Genome-wide screen uncovers novel pathways for tRNA processing and nuclear–cytoplasmic dynamics

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Transfer ribonucleic acids (tRNAs) are essential components of the protein synthesis machinery in all kingdoms of life, as they bring amino acids to ribosomes during translation. tRNAs are also involved in many critical processes, including cellular responses to stress, protein degradation, apoptosis, and retrovirus replication (Phizicky and Hopper 2010). Moreover, defects in tRNA processing are responsible for numerous human disorders (Kirchner and Ignatova 2015). The important roles of tRNAs have highlighted a need for a complete understanding of the steps that affect tRNA processing and subcellular dynamics.

To date, 94 gene products that participate in tRNA post-transcriptional processing have been identified in Saccharomyces cerevisiae. Seventy-three are involved in the 25 known tRNA nucleotide modifications, and 21 are involved in tRNA end processing and splicing (Hopper 2013; Sharma et al. 2015). Moreover, there are numerous additional genes encoding proteins that function in tRNA turnover and nuclear–cytoplasmic dynamic pathways that are shared with other types of RNA (Hopper 2013; Takano et al. 2015).

Although genes involved in tRNA biology have been studied extensively, many aspects of tRNA processing and subcellular dynamics remain unclear [Fig. 1A]. For example, RNA polymerase III [Pol III] mediates tRNA transcription, but so far only a single yeast Pol III regulator, Maf1, has been identified [Fluta et al. 2001], and it is not clear whether other regulators exist. After tRNA transcription, endonucleases RNase P and RNase Z and exonucleases catalyze tRNA leader and trailer removal...

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The steps that likely have missing gene products are highlighted by red numbers. Some of the novel gene products identified by this screen are highlighted in blue. The steps that likely have missing gene products are highlighted by red numbers. Some of the novel gene products identified by this screen are highlighted in blue.

A schematic view of a primary tRNA\textsuperscript{ile}\textsubscript{UAU} transcript with 5′ leader, 3′ trailer, exons, and intron and probe 1 used for Northern hybridization. Expected phenotypes of mutants that are defective in different processes of tRNA biology by Northern analysis of tRNA\textsuperscript{ile}\textsubscript{UAU}. Lane 2∗ indicates defects in exonucleolytic 3′ end trimming.

Primary tRNA transcript, 145 nucleotides (nt); (I) end-matured intron containing tRNA, 136 nt; (M) mature tRNA, 76 nt; (IN) intron, 60 nt; (H) 5′ exon, 37 nt. Numbers below each lane indicate the number of mutants discovered for each phenotype.

Strategies for candidate validation for unessential genes (blue) and essential genes (pink).

In order to identify gene products that function in tRNA production, processing, degradation, and subcellular movement, we conducted an unbiased genome-wide screen of nearly all annotated genes in \textit{S. cerevisiae}. We used one yeast haploid deletion collection (Winzeler et al. 1999) and two temperature-sensitive collections (Ben-Aroya et al. 2008; Li et al. 2011) to identify mutants with defects in tRNA processing. Previously, we reported that Xrn1, found by this screen, is a key component of the mechanism for tRNA intron turnover (Wu and Hopper 2014). Here, we provide the results of the entire screen that uncovered 162 novel gene products involved in various tRNA biology processes. These include nuclear pore complex (NPC) proteins, tRNA-aminoacyl synthetases.
(aaRS), cytoskeleton components, mitochondrial membrane proteins, and mRNA/ribosome export machinery. Importantly, we found that the Ran GTPase-dependent karyopherin Crm1/Xpo1 may provide a novel pathway for nuclear export of intron-containing tRNAs.

Results

Genome-wide screen to identify novel gene products involved in tRNA biology

To systematically identify gene products that function in tRNA biology [Fig. 1A], we conducted a genome-wide screen of nearly all annotated essential and unessential protein-encoding genes in *S. cerevisiae*. The yeast genome contains 6604 verified and dubious protein-encoding genes [YeastMine, *Saccharomyces* Genome Database, http://www.yeastgenome.org; Cherry et al. 2012]. We analyzed mutants from three yeast collections: the yeast *MATa* haploid collection that contains deletions of 4847 unessential protein-coding genes [Winzeler et al. 1999], a collection created by Boone and coworkers [Li et al. 2011] that contains 757 temperature-sensitive alleles of 476 essential genes, and a collection generated by Hieter and coworkers [Ben-Aroya et al. 2008] that contains 333 temperature-sensitive alleles of 332 essential genes. Together, these collections contain nearly all annotated unessential genes and 765 of 1105 (73.1%) annotated essential genes [Giaever et al. 2002], which in total comprise at least 85.0% of the yeast ORFs.

To conduct this genome-wide screen, we developed a method that consists of culturing yeast in 96-deep-well plates, a fast procedure for isolation of low-molecular-weight RNAs from the cultures, and a nonradioactive labeling method for RNA detection [Fig. 1B; Wu et al. 2013]. We chose to detect one tRNA encoded by an intron-containing gene, tRNA*UAU*, as it contains the largest intron [60 nucleotides (nt)], and its various processing intermediates have distinct sizes, thereby allowing assessment of all processing intermediates, byproducts, and mature tRNAs in single gel lanes. A probe complementary to the whole 5’ exon and nucleotides 1–30 of the intron [probe 1] was designed [Fig. 1C] to detect the primary tRNA transcript [P], end-matured intron-containing tRNA [I], mature tRNA [M], free introns [IN], tRNA halves [H], and other possible species [Fig. 1D]. RNAs were extracted from cells with deletions of unessential genes grown in rich medium at 23°C and cells with temperature-sensitive mutations after a 2-h shift to the nonpermissive temperature (37°C).

