Regulation of nuclear tRNA export in response to nutrient stress is not evolutionarily conserved and requires the TORC1 and PKA signaling pathways in *Saccharomyces cerevisiae*

by

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ABSTRACT

REGULATION OF NUCLEAR tRNA EXPORT IN RESPONSE TO NUTRIENT STRESS IS NOT EVOLUTIONARILY CONSERVED AND REQUIRES THE TORC1 AND PKA SIGNALING PATHWAYS IN SACCHAROMYCES CEREVISIAE

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University of Guelph, 2012                                                   Dr. D. Mangroo
Dr. G. van der Merwe

Saccharomyces cerevisiae are unicellular organisms that are highly adaptable to acute changes in nutrient availability. The two main signaling pathways that allow S. cerevisiae to sense and respond to changes in glucose availability in the environment are the conserved cAMP/PKA and AMPK/Snf1 kinase-dependent pathways. The conserved TORC1 pathway is primarily responsible for allowing cells to respond to the availability of nitrogen. Studies have shown that S. cerevisiae, but not mammalian and plant cells, regulate nuclear tRNA trafficking in response to nutrient stress. Here, we show that the yeast species of the Saccharomyces genus, but not Schizosaccharomyces pombe and Kluyveromyces lactis specifically regulate nuclear tRNA export in response to nutrient stress, providing further evidence that regulation of nuclear tRNA export in response to nutrient availability is not evolutionarily conserved. We also established that amino acid and nitrogen starvation affects nuclear export of a subset of tRNAs in S. cerevisiae. Inhibition of TORC1 signaling by rapamycin treatment, which simulates nitrogen starvation, also affects nuclear export of the same subset of tRNAs, suggesting that the TORC1 signaling pathway plays a role in regulating nuclear export of the tRNAs in response to nitrogen level. Regulation of nuclear export of these tRNAs by nitrogen deprivation is most likely due to
an effect on the function of the nuclear tRNA export receptors, as overexpression of the tRNA export receptor, Los1p, restores export of the tRNAs during nitrogen starvation. These findings suggest that the TORC1 signaling pathway may, in part, regulate nuclear export of the tRNAs by affecting the function of the tRNA export receptors.

In contrast to amino acid and nitrogen starvation, glucose depletion affects nuclear export of all tRNA species in *S. cerevisiae*. Evidence obtained suggests that nuclear retention of tRNA in cells deprived of glucose is due to a block in nuclear re-import of the nuclear tRNA export receptors. Retention of the receptors in the cytoplasm is not caused by activation of Snflp, but by the inactivation of PKA during glucose deprivation. Furthermore, regulation of nuclear re-import of the receptors is not due to phosphorylation of the tRNA export receptors by PKA. However, PKA phosphorylates known components of the tRNA export machinery. A model that is consistent with the data is that PKA and an unknown mechanism regulate the activity of these components or an unidentified protein(s) to control nuclear re-import of the receptors in response to glucose availability.
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<tr>
<td>2YT</td>
<td>2 X yeast tryptone</td>
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<tr>
<td>aaRS</td>
<td>aminoacyl-tRNA synthetase</td>
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<td>tRNA&lt;sub&gt;Tyr&lt;sup&gt;am&lt;/sub&gt;</td>
<td>amber suppressor tyrosyl-tRNA</td>
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<td>cAMP</td>
<td>adenosine 3', 5'-cyclic monophosphate</td>
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<td>GTPase-activating protein</td>
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<td>GluRS</td>
<td>glutamyl-tRNA synthetase</td>
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<td>hemaglutinin</td>
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<td>HEAT</td>
<td>Huntington, eukaryotic elongation factor 3 (eEF3), the regulatory A subunit of protein phosphatase 2A, TOR</td>
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<td>kDa</td>
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<td>NE</td>
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<td>ORF</td>
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<td>PAGE</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PIPES</td>
<td>piperazine-N,N'-bis(ethanesulfonic acid)</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<td>Pre-tRNA</td>
<td>precursor tRNA</td>
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<td>Ran binding protein 1</td>
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<td>RanGAP</td>
<td>Ran GTPase activating protein</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>snoRNA</td>
<td>small nucleolar RNA</td>
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<tr>
<td>TOR</td>
<td>target of rapamycin</td>
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<tr>
<td>TORC</td>
<td>TOR complex</td>
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<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
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<td>transfer RNA</td>
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<td>U Three snoRNA associated protein</td>
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<tr>
<td>YES</td>
<td>yeast extract with supplements</td>
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<tr>
<td>YPD</td>
<td>yeast peptone dextrose</td>
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1.0. Introduction

1.1. Overview

Eukaryotic cells possess an extensive network of regulatory mechanisms that control cell growth and proliferation. This network allows cells to adapt to changing environmental conditions by influencing protein translation and gene expression. Transfer RNA (tRNA) is an important molecule that acts as an adaptor between the mRNA codon and corresponding amino acid. tRNAs are synthesized in the nucleolus and must be exported to the cytoplasm to function in protein synthesis. Studies indicate that nuclear-cytoplasmic tRNA transport may play an important role in regulating the protein translation rate. For instance, eukaryotic cells have been shown to inhibit nuclear tRNA export in response to nutrient stress and DNA damage causing a decrease in protein translation in order to survive under these conditions (Ghavidel et al., 2007). In contrast, cancer cells display an increase in tRNA transcription resulting in a rapid rate of translation and cell proliferation (White, 2004; White, 2005). One strategy that is used to regulate translation through nuclear tRNA transport is to control the function of the proteins involved in the tRNA export process. For example, nuclear tRNA export requires export receptors to transport the tRNA across the nuclear pore complex, and DNA damage and nutrient stress have been shown to alter the localization of the tRNA export receptors making them unavailable for export thereby inhibiting tRNA export (Quan et al., 2007; Ghavidel et al., 2007). The proteins involved in transporting tRNAs from their point of synthesis in the nucleolus to the translation machinery in the cytoplasm are slowly being revealed. However, the regulatory mechanisms and signaling pathways controlling tRNA transport are poorly understood. This introduction will outline our current understanding of the *Saccharomyces cerevisiae* nuclear
tRNA export process and the possible signaling pathways involved in regulating nuclear-cytoplasmic tRNA trafficking.

1.2. The Nuclear Pore Complex

The nucleus is a unique compartment found in eukaryotic cells that allows for compartmentalization of specific cellular components in order to separate and regulate various processes. This compartment is surrounded by a nuclear envelope (NE) composed of two lipid bilayers with the outer membrane being contiguous with the endoplasmic reticulum. The presence of this membrane does not allow the free movement of molecules between the nucleoplasm and cytoplasm. Consequently, the inner and outer membranes join to form pores throughout the NE (Hetzer, 2010). Nuclear pore complexes (NPC) located within these pores create channels in the NE to enable molecules to move into and out of the nucleus in a controlled manner. These evolutionarily conserved large proteineous complexes consist of an 8-fold symmetrical cylinder composed of approximately 30 proteins known as nucleoporins (nups) (Rout et al., 2000; Wente and Rout, 2010). Filaments protrude into the nucleoplasm and are connected together at the distal end to form the nuclear basket. Eight cytoplasmic filaments extend beyond the cytoplasmic face of the NPC protruding into the cytoplasm (Alber et al., 2007). Due to the small number of proteins that make up the large NPC, multiple copies of each protein are present in the eight spokes that transverse the NE. Several nups are symmetrically distributed throughout the NPC while others are only present at specific locations within the complex to possibly allow for directional transport through the NPC (Rout et al., 2000). Proteins containing transmembrane α-helices form the membrane ring that anchors the NPC in the NE while the central cylindrical channel is formed from core structural components that bind to this ring. These core components interact with nups containing unstructured filaments made up of
phenylalanine-glycine (FG) repeats that line the central channel (Aitchison and Rout, 2012). The FG filaments are directed toward the interior of the NPC to create a selective gel-like barrier that enables NPCs to control and regulate movement of macromolecules across the NE (Frey et al., 2006; Frey and Gorlich, 2007; Frey and Gorlich, 2009).

1.3. β-karyopherins

NPCs allow for the diffusion of ions, small molecules and proteins (less than 40 kDa) between the nucleus and cytoplasm; however, molecules larger than 40 kDa and some RNAs below the size exclusion limit require carrier-mediated transport through the NPC. The majority of these carriers are members of the β-karyopherin family (Conti et al., 2006; Cook et al., 2009). Despite low sequence similarity, these proteins have several characteristic motifs known as HEAT repeats formed by two α-helices connected by flexible intervening loop regions. Structural studies indicate that β-karyopherins have a superhelical structure (Conti et al., 2006) and that cargoes bind to the concave surface of the C-terminal arch. Pockets between each HEAT motif interact with the FG repeats lining the central channel allowing translocation of the β-karyopherin through the NPC (Cook and Conti, 2010). β-karyopherins involved in nuclear import are known as importins while those required for nuclear export are called exportins. There are a select few that have the ability to act as both an importin and exportin. These unique proteins can interact directly with the cargo or indirectly in the presence of adaptor protein/molecule. Fourteen β-karyopherins have been identified in yeast while twenty have been discovered in mammalian cells (Macara, 2001; Mosammaparast and Pemberton, 2004). Some β-karyopherins are dedicated to the import or export of a single cargo whereas other β-karyopherins can interact and import or export multiple cargoes. This flexibility in cargo binding of β-karyopherins of the latter category also allows them to compensate for the loss of a β-
karyopherin, since deletion often does not result in growth impairment (Mosammaparast and Pemberton, 2004). Furthermore, studies suggest that β-karyopherins interact more strongly with nups located on the edges of the NPC. The asymmetrically distributed nups are proposed docking sites for importins on the nuclear face and exportins on the cytoplasmic face that may help establish directionality through the NPC (Liu and Stewart, 2005; Aitchison and Rout, 2012). A common characteristic of all β-karyopherins is their ability to bind RanGTPase (Gsp1p in yeast).

1.4. Ran GTPase

The directionality of β-karyopherin movement through the NPC is accomplished by the function of RanGTPase. Ran is a conserved small Ras-like GTPase that can exist in the GTP or GDP bound form and is essential for the majority of transport through the NPC. Interestingly, directionality is the result of a gradient whereby the majority of Ran bound to GTP is present in the nucleus whereas Ran in the GDP form is in the cytoplasm (Gorlich and Kutay, 1999). Evidence has shown that the direction of import and export can be reversed by inversing the location of RanGTP and RanGDP, indicating the importance of their location for translocation through the NPC (Nachury and Weis, 1999). The distribution of the two forms of Ran is accomplished by the chromatin bound Ran guanine nucleotide exchange factor (RanGEF, Prp20p in yeast) in the nucleus and the RanGTPase activating protein (RanGAP, Rna1p in yeast) in the cytoplasm (Becker et al., 1995; Akhtar et al., 2001). In the nucleus, RanGEF promotes the exchange of GDP for GTP by stabilizing the nucleotide free form of Ran. In contrast, RanGAP stimulates Ran’s intrinsic GTPase activity to convert GTP to GDP in the cytoplasm by binding and stabilizing the transition state (Stewart, 2007). The exportin in the export complex protects RanGTP from RanGAP preventing hydrolysis of GTP to GDP (Floer and Blobel, 1996).
Therefore, the stimulation of the GTPase activity of Ran also requires the binding of the Ran binding protein 1 (RanBP1, Yrb1 in yeast) as it prises apart the complex enabling RanGAP to interact with Ran (Butler and Wolfe, 1994; Bischoff and Gorlich, 1997; Seewald et al., 2003). The sequestration of these components in their respective compartments maintains the RanGTP gradient resulting in proper transport between the nucleus and the cytoplasm.

The most well known example of nuclear import involves importin-β. It is one of the β-karyopherins that involves an adaptor protein, in this case importin-α (Stewart, 2007). Cargo containing a classical nuclear localization signal (NLS) binds to importin-α resulting in exposure of the importin-β binding domain (IBB) of importin-α (Cingolani et al., 1999). Importin-β in turn binds the IBB found in the cargo bound importin-α and together they move into the nucleus. Upon entry into the nucleus, RanGTP binds to importin-β in the transport complex causing release of the cargo (Figure 1) (Vetter et al., 1999; Lee et al., 2005). The RanGTP-importin-β complex and importin-α bound to its exportin, Cse1, and RanGTP subsequently exit the nucleus through the NPC restoring the importin-β and importin-α in the cytoplasm for another round of import (Kutay et al., 1997; Cook et al., 2007).

In contrast, exportins need to be bound to RanGTP in order to properly interact and bind to their respective cargo. The superhelical structure of the exportin is either in a tightly bound state or open state which results in a distortion of the cargo binding site (Cook and Conti, 2010). In this conformation, the cargo is unable to interact with the exportin. Interaction with RanGTP in concert with the cargo molecule induces a conformational change in the exportin resulting in a functional cargo binding site (Conti et al., 2006). Therefore, exportins can only bind their cargo in the presence of RanGTP. The exportin of the trimeric export complex interacts with the FG nups of the NPC and moves towards the cytoplasm and interacts with the docking nups on the
cytoplasmic edge of the NPC (Mosammaparast and Pemberton, 2004; Aitchison and Rout, 2012). The cytoplasmic localized RanGAP in concert with RanBP1 activate the GTPase activity of Ran and GTP is hydrolyzed to GDP (Bischoff and Gorlich, 1997). As a result, a conformational change in Ran causes dissociation of the complex and release of the cargo into the cytoplasm (Bischoff and Ponstingl, 1995). To avoid depletion of Ran within the nucleus, RanGDP is recycled back through the NPC by Ntf2p where GDP is exchanged for GTP by the RanGEF (Figure 1) (Ribbeck et al., 1998). In addition, the exportin is able to translocate through the NPC to enter the nucleus for another round of export. The interruption in cargo binding in the absence of RanGTP inhibits exportins from re-binding and re-importing the cargo that has just been released into the cytoplasm (Mosammaparast and Pemberton, 2004). Therefore, the sequestering of RanGTP in the nucleus and RanGDP in the cytoplasm is required to inhibit the formation of a futile import/export cycle.
Figure 1. RanGTPase cycle involved in the general import and export of cargo through the NPC. The formation of the import complex between the importin and cargo destined for the nucleus allows for translocation through the NPC. The RanGTPase, Gsp1p-GTP, bind the importin resulting in release of the cargo. The export complex forms between the exportin, cargo and Gsp1p-GTP in the nucleus. After translocation through the NPC, release of the cargo and exportin is initiated by RanGAP (Rna1p) in the presence of RanBP1 (Yrb1p) activating the GTPase activity of Gsp1p to cause the hydrolysis of GTP to GDP. Ntf2p binds Gsp1p-GDP and transports it back into the nucleus where RanGEF (Prp20p) induces the Gsp1p nucleotide exchange from GDP to GTP.
1.5. Transcription and maturation of tRNA

Proper tRNA maturation is required to produce a tRNA molecule that can perform multiple rounds of protein translation. tRNAs are made as precursor tRNA (pre-tRNA) molecules by RNA Polymerase III transcription of tDNA genes in the nucleolus (Hopper et al., 2010). The precursor and mature tRNA molecule have an L-shaped three dimensional structure formed by an acceptor arm, D-arm, T\(\psi\)C arm, the anticodon arm and a variable arm (Arts et al., 1998b). To obtain mature tRNA, the pre-tRNA must undergo five maturation steps. To begin, the pre-tRNA is bound at the 3’ end by the La protein protecting it from digestion by endonucleases (Wolin and Cedervall, 2002). The 5’ leader sequence is removed in a single endonucleolytic cleavage step by the ribozyme, RNase P (Figure 2) (Frank and Pace, 1998). However, cells that lack the La protein may undergo 3’ end maturation before processing of the 5’ end (Wolin and Cedervall, 2002). Following removal of the 5’ leader sequence, the 3’ trailer sequence is removed by either the endonuclease, RNase Z, or an exonuclease (Schurer et al., 2001; Morl and Marchfelder, 2001). The nucleotides C, C and A are added to the 3’ end of the acceptor arm by the ATP(CTP):tRNA nucleotidyltransferase (Cca1p). The mechanism used by Cca1p for the addition of CCA is unknown but the process has been found to occur in the nucleus (Chen et al., 1990). Modifications of various bases within the tRNA molecule including methylation, addition of isopentyl groups and modification of uridine to pseudouridine (\(\psi\)) are performed within the nucleus (Wolin and Matera, 1999; Hopper and Phizicky, 2003). However, recent studies have suggested that further modification of some tRNAs occur in the cytoplasm following nuclear export of the tRNAs (Ohira and Suzuki, 2011).

Twenty percent of tDNA genes in *S. cerevisiae* and approximately 5% of tDNA genes in *Homo sapiens* contain an intron and therefore an additional splicing step is required for their
maturation. In mammalian cells the entire maturation process including the splicing of the intron occurs in the nucleus (Paushkin et al., 2004). In contrast, it has recently been reported that this splicing event occurs on the cytoplasmic surface of the mitochondria in *S. cerevisiae*. The subunits of the tRNA endonuclease were found to localize to the mitochondria by immunofluorescence and subcellular fractionation studies (Yoshihisa et al., 2003). Furthermore, inhibition of tRNA splicing on the mitochondria resulted in the cytoplasmic accumulation of unspliced tRNA (Yoshihisa et al., 2007). The catalytic subunits of the tRNA splicing endonuclease, Sen2p and Sen34p, remove the intron from the pre-tRNA. tRNA ligase then joins the 3’ and 5’ ends together leaving a 2’ phosphate at the junction site. This 2’ phosphate is subsequently removed by 2’-phosphotransferase, Tpt1p (Abelson et al., 1998; Yoshihisa et al., 2003). As a result of cytoplasmic splicing, the pre-tRNA must be transported out of the nucleus for splicing and then re-imported back into the nucleus for further modification and quality assurance by aminoacylation before the mature tRNA is re-exported to the cytoplasm for use in protein synthesis.
Figure 2. The tRNA maturation process for pre-tRNAs made from either intronless or intron-containing tDNA genes. Following synthesis, the pre-tRNAs go through several modification steps including trimming of the 5' leader and 3' trailer sequences multiple base modifications and in some cases removal of the intron. The mature tRNA undergoes a final quality assurance step of aminoacylation before export to the cytoplasm.
1.6. Nuclear aminoacylation of tRNA

Once the pre-tRNA have acquired mature 3’ and 5’ ends, the addition of CCA, base modifications and in some cases removal of the intron, the tRNA must be aminoacylated to function in protein synthesis. Aminoacyl-tRNA synthetases (aaRS) are able to discriminate between different tRNAs by identifying specific sequence determinants (Giege et al., 1998; Ling et al., 2009). This allows the aaRS to add the amino acid that correlates to the proper tRNA anticodon. Furthermore, this provides a quality assurance step in tRNA maturation, since the aaRS would be unable to interact with an improperly matured tRNA. It has been shown that several aaRS localize to nucleus in both yeast and mammals. Moreover, the *S. cerevisiae* tyrosyl-tRNA synthetase, Tys1p, contains a NLS sequence and recent studies have demonstrated that Tys1p localizes to the nucleolus, indicating that tRNA aminoacylation occurs in the nucleolus of *S. cerevisiae* (Azad et al., 2001; Steiner-Mosonyi and Mangroo, 2004; Strub et al., 2007). The tRNA aminoacylation step may also occur in the nucleolus of mammalian cells, since a large number of aminoacyl-tRNA synthetases were found in this compartment by a large-scale proteomic study (Nathanson and Deutscher, 2000; Ko et al., 2000). Furthermore, injection of tRNA$^{\text{Met}}$ and tRNA$^{\text{Tyr}}$ into the nuclei of *Xenopus laevis* oocytes confirmed that the tRNAs were aminoacylated in the nucleus prior to export (Lund and Dahlberg, 1998). In addition, several tRNA molecules were aminoacylated in the nucleus of both yeast and mammals (Lund and Dahlberg, 1998; Grosshans et al., 2000; Steiner-Mosonyi and Mangroo, 2004; Chafe and Mangroo, 2010). Moreover, injection of immature tRNAs delayed their nuclear export until the full maturation and aminoacylation process was completed (Lund and Dahlberg, 1998). These data suggest that tRNA aminoacylation in the nucleus is used as a mechanism to monitor the
quality of the matured tRNA since only properly matured tRNAs are aminoacylated and exported to the cytoplasm.

Numerous studies have also shown that the efficiency of nuclear tRNA export is dependent on nuclear tRNA aminoacylation. Inhibition of nuclear aminoacylation in *X. laevis* oocytes using a competitive inhibitor of the tyrosyl-tRNA synthetase resulted in a reduction in export of tRNA\textsuperscript{Tyr} (Lund and Dahlberg, 1998). Furthermore, amino acid starvation of amino acid auxotrophic *S. cerevisiae* strains, inhibition of the aminoacyl-tRNA synthetase by temperature sensitive mutations or the use of aminoacyl-tRNA synthetase specific inhibitors results in nuclear retention of the corresponding tRNA (Lund and Dahlberg, 1998; Sarkar *et al.*, 1999; Grosshans *et al.*, 2000; Azad *et al.*, 2001). Therefore, nuclear aminoacylation is required as a checkpoint to ensure that high quality and fully matured tRNAs are being exported to the cytoplasm for use in protein synthesis.

1.7. Nuclear import of newly spliced tRNA

Splicing of tRNA in the cytoplasm moved away from the central idea that synthesis and complete maturation of tRNA was solely occurring in the nucleus in eukaryotes. tRNA aminoacylation also occurs in the nucleus and is suggested to be providing a mechanism for proofreading the integrity of newly matured tRNAs. Therefore, it was hypothesized that newly spliced tRNAs in the cytoplasm are re-imported into the nucleus to undergo the final nuclear tRNA aminoacylation quality control step. The *kar1-1* mutant strain is defective in nuclear fusion, and when mated with a wild type strain the resulting heterokaryon cell shares the same cytosol but contains two nuclei (Rose and Fink, 1987). The *kar1-1* strain was used to perform heterokaryon shuttling assays to determine whether tRNA could in fact be imported into the nucleus. The *los1* mutant strain, which displays a defect in nuclear tRNA export due to loss of
the function of the nuclear tRNA export receptor Los1p, was fused to the kar1-1 strain. The *S. pombe* initiator tRNA$^{\text{Met}}$ or the *D. discoideum* nonsense suppressor tRNA$^{\text{Glu}}$, expressed in one strain prior to formation of heterokaryons, were observed in both nuclei that share the same cytosol (Shaheen and Hopper, 2005; Takano *et al.*, 2005). This suggests that the newly shared cytoplasmic tRNAs import into the nucleus of the strain that does not encode the exogenous tRNA. Furthermore, endogenous tRNA was also shown to accumulate in the nucleus after the inhibition of gene transcription by thiolutin in the los1 msn5 mutant strain, which has lost the function of both known nuclear tRNA export receptors. Under these conditions new tRNAs are not synthesized and therefore the tRNA present in the nucleus must have been imported from the cytoplasm (Takano *et al.*, 2005). These studies established that tRNA can be imported back into the nucleus.

The mechanism of nuclear import of newly spliced tRNA is controversial, since multiple studies have resulted in conflicting results. For instance, it was reported that nuclear import of tRNA was energy dependent but Ran independent, since depletion of ATP with NaN$_3$ and 2-deoxyglucose treatment in the los1 msn5 strain but not depletion of Rna1p using the temperature sensitive rna1-1 mutant strain after inhibition of tRNA synthesis abolished the nuclear retention of mature tRNA$^{\text{Tyr}}$ (Takano *et al.*, 2005). In contrast, another study showed that deletion of the known β-karyopherin, Mtr10p, also alleviated nuclear tRNA retention after amino acid starvation suggesting the involvement of Ran (Shaheen and Hopper, 2005). A third study showed that while depletion of Mtr10p affected nuclear import of its known cargo Npl3p, it did not affect nuclear import of newly spliced tRNA, as nuclear retention of matured spliced tRNAs was observed during amino acid starvation (Chafe *et al.*, 2011). Therefore, the mechanism responsible for nuclear import of newly spliced tRNAs has yet to be fully elucidated. However, nuclear
localization of a cytoplasmic tRNA$^{\text{Phe}}$ modification enzyme that catalyzes a reaction following splicing demonstrated that tRNA$^{\text{Phe}}$ was still being modified in the presence or absence of amino acids, suggesting that nuclear import of spliced tRNA occurs by a constitutive process (Murthi et al., 2010).

1.8. Protein components of nuclear-cytoplasmic tRNA transport

1.8.1. Los1p

Early studies of nuclear export of tRNA demonstrated that even though tRNA is smaller than the size exclusion limit of the NPC, they are exported to the cytoplasm by a carrier-mediated process (Zasloff, 1983). The S. cerevisiae $\beta$-karyopherin, Los1p, was initially shown to be involved in tRNA splicing, since a Los1p temperature sensitive mutation and deletion of LOS1 resulted in the accumulation of end trimmed unspliced precursor tRNAs (Hopper et al., 1980; Hurt et al., 1987). However, the mammalian homolog, Exportin-t (Xpo-t), was later identified as a bona fide nuclear export receptor specific for tRNA export (Kutay et al., 1998; Arts et al., 1998a). Subsequently, it was reported that deletion of LOS1 resulted in nuclear retention of tRNAs derived from both intronless and intron-containing pre-tRNAs at elevated temperatures but not mRNA (Sarkar and Hopper, 1998). Although end trimmed unspliced tRNAs accumulated in the nucleus when LOS1 was deleted, tRNA aminoacylation of mature tRNAs was not affected (Steiner-Mosonyi and Mangroo, 2004). Los1p was shown to be located primarily at the NPC and to interact with the NPC through Nup2p and Nsp1p (Shen et al., 1993; Hellmuth et al., 1998). Gsp1p-GTP within an export complex is not accessible to Rna1p which promotes the activation of the intrinsic GTPase activity of Gsp1p (Floer and Blobel, 1996). Therefore, the hydrolysis of GTP to GDP could be used as an indicator of whether a $\beta$-karyopherin and a cargo are able to form a proper export complex. It was discovered using this
GTPase protection assay that Los1p forms an export complex with Gsp1p-GTP only in the presence of tRNA (Hellmuth et al., 1998). These studies established that Los1p functions as an export receptor specific for tRNA transport similar to the mammalian homolog, Xpo-t.

The structure of the Schizosaccharomyces pombe Xpo-t revealed that Xpo-t binds to end matured tRNAs through structural features within the TψC and acceptor arms. Xpo-t requires Gsp1p-GTP to cause a conformational change within the β-karyopherin revealing an undistorted tRNA binding site that specifically fits end matured tRNAs independent of aminoacylation (Cook et al., 2009). The S. pombe Xpo-t structure does not interact with specific base sequences but with the phospho-ribose backbone within the tRNA molecule allowing it to bind all tRNAs (Arts et al., 1998b; Cook et al., 2009). In mammalian cells, maturation and splicing of the tRNAs occurs exclusively within the nucleus and end maturation is believed to follow splicing (Paushkin et al., 2004). Therefore, it was suggested that Xpo-t can act as a quality control step, since it will only interact with end-matured tRNA (Lipowsky et al., 1999; Cook et al., 2009). The discovery of the mitochondrial localization of the yeast tRNA splicing complex and the accumulation of unspliced tRNA in the los1 mutant strain has led to the proposal that Los1p initially exports the intron-containing tRNAs for splicing (Yoshihisa et al., 2007). Therefore, Los1p is a tRNA specific export receptor required for the export of both intron-containing pre-tRNAs and mature tRNAs.

