Transfer RNA Nuclear Export Pathways in *Saccharomyces cerevisiae*

A Senior Honors Thesis

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ABSTRACT

tRNAs are small RNA molecules that function by delivering amino acids to the ribosome during translation. The focus of this work is to understand how tRNA moves from its site of synthesis in the nucleus to its site of function in the cytoplasm in yeast. The β-importin protein family member, Los1, binds to end-processed tRNAs in the nucleus and exports them to the cytoplasm. However, since Los1 is unessential, additional nuclear export tRNA pathways must exist. Our lab has identified two additional β-importins, Msn5 and Crm1, which cause tRNA nuclear accumulation when mutated.

This study seeks to determine if Los1, Msn5, and Crm1 are the only proteins involved in tRNA nuclear export? Towards this goal, I have constructed yeast strains bearing multiple mutations of the known genes. Should the *crm1-1 msn5Δ los1Δ* triple mutant be viable, it will suggest there are other unidentified tRNA nuclear pathways. However, if the triple mutant is not viable, then possibly Los1, Msn5, and Crm1 are the only tRNA export pathways. Previously, the *los1Δ msn5Δ* double deletion was determined to be viable. The *crm1-1 msn5Δ* and *crm1-1 los1Δ* double mutant are viable and, interestingly, they both display increased temperature sensitivity when compared with either single mutant or the *los1Δ msn5Δ* double mutant. Therefore, the *crm1-1* mutation coupled with the deletion of *los1* or *msn5* likely further impairs the export of tRNA from the nucleus. Because the double mutants are viable, I am constructing the triple *crm1-1 msn5Δ los1Δ* mutant.

CHAPTER 1: INTRODUCTION

tRNAs are small RNA molecules that are responsible for delivering amino acids to the ribosome for polymerization into a polypeptide during translation. They are synthesized in the nucleus as a primary transcript containing extra sequences on the 3' and 5' ends. Approximately 25% of yeast tRNAs also contain introns located one nuceleotide 3' to the anti-codon. Maturation of tRNA involves nuclear removal of the 3' and 5' ends, removal of introns by the splicing endonuclease, and various other nucleotide modifications occurring in the nucleus and cytosol. In yeast, unlike mammalian cells, the splicing endonuclease is located on the surface of the mitochondria [31,32]. Functioning as an adaptor molecule, tRNA facilitate the translation of an mRNA transcript into proteins. These molecules are necessary for proteins synthesis, which occurs in the cytoplasm at the ribosomes, and therefore tRNA export from their nuclear site of synthesis to the cytoplasm is essential.

tRNA transport is a dynamic, regulated, multidirectional process that determines the nuclear-cytosolic distribution of tRNA within the cell. The identification of mutations and stressors that alter the nuclear-cytosolic distribution of tRNA have aided our understanding of tRNA nuclear export. A wild-type cell grown in rich media has an even distribution of tRNA throughout the cell. However, blocked tRNA export, defective tRNA 3' end formation, inhibition of aminoacylation, and nutrient deprivation will cause tRNA nuclear accumulation [29,23,24]. The current model of tRNA transport (Figure 1.1) has three distinctive steps: primary tRNA nuclear export, retrograde tRNA transport (import), and re-export. Due to the mitochondrial location of the splicing endonuclease [31,32], primary tRNA nuclear export is

distinguishable from re-export in yeast, as primary export defects cause accumulation of intron-containing pre-tRNA.

Los1, a β-importin protein family member, binds to end-processed tRNAs in the nucleus and exports them to the cytoplasm [1,6,12,15,17,21]. Deletion of *LOS1* causes accumulation of intron-containing tRNA indicating that Los1 has a role in the primary tRNA export pathway. As *los1* cells accumulate every family of tRNA within the nuclei and Los1 binds mature tRNA, it is also likely to function in re-export. Despite the importance of tRNA nuclear export, *LOS1* is surprisingly unessential [13]. The unessential nature of *LOS1* has been demonstrated in other organisms containing *LOS1* homologues such as *S. pombe* and *Arabidopsis* [20]. The nonessential character of *LOS1* indicates that loss of the gene is likely compensated for by other genes that function in a similar fashion. Moreover, there is evidence that certain arthropods have no endogenous *LOS1* homologue [25]. These data indicate that there is another protein or proteins involved in the nuclear export of tRNA.

Msn5 is another protein that may play a role in the export of nuclear tRNAs. Msn5 is a β-importin that functions in pre-microRNA and tRNA export in *Drosophila* [11,25]. In yeast, Msn5 has been indicated in exporting transcription factors to the cytoplasm in response to nutrient availability [4,8]. The likelihood of Msn5 affecting nuclear export is evidenced by *MSN5* deletions resulting in tRNA nuclear accumulation when coupled with *LOS1* deletions [28]. Furthermore, Msn5 has been demonstrated to co-purify with other proteins shown to play a role in tRNA nuclear-cytoplasmic dynamics, namely Cex1 and Utp8 proteins [19,27]. Despite the implication of Msn5 being involved in tRNA nuclear export, it is unlikely this is the only other protein involved, as *los1Δ msn5Δ* double mutants do not display increased growth defects.

Synthetic lethality, or at least synthetic growth defects, would be expected if Los1 and Msn5 were the only proteins involved in tRNA nuclear export.

It has been shown that under several circumstances, namely defects in tRNA 3'-end formation and aminoacylation and during amino acid starvation tRNA accumulates in the nucleus. Interestingly, these tRNAs that accumulate do not contain introns [2,5]. Because these spliced tRNAs accumulate in the nucleus and the tRNA splicing machinery is located on the surface of the mitochondria [31,32], it reasons that tRNA is capable of being exported from the nucleus to its site of function in the cytoplasm and is then able to be imported in a retrograde manner back into the nucleus. This hypothesis was tested and supported by data showing the mitochondrial tRNA splicing machinery does not relocate to the nucleus under the same conditions [23]. Utilizing heterokaryon assays, yeast cells modified to produce tRNA from another organism were mated with yeast cells that were incapable of nuclear fusion and capable only of endogenous tRNA production. The result was that both nuclei were able to accumulate tagged tRNA despite only one nuclei producing it. This is strong evidence for the retrograde pathway of tRNA transport [23,28]. Additionally, the accumulation observed was determined not to be due to new transcription via the use of a tRNA transcription inhibitor [29]. As a result, retrograde movement was demonstrated for multiple independent families of tRNAs [28].

The role of nutrient availability and tRNA accumulation was broadened by other studies demonstrating that yeast cells deprived of amino acid [23], glucose [29], and phosphate [14] all led to nuclear accumulation of cytoplasmic tRNA, and that these nuclear tRNAs are re-exported upon resupplying the cells with the deprived nutrient [14,23,29].

