Retrograde movement of tRNAs from the cytoplasm to the nucleus in *Saccharomyces cerevisiae*

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In eukaryotes, tRNAs transcribed in the nucleus function in cytoplasmic protein synthesis. The Ran-GTP-binding exportin, Los1p/Xpo-t, and additional pathway(s) mediate tRNA transport to the cytoplasm. Although tRNA movement was thought to be unidirectional, recent reports that yeast precursor tRNA splicing occurs in the cytoplasm, whereas fully spliced tRNAs can reside in the nucleus, require that either the precursor tRNA splicing machinery or mature tRNAs move from the cytoplasm to the nucleus. Our data argue against the first possibility and strongly support the second. Combining heterokaryon analysis with fluorescence *in situ* hybridization, we show that a foreign tRNA encoded by one nucleus can move from the cytoplasm to a second nucleus that does not encode the tRNA. We also discovered nuclear accumulation of endogenous cytoplasmic tRNAs in haploid yeast cells in response to nutritional deprivation. Nuclear accumulation of cytoplasmic tRNA requires Ran and the Mtr10/Kap111 member of the importin-β family. Retrograde tRNA nuclear import may provide a novel mechanism to regulate gene expression in eukaryotes.

amino acid deprivation | heterokaryon | Mtr10 | tRNA nuclear import | yeast

Eukaryotic precursor tRNAs (pretRNAs) contain 5′ and 3′ extra sequences and, for ≈25% of *Saccharomyces cerevisiae* (yeast) tRNA families, introns. To be fully functional, pretRNAs are subject to several maturation steps, including removal of the extra sequences, numerous nucleoside modifications, and addition of the 3′ terminal CCA nucleotides (1). In vertebrates, nearly all tRNA posttranscriptional processing occurs in the nucleus before export of tRNAs to the cytoplasm (1–3). In contrast, several lines of evidence show that in yeast, pretRNA intron removal occurs in the cytoplasm, not the nucleoplasm. First, the subunits of the heterotetrameric tRNA-specific splicing endonuclease, Sen15, Sen2, Sen34, and Sen54, colocalize with mitochondria (4, 5). Tethering of a noncatalytic subunit, Sen54, to mitochondria does not inhibit pretRNA splicing, whereas mutations prohibiting its mitochondrial association cause accumulation of unspliced pretRNAs (4). Moreover, cells with conditional mutations in a gene encoding a catalytic subunit, Sen2, accumulate unspliced pretRNAs in the cytoplasm (4). A previous report employing immunofluorescence and immune electron microscopy concluded that a large fraction of tRNA ligase, catalyzing second step of pretRNA splicing, resides in the nucleus (6); however, more recent reports show that there is a functional pool of tRNA ligase activity in the cytoplasm (7) and that carboxyl GFP-tagged ligase is cytosolic (5). Finally, a carboxyl GFP-tagged version of the enzyme catalyzing the third step of pretRNA splicing, removal of the residual 2′ phosphate (8), is distributed throughout the cytoplasm (5).

Splicing of pretRNAs in the cytoplasm provides an explanation for the pretRNA splicing defects observed when there are mutations in the tRNA nuclear export machinery, including the tRNA exportin, Los1p (9–11), the Ran cycle (12), or nuclear pore components (13), because pretRNAs retained in the nucleus would be unable to access the cytoplasmic splicing endonuclease. However, cytoplasmic tRNA splicing is not readily consistent with the other observations that mutations of genes encoding aminoacyl-tRNA synthetases, *tys1-1* and *ils1-1*, or the CCA adding enzyme, *cca1-1*, the inhibition of isoleucyl-tRNA synthetase, or amino acid deprivation result in nuclear pools of spliced, mature tRNAs (refs. 14–16 and M. L. Whitney and A.K.H., unpublished results). If tRNA transport is unidirectional, nuclear accumulation of spliced tRNA is in direct conflict with the data showing that pretRNA splicing occurs solely in the cytoplasm.

Two scenarios can explain why defects in the tRNA nuclear export machinery result in nuclear pools of unspliced pretRNAs, whereas defects in tRNA aminoacylation result in nuclear pools of spliced tRNAs. Either there is pretRNA splicing in the nucleus under the conditions that spliced tRNA resides in the nucleus, possibly due to shuttling of the splicing endonuclease complex to the nucleus, or tRNA must be able to relocate from the cytoplasm to the nucleus. We provide results that argue against nuclear pools of splicing endonuclease and present evidence supporting tRNA retrograde movement from the cytoplasm to the nucleus.