1.8.2. Msn5p

Although deletion of LOS1 results in a decrease in the efficiency of nuclear tRNA export, the cells are still viable indicating the presence of at least one other tRNA export receptor. The β-karyopherin Msn5p has been implicated in both nuclear import and export and recognizes a variety of cargoes including several phosphorylated proteins and precursors of microRNA.
(Kaffman *et al.*, 1998; Shibata *et al.*, 2006). The mammalian homolog, Exportin-5 (Xpo-5), was initially shown to participate in tRNA export when it was discovered that it was able to export the eukaryotic elongation factor 1A (eEF1A) only in the presence of aminoacylated tRNA (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). However, Xpo-5 has not been shown to independently transport tRNA across the NE. Although, deletion of *MSN5* did not affect the cellular distribution of tRNA, a synergistic effect on tRNA export resulting in nuclear accumulation was observed in the *los1 msn5* mutant strain, indicating the involvement of Msn5p in tRNA export (Takano *et al.*, 2005). Moreover, loss of the function of Msn5p did not reduce the efficiency of tRNA export by affecting tRNA aminoacylation since the tRNA aminoacylation status was not altered in the double mutant (Takano *et al.*, 2005). Recent reports have demonstrated that Msn5p is an authentic nuclear export receptor for tRNA (Eswara *et al.*, 2009; Murthi *et al.*, 2010). One study proposed that Msn5p may export aminoacylated tRNA when the tRNA is bound to eEF-1A similar to the mammalian homolog, Xpo-5 (Murthi *et al.*, 2010). However, a GTPase protection assay showed that Msn5p in combination with tRNA forms an export complex with the GTP bound form of Gsp1p (Eswara *et al.*, 2009). In contrast to Los1p, the function of Msn5p is not evolutionarily conserved (Park *et al.*, 2005; Shibata *et al.*, 2006). Furthermore, Msn5p does not participate in nuclear export of intron-containing pre-tRNAs, since accumulation of unspliced pre-tRNAs was not observed in the *msn5* mutant strain (Murthi *et al.*, 2010). Deletion of both the *LOS1* and *MSN5* genes is not lethal suggesting the existence of at least one more unidentified tRNA export receptor.
1.8.3. Cca1p

During tRNA maturation Cca1p adds the nucleotides C,C and A to the trimmed 3’ end of the tRNA acceptor arm subsequently priming the tRNA for proper aminoacylation and nuclear export (Chen et al., 1990; Wolfe et al., 1996; Lund and Dahlberg, 1998). Reports have also demonstrated that Cca1p not only adds CCA for aminoacylation but it also has the ability to add CCACCA to the 3’ end of aberrant tRNAs, consequently, targeting them for degradation (Dupasquier et al., 2008; Wilusz et al., 2011). Interestingly, the overexpression of Cca1p rescued the growth defect and alleviated the nuclear tRNA retention of tRNA^Met, which is encoded by an intronless gene, exhibited by the los1 mutant strain incubated at elevated temperatures. Therefore, it was proposed that Cca1p functions in a nuclear tRNA export pathway independent of Los1p. However, Cca1p overexpression was unable to alleviate the nuclear retention of tRNA^Tyr, which is made from an intron-containing precursor, in a tys1 Tys1-nls (mutant NLS) temperature sensitive mutant strain defective in nuclear aminoacylation of tRNA^Tyr (Steiner-Mosonyi et al., 2003). Therefore, it was suggested that Cca1p plays a role in the export of tRNAs encoded by intronless genes. Cca1p has been found to localize to the cytoplasm, nucleus and mitochondria. Cca1p tagged with the simian virus 40 NLS was shown to shuttle between the nucleus and cytoplasm in the heterokaryon assay using the kar1-1 mutant strain defective in nuclear fusion (Rose and Fink, 1987; Feng and Hopper, 2002). Taken together the data suggest that Cca1p participates in a Los1p-independent pathway that is responsible for nuclear export of tRNAs derived from intronless pre-tRNAs. However, to date Cca1p has not been shown to act as a bona fide nuclear tRNA export receptor. Thus, the exact role of Cca1p in nuclear tRNA export remains unclear.
1.8.4. Utp8p

The U3 protein 8 (Utp8p) was first identified along with 16 other Utp proteins in association with the U3 small nucleolar RNA (U3 snoRNA) forming the ribonucleoprotein (RNP) complex involved in the biogenesis and maturation of the 18S ribosomal RNA (rRNA) in the nucleolus (Dragon et al., 2002). However, other studies have shown that Utp8 also plays a crucial role in nuclear tRNA export in *S. cerevisiae*. Utp8p was first discovered as a tRNA-binding protein using a yeast tRNA three hybrid interaction screen involving a tRNA-MS2 hybrid RNA gene and the LexA DNA-binding domain fused to the MS2 coat protein (Steiner-Mosonyi *et al.*, 2003). Further reports verified that Utp8p was an authentic tRNA-binding protein since it was able to bind tRNA directly and saturably *in vitro*. Moreover, structural analysis and mutational studies revealed that a positively charged cleft in the C-terminal domain of Utp8p is required for tRNA binding (McGuire *et al.*, 2009). In support of Utp8p being involved in nuclear tRNA export, overexpression of the protein rescued growth of a strain harboring an export defective mutant amber suppressor tyrosyl-tRNA (tRNA\(^{\text{Tyr}}_{\text{am}}\)) with a G11:C24 mutation (Cleary and Mangroo, 2000; Steiner-Mosonyi *et al.*, 2003). Overexpression not only restored growth but alleviated the nuclear retention typically seen with the export defective mutant tRNA\(^{\text{Tyr}}_{\text{am}}\) (Steiner-Mosonyi *et al.*, 2003). Furthermore, depletion of Utp8p resulted in nuclear retention of both tRNAs derived from intronless and intron-containing pre-tRNA, but not export of mRNA or the small and large ribosomal subunits. tRNA maturation and nuclear aminoacylation was also not inhibited after depletion of Utp8p, suggesting that Utp8p is required specifically for nuclear tRNA export of aminoacylated tRNAs (Steiner-Mosonyi *et al.*, 2003). Furthermore, Utp8p overexpression rescued nuclear export of non-aminoacylated tRNA\(^{\text{Tyr}}\) in a mutant strain defective in the nuclear import of the tyrosyl-tRNA synthetase (Tys1p), which is required for
nuclear aminoacylation of tRNA_{Tyr} (Steiner-Mosonyi et al., 2003). The data led to the suggestion that Utp8p is an essential nucleolar protein involved in the nuclear export of aminoacylated and non-aminoacylated tRNAs derived from both intronless and intron-containing pre-tRNAs.

Further characterization of Utp8p demonstrated that it performs an intranuclear function in tRNA export as opposed to being the unidentified tRNA export receptor. A heterokaryon assay illustrated that Utp8p does not shuttle between the nucleus and the cytoplasm, a requirement for tRNA export receptors. Furthermore, the nuclear retention of tRNA seen after depletion of Utp8p was found to co-localize with the U18 snoRNA in the nucleolus (Steiner-Mosonyi and Mangroo, 2004). To further elucidate the function of Utp8p, large-scale tandem affinity purification (TAP) and mass spectrometric analyses were performed to identify associated proteins. Interestingly, Utp8 was found to interact with aminoacyl-tRNA synthetases (aaRSs) and the tRNA export receptor, Msn5p. Additional affinity purification analyses showed that Utp8p also interacts with Los1p, Cca1p and Gsp1p. In vitro analyses showed that Utp8p interacts directly with the tRNA synthetase Tys1p; the tRNA export receptors, Los1p, Msn5p and Cca1p; and the yeast RanGTPase, Gsp1p (Strub et al., 2007). As stated, recent reports have revealed that efficient nuclear tRNA export requires the nuclear pools of aaRSs (Azad et al., 2001; Steiner-Mosonyi and Mangroo, 2004). Therefore, the fact that Utp8p interacts with both the aaRS and the tRNA export receptors suggests that Utp8p may serve as a link between tRNA aminoacylation and translocation of the tRNA to the NPC. The interaction with Tys1p was found to occur in the nucleolus in vivo using a split GFP reconstitution system (Strub et al., 2007). Furthermore, Utp8p was found to co-localize with 4,6-diamidino-2-phenylindole (DAPI) in the nucleoplasm in a temperature sensitive nup120 mutant strain, which inhibits nuclear export because of clustering of the NPC at the non-permissive temperature (Aitchison et al., 1995; Strub
et al., 2007). Utp8p was also shown to form a complex with Los1p and Gsp1p that is dependent on the presence of tRNA (Strub et al., 2007). These studies suggest that Utp8p functions by picking up the aminoacylated tRNAs from the aaRS in the nucleolus and shuttling them through the nucleoplasm to the tRNA export receptor. In addition, in vitro binding studies strongly suggest that Utp8p also plays a critical role in establishing the receptor-tRNA-Gsp1p-GTP export complex by recruiting Gsp1p-GTP to the receptor-tRNA-Utp8p complex. However, the molecular details of the mechanism of Utp8p function are not fully understood.

1.8.5. Utp9p

The finding that Utp8p is required for nuclear tRNA export led to the possibility that other proteins of the U3 RNP complex that interact with Utp8p may also have a function in nuclear export of tRNA. Studies involving the depletion of the Utp proteins identified Utp9p and Utp22p but not Utp10p and Utp13p as being essential for nuclear tRNA export (Eswara et al., 2009; Eswara et al., 2012). These results suggest that only specific Utp proteins are associated with the tRNA export process while others perform a more prominent role in the biogenesis and maturation of 18S rRNA. As with Utp8p, loss of Utp9p function did not affect nuclear export of mRNA or tRNA maturation and aminoacylation, suggesting that it is required specifically for the export of mature tRNAs following aminoacylation (Eswara et al., 2009). Even though Utp9p binds directly to tRNA in a saturable manner, it did not have the ability to shuttle between the nucleus and the cytoplasm, suggesting that it does not function as a tRNA export receptor. Despite interacting directly with Utp8p as shown by tandem affinity purification (TAP) and in vitro protein binding studies, Utp9p does not interact with the aaRS, Tys1p, suggesting that it is required for export after Utp8p has obtained the aminoacylated tRNAs from the aaRS. Interestingly, depletion of Utp9p, resulted in accumulation of only mature tRNAs
derived from intron-containing pre-tRNAs (Eswara et al., 2009). Furthermore, Utp9p interacts directly in vitro with the tRNA export receptor, Msn5p, but not Los1p. Utp9p was also found to interact with the GTP bound form of Gsp1p in a tRNA-dependent manner in vitro (Eswara et al., 2009). Further studies discovered that the interaction between Utp9p and Gsp1p-GTP was tRNA-dependent and stronger after Utp9p was initially bound to Msn5p. Gsp1p interacts in a tRNA-independent manner if Gsp1p-GTP is bound to Utp9p before Msn5p. In contrast, Gsp1p-GTP showed no preferential binding when Utp8p was used in the same sequential in vitro binding assays (Eswara et al., 2009). These results suggest that mature tRNAs derived from intronless and intron-containing pre-tRNAs are exported by separate pathways. A model that is consistent with the data is that Utp9p functions with Utp8p in delivering mature spliced tRNAs made from intron-containing pre-tRNAs to Msn5p. Other evidence indicates that Utp9p aides in the formation of the Msn5p-tRNA-Gsp1p-GTP export complex, possibly by recruiting Gsp1p-GTP (Eswara et al., 2009).

1.8.6. Utp22p

Depletion of Utp22p also resulted in nuclear retention of tRNAs. Utp22p was found to specifically play a role in the export of tRNAs from the nucleus, since depletion did not affect the export of mRNA and the ribosomal subunits or nuclear tRNA aminoacylation (Eswara et al., 2012). However, unlike Utp9p, Utp22p showed nuclear accumulation of tRNAs derived from both intron-containing and intronless pre-tRNAs. Moreover, TAP and in vitro analyses showed that Utp22p interacts directly with Utp8p, Tys1p and Los1p but not Msn5p and Gsp1p. Therefore, the function of Utp22p may not be synonymous with the roles of Utp8p or Utp9p, but is required for the nuclear export of aminoacylated tRNAs going to the export receptor Los1p. Utp22p also interacted with Tys1p to a greater extent when Tys1p was bound to tRNA (Eswara
et al., 2012). However, since Utp22p does not specifically or saturably bind tRNA, it was suggested that Utp22p does not function in retrieving the aminoacylated tRNAs from the aminoacyl-tRNA synthetase. Studies have shown that Utp22p remains in the nucleolus and does not co-localize with Utp8p in the nup120 mutant strain where Utp8p was found to be in the nucleoplasm (Eswara et al., 2012). Therefore, it was hypothesized that Utp22p recruits Utp8p to the aaRS to collect aminoacyl-tRNAs and in concert with Utp8p delivers the aminoacyl-tRNAs to Los1p. However, unlike Utp9p, Utp22p does not assist in the formation of the tRNA-Los1p-Gsp1p export complex.

1.8.7. Cex1p

In addition to identifying Utp8p as a component of the nuclear tRNA export pathway, the yeast tRNA three hybrid interaction screen also uncovered a cytoplasmic component, cytoplasmic export protein 1 (Cex1p). Cex1p is an evolutionarily conserved protein consisting of an N-terminal kinase-like domain and a HEAT repeat domain. Like Utp8p, overexpression of Cex1p restored nuclear export of the export-defective G11:C24 yeast tyrosine amber suppressor tRNA mutant. Cex1p was also found to interact directly and saturably tRNA (McGuire and Mangroo, 2007). These findings led to the suggestion that Cex1p is a cytoplasmic component of the nuclear tRNA export process of S. cerevisiae. Cex1p was also found to be largely cytoplasmic with a small portion co-localizing with the nucleoporin Nup2p (McGuire and Mangroo, 2007). TAP and in vitro binding studies showed that Cex1p directly interacts with Nup116p but not Nup57p and Nup2p. Deletion of NUP116 resulted in the failure of Cex1p to co-localize with Nup2p (McGuire and Mangroo, 2007). Therefore, under steady state conditions, a fraction of Cex1p localizes to the NPC and is anchored there by interacting directly with Nup116p. TAP showed that Gsp1p, Los1p, Msn5p and the eukaryotic elongation factor eEF-1A
encoded by the *TEF2* gene interact with Cex1p. Furthermore, *in vitro* protein binding studies established that Cex1p directly interacts with Gsp1p-GTP, Los1p and Msn5p, but not Cca1p (McGuire and Mangroo, 2007; McGuire and Mangroo, 2012). In contrast, Cex1p does not interact with eEF-1A *in vitro*, suggesting that *in vivo* Cex1p may interact with eEF-1A through an adaptor protein (McGuire and Mangroo, 2007). The data suggest that Cex1p is a cytoplasmic component of the process involved in exporting aminoacyl-tRNAs from the nucleus. It was proposed that Cex1p collects the tRNA molecule from the export receptor at the cytoplasmic face of the NPC and assists in channelling them to eEF1A with the assistance of an unidentified protein. Correspondingly, the mammalian and plant homologs of Cex1p, SCYL1 and CTEXP, respectively, were shown to perform similar functions in nuclear tRNA export (Chafe and Mangroo, 2010; Johnstone *et al.*, 2011a).

Further investigation of the role Cex1p revealed that in addition to its direct interaction with Gsp1p-GTP, Cex1p was also able to interact directly with the yeast RanGAP, Rna1p (McGuire and Mangroo, 2012). Loss of the function of Rna1p in concert with deletion of Cex1p resulted in a synergistic block in nuclear tRNA export, suggesting that Cex1p may also perform a similar function to Rna1p. However, it was shown that Cex1p was unable to stimulate the intrinsic GTPase activity of Gsp1p even in the presence of tRNA, indicating that Cex1p was not a RanGAP (McGuire and Mangroo, 2012). Interestingly, deletion of the gene for the yeast RanBP1, Yrb1p, which aides Rna1p in accessing Gsp1p-GTP of the export complex consisting of the receptor, tRNA and Gsp1p-GTP, did not affect nuclear tRNA export. The use of the *in vitro* protection assay where the presence of an export complex bound to Gsp1p-GTP inhibits Rna1p activation of the GTPase activity of Gsp1p, demonstrated that Cex1p allows Rna1p to stimulate the GTPase activity of Gsp1p specifically when the export complex is Los1p-tRNA-
Gsp1p-GTP. Moreover, Rna1p interacts with Nup116p more strongly in the presence of Cex1p (McGuire and Mangroo, 2012). Since Rna1p is located in the cytoplasm, it was proposed that Cex1p recruits Rna1p to the NPC and facilitates Rna1p activation of the GTPase activity of Gsp1p in the receptor-tRNA-Gsp1p-GTP export complex to initiate dissociation of the tRNA export complex.

1.8.8. Arc1p

A synthetic lethal screen involving the los1 mutant strain demonstrated a lethal phenotype when the Arc1p gene was disrupted. This phenotype as well as the ability of either Arc1p or Los1p to rescue the los1 arc1 strain demonstrated a genetic interaction or functional overlap between these two proteins (Simos et al., 1996a). Since Los1p was previously identified as a tRNA export receptor, Arc1p was considered to possibly function in the nucleocytoplasmic tRNA transport process or in tRNA biogenesis. It was found that Arc1p, which is primarily located in the cytoplasm could be imported into the nucleus, but is maintained in the cytoplasm by the protein export receptor, Xpo1p, and was therefore considered an unlikely candidate for the unknown tRNA export receptor (Galani et al., 2005). Further studies found that Arc1p was required for the formation of a multienzyme complex involving two aminoacyl-tRNA synthetases, MetRS and GluRS in the cytoplasm of S. cerevisiae (Simos et al., 1996a). A similar larger mammalian complex involving the Arc1p homolog, p43, two other non-enzymatic proteins, p38 and p18, as well as nine synthetases was discovered, indicating that these complexes are evolutionarily conserved (Golinelli-Cohen et al., 2004). Free Arc1p was found to specifically bind a subset of intronless tRNA through its middle and C terminal domains. However, Arc1p preferentially binds to the elongator tRNA$^\text{Met}$ and tRNA$^\text{Glu}$ when it interacts with their corresponding aminoacyl-tRNA synthetases (Deinert et al., 2001). Furthermore, when
Arc1p was in a complex with MetRS and GluRS, it increased aminoacylation efficiency of the aminoacyl-tRNA synthetases by lowering their $K_m$ for their cognate tRNAs (Simos et al., 1996a; Deinert et al., 2001). Based on these findings it was suggested that Arc1p is responsible for delivering non-aminoacylated cytoplasmic tRNA to their cognate aminoacyl-tRNA synthetase, and to facilitate an efficient interaction between the synthetases and their cognate tRNAs (Simos et al., 1998). In addition, others have proposed that Arc1p may collect non-aminoacylated tRNA exiting the nucleus and delivering them to their cognate aaRSs.

Recently, it was determined that Arc1p has an N-terminal domain that binds the two aaRSs and a C-terminal tRNA-binding domain. Interestingly, Arc1p could only rescue growth of the los1 arc1 strain if both domains were localized to the cytoplasm (Golinelli-Cohen and Mirande, 2007). Furthermore, strict nuclear localization of MetRS by the expression of MetRS-NLS was lethal in an arc1 mes1 mutant strain. However, Arc1p could rescue growth of this strain by causing a portion of the synthetase to relocate to the cytoplasm (Golinelli-Cohen and Mirande, 2007). It was therefore suggested that Arc1p may also function as a cytoplasmic binding site that prevents aminoacyl-tRNA synthetases from being sequestered entirely in the nucleus for the nuclear tRNA aminoacylation quality assurance step.

### 1.9. Pathways of nuclear export of tRNA

Transport of tRNA from the point of transcription in the nucleus to the ribosome in the cytoplasm is believed to follow a channelling mechanism (Wolin and Matera, 1999). This method prevents the tRNA from dispersing within the cytoplasm and decreasing the efficiency of protein synthesis. Nuclear tRNA export involves multiple pathways for the export of aminoacylated and non-aminoacylated tRNAs (Grosshans et al., 2000; Steiner-Mosonyi and Mangroo, 2004).
1.9.1. Nuclear export of aminoacylated tRNAs

The nuclear export of aminoacylated tRNAs is proposed to be the primary export pathway due to the importance of nuclear aminoacylation for tRNA export. Mature tRNAs encoded by intronless tDNA genes are fully matured within the nucleus and then subjected to aminoacylation quality assurance in the nucleolus. It is possible that Utp22p functions in this pathway, since the function of Utp22p is essential and depletion results in nuclear retention of tRNAs made from intronless pre-tRNA (Eswara et al., 2012). It has been suggested that Utp22p recruits Utp8p to the aaRS to retrieve the aminoacylated tRNA from the aaRS. The Utp8p-Utp22p complex channels the aminoacyl-tRNAs to the export receptor Los1p after which Utp22p is released (Eswara et al., 2012). Subsequently, Utp8p loads Los1p with the aminoacylated tRNA and recruits Gsp1p-GTP to form the export complex, which then translocates through the NPC (Strub et al., 2007). However, since deletion of LOS1 is not lethal, another export receptor must be involved in the export of tRNAs from intronless pre-tRNAs. Once the export complex reaches the cytoplasmic face of the NPC, Cex1p collects the tRNAs and shuttles them to eEF-1A through an unknown associated protein. eEF-1A then delivers the aminoacylated tRNAs to the ribosome for protein synthesis (McGuire and Mangroo, 2007).
Figure 3. Nuclear export of aminoacylated mature tRNA derived from intronless pre-tRNA. Aminoacylated tRNAs are retrieved by Utp8p in the nucleolus and transported to the export receptor Los1p and an as yet unidentified receptor. tRNAs are collected from the export receptor by Cex1p and channelled to the ribosome through eEF-1A.
The nuclear export of aminoacylated tRNAs derived from intron-containing pre-tRNAs is more complicated due to the additional step of nuclear import. The tRNA synthesized by PolIII undergoes 5’ and 3’ end maturation and some modification before the intron-containing pre-tRNA is transported through the NPC to the cytoplasm by Los1p (Figure 4A) (Hopper et al., 1980; Hurt et al., 1987). Since Los1p is not essential, it suggests the involvement of an additional export receptor that can transport the end trimmed, intron-containing pre-tRNA. The pre-tRNA is transported to the spliceosome by an unknown mechanism for removal of the intron before being imported back into the nucleus, possibly by a β-karyopherin (Figure 4B) (Shaheen and Hopper, 2005). Once in the nucleus the tRNA undergoes further maturation and finally the quality assurance step of nuclear aminoacylation in the nucleolus. The re-export of retrograded tRNA can occur through multiple pathways. In the first pathway, Utp8p retrieves the aminoacylated spliced tRNA from the aaRS in the nucleolus and together with Utp9p transports the tRNA to the export receptor Msn5p for export to the cytoplasm (Figure 4C) (Eswara et al., 2009). As with Los1p, Msn5p is also not an essential protein and since deletion of both receptors is not lethal, an additional export receptor must participate in the re-export of mature aminoacylated spliced tRNA. Therefore, Utp9p may also facilitate export of retrograded tRNA through this unknown receptor. In the third pathway, Utp22p is likely also involved since depletion of Utp22p resulted in nuclear retention of mature tRNA made from intron-containing pre-tRNA (Eswara et al., 2012). Utp22p likely recruits Utp8p to retrieve the aminoacylated tRNA from the aaRS and Utp8p along with Utp22p deliver the tRNA to Los1p or the unknown receptor. The tRNA is passed on to Cex1p at the cytoplasmic face of the NPC where it will be shuttled to the ribosome for protein synthesis.
Figure 4. Nuclear export of mature aminoacylated tRNA derived from intron-containing pre-tRNA. Intron-containing tRNA is initially exported by Los1p or an unknown receptor and translocated to the spliceosome for removal of the intron (A). The spliced tRNA is imported back into the nucleus by an unknown mechanism where it undergoes further maturation and aminoacylation (B). Aminoacylated tRNAs are transported to the export receptors by Utp8p in conjunction with either Utp9p or Utp22p. Cex1p collects the tRNAs from the export complex and shuttles them to the ribosome through and associated protein and eEF-1A (C).
1.9.2. Nuclear export of non-aminoacylated tRNAs

Studies have reported that under optimal growth conditions, the pathways involving aminoacylated tRNAs are primarily responsible for nuclear tRNA export. However, the efficiency of tRNA export is significantly reduced but not eliminated when nuclear tRNA aminoacylation is inhibited, suggesting that non-aminoacylated tRNAs are exported to the cytoplasm but less efficiently (Azad et al., 2001). It is possible that nuclear export of non-aminoacylated tRNA may occur when the cells encounter suboptimal growth conditions such as low levels of nitrogen or carbon source. Utp8p has been shown to be involved in the export of non-aminoacylated tRNAs (Steiner-Mosonyi et al., 2003). Utp8p most likely collects the mature tRNA in the nucleolus and delivers them to the appropriate export receptors (Figure 5). Cca1p was found to be involved in the export of non-aminoacylated tRNAs derived from intronless genes (Feng and Hopper, 2002). It is not known whether Cca1p is as a bona fide tRNA export receptor or if it serves as an adaptor protein. However, Utp8p was found to interact directly with Cca1p (Strub et al., 2007). Therefore, for the export of mature tRNA made from intronless pre-tRNA, Utp8p delivers the tRNA to Cca1p. In the case of tRNA made from intron-containing pre-tRNA, Los1p has been predicted to be an export receptor, since the mammalian homolog, Xpo-t, cannot distinguish between aminoacylated and non-aminoacylated mature tRNAs (Arts et al., 1998b). This is consistent with data showing that loss of the function of Arc1p and Los1p is lethal (Simos et al., 1996a). However, Utp8p likely also delivers the tRNA to an unknown receptor. Following translocation through the NPC the non-aminoacylated tRNAs are passed on to Arc1p or its homologs to be aminoacylated by the aaRSs in complex with these proteins. The aminoacylated tRNAs are then channelled to eEF-1A and the ribosome for translation.
Figure 5. Nuclear export non-aminoacylated mature tRNA derived from intron-containing (A) and intronless pre-tRNA (B). For tRNAs derived from intron-containing pre-tRNA, Utp8p collects the nonaminoacylated tRNA and delivers it to the unknown export receptor or Los1p. For intronless tRNA, Utp8p delivers the tRNA to Cca1p. Following translocation the tRNA interacts with Arc1p and is aminoacylated by the aaRS before being transferred to eEF-1A and subsequently to the ribosome for protein synthesis.
1.10. Regulation of nuclear tRNA transport

Regulation of protein translation is an efficient mechanism for maintaining cellular survival under varying environmental conditions. tRNAs are synthesized in the nucleolus but function in protein synthesis in the cytoplasm. A possible mechanism for controlling protein translation could involve regulating the rate of nuclear tRNA export. Furthermore, several studies have demonstrated that tRNA transport is a regulated process (Whitney \textit{et al.}, 2007; Ghavidel \textit{et al.}, 2007). However, the signaling pathways that regulate nuclear tRNA export are largely unknown.

