Sharing the load: Mex67-Mtr2 co-functions with Los1 in primary tRNA nuclear export.

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Supplementary Materials and Methods

Plasmid constructions

We generated constructs to stoichiometrically co-express Mex67 and Mtr2 proteins from the multi-copy plasmid pYEX4T-1 (Whipple et al. 2011). MEX67 and MTR2 genes with their endogenous promoters were amplified from their respective ORF clones, obtained from molecular barcoded yeast open reading frame (MoBY-ORF) library (GE-Dharmacon) using primers containing specific restriction sites. The following amplicons were generated: a 2914 bp amplified MEX67 fragment having EcoRI site on its 5’ end and Xmal site on the 3’ end and 1713 bp MTR2 amplicon with a Xma I site at the 5’ end and a SalI site at the 3’ end. The enzyme used for amplification was Q5™ High Fidelity DNA Polymerase (NEB). The primers used in the amplification reactions are described in Table. S3. The fragments were digested with the respective restriction enzymes according to manufacturer’s protocol (NEB). pYEX4T-1 plasmid was digested with EcoRI and SalI and the ends were de-phosphorylated by Calf Intestinal Phosphatase (NEB) according to manufacturer’s protocol. The digested products were combined in a three-way ligation reaction at room temperature for 2h with T4 DNA ligase (NEB). The ligation mix was transformed in E. coli Mix and Go cells following manufacturer’s protocol (Zymoresearch). Plasmids were isolated from transformed colonies and accurate insertion of the MEX67 and MTR2 fragments were verified by diagnostic restriction digestions, PCR and sequencing.

Construction for multi-copy plasmid pRS426 co-expressing Protein A-tagged Mex67 at its C-terminal end and untagged Mtr2 was as follows: First, a MEX67 fragment containing 660 bp upstream sequence with endogenous promoter with EagI site at the 5’ end and EcoRI site at the end of the MEX67 stop codon was amplified from the yeast genome. This was ligated at the MCS of a pGemT vector (pIVY189). In parallel, a pRS426 vector containing the zz-domain of Protein A (pIVY105) was constructed by ligating pRS426 plasmid digested with HindIII and Xhol and a Protein A containing fragment derived from HindIII and Xhol digestion of a previously
described Protein A containing pGemT plasmid (Huang and Hopper 2015). Subsequently, the Mex67 fragment was digested from pIVY189 vector using enzymes EagI and EcoRI and then ligated into pIVY105, digested with the same enzymes to generate a pRS426 construct expressing Mex67 protein tagged with the zz-domain of Protein A (pIVY188). Finally, a MTR2 fragment with its endogenous promoter and SacI sites at both the ends was generated by PCR amplification. This SacI digested MTR2 fragment was ligated to SacI digested, dephosphorylated pIVY188 to generate a pRS426 construct co-expressing stoichiometric amounts of Protein A-tagged Mex67 and untagged Mtr2. The primers are described in Table S3. The plasmid was sequenced and the functionality of the tagged protein was verified by growth complementation assays (Fig. S10).

To create a pRS426 construct containing Protein A-tagged Mex67-5, site-directed mutagenesis was performed using mutagenic primers (described in Table S3) on pIVY189, converting Histidine400 to Tyrosine in MEX67 coding sequence and plasmid pIVY190 was created. The remaining steps to add C-terminal Protein A tag to Mex67-5 were the same as for Mex67.

**Yeast strains**

The mex67-5 mutant strain obtained from the ts mutant gene collection from Dr. C Boone’s laboratory (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (Li et al. 2011). mtr2 (MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 (or LYS2) met15Δ0 (or MET15) can1Δ::LEU2-MFA1pr::His3 yfg-ts::URA3) strain was derived from the yeast ts collection in Dr. Hieter’s laboratory (Ben-Aroya et al. 2010). We selected for URA3Δ variants of the mtr2 ts strain using 5-fluoroorotic acid selection (Boeke et al. 1984). ma1-1 and los1Δ strains were used for Northern hybridization experiments and FISH studies as described previously (Sarkar and Hopper 1998; Li et al. 2011; Winzeler et al. 1999). To generate the los1Δ mex67-5 double deletion strain, the HphMX4 gene deletion cassette (Goldstein and McCusker 1999) with flanking LOS1 sequences was amplified.
from pAG32 plasmid using primers IVY156 and IVY157 (Table. S3). The cassette was transformed into the mex67-5 ts strains containing a covering plasmid (pYEX4T-1) encoding MEX67, using standard protocols (Gietz and Schiestl 2008) and hygromycin resistant colonies were verified for los1Δ by PCR. Yeast strains expressing MORF-Los1 and RanGTP/GDP-locked mutants were described previously (Huang and Hopper 2015).

