Molecular Biology of the Cell
Vol. 21, 639–649, February 15, 2010

Regulation of tRNA Bidirectional Nuclear-Cytoplasmic Trafficking in *Saccharomyces cerevisiae*

Athulaprabha Murthi,*†‡ Hussam H. Shaheen,*†§ Hsiao-Yun Huang,*‖ Melanie A. Preston,¶ Tsung-Po Lai,* Eric M. Phizicky,¶ and Anita K. Hopper*#

*Department of Molecular Genetics, ‖Graduate Program in Molecular, Cellular, and Developmental Biology, #Center for RNA Biology, The Ohio State University, Columbus, OH 43210; and ¶Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642

Submitted July 7, 2009; Revised December 3, 2009; Accepted December 10, 2009
Monitoring Editor: Karsten Weis

tRNAs in yeast and vertebrate cells move bidirectionally and reversibly between the nucleus and the cytoplasm. We investigated roles of members of the β-importin family in tRNA subcellular dynamics. Retrograde import of tRNA into the nucleus is dependent, directly or indirectly, upon Mtr10. tRNA nuclear export utilizes at least two members of the β-importin family. The β-importins involved in nuclear export have shared and exclusive functions. Los1 functions in both the tRNA primary export and the tRNA reexport processes. Msn5 is unable to export tRNAs in the primary round of export if the tRNAs are encoded by intron-containing genes, and for these tRNAs Msn5 functions primarily in their reexport to the cytoplasm. The data support a model in which tRNA retrograde import to the nucleus is a constitutive process; in contrast, reexport of the imported tRNAs back to the cytoplasm is regulated by the availability of nutrients to cells and by tRNA aminoacylation in the nucleus. Finally, we implicate Tef1, the yeast orthologue of translation elongation factor eEF1A, in the tRNA reexport process and show that its subcellular distribution between the nucleus and cytoplasm is dependent upon Mtr10 and Msn5.

INTRODUCTION

Classically, the nucleus has been considered the cellular site for RNA production and the cytoplasm the site to convert the information encoded in RNA into proteins through the process of translation. Accordingly, mRNAs, rRNAs, and tRNAs would be born in the nucleus followed by their unidirectional transport to the cytoplasm for function. However, it is now clear that numerous RNAs move bidirectionally between the nucleus and the cytoplasm (for reviews see Hopper, 2006; Hopper and Shaheen, 2008). This work concerns tRNA nuclear-cytoplasmic dynamics in the yeast, *Saccharomyces cerevisiae*.

tRNA processing machineries are relatively conserved from Archea to vertebrates, but the subcellular distribution of the processing activities differs among organisms. In vertebrate cells, initial tRNA transcripts, if encoded by intron-containing genes, are first spliced and then processed at 5′ and 3′ termini, followed by their export to the cytoplasm (Lund and Dahlberg, 1998). tRNA nuclear export in vertebrate cells proceeds via the Ran pathway employing a member of the β-importin family, Exportin-t (Exp-t; Arts *et al.*, 1998a; Kutay *et al.*, 1998). Exp-t binds end-matured appropriately structured tRNAs and has similar affinities for intron-containing and intron-free tRNAs (Arts *et al.*, 1998b; Lipowsky *et al.*, 1999). The yeast orthologue of Exp-t, Los1, functions in tRNA nuclear export (Hellmuth *et al.*, 1998; Sarkar and Hopper, 1998). However, in contrast to vertebrates, in *S. cerevisiae* the tRNA splicing endonuclease is located on the cytoplasmic surface of mitochondria (Yoshihisa *et al.*, 2003). Therefore, 5′ and 3′ end processing in the nucleus precedes splicing, which follows tRNA nuclear export to the cytoplasm.

It was predicted that additional tRNA nuclear pathways exist in yeast because *LOS1* is an unessential gene and *los1*Δ mutants have a relatively normal growth phenotype (Hurt *et al.*, 1987). *LOS1* homologues in *Schizosaccharomyces pombe* (for reviews see Hopper and Shaheen, 2008) and plants (Hunter *et al.*, 2003) are also unessential, indicating that parallel tRNA nuclear export pathways may be widespread in nature. The β-importin family member Exportin-5 (yeast Msn5) has been implicated in tRNA nuclear export (Bohnsack *et al.*, 2002; Calado *et al.*, 2002; Takano *et al.*, 2005), in addition to its known functions in nuclear export of microRNAs (miRNA) in metazoans and nuclear export of particular phosphorylated nuclear-cytoplasmic shuttling proteins in yeast (Kaffman *et al.*, 1998; Yi *et al.*, 2003; Lund *et al.*, 2004; Zeng and Cullen, 2004; for review see Hopper, 1999). Exportin-5 binds short double-stranded RNA structures (Gwizdek *et al.*, 2003; Zeng and Cullen, 2004) and tRNAs in a RanGTP-dependent mechanism (Bohnsack *et al.*, 2002; Calado *et al.*, 2002; Shibata *et al.*, 2006); however, it is not thought to serve as the major tRNA exporter in vertebrates as Exp-t fulfills this role. Inhibition of Exp-t causes marked reduction of tRNA nuclear export (Arts *et al.*, 1998b; Lipowsky *et al.*, 1999), whereas knockdown of Exportin-5 has little effect on tRNA levels (Shibata *et al.*, 2006). Moreover, because complexes of Exportin-5 with aminoacylated-tRNA (aa-tRNA)

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–07–0551) on December 23, 2009.

† These authors contributed equally to this work.

Present addresses: ‡ National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892; § Merck and Co., Glycofi Department; 21 Lafayette Street, Suite 200, Lebanon, NH 03766.

Address correspondence to: Anita K. Hopper (hopper.64@osu.edu).

and RanGTP interact with translation elongation factor 1A (eEF1A; Tef1 and Tef2 in yeast), Exportin-5 has been proposed to serve as a mechanism to rid the nucleus of inadvertent pools of eEF1A that accumulate upon reassembly of nuclei after open mitosis (Bohnsack *et al.*, 2002). Similarly, the plant Exportin-5 orthologue HASTY appears not to affect tRNA levels (Park *et al.*, 2005). In contrast, for *Drosophila* which lacks an Exp-t homologue, Exportin-5 has been reported to serve for both miRNA and tRNA nuclear export (Shibata *et al.*, 2006 and references therein). The yeast orthologue, Msn5, has been shown to bind tRNA (Shibata *et al.*, 2006) and *los1*Δ *msn5*Δ double mutants accumulate more tRNA in the nucleus than cells with either single mutation (Takano *et al.*, 2005). In sum, Exportin-5 and its orthologues appear to have a significant role in tRNA nuclear export in some, but not all, organisms.

Previous data document that tRNA move bidirectionally in yeast—from the cytoplasm to the nucleus as well as from the nucleus to the cytoplasm (Shaheen and Hopper, 2005; Takano *et al.*, 2005). When deprived of nutrients, transcription of new tRNA is down-regulated (Ciesla *et al.*, 2007) and tRNAs that once resided in the cytoplasm accumulate in the nucleus via this tRNA retrograde process (Shaheen and Hopper, 2005; Hurto *et al.*, 2007; Whitney *et al.*, 2007). On reintroduction of nutrients, tRNAs harbored in the nucleus are once again exported to the cytoplasm by a process referred to as tRNA reexport (Whitney *et al.*, 2007). tRNA retrograde nuclear import and reexport occur in a similar manner in yeast and vertebrate cells (Shaheen *et al.*, 2007) and lentiviruses, such as HIV, appear to have usurped the tRNA retrograde import machinery to deliver the reverse transcription complex through nuclear pores in nondividing neuronal cells (Zaitseva *et al.*, 2006).

Here we investigate the regulation of tRNA import and reexport processes in yeast and the roles of importin-β members in the various steps. We report that tRNA nuclear import is a constitutive process and that the nuclear-cytoplasmic distribution of tRNAs is regulated by the ability of tRNAs to be reexported from the nucleus to the cytoplasm. We show that for tRNAs encoded by intron-containing genes, Msn5 serves a specialized role in tRNA reexport. In contrast, Los1 functions in both the primary tRNA nuclear export and the reexport processes for these tRNAs. Finally, we show that the subcellular distribution between the nucleus and the cytoplasm of translation elongation factor Tef1/2, whose function is required for efficient tRNA nuclear export (Grosshans *et al.*, 2000; McGuire and Mangroo, 2007), is dependent on Msn5 and Mtr10, as is the tRNA retrograde pathway.

MATERIALS AND METHODS

Strains and Media

Escherichia coli DH5α and SL-1 blue were used for propagation of recombinant DNA plasmids and was maintained in YT media with appropriate antibiotics. Most experiments used yeast strains BY4741 (*MATa his3*Δ *leu2*Δ *met15*Δ *ura3*Δ) and BY4742 (*MAT*α *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ). BY4741 and BY4742 are the parents to the deletion collections (Winzeler *et al.*, 1999; Open Biosystems, Huntsville, AL) possessing Kan^r replacements for endogenous genes encoding importin-β family members and *trm7*Δ. 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 Azad *et al.* (2001). To create *msn5*Δ *tef1*Δ and *los1*Δ *tef1*Δ strains, the endogenous *TEF1* ORFs in *msn5*Δ and *los1*Δ strains were deleted by gene replacement. BY4741 is also the parent for the collection of endogenously expressed Tef1-green fluorescent protein (GFP) yeast strains (Huh *et al.*, 2003; Invitrogen, Carlsbad, CA). *TEF1-GFP los1*Δ, *TEF1-GFP msn5*Δ, and *TEF2-GFP msn5*Δ strains were generated from the *TEF1-GFP* and *TEF2-GFP* strains by gene replacement. Yeast strain MS739 (*MAT*α *ura3-52 leu2-3, 112 ade2-101 kar1-1*) was provided by M. Rose (Princeton University).

