The Mitospecific Region of Mrp20 and its Importance for the Assembly of Mitochondrial Ribosomes in Saccharomyces cerevisiae

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THE MITOSPECIFIC REGION OF MRP20 AND ITS IMPORTANCE FOR THE ASSEMBLY OF MITOCHONDRIAL RIBOSOMES IN *Saccharomyces cerevisiae*

by

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ABSTRACT

THE MITOSPECIFIC REGION OF MRP20 AND ITS IMPORTANCE FOR THE ASSEMBLY OF MITOCHONDRIAL RIBOSOMES IN *Saccharomyces cerevisiae*

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Marquette University, 2011

Yeast mitochondrial ribosomes are composed of an rRNA scaffold, encoded by the mitochondrial genome and many different proteins, which, with the exception of one, are encoded by nuclear genes. These ribosomal proteins are imported into the mitochondrial matrix following their synthesis in the cytosol, however, little is known about the subsequent events which result in an assembled, translationally-competent ribosome. Many of the mitochondrial ribosomal proteins bear homology to bacterial ancestors. In addition to the acquisition of mitochondrial targeting signals, a number of these nuclearly-encoded ribosomal proteins have acquired additional domains, often at their C-termini, which are termed "mitochondrial-specific domains". The function(s) of these domains is currently unknown and it is postulated that they may be involved in the process of ribosomal assembly or for ensuring the targeting of the ribosome to the mitochondrial inner membrane where they are translationally-active.

Mrp20 protein is a nuclearly-encoded component of the mitochondrial large ribosomal subunit and shares homology with bacterial ribosomal protein L23, a protein located at the exit site of the ribosomal polypeptide tunnel. Mrp20 contains a C-terminal mitochondrial-specific domain of unknown function. In this study, we demonstrate that the C-terminal mitochondrial-specific region of Mrp20 is important to support the assembly of active mitochondrial ribosomes. It is proposed that the proteins at the exit site such as Mrp20 and MrpL40 are important for the assembly of mitochondrial ribosomes. Furthermore, the data presented here indicates that in the absence of the C-terminal region of the Mrp20 protein, the process of assembly of the ribosome becomes stalled, and the accumulation of a novel ribosome intermediate complex is observed. The characterization of this novel intermediate, which furthers our understanding of the assembly process of ribosomes in mitochondria, is presented.

PREFACE

Little is known about the early stages involved in the assembly of the large mitochondrial ribosomal subunit (54S) except the existence of an assembly intermediate, known as pre-54S particle. The 54S particle is tightly tethered to the inner mitochondrial membrane, however, it has not been investigated where the events prior to the assembly of 54S particle occur. Also, what keeps the 54S particle tightly tethered to the membrane is not clear. This study is focused on a component of the 54S particle, Mrp20, its role in ribosome assembly and mapping the early assembly events using a mutant derivative of the Mrp20 protein.

Mrp20, a nuclearly-encoded large ribosomal protein, is an essential component of the mitoribosomes. Mrp20 is homologous to the bacterial ribosomal protein L23, known to be located at the exit site of the 54S particle. In addition to the L23 domain, Mrp20 contains a C-terminal extension sequence called mitospecific region. The functional significance of this mitospecific region remains uncharacterized and is addressed in this study.

By creating a C-terminally truncated derivative of Mrp20, the Mrp20ΔC protein, the possible role of the mitospecific region of Mrp20 was tested for the assembly and function of mitoribosomes. It is shown here that the mitospecific region of Mrp20 is involved in the assembly of mitoribosomes and subsequently for the mitochondrial translation. Despite the defective ribosomal assembly, the steady states of ribosomal proteins (except Mrp20 and MrpL40) were not turned over and were found to be comparable to the wild type mitochondria. Based on these findings, the presence of a

subcomplex of ribosomal proteins in the *mrp20ΔC* mutant was proposed and that the subcomplex can prevent the turnover of ribosomal proteins in the absence of the assembled 54S particle. In the second part of the current study, the proposed ribosomal subcomplex was further characterized and its composition was investigated. The data here indicated the presence of two different subcomplexes formed in the *mrp20ΔC* mutant – one containing Mrp20ΔC and the ribosomal proteins at the exit site and another composed of proteins located at the interface of the small and large ribosomal subunits. From these observations, it is proposed that during ribosomal assembly, distinct subcomplexes may co-assemble to form a fully assembled 54S particle.

To address where early ribosomal assembly events take place, i.e. in the soluble matrix space or at the membrane surface, the *mrp20ΔC* and other ribosomal assembly mutants were further investigated by addressing the possible membrane association of the non-assembled ribosomal proteins. Contrary to a previous proposal, which suggests only fully assembled ribosomes are tethered to the membrane, data obtained in this study indicates that non-assembled ribosomal proteins and the ribosomal subcomplexes also have ability to tether to the inner membrane. It is thus proposed here that the inner membrane serves as a platform for early ribosome assembly events so that the need to target assembled ribosomes from the matrix to the inner membrane is circumvented.

Finally, the preliminary studies to identify proteins responsible for the tight association of ribosomal proteins with the inner membrane were initiated. It is proposed that large multimeric complexes in the inner membrane may recruit ribosomal proteins to large detergent-resistant platforms that may form ribosomal assembly sites in the membrane.

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Jasvinder Kaur, B.Sc. M.Sc.

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LIST OF PUBLICATIONS

- Jia, L[1]., **Kaur, J**. [1], and Stuart, R. A. Mapping of the Saccharomyces cerevisiae Oxa1-mitochondrial ribosome interface and identification of MrpL40, a ribosomal protein in close proximity to Oxa1 and critical for oxidative phosphorylation complex assembly.(2009) Eukaryotic Cell 8(11), 1792-1802 (co-author)

- **Kaur J** and Stuart, R.A, Truncation of the Mrp20 protein reveals novel ribosome assembly subcomplex in mitochondria. Accepted in *EMBO* reports Journal.

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LIST OF ABBREVIATIONS

AAC	ADP/ATP carrier
Å	Angstroms
ADP	adenosine 5’ diphosphate
Amp	ampicillin
ATP	adenosine 5’-triphosphate
BLAST	basic local alignment search toll
bp	base pair
CL	cardiolipin
^{o}C	degree Celsius
Cpr3	cyclophilin
COX	cytochrome oxidase
C-terminal	carboxyl terminal
Cyt b_1	cytochrome b_1
Δ	deletion
dATP	deoxyadenosine 5’ triphosphate
dCTP	deoxycytosine 5’ triphosphate
ddH_2O	double distilled water
dGTP	deoxyguanosine 5’ triphosphate
dNTP	deoxynucleoside 5’ triphosphate
dTTP	deoxythymidine 5’ triphosphate
DIG	digitonin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ECL	enzyme catalyzed light generation
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis-(2-aminoethyl ether)-tetraacetic acid
EM	electron microscopy
5-FOA	fluoro orotic acid
Fe-S	iron sulfur
g	gravity
GTP	guanosine triphosphate
HA	hemagglutinin
HEPES	N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid
His	Histidine
H_2O_2	hydrogen peroxide
hr	hour
HRP	horse radish peroxidase
IgG	immunoglobulin
kDa	kilo Dalton
KCl	potassium chloride
KH_2PO_4	potassium phosphate
KOH	potassium hydroxide
kV	kilo volts
L	liter
LB	Luria and Bertani broth

LiOAc	lithium acetate
LC-MS	Liquid chromatography mass spectrometry
µCi	micro Curi
µg	microgram
µl	microliter
M	molar
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester
Mba1	membrane-associated mitochondrial ribosomal receptor
mg	milligrams
$MgCl_2$	magnesium chloride
MgOAc	magnesium acetate
$MgSO_4$	magnesium sulfate
ml	milliliter
mM	milimolar
min	minute
mRNA	messenger ribonucleic acid
mt DNA	mitochondrial DNA
mitoribosomes	mitochondrial ribosomes
mts	mitochondrial targeting sequence
MRP	mitochondrial ribosomal protein
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
NADH	reduced nicotinamide adenine dinucleotide, reduced form
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
$NaPO_4$	sodium phosphate
Ni-NTA	nickel-nitrilotriacetic acid
N-terminal	amino-terminal
OD	optical density
ORF	open reading frame
Oxa1	oxidase assembly protein 1
OXPHOS	oxidative phosphorylation
pI	isoelectric point
$Pb(NO_3)_2$	lead nitrate
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEBP	phosphatidylethanolamine binding protein
Pi	inorganic phosphate
PMSF	phenylmethylsulphonyl fluoride
rpm	revolutions per minute
rho^0	rho zero
rho^-	rho minus
rho^+	rho plus
rRNA	ribosomal ribonucleic acid
RNase	ribonuclease
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SC	minimal media
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
Sec	secretary
SEM	sorbitol EDTA Mops buffer
SH	sobitol HEPES buffer
TBS	tris buffer saline
TCA	trichloroacetic acid
TE	tris EDTA buffer
tRNA	transfer ribonucleic acid
Tris	tris-(hydroxymethyl)-aminomethane
UTR	untranslated region
v/v	volume per volume
V	volt
WT	wild-type
w/v	weight per volume
Yip	yeast integration plasmid
YPD	yeast peptone dextrose
YPG	yeast extract peptone glycerol medium

Chapter 1 Introduction

Overview

The majority of the proteins comprising the oxidative phosphorylation (OXPHOS) complexes of the mitochondrial inner membrane are encoded by nuclear genome. The mitochondrial genome encodes only handful of OXPHOS proteins, each of them essential for the activity of their respective enzyme complexes. The mitochondrially-encoded proteins are synthesized on the mitochondrial ribosomes (mitoribosomes) and in a co-translational fashion they become inserted into the inner membrane of the mitochondria. Oxa1, an inner membrane protein is required for the insertion of these proteins into the inner membrane (Stuart, 2002). Studies from our lab and from a separate study indicate that Oxa1 can physically associate with the mitoribosomes (Jia *et al*, 2003; Szyrach *et al*, 2003). This physical interaction is thought to directly support the co-translational insertion of the mitochondrially-encoded proteins into the inner membrane. Oxa1 can be chemically cross-linked to two large ribosomal subunit proteins, Mrp20 and MrpL40, further demonstrating the close proximity of the ribosomes with the components of the inner membrane (Jia *et al*, 2003; Jia *et al*, 2009).

Both Mrp20 and MrpL40 are essential components of the mitoribosomes and each have a domain that shares homology with their bacterial ribosomal ancestors, the L23 and

L24 proteins, respectively (Fearon & Mason, 1992; Kitakawa *et al*, 1997). From the available crystal structure of bacterial ribosomes it is known that L23 and L24 proteins are both located at the exit site of the polypeptide tunnel of bacterial ribosomes (Ban *et al*, 2000; Nissen *et al*, 2000). During the evolution of mitoribosomes from their bacterial ancestors, many mitochondrial ribosomal proteins have acquired extension sequences (mostly at the C-termini), termed "mitospecific regions" in addition to their bacterial homology domains (O'Brien, 2002). The functional significance of these mitospecific regions is largely uncharacterized to date.

The study described in Chapter 3 (section 3.1) focused on investigating the function of the mitospecific region of the Mrp20 protein and its possible role in supporting the synthesis of mitochondrially-encoded proteins as well as in the stability and assembly of the mitochondrial ribosomal proteins. Therefore, relevant information on the mitochondrial translation system, the similarities and differences between bacterial and mitochondrial ribosomal proteins, will be described in this introduction section. In yeast, mitoribosomes are found to be tightly associated with the inner membrane,a similar behavior has also been observed for bovine mitoribosomes (Liu & Spremulli, 2000; Obbink *et al*, 1977; Watson, 1972). The association of ribosomal proteins with the inner membrane is proposed in this dissertation to be an important step in the process of ribosomal assembly. However, little is known about the assembly pathway or the identity of assembly intermediates of mitoribosomes. Chapter 3, section 3.2 focused on the finding that mutation in the mitospecific region of Mrp20 protein disrupts the process of ribosome assembly and results in the identification of novel ribosome assembly subcomplexes. The importance of the mitospecific region of Mrp20 for the association of

ribosomal proteins with the inner membrane will also be examined in Chapter 3, section 3.3. Finally, in section 3.4 of chapter 3, preliminary studies aimed at identifying proteins important for the association of ribosomal proteins with the inner membrane are presented. For this purpose, a combined overview of the literature on the association of mitoribosomes with the inner membrane, assembly of mitoribosomes and the known assembly factors involved in the assembly of bacterial and mitoribosomes will also be presented in this introduction.

1.1 Mitochondria

1.1.1 Mitochondrial compartments and function of mitochondria

All aerobic eukaryotic cells require mitochondria for cellular respiration. Mitochondria are double membrane organelles containing an outer membrane, an inner membrane and an aqueous internal compartment termed the matrix. The intermembrane space divides the outer and inner membrane. Mitochondria are known as the “power houses” of a cell because they are the primary source of ATP production through a process termed oxidative phosphorylation (OXPHOS). In addition to respiration, mitochondria are also important for other cellular processes such as cell signaling, cell differentiation and cell death (McBride *et al*, 2006). The OXPHOS process is mediated by the five multimeric complexes located in the inner membrane of mitochondria, namely, NADH-dehydrogenase (I), succinate dehydrogenase (II), cytochrome bc_1 complex (III), cytochrome *c* oxidase (COX, IV) and F_1-F_o ATP synthase (V). These respiratory

complexes are conserved from the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) to human with the exception of complex I, which is replaced by three NADH dehydrogenases in *S. cerevisiae* (Marres *et al*, 1991).

1.1.2 Origin of Mitochondria

According to the endosymbiotic theory, mitochondria are believed to originate from α-proteobacteria approximately two billion years ago (Dyall *et al*, 2004). The RNA and protein sequence similarities between the α-proteobacteria (such as *Paracoccus, Rickettsia and Bartonella* species) and mitochondria supports the endosymbiotic theory (Andersson *et al*, 1998; Gray *et al*, 1999; Lang *et al*, 1999). Based upon the endosymbiotic theory, most of the genes once encoded by bacteria were transferred from mitochondria to the nuclear genome of the host cell. The mitochondria retained only a small genome that is required for the synthesis of a limited number, but essential components of OXPHOS complexes. The lack of synthesis or the dysfunction of these mitochondrially-encoded proteins of the OXPHOS complexes can lead to respiratory deficiency and are often the underlying cause of some human diseases where the mitochondrial function is defective (DiMauro & Schon, 2008; Shoubridge, 2001). As a facultative anaerobe, yeast *S. cerevisiae* offers distinct advantages over other eukaryotes for biochemical and genetic studies of mitochondrial gene expression and respiratory metabolism.

1.1.3 Mitochondrial genome and proteome

The 85.8 kb *S. cerevisiae* mitochondrial genome encodes 8 proteins, as well as ribosomal RNAs (rRNA) and transfer RNAs (tRNA) for protein translation (Foury *et al*, 1998). The eight proteins are Cox1, Cox2, Cox3 of the COX complex; cytochrome *b* of the cytochrome bc_1 complex; Atp6, Atp8, and Atp9 of the F_1F_o-ATP synthase and the ribosome associated Var1 protein. The small and large ribosomal RNAs (15S and 21S rRNAs, respectively) of the mitoribosome are encoded by the mitochondrial genome, while all of the ribosomal proteins, except for Var1 of the small ribosomal subunit, are encoded by the nuclear genome. The *S. cerevisiae* mitochondrial genome also encodes 24 tRNAs and the 9S RNA component of RNase P (Borst & Grivell, 1978; Foury *et al*, 1998). The yeast cells that contain mitochondrial genome are called rho^+ (ρ^+), whereas those that have mutations/ deletions in the mitochondrial genome are known as rho^- (ρ^-) and those that have no mitochondrial genome are known as rho^0 (ρ^0).

Proteomic studies indicate that yeast mitochondria contain approximately 1000 different proteins which are involved in various cellular processes such as protein translocation, protein synthesis, genome maintenance, metabolism of amino acids, lipids, heme and iron, membrane dynamics, Fe-S cluster biosynthesis, apoptosis and signaling (Prokisch *et al*, 2004; Reinders *et al*, 2006; Sickmann *et al*, 2003). The mitochondrial proteome is mosaic in origin composed predominantly of nuclearly-encoded proteins and only eight mitochondrially-encoded proteins as mentioned previously (Figure 1). Many nuclearly-encoded mitochondrial proteins carry a N-terminal targeting signal that directs these proteins to specific compartments of the mitochondria following their synthesis in the cytosol (Figure 1A) (Neupert & Herrmann, 2007). The mitochondrially-encoded

proteins are synthesized within mitochondria by mitoribosomes in the matrix which are tethered to the inner membrane. The assembly of the multi-subunit mitochondrial complexes, in particular those that contain components encoded by the two genomes, such as many of the OXPHOS complexes, requires coordinated translation, translocation and assembly of proteins encoded by these two genomes (Figure 1B).

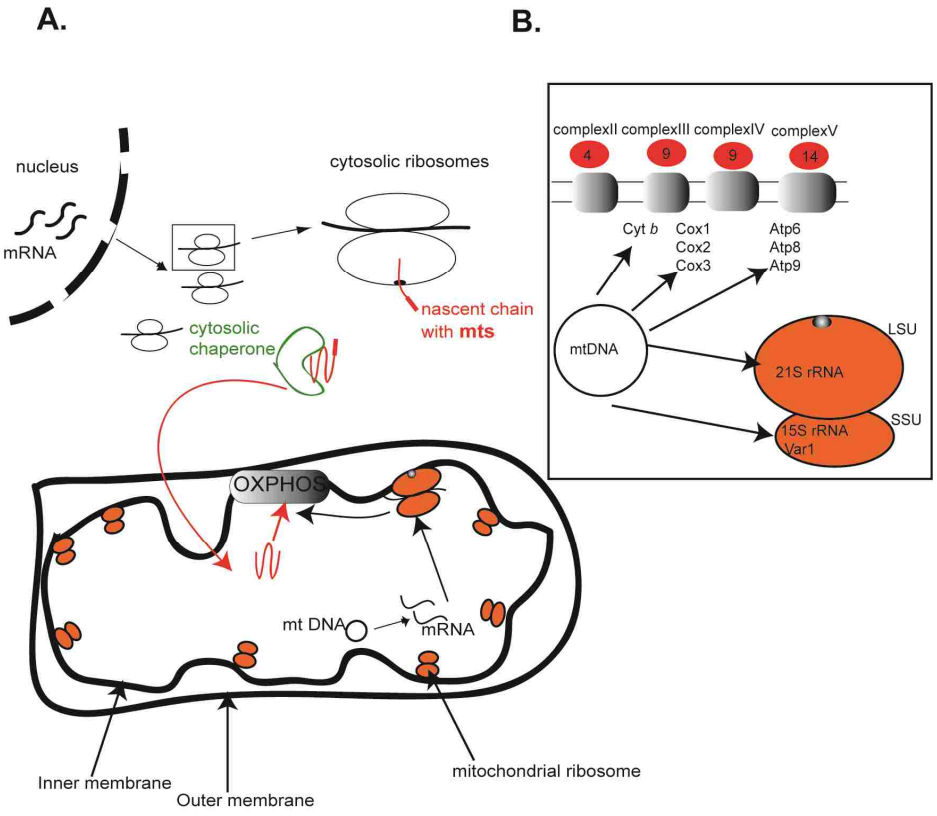

Figure 1: Biogenesis of the mitochondrial proteins in yeast (adapted from (Gruschke & Ott, 2010). (A) Nuclearly-encoded proteins destined for mitochondria are synthesized on the cytosolic ribosomes, in most cases with an N-terminal mitochondrial targeting sequence (mts). Following synthesis, the nascent proteins interact with cytosolic chaperones to maintain them in a "translocation-competent" state before they get imported. These proteins are components of the different compartments of mitochondria. The protein in the example is a component of an OXPHOS complex. Mitochondria have their own genome (mt DNA) that encodes for mRNA in the matrix. The translation of these mRNAs is initiated at the membrane by the membrane bound ribosomes. (B) The inner membrane contains four OXPHOS complexes. Complex II is exclusively made up of nuclearly-encoded proteins whereas complex III, IV and V are mosaic in origin containing proteins from both the nuclear and mitochondrial origin. The mitochondrial ribosomes contain all the proteins encoded by the nuclear genome except one small ribosomal protein, Var1, encoded by the mitochondrial genome. mt DNA also encodes 21S and 15S rRNA, components for the large and small ribosomal subunit respectively. mts- mitochondrial targeting signal. mt DNA- mitochondrial DNA; LSU - large ribosomal subunit; SSU- small ribosomal subunit.

1.2 Mitochondrial translational machinery and comparison from its bacterial ancestors

The mitoribosomes are closely related to bacterial ribosomes, displaying similar antibiotic sensitivities and similarities in their protein and RNA domains that contribute to the decoding and peptide bond formation (Spremulli *et al*, 2004). However, over a long period of evolution, mitoribosomes have acquired significant unique features both in terms of the structural composition and function.

1.2.1 Mitochondrial ribosomes differ in structural composition from their bacterial ancestors

The mitochondrial ribosomes have been shown to contain an increased protein mass and less rRNA molecules compared to their bacterial counterparts. The bacterial ribosomes contain 54 ribosomal proteins, however, mitoribosomes are reported to contain more proteins, ranging from 81 in most metazoans to 80 in yeast, to 63 in plants, but only 39 in apicomplexan parasites (Smits *et al*, 2007). Moreover, in most taxons, the mitoribosomes contain only two rRNA species – 15S and 21S rRNA in yeast and 12S and 16S rRNA in mammals in contrast to bacterial ribosomes that are comprised of 5S, 16S and 23S rRNA (Grivell, 1995; O'Brien *et al*, 2000). Thus, the protein:RNA ratio in mitoribosomes ranges from 1:1 in yeast to 2:1 in bovine in contrast to 1:2 in bacteria. The altered protein/RNA ratio in mitoribosomes has resulted in varying sedimentation values ranging from 70-74S in fungi, to 77-78S in higher plants and 55S in metazoans, in comparison to 70S for bacterial ribosomes. The expanded protein content in the mitoribosomes is attributed to two factors – i) acquisition of several new proteins that are unique to the

mitoribosomes with no homology to the known bacterial ribosomal proteins and ii) presence of the N- and C-terminal extension sequences in many mitoribosomal proteins, in addition to the domain that displays homology to the bacterial ribosomal protein (Sharma *et al*, 2003; Smits *et al*, 2007). These extension sequences (mostly present at the C-terminal region) are known as **mitospecific regions**. The importance of these mitospecific regions will be discussed further in section 1.3.3.

1.2.2 Mitoribosomes have adapted to specialized translation in comparison to the bacterial ribosomes

The bacterial ribosomes synthesize a diverse array of proteins, such as cytosolic, membrane-bound, secretory and non-secretory. In contrast, mitoribosomes are specialized to synthesize few proteins which, with the exception of one, are hydrophobic proteins and are components of the OXPHOS complexes. The mitoribosomes that synthesize these hydrophobic proteins are present in the matrix but tethered to the inner membrane (Spithill *et al*, 1978; Watson, 1972). Even the synthesis of the small ribosomal protein, Var1 is reported to occur at the membrane by membrane bound ribosomes (Fiori *et al*, 2003). The exclusive membrane bound nature of mitoribosomes is also evidenced from the early electron microscopic studies that demonstrate the localization of mitoribosomes on the inner membrane (Watson, 1972). The mitoribosomes are also found to be physically associated with the inner membrane proteins, Oxa1, Mba1 and Mdm38 further reflecting close intimacy between mitoribosomes and the inner membrane (Frazier *et al*, 2006; Jia *et al*, 2003; Ott *et al*, 2006; Szyrach *et al*, 2003). The molecular details of the mitoribosome association with the Oxa1 protein will be discussed in the

next section. Although, mitoribosomes have originated from eubacterial ancestors, the process of co-translational membrane insertion process proteins differs significantly in mitoribosomes from their bacterial ancestors and will be discussed below.

1.2.2.1 Insertion of proteins into membranes in bacteria and mitochondria

For the synthesis of membrane proteins in bacteria, cytosolic bacterial ribosomes need to be targeted to the membrane to support the co-translational insertion of the hydrophobic membrane proteins. To facilitate this insertion of membrane proteins, the soluble signal recognition particle (SRP) recognizes the signal sequence of nascent polypeptide as they emerge from the ribosomes and the SRP-ribosome complex is then targeted to the SecYEG translocase in the membrane. Another membrane protein called YidC (homolog of Oxa1 protein) is reported to facilitate the insertion of membrane proteins in the Sec-dependent as well as the Sec-independent manner. A soluble protein SecA (an ATPase motor) is also required to ensure the continuous insertion of some of the proteins into the membrane (Dalbey *et al*, 2010) (Figure 2A). After the co-translational insertion of membrane proteins, the membrane-bound bacterial ribosomes are thought to be released from the membrane and are cycle back to cytosol (Figure 2B). The molecular mechanism of co-translational insertion of membrane proteins in mitochondria differs significantly from the process in bacteria as the SRP-like protein and SecYEG-like proteins do not exist in the mitochondria of higher eukaryotes like fungi, plants and animals (Glick & Von Heijne, 1996).

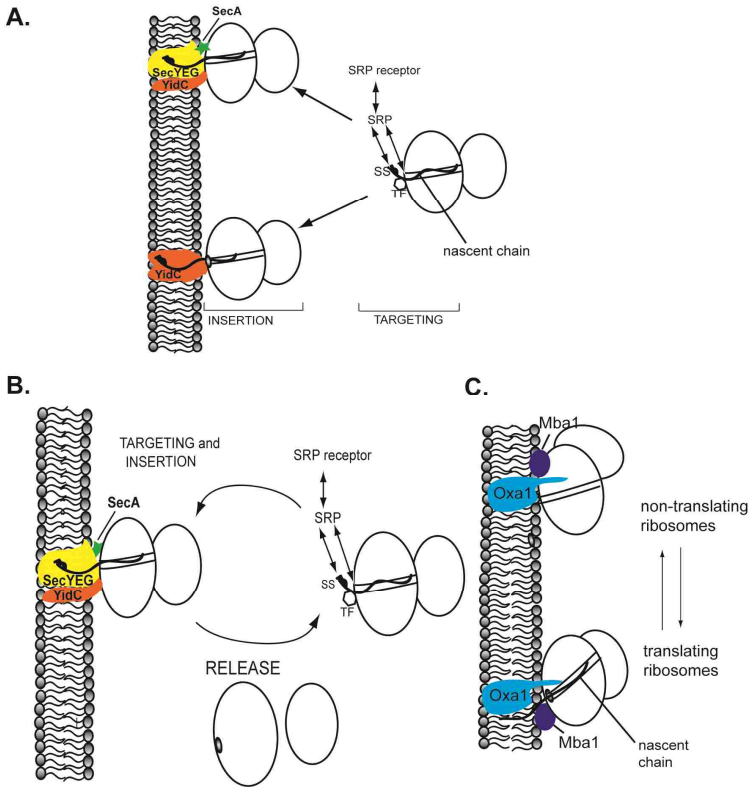

Figure 2: Comparison of the bacterial and mitochondrial translation machinery (adapted from Gruschke & Ott, 2010). (A) Targeting of bacterial ribosomes for the synthesis and insertion of proteins of the membrane proteins. The nascent polypeptides released from the cytosol are recognized by chaperone protein called trigger factor (TF). Nascent chains containing signal sequence (SS) are recognized by signal recognition particle (SRP) that further binds to SRP receptor in the cytosol. The SRP can also directly interact with ribosomes. Cytosolic ribosomes synthesizing hydrophobic proteins are targeted to the membrane where they can interact with membrane components, SecYEG and YidC or YidC only for the insertion of the hydrophobic proteins. SecA ATPase can also interact with ribosomes for the insertion of few hydrophobic proteins with the help of SecYEG complex (B) Recycling of the bacterial ribosomes. The cytosolic bacterial ribosomes translating membrane proteins are targeted to the membrane for co-translational insertion of the membrane proteins. After synthesis and insertion of the proteins, ribosomes are released from the membrane and recycled. (C) No recycling of mitochondrial ribosomes. The mitochondrial ribosomes are membrane tethered even in the absence of the translation and can interact with the inner membrane proteins, Oxa1 and Mba1. The co-translational insertion of the mitochondrially encoded hydrophobic proteins into the inner membrane is facilitated by Oxa1 and Mba1 protein.