Defects in any of the tRNA processing steps [numbered in Fig. 1A] are predicted to alter migration of tRNA species on polyacrylamide gels [Fig. 1D]. Figure 2 provides example Northern analyses of some of the identified mutants illustrated in Figure 1D. A mutant with increased transcription of the initial transcripts [Fig. 1A, step 1] is expected to accumulate species P [Figs. 1D [lanes 1,2], 2A [for a possible example]]. A mutant defective in 5’ or 3’ end processing [Fig. 1A, step 2] will accumulate P or species above or below P [Figs. 1D [lanes 1,2 or 2*], 2B,C]. A mutant defective in CCA addition to the 3′ end [Fig. 1A, step 3] will accumulate a RNA species that migrates slightly faster than CCA-containing tRNAs [Figs. 1D [lane 3], 2D]. Defects in initial nuclear export of tRNAs, delivery of tRNAs to the mitochondrial surface, or incorrect localization, assembly, or function of the SEN complex [Fig. 1A, steps 4, 5, or 6] will accumulate end-matured intron-containing tRNAs [I] [Figs. 1D [lanes 4,5,6], 2E–G [putative mutants]]. We previously reported discovery of mutants that are defective in intron turnover [Fig. 1A, step 7], as *xrn1Δ* and *rlg1-4* mutants accumulate free introns [IN] [Fig. 1D, lane 7; Wu and Hopper 2014]. Finally, a mutant that affects tRNA modification [Fig. 1A, step 9] may demonstrate altered migration of mature tRNAs and their processing intermediates [Figs. 1D [lane 9], 2I]. *los1A* or *sen2-42* cells, which accumulate end-matured intron-containing tRNA, and wild-type cells served as controls for the screen [Fig. 1D].

After the first round of the screen, 457 mutants were identified whose mutation led to tRNA processing defects. A second round of Northern analysis was performed for all initial mutants, and 242 candidates that had repeatable phenotypes were further validated. Since the yeast collections contain annotation errors or second site mutations [Grunenfelder and Winzeler 2002; Ben-Shitrit et al. 2012], the candidates were subjected to additional verification [Fig. 1E].

In the third round of the screen, candidates from the *MATa* haploid deletion collections were compared with the strain containing a deletion of the same gene in the
independently constituted MATα deletion collection (Figs. 1E, 2A–C,G for examples; Giaever et al. 2002). A candidate was considered verified if the corresponding mutant in the MATα deletion collection showed the same phenotype. Among verified deletion mutants, six were further confirmed by introduction of the yeast genomic tiling plasmid containing the corresponding wild-type gene [Jones et al. 2008] to assess whether the wild-type gene complements the mutant phenotype (Figs. 1E, 2H [for an example]; Supplemental Table S1). A total of 105 genes were validated, representing 2.2% of all unessential genes screened [Supplemental Table S1; see primary data in Supplemental Fig. S1].

To verify candidates from the Boone temperature-sensitive mutant collection (Li et al. 2011), three approaches were used (Fig. 1E). First, the Hieter laboratory (Ben-Aroya et al. 2008) generated numerous temperature-sensitive mutants by transferring mutant alleles from the Boone collection [Li et al. 2011] into the Hieter collection genetic background. If the same allele in two different genetic backgrounds demonstrated the same phenotype, this mutant allele was considered as verified (see Fig. 2E for an example and primary data for all mutants in Supplemental Fig. S2). Second, several gene candidates have multiple alleles available. If different alleles of the same gene confer the same phenotype [Supplemental Table S2; Supplemental Fig. S2], it strongly supports the notion that this gene plays a role in tRNA biology. Third, the remaining mutants that do not have either the same allele in different genetic backgrounds or multiple alleles were assessed by complementation assays using the vectors from the yeast genomic tiling collection [Jones et al. 2008] and/or the Molecular Barcoded Yeast ORF (MoBY-ORF) library [Ho et al. 2009] [Supplemental Table S2]. Using these approaches, 60 temperature-sensitive alleles of 50 essential genes were verified to affect tRNA biology [Supplemental Table S2], representing 10.5% of the 476 genes in the Boone collection.

Candidates identified in the Hieter temperature-sensitive collection were analyzed by complementation assays (Fig. 1E). Since the strains in the Hieter temperature-sensitive collection do not harbor the nutritional markers required for complementation tests using the available genome-wide library plasmid collections (Ben-Aroya et al. 2008), we selected for Ura− variants for the candidates using 5-fluoroorotic acid selection [Boeke et al. 1984]. The resulting ura3 mutants with temperature-sensitive alleles affecting tRNA biology were transformed with the MoBY-ORF vectors that contain wild-type versions of the relevant genes, and complementation was assessed by Northern analyses. This resulted in 20 validated mutants (Supplemental Table S3; see Northern analyses in Supplemental Fig. S3), representing 6.0% of the genes in the Hieter temperature-sensitive collection.

In summary, through this genome-wide screen, we identified 174 genes whose mutations affect tRNA migration by Northern analysis, screened by biological repeats, and validated with independent mutants and/or complementation assays [Supplemental Tables S1–S3; Supplemental Figs. S1–S3]. Forty percent of the identified genes are essential genes, and 60% are unessential (Fig. 3A). However, there is a significantly higher percentage of essential genes identified from the temperature-sensitive collections (10.5% from the Boone and 6.0% from the Hieter collections) than unessential genes from the deletion collection (2.2%).

Among the 174 identified genes, 12 were previously known to be involved in tRNA processing, modification, intron removal, and transport [Fig. 3B], whereas 162 are novel genes not known previously to affect levels of processing intermediates and/or migration of tRNAs. Functional gene ontology (GO) analysis of the 174 identified genes revealed a clear enrichment in RNA transport and nucleocytoplasmic transport factors. Other enriched GO categories include macromolecular complex subunit organization, chromatin modification, and establishment of ribosome localization (Fig. 3C), suggesting possible links between tRNA biology and these cellular processes. As expected, molecular functions related to RNA metabolic process were significantly enriched in the GO analysis [Fig. 3C].