Yeast strains were maintained on rich (YEPD) media with or without the antibiotic G418 (0.2 mg/ml) or on synthetic complete (SC) medium lacking appropriate nutritional ingredients for maintaining plasmids. For nutrient deprivation studies, SC medium lacking all amino acids was used (SC-aa). For studies employing galactose-inducible constructs, cells were grown in SC media with raffinose as the carbon source. At a cell density of OD_{600} = 0.4, fusion protein expression was induced for 2 h by addition of galactose to a final concentration of 2%. Cells were collected, resuspended in medium containing glucose as the sole carbon source, and further incubated for 1.5 h, in either SC or SC-aa medium.

Plasmids

PCR reactions were carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA).

DNAs were ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA). pIGout-NLS-(73-151)-Trm7-GFP and Nup49-mCherry were constructed as described (Lai *et al.*, 2009). pTef1-MORF was obtained from a collection of tagged yeast genes (Gelperin *et al.*, 2005).

Heterokaryon Analysis

Heterokaryon analysis was carried out essentially as described previously (Feng and Hopper, 2002). In brief, BY4741 cells harboring the plasmid encoding NLS-(73-151)-Trm7-GFP or control plasmids pGAL-H2B-GFP (Peng and Hopper, 2002) or pGAL-NLS-Cca1-GFP (Feng and Hopper, 2002) were grown overnight to a density of ~1.0×10^7 cells/ml in medium containing 2% raffinose. Galactose was added (final concentration, 2%), and the cells were grown for an additional 2 h. Induced cells were then washed twice and resuspended in medium containing 2% glucose and grown for 1 to 1.5 h. For most experiments, matings were initiated by mixing 3×10^6 cells with an equal number of MS739 cells by concentrating the cells on a 25-mm diameter, 0.45-μm pore size nitrocellulose filter and placing the filter on YEPD plates. After 1–2 h, cells were washed off the membrane and spotted on a slide for observation. DAPI (1 μg/ml) was used to locate DNA in the cells.

Microscopy

Epifluorescence imaging was accomplished using a Nikon Microscope Eclipse 90i (Nikon, Melville, NY) equipped with a CoolSNAP HQ^2 CCD camera (Photometrics, Tucson, AZ) and METAMORF Software (Molecular Devices, Sunnyvale, CA) or a Nikon Microphot-FX microscope equipped with a Sensys charge-coupled device camera (Photometrics) and QED software (QED Imaging, Pittsburgh, PA). To view cells by confocal microscopy, live cells were placed on a slide containing a thin layer of SC medium lacking leucine with 20% gelatin and 0.1 mM *n*-propylgallate and sealed under a coverslip with Valap as previously described (Wu *et al.*, 2006); the cells were viewed using 488- and 568-nm argon ion lasers employing a spinning disk apparatus (UltraView, PerkinElmer Life and Analytical Science, Waltham, MA), a 100×/1.4 NA objective lens (Nikon), and a cooled charged coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Maximum intensity projections of images were created using UltraView ERS software and image analyses of single optical 0.4-μm optical sections were performed using Image J (http://rsb.info.nih.gov/ij/). Adobe Photoshop was used for image assembly (San Jose, CA).

tRNA Purification and Analysis of Modified Nucleosides

$tRNA^{Phe}$ was purified from total yeast RNA using a 5′-biotinylated oligonucleotide (Integrated DNA Technologies, Coralville, IA) complementary to $tRNA^{Phe}$ as previously described (Jackman *et al.*, 2003). Binding of the biotinylated oligonucleotide to streptavidin (SA) magnetic particles (Roche, Indianapolis, IN) was performed according to the manufacturer's instructions. $tRNA^{Phe}$ was purified from 1 mg of total cellular RNA using 2 mg of bound beads (Tsurui *et al.*, 1994). Eluted tRNA was centrifuged at 13,000 rpm for 2 min to remove any residual beads and desalted/concentrated by centrifugation using a Centricon YM-10 (Amicon, Beverly, MA) with successive concentration and dilution into ddH_2O. About 10–15 μg of purified tRNA was recovered per milligram bulk low-molecular-weight RNA.

Purified $tRNA^{Phe}$ (1.25 μg) was treated with P1 nuclease in buffer containing 20 mM sodium acetate, pH 5.2, and 0.2 mM $ZnCl_2$ at 37°C for 2 h and then with 1 U of calf intestinal alkaline phosphatase (Roche) in 1× alkaline phosphatase reaction buffer (Roche) at 37°C for 1 h. The resulting nucleosides were resolved by HPLC (Waters [Milford, MA] Alliance Model 2690, equipped with Waters 996 photodiode array detector) using a reverse phase C18 column (supelcosil LC-18-T, 25 cm × 4.6 μm; Supelco, Bellefonte, PA) as described (Xing *et al.*, 2004). Individual spectra of the nucleosides were used to confirm the assignments. Quantification of modified nucleosides was carried out by measuring the area under each nucleoside peak at its known maximum absorbance. The areas of the main nucleosides (C, U, G, and A) were used to determine the total moles of tRNA in each injection by dividing the area observed by the known extinction coefficient for each nucleoside and then normalizing for the expected number of the tRNA nucleoside being analyzed. The moles of modified nucleotide in each injection were determined by dividing the area by the known extinction coefficient for each modified

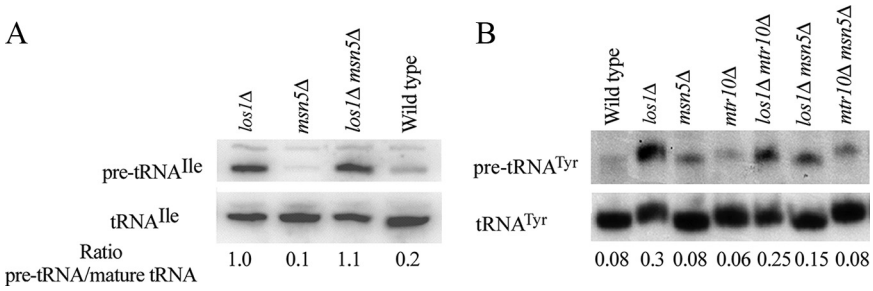

Figure 1. Los1 and Msn5 have different roles in tRNA nuclear export. (A) Northern analysis of pre-tRNA^{Ile} and mature tRNA^{Ile} in wild-type and mutant cells. (B) Northern analysis of pre-tRNA^{Tyr} and mature tRNA^{Tyr} in wild-type and mutant cells.

nucleoside and compared with the total moles of tRNA to obtain the ratio of modified nucleoside/tRNA.

In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed as previously described (Sarkar and Hopper, 1998) with the modifications detailed in (Stanford *et al.*, 2004). Each slide contained positive and negative controls for tRNA nuclear accumulation. All critical experiments were independently scored by at least two people, one of whom was unaware of the experimental details.

Northern Analysis

Low-molecular-weight RNAs were extracted from yeast cultures grown to densities similar to those used for FISH as described (Hopper *et al.*, 1980). Samples (5 μg) were electrophoretically separated at 4°C in 10% polyacrylamide gel containing 8 M urea and 1× TBE (0.09 M Tris, 0.09 M borate, 0.001 M EDTA). RNAs were electrophoretically transferred to Hybond N+ membranes (Amersham Pharmacia, Piscataway, NJ) using a Hoefer TE42 Transfer apparatus (Hoefer Scientific, San Francisco, CA) with 1× TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA). Northern analysis was conducted as previously described (Hurto *et al.*, 2007). The relative levels of precursor and mature tRNAs were determined using a Typhoon Trio Variable Mode Imager (GE Healthcare Bio-Sciences, Piscataway, NJ) and ImageQuant TL software (Molecular Dynamics).

Western Analysis

Tef1-MORF expression was assessed using chemiluminescence-based Western blot analysis following standard protocols. Yeast strains harboring the Tef1-MORF plasmid (Gelperin *et al.*, 2005) were cultured in SC lacking uracil with raffinose as the carbon source. Tef1-MORF was induced by addition of galactose (2% final concentration) to the cultures for 1 h. Protein extracts were prepared using a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% SDS, 0.5% Triton X-100, 20 mM EDTA, 2 mM PMSF, and 1× proteinase inhibitor cocktail. Aliquots of the extracts were resolved on a 10% polyacrylamide gel and were transferred to a nitrocellulose membrane using a semidry transfer device. The membrane was probed with anti-EF1α (clone CBP-KK1, Millipore, Billerica, MA) at a 1:2000 dilution. HRP-conjugated sheep anti-mouse IgG (GE Healthcare) was used at a 1:3000 dilution.

Immunofluorescence

Procedures were conducted as previously described (Tolerico *et al.*, 1999). We used a tagged version of Tef1 (Tef1-MORF; Gelperin *et al.*, 2005) and anti-HA to located Tef1 in yeast cells. After induction of Tef1-MORF by addition of galactose (2% final concentration) for 2 h to cultures grown with 2% raffinose, Tef1-MORF was located in cells by employing mouse monoclonal anti-HA (12CA5, Roche) at a 1:500 dilution. FITC-conjugated goat anti-mouse IgG (Jackson ImunoResearch Laboratories, West Grove, PA) was used at a 1:400 dilution to locate the primary antibody. Cells were counterstained with DAPI (0.1 μg/ml) to locate DNA.