In mitochondria, the co-translational insertion of hydrophobic proteins in the inner membrane is facilitated by the Oxa1 insertase with the help of membrane proteins, Cox18, Mba1 and Mdm38 (Frazier *et al*, 2006; Preuss *et al*, 2001; Saracco & Fox, 2002). In contrast to the bacterial ribosomes which initiate translation on the soluble ribosomes and then become targeted to the membrane to complete translation of membrane proteins (a process which is then coupled to membrane insertion), mitoribosomes are found to be tightly associated with the inner membrane (Gruschke *et al*, 2010). Thus the synthesis of their substrates destined for the insertion into the membrane does not require the prior targeting of soluble ribosomes to the membrane. The close association of mitoribosomes with the membrane is evidenced by the ability of membrane proteins, Oxa1, Mba1 and Mdm38 to physically interact with the mitoribosomes (Jia *et al*, 2003; Ott *et al*, 2006; Szyrach *et al*, 2003). Studies from our lab indicate that Oxa1 protein has the ability to cross-link with two ribosomal proteins, Mrp20 and MrpL40 (Jia *et al*, 2003; Jia *et al*, 2009). Interestingly, studies from our lab also demonstrate that Oxa1 can cross-link with Mrp20 protein independent of the nascent chain, indicating that the association of large ribosomal subunit is not dependent on the translation status of the ribosomes. Furthermore, the salt-sensitive association of the mitochondrial ribosomes with the inner membrane in bovine mitochondria has been demonstrated (Liu & Spremulli, 2000). Finally, there is no evidence to support that mioribosomes cycle on and off the membrane supporting the idea that unlike bacterial ribosomes, mitoribosomes remain tethered to the membrane (Figure 2C). However, it is unclear to date that how the mitoribosomes are tightly associated with the inner membrane.

Taken together, this section describes how mitoribosomes have diverged from the bacterial ribosomes in terms of their protein composition and function during the period of evolution. The changes in the protein dimensions, protein content and the function of ribosomes are thought to be an adaptation for the specialized translation system in mitochondria.

1.3 The polypeptide tunnel exit

The synthesis of nascent polypeptides takes place at the peptidyl transferase center of the ribosome, which is at the interface of the large and small subunits of the ribosomes. During translation, all kinds of polypeptides segments, small and big, hydrophilic and hydrophobic, basic or acidic must pass through a tunnel like structure called the polypeptide tunnel. This tunnel runs from the peptidyl transferase center in the large ribosomal subunit to its lower part called the exit site (Figure 3). The opening of this tunnel at its site is located at the bottom of the large ribosomal particle and is the region where the newly synthesized polypeptides leave the ribosomes for the first time and become exposed to a hydrophilic environment. This region is called the polypeptide tunnel exit and the rim to outside of the tunnel is called the exit site (Figure 3).

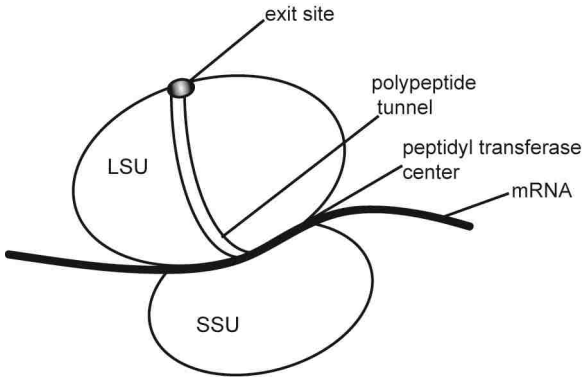

Figure 3: Polypeptide exit tunnel. The nascent polypeptides synthesized at the peptidyl transferase center (located at the interface of the small and large ribosomal subunit) pass through a tubular structure in the large ribosomal subunit called polypeptide tunnel. The nascent polypeptides are released from the opening in the large ribosomal subunit located distant from the peptidyl transferase center called “exit site”. LSU - Large ribosomal subunit; SSU- small ribosomal subunit.

The existence of the polypeptide tunnel was suggested by cryo electron microscopy (EM) studies and ribosomal crystallography (Bernabeu & Lake, 1982; Frank *et al*, 1995; Yonath *et al*, 1987). The bacterial ribosomal tunnel exit site and the RNA and protein components comprising it are well studied by a large number of crystal structure studies performed on the bacterial ribosomes (Ban *et al*, 2000; Harms *et al*, 2001; Nissen *et al*, 2000). A recent cryo EM of the bovine mitoribosome revealed for the first time that the tunnel exit of mitoribosomes has acquired some unique features and novel proteins in comparison to the bacterial ribosomes (Sharma *et al*, 2003). The current knowledge about the polypeptide tunnel, its composition and function in the bacterial and mitoribosomes will be discussed in the next two sections.

1.3.1 Polypeptide tunnel exit of bacterial ribosomes

The crystal structural studies performed on the bacterial ribosomes demonstrated that the polypeptide tunnel wall is predominantly made up of the 23S rRNA and looped out segments of the ribosomal proteins, L4, L22 and L23. The exit site, from where the nascent polypeptides are released is comprised of the rRNA and four ubiquitously conserved proteins- L22, L23, L24 and L29 (Ban *et al*, 2000; Harms *et al*, 2001; Nissen *et al*, 2000). Some of these conserved ribosomal proteins provide major interaction sites for various cytosolic factors involved in processing, folding and co- or post- translational targeting of the nascent polypeptides into the bacterial membrane or to the periplasm and beyond. For instance, the L23 and L29 ribosomal proteins serve as docking sites for the tight interaction of the SRP with SecYEG translocase in the inner membrane. L23 also interacts with the chaperone protein, trigger factor and hence couples protein biosynthesis

with chaperone-assisted protein folding events (Gu *et al*, 2003; Ullers *et al*, 2003). Recently, a cryo-EM study using bacterial ribosomes suggested that the ribosomal proteins, L22, L23 and L29 located at the exit site interact with the YidC protein, required for the insertion of some of the membrane proteins (Kohler *et al*, 2009). These interactions between the biogenesis factors and proteins of the ribosomal tunnel exit site are also found in eukaryotic cytosolic ribosomes showing their conserved nature. The cytosolic homolog of L23 protein, L23a has been shown to be the binding site for mammalian SRP as well as with the yeast Sec61 translocon (Beckmann *et al*, 2001; Halic *et al*, 2004).

Taken together, these studies collectively demonstrate that the exit tunnel proteins of the bacterial and eukaryotic cytosolic ribosomes provide a platform to support the biogenesis of newly synthesized polypeptides and the targeting of soluble ribosome to their respective membrane. Whether a similar nature of interaction between the ribosomal proteins and the translocase components is present in the mitochondria or not is discussed below.

1.3.2 Polypeptide tunnel exit of mitochondrial ribosomes

In contrast to the bacterial ribosome, limited information is available about the composition of mitoribosomes and its tunnel exit. The crystal structure of the mitoribosomes has not been yet elucidated. The first structural insight into the mitoribosomes was provided by a cryo EM study of bovine mitoribosomes (Sharma *et al*,

2003). This study provided some molecular details of mitoribosomes by its comparison to the crystal structures obtained previously from eukaryotic cytosolic and bacterial ribosomes. This EM based study revealed that the structural composition of the polypeptide tunnel in mitoribosomes was significantly different from the tunnels of the bacterial/eukaryotic cytosolic ribosomes. For example, the tunnel in bovine mitoribosomes was found to be more protein dense than that of bacterial ribosomes where the polypeptide tunnel is particularly rich in RNA. No counterpart of the bacterial ribosomal protein L29 was observed and there was significantly less exposure of rRNA domains at the exit site of the bovine mitoribosomes. While the tunnel exit site in bovine mitoribosomes is surrounded by homologs of bacterial ribosomal proteins – L22, L23 and L24, these proteins are much larger in mitochondrial than their bacterial counterparts. The large size of these proteins is attributed to the additional N- but more often C-terminal extensions, called **mitospecific regions,** whose functional significance remains largely uncharacterized. The cryo EM study of bovine mitoribosomes also indicated the presence of new ribosomal proteins in the vicinity of the L23 protein that do not have any prokaryotic counterparts. The identity of these mitoribosome-specific proteins was not defined in the cryo EM structure, but some recent biochemical studies performed in yeast and bovine mitoribosomes have identified novel mitoribosome-specific proteins located in the vicinity of the tunnel exit site that are discussed below (Gruschke *et al*, 2010; Haque *et al*, 2010) .

The chemical cross-linking studies in the yeast mitoribosomes identified the bacterial homologs of L22, L23, L24, and L29 (MrpL22, Mrp20, MrpL40 and MrpL4, respectively) at the exit site. It thus appears that except for L29 (homolog not found in

bovine mitoribosomes), the protein composition of the exit site in yeast and bovine mitoribosomes is conserved (Gruschke *et al*, 2010; Jia *et al*, 2003; Jia *et al*, 2009). The cross-linking studies further demonstrated that ribosomal proteins Mrp20 and MrpL40 (L23 and L24 homologs) can be cross-linked with the inner membrane translocase Oxa1, indicating their close vicinity to the exit site of the ribosomes (Jia *et al*, 2003; Jia *et al*, 2009). As mentioned previously, the Oxa1 protein mediates co-translational insertion of the hydrophobic membrane proteins with another integral membrane protein Mba1. An independent chemical cross-linking in yeast mitochondria demonstrated that the large ribosomal proteins, MrpL22 and MrpL4 (homologs of bacterial L22 and L29 proteins) are in the vicinity of the Mba1 protein, thus supporting their presence near the exit site in mitoribosomes (Gruschke *et al*, 2010). In addition to the conserved ribosomal proteins presence near the exit site in mitoribosomes, the chemical cross-linking done by Gruschke *et al* in yeast mitochondria, also identified three novel ribosomal proteins near the exit tunnel that do not have any known bacterial homologs. These mitoribosome-specific proteins are MrpL3, MrpL13, and MrpL27, and with the exception of MrpL13, they are conserved between fungi and mammals. The presence of novel mitoribosome-specific proteins in mammals was also demonstrated in a study designed to identify ribosomal proteins which interact with the C-terminal tail of mammalian Oxa1 protein. This study identified three novel ribosomal proteins, MRPL48, MRPL49 and MRPL51 that do not share any homology with bacterial ribosomal proteins (Haque *et al*, 2010). No homolog of MRPL48 and MRPL49 is found in yeast mitoribosomes, however, the Img2 protein of yeast mitoribosomes is predicted to be the homolog of MRPL51 protein. The

proteins that have been identified so far at the exit site of bacterial and yeast or bovine mitoribsomes is listed in Table 1.

Table 1 Proteins located at the exit site of the ribosomes in bacteria and their homologs in mitochondrial ribosome of yeast and mammals

Bacterial	**Yeast**	**Mammalian**
L22	MrpL22	MRPL22
L23	Mrp20	MRPL22
L24	MrpL40	MRPL24
L29	MrpL4	MRPL47 (predicted)
Not found	MrpL13 (fungal specific)	Not found
Not found	MrpL27	MRPL41
Not found	MrpL3	MRPL44
Not found	Img2 (predicted)	MRPL49 (mammalian specific)
Not found	Not found	MRPL48(mammalian specific)
Not found	Not found	MRPL51

Taken together, the structural and biochemical studies in the bovine and yeast mitoribosomes identified conserved ribosomal proteins at the exit site that share homology with the bacterial ribosomes. However, these mitochondrial ribosomal proteins are found to be larger in the size than their bacterial counterparts mainly because of their extended domains. Furthermore, cross-linking studies indicated that both yeast and mammalian mitoribosomes have also acquired some novel ribosomal proteins that do not share any homology with the bacterial ribosomal proteins. The functional significance of these mitospecific regions and the novel mitoribosomes specific proteins remains largely unknown and their possible role for the mitoribosomes will be discussed in next section.

1.3.3 Functional significance of mitospecific regions and mitoribosome-specific proteins in mitorobisomes

As outlined above, mitoribosomes contain novel ribosomal proteins, called mitoribosome-specific ribosomal proteins. Additionally, many ribosomal proteins with bacterial ancestors have acquired the N-and C-terminal mitospecific extension sequences in addition to their bacterial homology domain. The N-terminal extensions in many cases are cleavable mitochondrial targeting sequences required to ensure the targeting of the nuclearly-encoded ribosomal proteins into the mitochondria. The C-terminal mitospecific sequences (permanent extension sequences on the proteins), however are unique to each ribosomal protein, but often are conserved among their relatives.

What is the function of the mitoribosome-specific proteins or the mitospecific regions that some conserved proteins have acquired during evolution? One possibility could be that the new proteins or mitospecific regions may compensate for the loss of

rRNA components that act as scaffold for the ribosome stability. Since, during evolution mitoribosomes have lost rRNA components like 5S rRNA, the newly recruited sequences or proteins may stabilize the general structure of ribosomes. Furthermore, gain of new proteins or mitospecific region may be a result of adaptation of the specialized translation machinery in mitochondria. As previously mentioned, the mitoribosomes are found to be tightly tethered to the inner membrane, presumably to support co-translational insertion, and are thought not to cycle between the soluble and membrane populations. What keeps the ribosomes tethered to the membrane is, however, not clear. It is therefore conceivable that one of the functions of mitoribosome specific proteins or sequences is to ensure the tight association of ribosomes with the inner membrane. Finally, the mitospecific regions of ribosomal proteins may be important for the stable assembly of ribosomal proteins at the inner membrane. Such a notion is supported by the finding that the modulation in the mitospecific region of the MrpL40 protein, located at the exit site, compromises the ribosomal assembly (Jia *et al*, 2009). We propose here that the mitospecific region of Mrp20 and other ribosomal proteins at the exit site such as MrpL40 may be important for the assembly of mitoribsomes and their function. Therefore, in the present study, the functional relevance of the mitospecific region of the Mrp20 protein will be analyzed.

1.4 Assembly of mitoribosomes

1.4.1 Overview of assembly of the mitoribosomes

The 74S yeast mitoribosomes (54S large ribosomal subunit and 37S small ribosomal subunit) are composed of proteins encoded by two genetic systems, in contrast to bacterial and eukaryotic cytosolic ribosomes where all the ribosomal proteins and rRNA are produced by one genetic system (Grivell, 1995). In mitoribosomes, with the exception of a small ribosomal subunit, Var1, all other ribosomal subunits are encoded by the nuclear genome. The nuclearly-encoded subunits are imported into the mitochondria and in most cases, after the proteolytic maturation of their N-terminal mitochondrial targeting sequences, become assembled with the Var1 protein and the rRNA scaffold (both encoded by mitochondrial DNA). However, how such an intricate coordination between the two genetic systems and how the assembly of such a large oligomeric structure is achieved to result in the fully assembled ribosomes in the mitochondria is still unclear.

Little is known about the factors required for ribosomal assembly or the stages involved in the assembly of mitoribosomes in contrast to what is known about the bacterial ancestors. For instance, in bacterial ribosomes, conventional genetic studies have identified various assembly factors which include RNA helicases, RNA modifying enzymes (methyltransferases) and a large number of GTPases. On the other hand, to date only three GTPases, Mgt1 (Mitochondrial GTPase) in yeast and ERAL1 (Era G-protein-like 1) and NOA1 (Nitric oxide GTPase) in mammals have been identified in the mitoribosomes. These conserved GTPases are proposed to be important for the ribosomal biogenesis but their exact mechanism is not clear yet (Barrientos *et al*, 2003; Britton, 2009; Dennerlein *et al*, 2010; Kolanczyk *et al*, 2010).

Finally, a major question that remains unanswered in the field of mitoribosomes assembly is where the assembly of ribosomal proteins take place. As mentioned previously in Section 1.2.2, the mitoribosomes are found tightly associated with the inner membrane. However, it is not clear where the assembly process is initiated and at what stage during the formation of translationally-active ribosomes, do mitochondrial ribosomal proteins become tethered to the membrane.

1.4.2 Assembly of mitoribosomes is completed at the inner membrane

A recent study done by Nolden *et al*, has suggested that a late stage assembly intermediate of the large ribosomal subunit, termed the pre-54S particle, assembles and exists as a soluble component in the matrix, and only becomes targeted to the inner membrane to complete the final stage of assembly. The final steps of large ribosomal subunit assembly includes the incorporation of the membrane bound MrpL32 protein, a component of the large ribosomal subunit, which is first proteolytically matured by the inner membrane protease, the Yta10/Yta12 complex (Nolden *et al*, 2005). This study therefore demonstrates that the ribosomal large subunit assembly is completed at the inner membrane, however, little is known about the earlier steps which result in the production of the pre-54S particle. Furthermore, it is not yet investigated whether the pre-54S particle actually assembles as a soluble species in the matrix, or if the inner membrane serves as a platform for the early ribosome assembly events. In this dissertation, the possible role of the mitospecific region of Mrp20 protein for its involvement in the early ribosomal assembly events has been investigated.

1.5 Objectives of the research

1.5.1 Investigate the functional importance of the mitospecific region of Mrp20 protein

Mrp20 protein is an essential component of the large ribosomal subunit of mitoribosomes and contains a domain homologous to bacterial ribosomal protein L23, which is located at the tunnel exit site of the bacterial ribosomes. Based upon homology of Mrp20 with the bacterial L23 protein and its ability to cross-link with the Oxa1 protein, Mrp20 is also suggested to be located at the exit site of the yeast mitoribosomes. In addition to its homology with L23 protein, like many ribosomal proteins during the evolution of mitoribosomes, Mrp20 protein has acquired a mitospecific region at its C-terminus. However, the functional importance of this region of Mrp20 for the mitoribosomes is not well studied. In this study, data to indicate that the mitospecific region of the ribosomal proteins located at the tunnel exit site may be important for the stable assembly of ribosomal proteins into translationally competent ribosomes and for the OXPHOS function is presented. Chapter 3, section 3.1 comprises the results from a study investigating the functional importance of mitospecific region of the Mrp20 protein. A yeast strain carrying a truncation in the C-terminal region of Mrp20, the *mrp20*Δ*C* mutant was created. The mitochondria isolated from this strain were used to examine the stability of ribosomal proteins and the stability of subunits of OXPHOS complexes. The assembly of ribosomes was analyzed by biochemical approaches such as sucrose sedimentation analysis.

1.5.2 Identify the interacting partners of Mrp20ΔC protein and the presence of ribosomal subcomplex in the mrp20ΔC mutant

The pre-54S is the only ribosomal intermediate particle in the assembly pathway of mitoribosomes that has been identified and studied to date. Little is known about the events in early stages of ribosomal assembly.It is unknown whethere these are smaller and individual ribosomal subcomplexes that may be present prior to the formation of characterized pre-54S ribosomal particle. The identification of new ribosomal assembly subcomplexes or intermediates would be therefore important to further understand the process of mitoribosomes assembly. In this objective, using a *mrp20ΔC* mutant, a novel subcomplex of ribosomal proteins was identified. It was reasoned that although this subcomplex in the *mrp20ΔC* mutant could ensure the stability to ribosomal proteins but the progression to the fully assembled ribosomes was defective.

1.5.3 Investigate mrp20ΔC mutant to examine where the early ribosome assembly events occur relative to the inner membrane

The tight association of the completely assembled ribosomes with the inner membrane is well documented (Liu & Spremulli, 2000; van der Klei *et al*, 1994). However, it is not known if the earlier stages of ribosomal assembly process occur at the membrane or in the matrix. Previous studies have depicted the ribosome assembly intermediate, the pre-54S particle, as being assembled in the matrix and then targeted to the membrane for its assembly to the 54S large ribosomal subunit.Studies have been performed to analyze whether the pre-54S particle exist as soluble species. In this study using ribosomal assembly mutants such as *mrp20ΔC*, *Δyta10* and wild type rho^0, the membrane

association ability of the ribosomal proteins was analyzed with a goal to understand where the early ribosomal assembly events occur.

1.5.4 To identify the factors responsible for the association of mitoribosomes with the inner membrane

Mitoribosomes are membrane bound in a salt-sensitive fashion, i.e. ribosomal proteins can be released *in vitro* from the membrane with the increasing salt concentration. Previous studies in bovine mitoribosomes have suggested that ribosomes may form a detergent-resistant platform complex under low-salt conditions (Liu & Spremulli, 2000). Whether this detergent-resistant patch that recruits ribosomal proteins is composed of some protein complexes possibly in the inner membrane remains unanswered. In this study, we investigated the possibility in yeast mitochondria. A pilot study was initiated by using mass-spectrometry approach to identify the proteins that may be responsible for the detergent-resistant extraction behavior of ribosomal proteins.

Chapter 2 Materials and Methods

This chapter describes the chemicals and biological reagents as well as molecular biology, cell biology and protein chemistry methods used in the study.

2.1 Materials

2.1.1 Chemical reagents

Chemical reagents used in the study are listed in Table 2.

2.1.2 Oligonucleotides

Oligonucleotides used in this study in polymerase chain reactions (PCR) for construction of plasmids, generation of yeast mutants and verification of the mutant strains are listed Table 3.

2.1.3 Plasmids

The yeast integral plasmids, Yip351 GAL10-Mrp20ΔC, Yip351GAL10-Mrp20ΔCHis, Yip352GAL10-MrpL25$_{His}$, Yip352GAL10-MrpL27$_{His}$, Yip352GAL10- MrpL35$_{His}$ and Yip352GALPrx1$_{His}$, have been constructed by modification of the Yip351 and Yip352 plasmid (Hill *et al*, 1986). For cloning of Mrp20ΔC and Mrp20ΔC$_{His}$, their respective DNA fragments were amplified by PCR (primer pairs: ST556/ST557 and ST 734/ ST736, respectively) and cloned into the XbaI and PstI sites of the Yip351 vector downstream of

the GAL10 promoter. The DNA fragment encoding the His-tagged Mrp20ΔC protein was generated by incorporating 8 His-coding codons prior to the translational stop codon into the 3' reverse primer. A similar PCR strategy was used to amplify and clone the DNA fragments encoding 8 his-tagged MrpL25, MrpL27, MrpL35 and Prx1 proteins using primers as described in Table 3. The resulting PCR fragments were cloned into the XbaI/PstI (for Prx1) and BamHI/PstI (for MrpL25, MrpL27 and MrpL35) site of the Yip352 vector containing the GAL10 promoter.

The yeast centromeric plasmids, pRS316-MRP20 and pRS413-MRP20 have been constructed by modification of the pRS316 and pRS413 plasmids (Sikorski & Hieter, 1989). The 1.2 kb EcoRI/XbaI fragment of MRP20 gene containing 300bp 5' upstream ad 50bp 3'downstream (using primer pairs: ST601/ST602) was inserted into the corresponding polylinker site of the pRS316 or pRS413 plasmid.

The pET Duet-1 vector (Novagen Inc., Madison, WI) was used for bacterial co-expression studies. The pET Duet-1 $_{His}$MrpL40mt -Mrp20 construct was prepared by inserting ~0.6kb (270-891nt) EcoRI/HindIII fragment of MRPL40 gene and ~0.8kb (1-789nt) NdeI/XhoI fragment of MRP20 into the two polylinker sites of the vector. Similarly, pET Duet-1 Mrp20 – MrpL40mt construct was prepared by inserting ~0.8kb (1-789nt) NcoI/HindIII fragment of MRP40 gene and ~0.6kb (271-891nt) NdeI/XhoI fragment of MRPL40 into the two polylinker sites of the vector.

2.1.4 Saccharomyces cerevisiae (S.cerevisiae) strains

Yeast strains used in the study are listed in Table 4.

2.1.5 Antibodies

Antibodies used in this study are listed in Table 5.

2.2 Molecular biology techniques

The basic molecular biology techniques such as PCR reactions, restriction enzyme digestion of DNA, DNA precipitation, gel electrophoresis and gel purification of DNA, DNA ligation, preparation of *E.coli* competent cells, transformation of *E.coli*, colony PCR of *E.coli* cells, plasmid DNA extraction, DNA concentration determination, preparation of *S. cerevisiae* competent cells, transformation of yeast *S. cerevisiae* and genomic DNA isolation were done according to Stuart lab protocols adapted from the book, Molecular Cloning: A Laboratory Manual by Sambrook, Fritsch and Maniatis.

2.2.1 Site directed mutagenesis of MRP20

MRP20 sequence was mutagenized by polymerase chain reaction using protocol by the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The centromeric plasmid, pRS413-MRP20 was used as template for the PCR reaction. The base triplet TTT encoding phenylalanine 226 of MRP20 was replaced by GCC coding alanine (using primer pairs: ST655/ST656). Similarly, the base triplet CCT encoding proline 228 was changed to GCC coding for alanine (using primer pairs: ST657/ ST658). The exact sequences can be found in Table 3. The mutagenesis reaction was prepared following the manufacturer's protocol containing:

2.5µ10X reaction buffer

25 ng of dsDNA template

1µl (20pM) of forward oligonucleotide primer

1µl (20pM) of reverse oligonucleotide primer

0.5µl of dNTP mix (0.2mM of dATP, dCTP, dGTP, dTTP)

0.5µl of *Pfu ultra* DNA Polymerase (25 U/µl)

ddH_2O to a final volume of 25 µl

The cycle parameters for the QuikChange Site-Direct Mutagenesis method are as follows:

Segment	Cycles	Temperature	Time
1	1	95^{o}C	30 seconds
2	14	95^{o}C	30 seconds
		55^{o}C	1 minute
		68^{o}C	1 minute

DpnI Restriction Digestion of Parental Plasmid

The wild type parental pRS413-MRP20 (Amp^R) plasmid used for genetic manipulation was previously amplified and isolated from a Dam^+ strain of *E. coli*, thus yielding a methylated pRS413-MRP20 (Amp^R) plasmid. The QuikChange PCR kit was used to create a non-methylated mutated derivative of the pRS413-MRP20 plasmid which was used for subsequent cloning steps. The methylated template plasmid was digested by adding 1µl of DpnI restriction enzyme to the 50 µl QuikChange PCR reaction (outlined above) and incubating the sample at 37^{o}C for 1-2 hrs. Following the DpnI restriction digestion, the DpnI restriction enzyme was heat-killed by 10 minute incubation

at 65^{o}C. The sample was then incubated on ice for 1 minute and the non-methylated mutated pRS413-MRP20 (Amp^{R}) plasmid was transformed into bacterial competent cells (MH1) and plated on to LB +Amp plates (Table 5) for selection of positive transformants. DNA sequencing was also performed to verify the mutated residue and no additional mutations within the ORF of interest were present.

2.2.2 S. cerevisiae strains and genetic techniques

All strains used in this study were isogenic to W303-1A.

Construction of mrp20ΔC strain

For generation of the *mrp20ΔC* strain, the codons for amino acids 240-263 of the chromosomal MRP20 gene (primer pair: ST556/ST557 and the flanking primer pair: ST558/ST 559) were replaced by a translational stop codon followed by HIS3 auxotrophic cassette through homologous recombination method. The truncation in the C-terminal region was identified by PCR based analysis using primers ST560/ST561. Western blot analysis with Mrp20 antibody was performed to verify the expression of truncated Mrp20 protein.

Construction of MRP20 knock-out strain

Null mutant strain of MRP20 gene, *Δmrp20* was created by introduction of the 1.2 kb PCR amplified kanamycin resistance gene (KAN^{r}) flanked with 25bp upstream and 25bp downstream sequence of MRP20 gene (using ST601 and ST602) into the MRP20 locus of wild-type cells that were complemented with centromeric plasmid, pRS316-MRP20 to prevent the loss of mitochondrial DNA in wild type cells (cells with null mutants in ribosomal proteins have high frequency to lose mitochondrial DNA and go ρ^{0}. A

complete replacement of the MRP20 gene open reading frame (ORF) by the KAN^r PCR product was achieved using homologous recombination (Wach *et al*., 1994). The null *mrp20* strains were verified by PCR based method using ST621, ST622 primers.

Construction of yeast strains over-expressing Mrp20ΔC and His- tagged derivatives of Mrp20ΔC, MrpL25, MrpL27, MrpL35 and Prx1

For over-expression of the Mrp20ΔC and $Mrp20\Delta C_{His}$ proteins, the recombinant Yip351 plasmids as described in section 2.1.3 were linearized with the restriction enzyme BstEII and transformed into *mrp20ΔC* (HIS+) strain. HIS^+, LEU^+ transformants were selected, and the level of expression of the Mrp20ΔC protein was verified.