Materials and Methods

Strains and Media. The following yeast strains were used: SS330 (*MATa ade2-101 his3Δ200 tyr1 ura3-52*), MS739 (*MATα ura3-52 leu2-3*, *112 ade2-101 kar1-1*; provided by M. Rose, Princeton University, Princeton), BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*), and derivatives of BY4741 possessing Kan^r replacements for endogenous genes encoding importin-β family members (17) (Open Biosystems, Huntsville, AL). Because the strain lacking *MTR10* is not included in the yeast deletion collection, it was constructed in the BY4741 background by gene replacement, as described in ref. 18. American Type Culture Collection 201388 [*MATa SEN2-GFPHIS3MX6*::*his3Δ leu2Δ0 met15Δ0 ura3Δ0* (5)] was obtained from Invitrogen. EE1b-35 (*MATa RNA1 rnh1*::*URA3 ura3-52 ade1 tyr1 his7 his4* Gal$^-$) and EE1b-6 (*MATa rna1-1 rnh1*::*URA3 ura3-52 ade1 tyr1 his7 his4* Gal$^-$) containing *URA3*-tagged *RNA1* alleles are described in ref. 19. Strain MS739B (*MATα los1*::*Nat^r ura3-52 leu2-3*, *112 ade2-101 kar1-1*) was derived from MS739 by replacing *LOS1* with a PCR-generated *los1*::*Nat^r* cassette as described in ref. 20. Likewise, MS739-35 (*MATα RNA1 rnh1*::*URA ura3-52 leu2-3*,*-112 ade2-101 kar1-1*) and MS739-6 (*MATα rna1-1 rnh1*::*URA3 ura3-52 leu2-3*, *112 ade2-101 kar1-1*) were created by gene replacement of the endogenous *RNA1* locus with previously constructed *URA3*-tagged *RNA1* or *rna1-1* cassettes (19). Strain HHS1 was generated by crossing ts2-6c [*MATα ade2-101 his3Δ200 tyr1 ura3-52 tys1-1* (14)] with American Type Culture Collection 201388 followed by tetrad analysis to obtain a strain with both *tys1-1* and *SEN2-GFPHIS3MX6*::*his3Δ* alleles.

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Abbreviation: pretRNA, precursor tRNA.

See Commentary on page 11127.

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Plasmids. *Dictyostelium discoideum* $tRNA^{Glu}$ vector: pUF^{-}7 (encoding the *D. discoideum* tRNA suppressor, $tRNA^{Glu}$ under tetracycline-dependent expression) and pGalTR1 (encoding the Tet repressor) were obtained from T. Dingermann (Institut für Pharmazeutische Biologie Biozentrum, Frankfurt) (21). The $tRNA^{Glu-D}$ and TetR encoding genes were transferred to single low copy centromere-containing vectors, pRS416 (*URA3*-containing) and pRS415 (*LEU2*-containing) (22), generating pRS416$tRNA^{Glu-D}$ and pRS415$tRNA^{Glu-D}$, respectively. $tRNA^{Glu-D}$ is constitutively expressed from these plasmids when cells are grown in media with glucose as the carbon source.

Heterokaryon Analyses. Heterokayon analysis of tRNA movement was performed as follows: MS739 or MS739B cells, defective in nuclear karyogamy (*kar1-1*), were mated to *KAR1* strains BY4741 or BY4741*los1*. One or the other of the mating partners harbored pRS416$tRNA^{Glu-D}$. Equal numbers of cells of the two strains from log-phase liquid cultures were mixed and then immobilized by collection onto 0.45-μm nitrocellulose membranes (Millipore). Cells were allowed to mate at 30°C by placing the membranes on rich solid medium for ≈2 h. Heterokaryon zygotes then were diluted in liquid medium and incubated for 0–4 h. To examine the role of the Ran cycle in tRNA nuclear import strains MS739-35 (relevant genotype: *kar1-1 RNA1*) and MS739-6 (relevant genotype: *kar1-1 rna1-1*) carrying pRS415$tRNA^{Glu-D}$ were mated at 24°C with strains EE1b-35 (relevant genotype: *KAR1 RNA1*) or EE1b-6 (relevant genotype: *KAR1 rna1-1*), respectively. Zygotes were harvested and incubated in liquid rich medium for 25 min at the nonpermissive temperature for *rna1-1* (37°C). Half of the heterokaryon zygotes were maintained in rich medium, and the other half were transferred to medium lacking amino acids. Cultures were incubated further for 90 min at 37°C before fluorescence *in situ* hybridization (FISH).

FISH. Published procedures and probes were used to monitor $tRNA^{Tyr}$ and poly(A)-containing RNA (10). FISH02, CCAGTGTTAGAGACTAGAGTGTACCGACTACACCAATGA, was used to detect $tRNA^{Glu-D}$; $tRNA^{His}$ detection used oligonucleotide FISH-$tRNA^{His}$, TCCTAGAATCGAACCAGGGTTTCATCGGCCACAACGATGTGTACTAACCACTATACTAAG.

Microscopy. Fluorescence images for FISH studies were observed by using a Nikon Microphot-FX microscope. Images were captured by using a SenSys charge-coupled device camera (Photometrics, Tucson, AZ) using QED software (QED Imaging, Pittsburgh). Because the signal for Sen2-GFP was weak, we used a Nikon Eclipse E1000 microscope, capturing the images with an Orca Extended Range charge-coupled device camera (Hamamatsu) using IMAGE PRO PLUS software (Version 4.1, Media Cybernetics, Silver Spring, MD). Images were arranged with PHOTOSHOP 5.0 (Adobe Systems, San Jose, CA).

Results

To test whether the tRNA-splicing endonuclease complex might be nuclear under particular conditions, we compared the subcellular location of one of the catalytic subunits in cells grown in rich medium with its location in cells depleted for amino acids or in cells with a temperature-sensitive tyrosyl-tRNA synthetase, Tys1-1. We used a version of *SEN2* that is tagged at the C terminus with GFP (*SEN2-GFP*) and replaces the endogenous gene (5). Whereas free GFP is located diffusely throughout the cytoplasm, Sen2-GFP predominately colocalizes with mitochondria (5) (Fig. 1), consistent with the previously determined mitochondrial location of the tRNA-splicing endonuclease complex (4).