RESULTS

Msn5 and Los1 Function in Reexport of tRNAs from the Nucleus to the Cytoplasm

Because the yeast tRNA splicing endonuclease is located on the cytoplasmic surface of mitochondria (Yoshihisa *et al.*, 2003), a defect in the export of end-matured intron-containing pre-tRNA from the nucleus to the cytoplasm will result in inhibition of pre-tRNA splicing because the tRNA substrate and the splicing endonuclease are in two different subcellular locations. In fact, the components of the tRNA nuclear export machinery, Los1 and Rna1, were discovered by accumulation of end-matured, intron-containing pre-tRNAs in *los1-1*and *rna1-1* mutant cells (Hopper *et al.*, 1978; Hopper *et al.*, 1980) rather than by their defects in tRNA subcellular dynamics. Msn5 has been implicated in tRNA nuclear export; however, in contrast to *rna1-1* and *los1*Δ mutations, deletion of *MSN5* does not result in the accumulation of intron-containing pre-tRNA^{Ile}UAU (Figure 1A), pre-tRNA^{Tyr} (Figure 1B), pre-tRNA^{Phe} (not shown), or pre-tRNA^{Leu}CAA (not shown). One interpretation of these results is that Msn5 plays only a minor role in tRNA nuclear export, as has been suggested for its orthologue, Exportin-5, in vertebrate cells (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). However, results from FISH studies are not consistent with this interpretation. As expected, in wild-type cells neither tRNA^{His}, encoded by genes lacking introns, nor tRNA^{Tyr}, encoded by intron-containing genes, accumulate in nuclei (Figure 2A, 1 and 2). Also, as expected, there are nuclear pools of both of these tRNA species in *los1*Δ cells (Figure 2A, 3 and 4). Significantly, tRNA^{His} and tRNA^{Tyr} accumulate in nuclei of *msn5*Δ cells (Figure 2A, 5 and 6). Thus, *msn5*Δ cells have defective tRNA nuclear-cytoplasmic distribution, despite the fact that pre-tRNA splicing is not affected by deletion of *MSN5*.

One plausible reason why there are nuclear pools of spliced tRNAs in *msn5*Δ cells is that the nuclear tRNAs in these cells previously resided in the cytoplasm. In this case, in *msn5*Δ cells, intron-containing pre-tRNAs would have been exported to the cytoplasm via Los1 (and perhaps additional pathways), spliced on the surface of mitochondria, and imported into the nucleus by the tRNA retrograde process, but then reexported to the cytoplasm inefficiently. According to this scenario, Msn5 would be involved in the tRNA reexport process rather than the primary export process, at least for tRNAs encoded by intron-containing genes.

To test the idea that Msn5 functions in tRNA reexport, we evaluated pre-tRNA intron removal and tRNA subcellular distribution in *mtr10*Δ *msn5*Δ and *mtr10*Δ *los1*Δ double mutants under fed and nutrient-deprived conditions. Mtr10, a β-importin family member, directly or indirectly, functions in the retrograde movement of tRNA from the cytoplasm to the nucleus because nuclear accumulation of cytoplasmic tRNA upon nutrient deprivation is dependent on Mtr10 (Shaheen and Hopper, 2005; Hurto *et al.*, 2007; Whitney *et al.*, 2007). If Msn5 functions downstream of Mtr10 in the tRNA reexport process, then in a mutant background in which retrograde import fails to occur (i.e., *mtr10*Δ), there should be greatly reduced nuclear pools of tRNA upon nutrient deprivation whether or not Msn5 is functional. On the other hand, if Msn5 functions upstream of Mtr10, then the phenotype of *msn5*Δ *mtr10*Δ double mutants should resemble *msn5*Δ alone.

Cells with a *MTR10* deletion do not accumulate elevated levels of unspliced tRNA, whereas *los1*Δ *mtr10*Δ cells do

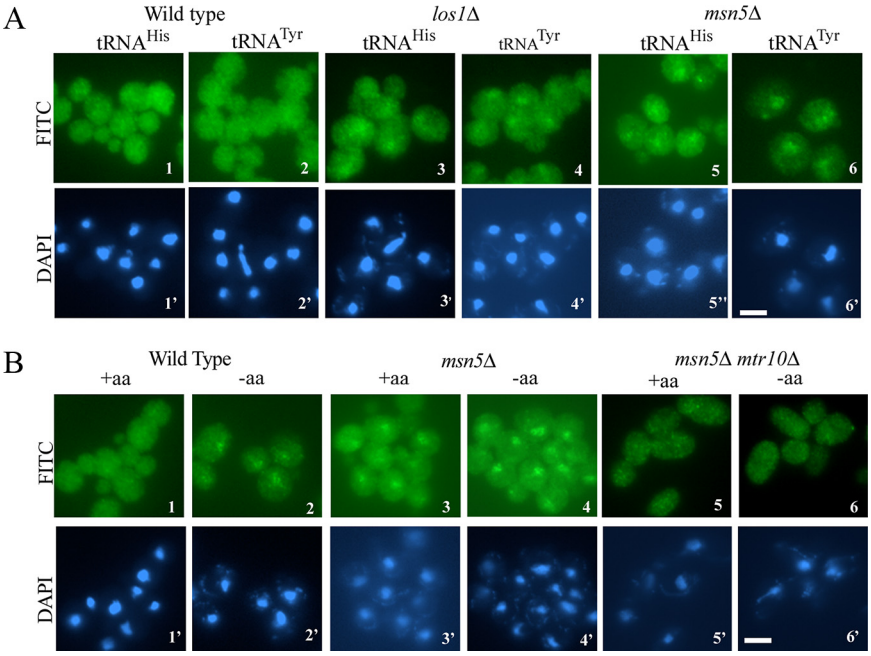

Figure 2. FISH analysis of the role of Msn5 in tRNA nuclear-cytoplasmic dynamics. (A) Locations of $tRNA^{His}$ and $tRNA^{Tyr}$ in wild-type (1 and 2), *los1*Δ (3 and 4), and *msn5*Δ (5 and 6) cells. 1–6, FISH signal; 1′–6′, same cells stained with DAPI. (B) FISH analysis of the location of $tRNA^{Tyr}$ in wild-type (1 and 2), *msn5*Δ (3 and 4), and *mtr10*Δ *msn5*Δ (5 and 6) cells in nutrient-rich (1, 3, and 5) and amino acid–deprived (2, 4, and 6) conditions. 1–6, FISH signal; 1′–6′, same cells stained with DAPI. White bar, 5 μm.

(Figure 1B); thus *los1*Δ is epistatic to *mtr10*Δ, as expected when the primary round of tRNA nuclear export is defective in *los1*Δ cells. Cells with deletions of both *MTR10* and *MSN5,* in contrast, do not accumulate greater levels of intron-containing pre-tRNAs than do wild-type cells (Figure 1B), again indicating that Msn5 does not function in primary export of intron-containing pre-tRNAs to the cytoplasm. Although *msn5*Δ mutants have nuclear pools of $tRNA^{Tyr}$, *msn5*Δ *mtr10*Δ double mutant cells fail to accumulate nuclear pools of tRNA regardless of nutrient availability (Figure 2B). The data support the idea that Msn5 functions downstream of Mtr10 in reexport of tRNAs that were spliced in the cytoplasm and subsequently reimported into the nucleus.

Previous studies showed that Exp-t binds end-matured intron-containing and intron-free tRNAs with similar efficiencies (Arts *et al.,* 1998b; Lipowsky *et al.,* 1999). If this is also the case for yeast Los1, then for tRNAs encoded by intron-containing genes, Los1 should function in their primary export pathway, transporting end-matured intron-containing pre-tRNAs from the nucleus to the cytoplasm as well as for their retrograde reexport, transporting mature tRNAs processed in the cytoplasm, and imported into nuclei back to the cytoplasm. Similarly, for tRNAs encoded by intron-free tRNA genes, Los1 should bind newly synthesized end-matured tRNAs as well as the population of tRNAs that have been imported from the cytoplasm. Thus, Los1 is expected to function in both types of tRNA nuclear export: initial export of newly synthesized end-matured tRNA and reexport of mature tRNAs retrograde imported from cytoplasmic pools.

If Los1 indeed functions in the tRNA reexport process as well as in the initial round of tRNA nuclear export, in cells deficient for both tRNA retrograde import (*mtr10*Δ) and primary/reexport steps (*los1*Δ) there might be a significant decrease in the level of nuclear tRNAs compared with cells deficient in Los1 alone. To test this, we compared the accumulation of nuclear pools of tRNA in wild-type, *los1*Δ, *mtr10*Δ, and *los1*Δ *mtr10*Δ double mutants. As expected, in wild-type cells both $tRNA^{Tyr}$ (Figure 3A), encoded by intron-containing tRNA genes, and $tRNA^{His}$ (Figure 3B), encoded by intron-free tRNA genes, are primarily cytoplasmic when cells are satiated (Figure 3, A and B, 1 and 1′), but exhibit significant nuclear pools when cells have been deprived of amino acids (Figure 3 A and B, 2 and 2′). As previously reported, cells lacking Los1 accumulate nuclear tRNA pools of both $tRNA^{Tyr}$ and $tRNA^{His}$ regardless of whether the cells are fed or deprived of nutrients (Figure 3, A and B, panels 3, 4, 3′, and 4′). Also, as previously reported, cells lacking Mtr10 do not have nuclear pools of tRNA regardless of the tRNA species or nutrient availability (Figure 3, A and B, 5, 6, 5′, and 6′). Consistent with the hypothesis that Los1 functions in tRNA reexport, *los1*Δ *mtr10*Δ do not possess significant nuclear pools of tRNA whether the cells are fed or nutrient deprived (Figure 3, A and B, 7, 8, 7′, and 8′). One interpretation of these results is that the nuclear pools of tRNA detected by FISH are derived primarily from cytoplasmic tRNA and that Los1 and Msn5 both participate in the tRNA reexport process. In sum, the data support a role for Los1 in both the initial tRNA export process and the reexport process, whereas, at least for tRNAs encoded by intron-containing genes, Msn5 functions primarily in the reexport process.

