Regulation of tRNA Subcellular Trafficking in *Saccharomyces cerevisiae*

**DISSEMINATION**

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By

Hsiao-Yun Huang

Graduate Program in Molecular, Cellular and Developmental Biology

The Ohio State University

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Dissertation Committee:

Dr. Anita K. Hopper, Advisor

Dr. Stephen A. Osmani

Dr. Daniel R. Schoenberg

Dr. Robin P. Wharton

Dr. Paul Herman
Eukaryotic tRNAs serve the essential function for protein synthesis in the cytoplasm. tRNA subcellular movement was long believed to be unidirectional, from the nucleus to the cytoplasm. Surprisingly, it is now known that mature tRNAs also move in a retrograde fashion from the cytoplasm to the nucleus via retrograde tRNA nuclear import, a process that is conserved from lower to higher eukaryotes. To investigate the mechanisms of bidirectional tRNA subcellular trafficking, we addressed important and fundamental issues regarding the retrograde pathway using yeast, *Saccharomyces cerevisiae*, as a model system: (1) What are the functions of known β-importins in tRNA subcellular trafficking? (2) What are the mechanisms by which the re-export step has specificity to processed and likely charged tRNAs? (3) How does the response to nutrient availability regulate the tRNA re-export step? We first successfully developed an optimized crosslinking-based co-immunoprecipitation strategy to detect *in vivo* tRNA transport complexes. *In vivo* genetic and biochemical data suggest that Los1 binds and exports both intron-containing pre-tRNAs and mature tRNAs. Therefore, Los1 functions in both primary tRNA nuclear export and re-export. For tRNAs encoded by intron-containing genes, Msn5 functions primarily in tRNA re-export to the cytoplasm. The specificity of Msn5 to mature tRNAs appears to be assisted by eEF1A via formation of a cooperative nuclear export complex. We also showed that there are multiple levels of
control connecting nutrient conditions to tRNA subcellular distribution: tRNA aminoacylation, aa signaling, and subcellular distribution of tRNA exportin.
This dissertation is dedicated to my family and the memory of my dear father.
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Vita

1997-2000 ........................ Kaohsiung Municipal Girls’ Senior High School, Taiwan
2000-2004 ........................... B.S., Life Science, National Central University, Taiwan
2004-2005 ........................... M.S., Life Science, National Central University, Taiwan
2005-2007 ........................... Research Assistant, National Central University, Taiwan
2007-2014 ........................... Graduate Research Associate, Graduate Program in
                                 Molecular, Cellular, and Developmental Biology,
                                 The Ohio State University

Publications


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### Fields of Study

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Figure A.2 Northern analysis of pre-tRNA\textsuperscript{Ile} and mature tRNA\textsuperscript{Ile} for tRNA processing candidates

Figure B.1 The subcellular distribution of tRNA tracker in wt and mutant cells

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Chapter 1 Introduction

Eukaryotic cells contain a variety of membrane bound organelles in their cytoplasm, including mitochondria, Golgi apparatus, vacuole, and nucleus. The nucleus which is surrounded by the nuclear envelope serves as the genetic information center that regulates a diversity of biological processes. Compartmentalization in eukaryotes provides opportunity for spatial and temporal regulation, and the import and export of proteins and nucleic acids through the nuclear pores are tightly controlled. The nuclear-cytoplasmic traffic serves not only to permit operation of the basal replication, transcription, and processing machinery but also to regulate cell cycle, transcriptional activation and repression, and other cellular processes.

Transfer RNA (tRNA) that is transcribed in the nucleus must be properly transported to the cytoplasm since it serves its essential role of delivering amino acid to the cytoplasmic protein synthesis machinery. Surprisingly, tRNA moves from the cytoplasm to the nucleus via retrograde nuclear import and can again access the cytoplasm via the re-export step. The tRNA retrograde pathway is conserved from yeast to vertebrates and it is responsive to nutrient availability. The mechanisms regulating tRNA subcellular movement remain poorly understood. Thus, the goal of this dissertation is to investigate
the regulation of tRNA nuclear-cytoplasmic trafficking, using *Saccharomyces cerevisiae* (budding yeast), a single cell eukaryote, as the model organism.

### 1.1 Overview of tRNA biology

tRNA serves an essential role in protein synthesis. Interestingly, eukaryotic tRNA also functions in other processes and signaling pathways, such as targeting protein for degradation, signaling in the general amino acid control (GAAC) pathway, and regulation of apoptosis by directly binding to cytochrome C (Varshavsky, 1997; Dever & Hinnebusch, 2005; Mei et al., 2010a). Alterations in the levels of tRNA transcripts and defects in tRNA processing and modifications result in several human diseases, including neuronal disorders (Lemmens et al., 2010), pontocerebellar hypoplasia (Budde et al., 2008), and cancer [(Pavon-Eternod et al., 2009; Maute et al., 2013; Pavon-Eternod et al., 2013); review: (Phizicky & Hopper, 2010; Torres et al., 2014)]. Due to the importance of tRNA, it is crucial to thoroughly understand its biogenesis. tRNA biogenesis involves multiple steps conserved throughout all eukaryotes, including transcription, post-transcriptional alterations, and nuclear-cytoplasmic trafficking. In addition to the regulation of each step of tRNA biogenesis, cells also employ multiple tRNA quality control mechanisms to prevent inappropriate substrates being used in the protein synthesis. The following introductions focus on tRNA post-transcriptional processing, tRNA subcellular trafficking, and tRNA quality control.
1.2 tRNA post-transcriptional processing

tRNAs are transcribed by RNA polymerase III as precursor molecules (pre-tRNAs). The pre-tRNAs undergo an elaborate set of post-transcriptional steps to generate mature tRNAs. These steps include: removal of both the 5′ leader and 3′ trailer sequences, nucleotide addition to all 3′ ends and to a 5′ end of one tRNA, tRNA\textsuperscript{His}, removal of introns for transcripts transcribed by intron-containing genes, and addition of nucleotide modifications (Fig. 1.1).

1.2.1 tRNA 5’ and 3’ processing

The majority of yeast pre-tRNAs transcripts contain about 12 extra leader nucleotides on the 5′ end and about 12 extra trailer nucleotides on the 3′ end (O’Connor & Peebles, 1991; Hopper & Phizicky, 2003). Maturation of tRNAs in nearly all organisms begins with removal of the 5′ extension by the endonuclease RNase P which is located in the nucleolus in yeast (Xiao et al., 2002). RNase P is a ribonucleoprotein complex comprised of a single RNA and a variable number of protein subunits in bacteria, Archaea, and eukaryotes (Jarrous & Gopalan, 2010). Recent studies have showed that there are extensive phylogenetic differences in RNase P structure. The RNA subunit is the catalytic component in bacteria which have one protein subunit (Guerrier-Takada et al., 1983; Torres-Larios et al., 2005) and in Archaea which have five protein components (Pannucci et al., 1999; Cho et al., 2010). In eukaryotes, the protein subunits have been shown to play a supportive role in catalyzing the removal of the 5′ end of tRNA (Crary et al., 1998; Kurz & Fierke, 2002). Yeast nucleolar RNase P consists of nine proteins (Pop1,
Pop3-8, Rpp1, and Rpr2) and a single essential RNA (RPR1) (Xiao et al., 2002). The source of eukaryotic RNase P catalysis remains unclear since RNA components have substantial differences in regions important for stability and catalysis (Marquez et al., 2005; Marquez et al., 2006). Interestingly, higher plant mitochondrial and nuclear forms of RNaseP are now known to be protein enzymes (Thomas et al., 2000; Gutmann et al., 2012). In addition to the canonical role in pre-tRNA processing, most of the proteins subunits of the yeast and human RNase P are shared with RNase MRP, involved in rRNA maturation (Xiao et al., 2002; Jarrous & Gopalan, 2010; Pinker et al., 2013).

3’ end maturation is far more complex than 5’ end processing of tRNA. Maturation of 3’ extensions from pre-tRNA requires both exo- and endo- nucleases (Li & Deutscher, 1996; Phizicky & Hopper, 2010; Skowronek et al., 2014). Yeast Rex1 is a 3’ to 5’ exonuclease that participates in the processing of pre-tRNA trailers as well as in the processing of other RNAs such as 5S rRNA, 5.8S rRNA, and snRNAs (van Hoof et al., 2000; Copela et al., 2008; Ozanick et al., 2009). RNase Z, Trz1, is the endonuclease that participates in 3’ end processing for both mitochondrial and nuclear encoded tRNAs (Chen et al., 2005; Maraia & Lamichhane, 2011; Daoud et al., 2012; Hopper, 2013; Skowronek et al., 2014). Recent studies show that the majority of tRNAs utilize both endonucleolytic cleavage and exonucleolytic trimming pathways, with a preference for the endonucleolytic cleavage (Skowronek et al., 2014). Exonucleolytic digestion occurs to some degree in wild-type cells and not only when endonucleolytic cleavage is inhibited. However, 3’ end processing of pre-tRNAs with longer 3’ trailers depends to a greater extent on endonuclease, probably due to an inefficient exonucleolytic pathway,
inhibited by secondary structures comprised within their longer 3′ extension (Skowronek et al., 2014).

In addition, pre-tRNA processing involves La protein that possesses chaperon activity. Generally, Pol III pauses at the tRNA gene terminator and generates a variable length 3′ oligo (U) tract on pre-tRNA. It seems that precursors with different 3′ ends display diverse affinities for La binding, which favors Trz1-mediated endonucleolytic cleavage and protects against exonucleolytic trimming (Yoo & Wolin, 1997). It is proposed that 3′ oligo (U) length is a primary determinant of La binding with subsequent steps distinguished by 3′ endo- vs. exo- nuclease, chaperon activities and nuclear surveillance [Review: (Maraia & Lamichhane, 2011)].

All tRNAs contain a 3′ terminal CCA sequence that is necessary for tRNA aminoacylation. In yeast and vertebrates, addition of 3′ CCA sequence is catalyzed by nucleotidyl transferase, while E. coli tRNAs are encoded with a CCA sequence. But E. coli also possesses the gene for the CCA adding enzyme, which functions in tRNA 3′ end repair (Zhu & Deutscher, 1987; Reuven & Deutscher, 1993). Yeast tRNA nucleotidyl transferase is encoded by CCA1 (Aebi et al., 1990). CCA1 encodes multiple isoforms, Cca1-I, Cca1-II, and Cca1-III, which are generated by alternative transcriptional and distinct translational start sites. These isoforms are differently distributed among mitochondria, the cytoplasm, and the nucleoplasm [Review: (Martin & Hopper, 1994)]. Addition of 3′ CCA sequence is normally catalyzed by the nuclear pool of CCA adding enzyme, while the cytoplasmic pool functions in tRNA 3′ repair. It is thought that the mitochondrial form functions in both biogenesis and 3′ end repair.
1.2.2 tRNA splicing

Analysis of bacterial, archael, and eukaryotic genomes revealed that tRNA genes often contain introns (Chan & Lowe, 2009). Removal of introns by tRNA splicing is essential as intron-containing pre-tRNAs cannot be aminoacylated, nor base pair with anticodons (O'Farrell et al., 1978). Yeast and vertebrate tRNA introns are always located one base 3′ to the anticodon, but introns appear in other locations in tRNA genes of Archaea [(Phizicky & Hopper, 2010); http://lowelab.ucsc.edu/GtRNAdb/]. Of the 274 yeast nuclear tRNA genes, 59 (>20%) contain an intron, while only 6% of human tRNA genes are encoded by intron-containing genes (Chan & Lowe, 2009). In S. cerevisiae, a total 10 tRNA families contain an intron. They vary from 14-60 nucleotides, but for a given family they are nearly identical in length and sequence (http://lowelab.ucsc.edu/GtRNAdb/). Thus, in order to generate a complete set of tRNAs for decoding, removal of introns by tRNA splicing is an essential process.

**Pre-tRNA splicing steps**- The pre-tRNA splicing reaction occurs in yeast including three steps, involving three essential enzymes (Phizicky et al., 1992; Culver et al., 1997; Trotta et al., 1997). The first step of the splicing is the removal of the intron from pre-tRNAs resulting in two tRNA halves (5′ half and 3′ half). This step is catalyzed by tRNA splicing endonuclease (Knapp et al., 1978). The yeast tRNA splicing endonuclease is a heteratetramer (Sen2, Sen34, Sen 15, and Sen54) (Trotta et al., 1997). Upon endonucleolytic cleavage, the tRNA 5′ half possesses a 2′, 3′ cyclic phosphate and the 3′ half possesses a 5′ hydroxyl. The two halves are ligated together in the second step by the
yeast tRNA ligase, Trl1 (Phizicky et al., 1986). The reaction results in the creation of a splice junction with a 3’, 5’ phosphodiester bond and a 2’ residual phosphate at the splice junction (Greer et al., 1983; Abelson et al., 1998). The residual 2’ phosphate at the splice junction is removed in the third step which is catalyzed by the 2’ phosphotransferase, encoded by yeast TPT1 (Spinelli et al., 1997). The complicated yeast tRNA splicing and ligation mechanism is conserved in plants (Gegenheimer et al., 1983; Schwartz et al., 1983; Culver et al., 1994; Englert & Beier, 2005). However, vertebrates and Archaea ligate the 5’ and 3’ halves directly by a 3’-5’ ligase activity, which bypass the need for a 2’ phosphotransferase [(Popow et al., 2011; Popow et al., 2012); review: (Hopper, 2013)]. Therefore, the step one of pre-tRNA splicing is conserved from Archaea, to yeast, to plants, to vertebrates, while the steps of completion of the splicing reaction are not conserved.

**Cellular distribution of splicing enzymes**- In vertebrates, pre-tRNA splicing occurs in the nucleoplasm (Fig. 1.1 A) (Melton et al., 1980; Lund & Dahlberg, 1998). Surprisingly, in yeast, tRNA splicing endonuclease is located on the outer surface of mitochondria (Yoshihisa et al., 2003), the tRNA ligase is distributed throughout the cytoplasm (Huh et al., 2003; Mori et al., 2010), and the 2’ phosphotransferase is located in both the nucleus and the cytoplasm (Dhungel & Hopper, 2012). Thus, yeast splicing occurs in the cytoplasm, suggesting that end-processed intron-containing pre-tRNAs must be exported from nucleus to cytoplasm to access to the tRNA splicing machinery (Fig. 1.1 B). Studies that re-engineered yeast cells to have a subcellular organization of the tRNA splicing machinery similar to vertebrate cells revealed that tRNA splicing endonuclease possesses
a novel function unrelated to splicing that requires all four subunits to be located in the cytoplasm. It has been suggested that tRNA splicing endonuclease might have an indirect role in pre-rRNA processing (Dhungel & Hopper, 2012).

### 1.2.3 tRNA modification

One of the remarkable features of tRNA from all organisms is that they are highly modified by numerous post-transcriptional steps. More than 100 different modifications and RNA modifying enzymes have been described (Czerwoniec et al., 2009). Some modifications are restricted to Archaea, bacteria, or eukaryotes, but many are shared. Thus, genomes encode a large number of enzymes responsible for catalyzing the correct modifications at the proper site of particular tRNAs. In *S. cerevisiae*, the majority of enzymes that modify tRNA have been identified (Phizicky & Hopper, 2010). The distributions of the modifications among tRNA families have been compiled at [http://modomics.genesilico.pl/sequences/list/tRNA](http://modomics.genesilico.pl/sequences/list/tRNA).

**Functions for tRNA modifications** - tRNA modifications serve numerous purposes, including (1) translation fidelity, (2) tRNA folding or stability, and (3) tRNA discrimination.

(1) Modifications can function in codon-anticodon interactions and reading frame maintenance. Modifications of the anticodon can affect decoding. The deamination of adenosine (A) to inosine (I) at wobble position 34 of tRNA provides an example of tRNA modification affecting decoding. As A only base pairs with U, but I base pairs with U, C, and A, tRNAs with I at the wobble position have a broader codon-anticodon interaction
capacity (Gerber & Keller, 1999). Modifications in the anticodon loop can also maintain the reading frames during translation. For example, mutation of the genes responsible for \( (yW) \) modification of tRNA\(^{Phe} \) at position 37 results in increases in -1 frameshifting during translation (Waas et al., 2007).

(2) Modifications in the main body of the tRNA can affect tRNA folding or stability. For example, when cells possess mutations of multiple modifications genes, such as \( pus1\Delta\ pus4\Delta, trm4\Delta\ trm8\Delta, \) or \( trm44\Delta\ tan1\Delta \) synthetic lethality or temperature sensitive growth phenotype occurs (Grosshans et al., 2001; Alexandrov et al., 2006). The temperature sensitive growth has been shown to be caused by tRNA instability (Kotelawala et al., 2008; Dewe et al., 2012), and turnover of unstable tRNA is mediated by the 5’ to 3’ rapid tRNA decay pathway (RTD) (Alexandrov et al., 2006). Thus, tRNA modifications are key determinants of tRNA stability.

(3) Modifications at various positions specifically provide tRNA discrimination. For instance, tRNA\(^{\text{iMet}} \) is modified at adenosine 64 (Ar(p)64) by Rit1 which only interacts with unique T stem-loop of tRNA\(^{\text{iMet}} \). Modified tRNA\(^{\text{iMet}} \) does not interact with eEF1A so that it functions only at an initiating AUG codon (Shin et al., 2011).

\text{tRNA modification enzymes also serve novel functions beyond tRNA discrimination, decoding, and tRNA stability. Mod5, which is responsible for modification of A\textsubscript{37} to iA\textsubscript{37}, regulates the sterol biosynthesis pathway (Benko et al., 2000). Sterol biosynthesis and modification of iA\textsubscript{37} utilize the same intermediate and the two pathways compete, thereby connecting tRNA modification with sterol metabolism (Benko et al., 2000).} \)
Cell biology of tRNA modifications- In eukaryotes, tRNA modifications occur in a preferred order (Nishikura & De Robertis, 1981) and are catalyzed at multiple cellular sites (Fig. 1.1). In yeast, some modification enzymes are restricted to the nucleus and particular subnuclear locations, such as the nucleoplasm or inner nuclear membrane, whereas others are restricted to the cytoplasm [Review: (Hopper, 2013)]. Trm6 and Trm61, responsible for m^1A_{58} modification that occurs on some tRNA initial transcripts, are located in the nucleus. For the group of enzymes that modify intron-requiring sites, they mainly reside in the nucleus where intron-containing pre-tRNAs are located (Hopper & Phizicky, 2003; Huh et al., 2003). However, not all of enzymes that modify spliced tRNA are localized in the cytoplasm. For example, part of the cellular pool of Mod5 (t^6A_{37}) which only modified spliced tRNA is located in the nucleolus (Tolerico et al., 1999) and Trm5 (m^1G_{37}, m^1I_{37}), which also modifies only spliced tRNA, is located in the nucleus and mitochondria (Lee et al., 2007; Ohira & Suzuki, 2011). Some modification enzymes able to modify either intron-containing or intron-lacking tRNAs are located in the nucleus, such as Trm1 (m^2G_{26}) (Lai et al., 2009); others appear to be primarily cytoplasmic, such as Sua5 (c^6A_{37}) (Huh et al., 2003). The subcellular locations of some modification enzymes remain unknown. Generally, the subcellular distribution of the tRNA modification enzymes dictates an ordered pathway for tRNA modification. Interestingly, ordered modifications appear to be required for the yW_{37} modification of tRNA^{Phe} (Ohira & Suzuki, 2011; Guy et al., 2012). The requirement of Gm_{34} and Cm_{32} modification for yW modification of tRNA^{Phe} represents that the specific tRNA modifications can direct the modification on a different residue (Guy et al., 2012).
Similarly, in *Thermus thermophilus* the $m^7G_{46}$ modification provides the introduction of other modifications via a tRNA modification network (Tomikawa et al., 2010).

### 1.2.4 tRNA aminoacylation

Before binding to ribosomes, tRNAs are aminoacylated, or charged, at the 3′ end of the CCA sequence. Charging is catalyzed by aminoacyl tRNA synthetases (aaRS). There are 20 aaRSs, each corresponding to a single amino acid. Since there are multiple tRNAs for its cognate amino acid, one aaRS generally recognize more than one tRNA substrate. Although the classical view was that tRNA aminoacylation solely occurs in the cytoplasm, several aaRSs are located in the nucleus in yeast and vertebrates (Lund & Dahlberg, 1998; Nathanson & Deutscher, 2000; Azad et al., 2001). Surprisingly, tRNA charging has been shown to occur in the nucleus and nuclear aminoacylation is important for tRNA nuclear export (Lund & Dahlberg, 1998; Sarkar et al., 1999; Azad et al., 2001). It is proposed that nuclear aminoacylation of tRNA serves as a proofreading mechanism to ensure that only properly processed tRNAs access the cytoplasmic protein synthesis machinery (Lund & Dahlberg, 1998). The regulation of tRNA aminoacylation status in tRNA subcellular movement is discussed in the following chapters.
1.3 tRNA subcellular trafficking

Pre-tRNAs in the nucleus are processed and exported to the cytoplasm where the translation machinery located. It is now known that tRNAs also move from the cytoplasm to the nucleus via retrograde nuclear import and can again access the cytoplasm via tRNA re-export (Shaheen & Hopper, 2005; Takano et al., 2005; Whitney et al., 2007). This tRNA retrograde pathway is conserved from yeast to vertebrates (Zaitseva et al., 2006; Shaheen et al., 2007; Barhoom et al., 2011; Miyagawa et al., 2012). The current model of tRNA subcellular trafficking includes three separate steps. First, newly transcribed and end-processed tRNAs are exported from the nucleus to the cytoplasm (primary tRNA nuclear export) (Fig. 1.2 A). Second, mature tRNAs are constitutively transported back to the nucleus via retrograde tRNA nuclear import (Fig. 1.2 B) (Shaheen & Hopper, 2005; Takano et al., 2005; Zaitseva et al., 2006; Shaheen et al., 2007; Murthi et al., 2010; Barhoom et al., 2011; Miyagawa et al., 2012). Third, mature tRNAs are re-exported to the cytoplasm (tRNA re-export) (Fig. 1.2 C) (Whitney et al., 2007).

1.3.1 Overview of nuclear-cytoplasmic transport

Movement of tRNAs between the nucleus and the cytoplasm proceeds via the Ran pathway with the association of importin-β family members. Cells encode numerous importin-β family members; the members of importin-β family which are dedicated to the nuclear export process are exportins, whereas the members that transport cargo from the cytoplasm to the nucleus are importins. The process of nuclear transport is controlled by small GTPase Ran, which binds to the β-importins to regulate association and
dissociation of the transport complexes (Strom & Weis, 2001; Fried & Kutay, 2003; Madrid & Weis, 2006). Ran exists in a GTP-bound form in the nucleus and a GDP-bound form in the cytoplasm. This asymmetric distribution results from the distinct subcellular localizations of the Ran cycle regulators, the nuclear guanine nucleotide exchange factor (RanGEF) which is responsible for the conversion of RanGDP to RanGTP states and the cytoplasmic GTPase activating protein (RanGAP) which stimulates GTP-bound Ran to hydrolyze to be GDP-bound Ran (Ohtsubo et al., 1989; Hopper et al., 1990; Bischoff & Ponstingl, 1991; Bischoff et al., 1994). Exportins bind cargo, directly or via an adaptor, and RanGTP in the nucleus and then move to the cytoplasm. RanGAP stimulates GTP hydrolysis of Ran to dissociate the export complexes and thereby release cargo in the cytoplasm (Fig. 1.3 A). In contrast, importins bind cargo, directly or via an adaptor, in the cytoplasm. The importin-cargo complex moves to the nucleus where it encounters RanGTP leading to release of the cargo in the nucleus (Fig. 1.3 B) [Review: (Chook & Suel, 2011)].

1.3.2 tRNA nuclear export

Los1- Nuclear export of tRNA utilizes the Ran pathway and the well conserved importin-β family member, Exp-t in vertebrates, Los1 in yeast, Xpo-t in Schizosaccharomyces pombe, or PAUSED in plants (Hopper et al., 1980; Arts et al., 1998b; Hellmuth et al., 1998; Kutay et al., 1998; Sarkar & Hopper, 1998; Hunter et al., 2003). Exp-t preferentially binds with the appropriately structured tRNA backbone as well as the mature tRNA 5’ and 3’ ends, but it has no preference for intron-containing or intron-less
tRNAs (Arts et al., 1998b; Lund & Dahlberg, 1998; Lipowsky et al., 1999). Crystallography studies of *S. pombe* Xpo-t in complex with tRNA and RanGTP elegantly demonstrate and support the early observations that Xpo-t wraps around tRNA, making contacts with the acceptor arm, the TΨC, and D-loops, leaving the anticodon loop exposed (Cook et al., 2009).

The *S. cerevisiae* Exp-t homologue is Los1, was first identified by *los1* mutant (Hopper et al., 1980; Hellmuth et al., 1998; Sarkar & Hopper, 1998). *los1Δ* cells accumulate end-processed intron-containing pre-tRNAs due to defects in tRNA nuclear export from the nucleus to the cytoplasm where the splicing machinery is located (Sarkar & Hopper, 1998; Yoshihisa et al., 2003; Yoshihisa et al., 2007). To date, Los1 is the only known exportin that functions in delivering intron-containing pre-tRNA to the cytoplasm (Fig. 1.2 A). Despite the defects in tRNA nuclear export, *los1Δ* cells are viable. In addition, *S. pombe* Xpo-t and *Arabidopsis* PAUSED are also unessential (Hunter et al., 2003; Cherkasova et al., 2011). In *Drosophila melanogaster*, no Exp-t homologue has been identified (Lippai et al., 2000). Thus, additional tRNA nuclear export pathways should exist in yeast, *Arabidopsis*, and *Drosophila*.

**Msn5**- A second importin-β family member, Expoerin-5 (Exp-5; yeast Msn5), is implicated in tRNA nuclear export. Vertebrate Exp-5 is thought to play a minor role in tRNA nuclear export, and it primarily functions in export of microRNA [Review: (Katahira & Yoneda, 2011)]. Yeast Msn5 has a well-defined role in nuclear export of particular phosphorylated proteins [Review: (Hopper, 1999)]. Msn5 appears to be involved in tRNA nuclear export because *msn5Δ* cells accumulate nuclear pools of tRNA
(Murthi et al., 2010) and \textit{msn5A los1A} cells have larger nuclear pool of tRNA than either mutant alone (Takano et al., 2005). In addition, Msn5 is able to bind tRNAs in a RanGTP dependent manner \emph{in vitro} (Shibata et al., 2006). The role of yeast Msn5 in tRNA nuclear export is detailed in the following chapters.

\subsection*{1.3.3 Retrograde import}

It was widely believed that tRNA movement was unidirectional from the nucleus to the cytoplasm. However, this view was challenged by surprising findings. First, yeast tRNA splicing occurs on the outer surface of the mitochondria instead of in the nucleus (Yoshihisa et al., 2003; Yoshihisa et al., 2007). Second, tRNAs can be aminoacylated in the nucleus by nuclear pools of aaRSs. When tRNA nuclear charging is defective, uncharged mature tRNAs accumulate in the nucleus (Lund & Dahlberg, 1998; Sarkar et al., 1999; Grosshans et al., 2000; Azad et al., 2001; Feng & Hopper, 2002; Murthi et al., 2010). These raise the question of how spliced uncharged tRNAs can reside in the nucleus in these aaRS mutants as tRNA splicing machinery resides in the cytoplasm. To explain this conundrum, it was proposed that tRNAs travel in a retrograde fashion from the cytoplasm to the nucleus. This premise was supported by at least three lines of evidence. First, in heterokaryons, foreign tRNA encoded by one nucleus can accumulate in the nucleus that does not encode the foreign tRNA (Shaheen & Hopper, 2005; Takano et al., 2005). Second, when new tRNA transcription is inhibited, tRNAs accumulate in the nucleus upon nutrient deprivation (Takano et al., 2005; Whitney et al., 2007). Third, mature tRNAs fail to accumulate in the nucleus in haploid cells bearing a deletion of
MTR10, an importin-β family member, presumably because Mtr10 is required for their import from the cytoplasm to the nucleus (Shaheen & Hopper, 2005; Murthi et al., 2010). However, it is unclear whether Mtr10 acts directly upon nuclear import by binding cytoplasmic tRNAs (Fig. 1.2 B).

Since the tRNA retrograde pathway was discovered in an organism for which pre-tRNA splicing occurs in the cytoplasm, it was important to investigate whether tRNA retrograde nuclear import occurs in other organisms. Several studies indicated that tRNA retrograde import is conserved in vertebrates. tRNA retrograde nuclear accumulation has been demonstrated in rat hepatoma cells upon amino acid deprivation (Shaheen et al., 2007), in Chinese hamster ovary cells upon inhibition of protein synthesis (Barhoom et al., 2011), and in heat-stressed human cells (Miyagawa et al., 2012). In addition, HIV appears to have usurped the tRNA retrograde import machinery to deliver the reverse transcription complex through nuclear pores in nondividing neuronal cells (Zaitseva et al., 2006). Thus, tRNA retrograde import is conserved between yeast and vertebrates.