For the over-expression of His-tagged MrpL25, MrpL27, MrpL35 and Prx1 proteins, the recombinant Yip352 plasmids containing the respective genes under GAL10 promoter (as described in section 2.1.3) were linearized with NcoI and transformed into *mrp20ΔC* strain, harboring the Yip351-GAL10 driven Mrp20ΔC expression system. URA^+, HIS^+, LEU^+ transformants were selected and the expression of the $MrpL25_{His}$ or $MrpL27_{His}$ or or $MrpL35_{His}$ or $Prx1_{His}$ and Mrp20ΔC proteins were confirmed.

Construction of yeast strains expressing mutated $Mrp20F_{226}A$ or $Mrp20P_{228}A$

The centromeric plasmid, pRS413 (with HIS3 auxotrophic marker) containing $MRP20F_{226}A$ or $Mrp20P_{228}A$ were transformed in the Δ*mrp20* yeast strain (*mrp20::*KAN^r*)* complemented with pRS316-MRP20 plasmid. The URA+HIS+ transformants were selected and analyzed for their growth on YP-glycerol containing media. The transformants were cured of pRS316-MRP20 plasmid using 5-fluoro orotic acid (5-FOA) selection procedure (Uracil encoding gene can convert 5-fluoro orotic acid

to 5-Fluro uracil that is toxic to the cells). URA-HIS+ transformants were selected and analyzed for the growth phenotype on glycerol- containing media (YPG) by replica printing.

2.2.3 DNA sequencing

DNA sequencing was performed at the Functional Biosystems Company, Madison.

Table 2 Chemical reagents used in this study

Source	Name
AAPER Alcohol and Chemical (Shelbyville, KY)	Ethanol
Angus (Niagara Falls, NY)	Yeast nitrogen base
Becton, Dickinson and Company (Sparks, MD)	Bacto™ Agar
	Bacto™ Peptone
	Bacto™ Tryptone
	Bacto™ Yeast extract
EM Science (Gibbstown, NJ)	Ammonium acetate
	Ammonium chloride (NH_4Cl)
	Ammonium persulfate (APS)
	Ammonium sulfate ($(NH_4)_2SO_4$)
	Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$)
	Dimethyl sulfoxide (DMSO)
	Ethylenediamine tetraacetic acid (EDTA)
	Ethylene glycol bis-(2-aminoethyl ether)-tetraacetic acid (EGTA)
	Glucose
	Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)
	Potassium chloride (KCl)
	Phenylmethylsulphonyl fluoride (PMSF)
	Sodium acetate
	Sodium chloride (NaCl)
	Sodium phosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$)
	Sucrose
	N,N,N',N'-tetramethylethylene diamine (TEMED)

	Triton X-100
Fisher Scientific (Fair Lawn, NJ)	Acetone
Fluka (Buchs, Germany)	Sodium carbonate anhydrous
GE Healthcare (Arlington Heights, IL)	Redivue L-[^{35}S]methionine
ICN biomedicals (Aurora, Ohio)	Geneticin disulfate salt (G418)
Invitrogen (Carlsbad, CA)	Acrylamide
	Agarose (Ultra pure)
Mallinckrodt (Paris, KY)	Potassium hydroxide (KOH)
Mallinckrodt Baker (Phillipsburg, NJ)	Acetic acid
	Sodium dodecyl Sulfate (SDS)
Pierce (Rockford, IL)	MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester)
Promega (Madison, WI)	dNTP (dATP, dCTP, dGTP, dTTP)
Roche (Mannheim, Germany)	Guanosine-5'-triphosphate (GTP)
	NADH
	Phosphoenolpyruvate (PEP)
Serva (Heidelberg, Germany)	Ponceau S
Shelton Scientific (Shelton, CT)	Phenol/chloroform/isoamylalchol (25:24:1)
Sigma (St. Louis, MO)	Adenine
	Adenosine 5'-triphosphate (ATP)
	5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol)
	6-aminohexanoic acid
	Ampicillin
	Bisacrylamide
	BIS-TRIS
	Bromphenol Blue
	Citric acid, monohydrate
	p-Coumaric acid
	L-Cysteine
	Ethidium bromide
	Galactose
	Glycerol
	Glycine
	L-Histidine
	Hydrogen peroxide (30% w/v, H_2O_2)
	Hydrogen chloride (HCl)
	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES)
	Imidazole
	α-ketoglutaric acid
	DL-Lactic acid

	Lead nitrate [$Pb(NO_3)_2$]
	L-Leucine
	Lithium Acetate (LiOAc)
	Magnesium Chloride ($MgCl_2$)
	2-mercaptoethanol
	Methionine
	Potassium phosphate (KH_2PO_4)
	2-Propanol
	Silver nitrate
	Sodium hydroxide (NaOH)
	Sodium bicarbonate ($NaHCO_3$)
	Sodium phosphate anhydrous (Na_2HPO_4)
	Sodium thiosulfate pentahydrate
	Sorbitol
	Trichloroacetic acid (TCA)
	Tricine
	Trizma base
	L-Tryptophan
	Uracil
US Biological (Swampscott, MA)	5- Fluoro Orotic Acid Dropout mix synthetic minus Ade, His, Leu, Trp, Ura, w/o yeast nitrogen base
	Tryptone
VWR (West Chester, PA)	Methanol

Table 3 Oligonucleotides used in this study

Name	Sequence
ST556 Mrp20ΔC S1	CGGGTGGCCCAGCCTTTTATACCTCGCTTCTTAAA ACGAGAGATAGACAATAAGCGATGACGTACGCT GCAGGTCGAC
ST557 Mrp20ΔC S2	GCTTATCTGGGTATTTGCGTGTGGGTTAACGAAG GGGTGGAATGGGAAACCTTATCGATGAATTCGAG CTCG
ST558 Mrp20ΔC S1’	GACGCCAACAAACCCGGCACAGCATTCGACGGA

Name	Sequence
	GTCGTGGGGCCTTACGAACGGGTGGCCCAGCCTTTTATACCTCGC
ST559 Mrp20ΔC S2’	CTGTTTAGAGATTATGTAGTTTGCTCCGTATATACAAGTGTGAGGGGTGCAAGATGCTTATCTGGGTATTTGCG
ST560 Mrp20 A1	CCCGAAGAACCCCGTCCGGA
ST561 Mrp20A2	CTGTTTAGAGATTATGTAGT
ST601 Mrp20-EcoRI For	GGG GAATTC CGACGAAGTGAGCTTGTCTAC
ST602 Mrp20-XbaI Rev	GGGTCTAGA GCAAGATGCTTATCTGG
ST603 Mrp20S1	GATTAGCAGGCTCCAACAGAAAAAAAAATAAAATAAAAATAAAAAGTACGCGTACGCTGCAGGTCGAC
ST604 Mrp20S2	GTATTTGCGTGTGGGTTAACGAAGGGGTGGAATGGGAAACCTTCTAATCGATGAATTCGAGCTCG
ST605 Mrp20A1	GGAGAAGAGATAGGTGGACAG
ST606 Mrp20A4	CTGTTTAGAGATTATGTAG
ST621Mrp20S1’	CGTAACGCTACTAACAAATAATGTGCTTTGATTAGCAGGCTCCAACAG
ST622 Mrp20S2’	GTTTGCTCCGTATATACAAGTGTGAGGGGTGCAAGATGCTTATCTGGGTATTTGCGTGTGGG
ST645 Xba_Mrp20ΔC	GGGTCTAGAATGCCACGATTGACGGTTGGAACG
ST646 PstI _Mrp20ΔC	GGGCTGCAGCTATCGCTTATTGTCTATCTCTCG
ST655 Mrp20FA For	CGAACGGGTGGCCCAGCCTGCCATACCTCGCTTCTTAAAACG
ST656 Mrp20FA Rev	CGTTTTAAGAAGCGAGGTATGGCAGGCTGGGCCACCCGTTCG
ST657 Mrp20PA For	GGGTGGCCCAGCCTTTTATAGCCCGCTTCTTAAAACGAGAG
ST658 Mrp20PARev	CTCTCGTTTTAAGAAGCGGGCTATAAAAGGCTGGGCCACCC
ST669 MrpL40EcoRI For	GGGGAATTCGAATTCATTCATATTAGATGAGAATGGGCCC
ST670 MrpL40HindIII Re	GGGAAGCTTCGTGAATAGTGAAGGGGATATACT
ST675 Mrp20HindIII Rev	GGGAAGCTTCTAATGTAGATCTTCTATGTACCTGTTCAGGGC
ST676 MrpL40NdeI	GGGCATATGAATTCATTCATATTAGATGAGAATGGGCCC
ST734 Mrp20ΔCHis Rev	GGGATGCATCTA*ATGGTGATGGTGATGGTGATGGT GT*CGCTTATTGTCTATCTCTCGTTTTAAGAAGCG
ST736 Mrp20ΔCHis Nde XbaI For	GGGTCTAGACATATGCCACGATTGACGGTTGGAACGAAGAATATG

Name	Sequence
ST738 Mrp20NcoI For	GGGCCATGGGCCCACGATTGACGGTTGGAACGAA GAATATG
ST739 MrpL40XhoI Rev	GGGCTCGAGCGTGAATAGTGAAGGGGATATACTG
ST748 Prx1 PstI Rev	GGGCTGCAGCTAATGGTGATGGTGATGGTGATGG TGTTATTTCGACTTGGTGAATC
ST751Prx1 XbaI For	GGGTCTAGAATGTTTAGTAGAATTTGTAGCGC
ST753 MrpL27 BamHI For	GGGGGATCCATGAAAGGCTCACCCATTTCTC
ST754 MrpL27 PstI Rev	GGGCTGCAGCTAATGGTGATGGTGATGGTGATGG TG TCCACGCTCCTTATAACATG
ST783 MrpL35 BamHI SpeI For	GGGGGATCCGGGACTAGTATGTTACGAAGATCTA TTCATACC
ST784 MrpL35 PstI Rev	GGGCTGCAGTCAATGGTGATGGTGATGGTGATGG TGCCTTCTAACTCTGCTAAAAACCCTTCC
ST785 MrpL25 BamHI XbaI For	GGGGGATCCGGGTCTAGAATGTCATACAAACAGT ATTTTGATAGTTTGCC
ST786 MrpL25 PstI Rev	GGGCTGCAGTCAATGGTGATGGTGATGGTGATGG TGGAACCAGCCTATACTTTGG

Table 4 Genotypes and sources of yeast strains used in this study

Name	Genomic markers	Reference
W303-1A	*MATa, ade2-1 his3-15 leu2-3,112 trp1-1 ura3-1*	Dr. Tzagoloff
W303-1B	*MATα, ade2-1 his3-15 leu2-3,112 trp1-1 ura3-1*	Dr. Tzagoloff
rho^0 W303-1A	*MATa, ade2-1 his3-15 leu2-3,112 trp1-1 ura3-1*	Dr. Tzagoloff
rho^0 W303-1B	*MATα, ade2-1 his3-15 leu2-3,112 trp1-1 ura3-1 OXA1Δ165::KAN^r*	Dr. Tzagoloff
Δ*oxa1*	*MATa, ade2-1 his3-15 leu2-3,112 trp1-1 ura3-1 oxa1::KAN^r*	Dr. Stuart
Δ*yta10*	*MATa ade2-1 his3-11,15 trp1-1 leu2,112 ura3-52 yta10::URA3*	Dr.Langer
Mrp20ΔC	*MATa, ade2-1 his3-15 leu2-3,112 trp1-1 ura3-1 MRP20ΔC23-HIS3*	This study
Δ*mrp20*+Mrp20F_{226}A	*MATa, ade2-1 HIS3-MRP20F_{226}A leu2-3,112 trp1-1 ura3-1 mrp20::KAN^r*	This study
Δ*mrp20*+Mrp20P_{228}A	*MATa, ade2-1 HIS3-MRP20P_{228}A leu2-3,112 trp1-1 ura3-1 mrp20::KAN^r*	This study

Mrp20ΔC +GAL-Mrp20ΔC	*MATa, ade2-1 his3-15 LEU2- Mrp20ΔC, 112 trp1-1 ura3-1 MRP20ΔC23-HIS3*	This study
Mrp20ΔC +GAL-Mrp20ΔCHis	*MATa, ade2-1 his3-15 LEU2-Mrp20ΔCHis,112 trp1-1 ura3-1 MRP20ΔC23-HIS3*	This study
Mrp20ΔC +GAL-Mrp20ΔC+ GAL-$Prx1_{His}$	*MATa, ade2-1 his3-15 LEU2-Mrp20ΔC,112 trp1-1 URA3-PRX1His MRP20ΔC23-HIS3*	This study
Mrp20ΔC +GAL-Mrp20ΔC+ GAL-$MrpL25_{His}$	*MATa, ade2-1 his3-15 LEU2-Mrp20ΔC,112 trp1-1 URA3-MRPL25His MRP20ΔC23-HIS3*	This study
Mrp20ΔC +GAL-Mrp20ΔC+ GAL-$MrpL27_{His}$	*MATa, ade2-1 his3-15 LEU2-Mrp20ΔC,112 trp1-1 URA3-MRPL27His MRP20ΔC23-HIS3*	This study
Mrp20ΔC +GAL-Mrp20ΔC+ GAL-$MrpL35_{His}$	*MATa, ade2-1 his3-15 LEU2-Mrp20ΔC,112 trp1-1 URA3-MRPL35His MRP20ΔC23-HIS3*	This study

Table 5 Antibodies used in this study

Targeted epitope	Source	Reference
Primary Antibodies		
AAC	Rabbit	Dr. Stuart
Cox1	Mouse	Invitrogen (Carlsbad, CA)
Cox2	Rabbit	Dr. Stuart
Cpr3	Rabbit	Dr. Stuart
Cyt b_2	Rabbit	Dr.Stuart
Rieske FeS	Rabbit	Dr.Stuart
Hemagglutinin tag	Rabbit	Covance
His tag	Rabbit	Bethyl (Montgomery, TX)
Mrp20	Mouse	Dr. Mason (Amherst, MA)
Mrp10	Rabbit	Dr. Tzagoloff (Columbia, NY)
MrpL4	Rabbit	Dr.Ott (Kaiserslautern)
MrpL32	Rabbit	Dr. Langer (Köln)
MrpL40	Rabbit	Dr.Stuart
MrpL36	Rabbit	Dr. Herrmann (Kaiserslautern)
Mrp49	Mouse	Dr. Boguta (Warsaw)
Mrp7	Mouse	Dr.Mason (Amherst, MA)
Su e of ATP synthase	Rabbit	Dr. Stuart
Tim17	Rabbit	Dr. Stuart

Secondary Antibodies		
Anti-mouse IgG, horseradish peroxidase linked whole antibody	Sheep	Amersham Biosciences (England, UK)
Anti-rabbit IgG (whole molecule)-peroxidase antibody	Goat	Sigma-Aldrich (St. Louis, MO)

2.3 Cell Biology

2.3.1 Growth of S. cerevisiae strains

For the isolation of the genomic DNA or the preparation of competent cells, yeast cells from a given strain were grown in YPAD medium. Only for the preparation of competent cells from Δ*mrp20* strain that was complemented with pRS316MRP20, cells were grown in SC medium lacking uracil. For the isolation of mitochondria by either by quick preparation or long preparation, cells were grown in YP-Lactate media containing 2% galactose. The details for the ingredients of the media can be found in Table 6.

2.3.2 Serial dilution method for the growth analysis

The serial method is used to study the cell growth on plates. Yeast cells were first maintained on YPAD plates at 30°C for 48 to 72 hours and then transferred from the plates into 500 µl sterile H_2O. The OD_{580nm} of the yeast cell suspension was determined and equivalent OD units (10 to 50 OD units) of the yeast cells were prepared in sterile H_2O. The yeast cells were further serially diluted, 1:10, 1:100, 1:1,000 and 1:10,000. 2 µl of each diluted sample was plated onto YPD, YPG or YPG+0.1% galactose (to induce the GAL10 promoter) medium and one of each type of plate was subsequently incubated

at 30°C for 24 to 72 hours. The resulting plates were scanned to document the growth of the yeast strains.

2.3.3 Isolation of mitochondria

Mitochondria were isolated from a given yeast strain to study various functions of the protein of interest. For isolation of mitochondria, three different procedures are used in our laboratory: Long preparation, which yields pure, intact and large quantities of mitochondria. Quick preparation was adapted for fast and easy isolation of mitochondria from yeast strains.

2.3.3.1 Long preparation procedure

Mitochondria from a given yeast strain was isolated from 4 L of appropriate medium (usually YP Lactate +2% galactose), which was usually harvested at mid-logarithmic phase. Mitochondria from yeast cells were isolated according to the method of (Daum *et al*., 1982) with modifications (Herrmann *et al*., 1994). Isolated mitochondria were resuspended in SEM buffer (250 mM sucrose, 2 mM EDTA, 10 mM MOPS-KOH pH 7.2). After protein determination (2.4.13), mitochondria were aliquoted at protein concentration of 10 mg/ml in 25 µl aliquots. After fast freezing in liquid nitrogen, aliquots were stored at -80°C. Traditionally aliquots of these mitochondria are thawed only once and used fresh.

2.3.3.2 Quick preparation procedure

Mitochondria were usually isolated from this procedure to screen the yeast transformants to examine the success of an expression of the protein of interest. For this purpose, 5 ml of yeast cells were grown overnight at 30°C in YPG with 2% galactose media. Cells were

harvested by centrifugation (2,200 rpm, 3 min, RT) and resuspended in a total of 1.2 ml SH buffer (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.2) containing freshly made PMSF (1 mM). Cell suspension was divided equally between microfuge tubes and glass beads (0.1 g) were added on ice. Cell suspension was vortex for 5-6 times each for 30 s, and in between samples was left on ice to cool down. After vortexing, samples were centrifuged (3,000 rpm, 3 min, 4°C) and supernatant which contained isolated mitochondria were removed into a fresh tube. Samples were pooled and centrifuged (12,000 rpm, 15 min at 4°C) and to the pellet 50 µl of 1X Laemmli buffer (2% SDS, 10% glycerol, 0.01% bromophenol blue, 2.5 % β-mercaptoethanol, 60mM Tris, pH 6.8) were added. Each sample was heated for 5 min at 95°C and was analyzed by SDS-PAGE, Western blotting and immunedecoration with the appropriate antibody.

2.3.4 Mitochondrial in organello translation

In organello labeling of mitochondrial translation products were essentially done as described previously (Hell *et al*, 2001). Mitochondria (100 µg protein) were incubated in translation buffer (0.6 M sorbitol, 150 mM KCl, 15 mM KH_2PO_4, 13 mM $MgSO_4$, 0.15 mg/ml all amino acids except methionine, 4 mM ATP, 0.5 mM GTP, 5 mM α-ketoglutarate, 5 mM phosphoenolpyruvate, 3 mg/ml fatty acid-free bovine serum albumin, and 20 mM Tris-Cl, pH 7.4) containing 0.6 units of pyruvate kinase (Roche, Indianapolis, IN) and 2 mM NADH. After 5 min of incubation at 25°C, 0.5 µl of [^{35}S] methionine (2-7.5 µCi) were added. Samples were incubated for 30 min at 25°C, and labeling was stopped by the addition of unlabeled methionine (30 mM) and puromycin (42 µg/ml). The samples were further incubated for 30 min to completely stop synthesis, and mitochondria were isolated by centrifugation, washed in 1 ml 0.6 M sorbitol, 20 mM

HEPES, pH 7.4, and lysed in 30 µl of 1X Laemmli buffer. Translation products were analyzed by SDS-PAGE and autoradiography.

2.3.5 Maintenance of yeast and bacterial strains

Bacterial stocks were prepared by mixing 500 µl of culture grown overnight in LB-Amp medium at 37°C with 500 µl of 100 % (v/v) glycerol sterilized in 2ml tubes and stored at -80°C. Yeast stocks were prepared by scraping a freshly grown culture from a plate (*e.g.* YPG or selective media) and resuspending the cells in 1 ml sterile 15% (v/v) glycerol. After mixing, tubes were immediately stored at -80°C.

2.4 Protein Biochemistry

2.4.1 Chemical cross-linking of proteins in isolated mitochondria

Mitochondria (200 µg of total protein) were suspended in 600 µl of SH buffer (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.2) and cross-linked by MBS (0.5 mM) for 30 min on ice. Excess MBS was quenched by addition of 30 µl of glycine (1 M, pH 8.0). Mitochondria was re-isolated by centrifugation (15,000 rpm, 15 min, at 4°C) and washed with 1 ml SH buffer (15,000 rpm, 15 min, at 4°C) for further analysis.

2.4.2 Mrp20 and cross-linked adduct immunoprecipitation

For the immunoprecipitation of Mrp20 and its cross-linked adducts, mitochondria (200µg total protein) following MBS cross-linking were solubilized in SDS (1%) buffer

and cooked (5 min at 95°C), followed by dilution into immunoprecipitation (IP) buffer (1% Triton X-100, 300 mM NaCl, 10 mM Tris-HCl, pH 7.4) for 30 min on ice. After a clarifying spin (20,860 *x* g; 15 min at 4°C), the supernatants were incubated overnight at 4°C with protein A-Sepharose beads and 30µl of culture supernatant containing the Mrp20 monoclonal antibody. The beads were washed three times with IP buffer and twice with IP buffer without Triton X-100. Bound proteins were eluted with SDS-sample buffer containing 5% (vol/vol) β-mercaptoethanol. The immunoprecipitated Mrp20 and cross-linked adducts were analyzed by SDS-PAGE, Western blotting, and immunedecoration with MrpL40-specific antiserum.

2.4.3 Ni NTA purification

For Ni-nitrilotriacetic acid (Ni-NTA) purification of His-tagged protein and their interacting partners, isolated mitochondria (200 µg total protein) were resuspended in 200 µl of lysis buffer (20mM HEPES, 160mM KCl, 1mM phenylmethylsulfonate fluoride, PMSF and 1% Triton X-100) for 30 min on ice. After a clarifying spin (9271 *x* g, 10 min at 4^{o} C), the supernatant was collected, supplemented with imidazole (30 mM final concentration) and incubated with Ni-NTA beads (pre-equilibrated in lysis buffer) for 1 h at 4^{o} C. The beads were then washed three times with lysis buffer containing imidazole and the bound proteins were eluted with SDS-sample buffer containing 5% (vol/vol) β-mercaptoethanol and 450mM Imidazole. Samples were analyzed by SDS-PAGE and Western blotting. To purify the interacting partners for mass spectrometry, 1 mg of mitochondria were used for the affinity purification step and the eluted proteins were

separated by SDS-PAGE following silver staining. The bands were excised from the silver gel and analyzed by mass spectrometry.

2.4.4 Recombinant co-expression of Mrp20 and mitospecific region of MrpL40 and Ni-NTA purification

pET Duet-1 constructs as described in section 2.1.3 with a T7 expression system were introduced in the BL21 *E.coli* strain (Novagen, Inc. Madison, WI). The cells (5ml in LB+AMP media) containing the recombinant plasmids were allowed to grow at 37^{o}C till they reached the $O.D_{600}$ of 0.6 and then induced with 0.5mM isopropylthio-β-*D*-galactoside (IPTG). After 4 hrs of induction at 37^{o}C, cells were collected and pelleted at room temperature. The pellet was resuspended in 600μL of buffer B (50mM Sodium phosphate and 300mM NaCl, pH-7.0, 1mM PMSF) and the cell lysate was sonicated in ice bath using Branson microtip250 sonifier for 30-40s (10sx3) with an interval of 90s. The sonicated supernatant was then subjected to clarifying spin at 9271 *x* g for 10 min at 4^{o}C. Following centrifugation, 5% of the supernatant (Total) was saved and rest was incubated with the Ni-NTA sepharose beads (pre-equilibrated with buffer B) and 20mM Imidazole for 1 hr at 4^{o}C with gentle rocking. After incubation, the beads were washed with buffer B containing 30mM Imidazole. The proteins were eluted with elution buffer (50mM Sodium phosphate, 300mM NaCl and 150mM Imidazole, pH-7.0). The eluted material (Bound) and the 5% supernatant (Total) were analyzed on SDS-PAGE followed by Western blotting and immunedecoration with MrpL40 and Mrp20 antiserum.

2.4.5 Triton X-100 solubilization of mitochondria and sucrose density sedimentation analysis

Sucrose gradient analysis of detergent-solubilized mitochondrial ribosomes was performed essentially as previously published (Williams *et al*, 2005; Williams *et al*, 2004). Mitochondria (300 μg protein) were solubilized with 300 μl of lysis buffer (0.5% Triton X-100, 10 mM Mg-acetate, 0.1 M NaCl, 20 mM HEPES-KOH, pH 7.4, 1 mM PMSF) for 30 min on ice. The lysate was clarified by centrifugation at 30,000 *x* g for 30 min at 4°C, and the supernatant was layered onto an 11-ml continuous sucrose gradient (15 to 30%) containing 500 mM NH_4Cl, 10 mM Tris-HCl, pH 7.4, 10 mM Mg-acetate, 7 mM β-mercaptoethanol, and 0.5 mM PMSF. Gradients were centrifuged at 20,500 rpm for 17 h at 4°C in a Beckman SW41 Ti rotor (Optima L90K ultracentrifuge). Fractions (750 μl) were collected, trichloroacetic acid precipitated, and subjected to SDS-PAGE and Western blot analysis.

2.4.6 Digitonin solubilization of mitochondria and sucrose gradient centrifugation

Mitochondria (300 μg of protein) were solubilized with 600 μl of lysis buffer (0.25% (w/v) digitonin, 20 mM HEPES-KOH pH 7.2, 0.5 mM PMSF) for 30 min on ice as previously published in (Jia *et al*, 2003). Following a clarifying spin (21, 000 *x* g, 15 min at 4°C), the supernatant was loaded on top of a linear 20–40% linear sucrose gradient (11 ml) (in 20 mM HEPES-KOH pH 7.2, 0.1% digitonin, 0.5 mM PMSF). Following centrifugation (26000 rpm, Beckman: SW41 Ti rotor, Optima L90K ultracentrifuge, 16 h at 4°C), fractions (0.9 ml) were harvested from the bottom of the gradient. Samples were TCA precipitated and subjected to SDS–PAGE and Western blot analysis.

2.4.7 Analysis of the detergent-resistant pellet

The wild type mitochondria (200µg) were solubilized in 600µl lysis buffer (20mM HEPES, 40mM or 160mM KCl, 1mM PMSF and 1% Triton X-100) were incubated on ice for 30 min. The solubilized lysate was centrifuged at 9,271 *x* g to result in a low-speed pellet (LP) and supernatant (LS). The supernatant fraction was TCA precipitated and harvested (2.4.15). Both pellet (LP) and TCA precipitated supernatant (LS) were solubilized with 1X Laemmlli buffer containing 5% (vol/vol) β-mercaptoethanol. When indicated, the low-speed pellet was further solubilized in 600µl of lysis buffer (20mM HEPES, 160mM KCl, 1mM PMSF and 1% Triton X-100) and subjected to sucrose sedimentation analysis as described in 2.4.5. In a similar manner, the low-speed supernatant (equilibrated to 160mM KCl) was also subjected to the sucrose sedimentation analysis. The fractions were harvested and processed as described in 2.4.15.

When indicated, the low-speed pellet obtained from Triton X-100 treatment was further solubilized with lysis buffer (20mM HEPES, 40mM or 160mM KCl, 1mM PMSF and 1% Triton X-114) and incubated at 37^{o}C for 5 min. The solubilized material was centrifuged at 16,000 *x* g (room temperature) for 10 min to result in a detergent and an aqueous phase. The fractions from both the phases were TCA precipitated (2.4.15) and analyzed by SDS-PAGE followed by Western blotting or silver staining.

2.4.8 Subfractionation of mitochondria

The protocol was essentially performed as previously published by Liu and Spermulli, 2000. The mitoplasts were prepared by resuspending 400 µg of isolated mitochondria in

the ice cold hypotonic buffer (900µl, 20mM HEPES.KOH, pH-7.4) for 30 min on ice followed by centrifufgation (9,271 *x* g). The isolated mitoplasts were then resuspended in 600µl of buffer A [20mM HEPES-KOH, pH-7.4, 40mM KCl (or 160mM KCl, as indicated), 1mM DTT, 1mM PMSF] by gentle vortexing. The resuspended mitoplasts were subjected to sonication on ice with a Branson sonifier 250 microtip (45% amplitude, 5 pulses of 6s each interrupted by 90s interval). The resulting sample was centrifuged for 10 min at 9,271 *x* g to give rise to a pellet fraction (the membranes) and a supernatant (the soluble proteins). To ensure that free ribosomes (i.e. non-membrane associated) would not pellet under these centrifugation conditions, the sonicated sample was treated with 160mM KCl and 1% Triton X-100, prior to the centrifugation step.