Concordantly, it has been found that tRNA nuclear accumulation does not occur in mutants lacking another β-importin family, Mtr10. In *mtr10Δ* cells deprived of amino acids,

phosphate, or glucose did not result in the nuclear accumulation of tRNA [14,23,29]. Mtr10 is responsible for shuttling tRNAs from the cytoplasm back into the nucleus in a retrograde fashion. The biological function of shuttling tRNAs back into the nucleus is still unknown, but possible hypotheses include sequestration for proofreading allowing the cell the opportunity to check for proper tRNA aminocylation. An alternative hypothesis is that the retrograde pathway functions as a mechanism to downregulate overall protein synthesis during stress conditions. Once these previously cytoplasmic tRNAs have been shuttled back to the nucleus they are able to be re-exported again by the same Los1, Msn5 and other unknown proteins.

The retrograde pathway is conserved in vertebrate cells. Employing rat hepatoma H4IIE, Shaheen *et al* (2007) showed that amino acid deprivation caused nuclear accumulation of tRNA (even with tRNA transcription inhibitors). The study showed that the nuclear accumulated tRNAs are re-exported once amino acids are supplied to the cells [24]. The data indicate the process in vertebrate functions in a similar manner to the studies involving *S. cerivisiae*.

Previously, our lab identified an essential gene, *CRM1*, which may play a role in the export of tRNAs from the nucleus. Crm1 is a β-importin known to play a major role in protein and RNA export from the nucleus [15]. During a screen of yeast with mutations in genes encoding all known exportins and importins implicated in RNA metabolism it was identified that a temperature sensitive *crm1-1* allele accumulated tRNA in the nucleus [2]. Since the *crm1-1* mutant displays tRNA nuclear accumulation at non-permissive temperature, it is a possible candidate for involvement in the export pathway.

The question we proposed is: Are Crm1, Los1 and Msn5 the only proteins involved in the process of tRNA nuclear export? As *CRM1* is an essential gene it cannot be deleted, so to answer the question a temperature sensitive mutant allele of *CRM1* was utilized, *crm1-1*. This

temperature sensitive (ts) mutation allows for a way to bypass the essential nature of the gene by temperature inactivation. At the non-permissive temperature, such a ts mutant acts as though the gene were deleted. Starting with a *crm1-1* strain, two different double mutants were created utilizing the genes known to be involved in the pathway.

The double mutants of *crm1-1 msn5Δ* and *crm1-1 los1Δ* displayed increased temperature sensitivity when compared to the wild type, *crm1-1*, *msn5Δ*, *los1Δ* single mutants, and the *los1Δ msn5Δ* double mutant (Figure 3.2). As the *crm1-1 msn5Δ* and *crm1-1 los1Δ* double mutants have been found to be viable I am creating the triple mutant *crm1-1 los1Δ msn5Δ*. Should the triple mutant be viable, it will indicate there are likely other unknown proteins involved the pathway. Conversely, should the triple mutant be lethal it is a strong indication that Crm1, Msn5, and Los1 are the major players in the pathway.

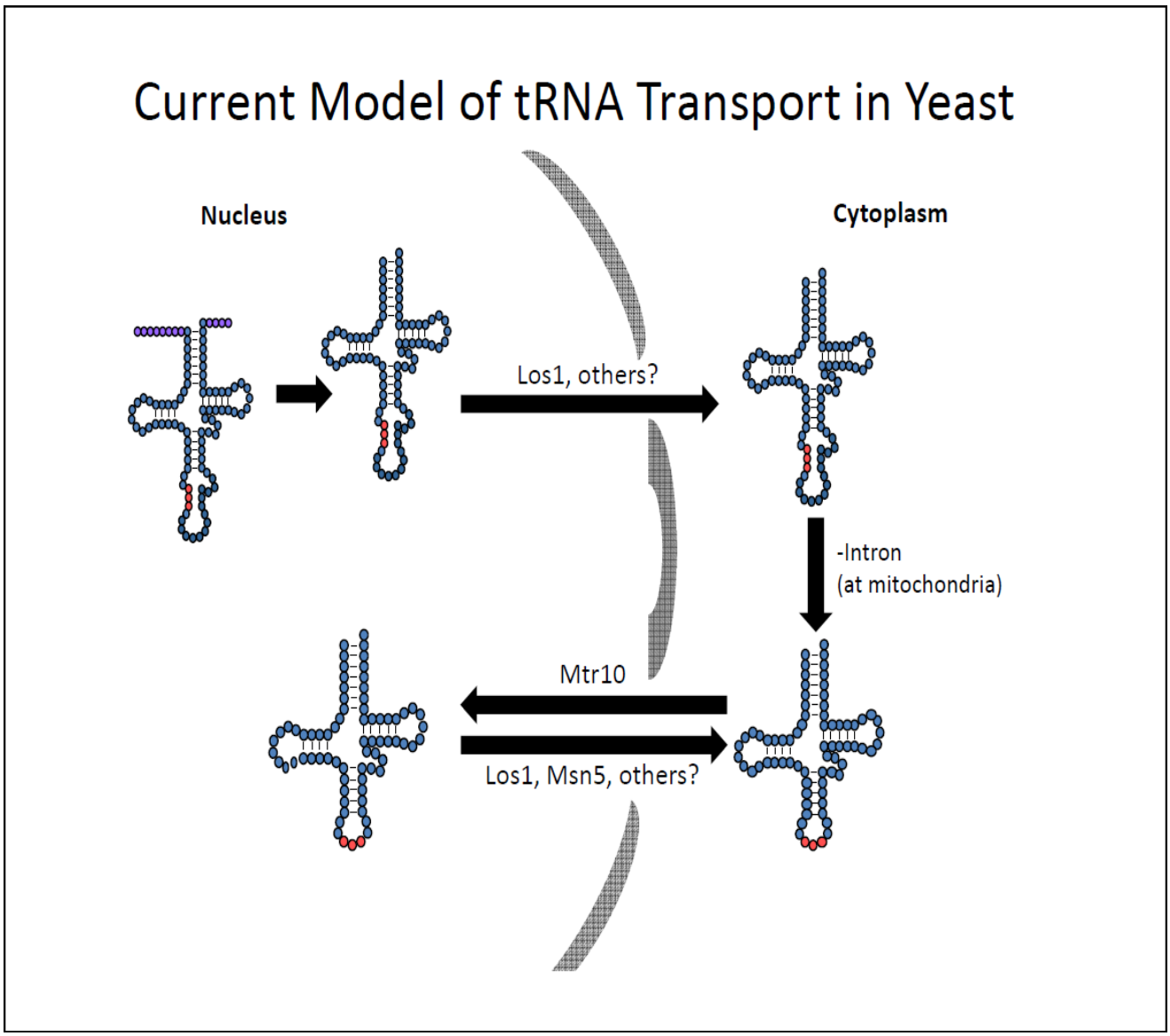
Current Model of tRNA Transport in Yeast
Nucleus
Cytoplasm
Los1, others?
-Intron
(at mitochondria)
Mtr10
Los1, Msn5, others?

FIGURE 1.1

CHAPTER 2: MATERIAL AND METHODS

Strains and Media

Strains used in this study are listed in Table 2.1. Strains were initially grown in yeast extract peptone dextrose media (YEPD). Transformants were selected by growing strains on YEPD+ cloNat (100 mg/L; Werner BioReagents), YEPD+ Hygromycin (6 mL/L; Cal Biochem), or -URA media (synthetic defined media lacking uracil).

