INTERSECTION OF RNA PROCESSING AND FATTY ACID SYNTHESIS AND ATTACHMENT IN YEAST MITOCHONDRIA

by

Melissa Suzanne Schonauer

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As members of the Dissertation Committee, we certify that we have read the dissertation

prepared by **Melissa Suzanne Schonauer**

entitled **INTERSECTION OF RNA PROCESSING AND FATTY ACID SYNTHESIS AND ATTACHMENT IN YEAST MITOCHONDRIA**

and recommend that it be accepted as fulfilling the dissertation requirement for the

Degree of Doctor of Philosophy

_____ Date: 10/24/2008
Carol Dieckmann

_____ Date: 10/24/2008
Frans Tax

_____ Date: 10/24/2008
Ted Weinert

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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DEDICATION

To my husband, Kurt.

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LIST OF ABREVIATIONS

YEPD	Glucose media
YEPG	Glycerol media
***rho*$^{+}$**	Wild-type mitochondrial genome
***rho*^{0}**	Mitochondria without DNA
COB	Mitochondrial cytochrome *b* gene
PET127	Nuclear gene that encodes Pet127
ts	Temperature-sensitive
cs	Cold-sensitive
RNase P	Ribonuclease P
RPM1	RNA component of mitochondrial RNase P
Rpm2	Nuclear-encoded protein component of mitochondrial RNase P
HTD2	Nuclear gene that encodes 3-hydroxyacyl thioester dehydratase 2 (Htd2)
FAS II	Type II fatty acid synthesis pathway
LIP3	Nuclear gene that encodes Lip3
LplA	*E. coli* lipoate-protein ligase
mtlplA	*E. coli lplA* gene fused to a mitochondrial targeting sequence
lip	lipoic acid synthesis and attachment
SCR1	Signal recognition particle RNA
GFP	Green fluorescent protein
ACP	Acyl carrier protein
PDH	Pyruvate dehydrogenase
α-KDH	α-ketoglutarate dehydrogenase
GC	Glycine cleavage enzyme
Lat1	E2 subunit of PDH
Kgd2	E2 subunit of α-KDH
Gcv3	H protein of GC

ABSTRACT

Intersections of distinct biological pathways in cells allow for nodes of metabolic regulation. This work describes the discovery of the intersection of two pathways in yeast mitochondria: RNA processing and fatty acid synthesis and attachment. Analysis of the components of the pathways is presented here along with a model illustrating the connection as a potential mode of regulation of mitochondrial gene expression.

A genome-wide screen of respiratory-deficient *Saccharomyces cerevisiae* deletion strains for defects in mitochondrial RNA processing revealed that two novel genes affect processing of mitochondrial tRNAs by RNase P. One gene encodes Htd2, an enzyme in the type II mitochondrial fatty acid synthesis pathway (FAS II). The other gene is described here as encoding Lip3, an enzyme involved in the synthesis and attachment of the co-factor lipoic acid, which is synthesized from a product of the FAS II pathway.

RPM1 is the mitochondrial-encoded RNA subunit of mitochondrial RNase P. The multigenic transcription unit containing RPM1 also contains $tRNA^{pro}$. Maturation of RPM1 necessitates processing of the tRNA by RNase P. Thus, RNase P is required for maturation of its own RNA component, constituting a positive feedback cycle. The present work demonstrates that a product of the FAS II pathway is necessary for the assembly or activity of RNase P, as deletion of any gene encoding an FAS II enzyme results in inefficient processing of $tRNA^{pro}$ from the transcript.

Analysis of the enzymes involved in the synthesis and attachment of lipoic acid to target proteins is also described here. Disruption of any of these enzymes affects protein

lipoylation and tRNA processing. Gcv3, a target of lipoylation, was found to be required for lipoylation as well as for efficient tRNA processing.

A second feedback cycle controlling pyruvate dehydrogenase activity and fatty acid synthesis may be functional under certain conditions. Pyruvate dehydrogenase, which provides acetyl-CoA for the FAS II pathway, requires lipoic acid for its activity. It is hypothesized that the two feedback cycles and the role of Gcv3 may provide switch-like regulation of mitochondrial gene expression in response to the nutritional state of the cell.

CHAPTER 1. INTRODUCTION

OVERVIEW

Mitochondria are an integral part of the eukaryotic cell since a number of essential functions are carried out within this organelle. Investigations into the inner workings of the mitochondrial compartment have been focused mostly on genetically identifying individual mitochondrial proteins and determining their function. A number of aspects of mitochondrial biology, though, have posed technical challenges that have been difficult, if not impossible, to overcome in the laboratory. The low abundance of mitochondrial proteins and the high percentage of mitochondrial proteins that are associated with the membrane have limited the success of biochemical experiments. Also, many resident proteins have evolved more than one function due to restricted space within the organellar compartment, which has made it difficult to assemble networks of mitochondrial molecular pathways within the context of the whole cell. However, in the last few years, technological advances in genomics and proteomics have increased our knowledge of the complete set of mitochondrial proteins in different organisms.

Well-established genetic tools available in the yeast *Saccharomyces cerevisiae* have aided the advancement of mitochondrial research. Unlike mammals, yeast are facultative anaerobes; they can either produce energy via respiration, which requires mitochondrial gene expression, or via fermentation, which does not (the mitochondrial genome encodes only factors necessary for respiration). Therefore, mutations in nuclear genes that encode factors required for mitochondrial gene expression, or *PET* genes, can

be made and their effects can be studied by maintaining the cells on fermentable medium. Mutations in nuclear *PET* genes result in respiratory deficiency and a *pet*ite colony phenotype when grown on fermentable medium (Tzagoloff and Dieckmann 1990). Studies that have been conducted by different labs over the past 20 years have established a catalog of *PET* gene functions that continues to be built upon today. The results of one genome-wide screen in search of novel nuclear *PET* genes were published in 1990 (Tzagoloff and Dieckmann 1990). Mutagenesis with ethyl methanesulfonate yielded 2,000 *pet* strains, which fall into 215 complementation groups. Many genes have been studied thoroughly (Ellis *et al.*, 2004, Helfenbein *et al.*, 2003, Souza *et al.*, 2000, Hell *et al.*, 2000), but it is an ongoing effort to characterize the genes described by the remaining complementation groups. Now, with the availability of the genome sequence of yeast, gene knockout screens have been employed to study mitochondrial function on a global scale in a cost- and time-efficient manner.

Here, I describe the results of a genome-wide screen that was conducted to gain further insight into the mechanisms governing the regulation of mitochondrial gene expression. A collection of yeast deletion mutants was surveyed for new *PET* genes involved in mitochondrial RNA metabolism. Two genes, *HTD2* and *LIP3*, were found to be required for efficient processing of mitochondrial tRNAs by mitochondrial RNase P. Htd2 is an enzyme in the type II mitochondrial fatty acid synthesis pathway (FAS II). I show that a product of the FAS II pathway, not Htd2 *per se*, is required for efficient tRNA processing. I also demonstrate that Lip3 is an enzyme involved in attaching the cofactor lipoic acid, which is synthesized from a product of the FAS II pathway, to target

proteins. I present evidence that one of the enzymes in the lipoylation pathway, Gcv3, is itself a target of lipoylation. In summary, this work presents the finding of a novel connection between RNA processing and fatty acid synthesis-lipoic acid attachment in yeast mitochondria. The mechanism governing the connection between these pathways is not understood, but a model is presented that hypothesizes that this intersection may allow for a new mode of control of mitochondrial gene expression in response to the nutritional state of the cell.

MITOCHONDRIAL FUNCTIONS

Mitochondria produce most of the energy in eukaryotic cells by oxidative phosphorylation. In addition to this bioenergetic function, the organelle also plays a role in apoptosis, ionic homeostasis, metabolism of amino acids and iron, and synthesis of cellular metabolites, fatty acids and cardiolipin (Atamna *et al.*, 2002, Wang 2001, Soetens *et al.*, 1998, Schlame and Greenberg 1997). The recent development of high-throughput genomic and proteomic experiments have generated large organellar and cellular datasets, and are leading us to a more complete catalog of mitochondrial proteins and function as well as to a deeper understanding of nuclear-mitochondrial interactions. These types of studies have led also to the unexpected finding that seemingly unrelated functions or pathways intersect, such as the findings that are presented in this work.

MITOCHONDRIAL BIOGENESIS

Mitochondria have small genomes that only code for a few proteins. Most mitochondrial proteins are encoded by nuclear genes, translated in the cytoplasm and targeted to the organelle. These imported proteins function in mitochondrial DNA replication, transcription, translation, RNA processing and metabolism, oxidative phosphorylation complex assembly, organelle fusion, fission and shape maintenance, and many of the aspects of metabolism listed above.

The oxidative phosphorylation system in the inner mitochondrial membrane of *Saccharomyces cerevisiae* consists of a chain of three respiratory complexes and ATP synthase (Schagger 2002). Most of the subunits of these complexes are encoded in the nucleus, while seven are encoded in mitochondrial DNA (mtDNA). The 75 kb circular mitochondrial genome of *S. cerevisiae* encodes factors required for cellular respiration: three subunits of respiratory chain complex IV (Cox1, Cox2, and Cox3), one subunit of complex III (Cyt *b*), three subunits of the ATP synthase (Atp6, Atp8, and Atp9), the RNA subunit of mitochondrial RNase P (RPM1), 24 tRNAs, two ribosomal RNAs, and a ribosomal protein (Var1) (Foury *et al.*, 1998, de Zamaroczy and Bernardi 1986). All of the respiratory complexes and the ATP synthase have many nuclear-encoded subunits as well. The production of the respiratory chain complexes and ATP synthase occur via a coordinated effort between the nucleus and the mitochondria.

Yeast nuclear-encoded mitochondrial factors that are required for mitochondrial gene expression, and thus for respiration, are termed *PET* genes. When mutated, they result in respiratory deficiency and a *pet*ite colony phenotype when grown on a

fermentable medium (Tzagoloff and Dieckmann 1990). Yeast strains that maintain wild-type mtDNA in the presence of *PET* gene mutations are termed *rho*$^{+}$ (*rho* denotes the mitochondrial genome), while strains that have deletions in or loss of mtDNA due to genome instability are *rho*$^{-}$ or *rho*^{0} petites, respectively. Mitochondrial genome instability results from defects in nuclear gene products required for mtDNA replication, transcription, and protein synthesis (Contamine and Picard 2000, Myers *et al.*, 1985).

MITOCHONDRIAL GENE EXPRESSION

Regulation

Mitochondrial biogenesis is controlled by the coordinated expression of the nuclear and mitochondrial genomes. The general mechanisms governing this regulation, however, are unknown, partially because the full-complement of interacting factors is not yet determined. One facet of mitochondrial biogenesis, mitochondrial RNA metabolism, is of particular interest, as several general mechanisms controlling this process have yet to be discovered. It is known that the regulation of gene expression within the organelle occurs mostly at the post-transcriptional level. There are 13 promoters in the mitochondrial genome of *S. cerevisiae*, and the transcriptional machinery in the organelle produces mostly multigenic transcripts that are then cleaved and processed into mature RNAs. Nuclear-encoded factors function in 5' and 3' RNA processing, splicing, RNA stability, translation initiation, and respiratory chain complex assembly (Dieckmann and Staples 1994). Some of the factors are message-specific and some are general.

The mitochondrial apocytochrome *b* (*COB*) mRNA is one model for studying general and message-specific factors involved in organellar gene expression. Fig. 1.1 illustrates this model. *COB* is co-transcribed with the upstream $tRNA^{glu}$ and processing of this tRNA frees the COB message for further processing. Pet127 is a general factor involved in 5' end trimming and maturation of COB pre-mRNA and other RNAs (Wiesenberger *et al.*, 2007, Wiesenberger and Fox 1997). Cbp1 is a specific factor that is required for message stability as well as for translation initiation by binding to the 5' UTR (Dieckmann *et al.*, 1984, Islas-Osuna *et al.*, 2002, Chen and Dieckmann 1994, Mittelmeier and Dieckmann 1993). Cbs1 and Cbs2 are translational activators that bind to the 5' UTR (Rodel *et al.*, 1986), and multiple splicing factors are required for removal of the five *COB* introns (Dieckmann and Staples 1994). *COB* contains a conserved dodecamer sequence, AAUAAUAUUCUU, that is found at the 3' end of this message and the other six mitochondrial precursor mRNAs (Butow *et al.*, 1989, Zassenhaus *et al.*, 1984). The RNAs are cleaved two bases downstream of the dodecamer, which is sufficient to form the mature 3' end (Hofmann *et al.*, 1993). The enzyme that processes the pre-mRNAs at this site has not been characterized yet.

RNase P

The tRNAs encoded in the mitochondrial genome have 5' leader sequences that are processed by mitochondrial RNase P (Walker and Engelke 2006, Frank and Pace 1998). RNase P is a ribonucleoprotein complex that consists of one protein and one RNA.

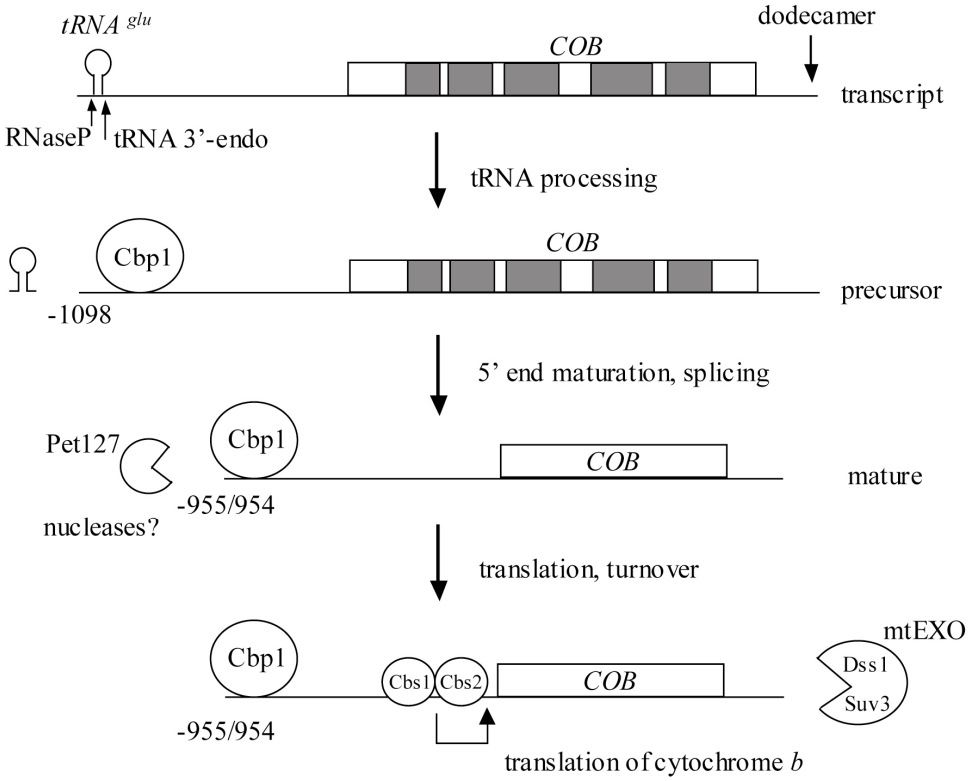


Figure 1.1. Processing of apocytochrome *b* (*COB*) mRNA. The *COB* gene is transcribed as a single transcription unit with *tRNA*^{glu}. tRNA^{glu} is processed by mitochondrial RNase P and the tRNA 3' endonuclease. Exons in the *COB* open reading frame are depicted as open boxes and introns are depicted as gray boxes. The *COB* precursor RNA is further processed at the 5' end in a Pet127-dependent reaction. Cbp1 protects COB mRNA from degradation, and Cbs1 and Cbs2 are translational activators of COB mRNA. mtEXO degrades RNAs in the 3' to 5' direction.

The protein component, Rpm2, is encoded in the nuclear genome, translated in the cytoplasm and imported into the organelle (Morales *et al.*, 1992). The RNA component, RPM1, is encoded in mtDNA (Underbrink-Lyon *et al.*, 1983). Its path toward becoming a functional component of RNase P is more complicated, as its maturation, like *COB* mRNA, depends on several processing steps. As illustrated in Fig. 1.2, *RPM1* is transcribed either from the FP promoter with the upstream $tRNA_f^{met}$ and the downstream $tRNA^{pro}$ or from the SP promoter with only $tRNA^{pro}$. Production of mature RPM1 RNA requires processing of $tRNA_f^{met}$ from the 5' end of the initial multigenic transcript by 3' tRNA endonuclease, and of $tRNA^{pro}$ from the 3' end of the transcript by RNase P. Following tRNA removal, the 5' extension sequence of RPM1 is processed in a Pet127-dependent reaction (Ellis *et al.*, 2005), and the 3' end is processed either by the Dss1/Suv3 3' to 5' exonuclease (Dziembowski *et al.*, 2003) and/or by other enzymes not yet characterized. Thus, the production of mature RPM1 RNA requires the activity of a variety of mitochondrial RNA processing factors, including RNase P itself. We have also found, as presented here, that a product of the type II mitochondrial fatty acid synthesis pathway (FAS II) also affects maturation of the RPM1 RNA (see below).

FATTY ACID SYNTHESIS AND ATTACHMENT PATHWAYS IN YEAST

The FAS II pathway

The FAS II pathway, which produces octanoic acid, the precursor to lipoic acid, is carried out by sequential action of individual enzymes in the mitochondrial compartment (Tehlivets *et al.*, 2007, Hiltunen *et al.*, 2005). The acyl chain is built on the acyl carrier

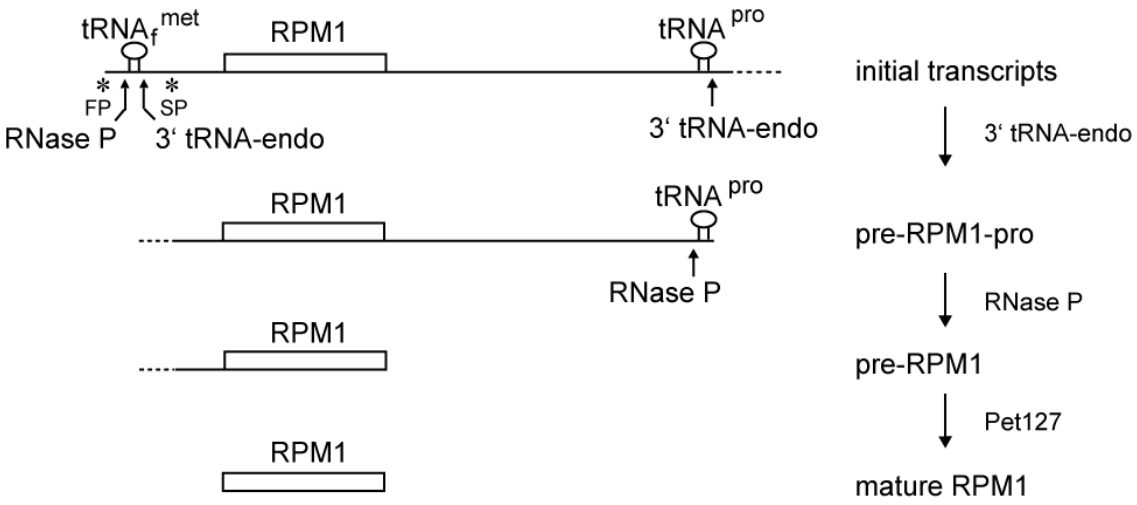
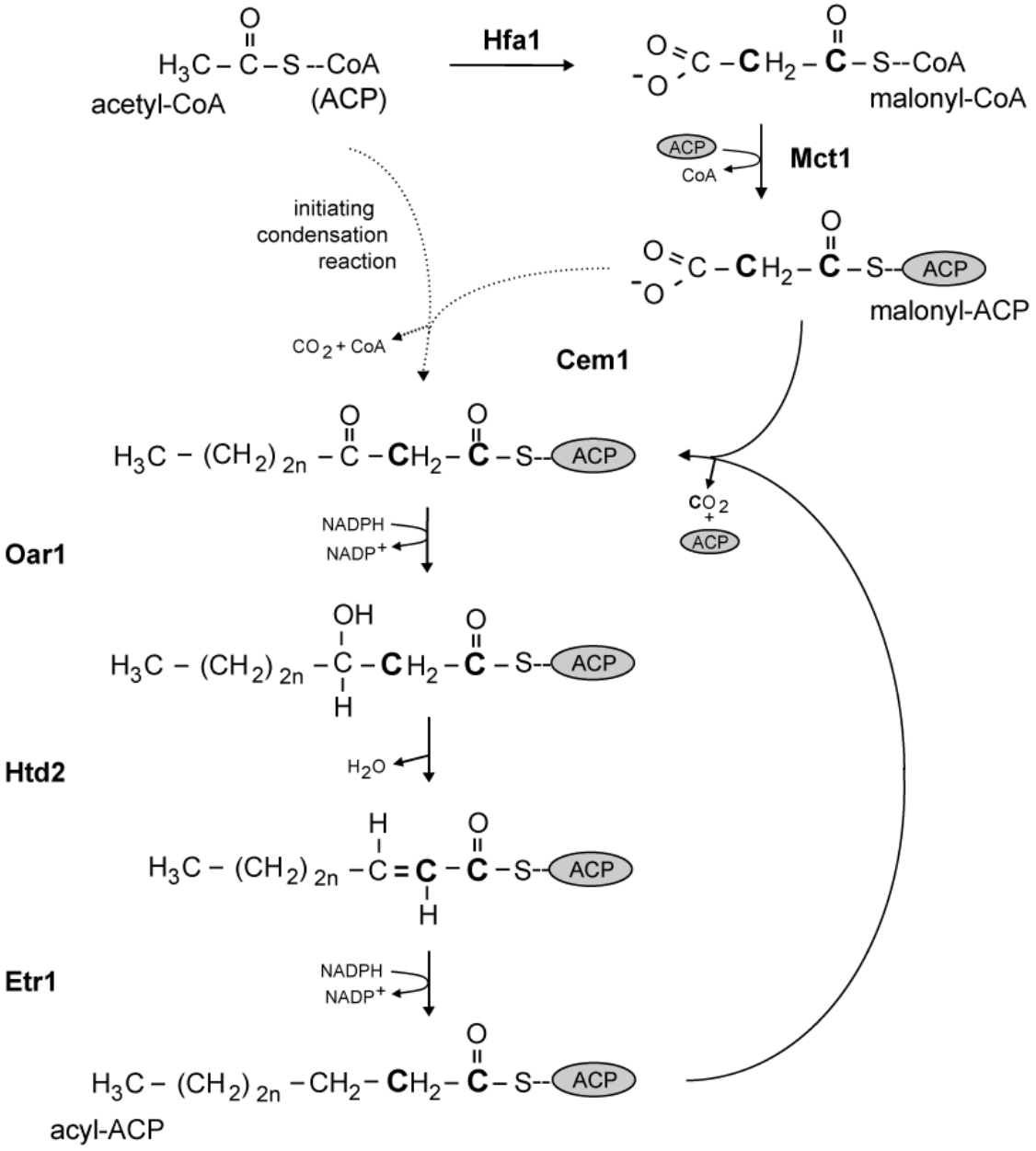


Figure 1.2. Processing of RPM1-containing transcripts. *RPM1* is transcribed in a single transcription unit with both upstream *tRNA$_f^{met}$* and downstream *tRNA^{pro}* from the FP promoter (5’ end represented by * FP) or with only the downstream *tRNA^{pro}* from the SP promoter (5’ end represented by * SP). 3’ tRNA endonuclease generates the high molecular weight precursor termed pre-RPM1-pro. The 5’ end of the precursor transcript is produced either by the SP promoter or by cleavage at the 3’ end of tRNA$_f^{met}$. The interval between the SP promoter and tRNA$_f^{met}$ is designated by a dotted line. RNase P processes the pre-RPM1-pro precursor RNA at the 5’ end of tRNA^{pro}, generating the pre-RPM1 intermediate. Further processing at the 5’ end of this intermediate in a Pet127-dependent reaction produces mature RPM1 RNA.

protein (ACP) in two-carbon increments. Fig. 1.3 illustrates the pathway and the enzymes involved. Because the type I fatty acid synthesis pathway, which occurs in the cytoplasm, produces the bulk of fatty acids for the cell (Smith 1994), the purpose of the FAS II pathway in eukaryotes has been under debate. Brody *et al.* (Brody *et al.*, 1997) and Wada *et al.* (Wada *et al.*, 1997) have proposed that the production of octanoic acid, the precursor to lipoic acid, is the sole purpose of this separate, organellar pathway. Indeed, we show that the FAS II pathway is the sole source of octanoic acid in the cell (Chapter 3). However, it is not clear whether longer fatty acids are produced by this pathway and, if so, what their function may be.

HTD2, which encodes an enzyme in the FAS II pathway, was found to be required for efficient processing of the pre-RPM1-pro precursor transcript by RNase P in the genomic screen described in Chapter 2. We show that deletion of any gene encoding an enzyme in the pathway, not *HTD2 per se*, causes a defect in RNA processing (Chapter 3). Therefore, a product of the FAS II pathway is required for efficient processing of the pre-RPM1-pro precursor transcript. Because the maturation of RPM1, the RNA subunit of RNase P, requires the activity of RNase P, it is hypothesized that a product of the FAS II pathway is necessary for the activity or assembly of RNase P. The mechanism underlying the connection between the FAS II and RNA processing pathways in mitochondria is not yet known.

Figure 1.3. The mitochondrial type II fatty acid synthesis pathway (FAS II) in yeast. Acyl carrier protein (ACP) (Brody *et al.*, 1997) is depicted as a gray oval. Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (Hfa1) (Hoja *et al.*, 2004). The malonyl moiety is transferred onto ACP by malonyl-CoA:ACP transferase (Mct1) (Schneider *et al.*, 1997a). Acetyl-CoA (ACP) and malonyl-ACP are condensed by β-ketoacyl-ACP synthase (Cem1) to form 3-ketoacyl-ACP (Harington *et al.*, 1993). It is not known whether acetyl-CoA or acetyl-ACP is utilized in the initiating condensation reaction, which is denoted by dotted arrows. The reduction to 3-hydroxyacyl-ACP is catalyzed by 3-ketoacyl:ACP-reductase (Oar1) (Schneider *et al.*, 1997a). The dehydration reaction producing *trans*-2-enoyl-ACP is catalyzed by 3-hydroxyacyl-thioester dehydratase (Htd2) (Kastaniotis *et al.*, 2004). *Trans*-2-enoyl-ACP is reduced by 2-enoyl thioester reductase (Etr1) (Torkko *et al.*, 2001). The resulting acyl-ACP molecule is condensed with malonyl-ACP by Cem1, cyclically elongating the acyl chain by two carbon units to produce octanoic acid. It is unclear whether longer fatty acids are produced by the mitochondrial FAS II pathway in yeast. The letter "n" in the acyl chains signifies the number of cycles, with n=0 representing the product of the initiating condensation reaction. The bold letters (**C**) denote the carbon atoms donated by malonyl-CoA in the most recent condensation reaction.


Hfa1
acetyl-CoA
(ACP)
malonyl-CoA
ACP
CoA
Mct1
initiating
condensation
reaction
malonyl-ACP
CO2 + CoA
Cem1
CO2
ACP
NADPH
NADP+
Oar1
Htd2
H2O
Etr1
acyl-ACP

Lipoic acid synthesis and attachment

As stated above, the mitochondrial FAS II pathway is the source of octanoic acid, which is the precursor for the synthesis of lipoic acid in yeast. Lipoic acid is a sulfur-containing cofactor required for the function of three multienzyme complexes in mitochondria that catalyze oxidative decarboxylation reactions (Perham 2000). The cofactor is covalently attached to a specific lysine residue within a conserved lipoylation domain in the E2 subunits of pyruvate dehydrogenase (PDH) (Niu *et al.*, 1988) and α-ketoglutarate dehydrogenase (α-KDH) (Repetto and Tzagoloff 1990), and in the H protein of the glycine cleavage enzyme (GC) (Nagarajan and Storms 1997). Lipoic acid serves as a "swinging arm" that shuttles reaction intermediates between active sites in the complexes.