Regulation of the tRNA Retrograde Process and a Possible Role for Tef1 in the Reexport Step

One likely explanation for the nutrient-independent tRNA nuclear accumulation in *msn5*Δ cells is that tRNA nuclear import is constitutive, but that efficient tRNA reexport requires that cells be satiated. Constitutive tRNA nuclear import is consistent with previous studies of tRNA dynamics in heterokaryons that documented movement of tRNAs encoded by one nucleus to a nucleus not encoding the tRNA. For heterokaryons in which both nuclei possessed a *LOS1* deletion, tRNA encoded by one nucleus accumulated in all nuclei, including the nuclei not encoding the tRNA, regardless of nutrient status (Shaheen and Hopper, 2005; Takano *et al.,* 2005). Nuclear accumulation of tRNA in *msn5*Δ haploid cells and in *los1*Δ heterokayarons under fed conditions is consistent with the idea that tRNA movement from the cytoplasm to the nucleus is a constitutive process and that

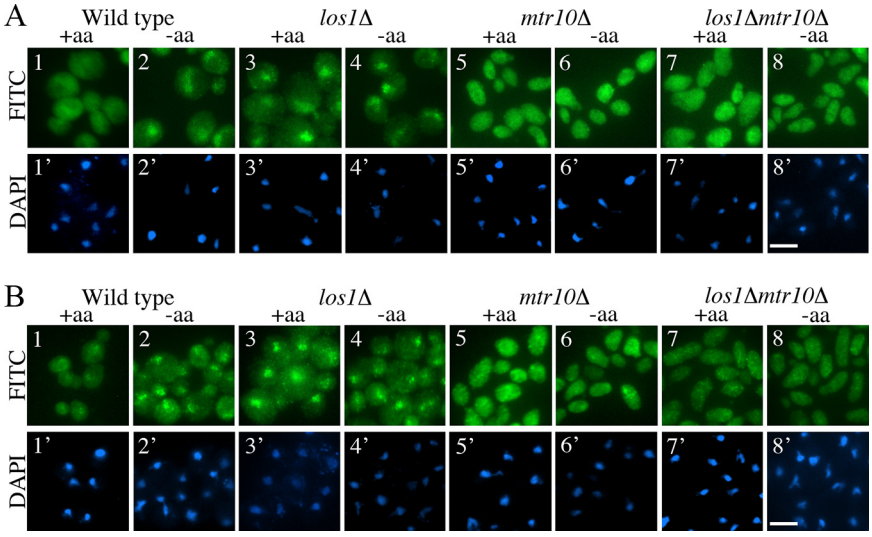

Figure 3. Los1 and Msn5 function in the tRNA reexport process. FISH analysis for $tRNA^{Tyr}$ (A) and $tRNA^{His}$ (B) in wild-type fed (panels 1) or amino acid deprived (panels 2), *los1*Δ (panels 3 and 4), *mtr10*Δ (panels 5 and 6), and *los1*Δ *mtr10*Δ (panels 7 and 8) cells. 1–8, location of the tRNAs; 1′–8′, the same cells stained with DAPI. White bar, 5 μm

tRNA nuclear accumulation results from defects in the tRNA reexport step. We developed an assay to determine whether the tRNA nuclear import process is indeed constitutive.

By employing a nuclear localized tRNA modification enzyme that modifies only spliced tRNA substrates we sought to determine whether mature, spliced tRNAs are constitutively imported into the nucleus. Some tRNA modifications (e.g., Cm_{32}, Gm_{18}, Gm_{34}, m^1G_{37} and i^6A_{37}) occur only on spliced tRNAs (Etcheverry *et al.*, 1979; Nishikura and De Robertis, 1981; Jiang *et al.*, 1997). Because pre-tRNA splicing is a cytoplasmic process, the only way for tRNAs encoded by intron-containing genes to acquire a modification that is catalyzed by a nucleus-localized, splicing-dependent enzyme is for the tRNAs to be exported to the cytoplasm and then imported back into the nucleus (see Figure 4A). Trm7 is a cytoplasmic unessential splicing-dependent modification enzyme that catalyzes 2′-O-methylation of C_{32} and G_{34} (Cm_{32} and Gm_{34}); Grosjean *et al.*, 1997; Jiang *et al.*, 1997; Pintard *et al.*, 2002). Deletion of *TRM7* does not affect tRNA processing or subcellular distribution (Supplementary Figure S1). If Trm7 were relocated from its normal cytoplasmic location to the nucleus, then, for a tRNA encoded by an intron-containing gene, Cm_{32} or Gm_{34} modification could monitor retrograde movement. If tRNAs were imported into the nucleus only when cells are deprived of nutrients, then Cm_{32} and Gm_{34} modifications would only occur upon nutrient deprivation. In contrast, if tRNA moves constitutively between the nucleus and the cytoplasm, then tRNAs would possess the modifications whether or not the cells are deprived of nutrients (Figure 4A).

Two prerequisites must be met for this strategy to work. First, Trm7 must be targeted and tethered in the nucleus so that there is no detectable activity in the cytoplasm and, second, Trm7 must remain catalytically active. We recently reported that Trm7-GFP provided with a nuclear localization sequence (NLS) and a peptide region (amino acids 73-151) from yeast Trm1 results in the relocation of Trm7 to the inner nuclear membrane (INM; Lai *et al.*, 2009). When this modified fusion protein, NLS-(73-151)-Trm7-GFP, is expressed in wild-type or *trm7*Δ cells it is located as one or a few spots on the INM in fed and nutrient-deprived cells (Figure 4B). Although the steady-state location of NLS-(73-151)-Trm7-GFP is nuclear, it is formally possible that this protein can transit between the nucleus and the cytoplasm. To assure that the NLS-(73-151)-Trm7-GFP is maintained in the nucleus, we used a heterokaryon assay. Cells encoding a galactose-regulated NLS-(73-151)-Trm7-GFP were mated to a yeast strain with the karyogamy deficient mutation, *kar1-1* (Vallen *et al.*, 1992), and we monitored whether NLS-(73-151)-Trm7-GFP could accumulate in a nucleus that does not encode this protein. Proteins were induced for 2 h by addition of galactose, and the cells were then transferred to glucose-containing medium to repress further protein expression. After 1.5 h, the cells were mated with the *kar1-1* strain. Heterokaryons were allowed to form, and then the locations of the proteins were assessed. The shuttling protein NLS-Cca1-GFP (Feng and Hopper, 2002) and the nonshuttling nuclear protein histone H2B-GFP (Peng and Hopper, 2002) provided controls. NLS-Cca1-GFP was located in both nuclei of heterokaryons (Figure 4C), as anticipated for a nuclear-cytoplasmic shuttling protein. In contrast, H2B-GFP was located in only single nuclei of heterokaryons (Figure 4C), as expected for a nonshuttling protein. NLS-(73-151)-Trm7-GFP, like H2B-GFP, located in single nuclei of heterokaryons (Figure 4C), demonstrating that NLS-(73-151)-Trm7-GFP does not shuttle between the nucleus and cytoplasm. The data also indicate that it is unlikely that there were cytoplasmic pools of NLS-(73-151)-Trm7-GFP that were too dilute to detect in wild-type and *trm7*Δ haploid cells because such pools should have generated a GFP signal in heterokaryon nuclei that did not encode this protein.

To determine whether tRNA nuclear import is constitutive, Cm_{32} modification of tRNA was assessed in tRNA isolated from satiated and amino acid–deprived wild-type and *trm7*Δ cells possessing NLS-(73-151)-Trm7-GFP or vector alone. Because Trm7 modifies C_{32} of tRNAs encoded by tRNA genes lacking introns as well as intron-containing genes (Pintard *et al.*, 2002), it was necessary to purify and analyze nucleoside modifications of a tRNA encoded by an intron-containing gene. $tRNA^{Phe}$ is modified by Trm7 at C_{32}, and it is encoded by an intron-containing gene. NLS-(73-151)-Trm7-GFP synthesis was induced for 2 h by addition of galactose to early log phase cells and $tRNA^{Phe}$ was purified from bulk tRNA by use of Biotin-tagged probes bound to streptavidin beads (Jackman *et al.*, 2003). After digestion to nucleosides the content of Cm_{32} was determined by HPLC (Xing *et al.*, 2004). As expected, $tRNA^{Phe}$ isolated from wild-type cells harboring the vector alone or expressing NLS-(73-151)-Trm7-GFP possessed 1 mol of Cm_{32} (1.0 observed) and 2.0 mol (1.85 observed) of the control modification m^5C per

