Sharing the load: Mex67–Mtr2 cofunctions with Los1 in primary tRNA nuclear export

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Eukaryotic transfer RNAs (tRNAs) are exported from the nucleus, their site of synthesis, to the cytoplasm, their site of function for protein synthesis. The evolutionarily conserved β-importin family member Los1 (Exportin-t) has been the only exporter known to execute nuclear export of newly transcribed intron-containing pre-tRNAs. Interestingly, *LOS1* is unessential in all tested organisms. As tRNA nuclear export is essential, we previously interrogated the budding yeast proteome to identify candidates that function in tRNA nuclear export. Here, we provide molecular, genetic, cytological, and biochemical evidence that the Mex67–Mtr2 (TAP–p15) heterodimer, best characterized for its essential role in mRNA nuclear export, cofunctions with Los1 in tRNA nuclear export. Inactivation of Mex67 or Mtr2 leads to rapid accumulation of end-matured unspliced tRNAs in the nucleus. Remarkably, merely fivefold overexpression of Mex67–Mtr2 can substitute for Los1 in *los1*Δ cells. Moreover, in vivo coimmunoprecipitation assays with tagged Mex67 document that the Mex67 binds tRNAs. Our data also show that tRNA exporters surprisingly exhibit differential tRNA substrate preferences. The existence of multiple tRNA exporters, each with different tRNA preferences, may indicate that the proteome can be regulated by tRNA nuclear export. Thus, our data show that Mex67–Mtr2 functions in primary nuclear export for a subset of yeast tRNAs.

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Transfer RNAs (tRNAs) function as adapters for protein synthesis by delivering amino acids to the ribosome for incorporation into nascent polypeptides. tRNAs, transcribed in the nucleolus as primary transcripts, undergo processing, modification, and subcellular trafficking to mature into functional tRNAs that participate in cytoplasmic translation. Nascent tRNAs are synthesized by RNA polymerase III with 5′ leader and 3′ trailer sequences, which are removed, and a trinucleotide CCA sequence is added to the processed 3′ ends (Aebi et al. 1990; Frank and Pace 1998; Haeusler et al. 2008; Skowronek et al. 2014). In the budding yeast *Saccharomyces cerevisiae*, 22% of tRNA genes also contain introns (Chan and Lowe 2009). The end-processed intron-containing tRNAs as well as intronless tRNAs are escorted from the nucleus via the primary tRNA nuclear export step. tRNAs with introns are then spliced on the surface of the mitochondria in yeast by the heterotetrameric tRNA splicing endonuclease (SEN) enzyme complex (Trotta et al. 1997; Yoshihisa 2003; Yoshihisa et al. 2007). Mature tRNAs have dynamic movement between the nucleus and the cytoplasm: Cytoplasmic tRNAs are constitutively imported back to the nucleus via the evolutionarily conserved tRNA retrograde nuclear import process, and these tRNAs are once again shuttled to the cytoplasm via the tRNA re-export step (Shaheen and Hopper 2005; Takano 2005; Zaitseva et al. 2006; Shaheen et al. 2007; Whitney et al. 2007).

Although tRNAs are smaller than the 40-kDa cutoff for passive diffusion through the nuclear pore complexes (NPCs), tRNA nuclear export is an active process requiring tRNA transport factors (Zasloff 1983; Arts et al. 1998a; Hellmuth et al. 1998; Kutay et al. 1998; Cook et al. 2009). One well-studied member of the family of β-importins—a Ran-binding protein involved in nuclear export of selected RNA and protein cargo—functions in the primary nuclear export of both intron-containing and

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intron-less tRNAs in yeast. This β-importin—yeast Los1 (Hopper et al. 1980), Exportin-t or Exp-t in vertebrates (Arts et al. 1998a; Kutay et al. 1998), Xpot in *Schizosaccharymoces pombe* (Cherkasova et al. 2012), and PAUSED in *Arabidopsis* sp. (Hunter et al. 2003)—is conserved among eukaryotic organisms. Los1 and its homologs bind both spliced and unspliced tRNA in a RanGTP-dependent manner (Arts et al. 1998a,b; Hellmuth et al. 1998; Kutay et al. 1998; Lund and Dahlberg 1998; Huang and Hopper 2015). Structural studies of *S. pombe* Xpot in complex with tRNA and RanGTP and biochemical data document that Los1/Xpot interacts with only correctly structured tRNAs with mature 5′ and 3′ ends (Arts et al. 1998b; Lund and Dahlberg 1998; Lipowsky et al. 1999; Cook et al. 2009). However, Los1 cannot be the sole primary tRNA nuclear exporter because *LOS1*, *XPOT*, and *PSD* are unessential genes (Hurt et al. 1987; Li and Chen 2003; Cherkasova et al. 2012). Also, in recent genome-wide screens to identify essential human genes, Exportin-t was scored to be nonessential in most human haploid cancer cell lines (Blomen et al. 2015; Hart et al. 2015; Wang et al. 2015). Moreover, *Drosophila melanogaster* lacks a homolog for Exportin-t (Lippai et al. 2000). Since the export of tRNAs from nucleus to cytoplasm is essential, there must be additional gene products to perform the crucial role of primary tRNA nuclear export.

Another β-importin family member, yeast Msn5 (Exportin-5 in vertebrates and HASTY in plants), provides one such potential alternate pathway for tRNA nuclear export (Bohnsack et al. 2002; Calado et al. 2002; Shibata et al. 2006; Huang and Hopper 2015). However, unlike *los1*Δ, *msn5*Δ cells do not accumulate end-processed intron-containing pre-tRNAs (Murthi et al. 2010), and overexpressed Msn5 is unable to suppress pre-tRNA accumulation defects observed in *los1*Δ cells (Huang and Hopper 2015). Moreover, although Msn5 binds spliced charged tRNAs, it interacts with intron-containing tRNAs with low affinity. Thus, Msn5 is unable to substitute for Los1 in primary tRNA nuclear export but rather participates in the tRNA nuclear re-export step (Murthi et al. 2010; Huang and Hopper 2015).

Recently, Ohira and Suzuki (2016) reported that, like mRNAs, initial tRNA transcripts can be capped at the 5′ ends. These capped pre-tRNAs can be exported to the cytoplasm for intron removal. Los1 is unable to recognize the termini of capped pre-tRNAs for nuclear export, as Los1 can bind only end-matured tRNAs with high affinity (Arts et al. 1998b; Lund and Dahlberg 1998; Lipowsky et al. 1999; Cook et al. 2009). Hence, capped intron-containing pre-tRNAs with unprocessed ends are likely exported to the cytoplasm via a Los1-independent nuclear export pathway.

To identify novel proteins involved in tRNA biology, particularly in nuclear export, splicing, and maturation of tRNAs, we recently conducted an unbiased comprehensive screen covering ~80% of the yeast proteome (Wu et al. 2015). This screen uncovered 162 previously unidentified proteins (and 12 previously known proteins) that are important in tRNA biology. Importantly, Crm1, best known for its role in nuclear export of proteins with leucine-rich motifs (Fornerod et al. 1997), was shown to affect primary pre-tRNA nuclear export and have synthetic negative genetic interactions with Los1 (Wu et al. 2015). The data indicated that Crm1 could function in tRNA nuclear export. However, further studies documenting tRNA binding with Crm1 are required to validate its direct role in nuclear–cytoplasmic dynamics. In the same screen, *MEX67* and *MTR2*, two essential genes, were identified; they accumulate end-processed intron-containing pre-tRNA—a proxy for defective tRNA nuclear export.