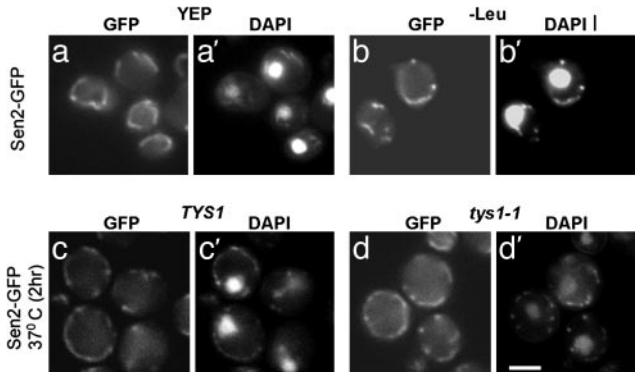

Fig. 1. Location of Sen2-GFP under conditions in which mature tRNAs accumulate in the nucleus. (*a* and *a′*) American Type Culture Collection strain 201388 possessing the chromosomally located *SEN2-GFP* allele was grown at 24°C on rich medium, then suspended in 1 μg/ml DAPI in H_2O. (*b* and *b′*) American Type Culture Collection 201388 in medium lacking leucine for 2 h at 24°C. (*c* and *c′*) American Type Culture Collection 201388 cells (*TYS1 SEN2-GFP*) shifted to 37°C for 2 h. (*d* and *d′*) HHS1 (*tys1-1 SEN2-GFP*) cells shifted to 37°C for 2 h. (*a*–*d*) GFP signal. (*a′*–*d′*) DAPI signal for mitochondrial and nuclear DNA. (Bar: 5 μm.)

Cells were incubated for 2 h at 24°C on rich medium or on medium lacking a single amino acid essential for the growth of this strain (leucine; Fig. 1) or all amino acids (data not shown). Deprivation of amino acids causes nuclear accumulation of mature tRNAs (ref. 16; also see below). Regardless of amino acid availability, no Sen2-GFP nucleoplasmic or nuclear peripheral-associated signals were detected (Fig. 1 *a* and *b*). The location of Sen2-GFP in *TYS1* wild-type (WT) and *tys1-1* mutant cells also was compared. Sen2-GFP could not be detected in nuclei of WT or mutant cells after incubation for 1–2 h at the *tys1-1* nonpermissive temperature (37°C) (Fig. 1 *c* and *d*), conditions that cause nuclear accumulation of spliced tRNAs in *tys1-1* cells (ref. 14; M. L. Whitney and A.K.H., unpublished results). Thus, we could find no evidence for nuclear pools of tRNA-splicing endonuclease under conditions that cause spliced tRNAs to accumulate in the nucleus.

If accumulation of spliced tRNAs in the nucleus is unlikely to be explained by movement of the splicing endonuclease from the cytoplasm to the nucleus, then the nuclear pools might result from movement of spliced tRNAs from the cytoplasm to the nucleus. To test this rather unorthodox possibility, we used a modified heterokaryon assay. In brief, a strain harboring the *kar1-1* mutation that prevents nuclear fusion in zygotes (for a review, see ref. 23) is mated with a strain without this mutation (*KAR1*) to generate a zygote with two nuclei within a shared cytoplasm. We used FISH to assess whether a tRNA encoded by one nucleus can move from the shared cytoplasm to a nucleus that does not encode this tRNA.

First, it was necessary to identify a reporter tRNA expressed by only one nucleus of a heterokaryon. Because each yeast tRNA family is essential, it was not feasible to delete endogenous tRNA genes from one of the parental strains. Therefore, we used a ''foreign'' tRNA. The *D. discoideum* intronless gene encodes a UAG nonsense suppressor $tRNA^{Glu}$ ($tRNA^{Glu-D}$) that is transcribed and processed into a tRNA that participates in translation in *S. cerevisiae* (21). We expressed the $tRNA^{Glu-D}$-encoding gene from a centromere-containing plasmid, pRS416$tRNA^{Glu-D}$, that is maintained in approximately one copy per nucleus (22) to assure a low rate of exchange of the DNA encoding $tRNA^{Glu-D}$ between heterokaryon nuclei. The average number of transfers varies from ≈0.002 per nucleus for entire chromosomes (24) to ≈0.2 per nucleus for the multicopy 2μ plasmid (25). To confirm that $tRNA^{Glu-D}$ is

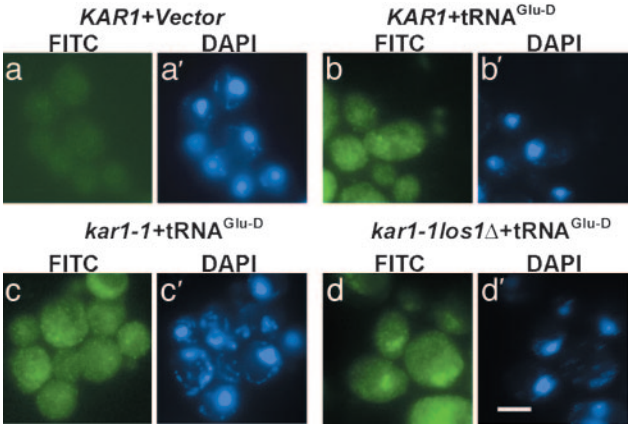

Fig. 2. Specific expression and detection of *D. discoideum* $tRNA^{Glu}$ in *S. cerevisiae*. FISH analysis by using a digoxgenin-labled $tRNA^{Glu\text{-}D}$ probe in BY4741 with vector alone (*a*), BY4741 with pRS416$tRNA^{Glu\text{-}D}$ (*b*), MS739 (*kar1-1*) with pRS416$tRNA^{Glu\text{-}D}$ (*c*), and MS739B (*kar1-1 los1*Δ) with pRS416$tRNA^{Glu\text{-}D}$ (*d*). (*a*′–*d*′) Location of nuclear DNA in the respective cells as determined by DAPI staining. (Bar: 5 μm.)