Strain Construction

The deletions were achieved by lithium acetate transformation [28]. Using 1.5 mL of culture, cells were grown overnight, pelleted and resuspended in 100µL OSB buffer (0.1M lithium acetate, 0.05 M DTT, 40% polyethelene glycol, 0.5 mg/mL single-stranded salmon sperm DNA). Then 10 µL of plasmid DNA or PCR product was added to each reaction. The reaction mixes were incubated for 20 min at 45°C. For nutritional markers the reaction mixture was plated following incubation directly onto -URA media. For drug selection markers the reaction mixture was allowed to grow non-selectively in 2mL YEPD media for 3-6 hours. The cultures were then pelleted in 1.5 mL eppendorf tubes and plated on drug selective media.

All oligonucleotides used in this study are listed in Table 2.2. To create the PCR product for replacing the endogenous *MSN5*, primers WHIT59 and WHIT60 were used to amplify the *natMX4* template by employing PCR [29]. Essentially, this utilizes the sequence homology of *MSN5* shared with the WHIT59 and WHIT60 primers to target the NAT^R gene to replace the endogenous MSN5 gene (Figure 1.2). The 3' ends of WHIT59 and WHIT60 share sequence homology with the template allowing for PCR amplification. The creation of the PCR product to replace the endogenous *LOS1* with HPH^R was done in a similar fashion (Figure 1.3). The same

strategy of homology was utilized, however primers RLH007A and RLH007B shared sequence homology with the *hphMX4* and the 3' ends shared homology with the template allowing for PCR amplification.

Subsequently, in order to create the *crm1-1 los1Δ* msn5Δ triple mutant the *crm1-1 los1Δ* and *crm1-1* msn5Δ double mutants are both utilized in a two angled approach (Figure 3.4). Each double mutant was transformed with a covering plasmid to complement for the loss of the deleted gene. If Crm1, Los1, and Msn5 are the major proteins involved in the pathway, it is likely that the cells will not be viable. Complementation allows for a way to bypass the potential lethality of the triple mutant by supplying a functional copy of one of the deleted genes. Once the triple mutant is transformed and confirmed, selection for the loss of the covering plasmid is necessary to assay the cells viability. If the triple mutant is viable, it indicates that there are other proteins involved in the pathway.

Preparation of Genomic DNA

Single colonies were selected from streaks of transformation candidates and grown to saturation in 2 mL of YEPD. Cells were pelleted and cell walls were digested by resuspension in 200 µL of zymolyase solution. DNA was purified by phenol/chloroform extraction and precipitated by ethanol treatment. The DNA was then dissolved in sterile water for use as a template during PCR.

Confirmation of Genomic DNA

To confirm the knockout of *MSN5* in the *crm1-1 msn5Δ* double mutant primers were selected and the genomic DNA was PCR amplified utilizing JJH001 and JJH002 or JJH003 and

JJH004 to confirm the deletion of *MSN5*. The method utilized one primer designed to hybridize within the gene and another to hybridize outside of the integration area. Agarose gel electrophoresis was used to analyze the PCR products (Figure 3.1). Candidates that have had their endogenous *MSN5* replaced will lack the band corresponding to these primers. JJH001 and RLH025C or JJH004 and RLH025A were used to confirm the proper integration of *natMX4*. Again, the strategy was to hybridize to a region inside the inserted gene and another region outside the recombination area. Candidates that have had a successful integration of *natMX4* will display a band corresponding to these primers. By utilizing both *natMX4* and *MSN5* primers it allows for both confirming the presence of *natMX4* and the confirming absence of *MSN5*. If both are successful it indicates proper integration.

Confirming the replacement of *LOS1* with *hphMX4* is achieved in the same manner (Figure 3.1). The difference being that primers 7 and 12 or RLH010A and RLH010B are used for detecting the presence of LOS1 and RLH025B and RLH010B are used for confirming the integration of *hphMX4*. Again, candidates that both lack the band corresponding to *LOS1* and possess the band corresponding to *hphMX4* are successful candidates.

Plasmids

Plasmids used in this study are listed in Table 2.3. The YCp MSN5 plasmid was constructed by digesting MSN5 with restriction enzymes XhoI and NheI and the resulting fragment was purified. pRS416 was digested at the multiple cloning site with XhoI and SpeI. The fragments were purified and ligated together using T4 DNA ligase (New England BioLabs) and the ligation product was transformed in chemical competent *E. coli*; colonies that displayed ampicillin resistance were analyzed further.

Yeast Growth Assay

Spot assays were performed by growing cultures in YEPD media to saturation. The optical density (A_{600}) of the cultures was obtained by spectrophotometry, and the density was equalized to the lowest optical density culture. A series of four serial dilutions of each culture were created with each diluted by ten fold. Five µL of each dilution was plated on five YEPD plates and incubated at 23°C, 30°C, 34°C, 37° or 39°C. Pictures were taken between 24-48 h after spotting. Photoshop CS3 (Adobe) was used for assembling the images.