Yeast Mex67 (metazoan NXF1, also known as TAP in humans) forms a heterodimer with Mtr2 (NXT1 or p15 in humans) and is best known for its function in the nuclear export of most mRNAs (Segref et al. 1997; Grüter et al. 1998; Santos-Rosa et al. 1998; Katahira et al. 1999; Herold et al. 2001). Mex67 is a multidomain protein consisting of an N-terminal domain (N), a leucine-rich repeat (LRR) domain, a middle nuclear transport factor 2 (NTF2-like; M) domain, and a C-terminal ubiquitin-associated (UBA)-like domain (Santos-Rosa et al. 1998; Sträßer et al. 2000). The Mex67 M domain binds Mtr2 to form a heterodimer complex that is recruited onto mRNAs designated for export through the concerted actions of various adapter proteins. Mtr2 is also structurally related to NTF2 proteins. Mex67 dimerization with Mtr2 is required for efficient mRNA nuclear export, in contrast to human TAP, for which mRNA nuclear export activity can be independent of p15 (Braun et al. 2002). Mex67–Mtr2 and their orthologs export cargoes in addition to mRNAs. For example, nuclear export of viral RNAs containing a constitutive transport element (CTE) in the 3′ end of the ssRNA genome is mediated by NXF1–NXT1 (Ernst et al. 1997b; Pasquinelli et al. 1997; Aibara et al. 2015). Mex67–Mtr2 is also known to export pre-60S and pre-40S ribosomal subunits and telomerase RNA TLC1 (Yao et al. 2007; Faza et al. 2012; Wu et al. 2014). Based on the repertoire of RNA cargoes that can be exported out of the nucleus by Mex67–Mtr2 and our data showing that *mex67-5* and *mtr2* temperature-sensitive mutants accumulate end-processed intron-containing pre-tRNAs at the nonpermissive temperature (Wu et al. 2015), we tested the hypothesis that Mex67–Mtr2 serves as a tRNA nuclear exporter in budding yeast. Here we provide molecular, genetic, cytological, and biochemical data that support this hypothesis and show that Mex67–Mtr2 cofunctions with Los1 in tRNA nuclear export.

Results

Temperature-sensitive mex67-5 and mtr2 mutant yeast cells rapidly accumulate end-processed intron-containing tRNAs at the nonpermissive temperature

Temperature-sensitive strains *mex67-5* and *mtr2* accumulate increased amounts of end-processed intron-containing tRNAs compared with wild-type cells when incubated for 2 h at the nonpermissive temperature (37°C) (Fig. 1A,B, $tRNA^{Ile}_{UAU}$; Supplemental Fig. S1B,

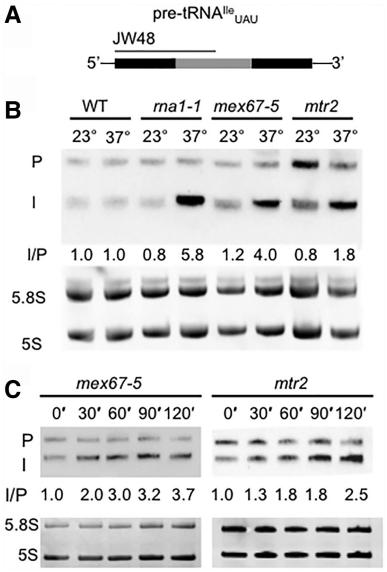

Figure 1. *mex67-5* and *mtr2* temperature-sensitive yeast cells rapidly accumulate end-processed intron-containing tRNAs at 37°C. (*A*) A schematic diagram (not drawn to scale) of the primary tRNA$^{Ile}_{UAU}$ transcript, with the 5′ leader and 3′ trailer as thin lines. (Black boxes) Exons; (gray box) intron. The probe (JW48) is diagrammed *above* the schematic drawing. (*B*) Northern hybridization, performed with probe JW48, of total small RNA isolated from log phase wild-type (WT), *rna1-1, mex67-5*, and *mtr2* yeast cells grown at 23°C or after a 2-h shift to 37°C. (P) Primary tRNA$^{Ile}_{UAU}$ transcripts with 5′ leaders, 3′ trailers, and introns (146–156 nucleotides [nt]); (I) 5′ and 3′ end-processed intron-containing tRNA$^{Ile}_{UAU}$ (136 nt). Ratios of signal intensities of I versus P (I/P) were calculated for each mutant and then normalized to the wild-type ratio incubated at the same temperature. Ethidium bromide staining of 5.8S and 5S rRNAs was used as a loading control for each lane. (*C*) Time-course Northern hybridization of pre-tRNA$^{Ile}_{UAU}$ for *mex67-5* and *mtr2* cells after the shift to 37°C at the indicated time points. 5S and 5.8S rRNA are the same as in *B*.

tRNA$^{Tyr}_{GUA}$). The temperature-sensitive mutant of the RanGTPase-activating (Rna1) protein, encoded by *rna1-1*, which is essential for RanGTPase-mediated nuclear export of macromolecules, served as a positive control for pre-tRNA accumulation due to tRNA nuclear–cytoplasmic exchange defects (Fig. 1B; Corbett et al. 1995; Sarkar and Hopper 1998).

Accumulation of the end-processed intron-containing pre-tRNA species (tRNA$^{Ile}_{UAU}$) in *mex67-5* and *mtr2* strains was complemented when the strains were transformed with multicopy plasmids expressing wild-type Mex67 and Mtr2, respectively (Supplemental Fig. S2). It should be noted that elevated levels of intron-containing primary tRNAs with 5′ leader and 3′ trailer sequences are observed in *mtr2* temperature-sensitive cells (Fig. 1B) as well as *mtr2* temperature-sensitive cells transformed with vector (Supplemental Fig. S2B) when grown at 23° C. The *mtr2* strain has a different genetic background compared with wild-type or *mex67-5* parental strains (see the Supplemental Material). As the *mtr2* strain was derived from meiosis of diploid cells, a wild-type isogenic parental strain is unavailable; it is likely that the observed accumulation of primary tRNA at 23°C is related to the *mtr2* strain's genetic background.

As anticipated, the plasmid-borne wild-type copies of the *MEX67* and *MTR2* genes also were able to complement the temperature-sensitive growth defects of *mex67-5* and *mtr2* cells, respectively (Supplemental Fig. S3). The *mex67-5* mutant strain is significantly more thermosensitive than the *mtr2* mutant strain; *mex67-5* cells with vector alone exhibited growth defects even at 30°C, consistent with a previous report (Hurt et al. 2000).

Since the Mex67–Mtr2 heterodimer functions in mRNA nuclear export, the pre-tRNA accumulation observed for *mex67-5* and *mtr2* cells at 37°C could be due to defective export of mRNAs that indirectly affect pre-tRNA processing. Such an indirect mechanism would be expected to have a delayed effect upon defective pre-tRNA splicing. Thus, Northern analyses were performed for *mex67-5* and *mtr2* cells at various times after shifting the cells to 37°C (Fig. 1C). *mex67-5* cells accumulated unspliced tRNA at the earliest time point (30 min), but *mtr2* cells accumulated detectable levels of pre-tRNAs only after the 60-min time point. Faster pre-tRNA accumulation in *mex67-5* cells versus *mtr2* cells could be due to the enhanced thermosensitivity of *mex67-5* cells (Supplemental Fig. S3). The relatively rapid accumulation of unspliced pre-tRNA$^{Ile}_{UAU}$ is consistent with direct roles of Mex67 and Mtr2 in affecting pre-tRNA processing.

Nuclear accumulation of tRNAs is observed in mex67-5 and mtr2 cells at the nonpermissive temperature

Since, in yeast, the SEN complex is located on the surface of the mitochondria, we considered three possible scenarios by which *mex67-5* cells could accumulate end-processed unspliced pre-tRNAs at 37°C: (1) After 5′ and 3′ end processing in the nucleus, nuclear export of tRNAs to the cytoplasm is inhibited. (2) pre-tRNAs exported out of the nucleus are inhibited in reaching the surface of mitochondria for splicing. (3) Inactivation of Mex67 or Mtr2 somehow affects SEN activity. According to the first scenario, inactivation of Mex67–Mtr2 would lead to the accumulation of tRNAs in the nucleus, whereas the second and third scenarios would not be expected to cause tRNA nuclear accumulation. To distinguish between the alternatives, we used fluorescent in situ hybridization (FISH) to determine the subcellular distribution of tRNA$^{Ile}_{UAU}$ and tRNA$^{Tyr}_{GUA}$.