The synthesis of lipoic acid from octanoic acid and the attachment of the cofactor to the target enzymes are well-defined in *E. coli*, but less so in yeast. In *E. coli*, the LipA enzyme adds two sulfur atoms to the octanoyl-ACP carbon chain produced in *de novo* fatty acid synthesis (Miller *et al.*, 2000), and LipB catalyzes the transfer of the lipoyl moiety from ACP to target proteins (Nesbitt *et al.*, 2005, Jordan and Cronan, Jr. 2003). In a separate pathway, the LplA enzyme activates and attaches free lipoic acid scavenged from the environment to target proteins (Morris *et al.*, 1995, Morris *et al.*, 1994). These two pathways are illustrated in Fig. 1.4. In yeast, the homologs of LipA and LipB, which are Lip5 and Lip2, respectively, have been partially characterized (Marvin *et al.*, 2001, Sulo and Martin 1993). We show here that the predicted amino acid sequence of *LIP3*, the second gene found in the screen, is homologous to *E. coli* LplA and is, indeed,

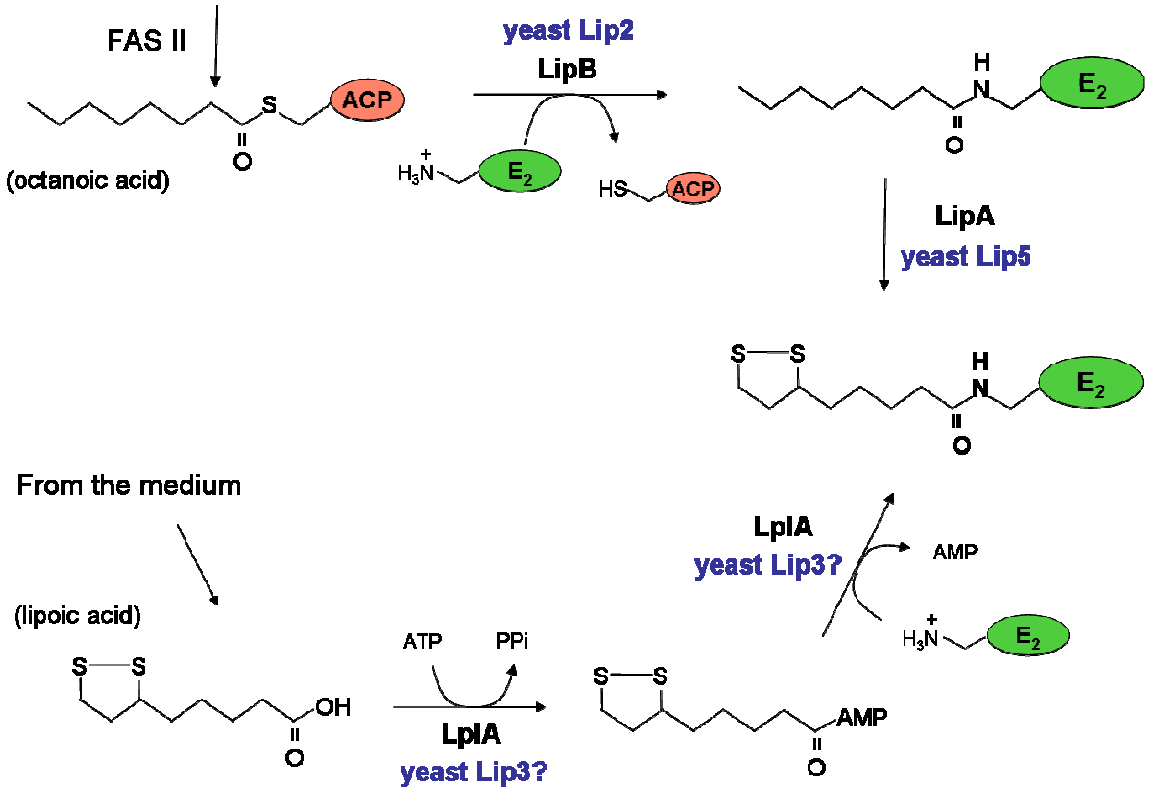


Figure 1.4. Lipoic acid synthesis and attachment in *E. coli*. Two pathways exist in *E. coli* for protein lipoylation. Octanoic acid produced by the fatty acid synthesis pathway is first transferred onto the acceptor lysine residue in the lipoyl domain of target proteins by LipB, and LipA then catalyzes the insertion of two sulfur atoms into the octanoyl group. Alternatively, free lipoic acid obtained from the medium is first activated by ATP to lipoyl-AMP, and the lipoyl group is then transferred onto the acceptor lysine residue. Both of these reactions are carried out by LplA. Bacterial enzymes are in black, yeast homologs are blue. The question mark after Lip3 denotes that it is not known whether Lip3 activates lipoic acid with AMP. The pink oval represents the acyl carrier protein (ACP). The green oval represents the target E2 and H proteins.

essential for lipoic acid synthesis and attachment in yeast (Chapter 4). However, it is not known whether Lip3 both activates free lipoic acid with AMP and transfers the lipoyl group to target proteins like E. coli LplA or whether Lip3 only transfers the lipoyl group previously activated by another enzyme, like the mammalian lipoyltransferases. We also show that there is only one lipoylation pathway in yeast and that Gcv3, one of the targets of lipoylation, is also required for lipoylation. In addition, Lip3 and the other enzymes involved in lipoic acid synthesis and attachment are required for efficient processing of the pre-RPM1-pro precursor RNA, albeit in a *t*emperature-*s*ensitive manner (Chapter 5).

SUMMARY OF CHAPTERS

As described in this introduction, the mechanisms that control mitochondrial biogenesis are not understood, and the full complement of proteins involved in this process is not known. Thus, I conducted a genome-wide screen for new *PET* genes involved in mitochondrial RNA metabolism in order to gain further insight into the regulation of mitochondrial gene expression. The major findings of this work are briefly outlined below. Chapter 6 contains a discussion of general conclusions and of future directions for this research.

Chapter 2. Genomic Screen

The details of the genomic screen and the results are described here. The main finding was that mutations in two genes of unknown function were found to affect processing of a tRNA from an initial multigenic transcript containing the mitochondrially encoded RNase P RNA, RPM1. One gene, *YHR067w*, was determined to encode Htd2, an enzyme in the mitochondrial type II fatty acid biosynthesis pathway (FAS II), by our collaborators at the University of Oulu in Oulu, Finland (Kastaniotis *et al.*, 2004). The second gene, *YJL046w*, was determined to encode Lip3, an enzyme involved in the attachment of the cofactor lipoic acid, which is synthesized from a product of the FAS II pathway.

Chapter 3. Manuscript I:

Intersection of RNA Processing and the Fatty Acid Synthesis Pathway in Yeast Mitochondria

The discovery of the connection between RNA processing and fatty acid synthesis in yeast mitochondria is the focus of this manuscript. Deletion of any gene encoding an enzyme in the FAS II pathway, not just *HTD2*, affects tRNA processing by mitochondrial RNase P. We conclude that a product of the FAS II pathway is required for assembly or activity of mitochondrial RNase P. We also show that the FAS II pathway is the sole source of octanoic acid, which is the precursor for the synthesis of lipoic acid. This manuscript has been accepted for publication in the November 2008 issue of the journal Molecular and Cellular Biology.

Chapter 4. Manuscript II:

Lipoic Acid Synthesis and Attachment in Yeast Mitochondria

The second gene found in the screen was determined to encode Lip3, an enzyme involved in the attachment of the enzyme cofactor lipoic acid, which is synthesized from the octanoic acid product of the FAS II pathway. Characterization of Lip3 and of the lipoic acid attachment pathway in yeast is described here. Four or five proteins are involved in one protein lipoylation pathway. One of these proteins is Gcv3, which is also a target of lipoylation.

Chapter 5. Further Investigations on RNA Processing

In an attempt to further understand the nature of the intersection between RNA processing and the fatty acid synthesis and lipoic acid attachment pathways, RNA processing was analyzed in various mutants grown at different temperatures.

CHAPTER 2. GENOMIC SCREEN

SUMMARY

In the search for new nuclear *PET* genes involved in mitochondrial RNA metabolism, a genome-wide screen was performed in which a set of yeast deletion mutants was surveyed for mitochondrial RNA aberrancies. Strains harboring deletions of two genes of unknown function, *YHR067w* and *YJL067w*, were found to be defective in the processing of tRNA from a multigenic transcript also containing RPM1, the RNA component of mitochondrial RNase P. Our collaborators determined that *YHR067w* encodes Htd2, an enzyme in the mitochondrial fatty acid synthesis pathway. We determined that *YJL067w* encodes Lip3, an enzyme involved in the synthesis and attachment of the cofactor lipoic acid to target proteins. Further analysis of these proteins is described in latter chapters.

INTRODUCTION

As discussed in Chapter 1, the full set of proteins required for mitochondrial gene expression and their function is not yet determined. The completion of the genome sequence of *S. cerevisiae* has opened up new avenues for global genomic and proteomic studies of mitochondrial proteins and mitochondrial functions. In particular, gene knockout screens have been useful for finding novel proteins involved in specific pathways. One advantage of these types of studies is that proteins known to function in certain, seemingly unrelated, pathways can be found to function in new pathways. This is particularly true for the mitochondrial compartment, in which numerous proteins are pleiotropic. In our case, the results of our screen did not reveal new functions for proteins, but rather, a novel connection between pathways.

MATERIALS AND METHODS

Strains and media

S. cerevisiae haploid deletion strains (BY4741) from the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) were obtained from Roy R. Parker. The genotypes of the BY4741 and the MY6 *rho*^{0} tester strains are listed in Table 3.1. Cells were grown in medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose (YEPD) or 3% glycerol (YEPG) instead of glucose. Solid media contained 2% (w/v) agar.

Screen for respiratory deficiency

Cells from one half of a 96-well plate were plated onto YEPD medium, and the cells were replica plated onto three more YEPD plates and four YEPG plates. One of each YEPD and YEPG plate were placed into 18°C, 24°C, 30°C, and 37°C incubators (cells were grown at 24°C to test whether 30°C is a restrictive temperature). Cells on YEPD medium were grown for 2-3 days, and cells on YEPD medium were grown for 3-5 days.

rho^0 test

The 84 strains that remained in our study were checked for the ability to maintain mitochondrial DNA (mtDNA) by mating to the MY6 tester strain, which lacks mtDNA (*rho*^{0}). Resulting heterozygous diploids were respiratory-competent if they contained

wild-type mtDNA or were respiratory-deficient if they had mutations in, or sustained loss of, mtDNA.

Isolation of whole-cell RNA

Total RNA was isolated from midlogarithmic cultures after growth in liquid YEPD medium at 18°C, 30°C or 37°C as described (Caponigro *et al.*, 1993).

Northern blot analysis

10 μg of total RNA was separated on a 1.2% (w/v) agarose gel in TB buffer (83 mM Tris base, 89 mM boric acid, pH 8.3). The RNAs were transferred onto a Nytran membrane (Schleicher & Schuell, Keene, NH) overnight in 20× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) and hybridized (6× SSC, 10× Denhardt's solution, 0.1% SDS, 50 μg/ml carrier DNA) overnight at 5-10°C below the T_m of the oligonucleotide probes. The blots were hybridized with two probes at a time, stripped and re-probed a total of five times. All blots were stripped and hybridized with the SCR1 loading control probe. Oligonucleotide probes (listed in Table 2) were ^{32}P end-labeled using T4 DNA kinase (Fermentas, Hanover, MD). Blots were analyzed on a PhosphorImager (Typhoon 9410, GE Healthcare, Piscataway, NJ).

RESULTS

The screen

A genome-wide screen of a collection of yeast mutants was conducted in an effort to uncover novel nuclear factors involved in mitochondrial RNA processing, protection and degradation. The EUROSCARF collection of haploid yeast deletion strains (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) was created by systematic deletion of each of the ~5000 non-essential open reading frames by homologous recombination with the kanamycin resistance gene (Winzeler *et al.*, 1999). The strains were first screened for respiratory-deficiency, and then a subset of the respiratory-deficient strains was then surveyed for aberrancies in mitochondrial RNAs.

The ~5000 strains were tested for respiratory-deficiency (inability to grow on rich glycerol medium), with the logic that any mutants with defects in RNA metabolism would not produce wild type respiratory chain proteins, and thus would not be able to respire. All of the strains were plated on glucose (YEPD), a fermentable carbon source, and glycerol (YEPG), a non-fermentable carbon source, and grown at 18ºC, 24ºC, 30ºC, and 37ºC. Strains were identified that were unable to grow on glycerol. These respiratory-deficient mutants were surveyed for mitochondrial RNA processing defects by Northern blotting. Oligonucleotide probes complementary to *ATP8/6*, *ATP9*, *COB*, *COX1, COX2, COX3,* and *VAR1* mRNAs, 15S and 21S rRNAs, and RPM1 RNA were used. The six multigenic transcripts that contain these genes are shown in Fig. 2.1. The goal was to characterize novel factors involved in maturation of 5' and 3' ends of mitochondrial

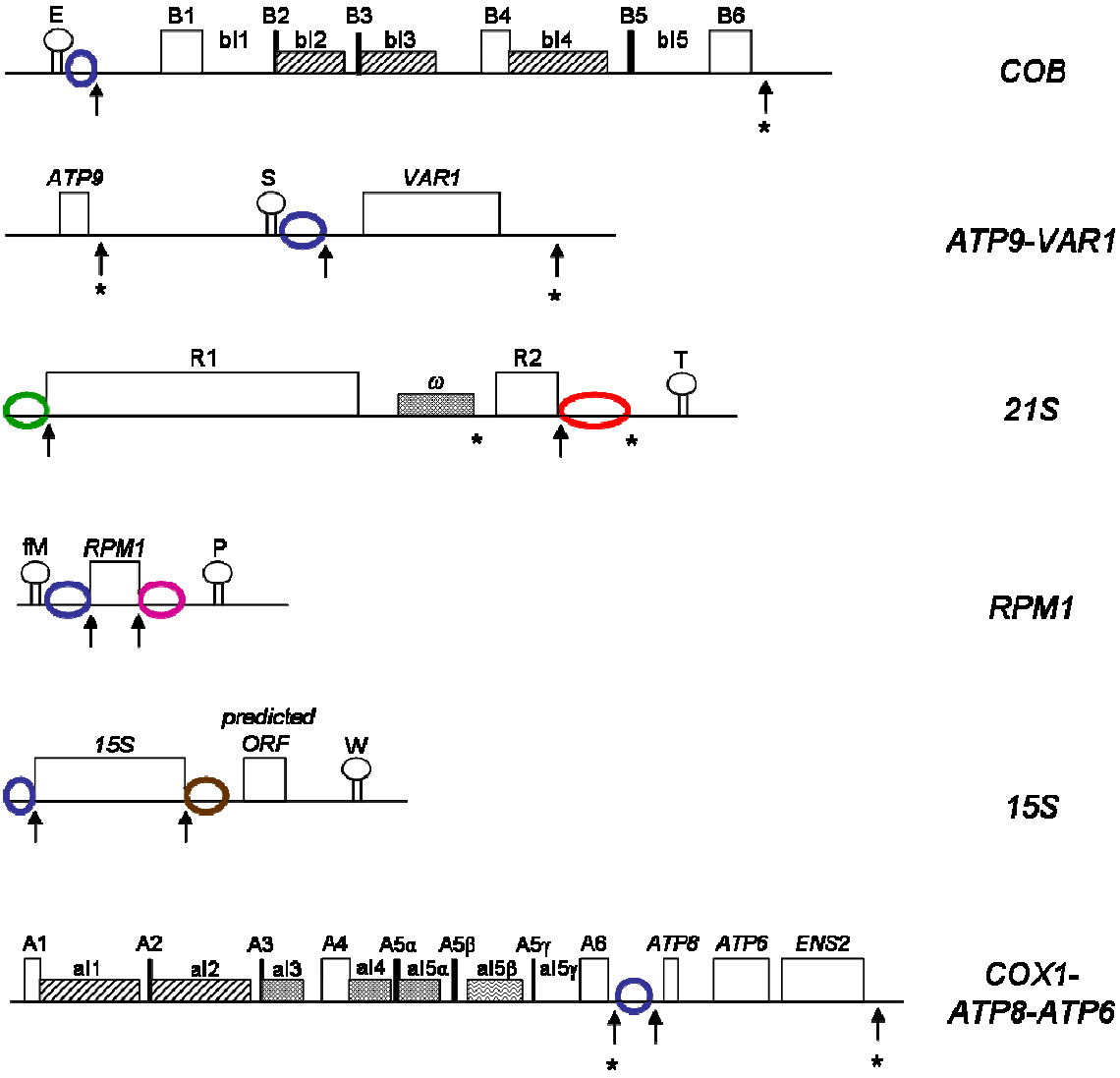


Figure 2.1. Mitochondrial transcription units in yeast. Open reading frames encoding exons or entire genes are shown as open boxes. Hatched boxes denote ORFs encoding maturases. Cross-hatched boxes denote ORFs encoding homing endonucleases. The wavy box denotes an uncharacterized intronic ORF. Lolipops denote tRNAs. Arrows indicate mature ends of transcripts. Asterisks indicate presence of the dodecamer. Colored circles represent regions of 5' and 3' end processing. The blue circles represent 5' extension sequences affected by Pet127 and Cbt1. All other circles represent 5' and/or 3' extension sequences affected by unknown enzymes. Transcripts of *COX2* and *COX3* are not shown as their 5' and 3' ends are not processed. Transcripts of tRNA clusters are not shown as tRNA 5' ends are processed by mitochondrial RNase P and 3' ends are processed by the 3' tRNA endonuclease.

precursor RNAs, mitochondrial mRNA stability, and/or *bona fide* mitochondrial splicing factors.

Although the glucose vs. glycerol growth assay was similar to that of two other groups that searched for respiratory-deficient knockout strains (Steinmetz *et al.*, 2002, Dimmer *et al.*, 2002), two features were added to the screen that are unique. First, conditional mutants were identified that could not grow on glycerol only at high temperature (37°C) or at low temperature (18°C). The rationale for searching for conditional respiratory-deficient mutants is that some deletion strains that have mitochondrial RNA phenotypes may only be respiratory incompetent at high or low temperatures. Strains that have deletions in the mitochondrially targeted genes *PET127* (Ellis *et al.*, 2005, Wiesenberger and Fox 1997) and *CBT1* (Ellis *et al.*, 2005, Rieger *et al.*, 1997) are defective in mitochondrial mRNA processing and are *t*emperature-*s*ensitive (*ts*) for respiration. Second, respiratory-deficient deletion strains with unstable mitochondrial genomes had intronless mtDNA re-introduced. Our rationale for searching for strains that can maintain intronless mtDNA is that some deleted genes could code for components of RNA turnover pathways. Cells with deletions in *DSS1* and *SUV3*, which encode subunits of the 3'-5' exonuclease involved in mitochondrial RNA turnover, can maintain stable genomes if all 13 introns are absent (Dziembowski *et al.*, 2003, Dziembowski *et al.*, 1998).

266 respiratory-deficient strains found, 84 retained in study

266 respiratory-deficient strains were identified in the screen. The *Saccharomyces* Genome Database (http://www.yeastgenome.org/) was used to determine the function of each of the 266 ORFs and thus to select which strains would be included for further analysis. 182 strains were immediately eliminated from further study as they contain deletions in well-characterized *PET* genes. The 84 strains that remained in our study contained deletions of uncharacterized genes or of genes encoding proteins that function outside of the mitochondrial compartment. The logic behind retaining this latter group of strains was that some of the genes may encode proteins with dual localization to mitochondria or that some genes were mischaracterized.

The 84 strains were checked for the ability to maintain mitochondrial DNA (mtDNA) by mating to the MY6 tester strain, which lacks mtDNA (*rho*^{0}). Resulting heterozygous diploids were respiratory-competent if they contained wild type mtDNA or were respiratory-deficient if they had mutations in or sustained loss of mtDNA. The strains fell into three classes; 12 are *t*emperature- and/or *c*old-*s*ensitive (*ts* and/or *cs*), 20 are respiratory-deficient and *rho*$^{+}$, and 52 are respiratory-deficient and *rho*^{0}. To study the effects of the deleted nuclear genes on mitochondrial RNA processing and stability, cells in the first class were grown at the non-permissive temperature in fermentable medium, total RNA was isolated and analyzed by Northern blot, and all RNAs, excluding tRNAs, encoded in the mitochondrial genome were surveyed for aberrancies. Cells in the second class were grown at 30°C, the normal growth temperature for yeast, and mitochondrial RNAs were surveyed in the same way. Cells in the third class of respiratory-deficiency

were forced to completely lose their mitochondrial DNA by ethidium bromide mutagenesis and intronless mtDNA was introduced via a *kar1* cross that does not allow fusion of nuclei. Strains that retain intronless mtDNA were not followed.

The 84 respiratory-deficient strains are organized in Table 2.1. The amino acid sequence translation of each ORF was analyzed by the MitoProt prediction program (Claros and Vincens 1996) to assess the likelihood that each polypeptide is targeted to mitochondria.

The *yhr067wΔ* and *yjl046wΔ* strains inefficiently process pre-RPM1-pro precursor RNA

A Northern blot showing the main findings of the screen are shown in Fig. 2.2. Total RNA from the wild-type, *yhr067wΔ* and *yjl046wΔ* strains was hybridized with a probe specific for RPM1, the RNA component of mitochondrial RNase P. The blot revealed defects in processing of $tRNA^{pro}$ from the initial RPM1-containing transcript in both deletion strains. The details of processing of RPM1-containing transcripts are described in the Introduction and Fig. 1.2. There was an accumulation of the pre-RPM1-pro precursor RNA and lower levels of the pre-RPM1 intermediate and of mature RPM1 in both mutant strains as compared to the wild-type strain. Deletion of both ORFs specifically affects processing of the RPM1-containing transcript, as processing of mitochondrial mRNAs or rRNAs was not affected (data not shown). No other Northern blots are shown here as no other respiratory-deficient deletion strains had significant mitochondrial RNA aberrancies.

Table 2.1 - ORFs deleted from the 84 respiratory-deficient strains

MitoProt[a]	ORF[b]	Name[c]	Function	*ts/cs*[d]	mtDNA[e]
0.025	*YAL035W*	*FUN12*	translation initiation factor activity	*ts*	
0.68	*YBL012C*	?	?		*rho*^{0}
0.14	*YBL044W*	?	?		*rho*^{0}
0	*YBR132C*	*AGP2*	carnitine transporter		*rho*^{0}
0.79	*YBR163W*	*DEM1*	?		
0.05	*YBR173C*	*UMP1*	part of 20S proteasome	*ts*	
0.922	*YCL023C*	?	?	*ts*	
0.06	*YCR004C*	*YCP4*	?		*rho*^{0}
0.12	*YCR061W*	?	?		*rho*^{0}
0.82	*YDL040C*	*NAT1*	N-term acetyltransferase		*rho*^{0}
0.02	*YDL057W*	?	?		*rho*^{0}
0.62	*YDL063C*	?	?		*rho*^{0}
0.99	*YDL104C*	*QRI7*	?		*rho*^{0}
0.02	*YDL113C*	*CVT20*	cytoplasm to vacuole targeting		*rho*^{0}
0.02	*YDL135C*	*RDI1*	Rho GTPase mediated signal transduction		*rho*^{0}
0.03	*YDL146W*	?	?		*rho*^{0}
0.03	*YDL167C*	*NRP1*	?		*rho*^{0}
0.26	*YDL198C*	*YHM1*	? Mito carrier protein ?		*rho*^{0}
0.31	*YDR042C*	?	?		
0.9	*YDR065W*	?	?		*rho*^{0}
0.32	*YDR078C*	*SHU2*	?		*rho*^{0}
0.06	*YDR138W*	*HPR1*	part of CCR4 deadenylase complex		
0.04	*YDR185C*	*?*	?	*ts*	
0.24	*YDR295C*	*PLO2*	?		
0.13	*YDR470C*	*UGO1*	mito fusion, mito carrier		
0.92	*YDR493W*	*FMP36*	?	cs/*ts*	
0.32	*YEL029C*	*BUD16*	bud site selection, cytoplasm		*rho*^{0}
0.04	*YER103W*	*SSA4*	HSP70 family heat shock protein		*rho*^{0}
0.09	*YER110C*	*KAP123*	importin, substrate docking to NPC		*rho*^{0}
0.05	*YER122C*	*GLO3*	Golgi-ER, ER-Golgi transport		*rho*^{0}
0.09	*YER169W*	*RPH1*	DNA damage, ZN finger domain		*rho*^{0}
0.98	*YGL038C*	*OCH1*	N-linked glycosylation		
0.98	*YGL064C*	*MRH4*	mito RNA helicase (no papers)		*rho*^{0}
0.81	*YGL107C*	*RMD9*	?		*rho*^{0}
0.38	*YGL135W*	*RPL1B*	cyto lg rib subunit, 60S protein		
0.038	*YGL206C*	*CHC1*	clathrin heavy chain, vesicle transport		*rho*^{0}
0.16	*YGL220W*	?	?		*rho*^{0}
0.91	*YGR102C*	?	?		*rho*^{0}
0.94	*YGR112W*	*SHY1*	req'd for expression of *COX1*		
0.06	*YGR257C*	*MTM1*	putative transporter, mito innermembrane		*rho*^{0}
0.22	*YGR262C*	*BUD32*	kinase, unknown		
0.31	*YHR030C*	*SLT2*	signal transduction in cell integrity, mating	*ts*	
0.63	***YHR067W***	*RMD12*	?		
0.32	*YHR116W*	?	?		
0.72	*YHR168W*	?	?		*rho*^{0}
0.1	*YIL088C*	?	?		*rho*^{0}
0.95	***YJL046W***	?	?		

Table 2.1 - ***Continued***

MitoProt[a]	ORF[b]	Name[c]	Function	*ts/cs*[d]	mtDNA[e]
0.22	*YJL079C*	*PRY1*	pathogen related protein	*ts*	
0.95	*YJR120W*	?	?		
0.98	*YJR122W*	*CAF17*	part of *CCR4* deadenylase complex		*rho*^{0}
0	*YLL006W*	*MMM1*	maintaining mito structure		
0.12	*YLL033W*	*GON2*	?		*rho*^{0}
0.15	*YLR091W*	?	?		*rho*^{0}
0.98	*YLR204W*	*QRI5*	?		
0.71	*YLR260W*	*LCB5*	sphingolipid long chain base kinase		*rho*^{0}
0.03	*YML088W*	*UFO1*	ubiquitin ligase, response to DNA damage		*rho*^{0}
0.97	*YMR066W*	*SOV1*	? synthesis of Var1 ?		
0.4	*YMR071C*	*TVP18*	?		
0.93	*YMR072W*	*ABF2*	mito genome maintenance		
0.74	*YMR097C*	*MTG1*	?		*rho*^{0}
0.49	*YMR098C*	?	?		*rho*^{0}
0.25	*YMR151W*	*YIM2*	?		
0.06	*YMR184W*	?	?		*rho*^{0}
0.35	*YNL081C*	*SWS2*	? putative mito rib sm subunit protein ?		*rho*^{0}
0.01	*YNL084C*	*END3*	cytoskeletal adaptor, cytokinesis, budding		*rho*^{0}
1.04	*YNL136W*	*EAF7*	?	*ts*	
0.22	*YNL160W*	*YGP1*	synthesized in starvation		*rho*^{0}
0.57	*YNL170W*	?	?		*rho*^{0}
0.07	*YNL225C*	*CNM67*	part of spindle pole body		*rho*^{0}
0.93	*YOL027C*	*MDM38*	mito organization	*ts*	
0.98	*YOR017W*	*PET127*	mito RNA processing	*ts*	
0.21	*YOR033C*	*EXO1*	5'-3' exonuclease		*rho*^{0}
0.91	*YOR205C*	*FMP38*	?		*rho*^{0}
0.31	*YOR241W*	*MET7*	folylpolyglutamate synthase activity		*rho*^{0}
0.02	*YOR304C-A*	?	?		*rho*^{0}
0.75	*YOR305W*	?	?		*rho*^{0}
0.01	*YOR315W*	?	?		*rho*^{0}
0.058	*YOR350C*	*MNE1*	?		
0.42	*YPL031C*	*PHO85*	kinase, phosphate and glycogen metabolism		*rho*^{0}
0.92	*YPL059W*	*GRX5*	glutaredoxin, protect against oxidative damage		*rho*^{0}
0.99	*YPL183W-A*	*GON5*	mito ribosome protein	*cs*	
0.13	*YPR072W*	*NOT5*	part of *CCR4* deadenylase complex		*rho*^{0}
0.37	*YPR116W*	?	?		*rho*^{0}
0.03	*YPR123C*	?	?	*cs*	

[a] Probability of gene product being imported into mitochondria using the MitoProt algorithm (Claros and Vincens 1996)
[b] ORFs encoding Rmd12/Htd2 (*YHR067W*) and Lip3 (*YJL046W*) are in bold
[c] Name and function determined by *Saccharomyces* Genome database
[d] *t*emperature and/or *c*old sensitive respiratory-deficiency
[e] Lack of mtDNA (*rho*^{0}) determined by mating to MY6 *rho*^{0} tester strain

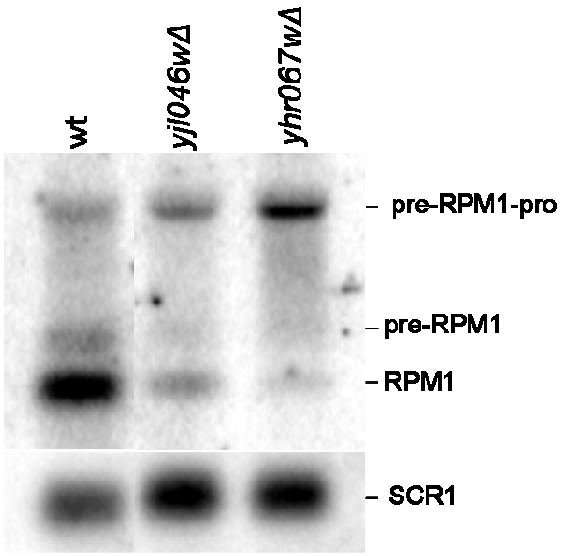


Figure 2.2. Northern blot analysis of RPM1-containing transcripts in strains *yjl046wΔ* and *yhr067wΔ*. A Northern blot of total RNA extracted from strains *yjl046wΔ* and *yhr067wΔ* was hybridized with a probe complementary to RPM1 RNA. pre-RPM1-pro and pre-RPM1 are the precursor RNAs, while RPM1 is the mature RNA. The blot was stripped and hybridized with a probe complementary to SCR1, the RNA subunit of the cytoplasmic Signal Recognition Particle, as a loading control.

DISCUSSION

The results of our screen are similar to other screens performed with the deletion collection in search of nuclear-encoded mitochondrial factors. While we found 266 respiratory-deficient strains, Steinmetz *et al*. identified 214 strains with severe respiratory growth defects (Steinmetz *et al.*, 2002) and Dimmer *et al*. identified 319 respiratory-deficient strains (Dimmer *et al.*, 2002). The mutagenesis screen performed by Tzagoloff and Dieckmann in 1990 (Tzagoloff and Dieckmann 1990) resulted in 215 *pet* strains. Taken together, the results of these studies suggest that the number of nuclear *PET* genes is about 300.