A recent report has demonstrated that DNA damage induced chemically or with UV radiation resulted in an increase in end-trimmed unspliced tRNAs. The defect in splicing was further attributed to a decrease in the rate of the initial export of end-trimmed intron-containing tRNAs and not inhibition of the spliceosome activity (Ghavidel \textit{et al.}, 2007). Furthermore, DNA damage caused Los1p, which is known to export intron-containing tRNAs to the cytoplasm for splicing, but not other β-karyopherins to relocate to the cytoplasm. More importantly, unspliced tRNAs did not accumulate in the \textit{mec1} and \textit{rad53} mutant strains (Ghavidel \textit{et al.}, 2007). Mec1p and Rad53p are known components of the cell cycle checkpoint signaling pathway that responds to DNA damage (Longhese \textit{et al.}, 2003). Therefore, nuclear tRNA export is being regulated through the redistribution of Los1p by the cell cycle checkpoint pathway in response to DNA damage. It is presently not known whether mammalian cells re-distribute the nuclear tRNA export receptor exportin-t in response to DNA damage.

Furthermore, several studies have shown that the cellular distribution of tRNA is also regulated in response to changes in levels of glucose, nitrogen, amino acids, or inorganic phosphate (Hurto \textit{et al.}, 2007; Whitney \textit{et al.}, 2007; Eswara \textit{et al.}, 2009; Pierce \textit{et al.}, 2010).
*S. cerevisiae* cells starved of amino acids or glucose showed nuclear accumulation of tRNA. Moreover, the tRNA was rapidly redistributed after the depleted nutrient was re-added to the growth media (Whitney et al., 2007). Low phosphate or nitrogen levels similarly resulted in the nuclear retention of tRNAs (Hurto et al., 2007; Eswara et al., 2009). The rapid response to nutrient availability suggested that nuclear tRNA transport was being controlled. Interestingly, nuclear import of tRNA occurs by a constitutive process, suggesting that changes in the rate of the nuclear export of tRNA may account for the nuclear retention observed after the depletion of nutrients (Murthi et al., 2010). Initial investigations have implicated several signaling pathways in regulating nuclear tRNA export. For instance, nuclear accumulation of tRNAs no longer occurred when the TORC1 pathway was inhibited by rapamycin, which simulates nitrogen deprivation, before the cells were starved of amino acids, suggesting that the TORC1 complex may not be involved in controlling tRNA trafficking between the nucleus and cytoplasm during nitrogen deprivation (Whitney et al., 2007). Deletion of *REG1*, the regulatory subunit of the protein phosphatase 1, Glc7p, renders Snf1p constitutively active and alleviates the nuclear tRNA retention observed during glucose starvation. This finding suggests that the Snf1 signaling pathway, which is turned on under glucose limiting conditions, may not be involved in regulating nuclear-cytoplasmic tRNA trafficking during glucose deprivation. A constitutively active PKA with attenuated activity also restored tRNA export during glucose starvation, suggesting that the PKA pathway, which is turned on when glucose is available, may play a role in the regulation of nuclear tRNA export during glucose deprivation (Whitney et al., 2007). Furthermore, deletion of *PHO88* which is involved in regulating the uptake of inorganic phosphate resulted in the nuclear accumulation of tRNA in a time and temperature sensitive manner (Hurto et al., 2007). Based on this finding, it was proposed that the PHO pathway is involved in the regulation of tRNA export.
in response to varying phosphate levels. Long term depletion of glucose and phosphate resulted in the redistribution of tRNA throughout the cell, suggesting that the cell adjusts the rate of nuclear tRNA export during prolonged nutrient stress (Hurto et al., 2007; Whitney et al., 2007).

The protein kinase Gcn2p is involved in the general amino acid signaling pathway and is activated when it binds uncharged tRNAs (Wek et al., 1995; Dong et al., 2000). As a result, Gcn2p phosphorylates eIF2α to inhibit protein translation in response to amino acid starvation (Simpson and Ashe, 2012). Deletion of GCN2 did not affect the nuclear retention of tRNA as a result of amino acid starvation, suggesting that this pathway also does not play a role in regulating nuclear tRNA export during amino acid deprivation (Whitney et al., 2007). Thus, the mechanism and the signaling pathways that are involved in regulating nuclear-cytoplasmic trafficking of tRNA is poorly understood. Interestingly, as with the cellular response to DNA damage, studies have reported that the location of export receptors including Los1p and Msn5p are altered in response to changes in glucose level, heat shock and salt stress (Quan et al., 2007). These findings suggest that signaling pathways may possibly regulate nuclear tRNA export by affecting the function and location of proteins involved in the tRNA export pathway.

1.11. Nutrient sensing pathways in *Saccharomyces cerevisiae*

Cell survival during poor environmental conditions depends on several signaling pathways that can sense changes in nutrient levels and elicit an appropriate cellular response. Low nutrient levels cause the cell to arrest or reduce nonessential cellular processes including transcription and translation. It also results in cell cycle arrest in G₁ or G₀ to accommodate the decrease in energy availability (Winderickx et al., 2003). The regulation of the cellular response to glucose and nitrogen starvation has been demonstrated to involve the protein kinase A (PKA),
Snf1p and target of rapamycin (TOR) signaling pathways. These are pathways that may possibly regulate nuclear tRNA export in response to nutrient stress.

1.1.1. Protein kinase A signaling pathway

The PKA signaling pathway is responsible for maintaining cell growth and proliferation in favorable environmental conditions. The complex is composed of two regulatory subunits and two catalytic subunits (Toda et al., 1987a; Toda et al., 1987b). In the presence of intracellular glucose, phosphorylation of glucose to glucose-6-phosphate by one of the hexokinases, Hxk1p, Hxk2p, or Glk1p, is required to signal the activation of the small G proteins, Ras1p and Ras2p (Colombo et al., 2004). The guanine nucleotide exchange factors (GEF), Cdc25p or Sdc25, activates the Ras proteins by stimulating the exchange of GDP for GTP (Figure 6) (Toda et al., 1985; Broek et al., 1987; Colombo et al., 2004). Consequently, the increase in Ras2p-GTP leads to its interaction and activation of adenylate cyclase (Cyr1p) (Toda et al., 1985). The transient increase in the concentration of cyclic AMP (cAMP) allows cAMP to bind the PKA regulatory subunit, Bcy1p (Johnson et al., 1987; Kuret et al., 1988). Subsequent nuclear localization of Bcy1p activates PKA by releasing the three partially redundant catalytic subunits Tpk1p, Tpk2p, and Tpk3p (Toda et al., 1987a; Toda et al., 1987b; Griffioen et al., 2000). It has recently been shown that different cellular localization of the catalytic subunits may account for the differences in their target specificity (Ptacek et al., 2005).

The phosphorylation of downstream targets, such as the transcription factors Msn2p and Msn4p, results in the repression of stress response and glucose-repressed genes and an increase in the expression of genes associated with metabolic processes such as ribosome biogenesis (Smets et al., 2010). However, a decrease in glucose correlates with activation of the intrinsic GTPase activity of Ras by GTPase activating proteins (GAP), Ira1 and Ira2 (Tanaka et al., 1990;
Colombo et al., 2004). GDP bound Ras is no longer capable of stimulating the production of cAMP by Cyr1p. Therefore, reduction in cAMP by the low and high affinity phosphodiesterases, Pde1p and Pde2p, allows Bcy1p to re-enter the cytoplasm through its interaction with Zds1p to inhibit PKA activity by binding to the catalytic subunits (Sass et al., 1986; Nikawa et al., 1987; Griffioen et al., 2001).

Working in parallel to the RasGTPase pathway to sense extracellular glucose is the Gpr1p/Gpa2p pathway. Gpa2p exchanges GDP for GTP after the G-protein coupled receptor, Gpr1p, senses an increase of extracellular glucose (Kraakman et al., 1999; Rolland et al., 2000). As a result, Gpa2p-GTP stimulates Cyr1p, increasing the production of cAMP and subsequent activation of PKA. During glucose starvation, the intrinsic GTPase activity of Gpa2p-GTP is stimulated by Rgs2p which reduces the Gpa2 mediated activation of Cyr1p. Active PKA in rich growth conditions allows the cell to exit G1 and progress through the cell cycle by phosphorylating and inhibiting the activity of the protein kinase Rim15p and nuclear import of the transcription factors Msn2p and Msn4p (Gorner et al., 1998; Gorner et al., 2002; Pedruzzi et al., 2003; Camerini et al., 2004). Inactivation of PKA during nutrient limiting conditions activates Rim15p which induces the function of the transcription factors Msn2/4p and Gis1p. Msn2/4p and Gis1p mediate the transcription of stress response element genes and post diauxic shift genes, respectively, consequently, allowing for survival during poor nutrient conditions (Smets et al., 2010).
Figure 6. The PKA signaling pathway in yeast. Activation of PKA occurs in the presence of glucose due to the activation of Ras2p and Gpa2p and subsequent production of cAMP by the adenylate cyclase, Cyr1p. cAMP binds the regulatory subunits and inhibits their interaction with the catalytic subunits Tpk1p, Tpk2p, and Tpk3p. In the absence of glucose, PKA is inactivated by binding of Bcy1p to the catalytic subunits following inactivation of Ras2p and Gpa1p and the reduction of cAMP by Pde1/2p.
1.11.2. Snf1p signaling pathway

Unlike the PKA pathway, the Snf1 pathway is activated by the depletion of glucose or growth on a nonfermentable carbon source. The Snf1p protein kinase is composed of an N-terminal kinase domain and a C-terminal regulatory domain. In the presence of glucose, the regulatory domain is bound to the catalytic resulting in autoinhibition (Jiang and Carlson, 1996). Decrease in glucose stimulates the Snf1p activating kinases, Sak1p, Elm1p and Tos3p to phosphorylate Snf1p on its activation loop at threonine-210 (Thr-210) within the kinase domain (McCartney and Schmidt, 2001) (Figure 7). Binding of the regulatory subunit, Snf4p, to the regulatory domain of Snf1p is required for full activation of the catalytic kinase domain as it allows the Snf1p activating kinases access to the phosphorylation site on the activation loop (Jiang and Carlson, 1997; McCartney and Schmidt, 2001; Momcilovic et al., 2008). Following activation, Snf1p interacts with one of three scaffolding proteins, Gal83p, Sip1p or Sip2p. These proteins regulate the localization and therefore the specific targets of Snf1p. For instance, Gal83p directs Snf1p to the nucleus while Sip1p targets Snf1p to the vacuole and the interaction with Sip2p maintains Snf1p in the cytoplasm (Vincent et al., 2001).

Activated Snf1p phosphorylates transcription factors such as Mig1p and Cat8p, consequently derepressing glucose-repressed genes and increasing the expression of gluconeogenic genes allowing cell survival on non-fermentable carbon sources or in low levels of glucose (Broach, 2012). Upon resupplementation of glucose, the regulatory protein, Reg1p, directs the protein phosphatase, Glc7p, to Snf1p (Sanz et al., 2000). Subsequently, Glc7p dephosphorylates and inactivates Snf1p. Deletion of Reg1p abolishes the interaction between Glc7p and Snf1p and Snf1p remains active even in the presence of glucose (Ludin et al., 1998; Sanz et al., 2000). The Glc7p-Reg1p complex is inhibited in glucose deprived conditions by the
phosphorylation of Reg1p by active Snf1p (Rubenstein et al., 2008). Recent reports have also found that Snf1p and Glc7p compete to interact with a common binding site on Reg1p and this competitive interaction may regulate the dephosphorylation of Snf1p. As a result, Snf1p is only dephosphorylated when Glc7p is bound to Reg1p and not when Snf1p is bound to Reg1p (Tabba et al., 2010). Dephosphorylation after the addition of a fermentable carbon source causes the regulatory domain to once again autoinhibit the catalytic domain. It is thought that the signal of glucose depletion is carried to Snf1p by the hexokinase, Hxk2p. However, it is unknown whether Hxk2p signals the availability of glucose by its phosphorylation of glucose, an association with Reg1p-Glc7p complex or binding to Mig1p (Sanz et al., 2000; Ahuatzi et al., 2004; Ahuatzi et al., 2007).

Figure 7. Activation of the Snf1p kinase requires phosphorylation of the activation loop by the Snf1p activating kinases Sak1p Elm1p and Tos3p in conjunction with Snf4p. Phosphorylation of Snf1p results in release from autoinhibition and binding to the β subunits Sip1p/Sip2p/Gal83p required for localization to specific compartments. Dephosphorylation involves the protein phosphatase, Glc7p and its regulatory subunit, Reg1p.
1.11.3. Target of rapamycin signaling pathway

The Target of rapamycin (TOR) pathway has been discovered in almost all eukaryotic species. It is involved in several processes required for normal cell growth including transcription, translation and ribosome biogenesis and is considered a central controller of cell proliferation. TOR has been implicated in the response to nutrient availability and the regulation of cell cycle progression from the G\textsubscript{1} phase (Brown \textit{et al.}, 1994). The evolutionarily conserved TOR protein has been characterized as a serine/threonine kinase belonging to the family of phosphatidylinositol kinase – related kinases (PIKK) (Crespo and Hall, 2002; Loewith \textit{et al.}, 2002). In \textit{S. cerevisiae}, two TOR proteins, Tor1p and Tor2p have been identified. In contrast, only a single TOR protein is present in other eukaryotes (Hay and Sonenberg, 2004). Two distinguishable complexes arise from specific proteins that associate with the TOR kinases and are subsequently named TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 may consist of Tor1p or Tor2p as well the proteins Lst8p, Tco89p and Kog1p. Lst8p and Kog1p are essential positive regulators of TORC1 activity (Hay and Sonenberg, 2004). TORC2, on the other hand, can only contain Tor2p along with Lst8p, Avo1p, Avo2p and Avo3p (Sarbassov \textit{et al.}, 2005). The functional separation of the complexes is based the unique role of TORC2 in controlling actin cytoskeleton distribution. Furthermore, TORC1 but not TORC2 is sensitive to the clinically important drug, rapamycin (Loewith \textit{et al.}, 2002). Rapamycin treatment arrests cells in G\textsubscript{1} resulting in decreased protein synthesis and ribosome biogenesis, increased expression of genes required for usage of poor nitrogen sources, formation of storage carbohydrates and initiation of autophagy (Brown \textit{et al.}, 1994; Barbet \textit{et al.}, 1996; Powers and Walter, 1999).
Activated TOR leads to a high level of transcription and translation. It has been proposed that the transcription factor TFIIIB and protein phosphatase 2A may be involved in inhibiting RNA Polymerase III after rapamycin treatment (Zaragoza et al., 1998). This would result in the reduced production of rRNA and tRNA. Studies have shown that ribosome maturation is also regulated by the TOR pathway (Powers and Walter, 1999; Honma et al., 2006). The nuclear GTP-binding protein Nog1 co-localizes and forms a complex with the 60S ribosomal proteins. When TORC1 is active the Nog1p complex moves from the nucleolus to the nucleoplasm. However, after treatment with rapamycin the complex remains in the nucleolus and late stage maturation no longer occurs (Honma et al., 2006).

Also, the protein phosphatase 2A is a part of the TOR signaling pathway along with the protein phosphatase 2A-like, Sit4p (Crespo and Hall, 2002). When nutrients are at a viable level, the yeast TORC1 phosphorylates Tap42p, a phosphatase type 2A associating protein. Recent evidence suggests that this protein remains bound to TORC1 at the plasma membrane. Phosphorylation of Tap42p inhibits the binding of the regulatory subunits of PP2A to the catalytic subunits Pph21p and Pph22p (Yan et al., 2006). Therefore, when the Tap42-PP2A complex is bound to TORC1, PP2A is inactive and unable to dephosphorylate its substrates. When cells are treated with rapamycin or subjected to nitrogen starvation, Tap42 dissociates from TORC1 and PP2A becomes active and Tap42 is subsequently dephosphorylated by PP2A holoenzyme (Yan et al., 2006). Activation of PP2A results in the dephosphorylation of 4E-BP reducing the rate of protein synthesis, nuclear accumulation of the transcription factor Msn2p and activation of genes in the general stress response (Santhanam et al., 2004).

Sit4p is another phosphatase known to associate with Tap42 and is regulated by the TOR pathway. However, Sit4p activates the transcription of genes required for the nitrogen stress
response through the dephosphorylation of the GATA transcription factors, Gln3p and Gat1p (Beck and Hall, 1999; Crespo and Hall, 2002). Under rich growth conditions these phosphorylated transcription factors are sequestered in the cytoplasm. The mechanism sequestering Gat1p in the cytoplasm is unknown but Gln3p is retained through its interaction with Ure2p (Scherens et al., 2006). Activation of Sit4p and PP2A by inhibition of TORC1 results in the dephosphorylation of Gln3p and Gat1p and their nuclear import where it can activate the expression of stress related genes and genes required for utilization of poor nitrogen sources (Cox et al., 2000; Magasanik and Kaiser, 2002; Tate et al., 2009; Tate et al., 2010). Therefore, the TOR signaling pathway is involved in the regulation of protein synthesis at both the transcriptional and translational levels.

1.12. Thesis objectives

Cells constantly adjust to changes in their environment by regulating cellular processes such as transcription and translation. Recent evidence has suggested that a possible mechanism for controlling gene expression and cellular growth involves the regulation of nuclear tRNA transport. Changes in glucose, amino acid or phosphate levels result in nuclear accumulation of tRNA derived from both intron-containing and intronless pre-tRNA in S. cerevisiae (Hurto et al., 2007; Whitney et al., 2007). It was previously hypothesized that the nuclear retention observed was the result of mature cytoplasmic tRNAs being rapidly re-imported back to the nucleus in response to nutrient depletion (Whitney et al., 2007). However, nuclear tRNA accumulation due to nutrient stress may also result from cells blocking nuclear export of tRNA rather than enhancing import. The accumulation of tRNA in the nucleus is also rapidly reversible after the depleted nutrient is re-added to the growth media, suggesting that the process of nuclear tRNA transport in response to nutrient stress is regulated (Hurto et al., 2007; Whitney et al., 2007).
This regulation of nuclear-cytoplasmic tRNA trafficking may aid in the cell surviving poor growth conditions by providing a means to adjust the rate of translation with respect to varying environmental conditions. Moreover, signaling pathways are known to regulate cellular processes in response to nutrient availability. However, little is known about the signaling pathways and mechanisms associated with the regulation of nuclear tRNA export.

In addition, previous studies have shown that amino acid starvation results in nuclear retention of tRNA in rat hepatoma cells suggesting that mammalian cells may also regulate nuclear-cytoplasmic tRNA trafficking in response to nutrient stress (Shaheen et al., 2007). However, reports from our laboratory demonstrated that amino acid and glucose starvation of rat hepatoma H4IIE, HeLa, and HEK293 cells do not result in nuclear accumulation of tRNA (Chafe et al., 2011). Also, nuclear retention of tRNA did not occur in plants cells deprived of nitrogen or sucrose (Johnstone et al., 2011b). These findings suggest that higher eukaryotes may not regulate nuclear tRNA transport in response to nutrient stress.

Therefore, we hypothesized that \textit{S. cerevisiae} controls nuclear tRNA export through known nutrient signaling pathways and that this method of regulating nuclear-cytoplasmic trafficking of tRNAs in response to nutrient stress is not evolutionarily conserved. (1) The first objective of this thesis is to investigate whether nutrient stress stimulates a blockage in nuclear export of tRNA or promotes nuclear re-import of mature cytoplasmic tRNAs from intronless and intron-containing pre-tRNAs. (2) The second objective is to investigate whether the regulation of nuclear-cytoplasmic tRNA trafficking in response to nutrient stress is evolutionarily conserved between different genera of yeasts. (3) The third objective is to identify signaling pathways controlling nuclear tRNA export in response to amino acid, nitrogen and glucose stress. In particular, we investigated whether the two opposing glucose sensing signaling pathways, PKA
and Snf1p, are involved in regulating tRNA trafficking during glucose starvation and if they are, delineate their possible mechanism of action.
2.0. Materials and Methods

2.1. Strains

Table 1: List of yeast strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. paradoxus</em></td>
<td>A4</td>
<td>Isolated from bark of <em>Quercus rubra</em> in Mont St-Hilaire, Quebec, Canada</td>
<td>Dr. E. Louis (Institute of Genetics, University of Nottingham, United Kingdom)</td>
</tr>
<tr>
<td><em>S. bayanus</em></td>
<td>DBY17007</td>
<td><em>MATa, hoΔ::KAN</em></td>
<td>Dr. G. van der Merwe (University of Guelph, Canada)</td>
</tr>
<tr>
<td><em>K. lactis</em></td>
<td>NK40</td>
<td><em>MATa, ade1 ade2 leu2 [k1', k2']</em></td>
<td>Dr. G. van der Merwe ATCC</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td>CHP428</td>
<td><em>h+ ura4-D18 leu1-32 ade6-M210 his7-366</em></td>
<td>Dr. G. van der Merwe</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>TB50a</td>
<td><em>MATa, his3, leu2, ura3, trp1, rme1 HMLa</em></td>
<td>Dr. G. van der Merwe</td>
</tr>
<tr>
<td></td>
<td>JH11-1c</td>
<td><em>MATa, his4, leu2, ura3, trp1, rme1 HMLa, TOR1-1</em></td>
<td>Dr. G. van der Merwe</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>BY4742</td>
<td><em>MATa, his3, leu2, lys2, ura3</em></td>
<td>Open biosystems</td>
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<tr>
<td><em>S. cerevisiae</em></td>
<td>SNF1-HA</td>
<td><em>MATa, his3, leu2, lys2, ura3, SNF1::HA-kanMX6</em></td>
<td>Dr. G. van der Merwe</td>
</tr>
<tr>
<td></td>
<td>sak1</td>
<td><em>MATa, his3, leu2, lys2, ura3, sak1::kanMX6</em></td>
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<tr>
<td></td>
<td>sch9</td>
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<tr>
<td></td>
<td>elm1</td>
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<tr>
<td></td>
<td>rim15</td>
<td><em>MATa, his3, leu2, lys2, ura3, rim15::kanMX6</em></td>
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<tr>
<td></td>
<td>UTP8-GFP</td>
<td><em>MATa, his3, leu2, met15, ura3, UTP8::GFP-HIS3</em></td>
<td>Invitrogen</td>
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<tr>
<td></td>
<td>LOS1-GFP</td>
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<td></td>
<td>MSN5-GFP</td>
<td><em>MATa, his3, leu2, met15, ura3, MSN5::GFP-HIS3</em></td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>SP1</td>
<td><em>MATa, his3, leu2, ura3, trp1, ade1, can1</em></td>
<td>Dr. I. Sadowski (University of British Columbia, Canada)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>RS13-58A-1</td>
<td><em>MATa, his3, leu2, ura3, trp1, ade1, can1, tpk1 w1, tpk2::HIS3, tpk3::TRP1</em></td>
<td>Dr. I. Sadowski</td>
</tr>
<tr>
<td></td>
<td>BY4743</td>
<td><em>MATa/MATa his3/his3, leu2 /leu2, met15/MET15, LYS2/lys2, ura3/ura3</em></td>
<td>Open biosystems</td>
</tr>
<tr>
<td></td>
<td>bcy1/bcy1</td>
<td><em>MATa/MATa his3/his3, leu2 /leu2, met15/MET15, LYS2/lys2, ura3/ura3, bcy1/bcy1::kanMX6</em></td>
<td>Open biosystems</td>
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</tbody>
</table>
2.2. Plasmids

The pYX242 plasmid containing the triose-phosphate promoter and termination sequence was obtained from Novagen. The MSN5 open reading frame (ORF) was amplified by PCR from *S. cerevisiae* genomic DNA. The pYX242-MSN5 plasmid was constructed by inserting the MSN5 ORF into the *NcoI* and *XmaI* sites of pYX242. The pYX242-UTP8, pYX242-LOS1, and pYX242-CEX1 plasmids were constructed as previously described (Steiner-Mosonyi *et al.*, 2003; McGuire and Mangroo, 2007). pRS315-RPL25 and pRS316-RPS2 were obtained from Dr. E. Hurt (University of Heidelberg, Germany). The plasmids YCp50, YCp50-RAS2, and YCp50-RAS2\textsuperscript{Val19} were provided by Dr. G. van der Merwe (University of Guelph, Canada). pET19b-LOS1, pET23d-MSN5, pET19b-UTP8, pET19b-CEX1, pET19b-UTP9 and pET23a-UTP22 were constructed as described previously (McGuire and Mangroo, 2007; Strub *et al.*, 2007; Eswara *et al.*, 2009).

2.3. Antibodies

**Table 2: List of antibodies used**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los1p</td>
<td>Rabbit polyclonal</td>
<td>Dr. E. Hurt</td>
</tr>
<tr>
<td>Msn5p</td>
<td>Rabbit polyclonal</td>
<td>Cedarlane (Custom)</td>
</tr>
<tr>
<td>Cex1p</td>
<td>Rabbit polyclonal</td>
<td>Invitrogen (Custom)</td>
</tr>
<tr>
<td>Utp8p</td>
<td>Rabbit polyclonal</td>
<td>Invitrogen (Custom)</td>
</tr>
<tr>
<td>Utp9p</td>
<td>Rabbit polyclonal</td>
<td>Cedarlane (Custom)</td>
</tr>
<tr>
<td>Nsp1p</td>
<td>Mouse monoclonal</td>
<td>MyBiosource (MBS130087)</td>
</tr>
<tr>
<td>HA</td>
<td>Rabbit polyclonal</td>
<td>Sigma</td>
</tr>
<tr>
<td>His</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz (G18)</td>
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<td>Actin</td>
<td>Mouse monoclonal</td>
<td>Abcam</td>
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<td>Phosphothreonine</td>
<td>Rabbit polyclonal</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Phospho-AMPKα Thr-172</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Mouse HRP conjugate</td>
<td>Sheep polyclonal</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Rabbit HRP conjugate</td>
<td>Donkey polyclonal</td>
<td>GE Healthcare</td>
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<tr>
<td>Mouse Alexa Fluor 488</td>
<td>Goat polyclonal</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Rabbit Alexa Fluor 594</td>
<td>Goat polyclonal</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
2.4. Nutrient Starvation

Cells were grown in synthetic complete media (SC) containing 2% glucose to mid-logarithmic phase at 30°C. The cells were washed twice with sterile water and resuspended in synthetic media lacking glucose, amino acids or nitrogen (media without amino acids and ammonium sulphate) and incubated at 30°C for the times indicated. Following glucose starvation, the culture was resupplemented with 2% glucose from a 40% glucose stock solution and incubated for an additional 20 min.

The *S. pombe* strain CHP428 was grown in complete Edinburgh minimal media (EMM) (MP Biomedicals) containing 2% glucose to mid-logarithmic phase at 30°C. The cells were washed twice with sterile water and resuspended in EMM lacking glucose, nitrogen (ammonium and amino acids) or amino acids and incubated at 30°C for the specified times.