2.4.9 Membrane floatation assay

The membrane floatation assay protocol was performed essentially as previously published (Prestele *et al*, 2009). Isolated mitoplasts from 600 µg of mitochondria were sonified in buffer A, as described earlier. The sonicated sample was adjusted to 2.2M sucrose in buffer A (final volume 1 ml), transferred into a centrifugation tube and layers (each 1ml) of 2 M,1.5 M and 1 M sucrose in (buffer A) were layered on top. The tubes were centrifuged in a Beckman MLS-50 rotor for 17 h at 45,000 rpm at 4^{o}C. Fractions (500 µl) were collected, trichloroacetic acid precipitated, and subjected to SDS-PAGE and western blot analysis.

2.4.10 SDS-PAGE

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels were prepared according to Laemmli (1970) as 9 cm x 15 cm separating layer and 1 cm x 15 cm stacking layer. For this study, 16% acrylamide and 0.2% bis-acrylamide SDS-PAGE gels were used (Laemmli, 1970). SDS-PAGE gel was assembled into electrophoresis chamber with 1X electrophoresis running buffer (2.9% glycine, 0.1% SDS, 50 mM Tris-Cl pH 8.9). Samples prepared with 1X Laemmli buffer and were directly loaded onto the gel. Molecular weight marker (Dalton Mark VIII-L standard Sigma) was also loaded on the gel for the estimation of molecular weight. The electrophoresis was carried out at constant current (25 mA) for approximately 2-2.5 hrs.

2.4.11 Western blotting

SDS-PAGE gel was usually transferred onto nitrocellulose membrane for immunedecoration. A modified version of the semi-dry blotting method described by Kyshe-Anderson (1984) was used. For this purpose, a sandwich was prepared as follows: two layers of Whatman paper 3mm, nitrocellulose membrane (0.2μ), gel and Whatman paper 3mm (all soaked in transfer buffer) was prepared. The sandwich was prepared on Western blot apparatus (Genemate) and was subjected to electrophoresis at 150 mA for 1.5 h. Following Western blotting, the nitrocellulose membrane was washed with H_2O and stained with Ponceau S solution (0.2% Ponceau S, 3% tricholoracetic acid) to visualize the proteins. The position of molecular weight standard was marked and the nitrocellulose membrane was washed with H_2O before immunedecoration.

2.4.12 Immunedecoration of nitrocellulose membrane

Nitrocellulose membrane was blocked with 5% milk powder (fat-free) solution prepared in 1X TBS (10 mM Tris-Cl pH 7.5, 150 mM NaCl) for 30 min at room temperature. The primary antibody of interest was usually prepared in 5% milk solution with a dilution of 1:1000 or 1:5000 depending on the recommendation of the provider. The incubation with primary antibody was performed for 1 hr at room temperature and was followed by three consecutive steps of washing the nitrocellulose membrane: 1X TBS for 10 min, 1X TBS with 0.05% (w/v) of Triton X-100 for 10 min and 1X TBS for 10 min.

The membrane was then incubated with secondary antibody (depending on the F_c-portion of primary antibody, usually goat anti-rabbit) prepared in milk solution (1:5000 dilution) for 1 hr at room temperature, which was coupled to horseradish peroxidase. The nitrocellulose membrane was again washed three times as described above.

An enzymatic chemiluminescence (ECL) based method was used to detect the horseradish peroxidase signal (Roswell and White 1978). Two ECL solutions were prepared as follows: solution 1 [0.1 M Tris-Cl pH 8.8, 66 mg/L Coumaric acid (dissolved in DMSO first), 0.44 m/L luminol (dissolved in DMSO)] and solution 2 (0.1 M Tris-Cl pH 8.8, 0.018% H_2O_2) was mixed in a ratio of 1:1 in dark room and the nitrocellulose membrane was soaked in the solution for 1 min and then exposed to X-ray film for different time intervals. X-ray films were developed using Agfa CP 1000 system.

2.4.13 Protein concentration determination

Protein determination was performed using the Bradford assay, which is a colorimetric based detection assay, commonly used to measure the concentration of proteins in

solution (Bradford, 1976). Protein standards (bovine gamma globulin IgG) were prepared in total 100 µl H_2O in an increasing concentration of 0, 0.04, 0.07, 0.15, 0.3 and 0.6 mg/ml. Unknown protein samples were diluted 1:10 with H_2O and 5 µl of the diluted sample was used for protein determination. 1 ml of Biorad Protein Assay Reagent (diluted 1:5) was added to the unknown and standard protein samples. The $OD_{595\ nm}$ of each sample was determined by spectrophotometer and the $OD_{595\ nm}$ of the standard samples were plotted against the protein concentration. The standard graph generated was used to calculate the concentration of unknown samples.

2.4.14 Steady state analysis of mitochondrial proteins

To analyze the steady state level of different mitochondrial proteins, 200 µg of isolated mitochondria (10 mg/ml in SEM buffer pH 7.2) was resuspended in equal volume of 2X Laemmli buffer and further diluted one-fold with 1X Laemmli buffer (final concentration 2.5 mg/ml). 30 µl of each sample was loaded onto SDS-PAGE and analyzed by Western blotting and immunedecoration with indicated antibody.

2.4.15 TCA precipitation of proteins

Trichloroacetic acid (TCA) precipitation was performed to concentrate the proteins from dilute sample. 200 µl of a protein solution is treated with 80 µl TCA (3M) and 12 µl deoxycholate (0.15%). The samples were mixed and incubated overnight at -80°C and the precipitated proteins were isolated by centrifugation (30,000 x g, 30 min, and 4°C). The pellet was washed with 1 ml cold acetone and the pellet was air-dried at 40°C to remove the traces of acetone. The precipitated protein was then solubilized in 30 µl of 1XLaemmli buffer and heated at 95°C for 5 min for SDS-PAGE analysis.

Table 6 Names and compositions of liquid media and plates used in this study

Media	Composition
Liquid media	
SOB + Mg^{2+}	20 g Bacto-Typtone, 5 g Bacto-Yeast Extract, 0.5 g NaCl, 950 ml ddH_2O, 10 ml 2M $MgCl_2$
LB	10 g Bacto-Tryptone, 5 g Bacto-Yeast Extract, 10 g NaCl, 950 ml ddH_2O
LB + AMP	100 ml LB Media, 100 µl Ampicillin (100 mg/ml)
YPAD	10 g Bacto-Yeast Extract, 20 g Bacto-Peptone, 5.5 ml Lactate (90%), 930 ml ddH_2O, 50 ml Glucose (40%), 100 mg Adenine, pH 5.5 with KOH
YP-Lactate	10 g Bacto-Yeast Extract, 20 g Bacto-Peptone, 5.5 ml Lactate (90%), 900 ml ddH_2O, pH 5.5 with KOH
SC-URA	1.45 g Yeast Nitrogen Base (without amino acids and ammonium sulfate), 5.0 g Ammonium Sulfate, 2.0 g Amino Acid Drop-out. Mix, 905 ml ddH_2O. 50 ml Glucose (40%), 3 ml histidine (2%), 10 ml tryptophan (0.8%), 20 ml leucine (1.31%), 15 ml Adenine (0.135%)
YP-Glycerol (YPG)	900 ml YP-Lactate Media, 100 ml Glycerol (30%)
Plates	
LB	20 g agar in 1000 ml LB liquid medium
LB + AMP	20 g agar in 1000ml LB+AMP liquid medium
SC Plates	1.45 g Yeast Nitrogen Base (without amino acids and ammonium sulfate), 5.0 g Ammonium Sulfate, 2.0 g Amino Acid Drop-out Mix, 20 g Agar, 460 ml ddH_2O, 50 ml Glucose (40%), 3 ml histidine (2%), 10 ml tryptophan (0.8%), 20 ml leucine (1.31%), 15 ml Adenine (0.135%) or 10 ml uracil (0.224%). Specific Amino Acids are eliminated when indicated.
YPAD	20 g agar in 1000 ml YPAD liquid medium
YPAD + G418	YPAD plates with G418
YPG	20 g agar in 1000 ml YPG medium
YPG + 0.1% Galactose	900 ml YP-Lactate liquid media, 20 g agar, 100 ml Glycerol (30%), 3.3 ml Galactose (30%)
SC-HIS+5-FOA Plates	1g 5-FOA (0.1%) dissolved in warm wáter and filter sterilized. Mix with media for SD plates (see above). All amino acids except histidine were added.

Chapter 3 Results

3.1 The mitospecific region of Mrp20 is important for the assembly of mitoribosomes and functional OXPHOS system

Introduction

Mrp20 is an essential component of the mitochondrial large ribosomal subunit (54S). Mrp20, a nuclearly encoded protein is 263 amino acids in length with a molecular weight of ~30.5 kDa. Mrp20 is located in the vicinity of the inner membrane of the mitochondria as shown chemically cross-linking of Mrp20 with Oxa1 protein and by immuno EM localization studies. (Prestele *et al*, 2009). The Mrp20 protein shares homology with the bacterial ribosomal protein L23. The crystal structure of the 50S subunit from the bacteria *Deinococcus radiodurans* shows that L23 is located next to the exit of the tunnel of the large ribosomal subunit from where the nascent polypeptides emerge (Harms *et al*, 2001). In bacteria, the L23 ribosomal subunit has been shown to physically interact with i) a chaperone protein called trigger factor, ii) components of the signal recognition particle (SRP) and iii) components of the SecYEG and YidC protein insertion machinery (Gu *et al*, 2003; Halic *et al*, 2004; Kohler *et al*, 2009; Ullers *et al*, 2003) In the process of co-translational insertion of membrane proteins in bacteria, the hydrophobic nascent polypeptides interact with the chaperone trigger factor and the translating ribosomes are targeted to membrane (to the SecYEG/YidC complex) with the help of SRP where the

nascent chains become inserted into the membrane. The bacterial L23 protein thus plays an important role in coordinating steps of protein synthesis with those of membrane insertion. However, in yeast and bovine mitoribosomes the homolog of SRP and trigger factor has not been found yet suggesting an alternate mechanism in the co-translational insertion of the mitochondrially encoded proteins (Glick & Von Heijne, 1996). It is not known if Mrp20 protein plays a direct role in the co-translational insertion of mitochondrially-encoded protein like its ancestor (L23), but presumably if it has any possible role in this process, it cannot be associated with SRP or trigger factor like proteins in mitochondria. It is thought that Mrp20 protein must have evolved in a different way during the evolution of mitoribosomes, a suggestion which is supported by the presence of an extra mitospecific region in its C-terminal region that may indicate the acquisition of a new function of Mrp20 protein.

BLASTp analysis of the Mrp20 protein reveal N- and C-terminal extension sequences in addition to the conserved L23 domain. The N-terminal sequence (residues 1-33) contains a cleavable mitochondrial targeting signal of this nuclearly-encoded protein. The C-terminal segment (residues 177-263) encompasses conserved extension sequence called a mitospecific region. The presence of mitospecific regions have been reported in many other mitoribosomal proteins. Notably, there is no similarity within the mitospecific regions themselves from different ribosomal proteins, suggesting that the various mitospecific regions may each play diverse roles in the function of the mitoribosomes. Not much is known about the functional relevance of the mitospecific region of different ribosomal proteins. So far, the mitospecific region of only two ribosomal proteins has been studied. The mitospecific regions of these two ribosomal

proteins, Mrpl36 and MrpL40 are suggested to be important for the translational fidelity of mitochondrially- synthesized proteins and the assembly of translationally active ribosomes, respectively (Jia *et al*, 2009; Prestele *et al*, 2009).

The objective of the present study is to understand the functional relevance of the mitospecific region of Mrp20 protein, a ribosomal protein predicted to be located at the exit site of the tunnel of the mitoribosomes and which may play a unique function in mitochondrial ribosomes. Using a yeast mutant bearing a C-terminal deletion, *mrp20*Δ*C,* the data presented here demonstrates that the mitospecific region of Mrp20 is important for mitochondrial translation. Moreover, the steady state levels of proteins of OXPHOS complexes were severely reduced in the mutant. The biochemical studies performed on the *mrp20*Δ*C* mutant demonstrate a defect in the assembly of stable ribosomes that underlies the observed loss in translation ability of the mitochondrial ribosomes. The C-terminal region of Mrp20 was found to be important for the stability of Mrp20 and indirectly of the MrpL40 protein and interestingly, these two proteins were found to physically associate with each other. Despite the observed defect in assembly of the mitoribosomal proteins, the level of ribosomal proteins tested in *mrp20*Δ*C* mutant (other than Mrp20 and MrpL40) were found comparable to wild type, mitochondria suggesting that the ribosomes in the *mrp20*Δ*C* mutant might have reached a partial assembly state sufficient to ensure the proteolytic stability of the ribosomal proteins measured.

Results

3.1.1 The mitospecific region of Mrp20 is important for cellular respiration

The mitospecific region of Mrp20 is conserved and predicted to form an α-helical domain (aa 229-254) (Figure 4A). To assess the relevance of the conserved mitospecific region of Mrp20 protein, a yeast strain expressing a truncated derivative of Mrp20, *mrp20ΔC*, was created. In the *mrp20ΔC* mutant, the last 23 amino acids of the protein were removed by an internal deletion in the chromosomal MRP20 gene (Figure 4A). A small truncation rather than deletion of the mitospecific region was performed since an attempt to create a yeast strain with the complete deletion of the mitospecific region (residues 177-263) was unsuccessful. In contrast to wild type cells, growth of *mrp20ΔC* yeast cells was strongly impaired on a non-fermentable carbon source (glycerol based media) at 30^{o}C suggesting a defect in the ability of the cells to respire (Figure 4B). As a negative control to monitor the growth ability, the wild type rho zero (ρ^0) cells were also analyzed in parallel. The ρ^0 cells do not contain mitochondrial DNA and thus are unable to synthesize mitochondrially-encoded proteins required for cellular respiration. The growth defect on glycerol based media (YPG) displayed by the *mrp20ΔC* cells is not due to instability of the mtDNA, because genetic crossing of the haploid *mrp20ΔC* cells (mating type a) with haploid ρ^0 cells (mating type α) resulted in respiration-competent diploid cells (Figure 4C). As a positive control, *Δoxa1* cells (shown previously to contain

mitochondrial DNA) when crossed with ρ^0 cells also produced respiration-competent diploid cells (Figure 4C). It was therefore concluded that *mrp20ΔC* cells are ρ^+ (i.e. contain mitochondrial DNA).

To test if the truncation of the mitospecific region of Mrp20 had any effect on the stability of Mrp20 protein, mitochondria from the yeast strain carrying the truncation in

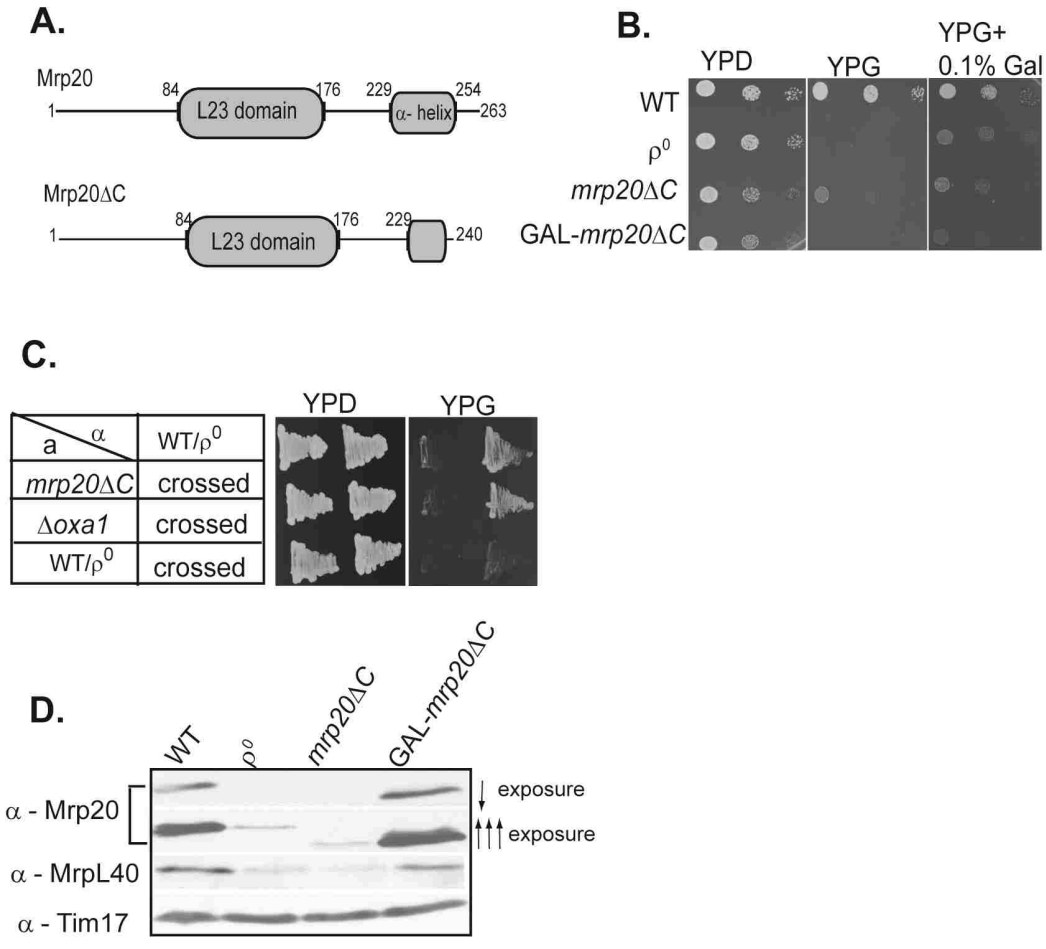

Figure 4: The mitospecific region of Mrp20 protein is important for cellular respiration and the stability of the Mrp20 protein. (A) The *S. cerevisiae* Mrp20 protein is depicted. The position of a conserved bacterial domain, L23 and the C-terminal mitospecific region encompassing α-helical domain is indicated (177-263 aa). A region of this mitospecific region (terminal 23 amino acids, aa) has been removed to create the Mrp20ΔC protein. (B) Deletion of the mitospecifc region (23 aa) impairs growth ability on non-fermentable carbon sources (YPG). Serial 10-fold dilutions were spotted on YP plates containing 3% glucose or 3% glycerol and 3% glycerol + 0.1% galactose were incubated for 3 days. (C) Yeast cells containing truncation in the C-terminal region of Mrp20 retain mtDNA. The indicated haploid strains were crossed to wild type ρ^0 strain. After selection, the diploid strains and their parental strains were tested for their ability to respire. The Δ*oxa1* strain was used as a positive control. (D) Mitochondrial proteins (50μg) from the indicated strains were separated by SDS-PAGE and analyzed by Western blotting with Mrp20-specific antibody. Two different exposures are shown for the Mrp20-specific antibody as indicated by ↓(short) and ⇑(long). Tim17 was used as a loading control.

the Mrp20 protein, *mrp20ΔC* mitochondria were isolated and analyzed by SDS-PAGE and Western blotting. The steady state levels of the Mrp20ΔC protein were observed to be strongly reduced, when compared with the full-length Mrp20 protein in the wild type mitochondria (Figure 4D). In ρ^0 mitochondria, the levels of Mrp20 protein were strongly reduced due to the fact that in the absence of fully assembled ribosomes (due to lack of rRNA), the ribosomal proteins are proteolytically unstable (Figure 4D).

The growth defect of *mrp20ΔC* cells could either be due to a direct consequence of compromising the mitospecific region of Mrp20, or indirectly due to the reduced levels of Mrp20 protein observed in the mutant mitochondria. To test the latter possibility, the Mrp20ΔC protein was over-expressed in *mrp20ΔC* yeast cells, by placing its expression under the control of strong galactose-inducible promoter, GAL10. This strain will be referred as GAL-*mrp20ΔC* from now onwards. Mitochondria isolated from the GAL-*mrp20ΔC* cells were analyzed by SDS-PAGE followed by western blotting and immunedecoration with Mrp20-specific antibody. The steady state levels of *Mrp20ΔC* in the GAL-*mrp20ΔC* were observed to be similar to those of endogenous full-length Mrp20 protein in wild-type mitochondria (Figure 4D). The growth defect on glycerol containing medium was not corrected, however, following over-expression of *Mrp20ΔC* (YPG + 0.1% Gal medium) (Figure 4B). This demonstrated that the growth phenotype in *mrp20ΔC* cells was directly due to the truncation in the mitospecific region of Mrp20 and not an indirect effect of reduced levels of the Mrp20 protein *per se*.

In summary, the mitospecific region of Mrp20 is important for the stability of Mrp20 protein and also plays an important role in the ability of the Mrp20 protein to support aerobic respiration.

3.1.2 The mrp20ΔC cells exhibit reduced translation and stability of OXPHOS proteins

Given that the *mrp20ΔC* cells and GAL-*mrp20ΔC* cells show a defect in cellular respiration, the ability of these cells to synthesize mitochondrially-encoded proteins was next tested. *In organello* translation using [^{35}S]methionine was performed in mitochondria isolated from wild type, *mrp20*Δ*C* and GAL-*mrp20*Δ*C* cells. Labeling of mitochondrial translation products with [^{35}S]methionine revealed that both *mrp20*Δ*C* and GAL-*mrp20*Δ*C* mitochondria display a strong defect in their ability to support the synthesis of all the eight mitochondrially-encoded proteins, when compared to the wild type control mitochondria, analyzed in parallel. Interestingly, the GAL-*mrp20*Δ*C* cells displayed a more pronounced defect in the synthesis of mitochondrially-encoded proteins than the *mrp20*Δ*C* cells (Figure 5A). The strongly reduced translational ability of the GAL-*mrp20*Δ*C* cells even when the levels of Mrp20 are not limiting remains unclear.

The steady state levels of subunits of OXPHOS complexes were next analyzed to determine the level of assembly of OXPHOS complexes in the *mrp20*Δ*C* mutant. A strong reduction in the steady state levels of Cox1 and Cox2 of COX complex, Rieske Fe-S protein of Cyt bc_1 complex and Su e of the F_1-F_0 ATP synthase complex was

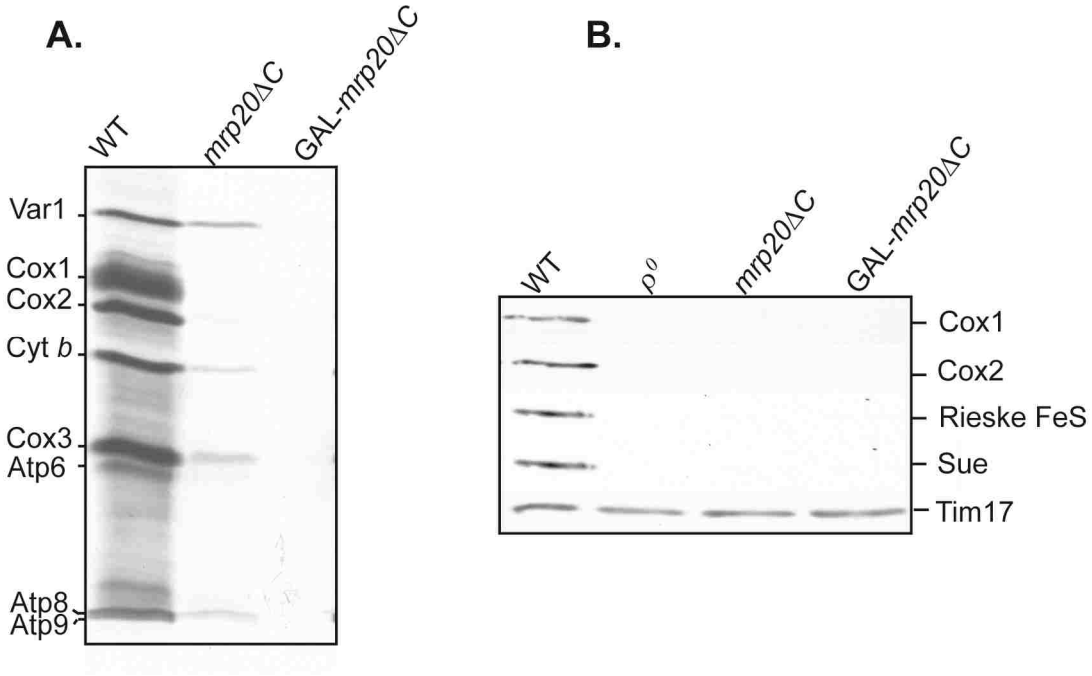

Figure 5: The deletion in the C-terminal region of the Mrp20 protein strongly compromises the mitochondrial translation and the stability of the OXPHOS proteins. (A) *In organello* translation was monitored in the mitochondria isolated from WT, *mrp20ΔC* and GAL10 -*mrp20ΔC* for 30 min at 25^{o}C following the addition of [^{35}S]methionine. Following a chase with excess cold methionine and puromycin for 30 min at 25^{o}C, mitochondria were reisolated, lysed in SDS-sample buffer, and subjected to SDS-PAGE followed by Western blotting and autoradiography. Abbreviations: Cox1, Cox2 and Cox3 - cytochrome *c* oxidase subunits 1, 2 and 3, respectively; Cyt *b* - cytochrome *b*; Atp6, Atp8, and Atp9, subunits 6, 8, and 9 of the F_o sector, respectively. (B) Mitochondria (50μg of protein) were subjected to SDS-PAGE, Western blotting and immunedecoration for the presence of subunits of the OXPHOS complex as indicated. Tim17 was used as a loading control.

observed in the *mrp20ΔC* and GAL-*mrp20ΔC* mutants (Figure 5B). These proteins were chosen for this analysis because they are susceptible to proteolytic turn over in the absence of the stable assembly of their respective complexes. As a negative control, ρ^0 mitochondria that do not have mitochondrial DNA and thus have defective assembly of OXPHOS complexes were also analyzed and were shown to have severely reduced levels of COX, Cyt *b* and ATP synthase complex subunits.

To summarize, the truncation in the mitospecific region of Mrp20 leads to severe defect in the ability to synthesize the mitochondrially-encoded proteins and the assembly of the OXPHOS complexes. A strong reduction in the translation of the key subunits of OXPHOS complexes thus explains a defect in respiration and inability to grow on non-fermentable carbon source of *mrp20ΔC* and GAL-*mrp20ΔC* cells.

3.1.3 Mrp20 and MrpL40 proteins can directly interact with each other

Steady state analysis of the Mrp20ΔC protein indicated that the C-terminal truncation caused instability of the Mrp20 protein, as the levels of Mrp20 protein were strongly reduced in the *mrp20ΔC* mitochondria. In addition, the levels of MrpL40, another large ribosomal subunit protein were also decreased in the *mrp20ΔC* mitochondria. Furthermore, the levels of MrpL40 protein were increased by the over-expression of Mrp20ΔC protein suggesting the dependence of the stability of MrpL40 on the Mrp20 protein (Figure 4D). In contrast, the steady state levels of other ribosomal proteins tested in this study were not reduced (see later Figure 8B). Previous findings from our lab have indicated that modulation in the mitospecific region of MrpL40 can lead to a reduction in

the levels of Mrp20 protein (Jia *et al*, 2009). Given the finding that disruption of the mitospecific region of either protein leads to reduced levels of the other, it is proposed that these two proteins can interact with each other and are thus interdependent on each other for their stability. The bacterial homologs of Mrp20 and MrpL40 (L23 and L24) are shown to be located close to each other based upon the crystal structure studies of the bacterial ribosome. It was therefore initially tested if these proteins are in vicinity to each other with the assembled 54S particle. For this purpose, a chemical cross-linking approach was used to probe the molecular environment of the MrpL40 protein in isolated wild type mitochondria using the sulfhydryl-amino-specific heterobifunctional, noncleavable, cross-linking reagent MBS (9.9-Å spacer arm). Following cross-linking, SDS-PAGE and Western blot analysis, the MrpL40 protein was found to form a cross-linked adduct of approximately 70 kDa, as revealed by decoration with MrpL40-specific antisera (Figure 6A). To test whether this 70 kDa MrpL40 adduct may represent cross-linking of MrpL40 to Mrp20, the ability of Mrp20 to form an MBS-generated cross-linked adduct of similar size was addressed in parallel. Mrp20 was found to form two dominant cross-linked adducts of approximately 62 and 70 kDa in wild type mitochondria (Figure 6B, left panel), the larger of which comigrated with the MrpL40 cross-linked adduct (Figure 6B, center panel). To demonstrate that this Mrp20 adduct represented a cross-linked product between Mrp20 and MrpL40, the Mrp20 and its cross-linked adducts were immunoprecipitated using an Mrp20 monoclonal antibody, analyzed by SDS-PAGE and Western blotting, and then probed with the MrpL40-specific antiserum (Figure 6B, right panel). An MrpL40-reactive band of 70kDa was observed in the Mrp20-immunoprecipitated material from the cross-linked samples. No MrpL40-

containing material was detected in the Mrp20 immunoprecipitate in the absence of prior cross-linking. It was concluded therefore that the 70-kDa MBS cross-linked adduct observed with both Mrp20- and MrpL40-specific antibodies represents an Mrp20-MrpL40 adduct. The ability of Mrp20 and MrpL40 to cross-link with each other demonstrates that the two proteins exist in vicinity to each other similar to their bacterial homologs, L23 and L24 respectively.