1.3.4 tRNA re-export is likely regulated

tRNA retrograde nuclear accumulation is responsive to nutrients as cells accumulate tRNA in the nucleus upon amino acids (aa), glucose, or phosphate deprivation (Grosshans et al., 2000; Shaheen & Hopper, 2005; Hurto et al., 2007; Whitney et al., 2007), while cells exhibit an even distribution of tRNA throughout the nucleus and cytoplasm under nutrient replete conditions. The subcellular distribution of tRNAs between the nucleus and cytoplasm is a result from the balance among the rates of
primary export, nuclear import, and re-export. Retrograde tRNA nuclear import is constitutive (Murthi et al., 2010), thus implicating regulation of nutrient-dependent nuclear accumulation of cytoplasmic tRNAs at the re-export step. The mechanisms regulating the tRNA re-export step remain unclear. Studies regarding mechanisms of the regulated tRNA re-export step and the regulations of tRNA subcellular movement in response to nutrient availability are reported in the following chapters.

### 1.3.5 Function of retrograde pathway

It is suggested that tRNA retrograde pathway likely serves several functions, including tRNA modification, regulation of translation, and tRNA quality control. tRNA retrograde movement is required for tRNA$^{Phe}$ wybutosine (yW) modification (Ohira & Suzuki, 2011). The yW modification occurs on the tRNA$^{Phe}$ that is encoded by an intron-containing gene. This modification functions in maintenance of reading frame in translation. The first step of yW biosynthesis is catalyzed by a nuclear protein, Trm5, and then additional steps of yW biosynthesis are catalyzed by four cytoplasmic proteins, Tyw1-4. Interestingly, Trm5 has strict substrate specificity for spliced tRNA. Since Trm5 is located in the nucleoplasm, tRNA$^{Phe}$ must first exit the nucleus to be spliced in the cytoplasm and then it returns to the nucleus to acquire modification catalyzed by Trm5. Thus, tRNA retrograde import serves an essential function on yW biogenesis of tRNAs.

When cells are starved for various nutrients or when tRNA nuclear charging is defective, the nuclear pool of imported tRNAs increases (Grosshans et al., 2000; Azad et al., 2001; Shaheen & Hopper, 2005; Hurto et al., 2007; Whitney et al., 2007).
Redistribution of tRNA from the cytoplasm to the nucleus upon nutrient deprivation could affect translation globally; however, no such mRNAs were identified by a genome-wide analysis. Instead, translation of a subset of mRNAs encoding enzymes involved in aa biosynthesis pathways were found to be down-regulated when either tRNA retrograde nuclear import or re-export are defective. This finding connects tRNA subcellular dynamics to cellular physiology (Chu & Hopper, 2013). Thus, tRNA retrograde process is important for regulation of aa biosynthesis pathways. In addition, this finding raises the question of why defects in tRNA nuclear export do not have a remarkably effect on protein synthesis. One possibility is that cells transcribe more tRNAs than required for protein synthesis as there is extensive turnover of pre-tRNAs by the exosome (Gudipati et al., 2012). Thus, due to excess production of tRNAs, the reduced cytoplasmic pools of tRNA in cells with defective tRNA export are likely still sufficient to maintain global translation.

tRNA retrograde pathway also serves to relocate aberrant tRNAs from the cytoplasm to the nucleus for repair and/or turnover (Kramer & Hopper, 2013). This function may provide an additional mechanism for tRNA quality control, discussed in detail below.
1.4 tRNA quality control mechanisms

Cells appear to employ multiple levels of quality control mechanisms that work in parallel to maintain a pool of appropriately processed, folded and functional tRNAs in the cytoplasm where they are able to interact with the translation machinery.

1.4.1 Exp-t serves a role in tRNA quality control

One of the quality control steps involves discrimination of precursor tRNAs during primary tRNA export. The vertebrate Exp-t proofreads tRNAs during export, by preferentially binding to and exporting appropriately structured tRNAs with mature 5′ and 3′ ends (Arts et al., 1998b; Lund & Dahlberg, 1998; Lipowsky et al., 1999). The crystallography structural studies evidence that *S. pombe* Xpo-t contacts the tRNA elbow region and acceptor arm, including the 3′ CCA overhang, and the binding site requires that this overhang is no longer than 4 nucleotides (Cook et al., 2009). This binding property of Exp-t provides a selection to preferentially export tRNAs that possess mature termini and are appropriately structured to the cytoplasm. Consequently, Exp-t (yeast Los1) serves a quality control function assuring that end-processed and folded tRNAs access the cytoplasmic protein synthesis apparatus (Fig. 1.4 A).

1.4.2 tRNA turnover pathways appear to function in tRNA quality control

tRNA turnover pathways appear to function in tRNA quality control, eliminating tRNAs that are inappropriately processed, incorrectly modified, or misfolded. Although tRNAs are highly stable molecules with half-lives estimated from ~9 hr to days
[(Gudipati et al., 2012); review: (Hopper, 2013)], there are at least two mechanisms involved in tRNA degradation.

First, defective pre-tRNAs can be adenylated by TRAMP complexes for targeting to the nuclear exosome. Defects in maturation, modification, and/or 3’ end processing are the substrates of TRAMP complexes. Substrates of the yeast TRAMP complex are polyadenylated and then subject to 3’ to 5’ exonucleolytic degradation by the nuclear exosome. Thus, the TRAMP complex collaborates with the nuclear exosome that monitors both appropriate tRNA modification and 3’ end maturation, which serves as a tRNA quality control in the nucleus.

A second mechanism of rapid tRNA degradation pathway (RTD) occurs when tRNAs are defective in their modifications and/or stability of the combined acceptor and T stems (Chernyakov et al., 2008). This degradation utilizes the 5’ to 3’ exonucleases Rat1 and Xrn1. The stability of the tRNA structure at the 5’ end dictates whether it can be subject to 5’ to 3’ degradation. Rat1 resides in the nucleus and Xrn1 in the cytoplasm, showing that RTD can happen either in the nucleus or the cytoplasm. Interestingly, recent studies reveal that there seems to be competition between the RTD pathway and translation factor eEF1A, possibly due to the interaction between tRNA and eEF1A that provides immunity from the RTD surveillance machinery (Dewe et al., 2012; Turowski et al., 2012).
1.4.3 tRNA retrograde nuclear pathway functions in quality control

Recent studies showed that retrograde tRNA nuclear import functions, in part, in proofreading tRNAs (Kramer & Hopper, 2013). Although Los1 provides an initial quality control by preventing improper tRNAs from escaping the nucleus, low levels of improper export of aberrant tRNAs have been detected (Kramer & Hopper, 2013). In addition, Los1 does not have complete fidelity for appropriately modified tRNAs because Los1 recognizes structures common to all tRNAs that are variously modified (Arts et al., 1998b; Lipowsky et al., 1999; Cook et al., 2009). Thus, Los1-mediated nuclear export prevents, although not completely, nuclear export of 5′, 3′ end-extended, tRNAs (Fig. 1.4 B) but not likely many hypomodified tRNAs (Fig. 1.4 C). Stable hypomodified tRNAs that are mistakenly been exported to the cytoplasm may be deleterious to the translation fidelity. Thus, sequestering aberrant cytoplasmic tRNAs, such as 5′ and 3′ end-extended spliced tRNAs (Fig. 1.4 B) and hypomodified tRNAs (Fig. 1.4 C), into the nucleus via tRNA retrograde pathway would ensure that only properly processed and modified tRNAs reside in the cytoplasm and interact with the translation machinery. Accumulation of aberrant tRNAs in mutant cells that are defective in tRNA nuclear traffic provides evidence that normally these aberrant tRNAs are imported into the nucleus where they are repaired or degraded. The tRNA retrograde nuclear pathway functions in parallel with the cytoplasmic 5′ to 3′ exonuclease quality control pathway because cells are not viable if they lack both the tRNA retrograde and cytoplasmic RTD pathways (Fig. 1.4 C, green arrow and green packman) (Kramer & Hopper, 2013). Therefore, tRNA retrograde
pathway serves an additional proofreading role to monitor both end-processing of pre-tRNA and the modification status of mature tRNAs.

### 1.5 Aims of this Study

Cytoplasmic tRNAs move bidirectionally between the nucleus and the cytoplasm. The imported cytoplasmic tRNAs accumulate in the nucleus upon nutrient deprivation. tRNA re-export is the step most likely regulated by nutrient availability. However, the mechanisms regulating tRNA subcellular movement remain poorly understood. Thus, the work presented here has three main objectives to investigate the regulation of tRNA subcellular dynamics using *S. cerevisiae* as the model organism: (1) Investigate the role of β-importins in tRNA subcellular dynamics; (2) Investigate the possible tRNA re-export models in regulating nuclear export of mature or charged tRNA; (3) Elucidate the mechanisms regulating tRNA subcellular trafficking in response to nutrient availability.
Figure 1.1. Summary of tRNA processing pathways in vertebrates and yeast. (A) In vertebrates, newly transcribed intron-containing tRNAs are spliced and then the ends (purple) are processed in the nucleus. Following CCA (green) addition, and nucleotide modifications (orange), tRNAs are exported to the cytoplasm. Additional modifications (brown) occur after export to the cytoplasm. (B) In yeast, pre-tRNAs are transcribed in the nucleus where leader and trailer sequences (purple) are removed prior CCA (green) addition. End-processed, partially modified intron-containing pre-tRNAs are exported to the cytoplasm. Splicing and additional modifications (brown) occur after export to the cytoplasm. Intron sequences are indicated by yellow circles.
**Figure 1.2. Current model of tRNA subcellular dynamics.** (A) End-processed and intron-containing pre-tRNAs as well as intron-less pre-tRNAs are exported to the cytoplasm by Los1 and at least one unknown exporter. Red arrows indicate tRNA primary nuclear export. Red text, β-importins function in primary export. (B) Cytoplasmic tRNAs constitutively return to the nucleus via retrograde nuclear import, directly or indirectly by Mtr10. Green arrow indicates retrograde tRNA nuclear import. (C) Imported cytoplasmic tRNAs are re-exported to the cytoplasm via re-export process by Los1 and at least one unknown exporter. Purple arrow indicates tRNA nuclear re-export. Purple text, β-importins function in tRNA re-export.
Figure 1.3. Model of Ran-dependent nuclear-cytoplasmic transport. (A) Exportin (yellow) binds cargo (red) and RanGTP (black) in the nucleus and then moves to the cytoplasm. RanGTP hydrolysis leads to the release of cargo into the cytoplasm. (B) Importin (blue) binds the cargo in the cytoplasm. After moving to the nucleus, binding of RanGTP leads to the release of the cargo into the nucleus.
Figure 1.4. tRNA processing and trafficking pathway. (A) Canonical retrograde pathway. Black arrows indicate the canonical pathway leading to mature cytoplasmic tRNAs that participate in translation. (B) Precocious nuclear export and retrograde import of end-extended spliced tRNAs. Red dotted arrow indicates precocious nuclear export before 5′ and 3′ processing; aberrant transcripts are spliced in the cytoplasm and return to the nucleus via retrograde nuclear import (red solid arrow). (C) Precocious nuclear export and retrograde import of hypomodified tRNAs. Green dotted arrows indicate precocious nuclear export before complete modification in the nucleus; hypomodified tRNAs are spliced in the cytoplasm and may participate in translation or may be destroyed by the 5′-3′ RTD exonuclease (green packman), or may return to the nucleus via retrograde import (green solid arrow). Magenta arrow indicates the tRNA position normally modified by m^2^G_{26}; the nucleotide missing this modification is indicated by a magenta circle.
Chapter 2 General Materials and Methods

2.1 Yeast strains and media

The following yeast strains and collection derived from yeast strain BY4741 (MATa his3Δ leu2Δ met15Δ ura3Δ) were used in this study: strains harboring endogenously GFP-tagged protein (Huh et al., 2003), the strains used from the temperature sensitive (ts) collection (Li et al., 2011) and the deletion collection [(Winzeler et al., 1999); Open Biosystems]. All of the employed yeast strains are listed in Table 2.1

Yeast strains were maintained in YEPD (yeast extract/peptone/glucose) media or in synthetic complete defined media (SC) lacking the appropriate nutritional ingredients for selection.

2.2 General method for plasmid construction

PCR techniques were employed to amplify desired DNA fragments from plasmids or yeast genomic DNA with oligos containing restriction sites at 5’ and 3’ ends of DNA, and employed PfuUltra (Stratagene) or Platinum Taq (invitrogen) high fidelity DNA polymerases. PCR products were subsequently ligated into pGEM-T vector (Promega) following manufacturer’s instructions and then transformed into E. coli cells. Once confirmed by DNA sequencing, plasmid construction was conducted by restriction
digestion of DNA fragments in buffers suggested by the manufacturer, usually for 2 hr at 37°C, and then resolved on agarose gel of appropriate percentage in 1X Tris-borate (TBE, 45 mM Tris-borate, 1 mM EDTA). Gel slices that contain the desired restriction fragments were isolated using QIAquick gel extraction kit (Qiagen) according to manufacturer’s instructions. Dephosphorylation of vector was performed by adding 1 µl CIP phosphatase (New England Biolabs) to the digestion reaction after 2 hr and adding an extra hour of incubation at 37°C. Then, DNA fragments of restriction products were ligated using T4 DNA ligase (New England Biolabs). E. coli XL1-Blue was utilized for recombinant DNA plasmid propagation and was maintained in Luria-Bertani (LB) media with antibiotics for selection.

2.3 Oligonucleotides and PCR

DNA oligonucleotides were synthesized by Sigma-Aldrich. The sequences of all employed oligonucleotides are in Table 2.2.

25, 50 or 100 µl PCR reactions were performed by setting up a master mix reaction containing 1X PCR Buffer, 10 mM dNTPs mixture (dATP, dCTP, dGTP and dTTP), 10 µM of each oligonucleotide, ~100 ng/µl of plasmid or genomic DNA and water to complete final volume. Standard PCR conditions during thermocycling were 94°C for 3 min, followed by 25-30 cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 1-5 min (extension, depending on the product size). Then, a final extension of 72°C for 5 min completed the reaction. Annealing temperatures for gradient PCR ranged from 52-62°C.
2.4 Sequencing

DNA sequencing was carried out by the Plant-Microbe Genomics Facility at The Ohio State University.

2.5 Preparation and chemical transformation of *E. coli* competent cells

*E. coli* (XL1-Blue) was cultured overnight on LB plate at 37°C. 10-12 colonies were transferred to 250 ml of Super Optimal Broth (SOB) media (2% Tryptone, 0.5% Yeast extract, 85mM NaCl, 2.5 mM KCl, 10 mM MgCl₂), and cultured at 18°C with vigorous shaking (230 rpm) until OD₆₀₀=0.55-0.75. The culture was incubated on ice for 10 min, and then harvested by centrifugation (Beckman Coulter Avanti J-26 XP) at 5,000 rpm for 10 min at 4°C. Cell pellets were resuspended in 80 ml of ice-cold TB solution (10 mM HEPES, 15 mM CaCl₂, 250 mM KCl, 55 mM MnCl₂). The cultures were incubated on ice for 10 min, and then centrifuged at 4°C for 10 min. The pellet was resuspended in 20 ml of ice-cold TB. 1.5 ml of dimethyl sulfoxide (DMSO) was subsequently added to the mixture and gently mixed by swirling. After incubating the mixture on ice for 10 min, cells were aliquoted into 100 µl and frozen in liquid nitrogen and then stored at -80°C.

100 µl of competent *E. coli* cells in a microtube was thawed on ice. 1 µl of plasmid DNA or ligation products was added to competent cells. The cells were kept on ice for 30 min. Heat shock was performed at 42°C for 45 sec followed by 1 min incubation on ice. Then, 800 µl of Luria-Bertani (LB) media was added to the cells. Cells were incubated in an air shaker at 37°C for 30 min. After recovery, cells were plated in LB media
containing 50 µg/ml of ampicillin or kanamycin for plasmid selection and grown overnight at 37°C to isolate single colonies.

2.6 Plasmid DNA Purification

Qiagen’s QIAprep Miniprep kit was used for all plasmid DNA purifications following manufacturer’s instructions. In brief, *E. coli* cells that contain plasmids were grown at 37°C for overnight in liquid culture with the appropriate antibiotic. The bacteria cells were pelleted and then lysed under alkaline conditions. The lysate was subsequently neutralized and adjusted to high-salt binding conditions. The solution that contained plasmids was then applied to the QIAprep column to absorb plasmids to the silica membrane in a high-salt buffer. Endonucleases were then removed with the first wash step. A second wash step with a buffer containing ethanol was used to remove salt. The plasmid DNA was then eluted from the binding column by centrifugation.

2.7 Preparation of yeast competent cells and yeast transformation

To obtain competent cells, 50 ml of yeast culture grown overnight to an OD of 0.6-0.8 at 600 nm, was pelleted in a 50 ml Falcon tube at 3,000 rpm using a Jouan tabletop centrifuge for 5 min. The pellet was washed once with distilled water. And cells were pellets again and resuspended into 1 ml of freshly prepared 1xTE-1xLiAc solution (10xTE= 100 mM Tris-HCl + 10 mM EDTA, pH 7.5 and 10x LiAc = 1 M LiAc, pH 7.5 by using acetic acid). Integration of DNA fragments into the yeast genome by homologous recombination was carried out by adding 10 µl of PCR product and 10 µl of
ssDNA into a 1.5 ml microtube containing 100 µl of competent cells. Microtubes were placed on a rolling platform and incubated at 30°C for 30 min. 600 µl of TE/Lithium Acetate/PEG mix (6.66 ml 60% PEG, 1 ml 1M LiAc, 1 ml 10x TE and 1.33 ml water) was added to the microtubes and content was mixed slowly. Microtubes were placed in the rolling platform at 30°C for 1 hr. Then, microtubes were placed in a water bath at 42°C for 15 min. Cells were pelleted at 4,000 rpm using a 5426 Eppendorf microcentrifuge for 30 sec and were resuspended in 1xTE. Cells were plated in selective media and incubated at 23°C or 30°C.

2.8 Isolation of plasmid DNA from yeast

The protocol was modified from a procedure provided by M. Jones (Chugai Institute for Molecular Medicine, Ibaraki, Japan); it employs the QIAprep Spin Miniprep Kit. 5 ml of yeast culture was grown at 23° or 30°C for overnight. Cells were harvested and resuspended in 250 µl of buffer P1 containing 0.1 mg/ml RNase A. Cells were broken down by adding 50-100 µl of glass beads to vortex for 5 min. 250 µl buffer P2 was added into the supernatant and then incubated at room temperature (RT) for 5 min. After incubation, 350 µl of buffer N3 was added into the mixture to neutralize the reaction. The reaction was centrifuged for 10 min at 21130 xg (Eppendorf centrifuge 5424) to get the clarified lysate. The clarified lysate was transferred to QIAprep Spin Column. The column was centrifuged for 1 min at 21130 xg. The column was washed with 0.75 ml of buffer PE. Afterward, 25 µl of plasmid DNA was eluted by centrifugation for 2 min at 21130 xg.
2.9 Northern analysis

Small RNAs were extracted from yeast cultures grown to early log phase (OD$_{600}$=0.3-0.5). Samples were electrophoretically separated at 4°C in 10% polyacrylamide gel containing 8 M urea and 1x TBE [0.09 M Tris, 0.09 M borate, 0.001 M ethylenediaminetetraacetic acid (EDTA)]. RNAs were electrophoretically transferred to Hybond N+ membranes (Amersham Pharmacia) using a Hoefer TE42 Transfer apparatus (Hoefer Scientific) with 1x TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA). Northern analysis was conducted as previously described (Wu et al., 2013). tRNAs were detected with a $\gamma^{32}$P-labeled probe or a DIG-labeled probe complementary to tRNA$^{\text{Ile}}$. The relative levels of precursor and mature tRNAs were determined using a Typhoon Trio Variable Mode Imager (GE Healthcare Bio-Sciences Corp) and ImageQuant TL software.

2.10 RT-PCR

First strand cDNA Synthesis was carried out using Superscript III reverse transcriptase (Invitrogen), according to the manufacturer’s protocol. Subsequent PCR reactions were carried out using 2 µl of cDNA, and 15 µl of each PCR reaction was electrophoretically separated on 2% agarose gel. PCR conditions were as following, 1min at 95 °C; various cycles of 30 sec at 95 °C, 20 sec at 52 °C and 20 sec at 72°C; 30 sec at 72°C.
2.11 Real-time RT-PCR

RNAs were treated with Turbo DNase (Ambion) according to the manufacturer protocol. 2 µl of RNA was used to prepare cDNAs for real-time PCR using Superscript III reverse transcriptase (Invitrogen). PCR was performed using SsoFast Eva Green Supermix (Bio Rad) and the CFX96 instrument (Bio Rad). Real time PCR conditions were as follows: 1min at 95 °C; various cycles of 30 sec at 95 °C, 20 sec at 52 °C and 20 sec at 72°C; 30 sec at 72°C. Standard curve were performed with 10-fold serial dilutions of gel-extracted RT-PCR product as template to determine the concentrations of samples. No template controls were analyzed for each primer sets, and no RT controls were analyzed for each sample with each primer set. Data was analyzed by CFX96 software (Bio Rad).

2.12 Indirect immunofluorescence

Indirect immunofluorescence (IF) was performed as described by Li et al., (1989) and Pringle, et al (1991). 15 ml of yeast culture were grown overnight to log phase in an air shaker at 23°C. Then, 1.2 ml of 37% formaldehyde was added to the cultures. Cultures were centrifuged at 3,000 rpm for 5 min using a Jouan tabletop centrifuge. Pellets were resuspended in 2.5 ml of solution A containing 40 mM K₂HPO₄-KH₂PO₄ and 500 µM MgCl₂. The solution was supplemented with 0.6 ml of 37% formaldehyde and cells were incubated at RT for 25 to 30 min depending on the primary antibody used. For use of primary monoclonal mouse anti-Myc antibody (9E10, 1:100 dilution, Santa Cruz Biotechnology) and polyclonal rabbit HA antibody (12CA5, 1:100 dilution, Roche)
cells were incubated for 25 min. After incubation, cells were washed twice with solution A and resuspended in solution B containing 40 mM K\textsubscript{2}HPO\textsubscript{4}-KH\textsubscript{2}PO\textsubscript{4}, 500 μM MgCl\textsubscript{2} and 1.2 M sorbitol. Cells were pelleted and resuspended in 500 µl of digestion solution (1 ml solution B, 55µl glusulase, 10µl mercaptoethanol and a toothpick tip of zymolyase 20T powder, MPbio) for cell wall removal (digestion) and were incubated at 37°C for 20-30 min. Digestion was monitored by light microscopy at different time points until about 60-70% of the cells had their wall removed. Digested cells were pelleted at 3,000 rpm for 3 min using a Jouan tabletop centrifuge. Cells were resuspended in solution B for washing (twice). After washing, cells were resuspended again in 200-500 µl of solution B, depending on cell density.

Cells were adhered to glass 12-well slides coated with poly-lysine (1:10 dilution from 0.1% stock, Sigma) for 1 min. 10 µl of sample was added to each well and aspirated after 1 min. To inhibit non-specific binding of antibodies, 10 µl of solution F was added to each well for blocking. Solution F contained 0.73 mM KH\textsubscript{2}PO\textsubscript{4} pH 7.4, 0.15 M NaCl, 0.015 NaN\textsubscript{3} and 0.2% BSA. After 1 hr, solution F was aspirated and primary antibody diluted (as mentioned above) in solution F, was added and incubated for 1 hr. After incubation, primary antibody was aspirated from the wells and washed seven times with solution F. Secondary antibody (FITC goat anti-mouse IgG, Jackson laboratories) diluted 1:400 in solution F was added to each well and incubated for 1 hr covered by aluminum foil. Secondary antibodies used were FITC-conjugated goat anti-mouse IgG. After incubation, wells were washed five times with solution F. To visualize DNA in the cells, 4’,6-diamidino-2-phenyllindole dihydrochloride (DAPI, 1:100,000 dilution in water from
a 10 mg/ml stock) was added to the wells and incubated for 1 min, then washed twice with water. Mounting media (50 mg of phenylenediamine in 5 ml PBS, pH 9, added to 25 ml glycerol) was added to the slide and then covered with a cover slip that was sealed by using nail polish and storage at -20°C before microscopy.

2.13 Fluorescence In situ hybridization

Fluorescence in situ hybridization (FISH) was performed as previously described (Sarkar & Hopper, 1998) with the modifications detailed in (Stanford et al., 2004). Each slide contained positive and negative controls for tRNA nuclear accumulation.

2.14 Microfluidics

To study the intracellular dynamics of GFP-tagged proteins in wt and ts strains, microfluidics system (Cell ASIC, EMD Millipore) was employed. The temperature was conducted by microfluidic control system (Cell ASIC, EMD Millipore). Changes in the media and incubation times were programmed using the ONIX™ FG Software (Cell ASIC, EMD Millipore), according to the manufacturer’s protocol.

2.15 Microscopy and imaging

To view live cells by confocal microscopy, cells were placed on a slide containing a thin layer of appropriate medium with 20% gelatin and 0.1 mM n-propylgallate as previously described (Wu et al., 2006). Alternatively, cells were analyzed using the microfluidics system. Monitoring of live cells was performed using a Nikon microscope.
equipped with a spinning disk confocal apparatus (UltraView, PerkinElmer Life and Analytical Science, Waltham, MA) and a cooled charged coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Cells were visualized using 488 nm (green) and 568 nm (red) argon ion lasers and a 100x/1.4 NA objective lens. Maximum intensity projections of images were created using UltraView ERS software and image analyses of single optical 0.4 µm optical sections were performed using ImageJ (http://rsb.info.nih.gov/ij/).

Epifluorescence imaging was accomplished using a Nikon Microscope Eclipse 90i and a 60x objective equipped with a CoolSNAP HQ2 CCD camera (Phometrics) and Nis-Elements software (3.1). Cells were visualized using Differential Interference Contrast (DIC), fluorescein isothiocyanate (FITC), DAPI and/or Texas red filters. Adobe Photoshop (San Jose, CA) was used for image assembly.

2.16 Harvesting yeast cells and cryogenic disruption

This protocol was modified according to (Alber et al., 2007). Yeast strains that carry Protein A ZZ domain tagged proteins were grown in appropriate selective media until they reached early or mid log phase (OD_{600}= 0.4-0.6). Cells were harvested and then washed with 50 ml ice-cold ddH_{2}O twice in a 50 ml tube. From this step on, cells were kept on ice. After washing with ice-cold ddH_{2}O, the cell pellets were subsequently resuspended in equal volume of the resuspension buffer [20 mM KOH- HEPES, pH 7.4, 1.2% PVP, 1 mM DTT, 1:100 of protease inhibitor cocktail (Calbiochem) and 1:100 of solution P (2% PMSF, 0.04% pepstatin A in 100% ethanol)]. Cells were centrifuged at
3,000 xg, 10 min at 4°C and the pellet was pushed through a plastic syringe into a 50 ml tube filled with liquid nitrogen, and the resulting frozen “noodles” were stored at -80°C.

To prevent any enzyme reactions during the disruption, all equipment for this procedure was pre-chilled by immersing into liquid nitrogen before using. The procedure was according to (Alber et al., 2007) with the following modifications. Frozen yeast noodles were transferred to the steel jar with the appropriate amount of steel balls. The steel jar was then placed to planetary ball mill PQ-N04 to perform grinding, 400 rpm, 2 min for each direction (clockwise and anti-clockwise) in 8 to 10 cycles. Between each cycle, the steel jar was removed and cooled in liquid nitrogen. The cell lysed by cryogenic grinding (cell grindate) was then transferred to pre-chilled 50 ml tube and stored at -80°C.

2.17 Conjugation of magnetic beads with rabbit IgG

Dynal magnetic beads were conjugated to antibodies according to the manufacturer’s instructions, but with several modifications as described (Alber et al., 2007) (http://www.rockefeller.edu/labheads/rout/protocols/Conjugation_of_Dynabeads.pdf). Conjugated beads were stored at 4 °C in 1xPBS, 0.02% NaN₃.

2.18 Affinity purification of protein A-tagged protein

Purifications were performed as described (Alber et al., 2007) with the optimizations. In brief, 0.5 g of frozen cell grindate was resuspended in 4.5 ml of the extraction buffer (20 mM Hepes, pH 6.1, 110 mM KOAc, 2 mM MgCl₂, 75 mM NaCl, 0.5 % Triton, 0.1%
Tween-20, 1 mM DTT) with 1:100 dilutions of protease inhibitor cocktail set IV (Calbiochem) and solution P. The cell extract was centrifuged at 3000 xg, 4 °C for 10 min. The soluble extract was incubated with IgG-conjugated magnetic beads (washed with extraction buffer 3 times before used) at 4 °C for 30 min. The magnetic beads were then collected with a magnet, washed six times with 1 ml of the ice-cold extraction buffer and once with 0.1 M NH₄OAc, 0.1 mM MgCl₂, 0.02% Tween-20. Protein was eluted with 700 µl of 0.5 M NH₄OH, 0.5 mM EDTA by incubation for 20 min at room temperature. The eluates were lyophilized in a SpeedVac (Thermo Savant) overnight. The pellets were resuspended in NuPAGE LDS sample buffer (Life technologies), separated on a 4-12 % NuPAGE Novex Bis-Tris precast gel (Life technologies) according to the manufacturer’s specifications. Proteins were visualized after staining with SypoRuby protein staining (Invitrogen).