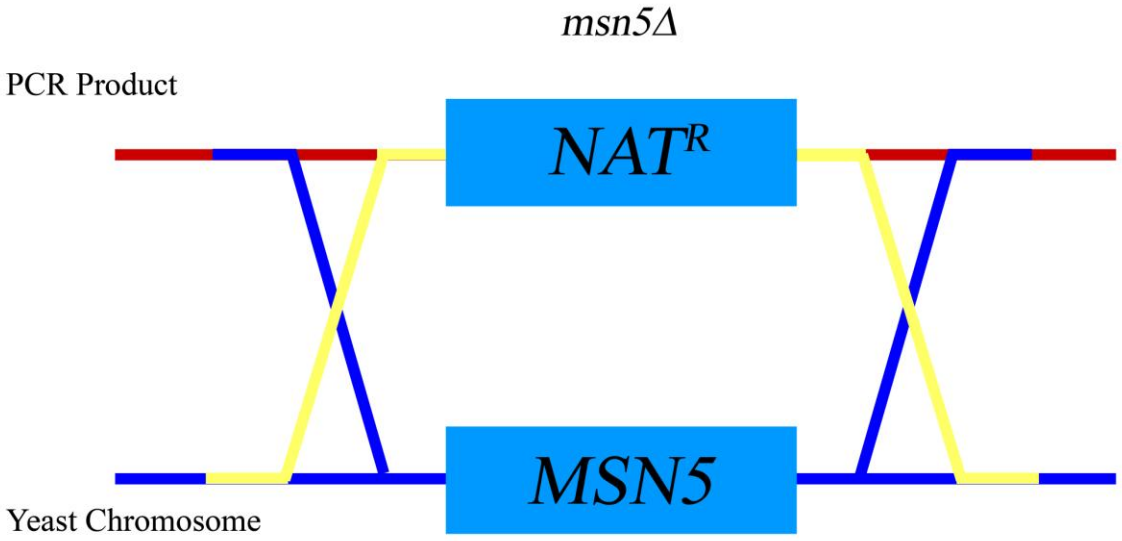

Figure 1.2: *NAT*^{R} replaces *msn5Δ* homologous recombination

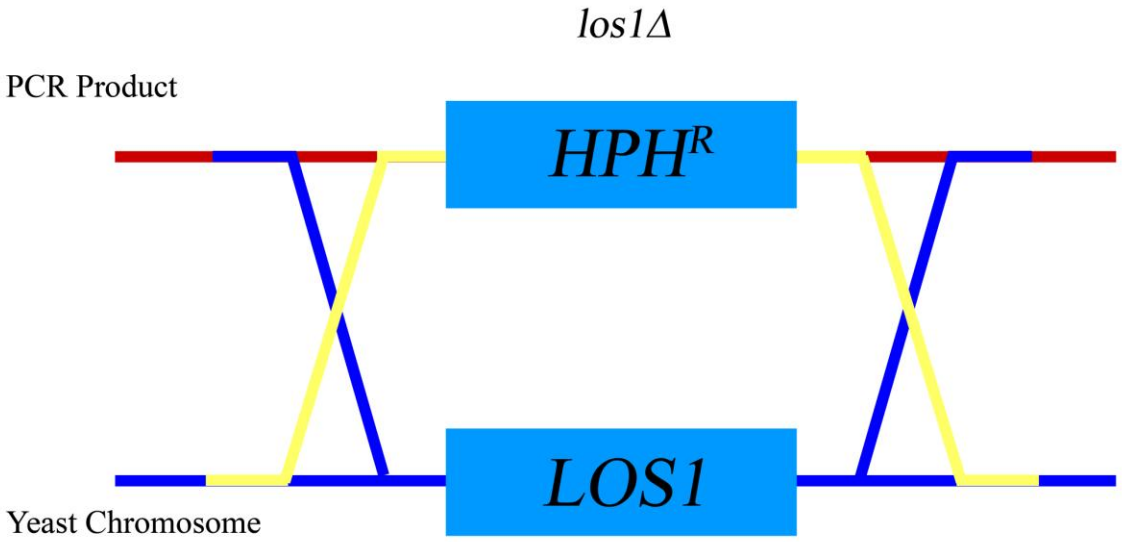

Figure 1.3: *HPH*^{R} replaces *los1Δ* homologous recombination

Table 2.1

Yeast Strain(s)	Genotype	Citation
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems, Huntsville, AL
crm1-1	*MATa CRM1::crm1-1G418 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	C. Boone (unpublished)
los1Δ	*MATa los1::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	*MATa* deletion collection (Invitrogen)
msn5Δ	*MATa msn5::kanMXhis3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	*MATa* deletion collection (Invitrogen)
los1Δ msn5Δ	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 los1Δ msn5Δ*	Gift from Ivy Huang
crm1-1 los1Δ	*MATa los1::nat^R CRM1::crm1-1G418 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This Study
crm1-1 msn5Δ	*MATa msn5::hph^R CRM1::crm1-1G418 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This Study

Table 2.2

Oligo Name	Sequence 5' to 3'	Citation
JJH001	CATTCGTGTAGCAATTGTTACTGTG	This Study
JJH002	GGCTTTCATTACTATAGGGACGG	This Study
JJH003	CAACACGGTGGATGAATATTTCAC	This Study
JJH004	CATTGCCAATGGTTTCCTATTTCGC	This Study
RLH007A	AAGTTGTCGGTAAGCAACCTATAGAACAAGTGTTTCCAGTCA AATCGAGGAACATGGAGGCCCAGAATACC	[27]
RLH007B	CATATTACCAATTTAAATATTAGTATAAATATAATTACAACT TTTACTCTCCAGTATAGCGA CCAGCATTCAC	[27]
RLH010A	CAGGTTACTCATTGTGGGATC	[27]
RLH010B	GCACTTTTA GTGCCATTACATG	[27]
Primer 7	TAAGAACCCAAGAAAaTC	R.L.Hurto (unpublished)
Primer 12	AGATTCTTGAAGTTGACA	R.L.Hurto (unpublished)
RLH025A	CCGGCGGATGGGGTTCAC	R.L.Hurto (unpublished)
RLH025B	CACTACATGGCGTGATTTCATATGCGCG	R.L.Hurto (unpublished)
RLH025C	GTGTCGGTGGTGAAGGACCCATCC	R.L.Hurto (unpublished)
WHIT 59	CGTTGATTGGAAGAAAAGTAATGGATTCCACAGGCGCTTCTC AGATTGTTACAGCTGAAGCTTCGTACGC	M. Whitney (unpublished)
WHIT60	CAGACCCACATTAAAACGCTTGATTATATGCATATTTACCGG CTGCCGACTGTGGATCTGATATCATCGA	M. Whitney (unpublished)

Table 2.3

Plasmid Name	**Characteristics**	**Citation**
pRS416	pBLUESCRIPTII URA3 CEN6 ARSH4 lacZ Amp^R	[26]
YCp 50	ARSI URA3 CEN4 Amp^R	[16]
MSN5 MORF	P_{GAL} URA3 HIS6-HA Amp^R MSN5	[3]
YEp MSN5	LEU2 lacZ Kan^R MSN5	Yeast Tiling Collection (Open Biosystems)
YCp LOS1	ARSI URA3 CEN4 Amp^R LOS1	[13]
YCp MSN5	pBLUESCRIPTII URA3 CEN6 ARSH4 lacZ Amp^R MSN5	This Study

CHAPTER 3: RESULTS

Phenotype of Double Mutants

The *MSN5* and *LOS1* genes are known to function in the export of nuclear tRNA to the cytoplasm. *CRM1* is an essential gene that may also be involved in the same pathway. However, since *CRM1* is an essential gene, a temperature sensitive (ts) strain, *crm1-1*, was utilized. Starting with the *crm1-1* strain, *MSN5* was knocked out by replacing the endogenous gene with a NAT^R drug resitance marker (Figure 3.4). This was accomplished by creating PCR products with sequence homology to the MSN5 gene directly upstream and downstream of the NAT^R marker and using homologous recombination (Figure 1.2). Genomic DNA of the *crm1-1 msn5Δ* double mutant was prepared. Primers were used to amplify the genomic DNA by PCR. The PCR product was analyzed and the *crm1-1 msn5Δ* double mutant was confirmed by agarose gel electrophoresis (Figure 3.1). The absence of an expected 715 bp band indicates *MSN5* is not present, and the presence of a 507 bp band indicates the integration of *natMX4*. Therefore, lanes 3 and 4 correspond to successful transformants.

The phenotype of the *crm1-1 msn5Δ* double mutant was assessed by spot assay to evaluate the growth of the double mutant. At 30°C, *crm1-1 msn5Δ* shows a reduced growth rate compared to the wild type, *losΔ, msn5Δ,* or the *los1Δ msn5Δ* double mutant. At 34°C, the *crm1-1 msn5Δ* double mutant also shows increased temperature sensitivity compared to those at 30°C, but the interesting difference at 34°C is that the *crm1-1 msn5Δ* mutant displays greater temperature sensitivity than the *crm1-1* strain.