**tRNA extraction**

Cells from the indicated strains were pelleted from 15 ml of log phase cultures (OD<sub>600</sub> 0.25-0.35) and resuspended. The temperature sensitive cells were shifted at 37°C for 2h and harvested before the OD<sub>600</sub> reached 0.6. Harvested cells were washed once with ice-cold TSE buffer (10 mM Tris-Cl, pH 7.5, 10 mM EDTA, 100 mM NaCl), and then pelleted and resuspended in 500 µl TSE. The cell suspensions were mixed with equal volumes of TSE-saturated phenol and shaken for 6 times at 42°C for 3’ each, with 1’ incubation on ice in-between. After centrifugation, the aqueous phase was separated and 1250 µl ethanol was added. Small RNAs were precipitated overnight at -80°C.

**In situ hybridization**

Fluorescent in situ hybridization was performed as described previously (Sarkar and Hopper 1998; Wu et al. 2015), with slight modifications. Overnight grown, early log phase (OD<sub>600</sub> 0.1-0.2) temperature sensitive yeast strains were shifted from 23°C to 37°C for 2h or 4h (as indicated), before the OD<sub>600</sub> reached 0.3. The concentrations of probes used for detecting intron-containing as well as spliced tRNA<sup>lle</sup>UAU (SRIM03 and SRIM04, respectively) were 1 picomoles/µl, whereas the concentration of probe (SRIM15), used to detect tRNA<sup>Tyr</sup>GUA, was 0.3 picomoles/µl. Single plane images were captured using a Nikon microscope equipped with a Ultra-view Vox spinning disk confocal apparatus (PerkinElmer Life and Analytical Science) and a cool charge-coupled device camera (Orca-AG, Hamamatsu). tRNA signals were visualized using a 561-nm (red) argon ion laser and DNA was stained by DAPI and visualized using a 405-
nm (blue) laser and a 60X/1.4 NA objective lens. Images were documented using Volocity software (Perkin-Elmer), and analyzed using Image J (NIH).

**DIG-labeling of probes**

3’ end-labeling of DNA probes used for Northern hybridization and FISH assays were conducted using the 2nd generation, DIG Oligonucleotide 3’-End Labeling Kit (Roche), following the manufacturer’s protocols.

**Conjugation of magnetic beads**

Magnetic beads (Dynabeads, ThermoFisher) were conjugated to IgG (Sigma) according to the protocol from the Rout Lab (Alber et al. 2007).

**In vivo co-immunoprecipitation assay**

*mex67*-5 yeast cells co-expressing either Protein A-tagged Mex67 with untagged Mtr2 or Protein A-tagged mutant Mex67-5 were grown in SC-URA media and incubated at 23°C to OD<sub>600</sub> of 0.3-0.4. The cells were then transferred to 37°C for 2h. Yeast cells co-expressing MORF-tagged Los1 with Ran locked mutants were grown as described previously (Huang and Hopper 2015). Chemical cross-linking of RNA-protein complexes was carried out by adding formaldehyde into the cultures to a final concentration of 0.3%. After 30’, cross-linking was quenched by addition of glycine to a final concentration of 66 mM. After 10’, harvested cells were rapidly frozen in liquid nitrogen and were cryolysed, using a planetary ball mill (Alber et al. 2007). 0.5 mg of frozen, ground cells were suspended in 5 ml of RNP-compatible extraction buffer (20 mM HEPES, pH 6.1, 110 mM KOAc, 2 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.5% Triton, 0.1% Tween-20, 1 mM DTT) with 1:100 dilutions of protease inhibitor cocktail set IV (EMD Millipore), 500 µg of PMSF and 1:1000 dilutions of RNase inhibitor (SUPERaseIN, Roche); 10 mM of GTP was added to the extracts of MORF-Los1 cells that contained RanGTP locked constructs. After centrifugation at 3000xg, 4°C for 10’, the soluble extract was clarified by rapidly passing through a 1.6 µ GD/X glass microfiber syringe filter (25 mm)(Whatmann). The lysate was then incubated with IgG-conjugated magnetic beads at 4°C for 30’. The beads were then collected with a
magnet, washed six times with 1 ml of ice-cold extraction buffer and once with ice-cold Wash buffer (0.1 M NH₄OAc, 0.1 mM MgCl₂, 0.02% Tween-20). The beads, suspended in wash buffer, were divided into two equal fractions. One fraction was used for protein extraction, by incubating the beads with freshly-prepared protein elution buffer (0.5 M NH₄OH, 0.5 mM EDTA) for 20’ at room temperature. The eluates were lyophilized overnight using a Speed Vac (Thermo Savant). The lyophilized pellets were suspended in SDS protein-loading buffer with freshly added 5% 2-β-mercaptoethanol (62.5 mM Tris-HCl, pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 10 % glycerol) and heated at 90°C for 30’ to disassemble the putative complexes cross-linked by formaldehyde. After centrifugation at 2000 rpm, the protein suspensions were separated on a 4-12% NuPAGE Novex Bis-Tris precast gels (Life technologies) according to the manufacturer’s specifications. A fraction of the proteins were stained with Sypro Ruby (Molecular Probes) according to the manufacturer’s specifications. The remaining proteins were used for Western analyses.

