that Rai1p is not imported into the nucleus by Kap123p, and they do not support the hypothesis that the genetic interaction reflects a physical interaction between Rai1p and Kap123p.

**Rai1p interacts with Rat1p and assembling 60S ribosomal subunits.** To determine the physical interactions made by Rai1p, an Rai1p-pA chimera was generated and affinity purified from whole-cell lysates using IgG-Sepharose. These experiments yielded a single major protein of approximately 120 kDa, which was identified as Rat1p by tandem mass spectrometry of excised gel slices (Fig. 2). This is in agreement with the data from Johnson’s group, which also established a physical interaction between Rai1p and Rat1p (58). In those studies, ∆rail strains were shown to accumulate 27A3 precursors and Rat1p activity was more robust in the presence of interacting Rai1p, suggesting that Rai1p and Rat1p function together in the trimming of the rRNA species to the 27S8s precursor (58). In agreement with this function for Rai1p, ∆rail and rat1-1 are synthetically lethal (Fig. 2). Interestingly, we also noted that Rai1p-GFP was mislocalized to the cytoplasm in rat1-1 cells (Fig. 3). One scenario to explain this interesting result would be that in the absence of a fully functional Rat1p, Rai1p is no longer effectively tethered to Rai1p in the nucleus and has become free to move within the nucleoplasm and, perhaps, exit the nucleus.

One potential mechanism for this movement and exit may be through an interaction with assembling ribosomes. We therefore tested for such an interaction. Ribosomes, polysomes, and ribosomal subunits were resolved by linear sucrose gradient centrifugation from a Rai1p-pA-expressing strain (RAI1-A) and fractions from the gradient were probed for Rai1p-pA. Under these conditions, Rai1p-pA was found only in the load fraction (data not shown). This suggested that if Rai1p interacts with ribosomal particles, it is either not stable under these conditions or occurs at levels below the level of detection. We therefore sought to increase the concentration of 60S subunits in the nucleus by inhibiting 60S subunit export. To accomplish this, a mutant version of Nmd3p was expressed in RAI1-A cells. Nmd3p was recently identified as an adaptor protein that, through its binding to both the 60S subunit protein rpL10 and the karyopherin Crm1p, mediates the Ran-dependent export of the pre-60S particle from the nucleus (20, 26). It has also been shown that deletion of the C-terminal 100 amino acids of Nmd3p inhibits ribosomal subunit export in a dominant negative fashion (26). Thus, polysomes were fractionated from RAI1-A cells expressing nmd3ΔA100 from a plasmid and the presence of Rai1p-pA in each fraction was determined by Western blotting. A monoclonal antibody to wild-type rpL3 (TCM1) was used to identify the 60S, 80S, and polysome fractions. Under these conditions, Rai1p-pA was detected in association with 60S (precursor) subunits (Fig. 4), but not with 40S subunits or polysome fractions. Moreover, affinity purification of Rai1p-pA from lysates derived from cells expressing nmd3ΔA100 also showed association of the 60S subunit marker protein rpL3 with the Rai1p chimera (Fig. 4). Further examination of this Rai1p-pA-immunopurified fraction by mass spectrometry demonstrated that several ribosomal proteins associated with Rai1p-pA under these conditions. This included...
22 60S ribosomal subunit proteins and 8 (early associating) 40S subunit proteins from a total of 33 identifications (Fig. 4B). In addition to Rat1p, also associated with the fraction were Las1p and Grc3p (14, 16); however, the potential roles of these proteins in ribosome assembly remain to be investigated.

The number of 60S subunit proteins identified by mass spectrometry suggests that pre-60S particles associate with Rai1p. The source of the relatively few 40S proteins identified is unclear. In proteomics approaches to identify components of purified subunit precursors, small numbers of ribosomal proteins from other subunits are commonly detected. In the study here, this may be because Rai1p can interact with the 60S particles as well as 80S particles, through the 60S subunit. Alternatively, as suggested by Harnipicharnchai et al. (24), some 40S subunit proteins may associate with 60S precursor particles. Interestingly, six of the eight small subunit proteins identified here were also identified in association with 66S precursor particles by Harnipicharnchai et al. (24).