2.5. Fluorescence in situ hybridization in *Saccharomyces* genus and *K. lactis*

Fluorescence *in situ* hybridization (FISH) for tRNA localization in strains of the *Saccharomyces* genus and *K. lactis* was performed as previously described (Grosshans *et al.*, 2000; Steiner-Mosonyi and Mangroo, 2004). Briefly, the cells from 50 ml of culture were fixed and treated with zymolyase-100T. The cells from each fixed sample were placed in multiple wells on poly-L-lysine (Sigma) coated slides. Pre-hybridization was performed at 37°C for 1 h in hybridization buffer (4 X SSC, 50% formamide, 10% dextran sulfate, 125 µg/ml *E. coli* tRNA, 500 µg/ml salmon sperm DNA, 0.5 units/µL RNasin, 10 mM DTT, and 1X Denhardt’s solution). Following pre-hybridization, the cells from each sample were probed with 5 pmol of 5’-end Cy3-labeled probe specific for either mature tRNA\(^{\text{Tyr}}\), tRNA\(^{\text{Gly}}\), tRNA\(^{\text{His}}\), or tRNA\(^{\text{Leu}}\) and the slides were incubated overnight at 37°C. A 5’-end Alexafluor 488-labeled 30-mer polydT oligonucleotide was used to detect mRNA. The slides were washed once with 2 X SSC, three
times with 1 X SSC and once with 0.5 X SSC for 5 min at room temperature. DNA was visualized by staining with DAPI. The slides were viewed under a 100X objective lens of a Nikon Eclipse 6600 microscope. Images were captured using the Coolsnapfx monochrome CCD digital camera (Roper Scientific) and processed using the Metamorph program (Universal Imaging). Quantification was performed by determining the percentage of cells showing nuclear accumulation from three independent fields of ~200 cells from two independent experiments. A p-value was calculated using Student’s two tailed paired t-test to determine whether the percentage was significantly different between two stains.

2.6. Fluorescence in situ hybridization in S. pombe

FISH was performed as previously described with the following modifications (Tani et al., 1996a). Cells were fixed for 15 min in 4% formaldehyde at 30°C in media before being resuspended in 0.1M Sörensen’s buffer (pH 6.0) containing 4% formaldehyde and fixed for 2.75 h at room temperature. Spheroplasts were formed by treating the cells for 20 min at 37°C with 1 mg/ml lysing enzyme (Sigma L1412) and 1 mg/ml Zymolyase 100T (MP Biomedicals) in PEMS buffer pH 6.9 (100 mM PIPES, 0.1 M MgCl₂, 1 mM EGTA, 1.2 M Sorbitol) and adhered to poly-L-lysine (Sigma) coated slides. Prehybridization was carried out at room temperature in hybridization buffer (4 X SSC, 50% formamide, 10% dextran sulphate, 125 μg/ml Escherichia coli tRNA, 500 μg/ml salmon sperm DNA, 0.5 U/μl RNasin (Promega) and 1×Denhardt’s solution) for 30 min followed by hybridization for 16-20 h at 42°C after the addition of 5 pmol of 5’-end Cy3-labelled probe specific for either mature tRNA^{Tyr}, tRNA^{His}, tRNA^{Gly}, intron-containing tRNA^{Tyr} or 5’-end fluorescein-labeled oligo dT. The slides were washed 3 times in 4 X SSC (20 min) with 5 μg of DAPI (Sigma) added to the third wash. This was followed by a 20 min wash in 2 X SSC and a 10 min wash in 1 X SSC. The slides were viewed using a Nikon.
Eclipse 6600 microscope with a 100X objective. Images were captured using the Coolsnapfx monochrome CCD digital camera (Roper Scientific) and processed using the Metamorph program (Universal Imaging).

2.7. Total RNA extraction from S. cerevisiae and K. lactis

The K. lactis and S. cerevisiae strains were grown in the specified media to OD₆₀₀ ~0.6-0.7 and total RNA was extracted as previously described (Cleary and Mangroo, 2000). Cells were harvested and the pellet was resuspended in 0.5 M NaCl, 0.2 M Tris-HCl pH 7.4, 10 mM EDTA. Glass beads (2 g) (Sigma) and an equal volume of phenol:chloroform were added to the suspension. The cells were lysed by vortexing the mixture 3 times for 30 sec with 30 sec on ice in between. The aqueous phase was removed following centrifugation at 1000 g for 10 min and the phenol was re-extracted with an equal volume of lysis buffer. The aqueous phase was extracted again with phenol:chloroform followed by an extraction with chloroform. The RNA was precipitated with 1/10 vol 3 M LiAc and 2.5 vol 95% EtOH overnight at -20°C. The precipitated RNA was pelleted at 4000 g for 30 min and resuspended in an appropriate volume of TE. Total RNA (5-10 µg) was loaded onto a 10% polyacrylamide gel containing 8M urea and separated using 1 X TBE. The RNA was transferred onto a Nytran Plus membrane and hybridization with 5’ end P³²-labelled probe specific for either tRNA_Tyr or tRNA_Gly was performed overnight at 37°C.

2.8. Total RNA isolation from S. pombe

The S. pombe strain CHP428 was grown in YES media (MP Biomedicals) to mid-logarithmic phase at 30°C. Total RNA extraction was performed as described previously (Lyne et al.). The cells were pelleted and snap frozen in a dry ice/ethanol bath. The pellet was thawed and washed once with DEPC treated water followed by resuspension in TES (10 mM Tris pH 7,
10 mM EDTA pH 8, 0.5% SDS). An equal volume of acidic phenol:chloroform, pH 5.2, (Fisher Scientific) was added and the suspension was incubated at 65°C for 1 h with vortexing for 10 sec every 10 min. The sample was cooled on ice for 1 min with 20 sec vortexing before centrifugation at 18000 g for 5 min at 4°C. Subsequently, the aqueous phase was extracted once more with an equal volume phenol:chloroform followed by an extraction with chloroform (Fisher Scientific). RNA was precipitated with 3 volumes of 95% ethanol and 75 mM NaAc, pH 5.2, overnight at -20°C. The precipitated RNA was washed once with 70% EtOH and dried before being resuspended in DEPC water. Total RNA (5 µg) was loaded onto a 10% or 15% polyacrylamide gel containing 8M urea and separated using 1 X TBE. Where indicated the gel was stained with ethidium bromide. The RNA was transferred onto a Nytran Plus membrane. Hybridization with 5’ end P³²-labelled probe specific for mature tRNA^Tyr, mature tRNA^Gly or intron-containing tRNA^Tyr was performed overnight at 42°C. The membrane was washed 4 times for 30 min with 3 X SET, 0.1% SDS at room temperature and subjected to autoradiography.
2.9. Oligonucleotides

Table 3: Oligonucleotides used for fluorescence in situ hybridization

<table>
<thead>
<tr>
<th>Species</th>
<th>tRNA</th>
<th>Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</td>
<td>5'-GCCAGTCGAACGCCGATCTCAAGATTTACGTCTTGGCCTTAAACCAACTTGGCTACC-3'</td>
</tr>
<tr>
<td></td>
<td>tRNA&lt;sub&gt;Gly&lt;/sub&gt;</td>
<td>5'-CGAACCCGGGGGGCCCAACGATGGAAGCTTGGATTTACC-3'</td>
</tr>
<tr>
<td></td>
<td>tRNA&lt;sub&gt;His&lt;/sub&gt;</td>
<td>5'-TCTTGAATCGAACCAGGTTTCATCGGCCCAACAAGGATGTGTACTAACCAACTTATTAG-3'</td>
</tr>
<tr>
<td></td>
<td>tRNA&lt;sub&gt;Leu&lt;/sub&gt;</td>
<td>5'-GCCATCTTACGATACCTGAGCTTGAATCGGCGC-3'</td>
</tr>
<tr>
<td><em>S. paradoxus</em></td>
<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</td>
<td>5'-GCCAGTCGAACGCCGATCTCAAGATTTACGTCTTGGCCTTAAACCAACTTGGCTACC-3'</td>
</tr>
<tr>
<td></td>
<td>tRNA&lt;sub&gt;Gly&lt;/sub&gt;</td>
<td>5'-CGAACCCGGGGGGCCCAACGATGGAAGCTTGGATTTACC-3'</td>
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<tr>
<td><em>S. bayanus</em></td>
<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</td>
<td>5'-GCCAGTCGAACGCCGATCTCAAGATTTACGTCTTGGCCTTAAACCAACTTGGCTACC-3'</td>
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<tr>
<td></td>
<td>tRNA&lt;sub&gt;Gly&lt;/sub&gt;</td>
<td>5'-CGAACCCGGGGGGCCCAACGATGGAAGCTTGGATTTACC-3'</td>
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<tr>
<td><em>K. lactis</em></td>
<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</td>
<td>5'-GCCAGTCGAACGCCGATCTCAAGATTTACGTCTTGGCCTTAAACCAACTTGGCTACC-3'</td>
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<td>5'-CGAACCCGGGGGGCCCAACGATGGAAGCTTGGATTTACC-3'</td>
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<td></td>
<td>tRNA&lt;sub&gt;Leu&lt;/sub&gt;</td>
<td>5'-GCCATCTTACGATACCTGAGCTTGAATCGGCGC-3'</td>
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<tr>
<td><em>S. pombe</em></td>
<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt;</td>
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<td>tRNA&lt;sub&gt;Tyr&lt;/sub&gt; intron*</td>
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<td>tRNA&lt;sub&gt;Gly&lt;/sub&gt;</td>
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<tr>
<td></td>
<td>tRNA&lt;sub&gt;His&lt;/sub&gt;</td>
<td>5'-GAATCGAACCAGCTTGGCAGATCAGCGCCCAACAAGGATGTGTACTAAC-3'</td>
</tr>
</tbody>
</table>

*Intron sequence displayed in lower case.

2.10. Immunofluorescence microscopy

The indicated strains were grown as described above and fixed at the required time points with 4% formaldehyde for 1 h at 30°C. Following fixation, 25 ml of the cell culture was harvested and the cells were washed 3 times with 0.1 M potassium phosphate, 1.2 M sorbitol pH 6.4. The cells were spheroplasted at 30°C for 30 min with 150 µg zymolyase 100T. The cells were washed once with 0.1 M potassium phosphate, 1.2 M sorbitol pH 6.4, and adhered on poly-L-lysine coated slides. The spheroplasts were dehydrated by immersing the slides in ice cold methanol for 5 min followed by ice cold ace tone for 30 sec. The slides were allowed to dry and the cells were rehydrated with 1 X PBS containing 1% BSA for 1 h at room temperature. The
cells were probed with the primary antibody for the protein of interest followed by AlexaFluor 488 or AlexaFluor 594 conjugated secondary antibodies (Invitrogen). The antibody incubations were performed for 1 h at room temperature. After incubation with each antibody, slides were washed 4 times for 5 min with 1 X PBS containing 1% BSA and mounted with mowial. The cells were viewed using a Nikon Eclipse 6600 microscope with a 100X objective lens. Images were captured using the CoolSnapfx monochrome CCD digital camera (Roper Scientific) and processed using the Metamorph program (Universal Imaging). To test specificity of secondary antibodies, the cells were probed with the AlexaFluor conjugated secondary antibodies in the absence of the primary antibodies. The fluorescent signal of the secondary antibodies was not detected in the absence of the primary antibodies.

2.11. mRNA Northern blot analysis

Total RNA was isolated as described above for S. cerevisiae. For the analysis of the expression level for HSP12, 10 µg of total RNA was separated by electrophoresis on a 1.2 % formaldehyde agarose gel using 1 X MOPS running buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0) at 80 V for 3 h. The RNAs were capillary transferred to a positively charged nylon membrane (Roche) with 20 X SSC overnight and UV crosslinked to the membrane. Prehybridization was performed by incubating the membrane with 15 ml of DIG Easy Hyb buffer (Roche) for 4 h at 50°C. DIG labeled probes specific for HSP12 and ACT1 were generated by PCR from genomic DNA (Novagen) using the Expand DNA polymerase (Roche) using gene specific primers and DIG DNA labeling mix containing DIG-11-dUTP. The probes were cleaned using a PCR clean-up kit (Qiagen) and denatured at 95°C for 5 min. The denatured probe was added to the membrane in 5 ml of DIG Easy Hyb buffer and incubated overnight at 50°C. The membrane was washed twice for 10 min at room temperature in low stringency buffer
(2 X SSC, 0.1% SDS) followed by 4 washes at 50°C in high stringency buffer (0.1 X SSC, 0.1% SDS). To detect the DIG labeled probe, the blot was blocked with blocking reagent (Roche) in 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 for 1 h at room temperature. The membrane was subsequently incubated with α-DIG-AP Fab fragments for 1 h and washed twice for 15 min with 0.1 M Maleic acid, pH 7.5, 0.15 M NaCl, 0.3% (v/v) Tween 20 followed by a 5 min wash in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Detection was conducted by autoradiography after the membrane was incubated for 5 min with CDP-Star solution.

2.12. Aminoacylation status of tRNA by Northern blot analysis

Cells were starved of glucose as described above and 1 l of cell culture was harvested at the appropriate time points. Nuclear and post-nuclear RNA was isolated under acidic conditions as previously described (Steiner-Mosonyi and Mangroo, 2004). Briefly, the cells were spheroplasted in 1.2 M sorbitol, 10 mM EDTA, 10 mM potassium phosphate, 0.1% 2-mercaptoethanol with 10 mg Zymolyase-100T for 25 min at 30°C. Spheroplasts were lysed using a Dounce homogenizer in 300 mM sodium acetate pH 5.0, 5 mM magnesium acetate, 0.1 % (v/v) NP-40 and 250 mM sucrose. The lysed cells were subjected to a sucrose gradient by overlaying the clarified lysate onto 300 mM sodium acetate pH 5.0, 5 mM magnesium acetate, 500 mM sucrose. After centrifugation at 8200 g for 20 min, total RNA was isolated from the nuclear and post-nuclear fractions with several acidic phenol and chloroform washes. The RNA was precipitated with ethanol and resuspended in 20 mM sodium acetate, pH 5.0.

Total RNA from the nuclear and post-nuclear fractions was separated by electrophoresis on a 6.5 % polyacrylamide containing 8 M urea at 500 V using 0.1 M sodium acetate buffer, pH 5.0, at 4°C. Marker for deacylated tRNA was made from a sample of RNA from each fraction by incubating total RNA in 200 mM Tris, pH 9.5 at 37°C for 1 h. Separated RNA was transferred
onto Nytran Plus membranes and prehybridized for 4 h at 37°C in hybridization buffer (4 X SET, 250 µg/ml salmon sperm DNA, 10 X Denhardt’s solution, and 0.1% SDS). Hybridization was conducted overnight at 37°C in hybridization buffer containing 5’-end 32P-labelled oligonucleotide (1-2 X 10⁶ cpm/ml). The membranes were washed 4 times for 30 min with 3 X SET, 0.1% SDS at room temperature and subjected to autoradiography.

2.13. Preparation of cell extract and Western blot analyses

Cells were grown in appropriate media to mid-logarithmic phase at 30°C and washed twice with sterile water. The cells were resuspended in media lacking glucose, nitrogen or amino acids. Cells from 25 ml of culture were pelleted and resuspended in NP-40 buffer (15 mM Na₂HPO₄, pH 7.2 buffer containing 10 mM NaH₂PO₄, 2% NP-40 (v/v), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, and complete, EDTA-free protease inhibitor cocktail (Roche), 1mM PMSF). The cells were lysed by vortexing 6 times for 30 sec with glass beads at 4°C followed by 30 sec on ice. The cell lysate was clarified by centrifugation at 21000 g for 10 min. Protein concentration was quantified using the Biorad DC protein assay and 20 µg of total protein from each sample was separated on a 4-12 % Novex Bis-Tris gel (Invitrogen) or 10% SDS-PAGE gel (Biorad). The proteins were transferred to Immobilon-P membrane (Millipore) and probed with the appropriate antibody in TBS-T with 0.25% skim milk or 0.5% BSA (phospho-antibodies). Subsequently, the membrane was incubated with horseradish peroxidase conjugated secondary antibodies and the signal detected with the ECL Western detection system (GE Healthcare).

2.14. Lambda phosphatase treatment of protein extracts

Cells from 25 ml of culture were pelleted and lysed in 25 mM Tris-HCl, pH 7.5 buffer containing 2% NP-40 (v/v), 150 mM NaCl, 2 mM EDTA, 1 mM MnCl₂ and EDTA-free ProteCEASE inhibitor cocktail (G biosciences), 1 mM PMSF with glass beads. Total protein (20
μg) was incubated with 40 U of lambda phosphatase (NEB) for 1 h at 30°C. The reaction was stopped by boiling for 10 min after the addition of NuPage loading dye and sample reducing agent.

2.15. Overexpression and purification of His tagged proteins

His affinity tagged Los1p, Msn5p, Utp8p, Utp9p, Utp22p, and Cex1p were purified as previously described (McGuire and Mangroo, 2007; Strub et al., 2007; Eswara et al., 2009). Briefly, *Escherichia coli* BL21 (DE3) Codon Plus RIL (Novagen) harbouring the pET expression vectors were grown in 1 l of 2YT medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C to an OD 600 ~0.4-0.5. The cells were incubated at 15°C for 15 h with 0.1-1.0 mM isopropyl β-D-thiogalactoside (IPTG) and harvested by centrifugation. The cells were resuspended in 30 ml of binding buffer (20 mM NaH₂PO₄, pH 7.5 containing 500 mM NaCl, 5 mM imidazole, protease inhibitor cocktail (EDTA-free; Roche)) and lysed with a French press at 70,000 kPa. The lysate was clarified at 27,000 g for 30 min before being applied onto a 1 ml HisTrap HP column (GE Healthcare). The columns were washed with 20 ml of 20 mM NaH₂PO₄, pH 7.5 buffer containing 500 mM NaCl and 50 mM imidazole and with 10 ml of 20 mM NaH₂PO₄, pH 7.5 buffer containing 500 mM KCl to remove tRNA. The proteins were eluted from the column with a gradient of increasing concentration of imidazole and dialyzed against IPP150 buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 % glycerol (w/v) and 0.1 % Nonidet P-40 (v/v)).

Los1p was further purified by gel filtration using a Superdex 200 16/60 column (GE Healthcare). Cex1p and Utp9p were further purified by loading the proteins dialyzed against 20 mM NaH₂PO₄, pH 7.5 buffer containing 100-260 mM NaCl onto a HiTrap Q HP ion exchange
column. The proteins were eluted with an increasing gradient of NaCl and dialyzed against IPP150, pH 7.5 containing 20 % glycerol (w/v) and stored at -80°C.

2.16. In vitro phosphorylation by PKA

Yeast recombinant protein was expressed and purified as described above. The catalytic subunit of bovine PKA was obtained from Sigma and reconstituted in 6 mg/ml DTT to a final concentration of 0.5 U/µL (Sigma P2645). His tagged recombinant protein was expressed and purified from *E. coli* BL21 (DE3) Codon Plus RIL cells as described above. The PKA phosphorylation assay was performed by combining 2 µg of recombinant protein with 1 U of bovine PKA (10 U/µg) and 5 µCi of $[^{32}\text{P}]$ATP in PKA phosphorylation buffer (50 mM potassium phosphate, pH 7.15, 5 mM NaF, 10 mM MgCl₂, 4.5 mM DTT, and both ProteCEASE and Phosphatase Arrest-I inhibitors (G biosciences)) to a total volume of 20 µL. A PKA substrate peptide (Sigma SCP0212) was used as a positive control for the phosphorylation reaction. The reactions were incubated for 30 min at 25°C before being terminated by the addition of SDS-PAGE sample buffer (NuPAGE, Invitrogen) and boiled for 10 min. The samples were separated on a 10% SDS-PAGE gel. The gel was dried and visualized using a Phosphor screen (Biorad).
3.0. Results

The results chapter is composed of two sections with section 3.1 detailing the investigation into the characterization of nuclear tRNA export in response to amino acid and nitrogen stress; the possible involvement of the TOR signaling pathway in regulating this response; and whether the regulation of nuclear tRNA export in response to nutrient stress is evolutionarily conserved. This data was previously published in *Mol Biol Cell*. 2009 Dec; 20 (23):5007-25 (Eswara et al., 2009) and *Nucleus*. 2010 May-Jun; 1 (3):224-30 (Pierce et al., 2010). The portion involving characterization of retrograde transport in various species of yeasts has been published in the journal *Mol. Biol. Cell* 2011 Sept; 22: 1091-1103 (Chafe et al., 2011) and *Biochem Cell Biol*. 2011 Dec; 89 (6):554-61 (Pierce and Mangroo, 2011). Only the work I contributed to each publication is included here.

In section 3.2, we identify the possible signaling pathways involved in regulating nuclear tRNA export in response to glucose starvation. In particular, we investigate the role of the PKA signaling pathway and its possible mechanism in regulating nuclear tRNA export during glucose starvation.
3.1. Nutrient stress affects nuclear export of tRNA in only yeast species of the Saccharomyces genus

3.1.1. Amino acid and nitrogen stress affects nuclear tRNA re-export of mature tRNAs made from intron-containing pre-tRNA in S. cerevisiae

Regulation of nuclear tRNA export has been linked to changes in glucose, nitrogen, amino acid or phosphate availability (Hurto et al., 2007; Whitney et al., 2007; Eswara et al., 2009). Nuclear accumulation of mature tRNAs derived from both intron-containing and intronless pre-tRNAs occurs upon depletion of glucose or amino acids (Whitney et al., 2007). These findings led others to propose that nutrient stress causes nuclear re-import of tRNAs in the cytoplasm (Whitney et al., 2007). Interestingly, the tRNA splicing complex in Saccharomyces cerevisiae has been found to be located on the cytoplasmic surface of the mitochondria (Yoshihisa et al., 2003). Consequently, tRNAs derived from intron-containing pre-tRNAs are exported to the cytoplasm for splicing and then imported back to the nucleus for further maturation and aminoacylation quality assurance (Yoshihisa et al., 2003; Ohira and Suzuki, 2011). Furthermore, recent reports have shown that newly spliced tRNAs are constitutively imported from the cytoplasm to the nucleus (Murthi et al., 2010). These findings combined with studies demonstrating that intronless pre-tRNAs are matured entirely in the nucleus of S. cerevisiae question the conclusion that nutrient stress results in retrograde transport of cytoplasmic tRNAs to the nucleus. An alternative explanation is that nutrient stress results in a block in nuclear re-export of mature spliced tRNAs made from intron-containing precursors, that were imported back into the nucleus for aminoacylation following splicing (retrogradely transported), and nuclear export of mature tRNAs derived from intronless pre-tRNAs. We therefore investigated whether nutrient stress causes nuclear re-import of cytoplasmic tRNAs, or
a block in nuclear export of tRNA. In addition, we tested whether regulation of the nuclear-cytoplasmic tRNA trafficking process by nutrient stress is evolutionarily conserved.

To verify that depletion of nitrogen or amino acids results in a general block in tRNA export, FISH using probes specific to mature tRNAs were employed to detect the nuclear-cytoplasmic distribution of mature tRNAs derived from both intron-containing and intronless precursors during amino acid starvation. The *S. cerevisiae* wild type BY4742 strain was grown in synthetic complete media and shifted to media lacking all amino acids. Consistent with previous reports (Whitney *et al.*, 2007), accumulation of mature spliced tRNA<sub>Tyr</sub> was observed after 30 min of amino acid deprivation and persisted over an additional 60 min period (Figure 8A). Surprisingly, tRNA<sub>Gly</sub> which is derived from an intronless precursor did not accumulate in the nucleus after 90 min of amino acid deprivation (Figure 8C). Furthermore, mature tRNA<sub>Leu</sub> (Figure 8B) derived from an intron-containing precursor but not tRNA<sub>His</sub> (Figure 8D) derived from an intronless pre-tRNA showed nuclear accumulation following amino acid starvation. The data indicates that amino acid starvation only affects nuclear re-export of mature retrogradely transported tRNAs derived from intron-containing precursor tRNAs (Chafe *et al.*, 2011). Thus, it is possible that nutrient stress such as amino acid deprivation may affect nuclear re-export of mature tRNAs made from intron-containing pre-tRNAs, but not nuclear export of mature tRNAs made from intronless precursors. Interestingly, the data also suggest that different pathways are used for nuclear export of mature tRNAs made from intronless pre-tRNAs and re-export of mature tRNAs derived from intron-containing precursors.
Figure 8. Amino acid deprivation affects nuclear re-export of retrograded tRNA derived from intron-containing precursor tRNAs but not tRNAs obtained from intronless precursor tRNA. The wild type BY4742 strain was grown in synthetic complete media to mid logarithmic phase before being transferred to media without amino acids. FISH analysis was used to monitor the localization of mature tRNA$^{\text{Tyr}}$ (A), tRNA$^{\text{Leu}}$ (B) tRNA$^{\text{Gly}}$ (C), and tRNA$^{\text{His}}$ (D). Arrows indicate cells displaying nuclear tRNA accumulation. The DNA was visualized by DAPI staining. Scale bar represents 5 µm.
To test whether the differential effect observed for nuclear export of tRNA is specific for amino acid starvation, cellular distribution of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} was also monitored during nitrogen starvation. \textit{S. cerevisiae} cells grown in synthetic complete media showed that tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} are distributed evenly throughout the cell. Cells starved of nitrogen (synthetic media without amino acids and ammonium sulfate) displayed nuclear accumulation of tRNA\textsuperscript{Tyr} (Figure 9A) but not tRNA\textsuperscript{Gly} (Figure 9B) over a 90 min time period (Eswara \textit{et al.}, 2009). Interestingly, further studies in our laboratory found that tRNA\textsuperscript{Ile(AAU)} derived from an intron-containing pre-tRNA but not tRNA\textsuperscript{Ile(UAU)} derived from an intronless precursor accumulated in the nucleus after amino acid starvation, providing strong evidence that only nuclear re-export of those mature tRNAs from intron-containing precursors are blocked during amino acid and nitrogen starvation (Chafe \textit{et al.}, 2011). Since studies have shown that nuclear re-import of newly spliced tRNAs occurs by a constitutive process (Murthi \textit{et al.}, 2010), these data suggest that amino acid and nitrogen starvation only affects nuclear re-export of mature retrograded tRNAs derived from intron containing pre-tRNAs and that this pathway may be regulated differently from that used to export mature tRNAs derived from intronless pre-tRNAs. The reason why our findings differ from those reported is not known. More importantly, these data are also consistent with our proposal that nutrient stress does not cause retrograde transport of cytoplasmic tRNAs to the nucleus.
Figure 9. Nitrogen stress blocks nuclear re-export of retrograded tRNA derived from intron-containing precursor tRNAs. The wild type BY4742 strain was grown in synthetic complete media to mid logarithmic phase before being transferred to media without amino acids and ammonium sulfate for the indicated times. Fluorescence in situ hybridization (FISH) analysis was used to monitor the location of mature tRNA$^{\text{Tyr}}$ (A), and tRNA$^{\text{Gly}}$ (B). Arrows indicate cells displaying nuclear tRNA accumulation. The DNA was visualized by using DAPI staining. Scale bar represents 5 µm.
3.1.2. Occurrence of nuclear retention of tRNA^His in the histidine, leucine auxotrophic S. cerevisiae strain starved of amino acids occurs later than that for tRNA^Tyr and tRNA^Leu