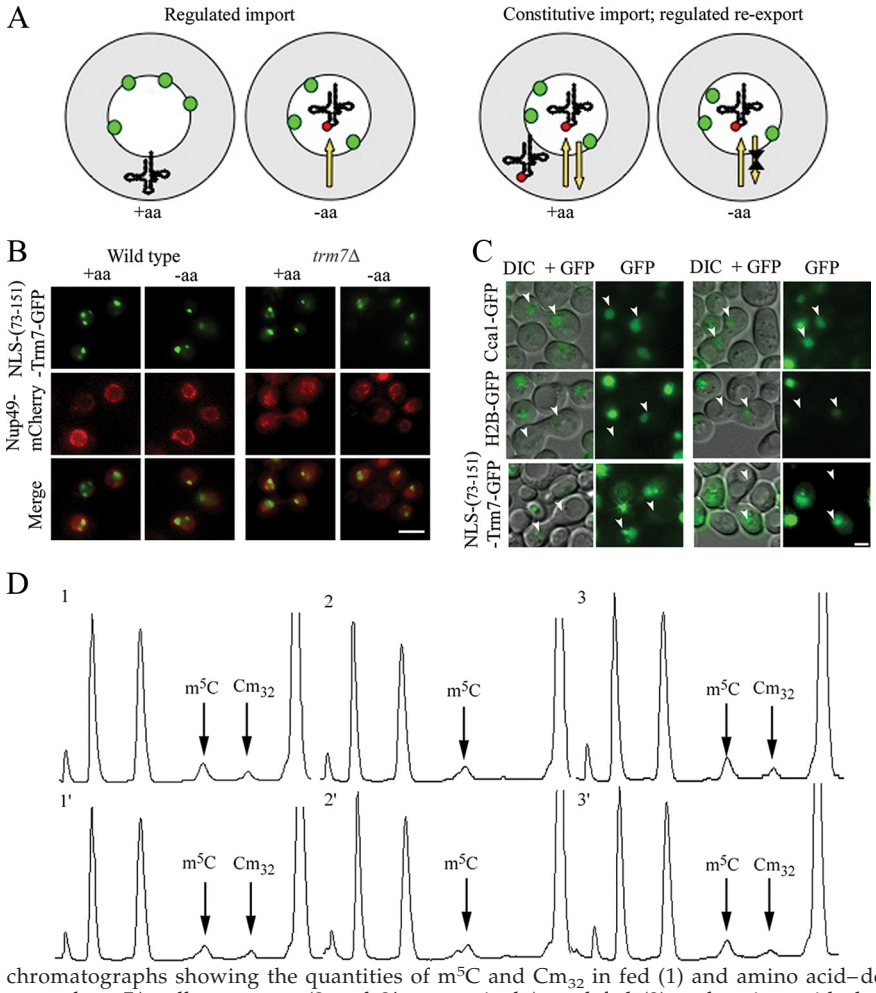

Figure 4. tRNA nuclear import is constitutive. (A) Strategy to follow the subcellular dynamics of $tRNA^{Phe}$ in fed and amino acid–deprived cells. Cells are drawn as circles with the cytoplasm colored light gray, and the nucleoplasm uncolored. The green balls represent the version of Trm7 [NLS-(73-151)-Trm7-GFP], which has been epitope-tagged and modified so that it is located at the INM. Trm7 specifies the tRNA 2′-*O*-methylcytosine modification at position 32 (Cm_{32}) indicated by the red ball on the tRNA cartoons. If $tRNA^{Phe}$ enters the nucleus only when cells are deprived of amino acids, then $tRNA^{Phe}$ will not bear Cm_{32} when cells are propagated in medium with all amino acids; if import is constitutive, then $tRNA^{Phe}$ will be modified in cells propagated in the presence and the absence of amino acids. (B) NLS-(73-151)-Trm7-GFP is located at the INM in wild-type and *trm7*Δ cells. Cells contain a plasmid encoding galactose-inducible NLS-(73-151)-Trm7-GFP and a plasmid encoding constitutively expressed nucleoporin, Nup49-mCherry, to mark the nuclear rim. NLS-(73-151)-Trm7-GFP is located as one or a few spots on the INM in both *trm7*Δ and wild-type cells when all amino acids are supplied or when the cells have been deprived for amino acids. White bar, 5 μm. (C) NLS-(73-151)-Trm7-GFP is stably maintained in the nucleus. Heterokaryon analysis was used to determine whether NLS-(73-151)-Trm7-GFP is maintained in the nucleus. Top row, NLS-Cca1-GFP can move from one nucleus to another in heterokaryons; middle row, H2B-GFP does not shuttle out of the nucleus that encodes this protein; bottom row, NLS-(73-151)-Trm7-GFP, like H2B-GFP, is located in only one of the nuclei of heterokaryons. White bar, 5 μm. (D) Representative HPLC chromatographs showing the quantities of m^5C and Cm_{32} in fed (1) and amino acid–deprived (1′) wild-type cells + vector, fed, and starved *trm7*Δ cells + vector (2 and 2′, respectively), and fed (3) and amino acid–deprived (3′) *trm7*Δ cells harboring the plasmid encoding NLS-(I73-151)-Trm7-GFP.

tRNA for tRNAs isolated from both satiated and amino acid–deprived cells (Figure 4D, 1 and 1′, Table 1, Supplemental Figure S2, and data not shown). The molar levels of Cm_{32} and m^5C as well as other modifications characteristic for $tRNA^{Phe}$ (data not shown) verify the method for purifying $tRNA^{Phe}$ from bulk tRNA. In agreement with the role of Trm7 in modifying $tRNA^{Phe}$ at C_{32}, $tRNA^{Phe}$ isolated from *trm7*Δ strains possessing vector alone contained ~2.0 mol of m^5C, but lacked detectable Cm_{32} modification (Figure 4D, 2 and 2′, Table 1, and Supplemental Figure S2). In three independent experiments for *trm7*Δ satiated cells harboring the plasmid encoding NLS-(73-151)-Trm7-GFP, $tRNA^{Phe}$ possessed 0.57, 0.62, and 0.68 mol of Cm_{32} modification (Figure 4D3, Table 1, and Supplemental Figure S2). The data show that NLS-(73-151)-Trm7-GFP retains catalytic activity. In multiple repeats of these studies as well as studies with shorter periods of induction of NLS-(73-151)-Trm7-GFP, the same molar levels (0.58, 0.65, and 0.72) of Cm_{32} modification were present in $tRNA^{Phe}$ isolated from cells subjected to amino acid deprivation as for cells provided with all amino acids (Figure 4D, 3′, Table 1, Supplemental Figure S2 and data not shown). Thus, there are equimolar quantities of Cm_{32} in tRNA regardless of nutrient availability to cells.

Table 1. Moles of Cm_{32} and m^5C in cells with and without amino acids and nuclear Trm7

Strain and condition	Avg. Cm_{32}	SD Cm_{32}	Avg. m^5C	SD m^5C
Wild type + vector + aa	0.995	0.078	1.860	0.028
Wild type + vector − aa	1.0300	0.099	1.835	0.007
*trm7*Δ + vector + aa	0.005	0.007	1.715	0.092
*trm7*Δ + vector − aa	0.020	0.028	1.870	0.085
*trm7*Δ + nuclear Trm7 + aa	0.623	0.055	1.837	0.038
*trm7*Δ + nuclear Trm7 − aa	0.650	0.070	1.783	0.047

The data are consistent with tRNA accessing the nucleus whether or not the cells are satiated, and they support the notion that tRNA retrograde import is a constitutive process, thus implicating regulation of nutrient-dependent nuclear accumulation of cytoplasmic tRNA at the reexport step. Regulation of tRNA reexport by nutrient availability could require tRNA aminoacylation in the nucleus as tRNA export is inhibited when tRNA nuclear charging is defective (Sarkar *et al.*, 1999; Grosshans *et al.*, 2000; Azad *et al.*, 2001). Although Msn5 appears to be dedicated to the tRNA reexport step of the retrograde pathway, it interacts with uncharged tRNA in vitro (Shibata *et al.*, 2006). So, if Msn5 regulates tRNA reexport in response to nutrient availability,

it may do so in conjunction with another protein(s) able to distinguish aa-tRNAs from uncharged tRNAs. Tef1 and Tef2 are candidates for this function.

TEF1 and *TEF2* encode identical eukaryotic translation elongation factor 1 alpha proteins (eEF1A in vertebrates). Tef1/2 or eEF1A bind aa-tRNAs and function in delivering aa-tRNAs to ribosomes (Schirmaier and Philippsen, 1984). Several lines of evidence have implicated Tef1/2 in tRNA nuclear-cytoplasmic dynamics: 1) *TEF2* was identified as a multicopy suppressor of *los1*Δ (Grosshans *et al.*, 2000); 2) *tef2* or *tef1* and *los1* (Grosshans *et al.*, 2000) and *tef1* and *msn5* (Figure 5A) deletions have synthetic growth defects; and 3) *tef2* mutants accumulate nuclear pools of tRNA (Grosshans *et al.*, 2000; McGuire and Mangroo, 2007). Because this translation factor interacts only with aa-tRNAs and defects in Tef1/2 do not cause accumulation of intron-containing pre-tRNAs (Grosshans *et al.*, 2000), Tef1/2 likely functions only in the tRNA reexport pathway, at least for tRNAs encoded by intron-containing genes. The Msn5 vertebrate orthologue, Exportin-5, forms a heterotetrameric complex with aa-tRNA, RanGTP, and eEF1A, resulting in coexport eEF1A and tRNA to the cytoplasm (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). The data for Tef1/2 and eEF1A in tRNA subcellular dynamics has been interpreted to mean that this well-studied cytoplasmic translation factor serves either to channel tRNA and other proteins exiting the nucleus to the translation machinery (Grosshans *et al.*, 2000; McGuire and Mangroo, 2007; Khacho *et al.*, 2008) or to rid the nuclear interior of inadvertent pools of eEF1A that accumulate during open mitosis (Bohnsack *et al.*, 2002). The possibility that Tef1/2 might have a more direct role in tRNA nuclear export has not been explored.

If Tef1/2 function directly in tRNA reexport, then there should be nuclear pools of these proteins. We used three methods to locate Tef1 in yeast grown in satiated and nutrient-deprived conditions. First we attempted to employ immunofluorescence (IF) using various commercially available antibodies raised against eEF1A. Although some of these could detect Tef1/2 as assessed by Western analyses (*Materials and Methods*; Supplemental Figure S3A), they were unable to detect Tef1 by IF. Second, we used live cell confocal imaging to assess nuclear pools of endogenous Tef1 and Tef2 encoded by Tef1-GFP and Tef2-GFP gene replacements (Figure 5, B and C, and data not shown; Huh *et al.*, 2003). Third, we used indirect IF to determine the subcellular distribution of a galactose-regulated HA-tagged multicopy functional version of Tef1, Tef1-MORF (Supplemental Figure S3, B–D; Gelperin *et al.*, 2005).

As assessed by epifluorescence and confocal imaging of live cells, Tef1-GFP and Tef2-GFP are excluded from the nucleus in wild-type and *los1*Δ cells (Figure 5, B and C, 1–4; and data not shown), both under fed and nutrient-deprived conditions (cf. panels 1 with 2 and 3 with 4). In stark contrast, Tef1 is not excluded from nuclei in *msn5*Δ cells (Figure 5B, panels 5 and 6, and data not shown) and instead is rather evenly distributed between the nucleus and the cytoplasm as determined by pixel intensity profiles of single 0.4-μm confocal sections of individual cells (Figure 5C). The data indicate that Tef1/2 can access the nuclear interior and that its distribution between the nucleus and cytoplasm is dependent, at least in part, upon the exportin, Msn5.