The *crm1-1 msn5Δ* double mutant was transformed with a covering plasmid, to allow for complementation of the deleted gene. The initial plasmid, MSN5 MORF [3], was a high copy plasmid and was under a GAL inducible promoter. In the past, members of our lab have

experienced synthetic growth defects as a result of high copies of *MSN5*. When the double mutant was transformed with the MSN5 MORF plasmid the cells displayed a phenotype of reduced growth (data not shown). It is likely that the overproduction of Msn5 as a result of GAL induction resulted in cellular toxicity. Utilizing a low copy plasmid with *MSN5* under the endogenous promoter may rescue the growth defect.

Therefore, a centromeric plasmid, YCpMSN5, was constructed by digesting the pRS416 plasmid [26] (a centromeric plasmid) and *MSN5* with the endogenous promoter from the YEpMSN5 (Open Biosystems). *MSN5*, with the endogenous promoter, was ligated into the pRS416 multiple cloning site. The plasmid construct was transformed into chemical competent *E. coli* cells and colonies displaying ampicillin resistance were isolated. The plasmid was transformed into the *crm1-1 msn5Δ* double mutant. YCpMSN5 did not result in the reduced growth displayed by the previous plasmid (data not shown), thereby improving the cells viability and transformation efficiency.

The *crm1-1 msn5Δ* double mutant harboring *YCpMSN5* was then used to attempt creation of the *crm1-1 msn5Δ los1Δ* triple mutant. This transformation was performed multiple times and resulted in either no candidates or false positive transformants. However, during the transformations to delete *LOS1* in the *crm1-1 msn5Δ* double mutant, the *crm1-1* single mutant was used as a control, and from the *crm1-1* control, candidates of *crm1-1 los1Δ* were confirmed as successful transformants. Genomic DNA of the *crm1-1 los1Δ* double mutant was prepared and primers were used to amplify the DNA by use of PCR. The PCR product was analyzed by agarose gel electrophoresis and the *crm1-1 los1Δ* double mutant was confirmed (Figure 3.1). The absence of the expected 674 bp band indicates *LOS1* is not present, and the presence of a 973 bp

band indicates the integration of *hphMX4* gene. Therefore, lane 2 indicates a successful transformant.

The phenotype of the *crm1-1 los1Δ* double mutant was ascertained by spot assay (Figure 3.2). The temperature sensitivity of *crm1-1 los1Δ* at 30°C is drastically increased compared to the wild type, *los1Δ, msn5Δ, crm1-1*, and *los1Δmsn5Δ*. Moreover, *crm1-1 los1Δ* also displays a reduced growth rate compared to *crm1-1 msn5Δ* at 30°C.

With the addition of *crm1-1 los1Δ* the two angled approach of transforming both double mutants is being used to expedite the process of creating the triple mutant. The *crm1-1 los1Δ* double mutant was transformed with a covering plasmid YCpLOS1, supplying the cell with a functional copy of *LOS1* under the endogenous promoter. If Crm1, Los1, and Msn5 are the only proteins involved in the pathway, a triple mutant would likely not be viable. YCpLOS1 allows for complementation to prevent this possible lethality. The triple mutant *crm1-1 los1Δ msn5Δ* has yet to be attained.

When the triple mutant is confirmed, the next step is to select for the loss of the covering plasmid. Both of the covering plasmids used contain a URA3 marker, which will cause cells grown on 5FOA media to produce a toxic compound. This will select for the loss of the plasmid. This raises the possibility that if Crm1, Los1 and Msn5 are the only genes involved in this critical pathway, then a triple mutant may not be viable without a covering plasmid.