We have established that amino acid and nitrogen starvation over a 90 min period result in nuclear accumulation of tRNA^Tyr and tRNA^Leu made from intron-containing pre-tRNAs but not tRNA^His and tRNA^Gly made from intronless pre-tRNAs. However, it has previously been shown that inhibition of nuclear tRNA aminoacylation blocks nuclear export of tRNAs in both *Xenopus* oocytes and *S. cerevisiae* (Lund and Dahlberg, 1998; Grosshans *et al.*, 2000). Therefore, tRNA^His should eventually accumulate in the nucleus of the histidine, leucine, and lysine auxotrophic *S. cerevisiae* strain starved of amino acids due to a decrease in tRNA^His nuclear aminoacylation. To investigate the possibility that tRNA^His would accumulate in the nucleus of the histidine auxotrophic strain, FISH was used to monitor the nuclear-cytoplasmic distribution of tRNA^Tyr, tRNA^Leu, tRNA^Gly and tRNA^His over a 360 min period. Nuclear accumulation of tRNA^Tyr (Figure 10A) and tRNA^Leu (Figure 10B) was again observed after 30 min of amino acid starvation and this nuclear localization persisted for the entire 360 min time period. As expected, nuclear accumulation of tRNA^His was observed in the histidine auxotrophic strain but only after the cells had been deprived of amino acids for 120 min (Figure 10D). Conversely, tRNA^Gly did not accumulate in the nucleus following the 360 min amino acid starvation (Figure 10C) (Chafe *et al.*, 2011). These data are also consistent with previous reports that nuclear tRNA aminoacylation is required for efficient nuclear export of tRNAs (Lund and Dahlberg, 1998; Grosshans *et al.*, 2000; Steiner-Mosonyi and Mangroo, 2004). Furthermore, the later onset of nuclear accumulation of tRNA^His compared to the tRNAs derived from intron-containing precursors suggest that the effect is due to a lack of nuclear tRNA aminoacylation and not a general inhibition of nuclear tRNA export. Moreover, these findings suggest that amino acid or nitrogen starvation only affects nuclear re-export of retrograded mature tRNAs made
from intron-containing precursors. This effect is unlikely to be related to a defect in nuclear aminoacylation of the tRNAs made from intron-containing precursors, as a block in nuclear export of tRNA\textsuperscript{Tyr} occurred considerably sooner than tRNA\textsuperscript{His} in the leucine, histidine auxotrophic strain, and that the aminoacylation status of mature tRNA\textsuperscript{Tyr} from intron-containing precursors was reported be unaffected during amino acid deprivation over a 60 min period (Whitney et al., 2007).
Figure 10. Nuclear retention of tRNA^{His} in the histidine, leucine auxotrophic strain occurs later than retention of tRNA^{Tyr} or tRNA^{Leu} during amino acid starvation. *S. cerevisiae* cells were grown in complete minimal media to mid-logarithmic phase before being transferred to media lacking all amino acids. FISH analysis was used to monitor the localization of mature tRNA^{Tyr} (A), tRNA^{Leu} (B) tRNA^{Gly} (C), and tRNA^{His} (D) during the 360 min time course. Arrows indicate cells displaying nuclear tRNA accumulation. The DNA was visualized by DAPI staining. Scale bar represents 5 μm.
3.1.3. **Nuclear retention of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Leu} in the histidine, leucine auxotrophic S. cerevisiae strain starved of histidine occurs later than that for tRNA\textsuperscript{His}**

Reports have demonstrated that removal of a single amino acid from the growth media, inhibition of an aminoacyl-tRNA synthetase, or inhibition of nuclear import of a catalytically active aminoacyl-tRNA synthetase results in nuclear retention of the cognate tRNA due to a block in aminoacylation of the tRNA in the nucleus (Sarkar \textit{et al.}, 1999; Grosshans \textit{et al.}, 2000; Azad \textit{et al.}, 2001; Steiner-Mosonyi and Mangroo, 2004). To verify further that the retention observed for tRNA\textsuperscript{His} during amino acid starvation was due to a reduction in aminoacylation of the tRNA in the nucleus, the histidine, leucine, and lysine auxotrophic strain was starved of only histidine and FISH was used to monitor the cellular distribution of tRNA\textsuperscript{Tyr}, tRNA\textsuperscript{Leu}, tRNA\textsuperscript{Gly} and tRNA\textsuperscript{His}. The cells were grown in synthetic complete media to exponential growth and transferred to media lacking only histidine. Consistent with amino acid starvation, nuclear accumulation of tRNA\textsuperscript{His} was observed after 120 min of histidine starvation (Figure 11D). Nuclear retention of tRNA\textsuperscript{Gly} was not seen for the duration of the 240 min time course (Figure 11C). Interestingly, nuclear accumulation of tRNA\textsuperscript{Tyr} (Figure 11A) and tRNA\textsuperscript{Leu} (Figure 11B) was observed after 240 min of histidine starvation (Chafe \textit{et al.}, 2011). These data also suggest that nuclear accumulation of tRNA\textsuperscript{His} was a result of a decrease in nuclear export due to a lack of aminoacylation of the tRNA in the nucleus. Furthermore, the appearance of nuclear accumulation of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Leu} when cells are starved of only histidine indicate that nuclear export of mature retrogradedly transported tRNAs from intron-containing pre-tRNAs are regulated in response to nutrient starvation.
Figure 11. Nuclear accumulation of tRNA^{His} occurs prior to retention of tRNA^{Tyr} or tRNA^{Leu} in the histidine, leucine auxotrophic strain starved of only of histidine. *S. cerevisiae* cells were incubated at 30°C in minimal media lacking histidine. FISH analysis was used to monitor the localization of mature tRNA^{Tyr} (A), tRNA^{Leu} (B), tRNA^{Gly} (C), and tRNA^{His} (D) at the indicated times for a 240 min time period. Arrows indicate cells displaying nuclear tRNA accumulation. DAPI staining of the DNA was used to visualize the location of the nucleus. Scale bar represents 5 µm.
3.1.4. Overexpression of the tRNA export receptor, Los1p, restores export of retrograded tRNAs during nitrogen starvation

Studies in our laboratory have shown that components of the nuclear tRNA export machinery are specifically involved in the export of either mature tRNAs derived from intron-containing precursors or mature tRNAs derived from intronless pre-tRNAs. For instance, depletion of the essential nucleolar protein, Utp9p, resulted in nuclear accumulation of tRNAs made only from intron-containing precursors (Eswara et al., 2009). Utp9p was also found to interact specifically with the export receptor Msn5p, but not with the export receptor Los1p. These data suggest that Utp9p along with Msn5p are involved in a pathway specific for the nuclear re-export of mature spliced tRNAs derived from intron-containing pre-tRNAs. However, the function of Msn5p is not essential and it has been previously reported that deletion of the export receptor Los1p, also results in retention of tRNAs derived from both intron-containing and intronless pre-tRNAs (Murthi et al., 2010). These findings suggest that nuclear re-export of retrogradedly transported mature tRNAs from intron-containing pre-tRNAs are facilitated by several pathways.

Previous studies have shown that a variety of stresses affect the function of nuclear import/export receptors in S. cerevisiae (Kodiha et al., 2004; Quan et al., 2007; Kodiha et al., 2008; Crampton et al., 2009). It is therefore possible that amino acid or nitrogen starvation is affecting the activity of the nuclear tRNA export receptors. To test this possibility, we investigated whether overexpression of Los1p restores nuclear export of tRNA^{Tyr} during nitrogen starvation. The S. cerevisiae BY4742 strain transformed with pYX242 (Figure 12A), pYX242-UTP8 (Figure 12B) or pYX242-CEX1 (Figure 12C) displayed nuclear accumulation of tRNA^{Tyr} following nitrogen starvation for 90 min. Overexpression of Los1p, however, restored nuclear re-export of tRNA^{Tyr} as indicated by the uniform nuclear-cytoplasmic distribution of tRNA^{Tyr} in
cells deprived of nitrogen (Figure 12D) (Pierce et al., 2010). The data confirm that Los1p is also involved in the nuclear re-export of retrogradely transported tRNAs made from intron-containing precursors, and suggest that amino acid or nitrogen starvation is, in part, affecting the function of the nuclear tRNA export receptors.

**Figure 12.** Nitrogen starvation appears to affect the activity of Los1p. The BY4742 strain containing pYX242 (A), pYX242-UTP8 (B), pYX242-CEX1 (C), or pYX242-LOS1 (D) was grown to mid-log phase and starved of nitrogen by incubating the cells in media without amino acids or ammonium sulfate. FISH analysis was used to monitor the localization of mature tRNA^Tyr_. Arrows indicate cells displaying nuclear tRNA accumulation. DAPI staining of the DNA was used to indicate the position of the nucleus. Scale bar represents 5 µm.
3.1.5. Nitrogen starvation does not affect the function of the tRNA export receptor, Los1p, by altering its cellular location

Various studies have shown that different stress conditions can affect the activity of export receptors by causing a change in their cellular location. For instance, Los1p has been shown to locate to the cytoplasm following DNA damage, heat shock and the switch to ethanol containing media (Quan et al., 2007; Ghavidel et al., 2007). Therefore, fluorescence microscopy was used to determine whether nitrogen starvation affects the cellular location of Los1p, thereby compromising its ability to participate in nuclear tRNA export. The Los1p-GFP strain was grown in synthetic complete media to mid log phase before it was transferred to media lacking nitrogen. The localization of Los1p-GFP was monitored by fluorescence microscopy at 30 min intervals over a 90 min time period. Los1p-GFP was found to be located at the NPC in fed and nitrogen starved cells at 30 min. Los1p-GFP remained at the NPC for an additional 60 min of nitrogen starvation (Figure 13). These data suggest that nitrogen deprivation does not affect nuclear tRNA export and the activity of the export receptor by altering the cellular location of Los1p at the NPC.
Figure 13. Nitrogen starvation does not affect the location of Los1p. The Los1p-GFP strain was grown to mid log phase in synthetic complete media to an OD$_{600}$~0.6. The cells were transferred to media lacking nitrogen (without amino acids and ammonium sulfate) and incubated at 30°C for the times indicated. Fluorescence microscopy was used to monitor the location of Los1p-GFP. Scale bar represents 5 µm.
3.1.6. Inhibition of the TOR signaling pathway affects nuclear export of tRNA from intron-containing pre-tRNA in *S. cerevisiae*

The TOR signaling pathway has been shown to be a central controller for cellular response to changes in nutrient availability. The inhibition of TORC1 with rapamycin simulates characteristics that are found in nitrogen deprived cells including cells arresting in G0, an increase in the expression of genes required for usage of poor nitrogen sources and a decrease in protein synthesis and ribosome biogenesis (Crespo, *et al.* 2002, Schmelzle, *et al.* 2000, Brown, *et al.* 1994). Therefore, we investigated whether TORC1 was involved in regulating nuclear re-export of retrogradedly transported tRNAs derived from intron-containing pre-tRNAs as observed in nitrogen starved cells. The *S. cerevisiae* TB50a strain was grown in YPD and treated with either 200 ng/ml rapamycin or the drug vehicle (90% ethanol 10% Tween-20). FISH was performed on samples taken at 15 min intervals for 60 min to monitor the nuclear-cytoplasmic distribution of tRNAs. We found that tRNA\textsuperscript{Tyr} (Figure 14A) and tRNA\textsuperscript{Gly} (Figure 14C) were distributed throughout the cell when the culture was treated with the drug vehicle for the 60 min time period. Treatment with rapamycin for 45 min to 60 min resulted in nuclear retention of tRNA\textsuperscript{Tyr} (Figure 14B), a tRNA derived from an intron-containing pre-tRNA, but not tRNA\textsuperscript{Gly}, a tRNA derived from an intronless pre-tRNA (Figure 14D). In addition, mature tRNA\textsuperscript{Tyr} was found to be evenly distributed in the cell in the presence or absence of rapamycin in the rapamycin-insensitive strain (Figure 15) (Pierce *et al.*, 2010). In contrast to previous studies (Whitney *et al.*, 2007), these results suggest that TORC1 may be involved in the regulation of nuclear re-export of retrogradedly transported tRNAs derived from intron-containing pre-tRNAs in response to nitrogen availability. While the mechanism by which TORC1 regulates nuclear re-export of retrogradedly transported tRNAs is unknown, it is possible that this signaling pathway
is controlling the activity of the nuclear tRNA export receptors. Furthermore, it is not known why these findings differ from those reported by others (Whitney et al., 2007).

**Figure 14.** Inhibition of TORC1 with rapamycin inhibits nuclear re-export of mature tRNA derived from intron-containing precursors but not nuclear export of tRNAs derived from intronless pre-tRNAs. The TB50a strain was grown to an OD$_{600}$ of 0.6-0.8 in YPD at 30°C and treated with 200 ng/ml rapamycin (+ rap) or the same volume of the drug vehicle (-rap) (10 % Tween20 and 90 % ethanol). Both control and rapamycin treated cells were allowed to continue growing for the specified times at 30°C until fixed with 4 % formaldehyde. FISH analysis was performed using 5 pmol of Cy3-labelled oligonucleotide specific for mature (A and B) tRNA$^{Tyr}$ or (C and D) tRNA$^{Gly}$. DAPI staining was performed to visualize the DNA. Cells were visualized using 100X objective lens of a Nikon Eclipse 6600 microscope. Images were captured using CoolSnapfx monochrome CCD digital camera and processed using Metamorph. Nuclear accumulation of tRNA is indicated by the arrow.
Figure 15. Nuclear tRNA export is not affected by rapamycin treatment in the rapamycin-insensitive strain JH11-1c. FISH was performed on cells treated with 200 ng/ml rapamycin (B) or the same volume of drug vehicle (A), consisting of 10% Tween20 and 90% ethanol, in YPD at 30°C. FISH analysis was performed using 5 pmol Cy3-labelled tRNA Tyr. DAPI staining was used to visualize the DNA. Images were captured using a Nikon Eclipse 6600 microscope and Coolsnapfx monochrome CCD digital camera and processed using Metamorph. Scale bar represents 5 µm.

Nuclear tRNA aminoacylation has been shown to be required for efficient export of mature tRNA from the nucleus. Aminoacylation can only occur with fully processed and properly matured tRNAs and is therefore part of a nuclear quality control mechanism. Consequently, Northern blot analysis was used to determine whether the TOR signaling pathway is affecting nuclear re-export of tRNAs made from intron-containing precursors by inhibiting nuclear aminoacylation of tRNA Tyr. Cells treated with rapamycin or the drug delivery vehicle were fractionated and total RNA from the nuclear and cytoplasmic fractions were isolated under acidic conditions to preserve the ester bond between the tRNA and the amino acid. The RNA was separated with a urea PAGE gel, transferred to Nytran Plus membranes and mature tRNA Tyr was detected with a 32P-end labelled oligonucleotide (Figure 16). A base treated sample of RNA
served as a marker for deacylated tRNA\textsuperscript{Tyr} (lanes 3 and 6). Treatment with the drug vehicle or rapamycin did not affect the ability of tRNA\textsuperscript{Tyr} to be aminoacylated in the cytoplasm (lanes 1 and 2) or nucleus (lanes 4 and 5) (Pierce \textit{et al.}, 2010). Consistent with previous reports that amino acid starvation does not affect aminoacylation of tRNA\textsuperscript{Tyr} (Whitney \textit{et al.}, 2007), the TOR signaling pathway also does not prevent nuclear tRNA aminoacylation. Therefore, the TOR signaling pathway is regulating nuclear re-export of mature retrogradedly transported spliced tRNAs after the aminoacylation quality control step. Importantly, these data establish that nuclear accumulation of retrogradedly transported tRNAs made from intron-containing pre-tRNAs by nutrient stress is due to a block in the nuclear re-export of retrograded tRNAs.

\textbf{Figure 16.} TORC1 is not regulating nuclear tRNA export by inhibiting tRNA aminoacylation. Total RNA was isolated from the TB50a strain under acidic conditions following treatment with 200 ng/ml rapamycin or the drug vehicle for 1 hr. Total RNA from nuclear and post-nuclear fractions were separated on a 6.5 % polyacrylamide gel containing 8 M urea and transferred to Nytran Plus membranes for Northern blot analysis to detect the aminoacylation status of tRNA\textsuperscript{Tyr}. Deacylated tRNAs were prepared by incubating total RNA from each fraction for 1 h at 37 °C with 100 mM Tris, pH 9.5.
3.1.7. *Amino acid and nitrogen starvation does not affect nuclear export of tRNA from intron-containing pre-tRNAs in S. pombe*

Data from this study established that nutrient stress such as amino acid deprivation results in a block in nuclear re-export of the retrogradely transported tRNA following aminoacylation. Also, studies reported indicate that amino acid starvation of rat hepatoma cells resulted in nuclear accumulation of tRNAs made from intron-containing pre-tRNAs (Shaheen et al., 2007). Interestingly, parallel studies in our laboratory indicate that nuclear-cytoplasmic tRNA trafficking in mammalian and plant cells is not affected by nutrient stress (Chafe et al., 2011; Johnstone et al., 2011b). These findings suggest that regulation of nuclear re-export of tRNAs made from intron-containing precursors in response to nutrient stress is either unique to *S. cerevisiae* alone or is a characteristic of all yeasts. As a result, we investigated whether other species of yeast have the ability to regulate nuclear tRNA export in response to nutrient stress. To test this possibility, we investigated the effect of amino acid or nitrogen deprivation on nuclear export of mature tRNAs derived from intron-containing pre-tRNAs in the fission yeast, *Schizosaccharomyces pombe*. While it is not known whether *S. pombe* carries out tRNA splicing in the cytoplasm, genome-scale localization studies have shown that several components of the tRNA splicing machinery are located in the cytoplasm (Matsuyama et al., 2006).

Cellular response to nutrient stress results in changes in the phosphorylation status of various proteins involved in signaling cascades. Therefore, to verify that amino acid or nitrogen starvation has an effect on *S. pombe* cells, the phosphorylation status of proteins phosphorylated at threonine residues was monitored by Western blot analysis using an α-phosphothreonine antibody (Figure 17A). Several phosphorylated proteins were detected in cell lysate prepared from *S. pombe* cells grown in complete Edinburgh minimal media (EMM) (lane 2). Cells subjected to amino acid (lane 3) or nitrogen deprivation (lane 4) for 120 min resulted in
increased phosphorylation of several proteins. Actin levels in the cell lysate show that approximately the same amounts of cell extracts were separated by SDS-PAGE. The results indicate that *S. pombe* cells respond to nitrogen or amino acid starvation.

FISH has previously been used to detect nuclear retention of mRNA in *S. pombe* cells subjected to heat shock (Tani *et al.*, 1996b). To confirm that the FISH conditions used are capable of detecting nuclear RNA, mRNA was detected using an oligo-dT probe in the nucleus of *S. pombe* cells subjected to heat shock at 42°C for 180 min (Figure 17C). To test whether tRNA can be detected in the nucleus of *S. pombe* cells by FISH, we monitored the cellular distribution of mature tRNA$_{\text{Tyr}}$ and the intron-containing pre-tRNA$_{\text{Tyr}}$. Northern blot analysis confirmed that the probes used for detection of mature and intron-containing tRNA$_{\text{Tyr}}$ detects the correct form in total RNA isolated from *S. pombe* cells (Figure 17B). It has been shown that intron-containing pre-tRNAs in *S. cerevisiae* are predominantly located in the nucleus. Consistent with these reports, the intron-containing pre-tRNA$_{\text{Tyr}}$ was detected in the nucleus of *S. pombe* cells while mature tRNA$_{\text{Tyr}}$ was predominantly cytoplasmic (Figure 17D). Therefore, the FISH method used is capable of detecting nuclear RNA in *S. pombe* (Pierce and Mangroo, 2011).
Figure 17. Amino acids or nitrogen deprivation affects *Schizosaccharomyces pombe*, and FISH was able to detect nuclear RNA in *S. pombe*. (A) Nutrient starvation caused increased phosphorylation of proteins at threonine residues. Cell extract was prepared from *S. pombe* cells grown in complete EMM (lane 2) and in EMM containing glucose but lacking amino acids (lane 3) or nitrogen (lane 4) for 2 h. Western blot analysis was used to detect proteins phosphorylated at threonine residues with α-phosphothreonine. Actin was used as a loading control. (B) The oligonucleotide used for FISH detects the corresponding tRNA by Northern blot analysis. Total RNA was isolated from *S. pombe* and 25 µg of RNA was separated by PAGE. The separated RNAs were detected by ethidium bromide staining of the gel and transferred to Nytran Plus membranes. The blot was probed with a 32P-labeled oligonucleotide complementary to mature or intron-containing tRNA Tyr. (C) FISH detects mRNA in the nucleus of *S. pombe* subjected to heat shock. The cells were incubated in complete EMM at 30 °C or 42 °C for 3 h, and FISH was used to detect the cellular location of mRNA. The arrows indicate the location of nuclei. (D) FISH detects intron-containing pre-tRNA Tyr but not mature tRNA Tyr in the nucleus of fed *S. pombe* cells. The cellular location of the mature tRNA and intron-containing pre-tRNA was monitored by FISH. DNA was visualized by DAPI staining. Scale bar represents 5 μm.
In contrast to *S. cerevisiae*, nuclear accumulation of tRNA$^{\text{Gly}}$, derived from an intronless pre-tRNA, or tRNA$^{\text{Tyr}}$, derived from an intron-containing pre-tRNA, was not observed in *S. pombe* cells starved of amino acids (Figure 18A) or nitrogen (Figure 18B) over a 120 min period. In addition, inhibition of aminoacylation of tRNA$^{\text{His}}$ in the *S. pombe* histidine auxotrophic strain by histidine starvation for 8 h resulted in nuclear accumulation of tRNA$^{\text{His}}$ (Figure 19A) but not tRNA$^{\text{Tyr}}$ (Figure 19B). This finding also verifies that FISH is capable of detecting nuclear accumulation of tRNA in *S. pombe* (Pierce and Mangroo, 2011). These results also suggest that aminoacylation is required for efficient nuclear tRNA export in *S. pombe* but, more importantly, that amino acid or nitrogen starvation does not block nuclear export of tRNAs made from intron-containing pre-tRNA. Since *S. pombe* is a fission yeast, these results suggest that regulation of nuclear export of mature tRNAs made from intron-containing pre-tRNAs may be limited to *S. cerevisiae* and other budding yeasts.
Figure 18. Amino acid or nitrogen deprivation does not affect nuclear export of tRNAs made from intronless precursors or tRNAs derived from intron-containing precursor tRNAs in *Schizosaccharomyces pombe*. *S. pombe* cells were grown to mid-log phase in synthetic complete media and then incubated in synthetic media without amino acids (A) or nitrogen (B). FISH was used to detect the localization of mature tRNA\textsubscript{YR} and tRNA\textsubscript{Gly} at the indicated times. Arrows indicate location of the nuclei visualized by DAPI staining of the DNA. Scale bar represents 5 µm.
Figure 19. Histidine starvation of the histidine, leucine auxotrophic *S. pombe* strain results in nuclear accumulation of mature tRNA$^\text{His}$ but not tRNA$^\text{Tyr}$. The *S. pombe* strain, CHP428 was incubated at 30°C in Edinburgh minimal media lacking histidine for 8 h. FISH analysis was used to monitor the localization of mature tRNA$^\text{His}$ (A) and tRNA$^\text{Tyr}$ (B). Arrows indicate the location of the nucleus visualized by DAPI staining. Scale bar represents 5 µm.
3.1.8. Amino acid starvation affects nuclear export of tRNA from intron-containing pre-tRNA in Saccharomyces genus but not K. lactis

To test whether regulation of nuclear export of mature tRNAs derived from intron-containing pre-tRNAs is specific for budding yeast or perhaps just to species of Saccharomyces, we investigated the effect of amino acid deprivation on nuclear-cytoplasmic trafficking of tRNA\textsuperscript{Tyr} in Kluyveromyces lactis, and two closely related species of Saccharomyces: S. bayanus and S. paradoxus (Figure 20). K. lactis is a Crabtree-negative budding yeast that differs from S. cerevisiae by not performing aerobic fermentation but aerobic respiratory growth (Gonzalez-Siso et al., 2000). FISH was used to monitor the distribution of tRNA\textsuperscript{Gly} and tRNA\textsuperscript{Tyr} during amino acid starvation. Nuclear accumulation of tRNA\textsuperscript{Gly} was not observed in K. lactis upon depletion of amino acids. In contrast to S. cerevisiae, amino acid deprivation of K. lactis cells did not affect nuclear-cytoplasmic distribution of tRNA\textsuperscript{Tyr} (Figure 21A). Northern blot analysis verified that the oligonucleotides used are able to detect mature tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} in total RNA isolated from K. lactis (Figure 21B). Furthermore, leucine starvation of the leucine auxotrophic K. lactis strain resulted in nuclear accumulation of tRNA\textsuperscript{Leu} but not tRNA\textsuperscript{Tyr} (Figure 21C) verifying that we are able to detect nuclear tRNA in K. lactis (Chafe et al., 2011). These data suggest that a decrease in nuclear tRNA aminoacylation results in reduced efficiency of nuclear tRNA export. In addition, these finding suggest that regulation of nuclear export of mature spliced tRNA is not specific for budding yeasts, but that it may be limited to yeasts of the genus Saccharomyces.
Figure 20. Phylogenetic tree demonstrating the evolutionary relationship between different species of yeast. The phylogenetic tree was generated from the comparison of 42 sequenced fungal genomes using the matrix representation with parsimony method (Fitzpatrick et al., 2006). Species of yeast used in this study are highlighted in red.
Figure 21. Amino acid deprivation does not affect nuclear export of tRNAs from intronless precursors or intron-containing precursor tRNAs in *Kluyveromyces lactis*. (A) *K. lactis* cells were grown to mid-log phase in synthetic complete media and then incubated in synthetic media without amino acids. FISH was used to detect the localization of mature tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} at the indicated times. (B) Northern blot analysis confirms that the oligonucleotides used are able to detect tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} in total RNA isolated from *K. lactis*. (C) Nuclear accumulation of mature tRNA\textsuperscript{Leu} but not tRNA\textsuperscript{Tyr} was observed in the leucine auxotrophic *K. lactis* strain deprived of leucine. The *K. lactis* strain, NK40, was grown to mid-log phase in synthetic complete media and transferred to media containing glucose but lacking leucine for 4 h. FISH analysis was used to monitor the localization of mature tRNA\textsuperscript{Leu} and tRNA\textsuperscript{Tyr}. Arrows indicate the location of the nuclei visualized by DAPI staining of the DNA. Scale bar represents 5 µm.
To determine whether regulation of nuclear export of tRNAs made from intron-containing precursors by nitrogen or amino acid starvation is exclusively a characteristic of all species of the *Saccharomyces* genus or is specific to *S. cerevisiae*, the cellular distribution of tRNA\(^{\text{Gly}}\) and tRNA\(^{\text{Tyr}}\) was detected by performing FISH analysis in *S. bayanus* and *S. paradoxus*. Cells were grown in synthetic complete media and transferred to media lacking all amino acids for the indicated times. As expected based on results from *S. cerevisiae*, nuclear accumulation of tRNA\(^{\text{Gly}}\) was not observed following amino acid starvation in either *S. paradoxus* (Figure 22A) or *S. bayanus* (Figure 22B). In contrast, nuclear accumulation of tRNA\(^{\text{Tyr}}\) was found in both *S. bayanus* (Figure 22A) and *S. paradoxus* (Figure 22B) cells starved of amino acids (Chafe et al., 2011). This finding suggests that regulation of nuclear export of tRNAs derived from intron-containing pre-tRNAs in response to nutrient availability may be limited to yeasts of the genus *Saccharomyces*. 
Figure 22. Amino acid deprivation causes nuclear retention of mature tRNA derived from intron-containing precursor but not tRNAs obtained from intronless precursor tRNA in *S. paradoxus* and *S. bayanus*. Cells were grown in synthetic complete media to mid logarithmic phase and then incubated in media containing glucose but lacking all amino acids. FISH analysis was used to monitor the localization of mature tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} in *S. paradoxus* (A) and *S. bayanus* (B). Arrows indicate cells displaying accumulation of tRNA in the nucleus. The DNA was visualized by DAPI staining. Scale bar represents 5 µm.
3.1.9. Glucose starvation affects nuclear tRNA export in species of the Saccharomyces genus but not in K. lactis and S. pombe