First, to verify the known defect of *mex67-5* and *mtr2* in mRNA export, we assessed the location of poly(A)$^+$-containing RNA in cells using a 50-nucleotide oligo(d)T probe, as described previously (Fig. 2B; Sarkar and Hopper 1998). For wild-type cells incubated for 4 h at 37°C (Fig. 2D, top panel), poly(A)$^+$-containing RNA was distributed throughout the cells. In contrast and as anticipated, for *mex67-5* and *mtr2* cells incubated for 4 h at 37°C (Fig. 2D, third and fourth rows), poly(A)$^+$-containing RNA was predominately nuclear. Similarly, poly(A)$^+$-containing RNA distributions were nuclear and cytoplasmic in

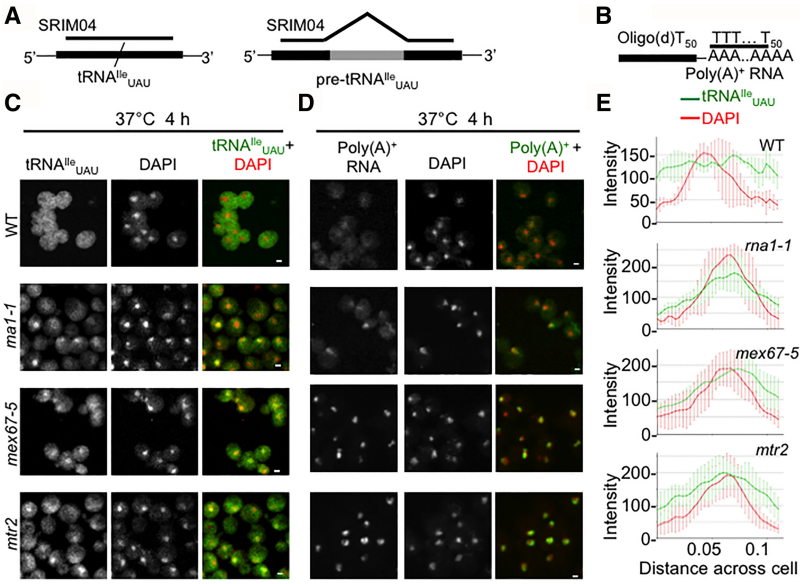

Figure 2. tRNA$^{Ile}_{UAU}$ accumulates in the nuclei of *mex67-5* cells at the nonpermissive temperature. (*A*) Schematic presentation of the probe SRIM04 used for FISH. Probe SRIM04 can hybridize to intron-containing as well as spliced tRNA$^{Ile}_{UAU}$, as depicted. (*B*) Schematic presentation of the probe oligo(dT)$_{50}$ used to determine the subcellular localization of poly(A)$^+$ RNA in the FISH assay. (*C*) Wild-type, *rna1-1*, *mex67-5*, and *mtr2* cells were grown at 23°C, and the log phase cells were shifted to 37°C. Cells were harvested at the indicated times after the temperature shift, and the subcellular localization of tRNA$^{Ile}_{UAU}$ was examined by FISH using probe SRIM04. Representative micrographs show subcellular localization for tRNA$^{Ile}_{UAU}$ (green in the merged panel), and the nuclear DNA was visualized by DAPI staining (red in the merged panel). Bars, 2 µm. (*D*) Subcellular localization of poly (A)$^+$ RNA was examined by FISH using probe oligo(dT)$_{50}$. Representative micrographs show the localization of poly(A)$^+$ RNA (green in the merged panel), and the nuclear DNA was visualized by DAPI staining (red in the merged panel). Bars, 2 µm. (*E*) Five random cells from the micrographs (Fig. 4C, below) of a single section were chosen (images of the cells quantitated are shown in Supplemental Fig. S6), and fluorescence intensities of FISH and DAPI fluorophores were calculated on a line drawn from one end to the other end of each cell across the nucleus. Each graph shows the intensity profile of individual fluorophores, where the values represent the relative intensity average ± SD from the five cells.

the *rna1-1* mutant strain when the cells were incubated for 4 h at 37°C (Fig. 2D, second row), confirming previous observations (Sarkar and Hopper 1998).

We next determined the subcellular localization of tRNAs in *mex67-5* and *mtr2* temperature-sensitive cells at nonpermissive temperatures. We used FISH probes SRIM04 and SRIM15 (Fig. 2A; Supplemental Figs. S4A, S5A; Supplemental Table. S1), which detect both the intron-containing and spliced forms of tRNA$^{Ile}_{UAU}$ and tRNA$^{Tyr}_{GUA}$, respectively (Sarkar et al. 1999; Wu et al. 2015). As expected, tRNA$^{Ile}_{UAU}$ and tRNA$^{Tyr}_{GUA}$ were distributed evenly throughout the cells when the wild-type cells were grown at 23°C (Supplemental Fig. S5B, tRNA$^{Tyr}_{GUA}$, top row) or shifted for 2 h to 37°C (Supplemental Fig. S4B, tRNA$^{Ile}_{UAU}$, top row) or 4 h (Fig. 2C, tRNA$^{Ile}_{UAU}$; Supplemental Fig. S5B, tRNA$^{Tyr}_{GUA}$, top row). In contrast and also as expected, *rna1-1* cells displayed an even distribution of tRNAs for cells grown at 23°C (Supplemental Fig. S5B, second row) and prominent nuclear pools of tRNA$^{Ile}_{UAU}$ and tRNA$^{Tyr}_{GUA}$ for cells incubated for 4 h at 37°C (Fig. 2C, tRNA$^{Ile}_{UAU}$; Supplemental Fig. S5B, tRNA$^{Tyr}_{GUA}$, second row). Consistent with Mex67 and Mtr2 functioning in pre-tRNA nuclear export, *mex67-5* and *mtr2* cells displayed robust nuclear accumulation of tRNA$^{Ile}_{UAU}$ and tRNA$^{Tyr}_{GUA}$ upon incubation for either 2 h (Supplemental Fig. S4B, tRNA$^{Ile}_{UAU}$) or 4 h (Fig. 2C, third and fourth rows, tRNA$^{Ile}_{UAU}$; Supplemental Fig. S5B, tRNA$^{Tyr}_{GUA}$) at 37°C. To quantitate tRNA subcellular distributions, we plotted the fluorescence intensities of the FISH and DAPI signals (arrows in Supplemental Fig. S6 indicate the cells analyzed). The maximum of the distribution of FISH fluorescence overlapped with DAPI fluorescence in *rna1-1* cells, whereas, in wild-type cells, the FISH fluorescence distribution was relatively flat without a distinct maximum (Fig. 2E, tRNA$^{Ile}_{UAU}$; Supplemental Fig. S5C, tRNA$^{Tyr}_{GUA}$). *mex67-5* and *mtr2* cells showed a distinct maxima of FISH fluorescence overlapping the nucleus, confirming the visual observation of tRNA nuclear accumulation. The data support the hypothesis that Mex67 and Mtr2 function in tRNA nuclear export.

Overexpression of the Mex67–Mtr2 heterodimer suppresses pre-tRNA nuclear accumulation in los1Δ cells

Los1 is the only known β-importin designated exclusively for tRNA export from the nucleus to the cytoplasm. If Mex67–Mtr2 is indeed a bona fide tRNA nuclear exporter, it may be able to substitute for the role of Los1 in tRNA subcellular dynamics. Robust nuclear accumulation of end-processed intron-containing tRNAs was observed in the presence of endogenous levels of Mex67–Mtr2 in *los1Δ* cells (Sarkar and Hopper 1998). This pre-tRNA nuclear accumulation may be a result of suboptimal amounts of tRNAs being exported out of the nucleus due to a limiting level of Mex67–Mtr2 for tRNA export under endogenous conditions, similar to what has been observed for Mex67–Mtr2-mediated pre-60S ribosomal subunit export (Lo and Johnson 2009). We assessed whether additional copies of Mex67–Mtr2 can rescue the phenotype of defective tRNA nuclear export of *los1Δ* cells.