It was further proposed that mitospecific regions of Mrp20 and MrpL40 proteins may support their interaction and consequently their stability. To this end, a direct interaction between the mitospecific region of MrpL40 and the Mrp20 protein was tested by reconstructing a bacterial expression vector that enables the co-expression of Mrp20 and the mitospecific region of MrpL40. The MrpL40 protein contains an N-terminal conserved KOW (Kyrpides, Ouzounis, Woese) motif (residues 62-89) and an additional C-terminal mitospecific region (aa 90-297) (Figure 6C). An N-terminal His-tagged version of the mitospecific region of MrpL40 protein ($_{His}$MrpL40mt) was co-expressed with full length Mrp20 protein in bacteria to test if Mrp20 could co-purify with MrpL40mt protein. Purification of the His-tagged MrpL40mt protein was achieved using Ni-NTA affinity chromatography. As a negative control for the experiment, a non-His tagged version of the mitospecific region of MrpL40 protein (MrpL40mt) was co-expressed with Mrp20 protein. The solubilized *E.coli* extract obtained from bacterial cells containing Mrp20 protein co-expressed with non-tagged MrpL40mt or His-tagged MrpL40mt were incubated with Ni-NTA beads. The pre-bound material and the material recovered on beads were analyzed by SDS-PAGE followed by Western blotting with MrpL40- and Mrp20- specific antibodies. The successful expression of Mrp20 and

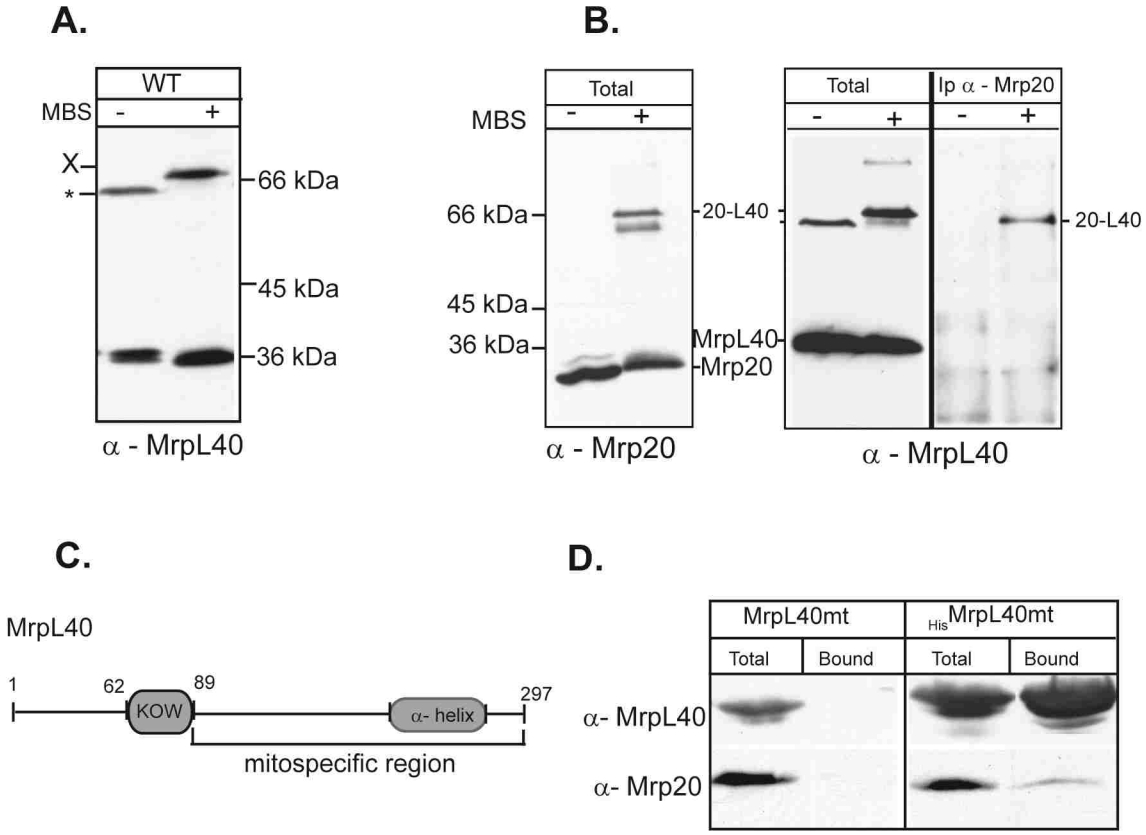

Figure 6: Mrp20 and MrpL40 exist in close proximity to each other and are interdependent on each other for their stability. (A) Wild-type (WT) mitochondria were subjected to cross-linking with MBS or mock treated, as indicated. Following quenching, mitochondria were reisolated and analyzed by SDS-PAGE, Western blotting, and imunodecoration with MrpL40 antisera. * indicate cross-reactivity signal from the MrpL40 antisera. X indicates a MrpL40-specific MBS adduct. (B) WT mitochondria were cross-linked (MBS or mock treated) and were either lysed directly in SDS-sample buffer ("Total") or were lysed in SDS-buffer, diluted in Triton X-100 buffer, and Mrp20 and cross-linked adducts were immunoprecipitated using Mrp20 monoclonal antibodies (IP-α Mrp20). Immunedecoration of the resulting Western blots was performed with either Mrp20- (left panel) or with MrpL40- specific antisera (center and right panels). The position of the Mrp20-MrpL40 adduct is represented by "20-L40". (C) The position of the conserved KOW motif and the mitospecific region of MrpL40 protein that was used for the expression in bacteria is represented. (D) Recombinant non-tagged and His-tagged mitospecific region of MrpL40 (MrpL40mt or $_{His}$MrpL40mt) was co-expressed with Mrp20 protein in the bacteria. The bacterial lysate from Mrp20-MrpL40mt or Mrp20-$_{His}$MrpL40mt was subjected to Ni-NTA affinity chromatography followed by Western blotting with indicated antibodies. Total - 5% of the solubilized lysate; Bound - the Ni-NTA eluted material.

MrpL40mt (non-tagged and his-tagged) was observed by analyzing total lysate before incubation with the beads (Figure 6D). Furthermore, the His-tagged MrpL40mt was specifically recovered in the beads compared to the non-tagged MrpL40mt (Figure 6D). Probing with Mrp20-antibody indicated that Mrp20 can be specifically co-purified with $_{His}$MrpL40mt on the Ni-NTA beads (Figure 6D).

Taking these results together, it was concluded that the C-terminal region of Mrp20 is important for its own stability, and thus indirectly for the stability of MrpL40, a protein like Mrp20, located in the vicinity of the exit tunnel. Both the chemical cross-linking approach and co-expression affinity purification studies using recombinant Mrp20 and $_{His}$MrpL40mt demonstrated a direct interaction between these two ribosomal proteins. It is therefore concluded that Mrp20 and MrpL40 are direct physical partners of each other at the exit site of the ribosomal tunnel.

3.1.4 The conserved residue Pro_{228}, but not Phe_{226}, in the mitospecific region of Mrp20 is important for cellular respiration

The Mrp20 protein contains a conserved C-terminal mitospecific region that is predicted to form an α-helical region (Figure 4A). As shown in previous results, deletion of the C-terminal region of the Mrp20 protein compromised the function and strongly reduced the stability of the Mrp20 protein (Figure 4D). Therefore, an attempt to perform site-directed mutagenesis in the conserved residues of the mitospecific region of the Mrp20 protein was done in order to perform more subtle change with the goal of not affecting the stability, but only the function of the Mrp20 protein. Multiple sequence alignment of

Mrp20 from yeast with other fungal homologs shows the presence of conserved amino acids in the mitospecific region (Figure 7A). In particular, three amino acids "Phe-Ile-Pro" (FIP, residues 226-228) show high conservation among different species and are located just before the conserved α-helical region. It may be possible that these hydrophobic residues "FIP" could be important for maintaining structural integrity or positioning of the helical region of Mrp20 protein. Hence, to analyze the importance of the helical domain of the mitospecific region, the conserved residues "F_{226}" and "P_{228}" were mutated to Ala residues (A), "A_{226} and $A_{228,}$" respectively. The mutated Mrp20 derivatives (*mrp20*F_{226}A or *mrp20*P_{228}A) were cloned into the centromeric plasmid (pRS413) and transformed in *Δmrp20* yeast cells, which expressed the full length Mrp20 protein from a gene harbored on a different centromeric plasmid, pRS316-MRP20. The strategy of co-expressing full length Mrp20 from the centromeric pRS316 plasmid was employed since it has been reported that null *Δmrp20* mutant cells frequently go ρ^0 (i.e. lose the mitochondrial DNA) and thus once they lose their mitochondrial DNA, they could not be complemented to grow on glycerol-based media. The growth phenotype of the *mrp20*F_{226}A or *mrp20*P_{228}A containing yeast cells was examined on the glycerol-containing media following the loss of wild type MRP20 plasmid (pRS316 has URA3 based auxotrophic marker) using the 5-fluoroorotic acid (5-FOA) chase procedure (expression of the URA3 is toxic to cell in presence of 5-FOA. The *mrp20*F_{226}A containing plasmid was able to complement the *Δmrp20* null mutant in the absence of the pRS316-MRP20 plasmid. Interestingly, the *mrp20*P_{228}A containing plasmid was unable to complement *Δmrp20* null mutant and the cells were unable to grow on a respiration competent media (Figure 7B).

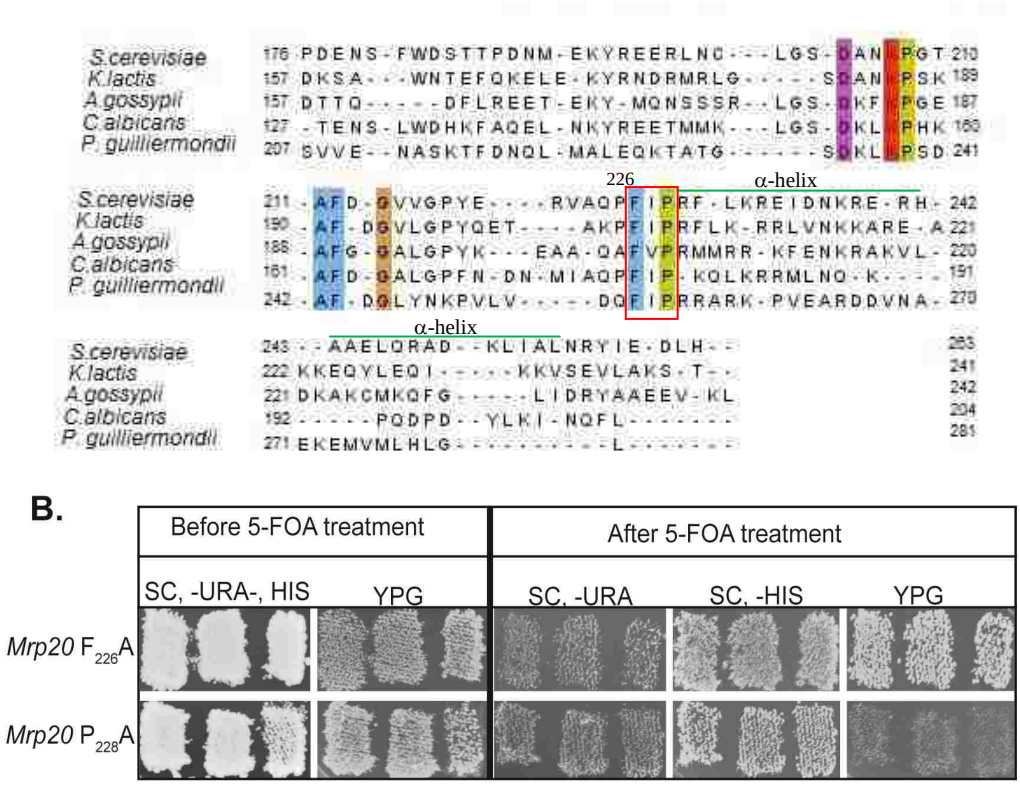

Figure 7: Mutagenesis of the conserved residue in the mitospecific region of the Mrp20 protein. Multiple sequence alignment of the mitospecific region of Mrp20 from *S. cerevisiae* and related fungal species. Amino acids with 100% identity are color shaded. The alpha helical domain of Mrp20 from *S. cerevisiae* is indicated by the green line (aa 229-254). The two identical residues present before the helical region that are mutated in this study are boxed in red (F_{226} and P_{228}). *S. cerevisiae - Saccharomyces cerevisiae; K. lactis - Kluveromyces lactis; A.gossypii - Ashyba gossypii; C.albicans - Candida albicans; P. guilleromondii - Pichia guilleromondii.*(B) Δ*mrp20* cells (complemented with the centromeric plasmid, pRS316-MRP20, with URA3 auxotrophic marker) were transformed with the centromeric plasmid, pRS413-Mrp20F_{226}A or pRS413-Mrp20P_{228}A with HIS3 auxotropic marker. Different transformants containing mutated plasmids were selected on minimal but fermentable media lacking URA and HIS (SC,-URA,-HIS; before 5-FOA treatment). The growth of cells containing both the plasmids was monitored on non-fermentable media containing glycerol (YPG). The centromeric URA3 plasmid containing wild type MRP20 gene (pRS316-MRP20) was chased out by plating on 5-fluro orotic acid (5-FOA) containing media which kills cells expressing URA3. Following 5-FOA treatment, the loss of the URA3 plasmid was confirmed by replica printing of the transformants onto minimal media lacking URA. The cells that retained mutated plasmids were selected on minimal media lacking HIS and the effect of the mutation was analyzed by replica printing on a glycerol-based media.

In summary, the conserved residue "F_{226}" is not essential for the function of the Mrp20 protein however, it appears that P_{228} might be important for the function of the Mrp20 protein or mutation of the Pro residue to Ala residue was detrimental for its function. In the future experiments, the *mrp20*P_{228}A mutant could be used to perform a multi-copy suppressor analysis with the goal to identify potential interacting partners (physical or functional) of the Mrp20 protein. Furthermore, a more extensive random mutagenesis of Mrp20 mitospecific region can be performed to identify the residues important for the Mrp20 for its ability to support cellular respiration.

3.1.5 The assembly of mitochondrial ribosomes is adversely affected in mitochondria harboring the truncated Mrp20ΔC protein

The defect in the synthesis of mitochondrially encoded proteins in *mrp20ΔC* mitochondria may be explained if translationally-competent ribosomes cannot assemble in these mutant mitochondria. To this end, the assembly state of the ribosomes in the Mrp20ΔC-containing mitochondria was analyzed and compared to those of the wild type control. Mitochondria isolated from GAL-*mrp20ΔC* and wild type control strains were detergent solubilized (1% Triton X-100) and after a clarifying spin, the solubilized protein complexes were subjected to sucrose density sedimentation analysis on a linear 15-30% sucrose gradient. In the wild type control mitochondria which contain assembled ribosomes, Western blotting and immunedecoration indicated that the large ribosomal proteins such as Mrp20, MrpL40, MrpL36 and Mrp49 co-migrated deep into the gradient (Fraction 10-11) indicating their co-assembly into the 54S particle (Figure 8A, upper

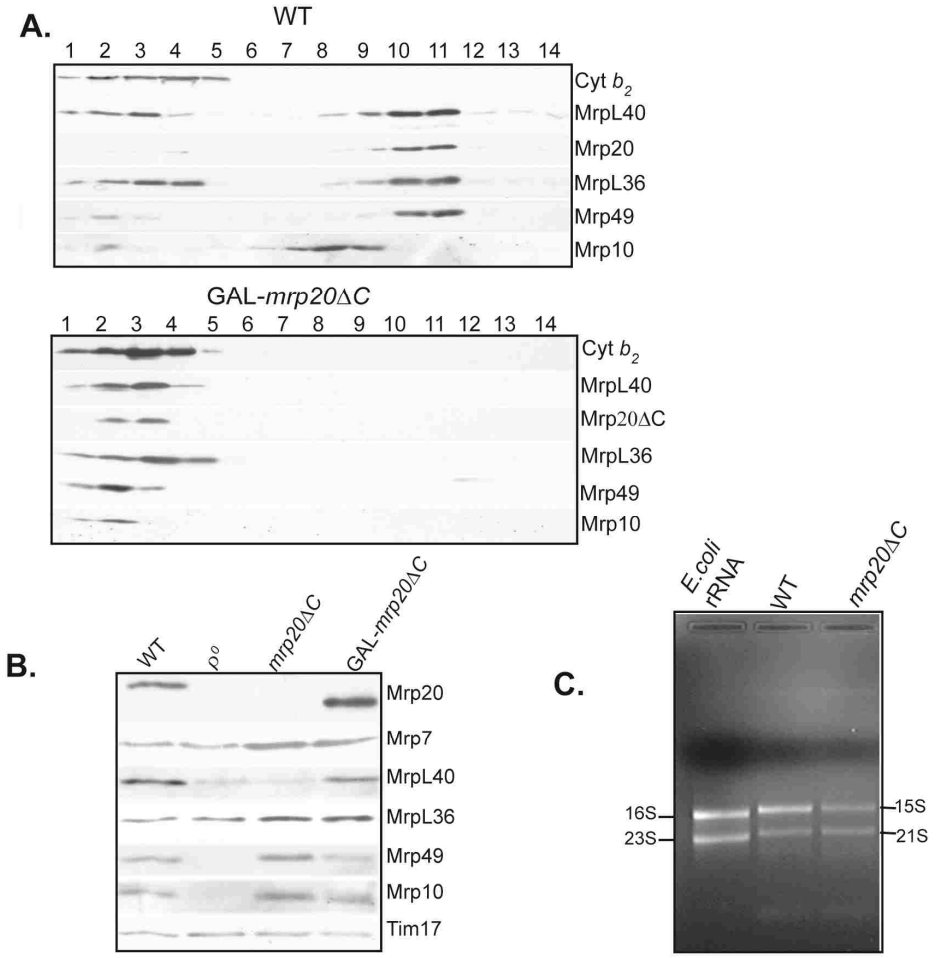

Figure 8: The truncation in the mitospecific region of Mrp20 has adverse effect on the stable assembly of mitoribosomes. (A) Wild type and GAL-*mrp20ΔC* mitochondria were lysed with a Triton X-100-containing buffer and subjected to clarifying spin. The resulting solubilized material was fractionated on a continuous sucrose gradient (15-30%). Fractions were collected (1 to 14, top to bottom of the gradient) and were analyzed by SDS-PAGE and Western blotting. Immunedecoration was performed with antibodies directed against large ribosomal proteins, MrpL40, Mrp20, MrpL36 and Mrp49, and the small ribosomal protein Mrp10, as indicated. The fractionation behavior of a control soluble (non-ribosomal) protein cytochrome b_2 (Cyt b_2) was also studied. (B) Mitochondria (50μg) were subjected to SDS-PAGE analysis and analyzed by Western blotting for various large ribosomal proteins and a small ribosomal protein, Mrp10, as indicated. Tim17 was used as a loading control. (C) Total RNA extracted from the Wild type and *mrp20ΔC* mitochondria was analyzed on 1% agarose gel. Purified ribosomal RNA from *E.coli* was analyzed in parallel. The gel was stained with ethidium bromide and photographed.

panel). The small 37S ribosomal subunit protein, Mrp10 is recovered in earlier fractions, prior to the large ribosomal proteins suggesting its assembled nature as well as indicating that in the present fractionation and centrifugation conditions the large and small ribosomal subunits do not stay together (Figure 8A, upper panel). As expected, the control soluble protein marker, cytochrome b_2 (Cyt b_2) was found towards the top of the gradient. A fraction of the large ribosomal proteins, MrpL36 and Mrp49 were also detected on the top of gradient. This observation is consistent with previously published results and indicates that a free population of these ribosomal proteins (not assembled into ribosomes) can exist stably in the matrix (Williams *et al*, 2005). In contrast to the wild type mitochondria, a strong defect in the stable assembly of ribosomal proteins was observed in GAL-*mrp20*Δ*C* mitochondrial sample. The ribosomal proteins analyzed in GAL-*mrp20*Δ*C* extract were all recovered towards the top of the gradient, indicating that they failed to assemble into stable 54S and 37S ribosomal complexes (Figure 8A, lower panel). It can be concluded therefore that the GAL-*mrp20*Δ*C* mutant mitochondria harbor assembly-defective ribosomes, which in turn may account for the observed impaired translational capacity of these mitochondria.

Steady state analysis of the ribosomal proteins in the GAL-*mrp20*Δ*C* mitochondria indicated that, the levels of ribosomal proteins such as Mrp20, MrpL40, MrpL36, Mrp49 and Mrp10 were similar to those of control wild type ρ^+ (i.e. mitochondrial DNA containing) mitochondria (Figure 8B). However, the levels of these ribosomal proteins were strongly reduced in the ρ^0 mitochondria, where the complete

assembly of ribosome is defective due to the absence of mitochondrial DNA and hence the coding capacities for the rRNA that acts as a scaffold for the ribosomal assembly.

Ribosomal proteins in ρ^0 mitochondria are often highly susceptible to proteolytic turnover in the absence of the assembling together with their rRNA scaffold and therefore do not accumulate to high steady state levels. Although the sucrose gradient centrifugation experiment indicated the absence of detergent stable assembled ribosomes in the *mrp20ΔC* mitochondria (not shown) and GAL-*mrp20ΔC* mitochondria (Figure 8A, lower panel), the observed presence of the ribosomal proteins, Mrp49 and Mrp10, at levels similar to wild type ρ^+ control mitochondria, indicated that these proteins must have achieved at least a partial assembly state in the *mrp20ΔC* and GAL-*mrp20ΔC* mutant mitochondria, i.e. sufficient to ensure their proteolytic stability. As mentioned before, when tested by genetic crossing, the *mrp20ΔC* mitochondria were demonstrated to contain their mtDNA, i.e. was ρ^+ (Figure 4D). Consistently, the presence of ribosomal RNA was also demonstrated in the *mrp20ΔC* mitochondria. However, the levels of large rRNA (21S) were relatively reduced in comparison to the wild type mitochondria (Figure 8C). It can be therefore concluded that the observed assembly defect in these mitochondria is not due to an absence of the coding capacity for rRNA, a conclusion consistent with the observed stability of the ribosomal protein, normally turned over when the rRNA is absent. Taking together the GAL-*mrp20ΔC* results, it can be concluded that the C-terminal region of Mrp20 is important for the stable assembly of mitoribosomes. Despite the observed defect in the ribosomal assembly, Mrp49and Mrp10 are not proteolytically turned over in the *mrp20ΔC* mitochondria and show steady state

levels comparable to those of wild type mitochondria. This suggests that they have achieved a partial assembled state.

3.2 Assembly of mitoribosomes is stalled in the GAL-mrp20ΔC mutant and a novel subcomplex of the large ribosomal subunit is observed

Introduction

The fully assembled mitochondrial ribosomes are comprised of a 54S large ribosomal subunit and a 37S small ribosomal subunit. Little is known about the assembly steps that occur prior to the completion of fully assembled and translationally-competent ribosomes at the inner membrane. The ribosomal assembly process has been proposed to involve the docking of a soluble pre-54S stable complex from the matrix onto the inner membrane, a step which precedes the incorporation of one of the final protein components, the MrpL32 protein (Nolden *et al*, 2005). At the inner membrane, the pre-54S ribosomal particle is proposed to associate with the membrane bound ribosomal protein, MrpL32 to form the functional 54S large ribosomal subunit. The presence of a detergent- stable pre-54S ribosomal particle was demonstrated in *Δyta10/Δyta12* mutant, where the proteolytic maturation of MrpL32 protein is defective, thus preventing its assembly into the pre-54S ribosomal particle. It is not known how and where the assembly of pre-54S ribosomal particle occurs or if the pre-54S particle exist as soluble species as suggested (Nolden *et al*, 2005). Whether ribosomal assembly involves coming together of individual ribosomal

proteins or ribosomal subcomplexes with the rRNA scaffold remains unanswered. Furthermore, it is not clear if the assembly events occur in the matrix or at the surface of the inner membrane.

The observed proteolytic stability of the ribosomal proteins tested in the *mrp20*Δ*C* and GAL-*mrp20*Δ*C* mitochondria, in contrast to their susceptibility observed in the ρ^0 mitochondria, indicated that these proteins may have achieved at least a partial assembly state (i.e. they have formed an assembly intermediate or subcomplex) securing their stability, in both *mrp20*Δ*C* and GAL-*mrp20*Δ*C* mitochondria. The possibility of this proposed subcomplex in the GAL-*mrp20*Δ*C* mutant was analyzed to gain more insight into the early steps of ribosome assembly.

The results shown here demonstrate the identification of a novel subcomplex in the GAL-*mrp20*Δ*C* mitochondria, the Mrp20ΔC subcomplex. The protein components of this Mrp20ΔC-containing subcomplex were identified and found to be enriched in proteins known to be located at the polypeptide exit tunnel of the 54S particle.

