MAMMALIAN MITOCHONDRIAL RIBOSOMAL PROTEINS :
A MATTER OF LIFE AND DEATH

A Dissertation in
Biochemistry, Microbiology and Molecular Biology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2010
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Human mitochondria are essential for cell survival while playing key roles in programmed cell death also called apoptosis. First of all, mitochondria are the main source of energy for the eukaryotic cell. Mitochondria produce more than 90% of the energy used by mammalian cells in a process referred to as oxidative phosphorylation. This demanding mechanism requires a lot of proteins, which are nucleus-encoded, synthesized in cytoplasm, and imported into mitochondria. However, it also requires 13 mitochondrial-encoded proteins. Mitochondria have their own 16.5 kb circular genome (mtDNA) and ribosome to translate these 13 proteins. Even though mammalian mitochondrial ribosomes (55S) differ from bacterial (70S) and mammalian cytoplasmic ribosomes (80S), many of the mitochondrial ribosomal proteins have bacterial homologs and similar functions. Some of the mitochondrial ribosomal proteins, which have no homologs in bacterial ribosomes, are involved in the process of apoptosis. Therefore, mitochondrial ribosome is essential not only for the translation of 13 proteins for energy production but also the regulation of apoptosis.

Here, two regulatory mechanisms involved in mitochondrial functions by mitochondrial ribosomal proteins were investigated; energy production by OXPHOS and apoptosis. First, we reported that the presence of different splice variants of MRPS29, which contained an uORF in the 5'-UTR in humans and the expression of MRPS29 could be translationally controlled by the uORF found in the 5'-UTR. Data presented in this thesis also suggest that reduction in MRPS29 expression by uORF may inhibit MRPS29-induced apoptosis. Therefore, the presence of uORF-MRPS29 mRNAs in human
potentially indicates a new mechanism for regulation of apoptosis.

Another regulatory mechanism via post-translational modification has been revealed by studying mitochondrial ribosomal L7/L12 stalk region. The mitochondrial ribosomal protein L10 (MRPL10) was identified as a major acetylated protein in the mitochondrial ribosome. We also found that ribosome associated SIRT3 was responsible for deacetylation of MRPL10 in a NAD\(^+\)-dependent manner. A mechanism by which the mitochondrial translation is regulated by reversible acetylation is proposed. Increased acetylation status of mitochondrial ribosome in SIRT3 knock-out (\(\text{Sirt}3^{-/-}\)) mice enhanced MRPL12 binding to the ribosome and recruitment of elongation factors to increase translation. On the other hand, over-expression of SIRT3 reduced the translation and MRPL12 binding to ribosomes by deacetylation of the mitochondrial ribosome. Therefore, acetylation of MRPL10 due to inhibition of SIRT3 could enhance its interaction with MRPL12, resulting in increased MRPL12 binding to ribosomes and the recruitment of elongation factors during translation. These findings constitute the first evidence for the regulation of mitochondrial protein synthesis through the reversible acetylation of the mitochondrial ribosome and identified MRPL10 as a novel substrate of NAD\(^+\)-dependent deacetylase, SIRT3.
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ABBREVIATIONS