We generated a multicopy plasmid that contains both *MEX67* and *MTR2* regulated by their endogenous promoters and transformed this plasmid or the vector into *los1Δ* cells (*los1Δ*+M–M and *los1Δ*+V, respectively).

RT-qPCR analyses revealed approximate fivefold increases in *MEX67* and *MTR2* mRNA levels in *los1*Δ+M–M cells compared with *los1*Δ with vector (*los1*Δ+V) (Supplemental Fig. S7). Northern analyses showed that *los1*Δ cells transformed with the vector (*los1*Δ+V) accumulated the end-processed intron-containing forms of all 10 tRNA species expressed from intron-containing tRNA genes, compared with the wild-type cells transformed with the vector (WT+V), which showed low levels of pre-tRNA species (Fig. 3). Remarkably, upon fivefold overexpression of Mex67–Mtr2, the level of the pre-tRNA species in *los1*Δ cells decreased significantly to a level similar to that in wild-type cells (cf. *los1*Δ +M–M and WT+V in Fig. 3). For some tRNAs (such as $tRNA^{Leu}_{UAG}$ and $tRNA^{Phe}_{UGG}$), overexpression of Mex67–Mtr2 or Los1 in *los1*Δ cells resulted in reduction of the levels of end-processed intron-containing tRNAs beyond that in wild-type cells, supporting previous reports that Los1 is limiting in wild-type yeast cells (Fig. 3B; Ghavidel et al. 2007) and indicating that endogenous Mex67–Mtr2 levels may be also limiting for tRNA export. The data document that as little as a fivefold excess of Mex67–Mtr2 suppresses the pre-tRNA splicing defects of *los1*Δ cells.

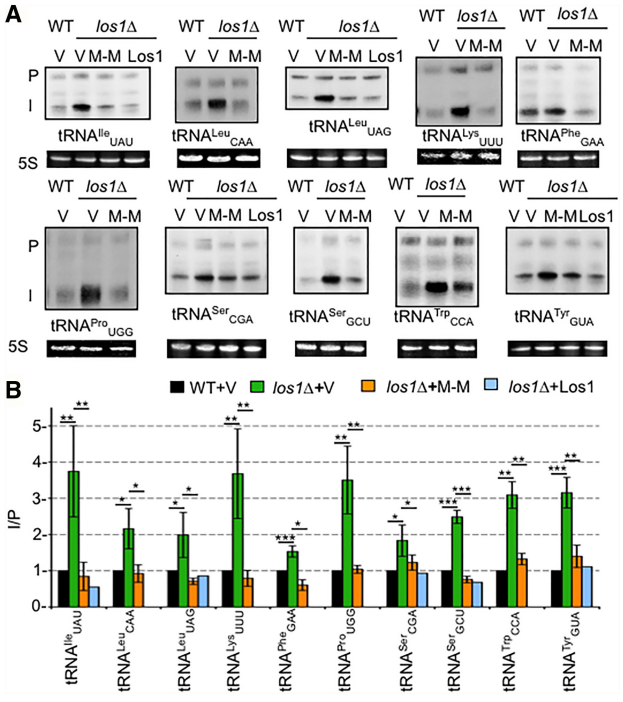

Figure 3. Suppression of accumulation of all 10 end-mature intron-containing tRNAs in *los1*Δ cells upon fivefold overexpression of Mex67–Mtr2 (*A*) Northern hybridization of small RNAs isolated from wild-type yeast cells transformed with vector (WT +V) and *los1*Δ cells transformed with vector (*los1*Δ+V), *MEX67* and *MTR2* (*los1*Δ+M–M), or *LOS1* (*los1*Δ+Los1). Specific intron-containing tRNAs, as indicated *below* each blot, were identified using complementary probes (Supplemental Table. S1). (P) Initial precursor intron-containing tRNAs; (I) end mature intron-containing tRNAs. 5S rRNA, visualized by SYBR Gold staining, served as the loading control. (*B*) I/P ratios were calculated for each of the intron-containing tRNAs (as indicated by specified colored bars *above* the bar graph) and normalized to WT+V (black column). I/P ratios with mean ± SD from three or more independent experiments were calculated, except for the I/P ratio of the *los1*Δ+Los1 strain (blue columns), which was performed only once for the indicated tRNA species.

We also assessed the effects of Mex67–Mtr2 overexpression on tRNA nuclear export. We conducted FISH analyses using a probe (SRIM03) complementary to the intron of $tRNA^{Ile}_{UAU}$ (Fig. 4A; Supplemental Table S1; Sarkar and Hopper 1998). SRIM03 detects intron-containing species of $tRNA^{Ile}_{UAU}$, which is present in low levels in the nucleus of all yeast cells due to constitutive nuclear transcription (Fig. 4B). Quantitation of the FISH fluorescence intensities showed that the maxima of FISH fluorescence overlap with DAPI fluorescence. As anticipated, increases in the nuclear FISH signal intensities were observed in the absence of Los1-mediated primary tRNA nuclear export compared with wild-type cells (*los1*Δ+V cells vs. WT+V cells in Fig. 4B). Accordingly, the average FISH signal intensities of *los1*Δ+V cells were higher than for WT+V cells (Fig. 4C). Overexpression of Mex67–Mtr2 in *los1*Δ cells (*los1*Δ+M-M) resulted in a significant decrease of $tRNA^{Ile}_{UAU}$ FISH signal intensities in most cells (Fig. 4B,C). Using the FISH probe SRIM04 that detects both intron-containing and mature forms of $tRNA^{Ile}_{UAU}$, we verified $tRNA^{Ile}_{UAU}$ nuclear accumulation in *los1*Δ+V cells and the rescue of this phenotype in *los1*Δ+M–M cells (Supplemental Fig. S8). Thus, by both Northern hybridization analyses of pre-tRNA accumulation and FISH analyses of the subcellular distribution of tRNA, the data document that fivefold overexpressed Mex67–Mtr2 can substitute for Los1. Interestingly, no synthetic-negative genetic interactions were observed between *mex67-5* and *los1*Δ at high growth temperatures, as the *los1Δ mex67-5* double mutant did not show pronounced growth defects when compared with either *mex67-5* or *los1*Δ single mutants when incubated at various semipermissive temperatures for *mex67-5* (Supplemental Fig. S9).

Mex67 binds $tRNA^{Ile}_{UAU}$

The Northern analyses and cytological and genetic data support the hypothesis that Mex67–Mtr2 functions in tRNA nuclear export. To assess whether Mex67–Mtr2 functions directly in tRNA nuclear export by forming export complexes with tRNAs, we used a modified form of a previously described in vivo coimmunoprecipitation assay (Huang and Hopper 2015). For this method, a multicopy plasmid encoding protein A-tagged Mex67 and untagged Mtr2 was transformed in *mex67-5* cells. The exogenously expressed tagged protein was functional, as it was able to complement the growth defects of *mex67-5* cells at 37°C (Supplemental Fig. S10). We also expressed protein A-tagged mutant Mex67-5 that possesses a His400-to-Tyr400 substitution in the M domain that led to weakened interactions with Mtr2 and resulted in loss of functional heterodimer formation and defective mRNA nuclear export (Segref et al. 1997; Santos-Rosa et al. 1998). Since *mex67-5* also showed potential nuclear