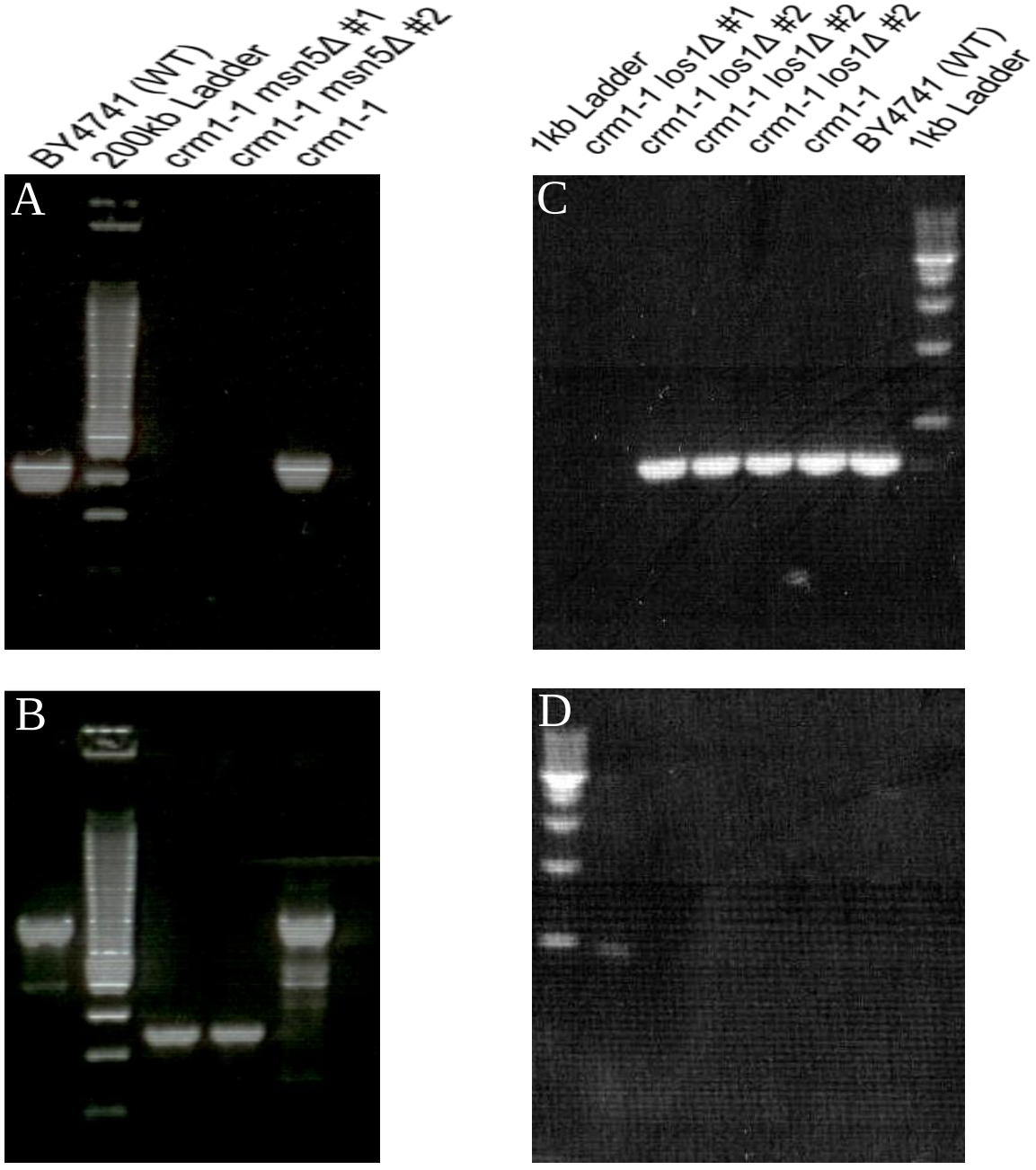

Figure 3.1: PCR Confirmation of *crm1-1 msn5Δ* and *crm1-1 los1Δ* candidates. (A) Absence of expected 715 bp band in lanes 3 and 4 indicate *MSN5* is not present. (B) Presence of expected 507 bp band in lanes 3 and 4 indicate integration of *natMX4*. Multiple bands present in WT and *crm1-1* are consistent with known nonspecific bands. (C) Absence of expected 674 bp band in lane 2 indicates *LOS1* is not present. (D) Presence of expected 973 bp band in lane 2 indicates integration of *hphMX4*.

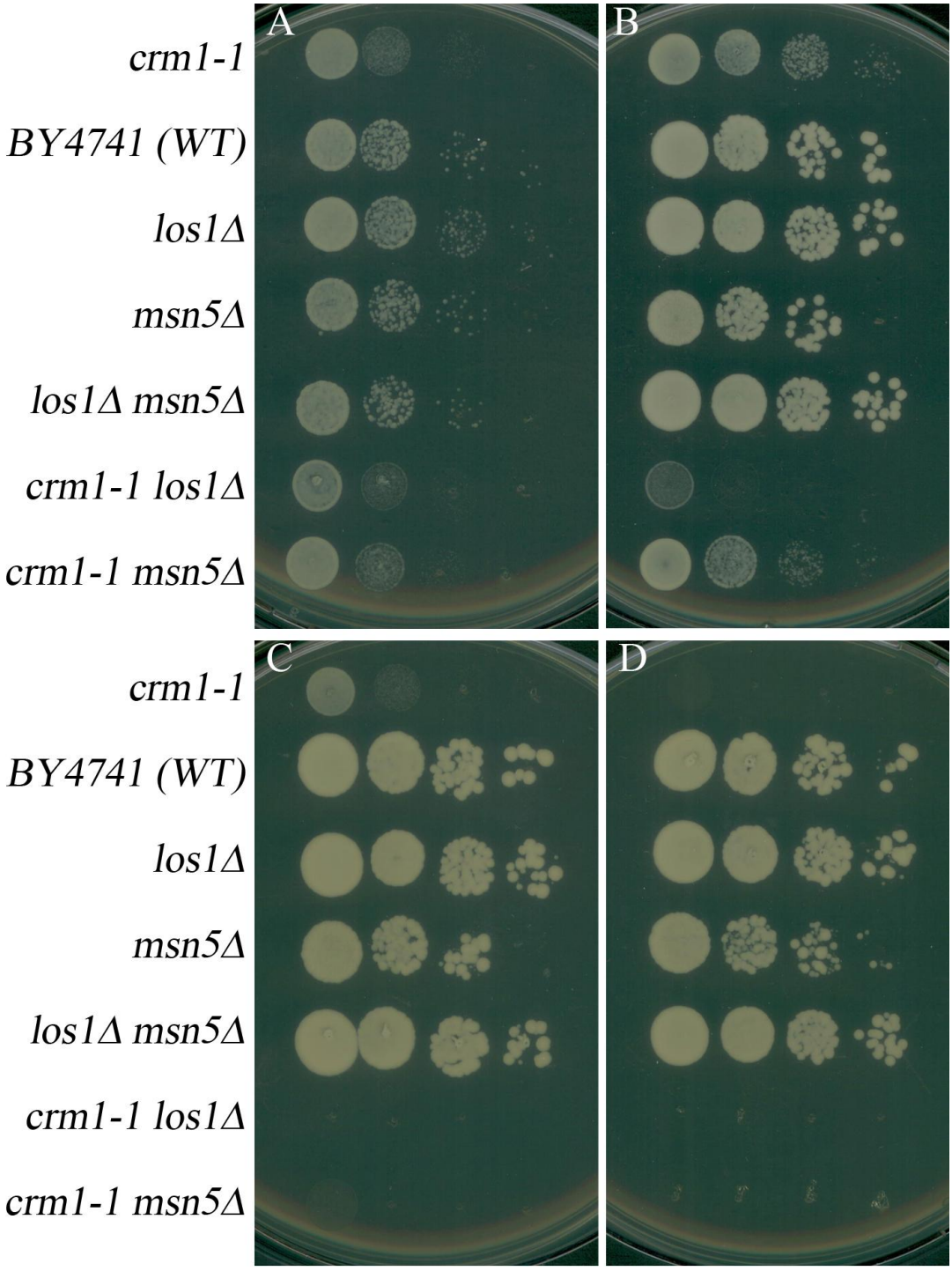

Figure 3.2: Yeast Growth Assay of wild type, single mutants and double mutants. (A) 23°C; all strains viable. (B) 30°C; *crm1-1 los1Δ* shows increased temperature sensitivity compared to single and double mutants. (C) 34°C; *crm1-1 los1Δ* and *crm1-1 msn5Δ* show increased temperature sensitivity compared to single and double mutants. (D) 37°C; *crm1-1* non-permissive temperature.