Results

3.2.1 Identification of a novel subcomplex of the large ribosomal proteins in the mrp20ΔC mutant

In order to identify a potential Mrp20ΔC-containing subcomplex of ribosomal proteins in the GAL-*mrp20*Δ*C* mitochondria, a histidine-tagged derivative of Mrp20ΔC called $Mrp20\Delta C_{His}$ was constructed and purified with Ni-NTA chromatography with the goal to

identify its interacting partner proteins. Eight histidine (His) residues were incorporated at the C-terminus of the Mrp20ΔC protein (Mrp20ΔC$_{His}$) and this His-tagged derivative was expressed under the control of the GAL10 promoter in the *mrp20ΔC* yeast strain, referred hereafter as GAL-*mrp20ΔC*$_{His}$. Mrp20ΔC$_{His}$ expression in the GAL-*mrp20ΔC*$_{His}$ strain was verified by immunoblotting with both the Mrp20 monoclonal and His-tag specific antibodies (Figure 9A). The Mrp20ΔC$_{His}$ protein migrated slightly larger than the over-expressed Mrp20ΔC protein, due to the presence of the C-terminal his-tag. This Mrp20ΔC$_{His}$ derivative was then used for affinity chromatography (Figure 9B). Wild type mitochondria and *mrp20ΔC* mitochondria harboring the GAL10-driven His-tagged or non-tagged versions of Mrp20ΔC were lysed in Triton X-100 containing buffer and subjected to affinity chromatography using Ni-NTA sepharose beads. Western blotting, using Mrp20-specific antibodies, demonstrated the specific recovery of Mrp20ΔC$_{His}$ on the Ni-NTA beads (Figure 9B). The Ni-NTA recovered material from parallel affinity purification samples was analyzed by silver staining in order to analyze if the Mrp20ΔC$_{His}$ protein was recovered in a complex with other proteins. In addition to Mrp20ΔC$_{His}$ protein, a number of co-purifying proteins were recovered on the Ni-NTA beads in a specific manner (Figure 9C). The bands corresponding to these proteins were excised and subjected to mass spectrometry for identification. The resulting analysis indicated the proteins to be MrpL35, MrpL40, MrpL4, MrpL25 and MrpL27, all large ribosomal proteins. In addition, one minor, but reproducible band, at approximately 28 kDa, was identified as Prx1, a mitochondrial peroxiredoxin protein. Using available antibodies against MrpL40, MrpL4 and Prx1, their specific recovery with the Mrp20ΔC$_{His}$ protein was verified (Figure 9D). MrpL40, MrpL4 and Prx1 were recovered

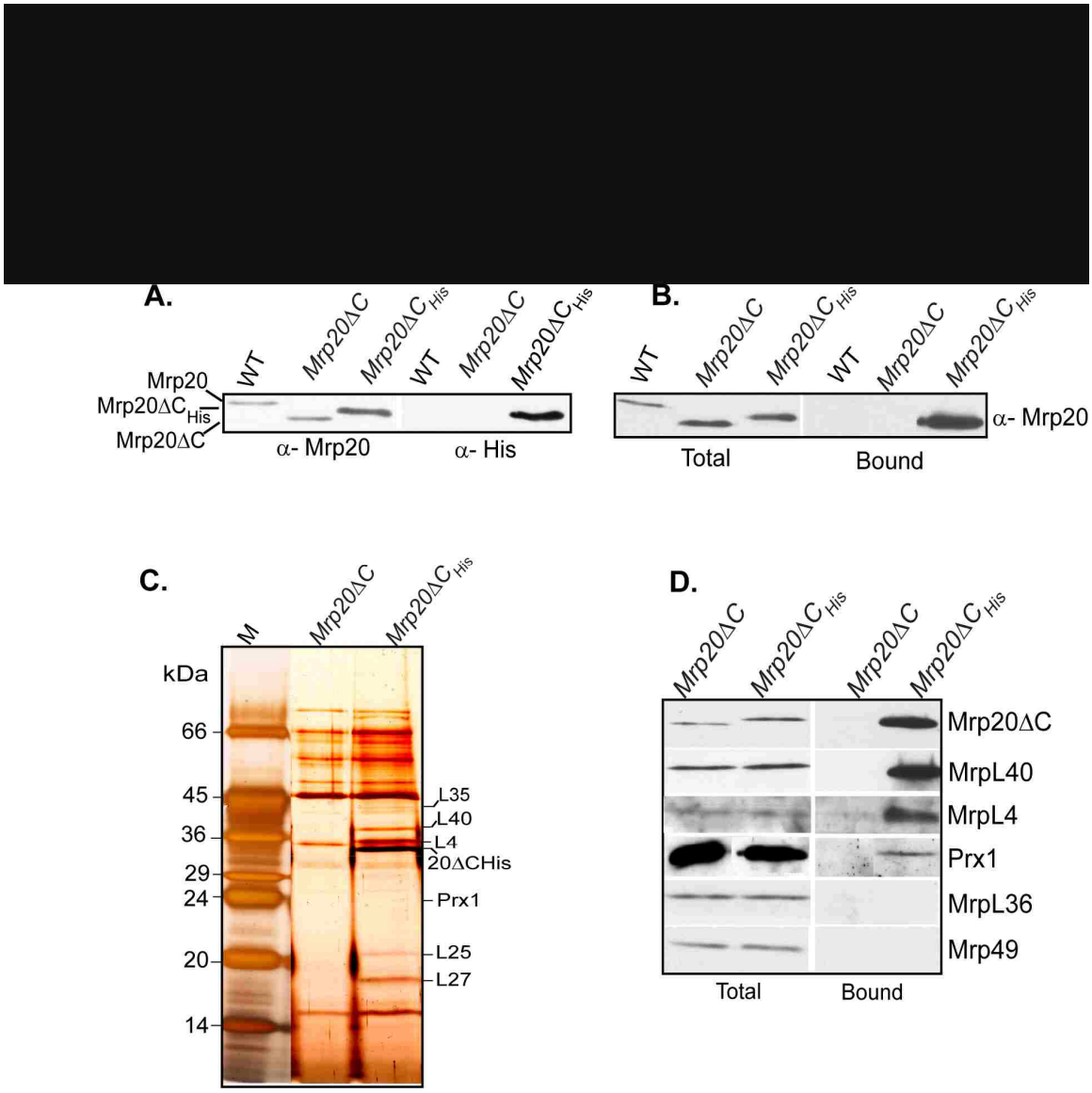

Figure 9: Isolation of Mrp20ΔC subcomplex. (A, B) Mitochondria isolated from the yeast strains expressing full length Mrp20 (WT), Mrp20ΔC or Mrp20ΔC$_{His}$ over-expressed in the *mrp20ΔC* yeast strain were analyzed by SDS-PAGE followed by western blotting with Mrp20- and His-specific antisera (A), or subjected to detergent solubilization and Ni-NTA purification followed by and Western blotting and immunedecoration (B). (C, D) Mrp20ΔC and Mrp20ΔC$_{His}$ mitochondria were subjected to Ni-NTA affinity chromatography as described above and the eluted material ("Bound") was analyzed by SDS-PAGE followed by silver staining. The proteins co-purified with Mrp20ΔC$_{His}$ in a specific manner were analyzed by LC-MS and are indicated in the figure. M- Molecular weight marker. The 5% lysate ("Total") and the eluted material ("Bound") was also analyzed by western blot assays using antibodies specific for large ribosomal proteins and a non-ribosomal protein Prx1.

on the Ni-NTA beads with the purified Mrp20ΔC$_{His}$ protein, but were not present in the control non-tagged GAL-*mrp20ΔC* or wild type samples. The ribosomal proteins MrpL36 and Mrp49 were not found in association with the Mrp20ΔC$_{His}$ protein, indicating that the Mrp20ΔC subassembly complex was limited in its composition and does not contain all of the large ribosomal subunit proteins.

The interaction of MrpL40 protein with the Mrp20ΔC-containing subcomplex is consistent with previously discussed results where the Mrp20 and MrpL40 proteins were shown to be in vicinity to each other. To verify if other candidate proteins (MrpL25, MrpL27, MrpL35 and Prx1) are true interacting partners of Mrp20ΔC containing subcomplex, these proteins were separately His-tagged and expressed in yeast cells harboring Mrp20ΔC protein, in order to perform a reciprocal pull-down experiment that will be described later (sections 3.2.2, 3.2.3 and 3.2.4).

3.2.2 *MrpL25 and MrpL27 were verified as the interacting partners of Mrp20ΔC-containing subcomplex*

MrpL25 and MrpL27 proteins are components of the large ribosomal subunit of mitochondrial ribosomes and are unique to mitoribosomes.In order to verify and characterize the proposed association of MrpL25 and Mrpl27 with the Mrp20ΔC subcomplex, these ribosomal proteins were His-tagged at the C-terminus. The expression of His8-tagged variant of MrpL25 and MrpL27 were driven by a GAL10 promoter in *mrp20ΔC* cells harboring the non-tagged, over-expressed Mrp20ΔC protein. Antibodies against His-tag were used to initially verify that MrpL25$_{His}$ and MrpL27$_{His}$ were present in mitochondria and could be recovered on the Ni-NTA beads following their Triton X-

100 solubilization (Figure 10A). Immunedecoration of the Ni-NTA purified material demonstrated that the Mrp20ΔC, MrpL4 and MrpL40 proteins were efficiently co-purified with both the $MrpL25_{His}$ and $MrpL27_{His}$ proteins from the GAL-*mrp20ΔC* mitochondria (Figure 10B) and in a similar manner as had been observed for the $Mrp20\Delta C_{His}$ protein. Furthermore, as had been also observed for the $Mrp20\Delta C_{His}$ pull-down, other large ribosomal subunit components such as Mrp7, MrpL36 and Mrp49, were not found in association with the $MrpL25_{His}$ or $MrpL27_{His}$ proteins.

Taking together these results, $MrpL25_{His}$ and $MrpL27_{His}$ were confirmed to be a part of the Mrp20ΔC- subcomplex, which consists of at least Mrp20ΔC, MrpL4, MrpL40, MrpL25, MrpL27 and Prx1 proteins.

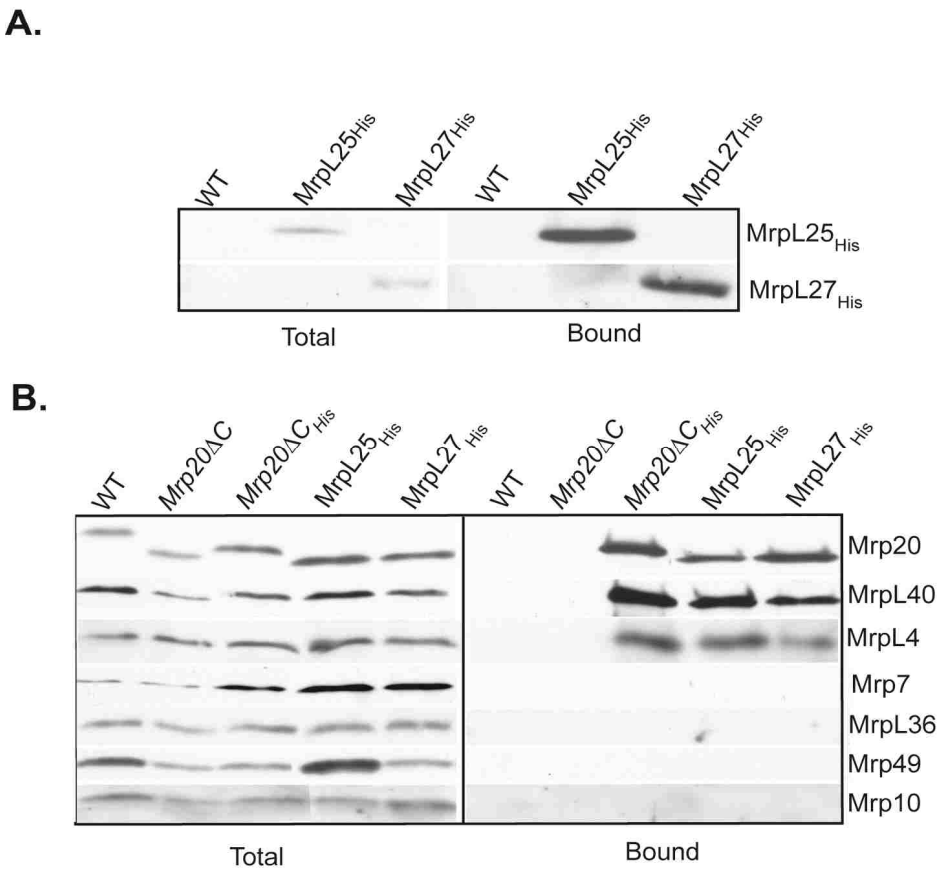

Figure 10: MrpL25 and MrpL27 are the interacting partners of the Mrp20ΔC subcomplex. (A, B) Mitochondria from GAL10-*mrp20ΔC* strain over-expressing His-tagged MrpL25 or MrpL27 and the control wild type mitochondria were detergent solubilized and subjected to the Ni-NTA affinity purification. The 5% lysate ("Total") and the eluted material ("Bound") was analyzed by SDS-PAGE followed by Western blotting with His-specific antibody (A) or immunedecorated with ribosomal proteins specific antibodies as indicated (B).

3.2.3 MrpL35 protein is not a part of the Mrp20ΔC containing subcomplex

MrpL35 protein, a component of the large ribosomal subunit is unique to mitochondrial ribosomes (mammalian homolog-MRPL38, Table 5) with no homology to any bacterial ribosomal protein. As mentioned previously, the analysis of Ni-NTA purified material from Mrp20ΔC$_{His}$ mitochondria by silver staining suggested the presence of a minor band of approximate size of 43kDa that was identified as MrpL35 protein by mass spectrometry analysis (Figure 9B). To analyze the possible association of MrpL35 with the Mrp20ΔC subcomplex, C-terminal His8-tagged construct of MrpL35 was created and expressed under an inducible GAL10 promoter in the yeast cells harboring Mrp20ΔC protein (referred hereafter as MrpL35$_{His}$). Immunedecoration with His-specific antibody confirmed the expression of MrpL35$_{His}$ in GAL-*mrp20ΔC* mitochondria (Figure 11A). Detergent lysis and Ni-NTA purification was then performed to test if the MrpL35$_{His}$ protein co-purified with the Mrp20ΔC subcomplex. Western blotting of the Ni-NTA purified material with antibodies specific for ribosomal proteins indicated that Mrp20ΔC, MrpL40 and Mrp49 were not recovered from beads containing purified MrpL35$_{His}$ protein (Figure 11A). However, two other large ribosomal proteins, Mrp7 and MrpL36 were found to be in association with MrpL35$_{His}$ (Figure 11A). From these results, it can be concluded that MrpL35 is not a part of the Mrp20ΔC-containing subcomplex, but rather forms a separate distinct subcomplex in these *mrp20ΔC* mitochondria, which contains at least the MrpL35, Mrp7 and MrpL36 proteins. Interestingly, the MrpL4

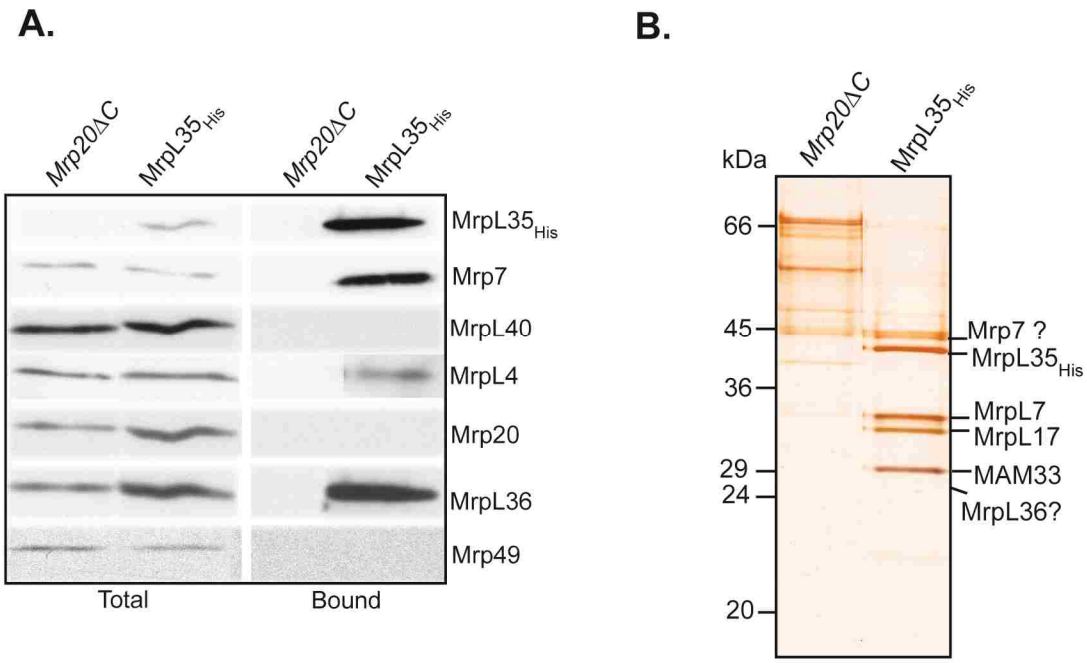

Figure 11: Isolation of the MrpL35$_{His}$ subcomplex. (A, B) Mitochondria isolated from *mrp20ΔC* cells over-expressing either only *Mrp20ΔC* or *Mrp20ΔC* and His-tagged MrpL35 protein were lysed in Triton X-100 containing buffer and subjected to Ni-NTA affinity chromatography. 5% of the solubilized lysate ("Total") and the Ni-NTA purified material ("Bound") from control and the His-tagged sample were analyzed by SDS-PAGE followed by Western blotting with His-specific and ribosomal proteins-specific antibodies (A) or bound material analyzed by silver staining (B). The expected position for Mrp7 and MrpL36 proteins that were found as interacting partners by Western blotting is indicated by (Mrp7?) and (MrpL36?).

protein, was found to associate both with the Mrp20ΔC as well as the MrpL35-containing subcomplex.

The protein composition of the $MrpL35_{His}$ subcomplex was further addressed. To this end, Ni-NTA purified material from *Mrp20Δ*C+$MrpL35_{His}$ mitochondria was further analyzed by SDS-PAGE followed by silver staining. In addition to $MrpL35_{His}$ protein, three dominant proteins were recovered in a specific manner with $MrpL35_{His}$-immobilized beads (Figure 11B). The large ribosomal proteins (Mrp7, MrpL4 and MrpL36) that were shown by immunedecoration to co-purify with $MrpL35_{His}$ (Figure 11A) were however not obvious in the silver-stained gel and thus possibly are present in sub-stoichiometric levels to the other proteins purified with $MrpL35_{His.}$ Mass spectrometry analysis of the dominant proteins from the silver-stained gel identified these proteins to be MrpL7 (33kDa), MrpL17 (32kDa) and MAM33 (30kDa) (Figure 11B). MrpL7 and MrpL17 are known to be essential components of the ribosomal 54S particles.. The third dominant protein identified to be Mam33, is an acidic protein of the mitochondrial matrix thought to be involved in oxidative phosphorylation but of an unknown function. The verification of these proteins members of the MrpL35 subcomplex and their functional significance is beyond the scope of the current study, but should be addressed in the future.

In summary, the results shown here suggest that the MrpL35 protein exists in a ribosomal subcomplex that is distinct from the Mrp20ΔC subcomplex. MrpL4, Mrp7 and MrpL36 proteins were demonstrated to associate with $MrpL35_{His}$ protein immobilized on Ni-NTA beads. Mass spectrometry analysis also suggested the presence of MrpL7, MrpL17 and Mam33 proteins with $MrpL35_{His}$ protein in this novel MrpL35 subcomplex.

3.2.4 Prx1, a mitochondrial peroxiredoxin protein is a part of Mrp20ΔC containing subcomplex

Prx1 is a mitochondrial peroxiredoxin protein involved in protection from oxidative stress in the mitochondria (Greetham & Grant, 2009). Prx1 protein was found to be component of the Mrp20ΔC$_{His}$ protein complex (Figure 9C, D). To further investigate and verify the association of Prx1 protein with the Mrp20ΔC subcomplex, a C-terminal His8-tagged variant of Prx1 was expressed under the control of GAL10-inducible promoter in yeast cells harboring Mrp20ΔC protein and in wild type yeast cells (Mrp20ΔC-Prx1$_{His}$ and WT-Prx1$_{His}$ respectively). Western blotting and immunedecoration with Prx1- and His-specific antiserum confirmed the over-expression of Prx1$_{His}$ in both GAL-*mrp20ΔC* and wild type cells (Figure 12A). Immunoblotting with Prx1 antibody indicated that the levels of Prx1$_{His}$ were approximately 30-fold higher than the endogenous Prx1 protein levels in both the *mrp20ΔC*-Prx1$_{His}$ and WT-Prx1$_{His}$ mitochondria (Figure 12A). Over-expression of Prx1$_{His}$ protein in the wild type cells did not appear to interfere with the ability of the cells to perform cellular respiration, as shown by the absence of any significant growth defect in WT-Prx1$_{His}$ cells compared to the wild type control (Figure 12B). As a negative control to monitor the growth phenotype, ρ^0 cells were analyzed in parallel (Figure 9B). To verify the association of Prx1 with the Mrp20ΔC subcomplex, a reciprocal pull-down experiment, using GAL-*mrp20ΔC*+ Prx1$_{His}$ and GAL-*mrp20ΔC* mitochondria, as negative control, was performed. Prx1$_{His}$ was shown to co-purify with Mrp20ΔC and MrpL40 proteins, further verifying the specificity of the previously

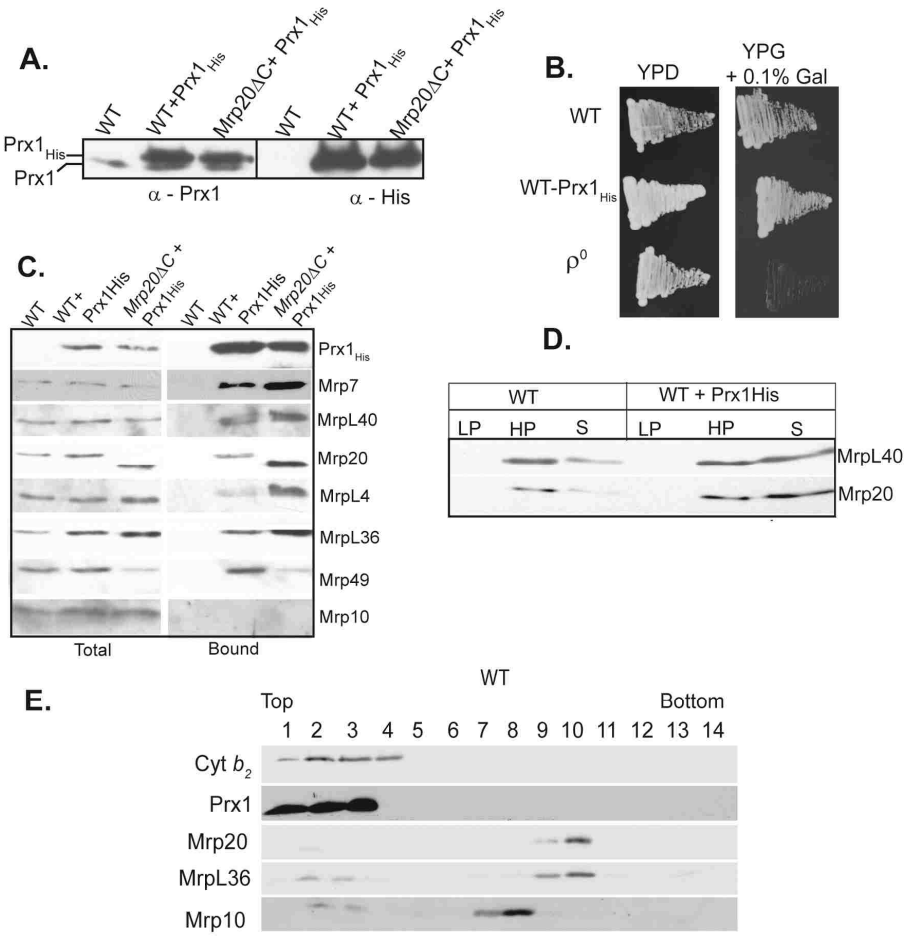

Figure 12: **Prx1 co-fractionates with non-assembled ribosomal proteins.** (A) Prx1 and $Prx1_{His}$ expression was analyzed in mitochondria (50μg) isolated from cells expressing wild-type Prx1 or over-expressing His-tagged Prx1 in the indicated strains. (B) Growth phenotype of indicated yeast strains was monitored on dextrose- or glycerol/galactose containing media (YPD and YPG+0.1% Gal) for three days at 30°C. (C) Triton X-100 lysis and Ni-NTA affinity purification was performed on mitochondria isolated from the indicated strains followed by Western blot analysis with antisera specific to His-tag and large ribosomal proteins. Total – 5% solubilized mitochondria, Bound- Ni-NTA eluted material. (D) Mitochondria from indicated strains were detergent solubilized under high-salt conditions and subjected to clarifying spin. The resulting pellet (LP) contains non-solubilized material. The solubilized material was then subjected to ultra-centrifugation, resulting in a high-pellet (HP) and supernatant (S). Samples were analyzed by SDS-PAGE followed by Western blotting with large ribosomal proteins as indicated. (E) Wild-type mitochondria were solubilized with digitonin containing lysis buffer and were fractionated on a linear sucrose gradient as described in Materials and Methods. Fractions were analyzed by SDS-PAGE and Western blotting with antibodies specific to Prx1, large ribosomal proteins (Mrp20 and MrpL36), small ribosomal protein (Mrp10) and the soluble protein marker Cyt b_2.

described Mrp20ΔC$_{His}$ results (Figure 12C). Interestingly, other large ribosomal proteins, Mrp7, MrpL36 and Mrp49 were also specifically recovered with Prx1$_{His}$ on the Ni-NTA beads. The small ribosomal protein, Mrp10 however was not found to be associated with the Prx1$_{His}$ protein. These data demonstrate that Prx1 can associate with more than one ribosomal subcomplex and that the Prx1 protein is not exclusively associated with the Mrp20ΔC subcomplex (Figure 12C).

The ability of Prx1$_{His}$ to co-purify with all the large ribosomal proteins may indicate that Prx1 normally associates with the assembled 54S ribosomal particles, or alternatively that that Prx1may associate with diverse ribosomal subcomplexes as they are undergoing assembly into the 54S particle.Prx1$_{His}$ was therefore over-expressed in the wild type cells (WT-Prx1$_{His}$) to test if Prx1 could also be found in association with the assembled 54S particle containing ribosomal proteins. Western blot analysis of the Ni-NTA purified material indicated that Prx1$_{His}$ from wild type mitochondria can co-purify with ribosomal proteins like Mrp20, MpL36, Mrp49, MrpL4, MrpL40 and Mrp7. The fraction of ribosomal proteins found with Prx1$_{His}$ from WT-Prx1$_{His}$ mitochondria, however, was significantly lower than was observed to co-purify with the Prx1$_{His}$ from *mrp20ΔC* mitochondria (Figure 12C). This suggests that the Prx1 protein has higher tendency to associate with the non-assembled ribosomal proteins or ribosomal subcomplexes as was observed using *mrp20ΔC* mitochondria. It may be possible that a small amount of the ribosomal proteins found with the Prx1$_{His}$ protein in the wild type mitochondria is due to the fact that over-expression of Prx1His may partially interfere with ribosomal assembly.

Therefore, a population of non-assembled ribosomes may exist in WT-Prx1$_{His}$ mitochondria in contrast to wild type mitochondria, with endogenous levels of Prx1. By differential ultra-centrifugation the assembly status of ribosomal proteins was therefore tested in WT-Prx1$_{His}$ mitochondria and compared directly with a wild type control mitochondria (i.e. harboring endogenously expressed Prx1). Under these centrifugation conditions, assembled ribosomes are recovered in a pellet fraction and soluble proteins or small soluble complexes (i.e. non-assembled ribosomal proteins) are retained in the supernatant. When the wild type mitochondrial extract was subjected to differential ultracentrifugation, the large ribosomal proteins such as Mrp20 and MrpL40 were largely found in the high-pellet fraction because they recovered with the assembled ribosomes and a non-assembled population of these proteins does not exist in wild type mitochondria (Figure 12D). However, in the WT-Prx1$_{His}$ mitochondrial extracts a significant fraction of Mrp20 and MrpL40 proteins, were found in the supernatant fraction following ultracentrifugation, indicating that they were not associated with assembled ribosomes. A population of non-assembled Mrp20 and MrpL40 proteins in the WT-Prx1$_{His}$ mitochondria, suggest that a defect in the assembly of ribosomal proteins in wild type mitochondria occurs upon over-expression of Prx1$_{His}$.

The proposal that Prx1 protein does not interact with the assembled 54S particle but only with the non-assembled ribosomal proteins or subcomplexes is supported by the fractionation of Prx1 protein relative to the assembled ribosomal proteins in wild type mitochondria. The Prx1 did not co-fractionate with assembled large ribosomal proteins even when wild type mitochondria were solubilized and analyzed by sucrose density centrifugation under milder conditions (0.25% digitonin and no salt) compared to the

conditions used for Ni-pull down assays (1% Triton and high salt). The inability of Prx1 to co-fractionate with the large ribosomal subunit suggests that Prx1 does not associate with the assembled 54S particle but rather is found in association with large ribosomal proteins present in subcomplexes i.e. that are not fully assembled into 54S particles.

Taking these results together, it can be concluded that Prx1 may act as a chaperone for the ribosomal subcomplexes during their assembly process and that over-expression of $Prx1_{His}$ can interfere with ribosomal assembly.

3.3 The Mrp20ΔC subcomplex is tethered to the inner membrane

Introduction

The prior results have demonstrated that truncation of the mitospecific region of Mrp20 protein causes a defect in the ribosome assembly pathway and results in the accumulation of stable ribosomal subcomplexes. The composition of these subcomplexes - the Mrp20ΔC subcomplex and the MrpL35 subcomplex have been described here. The completion of ribosomal assembly involves the association of pre-54S ribosomal particle with the inner membrane associated ribosomal protein, MrpL32. The assembled and translationally active ribosomes are tethered to the inner membrane, but it is currently unknown if the non-assembled ribosomal proteins (or assembly intermediates) can gain association with the membrane prior to the formation of an active 54S large ribosomal subunit. To further characterize the Mrp20ΔC subcomplex and possibly thereby to gain

more insight into the ribosome assembly process, the possible association of Mrp20ΔC subcomplex with the inner membrane was next addressed.