2.19 Western blot

Protein was assessed using chemiluminescence-based Western blot analysis following standard protocols as described (Chu & Hopper, 2013). The membrane was probed with primary antibodies: anti-Myc (9E10, Santa Cruz) at a 1:1500 dilution, anti-GFP (clone CBP-KK1, Roche) at a 1:1000 dilution, anti-Ran (ab4781, abcam) at a 1:1500 dilution, and anti-Rna1 at a 1:10,000 dilution (Hopper et al., 1990). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare, UK) at a 1:3000 dilution. Blots were developed using the Super Signal®
West Femto Maximum Sensitivity Substrate (Pierce). Protein signals were quantified using ImageJ (http://rsbweb.nih.gov/ij/).
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<td>CCT TCA TTA GGG CCC TAG CAA AAA AT T A</td>
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<tr>
<td>IVY32F</td>
<td>Smal of ARC1 at 201aa</td>
<td>CCC GGG ATG CCA GAA AAG CCA AAG CCA</td>
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<td>IVY33F</td>
<td>Smal of ARC1 at 132aa</td>
<td>CCC GGG ATG AAG AAG AAA AAG GCA CCT G</td>
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<td>IVY34R</td>
<td>AatII of ARC1 at 330aa</td>
<td>GAC GTC TAA GTG TTC CCA GAT CTT C T T</td>
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<td>IVY35R</td>
<td>HindIII of ARC1 at 350aa</td>
<td>AAG CTT TTC TTC TTC GTC TTT GAAGAT</td>
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<td>IVY36F</td>
<td>Sall at +1bp of mCherry</td>
<td>GTC GAC ATG GTG AGC AAG GGC GAG</td>
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<td>IVY37R</td>
<td>BglII at stop site of mCherry</td>
<td>AGA TCT CTT GTA CAG CTC GTC CAT</td>
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<td>IVY38F</td>
<td>Xbal at +1 of GST</td>
<td>GG CCG CCA CCG TCT AGA ATG TCC CCT ATA C</td>
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<td>KpnI at stop of GST</td>
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<td>IVY40F</td>
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<td>IVY42R</td>
<td>Smal at 427aa of Gle1</td>
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<td>IVY43</td>
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<td>IVY45</td>
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<td>IVY46</td>
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<td>IVY47</td>
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<td>TEF1:HPH conformation at</td>
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<td>TEF1:HPH conformation at</td>
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<td>IVY51R</td>
<td>TEF1:HPH at downstream 3’UTR</td>
<td>CACAACCCTGATGAAACGGACTG</td>
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<td>TGC TGC AGG AGC TGC AGC AGG TCC</td>
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<td>Smal of mCherry</td>
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<td>SacI of Mtr10</td>
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<td>KpnI of Mtr10</td>
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<td>XbaI at His-tag site of MORF</td>
<td>GAT GTG TCT AGA CAT CAT CAT CAC</td>
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<td>KpnI at MSN5</td>
<td>CTC TTT GAT GGT ACC TGA TAA TAA GGT GCC</td>
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<td>KpnI at 3'UTR of MSN5</td>
<td>GAA GAA TCT GGT ACC CTT ATT TAT ATC AG</td>
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<td>IVY67F</td>
<td>TEF2 KO conformation (upstream)</td>
<td>GGCAGAGATGATCGAGCGGTTTTTAGC</td>
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<td>IVY68F</td>
<td>TEF2 KO conformation (mid)</td>
<td>GCCATGGCAAGATGTGTTAAGCAGATC GGTGG</td>
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<tr>
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<td>GGT TTTGATGAGGGGCGTCTTCTTTTGGTTGAGTC</td>
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<td>GCA AAA AGT CCG CCG TTC TTA TTG TAG</td>
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<td>IVY100F</td>
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<td>TTAGTACTCCGTGTGCAATATATAAC GCCCGGCCATTTCCACGTATACAG TACGGTAGTCGACAAATGGGC gacggatatccgg</td>
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<td>TCA ATC AGT GCT TCT CAT GTG AAA CTT TTC TTT GTA TAT ACT TAA Ctggtgatctgatcatcaga</td>
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<tr>
<td>IVY141 F</td>
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<td>BamHI of GSP1 at-24 bp</td>
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<td>PstI of GSP1</td>
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<td>XmaI of Los1 at stop codon</td>
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<td>tRNA&lt;sup&gt;Leu&lt;/sup&gt; CAA</td>
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<td>conformation primer Kap95</td>
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Chapter 3 Regulation of tRNA Bidirectional Nuclear-Cytoplasmic Trafficking in

*Saccharomyces cerevisiae*


**Contribution**

Hsiao-Yun Huang performed the experiments for Figure 3.1, 3.2, 3.4, 3.5, and 3.6 A, C, and D in this chapter.

**3.1 Abstract**

tRNAs in yeast and vertebrate cells move bidirectionally and reversibly between the nucleus and the cytoplasm. We investigated roles of members of the β-importin family in tRNA subcellular dynamics. Retrograde import of tRNA into the nucleus is dependent, directly or indirectly, upon Mtr10. tRNA nuclear export utilizes at least two members of the β-importin family. The β-importins involved in nuclear export have shared and exclusive functions. Los1 functions in both the tRNA primary export and the tRNA re-export processes. Msn5 functions primarily in the nuclear re-export of tRNAs which are encoded by intron-containing genes. We implicate Tef1, the yeast orthologue of
translation elongation factor eEF1A, in the tRNA re-export process and show that its subcellular distribution between the nucleus and the cytoplasm is dependent upon Mtr10 and Msn5.

3.2 Introduction

Classically, the nucleus has been considered the cellular site for RNA production and the cytoplasm has been considered the site to convert the information encoded in RNA into proteins through the process of translation. Accordingly, mRNAs, rRNAs, and tRNAs would be born in the nucleus followed by their unidirectional transport to the cytoplasm for function. However, it is now clear that numerous RNAs move bidirectionally between the nucleus and the cytoplasm [Reviews: (Hopper, 2006; Hopper & Shaheen, 2008; Phizicky & Hopper, 2010)]. This work concerns tRNA nuclear-cytoplasmic dynamics in the yeast, S. cerevisiae.

tRNA processing machineries are relatively conserved from Archea to vertebrates, but the subcellular distribution of the processing activities differs among organisms. In vertebrate cells, initial tRNA transcripts, if encoded by intron-containing genes, are first spliced, then processed at 5′ and 3′ termini, followed by their export to the cytoplasm (Lund & Dahlberg, 1998). tRNA nuclear export in vertebrate cells proceeds via the Ran pathway employing a member of the β-importin family, Exportin-t (Exp-t) (Arts et al., 1998a; Kutay et al., 1998). Exp-t binds end-matured appropriately structured tRNAs and has similar affinities for intron-containing and intron-free tRNAs (Arts et al., 1998b; Lipowsky et al., 1999; Cook et al., 2009). The yeast orthologue of Exp-t, Los1, functions
in tRNA nuclear export (Hellmuth et al., 1998; Sarkar & Hopper, 1998). However, in contrast to vertebrates, in *S. cerevisiae* the tRNA splicing endonuclease is located on the cytoplasmic surface of mitochondria (Yoshihisa *et al.*, 2003). Therefore, 5′ and 3′ end processing in the nucleus precedes splicing which follows tRNA nuclear export to the cytoplasm.

It was predicted that additional tRNA nuclear export pathways exist in yeast because *LOS1* is an unessential gene and *los1Δ* mutants have a relatively normal growth phenotype (Hurt *et al.*, 1987). *LOS1* homologues in *S. pombe* ([Review: (Hopper & Shaheen, 2008)] and plants (Hunter *et al.*, 2003) are also unessential, indicating that parallel tRNA nuclear export pathways may be widespread in nature. The β-importin family member Exportin-5 (yeast Msn5) has been implicated in tRNA nuclear export (Bohnsack *et al.*, 2002; Calado *et al.*, 2002; Takano *et al.*, 2005), in addition to its known functions in nuclear export of micro-RNAs (miRNA) in metazoans and nuclear export of particular phosphorylated nuclear-cytoplasmic shuttling proteins in yeast [(Kaffman *et al.*, 1998; Yi *et al.*, 2003; Lund *et al.*, 2004; Zeng & Cullen, 2004); Review: (Hopper, 1999)]. Exportin-5 binds short double stranded RNA structures (Gwizdek *et al.*, 2003; Zeng *et al.*, 2005) and tRNAs in a RanGTP-dependent mechanism (Bohnsack *et al.*, 2002; Calado *et al.*, 2002; Shibata *et al.*, 2006); however, it is not thought to serve as the major tRNA exporter in vertebrates as Exp-t fulfills this role. Inhibition of Exp-t causes marked reduction of tRNA nuclear export (Arts *et al.*, 1998b; Lipowsky *et al.*, 1999) whereas knockdown of Exportin-5 has little effect on tRNA levels (Shibata *et al.*, 2006). Moreover, since complexes of Exportin-5 with aminoacylated-tRNA (aa-tRNA) and
RanGTP interact with translation elongation factor 1A (eEF1A; Tef1 and Tef2 in yeast), Exportin-5 has been proposed to serve as a mechanism to rid the nucleus of inadvertent pools of eEF1A that accumulate upon reassembly of nuclei after open mitosis (Bohsnack et al., 2002). Similarly, the plant Exportin-5 orthologue HASTY appears not to affect tRNA levels (Park et al., 2005). In contrast, for Drosophila which lacks an Exp-t homologue, Exportin-5 has been reported to serve for both miRNA and tRNA nuclear export [(Shibata et al., 2006) and references therein]. The yeast orthologue, Msn5, has been shown to bind tRNA in vitro (Shibata et al., 2006) and los1Δ msn5Δ double mutants accumulate more tRNA in the nucleus than cells with either single mutation (Takano et al., 2005). In sum, Exportin-5 and its orthologues appear to have a significant role in tRNA nuclear export in some, but not all, organisms.

Previous data document that tRNAs move bidirectionally in yeast – from the cytoplasm to the nucleus as well as from the nucleus to the cytoplasm (Shaheen & Hopper, 2005; Takano et al., 2005). Retrograde tRNA nuclear import and re-export occur in a similar fashion in yeast and vertebrate cells (Shaheen et al., 2007) and Lentiviruses, like HIV, appear to have usurped the tRNA retrograde import machinery to deliver the reverse transcription complex through nuclear pores in non-dividing neuronal cells (Zaitseva et al., 2006).

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

3.3 Materials and Methods

Strains and media

*E. coli* XL1-blue was used for propagation of recombinant DNA plasmids and was maintained in LB media with appropriate antibiotics. Most experiments employed yeast strains BY4741 (*MATα his3Δ leu2Δ met15Δ ura3Δ*) and BY4742 (*MATa his3Δ leu2Δ lys2Δ ura3Δ*). BY4741 and BY4742 are the parents to the deletion collections [(Winzeler et al., 1999); Open Biosystems] possessing Kan^r^ replacements for endogenous genes encoding importin-β family members. Because the strain lacking *MTR10* is not included in the yeast deletion collection, it was constructed in the BY4741 background by gene replacement, as described in (Azad et al., 2001). To create *msn5Δ tef1Δ* and *los1Δ tef1Δ* strains, the endogenous *TEF1* ORFs in *msn5Δ* and *los1Δ* strains were deleted by gene replacement. BY4741 is also the parent for the collection of endogenously expressed Tef1-GFP yeast strains [(Huh et al., 2003); Invitrogen]. *TEF1-GFP los1Δ, TEF1-GFP msn5Δ, and TEF2-GFP msn5Δ* strains were generated from the *TEF1-GFP* and *TEF2-GFP* strains by gene replacement.

Yeast strains were maintained on rich (YEPD) media or synthetic complete (SC) media lacking appropriate nutritional ingredients for maintaining plasmids. For nutrient
deprivation studies, SC media lacking all amino acids were used (SC-aa). For studies employing galactose-inducible constructs, cells were grown in SC media with raffinose as the carbon source. At a cell density of OD\textsubscript{600} = 0.4, fusion protein expression was induced for 2 hr by addition of galactose to a final concentration of 2%. Cells were collected, resuspended in media containing glucose as the sole carbon source, and further incubated for 1.5 hr, in either SC or SC-aa media.

**Plasmids**

PCR reactions were carried out using Pfu DNA polymerase (Stratagene). DNAs were ligated with T4 DNA ligase (New England Biolabs). Nup49-mCherry were constructed as described (Lai et al., 2009). pTef1-MORF was obtained from a collection of tagged yeast genes (Gelperin et al., 2005).

**Microscopy**

Epifluorescence imaging was accomplished using a Nikon Microscope Eclipse 90i equipped with a CoolSNAP HQ\textsuperscript{2} CCD camera (Phometrics) and METAMORF Software (Molecular Devices). To view cells by confocal microscopy, live cells were placed on a slide containing a thin layer of SC medium lacking leucine with 20% gelatin and 0.1 mM n-propylgallate and sealed under a coverslip with Valap as previously described (Wu et al., 2006); the cells were viewed using 488 and 568-nm argon ion lasers employing a spinning disk apparatus (UltraView, PerkinElmer Life and Analytical Science, Inc.), a 100/1.4 NA objective lens (Nikon), and a cooled charged coupled device camera (ORCA-
AG, Hamamatsu). Maximum intensity projections of images were created using UltraView ERS software and image analyses of single optical 0.4 µm optical sections were performed using ImageJ. Adobe Photoshop was used for image assembly.

**In situ hybridization**

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

**Northern analysis**

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

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

**Immunofluorescence**

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

### 3.4 Results

#### 3.4.1 Msn5 and Los1 function in re-export of tRNAs from the nucleus to the cytoplasm

To study whether Msn5 functions in tRNA nuclear export, we evaluated tRNA subcellular distribution in *msn5Δ* cells. In wild type (wt) cells neither tRNA\textsuperscript{His}, encoded by genes lacking introns, nor tRNA\textsuperscript{Tyr}, encoded by intron-containing genes, accumulate in nuclei (Fig. 3.1). Also, as previously reported (Shaheen & Hopper, 2005), there are nuclear pools of both of these tRNA species in *los1Δ* cells and their signal co-localize with DAPI staining which localized the nucleus (Fig. 3.1). Significantly, tRNA\textsuperscript{His} and tRNA\textsuperscript{Tyr} accumulate in nuclei of *msn5Δ* cells (Fig. 3.1). Thus, *msn5Δ* cells have defective tRNA nuclear-cytoplasmic distribution.

Since the yeast tRNA splicing endonuclease is located on the cytoplasmic surface of mitochondria (Yoshihisa *et al.*, 2003), a defect in the export of end-matured intron-containing pre-tRNA from the nucleus to the cytoplasm will result in inhibition of pre-tRNA splicing because the tRNA substrate and the splicing endonuclease are in two different subcellular locations. In fact, the components of the tRNA nuclear export machinery, Los1 and Rna1, were discovered by accumulation of end-matured, intron-containing pre-tRNAs in *los1-1* and *rna1-1* mutant cells (Hopper *et al.*, 1978; Hopper *et al.*, 1980) rather than by their defects in tRNA subcellular dynamics. Msn5 has been
implicated in tRNA nuclear export; however, in contrast to los1Δ mutations, deletion of MSN5 does not result in the accumulation of intron-containing pre-tRNA$^{\text{Ile}_{UAU}}$ (Fig. 3.2) and pre-tRNA$^{\text{Tyr}}$ (Murthi et al., 2010). Thus, msn5Δ cells have defective tRNA nuclear-cytoplasmic distribution (Fig. 3.1), despite the fact that pre-tRNA splicing is not affected by deletion of MSN5.

One plausible reason why there are nuclear pools of spliced tRNAs in msn5Δ cells is that the nuclear tRNAs in these cells previously resided in the cytoplasm. In this case, in msn5Δ cells, intron-containing pre-tRNAs would have been exported to the cytoplasm via Los1 (and perhaps additional pathways), spliced on the surface of mitochondria, and imported into the nucleus by the tRNA retrograde process, but then re-exported to the cytoplasm inefficiently. According to this scenario, Msn5 would be involved in the tRNA re-export process rather than the primary export process, at least for tRNAs encoded by intron-containing genes.

To test the idea that Msn5 functions in tRNA re-export, we evaluated tRNA subcellular distribution in mtr10Δ msn5Δ and mtr10Δ los1Δ double mutants under fed and nutrient deprived conditions. Mtr10, a β-importin family member, directly or indirectly, functions in the retrograde movement of tRNA from the cytoplasm to the nucleus since nuclear accumulation of cytoplasmic tRNA upon nutrient deprivation is dependent upon Mtr10 (Shaheen & Hopper, 2005; Hurto et al., 2007; Whitney et al., 2007). If Msn5 functions downstream of Mtr10 in the tRNA re-export process, then in a mutant background in which retrograde import fails to occur (i.e., mtr10Δ), there should be greatly reduced nuclear pools of tRNA upon nutrient deprivation whether or not Msn5
is functional. On the other hand, if Msn5 functions upstream of Mtr10, then the phenotype of \textit{msn5}Δ \textit{mtr10}Δ double mutants should resemble \textit{msn5}Δ alone. While \textit{msn5}Δ mutants have nuclear pools of tRNA\textsubscript{TY}, \textit{msn5}Δ \textit{mtr10}Δ double mutant cells fail to accumulate nuclear pools of tRNA regardless of nutrient availability (Fig. 3.3) (Murthi \textit{et al.}, 2010). The data support the idea that Msn5 functions downstream of Mtr10 in re-export of tRNAs that were spliced in the cytoplasm and subsequently re-imported into the nucleus.

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

If Los1 indeed functions in the tRNA re-export process as well as in the initial round of tRNA nuclear export, in cells deficient for both tRNA retrograde import (\textit{mtr10}Δ) and primary/re-export steps (\textit{los1}Δ) there might be a significant decrease in the level of nuclear tRNAs compared to cells deficient in Los1 alone. To test this, we compared the
accumulation of nuclear pools of tRNA in wild type, \textit{los1Δ, mtr10Δ,} and \textit{los1Δ mtr10Δ} double mutants. As expected, in wild type cells both tRNA\textsuperscript{Tyr} (Fig. 3.4 A), encoded by intron-containing tRNA genes, and tRNA\textsuperscript{His} (Fig. 3.4 B), encoded by intron-free tRNA genes, are primarily cytoplasmic when cells are satiated (Fig. 3.4 A and B, panel 1), but exhibit significant nuclear pools when cells have been deprived of amino acids (Fig. 3.4 A and B, panel 2) (Murthi \textit{et al.}, 2010). As previously reported, cells lacking Los1 accumulate nuclear tRNA pools of both tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{His} regardless of whether the cells are fed or deprived of nutrients (Fig. 3.4 A and B, panels 3 and 4). Also, as previously reported, cells lacking Mtr10 do not have nuclear pools of tRNA regardless of the tRNA species or nutrient availability (Fig. 3.4 A and B, panels 5 and 6). Consistent with the hypothesis that Los1 functions in tRNA re-export, \textit{los1Δ mtr10Δ} do not possess significant nuclear pools of tRNA whether the cells are fed or nutrient deprived (Fig. 3.4 A and B, panels 7 and 8) (Murthi \textit{et al.}, 2010). One interpretation of these results is that the nuclear pools of tRNA detected by FISH are derived primarily from cytoplasmic tRNA and that Los1 and Msn5 both participate in the tRNA re-export process. In sum, the data support a role for Los1 in both the initial tRNA export process and the re-export process, whereas, at least for tRNAs encoded by intron-containing genes, Msn5 functions primarily in the re-export process.
3.4.2 Regulation of tRNA retrograde process and a possible role for Tef1 in the re-export step

tRNA retrograde import is a constitutive process, and therefore accumulation of cytoplasmic tRNA in the nucleus upon nutrient deprivation is likely regulated at the re-export step (Murthi et al., 2010). Regulation of tRNA re-export by nutrient availability could require tRNA aminoacylation in the nucleus as tRNA export is inhibited when tRNA nuclear charging is defective (Sarkar et al., 1999; Grosshans et al., 2000; Azad et al., 2001). Although Msn5 appears to be dedicated to the tRNA re-export step of the retrograde pathway, it interacts with uncharged tRNA in vitro (Shibata et al., 2006). So, if Msn5 regulates tRNA re-export in response to nutrient availability in vivo, it may do so in conjunction with another protein(s) able to distinguish aa-tRNAs from uncharged tRNAs. Tef1 and Tef2 are candidates for this function.

*TEF1* and *TEF2* encode identical eukaryotic translation elongation factor 1 alpha proteins (eEF1A in vertebrates). Tef1/2 or eEF1A bind aa-tRNAs and function to deliver aa-tRNAs to ribosomes (Schirmaier & Philippsen, 1984). Several lines of evidence have implicated Tef1/2 in tRNA nuclear-cytoplasmic dynamics: (1) *TEF2* was identified as a multicopy suppressor of *los1Δ* (Grosshans et al., 2000); (2) *tef2* or *tef1* and *los1* (Grosshans et al., 2000) and *tef1* and *msn5* (Fig. 3.5 A) (Murthi et al., 2010) deletions have synthetic growth defects; and (3) *tef2* mutants accumulate nuclear pools of tRNA (Grosshans et al., 2000; McGuire & Mangroo, 2007). Since this translation factor interacts only with aa-tRNAs and defects in Tef1/2 do not cause accumulation of intron-containing pre-tRNAs (Grosshans et al., 2000), Tef1/2 likely function only in the tRNA
re-export pathway, at least for tRNAs encoded by intron-containing genes. The Msn5 vertebrate orthologue, Exportin-5, forms a heterotetrameric complex with aa-tRNA, RanGTP, and eEF1A, resulting in co-export eEF1A and tRNA to the cytoplasm (Bohnsack et al., 2002; Calado et al., 2002). The data for Tef1/2 and eEF1A in tRNA subcellular dynamics have been interpreted to mean that this well-studied cytoplasmic translation factor serves either to channel tRNA and other proteins exiting the nucleus to the translation machinery (Grosshans et al., 2000; McGuire & Mangroo, 2007; Khacho et al., 2008) or to rid the nuclear interior of inadvertent pools of eEF1A that accumulate during open mitosis (Bohnsack et al., 2002). The possibility that Tef1/2 might have a more direct role in tRNA nuclear export has not been explored.

If Tef1/2 directly functions in tRNA re-export, then there should be nuclear pools of these proteins. We employed three methods to locate Tef1 in yeast grown in satiated and nutrient deprived conditions. First we attempted to employ immunofluorescence (IF) using various commercially available antibodies raised against eEF1A. Although some of these could detect Tef1/2 as assessed by Western analyses (Fig. 3.6 A), they did not detect Tef1 by IF. Second, we employed live cell confocal imaging to assess nuclear pools of endogenous Tef1 encoded by Tef1-GFP gene replacements [Fig. 3.5 B and C; (Huh et al., 2003)]. Third, we employed indirect immunofluorescence (IF) to determine the subcellular distribution of a galactose-regulated HA-tagged multi-copy functional version of Tef1, Tef1-MORF [Fig. 3.6 B, C, and D (Gelperin et al., 2005)].

As assessed by epifluorescence and confocal imaging of live cells, Tef1-GFP are excluded from the nucleus in wild type and los1Δ cells (Fig. 3.5 B and C, panels 1-4),
both under fed and nutrient-deprived conditions (compare panels 1 with 2 and 3 with 4). In stark contrast, Tef1 is not excluded from nuclei in msn5Δ cells (Fig. 3.5 B, panels 5 and 6) and instead is rather evenly distributed between the nucleus and the cytoplasm as determined by pixel intensity profiles of single 0.4 µm confocal sections of individual cells (Fig. 3.5 C). The data indicate that Tef1/2 can access the nuclear interior and that its distribution between the nucleus and cytoplasm is dependent, at least in part, upon the exportin, Msn5 (Murthi et al., 2010).

A role for Msn5 in Tef1 subcellular location was confirmed by IF analyses of Tef1-MORF. As assessed by epifluorescence, Tef1-MORF was excluded from the nucleus in wild type cells (Fig. 3.6 B, panels 1). When cells were deprived of amino acids, a small portion of the Tef1-MORF pool appeared to localize around the nuclear membrane (Fig. 3.6 B, panels 2), consistent with earlier reports (Grosshans et al., 2000); it is unknown whether this is the outer or inner nuclear membrane. As for Tef1-GFP, the subcellular distribution of Tef1-MORF in los1Δ cells was similar to wild type cells (Fig. 3.6 B, panels 3 and 4). In cells that have the double deletion of the importin mtr10Δ and the exportin msn5Δ, Tef1 also did not accumulate inside the nucleus (Fig. 3.6 B, panels 7 and 8). In stark contrast, msn5Δ cells possessed significant apparent nucleoplasmic pools of Tef1-MORF (Fig. 3.6 B, panels 5 and 6). Confocal imaging of Tef1-MORF in wild type and msn5Δ cells containing a plasmid encoded nucleoporin, Nup49-mCherry, confirmed that Tef1-MORF is excluded from the nucleus in wild-type cells and nucleoplasmic in msn5Δ cells (Fig. 3.6 C and D) (Murthi et al., 2010). The IF results are consistent with the results from live imaging of endogenously expressed Tef1-GFP, even though there
are quantitative differences in the Tef1 nucleoplasmic pools between Tef1-GFP and Tef1-MORF, perhaps due to different expression levels or the different tags. The data indicate that Tef1’s entry into the nucleus is affected by Mtr10 and its exit is affected by Msn5, as are the entry and exit of tRNA, and they support the prerequisite for Tef1 nuclear-cytoplasmic dynamics if Tef1 were to serve a direct role in the tRNA re-export step of the retrograde process.

3.5 Discussion

We provide evidence that the two tRNA exportins, Los1 and Msn5, serve distinct but overlapping roles in yeast. It was previously appreciated that Los1 exports newly transcribed end-matured tRNA from the nucleus to the cytoplasm (primary export). Evidence to support this is: (1) accumulation of end-matured intron-containing pre-tRNA in los1Δ mutants (Hopper et al., 1980), (2) nuclear pools of tRNAs in los1Δ mutants (Sarkar & Hopper, 1998; Grosshans et al., 2000), and (3) in vitro binding of Los1 and tRNA with RanGTP (Hellmuth et al., 1998). Los1 also likely serves a proofreading role in nuclear export of newly synthesized tRNAs because its orthologue, Exp-t, will not interact with mutant tRNAs with aberrant secondary or tertiary structure (Arts et al., 1998a; Lipowsky et al., 1999; Cook et al., 2009). Two lines of evidence support the idea that Los1 participates in the tRNA re-export process as well as in the initial tRNA nuclear export step. First, in los1Δ X los1Δ heterokaryons, tRNAs encoded by one nucleus accumulate in nuclei not encoding the tRNA (Shaheen & Hopper, 2005; Takano et al., 2005); therefore, tRNA cytoplasmic tRNA derived from one nucleus can access a second
nucleus and, due to defects in re-export caused by absence of Los1, nuclear pools of the tRNA accumulate. Second, we show that haploid mtr10Δ los1Δ double mutant cells accumulate significantly less tRNA_Tyr and tRNA_His in the nucleus than do cells with only a los1Δ mutation. Thus, the tRNA_Tyr and tRNA_His detected by FISH in cells must come from two sources, newly synthesized tRNA and tRNA imported from the cytoplasm, and elimination of the latter results in significantly reduced nuclear pools. Our data showing that Los1 participates in both the initial export and re-export processes is consistent with the known interactions of the vertebrate homologue, Exp-t, and S. pombe Xpot with the TΨC and acceptor arms and the 5′ and 3′ termini of tRNAs, structures that are in common to end-matured newly synthesized intron-containing and imported mature tRNAs (Etcheverry et al., 1979; Lee & Knapp, 1985; Arts et al., 1998b; Lipowsky et al., 1999; Cook et al., 2009).

Unlike Los1, Msn5 appears not to function in the initial export process for tRNAs encoded by intron-containing tRNA genes. First, we do not detect nuclear pools of tRNA in mtr10Δ msn5Δ double mutants; second, and more important, there is no accumulation of intron-containing pre-tRNAs in msn5Δ mutants even though there are large nuclear pools of tRNAs. Since splicing occurs on the surface of mitochondria, the nuclear tRNAs encoded by intron-containing genes in msn5Δ cells must be derived from tRNAs that previously resided in the cytoplasm. We do not know whether Msn5 participates only in the re-export process for tRNAs encoded by genes lacking introns; however, it is difficult to imagine that Msn5 could distinguish between the new end-processed transcripts and the tRNA imported from the cytoplasm unless Msn5 monitors modifications that are
added solely in the cytoplasm. In sum, tRNA subcellular dynamics require at least three members of the importin-β family: Mtr10 which directly or indirectly functions in tRNA retrograde import, Los1 which directly functions in the initial and re-export steps, and Msn5 which, for tRNAs encoded by intron-containing genes, functions primarily in the tRNA re-export process. As los1Δmsn5Δ double mutants have no obvious growth defects (Takano et al., 2005), there are likely undiscovered additional mechanisms for tRNA nuclear export in yeast.