Ribosome assembly defects in rai1/kap123 cells. Together, the above data support and extend previous studies demonstrating a role for Rai1p in 60S ribosomal subunit biogenesis; however, they fail to explain the specific genetic interaction observed between rai1 and kap123. Northern blotting of rRNA was used to identify precursors that accumulate in rai1 and kap123 strains (Fig. 5). As expected, rai1 strains accumulated 35S and 27S rRNA species (27SA2 and 27SA3). Interestingly, these precursors accumulated slightly more in sf17 and Δkap123/Δrai1 cells. In addition, Δrai1, sf17, and Δkap123/Δrai1 cells accumulated 7S precursors and a fragment that corresponds to A17-E. The A17-E fragment is proposed to accumulate if the precise order of processing is disrupted (35) and may require Rat1p and/or Rai1p for trimming to 5.8S. On the other hand, the presence of 7S precursors suggests that exosome function is compromised in the absence of Rat1p, but remains intact in the absence of Kap123p. Interestingly, there was also a detectable increase in the 20S signal in cells lacking Kap123p, suggesting a weak 40S biogenesis defect. Together, these data suggest that the loss of Kap123p marginally alters both the 60S and 40S biogenesis pathways. However, because the rRNA processing defect in Δrai1 cells was not dramatically exacerbated or altered by the additional loss of Kap123p, it is not likely that the genetic interaction between kap123 and rai1 is due to a catastrophic block in either the 40S or 60S assembly pathway.

The ribosome and polysome profiles from each relevant strain were also examined (Fig. 6). In agreement with the results of Northern blotting analysis, Δrai1 cells also had a paucity of assembled 60S subunits. This was revealed by an overall decrease in the mature 60S/40S subunit ratio and by the accumulation of halfmers, small shoulders on 80S and polysome peaks which result from kinetically stalled 40S subunits that have threaded onto mRNA but are unable to initiate translation due to a lack of cytoplasmic 60S subunits. In contrast, cells lacking Kap123p showed relatively normal polysome profiles. Interestingly, free 40S subunits and halfmers did not accumulate to the same extent in Δkap123 cells as they did in Δkap123 cells, suggesting that the 40S/60S subunit ratio was somehow normalized in Δkap123 cells. This “nor-
FIG. 4. Rai1p interacts with 60S ribosomal subunits. (A) 60S ribosomal subunits were accumulated in the nucleus by expression of a plasmid encoding the nmd3Δ100 mutant allele in RAI1-A cells. Under these conditions, Rai1p-pA cofractionated with the 60S ribosomal subunit on sucrose gradients, as determined by immunoblotting the collected fractions to detect the pA tag. Detection of rpL3 served as an internal control to detect 60S particles. The top panel shows the corresponding polysome profile detected by spectrophotometry (OD254) of the fractionated sucrose gradient. (B) Top: Rai1p-pA immunopurifications were performed from wild-type cells and cells expressing nmd3Δ100. Proteins bound to Rai1p-pA were eluted with MgCl2 at the concentrations shown. The resulting fractions were immunoblotted to reveal the 60S marker rpL3, which remained associated with Rai1p-pA in cells expressing nmd3Δ100. Bottom: Rai1p-pA copurified with a number of 60S ribosomal subunit proteins from cells expressing the nmd3Δ100 allele. Proteins contained within the 1 M MgCl2 elution fraction were sequenced by tandem mass spectrometry, leading to the identification of 22 large and 8 small subunit proteins from a total of 33 identifications. Only proteins identified by two or more unique polypeptide matches were considered significant.
malizing effect of KAP123 deletion was confirmed by analysis of the 40S/60S subunit ratios (data not shown). Nevertheless, Δkap123/Δraii cells, like Δraii cells, showed a dramatic decrease in the total ribosomal content (Fig. 6). These data are consistent with the loss of Kap123p leading to an overall decrease in ribosome biogenesis efficiency, which is not specific to either subunit but upstream of Rai1p activity. Because Kap123p interacts with many early and late assembling ribosomal proteins, the observed epistasis likely reflects Kap123p’s role in the import of many proteins required for optimal ribosome assembly, perhaps including early assembly factors.