12S  rRNA of the small subunit of the mitochondrial ribosome
16S  rRNA of the large subunit of the mitochondrial ribosome
28S  small subunit of mitochondrial ribosome
30S  small subunit of bacterial ribosome
39S  large subunit of mitochondrial ribosome
50S  large subunit of bacterial ribosome
55S  intact mitochondrial ribosome
70S  intact bacterial ribosome
80S  intact cytoplasmic ribosome
aa-tRNA  aminoacyl tRNA
AIF  apoptosis inducing factor
ANT  adenine nucleotide translocator
Apaf1  apoptosis protease activating factor 1
A site  aminoacyl site for tRNA
ATP  adenosine triphosphate
ATP6  ATP synthase subunit 6
ATP8  ATP synthase subunit 8
Complex I  NADH dehydrogenase
Complex II  succinate dehydrogenase
Complex III  ubiquinol cytochrome c oxidoreductase
Complex IV  cytochrome c oxidase
Complex V  ATP synthase
COX1  cytochrome c oxidase subunit 1
COX2  cytochrome c oxidase subunit 2
COX3  cytochrome c oxidase subunit 3
Cyt b  ubiquinol cytochrome c oxidoreductase subunit
DAP3  death associated protein 3
DTT  dithiothreitol
EF-G  bacterial elongation factor G
EF-Ts  bacterial elongation factor Ts
EF-Tu  bacterial elongation factor Tu
EF-Gmt  mitochondrial elongation factor G
EF-Tumt  mitochondrial elongation factor Tu
E site  exit site for tRNA
fMet  formylated methionine
hNOA1  human nitric oxide associated protein 1
HSP60  heat shock protein 60
IB  immunoblotting
IF-1  bacterial initiation factor 1
IF-2  bacterial initiation factor 2
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<tr>
<td>IF-3</td>
<td>bacterial initiation factor 3</td>
</tr>
<tr>
<td>IF-2mt</td>
<td>mitochondrial initiation factor 2</td>
</tr>
<tr>
<td>IF-3mt</td>
<td>mitochondrial initiation factor 3</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>KP</td>
<td>kaemferol</td>
</tr>
<tr>
<td>MRP</td>
<td>mitochondrial ribosomal protein</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial genome</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NAM</td>
<td>nicotinamide</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>RF1</td>
<td>bacterial release factor 1</td>
</tr>
<tr>
<td>RF2</td>
<td>bacterial release factor 2</td>
</tr>
<tr>
<td>RF3</td>
<td>bacterial release factor 3</td>
</tr>
<tr>
<td>SCX</td>
<td>strong cation exchange chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SRL</td>
<td>sarcin-ricin loop</td>
</tr>
<tr>
<td>STS</td>
<td>staurosporine</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage dependent anion channel</td>
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ACKNOWLEDGEMENTS

First of all, I can not appreciate my advisors, Drs. Emine and Hasan Koc, enough for their support and encouragement to complete my Ph.D. When I first came to the United States for my studies, I was full of fear and anxiety about communicating in English. I can still remember the first day in the USA when we were at the airport. I kept saying “pardon”, whenever security asked about our documents. Since then, my wife has been teasing and calling me "Mr. Pardon". After I had settled down in the lab, Dr. Koc helped me a lot to adapt to the new environment and encouraged me to study a completely new field, proteomics. I was so much impressed with her passion for the research and learned a lot of protein chemistry techniques. She showed me how could be a Ph.D. by being a role model. During my Ph.D., I learned a lot of different techniques and experiments to investigate our curiosity in answering the some of the fundamental and basic questions about the mitochondria with her supports. I would also like to thank my former and present lab member, especially Jennifer and Huseyin. I would like to thank my dissertation committee for their support and guidance during my Ph. D. at Penn State University.

I would like to thank my family for their endless supports. As a graduate student, it is not easy to balance between family and lab to be a good father and husband. Especially I want to say “Thank you and I love you” to my wife, Ji-Yun. She sacrificed her life, especially a golden age, for me. She left her career and life behind in KOREA to support her husband. I could not pay back to her for all this scarifying life for me. I can not appreciate my little two angels, Ye-Na and Hannah. Their smiles made me happy and made it easier for me to continue to get my Ph. D., whenever I had a hard time. I appreciate my dad and mom who now have two doctors as sons. They always pray for me and my brother, Min-Suk. They always wake up early in the morning and go to church to pray for their sons, no matter if the weather is good or not. I believe their prayers made all of those possible even under some struggles and difficulties. I also appreciate my younger brother, Dr. Han, who has been served as first son instead of his older brother for my family.
Chapter 1