The 52 respiratory deficient strains with unstable mitochondrial genomes that were able to retain intronless mtDNA were not studied further as our attention quickly became focused on the *yhr067wΔ* and *yjl046wΔ* strains after completion of the screen. These remaining strains are available for future use.

At the time the screen was done, *YJL046w* and *YHR067w* were uncharacterized ORFs. Shortly after our finding of the RNA data, Alexander Kastaniotis, in the laboratory of Kalervo Hiltunen at the University of Oulu in Oulu, Finland, reported that *YHR067w* encodes 3-hydroxyacyl thioester dehydratase 2 enzyme (Htd2) in the mitochondrial type II fatty acid synthesis pathway (FAS II) (Kastaniotis *et al.*, 2004). This finding suggested a novel connection between an enzyme in the fatty acid synthesis pathway, or the pathway itself, and mitochondrial RNA processing. Chapter 3 presents an in-depth analysis of the intersection of these two pathways. Alignment of the predicted amino acid sequence of *YJL046w* revealed homology to known and predicted lipoate-protein ligases,

which are enzymes that catalyze covalent attachment of the cofactor lipoic acid to target enzymes. Lipoic acid is produced from its precursor molecule, octanoic acid, which is a product of the mitochondrial FAS II pathway. These data suggested a second link between production and attachment of lipoic acid and RNA processing in mitochondria. Chapter 4 presents an in-depth analysis of the lipoic acid synthesis and attachment pathway in yeast.

CHAPTER 3. INTERSECTION OF RNA PROCESSING AND THE FATTY ACID SYNTHESIS PATHWAY IN YEAST MITOCHONDRIA

STATEMENT BY AUTHOR

This chapter is an article that will be published in the November 2008 issue of the journal Molecular and Cellular Biology. I performed the research presented here and wrote this article with guidance from my advisor and our collaborators at the University of Oulu, Oulu, Finland.

SUMMARY

Distinct metabolic pathways can intersect in ways that allow hierarchical or reciprocal regulation. In a screen of respiratory-deficient yeast gene deletion strains for defects in mitochondrial RNA processing, we found that lack of any enzyme in the mitochondrial fatty acid biosynthetic pathway (FAS II) led to inefficient 5' processing of mitochondrial precursor tRNAs by RNase P. In particular, the precursor containing both the RNase P RNA (RPM1) and $tRNA^{pro}$ accumulated dramatically. Subsequent Pet127-driven 5' processing of RPM1 was blocked. The FAS II pathway defects resulted in loss of lipoic acid attachment to subunits of three key mitochondrial enzymes, which suggests that octanoic acid produced by the pathway is the sole precursor for lipoic acid synthesis and attachment. The protein component of yeast mitochondrial RNase P, Rpm2, is not modified by lipoic acid in the wild-type strain, and it is imported in FAS II mutant strains. Thus, a product of the FAS II pathway is required for RNase P RNA maturation,

which positively affects RNase P activity. In addition, a product is required for lipoic acid production, which is needed for the activity of pyruvate dehydrogenase, which feeds acetyl-CoA into the FAS II pathway. These two positive feedback cycles may provide switch-like control of mitochondrial gene expression in response to the metabolic state of the cell.

INTRODUCTION

Intersections of biological pathways previously thought to be distinct have emerged recently from genome-wide functional screens in model organisms (Yarunin *et al.*, 2005, Zewail *et al.*, 2003, Teixeira *et al.*, 2002). Through a genome-wide screen in *Saccharomyces cerevisiae*, we have discovered the intersection of mitochondrial type II fatty acid synthesis (FAS II) with mitochondrial RNA processing.

There are two distinct fatty acid synthesis pathways in eukaryotes. Fatty acid synthesis type I (FAS I) is catalyzed by cytosolic multifunctional enzyme complexes that produce the bulk of fatty acids in the cell (Smith 1994). Type II synthesis (FAS II) occurs in mitochondria and chloroplasts (as well as in prokaryotes) and is catalyzed by sequential action of individual enzymes (Tehlivets *et al.*, 2007, Hiltunen *et al.*, 2005). The mitochondrial FAS II pathway produces octanoic acid, which is the precursor molecule for the synthesis of lipoic acid. Lipoic acid is the "swinging arm" cofactor that serves to shuttle reaction intermediates between the active sites of several mitochondrial ketoacid dehydrogenase complexes (Perham 2000). In yeast, lipoic acid is covalently bound to conserved lysine residues on three different proteins, the E2 subunit of pyruvate dehydrogenase (PDH) (Niu *et al.*, 1988) and α-ketoglutarate dehydrogenase (α-KDH) (Repetto and Tzagoloff 1990), and on the H protein of the glycine cleavage enzyme (GC) (Nagarajan and Storms 1997).

Mitochondrial genes are expressed through a coordinated effort between the nuclear and mitochondrial genomes. Mitochondrial DNA in eukaryotes encodes a small number of proteins that are components of the respiratory chain complexes and ATP

synthase, as well as mitochondrial rRNAs and most tRNAs (Foury *et al.*, 1998, Anderson *et al.*, 1981). Other proteins required for mitochondrial function are encoded on nuclear chromosomes, translated in the cytoplasm, and imported into the organelle (Attardi and Schatz 1988). Mitochondrial genes are most often expressed in multigenic units and the initial transcripts are processed to produce mature RNAs (Dieckmann and Staples 1994, Tzagoloff and Dieckmann 1990). Mitochondrial tRNAs are cleaved from the initial multigenic transcripts by tRNA 3' endonuclease (Morl and Marchfelder 2001, Chen and Martin 1988) and mitochondrial RNase P, which processes the 5' leader sequences of tRNAs (Walker and Engelke 2006, Frank and Pace 1998). In yeast, mitochondrial RNase P is composed of the nuclear-encoded Rpm2 protein (Morales *et al.*, 1992), and mitochondrially-encoded *RPM1* RNA (Underbrink-Lyon *et al.*, 1983). As illustrated in Figure 1A, *RPM1* is transcribed either from the FP promoter with the upstream $tRNA_f^{met}$ and the downstream $tRNA^{pro}$ or from the SP promoter with only $tRNA^{pro}$ (Stribinskis *et al.*, 2001). Following tRNA removal, the 5' extension sequence of RPM1 is processed in a Pet127-dependent reaction (Ellis *et al.*, 2005), and the 3' end is processed either by the Dss1/Suv3 3' to 5' exonuclease (Dziembowski *et al.*, 2003) and/or by other enzymes not yet characterized. Because the production of mature RPM1 RNA requires the activity of a variety of mitochondrial RNA processing factors, including RNase P itself, RPM1 processing provides a good model for the study of mitochondrial RNA metabolism.

Here, we describe and analyze the unexpected finding that deletion of any gene in the mitochondrial mitochondrial type II fatty acid synthesis (FAS II) pathway results in inefficient 5' processing of precursors containing tRNAs by RNase P (Fig. 3.1A). A

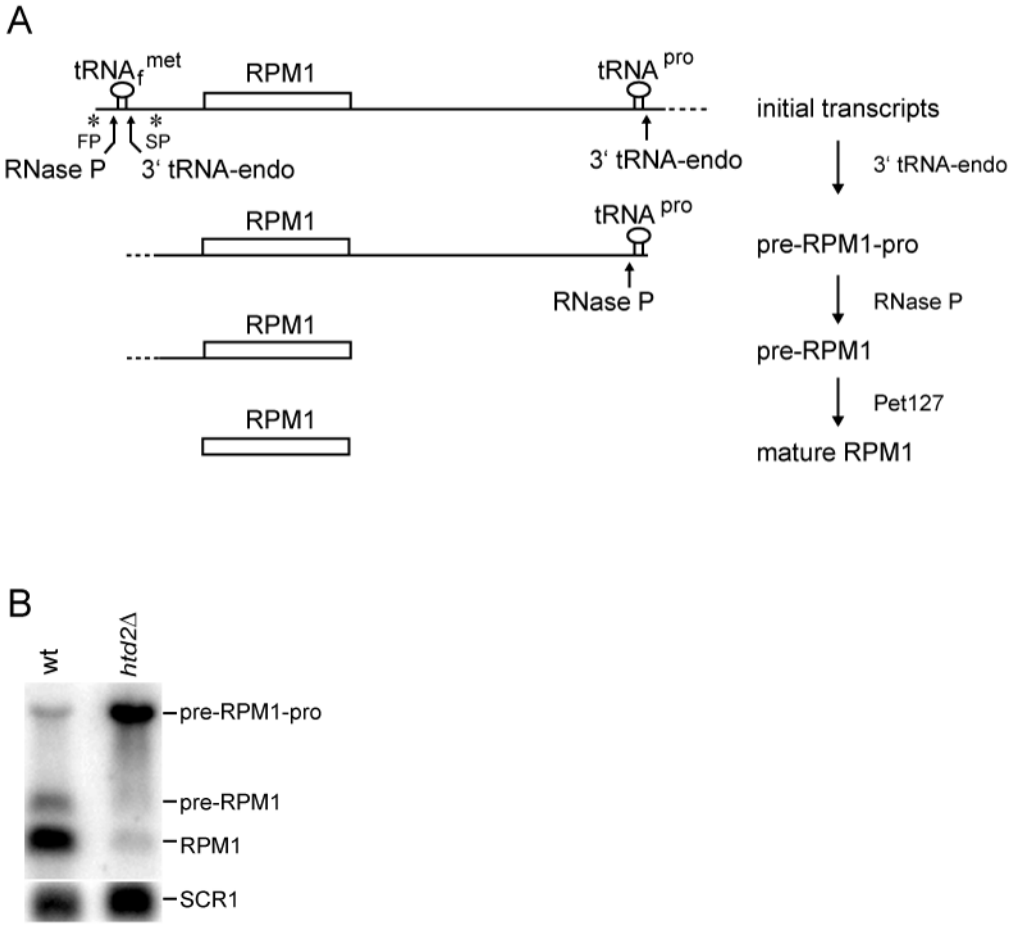


Figure 3.1. Processing of RPM1-containing transcripts. (A) *RPM1* is transcribed in a single transcription unit with both upstream $tRNA_f^{met}$ and downstream $tRNA^{pro}$ from the FP promoter (5' end represented by * FP) or with only the downstream $tRNA^{pro}$ from the SP promoter (5' end represented by * SP). 3' tRNA endonuclease generates the high molecular weight precursor termed pre-RPM1-pro. The 5' end of the precursor transcript is produced either by the SP promoter or by cleavage at the 3' end of $tRNA_f^{met}$. The interval between the SP promoter and $tRNA_f^{met}$ is designated by a dotted line. RNase P processes the pre-RPM1-pro precursor RNA at the 5' end of $tRNA^{pro}$, generating the pre-RPM1 intermediate. Further processing at the 5' end of this intermediate in a Pet127-dependent reaction produces mature RPM1 RNA. (B) A Northern blot of total RNA extracted from wild-type and *htd2Δ* was hybridized with a probe complementary to RPM1. A probe complementary to SCR1, the RNA subunit of the cytoplasmic Signal Recognition Particle, was used as a loading control.

model is presented for the regulation of mitochondrial gene expression by cellular catabolism.

MATERIALS AND METHODS

Strains and media

S. cerevisiae strains and their genotypes are listed in Table 3.1. The deletions in the strains of interest in the EUROSCARF collection were verified by PCR using a gene-specific 5' upstream primer and a Kanamycin-resistance gene 3' primer. Cells were grown in medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose (YEPD) or 3% glycerol (YEPG) instead of glucose. Solid media contained 2% (w/v) agar.

Disruption of open reading frames

The *HTD2*, *CEM1*, *CBP2*, *HFA1*, and *PET127* genes were deleted in BY4741 to confirm the phenotypes of the EUROSCARF strains. Each open reading frame was deleted by homologous recombination with the kanamycin resistance (*kanMX4*) cassette. *htd2Δ*::*kanMX4*, *cbp2Δ*::*kanMX4*, *hfa1Δ*::*kanMX4*, and *pet127Δ*::*kanMX4* constructs were generated by amplification of the *kanMX4* cassette using the corresponding primers listed in Table 3.2. The PCR products were gel-purified and transformed into the wild-type strain using the lithium acetate method (Gietz and Woods 2002) followed by selection for kanamycin resistance. The Rpm2-GFP *cbp2Δ* and Rpm2-GFP *htd2Δ* strains were made by transformation of the Rpm2-GFP wild-type strain (Huh *et al.*, 2003). The *cem1Δ*::*kanMX4* construct was generated by amplification of the *kanMX4* cassette using the CEM1-5'Kan and CEM1-3'Kan primers (Table 3.2), which are complementary to both *CEM1* untranslated regions and the *kanMX4* cassette. The resulting PCR product

Table 3.1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Reference
MY6/*rho*^{0}	*MATα*, *rho*^{0}, *lys1*	This study
BY4741	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF
cbp2Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cbp2*::*kanMX4*	This study
hfa1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, hfa1*::*kanMX4*	This study
mct1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, mct1*::*kanMX4*	EUROSCARF
cem1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cem1*::*kanMX4*	This study
oar1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, oar1*::*kanMX4*	EUROSCARF
htd2Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, htd2*::*kanMX4*	This study
etr1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, etr1*::*KANMX4*	EUROSCARF
pet127Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pet127*::*kanMX4*	This study
htd2Δpet127Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, htd2*::*kanMX4, pet127::LEU2*	This study
lpd1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lpd1*::*kanMX4*	EUROSCARF
lat1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lat1*::*kanMX4*	EUROSCARF
kgd2Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, kgd2*::*kanMX4*	EUROSCARF
gcv3Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, gcv3*::*kanMX4*	EUROSCARF
Rpm2-GFP wt	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, RPM2-GFP*::*HIS3*	(Huh *et al.*, 2003)
Rpm2-GFP *cbp2Δ*	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cbp2*::*kanMX4, RPM2-GFP*::*HIS3*	This study
Rpm2-GFP *htd2Δ*	*MAT***a**, *his3Δ1*, *leu2Δ0, met15Δ0, ura3Δ0, htd2*::*kanMX4, RPM2-GFP*::*HIS3*	This study

Table 3.2. Oligonucleotide probes and primers used in this study.

Oligonucleotide	Probe Number	Sequence 5'-3'
Probe[a]		
RPM1	3	ACTTTTTATTAATATATATATATGGACTCCTGCGGGG
tRNAfmet	1	GCAATAATACGATTTGAACG
tRNApro	5	AAGAAAGCGCCTGACCTTTTG
5' junction	2	ATAATATAAATATCTTATTCAAATTAAT
3' junction	4	AAATTAATTATGAATATGGATATTATATT
tRNAglu		AGGTGATGTCGTAACCATTAGACG
tRNAser		ATCACACTTTAAACCACTCAGTCAAC
SCR1		GTCTAGCCGCGAGGAAGG
Primer		
CEM1-5'Kan		CTGTCCTCGGTGTTGCCTAATTTTAAATTAAAGATCATTTCTTTACGATGTCCACGAGGTC
CEM1-3'Kan		TTAAGTTTTTGCTAATATTACCCTATATATATATTCAAATTATTATAATCGGTGTCGGTCTCG
HTD2-A		CTTTTAAATCATAGCCCAAA
HTD2-B		CACCTTTTTTTCAGTTCTTC
CBP2-A		ATAAGACAGTTCATTGTGGCA
CBP2-B		AAAGGAAAGAGTGAAGATTGC
HFA1-A		ATGAGATCTATAAGAAAATGGGC
HFA1-B		CTATCTCTTTCGCTTACTGTCC
PET127-A		TCATCTTTGAGTATATCACGTC
PET127-B		CATAATATGTACCAAGGGACT
PET127-up		CAGGGCACTTGAGAGAGCAC
PET127-down		CCCAACGCTGACTACTGTCT

[a]Probes are antisense.

was transformed into the wild-type strain using the same method as mentioned above. To make the *htd2Δpet127Δ* double deletion strain, the *PET127* ORF was amplified by PCR using the PET127-up and PET127-down primers and ligated to the pGEM-T Easy vector (Promega, Madison, WI). The *LEU2* gene from plasmid pUC18/*LEU2* was digested with *Xba*I and *Xma*I and ligated to the *Nhe*I and *Age*I sites in the *PET127* ORF. The *pet127*::*LEU2* construct was digested with *Not*I and the gel-purified fragment was transformed into the *htd2Δ*::*kanMX4* strain followed by selection for growth on plates lacking leucine.

The *hfa1Δ* mutation reported in Hoja *et al.* (Hoja *et al.*, 2004), which was made in a different strain background, causes a very slow-growth phenotype on glycerol. Since the mutation had little effect on respiratory growth at 30°C in our strain, the deletion was made anew in S288C and confirmed that our strain truly displayed a conditional phenotype on rich glycerol medium.

Isolation of whole-cell RNA

Total RNA was isolated from midlogarithmic cultures after growth in liquid YEPD medium at 30°C or 37°C as described (Caponigro *et al.*, 1993).

Northern blot analysis

10 or 30 µg of total RNA was separated on a 1.2% (w/v) agarose gel (a 2% agarose gel was used for tRNA analysis) in TB buffer (83 mM Tris base, 89 mM boric acid, pH 8.3). The RNAs were transferred onto a Nytran membrane (Schleicher &

Schuell) overnight in 20× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) and hybridized (6× SSC, 10× Denhardt's solution, 0.1% SDS, 50 µg/ml carrier DNA) overnight at 5-10°C below the T_m of the oligonucleotide probes. All blots were stripped and hybridized with the SCR1 loading control probe. Oligonucleotide probes (listed in Table 3.2) were ^{32}P end-labeled using T4 DNA kinase (Fermentas). Blots were analyzed on a PhosphorImager (Typhoon 9410, GE Healthcare).

Isolation and fractionation of mitochondria

Cells were grown to stationary phase in YEPD medium at 30°C. Mitochondria were prepared as described (Faye *et al.*, 1974), except that Zymolyase 20T (Seikagaku, Tokyo, Japan) was used instead of Glusulase to produce spheroplasts. Mitochondrial pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin A). For Western blots, Laemmli sample buffer was added to the resuspended mitochondrial pellets. For immunoprecipitations, the mitochondrial pellets were sonicated ten times for 10 sec each at 35% output (Fisher Sonic Dismembrator model 300). The mitochondrial matrix and membrane fractions were separated by ultracentrifugation for 30 min at ~100,000 × g in a TLA 100.3 rotor (Beckman Coulter, Fullerton, CA) at 4°C. Mitochondrial matrix supernatants were frozen at -20°C until used.

Western blot analysis

Approximately 40 µg of mitochondrial protein was resolved on SDS-polyacrylamide gels following the protocol described by Laemmli (Laemmli 1970). Proteins were transferred onto BioTrace polyvinylidene difluoride (PVDF) membrane (Pall, Pensacola, FL) at 22V overnight in transfer buffer (25 mM Tris base, pH 8.3, 200 mM glycine, 2% methanol, 0.01% SDS). The blots were preincubated in low salt buffer (150 mM NaCl, 40 mM Tris base, pH 8.0, 0.05% Tween-20) containing 5% (w/v) non-fat dried milk for 1 hour. For visualization of lipoylated proteins, the blots were reacted with anti-lipoic acid polyclonal antibody (Calbiochem, La Jolla, CA) diluted 1:7500 in a 1% milk solution in low salt buffer. Secondary antibody conjugated to horseradish peroxidase (HRP) (Pierce, Rockford, Il) was diluted 1:5000. Proteins were visualized using the ECL chemiluminescent substrate (Pierce). For visualization of Rpm2-GFP, the blots were reacted with anti-GFP monoclonal antibody (Covance, Berkeley, CA) diluted 1:2000. Secondary antibody conjugated to HRP (Pierce) was diluted 1:5000. Proteins were visualized using the SuperSignal Pico and Femto chemiluminescent substrates at a 70:30 ratio (Pierce). Anti-IDH (isocitrate dehydrogenase) antisera (a gift from Lee McAlister-Henn, University of Texas Southwestern Medical Center) was used as a loading control at a 1:500 dilution. Secondary antibody conjugated to HRP (Pierce) was diluted 1:5000, and proteins were visualized using ECL. Prestained RAINBOW molecular marker proteins (GE Healthcare) were used to estimate relative molecular mass.

Light microscopy

Strains were grown overnight in YEPD medium, diluted and grown until midlogarithmic phase. MitoTracker Red dye (CMXRos; Molecular Probes/Invitrogen, Carlsbad, CA) was added 45 minutes before harvesting the cells by centrifugation. The harvested cells were washed in minimal medium and the C-terminally tagged Rpm2–GFP chimera was localized by directly viewing the fluorescence signal through a GFP-optimized filter using a Leica DM-RXA microscope equipped with a mercury xenon light source. Images were captured by a Retiga EX digital camera (Q Imaging, Surrey, BC, Canada) and processed using Metamorph 6.0 software (Molecular Devices, Sunnyvale, CA).

Immunoprecipitation

0.2 mg Protein A-Sepharose was washed three times in wash buffer (150 mM KCl, 50 mM Tris pH 7.5, and 0.1% Triton X-100). All subsequent steps were done at 4°C. The mitochondrial matrix fraction (~3.5 mg of protein) was brought to 500 μL volume in IP buffer (150 mM KCl, 50 mM Tris pH 7.5, 0.1% Triton X-100, 0.1 mM DTT, and protease inhibitors as listed above). The sample was added to the Protein A-Sepharose beads for 3 hr at 4°C to pre-clear the supernatant. The beads were centrifuged and the sample was moved to a new tube containing newly washed Protein A-Sepharose beads, and polyclonal anti-GFP antibody (Molecular Probes) was added at 1:200. The tube was rotated at 4°C overnight. After centrifugation, the immunoprecipitation supernatant was collected and proteins were precipitated in 10% trichloroacetic acid

(VWR, West Chester, PA) and 80% acetone. The beads were washed twice in IP buffer for 30 sec and twice for 15 minutes each at 4°C, once in IP buffer without detergent for 30 sec, and once in IP buffer without detergent or salt for 30 sec. 100 µL of 1× loading buffer was added to the beads, which were then boiled for 5 minutes at 100°C. 30% of the immunoprecipitated and TCA precipitated protein was resolved on a 7-17% SDS-PAGE gradient gel and blotted as described above.

RESULTS

Mitochondrial pre-RPM1-pro precursor RNA accumulates in the *htd2Δ* strain

In an effort to uncover novel nuclear-encoded factors involved in mitochondrial RNA processing in yeast, the EUROSCARF haploid deletion strains (Frankfurt, Germany) were screened for respiratory-deficiency (inability to grow on rich glycerol medium, see Materials and Methods). Mutants that maintained stable mitochondrial genomes were then surveyed for mitochondrial RNA processing defects by Northern blot using probes specific for each of the mitochondrial multigenic transcripts. Hybridization of the Northern blots with a probe specific for RPM1, the RNA component of mitochondrial RNase P, revealed accumulation of the pre-RPM1-pro precursor RNA and lower levels of the pre-RPM1 intermediate and of mature RPM1 in strain *yhr067wΔ* (Fig. 3.1B). *YHR067w* encodes 3-hydroxyacyl thioester dehydratase 2 enzyme (Htd2) in the mitochondrial FAS II pathway (Kastaniotis *et al.*, 2004). This finding suggested a novel connection between an enzyme in the fatty acid synthesis pathway, or the pathway itself, and mitochondrial RNA processing.

Production of mature RPM1 RNA requires processing of $tRNA_f^{met}$ from the 5' end of the initial multigenic transcript by 3' tRNA endonuclease, and of $tRNA^{pro}$ from the 3' end of the transcript by RNase P (Fig. 3.1A). Following tRNA processing, the resulting transcript is trimmed at the 5' and 3' ends. To analyze the unprocessed sequences of the high molecular weight pre-RPM1-pro precursor RNA in the *htd2Δ* strain, Northern blots were hybridized with five probes complementary to different regions of the transcript (Fig. 3.2). Hybridization with a $tRNA^{pro}$-specific probe

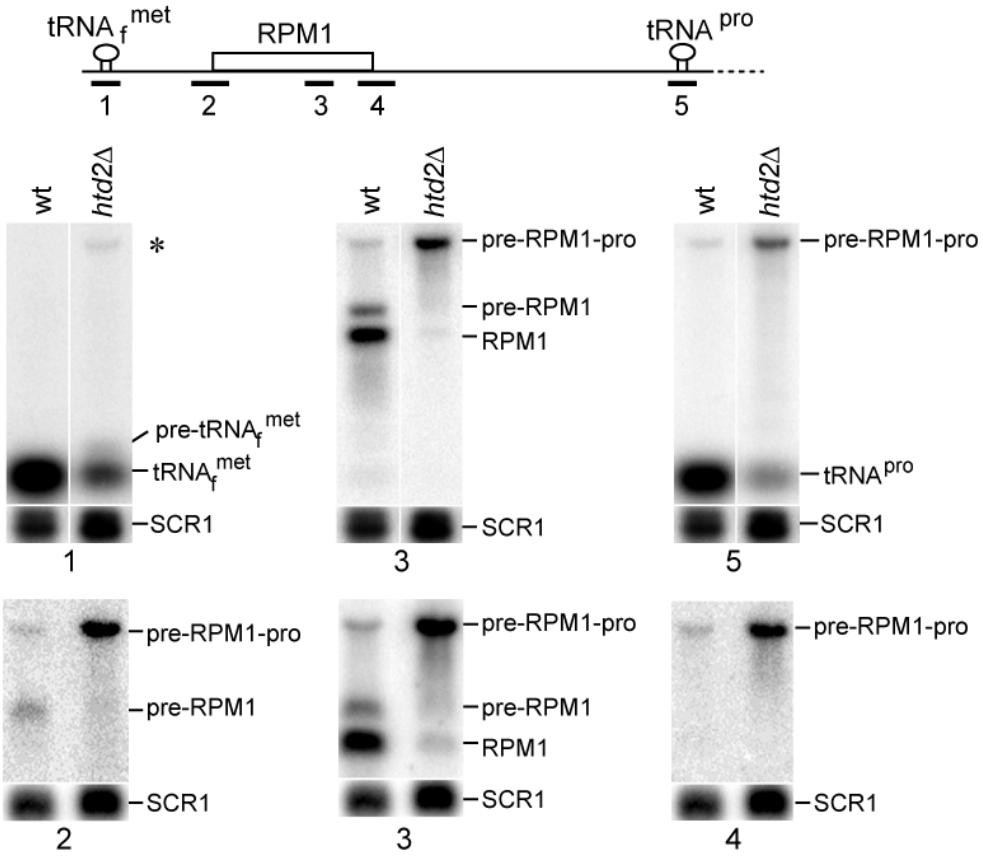


Figure 3.2. The initial RPM1-containing transcript is shown with the position of the probes used for Northern blot analysis. A Northern blot of total RNA extracted from the wild-type and *htd2Δ* strains was hybridized with a probe complementary to $tRNA_f^{met}$ (upper panel, probe #1). The asterisk denotes a high molecular weight pre-met-RPM1-pro RNA, which contains unprocessed $tRNA_f^{met}$. pre-$tRNA_f^{met}$ contains the presumed short leader sequence while mature tRNA is labeled as $tRNA_f^{met}$. The blot was stripped and probed for RPM1 (probe #3). pre-RPM1-pro and pre-RPM1 are the precursor RNAs, while RPM1 is the mature RNA. The blot was again stripped and probed for $tRNA^{pro}$ (probe #5). Mature tRNA is labeled as $tRNA^{pro}$. A Northern blot of 30 µg of total RNA extracted from wild-type and *htd2Δ* was hybridized with a probe complementary to sequence spanning the mature 5' end of RPM1 (lower panel, 5' junction probe, probe #2). The blot was stripped and probed for RPM1 (probe #3). The blot was again stripped and hybridized with a probe complementary to sequence spanning the mature 3' end of RPM1 (3' junction probe, probe #4). The 5' junction probe and the 3' junction probe were designed such that hybridization would only occur when both upstream and downstream sequence spanning the mature ends were present in the transcript. SCR1 was used as a loading control.

confirmed that the pre-RPM1-pro precursor contains unprocessed $tRNA^{pro}$ (Fig. 3.2, probe #5), which supports the conclusion that RNase P activity is compromised in the *htd2Δ* strain. Hybridization with a probe complementary to the sequence spanning the mature 5' end of RPM1 revealed that the pre-RPM1-pro precursor and pre-RPM1 intermediate RNAs also contain 5' extension sequences, the 5' ends of which were previously mapped to the SP promoter and the 3' end of the upstream $tRNA_f^{met}$ (Fig. 3.2, probe #2) (Stribinskis *et al.*, 2001). These results show that the *htd2Δ* mutation has two effects on processing of the pre-RPM1-pro precursor RNA: inefficient cleavage of $tRNA^{pro}$ by RNase P and impaired trimming of the 5' end of the transcript.