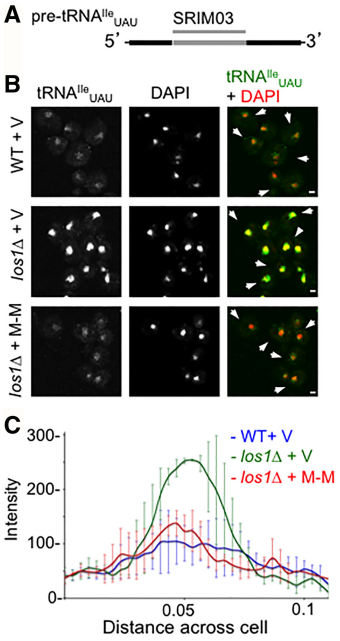

Figure 4. Nuclear accumulation of intron-containing tRNA$^{Ile}_{UAU}$ in the absence of Los1 is suppressed upon fivefold overexpression Mex67–Mtr2. (*A*) Schematic presentation of the probe SRIM03 used for FISH. (*B*) Wild-type yeast cells transformed with vector (WT+V), *los1*Δ cells transformed with vector (*los1*Δ+V), and los1Δ cells transformed with a plasmid encoding both *MEX67* and *MTR2* with their endogenous promoters (*los1*Δ+M–M) were grown at 23°C and harvested, and the accumulation of pre-tRNA species of tRNA$^{Ile}_{UAU}$ (green in the merged panel) was examined by FISH. Nuclear DNA is visualized by DAPI staining (red in the merged panel). Bars, 2 µm. (*C*) Five cells from each micrograph in *B* were analyzed for the distribution of fluorescence intensities of FISH and DAPI staining in each cell. In all cases, the maxima of the FISH fluorescence coincided with that of DAPI fluorescence of the nuclei. The intensity profiles of FISH fluorescence from the three strains were plotted. The relative intensities represent average ± SD from the five cells.

tRNA export defects (Fig. 2B,C), protein A-tagged *Mex67-5* may not bind tRNA and could serve as a negative control in our assay. Protein A-tagged *Mex67-5* protein was also unable to rescue temperature sensitivity of *mex67-5* cells at 37°C (Supplemental Fig. S10), showing that the protein A-tagged *Mex67-5* is inactive at 37°C. Since Mtr2 can serve as a multicopy suppressor for *mex67-5*, Mtr2 was not coexpressed with protein A-tagged *Mex67-5* (Supplemental Fig. S11A; Santos-Rosa et al. 1998). As positive and negative controls, we conducted contemporaneous pull-down studies of MORF-tagged Los1 protein with transient coexpression of Ran constructs encoding either induced RanGTP (Gsp1-G21V)-locked or RanGDP (Gsp1-T24N)-locked mutants, as described previously (Huang and Hopper 2015).

Formaldehyde cross-linked cells expressing the tagged proteins were immunoprecipitated using IgG-conjugated magnetic beads, and the enriched proteins were observed by Sypro-Ruby staining (Fig. 5A). Although protein A-tagged Mex67 and MORF-tagged Los1 proteins were substantially enriched in the pull-downs, enrichment of protein A-tagged mutant Mex67-5 protein was not observed. This lack of enrichment was not due to decreased production of Mex67-5 but rather was due to the instability of the mutant protein at 37°C (Supplemental Fig. S11B), consistent with previous studies showing that Mex67-5 is rapidly destroyed by ubiquitin ligase San1 and ubiquitin-conjugating enzymes Cdc34 and Ubc1 (Estruch et al. 2009).

Western blot analyses of the coimmunoprecipitated proteins using anti-Ran (Gsp1) showed, predictably, that Ran copurified with MORF-tagged Los1 in samples with Ran locked in the GTP-bound state (Supplemental Fig. S12) but not in samples with Ran locked in the GDP-bound state (data not shown), as reported previously (Huang and Hopper 2015). Since Mex67 and Mtr2 proteins are not β importins, no Ran copurification was detected with the protein A-tagged Mex67–Mtr2 heterodimer, also as expected (Supplemental Fig. S12).

To determine whether RNAs copurify with Mex67, the pull-down fractions were analyzed by RT–PCR (Fig. 5C–F). To support the qualitative observations, RT-qPCR analyses were performed in triplicates on copurified RNAs from the same yeast cultures of the described strains (Fig. 5B) and on coimmunoprecipitation experiments from a total of three independent biological replicates (Supplemental Fig. S13). Since Mex67–Mtr2 is the principal exporter of nuclear mRNAs, it is expected that mRNAs would copurify with protein A-tagged Mex67. Consistent with a previous report (Oeffinger et al. 2007), *PGK1* mRNA copurified with the Mex67–protein A pull-down fraction (Fig. 5C). On the other hand, since Los1 specifically binds tRNAs (Hellmuth et al. 1998), we anticipated that there would be no *PGK1* mRNA enrichment in cells expressing MORF-tagged Los1 and RanGTP-locked or RanGDP-locked forms. Consistent with the predictions, no detectible *PGK1* mRNA coenriched with MORF-tagged Los1 (Fig. 5C). RT-qPCR analyses confirmed that only the Mex67–Mtr2 heterodimer interacts with PGK1 mRNA (Fig. 5B; Supplemental Fig. S13).

To determine whether Mex67–Mtr2 binds intron-containing pre-tRNAs, RT–PCR assays were conducted with primers that specifically amplify unspliced pre-tRNA$^{Ile}_{UAU}$ (Fig. 5H; Supplemental Table S2). Unspliced pre-tRNA$^{Ile}_{UAU}$ copurified with Mex67 as well as with Los1 in cells expressing GTP-locked Ran but not Los1 in cells expressing GDP-locked Ran (Fig. 5D). Coenrichment of intron-containing pre-tRNA with Mex67 was also supported by RT-qPCR (Fig. 5B; Supplemental Fig. S13). Copurification of spliced tRNA$^{Ile}_{UAU}$ was detected by reverse transcription of bound tRNAs using a primer that spans the 5′ and 3′ exon junctions (Fig. 5I; Supplemental Table S2) followed by amplification of the cDNA. The data show that, like Los1 in the presence of RanGTP, Mex67 binds spliced tRNA$^{Ile}_{UAU}$ (Fig. 5E). This observation was also validated by RT-qPCR (Fig. 5B; Supplemental Fig. S13). Since splicing of tRNA$^{Ile}_{UAU}$ in yeast takes place on the mitochondrial surface only after the primary nuclear export step, copurification of mature tRNA$^{Ile}_{UAU}$ with Mex67 suggests that the heterodimer participates in