expressed in our strains and shares no sequence homology with endogenous yeast tRNAs, we performed Northern analysis. Radiolabeled probe complementary to $tRNA^{Glu\text{-}D}$ hybridizes only to RNA extracted from strains harboring the tRNA-encoding plasmid (data not shown) and is detected only in cells encoding $tRNA^{Glu\text{-}D}$ by FISH analysis (Fig. 2).

It was also necessary to establish conditions that allow detection of nuclear pools of tRNAs amidst the cytoplasmic pools as cells normally transport the vast majority of tRNA to the cytoplasm. We used two different means to generate $tRNA^{Glu\text{-}D}$ nuclear pools: (*i*) a *los1* null strain (Fig. 2*d*) [although there are one or more *los1*-independent tRNA nuclear export pathways, this strain accumulates nuclear pools of tRNAs encoded by both intron-containing and intron-free genes (10, 26)], and (*ii*) amino acid depletion [although the mechanism by which such conditions cause tRNA nuclear accumulation (16) is not completely understood, it likely results from reduced tRNA aminoacylation]. We reasoned that if $tRNA^{Glu\text{-}D}$ moved from the cytoplasm to the nucleus, movement could be monitored because *los1*Δ or amino acid deprivation would inhibit $tRNA^{Glu\text{-}D}$ nuclear export and would result in detectable nuclear pools of this tRNA.

If there is retrograde movement of tRNA from the cytoplasm to the nucleus, then tRNA encoded by only one of the nuclei of a heterokaryon should be detected in both nuclei in *los1*Δ cells or WT cells deprived of amino acids. In contrast, if tRNA movement is unidirectional, strictly nucleus to the cytoplasm, the tRNA will not be detected in the second nucleus. To distinguish between these possibilities, six types of heterokaryons were generated (Table 1). Mating *LOS1 kar1-1* strains with *LOS1 KAR1* strains generated types 1 and 2; for type 1 heterokaryons, the $tRNA^{Glu\text{-}D}$ was encoded by the *LOS1 kar1-1* haploid, whereas for type 2, it was encoded by the *LOS1 KAR1* haploid nucleus. Types 3 and 4 heterokaryons were generated by mating *los1*Δ *KAR1* with *LOS1 kar1-1* + $tRNA^{Glu\text{-}D}$ haploids and *los1*Δ *KAR1* + $tRNA^{Glu\text{-}D}$ with *LOS1 kar1-1*, respectively. Types 5 and 6 were generated by mating *los1*Δ *KAR1* with *los1*Δ *kar1-1* + $tRNA^{Glu\text{-}D}$ haploids and *los1*Δ *KAR1* + $tRNA^{Glu\text{-}D}$ with *los1*Δ *kar1-1* haploids, respectively. $tRNA^{Glu\text{-}D}$ was located by FISH immediately upon zygote formation and 2 and 4 h after zygote appearance.

Identical results were obtained for heterokaryon types 1 and 2 (Table 1 and Fig. 3*A a* and *b*, and data not shown). As anticipated, no nuclear $tRNA^{Glu\text{-}D}$ accumulation was observed at any time point when both parents possess WT *LOS1* alleles. Likewise, $tRNA^{Glu\text{-}D}$ was cytoplasmic in type 3 heterokaryons (*los1*Δ *KAR1* × *LOS1 kar1-1* + $tRNA^{Glu\text{-}D}$) at each time point (Table 1 and data not shown). This result is expected because the *LOS1* nucleus, capable of tRNA export, encodes the $tRNA^{Glu\text{-}D}$ and because *los1*Δ is recessive to *LOS1*. Identical results were obtained for type 4 heterokaryons (*los1*Δ *KAR1* + $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1*) harvested 2 and 4 h after zygote formation (Table 1, Fig. 3*A d* and *d*′ and data not shown). However, immediately upon zygote formation, ≈20% of the type 4 heterokaryons exhibited tRNA accumulation in one of the two nuclei (Table 1; see Fig. 3*A c* and *c*′ for an example). Nuclear $tRNA^{Glu\text{-}D}$ pools at this early time point likely result from a delay in providing the *los1*Δ nucleus with Los1p encoded by the *LOS1* nucleus. However, these results show that there are conditions for which the two nuclei can have different populations of tRNAs, further verifying the assay.

In striking contrast to types 1–4 matings, heterokaryons generated from matings of two *los1*Δ haploids (types 5 and 6) exhibited strong nuclear tRNA signals in both nuclei of the heterokaryons at all time points, irrespective of which nucleus encoded $tRNA^{Glu\text{-}D}$ (Table 1 and Fig. 3*A e–f*, data for type 6). In these same heterokaryons, poly(A)-containing RNA was primarily cytoplasmic (data not shown). The results support the hypothesis that tRNA encoded by one nucleus can gain access to a second nucleus and provide evidence for retrograde movement of tRNA from the cytoplasm to the nucleus.