The mitochondrial FAS II pathway is required for efficient processing of pre-RPM1-pro precursor RNA

We explored whether the defect in processing of pre-RPM1-pro RNA by RNase P was specific to a deletion of *HTD2* or if a disruption of any enzyme in the FAS II pathway resulted in the same phenotype (for details of the FAS II pathway, see Fig. 1.3). To answer this question, RNA from mutant strains, each harboring a deletion of a gene encoding a FAS II enzyme, was analyzed by Northern blot using the RPM1 probe (Fig. 3.3A). As disruption of the FAS II pathway leads to respiratory deficiency (Tehlivets *et al.*, 2007, Hiltunen *et al.*, 2005), RNA from *cbp2Δ*, which is defective in *COB* mRNA intron processing (McGraw and Tzagoloff 1983), was also analyzed as a control for a general effect of respiratory deficiency on tRNA processing. The Northern blots revealed that deletion of any of the FAS II pathway genes led to a decrease in mature RPM1 RNA

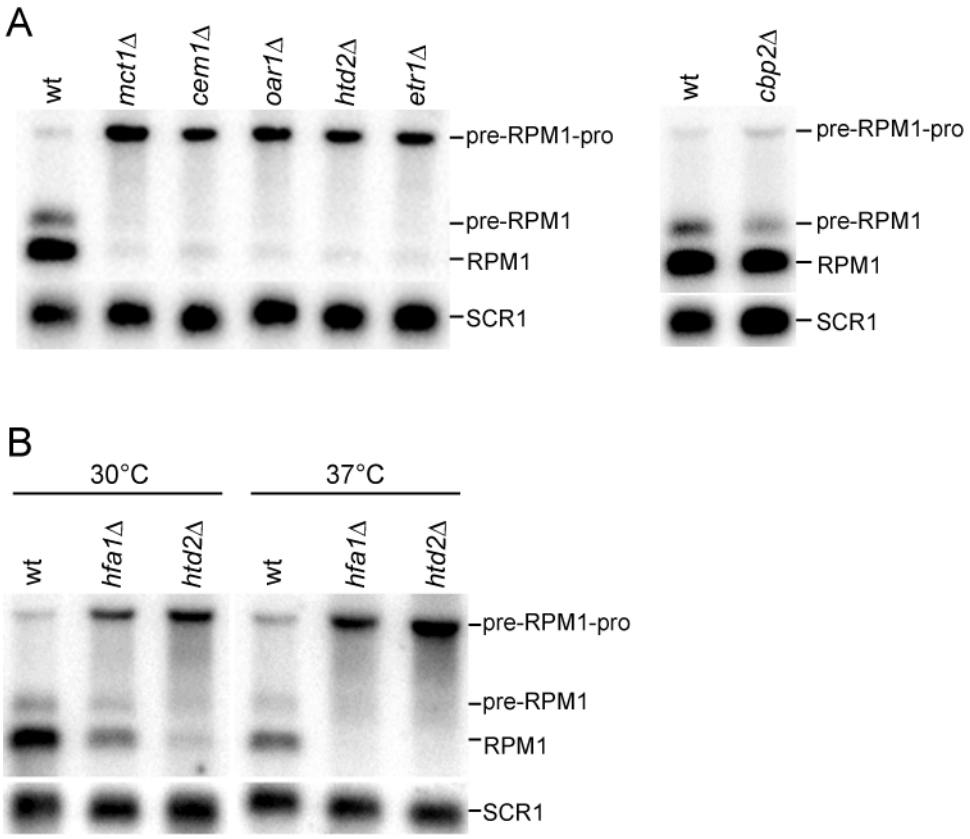


Figure 3.3. Northern analysis of RNase P activity on the pre-RPM1-pro precursor RNA in FAS II deletion and control strains. Northern blots of total RNA extracted from (A) FAS II deletion strains (see Fig. 1.3) and *cbp2Δ*, a respiratory-deficient control strain, grown at 30ºC, and (B) *hfa1Δ* and *htd2Δ* strains grown at 30ºC and 37ºC were hybridized with the RPM1 probe. The SCR1 probe was used as a loading control.

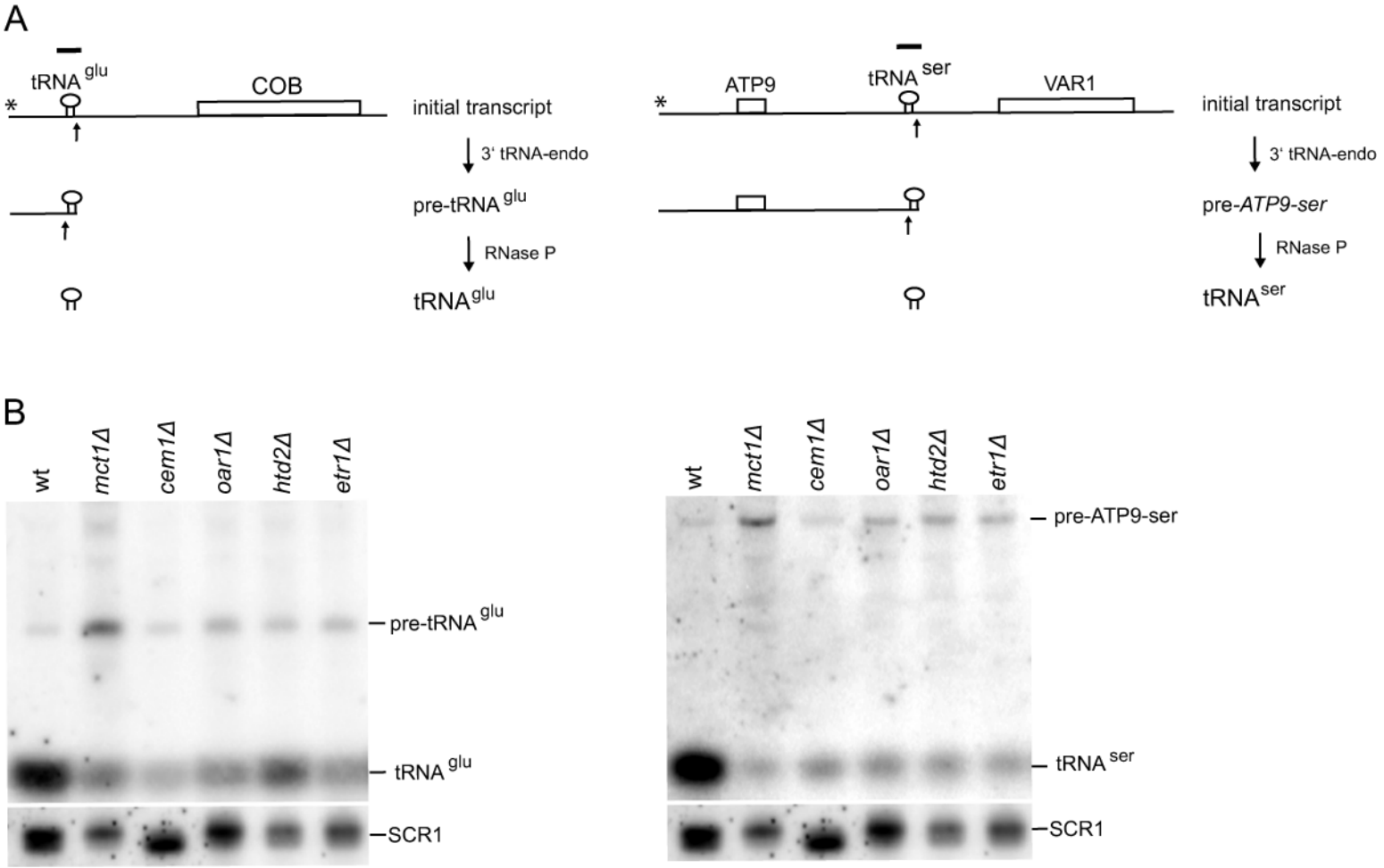
and the pre-RPM1 intermediate as well as accumulation of the pre-RPM1-pro precursor transcript as compared to the wild-type and *cbp2Δ* strains. Processing of mitochondrial-encoded mRNAs or rRNAs was not affected in the mutant strains (data not shown).

Unlike the FAS II mutant strains shown in Figure 3.3A, the *hfa1Δ* strain exhibited a conditional growth phenotype on respiratory medium. The pre-RPM1-pro RNA was inefficiently processed when *hfa1Δ* was grown at both the permissive temperature of 30°C and the non-permissive temperature of 37°C, but processing was more retarded at the higher temperature (Fig. 3.3B). That deletion of any of the FAS II pathways genes leads to inefficient removal of $tRNA^{pro}$ indicates that a product of the FAS II pathway is required for efficient processing of the pre-RPM1-pro precursor RNA by RNase P.

Disruption of the FAS II pathway affects processing of other mitochondrial tRNAs

Since action of RNase P on the pre-RPM1-pro precursor RNA in FAS II mutants is impeded, we examined other RNase P cleavage reactions. $tRNA^{glu}$ and $tRNA^{ser}$ were chosen as examples (Dieckmann and Staples 1994). These two tRNAs are also expressed in large multigenic transcripts (Fig. 3.4A). tRNA abundance and processing were assessed in wild-type and FAS II mutant strains by Northern blot (Fig. 3.4B). Levels of both mature $tRNA^{glu}$ and $tRNA^{ser}$ were decreased dramatically in the mutant strains, while levels of the precursor RNAs were increased somewhat, though the total of both species was lower than in wild-type. These data indicate that the FAS II pathway is generally required for efficient cleavage of mitochondrial tRNA 5' leader sequences by RNase P.

Figure 3.4. Northern analysis of mitochondrial tRNA processing by RNase P in FAS II deletion strains. (A) The primary transcripts containing $tRNA^{glu}$ and $tRNA^{ser}$ are shown as cartoons (5' ends are represented by *). The positions of the probes used for Northern blot analysis are shown above the transcripts. (B) A Northern blot was hybridized with a $tRNA^{glu}$ probe, stripped and hybridized with a $tRNA^{ser}$ probe. 3' tRNA endonuclease generates the precursor transcripts pre-$tRNA^{glu}$ and pre-ATP9-ser. RNase P processes the 5' ends of the tRNAs, producing mature $tRNA^{glu}$ and $tRNA^{ser}$. The asterisks denote transcription start sites. SCR1 was used as a loading control.


A
tRNA glu
COB
initial transcript
3' tRNA-endo
pre-tRNA glu
RNase P
tRNA glu
ATP9
tRNA ser
VAR1
initial transcript
3' tRNA-endo
pre-ATP9-ser
RNase P
tRNA ser
B
wt
mct1Δ
cem1Δ
oar1Δ
htd2Δ
etr1Δ
pre-tRNA glu
tRNA glu
SCR1
wt
mct1Δ
cem1Δ
oar1Δ
htd2Δ
etr1Δ
pre-ATP9-ser
tRNA ser
SCR1

The mitochondrial FAS II pathway is required for efficient 5' processing of pre-RPM1-pro RNA in a Pet127-driven reaction

The pre-RPM1-pro precursor, which accumulates in FAS II mutant strains, contains a 5' end extension sequence (Fig. 3.1). Thus, 5' processing of the intermediate in a Pet127-driven reaction that follows the RNase P processing step is severely retarded in these strains. Pet127 is a nuclear-encoded protein that is required for exonucleolytic processing of 5' end extension sequences of several mitochondrial precursor RNAs (Fekete *et al.*, 2008, Wiesenberger *et al.*, 2007, Wiesenberger and Fox 1997), including RPM1 (Ellis *et al.*, 2005). Deletion of *PET127* causes accumulation of precursor RNAs as well as temperature-sensitive respiratory-deficiency (Wiesenberger and Fox 1997). The blockage of this Pet127-dependent reaction in FAS II mutant strains is specific to the pre-RPM1-pro precursor; all other Pet127-dependent processing reactions (*COB*, *VAR1*, *ATP8*/6 mRNAs and 15S rRNA) are wild-type (data not shown). To analyze the 5' ends of pre-RPM1-pro and pre-RPM1 RNA in a *pet127Δ* strain, a Northern blot was hybridized with the RPM1 5' junction probe (Fig. 3.5A, probe #2). The probe revealed accumulation of the pre-RPM1 intermediate, which contains a 5' end extension, in the *pet127Δ* mutant compared to wild-type. Hybridization of the same blot with the RPM1 3' junction probe verified that the pre-RPM1 intermediate does not contain 3' extension sequence in *pet127Δ*. As expected, 5' processing of $tRNA^{pro}$ by RNase P was not affected in the *pet127Δ* mutant. The identities of the bands were verified by hybridization of the blot with the RPM1 probe (Fig. 3.5A, probe #3).

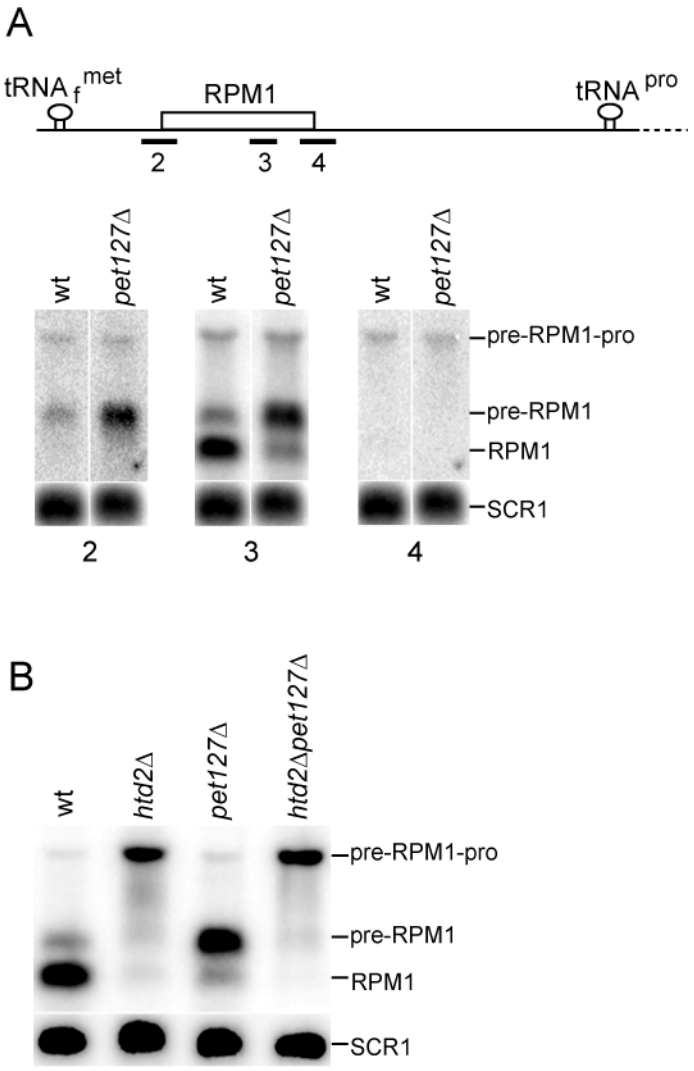


Figure 3.5. Northern analysis of pre-RPM1-pro and pre-RPM1 processing in *pet127Δ* strains. (A) The initial RPM1-containing transcript is shown with the position of probes used for Northern blot analysis. A Northern blot of 30 µg of total RNA from *pet127Δ* was hybridized with a probe complementary to sequence spanning the mature 5' end of RPM1 (5' junction probe, probe #2). The blot was stripped and probed for RPM1 (probe #3). The blot was again stripped and hybridized with a probe complementary to sequence spanning the mature 3' end of RPM1 (3' junction probe, probe #4). The 5' junction probe and the 3' junction probe were designed such that hybridization would only occur when both upstream and downstream sequence spanning the mature ends were present in the transcript. (B) A Northern blot of total RNA from *htd2Δ, pet127Δ* and *htd2Δpet127Δ* strains was hybridized with the RPM1 probe, stripped and hybridized with the SCR1 loading control probe.

Given that Pet127 is required for 5' end processing of RPM1 RNA, we tested whether a defect in the FAS II pathway is epistatic to *pet127Δ*. A Northern blot containing RNA from a *htd2Δpet127Δ* double mutant, as well the *htd2Δ* and *pet127Δ* single mutants, was hybridized with the RPM1 probe (Fig. 3.5B). The *htd2Δpet127Δ* double mutant accumulated the same longer pre-RPM1-pro precursor with a 5' end extension observed in the *htd2Δ* single mutant. These results indicate that *htd2Δ* is epistatic to *pet127Δ* and that trimming of the 5' end of pre-RPM1-pro in a Pet127-driven reaction requires prior FAS II pathway-dependent removal of $tRNA^{pro}$ from the 3' end of the precursor transcript.

The FAS II pathway is required for lipoylation of three mitochondrial proteins

Brody *et al*. (Brody *et al.*, 1997) and Wada *et al*. (Wada *et al.*, 1997) proposed that the mitochondrial FAS II pathway exists to provide the octanoic acid precursor for lipoic acid biosynthesis. To test this hypothesis, we analyzed the lipoylation state of three lipoic acid-modified mitochondrial proteins in wild-type and FAS II mutant strains. Western blots of mitochondrial proteins were probed with an antibody specific for lipoic acid (Humphries and Szweda 1998) (Fig. 3.6A). In wild-type, the anti-lipoic acid antibody detected all of the known lipoylated proteins, Lat1, the E2 subunit of pyruvate dehydrogenase (PDH), Kgd2, the E2 subunit of α-ketoglutarate dehydrogenase (α-KDH) complexes, and Gcv3, the H protein of the glycine cleavage enzyme (GC). In the *htd2Δ* strain, however, none of these lipoylated proteins were detected, even following overexposure of the film. These data are supportive of the long-standing hypothesis

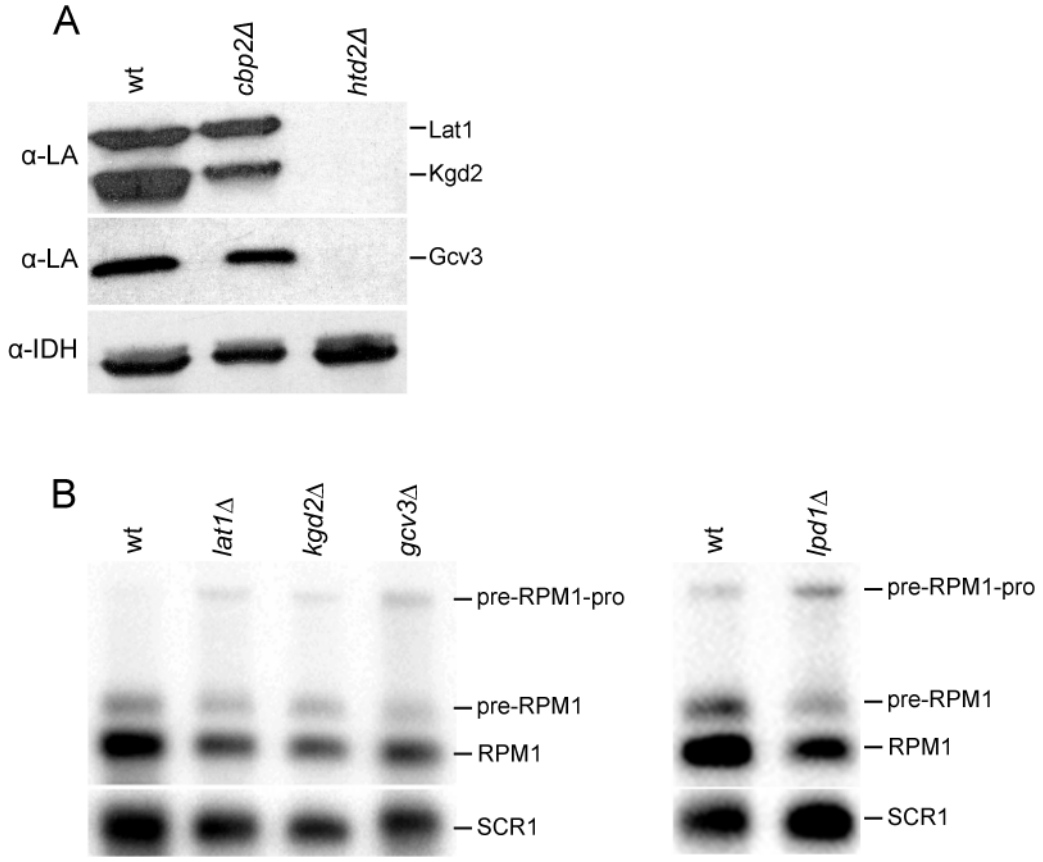


Figure 3.6. The FAS II pathway is the sole source of octanoic acid for protein lipoylation. (A) Western blot analysis of lipoylated proteins in an FAS II mutant strain. Mitochondrial extracts from wild-type, *cbp2Δ*, a respiratory-deficient control strain, and *htd2Δ* were analyzed by Western blot using an antibody directed against lipoic acid (α-LA). Lat1 and Kgd2 are the lipoylated E2 proteins of PDH and α-KDH, respectively, and Gcv3 is the lipoylated H protein of GC. Isocitrate dehydrogenase (antiserum α-IDH) was used as a loading control. (B) Northern blot analysis of pre-RPM1-pro processing in the *lat1Δ*, *kgd2Δ* and *gcv3Δ* strains as well as in the *lpd1Δ* strain was done using the RPM1 probe. Lpd1 is a common subunit shared by the three lipoic acid-dependent enzyme complexes. SCR1 was used as the loading control.

(Wada *et al.*, 1997, Brody *et al.*, 1997) that the FAS II pathway is the sole source of the octanoic acid precursor required for lipoylation of the E2 subunits and the H protein assayed here. These data also suggest that elimination of lipoylation may be sufficient to cause the respiratory-deficient phenotype of the FAS II mutant strains.

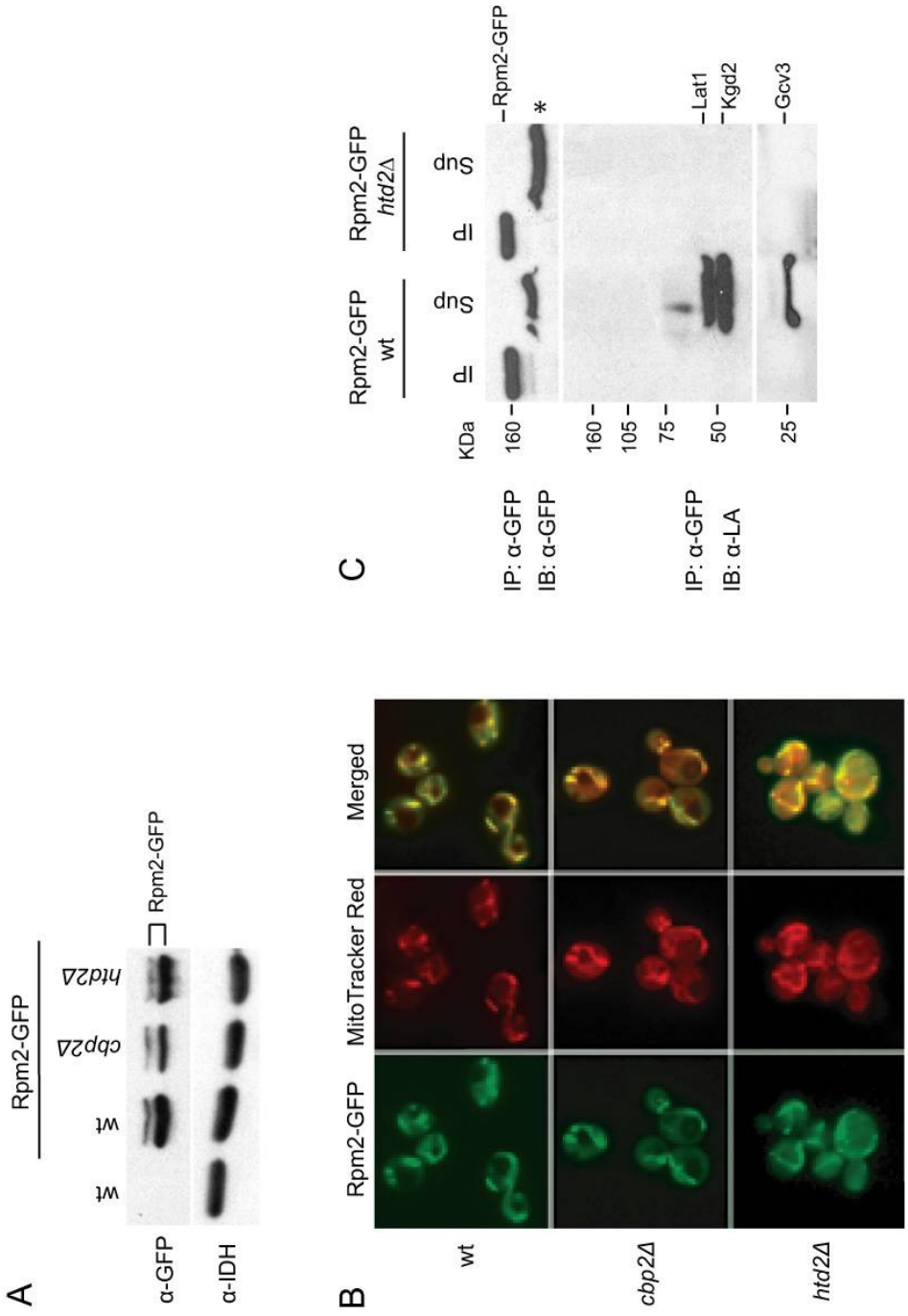
In light of these results, we asked whether enzymatic function of the lipoic acid-dependent mitochondrial multi-enzyme complexes is important for pre-RPM1-pro RNA processing. To address this question, we examined the RNA processing phenotype of strains harboring deletions of *LAT1*, *KGD2*, and *GCV3.* In these strains, processing of pre-RPM1-pro is partially affected (Fig. 3.6B), but the phenotype is not as severe as that of the FAS II mutant strains. We also analyzed RNA processing in *lpd1Δ* (the *LPD1* gene encodes dihydrolipoamide dehydrogenase, a subunit common to all three complexes (Roy and Dawes 1987)).These data indicate that enzymatic function of the lipoic acid-dependent complexes is not required for processing of the pre-RPM1-pro transcript by RNase P, but rather that synthesis of a fatty acid, such as octanoic or lipoic acid is necessary for efficient processing.

The tRNA processing defect is not due to mislocalization of Rpm2 in the *htd2*Δ strain

The tRNA processing defect in FAS II mutant strains may result from a reduction in RNase P activity due to either a partial blockage of import from the cytoplasm of Rpm2, the RNase P protein subunit, or to Rpm2 instability. Indeed, certain *rpm2* mutant alleles result in accumulation of the pre-RPM1-pro precursor RNA (Stribinskis *et al.*,

2001, Stribinskis *et al.*, 1996), a phenotype similar to that of the FAS II mutant strains. It is known that a disruption of the FAS II pathway affects mitochondrial morphology (Kastaniotis *et al.*, 2004, Torkko *et al.*, 2001), which may in turn affect import of Rpm2 from the cytoplasm. To investigate these possibilities, fluorescence microscopy and Western blotting were used to analyze localization and levels of an endogenously expressed Rpm2-GFP fusion protein in the wild-type as well as in the *htd2Δ* and *cbp2Δ* mutant backgrounds. The Rpm2-GFP wild-type strain respires, and displays wild-type RPM1 and tRNA processing (data not shown), suggesting that the Rpm2-GFP protein is imported into the mitochondrial compartment and is functional. Expression of Rpm2-GFP was analyzed by Western blot of mitochondrial extracts using an anti-GFP antibody (Fig. 3.7A). The protein detected in the wild-type and *htd2Δ* strains was of similar abundance, which indicates that a defect in fatty acid synthesis does not markedly affect steady-state levels of the Rpm2-GFP protein. In cells containing either an intact or a disrupted FAS II pathway, green fluorescent signal from Rpm2-GFP coincided with the red fluorescent signal from MitoTracker red dye that was added to the cells before harvesting (Fig. 3.7B). However, cells containing the *htd2Δ* mutation appeared to have more cytoplasmic green fluorescence relative to wild-type, indicating perhaps inefficient import of Rpm2-GFP or leaky mitochondrial membranes. These data show that Rpm2-GFP is localized primarily in mitochondria in the fatty acid biosynthetic deficient strain.

Figure 3.7. Analysis of Rpm2-GFP. Strains used were Rpm2-GFP wild-type, Rpm2-GFP *cbp2Δ*, a respiratory-deficient control strain, and Rpm2-GFP *htd2Δ*. (A) Rpm2-GFP protein levels were analyzed by Western blot of mitochondrial extracts using an antibody against GFP (α-GFP) to detect Rpm2-GFP. Isocitrate dehydrogenase (antiserum α-IDH) was used as a loading control. (B) Rpm2-GFP is localized to mitochondria. Cells harvested at mid-log phase were analyzed by fluorescence microscopy. MitoTracker Red was added to the cultures 45 minutes before harvesting. (C) Rpm2-GFP is not lipoylated. Proteins from mitochondrial matrix fractions from Rpm2-GFP wild-type and Rpm2-GFP *htd2Δ* were immunoprecipitated (IP) with anti-GFP antibody and Protein A-coupled beads. Non-immunoprecipitated protein (Sup) was TCA precipitated, and 30% of both the IP and Sup fractions were loaded onto a 7-17% gradient SDS-PAGE gel. Proteins were immunoblotted (IB) with anti-lipoic acid antibody (α-LA, lower panels). The blot was stripped and incubated with anti-GFP antibody (α-GFP, upper panel). The asterisk marks a non-specific background band.