If tRNA nuclear import is constitutive (Murthi et al., 2010), then the re-export process is likely regulated, since when cells are deprived for nutrients, cytoplasmic tRNAs accumulate in the nucleus (Shaheen & Hopper, 2005; Hurto et al., 2007; Shaheen et al., 2007; Whitney et al., 2007). tRNA re-export is likely regulated in response to the aminoacylation status of tRNAs because mutations or conditions that prohibit tRNA aminoacylation result in nuclear pools of mature tRNA (Lund & Dahlberg, 1998; Sarkar et al., 1999; Grosshans et al., 2000; Azad et al., 2001; Feng & Hopper, 2002; Gu et al., 2003; Gu et al., 2005). Since intron-containing tRNAs cannot be aminoacylated (O'Farrell et al., 1978) and Los1 exports intron-containing tRNAs, Los1-mediated tRNA re-export is unlikely to be responsive to conditions affecting tRNA aminoacylation. Rather, we propose that Msn5 is the tRNA exportin involved in regulated tRNA re-export. If so, Msn5 would be expected to preferentially bind and export aa-tRNAs. However, this is not consistent with the known binding specificities of the Exportin-5 family. The Exportin-5 orthologues bind short double stranded RNAs such as miRNAs (Gwizdek et al., 2003; Gwizdek et al., 2004; Lund et al., 2004; Zeng & Cullen, 2004; Shibata et al.,
as well as uncharged or aa-tRNAs, although there may be differences in binding affinities for tRNA among the various orthologues (Shibata et al., 2006).

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

A role for Tef1/2 in tRNA re-export would conveniently explain the presence of Tef1/2 in the nucleus in yeast. Because of its closed mitosis, yeast cannot inadvertently acquire Tef1/2 during mitosis, as proposed for nuclear pools of eEF1A in vertebrate cells
(Bohnsack et al., 2002; Calado et al., 2002). Because of the large size of Tef1-GFP (78 kDa, including the 28 kDa GFP tag) and Tef1-MORF [69 kDa, including the 19 kDa MORF tag (Gelperin et al., 2005)] nuclear Tef1 pools are also unlikely to arise from passive diffusion through the nuclear pore, which has a 40 kDa diffusion barrier (Shulga et al., 2000). Since the nuclear presence of Tef1/2 is dependent upon Mtr10 and Msn5 and parallels the subcellular distribution of tRNA and since mutations of TEF1/TEF2 cause nuclear accumulation of mature tRNA, the simplest explanation is that Tef1/2 function in tRNA nuclear re-export. Although it has been difficult to detect, in vivo, a quaternary complex of Msn5, aa-tRNA, RanGTP and Tef1 by conventional methodology, cross-linking-based efforts should provide a rigorous test in the future for this otherwise attractive model for tRNA re-export.
Figure 3.1. **FISH analysis of the role of Msn5 in tRNA nuclear-cytoplasmic dynamics.** Locations of tRNA\(^\text{His}\) and tRNA\(^\text{Tyr}\) in wild type (1, 2), los1\(^\Delta\) (3, 4), and msn5\(^\Delta\) (5, 6) cells. 1-6, FISH signal; 1’-6’, same cells stained with DAPI. White size bar = 5\(\mu\)m.

Figure 3.2. **Los1 and Msn5 have different roles in tRNA nuclear export.** Northern analysis of pre-tRNA\(^\text{Ile}\) and mature tRNA\(^\text{Ile}\) in wild type and mutant cells.
Figure 3.3. **FISH analysis of the location of tRNA^{Tyr}.** The subcellular distribution of tRNA in wild type (1-2), *msn5Δ* (3-4), and *mtr10Δ msn5Δ* (5-6) cells were examined in nutrient rich (1, 3, 5) and amino acid deprived (2, 4, 6) conditions. 1-6, FISH signal; 1’-6’, same cells stained with DAPI. White size bar = 5 µm.
Figure 3.4. Los1 and Msn5 function in the tRNA re-export process. FISH analysis for tRNA$_{Tyr}$ (A) and tRNA$_{His}$ (B) in wild-type fed (panels 1) or amino acid deprived (panels 2), los1$\Delta$ (panels 3 and 4), mtr10$\Delta$ (panels 5 and 6) and los1$\Delta$mtr10$\Delta$ (panels 7 and 8) cells. 1-8, location of the tRNAs; 1’-8’, the same cells stained with DAPI. White size bar = 5 $\mu$m.
Figure 3.5. Interactions between Los1 or Msn5 and Tef1. (A) Synthetic growth defects in losΔ tefΔ and msn5Δ tefΔ double mutants. Serial dilutions were made of the indicated yeast cultures. Aliquots (5 µl) of each dilution were spotted onto solid growth medium and incubated 2 to 3 days at various indicated temperatures. (B) Confocal images of the subcellular distribution of endogenously expressed Tef1-GFP in wild type and mutant fed and nutrient-deprived live cells. Wild type (panels 1 and 2), losΔ (panels 3 and 4), or msn5Δ (panels 5 and 6) mutant cells containing the constitutively expressed plasmid-encoded Nup49-mCherry were grown in SC media lacking leucine to maintain the plasmid. Cells in 1-1”, 3-3”, 5-5” were further incubated in media with all required nutrients, whereas cells in 2-2”, 4-4”, and 6-6” were deprived of amino acids for 1.5 hr. 1-6, location of Tef1-GFP; 1’-6’, location of Nup49-mCherry, demarking the nuclei rims, 1’’-6’’, overlay of 1-6 and 1’-6’, respectively. White size bar = 5 µm. (C) Pixel intensity profiles of the subcellular distribution of Tef1-GFP in wild type and mutant fed and nutrient deprived live cells. Pixel intensity profiles of 0.4µm sections are shown for three independent cells for each strain and condition. The cells in panel B that were scanned and plotted in panel C are indicated with the same shape arrows or arrowheads. Red lines and axes indicate Nup49-mCherry image intensities; green lines and axes indicate Tef1-GFP intensities.
Figure 3.5. Interactions between Los1 or Msn5 and Tef1.
Figure 3.6. Detection and subcellular dynamics of Tef1-MORF. (A) Induced levels of Tef1-MORF detected by Western analysis of the HA tag in the MORF addition. Wild-type and \textit{msn5}Δ cells containing the Tef1-MORF plasmid were cultured in repressed conditions (Glu; glucose as the carbon source) in the presence of all amino acids (+aa) or their absence (-aa). (B) Immunofluorescence analysis of the location of Tef1-MORF in wild type and mutant cells employing anti-HA as the primary antibody. Cells were first grown in media lacking uracil and containing raffinose as the carbon source; the expression of Tef1-MORF was induced by the addition of galactose to the media. After two hr Tef1 synthesis was terminated by the addition of glucose to the media. Cells in 1-1’, 3-3’, 5-5’, and 7-7’ were further cultured in complete media for 1.5 hr, whereas cells in 2-2’, 4-4’, 6-6’, and 8-8’ were deprived of amino acids for 1.5 hr. 1-8, FITC signal showing the location of the HA antigen; 1’-8’, the same cells stained with DAPI. (C) Confocal imaging of IF analysis of the subcellular distributions of Tef1-MORF and Nup49-mCherry. Tef1-MORF was expressed in wild type and \textit{msn5}Δ mutant fed and amino acid deprived cells as described for panel B. Top row: Tef1-MORF signal; middle row: Nup49-mCherry signal; bottom panel: merged Tef1-MORF and Nup49-mCherry signals. White size bar = 5 µm. (D) Pixel intensity profiles of the distributions of Tef1-MORF between the nucleoplasm and the cytoplasm and Nup49-mCherry at the nuclear envelop. Pixel intensity profiles are shown for two independent cells for each strain and condition. The cells in panel C that were scanned and plotted in panel D are indicated with the same shape arrows or arrowheads. Red lines and axes indicate Nup49-mCherry image intensities; green lines and axes indicate Tef1-GFP intensities.
Figure 3.6. Detection and subcellular dynamics of Tef1-MORF.
4.1 Abstract

In eukaryotes, mature tRNAs move bidirectionally between the nucleus and the cytoplasm. Three members of yeast importin-β family, Los1, Msn5, and Mtr10 have been implicated in tRNA subcellular movement. Here we developed formaldehyde crosslinking-based in vivo co-immunoprecipitation assays to study the in vivo interactions of β-importins with tRNAs. It has been proposed that Los1 participates in the primary and re-export of tRNAs to the cytoplasm, but Msn5 participates only in the re-export of tRNAs encoded by intron-containing genes. Consistent with this proposal, Los1 binds to both intron-containing and mature tRNAs, whereas Msn5 preferentially binds to mature tRNAs. Mtr10 affects tRNA nuclear import, but our data provide no evidence for Mtr10 binding to tRNA, implicating that Mtr10 indirectly functions in tRNA subcellular dynamics. In vitro studies report that Msn5 binds short dsRNAs and uncharged tRNA, in conflict with our in vivo genetic and biochemical data. To address this, we assessed whether other proteins aid in vivo specificity of Msn5 for spliced tRNA. Tef1 (vertebrate eEF1A), implicated in tRNA nuclear export, co-purifies with Msn5 in a RanGTP-dependent manner. Our studies and the studies in other organisms support the model that
Msn5 forms a cooperative quaternary complex with Tef1, RanGTP, and aa-tRNA to export mature, probably charged tRNAs to the cytoplasm.

4.2 Introduction

Eukaryotic tRNAs are transcribed in the nucleus but function in translation in the cytoplasm. Surprisingly, it was learned that tRNAs move bidirectionally between the nucleus and the cytoplasm (Shaheen & Hopper, 2005; Takano et al., 2005; Zaitseva et al., 2006; Shaheen et al., 2007; Barhoom et al., 2011; Miyagawa et al., 2012). Newly transcribed, end-processed tRNAs are exported from the nucleus to the cytoplasm via primary tRNA nuclear export. Cytoplasmic tRNAs are constitutively imported into the nucleus via retrograde tRNA nuclear import (Shaheen & Hopper, 2005; Takano et al., 2005; Zaitseva et al., 2006; Shaheen et al., 2007; Murthi et al., 2010; Barhoom et al., 2011; Miyagawa et al., 2012). Then, tRNAs can again access the cytoplasm via tRNA re-export (Shaheen & Hopper, 2005; Takano et al., 2005; Whitney et al., 2007).

Nuclear export of tRNAs proceeds via the Ran pathway. Ran is a small GTPase that regulates nuclear-cytoplasmic transport via its association with importin-β family members (Strom & Weis, 2001; Fried & Kutay, 2003; Madrid & Weis, 2006). Exportins are a subset of the importin-β family that are dedicated to nuclear export. Due to the asymmetric distribution of RanGAP in the cytoplasm and RanGEF in the nucleus (Ohtsubo et al., 1989; Hopper et al., 1990; Bischoff & Ponstingl, 1991; Bischoff et al., 1994), exportins cooperatively bind the cargo and RanGTP in the nucleus. After translocation to the cytoplasm, hydrolysis of the RanGTP, aided by the cytoplasmic
RanGAP, leads to disassembly of the export complex and delivery of the cargo to the cytoplasm.

In vertebrates, the member of the β-importin family that functions in tRNA nuclear export is Exportin-t (Exp-t). Homologues to Exp-t have been studied in budding yeast (Los1), fission yeast (Xpo-t), and plants (PAUSED) (Arts et al., 1998b; Kutay et al., 1998; Hunter et al., 2003; Cook et al., 2009). Studies of the binding capability of the vertebrate Exp-t and crystallography structural studies of the *Schizosaccharomyces pombe* Xpo-t homologue show that Exp-t preferentially binds to the appropriately structured tRNA backbone of tRNAs with mature 5′ and 3′ ends. The structural studies support earlier work showing that Exp-t has no preference for intron-containing or intron-less tRNAs (Arts et al., 1998b; Lund & Dahlberg, 1998; Lipowsky et al., 1999; Cook et al., 2009). Thus, Exp-t seems to serve a quality control function assuring that only tRNAs that are appropriately structured and possess mature termini access the cytoplasm and translation machinery.

The *S. cerevisiae* Exp-t homologue is Los1 (Hopper et al., 1980; Hellmuth et al., 1998; Sarkar & Hopper, 1998). In contrast to vertebrates, yeast splicing machinery is located on the surface of mitochondria (Yoshihisa et al., 2003; Yoshihisa et al., 2007). End-processed intron-containing pre-tRNAs are exported to the cytoplasm prior to intron removal. Therefore, deletion of *LOS1* results in the accumulation of end-processed intron-containing pre-tRNAs unable to access the cytoplasmic splicing machinery (Hopper et al., 1980; Sarkar & Hopper, 1998). Although the *in vitro* binding capability of Exp-t to tRNA is well-studied, *in vivo* complexes of Exp-t or Los1 with tRNA and
RanGTP have not yet been reported, possibly due to their transient nature (Hellmuth et al., 1998; McGuire & Mangroo, 2012).

Despite the defects in tRNA nuclear export, los1Δ cells are viable. S. pombe Xpo-t and Arabidopsis PAUSED are also unessential (Hunter et al., 2003; Cherkasova et al., 2011). In Drosophila melanogaster, no Exp-t homologue has been identified (Lippai et al., 2000). Thus, additional tRNA export pathways should exist in yeast, Drosophila, and Arabidopsis.

The vertebrate β-importin family member Exportin-5 (Exp-5) has also been implicated in the nuclear export of tRNA; however, it is thought to play only a minor role in tRNA nuclear export, and, instead, it primarily functions in the nuclear export of microRNAs [(Bohsack et al., 2002; Calado et al., 2002; Lund et al., 2004; Shibata et al., 2006; Mingot et al., 2013); review: (Leisegang et al., 2012)]. The yeast homologue, Msn5, is well-known to export particular phosphorylated transcription factors to the cytoplasm [Review: (Hopper, 1999)]. Msn5 is likely also involved in tRNA nuclear export because msn5Δ cells accumulate nuclear pools of tRNAs (Murthi et al., 2010) and msn5Δ los1Δ cells have larger nuclear pools of tRNAs than either mutant alone (Takano et al., 2005). In addition, Msn5 is able to bind tRNAs in a RanGTP-dependent manner in vitro (Shibata et al., 2006).

In contrast to los1Δ cells, deletion of MSN5 does not result in the accumulation of end-processed intron-containing pre-tRNAs (Murthi et al., 2010). Thus, one possible reason for why there are nuclear pools of tRNAs in msn5Δ cells is that the nuclear tRNAs in msn5Δ cells were previously spliced in the cytoplasm and imported into the nucleus.
via tRNA retrograde import. Therefore, Msn5 could function in tRNA re-export rather than primary tRNA nuclear export for the subset of tRNAs that are encoded by intron-containing genes (Murthi et al., 2010).

Yeast β-importin, Mtr10, is implicated in retrograde tRNA nuclear import as mtr10Δ cells fail to accumulate nuclear pools of tRNAs upon aa deprivation (Shaheen & Hopper, 2005; Murthi et al., 2010). However, it is unknown whether Mtr10 acts directly upon tRNA nuclear import by binding cytoplasmic tRNAs or whether it functions indirectly in retrograde tRNA nuclear import.

Here we employed crosslinking combined with co-immunoprecipitation (Co-IP) assays to study the interactions of β-importins and tRNAs in vivo. The data indicate that Los1 binds both intron-containing and mature tRNAs, while Mtr10 may not interact with tRNAs. Msn5 preferentially binds to mature tRNAs. We further demonstrated that Tef1, previously implicated in tRNA nuclear export (Grosshans et al., 2000; Bohnsack et al., 2002; Calado et al., 2002; Murthi et al., 2010; Mingot et al., 2013), interacts with Msn5 in the presence of Ran locked in the GTP-bound state but not GDP-bound state. Thus, it is likely that Msn5 and Tef1 function together to export mature tRNAs from the nucleus to the cytoplasm. Our studies argue against the possibility that Tef1 hands off aa-tRNA to Msn5 and support the alternative that Msn5-Tef1-aa-tRNA-RanGTP forms a quaternary re-export complex.
4.3 Material and Methods

Yeast Strains and Plasmids

Most experiments employed yeast strains BY4741 (MATa his3Δ leu2Δ met15Δ ura3Δ). Yeast strains were maintained in synthetic defined media (SC) lacking the appropriate nutritional ingredients for selection. Genomic Myc-tagged Tef1-Myc or GFP-tagged Npl3-GFP strains were generated by introducing PCR fragments, which contain homologous sequences with selection markers and Myc sequences or GFP sequences, respectively.

Multi-copy plasmid pMSN5-M was constructed by replacing the Scal/BamHI fragment of plasmid MSN5-MORF (Gelperin et al., 2005) with the EcoICRI/BamHI fragment of plasmid YEpMSN5 (Jones et al., 2008) that includes 400 bp upstream of MSN5 to the BamHI site within MSN5. Multicopy plasmid pLOS1-M was constructed by replacing the SacII/HindIII fragment of plasmid LOS1-MORF (Gelperin et al., 2005) with a PCR-amplified fragment from YEpLOS1 (Jones et al., 2008) that includes 400 bp upstream of LOS1 to the HindIII site within LOS1. Multicopy plasmid pMTR10-M was constructed as described (Kramer & Hopper, 2013). Multicopy plasmid pKap95-M was constructed by replacing the EagI/XhoI fragment of plasmid pRS426 with a PCR-amplified fragment from YEpkap95 (Jones et al., 2008) that includes 400 bp upstream and the open reading frame of KAP95 and with a PCR-amplified fragment including the protein A ZZ domain from pBG1805 (Gelperin et al., 2005). Plasmid with galactose-inducible Gsp1-G21V and Gsp1-T26N were constructed by site-directed mutagenesis as described (Kornbluth et al., 1994).
**Northern analysis**

Small RNA was isolated and separated by electrophoresis and transferred onto a Hybond N+ membrane (Amersham). tRNAs were detected with a $\gamma^{32p}$-labeled probe or a DIG-labeled probe complementary to tRNA$^{ile}$ as described (Wu et al., 2013). The relative levels of precursor and mature tRNAs were determined using ImageJ (http://rsb.info.nih.gov/ij/).

**Western Analysis**

Protein was assessed using chemiluminescence-based Western blot analysis following standard protocols as described (Chu & Hopper, 2013). The membrane was probed with primary antibodies: anti-Myc (9E10, Santa Cruz) at a 1:1500 dilution, anti-GFP (clone CBP-KK1, Roche) at a 1:1000 dilution, and anti-Ran (ab4781, abcam) at a 1:1500 dilution. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare, UK) at a 1:3000 dilution. Protein signals were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

**Co-immunoprecipitation assays**

Yeast strains that carry Protein A ZZ domain tagged proteins and inducible Ran locked mutants were grown in appropriated selective media at 30°C until they reached early or mid log phase (OD$_{600}$ of 0.4-0.6). Ran mutant proteins were induced by adding 2% galactose for 1 hr and then cultures were crosslinked by adding formaldehyde to a
final concentration of 0.3% for 30 min, quenched by adding glycine to the final concentration of 66 mM for 10 min. Harvested cells are rapidly frozen in liquid nitrogen and broken open in the solid phase by milling. Cryolysis is performed using a planetary ball mill. Co-immunoprecipitation (Co-IP) assays were performed as described (Alber et al., 2007) with the optimizations. In brief, 0.5g of frozen ground cells were resuspended in 4.5 ml of the extraction buffer (20 mM Hepes, pH 6.1, 110 mM KOAc, 2 mM MgCl₂, 75 mM NaCl, 0.5 % Triton, 0.1% Tween-20, 1 mM DTT, 5 µM GTP) with 1: 100 dilutions of protease inhibitor cocktail set IV (EMD Millipore) and 1: 1000 dilutions of RNase inhibitor (Roche). The suspension was isolated by centrifugation at 3000 xg, 4 °C for 10 minutes. The soluble extract was incubated with IgG-conjugated magnetic beads at 4 °C for 30 minutes. The magnetic beads were then collected with a magnet, washed six times with 1 ml of the ice-cold extraction buffer and once with 0.1 M NH₄OAc, 0.1 mM MgCl₂, 0.02% Tween-20. Protein was eluted with 0.5 M NH₄OH, 0.5 mM EDTA by incubation for 20 min at room temperature. The eluates were lyophilized in a SpeedVac (Thermo Savant) overnight. The pellets were resuspended in NuPAGE LDS sample buffer (Life technologies), separated on a 4-12 % NuPAGE Novex Bis-Tris precast gel (Life technologies) according to the manufacturer’s specifications.

**RNA isolation after immunoprecipitation assays**

After washing in 1 ml of 0.1 M NH₄OAc, 0.1 mM MgCl₂, 0.02% Tween-20, the magnetic beads were collected using a magnet, and resuspend in elution buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1% SDS, 10 mM DTT) following by 70 °C incubation
for 45 min. The samples were incubated with Proteinase K (20 mg/ml) (NEB) for 30 min at 30 °C. RNA was extracted using the same volume of acid phenol and then precipitated in 3 volumes of 100% EtOH with GlycoBlue (Ambion) at -80 °C. Pellets were resuspended in nuclease-free water.

**RT-PCR**

First strand cDNA Synthesis was carried out using Superscript III reverse transcriptase (Invitrogen), according to the manufacturer’s protocol. Subsequent PCR reactions were carried out using 2 µl of cDNA, and 15 µl of each PCR reaction was electrophoretically separated on 2% agarose gel.

**Real-time RT-PCR**

RNAs were treated with Turbo DNase (Ambion) according to the manufacturer protocol. 2 µl of RNA was used to prepare cDNAs for real-time PCR using Superscript III reverse transcriptase (Invitrogen). PCR was performed using SsoFast Eva Green Supermix (Bio Rad) and the CFX96 instrument (Bio Rad). Standard curve were performed with 10-fold serial dilutions of gel-extracted RT-PCR product as template to determine the concentrations of samples. No template controls were analyzed for each primer sets, and no RT controls were analyzed for each sample with each primer set. Data was analyzed by CFX96 software (Bio Rad).
**In Situ Hybridization**

Fluorescence *in situ* hybridization (FISH) was performed as previously described (Sarkar & Hopper, 1998) with the modifications detailed in (Stanford et al., 2004).

### 4.4 Results

#### 4.4.1 Msn5 is dedicated to tRNA nuclear re-export

It has been suggested that Msn5 participates in tRNA nuclear re-export but not primary tRNA nuclear export because, in contrast to *los1Δ* cells, *msn5Δ* cells do not accumulate end-processed intron-containing pre-tRNAs (Murthi et al., 2010). However, another interpretation of the data is that Msn5 plays only a minor role in primary tRNA nuclear export, consistent with the role of its mammalian orthologue, Exp-5, in tRNA export (Bohnsack et al., 2002; Calado et al., 2002). According to this proposal, in *msn5Δ* cells, Los1 would export the majority of the intron-containing pre-tRNAs to the cytoplasm, and thus, no end-processed intron-containing pre-tRNAs would accumulate, even though by FISH nuclear pools of tRNAs are detected in *msn5Δ* cells (Shaheen & Hopper, 2005; Murthi et al., 2010). To address this possibility, we employed *los1Δ* cells which accumulate end-processed intron-containing pre-tRNAs. If Msn5 plays only a minor role in primary tRNA nuclear export, one would expect that Msn5 should be able to suppress the defects of *los1Δ* cells when over-expressed, and thus with high cellular levels of Msn5 in *los1Δ* cells end-processed intron-containing pre-tRNAs should not accumulate or their levels should decrease. On the other hand, if Msn5 is dedicated to tRNA re-export for the category of tRNAs encoded by intron-containing genes, then
over-expressed Msn5 should not suppress the defect in primary tRNA export of los1Δ cells which results in accumulation of end-processed intron-containing pre-tRNAs.

To test the alternatives, we increased Msn5 expression levels by generating several Msn5 constructs. MORF- and TAP-tagged versions of Msn5 regulated by its own promoter were constructed in the yeast episomal plasmid (YEp), pRS426 vector (Sikorski & Hieter, 1989; Christianson et al., 1992), resulting in pMsn5-M and pMsn5-T. The MORF tag contains 6xHis, HA, and the IgG binding ZZ domain of Protein A (Gelperin et al., 2005) and the TAP tag consists of the calmodulin binding peptide and the Protein A ZZ domain (Ghaemmaghami et al., 2003). The tagged versions of Msn5 were created for the use in protein assays as there is no commercially available Msn5 antibody. To test whether the tagged versions of Msn5 are functional, we performed FISH. Unlike msn5Δ cells which accumulate tRNA in the nucleus, msn5Δ cells with the MORF-tagged Msn5 (pMsn5-M) evidence an even distribution of tRNAs between the nucleus and the cytoplasm (Fig. 4.1), thus showing that this tagged Msn5 complements the defects of msn5Δ cells and thereby maintains its function in tRNA nuclear export.

The pRS426 vectors reach a copy number of 5-20 per cell, and therefore yeast containing plasmids generated from this backbone can reach up to 20-fold elevated levels of protein expression compared to the endogenous level (Christianson et al., 1992). Thus, we investigated whether high levels of functional tagged Msn5 can suppress the defects in los1Δ cells by Northern analyses. los1Δ cells transformed with vectors accumulated elevated levels of unspliced tRNA, whereas wild type (wt) cells with vectors did not (Fig. 4.2 A, ratio I/P). As anticipated, the ratio of intron-containing pre-tRNAs to the primary
transcript decreased in los1Δ cells harboring a multi-copy plasmid with LOS1 (pLos1) (Fig. 4.2 A, ratio I/P). In contrast, los1Δ cells containing multi-copy plasmids with MSN5 (pMsn5-M or pMsn5-T) accumulated elevated levels of unspliced tRNA and showed no suppression of the los1Δ phenotype (Fig. 4.2 A, ratio I/P). Thus, the defects of los1Δ cells were not suppressed by high levels of Msn5, even though this tagged Msn5 in YEp vector is functional.

As Msn5 in YEp vector can reach up to 20-fold elevated expression and it is possible that such levels would be insufficient to suppress the los1Δ phenotype, we examined whether even higher expression levels of Msn5 would suppress the los1Δ phenotype. We constructed plasmids with untagged Msn5 in both the YEp (pMsn5) and the leu2d (pMsn5-leu2d) backbone to address the caveat that tagged versions of Msn5 may not be fully functional, even though they complemented the defects of msn5Δ cells. Due to a deletion of the LEU2 promoter, the plasmids with the leu2d backbone reach ~100 copies/cell in order for cells to grow in the synthetic media without leucine (Erhart & Hollenberg, 1983).

Consistent with the results from tagged Msn5 in the YEp vector, assessment of los1Δ cells harboring untagged Msn5 in the YEp vector (pMsn5) showed that intron-containing pre-tRNAs accumulated in the cells, compared to wt cells (Fig. 4.2 B, ratio I/P). In addition, even with higher expression levels of untagged Msn5 (pMsn5-leu2d) in los1Δ cells, intron-containing pre-tRNAs accumulated (Fig. 4.2 B, ratio I/P). Therefore, over-expression of active Msn5 constructs to levels of ~100-fold excess endogenous
levels cannot suppress the defects of los1Δ cells, and thus end-processed intron-containing pre-tRNAs accumulate in this deletion strain.

Taken together, the results support the model that Msn5 is unlikely to play a role, like Los1, in the primary nuclear export of intron-containing pre-tRNAs. Thus, we provide additional evidence to support the notion that Msn5 is dedicated to tRNA re-export but not primary nuclear export for tRNAs encoded by intron-containing genes.

4.4.2 Assays for in vivo detection and characterization of tRNA transport complexes

To investigate in vivo interactions of importin-β family members with tRNAs, we developed crosslinking-based Co-IP assays to analyze co-purified proteins and RNAs with β-importins. In eukaryotes, Ran regulates the association and dissociation of β-importins and cargos. Exportin binds its cargo in the presence of RanGTP and RanGTP hydrolysis to RanGDP disassembles the nuclear export complex. Thus, exportins should remain associated with cargo when hydrolysis of GTP to GDP is inhibited. In contrast, binding of importin to RanGTP leads to dissociation of cargo from importin-cargo complex. Therefore, importin-cargo complex should be enriched when Ran is predominantly in the GDP-bound form. To retain the transport complexes, plasmids encoding RanGTP (Gsp1-G21V) or RanGDP (Gsp1-T24N) locked mutant proteins were introduced in the yeast strains. Since expression of Ran locked mutant proteins results in dominant lethality (Kornbluth et al., 1994), the Ran constructs were engineered into galactose-inducible expression vectors, which can be maintained in the off-state in the absence of galactose and be highly induced by the addition of galactose to the media.
To titrate the induction time points for the Ran mutant proteins, we followed the subcellular locations of the tagged β-importins upon galactose induction. β-importins, Los1 and Msn5, maintained their normal distributions for at least 1 hr induction. Longer induction caused redistributions of these proteins that will be described in a separate communication (Huang and Hopper, in preparation) (Chapter 5). Therefore, RanGTP or RanGDP mutant proteins were induced for 1 hr in yeast cultures for Co-IP assays (Fig. 4.3 A).