To obtain enriched RNAs, half of the magnetic beads suspended in wash buffer were collected using a magnet, and re-suspended in RNA elution buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1% SDS, 10 mM DTT), followed by 70°C incubation for 45’. The samples were then incubated with Proteinase K (20 mg/ml) (NEB) for 30’ at 30°C. Enriched RNAs were extracted using an equal volume of acid phenol pH 4.4, then precipitated with 3 volumes of 100% EtOH and 1.5 ng of GlycoBlue™ co-precipitant (Ambion) and incubated overnight at -80°C. RNA pellets were re-suspended in nuclease-free water.

**Western analyses**

The antibodies and the concentrations used in western analyses were as follows: HRP-conjugated anti-protein A antibody (APA-HRP, Gallus) was used at 1:10,000 dilution, anti-Ran primary antibody (ab4781, Abcam) at 1:10,000 dilution, anti-Rna1 primary antibody at 1:10,000 dilution (Hopper et al. 1990). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG (GE Healthcare) (for anti-Ran and anti-Rna1
respectively) at 1:5000 dilution. Chemiluminiscent signals were generated by using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher) according to manufacturer’s protocol.

**RT-PCR**

Enriched RNAs from co-immunoprecipitation were treated with TurboDNase (Ambion) following manufacturer’s protocol. First strand cDNA was synthesised using SuperScript III reverse transcriptase (Invitrogen) following manufacturer’s protocol. PCR reactions were carried out using 1 µl of cDNA with GoTaq Flexi PCR system (Promega). The PCR conditions were as follows: For PGK1 mRNA and unspliced pre-tRNA\textsuperscript{Ile\textsubscript{UAU}}, 2 min at 95°C; 30 cycles of 30 s at 95°C, 20 s at 52°C, and 20 s at 72°C; 2 picomoles of primer sets (KC038 and KC039 for PGK1 mRNA and Ivy1 and Ivy3 pre-tRNA\textsuperscript{Ile\textsubscript{UAU}}). For spliced tRNA\textsuperscript{Ile\textsubscript{UAU}}, 2 min at 95°C; 26 cycles of 30 s at 95°C, 20 s at 52°C, and 20 s at 72°C; 20 s at 72°C; 2 picomoles of primer set Ivy1 and Ivy149 was used. For mitochondria encoded tRNA\textsuperscript{Ile\textsubscript{GUA}}, 2 min at 95°C; 40 cycles of 30 s at 95°C, 20 s at 52°C, and 20 s at 72°C; 20 s at 72°C; 10 picomoles of primer set KC034 and KC035 were used. 15 µl of PCR reactions were electrophoretically separated on 2% agarose gels.

**RT-qPCR**

DNase treatment and first strand cDNA synthesis were described for RT-PCR. RT-qPCR reactions were carried out using iTaq Universal SYBR Green supermix (Biorad) in a CFX96 instrument (Biorad). The PCR conditions were 2 min at 95°C; 40 cycles of 30 s at 95°C, 20 s at 52°C, and 20 s at 72°C; 30 s at 72°C 10 picomoles of primers were used to quantitate mitochondria encoded tRNA\textsuperscript{Ile\textsubscript{GUA}}, compared to 2 picomoles used for other RNA species. Standard curves were prepared with 10-fold serial dilutions of gel-extracted, RT-PCR products as templates to determine the concentrations of co-immunoprecipitated RNAs. RT-PCR
conditions used for making the templates for standard curves were specific to each RNA species.

To compare the amount of $MEX67$ and $MTR2$ mRNA over-expressed in los1Δ + M-M cells relative to los1Δ + V cells (Fig. S7), first strand $MEX67$ and $MTR2$ cDNAs were created by Superscript III from DNAase treated total RNA obtained from the two strains, following manufacturer’s protocol using primers KC041 and KC045, respectively. RT-qPCR was then performed using primer sets KC040, KC041 (for $MEX67$) and KC044, KC045 (for $MTR2$) using standardized protocols. In all cases, no template controls were analyzed for each primer set, and no RT controls were analyzed for each sample with each primer set. Data were analyzed by CFX96 software (Bio Rad).
Supplemental References