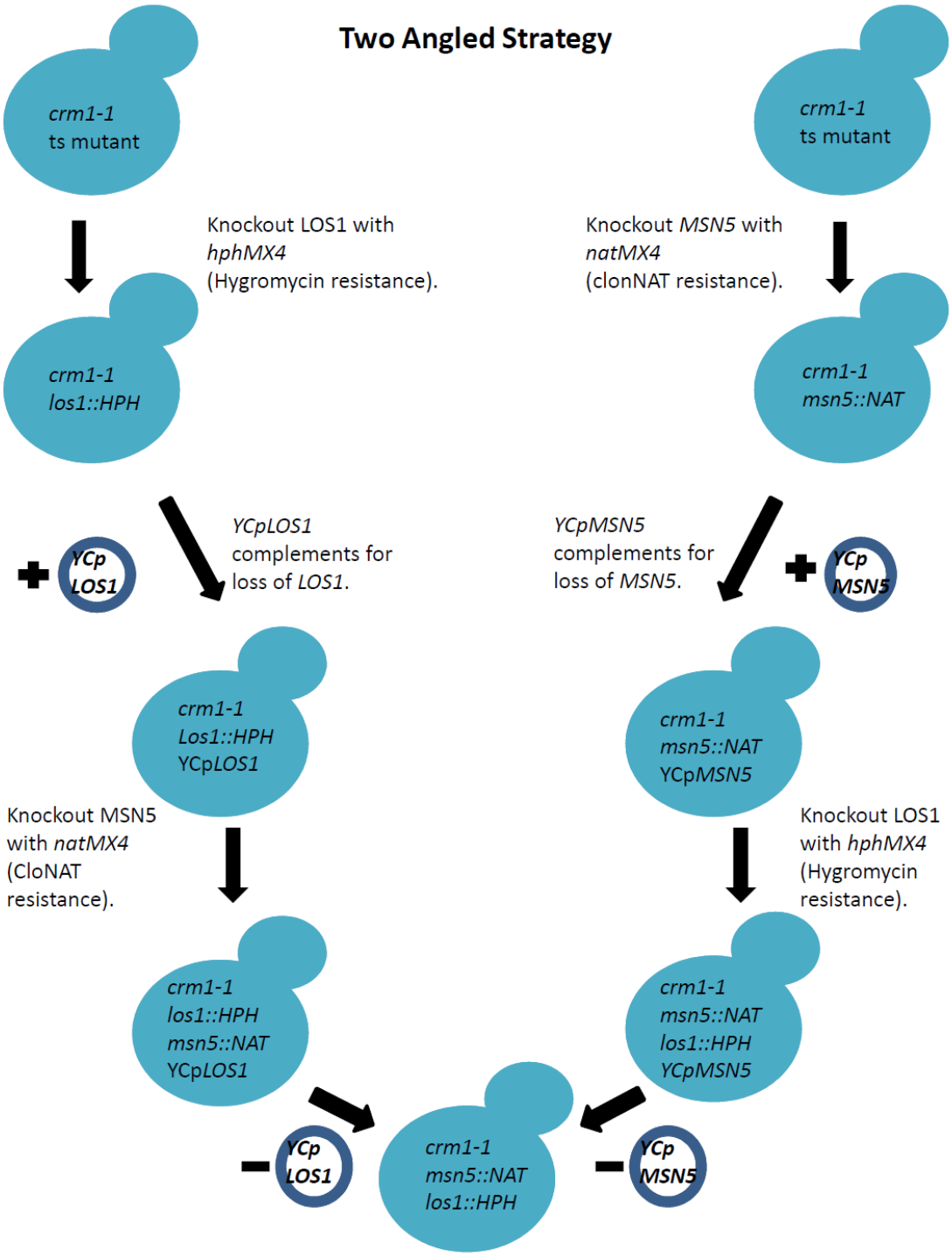
Two Angled Strategy
crm1-1
ts mutant
Knockout LOS1 with
hphMX4
(Hygromycin resistance).
crm1-1
los1::HPH
YCp
LOS1
YCpLOS1
complements for
loss of LOS1.
crm1-1
Los1::HPH
YCpLOS1
Knockout MSN5
with natMX4
(CloNAT
resistance).
crm1-1
los1::HPH
msn5::NAT
YCpLOS1
YCp
LOS1
crm1-1
msn5::NAT
los1::HPH
crm1-1
ts mutant
Knockout MSN5 with
natMX4
(clonNAT resistance).
crm1-1
msn5::NAT
YCpMSN5
complements for
loss of MSN5.
YCp
MSN5
crm1-1
msn5::NAT
YCpMSN5
Knockout LOS1
with hphMX4
(Hygromycin
resistance).
crm1-1
msn5::NAT
los1::HPH
YCpMSN5
YCp
MSN5

Figure 3.4

CHAPTER 4: DISCUSSION

Individually, the deletion of *MSN5* or *LOS1* results in no noticeable phenotype. These genes are unessential and furthermore the double deletion of *los1Δ msn5Δ* also has no significant phenotype. However, when *MSN5* or *LOS1* is deleted from the *crm1-1* mutant strain there is a substantial phenotype of temperature sensitivity. The spot assays conducted showed that the *crm1-1 msn5Δ* and *crm1-1 los1Δ* double mutants were more sensitive to temperature than *crm1-1* at 34°C. Interestingly, the *crm1-1 los1Δ* double mutant does not grow at 30°C, a substantial shift from both *crm1-1* and *crm1-1 msn5Δ* (Figure 3.2). The reduced growth rate of *crm1-1 los1Δ* at 30°C when compared to the *crm1-1 msn5Δ* double mutant is of significance. Los1 has been shown to be involved in both the export of newly synthesized tRNA and the re-export of tRNA that has been shuttled back to the nucleus. Msn5 has been shown to function in the re-export of tRNA; however, export of newly synthesized tRNA has not been demonstrated. If the main role of Msn5 is involved with only the re-export of tRNA, then deleting *LOS1* would should a more significant impact on nuclear-cytoplasmic dynamics than *MSN5*. The data produced in this study support the hypothesis that the main role of Msn5 is involved in the re-export of tRNA rather than the export of newly synthesized tRNA, based on the relative temperature sensitivity.

The function of Crm1 on nuclear-cytoplasmic dynamics is still unknown. Nonetheless, the data in this study would point towards Crm1 having a major role in the pathway. Previous data [2] have shown that the *crm1-1* single accumulates tRNA lacking introns. However, there was also a small amount of tRNA containing introns observed. Due to the previous data, as well as the double mutant phenotypes observed in this study, it suggests that Crm1 is involved in both export of newly synthesized tRNA and re-export of tRNA shuttled back into the nucleus.

It cannot be ruled out that stacking most any unessential deletion in one strain would result in synthetic growth defects. While that is possible, it would be likely that in this case the double mutant *los1Δ msn5Δ* would display increased temperature sensitivity in a similar fashion to *crm1-1 msn5Δ* and *crm1-1 los1Δ*. Since this is not the case, I argue that it is unlikely that the phenotype of increased temperature sensitivity in the *crm1-1 msn5Δ* and *crm1-1 los1Δ* double mutants are a result of multiple unrelated mutations. Rather, it is likely due to the genes in question having a role in the same pathway. Therefore, while not conclusive, it is reasonable to suggest that Crm1, Msn5 and Los1 have similar functions and the presence of one is at least partially able to compensate for mutations in another.

Unfortunately, due to time constraints the *crm1-1 los1Δ msn5Δ* triple mutant has yet to be created. The most important future goal of this research is to confirm a *crm1-1 los1Δ msn5Δ* triple mutant. From there, the next step would be to select for the loss of the covering plasmid. Since the covering plasmids used have a *URA3* marker, plating the triple mutant on 5FOA media results in a toxic intermediate and will select for cells that have lost the plasmid.

If loss of the plasmid results in viable cells it is likely there are other proteins involved in this pathway. Identifying further suspect genes could be performed by genome wide screens looking for nuclear accumulation of tRNA or by comparison of known genes and proteins to those in the Saccharomyces Genome Database. If candidates are unable to be obtained on 5FOA media it indicates that without a covering plasmid the cells are not viable. That conclusion would suggest that Crm1, Los1 and Msn5 are the major proteins functioning in this pathway.