A role for Msn5 in Tef1 subcellular location was confirmed by IF analyses of Tef1-MORF. As assessed by epifluorescence, Tef1-MORF was excluded from the nucleus in wild-type cells (Supplemental Figure S3B, panels 1). When cells were deprived of amino acids, a small portion of the Tef1-MORF pool appeared to localize around the nuclear membrane (Supplemental Figure S3B, panels 2), consistent with earlier reports (Grosshans *et al.*, 2000); it is unknown whether this is the outer or inner nuclear membrane. As for Tef1-GFP, the subcellular distribution of Tef1-MORF in *los1*Δ cells was similar to wild-type cells (Supplemental Figure S3B, panels 3 and 4). In cells that have the double deletion of the importin *mtr10*Δ and the exportin *msn5*Δ, Tef1 also did not accumulate inside the nucleus (Supplemental Figure S3B, panels 7 and 8). In stark contrast, *msn5*Δ cells possessed significant apparent nucleoplasmic pools of Tef1-MORF (Supplemental Figure S3B, panels 5 and 6). Confocal imaging of Tef1-MORF in wild-type and *msn5*Δ cells containing a plasmid encoded nucleoporin, Nup49-mCherry, confirmed that Tef1-MORF is excluded from the nucleus in wild-type cells and nucleoplasmic in *msn5*Δ cells (Supplemental Figure S3, C and D). The IF results are consistent with the results from live imaging of endogenously expressed Tef1-GFP, even though there are quantitative differences in the Tef1 nucleoplasmic pools between Tef1-GFP and Tef1-MORF, perhaps because of different expression levels or the different tags. The data indicate that Tef1's entry into the nucleus is affected by Mtr10 and its exit is affected by Msn5, as are the entry and exit of tRNA, and they support the prerequisite for Tef1 nuclear-cytoplasmic dynamics if Tef1 were to serve a direct role in the tRNA reexport step of the retrograde process.

DISCUSSION

We provide evidence that the two tRNA exportins, Los1 and Msn5, serve distinct but overlapping roles in yeast. It was previously appreciated that Los1 exports newly transcribed end-matured tRNA from the nucleus to the cytoplasm (primary export). Evidence to support this is as follows: 1) accumulation of end-matured intron-containing pre-tRNA in *los1*Δ mutants (Hopper *et al.*, 1980), 2) nuclear pools of tRNAs in *los1*Δ mutants (Sarkar and Hopper, 1998; Grosshans *et al.*, 2000), and 3) in vitro binding of Los1 and tRNA with RanGTP (Hellmuth *et al.*, 1998). Los1 also likely serves a proofreading role in nuclear export of newly synthesized tRNAs because its orthologue, Exp-t, will not interact with mutant tRNAs with aberrant secondary or tertiary structure (Arts *et al.*, 1998a; Lipowsky *et al.*, 1999; Cook *et al.*, 2009). Two lines of evidence support the idea that Los1 participates in the tRNA reexport process as well as in the initial tRNA nuclear export step. First, in *los1*Δ X *los1*Δ heterokaryons, tRNAs encoded by one nucleus accumulate in nuclei not encoding the tRNA (Shaheen and Hopper, 2005; Takano *et al.*, 2005); therefore, cytoplasmic tRNA derived from one nucleus can access a second nucleus and, because of defects in reexport caused by absence of Los1, nuclear pools of the tRNA accumulate. Second, we show that haploid *mtr10*Δ *los1*Δ double mutant cells accumulate significantly less $tRNA^{Tyr}$ and $tRNA^{His}$ in the nucleus than do cells with only a *los1*Δ mutation. Thus, the $tRNA^{Tyr}$ and $tRNA^{His}$ detected by FISH in cells must come from two sources, newly synthesized tRNA and tRNA imported from the cytoplasm, and elimination of the latter results in significantly reduced nuclear pools. Our data showing that Los1 participates in both the initial export and reexport processes is consistent with the known interactions of the vertebrate homologue, Exp-t, and *S. pombe* Xpot with the TΨC and acceptor arms and the 5′ and 3′ termini of tRNAs, structures that are in common to end-matured newly synthesized intron-containing and imported mature tRNAs (Etcheverry *et al.*, 1979; Lee and Knapp, 1985; Arts *et al.*, 1998b; Lipowsky *et al.*, 1999; Cook *et al.*, 2009).

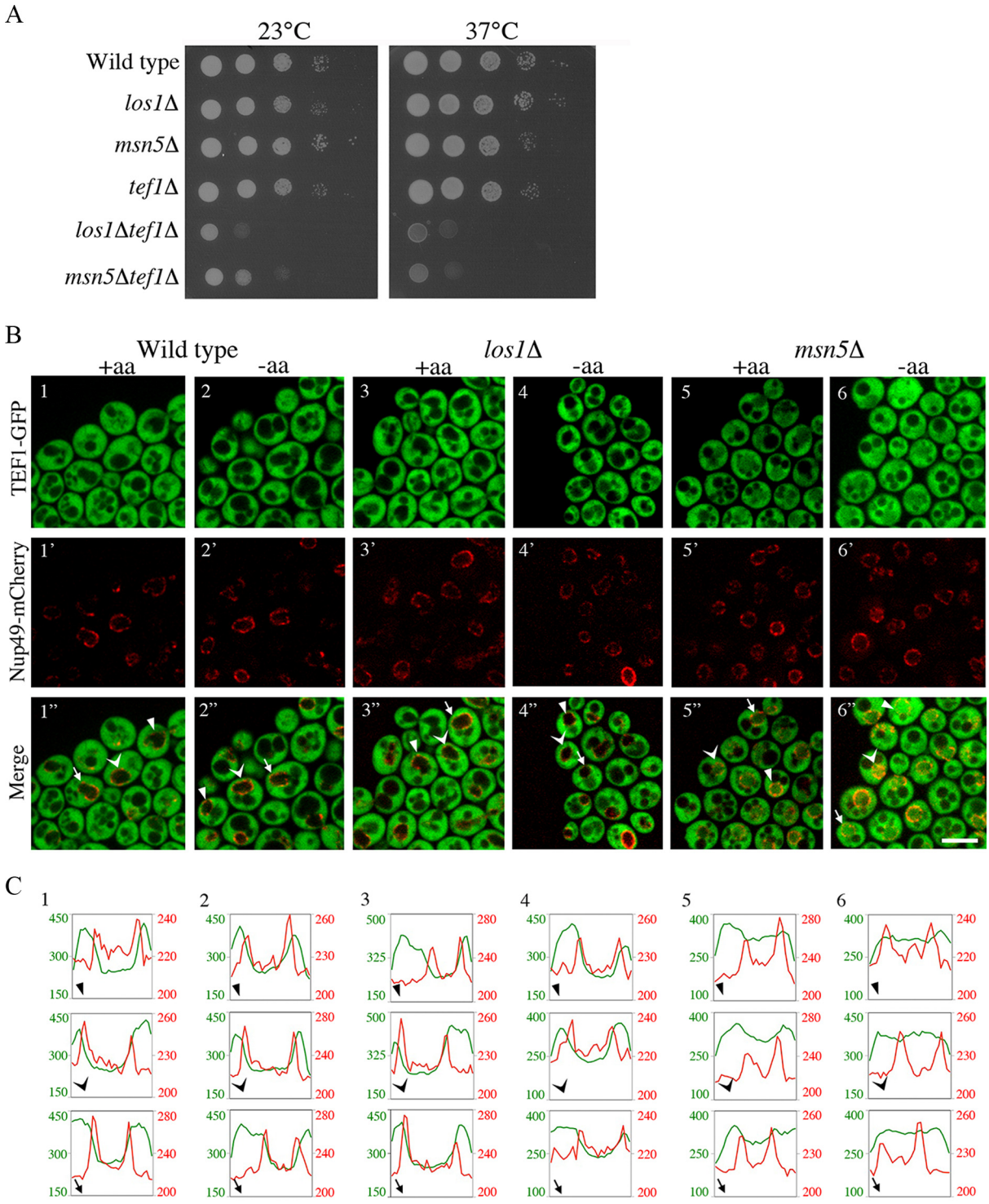

Figure 5. Interactions between Los1 or Msn5 and Tef1. (A) Synthetic growth defects in *los1*Δ *tef1*Δ and *msn5*Δ *tef1*Δ double mutants. Serial dilutions were made of the indicated yeast cultures. Aliquots (5 μl) of each dilution were spotted onto solid growth medium and incubated 2-3 d at various indicated temperatures. (B) Confocal images (0.4-μm sections) of the subcellular distribution of endogenously expressed Tef1-GFP in wild-type and mutant fed and nutrient-deprived live cells. Wild-type (panels 1 and 2), *los1*Δ (panels 3 and 4), or *msn5*Δ (panels 5 and 6) mutant cells containing the constitutively expressed plasmid-encoded Nup49-mCherry were grown in SC medium lacking leucine to maintain the plasmid. Cells in 1-1″, 3-3″, and 5-5″ were further incubated in medium with all required nutrients, whereas cells in 2-2″, 4-4″, and 6-6″ were deprived of amino acids for 1.5 h. 1–6, location of Tef1-GFP; 1′–6′, location of Nup49-mCherry, demarking the nuclei rims, 1″–6″, overlay of 1–6 and 1′–6′, respectively. White bar, 5 μm. (C) Pixel intensity profiles of the subcellular distribution of Tef1-GFP in wild-type and mutant fed and nutrient deprived live cells. Pixel intensity profiles of 0.4-μm sections are shown for three independent cells for each strain and condition. The cells in B that were scanned and plotted in C are indicated with the same shape arrows or arrowheads. Red lines and axes indicate Nup49-mCherry image intensities; green lines and axes indicate Tef1–GFP intensities.

Unlike Los1, Msn5 appears not to function in the initial export process for tRNAs encoded by intron-containing tRNA genes. First, we do not detect nuclear pools of tRNA in *mtr10*Δ *msn5*Δ double mutants; second, and more important, there is no accumulation of intron-containing pre-tRNAs in *msn5*Δ mutants even though there are large nuclear pools of tRNAs. Because splicing occurs on the surface of mitochondria, the nuclear tRNAs encoded by intron-containing genes in *msn5*Δ cells must be derived from tRNAs that previously resided in the cytoplasm. We do not know whether Msn5 participates only in the reexport process for tRNAs encoded by genes lacking introns; however, it is difficult to imagine that Msn5 could distinguish between the new end-processed transcripts and the tRNA imported from the cytoplasm unless Msn5 monitors modifications that are added solely in the cytoplasm. In sum, tRNA subcellular dynamics require at least three members of the importin-β family: Mtr10 which directly or indirectly functions in tRNA retrograde import, Los1 which directly functions in the initial and reexport steps, and Msn5 which, for tRNAs encoded by intron-containing genes, functions primarily in the tRNA reexport process. As *los1*Δ *msn5*Δ double mutants have no obvious growth defects (Takano *et al.*, 2005), there are likely undiscovered additional mechanisms for tRNA nuclear export in yeast.