To study whether β-importins, Msn5, Los1, and Mtr10, interact with tRNA in vivo, we employed crosslinking-based Co-IP assays. Kap95, which functions in nuclear import of NLS-containing proteins and has no reported function in RNA nuclear-cytoplasmic trafficking (Enenkel et al., 1995; Gilchrist & Rexach, 2003), served as a negative control. C-terminal MORF-tagged Msn5, Los1, Mtr10, and Kap95 proteins regulated by their endogenous promoters were constructed in the YEp vectors. FISH analyses were used to test the functionality of tagged Msn5, Los1, and Mtr10. As learned for MORF-tagged Msn5 (Fig. 4.1), tagged Los1 complements the defects of los1Δ cells and thereby maintains its function in tRNA nuclear export (Fig. 4.1). MORF-tagged Mtr10 is also able to complement the defects of mtr10Δ cells (Kramer & Hopper, 2013) (Fig. 4.4). Thus, tagged versions of Msn5, Los1, and Mtr10 are functional (Fig. 4.1 and 4.4).

To maintain the transient interacting complexes, formaldehyde crosslinking was employed (Fig. 4.3 A). Tagged β-importins containing Protein A ZZ domain were immunoprecipitated using IgG-conjugated magnetic beads. The retrieved proteins were incubated at 70°C for 45 min to reverse the crosslinking treatment. Heat-treated and
untreated extracts were analyzed by Sypro Ruby protein staining (Fig. 4.3 B and 4.5). The enriched proteins ranging from 110-165 kD were the expected size of these β-importins (Fig. 4.3 B, indicated by bracket). A <30 kD protein co-purified with Msn5, Los1, and Kap95. This is the expected size of Ran. Indeed, as examined by western blot analysis, Ran co-purified with Msn5, Los1, and Kap95 only in samples with Ran locked in the GTP-bound state (Fig. 4.3 C, lanes marked “T”), but not in samples with Ran locked in the GDP-bound state (Fig. 4.3 C, lanes marked “D”). Very little Ran co-purified with Mtr10 under the same conditions, possibly due to its low RanGTP binding affinity, as previous in vitro studies reported that the RanGTP binding constant of Mtr10 is roughly 3 orders of magnitude greater than Kap95 (Hahn & Schlenstedt, 2011).

Appropriate in vivo interactions for Kap95 were also documented by its putative association with Kap60. Kap95 forms a dimer with Kap60 to mediate nuclear import (Enenkel et al., 1995) and a 60 kD protein co-purified with Kap95 in a RanGDP-dependent manner (Fig. 4.3 B, white star), implicating that nuclear import complexes (Kap95-Kap60-cargo) were enriched. Taken together, the data support that export/import complexes were likely maintained throughout these in vivo experimental studies.

To determine whether tRNAs co-purify with the β-importins, the complexes obtained from Co-IP assays were treated with Proteinase K and DNase to enrich for putative RNAs. Because northern analyses were not sensitive enough to monitor the small amount of intron-containing pre-tRNAs (data not shown), RT-PCR analyses were used to assess whether mature tRNAs and/or intron-containing pre-tRNAs co-purified with β-importins. In addition, we obtained absolute quantification of the average of
putative RNA levels by employing real-time RT-PCR from three independent assays. In yeast, 10 tRNA families are transcribed with an intron located 3′ of nucleotide 37. However, many modifications, such as N1-methylguanosine, N3-methyluridine or N3-methylcytidine, block reverse transcriptase (Wittig & Wittig, 1978; Motorin et al., 2007). Since tRNA\textsubscript{Ile}\textsuperscript{UAU} and tRNA\textsubscript{Trp}\textsuperscript{CCA}, encoded by intron-containing genes, lack nucleotide modifications in the 5′ exon that block reverse transcriptase (Motorin et al., 2007; Machnicka et al., 2013), tRNA\textsubscript{Ile}\textsuperscript{UAU} and tRNA\textsubscript{Trp}\textsuperscript{CCA} were used to assess the quantity of intron-containing and mature tRNAs in the enriched complexes. Mature tRNAs were detected by reverse transcription using primers crossing the splice junction sequences (Fig. 4.6 A, primer 2) and followed by amplification using primers 1 and 2 (Fig. 4.6 A). Intron-containing pre-tRNAs were detected by reverse transcription using primers specific to the intron sequences (Fig. 4.6 A, primer 3) and then amplified by using primers 1 and 3 (Fig. 4.6 A). Primers specific to 5S rRNA were used to assess the amount of contaminating small RNAs, thereby serving as the negative control in the amplification studies.

4.4.3 Los1 functions in tRNA primary nuclear export and re-export

Mature tRNA\textsubscript{Ile} and tRNA\textsubscript{Trp} co-purified with Los1 when cells contained Ran in the GTP locked form (Fig. 4.6 B, lane 3), but not in the GDP locked form (Fig. 4.6 B, lane 4), supported by real-time RT-PCR (Fig. 4.6 D). We quantified the ratio of tRNA levels in the samples with locked RanGTP to the samples with locked RanGDP for each β-importin complex from Co-IP, resulting in a RanGTP/RanGDP ratio (Table 4.1, R). If β-
importin interacts with tRNA in a RanGTP-dependent manner, one would expect that the R ratio for this β-importin would be higher than the R ratio for the negative control Kap95. Indeed, the R ratio for Los1, ranging from 14.66 to 281.70, is much higher than the R ratio for Kap95 sample (R\textsubscript{kap95}, 0.47-2.27) for mature tRNAs (Table 4.1).

Intron-containing pre-tRNA\textsuperscript{Ile} and pre-tRNA\textsuperscript{Trp} also co-purified with Los1 and only in the presence of RanGTP locked mutant proteins (Fig. 4.6 C, lanes 3-4), also supported by quantification of enriched pre-tRNAs by real-time RT-PCR (Fig. 4.6 E and Table 4.1). Low levels of 5S rRNA were detected in Los1 enriched complexes containing both RanGTP and RanGDP locked forms (Fig. 4.6 F). Although the levels of 5S rRNA in Los1 samples with RanGTP are slightly higher than the sample with RanGDP, low levels of 5S rRNA were also detected in Kap95 samples (Fig. 4.6 F), probably due to low levels of contaminating RNAs in the Co-IP procedures.

Taken together, mature and intron-containing tRNAs co-purify with Los1 in a RanGTP-dependent manner, consistent with the \textit{in vitro} studies that Exportin-t is able to bind both mature and intron-containing tRNAs. Our studies provide the first \textit{in vivo} evidence for the yeast Los1-tRNA-RanGTP complex and support the notion that Los1 participates in the primary and re-export of tRNA to the cytoplasm.

4.4.4 No evidence for Mtr10 interaction with tRNA

Mtr10 is implicated in retrograde tRNA nuclear import as \textit{mtr10}Δ cells fail to accumulate nuclear tRNAs upon aa deprivation. In addition, unlike \textit{los1}Δ and \textit{msn5}Δ cells which accumulate nuclear tRNAs, \textit{mtr10}Δ \textit{los1}Δ and \textit{mtr10}Δ \textit{msn5}Δ cells do
not accumulate nuclear tRNAs (Murthi et al., 2010) and nuclear accumulation of tRNAs in mtr10Δ los1Δ or mtr10Δ msn5Δ cells is complemented when Mtr10 is exogenously expressed (Fig. 4.4). As Msn5 is thought to function in tRNA re-export, Mtr10 appears to function in the upstream step of Msn5. Therefore, Mtr10 is proposed to function in retrograde tRNA nuclear import, but whether Mtr10 functions, directly or indirectly, in tRNA nuclear import remains unknown.

To investigate whether Mtr10 directly interacts with tRNAs, we performed crosslinking-based Co-IP assays. We first assessed whether our in vivo crosslinking procedures are able to detect known Mtr10 binding proteins. Mtr10 is known to function as the Npl3 nuclear import receptor (Senger et al., 1998). Binding of RanGTP to import receptors leads to release of the import cargo from the complex into the nucleus. To assess binding of Mtr10 with Npl3, we introduced Npl3-GFP to cells that contain plasmid encoding functional MORF-tagged Mtr10 (pMtr10-M) (Gelperin et al., 2005; Kramer & Hopper, 2013) as well as plasmids encoding either the inducible locked RanGTP or RanGDP mutant proteins. As anticipated, Mtr10 co-purified Npl3-GFP when cells contained Ran locked in the GDP-bound state, but not when they contained Ran locked in the GTP-bound state (Fig. 4.3 D). However, assessment of enriched RNAs by RT-PCR showed that only very low levels of mature tRNA were detected in Mtr10 samples with either Ran mutant form. The detected tRNA levels in Mtr10 samples with RanGTP and RanGDP locked forms are similar (Fig. 4.6 B, lanes 5-6, and 4.6 D). Although low levels of amplified intron-containing pre-tRNAs were detected in Mtr10 samples, similar low levels of pre-tRNAs were also detected in the negative control Kap95 sample (Fig. 4.6 C,
lanes 5-8, and 4.6 E). Moreover, similar low levels of 5S rRNA were detected in Mtr10 samples with Ran in both GTP and GDP locked forms (Fig. 4.6 F, lanes 5-6). Finally, the R ratio for Mtr10 sample is similar to the R ratio for Kap95 sample in the quantification assay (Table 4.1). Thus, the amplified signals for RNAs co-purifying with Mtr10 are most likely due to contaminating RNAs. Although Npl3-GFP specifically co-purified with Mtr10, in the same samples, no statistically significant amount of tRNAs co-purified with Mtr10 in a either RanGTP or RanGDP-dependent manner. The data indicate that Mtr10 may not directly interact with tRNAs and function directly in retrograde tRNA nuclear import. However, we cannot eliminate the alternatives that Mtr10 does specifically interact with tRNAs with an affinity below our detection or that its interaction with tRNAs is unstable.

4.4.5 Msn5 preferentially interacts with mature tRNAs

Interaction of Msn5 with tRNA in vivo is poorly characterized. Data from genetic studies led to the working model that Msn5 exports only spliced tRNA, and perhaps only aa-tRNA [(Murthi et al., 2010); (Fig. 4.2, these studies)]. To test this working model, we investigated the in vivo interaction of Msn5 with tRNAs by analyzing the Msn5 complexes from Co-IP assays.

As analyzed by RT-PCR, mature tRNA^{ile} and tRNA^{Trp} co-purified with Msn5 from extracts with Ran locked in the GTP-bound state, whereas very little tRNA co-purified from extracts with Ran locked in the GDP-bound state (Fig. 4.6 B, lanes 1-2). The levels of mature tRNA^{ile} and tRNA^{Trp} in Msn5 samples with locked RanGTP were almost
equivalent to the levels in Los1 samples with locked RanGTP (Fig. 4.6 B, lanes 1 and 3). In contrast, the amount of detectable intron-containing pre-tRNAs in Msn5 samples were below the amount of detectable pre-tRNAs in the negative control Kap95 samples (Fig. 4.6 C, lanes 1-2, 7-8). Moreover, low levels of contaminating 5S rRNA were detected in Msn5 enriched complexes with both RanGTP and RanGDP locked forms (Fig. 4.6 F). Therefore, compared to co-purified pre-tRNAs with Los1, Msn5 evidenced markedly reduced co-purification of intron-containing pre-tRNAs.

Quantification of the enriched RNA levels by real-time RT-PCR supported the visual assessment. The levels of detectable mature tRNAs in Msn5 and Los1 samples with locked RanGTP were nearly identical (Fig. 4.6 D). Although very low levels of mature tRNAs were detected in Msn5 sample with RanGDP locked form, these levels were almost the same or below the negative control Kap95 (Fig. 4.6 D). In contrast to Los1, no statistically significant amount of pre-tRNA co-purified with Msn5 in a RanGTP-dependent manner (Fig. 4.6 E). Thus, Msn5 appears to interact with mature tRNAs in a RanGTP-dependent fashion.

To further investigate the specificity of Msn5 to mature tRNAs and pre-tRNAs, we compared the levels of enriched tRNA with Msn5 to that with Los1 by analyzing the RanGTP/RanGDP (R) ratio, as described above. For mature tRNA^{Ile}, the R ratio for Msn5 is ~32% of the ratio for Los1. However, for pre-tRNA^{Ile}, the R ratio for Msn5 is only ~9% of the ratio for Los1 (Table 4.1). Similarly, for mature tRNA^{Trp}, the R ratio for Msn5 is 25% of the ratio for Los1, whereas for pre-tRNA^{Trp}, the R ratio for Msn5 is only ~4% of the ratio for Los1 (Table 4.1). Therefore, Msn5 appears to interact less well than
Los1 with mature tRNAs and exceedingly poorly with intron-containing pre-tRNAs. One caveat is that these comparisons are highly influenced by detectable RNAs from extracts with locked RanGDP, as the levels of detectable RNAs in Msn5 samples with locked RanGDP are higher than the levels in Los1 samples. Taken together, compared to Los1, Msn5 preferentially binds to mature tRNAs.

4.4.6 Msn5 interacts with Tef1 in vivo

Our Co-IP studies provide evidence that Msn5 preferentially interacts with mature tRNAs vs. intron-containing tRNAs. In addition, our genetic data indicate that one level of control regulating the re-export step requires charged tRNA in the nucleus (Sarkar et al., 1999; Gu et al., 2005). However, in vitro Msn5 and its orthologues bind short duplex RNA (e.g. microRNA) as well as uncharged tRNA (Lund et al., 2004; Shibata et al., 2006). Hence, there is a contradiction between the in vitro and in vivo data. One way to rectify this contradiction would be the presence of a protein(s) in vivo that enhances the ability of Msn5 to bind to mature and/or aminoacylated tRNAs. A candidate protein for this function is the translation elongation factor eEF1A (yeast Tef1/2).

In mammalian cells, eEF1A has been implicated in tRNA export. Although eEF1A functions and localizes in the cytoplasm, it has been proposed that it can accidentally access the nucleus during open mitosis. Export of eEF1A is mediated by Exp-5, which binds aa-tRNAs and then recruits eEF1A from the nucleus to the cytoplasm (Bohsack et al., 2002; Calado et al., 2002; Mingot et al., 2013). In yeast, there are also several lines of evidence implicating Tef1 in tRNA nuclear export. Mutations of TEF1/TEF2 cause
nuclear accumulation of tRNAs (Grosshans et al., 2000); however, like for \textit{msn5}\textsuperscript{Δ} and unlike for \textit{los1}\textsuperscript{Δ} cells, there is no accumulation of unspliced tRNAs in cells with defects in Tef1/2. Therefore, Tef1/2 appear to function in the tRNA re-export step. Furthermore, Tef1/2 have synthetic negative genetic interactions with \textit{los1}\textsuperscript{Δ} and \textit{msn5}\textsuperscript{Δ} (Grosshans et al., 2000; Murthi et al., 2010). Finally, we showed that the subcellular distribution of Tef1 between the nucleus and cytoplasm depends on Msn5 (Murthi et al., 2010), supporting an interaction between Msn5 and Tef1 and also showing that Tef1 have dynamic nuclear-cytoplasmic distributions, even in an organism, like yeast, with a closed mitosis. Since Tef1 binds only with aa-tRNAs [Review: (Nissen et al., 1996)], we investigated whether Tef1 aids the specificity of Msn5 to export mature/charged tRNA to the cytoplasm. To test this proposal, we examined whether Msn5 interacts with Tef1 by \textit{in vivo} Co-IP methodologies.

An endogenously Myc-tagged Tef1 (Tef1-Myc) was generated by gene replacement. Since \textit{TEF1}/\textit{TEF2} are essential, the fact that the yeast strains with endogenously tagged Tef1-Myc along with depletion of \textit{TEF2} are viable provides evidence that this tagged version is active. Co-purification of Tef1-Myc with Msn5 was assessed by western analysis. Tef1-Myc co-purified with Msn5 in the extracts with an inducible RanGTP locked form, but not in the extracts with RanGDP locked form (Fig. 4.3 E). Thus, Msn5 interacts with Tef1 \textit{in vivo} in a RanGTP-dependent manner. In contrast, neither Los1, nor Kap95 interacts with Tef1 (Fig. 4.3 E), providing evidence for the specificity of Tef1 interaction with Msn5.
As Tef1 specifically co-purifies with Msn5, Tef1 may assist Msn5 to export mature and possibly only charged tRNA to the cytoplasm due to the specificity of Tef1 for aa-tRNAs. How might Tef1 provide specificity for Msn5 in tRNA nuclear export? Tef1 might form a quaternary complex with Msn5, RanGTP, and aa-tRNA. Alternatively, Tef1 could hand off aa-tRNA to Msn5 to form a tertiary complex, Msn5-aa-tRNA-RanGTP, for export. To distinguish between these possibilities, we assessed whether interaction between Msn5 and Tef1 is dependent upon tRNA by employing Co-IP of Msn5 from extracts treated with RNase A.

We investigated whether Msn5 exists in a quaternary complex with RanGTP, tRNA, and Tef1 by employing cells containing inducible RanGTP and treated with formaldehyde to crosslink Msn5 with its interacting partners. The extracts from the cells were either treated with RNase A or mock treated prior to purification of Msn5 by Co-IP assays (Fig. 4.7 A). Similar studies were conducted with cells containing tagged Kap95 which served as negative controls. Indeed, no interactions of Kap95 and Tef1 were detected (Fig. 4.7 B, lanes 1 and 3). As anticipated, Tef1 co-purified with Msn5 under mock treatment (Fig. 4.7 B, lane 2). Comparing the ratio of the co-purified Tef1 levels to the Msn5 levels, upon RNase addition the recruitment of Tef1 maintained ~92% of the relative Tef1 levels under mock conditions (Fig. 4.7 B, compare lanes 2 and 4). Under these experimental conditions RNase A treatment removed all detectable RNAs from the purified complexes as total RNA were assessed by RT-PCR of mature tRNA\textsuperscript{Ile} and intron-containing pre-tRNA\textsuperscript{Ile} (Fig. 4.7 C). Thus, Tef1 co-purifies with Msn5 even under
RNase treated conditions, supporting the notion that Msn5 forms a quaternary complex with Tef1, aa-tRNA, and RanGTP and arguing against the hand off model.

If Msn5 forms a quaternary complex, the quaternary complex could be formed by Msn5 interacting with aa-tRNA and then binding to Tef1 or could be assembled by Msn5 interacting with Tef1 as an adaptor for aa-tRNA. As all of the components in the quaternary complex could be crosslinked no matter how Msn5 assembles the complex, we tested these two possible complexes by employing RNase A treatment and Co-IP of Msn5 from extracts without crosslinking.

We first investigated whether Msn5 co-purifies Tef1-Myc in the conditions without crosslinking treatment. Surprisingly, in the presence of locked RanGTP, we were able to detect Tef1 in complex with Msn5 even in the absence of crosslinking (Fig. 4.7, lane 6). No Tef1-Myc co-purified with the negative control Kap95 in the same condition (Fig. 4.7 B, compare lanes 5, 6). Thus, crosslinking treatment is not required for the co-purification of Tef1 with Msn5.

To test the alternative possible ways of how the quaternary complexes assemble, the experiments were conducted by employing various RNase treatments and Co-IP assays. R indicates that the cell extract was treated with RNase A prior to IP assays, while R* indicates that IP assays were performed prior to RNase A treatment (Fig. 4.7 A). When the cell extract was treated with RNase A prior to binding with the beads (Fig. 4.7 A, condition “R”), the recruitment of Tef1-Myc was reduced to ~3% of the relative Tef1-Myc level compared to mock conditions (Fig. 4.7 B, compare lanes 6 and 10). Surprisingly, if the Msn5 complex was immunoprecipitated on beads first and then
treated with RNase (Fig. 4.7 A, condition “R*”), conditions in which Msn5 was highly enriched compared to other cellular proteins, ~35% of relative Tef1-Myc level was retained in complex with Msn5 compared to mock treatment (Fig. 4.7 B, compare lanes 6 and 8). Again, assessment of total RNA by RT-PCR showed that RNase effectively removed all detectable mature tRNA \textsuperscript{ile} and intron-containing tRNA \textsuperscript{ile} no matter the order of RNase addition and IP assays (Fig. 4.7 C). Partial retention of Tef1 in the Msn5 complex was reproducibly obtained when Msn5 was highly enriched prior to RNase treatment, whereas the complexes were nearly avoid of Tef1 when extracts were treated with RNase prior to binding to the beads. Thus, when Msn5 is enriched, the Msn5-Tef1 interaction is partially maintained upon RNase addition. Possible explanations for these results are discussed below.

Taken together, our studies argue against the possibility of Tef1 handing off aa-tRNA to Msn5 and support the proposal that Msn5 forms a quaternary complex with Tef1, RanGTP, and aa-tRNA. Therefore, Tef1 likely functions in the nucleus to aid the specificity of Msn5 to export aa-tRNAs.

4.5 Discussion

In organisms from yeast to vertebrates, tRNAs move bidirectionally between the nucleus and the cytoplasm. The mechanisms of this tRNA retrograde pathway are not entirely clear. Here we developed a methodology to study the \textit{in vivo} interaction of tRNA exportin/importin with tRNAs. We provide \textit{in vivo} biochemical evidence for the functions of tRNA exportin/importin in the tRNA retrograde pathway. As both mature
tRNAs and pre-tRNAs co-purify with Los1 in a RanGTP-dependent manner, it supports the notion that Los1 functions in both primary and re-export of tRNAs. In contrast, we fail to detect Mtr10-tRNA interaction, suggesting that Mtr10 might indirectly function in retrograde tRNA nuclear import. Msn5 preferentially interacts with mature tRNAs and this specificity might be aided by Tef1. Msn5 and Tef1 likely function cooperatively in tRNA re-export.

Formation of a Los1-tRNA-RanGTP complex has only been observed in an indirect in vitro assay but has not been reported in vivo, presumably attributed to its transient nature (Hellmuth et al., 1998). Here we show that Los1 is in complex with RanGTP and tRNAs by in vivo crosslinking-based Co-IP assays. Both intron-containing and mature tRNAs co-purify with Los1 in the presence of locked RanGTP but not when Ran is in the GDP locked form. The tRNA nuclear export function of Los1 is likely conserved among eukaryotes. Consistent with in vivo data, mammalian Exportin-t monitors the tRNA backbone and aminoacyl stem but it does not monitor the anticodon stem (Lipowsky et al, 1999). S. pombe Exporin-t wraps around the acceptor arm, TψC loop and D loop, but makes no contacts with the anticodon arm where the intron is located (Cook et al, 2009). Taken together, the data support the model that Los1/Exp-t functions in the primary nuclear export of end-processed intron-containing pre-tRNAs as well as re-export of spliced tRNAs for the category of tRNAs encoded by intron-containing genes (Fig. 4.8 A and C). In addition, for tRNAs encoded by intron-less genes, Los1 also participates in both their primary and re-export steps. The structural studies show that Los1 monitors the 3’ end of tRNA but it is unable to distinguish between uncharged and charged tRNA.
Thus, Los1 could function in the export of both uncharged and charged mature tRNAs (Fig. 4.8 C, purple and blue arrow).

Cells bearing a mutation of MTR10 fail to accumulate spliced tRNA in the nucleus when cells are nutrient deprived (Shaheen & Hopper, 2005; Murthi et al., 2010), presumably because retrograde tRNA nuclear import is defective. Mtr10 could function, directly or indirectly, in retrograde tRNA nuclear import. Surprisingly, Transportin 3 (Tnp3), the human homologue of Mtr10, binds viral capsid proteins and tRNAs in the presence of RanGTP (Zhou et al., 2011). Zhou et al. proposed that Tnp3 promotes a nuclear maturation process required for HIV-1 integration by displacing capsid proteins and tRNAs that remain bound to the viral pre-integration complex after nuclear entry (Zhou et al., 2011). Unlike Tnp3, Mtr10 is thought to function in tRNA nuclear import rather than nuclear tethering because the genetic data showed that mtr10Δ los1Δ or mtr10Δ msn5Δ cells do not accumulate nuclear tRNAs (Murthi et al., 2010). In our studies, we failed to detect either RanGTP or RanGDP-dependent interactions of Mtr10 with tRNA even though the construct was functional and even though we could detect the known Mtr10 cargo, Npl3. The most likely interpretation of the data is that Mtr10 does not directly interact with tRNAs. However, it is possible that Mtr10 binds tRNA with an affinity below our detection. If Mtr10 functions indirectly in the tRNA nuclear import step, it could import a cytoplasmic tRNA tether, or regulate cytoplasmic tRNA tethering function via importing the modulator of tRNA tether (Fig. 4.8 B).

It has been proposed that tRNA re-export is likely regulated in response to nutrient availability (Murthi et al., 2010). When tRNA charging is defective or when cells are
deprived for aa, uncharged tRNAs accumulate in the nucleus (Sarkar et al., 1999; Azad et al., 2001; Gu et al., 2005; Whitney et al., 2007; Murthi et al., 2010). Thus, one mechanism to regulate tRNA re-export requires tRNA aminoacylation in the nucleus (Sarkar et al., 1999; Gu et al., 2005) (Huang and Hopper, in preparation). Here we provide additional genetic and biochemical evidence to support the proposal that Msn5 functions to regulate tRNA re-export. Our in vivo biochemical studies show that Msn5 preferentially interacts with mature tRNAs; Msn5 specifically interacts with Tef1 and this interaction may aid Msn5 to preferentially export mature/charged tRNA to the cytoplasm.

How could Tef1 aid the specificity of Msn5 to mature tRNAs and possibly to aa-tRNAs? Our results argue against the possibility that Tef1 hands off aa-tRNA to Msn5. Rather, the data support the model that Msn5 forms a quaternary complex with Tef1, RanGTP, and aa-tRNA. Similarly, Exp-5, mammalian orthologue of Msn5, forms a cooperative quaternary complex with RanGTP, VA1 RNA, and protein ILF3. The affinity of VA1 RNA for Exp-5 is increased by the presence of ILF3 and formation of the complex results in the nuclear co-export of ILF3 and VA1 RNA (Gwizdek et al., 2004).

One of the possible models is that Msn5 interacts with aa-tRNA and then binds to Tef1 (Fig. 4.8 C, model 1). In mammalian cells, Exp-5 can recruit aa-tRNAs bound to the eEF1A (Bohsack et al., 2002; Calado et al., 2002; Mingot et al., 2013). The Exp-5-aa-tRNA-eEF1A-RanGTP complex has been proposed to export SNAG-containing proteins from the nucleus to the cytoplasm (Mingot et al., 2013). However, structural studies of Exp-5 have led to the prediction that Exp-5 binds to the 3' protruding end of tRNA (Okada et al., 2009). Since eEF1A binds the 3' end of tRNA when tRNA is
aminoacylated (Nissen et al., 1995; Nissen et al., 1996), in eEF1A-aa-tRNA complex the 3’ protruding end of aa-tRNA would be unavailable to interact with Exp-5. If the predicted Exp-5-tRNA interaction is correct, it is difficult to image that tRNA would serve as the bridge between Msn5 and Tef1 in the quaternary tRNA export complex.

In yeast, Msn5 functions in the nuclear export of particular phosphorylated proteins. Previous in vitro studies show that Msn5 directly interacts with its phosphorylated protein cargo, Pho4 (Kaffman et al., 1998), suggesting that Msn5 has ability to directly bind proteins. Therefore, it is rationally to consider the alternative that Msn5 may directly interact with Tef1 and then Tef1 interacts with aa-tRNAs (Fig. 4.8 C, model 2). According to this model, Tef1 would provide specificity for Msn5 to interact with aa-tRNAs in vivo. Our studies to investigate the possession of tRNA in the Msn5 quaternary complex assessed co-purification of Tef1 with Msn5 upon RNase treatment. Our assays show that when Msn5 is enriched, part of Tef1 pool co-purifies with Msn5 even upon RNase treatment (Fig. 4.7 B, compare lanes, 6 and 8). However, treatment of RNase prior to Msn5 enrichment greatly reduced the amount of Tef1 that co-purifies with Msn5 (Fig. 4.7 B, compare lanes 6 and 10). Since Tef1 has multiple cellular functions (Mateyak & Kinzy, 2010), Tef1 likely forms numerous different complexes. Therefore, prior to IP, in the presence of other cellular proteins, the Msn5-Tef1 interaction might be transient, particularly in the absence of tRNAs. Since Tef1 undergoes structural alteration upon binding aa-tRNA [Review: (Nissen et al., 1996)], it seems possible that this Msn5 complex might prefer intra-molecular interactions via protein (Msn5)-protein (Tef1)
interactions rather than protein (Msn5)-RNA (aa-tRNA) interactions (Fig. 4.8 C, model 2).