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Supplementary Figures

Fig. S1. *mex67-5* and *mtr2* temperature sensitive yeast cells accumulate end-processed, intron-containing tRNA$^{\text{Tyr}_{\text{GUA}}}$ at 37°C. (A) A schematic diagram (not drawn to scale) of primary tRNA$^{\text{Tyr}_{\text{GUA}}}$ transcript with 5’ leader and 3’ trailer as thin lines, the exons are black boxes and intron is gray box. The probe (KC031) is diagramed above the schematic drawing. (B) Northern hybridization of total small RNA isolated from wild-type (WT), *ma1-1*, *mex67-5* and *mtr2* yeast cells grown at 23°C or after 2 h shift to 37°C using probe KC031. P denotes primary tRNA$^{\text{Tyr}_{\text{GUA}}}$ transcripts with 5’ leader, 3’ trailer and intron; I denotes 5’, 3’ end-processed, intron-containing tRNA$^{\text{Tyr}_{\text{GUA}}}$. Ratios of signal intensities of I versus P (I/P) were calculated and then normalized to the WT ratio, incubated at the same temperature. Ethidium bromide staining of 5.8S and 5S rRNA was used as loading controls for each lane. The accumulation of slower migrating, end-extended, aberrant pre-tRNA$^{\text{Tyr}_{\text{GUA}}}$ species in cells grown at 37°C may be due to tRNA 3’ end-processing defects under thermal stress, as previously shown (Foretek et al. 2016).

Fig. S2. Accumulation of end-processed, intron-containing tRNA$^{\text{Ile}_{\text{UAU}}}$ in *mex67-5* and *mtr2* cells at non-permissive temperatures are complemented by plasmid-encoded Mex67 and Mtr2 respectively. Northern hybridization of RNAs isolated from: (Lanes 1 & 6) WT cells transformed with vector; (Lanes 2 & 7) *mex67-5* transformed with vector (Lanes 3 & 8) *mex67-5* cells transformed with plasmid containing *MEX67*; (Lanes 4 & 9) *mtr2* cells transformed with vector; (Lanes 5 & 10) *mtr2* cells transformed with plasmid containing *MTR2*. Log-phase culture cells of the respective strains were grown at 23°C (left) or transferred to 37°C for 2 h (right), as indicated. Ratios of signal intensities of I versus P (I/P) were calculated and normalized to the WT ratio grown at the same temperatures.

Fig. S3. Growth defects of *mex67-5* and *mtr2* cells at non-permissive temperatures are complemented by plasmid-encoded Mex67 and Mtr2. Ten-fold serial dilutions of *mex67-5* and *mtr2* cells containing vector, plasmid-encoded *MEX67* (pMEX67) or *MTR2* (pMTR2) were incubated on selective media at 23°C, 30°C, 34°C, or 37°C for 2 days.
Fig. S4. tRNA\textsuperscript{Ile\textsubscript{UAU}} accumulates in the nuclei of \textit{mex67-5} and \textit{mtr2} cells at the non-permissive temperature in 2h. (A) A schematic presentation of the probe SRIM04. (B) Early log-phase wild-type (WT), \textit{ma1-1}, \textit{mex67-5} and \textit{mtr2} cells were shifted to 37°C for 2h. tRNA\textsuperscript{Ile\textsubscript{UAU}} (green); nuclear DNA, visualized by DAPI (red) in the merged panel.

Fig. S5. tRNA\textsuperscript{Tyr\textsubscript{GUA}} accumulates in the nuclei in \textit{mex67-5} cells at the non-permissive temperature. (A) Schematic presentation of the probe SRIM15. (B) Wild-type (WT), \textit{ma1-1}, and \textit{mex67-5} cells were grown as for Fig. 2. tRNA\textsuperscript{Tyr\textsubscript{GUA}} (green); nuclear DNA, visualized by DAPI (red) in the merged panel. Bar, 2µ. (C) Five cells from each micrograph in B, 4h after the temperature shift to 37°C were analyzed for fluorescence intensities of tRNAs and DNA. Graphs show the relative intensity profiles of the fluorophores, ± S.D (vertical lines).

Fig. S6. Micrographs of WT, \textit{rna1-1}, \textit{mex67-5} and \textit{mtr2} cells harvested at 4h with analyzed cells marked with arrows. tRNA\textsuperscript{Ile\textsubscript{UAU}} FISH (green) and nuclear DNA (red) in the merged panel. Arrows mark the cells chosen for intensity analyses (in Fig. 2C). Bar, 2µ.

Fig. S7. RT-qPCR analyses of \textit{MEX67} and \textit{MTR2} mRNA quantities in \textit{los1\Delta} transformed with vector alone (\textit{los1\Delta} + V) or vector over-expressing \textit{Mex67-Mtr2} (\textit{los1\Delta} + M-M) cells. Bar graphs comparing fold-changes in \textit{MEX67} (vertical lines in bars) and \textit{MTR2} (horizontal lines in bars) mRNA expression levels in \textit{los1\Delta} cells transformed with either vector (maraschino bars), or \textit{MEX67-MTR2} co-expressed from multi-copy pYEX4T-1 plasmid (cantaloupe bars). Cells were grown in defined media lacking uracil.