60S subunit export is impaired in rai1/kap123 cells. To further investigate this specific Δraii/Δkap123 genetic interaction, we examined the localization of GFP fusions of three large subunit ribosomal proteins. It has been shown previously that carboxy-terminal GFP tags of some 60S ribosomal subunit proteins are faithfully integrated into functional ribosomes (27, 52); therefore, plasmids encoding C-terminal GFP chimeras of rpL2, rpL3, and rpL25 were constructed. When expressed in wild-type cells, each chimera cofractionated with 60S subunits in 7-to-42% sucrose gradients and yielded diffuse cytoplasmic GFP signals, thereby indicating that each of these fusions was integrated into 60S subunits (Fig. 7). While GFP-chimera reporters showed normal cytoplasmic distributions in Δrai1 or Δkap123 cells, double null Δkap123/Δraii strains exhibited a striking nuclear accumulation of the GFP signals from each 60S subunit reporter (Fig. 7). The ratio of nuclear to cytoplasmic 60S particles was quantified by isolating nuclei and comparing the abundance of 60S particles in the nuclear and cytoplasmic fractions by sucrose density gradient centrifugation under low Mg2+ conditions (Fig. 8). In addition, the presence of rpL3 in the nuclear and cytoplasmic fractions was assayed by Western blotting (Fig. 8). Taken together, these data suggest that the reporter proteins accumulated in the nucleus are assembled into pre-60S particles. Thus, Δkap123/Δraii cells exhibit a 60S, postassembly, nuclear export block that was not observed in cells harboring either mutation alone.

Nmd3p is a multicopy suppressor of sf17. The data presented above suggest that the genetic interaction between Δkap123 and Δraii is manifested at the late stages of 60S assembly. Because the only known role for Kap123p is in nuclear import, we hypothesized that an essential factor required for driving the export process was not imported efficiently in Δkap123 cells and that, in the absence of Rai1p, this factor becomes limiting. To investigate this possibility, sf17 cells were transformed with multicopy plasmids encoding two essential proteins required late in the 60S subunit assembly and that export Nmd3p (15, 26) and rpL10p (20) and the karyopherins Kap121p and Nmd5p. Interestingly, overexpression of Nmd3p, but not rpL10, Kap121p, or Nmd5p, was sufficient to rescue both the sectoring phenotype (data not shown) and the FOA sensitivity of sf17 cells (Fig. 9).

To determine if the nuclear localization of Nmd3p was altered as a result of loss of Kap123p, we assayed Nmd3p nuclear import in Δkap123 cells. Nmd3p is known to have one NLS and two NES sequences (20, 26). Galactose-inducible Nmd3p-GFP chimeras, either containing or lacking an NES (Nmd3-NLS-GFP and Nmd3-NLS/NEs-GFP), were expressed in kap123

FIG. 5. Northern analysis of rRNA processing. Normal rRNA processing is disrupted in strains lacking Rai1p and Kap123p. Equal quantities of total RNA from each strain were separated on a 1% agarose gel or a 10% Tris-borate-EDTA–urea gel in the case of short fragments and hybridized with the oligonucleotide probes listed in Materials and Methods. The position of each probe and the RNA species it is designed to detect are shown at the right.
and kap95 mutant strains (26). As shown in Fig. 9, Nmd3-NLS-GFP was nuclear in wild-type cells, but mislocalized to the cytoplasm in Δkap123 cells. In contrast, the reporter remained nuclear in kap95 cells, even after temperature shifts that have previously been shown to mislocalize classical NLS reporters (37).