A
Rpm2-GFP
wt
wt
cbp2Δ
htd2Δ
Rpm2-GFP
α-GFP
α-IDH
B
Rpm2-GFP
MitoTracker Red
Merged
wt
cbp2Δ
htd2Δ
C
Rpm2-GFP wt
Rpm2-GFP htd2Δ
TP
Sup
TP
Sup
KDa
160
IP: α-GFP
IB: α-GFP
Rpm2-GFP
*
160
105
75
IP: α-GFP
IB: α-LA
Lat1
Kgd2
50
25
Gcv3

Rpm2-GFP is not lipoylated

One simple hypothesis to explain the requirement of the FAS II-lipoic acid dependent pathways for tRNA processing is that Rpm2 is lipoylated, and that lipoylation is required for RNase P activity. To answer this question, Rpm2-GFP was immunoprecipitated (IP) from the soluble matrix fraction of mitochondrial extracts from wild-type, Rpm2-GFP wild-type and Rpm2-GFP *htd2Δ* strains using a polyclonal anti-GFP antibody. Lipoylation of the immunoprecipitated protein was then analyzed by Western blotting with the anti-lipoic acid antibody (Fig. 3.7C lower panels). No lipoylated proteins of the same mass as Rpm2-GFP were detected in the IP lanes. Also, no lipoylated proteins were observed in the Rpm2-GFP *htd2Δ* lanes, as expected. The blot was stripped and subsequently probed with a monoclonal anti-GFP antibody, which showed that most of the Rpm2-GFP protein had been immunoprecipitated from the GFP tagged strains (Fig. 3.7C upper panel). These data indicate that Rpm2-GFP is not lipoylated.

DISCUSSION

Here we show that disruption of the mitochondrial FAS II pathway in yeast results in inefficient processing at the 5' ends of mitochondrial tRNAs by RNase P. Specifically, in FAS II mutant strains, removal of $tRNA^{pro}$ from the 3' end of the pre-RPM1-pro transcript is inhibited, resulting in low levels of mature RPM1, the RNA subunit of mitochondrial RNase P. We conclude that a product of the FAS II pathway is required either for: 1) RNase P cleavage of all pre-tRNAs, or 2) maturation of the RNase P RNA itself, thus affecting assembly and/or activity of RNase P and subsequent processing of all other tRNA-containing multigenic transcripts. Our data reveal a novel connection between fatty acid metabolism and gene expression in mitochondria.

RNase P complexes exist in all kingdoms of life, although their components vary in homology and number (Walker and Engelke 2006, Xiao *et al.*, 2002). Mitochondrial RNase P activity has been found in vertebrates, fungi, protists, and plants (Walker and Engelke 2006), but mitochondrially encoded RNase P RNA has been identified only in a number of ascomycete fungi, the protist *Reclinomonas americana*, and the green alga *Nephroselmis olivacea* (Seif *et al.*, 2003). In these organisms, RNase P RNA is most often transcribed as part of a multigenic transcription unit that includes at least one tRNA gene (Seif *et al.*, 2003, Shu and Martin 1991). In some instances, RNase P RNA 5' and 3' ends are formed by tRNA processing enzymes, unlike in *S. cerevisiae*, where multiple processing events must occur to produce mature RPM1 RNA. Despite differences in the details of the pathway for processing the RNA component, RNase P plays a critical role

in the expression of mitochondrial genes and is in many cases involved in the maturation pathway of its own RNA subunit.

The purpose of the mitochondrial type II fatty acid synthesis pathway (FAS II) in eukaryotes has been under debate. Brody *et al*. (Brody *et al.*, 1997) and Wada *et al*. (Wada *et al.*, 1997) have proposed that the production of octanoic acid, the precursor to the cofactor lipoic acid, is the sole purpose of this separate, organellar pathway. The FAS II pathway may play a role in maintenance of mitochondrial morphology as deletion or overexpression of genes encoding pathway enzymes causes morphological defects (Kastaniotis *et al.*, 2004, Torkko *et al.*, 2001). It has also been suggested that the FAS II pathway may provide fatty acids for phospholipid repair (Schneider *et al.*, 1997b) or for insertion of membrane proteins (Harington *et al.*, 1994), but no evidence has been generated to support these hypotheses. While it has been shown for other organisms that the mitochondrial FAS II pathway is capable of the synthesis of fatty acids longer than C8 (Witkowski *et al.*, 2007), the physiological relevance of these fatty acids still remains to be demonstrated. Our results suggest that the mitochondrial fatty acid synthesis pathway is the sole source of octanoic acid used as the precursor for synthesis of lipoic acid.

There are two basic models one can build to explain the intersection of RNase P activity or assembly and fatty acid biosynthesis. The first model posits that the biosynthetic pathway provides a product that directly modifies the RNase P enzyme, either on the Rpm2 protein or RPM1 RNA component. The second model suggests that a pathway product indirectly affects RNase P assembly or activity.

In pursuit of the direct modification hypothesis, we have eliminated the possibility that Rpm2 is modified in a detectable amide linkage by lipoic acid, a downstream product of the FAS II pathway. However, the protein may be modified by octanoic acid or a longer fatty acid product of the pathway. Probing a Northern blot of mitochondrial RNA with the anti-lipoic acid antiserum did not detect a modification (data not shown). The antiserum may be ineffective in Northwestern detection or the RNA may be modified by another fatty acid. It is possible that fatty acid or lipoic acid could directly affect the conformation of the protein or RNA directly, but in a non-covalent association. We have no evidence yet for a direct covalent or non-covalent modification of the enzyme.

Since mitochondrial morphology is not wild-type in FAS II mutant strains (Kastaniotis *et al.*, 2004, Torkko *et al.*, 2001), we tested one example of an indirect effect on RNase P. We showed that Rpm2-GFP is imported into mitochondria (Fig. 3.7B), though there is some cytoplasmic staining, suggesting the process may not be as efficient as in wild type. Many other indirect scenarios have not been investigated yet, including covalent or non-covalent modification of other proteins that may interact with RNase P.

All FAS II mutant strains, except *hfa1Δ*, are respiratory deficient at all temperatures. All of the strains maintain stable mitochondrial genomes when grown at 30°C, which suggests that, despite the inefficiency of tRNA processing, enough mature tRNAs are produced to support mitochondrial translation, which is required for maintenance of mitochondrial DNA (mtDNA) (Contamine and Picard 2000, Myers *et al.*, 1985). When the mutant strains are grown at temperatures greater than 30°C, however,

tRNA processing is further blocked (see Fig. 3.3B, 37°C), and after ~50 generations of growth at 37°C, the strains lose their mtDNA (data not shown).

The *hfa1Δ* strain respires at 30°C but not at 37°C in our strain background (S288C). *HFA1* encodes mitochondrial acetyl-CoA carboxylase, which produces malonyl-CoA from acetyl-CoA (Brody *et al.*, 1997). The additional source of malonyl-CoA that supports respiration in the mutant strain at 30°C is not known. In a global tandem affinity purification (TAP) tag study on protein-protein interactions (Krogan *et al.*, 2006), Hfa1 purified with Acc1, the cytoplasmic acetyl-CoA carboxylase. It is possible that Acc1 is imported inefficiently into mitochondria and produces malonyl-CoA at a reduced rate compared to Hfa1. An alternative hypothesis is that malonyl-CoA is imported into the mitochondrial compartment from the cytosol. It has been shown that exogenous [^{14}C]malonyl-CoA supports fatty acid synthesis in mitochondria isolated from *Trypanosoma brucei* (Stephens *et al.*, 2007). It would be interesting to understand why increased temperature exacerbates the mitochondrial tRNA processing defect in the FAS II mutant strains.

Why does mitochondrial fatty acid biosynthesis and tRNA processing intersect? Our hypothesis is that this intersection has evolved to allow the cell to regulate mitochondrial gene expression in response to levels of cellular catabolites. The organization of the pre-RPM1-pro precursor RNA is particularly interesting in that it houses a tRNA downstream of RPM1. Since RNase P activity is required for the maturation of the RNase P RNA subunit, a positive feedback loop exists for RNase P maturation and activity (Fig. 3.8). A product of the FAS II pathway is involved in this

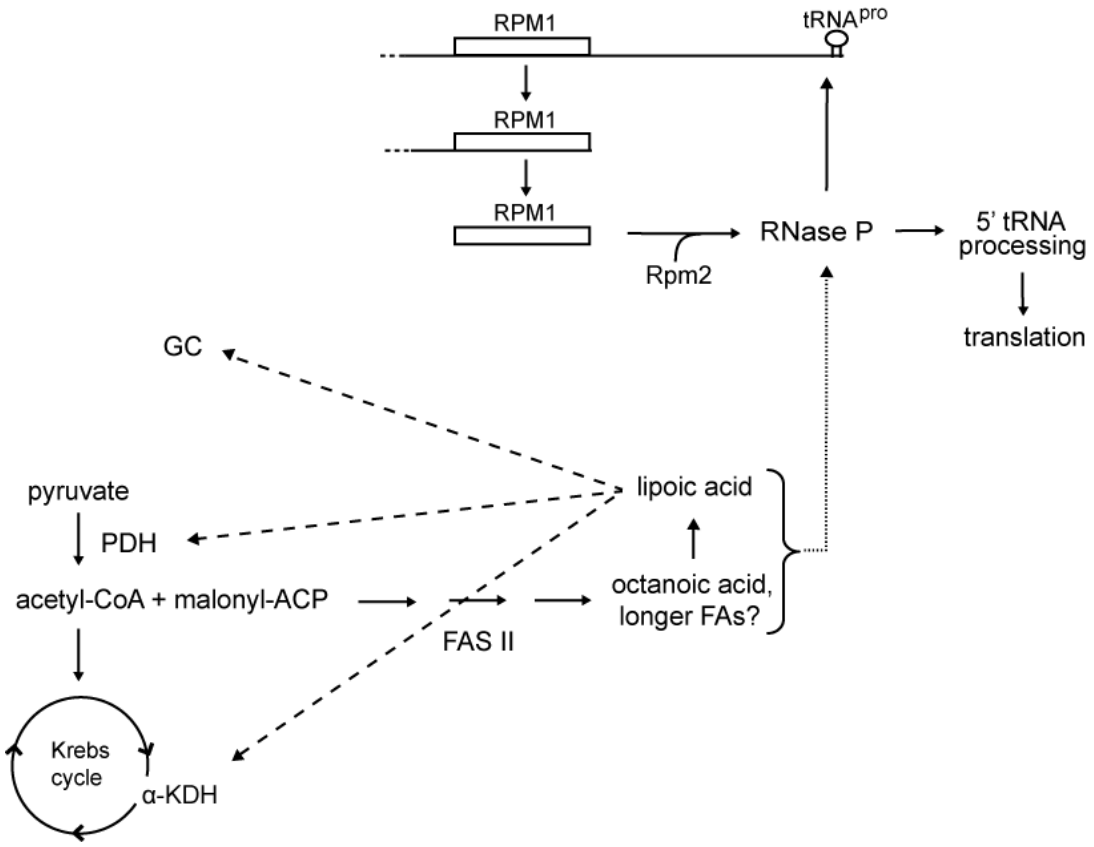


Figure 3.8. Positive feedback loops governing fatty acid-lipoic acid biosynthesis and RNase P activity. The intersection of the FAS II pathway and RNase P activity in yeast mitochondria is depicted as a cartoon. In the FAS II pathway, acetyl-CoA (or acetyl-ACP) and malonyl-ACP are condensed on an acyl carrier protein (ACP) and the carbon chain is elongated in several iterative steps to produce octanoic acid (Hiltunen *et al.*, 2005) (see Fig. 1.3). Other enzymes convert octanoic acid to lipoic acid (Sulo and Martin 1993) and attach the cofactor to target proteins (Marvin *et al.*, 2001). Lipoic acid is required for pyruvate dehydrogenase activity, creating one positive feedback loop. Fatty acid and/or lipoic acid is required for efficient RNase P activity. The second positive feedback loop is created because RNase P activity is required for the maturation of its own RNA subunit, RPM1, via an endonucleolytic cleavage of $tRNA^{pro}$ from the pre-RPM1-pro precursor RNA. The dashed arrows represent the requirement of lipoic acid for PDH, α-KDH and GC enzymatic activity. The dotted arrow represents the requirement of a product of the FAS II pathway for RNase P activity or assembly.

cycle because it is required for directly stimulating RNase P activity, and/or for efficient processing of the precursor RNA containing RNase P RNA. A second positive feedback loop could be operative under some conditions for pyruvate dehydrogenase activity and the FAS II pathway. PDH requires lipoic acid for the production of acetyl-CoA (Pronk *et al.*, 1996), which is fed into the FAS II pathway, which produces the octanoic acid precursor for lipoic acid synthesis (Fig. 3.8). However, cells containing inactive PDH lipoylate target proteins and have wild-type mitochondrial tRNA processing when grown in rich medium containing glucose, indicating that acetyl-CoA from another source, such as the pyruvate dehydrogenase bypass pathway (Boubekeur *et al.*, 1999), amino acid breakdown (Hazelwood *et al.*, 2008) or β-oxidation of fatty acids (Black and DiRusso 2007), can be fed into the FAS II pathway. The two feedback cycles described above may provide a switch-like character to turning on or off mitochondrial function in response to the availability of acetyl-CoA entering the organelle.

Recently, an intersection between the FAS II pathway and tRNA processing has emerged in mammals. The human 3-hydroxyacyl-thioester dehydratase 2 (HsHTD2) of the mitochondrial FAS II pathway was found to be encoded on a bicistronic cDNA downstream of RPP14, a protein subunit of RNase P (Autio *et al.*, 2008). This gene organization has been conserved over 400 million years of vertebrate evolution. It is currently under debate whether mammalian RNase P processes both nuclear-encoded and mitochondrial tRNA precursors (Puranam and Attardi 2001) or whether mammalian cells contain two distinct RNase P activities (Rossmanith and Karwan 1998). Clearly, though the nature of the connection between the mitochondrial FAS II pathway and tRNA

processing is different in yeast and vertebrates, both systems have the potential to be regulated by the availability of acetyl-CoA. The biological importance of the intersection of the FAS II pathway and RNase P function in tRNA processing is just beginning to be understood.

CHAPTER 4. LIPOIC ACID SYNTHESIS AND ATTACHMENT IN YEAST MITOCHONDRIA

STATEMENT BY AUTHOR

This chapter is a manuscript written by myself with input from my advisor to be submitted to the Journal of Biological Chemistry. I performed most of the research presented in this manuscript with guidance from my advisor. Our collaborators at the University of Oulu in Oulu, Finland, performed the enzyme activity assays and the lipoic acid content assays.

SUMMARY

Lipoic acid is a cofactor required for the function of three multi-enzyme complexes in yeast mitochondria: pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KDH), and the glycine cleavage enzyme (GC). It is covalently bound in an amide linkage to a conserved lysine residue in the lipoylation domain of Lat1, the E2 subunit of PDH, Kgd2, the E2 subunit of α-KDH, and Gcv3, the H protein of GC. The cofactor serves as a swinging arm that shuttles reaction intermediate between active sites. Although the function of lipoic acid has been well-studied, the mechanisms behind its biosynthesis and attachment remain unclear. We have shown that the type II mitochondrial fatty acid synthesis pathway (FAS II) is the only source of octanoic acid, the precursor for the synthesis of lipoic acid (Schonauer *et al.*, 2008). We show here, by Western blot analysis, that four enzymes, Lip2, Lip3, Lip5, and Gcv3, are involved in the

synthesis and attachment of lipoic acid in yeast mitochondria. Lip2 and Lip5, the homologs of the *E. coli* lipoyl(octanoyl)-protein transferase and lipoyl synthase, respectively, are required for lipoylation of all three lipoate-dependent subunits. Lip3, shown here to be homologous to the bacterial lipoate-protein ligase, is required for lipoylation of Lat1 and Kgd2. Gcv3, a target of lipoylation, also is required for lipoylation of Lat1 and Kgd2. In addition, we demonstrate here that the activity of Lip3 and Gcv3 is necessary for their roles in protein lipoylation. We propose that these four proteins may function in a lipoylation complex in mitochondria, and we present a model illustrating the role of the complex as a control point for mitochondrial gene expression in response to levels of acetyl-CoA.

INTRODUCTION

Lipoic acid (6,8-thioctic acid) is a sulfur-containing cofactor essential for the function of several multi-enzyme complexes that catalyze oxidative decarboxylation reactions in prokaryotic and eukaryotic organisms (Perham 2000, Herbert and Guest 1975). It is covalently attached via an amide linkage to a specific lysine residue on the surface of conserved lipoyl domains of the E2 subunits of pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KDH) and the branched chain α-keto acid dehydrogenase complexes, as well as of the H protein of the glycine cleavage enzyme (GC) (Reed and Hackert 1990). The lipoyl moiety serves as a swinging arm that shuttles reaction intermediates between active sites within the complexes (Perham 2000). Despite the well-characterized function of lipoic acid as a prosthetic group, the mechanisms of synthesis and attachment to proteins are the subject of ongoing investigations (Zhao *et al.*, 2005, Cicchillo and Booker 2005, Cronan *et al.*, 2005b, Morris *et al.*, 1995).

In *E. coli* there are two well-defined pathways for the synthesis and attachment of lipoic acid (Booker 2004). The first pathway follows *de novo* synthesis of octanoic acid on the acyl carrier protein, ACP (Jordan and Cronan, Jr. 1997). Lipoyl(octanoyl)-ACP:protein transferase (LipB) transfers either lipoic acid or octanoic acid from ACP to target lipoyl domains (Nesbitt *et al.*, 2005, Jordan and Cronan, Jr. 2003). Lipoyl synthase (LipA) catalyzes insertion of two sulfur atoms either before or after attachment of octanoic acid to the protein (Miller *et al.*, 2000). The preferred order of these two reactions has been shown to be attachment of octanoic acid by LipB first and insertion of sulfur by LipA second (Zhao *et al.*, 2003). The second pathway consists of a two-step

reaction carried out by lipoate-protein ligase (LplA). Lipoic acid, which can be supplied in the medium, is activated in an ATP-dependent manner to lipoyl-AMP and then the lipoyl group is transferred to the proteins (Morris *et al.*, 1994).

Lipoic acid synthesis and attachment to target proteins is less understood in eukaryotes. Homologs of the *E. coli* enzymes have been found in fungi, plants, protists and mammals, though many mechanistic details are unclear (Kang *et al.*, 2007, Ma *et al.*, 2006, Fujiwara *et al.*, 1999). In *S. cerevisiae*, the mitochondrial type II fatty acid biosynthetic pathway synthesizes octanoyl-ACP, which is the source for lipoic acid synthesis (Schonauer *et al.*, 2008). Lip2 and Lip5, the respective homologs of *E. coli* LipB and LipA, were shown to be required for PDH activity (Marvin *et al.*, 2001) and lipoic acid synthesis (Sulo and Martin 1993), indicating functional roles in *de novo* lipoic acid synthesis and attachment. However, there has been no report of an LplA-like lipoate-protein ligase homolog in yeast. In mammals, two separate enzymes are required for activation and transfer of free lipoic acid; lipoate-activating enzyme (Fujiwara *et al.*, 2001) and lipoyltransferase (Fujiwara *et al.*, 1994).

Here, we report the involvement of two additional enzymes in protein lipoylation in yeast mitochondria: Lip3, a lipoate-protein ligase homolog, which is required with Lip2 and Lip5 for lipoylation of Lat1 and Kgd2, and Gcv3, the H protein of the GC enzyme, which is absolutely required for lipoylation of any protein in yeast. Thus, we provide evidence that protein lipoylation in yeast has at least two novel features not previously recognized in bacteria.

MATERIALS AND METHODS

Strains and media

S. cerevisiae strains and their genotypes are listed in Table 4.1. The deletions in the strains of interest in the EUROSCARF collection were verified by PCR using a gene-specific 5' upstream primer and a Kanamycin-resistance gene 3' primer. Cells were grown in various media: YEPD [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose], YEPG [1% (w/v) yeast extract, 2% (w/v) peptone, and 3% (w/v) glycerol], WO -ura [0.67% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, 20 μg ml^{-1} methionine, 100 μg ml^{-1} leucine, and 20 μg ml^{-1} histidine], synthetic complete (SCD) [(Sigma-Aldrich, St. Louis, MO) containing 2% (w/v) glucose], and SCD lacking uracil (SC –ura). After plasmid transformation using the lithium-acetate method (Gietz and Woods 2002), uracil was omitted as a supplement in WO –ura medium to select for Ura^+ transformants. WO –ura glycerol plates contained 3% (w/v) glycerol and 0.01% (w/v) glucose as carbon sources. Solid media contained 2% (w/v) agar.

Disruption of open reading frames

The *LIP3*, *LIP2*, *LIP5*, and *GCV3* genes were deleted in BY4741 to confirm the phenotypes of the EUROSCARF strains. Each open reading frame was deleted by homologous recombination with the kanamycin resistance (*kanMX4*) cassette. *lip3Δ*::*kanMX4*, *lip2Δ*::*kanMX4*, *lip5Δ*::*kanMX*, and *gcv3Δ*::*kanMX4* constructs were generated by amplification of the *KanMX4* cassette using primers specific to the 5'

Table 4.1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
S. cerevisiae		
BY4741	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF
cbp2Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, cbp2*::*kanMX4*	(Schonauer *et al.*, 2008)
lip3Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lip3*::*kanMX4*	This study
lip2Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lip2*::*kanMX4*	EUROSCARF
lip5Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lip5*::*kanMX4*	This study
lat1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lat1*::*kanMX4*	(Schonauer *et al.*, 2008)
kgd2Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, kgd2*::*kanMX4*	(Schonauer *et al.*, 2008)
gcv3Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, gcv3*::*kanMX4*	This study
lpd1Δ	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lpd1*::*kanMX4*	EUROSCARF
Lip3-GFP wild-type	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, LIP3-GFP*::*HIS3MX6*	This study
Lip3-GFP K249L	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, LIP3*(*K249L*)*-GFP*::*HIS3MX6*	This study
Gcv3-GFP wild-type	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GCV3-GFP*::*HIS3MX6*	(Huh *et al.*, 2003)
Gcv3-GFP *Δkgd2*	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, kgd2*::*kanMX4, GCV3-GFP*::*HIS3MX6*	This study
Gcv3-GFP K102L	*MAT***a**, *his3Δ1*, *leu2Δ0, met15Δ0, ura3Δ0, GCV3*(*K102L*)*-GFP*::*HIS3MX6*	This study
Gcv3-GFP K102R	*MAT***a**, *his3Δ1*, *leu2Δ0, met15Δ0, ura3Δ0, GCV3*(*K102R*)*-GFP*::*HIS3MX6*	This study
E. coli		
JRG33-*lip9*	F$^-$, *Δ*(*gpt-pro*A)62, *lac*Y1, *lipA9sup*E44?, *gal*K2, *pur*B15, *his*G4, *rps*L35, *xyl-5*, *mtl*-1, *thi*-1, λ^-	(Herbert and Guest 1968)

upstream and 3' downstream regions of the genes. The PCR products were gel-purified and transformed into the wild-type strain using the lithium acetate method followed by selection for kanamycin resistance.

Construction of plasmids

The YEp352CTA1pr plasmid control was generated by removal of the *fabI* ORF in the previously described pYE352::mtFabI plasmid (Torkko *et al.*, 2001), leaving only the *CTA1* promoter. Plasmid YEp352*mtlplA* was generated by PCR amplification of the *E. coli lplA* ORF from genomic DNA using primers containing *Nco*I and *Xho*I restriction sites to replace the *fabI* ORF in pYE352::*mtfabI*. The final plasmid expresses a chimeric protein consisting of the yeast Coq3 mitochondrial localization sequence and the *lplA*-encoded *E. coli* lipoate-protein ligase from *CTA1* regulatory sequences (Kastaniotis *et al.*, 2004). The Lys133 to Leu mutation in LplA was generated by site-directed mutagenesis (Zheng *et al.*, 2004) in YEp352*mtlplA*, which yielded the YEp352*mtlplAK133L* plasmid. Plasmid Teasy*Lip3-GFP* was generated by ligation of 293 bp of *LIP3* 5' UTR sequence, the *LIP3* ORF without the stop codon, the *GFP-His3MX6* cassette from plasmid pFA6a-GFP(S65T)-His3MX6 (containing the *S. kluyveri HIS3* module) (Longtine MS…Pringle JR 1998 Yeast), and 40 bp of LIP3 3' UTR in the pGEM-T Easy vector (Promega, Madison, WI). The Lys249 to Leu mutation in Lip3-GFP was generated by site-directed mutagenesis in a plasmid containing the *LIP3* ORF, which then replaced the wild type *LIP3* ORF to produce the Teasy*LIP3*(*K249L*)*-GFP* plasmid. To generate the Teasy*GCV3*(*K102L*)*-GFP* and Teasy*GCV3*(*K102R*)*-GFP* plasmids, the *GCV3* ORF was

amplified by PCR and ligated to the pGEM-T Easy vector. The K102L and K102R mutations were made by site-directed mutagenesis. The GFP-containing constructs were excised from the pGEM-T Easy vector, gel-purified, and transformed into the wild-type strain or the Gcv3-GFP stain using the lithium acetate method followed by selection for kanamycin resistance. All PCR products were sequenced to check for PCR-induced mutations.

Isolation of and fractionation of mitochondria

For Western blot analysis, cells were grown to stationary phase in YEPD or WO –ura medium at 30°C. Mitochondrial fractions were isolated and prepared as described (Schonauer *et al.*, 2008). Post-mitochondrial supernatant (PMS) fractions were collected immediately following centrifugation of mitochondria. Laemmli sample buffer was added to the mitochondrial pellets resuspended in TE buffer plus protease inhibitors, and to the PMS fractions for analysis by gel electrophoresis and Western blot. For enzyme activity assays, cells were grown to OD_{600nm} 6.0-6.5 in YEPD at 30°C. Mitochondrial fractions were prepared in the same manner. Mitochondrial pellets were suspended in breaking buffer (0.1 M KPO_4 buffer pH 7.4, 1 mM EDTA, 10 μM thiamine pyrophosphate, 0.2 mM phenylmethanesulfonyl fluoride, 0.5 mg l^{-1} leupeptin, 0.68 mg l^{-1} pepstatin A) and broken by the addition of Triton X-100 to a final concentration of 1%. Mitochondrial membranes and intact mitochondria were removed by centrifugation for 20 minutes at 47,800 × g.

Western blot analysis

Approximately 40 µg of mitochondrial and post-mitochondrial supernatant (PMS) protein was resolved on SDS-polyacrylamide gels following the protocol described by Laemmli (Laemmli 1970). Generally, protein transfer and antibody incubations were performed as described (Schonauer *et al.*, 2008). Except, for visualization of Lip3-GFP and Gcv3-GFP, the blots were reacted with anti-GFP monoclonal antibody (Covance, Berkeley, CA) diluted 1:4000 and proteins were visualized using ECL chemiluminescent substrate (Pierce). Anti-IDH (isocitrate dehydrogenase) antisera was a gift from Lee McAlister-Henn, University of Texas Southwestern Medical Center.

Enzyme assays

Protein concentrations in mitochondrial extracts were estimated by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Bovine albumin (Sigma-Aldrich) was used as a standard. A modification of the method described by Kresze and Ronft (Kresze and Ronft 1981) was used to assay PDH and α-KDH activities. The activities were measured by following the formation of NADH at 340 nm. The reaction mixture contained 0.1 M KPO_4 buffer pH 8.0, 0.05 % Triton X-100, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM DTT, 0.5 mM EDTA, 0.2 mM thiamine pyrophosphate, 5 mM sodium pyruvate or 5 mM α-ketoglutaric acid, 2.5 mM NAD^+ and enzyme in a total volume of 0.99 ml. The reactions were initiated by the addition of 10 µl of 15 mM CoASH. Fumarase activity has been described earlier (Remes *et al.*, 1992). The activities were measured at room temperature and are based on the initial rates.

Light microscopy

Localization of Lip3-GFP was attempted by fluorescence microscopy as previously described (Schonauer *et al.*, 2008).

Lipoic acid analysis

Lipoic acid content of yeast strains was monitored by a biological assay described previously (Brody *et al.*, 1997, Hayden *et al.*, 1993)using the lipoic acid-deficient JRG33-*lip9 E. coli* strain, with minor modifications to the protocol. Yeast strains were grown in 50 ml of SCD media instead of YEPD, and acid hydrolysis was carried out in 0.5 ml 9 N H_2SO_4. JRG33-*lip9* cultures were inoculated to an initial $OD_{600\ nm}$ of 0.015 in 2 ml of 1 × basal growth medium (Herbert and Guest 1970) containing 50 mM sodium succinate and grown for 36 - 48 hours. The growth response of the strain was linear between 0.05 and 0.5 ng ml^{-1} lipoic acid in the cultures. Each result represents the mean ± standard deviation of lipoic acid content from three different yeast cultures.