However if candidates can be obtained on 5FOA media it suggests that there are other proteins involved in the export of nuclear tRNAs. It would not be unreasonable to suggest cells would have redundancy or similar functioning proteins for maintaining a vital function. tRNA

plays an essential role in cell biology due to the role in protein synthesis. It would seem to be advantageous for a cell to have redundancy in this pathway to allow for complex regulation of tRNA nuclear export pathways.

Possible Future Experiment

Should the triple mutant prove to be lethal without a covering plasmid there are options that may allow for further study of the mutant. One option would be to select for suppressors that would rescue viability by returning partial function of the pathway. Another option would be by deleting *MTR10* (Fig. 1.1), that functions in the import of once cytoplasmic tRNAs, it may be possible to circumvent the lethality of the triple mutant. The idea is thatiIf cytoplasmic tRNAs are required for viability, knocking out the re-import mechanism (*MTR10*) may allow enough tRNA to be retained in the cytoplasm for viability to be maintained. If this *crm1-1 los1Δ msn5Δ mtr10Δ* quadruple mutant is viable it will allow for further study of the pathway and deeper elucidation of tRNA nuclear and cytoplasm dynamics.

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REFERNCES

1 Arts, G.J., Fornerod M. and Mattaj W. (1998) Identification of a nuclear export receptor for tRNA. Curr. Biol. 8, 305–314

2 Feng, W. and Hopper, A.K. (2002) A Los1p-independent pathway for nuclear export of intronless tRNAs in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 99, 5412–5417

3 Gelperin DM, White MA, Wilkinson ML, Kon Y, Kung LA, Wise KJ, Lopez-Hoyo N, Jiang L, Piccirillo S, Yu H, Gerstein M., Dumont M., Phizicky E., Snyder M. and Grayhack E. (2005) Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes Dev. 19:2816–2826.

4 Gorlich, D. and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. 15, 607–660

5 Grosshans, H., Hurt E. and Simos G. (2000) An aminoacylation-dependent nuclear tRNA export pathway in yeast. Genes Dev. 14, 830–840

6 Hellmuth, K., Lau D., Bischoff R., Kunzler M., Hurt E. and Simos G. (1998) Yeast Los1p has properties of an exportinlike nucleocytoplasmic transport factor for tRNA. Mol. Cell. Biol. 18, 6374–6386

7 Goldstein, A.L. and J.H. McCusker. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae.* Yeast. 14: 1541-1553.

8 Hopper, A.K. (1999) Nucleocytoplasmic transport: inside out regulation. Curr. Biol. 9, R803–R806

9 Hopper, A.K. and Phizicky, E.M. (2003) tRNA transfers to the limelight. Genes Dev. 17, 162–180

10 Hopper, A.K., and Shaheen, H.H. (2008). A decade of surprises for tRNA nuclear-cytoplasmic dynamics. Trends Cell Biol. 18, 98–104.

11 Huh, W.K., Falvo J., Gerke L., Carroll A., Howson R., Weissman J. and O'Shea E. (2003) Global analysis of protein localization in budding yeast. Nature 425, 686–691

12 Hunter, C.A., Aukerman M., Sun H., Fokina M. and Poethig R. (2003) PAUSED encodes the Arabidopsis exportin-t ortholog. Plant Physiol. 132, 2135–2143

13 Hurt, D.J., Wang S., Lin Y.H. and Hopper A.K. (1987) Cloning and characterization of LOS1, a Saccharomyces cerevisiae gene that affects tRNA splicing. Mol. Cell. Biol. 7, 1208–1216

14 Hurto, R.L., Tong A., Boone C. and Hopper, A.K. (2007) Inorganic phosphate deprivation causes tRNA nuclear accumulation via retrograde transport in Saccharomyces cerevisiae. Genetics 176, 841–852

15 Hutten S., Kehlenbach R.H., (2007) CRM1-mediated nuclear export: to the pore and beyond, Trends Cell Biol. 17, 193–201.

16 Kuo, C.-L., and J. L. Campbell. (1983) Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. 3:1730-1737.

17 Li, J. and Chen, X. (2003) PAUSED, a putative exportin-t, acts pleiotropically in Arabidopsis development but is dispensable for viability. Plant Physiol. 132, 1913–1924

18 Lund, E. and Dahlberg, J.E. (1998) Proofreading and aminoacylation of tRNAs before export from the nucleus. Science 282, 2082–2085

19 McGuire, A.T. and Mangroo, D. (2007) Cex1p is a novel cytoplasmic component of the Saccharomyces cerevisiae nuclear tRNA export machinery. EMBO J. 26, 288–300

20 Melton, D.A., Robertis E.M. and Cortese R. (1980) Order and intracellular location of the events involved in the maturation of a spliced tRNA. Nature 284, 143–148

21 Sarkar, S. and Hopper, A.K. (1998) tRNA nuclear export in Saccharomyces cerevisiae: in situ hybridization analysis. Mol. Biol. Cell 9, 3041–3055

22 Schiestl, R.H., and R.D. Gietz. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. 16:339-346.

23 Shaheen, H.H. and Hopper, A.K. (2005) Retrograde movement of tRNAs from the cytoplasm to the nucleus in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 102, 11290–11295

24 Shaheen, H.H., Horetsky R., Kimball S., Murthi A., Jefferson L. and Hopper A.K. (2007) Retrograde nuclear accumulation of cytoplasmic tRNA in rat hepatoma cells in response to amino acid deprivation. Proc. Natl. Acad. Sci. U. S. A. 104, 8845–8850

25 Shibata, S., Sasaki M., Miki T., Shimamoto A., Furuichi Y., Katahira J. and Yoneda Y. (2006) Exportin-5 orthologues are functionally divergent among species. Nucleic Acids Res. 34, 4711–4721

26 Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122 (1), 19-27.

27 Strub, B.R., Eswora M., Pierce J. and Mangroo D. (2007) Utp8p is a nucleolar tRNA-binding protein that forms a complex with components of the nuclear tRNA export machinery in Saccharomyces cerevisiae. Mol. Biol. Cell 18, 3845–3859

28 Takano, A., Endo T. and Yoshihisa T. (2005) tRNA actively shuttles between the nucleus and cytosol in yeast. Science 309, 140–142

29 Whitney, M.L., Hurto R.L., Shaheen H. and Hopper A.K. (2007) Rapid and reversible nuclear accumulation of cytoplasmic tRNA in response to nutrient availability. Mol. Biol. Cell 18, 2678–2686

30 Yoshida, K. and Blobel, G. (2001) The karyopherin Kap142p/Msn5p mediates nuclear import and nuclear export of different cargo proteins. J. Cell Biol. 152, 729–740

31 Yoshihisa, T., Yunoki-Esaki K., Ohshima C., Tanak N. and Endo T. (2003) Possibility of cytoplasmic pre-tRNA splicing: the yeast tRNA splicing endonuclease mainly localizes on the mitochondria. Mol. Biol. Cell 14, 3266–3279

32 Yoshihisa, T., Ohshima C., Yunoki-Esaki K. and Endo T. (2007) Cytoplasmic splicing of tRNA in Saccharomyces cerevisiae. Genes Cells 12, 285–297