It might be possible to test the validity of model 2 by depletion of Tef1/2 from cell extracts. If Tef1/2 are required for Msn5 in complex with tRNAs, the levels of co-purified tRNAs from Msn5 enriched complexes would be affected when Tef1/2 are depleted. Since Tef1/2 are essential, construction of conditional double mutants is required. We attempted to construct a yeast strain harboring a heat-inducible degradation signal, degron, fused with Tef2 and also harboring the depletion of TEF1 gene. The target protein fused with temperature-activated degron and regulated by copper inducible promoter can be rapidly degraded at the non-permissive temperature. To maintain the strain viability during construction, we introduced a maintenance plasmid encoding TEF1. However, tef1Δ cells harboring Tef2-degron gene replacement cannot survive without maintenance plasmid encoding TEF1. It is likely due to the levels of Tef2-degron regulated by copper inducible promoter is not sufficient to support cell growth when TEF1 is depleted or due to this Tef2-degron protein is subject to rapid degradation, even at the permissive temperature. To overcome this difficulty, we have designed a new approach to deplete Tef1/2 from extracts by providing tef1Δ strain with increased levels of Tef2-degron or to construct yeast strain harboring degrons fused to both Tef1 and Tef2.

Our working model for tRNA subcellular dynamics (Fig. 4.8) provides a mechanism in which tRNA exportin has specificity to mature tRNAs and/or pre-tRNAs. It is likely that tRNA exportin derives its tRNA binding specificity via interaction with other protein factors, such as Tef1/2. As no evidence supports Mtr10 directly interacting and importing
tRNAs to the nucleus by our assays, the protein that functions in retrograde tRNA nuclear import remains unknown. Thus, further investigations are required to identify the missing players in bidirectional tRNA subcellular dynamics.
Table 4.1. Ratio of tRNA levels in samples with locked RanGTP to RanGDP for each β-importin

<table>
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<th>RanGTP/RanGDP (R)*</th>
<th>tRNA&lt;sup&gt;pre&lt;/sup&gt;</th>
<th>pre-tRNA&lt;sup&gt;pre&lt;/sup&gt;</th>
<th>tRNA&lt;sup&gt;trp&lt;/sup&gt;</th>
<th>pre-tRNA&lt;sup&gt;trp&lt;/sup&gt;</th>
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<td>0.57</td>
<td>0.47</td>
<td>0.48</td>
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<tr>
<td>Los1</td>
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<td>68.08</td>
<td>281.70</td>
<td>48.35</td>
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<tr>
<td>Mtr10</td>
<td>0.49</td>
<td>0.48</td>
<td>0.50</td>
<td>0.57</td>
</tr>
<tr>
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<td>6.17</td>
<td>71.37</td>
<td>2.18</td>
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<td>0.320</td>
<td>0.091</td>
<td>0.253</td>
<td>0.045</td>
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</table>

*Cq values for reaction without reverse transcriptase were 2<sup>12</sup> to 2<sup>18</sup>-fold higher than samples with RT for amplification.
**Figure 4.1. FISH analysis of the subcellular distribution of tRNA^His.** *msn5Δ* cells with exogenously expressed Msn5 and *los1Δ* cells with exogenously expressed Los1 are examined to study whether tagged Msn5 and Los1 are functional. DAPI staining of DNA shows the location of the nucleus. Bar = 5 µm.
Figure 4.2. Over-expression of Msn5 does not alleviate los1Δ defects. Northern analyses of pre-tRNA^{ile} and mature tRNA^{ile} in wt and los1Δ cells. (A) Cells with over-expressed tagged Msn5. (B) Cells with over-expressed untagged Msn5. P, primary tRNA transcript; I, end-processed intron-containing tRNA; M, mature tRNA. 5S rRNA serves as the loading control.
Figure 4.3. Detection of proteins co-purified with β-importins. (A) Flowchart of experimental designs. (B) Enriched proteins were detected by Sypro Ruby protein staining. Lanes 1,2, Msn5; lanes 3,4, Los1; lanes 5,6, Kap95; lanes 7,8, Mtr10; lanes 1,3,5,7, cells with Ran locked in the GTP-bound state; lanes 2,4,6,8, cells with Ran locked in the GDP-bound state. (C) Enriched proteins were immunoblotted with α-Ran antibody, (D) α-GFP antibody, and (E) α-Myc antibody.
Figure 4.4. FISH analysis of tRNA subcellular distribution in wt and mutant cells.
(A) tRNA$^{\text{His}}$ and (B) tRNA$^{\text{Tyr}}$ in wt and mutant cells with exogenously expressed Mtr10 are examined under fed and aa deprivation conditions. DAPI staining of DNA shows the location of the nucleus.
Figure 4.5. Comparison of crosslinked protein complexes and reverse crosslinked proteins after Co-IP assays. Enriched proteins were detected by Sypro Ruby protein staining. Lanes 1, 2, 3, proteins retrieved from crosslinking-based Co-IP assays; lanes 4, 5, 6, proteins retrieved from crosslinking-based Co-IP assays followed by 70 °C incubation to reverse the crosslinking. Putative complexes are indicated by red bracket on lanes 1-3.
Figure 4.6. tRNAs co-purify with Los1 and Msn5 but not with Mtr10 and Kap95. (A) Primer 1 and 2 are used to detect mature tRNA and primer 1 and 3 are used to detect intron-containing pre-tRNA. Black lines, exon; gray line, intron; slash, splice junction. M, mature tRNA; I, intron-containing tRNA. (B) Enriched RNAs detected by RT-PCR for mature tRNA^{ile} and tRNA^{Trp}. (C) Enriched RNAs detected by RT-PCR for intron-containing pre-tRNA^{ile} and pre-tRNA^{Trp}. (D) Enriched RNAs analyzed by real-time RT-PCR from three independent experiments for tRNA^{ile} and tRNA^{Trp}. (E) Real-time RT-PCR analyses for pre-tRNA^{ile}, and pre-tRNA^{Trp}. (F) RT-PCR and real-time RT-PCR analyses for 5S rRNA.
Figure 4.7. **Msn5 interacts with Tef1 in vivo.** (A) Flowchart of experimental designs. (B) Enriched proteins were immunoblotted with α-proteinA and α-Myc antibody. T/M, ratio of co-purified Tef1 to Msn5 protein (C) Total RNAs were analyzed by RT-PCR for mature tRNA^{Ile} and intron-containing pre-tRNA^{Ile}. 
Figure 4.8. The working model of tRNA subcellular dynamics for tRNAs encoded by intron-containing genes. (A) Red arrow indicates tRNA primary nuclear export. Blue line of tRNA, exon sequences; red line of tRNA, intron sequences. T, Ran in the GTP-bound state. (B) Green arrow indicates retrograde tRNA nuclear import. (C) Purple arrow indicates tRNA nuclear re-export. Blue arrows represent the re-export is dependent on the nuclear charging status of tRNAs.
Chapter 5 Separate responses to glucose and amino acid availability regulate tRNA subcellular traffic

5.1 Abstract

Cytoplasmic tRNAs move bidirectionally between the nucleus and the cytoplasm. tRNAs imported from the cytoplasm accumulate in nuclei upon nutrient deprivation, likely due to inhibition of tRNA nuclear re-export. Although tRNA nuclear accumulation occurs upon amino acid (aa) and glucose deprivation, cells appear to utilize different mechanisms to regulate the responses to aa and glucose availability. Subcellular distributions of tRNA exportins, Los1 and Msn5, which shuttle between the nucleus and the cytoplasm, are regulated in response to glucose availability. In nutrient replete conditions, Los1 and Msn5 are primarily nuclear. Upon aa deprivation they maintain their primarily nuclear distribution. However, upon glucose deprivation they rapidly become primarily cytoplasmic. Redistribution of tRNA exportins likely provides one mechanism for tRNA nuclear distribution upon glucose deprivation. Interestingly, response to glucose availability regulates the subcellular distribution of all tested β-importins due to alteration of the nuclear-cytoplasmic RanGTP gradient. Furthermore, two separate mechanisms are implicated in regulating the responses to aa availability, aminoacylation
status of tRNA and aa signaling. Thus, tRNA subcellular movement is regulated by diverse nutrient-sensitive mechanisms.

5.2 Introduction

In eukaryotes, tRNAs that are transcribed in the nucleus function in the cytoplasm. Initial tRNA transcripts undergo numerous post-transcriptional alterations, including removal of both the 5′ leaders and 3′ trailers, CCA nucleotide addition to the 3′ ends, removal of introns from the subset of pre-tRNAs transcribed by intron-containing genes, and addition of 25 distinct nucleotide modifications [Review: (Phizicky & Hopper, 2010)]. The processing events are similar between vertebrates and yeast but the subcellular locations where the events occur and order of the events are not conserved. In vertebrates, newly transcribed intron-containing tRNAs are spliced and then the ends are processed in the nucleus (Lund & Dahlberg, 1998; Paushkin et al., 2004). Following CCA addition, nucleotide modifications, and aminoacylation, tRNAs are exported to the cytoplasm (Lund & Dahlberg, 1998). In contrast, in yeast *Saccharomyces cerevisiae*, 5′ leaders and 3′ trailers are removed in the nucleus before the splicing occurs in the cytoplasm (Yoshihisa et al., 2003; Yoshihisa et al., 2007; Dhungel & Hopper, 2012). Thus, in yeast end-processed intron-containing pre-tRNAs are exported to the cytoplasm prior to removal of introns (Yoshihisa et al., 2003; Yoshihisa et al., 2007; Murthi et al., 2010).

It is now known that tRNAs move from the cytoplasm to the nucleus via retrograde nuclear import and can again access the cytoplasm via tRNA re-export (Shaheen &
Hopper, 2005; Takano et al., 2005; Whitney et al., 2007; Eswara et al., 2009). This tRNA retrograde nuclear import and re-export cycle is conserved from yeast to vertebrates (Zaitseva et al., 2006; Shaheen et al., 2007; Barhoom et al., 2011; Miyagawa et al., 2012). The current model of tRNA subcellular trafficking includes three steps. First, newly transcribed and end-processed tRNAs are exported from the nucleus to the cytoplasm (primary tRNA nuclear export). Second, mature cytoplasmic tRNAs are constitutively transported back to the nucleus via retrograde tRNA nuclear import (Shaheen & Hopper, 2005; Takano et al., 2005; Zaitseva et al., 2006; Shaheen et al., 2007; Murthi et al., 2010; Barhoom et al., 2011; Miyagawa et al., 2012). Third, mature tRNAs are re-exported to the cytoplasm (tRNA re-export) (Whitney et al., 2007; Eswara et al., 2009).

Export of tRNAs from the nucleus to the cytoplasm proceeds via the Ran pathway with the association of importin-β family members. In vertebrates, the importin-β family member that functions in tRNA nuclear export is Exportin-t (Arts et al., 1998b; Kutay et al., 1998). Binding of Exportin-t to tRNA is RanGTP dependent. After nuclear export, tRNA is released from the Export-t-tRNA-RanGTP complex by the action of cytoplasmic RanGAP which stimulates hydrolysis of RanGTP to RanGDP. Earlier observations showed that Exportin-t preferentially binds to the appropriately structured tRNA backbone with mature tRNA 5’ and 3’ ends but has no preference for intron-containing or intron-less tRNAs (Arts et al., 1998b; Lund & Dahlberg, 1998; Lipowsky et al., 1999). These findings have been supported by crystallography structural studies of the Schizosaccharomyces pombe Exportin-t homologue in complex with tRNA and RanGTP (Cook et al., 2009). The S. cerevisiae Exportin-t homologue is Los1 (Hopper et al., 1980;
End-processed intron-containing pre-tRNAs accumulate in los1Δ cells due to defects in primary nuclear export of pre-tRNAs to the cytoplasm where the splicing machinery resides (Sarkar & Hopper, 1998; Yoshihisa et al., 2003; Yoshihisa et al., 2007; Murthi et al., 2010).

The importin-β family member, Exportin-5/Msn5, is also implicated in nuclear export of tRNA (Bohnsack et al., 2002; Calado et al., 2002; Shibata et al., 2006; Murthi et al., 2010). Vertebrate Exportin-5 primarily functions in nuclear export of microRNAs (Lund et al., 2004), and it has been proposed that it plays a minor role in tRNA nuclear export [(Bohnsack et al., 2002; Calado et al., 2002; Mingot et al., 2013); review: (Leisegang et al., 2012)]. The yeast homologue, Msn5, has a well-known role in the export of particular phosphorylated transcription factors to the cytoplasm [Review: (Hopper, 1999)]. In addition, data support a role for Msn5 in tRNA nuclear export: (1) Msn5 is able to bind tRNA in a RanGTP dependent manner in vitro (Shibata et al., 2006); (2) msn5Δ cells accumulate nuclear pools of tRNA (Murthi et al., 2010); and (3) msn5Δ los1Δ cells accumulate a larger nuclear pool of tRNA than either mutant alone (Takano et al., 2005). However, in contrast to los1Δ cells, deletion of MSN5 does not result in accumulation of end-processed intron-containing pre-tRNAs (Murthi et al., 2010). One interpretation of the data is that the tRNAs that accumulate in nuclei in msn5Δ cells were previously spliced in the cytoplasm and re-entered the nucleus via retrograde tRNA import step. The working model is that Msn5 participates in tRNA nuclear re-export, but not primary tRNA nuclear export for tRNAs that are encoded by
intron-containing genes whereas Los1 likely functions in both primary nuclear export and re-export step for this class of tRNAs (Murthi et al., 2010).

tRNA retrograde nuclear accumulation occurs in response to loss of nutrients as cells exhibit nuclear accumulation of spliced tRNAs upon amino acids (aa), glucose, or inorganic phosphate deprivation (Grosshans et al., 2000; Shaheen & Hopper, 2005; Hurto et al., 2007; Whitney et al., 2007), while cells exhibit an even distribution of tRNA throughout the nucleus and cytoplasm under nutrient replete conditions. The subcellular distribution of tRNAs between the nucleus and the cytoplasm is a result from the balance among the rates of primary export, nuclear import, and re-export. Since retrograde tRNA nuclear import is constitutive (Murthi et al., 2010), re-export is likely to be the regulated step.

The mechanisms regulating tRNA nuclear-cytoplasmic distribution remain unclear. There are multiple possible mechanisms by which tRNA subcellular distribution is regulated, including nutrient-regulated tethering of tRNAs or tRNA importins/exportins, as well as participation of possible interacting cofactors of β-importins in response to nutrient conditions. Previous studies have shown that the subcellular distributions of the nuclear-cytoplasmic shuttling proteins, Los1 and Msn5, are affected by carbon source and stress conditions (Ghavidel et al., 2007; Karkusiewicz et al., 2011; Pierce et al., 2013). In cells grown with a fermentable sugar as the carbon source, Los1 is located primarily in the nucleus where it is able to bind tRNAs in a RanGTP dependent manner. In contrast, in cells grown in a non-fermentable carbon source, under glucose deprivation, or when the cells are exposed to DNA damaging agents, Los1 is primarily cytoplasmic.
separated from the tRNAs awaiting nuclear export (Ghavidel et al., 2007; Karkusiewicz et al., 2011; Pierce et al., 2014). The subcellular distribution of Msn5 is also affected by stress. Msn5 is concentrated in nuclei of unstressed cells, but it is located in the cytoplasm upon exposure to ethanol, heat, starvation, or severe oxidative stress (Quan et al., 2006; Quan et al., 2007; Pierce et al., 2014).

As Los1 and Msn5 bind RanGTP to form nuclear export complexes and they have distinct binding affinities for RanGTP, alteration of the nuclear-cytoplasmic RanGTP gradient could affect the subcellular distribution of Los1 and Msn5. Binding of Los1 to RanGTP has been undetectable in the absence of its cargo in vitro (Hellmuth et al., 1998; Hahn & Schlenstedt, 2011), while the dissociation constant of the Msn5/RanGTP complex is 52 nM, which is considered to be in the category of the medium binding affinity for importin-β family members (Hahn & Schlenstedt, 2011).

Here we investigated the regulation of endogenous levels of Los1 and Msn5 under nutrient deprivation conditions known to affect tRNA nuclear-cytoplasmic distribution. We provide evidence that response to aa availability is different from the response to glucose availability. Response to glucose availability is likely regulated by the subcellular distributions of tRNA exportins. Both Los1 and Msn5 are primarily nuclear under nutrient replete conditions. Upon aa deprivation Los1 and Msn5 maintain their primarily nuclear distribution. However, upon glucose deprivation Los1 and Msn5 are located primarily in the cytoplasm. The altered subcellular distribution of tRNA exportins between the nucleus and the cytoplasm is likely responsible for tRNA nuclear accumulation upon glucose deprivation. Investigation of the subcellular distribution of
other importin-β family members upon glucose deprivation indicates that the response to glucose availability appears to be a general phenomenon. This response is likely coordinated with RanGTP gradient and RanGTP binding affinities of the β-importins.

5.3 Materials and Methods

Yeast Strains and Plasmids

Most experiments employed yeast strains BY4741 (*MATα his3Δ leu2Δ met15Δ ura3Δ*). BY4741 is the parent for the collection of yeast strains that endogenously express GFP-tagged proteins [(Huh et al., 2003); Invitrogen]. The strain containing the *rnal-1* mutation is derived from BY4741 (Li et al., 2011). Yeast strains were maintained in synthetic defined media (SC) lacking the appropriate nutritional ingredients for selection. Genomic GFP-tagged Msn5-GFP, Los1-GFP or Kap95-GFP ts strains containing the *rnal1-1* mutation were generated by introducing DNA fragments, which contain homologous sequences with GFP sequences and *HIS3* selection markers (Longtine et al., 1998), to *rnal1-1*.

Plasmid Nup49-mCherry was constructed as described (Lai et al., 2009). The SacI/NaeI fragment of plasmid Nup49-mCherry was subcloned into plasmid pRS426 to construct plasmid pIVY48. Plasmid with galactose-inducible Gsp1-G21V and Gsp1-T26N were constructed by site-directed mutagenesis as described (Kornbluth et al., 1994).
Northern analysis

Small RNAs were isolated and separated by electrophoresis and transferred onto a Hybond N+ membrane (Amersham) as described (Murthi et al., 2010). tRNAs were detected with a DIG-labeled probe complementary to tRNA\textsuperscript{Ile} as described (Wu et al., 2013). The relative levels of precursor and mature tRNAs were determined using ImageJ (http://rsb.info.nih.gov/ij/).

In Situ Hybridization

Fluorescence \textit{in situ} hybridization (FISH) was performed as previously described (Sarkar & Hopper, 1998) with the modifications detailed in (Stanford et al., 2004).

Western Analysis

GFP fusion protein expression was assessed using chemiluminescence-based Western blot analysis following standard protocols as described (Chu & Hopper, 2013). The membrane was probed with primary antibodies: 1:1000 dilution anti-GFP (clone CBP-KK1, Roche) and anti-Rna1 at a 1:10,000 dilution (Hopper et al., 1990). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (GE Healthcare, UK) at a 1:3000 dilution. Protein signals were quantified using ImageJ (http://rsbweb.nih.gov/ij/).
Immunofluorescence

Immunofluorescence was conducted as previously described (Tolerico et al., 1999). The location of multi-copy Msn5-Myc and Los1-Myc was determined by employing mouse monoclonal anti-Myc (12CA5, Roche) at a 1:100 dilution. FITC-conjugated goat anti-mouse IgG (Jackson ImunoResearch Labs) was used at a 1:400 dilution to locate the primary antibody. Cells were counterstained with DAPI (0.1 µg/ml) to locate DNA.

Microfluidics

To study the intracellular dynamics of GFP-tagged proteins in wt and ts strains, microfluidics system (Cell ASIC) was employed. The temperature was conducted by microfluidic control system (Cell ASIC). Changes in the media and incubation times were programmed using the ONIX™ FG Software (Cell ASIC), according to the manufacturer’s protocol.

Microscopy and imaging

To view live cells by confocal microscopy, cells were placed on a slide containing a thin layer of appropriate medium with 20% gelatin and 0.1 mM n-propylgallate as previously described (Wu et al., 2006). Alternatively, cells were analyzed using the microfluidics system. Monitoring of live cells was performed using a Nikon microscope equipped with a spinning disk confocal apparatus (UltraView, PerkinElmer Life and Analytical Science) and a cooled charged coupled device camera (ORCA-AG, Hamamatsu). Cells were visualized using 488 nm (green) and 568 nm (red) argon ion
lasers and a 100x/1.4 NA objective lens. Maximum intensity projections of images were created using UltraView ERS software and image analyses of single optical 0.4µm optical sections were performed using ImageJ (http://rsb.info.nih.gov/ij/). Epifluorescence imaging was accomplished using a Nikon Microscope Eclipse 90i equipped with a CoolSNAP HQ2 CCD camera (Phometrics) and Nis-Elements software (3.1). Adobe Photoshop was used for image assembly.

**Protein purification**

Yeast cultures were grown at 30 °C to OD$_{600}$ of 0.4-0.6. Harvested cells are rapidly frozen in liquid nitrogen and broken open in the solid phase by milling. Cryolysis is performed using a planetary ball mill. Co-IP assays were performed as described (Alber et al., 2007) with the optimizations. In brief, 0.5 g of frozen ground cells were resuspended in 4.5 ml of the extraction buffer (20 mM Hepes, pH 6.1, 110 mM KOAc, 2 mM MgCl$_2$, 150 mM NaCl, 0.5 % Triton, 0.1% Tween-20, 1 mM DTT) with 1: 100 dilutions of protease inhibitor cocktail set IV (Calbiochem). The suspension was isolated by centrifugation at 3000 xg, 4 °C for 10 min. The soluble extract was incubated with IgG-conjugated magnetic beads at 4 °C for 30 min. The magnetic beads were then collected with a magnet, washed six times with 1ml of the ice-cold extraction buffer and once with 0.1 M NH$_4$OAc, 0.1mM MgCl$_2$, 0.02% Tween-20. Protein was eluted with 0.5 M NH$_4$OH, 0.5 mM EDTA by incubation for 20 min at room temperature. The eluates were lyophilized in a SpeedVac (Thermo Savant) overnight. The pellets were resuspended in NuPAGE LDS sample buffer (Life technologies), separated on a 4-12 %
NuPAGE Novex Bis-Tris precast gel (Life technologies) according to the manufacturer’s specifications.

Mass spectrometry analysis

Protein bands within gels were excised and the samples were sent to the Ohio State University Campus Chemical Instrument Center to perform Mass spectrometry analysis.

5.4 Results

5.4.1 tRNA re-export is regulated by nutrient availability

As retrograde tRNA nuclear import is constitutive (Murthi et al., 2010), we previously propose that the tRNA re-export step is regulated by nutrient availability. To investigate this proposal, here we performed Northern analyses to examine tRNA species in cells that are fed or deprived of aa or glucose. Since the yeast splicing machinery is located on the cytoplasmic surface of mitochondria (Yoshihisa et al., 2003; Yoshihisa et al., 2007), defects in primary nuclear export result in the accumulation of end-processed intron-containing pre-tRNAs. Thus, if the response to nutrient availability modulates the primary tRNA nuclear export step, end-processed intron-containing pre-tRNAs should accumulate upon nutrient deprivation, whereas if there are defects in the re-export step, spliced tRNAs should accumulate. RNAs were isolated from fed and nutrient deprived cells. Consistent with previous studies, under nutrient replete conditions cells deleted for LOS1 accumulated elevated levels of unspliced tRNA whereas wild type (wt) cells and cells deleted for MSN5 did not (Fig. 5.1, ratio of I/P). Wt cells deprived for aa or glucose

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rapidly accumulate tRNA in the nucleus (Whitney et al., 2007). Northern analysis of tRNA in wt cells deprived of aa or glucose for 30 min or 2 hr showed that the levels of primary and end-processed intron-containing pre-tRNAs decreased compared to fed conditions (Fig. 5.1, ratio of (P+I)/M); this decrease likely results from decreased transcription (Upadhya et al., 2002; Ciesla et al., 2007). Unlike los1Δ cells which are defective in primary tRNA export, the ratio of end-processed intron-containing pre-tRNAs to the primary transcript did not increase when wt or msn5Δ cells were deprived of nutrients (Fig. 5.1, ratio of I/P). The data provide further support of the proposal that tRNA nuclear re-export rather than primary export is regulated by nutrient availability.

5.4.2 Subcellular distributions of Msn5 and Los1 are not altered upon aa deprivation

The mechanisms regulating tRNA re-export in response to nutrient availability remain unclear [Review: (Hopper, 2013)]. Possible mechanisms include alteration of the tRNA exportins or their interacting proteins. Thus, we investigated whether the tRNA exportins are affected by nutrient status. We examined the steady state protein levels and subcellular distributions of Los1 and Msn5 under nutrient replete and depletion conditions. Endogenously tagged Msn5 and Los1 were generated by Msn5-GFP and Los1-GFP gene replacements (Huh et al., 2003). To test whether GFP-tagged tRNA exportins are functional, we performed FISH. The studies show that cells with Msn5-GFP or Los1-GFP gene replacements evidence a distribution of tRNAs between the nucleus and the cytoplasm that is indistinguishable from the distribution in wt cells and unlike
cells that possess mutations of either LOS1 or MSN5 (Fig. 5.2). Thus, GFP-tagged versions of these proteins maintain their function in tRNA nuclear export.

We determined the steady state expression levels of the functional tagged tRNA exportins in nutrient replete and depletion conditions by western blotting, as normalized to the control protein, Rna1. Cultures were deprived of all aa for various times (15, 30, 60, or 120 min). Comparing the normalized protein levels from cells that were aa deprived to the levels under fed conditions (ie, Msn5/Rna1 under experimental conditions to Msn5/Rna1 under fed conditions), Msn5-GFP levels were unaltered throughout the time course (Fig. 5.3 A, top, lanes 1-5, and 5.3 B). In contrast, Los1-GFP levels decreased ensuing 1 hr of aa deprivation, reaching ~46% of fed levels by 2 hr of aa deprivation (Fig. 5.3 A, bottom, lanes 1-5, and 5.3 B). Thus, aa deprivation does not markedly affect Msn5-GFP steady state levels, but does affect Los1-GFP levels after extended periods of aa deprivation.

To determine whether nutrient availability influences the steady state subcellular distributions of Msn5 and Los1, we employed confocal imaging of live cells harboring endogenous Msn5-GFP and Los1-GFP and exogenously expressed nuclear pore protein, Nup49-mCherry, to demarcate the nuclear boundary. Msn5-GFP was predominantly nuclear under fed conditions. When wt cells were deprived of aa for 0 to 2 hr, Msn5-GFP maintained this predominately nuclear location (Fig. 5.3 C, compare panels 1-3). To obtain a graphic display of the subcellular distribution of Msn5 in the different conditions, we generated pixel intensity profiles of single confocal section of individual cells (Fig. 5.3 D). Because Msn5-GFP is not observed in the vacuole (Fig. 5.3 D, white arrow), the
plot profiles avoid the area of vacuole (Fig. 5.3 C, white arrow and 5.3 D). Figure 5.3 C depicts the images of single confocal section upon nutrient deprivation for 15 or 60 min, whereas figure 5.3 D provides intensity plot profiles for all time points.

The subcellular distribution of Los1 was also insensitive to aa availability as Los1-GFP localized to the nuclear periphery and the nucleoplasm under fed conditions and throughout the time course, from 0 to 2 hr, of aa deprivation (Fig. 5.3 E and F). Thus, the subcellular distributions of Msn5 and Los1 are unaltered upon acute aa deprivation.

Since tRNA nuclear accumulation occurs within 10 min of aa removal (Whitney et al., 2007) and at this time point the expression levels and subcellular distributions of both Msn5 and Los1 are unchanged, we investigated whether these proteins are post-translationally modified under nutrient replete conditions or upon aa deprivation. To identify modifications, we purified the tagged versions of Msn5 and Los1 from cells grown under fed or 1 hr aa deprived conditions and then analyzing the resulting protein modifications by mass spectrometry. We obtained only partial protein sequence coverage (~70% coverage for Msn5 and ~60% coverage for Los1); none of the peptides had detectable levels of phosphate or ubiquitin modifications (Table 5.1). Although acetylated or methylated peptides were found, the relative levels of these peptides from fed and aa starved conditions were unaltered for Msn5 and Los1 (Table 5.1). Thus, neither steady state protein levels, nor subcellular distributions, nor assessable protein modifications of Los1 or Msn5 appears to account for tRNA nuclear accumulation upon acute aa deprivation. Below, we discuss other possible mechanisms.
5.4.3 Steady state subcellular distributions of Msn5 and Los1 are altered upon glucose deprivation

To investigate whether Msn5 and Los1 are regulated upon glucose deprivation, we examined their steady state levels upon glucose deprivation. Comparing normalized protein levels from cells that were glucose deprived to the levels under fed conditions, Msn5-GFP maintained at least 72% of protein levels (Fig. 5.3 A, top, lanes 1, 5-9 and 5.3 B, left panel, lanes 1, 5-9). Los1-GFP levels were maintained at ≥80% upon glucose deprivation (Fig. 5.3 A, bottom, lanes 1, 5-9 and 5.3 B, right panel, lanes 1, 5-9). Thus, glucose deprivation does not dramatically alter the steady state levels of Msn5 or Los1. Consistent with the notion that the assessable modifications of Msn5 and Los1 are unaltered upon aa deprivation, the relative levels of assessable modified peptides from fed and glucose deprived conditions were unchanged for both purified Msn5 and Los1 (Table 5.1).