Results

3.3.1 Ribosomal proteins are membrane associated prior to their assembly into the pre-54S ribosomal particle

Mitochondria isolated from the *mrp20ΔC* mutant, and harboring the over-expressed Mrp20ΔC protein, were subjected to a mild sonication treatment under low salt conditions and the mitochondrial membranes were recovered by low speed centrifugation (Figure 13A, left panel). The ribosomal proteins, such as *Mrp20ΔC*, MrpL40 and MrpL36, all co-fractionated with the integral inner membrane marker, the ADP/ATP carrier protein (AAC). The soluble mitochondrial matrix marker protein Cpr3 was recovered exclusively in the soluble supernatant fraction, indicating the sonication conditions successfully disturbed the integrity of the mitochondrial membrane system. The absence of ribosomal proteins in the supernatant fraction indicated that the non-assembled ribosomal proteins in the GAL-*mrp20ΔC* mitochondria are not located as soluble proteins in the matrix fraction, but rather are tethered to the inner membrane, despite the fact that they are not assembled into translationally-active ribosomes. The observed association of these ribosomal proteins with the membrane was partially sensitive to salt, because if the sonication step was performed in the presence of higher salt concentration, a significant fraction of these proteins were released from the membrane and recovered with Cpr3 in the supernatant fraction (Figure 13A, left panel).

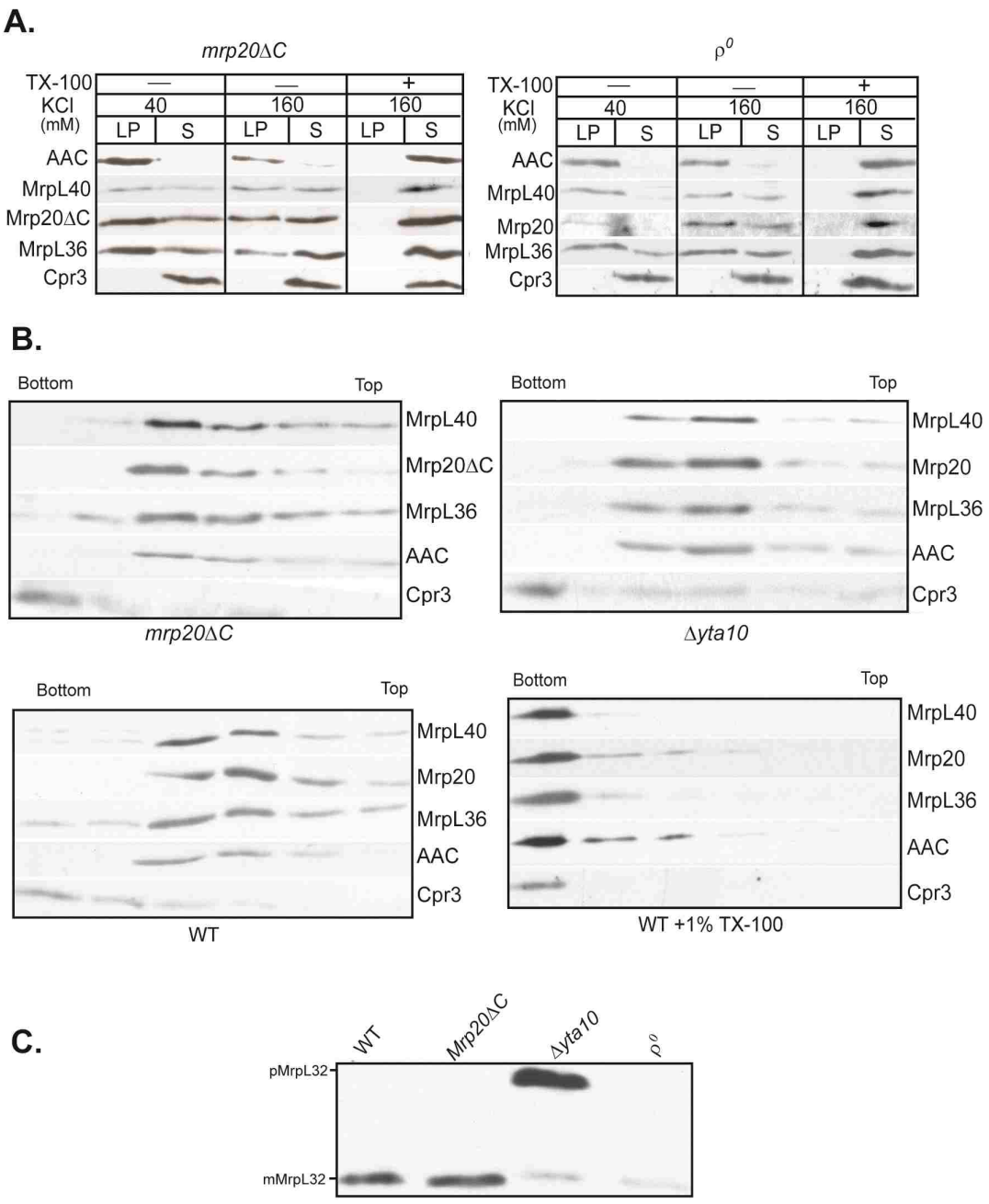

Figure 13: Membrane association of the ribosomal proteins is not dependent on the mitospecific region of Mrp20 protein and the assembly status of ribosomes. (A) The membrane pellet and the soluble supernatant fractions were obtained by sonicating the GAL10-*mrp20ΔC* (left) or ρ^0 (right) mitochondria under different salt concentration followed by low-speed centrifugation. Detergent treatment was performed prior to the centrifugation only for high-salt sonicated samples in parallel. The fractionation of ribosomal proteins was analyzed by western blotting with large ribosomal proteins

specific antibodies. AAC and Cpr3 were used as the membrane and the soluble protein markers respectively. AAC- ADP/ATP carrier protein, LP- low-speed pellet, Sup- supernatant (B) Mitochondria isolated from GAL10-*mrp20*Δ*C*, Δ*yta10* and wild type, as indicated, were sonicated under low salt conditions and subjected to membrane floatation assay. Proteins in the fractions were precipitated by the addition of 12% trichloroacetic acid and analyzed by Western blotting with antibodies against the proteins indicated. The sonicated lysate of the wild type mitochondria was treated with 1% Triton X-100 prior to the membrane floatation assay (lower right panel). Fractions were collected and processed as described above. (C) MrpL32 processing is normal in GAL10- *mrp20*Δ*C* mitochondria. Steady state levels of MrpL32 protein in the indicated strains. p, precursor; m, mature.

The recovery of the ribosomal proteins in the pellet fraction with AAC was dependent on their association with the membrane, because when the detergent Triton X-100 and high salt was added to solubilize the membrane, following sonication and prior to the differential centrifugation step, the ribosomal proteins were recovered exclusively in the supernatant fraction, together with the solubilized AAC protein. A similar behavior of the non-assembled ribosomal proteins was observed when the sonication experiments were performed with ρ^0 mitochondria. A significant fraction of Mrp20, MrpL40 and MrpL36 proteins from ρ^0 mitochondria also co-fractionated with the membrane pellet fraction following sonication, but as was observed with the Mrp20ΔC mitochondria, these non-assembled ribosomal proteins were recovered in the supernatant if detergent was added to lyse the ρ^0 membranes prior to the differential centrifugation step (Figure 13A, right panel).

To further demonstrate that the recovery of the non-assembled ribosomal proteins from the GAL-*mrp20ΔC* and ρ^0 mitochondria with the membrane pellet fraction truly reflected their membrane association, a membrane floatation approach was next employed. Following sonication in the presence of low salt, GAL-*mrp20ΔC* mitochondrial samples were placed at the bottom of a sucrose gradient and subjected to ultracentrifugation where the membranes were allowed to float up in the gradient (Figure 13B). Non-membrane associated proteins (soluble proteins, e.g. Cpr3, or aggregated

material) remained at the bottom of the gradient. The ribosomal marker proteins were observed to co-migrate with the inner membrane marker protein AAC, and they differed in their profile from the soluble matrix marker protein Cpr3, which was retained at the bottom of the gradient (Figure 13B, upper left panel). The observed floatation of the non-assembled ribosomal proteins in the GAL-*mrp20ΔC* sample was similar to that of the behavior of the assembled ribosomes in the wild type ρ^+ control sample analyzed in parallel, which also were observed to migrate with the floated membranes (Figure 13B, lower left panel). As a control to monitor if the ribosomal proteins float because of their association with the membrane, detergent was added to the wild type sample prior to the sucrose gradient. All the ribosomal proteins were recovered at the bottom of the gradient, together with the solubilized inner membrane proteins and matrix markers (Figure 13B, lower right panel). The wild type result is consistent with previously published data where a similar floatation approach was used to demonstrate the tight association of assembled ribosomes with the inner membrane (Prestele *et al*, 2009).

Using the sonication and floatation approach, the membrane association of ribosomal proteins in mitochondria isolated from a late stage ribosome assembly mutant, the *yta10* null strain was also addressed. Mitochondria deficient in the inner membrane Yta10/Yta12 protease fail to proteolytically mature the large ribosomal subunit protein MrpL32 (Figure 13C), and thus a final stage in the assembly of the large ribosomal subunit is prevented and a stable pre-54S ribosomal particle has been shown to accumulate (Nolden *et al*, 2005). As was observed in mitochondria from the early stage ribosome assembly mutants, the ρ^0 and the GAL-*mrp20ΔC*, sonication and floatation experiments indicated that in the Δ*yta10* sample, the ribosomal proteins also co-

fractionated with the inner membrane marker protein AAC in the gradient and were not recovered at the bottom of the gradient with the soluble marker protein, Cpr3 (Figure 13B, upper right panel).

Finally, in the GAL-*mrp20ΔC* mutant that was proposed to accumulate different ribosomal subcomplexes, the MrpL32 protein was found to be normally processed in contrast to the Δ*yta10* mitochondria where defective processing of MrpL32 protein leads to accumulation of the precursor form of the protein. Furthermore, the steady state levels of MrpL32 protein in the GAL-*mrp20ΔC* mutant were comparable to those of wild type mitochondria and in contrast to the ρ^0 mitochondria, where the MrpL32 protein is proteolytically unstable in the absence of ribosomal assembly (Figure 13C).

On the basis of these diverse ribosomal assembly defective mitochondria, it can be concluded that the association of ribosomal proteins with the inner membrane is not dependent on their prior assembly together with the rRNA into a pre-54S or the final 54S particle. Furthermore, the ability of ribosomal proteins in the GAL-*mrp20ΔC* mitochondria demonstrated that an intact mitospecific region of Mrp20 is not required to ensure association of Mrp20 or associated ribosomal proteins with the inner membrane.

3.4 Ribosomal proteins are associated with a detergent-resistant scaffold

Introduction

The mitochondrial inner membrane is considered to be the most protein-rich cellular membrane. A tight association of the mitoribosomes with the inner membrane has been reported for yeast, as well as bovine mitoribosomes. The ribosomal proteins of the bovine mitoribosomes were shown to be tightly associated with the inner membrane and a fraction of the ribosomal proteins remained in a “detergent resistant” fraction even under high-salt conditions (Liu & Spremulli, 2000). These evidences indicate that ribosomal proteins are permanently associated with the inner membrane but little is understood about what are the factors responsible for such a tight association. It is possible that there are some detergent resistant patches of protein or lipids in the inner membrane to which the ribosomal proteins could be associated making them detergent inaccessible. In this study, it is proposed that inner membrane proteins or lipids may interact with the ribosomal proteins to recruit them to the “detergent resistant” complexes in the yeast mitochondria that can act as a platform for the assembly of mitoribosomes.

Results

The detergent resistant extraction behavior of ribosomal proteins in wild type mitochondria under low salt and high salt conditions was investigated. Following Triton X-100 solubilization under low salt conditions, low-speed centrifugation was performed to obtain a “detergent resistant” pellet fraction and solubilized supernatant fraction. As expected, the detergent solubilization of mitochondria resulted in the release of the integral membrane proteins such as, AAC to the supernatant fraction, along with the soluble proteins marker protein, such as the Cpr3 (Figure 14A). Since mitochondrial

ribosomes are found associated with the inner membrane, one would expect that the detergent treatment would also release ribosomal proteins from the inner membrane into the soluble supernatant fraction and that the higher speed ultra-centrifugation conditions are required to pellet the soluble ribosomal proteins. However, as shown in Figure 14A (left panel), a significant fraction of the ribosomal proteins such as Mrp20 and MrpL40 were recovered in the low-speed detergent resistant pellet fraction. The absence of membrane protein, AAC from this pellet fraction indicated that the membrane solubilization was complete. When the detergent solubilization of mitochondria was performed under high salt conditions, the ribosomal proteins were all recovered in the supernatant fraction, and their absence from the low-speed pellet fraction was noticeable (Figure 14A, right panel). To monitor if the "detergent resistant" low-speed pellet contains assembled ribosomal proteins or an aggregate of ribosomal proteins that may have pelleted under low-speed, in a separate experiment, the low-speed pellet (from low salt conditions) was further treated with detergent in the presence of high salt and then subjected to the sucrose sedimentation analysis in parallel with low-speed supernatant fraction. The salt-extracted ribosomal proteins such as Mrp20 and MrpL40 were observed to be present as the 54S assembled proteins because they co-migrated deep into the gradient demonstrating their assembled nature (Figure 14B upper panel). As expected, the solubilized ribosomal proteins in the supernatant fraction (obtained after low-salt treatment) were also found to be assembled in nature as show by the sucrose sedimentation analysis (Figure 14B, lower panel). Thus, it can be concluded that the mitoribosomes present in the "detergent resistant" low-speed pellet are not aggregated ribosomal proteins but indeed represent 54S assembled ribosomal particles.

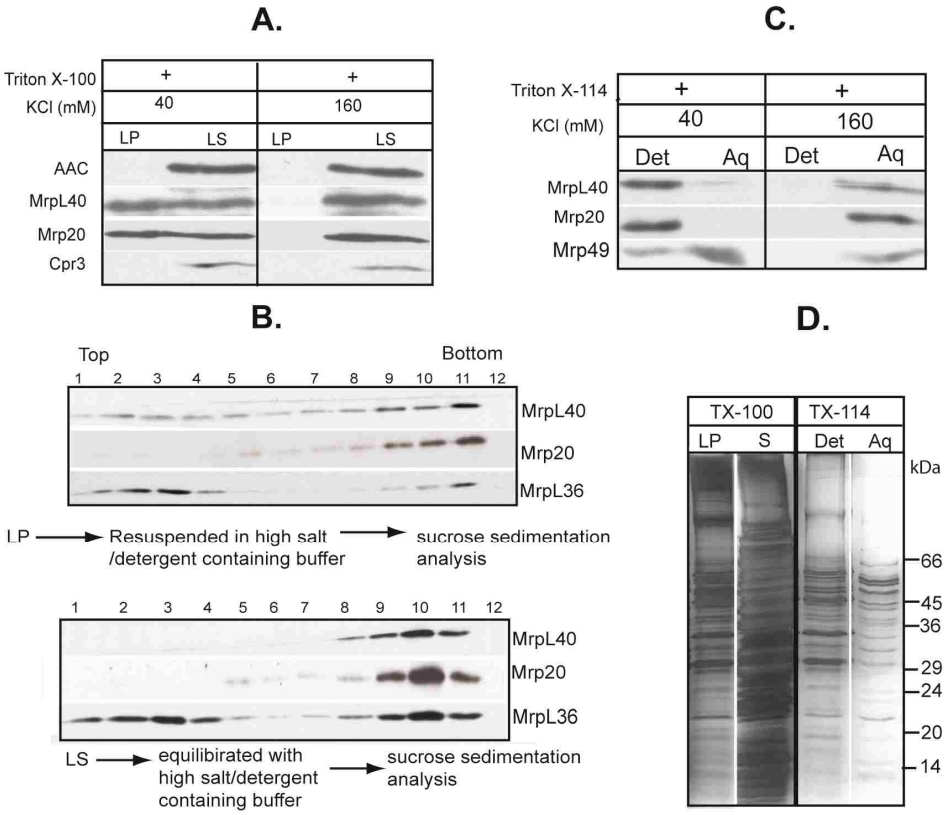

Figure 14: Ribosomal proteins are present in a detergent-resistant fraction in a salt-sensitive fashion. (A) Left panel: Mitochondria isolated from wild-type cells were Triton X-100 solubilized under low- and high-salt conditions. The solubilized material was subjected to low-speed centrifugation to result in a low-speed pellet (LP) and a supernatant (LS).The fractions were subjected to SDS-PAGE followed by Western blotting. (B) In a separate experiment the low- salt pellet (resuspended in high salt and detergent containing buffer) and the low-salt supernatant (equilibirated with high-salt and detergent) were further analyzed by sucrose sedimentation analysis on 15-30% linear sucrose gradient. Fractions were collected, harvested and analyzed by SDS-PAGE followed by immunoblotting with ribosomal antibodies. (C) Wild-type mitochondria were solubilized in Triton X-114 at 37^{o}C under low-salt and high-salt conditions. Low-speed centrifugation from the solubilized supernatant resulted in a detergent phase (Det) and an aqueous phase (Aq). The fractions were analyzed by SDS-PAGE followed by Western blotting with indicated antibodies. (D) Wild type mitochondria (300 μg) were solubilized in low-salt Triton X-100 containing buffer and divided into two equal halves and then subjected to the low speed centrifugation to result in low-speed pellet (LP) and low-speed supernatant (LS). The LP from first half was solubilized by SDS-sample buffer. The LP from other half was further resuspended in Triton X-114 containing buffer (low salt) at 37^{o}C and then subjected to low-speed centrifugation to result in a detergent phase (Det) and aqueous phase (Aq). The LS, Det and Aq fractions were TCA precipitated and analyzed on SDS-PAGE along with the LP. Western blot analysis with indicated antibodies was performed.

To investigate if the ribosomal proteins present in the low-salt pellet were associated with any hydrophobic membrane proteins, the "detergent resistant" low-speed pellet from the low-salt Triton X-100 extraction was independently treated with the detergent Triton X-114 at 37^{o}C. The non-ionic detergent Triton X-114, when warmed undergoes phase-partition into a detergent phase, containing extracted hydrophobic proteins and an aqueous phase, containing extracted hydrophilic proteins. These two phases can be separated from each other by centrifugation at room temperature, and can be separately analyzed. The low-speed pellet thus obtained after Triton X-100 solubilization was treated with Triton X-114 either under low-salt (40mM) or high-salt (160mM) conditions, warmed to 37^{o}C and then centrifuged to obtain a detergent and an aqueous phase. Both detergent and aqueous fractions were subsequently analyzed on SDS-PAGE followed by Western blotting and immunedecoration with ribosomal-specific antibodies. Under low-salt conditions, the ribosomal proteins, Mrp20 and MrpL40 proteins were recovered in the detergent fraction, whereas ribosomal protein such as Mrp49 was largely released into the aqueous phase indicating a different behavior of the ribosomal proteins (Figure 14C). The retention of Mrp20 and MrpL40 protein in the detergent phase under low-salt conditions suggests that these proteins may be in association with the hydrophobic integral membrane proteins and thus are recovered in this fraction. In the presence of high-salt, all of the ribosomal proteins are retained in the aqueous phase. The release of these proteins from the detergent phase under the high-salt conditions suggests that hydrophilic based protein-protein interactions with membrane protein(s) may have been responsible for their observed retention in the detergent phase under low-salt conditions.

Next, a pilot study was initiated to identify the proteins that could be responsible for retention of ribosomal proteins in the detergent phase under low-salt conditions. For this purpose, the Triton X-114 detergent phase fraction (as described above) was analyzed by SDS-PAGE followed by silver staining (Figure 14D). In a parallel experiment, this low-salt detergent phase fraction was subjected to mass-spectrometry analysis to identify the proteins that comprise the detergent phase. Our goal specifically was to identify membrane proteins that may be responsible for ensuring retention of ribosomal proteins with this low-speed detergent phase fraction. Due to the large number of proteins present in the detergent fraction, there were multiple proteins identified by mass-spectrometry data. Many of these proteins belonged to the abundant complexes in mitochondria such as pyruvate dehyrogenase (PDH) complex and the F_1-F_o ATP synthase complex, that are composed of multiple subunits. The presence of the matrix localized PDH complex proteins in the detergent phase fraction could be an artifact because of the abundant nature of this protein complex. Although, the ATP synthase complex is membrane localized, the presence of the subunits of the ATP synthase complex in this detergent fraction was thought to be an artifact since Western blot analysis with F_1 subunit of this complex was not observed in the detergent fraction (data not shown). In addition to these large soluble complexes, the mass-spectrometry analysis also revealed the presence of membrane proteins such as Phb1 and Phb2 (component of multimeric Prohibitin complex) and interestingly, the large ribosomal protein, MrpL35. These are preliminary results and further studies should be done to validate and further characterized this data. The ribosomal proteins such as Mrp20 and MrpL40 that were previously shown to be recovered in the detergent phase through immunological

detection were not identified in mass-spectrometry approach, demonstrating that an exhaustive analysis has not been completed. Some of the proteins indicated through this mass-spectrometry analysis to be present in the detergent fraction are listed in Table 7.

In summary, results shown here demonstrate that a large fraction of ribosomal proteins are not solubilized by Triton X-100 under low-salt conditions and are recovered in the “detergent resistant” low-speed pellet fraction. Further analysis of this low-speed pellet with Triton X-114 demonstrated that some ribosomal proteins such as Mrp20 and MrpL40 are associated with hydrophobic membrane proteins and thus retained in detergent phase in a salt-sensitive fashion. Mass spectrometry analysis of the low-speed detergent phase fraction obtained after Triton X-114 revealed proteins such as Phb1, Phb2 and MrpL35 that will be of particular interest to study in future to explore their possible role in the retention of ribosomal proteins in the detergent phase.

Table 7 Mass spectrometry analysis of the detergent phase obtained after Triton X-114 treatment

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Candidate proteins	Peptide count	% coverage	Component of the complexes
PDB1	10	29	E1 beta subunit of Pyruvate dehydrogenase
LAT1	9	22	E2 subunit of Pyruvate dehydrogenase
ATP1	5	11	ATP Synthase
PDA1	7	14	Pyruvate dehydrogenase
POR1	6	26	Mitochondrial porin
ATP2	7	18	ATP Synthase
PHB1	4	18	Prohibitin complex
PET9	3	10	ADP/ATP Carrier
PHB2	2	9	Prohibitin complex
MIR1	3	11	Mitochondrial phosphate carrier
QCR2	2	7	Cytochrome *c* reductase
MRPL35	2	6.5	Mitochondrial large ribosomal subunit
MGM101	2	7.8	Mitochondrial genome maintenance

Chapter 4 Discussion

Introduction

A large number of ribosomal proteins in the mitoribosomes have acquired C-terminal extension sequences termed mitospecific regions with an unknown function. One of the possible roles of the mitospecific region is thought to be the stabilization of ribosomal proteins and to support ribosomal assembly process. The primary objective of the present study was to understand the functional importance of the mitospecific region of a ribosomal protein, Mrp20 for the assembly of mitoribosomes in yeast mitochondria. Mrp20 is a nuclearly encoded protein located at the exit site of the large ribosomal subunit of mitoribosomes.

Using a truncated derivative of the Mrp20 protein, Mrp20ΔC, it was found that the mitospecific region of Mrp20 is important for the assembly of mitoribosomes. Further analysis of the *mrp20ΔC* mutant revealed the presence of a subcomplex of ribosomal proteins being accumulated in the mutant and that the subcomplex was deficient in proceeding to the 54S particle. Using the novel ribosomal assembly mutant, *mrp20ΔC,* the location of early ribosomal assembly events was analyzed.

The mitoribosomes are tightly associated with the inner membrane, however, little is known about the factors responsible for this association. One aspect of this study was

focused to identify the proteins/factors responsible for the tight association of ribosomes with the inner membrane.

4.1 Proteins at the exit site of mitoribosomes are important for ribosomal assembly and its function

The mitospecific region of Mrp20 is required for ribosomal assembly and OXPHOS function

The present study investigates the role of the mitospecific region of the Mrp20 protein in the assembly and function of mitoribosomes. Mitoribosomes have significantly deviated from their bacterial ancestors in terms of the protein composition and the function (Borst & Grivell, 1978; Yang *et al*, 1985). The additional presence of extra N- or C-terminal sequences in many ribosomal proteins is one of the features that mitoribosomes have adapted during their evolution from bacteria (O'Brien, 2002). Notably, the unique mitospecific sequences of ribosomal proteins are mostly conserved among their relatives suggesting these sequences play an important and diverse role in the function of the mitoribosomes. Whether the mitospecific regions are important for the mitoribosome-specific functions such as co-translational synthesis of mitochondrially-encoded proteins; compensation for the loss of rRNA; stabilization of the ribosomal proteins; or ribosomal assembly is largely unknown.

Mrp20 is a component of the large ribosomal subunit located at the exit site of the polypeptide tunnel. Sequence analysis of Mrp20 indicates the presence of a C-

terminal mitospecific region (of approximately 100 aa in length) in addition to the conserved bacterial L23 domain. It is demonstrated here that truncation of the mitospecific region of the yeast Mrp20 protein is deleterious for the assembly of the large (54S) ribosomal subunit in yeast. Specifically, yeast mutants harboring a C-terminally truncated Mrp20 protein (*mrp20ΔC* mutant) are respiratory inactive. This respiratory deficiency of the *mrp20ΔC* mutant is due to the failure to synthesize key subunits of the mitochondrial OXPHOS enzymes that are encoded by the mitochondrial genome, caused by the inability of their mitochondria to assemble translationally active ribosomes.

Similarly, our previous studies (Jia *et al*, 2009) have indicated that the mitospecific region of MrpL40, another component of the large ribosomal subunit is involved in the assembly of large ribosomal subunit. MrpL40, like Mrp20 is located at the exit site of the mitoribosomes and can cross-link with the inner membrane protein, Oxa1. In contrast to Mrp20 and MrpL40, the functional relevance of the mitospecific regions of MrpL4 and MrpL22 (also located at the exit site) remains unknown.

Collectively, these results indicate that the mitospecific regions of the Mrp20 and MrpL40 proteins are important for the ribosomal assembly. Based upon the location of both these proteins at the exit site, it is tempting to speculate that mitospecific region of Mrp20 and MrpL40 and may be other proteins at the ribosomal exit site function together for the assembly of mitoribosomes or mitoribosome-specific functions.

Mrp20 and MrpL40 are physically and functionally related

It is reported here that Mrp20 and MrpL40 proteins exist in close vicinity to each other and are interdependent on each other for stability. An independent study also later reported the ability of Mrp20 to be cross-linked with MrpL40 in yeast mitochondria and thus further substantiated our previously published results that are presented here (Gruschke *et al*, 2010). In this study, it was shown that Mrp20 can chemically cross-link with MrpL40 in intact wild-type mitochondria indicating their close proximity in the assembled ribosome (Jia *et al*, 2009). The purified Mrp20 protein was shown here to directly interact with the recombinant mitospecific region of the MrpL40 protein suggesting these two proteins are physical neighbors in the assembled ribosome. Furthermore, the truncation in Mrp20 leads to instability in both Mrp20 and MrpL40. Likewise, modulation of the mitospecific region of MrpL40 was shown to specifically cause a reduction in the steady state levels of Mrp20. From these results, it can be concluded that truncation in the mitospecific region of either protein (Mrp20 or MrpL40) disturbs their physical interaction and subsequently affects the stability of both the proteins.

What could be the significance of the interaction of Mrp20 and MrpL40 for the mitoribosomes? From the work presented here, it can be speculated that Mrp20 in association with the MrpL40 protein may function to secure the assembly and/or activity of the mitoribosomes to specific locations of the inner membrane, where the synthesis and coupled insertion of mitochondrially-encoded proteins could occur.