These results suggest that, in addition to the previously reported role in the import of several ribosomal proteins, Kap123p also imports Nmd3p, an essential late-acting assembly and export factor. The presence of a fraction of cells with some nuclear staining, along with cytoplasmic staining in the Δkap123 background, suggests that Nmd3p, like other essential Kap123p cargoes, can enter the nucleus by alternate means, albeit with reduced efficiency. Under normal conditions, the import defect associated with the loss of Kap123p alone does not appear to significantly compromise ribosome biogenesis. However, in the absence of Ral1p, the limited nuclear quantities of late-acting factors such as Nmd3p are not sufficient to support efficient export, leading to the accumulation of partly assembled 60S subunits in the nucleus and a growth defect that greatly exceeds that observed for either deletion alone.

DISCUSSION

Here, we used a synthetic fitness genetic screen to identify components that support Kap123p function and, when mutated, render cells dependent on the presence of Kap123p. Not surprisingly, this screen yielded several karyopherins: Kap121p, Sxm1p/Kap108p, and Nmd5p/Kap119p. Indeed, Kap121p has previously been shown to compensate for the loss of Kap123p, likely by importing several ribosomal proteins in its absence (46, 48). In addition, Sxm1p/Kap108p has previously been shown to interact physically with the ribosomal proteins rpl11A/B, rpl25, and rpl31A/B (42), and overproduction of this karyopherin suppresses a kap121ts mutant phenotype (41, 49), suggesting further complementarity between Kap123p, Kap121p, and Sxm1p/Kap108p. Moreover, the high degree of similarity between Sxm1p/Kap108p and Nmd5p/Kap119p (4) and the identification of NMD5 as genetically interacting with KAP123 suggest a similar and likely overlapping function for this karyopherin. Interestingly, the use of several karyopherins to import different ribosomal proteins by yeast cells seems to be conserved in metazoans. Numerous vertebrate karyopherins, including Kap β/importin β, Kap β2/transportin, RanBP5, RanBP7, and importin 11, have been implicated in ribosomal protein import (22). Furthermore, a metazoan Kap123p orthologue, importin β4, has recently been characterized (29). Thus, data from different systems suggest that ribosomal protein import and assembly makes use of a variety of import (and export) karyopherins and that their functional overlap may permit the loss of individual factors. However, it remains unclear to what extent these karyopherins functionally overlap under normal conditions and whether this multiple redundancy is exploited by cells to globally control the transport of classes of different molecules.