Fig. S8. Nuclear accumulation of spliced tRNA\textsuperscript{Ile\textsubscript{UAU}} in \textit{los1\Delta} cells is suppressed by over-expression of \textit{Mex67-Mtr2}. (A) Schematic presentation of probe SRIM04. (B) Wild type yeast cells transformed with vector (WT+V), \textit{los1\Delta} cells transformed with vector (\textit{los1\Delta}+V), or \textit{los1\Delta} cells co-expressing \textit{MEX67} and \textit{MTR2} (\textit{los1\Delta}+M-M) were grown at 23°C and the subcellular distribution of spliced tRNA\textsuperscript{Ile\textsubscript{UAU}} was examined by FISH. Bar, 2µ. (C) Five random cells from each micrograph from the experiment in panel B were analyzed for the distribution of
fluorescence intensities of FISH and DAPI staining. The background corrected intensity profiles of FISH fluorescence from the three strains were plotted as a scatter plot, where the values represent the relative average ± S.D. (vertical lines).

**Fig. S9. No negative synthetic genetic interactions detected between los1Δ and mex67-5 at mex67-5 elevated temperatures.** WT, los1Δ, mex67-5 and los1Δmex67-5 cells were grown to stationary phase and then serially diluted, spotted on complete media and incubated at 23°C, 26°C, 28°C, 30°C, 34°C and 37°C for 2 days.

**Fig. S10. Protein A-tagged Mex67 is functional.** Serial dilutions of mex67-5 cells containing vector or plasmid-encoded untagged Mex67 (pMex67), Protein A- tagged Mex67 (pMex67-A), Protein A-tagged Mex67-5 (pMex67-5-A), were spotted on defined medium and incubated at the indicated temperatures for 2 days.

**Fig. S11. Over-expressed Mtr2 can suppresses growth defects of mex67-5 cells and Mex67-5 is unstable at 37°C.** (A) WT cells transformed with vector (WT+V) and mex67-5 cells transformed with vector (mex67-5 + V), or a multi-copy plasmid co-expressing ProteinA-tagged Mex67 with untagged Mtr2 (mex67-5 + pMex67-A+Mtr2) , or multi-copy plasmid co-expressing ProteinA-tagged Mex67-5 with untagged Mtr2 (mex67-5 + pMex67-5-A+ Mtr2), were grown on selective media at 23°C, 30°C, 34°C and 37°C for 2 days. (B). Western blot of total cell lysate of mex67-5 cells either co-expressing Protein A-tagged Mex67 and untagged Mtr2 from a single multi-copy plasmid (pMex67-A+Mtr2) or expressing Protein A-tagged Mex67-5. The log phase cells were transferred to 37°C and incubated for 0 or 2h. IgG antibodies were used to detect respective ProteinA-tagged proteins in the lysate and anti-Rna1 antibodies were used to detect endogenous Rna1 levels which served as the loading control.

**Fig. S12. Ran purifies with Los1 but not with Mex67.** Proteins enriched by co-immunoprecipitation were detected by Western Blot using IgG antibody (top panel) and anti-Gsp1 (Ran) antibody (bottom panel).
Fig. S13. RT-qPCR analyses for PGK1 mRNA, unspliced pre-tRNA\textsubscript{Ile\textsubscript{UAU}}, spliced tRNA\textsubscript{Ile\textsubscript{UAU}} and mitochondrial tRNA\textsubscript{Ile\textsubscript{GAU}}, co-enriched with RNA exporters in three independent biological triplicates of co-immunoprecipitation assays. RT-qPCR analyses for PGK1 mRNA (horizontal lines in bars), unspliced tRNA\textsubscript{Ile\textsubscript{UAU}} (vertical lines in bars), spliced tRNA\textsubscript{Ile\textsubscript{UAU}} (diagonal lines in bars) and mitochondrial encoded tRNA\textsubscript{Ile\textsubscript{GAU}} (solid bars). The numbers along X-axis refer to the strains specified by lane numbers and are also color-coded. (Lane 1, Turquoise) mex67-5 cells expressing ProteinA-tagged Mex67-Mtr2; (Lane 2, Tangerine) mex67-5 cells expressing ProteinA-tagged Mex67-5; (Lane 3, Spring) WT cells co-expressing MORF-tagged Los1 and RanGTP-locked mutant; and (Lane 4, Blueberry) WT cells co-expressing MORF-tagged Los1 and RanGDP-locked mutant. The numbers along Y-axis denote absolute amounts (femtograms/µl) of RNA species enriched by co-immunoprecipitation assay. Bars denote values that represent average ± S.D. of amounts of RNA enriched from co-immunoprecipitation assays from three independent biological replicates.