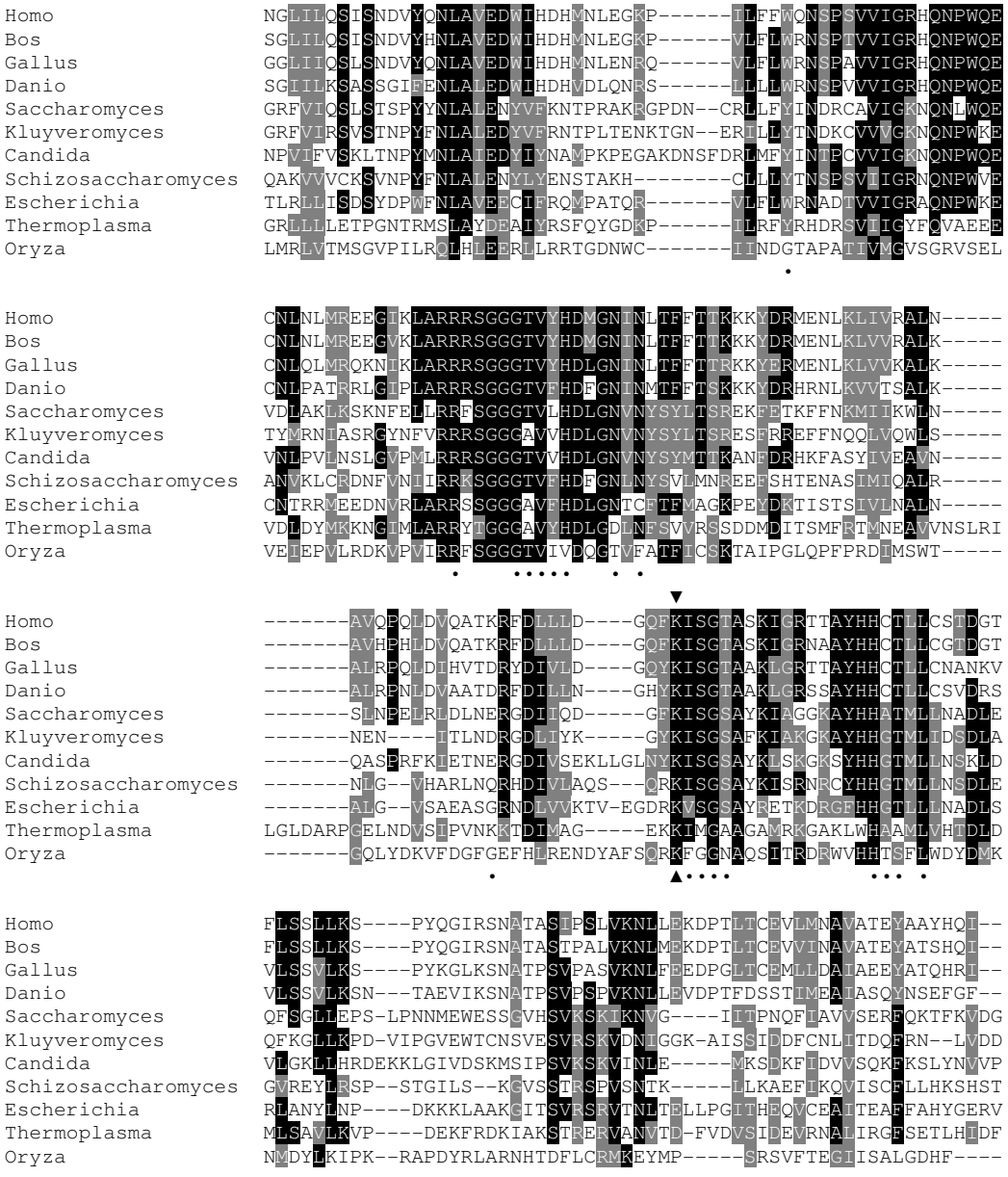
RESULTS

Lip3 is homologous to lipoyltransferases

The EUROSCARF collection of haploid *S. cerevisiae* strains carrying deletions in non-essential nuclear genes was screened in search of novel nuclear factors that affect mitochondrial RNA processing. The *yjl046wΔ* strain was found to be defective in the processing of 5' leader sequences of mitochondrial tRNAs. This phenotype is similar to mutants defective in the fatty acid synthesis pathway, which leads up to the biosynthesis of lipoic acid (Schonauer *et al.*, 2008). Alignment of the predicted amino acid sequence of *YJL046w* using ClustalW2 (Larkin *et al.*, 2007) revealed homology to lipoate-protein ligases in other organisms (Fig. 1). In addition, the MitoProt program for predicting whether proteins are targeted to mitochondria (Claros and Vincens 1996) assigned a 95% probability score for mitochondrial localization of the *YJL046w* gene product. Here we focus on the function of the gene product in mitochondrial protein lipoylation.

Lipoate-protein ligases are a family of proteins that attach the cofactor lipoic acid to target enzymes (Reche 2000). We wondered whether the *YJL046w* gene product functions in lipoic acid attachment in yeast. Four lipoic acid-deficient (*lip*) strains found previously (Tzagoloff and Dieckmann 1990) were crossed to the *yjl046wΔ* strain and the diploids were tested for complementation of respiratory deficiency. The *yjl046wΔ* strain did not complement the *lip3* mutation in the G49 lipoic acid-deficient mutant strain. Sequencing of *YJL046w* in the G49 strain revealed a premature stop codon at position 75.

Figure 4.1. Amino acid sequence alignment of lipoyltransferases. The predicted amino acid sequence of *Saccharomyces cerevisiae* Lip3 starting with residue 115 and ending with residue 330 is aligned with lipoyltransferases from *Homo sapien*, *Bos Taurus*, *Gallus gallus*, *Danio rerio*, *Kluyveromyces lactis*, *Candida albicans*, *Schizosaccharomyces pombe*, *Escherichia coli*, *Theroplasma acidophilum*, *Oryza sativa*. The residues shown are based on the structure of the N-terminal domain of *B. taurus* lipoyltransferase, which contains the lipoyl-AMP binding pocket (Fujiwara *et al.*, 2007). Residues that interact with lipoyl-AMP within 4.0 Å are marked by a dot. The arrowheads denote the highly conserved lysine residue. The sequence alignment was carried out using ClustalW2 (Larkin *et al.*, 2007).


Homo
Bos
Gallus
Danio
Saccharomyces
Kluyveromyces
Candida
Schizosaccharomyces
Escherichia
Thermoplasma
Oryza

We conclude that the *YJL046w* gene product, Lip3, is required for lipoic acid biosynthesis and/or, more likely, attachment.

Lip3 is required for activity of PDH and α-KDH

As a first step in the examination of the role of Lip3 in protein lipoylation, the total lipoic acid content of the *lip3Δ* strain was measured after acid hydrolysis to release the cofactor from mitochondrial enzymes (Brody *et al.*, 1997, Hayden *et al.*, 1993). The mutant strain contained approximately 10-fold less lipoic acid than the wild-type strain (Table 4.2), which suggests that Lip3 is involved in lipoic acid attachment. The respiratory deficient *cbp2Δ* strain, which is defective in *COB* mRNA intron processing (McGraw and Tzagoloff 1983), was also analyzed as a control for possible general effects of respiratory deficiency on lipoylation. The *cbp2Δ* strain contained slightly less lipoic acid than wild type, as reported previously for another respiratory deficient strain (Sulo and Martin 1993).

Since lipoic acid is required for activity of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KDH), the *lip3Δ* strain was assayed for these two lipoic acid-dependent enzyme activities. No PDH or α-KDH activity was detected in the *lip3Δ* strain (Table 4.3), confirming that Lip3 is essential for activity of both multi-enzyme complexes. Fumarase activity was assayed as a representative of all non-lipoic acid-dependent enzymes.

Table 4.2. Lipoic acid content in the *cbp2Δ* and *lip3Δ* strains

Strain	Lipoic acid content ng/g cells
	WO
wt	146.90 ± 28.33
cbp2Δ	126.90 ± 30.62
lip3Δ	10.20 ± 3.11

Lipoic acid assays were performed as described in the Materials and Methods section. The yeast strains were grown in WO medium. The growth response of the JRG33-*lip9* strain was followed by measuring turbidity at A_{600nm}. Each result represents the mean ± standard deviation of lipoic acid content from three different yeast cultures.

Table 4.3. PDH and α-KDH activity in the *lip3Δ* strain

Strain	Specific activity		
	A_{340} min^{-1} mg^{-1}		A_{240} min^{-1} mg^{-1}
	PDH	α-KDH	Fumarase
wt	86 ± 2	95 ± 0	1.44 ± 0.06
lip3Δ	0	0	0.96 ± 0.11

Mitochondrial extracts were prepared as described in the Materials and Methods section. Specific enzyme activities for pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KDH) are expressed as nanomoles of NADH formed at 340 nm min^{-1} mg^{-1} of mitochondrial proteins. Specific enzyme activity for fumarase is expressed as micromoles of fumarate formed at 250 nm min^{-1} mg^{-1} of mitochondrial proteins. Fumarase activity was measured as a control to ensure the mitochondria were functional. The experiment was performed in triplicate and the data shown are the average of the measurements ± standard error.

Four genes are involved in protein lipoylation in yeast

Three proteins in yeast are known to be modified by lipoic acid: Lat1, the E2 subunit of PDH, Kgd2, the E2 subunit of α-KDH and Gcv3, the H protein of the glycine cleavage enzyme (GC). Since all of these multi-enzyme complexes are mitochondrial, we analyzed the lipoylation state of mitochondrial proteins in the *lip3Δ, lip2Δ,* and *lip5Δ* mutants on Western blots challenged with anti-lipoic acid antiserum (Humphries and Szweda 1998). No lipoylated Lat1 or Kdg2 were detected in any of the three mutant strains compared to the wild-type and *cbp2Δ* strains (Fig. 4.2A). However, lipoylated Gcv3 was detected in the *lip3Δ* strain. These data suggest that all three enzymes, Lip3, Lip2 and Lip5, are required for lipoylation of Lat1 and Kgd2, but only Lip2 and Lip5 are required for lipoylation of Gcv3.

To verify the identity of the bands on the Western blot, mitochondrial proteins from the *lat1Δ*, *kgd2Δ*, and *gcv3Δ* strains were analyzed with the anti-lipoic acid antiserum (Fig. 4.2B). As expected, lipoylated Lat1 was the only protein not detected in the *lat1Δ* strain as compared to wild-type. Likewise, lipoylated Kgd2 was the only protein not detected in the *kgd2Δ* strain. Shockingly, however, no lipoylated proteins were recognized by the antiserum in the *gcv3Δ* strain. These results show that Gcv3, a target of lipoylation, is absolutely required for lipoylation of Lat1 and Kgd2. No other lipoylated proteins were detected in the *gcv3Δ* lane even after prolonged overexposure of the Western blot (data not shown).

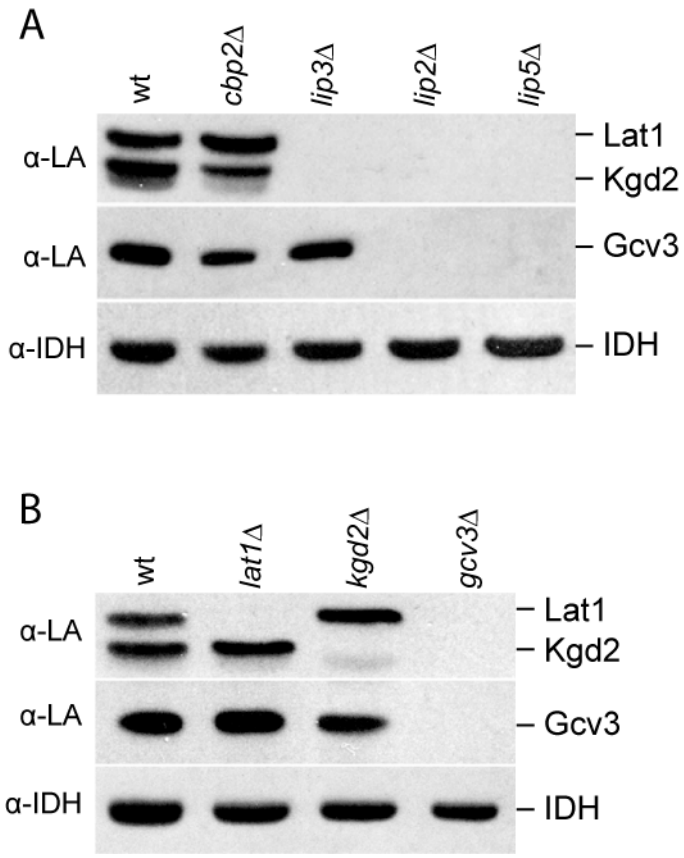


Figure 4.2. Analysis of lipoylated proteins in lipoylation mutant and control strains. Mitochondrial extracts from *A*, wild-type, *cbp2Δ*, a respiratory-deficient control strain, and the lipoic acid synthesis-attachment mutant strains *lip3Δ*, *lip2Δ*, and *lip5Δ*, and *B*, wild-type, *lat1Δ*, *kgd2Δ*, and *gcv3Δ* strains were analyzed by Western blot using an antibody directed against lipoic acid (α-LA). Lat1 and Kgd2 are the lipoylated E2 subunits of PDH and α-KDH, respectively, and Gcv3 is the lipoylated H protein of GC. Mitochondrial isocitrate dehydrogenase (antiserum α-IDH) was used as a loading control.

Localization of Lip3-GFP

A strain was constructed that expresses a *LIP3-GFP* fusion at the *LIP3* genomic locus, and it proved to be respiratory competent. To determine whether the Lip3-GFP fusion protein is localized to mitochondria, mitochondrial and post-mitochondrial supernatant (PMS) fractions were analyzed by Western blot using an anti-GFP antibody (Fig. 4.3A, top panel). A protein of approximately 105kDa, which corresponds to the predicted molecular mass of the Lip3-GFP polypeptide, was detected in the Lip3-GFP strain and not in the wild-type strain (lanes 1-4). Moreover, the polypeptide was only recognized in the mitochondrial fraction, not in the PMS fraction, of the Lip3-GFP strain. A protein of similar mass in the PMS fractions is a background band that is recognized by the antibody regardless of Lip3 fusion to GFP. These data show that the Lip3-GFP protein is expressed and is targeted to mitochondria.

The Lip3-GFP fusion protein was not visible by fluorescence microscopy using a Leica DM-RXA microscope, nor by Huh *et al.* as part of a global GFP tag study on protein localization (Huh *et al.*, 2003). These independent results indicate a low level of Lip3 expression in yeast mitochondria.

The conserved lysine in the lipoate-protein ligase active site is required for lipoylation

To investigate the function of the Lip3-GFP fusion protein, we analyzed the lipoylation state of the target proteins from the mitochondrial and PMS fractions on the

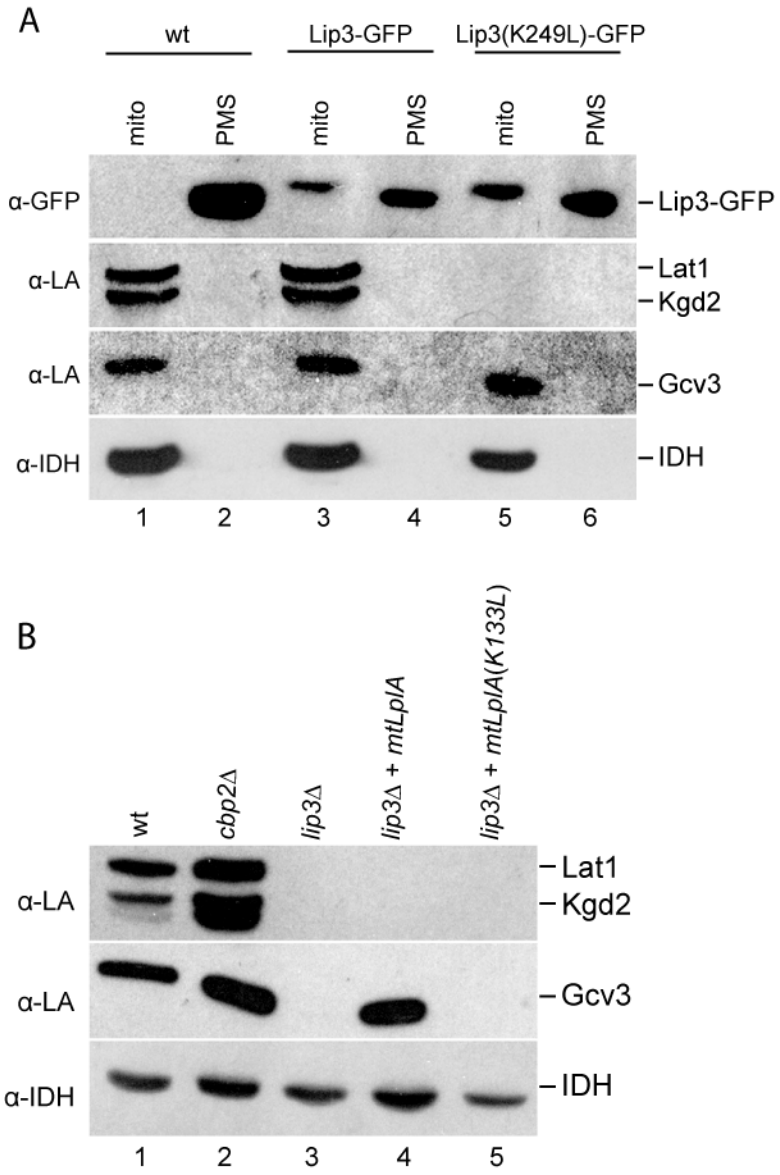


Figure 4.3. Functional analysis of Lip3. (A) Mitochondrial (mito) and post-mitochondrial supernatant (PMS) fractions from wild-type, Lip3-GFP, and Lip3(K249L)-GFP strains were analyzed by Western blot using the α-LA antibody. An anti-GFP antibody (α-GFP) was used to detect Lip3-GFP (top panel). (B) Analysis of mitochondrially localized *E. coli lplA* (*mtlplA*) in the *lip3Δ* strain. Western blot analysis of lipoylated proteins in mitochondrial extracts from wild-type, *cbp2Δ*, and *lip3Δ* forced to maintain the YEp352CTA1pr multicopy plasmid, *lip3Δ* expressing *mtlplA* in YEp352, and *lip3Δ* expressing *mtlplA*(*K133L*) in YEp352 using the α-LA antibody. The α-IDH antiserum was used as a loading control.

same Western blot. All three lipoic acid-modified proteins, Lat1, Kgd2 and Gcv3, were only detected in the mitochondrial fractions of the wild-type and Lip3-GFP strains (Fig. 4.3A, lanes 1 and 3). These results suggest that Lip3-GFP is functional in the lipoic acid synthesis-attachment pathway.

We next tested whether the activity of Lip3 is necessary for its role in protein lipoylation. The sequence alignment shown in Figure 1 revealed that a lysine residue (Lys249) that is strictly conserved among the LplA, LipB, and biotinyl protein ligase (BPL) protein families (Reche 2000), is also conserved in the Lip3 amino acid sequence. This lysine residue is in the lipoyl-AMP binding pocket and hydrogen bonds with the carbonyl oxygen atom of lipoyl-AMP, facilitating transfer of the lipoyl group to the epsilon amino group of the specific lysine acceptor in target apoproteins (Fujiwara *et al.*, 2007, Fujiwara *et al.*, 2005, Kim *et al.*, 2005). We mutated Lys249 to Leu in the Lip3-GFP fusion protein expressed at the *LIP3* locus. Mitochondrial localization and stability of the mutant form of the protein was confirmed by Western blot using the anti-GFP antibody (Fig. 4.3A, top panel, lanes 5-6). The anti-lipoic acid antiserum did not detect any lipoylated Lat1 or Kgd2 protein in the mitochondrial fraction of the strain expressing the Lip3(K249L)-GFP mutant protein (lane 5), as compared to strains expressing wild-type Lip3 (lane 1) or Lip3-GFP (lane 3). However, lipoylated Gcv3 was detected (lane 5). This result suggests that the Lys249 to Leu mutation in Lip3-GFP affects the activity of the enzyme, and that the activity of Lip3-GFP is required for lipoylation of Lat1 and Kgd2, but not for Gcv3. This phenotype is equivalent to that of the *lip3* deletion mutant strain.

***E. coli lplA* partially complements the *lip3Δ* mutation**

In an attempt to verify the function of yeast Lip3 as a lipoyl protein ligase, and since the amino acid sequence of Lip3 shares 27% identity with that of *E. coli* LplA, we examined whether LplA could complement the *lip3Δ* null mutation. *E. coli lplA* was fused to a mitochondrial targeting sequence (*mtlplA*), and expressed on a multicopy plasmid in the *lip3Δ* strain. Western blot analysis of protein lipoylation in mitochondrial extracts of the *lip3Δ* strain harboring the high copy plasmid expressing *mtlplA* rescued lipoylation of Gcv3 but not of Lat1 or Kgd2 (Fig. 4.3B, lane 4), whereas the strain without the *mtlplA* construct contained no lipoylated proteins (Fig. 4.3B, lane 3). All three targets of lipoylation were detected in the wild-type and *cbp2Δ* strains containing the plasmid alone (Fig. 4.3B, lanes 1-2). Additionally it should be noted that overexpression of *mtlplA* does not rescue the respiratory incompetence of the *lip3Δ* strain (the strain does not grow on medium with glycerol as the carbon source).

Since Gcv3 is lipoylated in the *lip3Δ* strain overexpressing *mtlplA*, we examined the lipoic acid content in addition to the Western blot analysis. The *lip3Δ* control strain contained about 10-fold less lipoic acid than the wild-type strain (both strains were also forced to maintain the plasmid with no insert) (Table 4.4). Overproduction of mtLplA increased the amount of lipoic acid in the *lip3Δ* strain by approximately 40 percent. These data suggest that mitochondrially-targeted *E. coli* LplA is functional in the lipoic acid attachment pathway in yeast, but that only one of three targets is recognized as substrate.

Table 4.4. Lipoic acid content in the *lip3Δ* and *lip3Δ + mtlplA* strains

Strain	Lipoic acid content ng/g cells
	WO -ura
wt	123.20 ± 25.06
cbp2Δ	114.50 ± 38.20
lip3Δ	15.60 ± 3.80
lip3Δ + mtlplA	20.00 ± 3.27

Lipoic acid assays were performed as described in the Material and Methods section. The wild-type, *cbp2Δ*, and *lip3Δ* control strains forced to maintain the YEp352CTA1pr multicopy plasmid and the *lip3Δ* strain expressing mitochondrially targeted *E. coli lplA* (*mtlplA*) in YEp352 were grown in WO medium lacking uracil. The growth response of the JRG33-*lip9* strain was followed by measuring turbidity at A_{600nm}. Each result represents the mean ± standard deviation of lipoic acid content from three different yeast cultures.

To test whether the activity of mtLplA is required for Gcv3 lipoylation, the strictly conserved lysine residue within the lipoyl-AMP binding pocket of LplA, Lys133, was changed to leucine (this mutation is analogous to the K249L mutation in Lip3-GFP). Unlike wild type mtLplA, the K133L mutant could not rescue lipoylation of Gcv3 (Fig. 4.3B, lane 5). These data suggest that the activity of mitochondrially targeted *E. coli* LplA is required for lipoylation of Gcv3, but cannot rescue lipoylation of Lat1 or Kdg2 in the *lip3Δ* strain.

Lipoylated Gcv3 is required for lipoylation of Lat1 and Kgd2

Since Gcv3, a target of lipoylation, is also required for lipoylation, (Fig. 4.2B), we explored whether the lipoylated form of Gcv3, in contrast to the apoprotein form, is required for this activity. To test this hypothesis, the specific lysine residue that is covalently modified by lipoic acid within the lipoylation domain of Gcv3, Lys102, was mutated to leucine and to arginine. The K102R mutation was made to preserve the positive charge while rendering the residue inactive as a lipoic acid acceptor. Both mutations were made in a strain that expresses a GFP tagged version of Gcv3 at the GCV3 locus. The strain that expresses wild-type Gcv3-GFP is respiratory competent and has wild-type levels of lipoylated Lat1 and Kgd2 (Fig. 4.4A, lane 2). The molecular weight of the Gcv3-GFP polypeptide is similar to that of the Kgd2 polypeptide, making lipoylated Gcv3-GFP (lane 2, marked with an asterisk) difficult to distinguish from lipoylated Kdg2. Therefore, the *KGD2* gene was deleted from the Gcv3-GFP strain, and lipoylated Gcv3-GFP (lane 3, marked with as asterisk) was visible. Interestingly, the

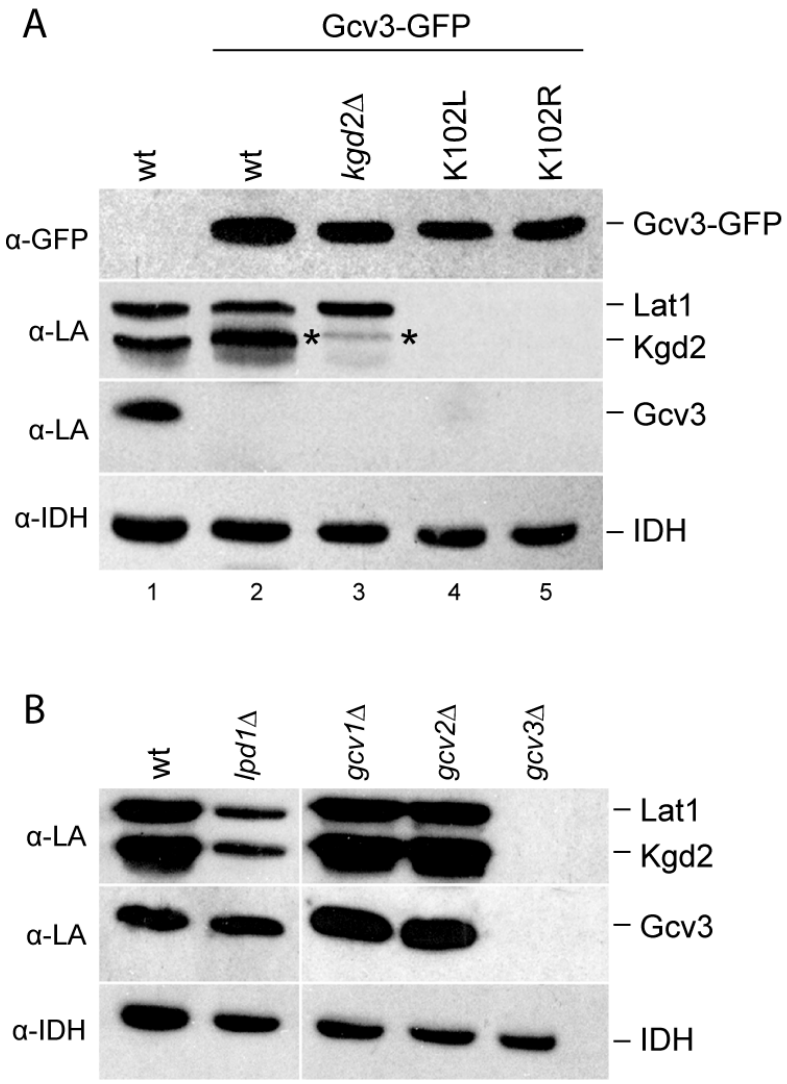


Figure 4.4. Analysis of Gcv3-GFP and GC mutant strains. (A) Mitochondrial extracts from wild-type, Gcv3-GFP, Gcv3-GFP *kgd2Δ*, Gcv3(K102L)-GFP, and Gcv3(K102R)-GFP strains were analyzed by Western blot using the α-LA antibody. The α-GFP antibody was used to detect Gcv3-GFP (top panel). The asterisk in the Gcv3-GFP *kgd2Δ* lane marks lipoylated Gcv3-GFP. The α-IDH antiserum was used to detect IDH as a loading control. (B) Mitochondrial extracts from wild-type, *gcv1Δ*, *gcv2Δ*, *gcv3Δ*, and *lpd1Δ* strains were analyzed by Western blot using the α-LA antibody. The α-IDH antiserum was used to detect IDH as a loading control.

K102L and K102R mutations eliminated lipoylation of Lat1 and Kgd2 (lanes 4-5), suggesting that the lipoylated form of Gcv3 is required for lipoylation of the E2 subunits.

We next wondered whether glycine cleavage (GC) activity *per se* is required for lipoylation of target proteins. Mitochondrial extracts from the *gcv1Δ* and *gcv2Δ* strains, which are deficient in the T and P proteins of the GC enzyme (Piper *et al.*, 2000), respectively, and the *lpd1Δ* strain, which lacks the L protein, were analyzed by Western blot using the anti-lipoic acid antiserum (Fig. 4.4B). The *LPD1* gene encodes dihydrolipoamide dehydrogenase, a subunit common to all three lipoic acid-dependent multienzyme complexes (Reed and Hackert 1990). All three lipoylated proteins were recognized in the *gcv1Δ*, *gcv2Δ*, and *lpd1Δ* strains, as compared to the *gcv3Δ* strain. There was a decrease in the levels of lipoylated proteins in the *lpd1Δ* strain. These data show that GC activity *per se* is not required for protein lipoylation.

DISCUSSION

Here we show that four enzymes are involved in protein lipoylation in yeast mitochondria; one is a target of lipoic acid modification itself. Lip2 and Lip5, the homologs of *E. coli* LipB and LipA, are required for lipoylation of all three lipoic acid-modified proteins: Lat1, Kgd2, and Gcv3. Lip3, the homolog of lipoate protein ligase (LplA) in *E. coli*, is required for lipoylation of Lat1 and Kgd2. The invariant Lys249 residue in the active site of Lip3 is essential for this function. Finally, lipoylated Gcv3 is required for lipoylation of Lat1 and Kgd2. We conclude that lipoic acid synthesis and attachment is substantially different in yeast than in *E. coli*.

Lipoic acid attachment is best characterized in *E. coli*. Attachment of lipoic acid to apoproteins is catalyzed by two different enzymes, LipB and LplA. LipB transfers the lipoyl (or octanoyl) group endogenously synthesized on acyl carrier protein (ACP) onto the acceptor lysine residue of target apoproteins (Nesbitt *et al.*, 2005, Jordan and Cronan, Jr. 2003). LplA catalyzes the attachment of exogenous free lipoic acid in a two-step reaction. ATP and lipoic acid are joined in formation of a lipoyl-AMP intermediate, and then the lipoyl group is transferred to the acceptor lipoyl domain of the targets. The crystal structure of *E. coli* LplA, with and without lipoic acid bound, has been solved (Fujiwara *et al.*, 2005). The overall structure of *E. coli* LplA includes an N-terminal domain and a C-terminal domain with a substrate-binding pocket in the N-terminal domain. Lipoic acid binds weakly to the hydrophobic core of the pocket. The weak binding facilitates transfer of the lipoyl group to target proteins.