Beyond redundant import pathways, the genetic screen revealed an interaction between the ribosome assembly factor Rai1p and KAP123. In an attempt to understand the molecular bases for this interaction, we investigated how the loss of both proteins specifically affected the process of ribosome biogenesis. As shown previously, cells lacking Rai1p revealed a 60S assembly defect, accumulating 27S rRNA and halfmers in polysome profiles. Remarkably, the loss of Kap123p had little effect on ribosome assembly or export, as we detected no obvious rRNA accumulation or ribosomal subunit export defects in cells lacking Kap123p, but cells lacking both Rai1p and Kap123p displayed a more complex phenotype. These cells showed a moderate augmentation of the 27S rRNA defect, normalization of the 40S/60S ratio, an overall decrease in the number of ribosomes, and an accumulation of assembled pre-60S subunits in the nucleus. Furthermore, although other karyopherins import ribosomal proteins, the genetic interaction between Rai1p and Kap123p was specific: out of the five yeast Kaps tested, including all those known to import ribosomal proteins, only KAP123 was able to rescue sf17 cells and only kap123/rai1 mutants displayed pre-60S ribosomal subunit export defects. Together these data suggest that, in these cells, the 60S biogenesis program was attenuated at a late, preassembly, preexport step.
FIG. 7. Cells lacking both Kap123p and Rai1p are defective in 60S subunit export. (A) The faithful integration of GFP reporters into ribosomal particles was confirmed by sucrose gradient centrifugation and subsequent immunoblotting against GFP-tagged reporters and endogenous rpL3/Tcm1p as an internal control. Shown is the profile for rpL2-GFP in an otherwise wild-type background, which is present in 60S, 80S, and polysome fractions, but not the 40S peak. rpL3-GFP and rpL25-GFP profiles were similar (data not shown). (B) Localization of ribosomal-GFP reporters and Nop1-GFP was determined by direct fluorescence microscopy of live cells. Fluorescent signal was detectable throughout the cell in wild-type (WT), Δkap123, and Δrai1 cells but appeared to accumulate in the nucleus and in some cases to the nucleolus in Δkap123/Δrai1 cells. Bar, 5 μm.
It is evident that cells lacking Rai1p are defective in 60S assembly, but why should the additional loss of Kap123p, whose only known function is in nuclear import, specifically cause an export defect? Consider ribosome assembly as a simple series of chemical reactions; the removal of products at each step contributes to the progression of the entire pathway. Alternatively, the failure to remove products at any step causes the accumulation of intermediates. Thus, considering that Rai1p interacts physically with Rat1p, a late-acting exonuclease, and that Rai1p was detected associated with assembled pre-60S subunits, we hypothesized that the protein(s) required at the late stages of biogenesis is not imported efficiently in kapat123 strains and that, in combination with a mutation in RAII, products downstream of Rai1p function were not efficiently processed to the next step, leading to the accumulation of (partially) assembled subunits. Thus, we speculate that the specific genetic interaction observed between KAP123 and RAII is due to a reduced efficiency of 60S subunit assembly, contributed by a lack Rai1p function, as well as an inability to import critical ribosomal assembly and export factors. Here we show that one such factor is Nmd3p. Overexpression of NMD3 rescued the slow-growth phenotype observed in sf17 (rail1-1/ kap123), rail1/rail1, and rail1/kap123 cells (data not shown), and direct visualization of an Nmd3p-GFP chimera demonstrated that efficient Nmd3p import into the nucleus requires Kap123p. While the mislocalization of Nmd3p is evident in kapat123 cells, deletion of NMD3 is lethal; thus, it is likely that other factors can also import Nmd3p in the absence of Kap123p. Furthermore, it is also likely that inefficient import of other factors contributes to the genetic interaction observed here. One such candidate is rpl10, also a late-acting assembly and export factor for 60S subunits imported by Kap123p (20). Nevertheless, because NMD3, but not RPL10, expression is sufficient to suppress the growth defects associated with rail1/kap123 cells, it is apparent that Nmd3p mislocalization is a primary cause of the rail1/kap123 genetic interaction.

Surprisingly, among the mutants assayed, only rail1-1 cells mislocalized Rai1p-GFP from the nucleus to the cytoplasm. Considering the tight in vitro binding between Rat1p and Rai1p, we propose that the steady-state localization of Rai1p to the nucleus is a result of its interaction with Rat1p. Furthermore, it is interesting to speculate that Rai1p may be used by Rat1p to tether the assembling subunit, but that, in the absence of a functional Rat1p, Rai1p may exit the nucleus with the ribosomal subunit. It has previously been shown that loss of active Rat1p can be complemented by directing Xrn1p, a normally cytoplasmic exonuclease, to the nucleus (30). It is not yet known if quality control mechanisms exist to prevent incompletely assembled ribosomal subunits from exiting the nucleus, but it seems possible that under conditions where Rai1p becomes cytoplasmic the subsequent maturation of unprocessed rRNA could occur in the cytoplasm, under the direction of Xrn1p. It will be interesting to determine if the function of Xrn1p is also augmented by Rai1p and if the export defect observed here also results from an active quality control mechanism.

The synthetic fitness screen employed here revealed a complex genetic interaction between KAP123, a nuclear import factor, and RAII, a ribosome biogenesis factor, which manifests itself in a ribosome subunit export defect. The data support a model where a cause of the defect is an inability to import sufficient quantities of the essential export factor Nmd3p to overcome the loss of Rai1p. It is particularly intriguing to speculate that the coordination of the late steps of 60S assembly...
biogenesis and nuclear export involve a direct link between Ral1p and Nmd3p, perhaps during the loading of ribosomes with Nmd3p. However, this remains to be investigated. The findings presented here underscore the integration of ribosome assembly and nucleo-cytoplasmic exchange; however, a good understanding of the entanglement between these two pathways demands further identification and characterization of ribosome assembly factors and an understanding of their relationships with the nuclear import-export apparatus.

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