Although the above experiments support retrograde tRNA movement, they were conducted with a mutant yeast strain with defects in tRNA nuclear export. Therefore, we assessed the effect of amino acid deprivation on $tRNA^{Glu\text{-}D}$ subcellular

Table 1. Distribution of $tRNA^{Glu\text{-}D}$ in heterokaryons

Type	Cross	*T* = 0 h	*T* = 2–4 h
1	*LOS1 KAR1* × *LOS1 kar1-1* + $tRNA^{Glu\text{-}D}$		
2	*LOS1 KAR1* + $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1*		
3	*los1ΔKAR1* × *LOS1 kar1-1* + $tRNA^{Glu\text{-}D}$		
4	*los1ΔKAR1* + $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1*	20% 80%	
5	*los1ΔKAR1* × *los1Δkar1-1* + $tRNA^{Glu\text{-}D}$		
6	*los1ΔKAR1* + $tRNA^{Glu\text{-}D}$ × *los1Δkar1-1*		

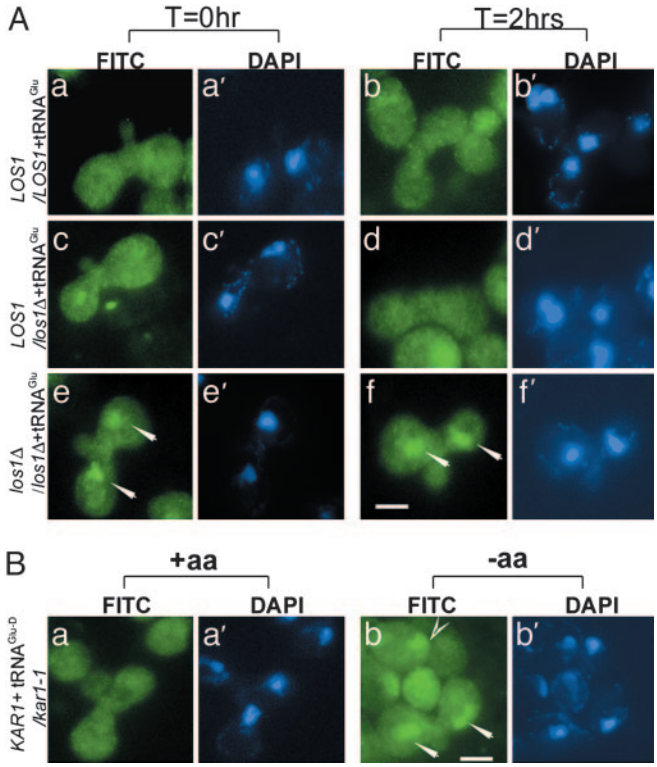

Fig. 3. FISH analysis showing $tRNA^{Glu\text{-}D}$ moves from the shared cytoplasm to the second nucleus of heterokaryon zygotes. (*A*) *los1*Δ cells. (*a*, *c*, and *e*) Cells were fixed immediately after zygote formation ($T = 0$ h) followed by FISH analysis using the $tRNA^{Glu\text{-}D}$ probe. (*a*) *LOS1 KAR1* + $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1* heterokaryons. (*c*) *los1*Δ *KAR1*+ $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1* heterokaryons. (*e*) Heterokaryon zygote generated by mating *los1*Δ *KAR1* + $tRNA^{Glu\text{-}D}$ and *los1*Δ *kar1-1* strains. (*a*′, *c*′, and *e*′) Same cells stained with DAPI to show locations of nuclei. (*b*, *d*, and *f*) Heterokaryons generated as for *a*, *c*, and *e*, fixed after growth for 2 h at 24°C and 2-h incubation at 37°C ($T = 2$ h). (*b*′, *d*′, and *f*′) Location of nuclear DNA in respective heterokaryons. Arrows point to the two nuclei of a heterokaryon, both containing $tRNA^{Glu\text{-}D}$. (Bar: 5 μm.) (*B*) Heterokaryons subjected to amino acid deprivation. (*a* and *a*′) *LOS1 KAR1* + $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1* heterokaryons incubated in rich medium at 24°C for 90 min. (*b* and *b*′) Heterokaryons of the same genotype as incubated in medium lacking amino acids at 24°C for 90 min. Filled arrows, $tRNA^{Glu\text{-}D}$ in two nuclei of a heterokaryon; open arrow, an unmated haploid accumulating $tRNA^{Glu\text{-}D}$ under amino acid deprivation. (Bar: 5 μm.)