**Figure 3.** Novel gene products in tRNA nuclear–cytoplasmic trafficking

Among the nucleocytoplasmic transport factors identified, there is an overrepresentation of NPC and the RanGTPase cycle proteins [Table 1]. Nuclear pores are the portals of the nuclear envelope for nuclear–}

Wu et al.
cytoplasmic transport of proteins and RNAs. Previous studies reported that mutations of genes encoding five nuclear pore proteins [Nups; Nup49, Nup116, Nup145, Nup133, and Nsp1] cause accumulation of intron-containing tRNAs, whereas mutations of three others [Nup1, Nup85, and Nup100] have no apparent effect on pretRNA splicing (Sharma et al. 1996; Simos et al. 1996). Of the ~30 genes encoding Nups [Wente and Rout 2010], 22 are available in the mutant collections that we screened. Consistent with previous reports, nup133Δ but not nup1Δ, nup85Δ, or nup100Δ cells accumulate intron-containing tRNA^Leu^UAU [Nup49, Nup116, Nup145, and Nsp1 are not represented in the mutant collections. Interestingly, cells harboring mutations of genes coding five additional Nups (Ndc1, Nup57, Nup159, Nup170, and Nup192), previously unknown to affect tRNA splicing, accumulate intron-containing tRNA^Leu^UAU [see Supplemental Figs. S1–S3]. Mutants harboring four different alleles of the nuclear envelop protein Brl1 also cause accumulation of unspliced tRNA [Supplemental Fig. S2]. The combined current and previous analyses show that mutations of 10 of the 26 assayed genes encoding mutant Nups affect pre-tRNA splicing [Table 1; Supplemental Figs. S1–S3].

The small GTPase Ran is required for nuclear-cytoplasmic transport of most macromolecules [Gorlich and Kutay 1999]. In yeast, the Ran GTPase system includes the GTPase Ran encoded by Gsp1 and Gsp2, the Ran GTPase-activating protein encoded by RNA1, the Ran guanine nucleotide exchange factor RCC1 encoded by PRP20/SRM1, and the Ran-binding protein RanBP1 encoded by Yrb1. We did not observe tRNA processing defects for the deletion mutants of either Gsp1 or Gsp2, likely due to their functional redundancy. However, we identified the other three genes, RNA1, PRP20/SRM1, and Yrb1, whose mutations cause the same phenotype —accumulation of end-matured intron-containing tRNAs [Table 1; Supplemental Fig. S2]. Previous studies reported that mutation of RNA1 [rna1-1] and PRP20 causes accumulation of intron-containing pre-RNAs [Hopper et al. 1978; Kadowaki et al. 1993; Sarkar and Hopper 1998]. Our results show, in addition, that Yrb1 is required for tRNA splicing.

We also identified two members of the importin-β family, Los1 and Crm1 [also called Xpo1, vertebrate exportin-1] [Table 1; Fig. 4]. los1Δ cells accumulate end-processed intron-containing tRNAs in the nucleus [Hopper et al. 1980; Sarkar and Hopper 1998; Murthi et al. 2010], and, as anticipated, we uncovered los1Δ as a mutant that is defective in pre-tRNA splicing.

The Ran GTPase-dependent karyopherin Crm1/Xpo1 may provide a second nuclear export pathway. Crm1 functions in nuclear export of proteins with leucine-rich nuclear export signals, preribosomes, and telomerase RNA [Fornerod et al. 1997; Stade et al. 1997; Woolford and Baserga 2013; Wu et al. 2014]. Through our screen, we learned that crm1-1 cells accumulate end-matured

### Table 1. Discovered gene products in tRNA nuclear–cytoplasmic transport

| Gene                  | NPC and nuclear envelope proteins | mRNA export machinery | Importin-β proteins | Essential/Unessential | Mutant phenotypes | References |
|-----------------------|----------------------------------|-----------------------|---------------------|-----------------------|-------------------|------------|
| NDC1                  | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 73; Supplemental Fig. S2-1, ABGel 92 | This study        |
| NUP57                 | Essential                        | Type 4/5/6            | Supplemental Fig. S3 | Sharma et al. 1996    |
| NUP133                | Unessential                      | Type 4/5/6            | Supplemental Fig. S1-2, JWGel 192 | This study        |
| NUP159                | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 79; Supplemental Fig. S2-1, ABGel 92 | This study        |
| NUP170                | Unessential                      | Type 4/5/6            | Supplemental Fig. S1-2, JWGel 191 | This study        |
| NUP192                | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 74 | This study        |
| SEC13                 | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 72 | This study        |
| BRL1                  | Essential                        | Type 4/5/6            | Supplemental Fig. S2-1, ABGel 118 | This study        |
| RNA1                  | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 73 | Hopper et al. 1978 |
| PRP20/SRM1            | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 81 | Kadowaki et al. 1993 |
| YRB1                  | Essential                        | Type 4/5/6            | Supplemental Fig. S2, ABGel 79 | This study        |
| MEX67                 | Essential                        | Type 4/5/6            | Fig. 2E; Supplemental Fig. S2, ABGel 81 | This study        |
| MTR2                  | Essential                        | Type 4/5/6            | Supplemental Fig. S3 | This study        |
| CRM1                  | Essential                        | Type 4/5/6            | Fig. 4; Supplemental Fig. S2-1, ABGel 83 | This study        |
| LOS1                  | Unessential                      | Type 4/5/6            | NA                   | This study        |