The biological function of the tRNA retrograde pathway is unknown, but two nonexclusive roles have been proposed: tRNA proofreading and down-regulation of protein synthesis under nutrient deprived conditions (Shaheen and Hopper, 2005). Yeast cells possess two different tRNA turnover machineries that monitor hypomodified tRNAs and tRNAs with damaged 3′ termini (Kadaba *et al.*, 2006; Chernyakov *et al.*, 2008; Copela *et al.*, 2008; Ozanick *et al.*, 2009). One of these pathways is located exclusively in the nucleus and the other is located in both the nucleus and the cytoplasm (Kadaba *et al.*, 2006; Chernyakov *et al.*, 2008). The retrograde pathway could target damaged or otherwise inappropriate cytoplasmic tRNAs to the nucleus for repair or turnover. If so, one would expect that tRNA import would be a constitutive process for continuous monitoring of the tRNA pool. Previous data support the notion that tRNA nuclear import is constitutive (Shaheen and Hopper, 2005; Takano *et al.*, 2005), and here we devised a new assay that provides an additional line of evidence supporting constitutive import, as required for proofreading. Whether the retrograde process also functions in regulating protein synthesis upon nutrient deprivation remains to be determined.

If tRNA nuclear import is constitutive, then the reexport process is likely regulated, since when cells are deprived for nutrients, cytoplasmic tRNAs accumulate in the nucleus (Shaheen and Hopper, 2005; Hurto *et al.*, 2007; Shaheen *et al.*, 2007; Whitney *et al.*, 2007). tRNA reexport is likely regulated in response to the aminoacylation status of tRNAs because mutations or conditions that prohibit tRNA aminoacylation result in nuclear pools of mature tRNA (Lund and Dahlberg, 1998; Sarkar *et al.*, 1999; Grosshans *et al.*, 2000; Azad *et al.*, 2001; Feng and Hopper, 2002; Gu *et al.*, 2005). Because intron-containing tRNAs cannot be aminoacylated (O'Farrell *et al.*, 1978) and Los1 exports intron-containing tRNAs, tRNA reexport by Los1 is unlikely to be responsive to conditions affecting tRNA aminoacylation. Rather, we propose that Msn5 is the tRNA exportin involved in regulated tRNA reexport. If so, Msn5 would be expected to preferentially bind and export aa-tRNAs. However, this is not consistent with the known binding specificities of the Exportin-5 family. The Exportin-5 orthologues bind short double-stranded RNAs such as miRNAs (Gwizdek *et al.*, 2003, 2004;Lund *et al.*, 2004; Zeng and Cullen, 2004; Shibata *et al.*, 2006) as well as uncharged or aa-tRNAs, although there may be differences in binding affinities for tRNA among the various orthologues (Shibata *et al.*, 2006).

Given the binding studies, it is difficult to understand how Msn5 might interact with and export primarily mature and, perhaps, only aa-tRNAs. However, studies of the interaction of VA1 RNA and ILF3 with Exportin-5 may provide an explanation. Exportin-5 forms a cooperative quaternary complex with RanGTP, VA1 RNA, and protein ILF3. The affinity of VA1 RNA for Exportin-5 is increased by the presence of ILF3 and formation of the complex results in the nuclear coexport of ILF3 and VA1 RNA (Gwizdek *et al.*, 2004). Analogously, in vivo Msn5 may interact with aa-tRNA via cooperative binding with other proteins resulting in its substrate specificity. Here we suggest that Tef1/2 may serve this function and show that Tef1 has nuclear-cytoplasmic dynamics that are consistent with such a function. If so, then the interaction of Tef1/2 with the Msn5 complex in yeast would be different from the interaction of Exportin-5 with eEF1A in vertebrate cells because eEF1A does not interact with Exportin-5 in the absence of aa-tRNA (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). Thus, for vertebrate Exportin-5, eEF1A has been proposed to be merely a passenger with the tRNA complex and one would not expect down-regulation of tRNA nuclear export upon down-regulation of eEF1A, in contrast to the negative consequences of *TEF1/2* deletions upon tRNA reexport in yeast (Grosshans *et al.*, 2000; McGuire and Mangroo, 2007).

A role for Tef1/2 in tRNA reexport would conveniently explain the presence of Tef1/2 in the nucleus in yeast. Because of its closed mitosis, yeast cannot inadvertently acquire Tef1/2 during mitosis, as proposed for nuclear pools of eEF1A in vertebrate cells (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). Because of the large size of Tef1-GFP (78 kDa, including the 28-kDa GFP tag) and Tef1-MORF (69 kDa, including the 19 kDa MORF tag; Gelperin *et al.*, 2005) nuclear Tef1 pools are also unlikely to arise from passive diffusion through the nuclear pore, which has a 40-kDa diffusion barrier (Shulga *et al.*, 2000). Because the nuclear presence of Tef1/2 is dependent on Mtr10 and Msn5 and parallels the subcellular distribution of tRNA and because mutations of *TEF1/TEF2* cause nuclear accumulation of mature tRNA, the simplest explanation is that Tef1/2 function in tRNA nuclear reexport. Although it has been difficult to detect, in vivo, a quaternary complex of Msn5, aa-tRNA, RanGTP, and Tef1 by conventional methodology, cross-linking-based efforts should provide a rigorous test in the future for this otherwise attractive model for tRNA reexport.

ACKNOWLEDGMENTS

We thank H.-Y. Chu, R. Hurto, and N. Dhungel for comments on the manuscript and all the students and postdoctoral scholars in the Hopper and Phizicky laboratories for stimulating conversations and ideas. This work was supported by grants from the National Institutes of Health (NIH): GM27930 to A.K.H. and GM52347 to E.M.P. M.A.P. was supported by NIH Cellular, Biochemical, and Molecular Science Training Grant GM068411.

REFERENCES

Arts, G. J., Fornerod, M., and Mattaj, I. W. (1998a). Identification of a nuclear export receptor for tRNA. Curr. Biol. *8*, 305–314.

Arts, G. J., Kuersten, S., Romby, P., Ehresmann, B., and Mattaj, I. W. (1998b). The role of exportin-t in selective nuclear export of mature tRNAs. EMBO J. *17*, 7430–7441.

Azad, A. K., Stanford, D. R., Sarkar, S., and Hopper, A. K. (2001). Role of nuclear pools of aminoacyl-tRNA synthetases in tRNA nuclear export. Mol. Biol. Cell *12*, 1381–1392.

Bohnsack, M. T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E., and Gorlich, D. (2002). Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. EMBO J. *21*, 6205–6215.

Calado, A., Treichel, N., Muller, E. C., Otto, A., and Kutay, U. (2002). Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. EMBO J. *21*, 6216–6224.

Chernyakov, I., Whipple, J. M., Kotelawala, L., Grayhack, E. J., and Phizicky, E. M. (2008). Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5′-3′ exonucleases Rat1 and Xrn1. Genes Dev. *22*, 1369–1380.

Ciesla, M., Towpik, J., Graczyk, D., Oficjalska-Pham, D., Harismendy, O., Suleau, A., Balicki, K., Conesa, C., Lefebvre, O., and Boguta, M. (2007). Maf1 is involved in coupling carbon metabolism to RNA polymerase III transcription. Mol. Cell. Biol. *27*, 7693–7702.

Cook, A. G., Fukuhara, N., Jinek, M., and Conti, E. (2009). Structures of the tRNA export factor in the nuclear and cytosolic states. Nature *461*, 60–65.

Copela, L. A., Fernandez, C. F., Sherrer, R. L., and Wolin, S. L. (2008). Competition between the Rex1 exonuclease and the La protein affects both Trf4p-mediated RNA quality control and pre-tRNA maturation. RNA *14*, 1214–1227.

Etcheverry, T., Colby, D., and Guthrie, C. (1979). A precursor to a minor species of yeast tRNASer contains an intervening sequence. Cell *18*, 11–26.

Feng, W., and Hopper, A. K. (2002). A Los1p-independent pathway for nuclear export of intronless tRNAs in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *99*, 5412–5417.

Gelperin, D. M., *et al*. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes Dev. *19*, 2816–2826.

Grosjean, H., Szweykowska-Kulinska, Z., Motorin, Y., Fasiolo, F., and Simos, G. (1997). Intron-dependent enzymatic formation of modified nucleosides in eukaryotic tRNAs: a review. Biochimie *79*, 293–302.

Grosshans, H., Hurt, E., and Simos, G. (2000). An aminoacylation-dependent nuclear tRNA export pathway in yeast. Genes Dev. *14*, 830–840.

Gu, W., Hurto, R. L., Hopper, A. K., Grayhack, E. J., and Phizicky, E. M. (2005). Depletion of *Saccharomyces cerevisiae* tRNA(His) guanylyltransferase Thg1p leads to uncharged tRNAHis with additional m(5)C. Mol. Cell. Biol. *25*, 8191–8201.

Gwizdek, C., Ossareh-Nazari, B., Brownawell, A. M., Doglio, A., Bertrand, E., Macara, I. G., and Dargemont, C. (2003). Exportin-5 mediates nuclear export of minihelix-containing RNAs. J. Biol. Chem. *278*, 5505–5508.

Gwizdek, C., Ossareh-Nazari, B., Brownawell, A. M., Evers, S., Macara, I. G., and Dargemont, C. (2004). Minihelix-containing RNAs mediate exportin-5-dependent nuclear export of the double-stranded RNA-binding protein ILF3. J. Biol. Chem. *279*, 884–891.