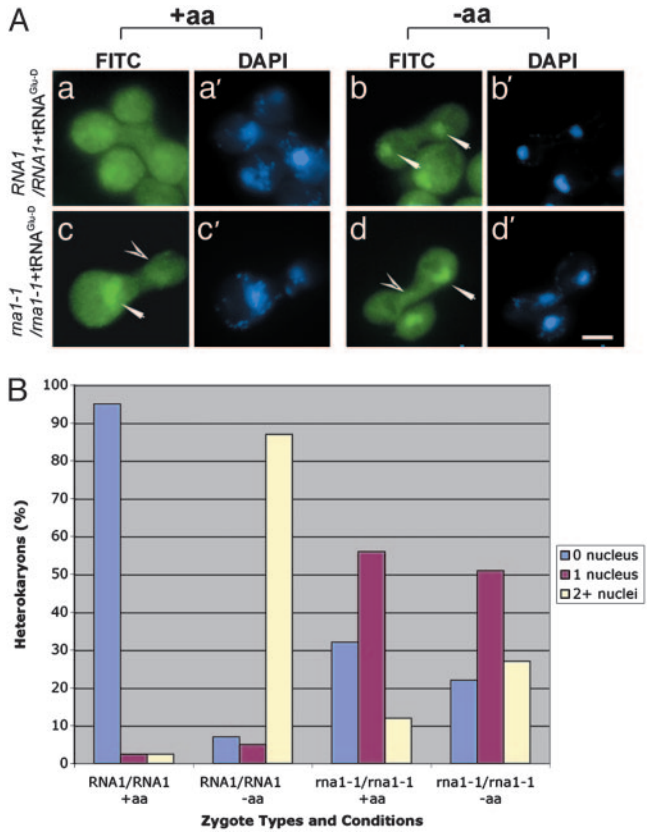

Fig. 4. Role of the Ran cycle in tRNA nuclear import. (*A*) FISH analysis to evaluate the dependence of tRNA nuclear import upon the Ran cycle. (*a* and *b*) *RNA1 KAR1* cells (EE1b-35) were mated in rich medium at 24°C with *RNA1 kar1-1* + $tRNA^{Glu\text{-}D}$ cells (MS739-35) to generate heterokaryons with two *RNA1* nuclei, only one of which encodes $tRNA^{Glu\text{-}D}$. (*c* and *d*) *rna1-1 KAR1* cells (EE1b-6) were mated with *rna1-1 kar1-1* + $tRNA^{Glu\text{-}D}$ cells (MS739-6) to generate heterokaryons with two *rna1-1* nuclei, only one of which encodes $tRNA^{Glu\text{-}D}$. After 25 min at 37°C, the culture was split, with half incubated 90 min in medium with amino acids (*a* and *c*) and half incubated in medium lacking amino acids (*b* and *d*), followed by FISH analyses. Filled arrows point to nuclei that accumulate $tRNA^{Glu\text{-}D}$; open arrows point to nuclei with little detectable $tRNA^{Glu\text{-}D}$. (Bar: 5 μm.) (*B*) Graphic display of tRNA nuclear accumulation. For each mating and condition, ≈50 heterokaryons were assessed and scored as follows: 0, none of the nuclei possessed $tRNA^{Glu\text{-}D}$; 1, one or more of the nuclei in the heterokaryon had no or little $tRNA^{Glu\text{-}D}$ signal; 2 or >2, all of the nuclei possessed $tRNA^{Glu\text{-}D}$.

location in strains with no mutations affecting tRNA nuclear export. Type 2 (*LOS1 KAR1* + $tRNA^{Glu\text{-}D}$ × *LOS1 kar1-1*) heterokaryons were incubated in rich or amino acid-depleted media for 90 min at 24°C, followed by FISH analysis. As anticipated, $tRNA^{Glu\text{-}D}$ was distributed throughout the cytoplasm for heterokaryons exposed to rich medium (Fig. 3*B a* and *a*′). Also, as anticipated, upon amino acid deprivation, approximately half of the unmated haploid cells (those harboring the tRNA-encoding plasmid) contained nuclear pools of $tRNA^{Glu\text{-}D}$ (Fig. 3*B b* and *b*′, open arrow). Remarkably, for heterokaryons derived from cells in which only one nucleus encodes $tRNA^{Glu\text{-}D}$, both nuclei accumulated this tRNA under conditions of amino acid deprivation (Fig. 3*B b* and *b*′, filled arrows; Fig. 4 provides additional examples and a tabulation of numerous heterokaryons). The data support movement of tRNA from the shared cytoplasm to the nucleus when cells are depleted for amino acids and can account for the nuclear pools of endogenous spliced tRNAs under these conditions.

Because $tRNA^{Glu\text{-}D}$ accumulates in the nucleus rather than being distributed throughout the cell, we anticipated that retrograde tRNA nuclear import may be an active process. To explore whether tRNA nuclear accumulation depends on the RanGTPase pathway used for active nucleus/cytoplasmic transport of most macromolecules, we assessed whether $tRNA^{Glu\text{-}D}$ travels retrograde from the joint cytoplasm to a nucleus that does not encode it in heterokaryons with a defective Ran cycle. The *rna1-1* mutation encodes a temperature-sensitive RanGAP that prohibits protein nuclear import both *in vivo* and *in vitro* (27), as well as tRNA nuclear export (10). We created *kar1-1 rna1-1* + pRS415$tRNA^{Glu\text{-}D}$ and *kar1-1 RNA1* + pRS415$tRNA^{Glu\text{-}D}$ isogenic strains and mated them to isogenic haploid *KAR1 rna1-1* or *KAR1 RNA1* strains, respectively. The resulting heterokaryons were shifted to 37°C (nonpermissive temperature for *rna1-1*) for 25 min and then incubated either in the presence or absence of amino acids for 90 min at 37°C before determining the subcellular distribution of $tRNA^{Glu\text{-}D}$ by FISH.