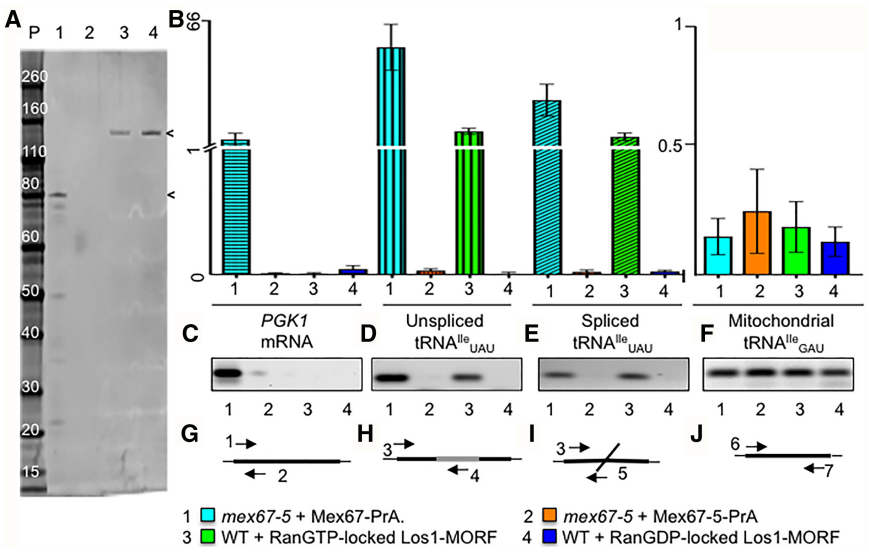

Figure 5. tRNAs copurify with Mex67. (*A*) Sypro Ruby staining of proteins enriched by coimmunoprecipitation. (Lane *1*) *mex67-5* cells coexpressing protein A-tagged Mex67 and untagged Mtr2 from a single multicopy plasmid. (Lane *2*) *mex67-5* cells expressing plasmid-borne protein A-tagged Mex67-5. (Lane *3*) Wild-type cells coexpressing plasmid-borne and plasmid-inducible RanGTP-locked MORF-tagged Los1. (Lane *4*) Wild-type cells coexpressing plasmid-borne and plasmid-inducible RanGDP-locked MORF-tagged Los1. (Black arrowheads) Enriched tagged proteins. (Lane *P*) Protein size markers. (*B*) RT-qPCR analyses for *PGK1* mRNA (horizontal lines in bars), unspliced $tRNA^{Ile}_{UAU}$ (vertical lines in bars), spliced $tRNA^{Ile}_{UAU}$ (diagonal lines in bars), and mitochondrial encoded $tRNA^{Ile}_{GUA}$ (solid bars). The numbers along the *X*-axis refer to the same strains as specified in the lanes in *A* and are also color-coded. (Turquoise) Lane *1*; (tangerine) lane *2*; (green) lane *3*; (blueberry) lane *4*. The numbers along the *Y*-axis denote absolute amounts (in femtograms) of RNA species enriched by coimmunoprecipitation assay. Bars denote values that represent the average ± SD of triplicate RT-qPCR assays from the same round of coimmunoprecipitation experiments of all four strains as in *A*. (*C–F*) RT–PCR analyses of enriched *PGK1* mRNA, unspliced $tRNA^{Ile}_{UAU}$, spliced $tRNA^{Ile}_{UAU}$, and mitochondrial-encoded $tRNA^{Ile}_{GAU}$, respectively, in coimmunoprecipitation assays. The numbers *below* the panel signify lane numbers as in *A*. (*G–J*) Schematic representation of pulled-down *PGK1* mRNA, unspliced $tRNA^{Ile}_{UAU}$, spliced $tRNA^{Ile}_{UAU}$, and mitochondrial-encoded $tRNA^{Ile}_{GAU}$, respectively, showing regions of complementarity with the specific primers (Supplemental Table S2) used for the RT–PCR and RT-qPCR assays. (Black lines) 5′ leader and 3′ trailer ends; (black boxes) exons; (gray box) intron; (slash) splice junction.

both the primary and the nuclear re-export steps in tRNA subcellular dynamics.

We conducted additional controls to verify that the observed enrichment of RNAs was not due to amplification of contaminating RNAs cross-linked to Mex67–Mtr2 complexes. Mex67–Mtr2 and Los1 are nuclear–cytoplasm shuttling proteins not associated with the mitochondrial matrix in vivo. Therefore, mitochondrial-encoded tRNAs detected in the pull-down experiments should represent RNA contamination. Low levels of mitochondrial $tRNA^{Ile}_{GAU}$ could only be detected by using high concentrations of PCR probes and increasing the number of PCR cycles. Similar levels of mitochondrial $tRNA^{Ile}_{GUA}$ were present in the pull-down fractions for each of the four strains, indicating equivalent low levels of contaminating tRNAs in the coimmunoprecipitated samples (Fig. 5B,F; Supplemental Fig. S13). Thus, the enrichment of mRNA and tRNAs observed for tagged Mex67 and Los1 pull-down fractions demonstrates the specificity of our coimmunoprecipitation assay.

Only a subset of the 10 intron-containing pre-tRNA species accumulates to high levels in mex67-5 and mtr2 cells at 37°C

A role for Mex67–Mtr2 in tRNA nuclear export was first evidenced by the accumulation of end-processed intron-containing pre-$tRNA^{Ile}_{UAU}$ and pre-$tRNA^{Tyr}_{GUA}$ in *mex67-5* and *mtr2* cells at 37°C and was further supported by the overexpression studies showing that fivefold elevated levels of Mex67–Mtr2 suppressed tRNA accumulation defects for all 10 intron-containing tRNA families of *los1Δ* cells. This led to the prediction that all 10 families of intron-containing tRNAs would be similarly affected in *mex67-5* and *mtr2* cells. We thus extended Northern hybridization analyses to the eight remaining intron-containing tRNA families. Surprisingly, only a subset of the end-processed intron-containing pre-tRNA families accumulated pre-tRNAs to significant levels in *mex67-5* and *mtr2* cells at 37°C (Fig. 6; Supplemental Fig. S14). End-processed intron-containing $tRNA^{Ile}_{UAU}$, $tRNA^{Tyr}_{GUA}$, $tRNA^{Trp}_{CCA}$, and $tRNA^{Pro}_{UGG}$ accumulated increased levels of pre-tRNA species in *mex67-5* and *mtr2* cells compared with wild-type cells when incubated for 2 h at 37°C. Significant amounts of pre-tRNA accumulation were not detected for the remaining six intron-containing tRNA families in *mex67-5* and *mtr2* cells. Since Los1 is active in *mex67-5* and *mtr2* cells at 37°C, accumulation of particular pre-tRNA species observed in *mex67-5 LOS1* and *mtr2 LOS1* cells at 37°C may reflect the efficiency (or the lack of thereof) of Los1-mediated nuclear export of these tRNA species. Thus, Los1 collaborates with Mex67–Mtr2 for efficient export of these four tRNA species. The data are consistent with Mex67–Mtr2 providing support for the nuclear export of a subset of tRNAs under standard growth conditions.

Discussion

The primary nuclear export of end-processed partially modified intron-containing as well as intronless pre-

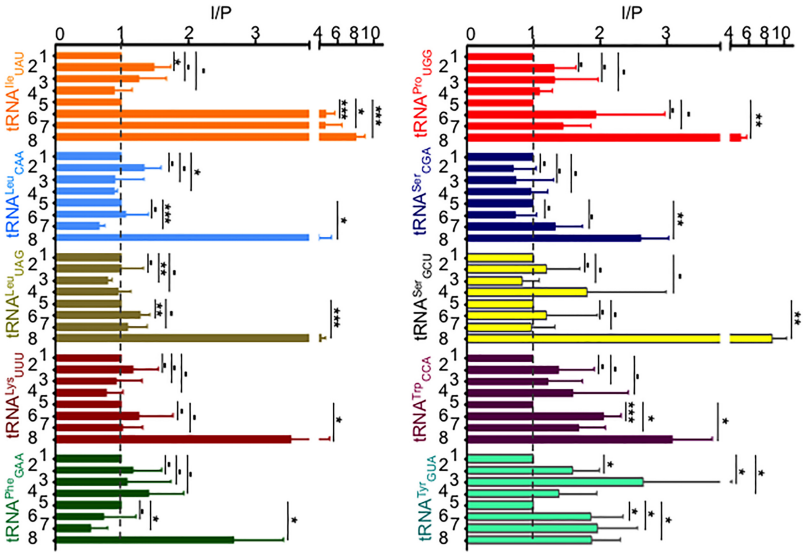

Figure 6. Inactivation of Mex67 or Mtr2 leads to the accumulation of end-matured intron-containing tRNAs for a subset of tRNA families. Small RNAs were purified from the following strains and incubated at the indicated temperatures to perform Northern hybridization: wild-type (1), *mex67-5* (2), *mtr2Δ* (3), and *rna1-1* (4) at 23°C and wild-type (5), *mex67-5* (6), *mtr2Δ* (7), and *rna1-1* (8) at 37°C for 2 h (representative Northern blots and specific probes are shown in Supplemental Fig. S14). I/P ratios of each tRNA species from cells at 23°C and 37°C were normalized to wild-type cells at 23°C and 37°C, respectively. Values indicate the average ± SD of normalized I/P ratio of each tRNA species, where $n = 3$ or more independent experiments. (***) $P < 0.0005$; (**) $P < 0.005$; (*) $P < 0.05$; (–) $P > 0.05$ (which is considered as nonsignificant), as determined by Student's *t*-test.

tRNAs constitutes the first of the three tRNA nuclear–cytoplasmic shuttling steps required for tRNA biosynthesis in eukaryotic cells (Phizicky and Hopper 2010, 2015; Hopper 2013). Yeast Los1 and the vertebrate and plant homologs Exportin-t and PAUSED were the first tRNA nuclear exporters to be discovered (Hopper et al. 1978, 1980; Arts et al. 1998a; Hellmuth et al. 1998; Kutay et al. 1998; Sarkar and Hopper 1998). However, genetic studies provide evidence that Los1/Exportin-t/PAUSED cannot be the sole tRNA nuclear exporters. To identify Los1-independent tRNA nuclear exporters, we interrogated the budding yeast proteome for mutants defective in tRNA nuclear export (Wu et al. 2015). Here we report that the Mex67–Mtr2 heterodimer identified in the screen functions in primary tRNA nuclear export.