Fig. S14. Inactivation of Mex67 or Mtr2 results in accumulation of end-matured, intron-containing tRNAs for a subset of the ten intron-containing tRNA families. WT, \textit{ma1-1}, \textit{mex67-5}, and \textit{mtr2} cells were grown over-night at 23°C and then shifted to 37°C for 2h, RNAs were isolated, and analyzed by Northern hybridization using probes specific to each of the ten intron-containing tRNAs (Table S1). For each tRNA family, one representative Northern blot, chosen from three independent biological replicates, is shown. The band intensities of P and I species and the I/P ratios for each tRNA families were calculated for each tRNA family, and normalized to WT cells at 0h [1-WT, 2-\textit{mex67-5}, 3- \textit{mtr2}, 4- \textit{ma1-1} (in black)], or at 2h [5-WT, 6-\textit{mex67-5}, 7- \textit{mtr2}, 8- \textit{ma1-1} (in red)] after temperature shift.
Table S1

DNA oligonucleotide probes employed

| Name | Function                  | Sequence                                                                                       | Used in                                      |
|------|---------------------------|-----------------------------------------------------------------------------------------------|----------------------------------------------|
| JW48 | Probe to detect tRNA\textsuperscript{Ile} \textsubscript{UAU} | GGCACAGAAACTCCGGAACCGAATGGGTGCTATA AGCAGGAAGCTCTAACC ACTGAGCTACACGAGC | Northern Hybridization (Figs. 1, S2, S14)  |
| KC031| Probe to detect tRNA\textsuperscript{Tyr} \textsubscript{GAU} | CCCGATCTCAAGATTTTCGTAGTGATAAAATTACAGT CTTGCCGCTTTAAACC | Northern Hybridization (Figs. 3, S1, S14)  |
| SRIM04| Probe to detect tRNA\textsuperscript{Ile} \textsubscript{UAU} | GTGGGGATGACGCCACGGTCGCGTTATAA GCACGAAGCTCTAAACCACACTGAGCTACA | FISH (Figs. 2, S4, S6, S8)                  |
| SRIM03| Probe to detect tRNA\textsuperscript{Ile} \textsubscript{UAU} | CGTTGCTTTAAGGCCTGGTTAAGGCTTTG GCACAGAAACTCCGGAACCGAATGTTGCTATA | FISH & Northern Hybridization (Figs. 4, S14) |
| SRIM15| Probe to detect tRNA\textsuperscript{Tyr} \textsubscript{GAU} | GCGAGTCGACGCGATCTCAAGATTACAGTC TTGCCGCTTTAACCACACTTGGCTACC | FISH (Figs. S5)                             |
| SM12 | Probe to detect tRNA\textsuperscript{Leu} \textsubscript{CAA} | GTTACCTGCGGTCAAGATTTCTTGGTAATGCAGGC GCCTTAGACCG | Northern Hybridization (Figs. 3, S14)       |
| SM13 | Probe to detect tRNA\textsuperscript{Leu} \textsubscript{UGA} | ATTTAGAGGTTAATCCACCTAAATCTGACCGCT TAAACC | Northern Hybridization (Figs. 3, S14)       |
| SM15 | Probe to detect tRNA\textsuperscript{Lys} \textsubscript{UUU} | CCTTGCTTAAGCGAATTCGCTTTAAAGCCGAACG CTCTACC | Northern Hybridization (Figs. 3, S14)       |
| SM10 | Probe to detect tRNA\textsuperscript{Pro} \textsubscript{GAA} | TAACTTGACGGAAGTATTTCTTCAATCTGGCGCT CTCC | Northern Hybridization (Figs. 3, S14)       |
| SM18 | Probe to detect tRNA\textsuperscript{Ser} \textsubscript{CGA} | CCCAAAGCGGAATCTACACTAGACCAACCACCGC CC | Northern Hybridization (Fig. 3, S14)       |
| SM20 | Probe to detect tRNA\textsuperscript{Ser} \textsubscript{CGA} | AGCCGAACCTTTATTTTATCCATTCGAGCTCTCGCT TAAACCACCTGGCCGCTATAGTGGCC | Northern Hybridization (Fig. 3, S14)       |
| SM21 | Probe to detect tRNA\textsuperscript{Ser} \textsubscript{GCU} | AATTGCTTTCTGAGAAATAGCAGGGCATCGCC TTAACCACCTCGGGCCACCTGGGAC | Northern Hybridization (Figs. 3, S14)       |
| SM11 | Probe to detect tRNA\textsuperscript{Trp} \textsubscript{CCA} | CGTGGAAATTTCAGATTTAATTGGAGGTCGAAAG CTCTACC | Northern Hybridization (Figs. 3, S14)       |
### Table S2
Primer sequences for RT-PCR and RT-qPCR assays