The crystal structure of LplA in the archaeon *Thermoplasma acidophilum* (Ta LplA) also has been solved (Kim *et al.*, 2005). In addition to the apoprotein form, the structures of LplA complexed with ATP and with lipoyl-AMP were also determined. Similar to *E. coli* LplA, Ta LplA catalyzes both the activation and transfer of lipoic acid, however it comprises only one domain, which is homologous to the N-terminal domain of *E. coli* LplA. Lipoyl-AMP adopts a U-shaped structure within the bifurcated binding pocket. The lipoyl group of the intermediate is buried within a hydrophobic channel and the AMP moiety forms hydrogen bonds with residues in the other tunnel of the two-lobed pocket. The bent arrangement orients the carbon atom subject to nucleophilic attack at the surface of Ta LplA. The carbonyl oxygen atom of the lipoyl group interacts with Lys145 (the invariant lysine residue analogous to Lys133 in *E. coli* and Lys249 in *S. cerevisiae*), in the binding pocket. This interaction likely aids nucleophilic attack of the exposed carbon atom of lipoyl-AMP by the ε-amino group of the acceptor lysine, resulting in an amide linkage.

In eukaryotes, two separate enzymes are required for catalysis of the two reactions carried out by lipoate-protein ligases in prokaryotes. Lipoate-activating enzyme, which is a medium-chain acyl-CoA synthetase, activates lipoic acid with GTP (Fujiwara *et al.*, 2001). Lipoyltransferase catalyses the transfer of the lipoyl group to the acceptor lysine residue of target proteins (Fujiwara 1994 or 1997?). Similar to *E. coli* LplA, the crystal structure of bovine lipoyltransferase (bLT) reveals that bLT consists of both an N-terminal domain and a C-terminal domain (Fujiwara *et al.*, 2007). Interestingly, the recombinant protein purified from *E. coli* contained endogenous lipoyl-AMP in the N-

terminal domain, indicating a high affinity of bLT for lipoyl-AMP. The invariant lysine residue in bLT (Lys135) forms similar bonds with lipoyl-AMP as does Lys145 of Ta LplA and, most likely, Lys133 of *E. coli* LplA. The gene encoding the human lipoyltransferase has been isolated (Fujiwara *et al.*, 1999) and amino acid sequence alignment reveals regions of significant homology with other LplA-like enzymes, including the invariant lysine residue (Fig. 4.1). It seems likely that the mammalian lipoyltransferase transfers lipoic acid obtained from the diet or from intestinal bacteria to target proteins, but recent reports of a mitochondrial ACP (Cronan *et al.*, 2005a) and of lipoic acid synthase (Yi and Maeda 2005, Morikawa *et al.*, 2001) indicate the existence of a lipoic acid biosynthetic pathway, which may be important for development or for certain tissues.

Studies on lipoic acid metabolism in yeast have been minimal. Lip2, Lip5, Lip3, and Gcv3 were all first identified in studies on other cellular processes (Marvin *et al.*, 2001, Nagarajan and Storms 1997, Sulo and Martin 1993). Here we have shown that they all function in the synthesis and/or attachment of lipoic acid. We have built a model that posits that Lip2, Lip5, Lip3, Gcv3, and possibly Lpd1 function in a lipoylation complex (Fig. 4.5). In independent support of this model, Lip2 was co-purified with Gcv3 in a global tandem affinity purification (TAP) tag study for protein interactions (Huh *et al.*, 2003). Our hypothesis is that some Gcv3 functions in the glycine cleavage enzyme and some participates in the lipoylation complex. Similarly, since deletion of *LPD1*, which codes for the dihydrolipoamide dehydrogenase subunit common to all three lipoic acid-dependent enzyme complexes, causes a decrease in protein lipoylation (Fig. 4.4B), some

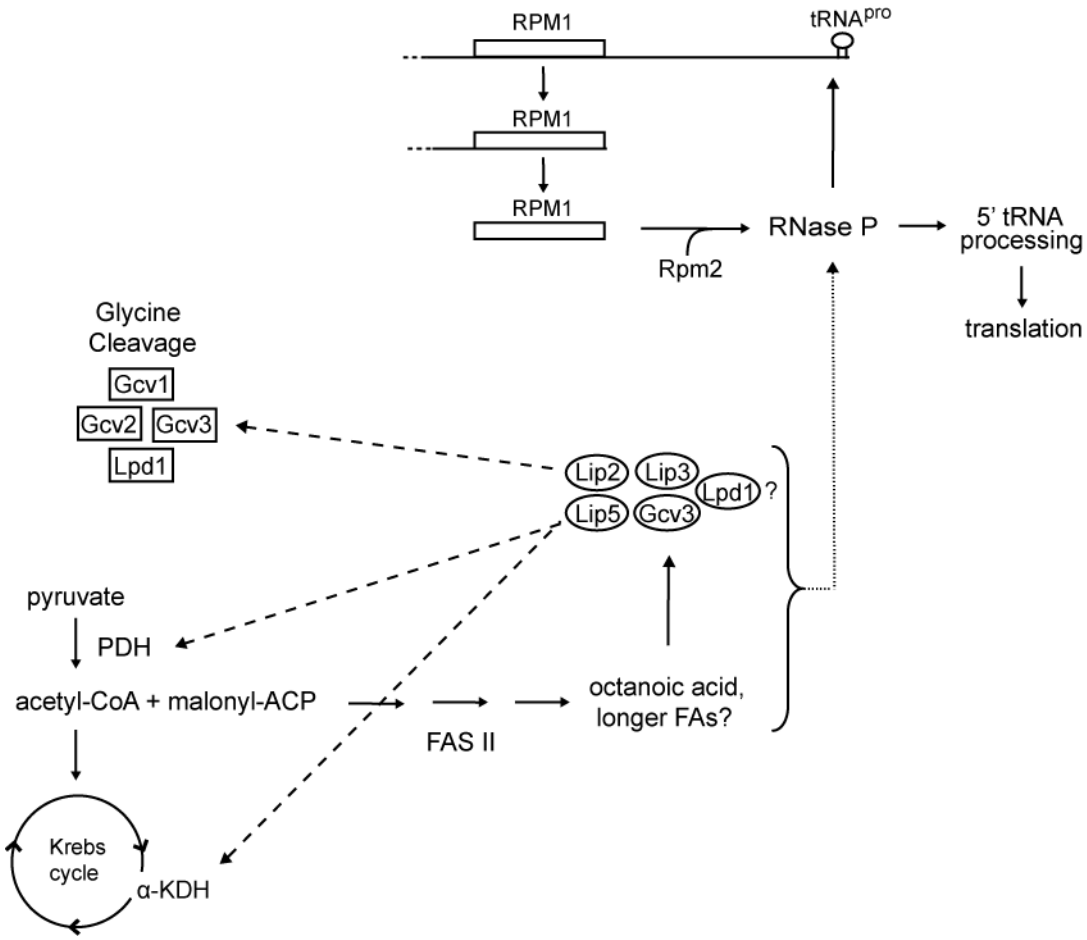


Figure 4.5. Model of protein lipoylation in yeast. The FAS II pathway produces octanoic acid, the precursor for lipoic acid synthesis. Lip2, Lip3, Lip5, Gcv3, and Lpd1 may function in a lipoylation complex. We previously reported that the mitochondrial type II fatty acid synthesis pathway (FAS II) and tRNA processing intersect (Schonauer *et al.*, 2008), and we hypothesize that this intersection exists as a point of regulation for mitochondrial gene expression. A product of FAS II is required for assembly or activity of mitochondrial RNase P, which processes the 5' leader sequences of tRNAs. Because RNase P activity is required for the maturation of its own mitochondrially encoded RNA subunit, a positive feedback loop exists for RNase P maturation and activity in response to the FAS II pathway. Pyruvate dehydrogenase, which requires lipoic acid for its function, produces acetyl-CoA, which is fed into the FAS II pathway. The dashed arrows represent the requirement of lipoic acid for PDH, α-KDH and GC enzymatic activity. The dotted arrow represents the requirement of a product of the FAS II pathway for RNase P activity or assembly.

Lpd1 may also function in the lipoylation complex. Alternatively, the enzymes may function separately as part of one lipoylation pathway in which Lip2 and Lip3 are both required for the transfer of lipoic acid, and lipoylation of Gcv3 occurs prior to the lipoylation of Lat1 and Kgd2.

How is Gcv3, a target of lipoylation, required for lipoylation? Since lipoylated Gcv3 is required for lipoylation of Lat1 and Kgd2, Gcv3 may act as a sensor for the metabolic state of the cell. When acetyl-CoA is abundant, our lipoylation complex model proposes that octanoic acid produced by the FAS II pathway is available for lipoylation of Gcv3 by Lip2 and Lip5. Lipoylated Gcv3 in turn may be required either for assembly of the active lipoylation complex or for conformational change of one or more enzymes in the complex. The assembled, active complex then lipoylates Lat1 and Kgd2, leading to cellular respiration. Under conditions of low availability of acetyl-CoA, low levels of lipoylated Gcv3 may act as a switch to turn off PDH and α-KDH activities by blocking lipoylation of Lat1 and Kgd2. Because PDH requires lipoic acid for the production of acetyl-CoA that is fed into the FAS II pathway, lower levels of octanoic acid and, therefore, lipoic acid are produced. However, Kgd2 and Gcv3 are lipoylated in the *lat1Δ* strain, indicating that, when the cells are grown in rich glucose medium, acetyl-CoA from another source, such as amino acid breakdown, β-oxidation of fatty acids, or the pyruvate dehydrogenase bypass pathway, can be fed into the FAS II pathway. Inactive PDH and α-KDH lead to a decrease in or loss of cellular respiration, and mitochondrial gene expression may also be affected.

Lipoylation of Gcv3 is also affected by the growth medium. Lip2 and Lip5 lipoylate Gcv3 in the *lip3Δ* strain when it is grown in rich YEPD medium (Fig. 4.2A), which contains fatty acids and glycine. Glycine induces expression of the glycine cleavage enzymes (Gelling *et al.*, 2004). However, Lip2 and Lip5 are unable to lipoylate Gcv3 when the strain is grown in minimal medium lacking any fatty acids or amino acids. Since Gcv3 is lipoylated in the wild-type strain grown on minimal medium (Fig. 4.3B), lack of lipoylated Gcv3 in the *lip3Δ* strain is likely not due to the absence of Gcv3 expression. The FAS II pathway is presumably intact in the *lip3Δ* strain, thus octanoic acid should be available for lipoylation of Gcv3 by Lip2 and Lip5.

Interestingly, overproduction of mitochondrially targeted *E. coli* LplA (mtLplA) in the *lip3Δ* strain rescues lipoylation of Gcv3 (Fig. 4.3B). It is not known whether *E. coli* mtLplA directly lipoylates Gcv3 with lipoic acid that is transferred from ACP, if mtLplA stimulates the octanoyl- or lipoyltransferase activity of Lip2 or if mtLplA binds lipoyl-AMP made by another enzyme. Despite lipoylation of Gcv3 in this strain, neither Lat1 nor Kgd2 are lipoylated. This result indicates that Lip3 is required either for lipoylation of Lat1 and Kgd2 directly, or for the complete function of the proposed lipoylation complex.

Together, our results suggest that proteins are lipoylated in yeast mitochondria via one pathway or lipoylation complex, unlike *E. coli*, which has two redundant lipoylation pathways. Future investigations will reveal whether protein lipoylation in mammalian mitochondria is similar to that in yeast. Of particular interest will be discovering whether the eukaryotic pathway/complex is regulated in response to the availability of acetyl-

CoA, and then in turn serves to regulate mitochondrial gene expression. One piece of evidence reported by Autio *et al*. (Autio *et al.*, 2008) suggests that mitochondrial gene expression may be regulated by the availability of acetyl-CoA in human cells. A bicistronic cDNA was found to encode an FAS II enzyme and an RNase P protein subunit. This gene arrangement has been conserved throughout vertebrate evolution from fish to human. The link between mitochondrial fatty acid synthesis and RNA processing in yeast (Schonauer *et al.*, 2008) and human (Autio *et al.*, 2008) along with the finding that protein lipoylation in yeast occurs via one pathway/complex suggest that eukaryotic mitochondrial function is highly regulated in response to cellular metabolism.

CHAPTER 5. FURTHER INVESTIGATIONS ON RNA PROCESSING

SUMMARY

Processing of 5' mitochondrial tRNA precursors by RNase P is inefficient in strains harboring defective in the FAS II pathway (Chapter 3) or in the synthesis and attachment of lipoic acid (this chapter). However, the RNA phenotype is not the same when both sets of mutants are compared. FAS II mutant strains strongly accumulate pre-RPM1-pro precursor RNA when grown at 30°C, while less precursor is accumulated in strains defective in lipoic acid synthesis and attachment (*lip* mutants), including the *gcv3Δ* strain (this chapter). Growth of the *lip* mutant strains at higher temperature (34°C) results in greater accumulation of the precursor, indicating a temperature-dependent effect on the maturation, assembly and/or activity of mitochondrial RNase P in these mutants. This chapter illustrates the analysis of RNase P-mediated pre-RPM1-pro processing in the *lip* mutants described in Chapter 4.

INTRODUCTION

Two mutant strains, *htd2Δ* and *lip3Δ*, were found in the genomic screen described in Chapter 2 to be defective in 5' processing of mitochondrial precursor tRNAs by mitochondrial RNase P. Subsequent experiments revealed: (*i*) that a product of the type II mitochondrial fatty acid synthesis pathway (FAS II), not Htd2 *per se*, is required for efficient processing of tRNAs (Chapter 3) and (*ii*) that Lip3 is involved in the synthesis and attachment of lipoic acid (Chapter 4). RNA processing was not analyzed in strains defective in lipoic acid synthesis attachment (*lip* mutants), as the experiments described in that chapter were directed towards analysis of protein lipoylation. Thus, investigation of RNA processing in the *lip* mutant strains is presented here.

The tRNA processing defect is severe in FAS II mutant strains grown at 30°C. The pre-RPM1-pro precursor RNA that accumulates is about 90% of the RPM1-containing RNA in these strains. However, $tRNA^{pro}$ processing is not as defective in *lip* mutant strains. As seen in Figure 2.2, the *lip3Δ* strain does not accumulate as much of the high molecular weight precursor as does the *htd2Δ* strain. Similarly, the RNA processing phenotype is not as severe in the *lip5Δ* strain, and it is almost wild type in the *lip2Δ* strain (this chapter). In an attempt to understand the difference in $tRNA^{pro}$ processing between FAS II and *lip* mutant strains, the cells were grown at higher temperatures and RNA was analyzed by Northern blot. The results show that the processing defect in the *lip* mutant strains, including *gcv3Δ*, is more severe after growth at 34°C. A similar phenotype is observed after the introduction of mutations that affect the activity of the Lip3-GFP and Gcv3-GFP proteins. Together, the data indicate an affect of temperature on RNase P

maturation, assembly and/or activity when lipoic acid synthesis and attachment are disrupted.

MATERIAL AND METHODS

Strains and media

S. cerevisiae strains and their genotypes are listed in Tables 3.1 and 4.1. The genotypes of the double deletion strains are *lipΔ2lip3Δ* (*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, lip3*::*URA3, lip2*::*kanMX4*), *lip3Δlip5Δ* (*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, lip3*::*URA3, lip5*::*kanMX4*), and *lip2Δlip5Δ* (*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, lip5*::*URA3, lip2*::*kanMX4*). Cells were grown in various media: YEPD [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose], YEPG [1% (w/v) yeast extract, 2% (w/v) peptone, and 3% (w/v) glycerol], and WO -ura [0.67% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, 20 μg ml^{-1} methionine, 100 μg ml^{-1} leucine, and 20 μg ml^{-1} histidine]. After plasmid transformation using the lithium-acetate method (Gietz and Woods 2002), uracil was omitted as a supplement in WO –ura medium to select for URA^{+} transformants.

Isolation of whole-cell RNA

Total RNA was isolated from midlogarithmic cultures after growth in liquid YEPD medium at 30°C or 34°C as described (Caponigro *et al.*, 1993).

Northern blot analysis

10 μg of total RNA was separated on a 1.2% (w/v) agarose gel in TB buffer (83 mM Tris base, 89 mM boric acid, pH 8.3). The RNAs were transferred onto a Nytran membrane (Schleicher & Schuell) overnight in 20× SSC (3 M sodium chloride, 0.3 M

sodium citrate, pH 7.0) and hybridized (6× SSC, 10× Denhardt's solution, 0.1% SDS, 50 μg/ml carrier DNA) overnight at 5-10°C below the T_m of the oligonucleotide probes. All blots were stripped and hybridized with the SCR1 loading control probe. RPM1 and SCR1 oligonucleotide probes (listed in Table 3.2) were ^{32}P end-labeled using T4 DNA kinase (Fermentas). Blots were analyzed on a PhosphorImager (Typhoon 9410, GE Healthcare).

RESULTS

pre-RPM1-pro precursor RNA accumulates in *lip* mutant strains grown at 34°C

Upon discovering that deletion of *YJL046w*, or *LIP3*, affects processing of the pre-RPM1-pro precursor RNA, we hypothesized that disruption of the other enzymes involved in the synthesis and attachment of lipoic acid would result in the same phenotype. We therefore tested the RNA processing phenotype in strains containing deletions of *LIP2* and *LIP5*, the products of which are homologous to *E. coli* LipB and LipA. A Northern blot of total RNA isolated from the wild-type, *lip3Δ*, *lip2Δ*, and *lip5Δ* strains grown at 30°C was hybridized with the probe specific for RPM1 (Fig. 5.1A, left panel). RNA from *cbp2Δ*, which is defective in *COB* mRNA processing (McGraw and Tzagoloff 1983), was also analyzed as a control for a general effect of respiratory deficiency on RNA processing. The *lip2* mutation had minimal effect on pre-RPM1-pro processing, while accumulation of the pre-RPM1-pro precursor was greater in the *lip3Δ* and *lip5Δ* strains as compared to the wild-type and *cbp2Δ* strains. Surprisingly, the RNA processing defect in the *lip* mutant strains was less dramatic than that in the FAS II mutant strains (see Chapter 3). However, the results suggest that synthesis and attachment of lipoic acid does affect tRNA processing by mitochondrial RNase P.

Since less pre-RPM1-pro precursor RNA accumulates in the *lip* mutant strains than in the FAS II mutant strains, we asked whether temperature affects the severity of the processing defect in the *lip* mutant strains. To answer this question, RNA processing was analyzed in the *lip* mutant strains grown at 34°C (Fig. 5.1A, right panel). There was a greater accumulation of the pre-RPM1-pro precursor when the strains were grown at

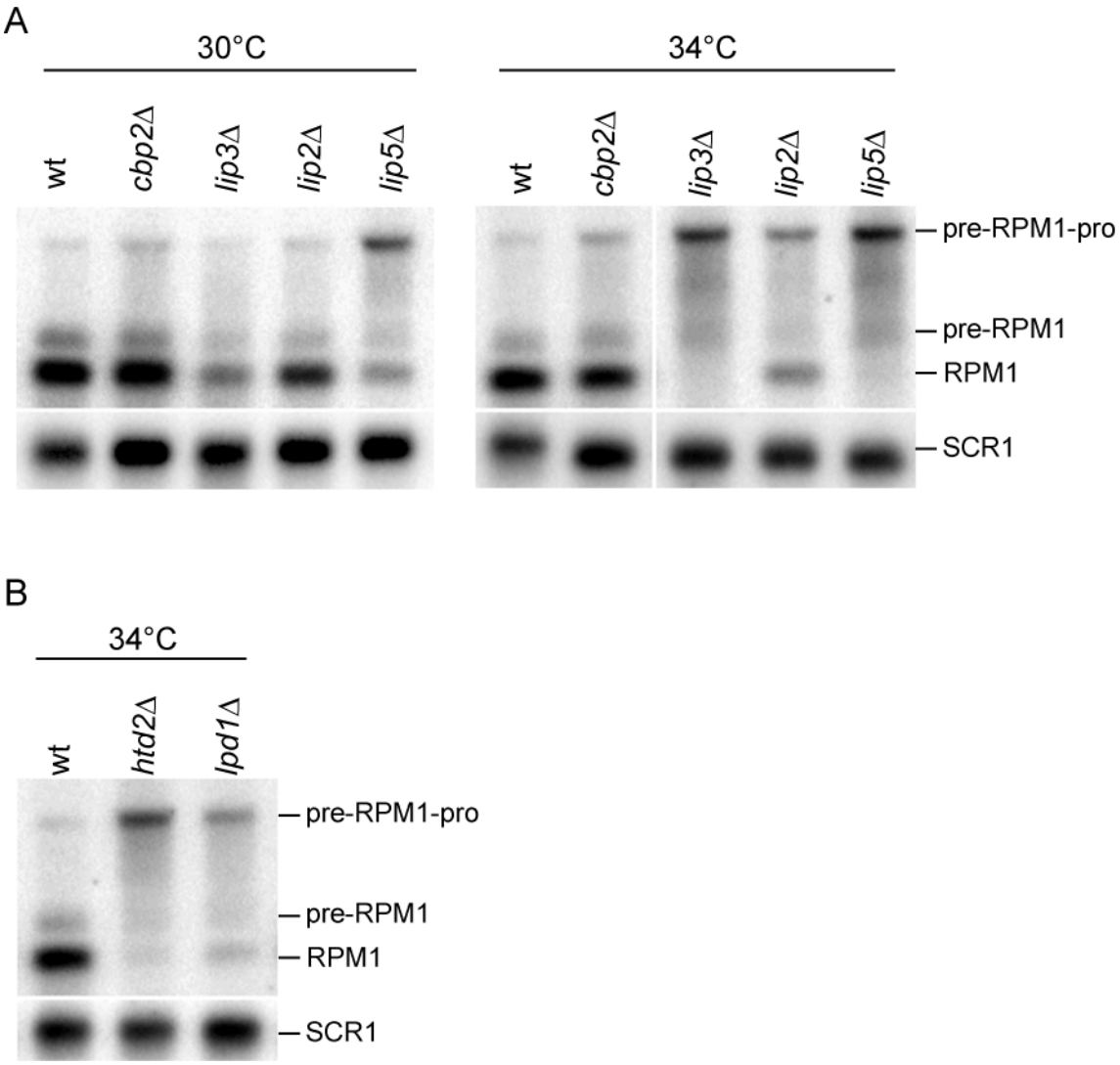


Figure 5.1. Northern blot analysis of pre-RPM1-pro processing in *lip* deletion strains grown at 30°C and 34°C. (A) A Northern blot of total RNA extracted from wild-type, *cbp2Δ*, and *lip* mutant strains (*lip3Δ*, *lip2Δ* and *lip5Δ*) grown at 30°C (left panel) and 34°C (right panel) was hybridized with the RPM1 probe. (B) A Northern blot of total RNA extracted from the *htd2Δ* and *lpd1Δ* strains grown at 34°C was probed with the RPM1 probe. SCR1 was used as a loading control.

higher temperature. RNA processing was also analyzed in the *htd2Δ* strain after growth at 34°C (Figure 5.1B). There was no observable change in RNA processing in this strain, as the defect is severe even after growth at lower temperature (Chapter 3). We also analyzed RNA processing in the *lpd1Δ* strain (*LPD1* encodes dihydrolipoamide dehydrogenase, a subunit common to all lipoic acid-dependent multi-enzyme complexes) and found that higher temperature did have an effect on RNA processing in this strain. These results show that the severity of the pre-RPM1-pro processing defect is *t*emperature-*s*ensitive (*ts*) in the *lip* and *lpd1Δ* mutant strains. The effect of temperature on RNA processing in these strains contrasts the inefficient processing phenotype found in the FAS II mutant strains grown at 30°C.

We next wondered whether deletion mutations in two *lip* genes cause a more severe RNA processing defect than that in strains with single mutations. To test this idea, the three possible combinations of *lip* double deletions were made: *lip2Δlip3Δ*, *lip3Δlip5Δ* and *lip2Δlip5Δ*. A Northern blot containing RNA from these double deletion mutant strains grown at 30°C and 34°C was hybridized with the RPM1 probe (Fig. 5.2). The pattern of epistasis is complex. *lip3Δ* is epistatic to *lip2Δ* at 30°C and 34°C in the double mutant. At both temperatures, the phenotype of *lip3Δ* is more severe than that of *lip2Δ*. *lip5Δ* is epistatic to *lip3Δ* at 30°C, but the phenotype of *lip3Δ* and *lip5Δ* is the same at 34°C (very severe), so epistatis cannot be assigned. The most unusual result, and one for which we have no explanation, is the attenuation of the severity of the phenotype of *lip5Δ* by combination with *lip2Δ*. At 30°C, the *lip2Δlip5Δ* double mutant has a phenotype midway between the

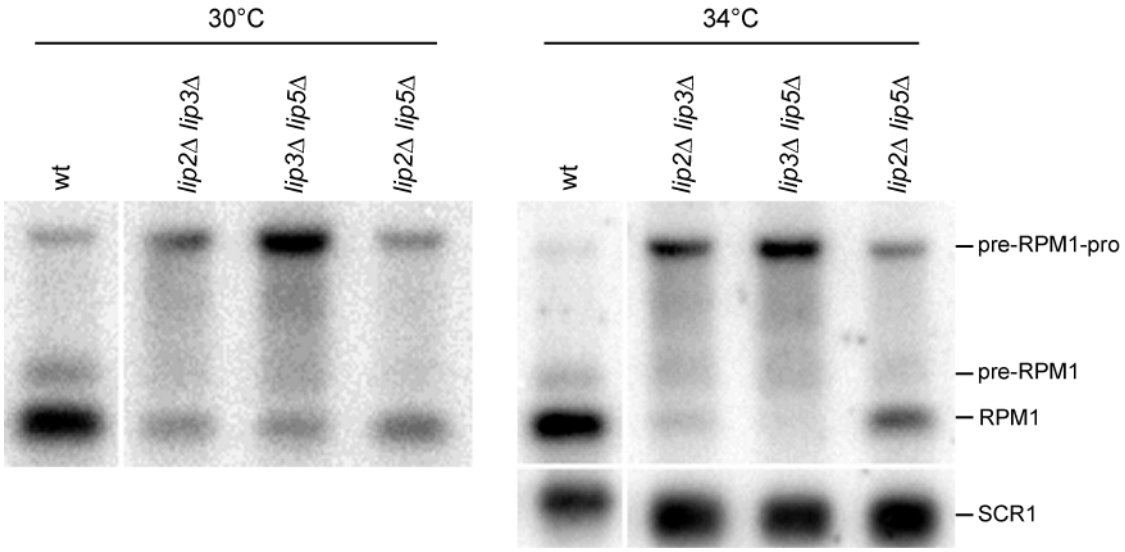


Figure 5.2. Northern blot analysis of pre-RPM1-pro processing in *lip* double deletion strains grown at 30°C and 34°C. A Northern blot of total RNA extracted from the wild-type, *lip2Δlip3Δ*, *lip3Δlip5Δ* and *lip2Δlip5Δ* strains grown at 30°C and 34°C was hybridized with the RPM1 probe. SCR1 was used as a loading control.

severity of each single mutant strain. At 34°C, the phenotype of the double is equivalent to *lip2Δ*.

Activity of Lip3-GFP is required for pre-RPM1-pro processing

In an effort to characterize Lip3, a strain that expresses a GFP tagged version of the protein was generated (Chapter 4). Western blot analysis using the α-GFP antibody confirmed expression and localization of Lip3-GFP to the mitochondrial compartment (see Chapter 4). The α-lipoic acid antibody detected all three lipoylation targets, verifying that the Lip3-GFP fusion protein is functional in the lipoylation pathway. RNA processing is also wild type in the Lip3-GFP strain (Fig. 5.3). Mutation of the invariant lysine residue (Lys249) within the proposed active site of Lip3 eliminated lipoylation. Given that the activity of Lip3-GFP is required for its role in lipoylation, we tested the hypothesis that its activity is also required for RNA processing. Northern blots containing RNA from the Lip3-GFP and Lip3(K249L)-GFP strains grown at 30°C and 34°C was hybridized with the RPM1 probe (Fig. 5.3). The blots revealed that the mutant strain is defective in pre-RPM1-pro processing at both temperatures, and that higher temperature has a greater effect on processing. These data show that the activity of Lip3-GFP is critical for efficient processing of the pre-RPM1-pro precursor RNA by RNase P.