In contrast to the unaltered nuclear-cytoplasmic distribution of Msn5-GFP and Los1-GFP upon aa deprivation, both proteins are redistributed upon glucose deprivation. Cells were deprived of glucose for various times (15, 30, 60, or 120 min). Msn5-GFP changed from primarily nucleoplasmic to cytoplasmic upon 15 min of glucose deprivation and remained cytoplasmic throughout the time course (Fig. 5.3 C, compare panels 1, 4, 5). Consistent with the visual images, the profiles showed that under fed conditions Msn5-GFP (green) was distributed between two Nup49-mCherry peaks (red) (Fig. 5.3 D). By 15 min of glucose deprivation, Msn5-GFP signal redistributed to primarily cytoplasmic (Fig. 5.3 D).
Under fed conditions, Los1-GFP localized to the nuclear periphery and the nucleoplasm. However, when cells were deprived of glucose for as short as 15 min, part of the Los1-GFP pool was localized to the cytoplasm whereas a portion of the pool remained at the nuclear periphery (Fig. 5.3 E, compare panels 1, 4, 5). As examined by pixel intensity profile, Los1-GFP levels increased in the cytoplasm and decreased in the nucleoplasm throughout the time course of glucose deprivation (Fig. 5.3 F). Although part of Los1-GFP pool remained at the nuclear periphery, we were unable to distinguish by confocal imaging between the outer or inner nuclear periphery (Fig. 5.3 F). Although the subcellular distribution of Los1-GFP was altered by glucose availability, the change was somewhat more subtle than that evidenced for Msn5-GFP. Thus, the subcellular distribution of these two endogenously expressed β-importins between the nucleus and the cytoplasm changes upon glucose deprivation.

As the previously published localization studies of Msn5 and Los1 were performed employing fixed cells and in some cases, high-copy protein expression levels (Quan et al., 2006; Ghavidel et al., 2007; Quan et al., 2007; Karkusiewicz et al., 2011; Pierce et al., 2013), we employed indirect immunofluorescence (IF) to compare the subcellular distribution of Myc-tagged multi-copy functional Msn5-Myc and Los1-Myc by IF to endogenous Msn5-GFP and Los1-GFP. Consistent with the results from live cell imaging of endogenously expressed proteins, multi-copy Msn5-Myc analyzed by IF was located primarily to the nucleus under fed and aa deprivation conditions (Fig. 5.4). However, under fed and aa deprivation conditions multi-copy Los1-Myc was primarily nucleoplasmic by IF, but at endogenous levels it primarily localized at the nuclear rim;
perhaps the nucleoplasmic pool for Los1-Myc results from its over-expression. Consistent with live cell imaging, after 1 hr of glucose deprivation, both Msn5-Myc and Los1-Myc became primarily cytoplasmic (Fig. 5.4). We note that the cytoplasmic pool of multi-copy Los1-Myc upon glucose deprivation examined by IF was higher than for endogenously expressed Los1-GFP, also likely due to its elevated expression (Fig. 5.4). Taken together, the data show that the steady state distributions of Msn5 and Los1 between the nucleus and cytoplasm are altered upon glucose deprivation, but not upon aa deprivation.

5.4.4 Altered subcellular distribution of tRNA exportins upon glucose deprivation is rapid and reversible

Nuclear accumulation of tRNA in response to acute glucose starvation is rapid and reversible (Whitney et al., 2007). One way this could occur is via the redistribution of the steady state pools of tRNA exportins, Los1 and Msn5, from the nucleus to the cytoplasm upon removal of glucose. If tRNA nuclear accumulation is, in fact, due to the altered subcellular distribution of the exportins, then one would expect that the subcellular dynamics of the exportins should be fast, preceding tRNA movement defects, and reversible. To test this prediction, we conducted live cell imaging using microfluidics. Microfluidics permitted appropriate aeration and immediate media exchange, thus allowing of tracking live cells in various growth conditions. We first located Msn5-GFP under fed condition, and then followed the response of Msn5-GFP upon glucose deprivation in the same microfluidic plate. Under fed conditions, the steady state
distribution of Msn5-GFP was predominantly nuclear, as expected (Fig. 5.5 A). The steady state distribution of Msn5-GFP became more cytoplasmic after 5 min of glucose removal (Fig. 5.5 A). To test reversibility, we reintroduced glucose to cells that had been deprived of glucose for 10 min. Within 10 min of refeeding the majority of the Msn5 pool was again nuclear (Fig. 5.5 A). Similarly, part of the Los1-GFP pool moved from the nuclear periphery to the cytoplasm after 5 min of glucose removal (Fig. 5.5 B). The subcellular dynamics of Los1-GFP was also reversible, even though the redistribution of Los1-GFP was more subtle than that of Msn5-GFP (Fig. 5.5 B). Thus, redistribution of tRNA exportins in response to acute glucose starvation is rapid, reversible, and their subcellular dynamics is consistent with the hypothesis that their redistributions may account for altered tRNA location upon glucose deprivation.

5.4.5 Steady state subcellular distributions of other importin-β family members are altered upon glucose deprivation

To learn whether redistribution of Los1 and Msn5 upon glucose deprivation is a general phenomenon that also occurs for other members of importin-β family, we expanded our analyses to other family members. We studied one additional exportin, Crm1, and two importins, Mtr10 and Kap95.

To investigate whether these family members were modulated by nutrient availability, endogenously GFP-tagged constructs were generated by gene replacement (Huh et al., 2003). Since CRMI and KAP95 are essential genes, the fact that the yeast strains with endogenously tagged Crm1-GFP and Kap95-GFP are viable and grow
similarly to wt cells provides evidence that these tagged versions are active. \textit{MTR10} is not essential but \textit{mtr10Δ} cells exhibit altered cellular morphology (Murthi et al., 2010). Because cells harboring the Mtr10-GFP replacement possess normal cellular morphology (data not shown), this tagged version of Mtr10 is also functional.

We first determined the levels of each of these proteins by western blotting, comparing the level of the \(β\)-importins to Rna1. Cultures were harvested at four time points (15, 30, 60, and 120 min) after aa or glucose deprivation. The steady state levels of all these tagged \(β\)-importin family members appeared to be relatively constant (Fig. 5.6 A).

Crm1 functions in the nuclear export of proteins, RNA, and ribosome subunits (Maurer et al., 2001; Johnson et al., 2002; Verheggen & Bertrand, 2012). To address whether nutrient deprivation regulates the subcellular distribution of Crm1-GFP, we observed the location of Crm1-GFP in cells deprived of aa or glucose for 0, 15 and 60 min. As determined by confocal imaging, Crm1-GFP was predominantly nuclear under nutrient replete conditions. When cells were deprived of aa for 15 or 60 min, Crm1 retained its primarily nuclear location (Fig. 5.6 B). However, upon glucose deprivation for 15 min, part of the Crm1-GFP signal was located in the cytoplasm; after longer periods of glucose deprivation Crm1-GFP pool gradually became less nucleoplasmic and more cytoplasmic, although some protein was retained at the nuclear periphery (Fig. 5.6 B). Assessment of Crm1-GFP distribution by intensity plot profiles confirmed the visual assessment (Fig. 5.6 C). Thus, similar to Msn5 and Los1, glucose availability, but not aa availability, affects the steady state subcellular distribution of Crm1-GFP.
Mtr10 mediates nuclear import of the mRNA binding protein Npl3 (Kadowaki et al., 1994; Pemberton et al., 1997; Senger et al., 1998). Also, cells bearing a deletion of MTR10 fail to accumulate the RNA subunit of telomerase or tRNA nuclear pools upon aa deprivation, presumably because Mtr10 is required, directly or indirectly, for their import from the cytoplasm to the nucleus (Ferrezuelo et al., 2002; Shaheen & Hopper, 2005; Murthi et al., 2010). Mtr10 redistributes from the cytoplasm to the nuclear periphery upon DNA replication stress (Tkach et al., 2012). Examination of Mtr10-GFP subcellular distribution showed that the majority of the endogenous Mtr10-GFP pool was evenly distributed in the cytoplasm of cells that received all nutrients or were deprived of aa for 15 or 60 min (Fig. 5.6 B). In contrast, in cells deprived of glucose for 15 min Mtr10-GFP was primarily located at the nuclear rim and in the nucleoplasm with residual cytoplasmic pools (Fig. 5.6 B); these distributions were supported by intensity plot profiles (Fig. 5.6 C).

The well characterized Kap95 functions in nuclear import of NLS-containing proteins (Enenkel et al., 1995; Gilchrist & Rexach, 2003). Consistent with earlier studies, we found endogenous Kap95-GFP to be primarily cytoplasmic with some nuclear rim signal under nutrient replete conditions. Upon 15 or 60 min deprivation of aa, Kap95 maintained its steady state cytoplasmic pools (Fig. 5.6 B). However, upon 15 min glucose deprivation the Kap95-GFP pool was concentrated at the nuclear rim, colocalizing with the nuclear pore protein, Nup49 (Fig. 5.6 B). The cytoplasmic Kap95-GFP pool dramatically decreased upon prolonged glucose deprivation compared to fed and aa deprivation conditions (Fig. 5.6 B). Intensity plot profiles of confocal imaging supported
the visual results (Fig. 5.6 C). Thus, like Msn5 and Los1, all tested members of importin-β family, both importins and exportins, invert their steady state nuclear-cytoplasmic distributions upon glucose but not upon aa deprivation.

5.4.6 Collapse of the RanGTP gradient affects the steady state subcellular distributions of importin-β family members

Assembly and disassembly of importin-β-cargo transport complexes are regulated by the small GTPase, Ran (Strom & Weis, 2001; Fried & Kutay, 2003; Madrid & Weis, 2006). Ran exists primarily in a GTP-bound form in the nucleus and in a GDP-bound form in the cytoplasm. This asymmetric distribution results from the distinct subcellular localizations of the Ran cycle regulators, the nuclear guanine nucleotide exchange factor (RanGEF, RCC1 in vertebrates, Prp20 in yeast) and the cytoplasmic GTPase activating protein (RanGAP in vertebrates, Rna1 in yeast) (Ohtsubo et al., 1989; Hopper et al., 1990; Bischoff & Ponstingl, 1991; Bischoff et al., 1994). Under normal growth conditions Ran is predominantly nuclear. However, when cells are exposed to starvation, heat, ethanol or hydrogen peroxide, Ran equilibrates between nucleus and cytoplasm (Stochaj et al., 2000). As the subcellular distributions of all tested β-importins were altered in response to loss of glucose, we tested the possibility that this redistribution is coordinated with alterations of the RanGTP gradient.

Yeast contains two paralogues of the mammalian Ran, encoded by the GSP1 and GSP2 genes. We examined the subcellular distribution of endogenously tagged Gsp2-GFP under fed and nutrient deprivation conditions. As determined by confocal imaging,
Gsp2-GFP was predominantly nuclear when cells were in nutrient replete conditions. In cells deprived of aa for 15 or 60 min, Gsp2-GFP retained its primarily nuclear location (Fig. 5.7 A). However, upon glucose deprivation for 15 or 60 min, the steady state Gsp2-GFP pool was primarily cytoplasmic (Fig. 5.7 A).

Removal of glucose from cells results in a rapid decrease in ATP level and an immediate increase in AMP/ATP ratio (Wilson et al., 1996; Ashe et al., 2000). Levels of GTP are intrinsically linked to ATP levels, because GDP is converted to GTP by nucleoside diphosphate kinase, which uses ATP as the phosphate donor (Parks & Agarwal, 1973; Kikkawa et al., 1990). Shortage of GTP-bound Ran upon ATP depletion results in inhibition of Ran-dependent nuclear transport (Schwoebel et al., 2002). Thus, glucose depletion may cause a decrease in GTP level and then result in shortage of RanGTP which in turn might affect nuclear-cytoplasmic shuttling of β-importins. To test whether collapse of the RanGTP gradient per se causes redistributions of β-importins, we employed yeast strains with an inducible Ran locked in the GTP- or GDP-bound state. Gsp1 with G21V mutation would bind to, but not hydrolyze GTP, thus stabilizing the GTP-bound form of Ran. The T24N mutation of Gsp1 is defective in GTP binding, thus it remains predominantly in the GDP-bound form (Kornbluth et al., 1994). Because expression of Gsp1-G21V and Gsp1-T24N in yeast cells causes dominant lethality (Schlenstedt et al., 1995), we constructed galactose-inducible Gsp1-G21V or Gsp1-T24N to inhibit their expressions until we intended to disrupt the RanGTP gradient. These plasmids encoding Ran mutant proteins were introduced into yeast strains harboring endogenous Msn5-GFP and Kap95-GFP.
As assessed by confocal live cell imaging, without galactose induction of the RanGTP or RanGDP locked mutant proteins, Msn5-GFP was predominantly nuclear, as expected (Fig. 5.7 B). In contrast, after 2 hr galactose (Gal) induction, Msn5-GFP was primarily cytoplasmic either with inducible Ran locked in the GTP or in the GDP-bound form (Fig. 5.7 B). Assessment of Kap95-GFP subcellular distribution showed that Kap95 was predominantly cytoplasmic and part of signal associated at the nuclear rim prior to galactose induction. However, after 2 hr induction of Ran mutant proteins, the Kap95-GFP pool remarkably increased to the nuclear periphery and the cytoplasmic pool decreased in cells with RanGTP locked form, whereas Kap95 was concentrated at the nuclear rim in cells with RanGDP locked form (Fig. 5.7 B). Thus, the distributions of both an exportin and an importin between the nucleus and the cytoplasm were inverted in cells with either RanGTP or RanGDP locked form. The results show that defects in RanGTP gradient either by introducing RanGTP or RanGDP locked forms affect the subcellular distributions of β-importins.

In the above experiments we observed nuclear-cytoplasmic redistributions of the β-importins after 2 hr, but not within 15 min, the time at which cells respond to glucose deprivation. The slowed kinetics is likely due to the facts that by necessity cells contain endogenous wt Ran and that production of the mutant proteins requires galactose induction. To address this issue, we altered RanGAP employing the rna1-1 strain that encodes a temperature-sensitive (ts) RanGAP (Hopper et al., 1990). Rna1 is necessary for GTP hydrolysis of RanGTP to RanGDP in the cytoplasm (Hopper et al., 1990; Becker et al., 1995; Bischoff et al., 1995; Corbett et al., 1995). Endogenously expressed GFP-
tagged β-importins, including Msn5, Los1, and Kap95, were generated in the rna1-1 strain by homologous recombination.

In both wt and rna1-1 cells Msn5 was predominantly nuclear at the permissive temperature (23°C); however, there was a cytoplasmic pool in rna1-1 cells, perhaps due to a defect even at 23°C (Fig. 5.8 A). After 15 min at non-permissive temperature (37°C), in wt cells Msn5-GFP remained primarily nuclear, although some cytoplasmic signal could be detected. In stark contrast, after 15 min at 37°C, Msn5-GFP in rna1-1 cells was primarily cytoplasmic (Fig. 5.8 A). Intensity plot profiles confirmed that there was much less nuclear signal and more cytoplasmic signal in rna1-1 cells at 37°C than wt cells (Fig. 5.9 A).

Los1-GFP in both wt and rna1-1 cells was located at the nuclear periphery at 23°C. After 15 min at 37°C, Los1-GFP in wt cells retained its primarily nuclear periphery distribution, although some cytoplasmic signal was detected (Fig. 5.8 B). However, in rna1-1 cells within 15 min at 37°C Los1-GFP was primarily cytoplasmic with significantly decreased nuclear rim signal (Fig. 5.8 B), supported by intensity plot profiles (Fig. 5.9 B).

In both wt and rna1-1 strains at 23°C, Kap95-GFP was predominantly cytoplasmic and part of signal was associated with the nuclear rim (Fig. 5.8 C). Upon shifting wt cells to 37°C for 15 or 60 min, Kap95-GFP remained predominantly cytoplasmic. In contrast, the Kap95-GFP signal remarkably increased at the nuclear periphery and decreased in the
cytoplasm in *rna1-1* cells after 15 min at 37°C (Fig. 5.8 C), confirmed by intensity plot profiles (Fig. 5.9 C).

Previous studies have shown that intron-containing tRNAs accumulate when cells possess mutations of the regulation of the Ran pathway (RanGAP-*rna1-1* and RanGEF-*prp20*) (Hopper et al., 1978; Kadowaki et al., 1993) and tRNAs accumulate in the nucleus in *rna1-1* cells (Sarkar & Hopper, 1998). Our studies provide additional insight for these tRNA export defects as tRNAs or pre-tRNAs to be exported and tRNA exportins are primarily in two separate subcellular compartments in *rna1-1* cells at the non-permissive temperature.

Taken together, the collapse of the RanGTP gradient causes remarkably inverse subcellular distributions of β-importins between the nucleus and the cytoplasm. Either increased cellular RanGTP or RanGDP levels results in similar consequence. In *rna1-1* cells at 37°C, nuclear-cytoplasmic distributions of the β-importins altered within 15 min, the time at which cells respond to glucose deprivation. Thus, collapse of the RanGTP gradient results in similar kinetics as glucose deprivation for redistributed β-importins and the data support the notion that redistribution of β-importins upon glucose deprivation is likely due to collapse of the RanGTP gradient.

**5.5 Discussion**

The tRNA retrograde pathway is conserved from yeast to vertebrates and is responsive to nutrient availability. Here, we provide additional evidence that tRNA nuclear re-export is likely the step that is affected in response to nutrient availability. We
also provided, for the first time, confocal live cell imaging of endogenously tagged functional β-importins under conditions known to affect tRNA subcellular distribution. Our studies show that tRNA nuclear re-export is subject to complex regulation as cells utilized different mechanisms in response to glucose vs. aa availability for tRNA subcellular dynamics.

Response to glucose availability regulates the subcellular distributions of Los1 and Msn5 between the nucleus and the cytoplasm. Under glucose replete conditions Los1 and Msn5 are primarily in the nucleus where they are able to interact with tRNAs and export them to the cytoplasm. When cells are deprived of glucose, Msn5, with kinetics that are identical to tRNA nuclear accumulation, becomes primarily cytoplasmic and hence unable to access nuclear tRNAs and deliver them to the cytoplasm; upon glucose deprivation, part of Los1 redistributes to the cytoplasm and part of Los1 remains at the nuclear periphery. Los1 functions in both primary tRNA nuclear export and re-export, whereas Msn5 appears to be dedicated to the tRNA nuclear re-export step for tRNAs encoded by intron-containing genes (Murthi et al., 2010). Thus, their overlapping but distinct functions may provide a mode for regulating primary tRNA export and re-export. Upon glucose deprivation, partial maintenance of Los1 at the nuclear periphery appears to sufficiently export the decreased levels of intron-containing pre-tRNAs, perhaps due to decreased transcription, and thereby primary tRNA nuclear export is not defective. As cytoplasmic Msn5 and Los1 are separated from the tRNAs awaiting nuclear export, their redistribution upon glucose removal may provide an explanation for nuclear accumulation of imported cytoplasmic tRNAs, and thus tRNA re-export is affected.
Therefore, our data are consistent with the hypothesis that the subcellular distributions of tRNA exportins regulate tRNA nuclear re-export in response to glucose availability.

All tested β-importins evidence altered distributions between the nucleus and the cytoplasm upon glucose deprivation but not aa deprivation. We investigated the possibility that the redistribution of β-importins upon glucose deprivation is due to the collapse of the RanGTP gradient. Indeed, Ran is predominantly nuclear under fed and aa deprived conditions but primarily cytoplasmic after 15 min of glucose removal (Fig. 5.7 A). Since Ran regulators are asymmetrically distributed, RanGEF in the nucleus and RanGAP in the cytoplasm (Ohtsubo et al., 1989; Hopper et al., 1990; Corbett et al., 1995), under fed conditions Ran is primarily in its GTP-bound state in the nucleus (Fig. 5.10 A), whereas upon glucose deprivation Ran is likely predominantly in its GDP-bound state in the cytoplasm (Fig. 5.10 B). Therefore, response to glucose deprivation appears to cause the collapse of the RanGTP gradient across the nuclear envelop. We hypothesize that altered subcellular distributions of β-importins upon glucose deprivation is due to the collapse of the RanGTP gradient. Consistent with the hypothesis, collapse of the RanGTP gradient causes inverted subcellular distributions of β-importins (Fig. 5.7 B and 5.8).

The individual β-importins demonstrated somewhat different patterns in subcellular redistribution upon glucose deprivation. Kap95 and Msn5 displayed dramatic redistribution, whereas Mtr10, Los1, and Crm1 showed subtle changes in nuclear-cytoplasmic redistribution. How do cells differentially regulate the subcellular distribution of individual β-importins upon glucose deprivation? One possible explanation could relate to the distinct binding affinities of individual β-importins for
RanGTP, as the affinities of β-importins for RanGTP differ significantly (Hahn & Schlenstedt, 2011). In yeast, the RanGTP binding affinity for Msn5 is higher than Los1 and Crm1, in fact that binding of Crm1 and Los1 to RanGTP is undetectable in the absence of their cargo in vitro (Hellmuth et al., 1998; Maurer et al., 2001; Hahn & Schlenstedt, 2011). As Msn5 displays more dramatic redistribution upon glucose removal than Los1 or Crm1 does (Fig. 5.10 C and D), the differential redistributions of importin-β members upon glucose deprivation likely relate to their RanGTP binding affinities. Consistently, Kap95 has strong affinity for RanGTP and its subcellular distribution is dramatically altered upon glucose deprivation (Fig. 5.10 E and F). In contrast, Mtr10 has weaker affinity for RanGTP and it exhibits only a subtle redistribution upon glucose removal (Fig. 5.10 E and F). Therefore, divergent binding affinities of β-importins for RanGTP may provide a model for their individual responses (Fig. 5.10 C-F) in which the differential redistributions of β-importins upon glucose deprivation correlate with their RanGTP binding affinities.

Cells appear to utilize different mechanisms responding to glucose vs. aa availability as the subcellular distributions of Msn5 and Los1 are altered upon glucose but not upon aa deprivation. Since tRNA nuclear accumulation occurs within 10 min of aa removal (Whitney et al., 2007) and at this time point the steady state levels and subcellular distributions of Los1 and Msn5 resemble fed conditions, tRNA nuclear accumulation is unlikely due to a decreased Los1 and Msn5 protein levels, even though we detect decreased Los1 levels after extended times of aa deprivation. Therefore, an additional mechanism(s) should account for the aa-sensitive nuclear accumulation of tRNAs.
As tRNAs can be aminoacylated in the nucleus by the nuclear pool of aminoacyl-tRNA synthetases (aaRS), uncharged tRNA may accumulate in the nucleus when tRNA charging is defective or when cells are deprived for aa (Lund & Dahlberg, 1998; Sarkar et al., 1999; Grosshans et al., 2000; Azad et al., 2001; Feng & Hopper, 2002; Whitney et al., 2007; Murthi et al., 2010). The notion that tRNA charging defects inhibit nuclear export of cognate tRNA is supported by two separate studies. Charging defects in methionyl-tRNA synthetase caused defective nuclear export of cognate tRNA\textsuperscript{Met}, however, nuclear export of non-cognate tRNA\textsuperscript{Ile} and tRNA\textsuperscript{Tyr} were not affected (Sarkar et al., 1999). Similarly, depletion of yeast \textit{THG1}, which is responsible for the addition of G\textsubscript{1} to the 5′ end of tRNA\textsuperscript{His}, results in uncharged tRNA\textsuperscript{His} as G\textsubscript{1} is a critical determinant for tRNA\textsuperscript{His} aminoacylation (Gu et al., 2005). \textit{thg1Δ} cells accumulate tRNA\textsuperscript{His} but not tRNA\textsuperscript{Tyr} in the nucleus (Gu et al., 2005). Defects of two other aaRS, isoleucyl-tRNA synthetase and tyrosyal-tRNA synthetase, were reported to affect nuclear export of both cognate and non-cognate tRNAs; however, the effects of these two mutations upon tRNA charging have not been reported. Therefore, at least in the cases of tRNA\textsuperscript{Met} and tRNA\textsuperscript{His}, nuclear aminoacylation status of tRNA affects nuclear export of cognate tRNAs but not the export of non-cognate tRNAs that have been assessed.

Upon aa deprivation tRNA\textsuperscript{Tyr}, tRNA\textsuperscript{Leu}, tRNA\textsuperscript{Ile}, tRNA\textsuperscript{Met} and tRNA\textsuperscript{His} accumulate in BY4741 cells (Grosshans et al., 2000; Shaheen & Hopper, 2005; Hurto et al., 2007; Whitney et al., 2007; Murthi et al., 2010). Unlike vertebrates, yeasts are able to produce all 20 aa unless they harbor mutations in particular aa biosynthesis pathways. BY4741 cells are auxotrophic for Leu and His. Upon aa deprivation cells are able to produce
prototropic amino acid, such as Tyr, Ile, and Met, and thus tRNA\textsuperscript{Tyr}, tRNA\textsuperscript{Ile}, tRNA\textsuperscript{Met} remain charged (Whitney et al., 2007). However, all these tRNAs accumulate in the nucleus when cells are depleted of all aa, suggesting that, in addition to the charging status of tRNA, a separate mechanism which is sensitive to aa availability should account for the nuclear accumulation of these charged tRNAs.

As nuclear accumulation of tRNA upon acute aa removal is rapid and reversible (Whitney et al., 2007), other possible mechanisms include aa signaling resulting in β-importin modifications, alteration of β-importin interacting proteins, and/or tethering of components in the various subcellular compartments. Inhibition of target of rapamycin (TOR) pathway by rapamycin is thought to mimic starvation and its role in regulating tRNA subcellular trafficking has been investigated. Although rapamycin treatment in mammalian cells does not affect nuclear tRNA export (Chafe et al., 2012), there are contradictory data regarding its affect in yeast (Whitney et al., 2007; Pierce et al., 2010). Further investigations are necessary to determine whether the TOR or other signaling pathways directly control subcellular movement of tRNAs in response to aa availability.