*See Figure 1 for an explanation of type 4/5/6. [NA] Not applicable, as los1Δ is a control for numerous gels.
intron-containing tRNA\textsubscript{UAU} and tRNA\textsubscript{Tyr} at the nonpermissive temperature [Fig. 4A,B]. To verify the phenotype of CRM1Δ cells identified in the Boone collection (B. crm1-1), we analyzed other strains that contain the same crm1-1 allele in different genetic backgrounds: the crm1-1 mutant in the Hieter temperature-sensitive collection background [H. crm1-1] and the original xpo1-1 allele [xpo1-1] [Stade et al. 1997]. Northern analysis shows that RNAs isolated from crm1-1 temperature-sensitive strains grown at 23°C generally possess slightly elevated levels of end-processed intron-containing tRNAs compared with wild-type and rna1-1 cells [Fig. 4A,B, cf. lanes 2,3,4 and 1,5]. After the shift to the nonpermissive temperature (37°C) for 2 h, there was no change in the distribution of tRNA species in wild-type cells [Fig. 4A,B, lanes 6]. However, the three crm1-1 temperature-sensitive strains as well as the rna1-1 strain [Fig. 4A,B, lanes 7–10] accumulated elevated levels of end-matured intron-containing tRNAs [I] as compared with the initial transcripts [P].

We validated that accumulation of species I is due to mutation of CRM1 by complementation assays. When Crm1 was exogenously expressed in crm1-1 cells from a multicopy plasmid [Jones et al. 2008], the level of end-matured intron-containing pre-tRNA\textsubscript{UAU} returned to the level observed in wild-type cells, whereas crm1-1 cells transformed with vector alone accumulated end-matured intron-containing tRNA\textsubscript{UAU} [Fig. 4C, cf. lanes 3,7 and 4,8]. The growth defect of crm1-1 cells after temperature shift was also complemented [Supplemental Fig. S4]. The data provide evidence that Crm1 affects tRNA biogenesis.

To determine whether the observed phenotype of crm1-1 may be an indirect consequence of exposing the mutant cells to 37°C for 2 h, we analyzed RNAs from cells exposed to 37°C for shorter times. crm1-1 cells accumulated end-matured intron-containing tRNAs after the shift to 37°C for 30 min [Fig. 4D]. The rapid effect on tRNAs indicates that Crm1 may play a direct role in tRNA nuclear export.

If Crm1 functions in parallel with Los1 to export intron-containing tRNA to the cytoplasm, there may be genetic interactions between crm1-1 and los1Δ. To test this, we used a crm1-1 los1Δ double mutant and assessed growth at various temperatures. At 23°C, the two single mutants (crm1-1 and los1Δ) and the crm1-1 los1Δ double mutant grew similarly to wild-type cells. The double mutant crm1-1 los1Δ cells grew less well than wild-type cells at 30°C. Importantly, although both single mutants (crm1-1 and los1Δ) are viable at 34°C, the double mutant crm1-1 los1Δ did not grow at 34°C [Fig. 4E], indicating that LOS1 genetically interacts with CRM1. The data demonstrate that cells are not viable if both pathways are blocked, further supporting that Crm1 may function in parallel with Los1 in tRNA nuclear export.

If Crm1 is involved in tRNA nuclear export, loss of its function should cause tRNA nuclear accumulation. We therefore evaluated the subcellular location of tRNA\textsubscript{Tyr}.

**Figure 4.** Crm1 is likely a new nuclear exporter for intron-containing tRNAs. (A) Temperature-sensitive mutation of the CRM1 gene leads to accumulation of end-matured intron-containing tRNA\textsubscript{UAU}.

Northern analysis of RNAs from wild-type, rna1-1, and crm1-1 strains of different genetic backgrounds was performed using probe 1 for tRNA\textsubscript{UAU}. (I/P) Ratio of species I divided by P and normalized to wild-type, rna1-1, and crm1-1 cells grown under the same conditions. (B) Temperature-sensitive mutation of the CRM1 gene leads to accumulation of end-matured intron-containing tRNA\textsubscript{Tyr}.

Northern analysis of RNAs from wild-type, rna1-1, and crm1-1 strains of different genetic backgrounds was performed using probe KC031. (I/P) Ratio of species I divided by P and normalized to wild-type cells grown under the same conditions. (C) Complementation of the crm1-1 temperature-sensitive phenotype by YEpCrm1. Northern analysis of RNAs from wild-type cells, Boone collection crm1-1 cells, and Boone collection crm1-1 cells transformed with YEpCrm1 or vector was performed using probe 1. (D) Northern analysis of RNAs from wild-type, crm1-1, los1Δ, crm1-1 los1Δ, and rna1-1 cells after 30 min of incubation at 37°C using probe 1. [E] Genetic interactions between LOS1 and CRM1. Serial dilutions of wild-type, crm1-1, los1Δ, and crm1-1 los1Δ cells were applied to YEPE medium, and the plates were incubated for 2 d at 23°C, 30°C, 34°C, and 37°C.
in crm1-1, wild-type, and rna1-1 cells by fluorescence in situ hybridization [FISH]. As expected, wild-type cells after the shift to 37°C for 0, 2, or 4 h exhibited an even distribution of tRNA^Tyr throughout cells [Fig. 5, top row]. rna1-1 cells exhibited nucleoplasmic accumulation of tRNA^Tyr after 2 or 4 h at 37°C, as previously reported [Fig. 5, middle row; Sarkar and Hopper 1998]. Surprisingly, for crm1-1 cells after the shift to 37°C for 2 h, the nucleoplasm appeared void of signal, and some cells accumulated tRNA^Tyr at the nuclear periphery [Fig. 5, bottom row, example cells are marked with arrowheads]. After 4 h at 37°C, most of the cells demonstrated tRNA^Tyr accumulation at the nuclear periphery [Fig. 5, bottom row, example cells are marked with arrowheads]. A similar nuclear peripheral location in crm1-1 cells was observed using a probe complementary to tRNA^{Ile}_{UAU} mature sequences (SRIM04) [Supplemental Material], although the signals were weaker, likely due to low tRNA expression (two tRNA^{Ile}_{UAU} genes vs. eight tRNA^Tyr genes) [Supplemental Fig. S5A]. We also attempted to locate only intron-containing pre-tRNA using a probe complementary to the tRNA^{Ile}_{UAU} intron (SRIM03) [Supplemental Material]. Although the signals are weak, the rna1-1 signal is nucleoplasmic before and after the shift to 37°C, whereas the signal for crm1-1 is nucleoplasmic before the shift to 37°C and is both nucleoplasmic and spread to the nuclear periphery after the shift to 37°C [Supplemental Fig. S5B]. Thus, loss of Crm1 function appears to cause tRNAs to accumulate at the nuclear periphery instead of the nucleoplasm. Together, the data support a role for Crm1 in tRNA nuclear/cytoplasmic dynamics.