As expected, $tRNA^{Glu\text{-}D}$ was distributed throughout the cytoplasm when heterokaryons with two *RNA1* nuclei were provided with amino acids (Fig. 4*A a* and *a*′ and *B*). Even though only one nucleus encodes $tRNA^{Glu\text{-}D}$, the majority of hetero-

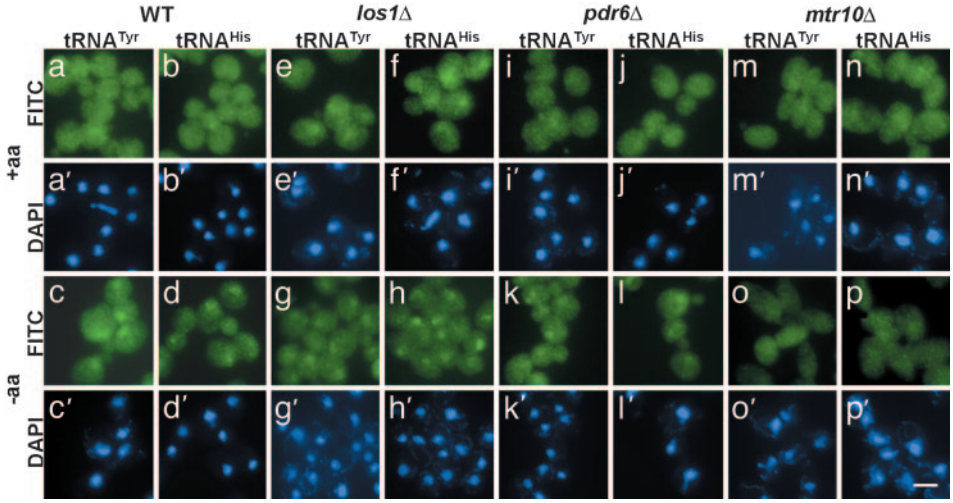

Fig. 5. Analysis of the role of importin-β family members in tRNA nuclear accumulation upon amino acid deprivation. The indicated cell cultures were grown in rich medium, and the cultures were split, with half provided with amino acids and half in medium lacking amino acids. After 2 h, the cultures were processed for FISH. (*a–d* and *a′–d′*) WT cells, treated as indicated. (*e–h* and *e′–h′*) *los1*Δ. (*i–l* and *i′–l′*) *pdr6*Δ. (*m–p* and *m′–p′*) *mtr10*Δ. (Bar: 5 μm.)

karyons contained $tRNA^{Glu\text{-}D}$ in both nuclei when they were deprived of amino acids, as expected for tRNA retrograde nuclear import in WT strains (Fig. 4 *A b* and *b′* and *B*). In contrast, for the majority of heterokaryons generated by mating two *rna1-1* strains, only one nucleus (presumably the nucleus encoding $tRNA^{Glu\text{-}D}$) possessed $tRNA^{Glu\text{-}D}$ nuclear pools in either in the presence or absence of amino acids (examples in Fig. 4*A c* and *d*; tabulated in Fig. 4*B*). This finding is the anticipated result if the Ran cycle is required for tRNA retrograde nuclear import because, at the nonpermissive temperature, newly transcribed $tRNA^{Glu\text{-}D}$ will not exit the *rna1-1* nucleus encoding it (10), and previously made $tRNA^{Glu\text{-}D}$ residing in the cytoplasm before the temperature shift would fail to move to either nucleus.

Because analysis of Ran dependence is complicated in the heterokaryon assay, we verified the results by independent means. If tRNA retrograde nuclear import is indeed Ran-dependent, then it should require 1 or more of the 14 members of the importin-β Ran-binding proteins that carry cargo into and out of the nucleus (for a review, see ref. 28). To determine whether tRNA nuclear import depends on a member of this family, we determined the consequences of deletions of genes encoding the nine unessential family members upon the subcellular distribution of endogenous tRNA in haploid cells in the presence and absence of amino acids. We anticipated that in the absence of amino acids, most of these haploid stains would possess tRNA nuclear pools generated by both newly synthesized tRNA and by tRNA imported from the cytosol. In contrast, if an importin-β family member is required for tRNA nuclear import, the nuclei should have no contribution of tRNA from the cytoplasm and therefore would have diminished tRNA pools.

For each of three independent experiments, the subcellular distributions of $tRNA^{Tyr}$ (Fig. 5), encoded by an intron-containing gene, and $tRNA^{His}$ (Fig. 5) and $tRNA^{Met}$ (data not shown), encoded by genes lacking introns, were monitored in the nine mutant strains with deletions of individual importin-β family members. As anticipated, all three tRNAs are cytoplasmic when WT haploid cells are provided with amino acids but accumulate in the nucleus when the cells are deprived of amino acids (Fig. 5 *a–d*). Also as anticipated, tRNAs accumulate in the nucleus in cells lacking the Los1 importin-β family member, both in the presence and absence of amino acids (Fig. 5 *e–h*). Of the strains lacking the remaining other eight unessential importin-β family members (*msn5*Δ/*kap142*Δ, *nmd5*Δ/*kap119*Δ, *kap114*Δ, *pdr6*Δ/*kap122*Δ, *sxm1*Δ/*kap108*Δ, *kap123*Δ, *kap120*Δ, and *mtr10*Δ/*kap111*Δ), seven accumulate nuclear pools of tRNA when deprived for amino acids (Fig. 5 *i–l*, data shown only for *pdr6*Δ). In stark contrast, cells depleted for *MTR10* fail to accumulate nuclear tRNAs in response to amino acid deprivation (Fig. 5 *m–p*). In addition to providing support for Ran dependence of tRNA retrograde nuclear import, the data extend the observations of tRNA retrograde nuclear import from a foreign tRNA in heterokaryons to endogenous tRNAs in haploid cells.