Our work suggests that there are multiple levels of responses to nutrient availability regulating tRNA re-export. Response to glucose availability is likely regulated by the subcellular distributions of tRNA exportins. Mechanisms regulating tRNA re-export in response to aa availability appears to be complex. Nuclear aminoacylation status of tRNA provides one regulatory mechanism of tRNA nuclear re-export. For mes\textsuperscript{I-1} and thg\textsuperscript{1A} mediated defects in tRNA charging, it seems that only cognate tRNA nuclear export is affected. In contrast, all tested tRNAs accumulate in the nucleus upon acute aa
deprivation (Grosshans et al., 2000; Shaheen & Hopper, 2005; Hurto et al., 2007; Whitney et al., 2007; Murthi et al., 2010; Hurto & Hopper, 2011). Therefore, cells appear to be able to sense the difference of the nutrient availability and utilize different mechanisms in response to different stresses. Interestingly, all of the studied β-importins demonstrated inverted subcellular distributions between the nucleus and the cytoplasm upon glucose deprivation, likely to due to the collapse of the RanGTP gradient. The individual redistributions of β-importins appear to be correlated with their RanGTP binding affinities.
Table 5.1. Post-translational modifications of Msn5 and Los1

1. Msn5

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Theoretical m/z</th>
<th>Measured m/z</th>
<th>Mass error (PPM)</th>
<th>Relative Abundance (%)</th>
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<tr>
<td><strong>K\text{430}-Acetylation of Msn5</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>430KFAEIDFQSK\text{439} \text{a}</td>
<td>606.8166 \text{2+}</td>
<td>606.8231 \text{2+}</td>
<td>10.8</td>
<td>96.46 97.72 98.22</td>
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<tr>
<td>430K\text{Acetylation}FAEIDFQSK\text{439} \text{b}</td>
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<td>627.8206 \text{2+}</td>
<td>-2.07</td>
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<th>Measured m/z</th>
<th>Mass error (PPM)</th>
<th>Relative Abundance (%)</th>
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<tr>
<td>997IVID\text{(CAM)}CVGQGNANPNSAK\text{1013} \text{a}</td>
<td>878.9360 \text{2+}</td>
<td>878.9393 \text{2+}</td>
<td>3.8</td>
<td>99.98 99.98 99.95</td>
</tr>
<tr>
<td>997IVID\text{(CAM)}CVGQGNANPNSAK\text{DiMet}\text{hyl1013} \text{b}</td>
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(continued)
2. Los1

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<th>K_{426}-Methylation of Los1</th>
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<th>Measured m/z</th>
<th>Mass error (PPM)</th>
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<td></td>
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<td>415DFLDNFQQIC\textsubscript{(CAM)}\textsuperscript{b}FK\textsubscript{(Me)426}</td>
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<td>2.39</td>
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<table>
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<th>E_{1047} or E_{1049}--- Methylation or K\textsubscript{1052}- Methylation of Los1</th>
<th>Peptide Sequence</th>
<th>Theoretical m/z</th>
<th>Measured m/z</th>
<th>Mass error (PPM)</th>
<th>Relative Abundance (%)</th>
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</thead>
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<td></td>
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<td>1040.9964\textsuperscript{2+}</td>
<td>1041.0012\textsuperscript{2+}</td>
<td>-4.6</td>
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<tr>
<td>or 1035ELFIVSSNPTTN\textsubscript{b} E\textsubscript{(Me)}\textsubscript{a}NEC\textsubscript{(CAM)}\textsubscript{b}\textsuperscript{b}VK\textsubscript{1052}</td>
<td>1048.0042\textsuperscript{2+}</td>
<td>1048.0038\textsuperscript{2+}</td>
<td>-0.38</td>
<td>3 2 2</td>
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<tr>
<td>1035ELFIVSSNPTTNE\textsubscript{b} NEC\textsubscript{(CAM)}\textsubscript{b}\textsuperscript{b}VK\textsubscript{(Me)1052}</td>
<td>1048.0042\textsuperscript{2+}</td>
<td>1048.0038\textsuperscript{2+}</td>
<td>-0.38</td>
<td>5 5 5</td>
<td></td>
</tr>
</tbody>
</table>

- Relative Abundance of unmodified peptide = Peak Area_{(unmodified)} / (Peak Area_{(modified)} + Peak Area_{(unmodified)}) \times 100
- Relative Abundance of modified peptide = Peak Area_{(modified)} / (Peak Area_{(modified)} + Peak Area_{(unmodified)}) \times 100
Figure 5.1. tRNA nuclear re-export is likely regulated in response to nutrient availability. Northern analysis of pre-tRNA\textsubscript{Ile} and mature tRNA\textsubscript{Ile} in wt and mutant cells under fed and nutrient deprived conditions. P, primary tRNA transcript; I, end-processed intron-containing pre-tRNA; M, mature tRNA. Ratio for I/P, end-processed intron-containing pre-tRNA to primary transcript. Ratio for (P+I)/M, both precursor tRNAs to mature tRNA. 5S rRNA serves as the loading control.
Figure 5.2. FISH analysis of the subcellular distribution of tRNA<sup>Tyr</sup> in wt, Msn5-GFP, and Los1-GFP cells. DAPI staining of DNA shows the location of the nucleus. Bar = 5 µm.
Figure 5.3. Redistribution of Msn5 and Los1 from the nucleus to the cytoplasm upon glucose but not aa deprivation. The time points of aa and glucose deprivation are indicated. (A) Western blot analysis showing Msn5-GFP and Los1-GFP expression. Rna1 is the loading control. (B) Quantification of Msn5-GFP and Los1-GFP expression by western blot analysis from three independent experiments. Relative protein levels were obtained by determining the amount of GFP-tagged protein normalized to Rna1 for each condition compared to that in fed condition. The numbers of x-axis in B represent the experimental conditions in A, lanes 1-9. (C) Confocal images of the subcellular distribution of endogenously expressed Msn5-GFP after the indicated times of removal of aa or glucose. (D) Intensity plot profiles of the subcellular distribution of Msn5-GFP. Intensity profiles of single sections are shown for two independent cells for each condition. The cells in C that are scanned and plotted in D are indicated with the same shape arrowheads. Red lines: Nup49-mCherry intensities; green lines: Msn5-GFP intensities. (E) Subcellular distribution of Los1-GFP after the indicated times of removal of aa or glucose. (F) Intensity plot profiles of the subcellular distribution of Los1-GFP. The cells in E that are scanned and plotted in F are indicated with the same shape arrowheads. Red lines: Nup49-mCherry intensities; green lines: Los1-GFP intensities. Bar = 5 μm.
Figure 5.3. Redistribution of Msn5 and Los1 from the nucleus to the cytoplasm upon glucose but not aa deprivation.
Figure 5.4. IF analysis of the subcellular distribution of multi-copy Msn5-Myc and Los1-Myc. Bar = 5 µm.
Figure 5.5. Redistribution of tRNA exportins upon glucose deprivation is rapid and reversible. (A) Confocal imaging of endogenously tagged Msn5-GFP and (B) Los1-GFP after the indicated times of removal or re-introduction of glucose by microfluidics. Bar= 5 µm.
Figure 5.6. Crm1, Mtr10, and Kap95 exhibit inverted subcellular distribution upon glucose but not aa deprivation. (A) Western blot analysis showing Crm1-GFP, Mtr10-GFP, and Kap95-GFP steady state levels under fed conditions and aa or glucose depletion conditions for the indicated times. Rna1 is the loading control. Ratio: relative protein level normalized to Rna1 in each condition compared to that in fed condition (B) Confocal images of the subcellular distribution of endogenously tagged Crm1-GFP, Mtr10-GFP, and Kap95-GFP under fed conditions and aa or glucose depletion conditions for the indicated times. Bar = 5 μm. (C) Intensity plot profiles of the subcellular distribution of β-importin-GFP. The cells in B that are scanned and plotted in C are indicated with the same shape arrowheads. Red lines indicate Nup49-mCherry intensities; green lines indicate β-importin-GFP intensities.
Figure 5.6. Crm1, Mtr10, and Kap95 exhibit inverted subcellular distribution upon glucose but not aa deprivation.
Figure 5.7. Redistribution of β-importins upon glucose deprivation is coordinated with the RanGTP gradient. (A) Microfluidics to study the subcellular dynamics of endogenously tagged Gsp2-GFP under fed and nutrient deprivation conditions for the indicated times. (B) Confocal imaging of Msn5-GFP and Kap95-GFP in cells with galactose-inducible Ran locked in the GTP- or GDP-bound state. “-“, indicates the locations of proteins prior to addition of galactose. “+”, indicates the locations of proteins after addition of galactose for 2 hr. Bar = 5 µm.
Figure 5.7. Redistribution of β-importins upon glucose deprivation is coordinated with the RanGTP gradient.
Figure 5.8. Collapse of the RanGTP gradient affects the distribution of β-importins.

The RanGTP gradient was altered by inactivation of RanGAP in the \textit{rnl1-1} strain. Microfluidics to study the subcellular dynamics of endogenously tagged (A) Msn5-GFP, (B) Los1-GFP, and (C) Kap95-GFP in wt and \textit{rnl1-1} ts mutant cells at permissive (23°C) and non-permissive (37°C) temperatures for the indicated times. Bar = 5 μm.
Figure 5.8. Collapse of the RanGTP gradient affects the distribution of β-importins.
Figure 5.9. Intensity plot profiles of the distribution of β-importins. (A) Msn5-GFP, (B) Los1-GFP, and (C) Kap95-GFP in wt and rna1-1 ts mutant are examined. The cells in Fig. 5.8 were scanned and plotted in Fig. 5.9 and are indicated with the same shape arrowheads. Red lines: Nup49-mCherry intensities; green lines: β-importin-GFP intensities.
Figure 5.10. Working model for the mechanism of the redistributions of \( \beta \)-importins.

(A) RanGTP gradient maintains under fed condition. T, RanGTP; D, RanGDP; N, nucleus; C, cytoplasm. (B) Upon glucose deprivation, redistributed Ran may cause the collapse of the RanGTP gradient. (C) Exportins are primarily nuclear under fed condition. (D) Exportins are redistributed to the cytoplasm upon glucose deprivation. (E) Importins are primarily cytoplasmic under fed condition. (F) Importins are redistributed upon glucose deprivation. Black triangle indicates the RanGTP binding affinity of \( \beta \)-importins from strong (left) to weak (right). Red arrows indicate greater shuttling of \( \beta \)-importins than shuttling of other \( \beta \)-importins in blue arrows. Solid arrows indicate normal shuttling under fed condition, while dotted arrows indicate aberrant shuttling under glucose deprivation.
tRNAs function in protein synthesis. In addition to their essential role, tRNAs function in regulating apoptosis and cell proliferation [(Pavon-Eternod et al., 2009; Pavon-Eternod et al., 2013); review: (Phizicky & Hopper, 2010)]. tRNAs are transcribed by RNA polymerase III which is inhibited by the critical tumor suppressors p53 and RB3 and activated by the proto-oncogene product c-Myc and Ras (Felton-Edkins et al., 2003; White, 2005). tRNAs are highly expressed in tumor cells and over-expression of tRNA\textsubscript{Met} is sufficient to lead to cancer development and progression (Pavon-Eternod et al., 2009; Pavon-Eternod et al., 2013). Furthermore, tRNAs regulate apoptotic sensitivity at the level of cytochrome C-mediated apoptosome formation by binding to cytochrome C (Mei et al., 2010a; Mei et al., 2010b). Therefore, elevated levels of tRNAs not only increase the efficiency of protein synthesis and cell proliferation, but also regulate apoptosis to promote uncontrolled cell growth. As inappropriate tRNA cellular levels are implicated in a number of human diseases [Review: (Phizicky & Hopper, 2010)], it is important to fully understand the mechanisms and the regulation of tRNA processing and subcellular trafficking.

tRNAs are dynamic within cells. In contrast to previous dogma that tRNA subcellular movement is unidirectional- from the nucleus to the cytoplasm, tRNAs move
bidirectionally between the nucleus and the cytoplasm. Much remains to be learned about how tRNAs travel between the nucleus and the cytoplasm. By employing genetic and biochemical approaches, we gained insight into how the known β-importins, Los1, Msn5, and Mtr10, function in the tRNA retrograde pathway. Furthermore, our cell biology studies shed light on how cells regulate tRNA subcellular distribution in response to nutrient availability.

Prior to the studies in this dissertation, the in vivo tRNA transport complexes had not been detected. The difficulty in conducting biochemical analyses of the in vivo complexes was due in part to the transient nature of the complexes and due to the fact that small GTPase Ran must be held in the GTP-bound state and the ester bond between the amino acid and tRNA (aa-tRNA) is subject to alkaline hydrolysis at neutral pH. In this study, we successfully developed a cross-linking based Co-IP strategy to detect in vivo tRNA transport complex (Chapter 4). We provide the first in vivo biochemical evidence for the interactions of Los1 with tRNAs. Both mature and intron-containing tRNAs co-purify with Los1 in a RanGTP-dependent manner, supporting the notion that Los1 functions in both primary export of pre-tRNA and re-export of mature tRNA to the cytoplasm for the tRNAs encoded by intron-containing genes (Fig. 6.1 A and C). The function of Los1 in tRNA nuclear export is conserved between yeast to vertebrates. Consistent with our data, mammalian Exportin-t monitors the tRNA backbone and aminoacyl stem but it does not monitor the anticodon stem (Arts et al., 1998b; Lipowsky et al., 1999). Crystallography structural studies of S. pombe Exportin-t also suggest that Exportin-t measures tRNA folding like a “molecular ruler” in which it recognizes
structures common to all tRNAs (Cook et al., 2009). Exportin-t contacts around the acceptor arm, TψC loop and D loop, but makes no contacts with the anticodon arm where the intron locates [(Cook et al., 2009); review: (Hopper, 2013)]. Conceivably, Los1/Exportin-t functions in primary tRNA nuclear export and re-export step (Fig. 6.1 A and C).

Our genetic data indicate that Mtr10 functions in retrograde tRNA nuclear import (Shaheen & Hopper, 2005; Murthi et al., 2010). However, we fail to detect either RanGTP or RanGDP-dependent bindings of Mtr10 with tRNAs (Chapter 4). If Mtr10 does not directly bind to cytoplasmic tRNAs, Mtr10 may affect tRNA nuclear import by other pathway, such as importing a tRNA adaptor or regulating the cytoplasmic tRNA tether. If Mtr10-adaptor-tRNA complexes exist and are maintained in our procedure, we expect to detect co-purified tRNAs with Mtr10 complexes. However, we fail to detect co-purified tRNAs, even though our biochemical studies employed formaldehyde crosslinking to maintain transport complexes. We cannot eliminate the possibility that Mtr10 binds to tRNAs with an affinity below our detection or the interaction of Mtr10 with tRNAs is unstable. Alternatively, Mtr10 may function indirectly in tRNA nuclear import, such as importing a cytoplasmic tRNA tether or regulating the function of tether (Fig. 6.1 B). Therefore, analysis of co-purified proteins with Mtr10 by mass spectrometry will shed light on whether and how it plays a role in retrograde tRNA nuclear import.

Msn5 appears to connect nutrient status with tRNA re-export by assembling a complex that depends upon aminoacylation of nuclear tRNAs. As in vivo data show that mature tRNAs and the translation factor, eEF1A, co-purify with Msn5 in a RanGTP-
dependent manner, our work implicates eEF1A in tRNA nuclear re-export because we also showed, contrary to dogma, that eEF1A shuttles in/out of the nucleus (Murthi et al., 2010). Thus, we purpose that eEF1A functions in aiding Msn5 specificity to aa-tRNAs in the re-export step. Our data support the notion that Msn5 forms a quaternary complex with eEF1A, aa-tRNA, and RanGTP. However, we were unable to distinguish whether Msn5 interacts with aa-tRNA and then binds to eEF1A or whether Msn5 interacts directly with eEF1A as an adaptor for aa-tRNA (Fig. 6.1 C). This tRNA re-export complex reveals an additional function for translation elongation factor eEF1A in the nucleus. Whether eEF1A is required for Msn5 binding to tRNA could be tested by depletion of eEF1A. Since eEF1A is essential, constructions of conditional mutants will be required for further investigations.

Los1 and Msn5 are the only two known yeast β-importin members shown to function in tRNA nuclear export. However, the los1Δ msn5Δ double mutant is viable. Because it is essential that tRNA be delivered to the cytoplasm for translation, additional tRNA nuclear export pathways should exist in yeast (Fig. 6.1). In addition, many enzymes and the regulatory factors involved in the processing of pre-tRNA, mature tRNA, and intron remain unclear. Genome-wide studies assessing other potential factors involved in tRNA subcellular dynamics and tRNA processing by Northern analyses could shed light on aspects of tRNA primary export, splicing, and intron turnover (Appendix A). To study tRNA subcellular dynamics in each of the ~6200 yeast mutant strains, development of tRNA tracker could not only substitute for the laborious fluorescence in situ hybridization procedures currently used to track tRNA in cells but also allow us to
identify novel proteins involved in the tRNA nuclear export and retrograde import pathway (Appendix B).

In addition, synthetic genetic array (SGA) analysis, which has been widely used to identify function-related genes, offers an efficient approach for the systematic construction of double mutants and enables a global analysis of synthetic genetic interaction. Thus, SGA analysis could be used for genome-wide genetic interactions with $los1\Delta$ $msn5\Delta$ mutant cells. Candidates which are synthetic sick or lethal with $los1\Delta$ $msn5\Delta$ mutants may function in the tRNA re-export process. Alternatively, potential protein (X) that possibly functions in the tRNA export step could be examined by the construction of $los1\Delta$ $msn5\Delta$ $X\Delta$ triple mutants. For example, nuclear protein, Sol1/2, has been implicated in tRNA re-export. In $sol1\Delta$ and $sol2\Delta$ cells tRNAs accumulate in the nucleus and mutants of $SOL$ genes have no defects in pre-tRNA splicing (Shen et al., 1996; Stanford et al., 2004). Whether Sol1 has synthetic negative interaction with $los1\Delta$ $msn5\Delta$ was examined, the data indicate that Sol1 has no synthetic lethal interaction with $los1\Delta$ $msn5\Delta$ and might not directly function in tRNA nuclear export (Appendix C). Thus, further studies will be necessary to discover missing players. If enzymes that contribute to tRNA processing and trafficking in yeast are identified in the near future, it would lead to new questions of whether the functions of those factors are conserved in higher eukaryotes.

Cells utilize multiple mechanisms regulating tRNA subcellular dynamics in response to diverse nutrient deprivation (Chapter 5). We show that tRNA subcellular distribution in response to glucose availability is regulated by the subcellular distributions
of Msn5 and Los1. Response of tRNA subcellular distribution to aa availability is regulated by nuclear tRNA aminoacylation for cognate tRNA. However, neither tRNA charging, nor subcellular distributions of Msn5 or Los1 appears to account for nuclear accumulation of non-cognate tRNA upon aa deprivation. Other possible mechanisms include aa signaling resulting in β-importin modifications, alteration of β-importin interacting proteins, and/or tethering of components in the various subcellular compartments. Although no detectable phosphorylated or ubiquitinated peptides of Los1 and Msn5 have been identified in this study, studies from our colleagues implicate that Los1 and Msn5 might be ubiquitinated (A. Domanska personal communication). Future studies could assess whether levels of ubiquitination of Los1 and Msn5 are altered in response to nutrient availability. In addition, it is known that eEF1A possesses post-translational modifications (Chi et al., 2007; Lin et al., 2010). Since Msn5 interacts with eEF1A in tRNA nuclear re-export, it is possible that the modification status of eEF1A is affected by aa availability, thereby altering the tRNA nuclear export complex. Future investigations could examine the post-translational modification status of eEF1A and the interaction of eEF1A and Msn5 under nutrient replete and depleted conditions.

Bidirectional tRNA traffic likely serves multiple functions in tRNA modification, regulation of translation, and tRNA quality control [(Ohira & Suzuki, 2011; Chu & Hopper, 2013; Kramer & Hopper, 2013); review: (Hopper, 2013)]. Our contribution adds new insights into this conserved tRNA retrograde pathway. In particular, our working model for tRNA subcellular dynamics provides a mechanism in which tRNA exportin has specificity to mature tRNAs and/or pre-tRNAs (Fig. 6.1). It is likely that tRNA exportin
derives its tRNA binding specificity via interaction with other protein factors, such as Tef1/2. This model opens this field to new ideas and more interesting questions to elucidate. It will be interesting to identify the missing players in bidirectional tRNA subcellular dynamics, to determine whether Mtr10 functions in retrograde tRNA nuclear import via a tRNA adaptor protein or cytoplasmic tRNA tether, to delineate how Msn5 forms a cooperative export complex with RanGTP, Tef1 and aa-tRNA, and finally to identify the signaling pathways that affect tRNA subcellular dynamics in response to nutrient availability.
Figure 6.1. The working model of tRNA subcellular dynamics in yeast. (A) Red arrows indicate tRNA primary nuclear export. Red text, β-importins function in primary export. (B) Green arrow indicates retrograde tRNA nuclear import. Green dotted arrow indicates indirect retrograde tRNA import pathway mediated by Mtr10. (C) Purple arrow indicates tRNA nuclear re-export. Purple text, β-importins function in re-export. Blue text, proteins function cooperatively in re-export.
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Appendix A. Genome-wide strategy to discover missing players in tRNA biogenesis

(Contribution to Wu et al., 2013)

In eukaryotes, tRNAs are transcribed in the nucleus and function in the cytoplasm. It is now known that tRNA subcellular movement is not strictly unidirectional from the nucleus to the cytoplasm [(Shaheen and Hopper, 2005; Takano et al., 2005); review: (Hopper, 2013)]. Although many factors involved in tRNAs subcellular dynamics have been investigated, much remains to be learned about tRNA processing and trafficking. Thus, we performed genome-wide analyses to identify novel gene products involved in tRNA biology, such as tRNA transcription regulation, additional tRNA export pathway, regulation of tRNA splicing, and intron degradation.

To investigate the processing pattern of pre-tRNA, mature tRNA, and intron, we employed Northern analyses to analyze small RNAs isolated from the yeast genome-wide deletion collection for non-essential genes and the yeast temperature-sensitive (ts) collection for essential genes (Li et al., 2011; Winzeler et al., 1999). Because the yeast deletion and ts collections are available in the 96-well microtiter plates, we could easily propagate cells for RNA analyses by transferring the collections to 96-deepwell plates. Small RNAs were extracted by phenol extraction. However, when yeasts in the 96-deepwell plates were cultured to stationary phase, only low quantity of primary transcripts and end-processed intron-containing pre-tRNAs were observed (Fig. A.1 A, P
and I). As limited aeration and circulation in 96-deepwell as well as growth phase of yeast cells could be the cause of the low yield of pre-tRNAs, we optimized conditions for culturing yeast cell in 96-deepwell plates to yield RNA of high quality and quantity from small cell cultures at early log phase (OD\textsubscript{600} = ~0.3) from a single well. In addition, due to the high height/width ratio (44.1 mm/ 9 mm) of the deep wells, shaking of the deepwell plate horizontally failed to circulate and aerate cells, therefore causing sedimentation of cells in the well bottom. To overcome this obstacle, plates were placed at a 60-degree angle on a platform shaker incubator at 220 rpm. Moreover, in order to facilitate the air circulation in the cell culture as well as to avoid any contamination, sterile breathable seal films instead of plastic seals were used to cover the plates (Wu et al., 2013). Under optimized growth conditions, the levels of tRNAs isolated from yeast cultured in 96-deepwell plates (candidates) are indistinguishable to the levels of tRNAs extracted from wt and los1\textDelta cells in normal 15 ml larger culture (Fig. A.1 B). Thus, the optimized conditions for small RNA isolation from cells propagated in 96-deepwell plates are appropriate to genome-wide analysis.

Since we developed a procedure for small RNA isolation from cells propagated in 96-deepwell plates, Northern analysis was employed to analyze the tRNA processing patterns in each of the mutant cells. Candidates that evidence higher molar yield of intron-containing pre-tRNAs relative to mature tRNAs are expected to have defects either in the pre-tRNA processing machinery, mitochondria localization of splicing machinery, or pre-tRNA nuclear export; candidates that evidence higher molar yield of primary transcript relative to intron-containing pre-tRNAs are expected to have defects in the
regulation of 5’ or 3’ processing of pre-tRNA (Fig. A.2 and Table A.1). Further analysis will be used to confirm the biological roles of these candidates.
Table A.1. Candidates from genome-wide Northern analysis

<table>
<thead>
<tr>
<th>Gene Ontology term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>mitochondrial membrane</td>
<td></td>
</tr>
</tbody>
</table>

| P-value | 0.02263 |

<table>
<thead>
<tr>
<th>Standard name</th>
<th>Systematic name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEM1</td>
<td>YAL048C</td>
<td>Evolutionarily-conserved tail-anchored outer mitochondrial membrane GTPase which regulates mitochondrial morphology; cells lacking Gem1p contain collapsed, globular, or grape-like mitochondria; not required for pheromone-induced cell death</td>
</tr>
<tr>
<td>PUF3</td>
<td>YLL013C</td>
<td>Protein of the mitochondrial outer surface, links the Arp2/3 complex with the mitochore during anterograde mitochondrial movement; also binds to and promotes degradation of mRNAs for select nuclear-encoded mitochondrial proteins</td>
</tr>
<tr>
<td>YPT7</td>
<td>YML001W</td>
<td>Rab family GTPase; GTP-binding protein of the rab family; required for homotypic fusion event in vacuole inheritance, for endosome-endosome fusion, similar to mammalian Rab7</td>
</tr>
<tr>
<td>CYT1</td>
<td>YOR065W</td>
<td>Cytochrome c1, component of the mitochondrial respiratory chain; expression is regulated by the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex</td>
</tr>
</tbody>
</table>

http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl
Figure A.1. Northern analysis of pre-tRNA$^{\text{lle}}$ and mature tRNA$^{\text{lle}}$. (A) RNAs isolated from cells cultured to stationary phase. (B) RNAs isolated from cells cultured to log phase. P, primary transcript. I, end-processed intron-containing tRNA. M, mature tRNA.
Figure A.2. Northern analysis of pre-tRNA\textsuperscript{Ile} and mature tRNA\textsuperscript{Ile} for tRNA processing candidates. P, primary transcript. I, end-processed intron-containing tRNA. M, mature tRNA.
Appendix B. Development of tRNA tracker

(Unpublished)

In the past decade, there have been many discoveries regarding tRNA nuclear-cytoplasmic dynamics. To further understand the diverse processes of tRNA, it is crucial to perform genome-wide screening to identify the novel proteins participating in tRNA biosynthesis. Thus, I attempted to develop a strategy to track tRNA in cells and to study tRNA subcellular dynamics in each of the ~6200 yeast mutant strains. In addition, tRNA tracker, if successful, could be used to follow tRNA subcellular trafficking in the different nutrition deprived conditions. Thus, the studies could gain insight of the regulation of tRNA movement in response to the availability of amino acid, glucose, and other nutrients.

I designed an in vivo tRNA tracker that could be used to track cellular movement of tRNA in live cells by employing a specific tRNA binding protein, Trbp111, fused to a fluorescent protein, mCherry. Trbp111 encoded by the thermophile, *Aquifex aeolicus*, specifically binds tRNA with high affinity (32nM). I anticipate that it should bind tRNA anywhere in yeast cells if it has access to particular subcellular locations. Thus, I modified Trbp111 so that it would be able to enter and exit the nucleus. To do so, I provided Trbp111 with a nuclear localization sequence (NLS) from the histone H2B protein and a nuclear export sequence (NES) from the yeast Gle1 protein. To learn
whether NLS-NES-Trbp111-mCherry indeed reports the subcellular distribution of tRNA, I introduced a plasmid encoding this protein into wild-type yeast strains and yeast strains that have defects in export of tRNA from the nucleus to the cytoplasm, such as los1Δ and msn5Δ strains. The constructs have a regulated GAL1 promoter to prevent the possibility of a negative effect on cell growth. (Fig. B.1). However, the expression signal in wt cells needed to be improved. We modified tracker by tagging two copies of fluorescence protein, mCherry. Although the signal of this modified Trbp111-2x mCherry increased (Fig. B.2), part of the fusion proteins was cytoplasmic and part of fusion proteins was nuclear in wt cells, while it appeared to have a large nuclear pool in los1Δ cells. As we expected that fusion proteins should be primarily cytoplasmic in wt cells, we attempted to modify tRNA tracker to correctly report the subcellular localization of tRNAs.

Since the tRNA tracker fusion protein may change the native structure of Trbp111, which affects the binding efficiency to tRNA, we sought to reconstruct the tracker by inserting linker sequences between Trbp111 and mCherry to maintain the native structure of Trbp111. The unstructured peptide, AAPAAPAGQEAAAPAPAAQAGPAAAPAA, was used as a linker sequence. However, the nuclear/cytoplasmic signal levels between the wt and los1Δ cells were still not remarkably different (Fig. B.3), suggesting that there was not much improvement by inserting linker sequences. Thus, further optimizations or employment of other tRNA binding proteins or oligos will be required before this in vivo tRNA tracker can be used to report tRNA subcellular dynamics.
Figure B.1. The subcellular distribution of tRNA tracker in wt and mutant cells. (A) Construction scheme of the tRNA tracker. (B) Epifluorescence imaging of the subcellular distribution of galactose-inducible Trbp111-mCherry in BY4742 (wt), los1Δ, and msn5Δ cells.
Figure B.2. The subcellular distribution of modified tRNA tracker in wt and mutant cells. (A) Construction scheme of the modified tRNA tracker. (B) Epifluorescence imaging of the subcellular distribution of galactose-inducible Trbp111-2x mCherry in BY4742 (wt) and los1Δ cells. Location of Pus1-GFP indicates the nucleus.
Figure B.3. The subcellular distribution of optimized tRNA tracker in wt and mutant cells. (A) Construction scheme of the optimized tRNA tracker. (B) Epifluorescence imaging of the subcellular distribution of galactose-inducible Trbp111-linker-2x mCherry in BY4742 (wt) and los1Δ cells.
Appendix C. Examination of the role of Sol1 in tRNA nuclear re-export

Synthetic genetic analysis has been widely used to identify function-related genes by the construction of double or triple mutants. Nuclear protein, Sol1/2, has been implicated in tRNA re-export. In sol1Δ and sol2Δ cells tRNAs accumulate in the nucleus and mutants of SOL genes have no defects in pre-tRNA splicing (Shen et al., 1996; Stanford et al., 2004). To examine whether Sol1 functions in the tRNA re-export step, I generated the yeast strain with los1Δ msn5Δ sol1Δ triple mutant. To maintain strain viability, maintenance plasmid containing LOS1 with URA3 marker was employed. Whether Sol1 has synthetic negative interaction with los1Δ msn5Δ was tested by 5-FOA (5-Fluoroorotic acid) selection. Since yeast enzyme ODCase can convert 5-FOA into the toxic compound, 5-fluorouracil, causing death, cells tend to lose the URA3-marked maintenance plasmid in media containing 5-FOA. Assessment of los1Δ msn5Δ sol1Δ triple mutant showed that this strain can lose the maintenance plasmid harboring LOS1 and can grow on 5-FOA plate, suggesting that los1Δ msn5Δ sol1Δ triple mutant is viable even after losing the maintenance plasmid. Thus, Sol1 has no synthetic lethal interactions with los1Δ msn5Δ and might not directly function in tRNA nuclear export (Fig. C.1).
Figure C.1. **Sol1 has no synthetic lethal interactions with los1Δ msn5Δ.** (A) los1Δ msn5Δ double mutant and (B) los1Δ msn5Δ sol1Δ triple mutant are viable on SC media supplemented with 5-FOA. Plates were incubated at 23 °C for 4 d.