| RNA species detected/measured | Primer pairs | Sequence |
|-------------------------------|--------------|----------|
| PGK1 mRNA                    | KC038        | ATGTCTTTATCTTCAAAGTTTGTCCTGCAAG |
|                               | (Primer 1 in Fig. 5G, S13) |           |
|                               | KC039        | GATCTTCTTACCCTCAATGGAGCTTGTTG |
|                               | (Primer 2 in Figs. 5G, S13) |           |
| Un-spliced tRNA\[^\text{UAU}\] | Ivy1         | GCTCGTGTAGCTCAGTGGTTAG |
|                               | (Primer 3 in Figs. 5H, S13) |           |
|                               | Ivy3         | CTTTTAAGGCTGTGTTGAAG |
|                               | (Primer 4 in Figs. 5H, S13) |           |
| Spliced tRNA\[^\text{UAU}\]  | Ivy1         | GCTCGTGTAGCTCAGTGGTTAG |
|                               | (Primer 3 in Figs. 5I, S13) |           |
|                               | Ivy149       | ACGTGTGCATTATAAGCAGCAG |
|                               | (Primer 5 in Figs. 5I, S13) |           |
| Mitochondrial tRNA\[^\text{GAU}\] | KC034        | GAA ACT ATA ATT CAA T |
|                               | (Primer 6 in Figs. 5J, S13) |           |
|                               | KC035        | GAAACTAACAGGGATT |
|                               | (Primer 7 in Figs. 5J, S13) |           |
| MEX67 mRNA                    | KC040        | GAATAAGATTAGCGTGAGGAAGCT |
|                               | (Fig. S7)    |           |
|                               | KC041        | CCTTCCACATGAGCGTCATAAAC |
| MTR2 mRNA       | KC044   | TTACGAAGAAGATATTAGC |
|-----------------|---------|---------------------|
| (Fig. S7)       |         |                     |
| KC045           |         | ATAGTCTACTCCTGTTAG  |
| (Fig. S7)       |         |                     |
### Table S3

#### Primer sequences for Cloning

| Name   | Function                                                                 | Sequence                                                                 | Used in |
|--------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|---------|
| KC12   | Forward primer to introduce EcoRI site to Mex67 5’end                     | AAAAAAAAAAGAATTCCGGTGAAGATCCATATTCTC TGC                                 | Cloning |
| KC013  | Reverse primer to introduce Xmal site to Mex67 3’end                       | AAAAAAAAAACCCGGGAATAGCAAAACCAAGGA AAGG                                   | Cloning |
| KC014  | Forward primer to introduce Xmal site to Mtr2 5’end                        | AAAAAAAAAACCCGGGTTCGTGGAGTTCCATGTA TA AGG                                | Cloning |
| KC015  | Reverse primer to introduce Sal I site in Mtr2 3’end                       | AAAAAAAAGGTCACTCCCCTACTCTAAACATTGGT TTCTC                                | Cloning |
| KC019  | Reverse primer to introduce ScaI site to Mtr2 3’end                       | AAAAAAAAGAGCTCCCTACTCTAAACATTGGT TTCTC                                  | Cloning |
| KC027  | Forward primer to introduce ScaI at Mtr2 5’end                            | AAAAAAAAGAGCTCTTCTGTGGAGTTCCATGTA TAAGG                                 | Cloning |
| IVY156 | LOS1:HPH KO upstream (pAG32)                                               | AAGTTGTCCGGTAAAGCAACCTATAAGAAACAAGTGT TCCAGTCAAATCGAGGAGCAGGCAGTCGACGGATCC CCGG | Cloning |
| IVY157 | LOS1:HPH KO downstream (pAG32)                                             | CATATTACCAATTAAAAATATTAGTATAAATATAATT ACAACTTTTTACTCTTCGGAGATCAGATCAGTCGA | Cloning |
| IVY212 | Forward primer for ProteinA-Mex67 mutation | TTCGTTATTACGGGTACGGATTTTTTGAA | Site-directed mutagenesis |
|--------|--------------------------------------------|--------------------------------|--------------------------|
| IVY213 | Forward primer for Protein A-Mex67-5 mutation | TTC AAA AAA TCC GTA CAA CGT AAT AAC GAA | Site-directed mutagenesis |

* Underlined sequences indicate introduced restriction sites.
### A

![Diagram of pre-tRNA structure with KC031 and GUA residue highlighted.]