***E. coli lplA* rescues the RNA processing defect in the *lip3Δ* strain**

Given that overproduction of *E. coli* LplA, the homolog of Lip3, is able to rescue lipoylation of Gcv3 in the *lip3Δ* strain (Fig. 4.3B), its ability to rescue the RNA

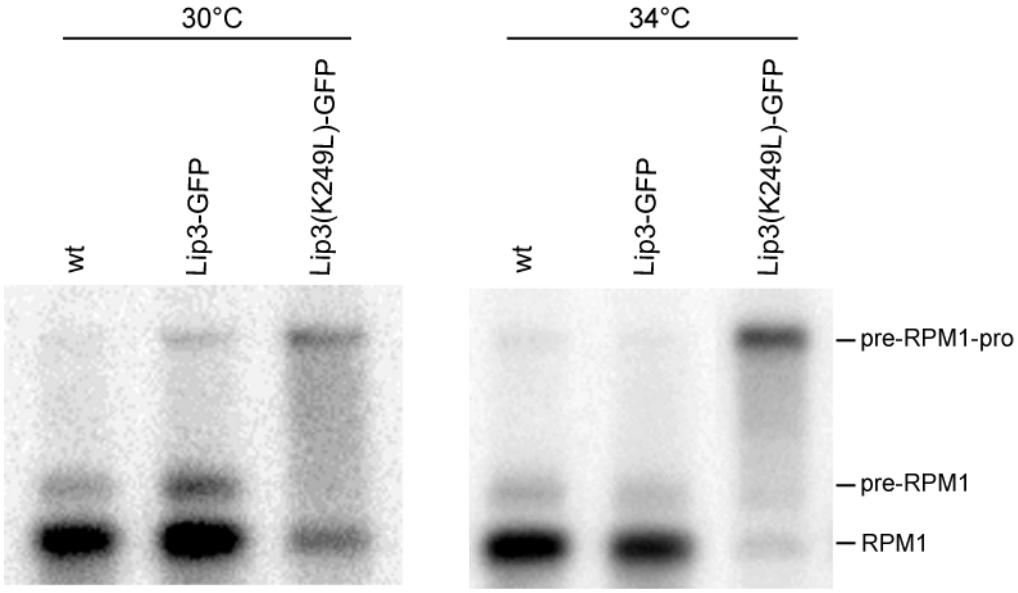


Figure 5.3 Northern blot analysis of pre-RPM1-pro processing in Lip-GFP strains grown at 30°C and 34°C. A Northern blot of total RNA extracted from the wild-type, Lip3-GFP and Lip3(K249L)-GFP strains grown at 30°C and 34°C was hybridized with the RPM1 probe.

processing defect was also tested. Analysis of RNA processing in the *lip3Δ* strain forced to maintain the YEp352 multicopy plasmid by growth at 30°C and 34°C on minimal medium revealed inefficient pre-RPM1-pro processing as compared to the wild-type strain forced to maintain the plasmid (Fig. 5.4A and B). Surprisingly, overproduction of mitochondrially targeted LplA completely rescued RNA processing in the *lip3Δ* strain grown at both temperatures, though the rescue was not as robust at 34°C as at 30°C. These results suggests that overexpressed *E. coli* LplA is able to lipoylate some protein that affects RNase P function, or it is able to lipoylate RNase P itself.

To test whether the activity of mtLplA is required for pre-RPM1-pro processing, the strictly conserved lysine residue within the lipoyl-AMP binding pocket (Lys133) of LplA was changed to a leucine (this mutation is analogous to the Lys249 to Leu mutation in Lip3-GFP). Unlike wild type mtLplA, the K133L mutant could not rescue RNA processing in the *lip3Δ* strain after growth at 34°C (Fig. 5.4B) (growth at 30°C is not shown). These results suggest that the activity of mitochondrially targeted *E. coli* LplA is required for lipoylation of Gcv3, but cannot rescue lipoylation of Lat1 or Kdg2 in the *lip3Δ* strain.

pre-RPM1-pro processing is defective in the *gcv3Δ* strain grown at 34°C

Given that higher temperature increases the severity of the RNA processing defect in the *lip* mutant strains, we wondered whether higher temperature also affected RNA processing in the *gcv3Δ* strain, since Gcv3 is also involved in the synthesis and

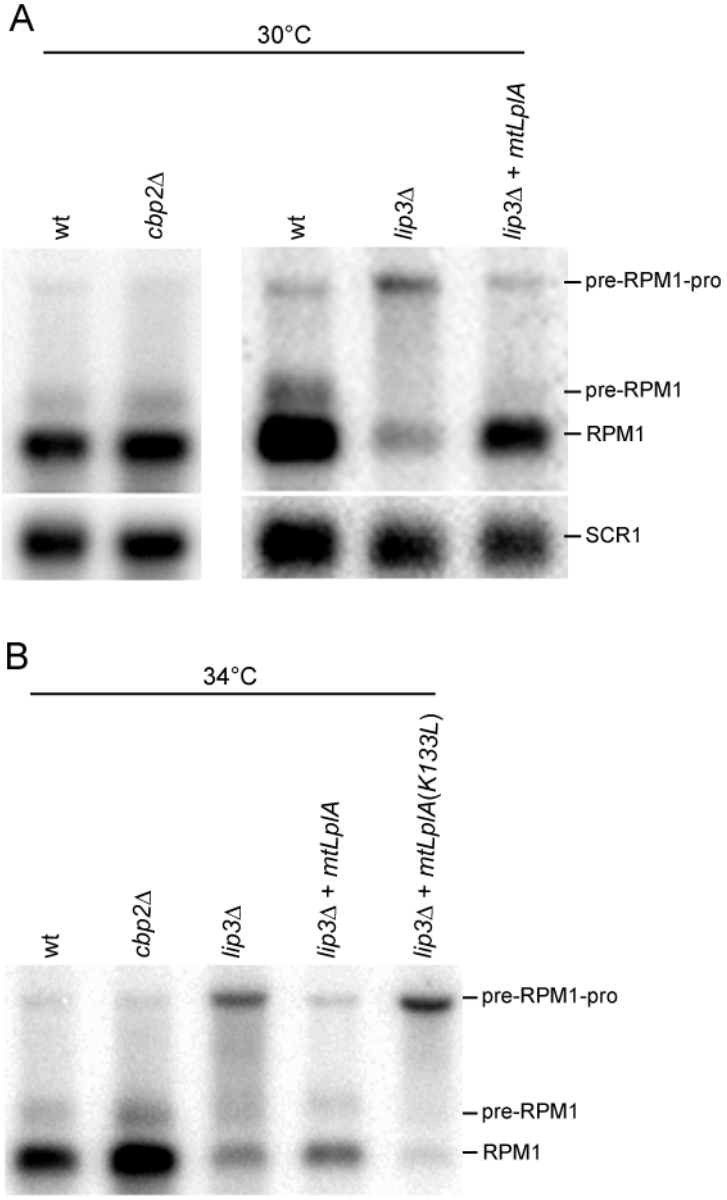


Figure 5.4. Northern blot analysis of pre-RPM1-pro processing in the *lip3Δ* strain overexpressing *E. coli mtlplA*. (A) Northern blots of total RNA extracted from wild-type, *cbp2Δ*, and *lip3Δ* forced to maintain the YEp352CTA1pr multicopy plasmid and *lip3Δ* expressing *mtlplA* in YEp352 grown at 30°C was hybridized with the RPM1 probe. (B) A Northern blot of the same strains plus *lip3Δ* expressing *mtlplA*(*K133L*) in YEp352 grown at 30°C was hybridized with the RPM1 probe. SCR1 was used as a loading control.

attachment of lipoic acid. RNA extracted from the *gcv3Δ* strain grown at 30°C and 34°C was analyzed by Northern blot (Fig. 5.5A). Growth at the higher temperature resulted in strong accumulation of the pre-RPM1-pro precursor. RNA extracted from the *lat1Δ* and *kgd2Δ* strains was also analyzed at both temperatures. In these strains, processing is only slightly affected as compared to the *gcv3Δ* strain.

We next wondered whether glycine cleavage (GC) activity is required for processing of the pre-RPM1-pro precursor. To test this, RNA extracted from the *gcv1Δ* and *gcv2Δ* strains was analyzed by Northern blot. Figure 5.5B shows that RNA processing is wild type in these mutant strains. Thus, Gcv3 itself, not GC activity, is required for RNA processing.

Lipoylated Gcv3 is required for pre-RPM1-pro processing

Given that lipoylated Gcv3 is required for lipoylation of Lat1 and Kgd2 (Fig. 4.4), we wondered whether lipoylated Gcv3 is also required for pre-RPM1-pro processing. To address this question, we utilized the strains expressing wild type and mutated forms of GFP-tagged Gcv3. RNA extracted from the Gcv3-GFP wild-type, Gcv3(K102L)-GFP and Gcv3(K102R)-GFP strains was hybridized with the RPM1 probe (Lys102 is the acceptor lysine residue in the lipoylation domain). Figure 5.6 demonstrates that the Gcv3-GFP strain has wild type RNA processing when grown at 30°C and 34°C. However, mutation of the Lys102 acceptor residue, which causes elimination of lipoylation of all proteins, results in defective pre-RPM1-pro processing when the strains are grown at 34°C. Thus, the effect of temperature on RNA processing in the Lys102 mutants is the

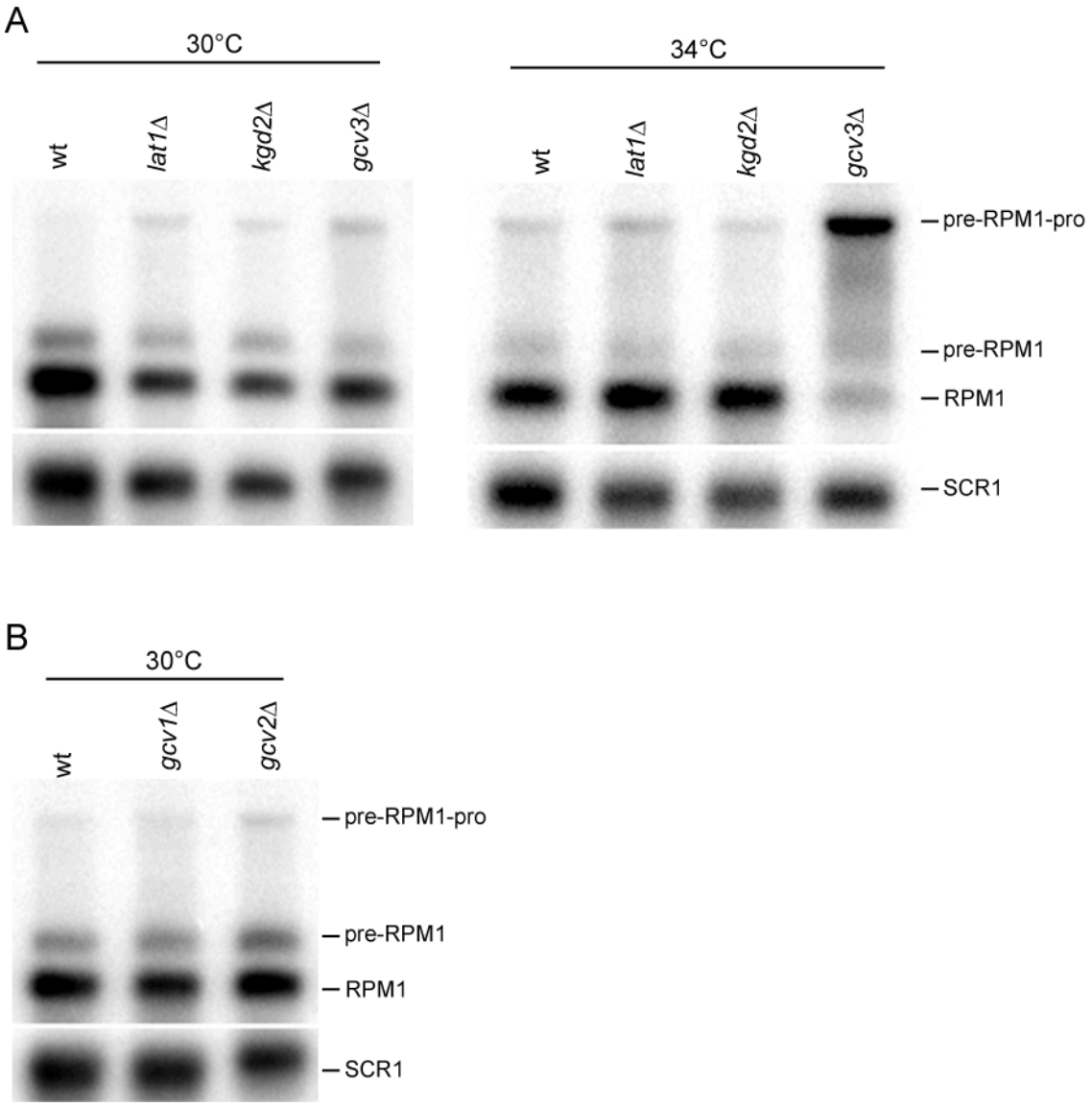


Figure 5.5. Northern blot analysis of pre-RPM1-pro processing in lipoylation target mutant and control strains. (A) A Northern blot of total RNA extracted wild-type, *lat1Δ*, *kgd2Δ*, and *gcv3Δ* strains grown at 30°C and 34°C was hybridized with the RPM1 probe. Lat1 and Kgd2 are the lipoylated E2 subunits of PDH and α-KDH, respectively, and Gcv3 is the lipoylated H protein of the GC enzyme. (B) A Northern blot of total RNA extracted from wild-type, *gcv1Δ* and *gcv2Δ* strains grown at 30°C was hybridized with the RPM1 probe. Gcv1 and Gcv2 are subunits of the GC enzyme. SCR1 was used as a loading control.

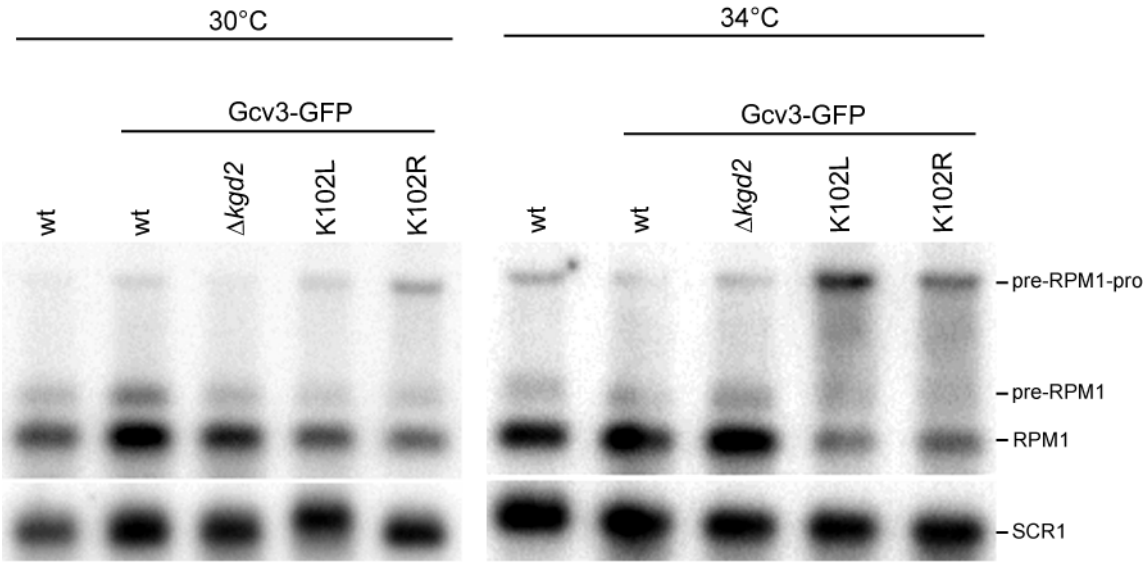


Figure 5.6. Northern blot analysis of pre-RPM1-pro processing in Gcv3-GFP strains grown at 30°C and 34°C. A Northern blot of total RNA extracted the wild-type, Gcv3-GFP, Gcv3-GFP *kgd2Δ*, Gcv3(K102L)-GFP and Gcv3(K102R)-GFP strains grown at 30°C and 34°C was hybridized with the RPM1 probe. SCR1 was used as the loading control.

same as that in the *gcv3* null strain. These data indicate that lipoylated Gcv3 is required for efficient processing of tRNA by mitochondrial RNase P.

DISCUSSION

Strains defective in the synthesis and attachment of lipoic acid are *t*emperature-*s*ensitive for pre-RPM1-pro processing. Specifically, growth of *lip2Δ*, *lip3Δ*, *lip5Δ*, and *gcv3Δ* at higher temperature (34°C) results in greater accumulation of the precursor RNA than growth at 30°C. We also found that the *lpd1Δ* strain has a more severe RNA processing phenotype after growth at the higher temperature. We conclude that mitochondrial RNase P maturation, assembly and/or activity is *t*emperature-*s*ensitive when synthesis and attachment of lipoic acid are disrupted.

The FAS II mutant strains grown at 30°C accumulate about 90% of RPM1-containing RNA in the pre-RPM1-pro form. After growth of the *htd2Δ* strain at 34°C, the ratio of precursor to mature RPM1 RNA remains largely the same. This result suggests that disruption of fatty acid production by the FAS II pathway affects RNase P cleavage of $tRNA^{pro}$ from the precursor at normal or high growth temperature.

The RNA phenotype observed in the *lip* mutant strains is different for each strain after growth at 30°C; the *lip2Δ* and *gcv3Δ* strains have almost wild type RNA processing, the *lip3Δ* strain is slightly more defective, and the *lip5Δ* strain accumulates about 50% of the RNA in the pre-RPM1-pro precursor form. However, each strain accumulates more of the precursor RNA after growth at 34°C. In each of these strains, it is assumed that fatty acid production by the FAS II pathway is not affected. Therefore, disruption of lipoic acid synthesis from the octanoic acid precursor and its attachment to target proteins also affects RNase P activity, but in a *t*emperature-*s*ensitive manner.

There are two hypotheses to explain how the synthesis and attachment of lipoic acid affect RNase P activity. One is that a fatty acid other than lipoic acid directly or indirectly affects RNase P assembly or activity. Since RNA processing is only partially defective in the *lip2Δ* and *lip5Δ* strains, which are completely defective in protein lipoylation, lipoic acid itself may not affect RNase P activity. At 30°C, the lipoylation complex (presented in Fig. 4.5) may be stable enough to promote RNA processing, even though lipoylation is defective. At 34°C, the lipoylation complex may be unstable, causing a defect in RNA processing. The second hypothesis posits that lipoic acid itself directly or indirectly affects RNase P assembly or activity. At 30°C, another fatty acid may be able to partially substitute for the lack of lipoic acid in the *lip2Δ* and *lip5Δ* strains, but this rescue is less efficient at 34°C.

The *lip3Δ* strain is unique in that Gcv3 is lipoylated when the strain is grown at 30°C (Fig. 4.1A), and RNA processing is only partially defective. At 34°C, levels of lipoylated Gcv3 are dramatically reduced (data not shown), and the RNA processing defect is more severe. Mutation of the invariant lysine residue (Lys249) in the proposed lipoyl binding pocket of Lip3 causes the same lipoylation and RNA processing phenotypes as the null mutation. Lipoylated Gcv3 may somehow affect RNA processing. Deletion of *GCV3* as well as mutation of the acceptor lysine residue that gets modified by lipoic acid in Gcv3 (Lys102) result in inefficient RNA processing at 34°C. More experiments need to be done to address this question.

The observation that overexpression of *E. coli* LplA in the *lip3Δ* strain rescues the defect in RNA processing suggests that the bacterial protein is able to promote RNase P

maturation, assembly and/or activity. Since lipoylation of Gcv3 is also restored in this strain, our hypothesis is that *E. coli* LplA is able to lipoylate some protein that affects RNase P function, or that it is able to lipoylate RNase P itself.

The proposed lipoylation complex consists of Lip2, Lip3, Lip5, Gcv3, and possibly Lpd1 (deletion of *LPD1* only partially affects protein lipoylation and RNA processing). Gcv3 may act as a sensor for the metabolic state of the cell to regulate mitochondrial gene expression. Lipoylated Gcv3 is required for lipoylation of Lat1 and Kgd2 (the E2 subunits of PDH and α-KGD, respectively) and for efficient pre-RPM1-pro processing at 34°C. Upon disruption of one of the enzymes in the lipoylation complex, lipoylated or non-lipoylated forms of Gcv3 may respond differently, which may ultimately affect RNase P activity and, thus, mitochondrial gene expression.

CHAPTER 6. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Further work should provide insight into the mechanism governing the intersection between RNA processing and fatty acid synthesis-attachment in yeast mitochondria. This dissertation described the discovery of the connection between these two pathways, which was an unexpected finding of a genome-wide screen for novel nuclear factors involved in mitochondrial RNA processing. Two genes were found to affect 5' processing of precursor tRNAs from an initial multigenic transcript also containing RPM1, the RNA component of mitochondrial RNase P. One gene encodes Htd2, an enzyme in the mitochondrial fatty acid synthesis pathway (FAS II), which is well-studied in yeast. The second gene encodes Lip3, an enzyme we found to be involved in the synthesis and attachment of lipoic acid, which is poorly understood in yeast. Subsequent experiments focused on characterizing which components of the two pathways affect the intersection. The majority of the experiments were designed to determine: (1) whether Htd2 or a product of the FAS II pathway is required for RNA processing, (2) the enzymes that are involved in the synthesis and attachment of lipoic acid in yeast, and (3) how the synthesis and attachment of lipoic acid to target proteins affects RNA processing. The results of the experiments indicate: (*i*) that a product of the FAS II pathway is crucial for efficient processing of $tRNA^{pro}$ by mitochondrial RNase P and (*ii*) that, after determining which proteins are involved, only one lipoic acid synthesis-attachment pathway exists in yeast.

The details of how the production of fatty acids affect tRNA processing are not yet known. We have shown that 5' processing of several pre-tRNAs by RNase P is defective in FAS II mutants (Schonauer *et al.*, 2008), but the defect in processing of $tRNA^{pro}$ from the pre-RPM1-pro transcript is most severe. Since activity of RNase P is required to release $tRNA^{pro}$ from the transcript, activity of RNase P is also required for the maturation of its own RNA subunit, RPM1. This positive feedback loop for RNase P activity may be controlled by the production of fatty acid in the mitochondria, as illustrated in the models presented in Chapters 3 and 4. Our hypothesis is that a fatty acid produced by the FAS II pathway is either necessary for assembly or for activity of mitochondrial RNase P. It is not known whether the fatty acid is lipoic acid, octanoic acid, or a longer acyl chain.

As shown in this work, five proteins were found to be involved in the synthesis of the cofactor lipoic acid from its precursor molecule, octanoic acid, and the attachment of lipoic acid to target proteins. This is fundamentally different from *E. coli*, which has two pathways. One of the proteins, Lip3, was found to be homologous to lipoate-protein ligases in *E. coli* and other organisms, which transfer free lipoic acid scavenged from the environment to target proteins. It is not known whether Lip3 is able to both activate and attach lipoyl-AMP as prokaryotic lipoate-protein ligases do (Fujiwara *et al.*, 2005, Kim *et al.*, 2005), or whether it is only able to attach the lipoyl group after activation by another enzyme, as the bovine lipoyltransferase does (Fujiwara *et al.*, 1994). Another protein found to be a part of the pathway was, surprisingly, Gcv3, which is a target of lipoylation. No other target of lipoylation has ever been found to be required for lipoylation. Our

hypothesis for how the pathway functions is that the four proteins absolutely required for lipoylation, Lip2, Lip3, Lip5 and Gcv3, function in a complex and that a fifth protein, Lpd1, that moderately affects lipoylation, is loosely or temporally associated with the complex.

Experiments that will test and expand upon the hypotheses presented here are described below. The specific aims are as follows:

(1) To investigate whether a fatty acid produced by the FAS II pathway directly or indirectly affects mitochondrial RNase P function, and to determine which species of fatty acid affects tRNA processing.

(2) To determine whether a fatty acid is required for assembly or activity of mitochondrial RNase P.

(3) To investigate whether the proteins involved in lipoic acid synthesis and attachment function in a complex.

(4) To investigate the enzymatic activity of Lip3.

DOES FATTY ACID PRODUCED BY THE FAS II PATHWAY DIRECTLY OR INDIRECTLY AFFECT MITOCHONDRIAL RNase P FUNCTION? WHAT SPECIES OF FATTY ACID AFFECTS tRNA PROCESSING?

Since a defect in the FAS II pathway leads to inefficient 5' processing of mitochondrial precursor tRNAs, mitochondrial RNase P function must be affected in these mutants. A number of experiments are proposed to test whether a product of the FAS II pathway directly modifies or affects conformation of RNase P.

Direct - Rpm2

To test whether a fatty acid directly modifies Rpm2, the protein component of mitochondrial RNase P, the Rpm2-GFP fusion protein can be immunoprecipitated from the wild-type and FAS II mutant strains using the α-GFP antibody, purified from an SDS-PAGE gel, and analyzed by mass spectrometry. We have already tested the possibility that Rpm2-GFP is modified by lipoic acid by immunoprecipitation using the α-GFP antibody and immunodetection using the α-lipoic acid antibody (Chapter 3), and we found that no lipoic acid was recognized. However, it is possible that the lipoyl moiety could be buried and inaccessible. It is also feasible that Rpm2 could be modified by octanoic acid or a longer fatty acid. Thus, ^{14}C-labeled octanoic acid, lipoic acid and longer fatty acids can be added to the growth medium, and Rpm2-GFP can be analyzed in the scintillation counter after immunoprecipitation. A similar assay could be done *in vitro*. Rpm2-GFP can be transcribed and translated and added to mitochondrial extracts from the wild-type, *lip3Δ* and *lip3Δ* + *mtLplA* strains (overexpression of mitochondrially localized *E. coli lplA* rescues lipoylation of Gcv3 in *lip3Δ*). Unlabeled octanoic acid, lipoic acid, and longer fatty acids (plus ATP) will be added to the extracts and Rpm2-GFP will be immunoprecipitated, gel-purified, and analyzed by mass spectrometry. Similarly, ^{14}C-labeled fatty acids can be added and precipitated Rpm2-GFP can be analyzed by scintillation counting.

Direct – RPM1

To test whether a fatty acid directly modifies RPM1, the RNA component of mitochondrial RNase P, ^{14}C-labeled octanoic acid, lipoic acid, and longer fatty acids can be added to the growth medium or to mitochondrial extracts as described above, and RNA can be isolated and analyzed. To test whether a fatty acid affects folding of RPM1, the RNA will be chemically modified in, and extracted from, the wild-type and FAS II-lipoic acid biosynthesis mutant strains. Primer extension can be used to detect sites of modification in processed and unprocessed RPM1 transcripts.

Indirect

It is possible that other proteins, modified by fatty acid, influence the function of mitochondrial RNase P. To look for these proteins, mitochondria can be isolated from the wild-type, *lip3Δ* and *lip3Δ + mtLplA* strains grown in the presence of ^{14}C-labeled malonyl-CoA (or malonate), which is fed into the FAS II pathway. Mitochondrial proteins can be analyzed by 2D gel electrophoresis and autoradiography followed by mass spectrometry.

IS FATTY ACID REQUIRED FOR ASSEMBLY OR ACTIVITY OF MITOCHONDRIAL RNase P?

The simplest explanation for the tRNA processing defect in FAS II mutant strains is that nuclearly encoded Rpm2, the protein component of mitochondrial RNase P, is not imported into the organelle. However, we have ruled out this possibility (see Chapter 3).

Therefore, a product of the FAS II pathway is required either for assembly or activity of RNase P. To test the assembly hypothesis, RNase P can be immunoprecipitated with the α-GFP antibody from the wild-type and *htd2Δ* strains. If RNase P is assembled, RPM1 RNA would precipitate with the Rpm2-GFP fusion protein. RPM1 can be extracted and precipitated and the presence of pre-RPM1 can be analyzed by Northern blot, 5' primer extension, RT-PCR, or fluorescence labeling. To test the activity hypothesis, RNase P immunoprecipitated from the wild-type and *htd2Δ* strains can be incubated with mitochondrial extracts and mitochondrial tRNAs can be analyzed by Northern blot and primer extension.

IS THERE A LIPOYLATION COMPLEX?

The three proteins that are modified by the cofactor lipoic acid in yeast are Lat1, the E2 subunit of PDH, Kgd2, the E2 subunit of α-KGD, and Gcv3, the H protein of the glycine cleavage enzyme. Since five proteins are involved in lipoylation of these proteins, our hypothesis is that they function in a lipoylation complex. Lip2 and Lip5 are essential for lipoylation of all three proteins, Lip3 is required for lipoylation of Lat1 and Kgd2, and lipoylated Gcv3 is itself necessary to lipoylate Lat1 and Kdg2. Lpd1, the common subunit of all three lipoylated complexes, is also involved in, but is not required for, lipoylation of all three targets (upon deletion of *LPD1*, levels of lipoylated proteins are decrease approximately two-fold). One piece of evidence that supports the idea of a complex is that Lip2 and Gcv3 were found to physically associate in a global TAP-tag study (Huh *et al.*, 2003).

To test our hypothesis that that five proteins function in a lipoylation complex, a series of immunoprecipitations can be done. Strains expressing GFP-tagged fusions of each of the five proteins already exist. Each GFP fusion protein can be immunoprecipitated with α-GFP antibody, and any proteins that co-precipitate can be analyzed by mass spectrometry. Alternatively, co-immunoprecipitations can be done with multiple tags, and the proteins can be analyzed by immunoblotting using available antibodies.

WHAT IS THE ENZYMATIC ACTIVITY OF Lip3?

Data presented in this work demonstrate that the Lip3 protein is involved in the synthesis and attachment of lipoic acid to target proteins in yeast. Three pieces of evidence support Lip3 as a lipoyltransferase: (1) the amino acid sequence of Lip3 aligns with lipoate protein ligases and lipoyltransferases characterized in other organisms, (2) deletion of *LIP3* eliminates lipoylation of two target proteins, and (3) mutation of the proposed lipoyl binding domain of Lip3 eliminates lipoylation of two target proteins. However, biochemical studies are required to determine the enzymatic activity of Lip3. Specifically, experiments should address whether Lip3 activity is similar to that of *E. coli* LplA, which both activates free lipoic acid with AMP and transfers the lipoyl group to target lysine residues, or to that of *B. taurus* bLT, which only transfers the lipoyl group previously activated by another enzyme. Recombinant Lip3 can be expressed in and purified from *E. coli*, and then tested for lipoyltransferase activity in the presence of purified apoH protein from *E. coli* plus lipoyl-AMP or lipoic acid and ATP. H protein

can then be run on nondenaturing PAGE gels and stained, as lipoylated H protein will migrate differently from apoH protein.

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