We also identified the two components of the general mRNA nuclear exporter heterodimer Mex67/Mtr2 (vertebrate TAP/p15) that mediates translocation of mRNAs through the NPC [Nino et al. 2013], as their mutants accumulate end-processed intron-containing tRNAs at the nonpermissive temperature (Table 1; Fig. 2E; Supplemental Figs. S2, S3; Supplemental Tables S2, S3). Further studies are required to address the possibility that Mex67/Mtr2 serves as yet another nuclear export pathway for intron-containing tRNAs.

Possible dynamics of tRNAs and SEN in the cytoplasm

We identified categories of mutants that accumulate end-processed unspliced tRNA^{Ile}_{UAU}, possibly due to defects in tRNA dynamics post-nuclear export to the cytoplasm. The first category includes mutations of genes encoding actin cytoskeleton components ACT1, MYO2, and PFY1 (Table 2; Fig. 2F; Supplemental Fig. S2). Since, upon nuclear export, intron-containing tRNAs must localize to mitochondria for splicing, but intron-containing tRNAs are nearly undetectable in the cytoplasm [Sarkar and Hopper 1998], there may be a mechanism to rapidly deliver tRNAs to mitochondria. As there is extensive precedence for the role of the actin cytoskeleton in RNA subcellular transport [Buxbaum et al. 2015], one possible role for Act1, Myo2, and Pfy1 in tRNA processing is to deliver intron-containing tRNAs to mitochondria.

The second category of genes possibly involved in tRNA cytoplasmic processing includes TOM70 and SAM37 that encode mitochondrial outer membrane proteins responsible for targeting proteins to mitochondria [Stojanovski et al. 2012]. tom70a and sam37a mutations cause accumulation of unspliced tRNAs (Table 2; Fig. 2G,H; Supplemental Fig. S1). Although the heterotetrameric SEN complex is located on the mitochondrial cytoplasmic surface [Yoshihisa et al. 2003], the mechanisms for its localization, assembly, and function remain unknown. It is possible that Tom70 and Sam37 are required for proper localization or function of the SEN complex on mitochondria.

Unanticipated phenotypes of alterations of genes involved in tRNA biology

We uncovered genes involved in tRNA biogenesis and/or processing that were not expected to affect accumulation of tRNA processing intermediates. One group in this category encodes aaRSs. tRNA nuclear re-export by Msn5 requires that the tRNA cargo be spliced and aminoacylated [Huang and Hopper 2015]; thus, one would anticipate that aaRS mutations would cause nuclear accumulation of mature cognate tRNAs but not defects in pre-tRNA processing. Contrary to this prediction, the Ile RS temperature-sensitive mutation [ils1-1] resulted in accumulation of end-processed unspliced tRNA^{Ile}_{UAU}. More surprisingly, four aaRS-encoding genes also resulted in accumulation of noncognate end-processed unspliced tRNA^{Ile}_{UAU}. The temperature-sensitive collections contain 16 of the 20 aaRS genes. Of these, only four [cdc60, frs2, gln4, and ths1] cause accumulation of end-matured unspliced noncognate tRNA^{Ile}_{UAU} (Table 2; Supplemental Figs. S2, S3). It is unknown what these aaRSs have in common or whether the unspliced tRNAs are located in the nucleus [which would occur via a defect in primary tRNA nuclear export] or the cytoplasm [which would occur if tRNAs or the SEN complex failed to reach mitochondria]. It is also

Figure 5. tRNAs accumulate at the nuclear periphery in crm1-1 cells. Wild-type, rna1-1, and crm1-1 cells were cultured at 23°C, shifted to 37°C, and harvested at 0, 2, and 4 h after the temperature shift. The subcellular localization of tRNA^Tyr was examined by FISH using probe SRIM15. Nuclei were visualized by DAPI staining of DNA [highlighted in blue in the merged panels]. Arrowheads highlight example cells with tRNA signal at the nuclear periphery.
possible that this phenotype is due to novel functions of aaRSs (Guo et al. 2010).

Another group of genes known to be involved in tRNA biology, but not anticipated to affect tRNA processing, includes those encoding tRNA modification enzymes. Surprisingly, cells with mutations of *DUS2*, encoding a dihydrouridine synthetase, and *NCS2* and *SAP190*, involved in mcms*s*U34 modification of tRNA\(^{\text{Lys}}\)\(^{\text{UUU}}\), tRNA\(^{\text{Glu}}\)\(^{\text{UUC}}\), and tRNA\(^{\text{Gln}}\)\(^{\text{UUG}}\) [Karlsborn et al. 2014], accumulate the tRNA\(^{\text{Ile}}\)\(^{\text{UAU}}\) primary transcript (Table 2; Supplemental Fig. S1). For *ncs2Δ* and *sap190Δ* cells, the phenotypes may be due to the indirect role of mcms*s*U34 in regulating translation of proteins involved in transcription and other processes [Karlsborn et al. 2014; Nedialkova and Leidel 2015].