### B

|        | WT   | rna1-1 | mex67-5 | mtr2  |
|--------|------|--------|---------|-------|
| 23°C   | 1.0  | 1.0    | 1.2     | 1.0   |
| 37°C   | 1.0  | 4.4    | 4.5     | 0.8   |
| I/P    | 1.0  | 1.2    | 4.4     | 0.8   |
| 5.8S   |      |        |         |       |
| 5S     |      |        |         |       |

Note: The image shows a figure with a diagram of pre-tRNA structure and a table comparing the effects of different mutants (WT, rna1-1, mex67-5, mtr2) at 23°C and 37°C.
A

pre-tRNA^{lle}_{UAU}

JW48

- - - - 3'

B

|          | 23°C   | 37°C   |
|----------|--------|--------|
| WT       |        |        |
| +V       |        |        |
| +V +pMEX67 |      |        |
| mtr2     | +V +pMTR2 |       |
| mex67-5  | +V     | +V +pMEX67 |
| mtr2     |        | +V +pMTR2 |

|          | 23°C   | 37°C   |
|----------|--------|--------|
| WT       |        |        |
| +V       |        |        |
| +V +pMEX67 |      |        |
| mtr2     | +V +pMTR2 |       |
| mex67-5  | +V     | +V +pMEX67 |
| mtr2     |        | +V +pMTR2 |

Chatterjee _Fig. S2_
A

SRIM04

5'——3'

SRIM04

5'——3'

B

37°C 2 h

tRNA\textsubscript{Ile}\textsubscript{UAU}  DAPI  tRNA\textsubscript{Ile}\textsubscript{UAU} + DAPI

WT

ma1-1

mex67-5

mtr2
Fig. S5

A

B

37°C 0h

37°C 4h

WT

ma1-1

mex67-5

C

37°C 4 h

WT

ma1-1

mex67-5

Chatterjee
37°C 4 h

| tRNA_{Ile}^{UAU} | DAPI | tRNA_{Ile}^{UAU+} |
|------------------|------|-------------------|
| WT               |      |                   |
| ma1-1            |      |                   |
| mex67-5          |      |                   |
| mtr2             |      |                   |

Chatterjee Fig. S6
The figure shows the fold over-expression of MEX67 and MTR2 in different conditions:

- los/Δ V
- los/Δ M-M
- los/Δ + V
- los/Δ M-M

The y-axis represents the fold over-expression, ranging from 0 to 8.
A

SRIM04

5’—

/ 3’

SRIM04

5’—

3’

B

WT + V

tRNA\textsuperscript{\text{Ile}}\textsubscript{UAU}  DAPI  tRNA\textsuperscript{\text{Ile}}\textsubscript{UAU} + DAPI

los1\Delta + V

tRNA\textsuperscript{\text{Ile}}\textsubscript{UAU}  DAPI  tRNA\textsuperscript{\text{Ile}}\textsubscript{UAU} + DAPI

los1\Delta + M-M

tRNA\textsuperscript{\text{Ile}}\textsubscript{UAU}  DAPI  tRNA\textsuperscript{\text{Ile}}\textsubscript{UAU} + DAPI

C

Intensity

0.0 0.05 0.1

Distance across cell

WT + V

Intensity

0.0 0.05 0.1

Distance across cell

los1\Delta + V

Intensity

0.0 0.05 0.1

Distance across cell

los1\Delta + M-M
mex67-5+ vector
mex67-5+ pMex67
WT+ pMex67-A
mex67-5+ pMex67-A
mex67-5+ pMex67-5-A

23°C  30°C  34°C  37°C
**A**

|       | 23°C | 30°C | 37°C |
|-------|------|------|------|
| WT + V | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) |
| mex67-5 + V | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) |
| mex67-5 + pMex67-A+Mtr2 | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) |
| mex67-5 + pMex67-5-A+Mtr2 | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) |

**B**

|       | 23°C | 37°C | 23°C | 37°C |
|-------|------|------|------|------|
| mex67-5 + pMtx67-A+Mtr2 | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) |
| mex67-5 + pMtx67-5-A | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) | ![Image](imagelink) |

Protein A

Rna1
|        | mex67-5 | WT          |
|--------|---------|-------------|
| Mex67  | -A      | RanGTP-locked|
| Mex67-5| -A      | Los1-MORF   |

**Immunoblot:**

- **MORF-Los1**
- **Mex67-A**
- **Ran**
Figure S13

**PGK1 mRNA**

- **Unspliced tRNA^{ile}_{UAU}**: 1. *mex67-5 + Mex67-PrA.* 2. *mex67-5 + Mex67-5-PrA* 3. *WT + RanGTP-locked Los1-MORF* 4. *WT + RanGDP-locked Los1-MORF*

- **Spliced tRNA^{ile}_{UAU}**

- **Mitochondrial tRNA^{ile}_{GAU}**