Changes in glucose availability elicits a global response in \textit{S. cerevisiae} including an alteration in the nuclear-cytoplasmic distribution of tRNAs. In particular, glucose starvation of \textit{S. cerevisiae} cells causes a general block in nuclear export of mature tRNAs derived from both intron-containing and intronless pre-tRNAs (Whitney \textit{et al.}, 2007). However, nuclear accumulation of tRNAs was not observed in mammalian and plant cells starved of glucose and sucrose respectively (Chafe \textit{et al.}, 2011; Johnstone \textit{et al.}, 2011b). Consequently, we investigated whether nuclear-cytoplasmic trafficking is affected during glucose starvation in all yeasts or is a characteristic of species of the \textit{Saccharomyces} genus as seen with amino acid starvation. The cellular location of tRNA^{Tyr} and tRNA^{Gly} in each species was monitored by FISH after cells were switched from synthetic complete media to media lacking glucose for the indicated times. Glucose deprivation for 60 min resulted in nuclear accumulation of tRNA^{Tyr}, tRNA^{Leu}, tRNA^{Gly} and tRNA^{His} in \textit{S. cerevisiae} (Figure 23). Similarly, nuclear accumulation of both tRNA^{Tyr} and tRNA^{Gly} were observed in \textit{S. paradoxus} (Figure 24A) and \textit{S. bayanus} (Figure 24B) after 30 min of glucose starvation. Retention was also observed for an additional 60 min in each of the three \textit{Saccharomyces} species. In contrast, glucose deprivation did not affect nuclear-cytoplasmic trafficking of tRNA^{Tyr} and tRNA^{Gly} in \textit{K. lactis} over a 90 min period (Figure 24C). Furthermore, glucose starvation of \textit{S. pombe} cells did not result in nuclear accumulation of tRNA^{Tyr} and tRNA^{Gly} after 120 min (Figure 24D) (Pierce and Mangroo, 2011; Chafe \textit{et al.}, 2011). These findings indicate that the general block in nuclear tRNA export in response to glucose starvation is common to species of the \textit{Saccharomyces} genus. Taken together, these findings suggest that like plant and mammalian cells, \textit{K. lactis} and \textit{S. pombe} do not block nuclear export of tRNAs in response to nutrient stress, and that the regulation of the nuclear tRNA export process is not
evolutionarily conserved. However, it is not understood why *Saccharomyces* species, but not other yeasts and higher eukaryotes regulate nuclear tRNA export in response to nutrient stress. Furthermore, it is also not known why the pathways that facilitate nuclear export of mature tRNAs derived from intron-containing precursors but not the pathway responsible for nuclear export of mature tRNAs made from intronless pre-tRNAs are regulated by nitrogen or amino acid starvation in *Saccharomyces* species. Moreover, it is unclear why amino acid or nitrogen deprivation only affects nuclear export of mature tRNAs from intron-containing pre-tRNAs whereas glucose deprivation affects nuclear export of tRNAs made from intron-containing and intronless precursors.
Figure 23. Glucose deprivation causes nuclear retention of tRNA from both intron-containing and intronless precursor tRNAs in *S. cerevisiae*. Cells were grown in synthetic complete media to mid logarithmic phase and then incubated in media lacking glucose for 60 min at 30°C. Cells were resupplemented with 2 % glucose for 20 min. FISH analysis was used to monitor the localization of mature tRNA\textsuperscript{Tyr} (A), tRNA\textsuperscript{Leu} (B), tRNA\textsuperscript{His} (C), and tRNA\textsuperscript{Gly} (D). Arrows indicate cells displaying accumulation of tRNA in the nucleus. The DNA was visualized by DAPI staining. Scale bar represents 5 µm.
Figure 24. Glucose deprivation causes nuclear retention of tRNA from both intron-containing and intronless precursor tRNAs in *S. paradoxus* and *S. bayanus* but not in *S. pombe* and *K. lactis*. Cells were grown in synthetic complete media to mid logarithmic phase and then incubated in media without glucose. FISH analysis was used to monitor the localization of mature tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} in *S. paradoxus* (A), *S. bayanus* (B), *K. lactis* (C), and *S. pombe* (D). Arrows indicate cells displaying accumulation of tRNA in the nucleus. The DNA was visualized by DAPI staining. Scale bar represents 5 µm.
3.2. *Protein Kinase A is part of a mechanism that regulates nuclear re-import of the nuclear tRNA export receptors Los1p and Msn5p to the nucleus*

Nuclear tRNA export is affected in response to various stress conditions including the depletion of glucose. The majority of processes that respond to changes in environmental conditions such as glucose levels are regulated by specific signaling pathways. Therefore, to understand how the cell is able to control the rate of nuclear export of tRNAs, we investigated the possible role of known glucose signaling pathways in regulating nuclear tRNA export in response to changes in glucose levels. Two pathways of interest that are particularly involved in the response to glucose are the PKA and Snf1p pathways. In the presence of glucose, PKA is activated and represses glucose repressed-genes and promotes cell growth, while Snf1p is inactive. In contrast, low glucose levels activate Snf1p allowing it to derepress glucose-repressed genes and promote processes required for cell survival in low glucose or a nonfermentable carbon source. In contrast, PKA is inactive under these conditions. Therefore, these two opposing pathways are possible candidates for being involved in the regulation of nuclear tRNA export. Thus, we aimed to ascertain which one of the two pathways is involved in regulating nuclear tRNA export in response to glucose availability, and to obtain insights into the mechanism of this regulation.
3.2.1. Glucose starvation specifically affects nuclear tRNA export in *S. cerevisiae*

Nuclear export of mature tRNAs made from intronless and intron-containing precursors has been shown to be affected when *S. cerevisiae* is deprived of glucose (Whitney *et al.*, 2007) (Figure 23). However, there is the possibility that glucose depletion may result in a general defect in nuclear export processes. To test whether other export processes are affected by glucose deprivation, the cellular location of mRNA was examined. FISH analyses of wild type cells grown in the presence of glucose showed that mRNA is uniformly distributed throughout the cell (Figure 25). This nuclear-cytoplasmic distribution of mRNA did not change after 60 min of glucose starvation or after the re-addition of glucose. Therefore, glucose deprivation does not affect the nuclear-cytoplasmic trafficking of mRNA in *S. cerevisiae*. The data also suggest that glucose starvation specifically affects the nuclear-cytoplasmic distribution of tRNAs made from intronless and intron-containing pre-tRNAs.

![Image](image_url)

**Figure 25.** Glucose starvation does not affect nuclear mRNA export. The wild type BY4742 strain was grown in synthetic complete media to mid-log phase and transferred into media containing all supplements but lacking glucose for 60 min at 30°C. Cells were resupplemented with 2% glucose for 20 min. FISH analysis using an Alexa-488 labeled oligo-dT probe was used to monitor localization of mRNA. DAPI staining of the DNA was used to visualize location of the nuclei. Scale bar represents 5 µm.
3.2.2. Aminoacylation status of tRNA in the nucleus and cytoplasm is not affected by depletion of glucose

Nuclear tRNA aminoacylation is required for efficient export of mature tRNAs from the nucleus, since it is the final quality assurance step in the tRNA maturation process (Lund and Dahlberg, 1998; Sarkar et al., 1999; Steiner-Mosonyi and Mangroo, 2004). Previous reports have shown that tRNA aminoacylation is not affected by glucose starvation (Whitney et al., 2007). However, this study did not assess whether aminoacylation of tRNAs in the nucleus was affected during glucose starvation. Therefore, to begin understanding how glucose deprivation affects nuclear tRNA export, Northern blot analysis was used to assess whether mature tRNAs derived from intronless and intron-containing pre-tRNAs are aminoacylated in the nucleus following glucose starvation. Total RNA was isolated under acidic conditions from nuclear and cytoplasmic fractions after wild type cells grown to exponential phase were subjected to glucose deprivation for 60 min. Northern hybridization was performed using 5’-end 32P-labelled probes specific for mature tRNA^{Tyr} and tRNA^{His} (Figure 26). To obtain a marker for deacylated tRNAs, total RNA from each fed sample was treated with base to cleave the ester linkage between the tRNA and amino acid. Aminoacylation of tRNA^{Tyr} and tRNA^{His} in the presence or absence of glucose was not affected in the cytoplasm (lanes 1-4) or the nucleus (lanes 5-8). Thus, the cellular reduction in nuclear tRNA export in response to glucose starvation is not likely the result of an inhibition of nuclear tRNA aminoacylation. Nuclear tRNA export is therefore regulated at a step following nuclear aminoacylation.
Figure 26. The nuclear accumulation of tRNA during glucose starvation does not result from the inability of tRNA to be aminoacylated in the nucleus or cytoplasm. Nuclear and post-nuclear fractions were isolated from the BY4742 strain under acidic conditions before (Fed), and after glucose starvation for 60 min (starved), or following incubation of starved cells in glucose-containing medium for 20 min (Refed). Total RNA was isolated from the nuclear and post-nuclear fractions under acidic condition and separated on a 6.5% polyacrylamide gel containing 8 M urea. The separated RNAs were transferred to Nytran Plus membranes for Northern blot analysis to detect the aminoacylation status of tRNA^{Tyr} derived from intron-containing pre-tRNAs (top panels) and tRNA^{His} derived from intronless pre-tRNAs (bottom panels). Deacylated tRNAs were prepared by incubating total RNA from each fraction for 60 min at 37˚C in 100 mM Tris, pH 9.5.
3.2.3. The tRNA export receptors, Los1p and Msn5p, are retained in the cytoplasm during glucose starvation

Previous studies have shown that a number of export receptors, including the nuclear tRNA export receptors accumulate in the cytoplasm of cells exposed to limiting amounts of glucose (Quan et al., 2007). Thus, it is possible that regulation of nuclear tRNA export in response to glucose stress may, in part, be due to an effect on nuclear re-import of the nuclear tRNA export receptors after a round of tRNA export. To verify that glucose deprivation affects translocation of the tRNA export receptors from the cytoplasm to the nucleus, the cellular location of Los1p (Figure 27A) and Msn5p (Figure 27B) was monitored by immunofluorescence in wild type cells deprived of glucose. As reported previously (Huh et al., 2003), Los1p was localized primarily at the NPC and Msn5p was found in the nucleoplasm in glucose fed cells based on their localization with respect to the nucleoporin, Nsp1p. In contrast, cytoplasmic retention of both Los1p and Msn5p was detected in a large percentage of cells deprived of glucose for 60 min. Los1p and Msn5p re-localized to the NPC and nucleoplasm, respectively, when the starved cells were supplemented with glucose for 20 min. However, localization of Nsp1p to the NPC was not affected by glucose deprivation. These findings suggest that nuclear accumulation of tRNAs in response to glucose starvation may be due to the inhibition of nuclear re-import of the tRNA export receptors, Los1p and Msn5p.
The tRNA export receptors Los1p and Msn5p are retained in the cytoplasm in the wild type strain during glucose starvation. Wild type cells were grown in synthetic complete media until mid-log phase at which time they were transferred to media lacking glucose for 60 min. Glucose was added at a final concentration of 2% and the cells were incubated for an additional 20 min at 30°C. The location of Los1p and Msn5p was monitored by immunofluorescence. Los1p (A) and Msn5p (B) were detected using rabbit polyclonal α-Los1p and α-Msn5p, respectively. The NPC was visualized by staining with α-Nsp1. Scale bar represents 5 µm.

A decrease in protein level could account for the decrease in fluorescent signal for both proteins at the NPC and nucleus. Therefore, we investigated whether the decrease in the signal of the nuclear tRNA export receptors at the proper location was due to a reduction in the cellular levels of the tRNA export receptors. Western blot analysis of total cell lysate extracted from glucose-fed cells (lanes 1) and cells starved of glucose for 60 min (lanes 2) show that the levels of Los1p (Figure 28A, upper panel) and Msn5p (Figure 28B, upper panel) were not affected during glucose starvation. Furthermore, the level of Los1p and Msn5p was not affected in starved cells incubated in medium containing glucose for 20 min (lanes 3). Approximately the same amount of protein was loaded for each cell extract based on the level of actin (bottom.
panels). These findings confirm that glucose deprivation affects translocation of the two receptors back to the nucleus, but not their cellular protein level.

Figure 28. Depletion of glucose does not affect the protein level of Los1p (A) and Msn5p (B) in total cell lysate. Western blot analysis of total cell lysate using α-Los1p and α-Msn5p was performed on samples taken from cells grown to exponential growth, depleted of glucose for 60 min and resupplemented with 2% glucose for 20 min. Actin was used as a loading control.

3.2.4. Overexpression of the tRNA export receptors rescues nuclear tRNA transport during glucose deprivation

To ascertain whether the effect of glucose stress on nuclear tRNA export is caused by regulation of the function of the nuclear tRNA export receptors by an alternative strategy, we investigated whether overexpression of the tRNA export receptors restore nuclear export of tRNAs in wild type cells deprived of glucose (Figure 29). For this analysis, FISH was used to monitor the cellular location of tRNA^{Tyr} made from an intron-containing precursor and tRNA^{Gly} derived from an intronless pre-tRNA in wild type cells harbouring pYX242 alone or pYX242 carrying the Los1p, Msn5p, Cex1p, or Utp8p gene. Western blot analyses confirm that Los1p (Figure 29B), Msn5p (Figure 29C), Cex1p (Figure 29D) and Utp8p (Figure 29E) are overproduced in BY4742 (compare lanes 1 and 2). The level of actin indicates that approximately the same amount of cell extract was used. Both tRNA^{Tyr} (upper panel) and
tRNA<sup>Gly</sup> (lower panel) were found to be uniformly distributed throughout the cells of the BY4742 pYX242 (Figure 29A), BY4742 pYX242-LOS1 (Figure 29B), BY4742 pYX242-MSN5 (Figure 29C), BY4742 pYX242-CEX1 (Figure 29D) and BY4742 pYX242-UTP8 (Figure 29E) strains grown in media containing 2 % glucose. Glucose deprivation of BY4742 pYX242 (Figure 29A) and BY4742 pYX242-CEX1 (Figure 29D) resulted in a similar percentage of cells showing nuclear retention of tRNA<sup>Tyr</sup> and tRNA<sup>Gly</sup> (Figure 29F). In contrast, a low percentage of glucose-deprived BY4742 pYX242-LOS1 (Figure 29B), BY4742 pYX242-MSN5 (Figure 29C) and BY4742 pYX242-UTP8 (Figure 29E) cells show nuclear retention of the two tRNAs. The data suggest that overexpression of nuclear components of the nuclear tRNA export pathway alleviates the nuclear tRNA accumulation observed during glucose starvation. These findings suggest that glucose availability affects the function of the export receptors most likely by regulating the return of the receptors to the nucleus. The data also suggest that the function of Utp8p in the nucleus, but not the function of Cex1p in the cytoplasm may be controlled by glucose availability.
Figure 29. Overexpression of tRNA export receptors alleviates the decrease in nuclear tRNA transport during glucose depletion. Nuclear accumulation of mature tRNA was monitored using FISH in cells grown to mid-logarithmic phase and starved of glucose for 60 min. BY4742 strain containing the empty vector pYX242 (A) or pYX242 containing components of the nuclear tRNA export pathway for overexpression of Cex1p (D) display similar amounts of nuclear accumulation of tRNA in response to glucose depletion. In contrast, overexpression of the export receptors Los1p (B) and Msn5p (C) and the intranuclear component Utp8p (E) showed a decrease in the amount of cells showing nuclear accumulation. Scale bar represents 5 µm. Total cell lysate was extracted from exponentially growing cells in media containing 2% glucose. Western blot analysis of 20 µg of cell extract shows overexpression of each protein in comparison with the strain containing only pYX242. Actin was used as a loading control. (F) The average number of cells from 3 independent fields of ~200 cells displaying nuclear accumulation of tRNA^{Tyr} and tRNA^{Gly} after 60 min of glucose starvation is indicated in the bar graph. Standard deviation and results from Student’s two tailed t-test are shown, *p<0.05; **p<0.005.
3.2.5. Glucose starvation does not affect the localization of Utp8p to the nucleolus

In view of the finding that overexpression of Utp8p also alleviated the block in nuclear tRNA export during glucose starvation, we investigated by fluorescence microscopy whether localization of Utp8p to the nucleolus is affected during glucose deprivation. The Utp8p-GFP strain was grown in synthetic complete media to an OD$_{600} \sim 0.6$. The cells were then incubated for a 90 min period in complete media lacking glucose and the location of Utp8p-GFP was determined at 30 min intervals by fluorescence microscopy. The localization of Utp8p-GFP was not altered for the entire 90 min period in the glucose deprived cells (Figure 30). The data indicate that nuclear import and localization of Utp8p to the nucleolus was not affected during glucose deprivation. This finding also suggests that glucose deprivation does not inhibit general nuclear import and is specifically affecting proper localization of the nuclear tRNA export receptors. Thus, we chose to focus on delineating the mechanism responsible for controlling nuclear re-import of the nuclear tRNA export receptors.
Figure 30. The location of Utp8p is not affected by glucose starvation. Glucose fed cells were grown to an OD_{600}~0.6 and incubated in media lacking glucose for the times indicated. Fluorescence microscopy was used to monitor the location of Utp8p-GFP in wild type cells. Scale bar represents 5 µm.
3.2.6. Deletion of components of the Snf1 signaling pathway does not affect nuclear accumulation of mature tRNA in response to glucose availability

Several signaling pathways are responsible for the cellular response to changes in glucose levels. In particular, the Snf1p signaling pathway is a major pathway that regulates the derepression of glucose repressed genes in *S. cerevisiae*. The Snf1p kinase is active in cells exposed to nonfermentable carbon sources or limiting glucose levels (Celenza and Carlson, 1989). Thus, we investigated whether activation of the Snf1p pathway when glucose is absent is responsible for inhibition of nuclear tRNA export by influencing nuclear re-import of the nuclear tRNA export receptors. Activation of Snf1p requires phosphorylation of Thr-210 in the activation loop by one of the Snf1p activating kinases: Sak1p, Elm1p or Tos3p (McCartney and Schmidt, 2001). The Snf1p mammalian homolog, AMP-activated protein kinase (AMPK), is also phosphorylated in the activation loop at the threonine residue 172 when turned on during glucose deprivation (Woods *et al.*, 2003). Previous reports have shown that an antibody towards Thr-172 of AMPK is also able to detect the phosphorylation of Snf1p at Thr-210 (Liu *et al.*, 2011). Therefore, to ascertain whether the Snf1p kinase is active under the experimental conditions used for FISH, the phosphorylation status of Thr-210 in the activation loop of Snf1p was monitored by Western blot analysis with α-phospho-AMPKα (Thr-172) (Figure 31). The Snf1p-3HA strain was cultured in synthetic complete media until mid-log phase before being incubated in synthetic media lacking glucose for 60 min. The starved cells were also resupplemented with 2% glucose and incubated for an additional 20 min at 30°C. Cell lysate was extracted by glass bead lysis in the presence of protease and phosphatase inhibitors and separated by SDS-PAGE. Snf1p was phosphorylated to a small degree in synthetic complete media (top row, lane 1) or when starved cells were resupplemented with glucose (top row, lane 5). In contrast, Snf1p phosphorylation increased after 60 min of glucose starvation (top row lane 3) as compared to Snf1p from fed or
refed cells. The authenticity of the phosphorylation was established by treating the cell extract with \( \lambda \)-phosphatase at 30°C for 60 min to remove the phosphate moiety from phosphorylated proteins (top row lanes 2, 4, and 6). Western blot analysis with \( \alpha \)-HA showed a decrease in total amount of Snf1p in the cell lysate from glucose starved cells. The amount of actin loaded was also lower in the glucose starved lysate, indicating that the difference in Snf1p protein level is due to unequal loading (bottom row). However, these findings suggest that Snf1p is activated after cells are starved of glucose for 60 min and is turned off when the starved cells are provided with glucose.

**Figure 31.** Depletion of glucose results in activation of Snf1p kinase by phosphorylation of Thr-210 in the activation loop. Total cell extract was isolated from cells grown in complete synthetic media, complete synthetic media lacking glucose for 60 min and media resupplemented with 2 % glucose for 20 min. Phosphorylation of Snf1p at Thr-210 was determined by Western blot analysis on equal amounts of total cell extract using \( \alpha \)-phospho-AMPK\(\alpha\) (Thr-172). Proteins were dephosphorylated by treating 20 µg of cell extract with 40 U of \( \lambda \)-phosphatase for 60 min at 30°C. Total Snf1p-HA present was detected with \( \alpha \)-HA. Actin was used as a loading control.
To test whether Snf1p was involved in the regulation of nuclear tRNA export in response to glucose starvation, FISH was used to monitor the cellular location of tRNAs in mutant strains lacking a component of the Snf1p pathway when deprived of glucose. The mutant strains tested were \textit{snf1}, \textit{snf4}, \textit{sak1}, and \textit{elm1}. Depletion of glucose results in an increase in the interaction between the regulatory subunit, Snf4p, and the regulatory domain of Snf1p (McCartney and Schmidt, 2001). This interaction alleviates the autoinhibition of the Snf1p regulatory domain and facilitates full activation of Snf1p (Momcilovic \textit{et al.}, 2008). Sak1p and Elm1p are two of the three kinases that are required to phosphorylate the activation loop at the Thr-210 residue within the catalytic domain of Snf1p (Hong \textit{et al.}, 2003; Sutherland \textit{et al.}, 2003). As observed previously, starvation of the wild type strain of glucose for a 60 min period (Figure 32A) resulted in nuclear retention of mature tRNA\textsuperscript{Tyr} (top) and tRNA\textsuperscript{Gly} (bottom). Nuclear export of both tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} was restored when the starved cells were supplemented with 2% glucose for 20 min. As with the wild type strain, nuclear retention of both tRNA\textsuperscript{Tyr} (top) and tRNA\textsuperscript{Gly} (bottom) was detected in \textit{snf1} (Figure 32B), \textit{snf4} (Figure 32C), \textit{sak1} (Figure 32D), and \textit{elm1} (Figure 32E) cells deprived of glucose. The percentage of mutant cells displaying nuclear retention of tRNA was not significantly different from the percentage of wild type cells showing nuclear retention (Figure 32F). As observed with wild type cells, inhibition of nuclear export of the tRNAs was alleviated when the starved mutant strains were provided with glucose. These findings suggest that the Snf1p signaling pathway may not play a role in controlling nuclear tRNA export in response to glucose starvation.
Figure 32. The Snf1 signaling pathway does not regulate nuclear transport of mature tRNA in response to glucose availability. Deletion of components involved in the Snf1p signaling pathway did not affect nuclear retention of tRNA during glucose starvation or redistribution of tRNA after the addition of glucose to the starved cells. The exponentially growing glucose fed cells were subsequently starved of glucose for 60 min and resupplemented with 2% glucose for 20 min. FISH analysis was used to observe the distribution of mature tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} in wild type (A), snf1 (B), snf4 (C), sak1 (D) and elm1 (E) strains. Arrows indicate the cells displaying nuclear tRNA accumulation. DNA was visualized using DAPI. Scale bar represents 5 \textmu m. (F) The average number of cells from 3 independent fields of ~200 cells displaying nuclear accumulation of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} after 60 min of glucose starvation is indicated in the bar graph. Standard deviation and results from Student’s two tailed $t$-test are shown, *$p<0.05$; **$p<0.005$. 
3.2.7. Constitutively active PKA alleviates accumulation of tRNA in the nucleus following glucose starvation