We provide four lines of evidence that Mex67–Mtr2 serves to export tRNA from the nucleus to the cytoplasm in the initial tRNA export step: (1) By Northern analysis, we showed that *mex67-5* and *mtr2* temperature-sensitive mutants accumulate end-matured intron-containing pre-tRNAs at the nonpermissive temperature—a proxy for defective pre-tRNA initial nuclear export. (2) By RNA FISH, we showed that there are increased nucleoplasmic pools of tRNAs in *mex67-5* and *mtr2* cells at the nonpermissive temperature. (3) By genetic interaction studies, we showed that Mex67–Mtr2 can substitute for Los1 when fivefold in excess. (4) By biochemical studies, we showed that Mex67 binds intron-containing tRNAs in vivo. Despite these strong lines of evidence for a role in tRNA nuclear export, we did not detect negative genetic synthetic defects in *los1Δ mex67-5* double-mutant cells by scoring growth at various semipermissive temperatures for *mex67-5*. It is possible that negative genetic interactions may be evidenced under other conditions, such as nutrient deprivation (Huang and Hopper 2014, 2016) or aging (Lord et al. 2017). It is also possible that Crm1 or yet another unidentified tRNA nuclear exporter maintains the viability of these *los1Δ mex67-5* cells by exporting sufficient cytoplasmic tRNA even in the absence of both Los1 and Mex67.

The major biological role of the Mex67 in yeast, flies, and vertebrates is for mRNA nuclear export (Santos-Rosa et al. 1998; Katahira et al. 1999; Kelly and Corbett 2009; Stewart 2010; Katahira 2012), although the complex has also been implicated in nuclear export of pre-40S and pre-60S ribosomes, viral RNAs, and telomerase RNA (Ernst et al. 1997b; Pasquinelli et al. 1997; Yao et al. 2007; Faza et al. 2012; Wu et al. 2014). Thus, the data reported here extend the biological role of the yeast Mex67–Mtr2 heterodimer to include tRNA nuclear export. There are hints that the vertebrate homolog of Mtr2 (Nxt1) may also function in tRNA nuclear export, as it has been reported to stimulate nuclear tRNA export in permeabilized HeLa cells (Ossareh-Nazari et al. 2000).

The question arises of why there would be multiple tRNA nuclear exporters (Fig. 7). Biochemical and structural studies have shown that Los1/Exportin-t binds the tRNA backbone elbow and acceptor stem but has no interactions with the anti-codon stem–loop or introns (Arts et al. 1998b; Lund and Dahlberg 1998; Lipowsky et al. 1999; Cook et al. 2009). Since the tRNA interaction site for Los1 is common to all appropriately structured and end-matured tRNAs, one would predict that Los1/Exportin-t would bind all tRNAs with similar efficiencies and therefore would export all tRNA families similarly. However, it has been reported previously that immobilized human Exportin-t binds different tRNA species with different affinities (Li and Sprinzl 2006). Here, we show that yeast Los1 appears to affect individual isoacceptor tRNAs differently. We determined the relative levels of end-processed intron-containing pre-tRNAs for each of the 10 families of intron-containing tRNAs in *los1Δ* cells as compared with wild-type cells. If Los1 exported each of the intron-containing pre-tRNAs with similar efficiency, one would predict that the fold increase of accumulation of end-processed intron-containing tRNA among the tRNA families would be similar. In contrast to this prediction, the levels differed from a low I/P (5′ and 3′ end-processed intron-containing to primary transcripts

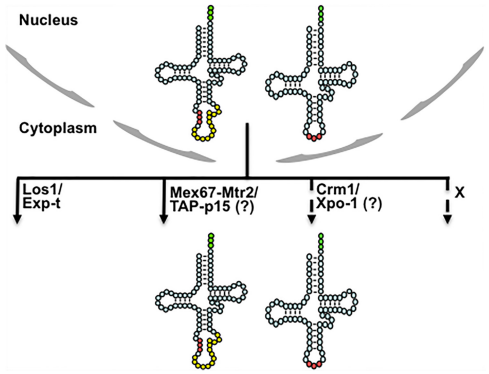

Figure 7. Multiple parallel pathways for primary tRNA nuclear export in yeast. Following 5′ and 3′ end processing, both intron-containing and intronless newly synthesized pre-tRNAs are escorted out of the nucleus by multiple parallel primary tRNA nuclear export pathways in yeast. (Yellow circles) Intron sequences; (blue circles) exons; (red circles) the anti-codon; (green circles) the added CCA trinucleotide. For simplicity, tRNA nucleotides modified in the nucleus prior to export are not indicated. The proteins involved in the primary tRNA nuclear export pathways are indicated. Solid arrows denote proteins with a verified role in the primary tRNA nuclear export, and dashed arrows denote possible roles in the process. The "X" next to one of the dashed arrows denotes yet unidentified tRNA nuclear export pathways. Since the roles of vertebrate orthologs of Mex67–Mtr2 and Crm1 in the primary nuclear tRNA export process are unknown, they are marked with question marks.

with 5′ leaders, 3′ trailers, and introns) ratio of ~1.5-fold for $tRNA^{Phe}_{GAA}$ and $tRNA^{Ser}_{CGA}$ to a high of >3.5-fold for $tRNA^{Ile}_{UAU}$, $tRNA^{Lys}_{UUU}$, and $tRNA^{Pro}_{UGG}$ (Fig. 3). Thus, nuclear export of some tRNAs, such as $tRNA^{Ile}_{UAU}$, $tRNA^{Lys}_{UUU}$, and $tRNA^{Pro}_{UGG}$, appears to be highly dependent on Los1, whereas nuclear export of others, such as $tRNA^{Phe}_{GAA}$ and $tRNA^{Ser}_{CGA}$, appears to be at least partially Los1-independent. The dependence of $tRNA^{Tyr}_{GUA}$, $tRNA^{Ile}_{UAU}$, and $tRNA^{Trp}_{CCA}$ on Los1 for tRNA export was also demonstrated in *nup100*Δ yeast cells, as these three tRNA species were found to accumulate in the nuclei of *nup100*Δ mutants even in the presence of endogenous levels of Los1 (Lord et al. 2017). So, one role for Los1-independent nuclear export pathways might be to aid efficient nuclear export for a subset of tRNAs. However, pre-$tRNA^{Phe}_{GAA}$ and pre-$tRNA^{Ser}_{CGA}$ that fail to accumulate to high levels in *los1*Δ cells also fail to accumulate to high levels in *mex67-5* and *mtr2* temperature-sensitive cells, suggesting that nuclear export of these two pre-tRNA species may also rely on a third exporter. We were unable to determine the commonalities among members of the intron-containing pre-tRNAs that accumulate in *mex67-5* or *mtr2* cells. However, the observed differences in the levels of pre-tRNA accumulation are unlikely to be due to the instability of these pre-tRNAs at 37°C because all of the members of the 10 intron-containing tRNA families accumulate to elevated levels in *rna1-1* cells under the same growth conditions.

Another reason that cells may have evolved multiple tRNA nuclear exporters is to aid responses to environmental conditions. For example, Los1 cellular level, which is limiting (Ghavidel et al. 2007), is reduced in response to acute amino acid deprivation (Huang and Hopper 2014). Since end-matured intron-containing pre-tRNAs do not accumulate upon amino acid deprivation, perhaps Mex67–Mtr2 (or Crm1 or an unidentified tRNA nuclear exporter) compensates for reduced levels of Los1 under these conditions. As there is likely to be substrate preference among the various tRNA exporters, their regulation in response to particular environmental conditions or stresses could influence the cytoplasmic pools of particular tRNAs—and hence the proteome—under various conditions.