Mutations of genes encoding Pol III transcription factors would be expected to cause decreased levels of primary tRNA transcripts and all subsequent intermediates in the processing pathway. In contrast to the expectation, we identified mutations of genes encoding Pol III subunits Rpc11, Rpc25, and Rpo31 and the Pol III transcription factor TFIIC subunit Tfc8 that have increased levels of primary tRNA\(^{\text{Ile}}\)\(^{\text{UAU}}\) transcripts (Table 2; Supplemental Figs. S2, S3). Another prominent GO category consists of the chromatin modification factors (Fig. 3C). Mutations of these cause the same phenotype of accumulation of the tRNA primary transcript (Table 2; Fig. 2A; Supplemental Fig. S1). We observed an overrepresentation of key subunits of the major histone acetylation (HAT) and deacetylation (HDAC) complexes, including Gcn5 and Ngg1 of the SAGA HAT complex, Eaf7 and Yaf9 of the NuA4 HAT complex, Hos2 and Set3 of the Set3 HDAC complex,

| Table 2. Novel genes that affect tRNA processing |
|-----------------|-----------------|-----------------|
| Gene symbol     | Essential/unessential | Mutant phenotypes\(^a\) | Northern data location (figure number and gel number) |
| Cytoskeleton    |                  |                  |                                                        |
| *ACT1*          | Essential        | Type 4/5/6       | Fig. 2F, Supplemental Fig. S2, ABGel 73, Supplemental Fig. S2-1, ABGel92 |
| *MYO2*          | Essential        | Type 4/5/6       | Supplemental Fig. S2, ABGel 73, Supplemental Fig. S2-1, ABGel92 |
| *PEY1*          | Essential        | Type 4/5/6       | Supplemental Fig. S2, ABGel 73 |
| Mitochondrial protein targeting |                  |                  |                                                        |
| *SAM37*         | Unessential      | Type 4/5/6       | Supplemental Fig. S1-2, JWGel 193L |
| *TOM70*         | Unessential      | Type 4/5/6       | Fig. 2G,H, Supplemental Fig. S1-3, JWGel 195 |
| aaRS            |                   |                  |                                                        |
| *ILS1*          | Essential        | Type 4/5/6       | Supplemental Fig. S2-1, KCGel |
| *CDC60*         | Essential        | Type 4/5/6       | Supplemental Fig. S2, ABGel 81 |
| *FRS2*          | Essential        | Type 4/5/6       | Supplemental Fig. S3 |
| *GLN4*          | Essential        | Type 4/5/6       | Supplemental Fig. S3 |
| *THS1*          | Essential        | Type 4/5/6       | Supplemental Fig. S3 |
| tRNA modification |                 |                  |                                                        |
| *DUS2*          | Unessential      | Type 1/2         | Supplemental Fig. S1-2, JWGel 194 |
| *NCS2*          | Unessential      | Type 1/2         | Supplemental Fig. S1-3, JWGel 195 |
| *SAP190*        | Unessential      | Type 1/2         | Supplemental Fig. S1-3, JWGel 196 |
| Pol III transcription |              |                  |                                                        |
| *RPC11*         | Essential        | Type 1/2         | Supplemental Fig. S3 |
| *RPC25*         | Essential        | Type 1/2         | Supplemental Fig. S3 |
| *RPO31*         | Essential        | Type 1/2         | Supplemental Fig. S2, ABGel 81, Supplemental Fig. S2-1, ABGel92 |
| *TFC8*          | Essential        | Type 1/2         | Supplemental Fig. S3 |
| Histone acetylation and deacetylation |              |                  |                                                        |
| *BDF1*          | Unessential      | Type 1/2         | Supplemental Fig. S1-3, JWGel 198 |
| *EAF7*          | Unessential      | Type 1/2         | Supplemental Fig. S1-3, JWGel 195 |
| *GCN5*          | Unessential      | Type 1/2         | Fig. 2A; Supplemental Fig. S1-3, JWGel 199 |
| *HOS2*          | Unessential      | Type 1/2         | Supplemental Fig. S1-2, JWGel 192 |
| *NGG1*          | Unessential      | Type 1/2         | Supplemental Fig. S1-2, JWGel 192 |
| *SET3*          | Unessential      | Type 1/2         | Supplemental Fig. S1-2, JWGel 190 |
| *SHG1*          | Unessential      | Type 1/2         | Supplemental Fig. S1-2, JWGel 192 |
| *SPT8*          | Unessential      | Type 1/2         | Supplemental Fig. S1, ABGel 119 |
| *UME6*          | Unessential      | Type 1/2         | Supplemental Fig. S1-2, JWGel 192 |
| *YAF9*          | Unessential      | Type 1/2         | Supplemental Fig. S1-3, JWGel 195 |

\(^a\)See Figure 1 for an explanation of types 4/5/6 and 1/2.
and Ume6, which recruits the Rpd3 HDAC complex to target elements (Kurdisani and Grunstein 2003).

In summary, through an unbiased genome-wide screen, we uncovered scores of novel gene products that are involved in tRNA processing and intracellular trafficking, providing insights into the complexity of tRNA biology, extensive links between tRNAs and various cellular processes, and likely pathways for tRNA nuclear export that function in parallel with Los1.

**Discussion**

As the first comprehensive analysis of the role of nearly the entire proteome in tRNA biology, this study discovered 174 gene products that function in tRNA transcription, processing, turnover, and subcellular movements. The percentage of essential genes identified is greater than the percentage of unessential genes identified, underscoring that essential genes more frequently participate in tRNA biology than unessential genes.