The PKA pathway is an additional pathway that plays a role in the cellular response to glucose starvation. In contrast to the Snf1p pathway, the PKA pathway is active when glucose is available and turned off when glucose is limiting. Ras2p is a G protein that activates the PKA pathway. When glucose is available, Ras2p in its GTP bound state stimulates the activity of the adenylate cyclase, Cyr1, subsequently producing cyclic AMP (cAMP) (Broek et al., 1985). cAMP binds to the PKA regulatory subunit Bcy1p, causing Bcy1p to release the three partially redundant PKA catalytic subunits, Tpk1p, Tpk2p and Tpk3p, resulting in triggering activation of PKA (Toda et al., 1987a; Toda et al., 1987b; Griffioen et al., 2000). Previous studies have shown that expression of a constitutively active Ras2p mutant protein, Ras2pVal19, results in a high level of Ras2p-GTP and constitutive activation of PKA in the absence of glucose (Dalley and Cannon, 1996). Thus, to investigate the involvement of the PKA pathway in controlling nuclear tRNA export, we investigated whether localization of tRNA is affected when the PKA pathway remained active during glucose deprivation by expression of Ras2pVal19 in wild type cells (Figure 33). Previous reports have shown that PKA negatively regulates transcription of HSP12 in response to glucose starvation (Varela et al., 1995). Northern blot analyses on total RNA isolated from wild type cells harboring YCp50, YCp50-Ras2p or YCp50-Ras2pVal19 starved of glucose for 60 min indicate that transcription of HSP12 is repressed in glucose deprived cells expressing Ras2pVal19 (Figure 33E lane 6 upper panel) but expressed in cells harboring the empty vector (lane 4 upper panel) or expressing the wild type Ras2p (lane 5 upper panel). Actin mRNA level indicates that approximately the same amount of total RNA was used for the analyses (Figure 33E lower panel). These findings demonstrate that PKA is turned on in wild type cells expressing Ras2pVal19. As observed previously, nuclear tRNA export is not
affected in glucose fed cells containing the empty vector (Figure 33A) or expressing wild type
Ras2p (Figure 33B) or Ras2p$^{\text{Val19}}$ (Figure 33C). However, during glucose deprivation
approximately 43% of cells expressing wild type Ras2p retained tRNA$^{\text{Tyr}}$ and 36% retained
tRNA$^{\text{Gly}}$ in the nucleus. In contrast, only 25% of cells expressing the constitutively active Ras2p
mutant protein retained tRNA$^{\text{Tyr}}$ and tRNA$^{\text{Gly}}$ in the nucleus during glucose deprivation (Figure
33D). This finding, in contrast to studies reported previously (Whitney et al., 2007), suggests
that the PKA signaling pathway plays a role in regulating nuclear tRNA export.
Figure 33. Activation of PKA by the expression of constitutively active Ras2p^{Val19} decreases the percentage of cells showing nuclear accumulation of tRNA after glucose depletion. FISH using a Cy3-labelled oligonucleotide complementary to mature tRNA was used to determine the localization of mature tRNA^{Tyr} and tRNA^{Gly} in cells transformed with the empty vector (A) YCp50, (B) YCp50-Ras2 expressing wild type Ras2p or (C) the YCp50-Ras2^{Val19} expressing the constitutively active mutant Ras2p^{Val19}. The cells from each strain were grown in synthetic media containing 2% glucose until mid-log phase before being incubated in media lacking glucose for 60 min. Starved cells were resupplemented with 2% glucose for 20 min. Scale bar represents 5 µm. (D) The percentage of cells showing nuclear accumulation of tRNA^{Tyr} and tRNA^{Gly} after 60 min of glucose depletion was determined from 3 independent fields of ~200 cells. Standard deviation and results from Student’s two tailed t-test are shown, *p<0.05; **p<0.005. (E) Expression of Ras2p^{Val19} results in decreased expression of HSP12 after 60 min of glucose starvation, indicating that PKA is active under these conditions. Northern blot analysis of the expression of HSP12 was performed on total RNA isolated from samples taken from each strain for the indicated conditions.
As stated, in the presence of glucose, cAMP binds to the PKA regulatory subunit Bcy1p, causing Bcy1p to locate to the nucleus allowing for activation of PKA. As a result, deletion of Bcy1p results in PKA being constitutively active in the absence of glucose. Thus, to test more directly that the PKA signaling pathway is involved in controlling nuclear tRNA export, nuclear-cytoplasmic tRNA trafficking was monitored by FISH in a bcy1 mutant strain (Figure 34B) and a bcy1 mutant strain lacking functional Tpk2p and Tpk3p but producing Tpk1p with reduced activity (tpk1\textsuperscript{wl} tpk2 tpk3 bcy1) (Figure 34C). The level of expression of HSP12 (Figure 34 D and E upper panels) was used to ascertain whether PKA is active and the amount of actin mRNA (Figure 34 D and E lower panels) was used to monitor the amount of RNA analysed. Northern blot analysis indicates that HSP12 is not expressed in the glucose fed, starved and refed bcy1 mutant strain (Figure 34D lanes 2, 4, and 6). HSP12 is also not expressed in the isogenic glucose fed wild type strain (Figure 34 D and E lane 1), but is expressed when the cells are starved of glucose (Figure 34 D and E lane 3). Consistent with previous findings, the PKA mutant with attenuated activity expressed HSP12 in the presence and absence of glucose suggesting that the attenuated activity is unable to repress the expression of HSP12 in the presence of glucose (Varela et al., 1995) (Figure 34E lane 2). Nevertheless, these findings indicate that PKA is active in the bcy1 mutant strain irrespective of whether glucose is present or absent. Glucose deprivation of the isogenic wild type strain (Figure 34A) results in nuclear accumulation of tRNA\textsuperscript{Tyr} (upper) and tRNA\textsuperscript{Gly} (lower) in the nucleus. Nuclear export of both tRNAs is restored when the starved cells are provided with glucose. Nuclear retention of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} was not observed in glucose fed bcy1 (Figure 34B) and tpk1\textsuperscript{wl} tpk2 tpk3 bcy1 (Figure 34C) cells. However, in contrast to wild type cells, tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} were detected primarily in the cytoplasm of bcy1 (Figure 34B) and tpk1\textsuperscript{wl} tpk2 tpk3 bcy1 (Figure 34C) cells starved of glucose.
for 60 min, and distribution of the tRNAs remained unchanged when the starved mutant cells were provided with glucose. Despite the inability of the *tpk1*<sup>wt</sup> *tpk2* *tpk3* *bcy1* mutant strain to repress expression of *HSP12* in the presence of glucose, the low PKA activity was able to alleviate the nuclear accumulation of tRNAs in response to glucose starvation. These findings also suggest that PKA is involved in regulating nuclear export of tRNA. Thus, it is possible that return of the nuclear tRNA export receptors to the nucleus is facilitated by a mechanism that involves PKA when glucose is available, which then becomes inactive when glucose is limiting and the PKA pathway is turned off.
Figure 34. tRNA does not accumulate in the nucleus after glucose starvation when PKA is constitutively active. PKA remains active upon deletion of the regulatory subunit, Bcy1p. The exponentially growing wild type strain, and the bcy1 and tpk\textsuperscript{wt} tpk2 tpk3 bcy1 mutant strains were grown in synthetic complete media before being starved of glucose for 60 min. Glucose was added to glucose starved cells and they were incubated at 30°C for an additional 20 min. FISH using a Cy3-labelled oligonucleotide was used to monitor mature tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} in the wild type strain (A), the bcy1 mutant strain (B) and the mutant strain containing the catalytic subunit, Tpk1p with attenuated activity (tpk\textsuperscript{wt} tpk2 tpk3 bcy1) (C). Nuclei displaying tRNA accumulation are indicated with arrows. Scale bar represents 5 µm. Increase in transcription of HSP12 in wild type cells but not in bcy1 mutant cells demonstrates that PKA remains active in the bcy1 mutant during glucose deprivation. Total RNA was isolated from fed, glucose starved and refeed cells and Northern blot analyses were used to determine the expression of HSP12 in the bcy1 (D) and tpk\textsuperscript{wt} tpk2 tpk3 bcy1 (E) mutant strains. The ACT1 mRNA was used as a loading control.
3.2.8. *Deletion of SCH9 does not affect regulation of nuclear transport of mature tRNA in response to glucose availability*

Similar to the PKA, Snf1 and TOR signaling pathways, the Sch9p signaling pathway has been shown to have significant importance in nutrient mediated cellular response. Recent reports demonstrate that Sch9p plays a role upstream of PKA by regulating localization of the PKA regulatory subunit, Bcy1p (Zhang *et al.*, 2011). In contrast, other studies have shown that PKA and Sch9p may also act in parallel (Roosen *et al.*, 2005). To test whether Sch9p may be acting upstream of the PKA signaling pathway in regulating nuclear tRNA export in response to glucose starvation, FISH was used to monitor mature tRNAs in an *sch9* mutant strain. Nuclear accumulation of tRNAs in wild type (Figure 35A) and *sch9* (Figure 35B) cells was not observed when the cells were grown initially in synthetic complete media or when supplemented with 2 % glucose following glucose deprivation. However, tRNAs accumulate in the nucleus in both the wild type and *sch9* strains after starvation of glucose for 60 min, suggesting that Sch9p does not play a role in regulating nuclear tRNA export. Furthermore, this finding suggests that PKA regulation of nuclear tRNA export in response to glucose starvation is independent of Sch9p.
Figure 35. The Sch9p protein kinase does not regulate nuclear transport of mature tRNA in response to glucose availability. Deletion of Sch9p resulted in nuclear accumulation of tRNA after cells were cultured in complete synthetic media lacking glucose for 60 min. The culture was resupplemented with 2 % glucose for 20 min. FISH analysis was used to observe the nuclear-cytoplasmic distribution of tRNA in wild type (A) and sch9 (B) strains. Arrows indicate nuclei showing nuclear tRNA accumulation. DNA was visualized using DAPI staining. Scale bar represents 5 µm. (C) The percentage of cells from 3 independent fields of ~200 cells displaying nuclear accumulation of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Gly} after 60 min of glucose starvation is indicated in the bar graph. Standard deviation and results from Student’s two tailed t-test are shown, *p<0.05; **p<0.005.
3.2.9. The tRNA export receptors, Los1p and Msn5p, are not retained in the cytoplasm during glucose starvation when PKA is constitutively active

As reported previously, cytoplasmic retention of both Los1p and Msn5p was detected in a large percentage of wild type cells deprived of glucose (Figure 27). Moreover, proper re-localization of both receptors was observed when the starved cells were supplemented with glucose. Furthermore, constitutively active PKA alleviates the nuclear retention of tRNAs observed during glucose starvation. Thus, PKA may be playing a role in facilitating nuclear re-import of the nuclear tRNA export receptors in response to glucose availability. To test this possibility, the location of the tRNA export receptors after glucose starvation in the bcy1 and \( tpk1^{wl} \) \( tpk2 \) \( tpk3 \) bcy1 strains was determined by immunofluorescence. As expected, Los1p is localized to the NPC in the wild type (Figure 36A), bcy1 (Figure 36B), and \( tpk1^{wl} \) \( tpk2 \) \( tpk3 \) bcy1 (Figure 36C) strains fed with glucose as seen by its colocalization with the nucleoporin, Nsp1p. Interestingly, while Msn5p was found to be localized to the nucleus in wild type (Figure 36D) and \( tpk1^{wl} \) \( tpk2 \) \( tpk3 \) bcy1 (Figure 36F), Msn5p was observed to be at the NPC in the bcy1 strain (Figure 36E). Los1p and Msn5p were retained in the cytoplasm in wild type cells after glucose starvation for 60 min, and their localization to the NPC and nucleus, respectively, was restored upon readdition of 2% glucose for 20 min. In contrast, localization of Los1p and Msn5p did not change in response to glucose deprivation or resupplementation in both the bcy1 and \( tpk1^{wl} \) \( tpk2 \) \( tpk3 \) bcy1 strains. Therefore, the tRNA export receptors do not change localization in the absence of glucose when PKA is constitutively active. Moreover, these findings suggest that PKA is part of a mechanism that regulates nuclear tRNA export by controlling nuclear re-import of the tRNA export receptors, Los1p and Msn5p, in response to glucose availability.
Figure 36. tRNA export receptors Los1p and Msn5p are retained in the cytoplasm in the wild type strain during glucose starvation but not when PKA is constitutively active. Glucose fed cells were grown to an OD_{600}~0.6 and incubated in media lacking glucose for 60 min. Following starvation, glucose was added at a 2% final concentration and the cells were grown for an additional 20 min at 30°C. Immunofluorescence using α-Los1p to detect Los1p was performed to monitor the location of the export receptors in the wild type strain (A) and the bcy1 (B) and the mutant strain containing the catalytic subunit, Tpk1p, with attenuated activity (tpk^{w1} tpk2 tpk3 bcy1) (C) mutant strains following glucose starvation. Also, α-Msn5p was used to determine the location of Msn5p in wild type (D), bcy1 (E) and tpk^{w1} tpk2 tpk3 bcy1 (F) cells starved of glucose. The nuclear pore complex was visualized using antibodies towards the nucleoporin, Nsp1p. Scale bar represents 5 µm.
3.2.10. Nuclear retention of tRNAs in the absence of glucose is not due to the cells entering the stationary phase, \( G_0 \)

When glucose is available, PKA phosphorylates the Rim15p protein kinase, which results in Rim15p being sequestered to the cytoplasm by its interaction with 14-3-3 protein (Reinders et al., 1998; Wanke et al., 2005). Consequently, the cells are able to grow and divide. However, when cells are starved of glucose, Rim15p becomes dephosphorylated, dissociates from the 14-3-3 protein and translocates to the nucleus. Rim15p in the nucleus activates the transcription factors responsible for expression of stress responsive genes and the cells enter the stationary phase (\( G_0 \)) (Reinders et al., 1998; Pedruzzi et al., 2003). It is therefore possible that the nuclear tRNA export process is inhibited because the cells have entered \( G_0 \). To investigate this possibility, we tested whether nuclear tRNA export is affected in a \( rim15 \) mutant strain starved of glucose. For this analysis, wild type and \( rim15 \) cells were synchronized by treatment with 15 \( \mu \text{g/ml} \) nocodazole for 2 h and allowed to grow for an additional 4 h after being released from cell cycle arrest by removal of the nocodazole. The cells were then starved of glucose for 90 min and the cellular location of tRNA was monitored by FISH. Both the wild type and \( rim15 \) strains synchronized with nocodazole showed uniform distribution of tRNA\(^\text{Tyr} \) and tRNA\(^\text{Gly} \) in the presence of glucose before and after glucose deprivation (Figure 37A). Furthermore, nuclear retention of tRNAs was observed in a higher percentage of synchronized wild type and \( rim15 \) cells starved of glucose (Figure 37B). These data suggest that blocking the cells from entering the stationary phase by deletion of Rim15p did not prevent inhibition of nuclear tRNA export.
**Figure 37.** Rim15p and arresting cells in G₀ is not required for PKA regulation of tRNA export during glucose starvation. Wild type and the rim15 mutant strains were synchronized by addition of 15 µg/ml of nocodazole for 120 min and then released and allowed to grow for an additional 240 min or treated with the drug vehicle, DMSO. Following synchronization, cells were cultured in complete synthetic media lacking glucose for 60 min and resupplemented with glucose for 20 min. FISH analysis with Cy3-labeled oligonucleotides toward mature tRNAs was used to observe the nuclear-cytoplasmic distribution of mature tRNA in wild type and rim15 strains. Nuclear accumulation of tRNA was observed in both wild type and rim15 when synchronized or treated with the drug vehicle (A). Arrows indicate nuclei showing nuclear tRNA accumulation. DNA was visualized using DAPI staining. Scale bar represents 5 µm. (B) The percentage of cells showing nuclear accumulation of tRNA\textsubscript{Tyr} and tRNA\textsubscript{Gly} after 60 min of glucose depletion was determined from 3 independent fields of ~200 cells. Standard deviation and results from Student’s two tailed t-test are shown, *p<0.05; **p<0.005.
To further verify that the nuclear tRNA accumulation observed is not dependent on the cell cycle, FISH was used to monitor localization of tRNA$^{\text{Tyr}}$ after cells were arrested in different stages of the cell cycle. Wild type cells were grown in YPD to mid-log phase and arrested in G1, S and G2/M phases of the cell cycle with $\alpha$-factor, hydroxyurea and nocodazole, respectively. The $cdc15-2b$ mutant strain contains a temperature sensitive allele of Cdc15p, a protein kinase required for exit from mitosis (Bardin et al., 2003). Therefore, $cdc15-2b$ cells proceeds normally through the cell cycle at the permissive temperature of 23°C but arrests in late mitosis when incubated at 37°C. Arresting wild type and $cdc15-2b$ strains in their respective stages of the cell cycle did not result in nuclear accumulation of tRNA$^{\text{Tyr}}$ (Figure 38). Thus, it would appear that inhibition of the nuclear tRNA export process is caused by a mechanism that is unrelated to cell cycle regulation. Taken together, these findings indicate that regulation of nuclear tRNA export by PKA is not a result of cells arresting in G0 because of the inactivation of PKA during glucose starvation.
Figure 38. Arresting cells in different stages of the cell cycle does not result in nuclear retention of mature tRNA\textsuperscript{Tyr}. Cells were grown in YPD until exponential growth and arrested in G1, S, or G2/M phase with 100 µg/ml α-factor, 100 mM hydroxyurea, or 15 µg/ml nocodazole, respectively. To arrest the culture in late mitosis, the 
\textit{cdc15-2b} strain was incubated in YPD at 37°C for 180 min. FISH analysis was used to observe the nuclear-cytoplasmic distribution of tRNA\textsuperscript{Tyr} when cells were fixed after being arrested in each stage of the cell cycle. Nuclei were located by DAPI staining of the DNA. Scale bar represents 5 µm.
3.2.11. Translocation of the nuclear tRNA export receptors to the nucleus is not directly controlled by phosphorylation by PKA

PKA is a kinase that phosphorylates proteins at serine and threonine residues. Thus, a mechanism by which PKA could facilitate re-import of the receptors to the nucleus is by phosphorylating the tRNA export receptors directly when glucose is available. Alternatively, PKA may function by phosphorylating proteins associated with translocation of the tRNA export receptors to the nucleus. Consequently, inactivation of PKA in the absence of glucose may allow dephosphorylation of these components and subsequent block in the translocation of the receptors to the nucleus. Several large-scale studies aimed at identifying phosphorylated proteins in *S. cerevisiae* suggest that Los1p and Msn5p are not subjected to phosphorylation (Bodenmiller et al., 2008). In contrast, Utp22p and Cex1p, which are known components of the nuclear tRNA export process, were found to be phosphorylated and contain possible PKA phosphorylation motifs (Bodenmiller et al., 2008). Nevertheless, we investigated whether PKA is able to phosphorylate Los1p and Msn5p as well as several known components of the tRNA export pathway using an *in vitro* PKA phosphorylation assay. It has previously been shown that the catalytic subunit from bovine PKA is able to phosphorylate recombinant yeast proteins (Chang et al., 2004; Portela et al., 2006). Thus, recombinant bovine PKA and $\gamma^{32}$P-ATP were incubated for 30 min at 23 °C with His tagged recombinant Los1p, Msn5p, Utp8p, Utp9p, Utp22p and Cex1p. The samples were separated on a SDS-PAGE gel and phosphorylation detected using a Phosphor screen (Figure 39). Phosphorylation of a peptide containing a PKA phosphorylation site (PKA substrate) was observed in the presence of PKA and $\gamma^{32}$P-ATP (lane 1) but not when PKA was absent indicating that the assay can specifically detect direct PKA phosphorylation (lane 2). Los1p (lanes 3 and 4 upper panel), Msn5p (lanes 5 and 6 upper panel) and Utp8p (lanes 9 and 10 upper panel) phosphorylation was not detected in the presence or absence of PKA. In
contrast, Utp22p (lanes 7 and 8 upper panel), Utp9p (lanes 11 and 12 upper panel) and Cex1p (lanes 13 and 14 upper panel) were found to be phosphorylated only in the presence of the PKA catalytic subunit. Western blot analysis (Figure 39 middle panels) and Coomassie blue staining (Figure 39 lower panels) show that equal amounts of each recombinant protein was subjected to incubation with $[^{32}\text{P}]{\text{ATP}}$ in the presence or absence of PKA. These findings suggest that PKA does not regulate localization of the tRNA export receptors, Los1p and Msn5p, by direct phosphorylation. It is possible that PKA phosphorylation of the nuclear (Utp9p and Utp22p) and cytoplasmic (Cex1p) components of the nuclear tRNA export pathway that directly interact with the tRNA export receptors during nuclear tRNA export plays a role in nuclear re-import of the tRNA export receptors
Figure 39. PKA phosphorylates components of the tRNA export pathway but not the tRNA export receptors in vitro. Utp22p, Utp9p and Cex1p were found to be phosphorylated in vitro by PKA. The export receptors Los1p and Msn5p as well as the intranuclear component Utp8p were not phosphorylated by PKA. 2 µg of purified His-tagged recombinant protein was combined with 1 U of bovine PKA and 5 µCi of \([\gamma-^{32}P]ATP\) at 25°C for 30 min in PKA phosphorylation buffer. Samples were separated by SDS-PAGE and imaged using a Phosphor Imager. A peptide containing a PKA phosphorylation site was used as a positive control for the in vitro phosphorylation. Western blot analysis and Coomassie staining indicate equal loading of protein in the presence and absence of PKA.
4.0. Discussion

The tDNA genes encoding tRNAs may either be intronless or contain an intron that must be spliced before the tRNA is fully mature for use in protein synthesis. Initially, it was believed that tRNA splicing in *Saccharomyces cerevisiae* like mammalian cells occurs in the nucleus (Paushkin *et al.*, 2004), and most likely at the nuclear envelope, as previous studies have shown that a number of the components of the tRNA splicing machinery are located at the nuclear membrane, and several nucleoporin mutants exhibited a defect in tRNA splicing in *S. cerevisiae* (Peebles *et al.*, 1983; Clark and Abelson, 1987; Simos *et al.*, 1996b). However, the tRNA spliceosome and consequently tRNA splicing in *S. cerevisiae* was found to be occurring on the cytoplasmic surface of the mitochondrial membrane (Yoshihisa *et al.*, 2003; Yoshihisa *et al.*, 2007). As a result, studies have determined that tRNAs derived from intron-containing precursor tRNA (pre-tRNA) are initially exported to the cytoplasm where they are spliced, imported back into the nucleus for further maturation and nuclear aminoacylation and then re-exported to the cytoplasm for use in protein synthesis (Shaheen and Hopper, 2005; Takano *et al.*, 2005; Murthi *et al.*, 2010; Ohira and Suzuki, 2011).

Interestingly, changes in the levels of nutrients such as glucose, amino acids or phosphate have been shown to cause nuclear accumulation of tRNAs made from intronless and intron-containing pre-tRNAs (Hurto *et al.*, 2007; Whitney *et al.*, 2007). These findings led to the proposal that nutrient stress caused nuclear import of mature cytoplasmic tRNAs (Figure 40). However, this study clearly indicates that nutrient stress does not cause retrograde transport of cytoplasmic tRNAs to nucleus. Instead the data indicate that nutrient stress causes a block in nuclear export of tRNAs to the cytoplasm. Furthermore, in contrast to previous studies, we only observed nuclear retention of mature tRNAs derived from intron-containing pre-tRNAs in
response to amino acid or nitrogen starvation (Figure 8 and 9). The reason why our findings differ from previous reports is not known; however, our results are consistent with studies showing that newly spliced tRNAs are imported into the nucleus by a constitutive process (Murthi et al., 2010). Moreover, our data also show that \textit{S. cerevisiae} does not regulate nuclear export of tRNAs made from intronless precursors, which undergo maturation completely in the nucleus, in response to amino acid and nitrogen starvation. Amino acid starvation does eventually result in nuclear accumulation of the intronless derived, tRNA\textsuperscript{His}, in the histidine auxotrophic \textit{S. cerevisiae} strain (Figure 10D). Retention of tRNA\textsuperscript{Leu} and tRNA\textsuperscript{Tyr}, which are both derived from intron-containing pre-tRNA, occurred much sooner than retention of tRNA\textsuperscript{His} (Figure 10 A and B). The late onset of inhibition of tRNA\textsuperscript{His} export is clearly due to a block in aminoacylation of tRNA in the nucleus, since inhibition of the nuclear aminoacylation step has been shown to result in a reduction in the efficiency of nuclear export of mature tRNAs (Lund and Dahlberg, 1998; Azad et al., 2001). The retention observed with tRNA\textsuperscript{Leu} could be attributed to a decrease in nuclear aminoacylation because the strain used is also auxotrophic for leucine. However, the strain is prototrophic for tyrosine and tRNA\textsuperscript{Tyr} remains aminoacylated for the entire duration of amino acid starvation (Whitney et al., 2007). Therefore, nuclear retention of tRNA\textsuperscript{Tyr} is not the result of an effect on aminoacylation of the tRNA in the nucleus. Also, the glycine prototrophic strain does not show accumulation of tRNA\textsuperscript{Gly} which is also derived from an intronless pre-tRNA during amino acid starvation. If \textit{S. cerevisiae} were regulating both tRNAs derived from intronless and intron-containing pre-tRNAs in the same manner in response to amino acid and nitrogen starvation then nuclear retention of all tRNAs would occur at the same time.
Figure 40. Model showing nuclear import of cytoplasmic tRNAs in response to nutrient starvation. Previous studies suggest that depletion of various nutrients including glucose and amino acids results in a rapid re-import of cytoplasmic tRNAs to the nucleus resulting in nuclear accumulation of tRNA.

tRNAs derived from intron-containing tRNAs were also regulated when cells were starved of a single amino acid (Figure 11 A and B). Although, the retention of tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Leu} but not tRNA\textsuperscript{Gly} occurred later than tRNA\textsuperscript{His} when the histidine auxotrophic strain was starved of histidine, it indicates that starvation of a single amino acid causes retention of the cognate tRNA but also affects the nuclear export of only the tRNAs derived from intron-containing tRNAs (Figure 11). The delay in accumulation of tRNAs from intron-containing pre-tRNAs may be attributed to histidine starvation not being as severe as amino acid starvation. Furthermore, studies in our laboratory also showed that the cellular distribution of tRNA\textsuperscript{Ile(AAU)}
derived from an intronless pre-tRNA was not affected by amino acid starvation while tRNA$_{\text{Ile(UAU)}}$ derived from an intron-containing tRNA accumulated in the nucleus during amino acid starvation (Chafe et al., 2011). This finding indicates that it is the presence of the intron and not the species of tRNA that specifies whether export of a tRNA is regulated by amino acid or nitrogen starvation. Therefore, amino acid and nitrogen stress cause the cell to regulate nuclear re-export of mature tRNAs made from intron-containing pre-tRNAs but not those derived from intronless tRNAs.

Recent evidence has demonstrated that certain components of the tRNA export pathway strictly participate in the nuclear re-export of tRNAs derived from intron-containing pre-tRNAs, suggesting that the two types of tRNAs are segregated into different export pathways (Eswara et al., 2009). This segregation of the two pathways would provide targets for regulating nuclear export of a specific subset of tRNAs. Why _S. cerevisiae_ regulates nuclear export of mature tRNAs from intron-containing pre-tRNAs, but not those from intronless precursors is not presently understood. However, this regulation of nuclear export of mature tRNAs made from intron-containing pre-tRNAs may, in part, be related to an unrealized function of nuclear tRNA$_{\text{Tyr}}$ and tRNA$_{\text{Trp}}$ in allowing cells to sense nitrogen or amino acid availability, as tRNA$_{\text{Trp}}$ and tRNA$_{\text{Tyr}}$ are encoded by only intron-containing tDNA genes while all other tRNAs that are encoded by intron-containing tDNA genes are also encoded by intronless tDNA genes.

Rat hepatoma H4IIE cells have been shown to accumulate tRNA in the nucleus in response to amino acid starvation (Shaheen et al., 2007). Based on this finding it was proposed that mammalian cells are also able to retrogradely transport cytoplasmic tRNAs to the nucleus in response to nutrient stress. However, parallel studies from our laboratory to determine whether nuclear tRNA import occurred in other mammalian cell lines showed that amino acid or glucose
starvation of rat hepatoma H4IIE, HeLa, and HEK293 cells do not result in nuclear accumulation of tRNA (Chafe et al., 2011). Furthermore, nuclear retention of tRNA did not occur in plant cells deprived of nitrogen or sucrose (Johnstone et al., 2011b). Mammalian and plant cells may not have the ability to respond to acute changes in environmental conditions due to the presence of vasculature systems providing a constant supply of nutrients throughout the organism to the individual cells. In this study, we also showed that the yeasts, Schizosaccharomyces pombe (Figure 18) and Kluyveromyces lactis (Figure 21), like mammalian and plant cells do not accumulate tRNA in response to amino acid or glucose starvation (Figure 24). However, nuclear aminoacylation was shown to be required for efficient nuclear export of mature tRNAs since nuclear accumulation was still observed after each species was starved of an amino acid for which they were auxotrophic (Figure 19 and 21C). In contrast, two species from the Saccharomyces genus, S. paradoxus and S. bayanus, showed nuclear retention of tRNA during amino acid and glucose starvation. Consistent with S. cerevisiae, S. paradoxus and S. bayanus also only showed nuclear accumulation of tRNAs derived from intron-containing pre-tRNAs in response to amino acid starvation (Figure 22). Furthermore, all three species but not S. pombe and K. lactis showed nuclear accumulation of tRNAs derived from both intronless and intron-containing pre-tRNAs during glucose starvation (Figure 23 and 24). Therefore, only species from the Saccharomyces genus regulate nuclear export of tRNAs in response to nutrient stress and the response is not evolutionarily conserved.