Hellmuth, K., Lau, D. M., Bischoff, F. R., Kunzler, M., Hurt, E., and Simos, G. (1998). Yeast Los1p has properties of an exportin-like nucleocytoplasmic transport factor for tRNA. Mol. Cell. Biol. *18*, 6374–6386.

Hopper, A. K. (1999). Nucleocytoplasmic transport: Inside out regulation. Curr. Biol. *9*, R803–R806.

Hopper, A. K. (2006). Cellular dynamics of small RNAs. Crit. Rev. Biochem. Mol. Biol. *41*, 3–19.

Hopper, A. K., Banks, F., and Evangelides, V. (1978). A yeast mutant which accumulates precursor tRNAs. Cell *14*, 211–219.

Hopper, A. K., Schultz, L. D., and Shapiro, R. A. (1980). Processing of intervening sequences: a new yeast mutant which fails to excise intervening sequences from precursor tRNAs. Cell *19*, 741–751.

Hopper, A. K., and Shaheen, H. H. (2008). A decade of surprises for tRNA nuclear-cytoplasmic dynamics. Trends Cell Biol. *18*, 98–104.

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686–691.

Hunter, C. A., Aukerman, M. J., Sun, H., Fokina, M., and Poethig, R. S. (2003). PAUSED encodes the Arabidopsis exportin-t ortholog. Plant Physiol. *132*, 2135–2143.

Hurt, D. J., Wang, S. S., Lin, Y. H., and Hopper, A. K. (1987). Cloning and characterization of LOS1, a *Saccharomyces cerevisiae* gene that affects tRNA splicing. Mol. Cell. Biol. *7*, 1208–1216.

Hurto, R. L., Tong, A. H., Boone, C., and Hopper, A. K. (2007). Inorganic phosphate deprivation causes tRNA nuclear accumulation via retrograde transport in *Saccharomyces cerevisiae*. Genetics *176*, 841–852.

Jackman, J. E., Montange, R. K., Malik, H. S., and Phizicky, E. M. (2003). Identification of the yeast gene encoding the tRNA m1G methyltransferase responsible for modification at position 9. RNA *9*, 574–585.

Jiang, H. Q., Motorin, Y., Jin, Y. X., and Grosjean, H. (1997). Pleiotropic effects of intron removal on base modification pattern of yeast tRNAPhe: an in vitro study. Nucleic Acids Res. *25*, 2694–2701.

Kadaba, S., Wang, X., and Anderson, J. T. (2006). Nuclear RNA surveillance in *Saccharomyces cerevisiae*: Trf4p-dependent polyadenylation of nascent hypomethylated tRNA and an aberrant form of 5S rRNA. RNA *12*, 508–521.

Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S., and O'Shea, E. K. (1998). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482–486.

Khacho, M., Mekhail, K., Pilon-Larose, K., Pause, A., Cote, J., and Lee, S. (2008). eEF1A is a novel component of the mammalian nuclear protein export machinery. Mol. Biol. Cell *19*, 5296–5308.

Kutay, U., Lipowsky, G., Izaurralde, E., Bischoff, F. R., Schwarzmaier, P., Hartmann, E., and Gorlich, D. (1998). Identification of a tRNA-specific nuclear export receptor. Mol. Cell *1*, 359–369.

Lai, T. P., Stauffer, K. A., Murthi, A., Shaheen, H. H., Peng, G., Martin, N. C., and Hopper, A. K. (2009). Mechanism and a peptide motif for targeting peripheral proteins to the yeast inner nuclear membrane. Traffic *10*, 1243–1256.

Lee, M. C., and Knapp, G. (1985). Transfer RNA splicing in *Saccharomyces cerevisiae*. Secondary and tertiary structures of the substrates. J. Biol. Chem. *260*, 3108–3115.

Lipowsky, G., Bischoff, F. R., Izaurralde, E., Kutay, U., Schafer, S., Gross, H. J., Beier, H., and Gorlich, D. (1999). Coordination of tRNA nuclear export with processing of tRNA. RNA *5*, 539–549.

Lund, E., and Dahlberg, J. E. (1998). Proofreading and aminoacylation of tRNAs before export from the nucleus. Science *282*, 2082–2085.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95–98.

McGuire, A. T., and Mangroo, D. (2007). Cex1p is a novel cytoplasmic component of the *Saccharomyces cerevisiae* nuclear tRNA export machinery. EMBO J. *26*, 288–300.

Nishikura, K., and De Robertis, E. M. (1981). RNA processing in microinjected *Xenopus* oocytes. Sequential addition of base modifications in the spliced transfer RNA. J Mol Biol *145*, 405–420.

O'Farrell, P. Z., Cordell, B., Valenzuela, P., Rutter, W. J., and Goodman, H. M. (1978). Structure and processing of yeast precursor tRNAs containing intervening sequences. Nature *274*, 438–445.

Ozanick, S. G., Wang, X., Costanzo, M., Brost, R. L., Boone, C., and Anderson, J. T. (2009). Rex1p deficiency leads to accumulation of precursor initiator tRNAMet and polyadenylation of substrate RNAs in *Saccharomyces cerevisiae*. Nucleic Acids Res. *37*, 298–308.

Park, M. Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R. S. (2005). Nuclear processing and export of microRNAs in Arabidopsis. Proc. Natl. Acad. Sci. USA *102*, 3691–3696.

Peng, G., and Hopper, J. E. (2002). Gene activation by interaction of an inhibitor with a cytoplasmic signaling protein. Proc. Natl. Acad. Sci. USA *99*, 8548–8553.

Pintard, L., Lecointe, F., Bujnicki, J. M., Bonnerot, C., Grosjean, H., and Lapeyre, B. (2002). Trm7p catalyses the formation of two 2′-O-methylriboses in yeast tRNA anticodon loop. EMBO J. *21*, 1811–1820.

Sarkar, S., Azad, A. K., and Hopper, A. K. (1999). Nuclear tRNA aminoacylation and its role in nuclear export of endogenous tRNAs in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *96*, 14366–14371.

Sarkar, S., and Hopper, A. K. (1998). tRNA nuclear export in *Saccharomyces cerevisiae*: in situ hybridization analysis. Mol. Biol. Cell *9*, 3041–3055.

Schirmaier, F., and Philippsen, P. (1984). Identification of two genes coding for the translation elongation factor EF-1 alpha of *S. cerevisiae*. EMBO J. *3*, 3311–3315.

Shaheen, H. H., and Hopper, A. K. (2005). Retrograde movement of tRNAs from the cytoplasm to the nucleus in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *102*, 11290–11295.

Shaheen, H. H., Horetsky, R. L., Kimball, S. R., Murthi, A., Jefferson, L. S., and Hopper, A. K. (2007). Retrograde nuclear accumulation of cytoplasmic tRNA in rat hepatoma cells in response to amino acid deprivation. Proc. Natl. Acad. Sci. USA *104*, 8845–8850.

Shibata, S., Sasaki, M., Miki, T., Shimamoto, A., Furuichi, Y., Katahira, J., and Yoneda, Y. (2006). Exportin-5 orthologues are functionally divergent among species. Nucleic Acids Res. *34*, 4711–4721.

Shulga, N., Mosammaparast, N., Wozniak, R., and Goldfarb, D. S. (2000). Yeast nucleoporins involved in passive nuclear envelope permeability. J. Cell Biol. *149*, 1027–1038.

Stanford, D. R., Whitney, M. L., Hurto, R. L., Eisaman, D. M., Shen, W. C., and Hopper, A. K. (2004). Division of labor among the yeast Sol proteins implicated in tRNA nuclear export and carbohydrate metabolism. Genetics *168*, 117–127.

Takano, A., Endo, T., and Yoshihisa, T. (2005). tRNA actively shuttles between the nucleus and cytosol in yeast. Science *309*, 140–142.

Tolerico, L. H., Benko, A. L., Aris, J. P., Stanford, D. R., Martin, N. C., and Hopper, A. K. (1999). Saccharomyces cerevisiae Mod5p-II contains sequences antagonistic for nuclear and cytosolic locations. Genetics *151*, 57–75.

Tsurui, H., Kumazawa, Y., Sanokawa, R., Watanabe, Y., Kuroda, T., Wada, A., Watanabe, K., and Shirai, T. (1994). Batchwise purification of specific tRNAs by a solid-phase DNA probe. Anal. Biochem. *221*, 166–172.

Vallen, E. A., Hiller, M. A., Scherson, T. Y., and Rose, M. D. (1992). Separate domains of KAR1 mediate distinct functions in mitosis and nuclear fusion. J. Cell Biol. *117*, 1277–1287.

Whitney, M. L., Hurto, R. L., Shaheen, H. H., and Hopper, A. K. (2007). Rapid and reversible nuclear accumulation of cytoplasmic tRNA in response to nutrient availability. Mol. Biol. Cell *18*, 2678–2686.

Winzeler, E. A., *et al*. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science *285*, 901–906.

Wu, J. Q., Sirotkin, V., Kovar, D. R., Lord, M., Beltzner, C. C., Kuhn, J. R., and Pollard, T. D. (2006). Assembly of the cytokinetic contractile ring from a broad band of nodes in fission yeast. J. Cell Biol. *174*, 391–402.

Xing, F., Hiley, S. L., Hughes, T. R., and Phizicky, E. M. (2004). The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. J. Biol. Chem. *279*, 17850–17860.

Yi, R., Qin, Y., Macara, I. G., and Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. *17*, 3011–3016.

Yoshihisa, T., Yunoki-Esaki, K., Ohshima, C., Tanaka, N., and Endo, T. (2003). Possibility of cytoplasmic pre-tRNA splicing: the yeast tRNA splicing endonuclease mainly localizes on the mitochondria. Mol. Biol. Cell *14*, 3266–3279.

Zaitseva, L., Myers, R., and Fassati, A. (2006). tRNAs promote nuclear import of HIV-1 intracellular reverse transcription complexes. PLoS Biol. *4*, e332.

Zeng, Y., and Cullen, B. R. (2004). Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. Nucleic Acids Res. *32*, 4776–4785.