Given the scope of the mutant collection and the method applied, the screen is likely to have missed a fraction of gene products that play a role in tRNA biology. First, we would not be able to identity the genes whose mutants are not available in the collections used for the screen. For example, we did not find three of the four SEN complex subunits [Sen15, Sen34, and Sen54] [Trotta et al. 1997], as mutations of these genes do not exist in the mutant collection of essential genes. Second, duplicated genes or genes with redundant functions might not be identified; for example, GSP1 and GSP2, both of which encode Ran. Neither gsp1Δ nor gsp2Δ scored in our assay, even though Ran pathway defects cause accumulation of end-processed intron-containing tRNAs [Hopper et al. 1978; Kadowaki et al. 1993; Sarkar and Hopper 1998]. Third, mutations of genes involved in tRNA retrograde import or re-export would not be revealed, as the imported tRNAs and cytoplasmic tRNAs are exactly the same size and cannot be differentiated by Northern analysis. Moreover, since this screen was conducted using only tRNA^UAc^, we could have missed gene products specific for particular tRNA families. For example, 3′ end processing steps differ among tRNA families [Skowronek et al. 2014], and Thg1 catalyzes a G^−1^ addition to only tRNA^His^ [Gu et al. 2003]. Also, some steps in tRNA biology, such as generation of tRNA half molecules by Rny1, occur only under particular stress conditions [Thompson and Parker 2009]; we would have missed mutations of the relevant genes because our screen was conducted for early log phase cells grown in rich YEPD medium. Furthermore, annotation errors in the MATa deletion collection could result in false negative results, as we did not perform the entire mutant hunt on the independent MATa deletion collection. Finally, we only screened protein-encoding genes, and it is possible that RNA-encoding genes might affect particular steps of tRNA biology. Nonetheless, this unbiased screen covered nearly all annotated yeast genes and revealed a role for 162 novel proteins in tRNA processing, intron turnover, and subcellular dynamics (Fig. 2B; Supplemental Figs. S1–S3; Supplemental Tables S1–S3).

**Ran, NPC, and tRNA nuclear–cytoplasmic trafficking**

A primary motivation for conducting this unbiased genome-wide screen was to gain understanding of the pathways for tRNA nuclear export and their mechanisms of action. This goal was met by the discoveries of Nups, a RanGTPase-associated protein, and two possible new nuclear export pathways for end-matured intron-containing tRNAs.

Nuclear–cytoplasmic transport of tRNAs occurs through nuclear pores composed of ~30 Nups [Wente and Rout 2010]. Six of the 22 available nucleoporin mutants accumulate end-matured unspliced tRNAs, including previously reported nup133Δ [Sharma et al. 1996; Simos et al. 1996] and five Nups not previously known to affect tRNA nuclear export. These genes encode Nups present in nearly all NPC substructures—the inner ring [Nup170], outer ring [Nup133], transmembrane ring [Ndc1], symmetric FG Nups [Nup57], and cytoplasmic FG Nups [Nup159] [Wente and Rout 2010]—suggesting the importance of each substructure in tRNA export. Since less than half of the NPC components were found to affect tRNA splicing, the identified Nups may have more direct interactions with the tRNA export complex than other Nups. It is also possible that other unidentified Nups perform redundant functions in tRNA nuclear export and therefore did not score in the screen, as there is evidence for their functional redundancy [Terry and Wente 2009].

We uncovered mutants defective in the Ran-GTPase cycle—rna1-1 [RanGAP], prp20/srm1-ts [RanGEP], and yrb1-51 [RanBP]—to be defective in pre-tRNA splicing, further establishing, as anticipated, the role of the whole Ran GTPase cycle in tRNA nuclear export. Ran-binding importin-β family members Los1 and Msn5 function in tRNA nuclear export. los1 mutants accumulate end-processed intron-containing tRNAs [Hopper et al. 1980] due to reduced primary tRNA nuclear export [Sarkar and Hopper 1998; Yoshihisa et al. 2003]. Since Msn5 does not export intron-containing tRNAs [Huang and Hopper 2015], Los1 is the only known protein for initial export of intron-containing tRNAs [Murthi et al. 2010]. However, S. cerevisiae Los1, the Schizosaccharomyces pombe homolog Xpot, and the plant homolog PAUSED are all unnecessary for cell viability, supporting the existence of alternative tRNA nuclear export pathways in these organisms [Hurt et al. 1987; Li and Chen 2003; Cherkasova et al. 2012].

The four yeast members of the importin-β family that serve as nuclear exportins [Los1 [exportin-t], Msn5 [exportin-5], Cse1 [CAS], and Crm1 [exportin-1]] are all represented in the mutant collections that we screened. Of these, Los1 and Crm1 affect tRNA biology, as assessed by Northern analysis. We identified Crm1 as a possible exporter for intron-containing tRNAs. Like los1Δ cells, crm1-1 temperature-sensitive mutants accumulate end-matured intron-containing tRNAs after the shift to the nonpermissive temperature. crm1-1 cells rapidly accumulate the pre-tRNAs, suggesting a direct role of Crm1 in tRNA biology. Furthermore, crm1-1 los1Δ double mutant cells do not grow at temperatures at which either single
mutant can grow, suggesting that cells require at least one of these pathways for tRNA nuclear export. We did not observe enhanced accumulation of end-matured intron-containing tRNAs in the crm1-1 los1Δ double mutant [Fig. 4D, lanes 4, 9] compared with the single mutants [Fig. 4D, lanes 2, 3, 7, 8]. The reason for this is unknown, but one possibility is that other tRNA nuclear pathways [for example, the Met67/Mtr2 pathway] may be up-regulated in the double mutant to compensate for the loss of two exporters.