Finally, there is a possibility that Mex67–Mtr2 functions in nuclear export of capped primary tRNA transcripts. Recently, novel spliced tRNAs with 5′ methyl-capped leaders and 3′ trailer sequences were identified in yeast and human cells (Ohira and Suzuki 2016). As biochemical and structural studies show that Los1 is unable to bind tRNAs with unprocessed ends in yeast (Arts et al. 1998b; Lipowsky et al. 1999; Cook et al. 2009), these end-extended tRNAs must have been exported to the cytoplasm for splicing via a Los1-independent nuclear exporter. Since Mex67–Mtr2 can efficiently export 5′-capped mRNA cargoes, one possibility is that capped pre-tRNAs are exported via Mex67–Mtr2. If this were the case, one might predict that initial tRNA transcripts with unprocessed termini and containing introns would accumulate in *mex67-5* or *mtr2* temperature-sensitive cells. However, by Northern hybridization studies, we did not detect accumulation of such pre-tRNAs in the mutants at the nonpermissive temperature (Fig. 1B). Rather, *mex67-5* and *mtr2* temperature-sensitive cells accumulate end-processed intron-containing pre-tRNAs. It is still feasible that Mex67–Mtr2 does function in the nuclear export of 5′-capped initial tRNA transcripts as well as mature tRNAs, and, if so, perhaps the putative increased nuclear retention of such pre-tRNAs in *mex67-5* or *mtr2* temperature-sensitive cells allows sufficient time to complete end processing. Further studies are warranted to determine whether Mex67–Mtr2 and its orthologs play a role in the nuclear export of these capped tRNAs in yeast and human cells.

Mex67–Mtr2 generally binds RNAs via adaptor proteins such as Yra1, Npl3, Pab1, and Nab2 (Kelly and Corbett 2009). However, none of the known Mex67–Mtr2 adaptors was uncovered in our proteome-wide interrogation for defects in tRNA biology (Wu et al. 2015). There also is precedence for Mex67/Nxf1–Mtr2/Nxt1 to interact directly with RNAs such as heat-shock mRNAs (Zander et al. 2016) and 5S rRNA (Yao et al. 2007). Moreover, Nxf1–Nxt1 directly binds unspliced RNA of retroviruses that contain a CTE (Ernst et al. 1997a; Pasquinelli et al. 1997). The CTE folds into an extended twofold symmetric stem–loop structure that is characterized by an internal loop, a terminal loop, and a tetranucleotide bulge adjacent to a terminal loop (Tabernero et al. 1996, 1997; Ernst et al. 1997b). Since bulges and loops are ubiquitous structural features of tRNAs, it is possible that the Mex67–Mtr2 heterodimer recognizes comparable identity elements in folded tRNAs and directly binds tRNA for nuclear export.

It is equally possible that interactions between tRNA and Mex67–Mtr2 are adapter protein-mediated, as yeast Mex67–Mtr2 purified from *Escherichia coli* was unable to bind to yeast tRNAs (Santos-Rosa et al. 1998; Yao et al. 2007). Our in vivo coimmunoprecipitation assay using protein A-tagged Mex67 should enrich for a mixture of tRNA and mRNA export complexes, and therefore it is unknown whether any adapter proteins participate in the tRNA export pathways or whether adapter proteins are required for nuclear tRNA export. Future investigations into the mechanism of nuclear tRNA export by Mex67 and Mtr2 are warranted.

In summary, there are at least two (Los1 and Mex67–Mtr2)—and possibly a third (Crm1) and fourth (unidentified [X])—primary nuclear tRNA exporters in budding yeast (Fig. 7). There also appears to be multiple parallel pathways (Los1, Mex67–Mtr2, and Msn5) for tRNA nuclear re-export. Moreover, several proteins with varied functions, such as Ssa2, Mtr10, Dhh1, and Pat1, potentially provide parallel pathways for retrograde import of cytoplasmic tRNAs to the nucleus (Senger et al. 1998; Shaheen and Hopper 2005; Hurto and Hopper 2011; Takano et al. 2015). Like for tRNA subcellular dynamics, multiple pathways are used for preribosome nuclear export, as both Crm1/Exportin-1 and Nxf1–Nxt1 export the pre-40S and pre-60S subunits (Gadal et al. 2001; Okamura et al. 2015). Also, although most mRNAs use the Mex67–Mtr2-dependent export machinery, a subset of mRNAs encoding stress proteins is exported by Crm1/Exportin-1 (Natalizio and Wente 2013; Okamura et al. 2015). It will be interesting to learn the substrate specificity of the parallel pathways, whether particular pathways are used under different environmental conditions or stresses, and whether each of the pathways has the same fidelity in RNA/RNP nuclear export.

Materials and methods

Yeast strains and media

The *mex67-5* and *mtr2* strains were obtained from the temperature-sensitive mutant collections, kindly provided by Dr. C. Boone (University of Toronto) and Dr. P. Hieter (The University of British Columbia). The Molecular Barcoded Yeast (MoBY)-ORF library used for complementation assays was purchased from Open Biosystems. Yeast strains were grown and maintained in synthetic defined medium (SC) lacking appropriate selection ingredients or in complete medium (YEPD) where applicable. Strains and plasmid constructions are described in more detail in the Supplemental Material.

Northern hybridization

Two micrograms of total RNA was separated by electrophoresis on urea-PAGE and transferred onto a Hybond N^+ membrane (Amersham), and specific tRNAs were detected by probing the membranes with digoxigenin-labeled probes as indicated in the figures and described previously (Wu et al. 2013). Equal loading of total small RNA in gels was verified by staining the gels using SYBR Gold nucleic acid gel stain (ThermoFisher) or ethidium bromide (as indicated in figures). Intensities of various tRNA species were quantified using ImageQuant (Perkin-Elmer). The statistical significance of an experiment was determined by performing an unpaired (homoscedastic) Student's *t*-test with two-tailed distribution across all of the biological replicates of an experiment in Microsoft Excel.

FISH

Yeast cells grown overnight at 23°C to early log phase (OD_{600} 0.15–0.3) were collected, and FISH was performed as described previously (Sarkar and Hopper 1998; Wu et al. 2015) with some modifications. Details of the methods and the probes used are in the Supplemental Material. To quantitate the FISH signal intensities, five random cells from an image were chosen. An arbitrary line on the image was drawn (using ImageJ) from one edge of the cell to the opposite edge, avoiding the vacuoles. Using the "plot profile" analysis of the various points at a regular interval on that line, the pixel intensities of the individual fluorescence signals were determined. Pixel values representing the average ± SD were plotted as scatter plot in Excel (Microsoft). For some points, the SDs of pixel intensities of flurophores between cells from biological replicates were too small for the visualization of error bars in the plot profile analysis.

Growth assays

Four microliters of aliquots from serial dilutions of the indicated yeast cultures, consisting of approximately the same number of cells, was spotted on complete or selective medium as indicated. The plates were incubated for 2 d at the indicated temperatures.

RNA coimmunoprecipitation assays

In vivo cross-linked tagged proteins (as indicated in Fig. 5; Supplemental Fig. S13) were coimmunoprecipitated using IgG-conjugated Dynabeads by using a modification of procedures described previously in Huang and Hopper (2015). Details of this procedure are in the Supplemental Material.

Western analyses

Enriched proteins by coimmunoprecipitation were observed using chemiluminescence-based Western blot analysis as described previously (Chu and Hopper 2013; Huang and Hopper 2015). The protein signals were quantified using ImageJ.

RT–PCR and RT-qPCR

RT–PCR and RT-qPCR were carried out by protocols described previously (Huang and Hopper 2015) with some modifications as detailed in the Supplemental Material.

Acknowledgments

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