4.2 Ribosomal assembly subcomplexes

Little is known about the events that bring together ribosomal proteins and rRNA to form stable large (54S) and small (37S) ribosomal subunits of the mitoribosomes. One of the large ribosomal subunit components, MrpL32, has been identified to be assembled at the later stage of the assembly process of 54S particle in yeast mitoribosomes. The processing of MrpL32 protein by inner membrane proteases, Yta10/Yta12 is required for the assembly of 54S particle (Nolden *et al*, 2005). Thus, an accumulation of pre-54S particle has been reported in the Δyta10/Δ*yta12* mutant and that the pre-54S particle is thought to contain all the large ribosomal proteins except a membrane tethered ribosomal protein, MrpL32. The presence of other ribosomal precursor particles or subcomplexes in the assembly process of the mitoribosomes has not been previously described. On the contrary, in the bacterial or eukaryotic cytosolic ribosomes assembly pathway, a number of assembly intermediates which accumulate in the absence of assembly factors have been characterized. The composition of such assembly intermediates have also been identified by advanced proteomic studies (Jiang *et al*, 2006; Merl *et al*, 2010; Talkington *et al*, 2005). However, the presence of such assembly intermediates (other than pre-54S) or subcomplexes and their composition is not known in mitoribosomes.

The availability of the *mrp20ΔC* mutant (from this study) that is defective in the assembly of mitoribosomes has provided a novel tool to gain new insights into some early events in the ribosome assembly process. The *mrp20ΔC* mutant is quite unique from the previously reported mitoribosome assembly mutants. Despite displaying strongly reduced mitochondrial protein synthesis capacity, unlike many other ribosomal

mutants, *mrp20ΔC* mitochondria retained their ρ^+ status and the levels of ribosomal proteins analyzed, with the exception of Mrp20 and MrpL40, were similar (or even slightly elevated), to those of the wild type ρ^+ mitochondria. Yeast mutants defective in mitochondrial protein synthesis have been reported to lose their mt DNA with high frequency, i.e. change from ρ^+ to ρ^-/ρ^0 status (Myers *et al*, 1985) and the non-assembled ribosomal proteins are present at strongly reduced levels, most likely due to their proteolytic instability in the absence of their assembly. The normal levels of the ribosomal proteins in the *mrp20ΔC* mitochondria suggested that at least these proteins had proceeded far enough in their assembly pathway to ensure their proteolytic stability and in contrast to ρ^0 mitochondria, where the ribosomal proteins are proteolytically unstable. Thus, the availability of the *mrp20ΔC* mutant with its stable levels of ribosomal proteins was really exciting as this assembly mutant was blocked at a stage upstream of the known ribosomal assembly mutant, *Δyta10/Δyta12* (containing pre-54S particle) and downstream from the ρ^0, (deficient in ribosomal assembly due to absence of rRNA and contain reduced levels of ribosomal proteins). The *mrp20ΔC* mutant thus represented a useful tool to study the early events in the ribosomal assembly pathway.

Here, the identification of a novel subcomplex of ribosomal proteins that accumulate in the absence of the mitospecific region of the Mrp20 protein is reported. Using His-tagged Mrp20ΔC protein, the Mrp20ΔC subcomplex was purified and its protein composition was determined. Additionally, the accumulation of another protein subcomplex in the *mrp20ΔC* mutant, the MrpL35 subcomplex, is also presented.

Mrp20ΔC subcomplex

In the absence of complete assembly of the 54S particle, the *mrp20ΔC* mutant accumulates a ribosomal assembly subcomplex, containing the Mrp20ΔC protein and a number of other ribosomal proteins. These proteins were identified to be large ribosomal proteins, MrpL40, MrpL4, MrpL25 and MrpL27. Proteins of the small ribosomal subunit were notably absent from this complex. A number of these proteins were previously identified in chemical cross-linking approaches used to identify proteins located in the proximity of the tunnel exit of mitochondrial ribosomes (Jia *et al*, 2003; Jia *et al*, 2009; Gruschke *et al*, 2010). Using ribosomal proteins in the yeast mitoribosomes such as Mrp20 and MrpL40 that show homology to the conserved bacterial ribosomal proteins (L23 and L24, respectively) located at the exit site of bacterial ribosomes, these chemical cross-linking approaches identified a network of ribosomal proteins including Mrp20, MrpL4, MrpL40, Mrp27, MrpL22, MrpL13 and MrpL3. In the present study, the ribosomal subcomplex in the *mrp20ΔC* mutant contains a number of these exit site proteins, namely Mrp20, MrpL4, MrpL40 and MrpL27, thus supporting the proposal that this subcomplex represents a true assembly intermediate enriched in proteins sharing a common location within the ribosome, i.e. the exit site of the ribosomal exit tunnel. In addition, a novel ribosomal protein, MrpL25 was found to be a component of the Mrp20ΔC subcomplex. This protein had not been previously identified as a neighbor of Mrp20 and these other exit site proteins. MrpL25 represents one of the mitoribosome-specific proteins, which are not conserved from the bacterial ribosomes and indeed, blastp search would indicate this protein to be exclusively found in fungal mitochondria

(Smits *et al*, 2007). Its presence is required to support aerobic growth indicating its function must be specialized to the fungal mitoribosomes. From the demonstrated association of MrpL25 with the Mrp20ΔC subcomplex in this study, it can be speculated that the MrpL25 protein may also be located at or near the exit site of the polypeptide tunnel.

It is important to note that the Mrp20ΔC subcomplex is limited in composition, as other large ribosomal subunit proteins, such as MrpL36 and Mrp7 were not found in this subcomplex. The MrpL36 and Mrp7 proteins represent homologs of the bacterial large ribosomal subunit proteins, L31 and L27 respectively, both of which are located at the interface of the large and small ribosomal subunits, i.e. distant from the exit site of the polypeptide tunnel of the large ribosomal subunit (Harms *et al*, 2001). The absence of MrpL36 and Mrp7 from the Mrp20ΔC subcomplex, which contains proteins comprising the exit site of the polypeptide tunnel, may be indicative that different regions of the large subunit may independently assemble into distinct subcomplexes, prior to their unification into a complete 54S particle.

From the present study, it may be concluded that proteins known to be located at or near the exit site of the ribosome nascent chain tunnel initially co-assemble to form a subcomplex. This subcomplex is thought to secure stability to the ribosomal proteins in the absence of their further assembly into stable 54S ribosomal particles. The exact function of the mitoribosome-specific proteins such as MrpL25 and MrpL27 is not known but it is plausible that these proteins may ensure the tight association of ribosomes

with the inner membrane or can interact with assembly factors required for ribosomal assembly.

Finally, an intact mitospecific region of Mrp20 is not required for the formation of the Mrp20ΔC-subcomplex. From the results presented here, it can be proposed that the presence of the mitospecific region of the Mrp20 protein may be critical for the interaction of this Mrp20-subcomplex with either an assembly factor, and/or with specific component(s) of another ribosome assembly intermediate required to ensure the next step in the 54S assembly.

MrpL35 subcomplex

The *mrp20ΔC* mutant was defective in the assembly of the 54S large ribosomal subunit and further analysis of the mutant using His-tagged Mrp20ΔC protein revealed the presence of a Mrp20ΔC subcomplex. Interestingly, a second subcomplex, termed here as MrpL35 subcomplex, which accumulated in the *mrp20ΔC*, was also identified in this study. This subcomplex was shown to be composed of the large ribosomal proteins, MrpL35, MrpL4, Mrp7, MrpL36 and possibly, MrpL7 and MrpL17. The preliminary studies also suggested a non-ribosomal protein, Mam33 to be associated with this MrpL35- containing subcomplex.

The MrpL35 protein is a constituent of the large ribosomal subunit and essential for mitochondrial translation. BLASTp analysis indicates that MrpL35 is mitochondrial-specific protein conserved from fungi to mammals (where it is known as MRPL38), but does not share any homology with the known bacterial ribosomal proteins (Smits *et al*,

2007). Furthermore, both the fungal and mammalian homolog of MrpL35 contains a putative C-terminal phosphatidyethanolamine binding protein (PEBP) domain. The functional relevance of the PEBP domain of MrpL35 for the mitoribosomes is not known but it will be interesting to analyze if the PEBP domain of MrpL35 is required for the association of mitoribosomes with the mitochondrial inner membrane that is rich in phosphatidyethanolamine (PE).

The finding that MrpL36 and Mrp7, large ribosomal proteins (not component of the Mrp20ΔC subcomplex) were specifically associated with the MrpL35 further supports the previously proposed hypothesis that different regions of ribosomal proteins may assemble independently. However, the ability of MrpL4 protein, (a component of the exit site) to associate with Mrp20ΔC as well as MrpL35 subcomplex was intriguing and raises the possibility that in the final assembled ribosomes Mrp20ΔC and MrpL35 subcomplex are in vicinity to each other and MrpL4 may be present at the interface between these two subcomplexes. Finally, future studies should be done to validate the association of ribosomal proteins such as MrpL7, MrpL17 and non-ribosomal protein, Mam33 with the MrpL35 subcomplex.

Prx1 association with ribosomal subcomplexes

Using His-tagged Mrp20ΔC, the association of a mitochondrial peroxiredoxin protein, Prx1, with the Mrp20ΔC subcomplex was observed. The reciprocal affinity purification of $Prx1_{His}$ from the *mrp20ΔC* mitochondria indicated Prx1 to be in association with not only the Mrp20ΔC subcomplex, but also the MrpL35 subcomplex, a second subcomplex

found to accumulated in the *mrp20ΔC* mutant. Using milder detergent solubilization conditions (digitonin and low salt, as opposed to Triton X-100 and high salt used for the Ni-NTA purifications here) and sucrose gradient centrifugation, no evidence to support an association between Prx1 and the assembled 54S particle from wild type mitochondria could be obtained. Interestingly, $Prx1_{His}$ was found to be associated with large ribosomal proteins in the wild type mitochondria. The studies here indicated that over-expression of $Prx1_{His}$ disturbs ribosomal assembly. Thus, $Prx1_{His}$ association with ribosomal proteins in wild-type mitochondria may reflect the increased population of ribosomal assembly subcomplexes formed when over-expression of $Prx1_{His}$ disturbed ribosomal assembly.

Peroxiredoxins are ubiquitous thiol-specific proteins that have multiple functions for stress protection as antioxidants and molecular chaperones and in the regulation of signal transduction (Greetham & Grant, 2009). Mitochondrial peroxidoxin, Prx1, is involved in the anti-oxidative stress in response to the reactive oxygen species (ROS) by decomposing hydrogen peroxide (H_2O_2) and was found to be important in suppressing mitochondrial DNA mutations caused by oxidative damage. A cytosolic homologue of Prx1 in yeast, Tsa1 (Thiol specific antioxidant) has been shown to be associated with cytosolic ribosomes where it is suggested to play both an antioxidant and a chaperone function to maintain the integrity of the ribosomes by preventing the aggregation of ribosomal proteins under stress conditions (Trotter *et al*, 2008).

To our knowledge, this is the first report showing association of Prx1 with large ribosomal subunit proteins that are not able to assemble into the 54S ribosomal particle. On the basis of this current finding, it is reasonable to propose that Prx1 may be involved

in the assembly of ribosomal subcomplexes and that Prx1 acts as a chaperone to prevent their oxidative damage, prior to assembly, but dissociate following the final assembly of mitochondrial ribosome. However, the association of Prx1 with the non-assembled ribosomal proteins or ribosomal subcomplexes does not appear to be an essential in the assembly process as Δ*prx1* cells are able to grow on glycerol-based media like wild-type cells suggesting that the assembled and functional ribosomes can be formed in the absence of Prx1 protein.

4.3 Inner membrane acts as a platform for the assembly of mitoribosomes

A large number of reports demonstrate the tight association of the assembled and functional ribosomes with the inner membrane. But a little is known where the assembly events prior to the formation of 54S occur, i.e. in the soluble matrix region or anchored to the inner membrane. The detergent stable pre-54S ribosomal assembly intermediate (that accumulates in the Δ*yta10*/Δ*yta12* mutant) has been depicted to accumulate in the matrix that is later targeted to the inner membrane to associate with MrpL32 and form a stable 54S particle (Nolden *et al*, 2005). However, it was never investigated where the pre-54S particle actually assembled.

Although mitoribosomes have originated from the bacterial ancestors, the translation machinery of mitochondria is specialized to synthesize mainly hydrophobic integral membrane proteins in contrast to the bacterial ribosome, that synthesize both soluble and membrane proteins. In bacteria, the assembly of bacterial ribosomes takes

place in the soluble cytosolic region and the assembled ribosomes are targeted to the membrane with the help of proteins like SRP for the co-translational insertion process of membrane proteins. After the translation of the membrane proteins, the bacterial ribosomes are possibly cycled back to the cytosol (Dalbey *et al*, 2010). However, in mitoribosomes, the SRP-like protein is not present and the mitoribosomes are thought to be permanently associated with the membrane thus circumventing the need for a soluble population of ribosomes which need targeting to the inner membrane . It is not clear though, if only assembled ribosomes are tethered to the membrane or if the entire assembly pathway takes place at the membrane. Using ribosomal assembly yeast mutants, the location of the early assembly events of mitoribosomes was investigated in this study.

Membrane association of ribosomal proteins in the ribosomal assembly mutants

In the absence of assembly of the 54S (or stable pre54S) particles, the results here demonstrated that the large ribosomal subunit proteins are still found in association with the mitochondrial inner membrane. Using both differential centrifugation and membrane floatation assay, the ribosomal proteins were shown to co-fractionate with the inner membrane in the ρ^0 and *mrp20ΔC* mitochondria, where ribosome assembly is blocked at early stages, as well as in the Δ*yta10* null mitochondria, where a late stage (stable pre54S) ribosomal particle has been shown to accumulate (Nolden *et al*, 2005). Furthermore, the Mrp20ΔC protein which is anchored to the membrane in the absence of complete ribosome assembly is present in its subcomplex, because if the mitochondrial

membranes are purified to remove the mitochondrial soluble proteins prior to detergent solubilization, the affinity purified Mrp20ΔC$_{His}$ protein, which had been membrane bound, was found to be in association with the MrpL25, MrpL27, MrpL4 and MrpL40 proteins (results not shown). A significant portion of the MrpL36 and Mrp7 proteins were also membrane anchored in the *mrp20ΔC* mitochondria, suggesting that the MrpL35 subcomplex and its ribosomal proteins may also associate with the membrane, but this remains to be further tested. It could be possible that the PEBP domain of MrpL35 is important for the association of the proteins of this subcomplex for their association with the inner membrane. These results indicate that the process of ribosome assembly occurs on the surface of the inner membrane.

Accumulating evidence has demonstrated that the assembled mitoribosomes are tightly associated with the inner membrane (Spithill *et al*, 1978; van der Klei *et al*, 1994) but if the non-assembled ribosomal proteins or ribosomal assembly intermediates can also associate with the membrane is not currently known. It has also been shown that non-translating ribosomes (but assembled) still have the ability to associate with Oxa1 and Mba1 suggesting that the ribosomes may be permanently associated with the inner membrane (Jia *et al*, 2003; Ott *et al*, 2006)

To our knowledge, this is the first report indicating the association of ribosomal proteins with the inner membrane prior to their assembly into the translationally-active ribosomes. The finding that not only fully assembledlarge ribosomal subunit but even the ribosomal subcomplexes or non-assembled ribosomal proteins can associate with the inner membrane is intriguing as it supports the hypothesis that the inner membrane, the site of ultimate translational activity, acts as platform for all the assembly events of

mitoribosomes. Furthermore, the present data also supports the idea that mitoribosomes do not need to recycle back into the matrix, i.e. a soluble population may not exist, but rather they are permanently tethered to the inner membrane. The tight association of the mitoribosomes is important for the co-translational insertion of the integral membrane proteins. Even the synthesis of a soluble small ribosomal protein, Var1, is also synthesized at the membrane suggesting that there is no requirement of the soluble matrix-located ribosomes (Fiori *et al*, 2003). The organization of the assembly events of the mitoribosomes thus significantly differs from their bacterial counterparts, where, the assembly of ribosomes occurs in the soluble cytosol and the assembled ribosomes are targeted to the plasma membrane to facilitate the co-translational insertion of the membrane proteins.

Based on the results discussed here, it is reasonable to propose that staging the assembly process of mitoribosomes on the inner membrane will circumvent the need to target an assembled matrix-localized large ribosomal subunit to the membrane when translation is required. It is tempting to speculate that certain inner membrane proteins or lipids may contribute to form specialized ribosome assembly sites in the inner membrane to which ribosomal proteins become targeted to following their import into mitochondria. It is possible that recruitment to a specialized location in the inner membrane can serve as nucleation sites for the initial assembly of ribosomal subcomplexes, such as the Mrp20-MrpL4-MrpL40-MrpL25-MrpL27 subcomplex or MrpL35-MrpL36-Mrp7, prior to the complete 54S complex assembly.

The observation that Mrp20ΔC and MrpL35 subcomplexes were located on the membrane surface, demonstrates however, that the mitospecific region of Mrp20 is not

required to target the Mrp20ΔC protein and either of its subcomplexes to the surface of the inner membrane. The association of the Mrp20 protein with the membrane was, however, partially affected when the C-terminal mitospecific region was truncated, as a fraction the Mrp20ΔC protein was recovered in soluble mitochondrial fraction under conditions when the full-length Mrp20 protein was recovered entirely with the mitochondrial membrane fraction.

It is therefore concluded from this study that an intact mitospecific region of Mrp20 may support, but is not required to target the protein to the inner membrane. The mitospecific region of Mrp20 is more likely to support the assembly of subcomplexes into 54S particle by interacting with ribosomal assembly factors. In the future it will also be important to study if the Mrp20ΔC subcomplex can interact with inner membrane proteins such as Oxa1 or Mba1 that have been shown to interact with Mrp20 and MrpL40 in the assembled 54S particle (Jia *et al,*2003 ; Jia *et al,*2009; Ott *et al,* 2006). This will allow one to understand whether the early ribosomal events at the membrane occur at the site where insertion takes place or at a different site in the inner membrane.

4.4 Detergent resistant extraction of ribosomal proteins

Yeast mitoribosomes have been shown to be tightly associated with the inner membrane and released only under high salt and detergent conditions(Obbink *et al,* 1977). What is the nature of tight association of mitoribosomes with the inner membrane? A biochemical study with ribosomal proteins and their ability to tether with the inner membrane under

different salt conditions was recently performed in bovine mitoribosomes (Liu and Spremulli, 2000). This study reported that under low-salt conditions, 50% of the large ribosomal subunits were membrane tethered. Interestingly, when the mitochondria were treated with buffers containing high salt and detergent (Triton X-100) to lyse the membrane, approximately 15% of the ribosomal proteins were recovered in a detergent-resistant fraction (Liu and Spremulli, 2000) which pelleted under centrifugation conditions where free solubilized ribosomes would not pellet. Thus, it appears that there is a large detergent-resistant patch or scaffold of membrane proteins or lipids to which the ribosomal proteins can associate (Liu and Spremulli, 2000).

Using wild type mitochondria from yeast, a study with a two-fold objective was initiated here. First, experiments were designed to test if yeast mitoribosomes behave in the same manner as bovine mitoribosomes with respect to the detergent resistant behavior of the ribosomal proteins and second, embark on a strategy to identify the components of such detergent-resistant fraction.

In this study, it is demonstrated that ribosomal proteins in yeast mitoribosomes, like bovine ribosomal proteins, are extracted in a detergent-resistant patch that is pelletable under low speed centrifugation conditions. Notably, the ribosomal proteins under low-speed centrifugation did not pellet independently as all of the ribosomal proteins were recovered in the solubilized supernatant fraction when the mitochondria were detergent solubilized under high salt conditions. Furthermore, the ribosomal proteins that were part of this detergent-resistant low speed pellet were found to represent fully assembled ribosomes, as evidenced by sucrose sedimentation analysis. These results

confirm that this detergent-resistant pellet contain assembled ribosomal proteins and not aggregated ribosomal proteins. Since the ribosomal proteins could be released from the detergent resistant pellet by the addition of high-salt, it is proposed that protein-protein interactions may be responsible for retaining the ribosomes in the detergent-resistant platform fraction. It is possible that some large oligomeric membrane proteins complexes may form special phospholipid rich (such as phosphatidylethanolamine, (PE) or cardiolipin, (CL) present in the inner membrane) microdomains to which ribosomal proteins are associated, that are not solubilized by the detergent.

In the second part of this study, the composition of the detergent-resistant pellet was further investigated to examine if this pellet contains any integral membrane proteins that may be responsible for recruiting and binding the ribosomal proteins. When the low-speed detergent resistant pellet (Triton X-100) was further extracted with Triton X-114, partitioning of hydrophobic membrane proteins (to the detergent phase) and hydrophilic soluble proteins (to the aqueous phase) was achieved. Mass-spectrometry analysis of the detergent phase fraction revealed membrane proteins such as Prohibitin 1 and 2 (Phb1 and Phb2), the ribosomal protein MrpL35, in addition to the components of the pyruvate dehydrogenase complex and the ATP synthase complex. The latter are very abundant complexes and their presence in the mass spectrometry results may be due to contamination.

The prohibitin (Phb1 and Phb2) proteins form a multimeric ring structure (~2 MDa) and are proposed to act as a scaffold for the organization of proteins in the inner membrane (Tatsuta *et al*, 2005). This multimeric prohibitin complex in the inner

membrane is also proposed to recruit lipid domains enriched in PE and CL in the inner membrane (Osman *et al*, 2009b). Genetic analysis reveals a functional relationship between prohibitins and enzymes of phospholipid metabolism (Osman *et al*, 2009a) . It is therefore an attractive possibility that mitoribosomes are associated with the multimeric ring complex of Phb1/Phb2 proteins in the inner membrane that may render the ribosomal proteins detergent resistant. It is tempting to speculate that association of some of the ribosomal proteins with a detergent resistant patch in the inner membrane may act as a nucleation center for the assembly of ribosomal proteins. Furthermore, the identification of MrpL35 as a potential candidate in the detergent resistant fraction is very attractive because as previously mentioned MrpL35 is reported to contain a PE binding protein (PEBP) domain in its C-terminal region. It is therefore possible that MrpL35 protein may associate with the inner membrane (rich in PE and CL) and the Phb1 and Phb2 proteins in the inner membrane and is one of the factors responsible for the tethering of ribosomes to the inner membrane. However, the possibility of other ribosomal proteins associating with Phb1/Phb2 or the inner membrane cannot be excluded. Current efforts are underway in our lab to understand the importance of the PEBP domain of the MrpL35 protein. Moreover, further studies should be done in the future to identify other membrane proteins or the lipids that are involved in the detergent resistant extraction behavior of ribosomal proteins as this may unveil how mitoribosomes are tightly associated with the inner membrane.

4.5 Proposed model for the assembly of mitochondrial ribosomes

Based upon findings reported here for the *mrp20ΔC* mutant, a model for the early ribosomal assembly events, and the possible role of the mitospecific region of Mrp20 protein in the assembly process, is proposed. In this model, the inner membrane acts as a platform for the formation of ribosomal subcomplexes in the early assembly stages and is where the completion of the assembly of large ribosomal assembly (54S) occurs. It is proposed that the ribosomal proteins that constitute different locations in the final assembled ribosome can initially co-assemble to form independent subcomplexes at the inner membrane. For instance, large ribosomal proteins that are located at the exit site of the ribosomes (such as Mrp20, MrpL40, MrpL22, MrpL4, MrpL25 and MrpL27) begin their assembly at specific locations in the inner membrane at or close to the site of translation and insertion of mitochondrially-encoded proteins. Likewise, proteins located at the interface of small and large ribosomal subunits (such as MrpL36, Mrp7, MrpL35 and possibly MrpL7 and MrpL17) form a different subcomplex. The components (such as membrane proteins or assembly factors) of the detergent-resistant complex may guide where the ribosomal assembly process should initiate at the membrane. Prx1 protein is proposed to be associated with these subcomplexes during the assembly process to prevent their aggregation or their oxidative damage. The ribosomal subcomplexes formed at the membrane are further stabilized by the rRNA that is thought as a scaffold to ensure their structural and proteolytic stability. These subcomplexes are proposed to associate with as yet unidentified assembly factors or membrane proteins that ensure the progression of the assembly of ribosomes to form a pre-54S ribosomal particle at the

membrane. The membrane associated MrpL32 protein finally associates with the pre-54S ribosomal particle to form a stable 54S ribosomal particle at the membrane which further recruits 37S ribosomal particle to form fully assembled and translationally active (74S) ribosomes at the inner membrane.

From the data discussed in this dissertation, it is proposed that in the *mrp20*Δ*C* mutant, the ribosomal assembly is stalled at anearly stage that results in the accumulation of a stable subcomplex of the large ribosomal subunit components. It is likely that the mitospecific region of the Mrp20 protein could be required for the interaction of Mrp20 containing subcomplex with ribosomal assembly factors or with the membrane proteins that are important for staging the progression of ribosomal assembly.

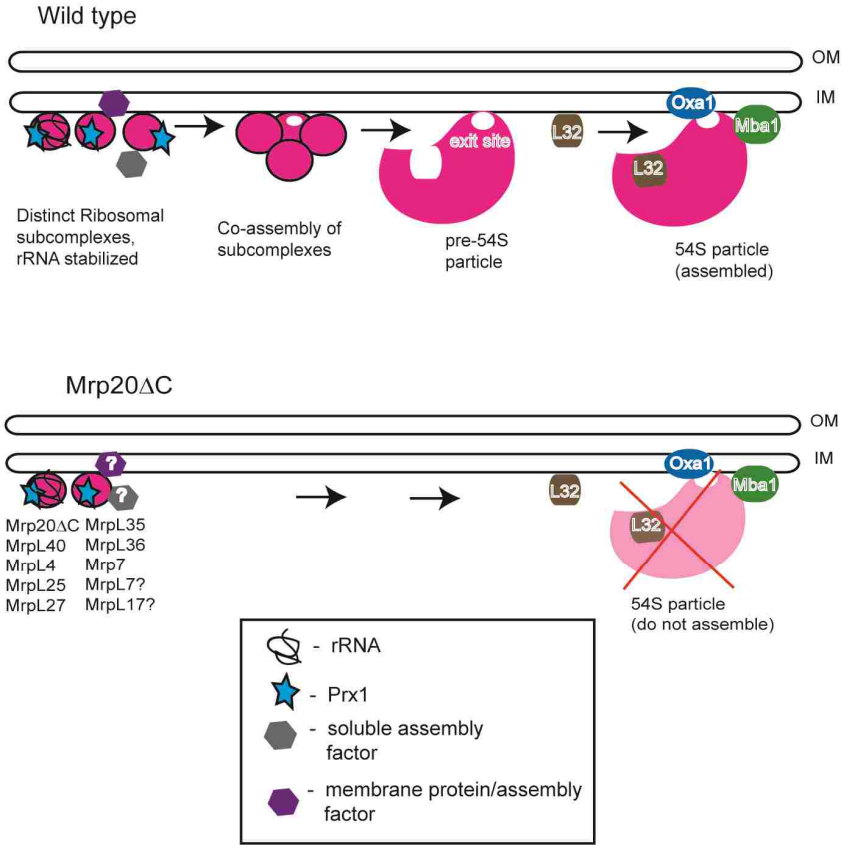

Figure 15: The assembly pathway of mitribosomes occurs at the membrane in the wild type and *mrp20ΔC* mitochondria. Upper panel – In wild type mitochondria, distinct subcomplexes of ribosomal proteins (stabilized by rRNA) are proposed to be associated with the inner membrane. Prx1 protein is thought to act as a chaperone for the non-assembled ribosomal subcomplexes. The assembly of these subcomplexes proceeds possibly by interacting with soluble/membrane assembly factors. The subcomplexes consisting of ribosomal proteins at the exit site are proposed to co-assemble with other subcomplexes at a site in the inner membrane where the translation and insertion of the mitochondrially encoded proteins will occur. The assembly process then continues to form pre-54S and 54S ribosomal particle at the inner membrane. Lower panel – In the *mrp20ΔC* mitochondria, two different subcomplexes, Mrp20ΔC and MrpL35 subcomplex have been identified. The ribosomal proteins identified with these subcomplexes are listed. Prx1 can associate with both the subcomplexes. In the absence of the mitospecific region of Mrp20, the assembly process is stalled at the early stages and assembled 54S ribosomal particle are not present. It may be possible that the intact mitospecific region is important to interact with the soluble/membrane assembly factors to carry out further assembly process.

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