An interesting characteristic of the Saccharomyces genus is their unique preference to perform fermentative growth in the presence of a fermentable carbon source such as glucose even though it is less energetically favorable. As a result, the majority of pyruvate synthesized from glycolysis is converted into ethanol and does not enter the citric acid cycle to produce
metabolites needed to make amino acids. Therefore, the lower energy level and reduced pools of precursors most likely affect the capacity of the Saccharomyces species to make amino acids for quality assurance of newly matured tRNAs and protein synthesis. Saccharomyces may therefore be more sensitive to changes in environmental conditions due to the reduced energy levels and metabolite pools. The regulation of nuclear tRNA export may provide an additional mechanism that Saccharomyces use to coordinate nutrient availability with protein synthesis in order to survive poor environmental conditions. Interestingly, Saccharomyces are able to perform anaerobic fermentative growth while the other yeast species S. pombe and K. lactis always require oxygen for growth (Visser et al., 1990; Nagy et al., 1992). Cancer cells under hypoxic conditions may also have the ability to regulate nuclear tRNA export in response to nutrient availability, since they have been shown to use respiration-independent pathways to produce energy instead of the citric acid cycle and electron transport chain (Vander Heiden et al., 2009).

The mechanisms controlling tRNA export in response to nutrient availability are poorly understood. Signaling pathways are known to regulate the cellular reaction to changes in nutrient levels. However, currently little is known about the signaling pathways controlling nuclear tRNA export. The TORC1 signaling pathway is a central controller that regulates gene expression and cellular growth in response to nitrogen and amino acid availability (Wullschleger et al., 2006). Furthermore, previous studies reported that inhibiting the TOR signaling pathway with rapamycin, which simulates nitrogen starvation, in cells prior to amino acid starvation did not show nuclear retention of tRNA during amino acid starvation (Whitney et al., 2007). In contrast to the studies reported, our investigation revealed that inhibition of the TORC1 signaling pathway by rapamycin resulted in nuclear accumulation of mature tRNAs encoded by intron-containing genes but not tRNAs made from intronless pre-tRNAs (Figure 14) while not affecting
the nuclear tRNA aminoacylation step (Figure 16). Also, a rapamycin insensitive TORC1 strain did not accumulate tRNA following rapamycin treatment suggesting that the effect observed was specifically due to the inhibition of TORC1 (Figure 15). Thus, these results support our finding that nitrogen starvation results in accumulation of only tRNAs derived from intron-containing pre-tRNAs. The data also indicate that the TORC1 signaling pathway is involved in regulating nuclear re-export of mature retrogradedly transported spliced tRNAs after the aminoacylation quality control step in response to nitrogen availability (Figure 41).

**Figure 41.** Model of TORC1 regulating nuclear tRNA export of tRNAs derived from intron-containing pre-tRNAs in response to nitrogen and amino acid levels. Active TORC1 stimulates the nuclear export of tRNAs derived from intron-containing pre-tRNA through and unknown mechanism when there is sufficient nitrogen or amino acids. Inactivation of TORC1 during nitrogen or amino acids starvation results in a reduction in nuclear tRNA export.
Recent studies have reported that the tRNA export receptors are relocated to the cytoplasm in response to various stress conditions including DNA damage, heat shock, low glucose levels and the switch to ethanol as the sole carbon source (Quan et al., 2007; Ghavidel et al., 2007). However, the mechanism responsible for the re-location of the tRNA export receptors in response to stress was not determined. Relocation of the export receptors to the cytoplasm would result in inefficient nuclear export of tRNA and represents a possible mechanism for controlling nuclear tRNA export. In this study, we demonstrated that the steady state localization of the tRNA specific export receptor, Los1p, was not affected by nitrogen starvation. However, overexpression of Los1p in nitrogen starved cells restored nuclear export of mature spliced tRNAs during nitrogen starvation. This definitively shows the involvement of Los1p in the nuclear re-export of mature tRNAs derived from introning-containing pre-tRNAs under normal growth conditions. The data also suggest that the function of Los1p may be compromised by another mechanism during nitrogen starvation that does not involve altering its cellular location. Alternatively, the overexpression of Los1p may compensate for the inhibition of a Los1p independent pathway involving another receptor such as Msn5p, for the re-export of mature retrogradedly transported tRNAs. However, further investigation into these possibilities is required. Therefore, while the mechanism by which TORC1 regulates nuclear re-export of retrogradedly transported tRNAs is unknown, it is possible that this signaling pathway is controlling the activity of the nuclear tRNA export receptors by a means other than affecting their localization.

Changes in glucose levels have been shown to regulate nuclear export of mature tRNAs from intronless pre-tRNAs and the re-export of mature spliced tRNAs without affecting nuclear aminoacylation (Whitney et al., 2007). The severity of glucose starvation may account for the
more rigorous regulation of tRNA export. Depletion of glucose severely limits cellular growth since cells no longer have a rich carbon source and must rely on gluconeogenesis and glycogen stores for survival (Francois and Parrou, 2001). Glucose depletion also rapidly inhibits translation by removing polysomes from mRNAs (Ashe et al., 2000). Sequestering the tRNAs in the nucleus makes them unavailable for protein synthesis. Therefore, it is possible that blocking nuclear tRNA export may provide an additional means by which the cell can control protein synthesis during glucose starvation. Interestingly, a higher percentage of cells show nuclear accumulation of tRNA\textsuperscript{Tyr} derived from an intron-containing pre-tRNA than tRNA\textsuperscript{Gly} which is made from an intronless pre-tRNA during glucose starvation. Therefore, even though glucose starvation results in a global inhibition of tRNA export, it is not clear why nuclear export of retrogradely transported tRNAs is more sensitive than nuclear export of tRNAs made from intronless pre-tRNAs during glucose deprivation. It is possible that nuclear export of mature tRNAs made from intronless pre-tRNAs is primarily exported by an unidentified tRNA export pathway that is not strictly regulated by glucose availability. This interpretation is consistent with the observation that nitrogen or amino acid deprivation does not affect nuclear export of mature tRNAs made from intronless precursors.

Glucose deprivation appears to be affecting the function of several components of the nuclear tRNA export machinery. Overexpression of the export receptors, Los1p and Msn5p, and the intranuclear component, Utp8p, but not the cytoplasmic component of nuclear tRNA export, Cex1p, was found to partially restore nuclear export of tRNA made from both intronless and intron-containing pre-tRNAs during glucose starvation (Figure 29). Since nuclear export of tRNAs is facilitated by redundant pathways, the partial rescue of nuclear retention of tRNAs derived from both intronless and intron-containing pre-tRNAs suggests that glucose starvation
causes the inhibition of more than one nuclear tRNA export pathway. Therefore, full restoration of nuclear export of tRNA during glucose starvation likely requires the overexpression of several components of the tRNA export pathways. Also, this provides evidence suggesting that Msn5p may also function in the export of mature tRNAs from intronless pre-tRNA in a Utp9p independent pathway. Furthermore, glucose availability may control the function of Utp8p in the nucleus, but not the function of Cex1p in the cytoplasm. In contrast to nitrogen starvation, the tRNA export receptors were relocated to the cytoplasm during glucose deprivation (Figure 27). The decrease in the nuclear signal of Msn5p and the signal at the NPC for Los1p did not result from a decrease in the cellular levels of the two proteins (Figure 28). Our findings confirm that glucose deprivation affects translocation of the two receptors back to the nucleus after a round of nuclear tRNA export. Interestingly, while the tRNA export receptors are relocated to a different cellular location, nucleolar import and localization of Utp8p was not affected during glucose deprivation (Figure 30). This suggests that glucose starvation is specifically affecting nuclear re-import of the nuclear tRNA export receptors. Consistent with this conclusion is finding that proper localization of Nsp1p was also not affected by glucose depletion. Further investigation into the mechanism regulating the function of Utp8p is required. However, this study focused on the signaling events controlling the nuclear re-import of the export receptors, Los1p and Msn5p, in response to glucose starvation.

Previous studies have established that the cellular response to glucose availability involves two opposing signaling pathways; PKA and Snf1p. In the presence of glucose the Snf1p pathway is inactivated while the PKA pathway is active and promotes cell growth and proliferation. Depletion of glucose causes the inactivation of PKA by the regulatory subunit, Bcy1p, resulting in the expression of stress response genes and cell entry into the stationary
phase G0 (Pedruzzi et al., 2003; Broach, 2012). In contrast, Snf1p is activated by one of the three Snf1p activating kinases, Sak1p, Elm1p and Tos3p in conjunction with Snf4p causing expression of glucose repressed genes (Smets et al., 2010). Therefore, we investigated whether the PKA or Snf1p pathways are involved in regulating the nuclear re-import of the tRNA export receptors in response to glucose starvation. We found that expression of the constitutive active Ras2p_{Val19} (Figure 33C) resulted in a significant reduction in nuclear tRNA retention while deletion of BCY1 completely abolished the nuclear tRNA accumulation normally observed during glucose starvation (Figure 34B). Furthermore, in support of PKA facilitating the re-import of the tRNA export receptors, Los1p and Msn5p were no longer found in the cytoplasm in the strains with a constitutively active PKA (Figure 36 B and E). Interestingly, the tpk1^{rl} tpk2 tpk3 bcy1 mutant strain with attenuated PKA activity also showed even nuclear-cytoplasmic distribution of tRNAs (Figure 34C) and proper localization of the tRNA export receptors during glucose starvation (Figure 36 C and F) even though it could not repress the expression of the reporter gene, HSP12, in the presence of glucose (Figure 34E). This suggests that the regulation of the export receptors is more sensitive to the activity of PKA than the transcription factors responsible for the expression of HSP12. Therefore, PKA regulates nuclear export of tRNAs derived from intron-containing and intronless pre-tRNAs in response to glucose availability by regulating the re-import of the tRNA export receptors (Figure 42).
Figure 42. Model showing the regulation of nuclear tRNA export by PKA in response to glucose availability. Active PKA stimulates the nuclear export of tRNAs derived from both intron-containing and intronless pre-tRNA in the presence of glucose. Deprivation of glucose results in inactivation of PKA and subsequent decrease in nuclear export of tRNAs derived from both intron-containing and intronless pre-tRNAs.

A recent study reported that PKA negatively regulates the Snf1p pathway by phosphorylating and inhibiting the main activating kinase, Sak1p (Barrett et al., 2012). As a result, not only could the activation of Snf1p in response to glucose starvation result in nuclear accumulation of tRNA but alternatively, the downstream effect PKA is having on the nuclear re-import of the tRNA export receptors may be mediated by the Snf1p pathway. However, deletion of key components of the Snf1p pathway required for the activation of Snf1p as well as the Snf1p kinase itself, did not affect the nuclear retention of tRNA during glucose starvation.
(Figure 32). Furthermore, interruption of the Snf1p pathway did not affect the redistribution of tRNA after the addition of glucose to starved cells. Therefore, the activation of Snf1p does not contribute to the regulation of nuclear tRNA export during glucose deprivation. Also, PKA is not regulating the re-import of the export receptors by regulating the activity of the Snf1p kinase in the presence of glucose.

Additionally, the Sch9p protein kinase is regulated in response to the availability of different nutrients including nitrogen and glucose. In rich nutrient conditions, active Sch9p is phosphorylated and regulates cell growth and the expression of genes required for ribosome biogenesis (Broach, 2012). Interestingly, it has been reported that Sch9p may regulate the phosphorylation and subsequent nuclear localization of the PKA regulatory subunit, Bcy1p, suggesting that Sch9p is functioning upstream of PKA (Zhang et al., 2011; Zhang and Gao, 2012). However, another study has shown that Sch9p may also act in parallel to PKA for certain functions (Roosen et al., 2005). We demonstrated that, like wild type cells, starving the sch9 mutant strain of glucose resulted in a similar percentage of cells showing nuclear accumulation (Figure 35). Therefore, in the case of nuclear tRNA export, Sch9p is not acting upstream of the PKA signaling pathway and is not involved in regulating the nuclear export of tRNA.

When glucose is abundant, PKA phosphorylates the Rim15p protein kinase. Rim15p is subsequently sequestered in the cytoplasm through an interaction with the 14-3-3 protein, Bmh2p, and cells grow and divide. Inhibition of PKA results in the dephosphorylation of Rim15p and its entry into the nucleus where it induces arrest in G₀ (Reinders et al., 1998) and expression of stress responsive genes through the transcription factors Msn2/4p and Gis1p (Reinders et al., 1998; Pedruzzi et al., 2003). We hypothesized that the nuclear tRNA export process is inhibited by Rim15p dependent entry into G₀ due to inactivation of PKA. However,
the percentage of cells showing nuclear accumulation between wild type and the \textit{rim15} mutant were not significantly different during glucose deprivation (Figure 37). Therefore, entry into $G_0$ is not required for inhibition of nuclear tRNA export or nuclear re-import of the tRNA export receptors. Although arresting cells at different stages of the cell cycle does not affect the nuclear tRNA export (Figure 38), a significantly higher percentage of synchronized wild type cells show nuclear retention of tRNA in response to glucose starvation (Figure 37B). This suggests that $S.\ cerevisiae$ cells are more sensitive to environmental changes when in a particular stage of the cell cycle. $S.\ cerevisiae$ exhibit a long $G_1$ where they are accumulating the biomass and protein content before entering S phase and budding (Forsburg and Nurse, 1991). It is likely during this stage that the cells regulate nuclear tRNA export as they are most susceptible to changes in nutrient availability. Therefore, it is possible that inhibition of nuclear tRNA export in response to nutrient availability is dependent on the cell cycle.

PKA is a kinase that is known to regulate protein function through phosphorylation of serine and threonine residues present on target proteins. Therefore, PKA may be facilitating the nuclear re-import of the tRNA export receptors when glucose is available by several mechanisms. Large-scale studies to identify phosphorylated proteins in $S.\ cerevisiae$ suggest that the export receptors Los1p and Msn5p are not phosphorylated (Bodenmiller \textit{et al.}, 2008). However, Utp22p and Cex1p were both suggested to have conserved PKA phosphorylation motifs. Therefore, to elucidate a possible mechanism, we investigated whether known components of the tRNA export pathway including the known tRNA export receptors can be directly phosphorylated by PKA \textit{in vitro}. Our data show that Utp8p, Los1p and Msn5p were not phosphorylated by PKA while PKA directly phosphorylated Utp22p, Utp9p and Cex1p (Figure 39). Therefore, PKA is not regulating the re-import of the tRNA export receptors or affecting the
function of Utp8p by direct phosphorylation. While the role of PKA is not understood, several scenarios are possible for the regulation of the re-import of the tRNA export receptors by PKA: 1) Los1p and Msn5p interact directly with a PKA phosphorylated protein that facilitates their re-import into the nucleus when glucose is available (Figure 43A). 2) The tRNA export receptors are retained in the cytoplasm by a dephosphorylated protein that in its PKA phosphorylated form cannot interact with Los1p and Msn5p but is dephosphorylated when PKA is inactivated (Figure 43B). 3) PKA phosphorylation regulates a phosphatase that dephosphorylates the proteins in 1 and 2 that may be phosphorylated by PKA or another kinase (Figure 43A and B). Previous studies have shown that phosphorylation of nucleoporins of the NPC affects nuclear-cytoplasmic trafficking of nuclear import/export receptors (Crampton et al., 2009; Kodiha et al., 2010). 4) PKA may phosphorylate and inhibit a kinase when glucose is available. However, dephosphorylation of the kinase by an unknown phosphatase after the inactivation of PKA would allow the kinase to phosphorylate a nucleoporin, preventing import of the nuclear tRNA export receptors (Figure 43C).
Figure 43. Model of the mechanisms PKA may use to regulate the re-import of the nuclear tRNA export receptors. (A) Phosphorylation of a cytoplasmic component by PKA facilitates re-import of the tRNA export receptors. (B) PKA phosphorylation of a cytoplasmic component prevents its direct interaction with the tRNA export receptors in the presence of glucose. Inactivation of PKA results in the component being able to interact and sequester the nuclear tRNA export receptors in the cytoplasm when glucose is not available. (C) PKA may phosphorylate an unknown kinase to prevent the kinase from phosphorylating a nucleoporin in the NPC. Phosphorylation of the nucleoporin by the unknown kinase would prevent re-import of the nuclear tRNA export receptors.

Utp9p has been shown to be specifically involved in the Msn5p-dependent re-export of mature spliced tRNAs while Utp22p is involved in the Los1p-dependent export of tRNAs made from both intronless and intron-containing pre-tRNAs (Eswara et al., 2009; Eswara et al., 2012). Utp9p and Utp22p are essential proteins that interact with the export receptors in the nucleus but
do not shuttle between the nucleus and cytoplasm. Therefore, although these proteins interact with the export receptors, it is unlikely that they are required for retaining Los1p and Msn5p in the cytoplasm due to the spatial separation during glucose starvation. However, their phosphorylation may play a role in regulating the function of Utp8p in the nucleus in response to glucose starvation. Regulating this intranuclear step of tRNA export would provide an additional level for controlling tRNA export in response to glucose starvation along with retaining the tRNA export receptors in the cytoplasm.

Cex1p is a non-essential cytoplasmic component involved in the export of aminoacylated tRNAs derived from both intron-containing and intronless pre-tRNAs and interacts with both Los1p and Msn5p (McGuire and Mangroo, 2007). Furthermore, Cex1p is proposed to be required for the recruiting the RanGAP, Rna1p, to the NPC and facilitate Rna1p activation of the GTPase activity of Ran and disassembly of the receptor-tRNA-RanGTP export complex (McGuire and Mangroo, 2012). As a result, the phosphorylation status of Cex1p may interfere with this function resulting in inefficient disassembly of the export complex and retention of the export receptors in the cytoplasm. However, overexpression of Cex1p did not alleviate nuclear tRNA accumulation in response to glucose starvation. Thus, it is unlikely that Cex1p plays a role in inhibiting the re-import of the tRNA export receptors in response to glucose starvation.

Regulation of nuclear tRNA export in response to nutrient availability is not evolutionarily conserved. The yeast species of the *Saccharomyces* genus, but not *S. pombe* and *K. lactis*, regulate nuclear tRNA export by unique mechanisms in response to changes in nutrient availability. Furthermore, the nuclear re-export of retrogradely transported spliced tRNAs is segregated from the pathway for export of mature tRNAs from intronless pre-tRNAs. As a result, nitrogen starvation causes a block in nuclear re-export of mature spliced tRNAs and not those
derived from intronless pre-tRNAs. Moreover, the TORC1 signaling pathway regulates the nuclear re-export of mature retrogradedly transported tRNAs in response to amino acid and nitrogen availability by affecting the function of the tRNA export receptor, Los1p, and most likely Msn5p. However, the TORC1 pathway does not regulate the function of Los1p by altering its cellular location. In contrast, glucose depletion causes a global inhibition of the export of tRNAs derived from both intronless and intron-containing tRNAs. Furthermore, nuclear tRNA accumulation during glucose starvation is not dependent on the cells entering the stationary phase, G₀, but results from the cytoplasmic retention of the tRNA export receptors, Los1p and Msn5p. The inhibition of nuclear re-import of the tRNA export receptors following a round of tRNA export is due to inactivation of PKA and not the activation of the Snf1p signaling pathway. However, this regulation is not due to PKA directly phosphorylating the export receptors. Moreover, PKA phosphorylates known components of the nuclear tRNA export pathway in vivo. Therefore, PKA may be facilitating nuclear re-import of the known tRNA export receptors by several mechanisms involving the phosphorylation of an associated protein.

These findings confirm *S. cerevisiae* as a highly adaptable organism that uses complex mechanisms to control nuclear transport of tRNAs to survive changes in environmental conditions. Our data suggest that nuclear-cytoplasmic trafficking of tRNAs in response to nutrient availability is strictly controlled by several known signaling pathways that regulate the function of the proteins involved in the transport process. The regulation of nuclear tRNA export requires the conserved PKA and TOR pathways in *S. cerevisiae* and is dependent on the type of nutrient stress. This regulation is not evolutionarily conserved and appears to occur only in a subset of yeast species. This study provides evidence that there are fundamental differences among diverse organisms in the regulation of nuclear tRNA transport. Therefore, other species of
yeast may also regulate nuclear tRNA transport through unique pathways and mechanisms. Nuclear tRNA transport is an essential process required for cell growth and proliferation. Several yeast species have been associated with infectious disease such as *Candida albicans* (Giri and Kindo, 2012). Therefore, understanding the components and regulatory pathways involved in nuclear tRNA export that are specific for a yeast species such as *C. albicans* may provide unique targets to inhibit their cell growth to prevent further spread of fungal infections while not affecting the host species.

In contrast, *S. cerevisiae* is required in industry for the production of bread, wine and beer. Activation of the identified signaling pathway may stimulate nuclear tRNA export and increase protein synthesis and the rate of cell growth. As a result, strains with an increased rate of nuclear tRNA export and subsequently faster cell growth may increase the rate of fermentation providing a more efficient and cost effective fermentation. Therefore, identifying the differences in the regulation and signaling pathways involved in nuclear tRNA transport is of importance in providing organism specific targets that may help in industrial or health related situations.

The signaling pathways regulating nuclear tRNA transport in higher eukaryotes is poorly understood. Although the regulation of nuclear tRNA transport in response to nutrient availability is not evolutionarily conserved, several components involved in nuclear tRNA export in *S. cerevisiae* are conserved in higher eukaryotes, for example, Cex1p (Chafe and Mangroo, 2010; Johnstone *et al.*, 2011a). Although, higher eukaryotes are not subjected to acute changes in environmental conditions, the mechanisms and proteins involved in the regulation of nuclear tRNA transport in *S. cerevisiae* may provide insight into the methods used by higher eukaryotes in response to other cellular stresses, such as DNA damage. This may provide targets to inhibit
nuclear tRNA transport and consequently result in the death of abnormal cells. Therefore, understanding the mechanisms controlling nuclear-cytoplasmic tRNA trafficking in *S. cerevisiae* may also shed light on possible techniques that can be used to influence cell growth in various organisms.

### 5.0 Future directions

The mechanisms regulating nuclear tRNA export in response to nutrient availability are still largely unknown. Based on our studies, nuclear tRNA export is controlled by the TORC1 and PKA pathways in response to amino acid/nitrogen and glucose starvation, respectively. Surprisingly, nitrogen starvation and inhibition of TORC1 signaling by rapamycin treatment results in nuclear accumulation of tRNAs derived only from intron-containing pre-tRNAs. This is, in part, due to regulation of the function of the nuclear tRNA export receptor, Los1p and possibly Msn5p. Thus, studies should be conducted to ascertain whether TORC1 is responsible for regulating the function of the tRNA export receptors. Initially, it could be tested whether overexpression of Los1p or Msn5p restores nuclear tRNA export when TORC1 is inhibited with rapamycin. Protein interaction studies and phosphorylation assays can be performed to determine whether TORC1 is directly affecting the function of the tRNA export receptors or whether another protein(s) is mediating this interaction.

Our studies show that Utp9p and Utp22p may be regulated by the PKA signaling pathway in order to control nuclear tRNA export in response to glucose starvation. Therefore, it is possible that Utp9p and Utp22p may also be involved in a mechanism regulating nuclear tRNA export during the depletion of other nutrients. Monitoring the cellular tRNA distribution when Utp9p and Utp22p are overexpressed during rapamycin treatment may allow us to determine whether these known proteins function in regulating the tRNA export receptors.
through their specific and direct interaction with Msn5p and Los1p, respectively. However, if
TORC1 is not directly affecting the export receptors or affecting them through Utp9p and
Utp22p, the proteins that interact with Los1p or Msn5p specifically during amino acid/nitrogen
starvation and rapamycin inhibition of TORC1 can be indentified by affinity purification and
mass spectrometry analyses. These proteins may play a role in mediating the response from
TORC1 to the export receptors. Monitoring the nuclear-cytoplasmic distribution of tRNA during
rapamycin treatment after depletion of the identified interacting partners would provide insight
into whether they are directly involved in the regulation of nuclear tRNA export by TORC1. In
addition, in vitro and in vivo binding and phosphorylation assays can be performed to determine
if the identified protein is a direct downstream effector of TORC1. Also, monitoring cellular
tRNA distribution after deletion of other known downstream effectors of the TORC1 pathway
that respond to amino acid and nitrogen starvation should identify components involved in
nuclear tRNA export. These studies may potentially shed light on how amino acid starvation and
TORC1 regulate the function of the tRNA export receptors and consequently nuclear tRNA
export.

We have shown that the cell cycle does not regulate nuclear-cytoplasmic trafficking of
tRNA. However, synchronized cells show an increased vulnerability to changes in nutrient stress
suggesting that the cells in a certain stage of the cell cycle respond differently than cells in a
different stage. Therefore, arresting cells and subsequently starving them would identify the cell
cycle stage that is most sensitive to changes in nutrient levels.

Based on these studies, we propose that PKA facilitates nuclear re-import of the tRNA
export receptors by phosphorylating a receptor-binding protein, consequently preventing its
interaction with the receptors. However, when cells are starved of glucose and PKA is
inactivated, the protein(s) becomes dephosphorylated and interacts with the receptors to retain them in the cytoplasm. Therefore, it would be of interest to identify the phosphatase that dephosphorylates the protein associated with the export receptor. Phosphatases involved with the export receptors could be identified by determining the phosphatase deletion mutant that no longer relocates Los1p to the cytoplasm during glucose starvation. Furthermore, proteomic studies could be conducted to identify proteins that associate with Los1p and Msn5p in fed cells and cells deprived of glucose. The loss of an interactor(s) or appearance of an interactor(s) in fed or glucose starved cells will lead to the identification of the protein proposed to affect nuclear re-import of the nuclear tRNA export receptors.

Although the in vitro phosphorylation data is intriguing, further investigation into the in vivo phosphorylation status of Utp22p, Utp9p and Cex1p in the presence and absence of glucose is required to determine if their phosphorylation status changes in response to varying levels of glucose and if they are bona fide in vivo substrates for the yeast PKA. Furthermore, mutation of the conserved PKA motifs found in Utp22p and Cex1p and monitoring cellular tRNA distribution would provide evidence as to whether their phosphorylation plays a role in regulating nuclear tRNA by facilitating nuclear re-import of the tRNA export receptors. Studies have shown that each of the three catalytic subunits, Tpk1p, Tpk2p and Tpk3p have a distinct subset of substrates (Robertson and Fink, 1998; Giaever et al., 2002). Therefore, observing the localization of the tRNA export receptors in deletion mutants for each of the subunits or creating double mutants would provide insight as to whether a single subunit or multiple subunits are responsible for facilitating re-import of the export receptors.
6.0. References


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