Curiously, crm1-1 cells accumulate tRNAs at the nuclear periphery, unlike rna1-1 and los1Δ cells that accumulate tRNAs in the nucleoplasm. The staining pattern is similar to the pattern that we previously reported for the nucleoporin Nsp1 [Murthy and Hopper 2005]. Possibly, there is a failure of tRNAs to move through the NPC or a failure to release tRNAs from the cytoplasmic face of the NPC in crm1-1 cells. Further in vivo biochemical studies to investigate direct interaction of end-matured intron-containing tRNAs with Crm1 are required to unravel its role in tRNA nuclear–cytoplasmic dynamics.

Mex67 and Mtr2 are components of the nuclear export machinery of mRNAs, 60S ribosomal subunits, and the telomerase RNA [Yao et al. 2007; Wu et al. 2014]. We found that mex67-ts5 and mtr2 mutants accumulate end-matured intron-containing tRNAs, the same phenotype as los1Δ and crm1-1 cells [Hopper et al. 1980; Sarkar and Hopper 1998]. Future studies are required to test the tantalizing possibility that the Mex67/Mtr2 complex provides another nuclear export pathway for tRNAs. In sum, a major discovery from this work is the possibility that three pathways operate in parallel to export intron-containing pre-tRNAs from the nucleus to the cytoplasm [Fig. 1A].

**Targeting tRNAs and the SEN complex to mitochondria for tRNA splicing**

Unlike the human SEN complex that is located in the nucleus [Paushkin et al. 2004], the yeast SEN complex is located on the outer surface of mitochondria [Yoshihisa et al. 2003]. Therefore, after nuclear export, intron-containing tRNAs must locate to mitochondria. However, no gene products have previously been implicated in this process. We identified mutations of three genes encoding cytoskeleton proteins that accumulate end-matured intron-containing tRNAs. These proteins include actin, a myosin V motor [Myo2], and profilin [Pfy1]. actin-mediated RNA subcellular transport requires myosins. Yeast has two myosin V motors: Myo2 and Myo4. Myo4 functions in delivery of ASH1 mRNA to daughter cells [Takizawa et al. 1997]. Possibly, Myo2 has a similar function for tRNA transport to mitochondria. In Drosophila, profilin is required for localizing the oskar mRNA to the posterior pole of the developing oocyte [Manseau et al. 1996], and so Pfy1 may also serve in tRNA subcellular dynamics. It is also possible that these proteins are involved in proper localization and function of the SEN complex or their encoding mRNAs to mitochondria.

The mechanisms for targeting each of the four subunits of SEN to mitochondria for proper assembly and function of the SEN complex remain unclear. We identified two genes encoding mitochondrial outer membrane proteins [TOM70 and SAM37] that participate in targeting proteins to mitochondria [Stojanovski et al. 2012]. We are examining the hypothesis that they are required for the proper localization of the SEN subunits to the mitochondria surface.

**Links between tRNAs and other cellular processes**

Our screen results also revealed links between tRNAs and many other cellular processes, such as histone acetylation/deacetylation. It is remarkable that the identified HAT and HDAC genes are related to each other physically and genetically [Lin et al. 2008] and that mutations of these genes cause the same result: accumulation of primary tRNA transcripts. While understanding the biological relevance of HAT and HDAC proteins requires further investigation, an interesting possibility is that these proteins ensure the chromatin organization needed for proper localization of tRNA genes in the nucleolus [Thompson et al. 2003] or access to the tRNA 5′ end processing enzyme RNase P [Bertrand et al. 1998].

In conclusion, our genome-wide screen discovered 162 novel gene products involved in tRNA biology, 116 of which have human orthologs. These include two possible novel tRNA nuclear exporters (Crm1 and Mex67/Mtr2), new roles of genes known to affect tRNAs, and links between tRNA biology and various cellular processes. The results not only depict a global view of tRNA biology in yeast but also could provide insights into tRNA biology in other higher organisms. Future work will be needed to address the roles of the identified gene products in tRNA biogenesis and subcellular movement.

**Materials and methods**

**Strains**

The S. cerevisiae MATa deletion collection [BY4741 background: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0] and the MATa deletion collection [BY4742 background: MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0] were purchased from Open Biosystems. Two temperature-sensitive collections were gifts from Dr. C. Boone and Dr. P. Hieter. The xpo1-1 temperature-sensitive strain was a gift from Dr. C. Guthrie. The yeast genomic tiling collection was purchased from Open Biosystems. The MoBY-ORF library was purchased from Thermo Scientific Open Biosystems. Standard methods for culturing yeast were used.

**High-throughput small RNA isolation and Northern analysis**

Yeast mutant strains were grown in the 96-deep-well plates in YEPD to early log phase, and small RNAs were isolated using a high-throughput RNA isolation method described previously [Wu et al. 2013]. The temperature-sensitive mutants were shifted to nonpermissive temperature [37°C] for 2 h before RNA extraction. tRNAs were analyzed by a nonradioactive Northern method.
using DIG-labeled probes as described [Wu et al. 2013] using probes 1 and KC031 (see the Supplemental Material).

Growth assays

Five microliters of aliquots from serial dilutions of the indicated yeast cell cultures was spotted on rich or selective medium. The plates were incubated for 2 d at the indicated temperatures.

FISH

Cells were grown in 15 mL of medium overnight at 23°C to early log phase (OD600 = 0.15–0.3). Temperature-sensitive cells were shifted to 37°C for 2 or 4 h before OD600 reached 0.3. Cells were collected, and FISH was performed as previously described using probe SRM15 for tRNAVT [Sarkar and Hopper 1998] with the previously described modifications [Stanford et al. 2004]. Images were captured using a Nikon microscope equipped with an Ultra-Vox spinning disk confocal apparatus (PerkinElmer Life and Analytical Science) and a cooled 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 63×/1.4 NA objective lens. Images were captured using Velocity software and National Institutes of Health ImageJ and assembled using Adobe Photoshop C6.

Oligonucleotides

The sequences of the oligonucleotides used are provided in the Supplemental Material.

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