Discussion

Because tRNAs function in cytoplasmic protein synthesis, their transport to the cytoplasm is essential. However, movement of tRNAs from the cytoplasm to the nucleus was thought not to occur. Nevertheless, complex nuclear/cytoplasmic dynamics have considerable precedence for other types of RNA. For example, vertebrate U1, U2, and U5 and U4 small nuclear RNAs normally egress from the nucleus to the cytoplasm for additional modifications and assembly into RNA–protein complexes before reimport into the nucleus where the small nuclear RNAs fulfill premRNA splicing functions (for a review, see ref. 29).

We envision two different, but nonexclusive, roles for tRNA nuclear import. The first, a constitutive process, tRNA proofreading in the nucleus, is based on the proposal that cytoplasmic maturation of small nuclear RNAs serves this type of function by preventing aberrant small nuclear RNAs from accessing premRNAs (29). For tRNAs, proofreading in the nucleus would prevent faulty tRNAs from interacting with the translation machinery. Accordingly, nuclear tRNA import would be required because parts of the yeast tRNA biogenesis pathway (e.g., splicing and some modifications) are located in the cytoplasm (1, 4). The recent demonstration of a nucleus-located tRNA degradation system in yeast that recognizes aberrant tRNAs (30) provides support for this model.

A second possible role for tRNA nuclear import would be regulatory in which the nucleus serves as a reservoir for cytoplasmic tRNA under certain physiological conditions. Accordingly, upon appropriate signaling events, nuclear tRNA pools could result from either constitutive nuclear import, followed by down-regulation of tRNA nuclear reexport or by up-regulation of tRNA import to the nucleus. Most conditions that result in nuclear accumulation of spliced tRNA affect the ability of tRNA

to be aminoacylated (14–16). Even *los1*Δ causes up-regulation of Gcn4, which generally occurs when there are insufficient cellular pools of aminoacylated tRNAs (31). However, as defects in the yeast translation elongation factor, eEF-1A, responsible for delivery of aminoacylated tRNAs to ribosomes, also cause nuclear accumulation of spliced tRNAs in yeast (16), tRNA aminoacylation may not be the sole signal for nuclear accumulation of cytoplasmic tRNA. There is considerable evidence that tRNA aminoacylation in the nucleus is important for tRNA nuclear export, both in vertebrates and in yeast (2, 14–16, 18). One model consistent with the data is that uncharged tRNA or tRNA unable to interact with eEF-1A would move to the nucleus and remain there until conditions permit nuclear tRNA aminoacylation, providing a nutritional checkpoint before tRNA reexport. New tools must be generated to learn whether tRNA nuclear import is constitutive and/or regulated.

Two lines of evidence support a role for RanGTPase in tRNA nuclear import. First, when deprived for amino acids, *rna1-1* × *rna1-1* heterokaryons with a defective Ran cycle accumulate tRNA primarily in one nucleus, presumably the nucleus encoding $tRNA^{Glu\text{-}D}$, whereas cells with a normal Ran cycle accumulate this tRNA in both nuclei. However, low levels of $tRNA^{Glu\text{-}D}$ sometimes could be detected in the second nucleus of *rna1-1* × *rna1-1* heterokaryons (see Fig. 4*B* for a tally); this result may be due to failure to reexport tRNA that was constitutively imported from the cytoplasm before the temperature shift. The second line of evidence is the identification of an importin necessary for tRNA nuclear accumulation. We demonstrated that endogenous tRNAs accumulate in nuclei when WT cells or eight of nine strains lacking an unessential member of the importin-β import/export receptor family are deprived of amino acids, but tRNA nuclear accumulation does not occur when cells lacking *MTR10* are deprived of amino acids.

Mtr10 has been reported to be essential (17); however, we and others (32, 33) find that strains lacking *MTR10* grow slowly. Interestingly, Mtr10 has been previously implicated in RNA nucleus/cytoplasm distribution. It has a well-characterized role as the import receptor for the shuttling protein Npl3 involved in mRNA nuclear export (32). More relevant to our studies, Mtr10 was implicated in nuclear import of the RNA component of yeast telomerase, Tlc1 (33). A role for Mtr10 in retrograde tRNA nuclear import in response to amino acid deprivation provides strong support for the idea that the process is Ran-dependent. However, because Takano *et al.* (34) report that tRNA nuclear accumulation detected in cells provided with amino acids is Ran-independent, Mtr10 and Ran could be required for signaling tRNA retrograde nuclear import rather than for the import process itself.

Although yeast is the first organism shown to have capability for tRNA movement from the cytoplasm to the nucleus, the recent demonstration of intron-containing $pretRNA^{Tyr}$ in nuclei of *Arabidopsis* with a deleted exportin-t homologue, *psd* (35), could mean that for *Arabidopsis*, as for *S. cerevisiae*, tRNA splicing occurs in the cytoplasm. It will be interesting to learn the subcellular distribution of the plant tRNA splicing endonuclease complex and whether other eukaryotes are capable of retrograde tRNA nuclear import.

Note. While this work was under review, Takano *et al.* (34) also reported tRNA retrograde nuclear import by using a different tRNA reporter expressed in *los1*Δ *KAR1* × *los1*Δ *kar1-1* heterokaryons.

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