Introduction

1.1 Mitochondrion

Mitochondria are responsible for providing more than 90% of the energy used by mammalian cells, which is generated by a process known as the oxidative phosphorylation (OXPHOS). The OXPHOS is a process of electron flow through the electron transport chain to generate adenosine triphosphate (ATP). Five different enzyme complexes located on the inner membrane are responsible for the synthesis of ATP (Fig. 1-1). Many mitochondrial protein components involved in OXPHOS are encoded by the nuclear DNA, translated by cytosolic ribosomes, and transported into the mitochondria. In addition to imported nuclear encoded proteins, mitochondria have their own genome and translational machinery/ribosomes for synthesis of 13 essential proteins of the electron transfer chain and ATP synthase complexes (Fig. 1-1 and 1-2). Major biochemical pathways including the tricarboxylic acid (TCA) cycle and fatty acid oxidation are also located in the mitochondrial matrix to provide reduced forms of cofactors, NADH (nicotinamide adenine dinucleotide) and FADH$_2$ to support oxidative phosphorylation (1). In brief, electrons from NADH or succinate by oxidation reach ubiquinone (also called coenzyme Q or Q) through Complex I (NADH dehydrogenase) or Complex II (succinate dehydrogenase), respectively (2,3). The fully reduced ubiquinone (also called ubiquinol or QH$_2$) carries electrons to Complex III (cytochrome c reductase). Complex III mediates the transfer of electrons from QH$_2$ to another electron carrier, cytochrome c (cyt c) (4). The final electron transport chain is Complex IV (cytochrome c oxidase), which transfers electrons from reduced cyt c to oxygen to generate H$_2$O (5,6). Electron flow through Complexes I, III, and IV or Complexes II, III,
and IV is accompanied by proton transfer from the matrix to the intermembrane space to generate an electrical chemical gradient ($\Delta \psi$). Finally the proton-motive force transports protons back into the matrix to provide energy for ATP synthesis through Complex V (ATP synthase) (7). Therefore, mutations in OXPHOS complexes contribute to numerous diseases (8). In addition, some cancer cells prefer glycolysis followed by lactic acid fermentation in the cytoplasm instead of OXPHOS in the mitochondria to generate ATP (9). This observation was first made by Dr. Otto Warburg and has been called the “Warburg effect” (10). In glycolysis, a six-carbon molecule, glucose, is converted to two three-carbon molecules, pyruvate, by a sequential enzyme-catalyzed reactions in the cytoplasm. Although glycolysis is a less efficient than OXPHOS in generating ATP, which produces only two ATPs and two NADHs, some cancer cells depend on glycolysis for their energy production. For example, different expression levels of hexokinase, which is involved in first step of glycolysis, have been observed in some cells to enhance glycolysis. Especially, under low oxygen conditions also called hypoxia, cells induce the expression of hexokinase and cells can produce ATP anaerobically, which compensates for impaired OXPHOS in mitochondria (11,12). Some cancer cells also elevate glycolysis even under non-hypoxia conditions by increasing the expression of hexokinases (13).

Besides energy production, the proton gradient from the electron transport chain is used to generate heat in brown fat of new born babies and hibernating animals to maintain their body temperature. Uncoupling proteins (UCP) are located on the inner membrane to provide an alternative path for the protons to enter the matrix and therefore can play a role in generating heat (14).
Figure 1-1. Structure and function of mitochondrion. Mitochondrion has its own genome (mtDNA) and ribosome (mtRibosome). Details of pathways for the mitochondrial function are described in text. Briefly, pyruvate generated by glycolysis enters the mitochondria through pyruvate dehydrogenase (PDH) and is converted to Acetyl-coA. The TCA cycle is initiated by combining Acetyl-coA and oxaloacetate. NADH and succinate, which are generated by the TCA cycle, give electrons to reach ubiquinone (Q) through Complex I and Complex II, respectively. Electron flow is accompanied by proton transfer from the matrix to intermembrane space and generate electrical chemical gradient (Δψ). ATP is generated by ATPase (Complex V) using proton-motive force. Small molecules including ATP can diffuse through adenine nucleotide translocator (ANT) and voltage dependent anion channel (VDAC). This figure is adapted from (1).
1.2 Mitochondrial genome

Mammalian mitochondria have their own 16.5 kb circular genome (mtDNA) which resides in the matrix (15). The mitochondrial genome encodes 37 genes, including 22 mitochondrial tRNAs, 13 mRNAs, and 2 rRNAs (15-17). In humans, mitochondrial genome encodes 7 proteins (ND1, 2, 3, 4, 4L, 5, and 6) of the 43 subunits of Complex I; 1 protein (cyt b) of the 11 subunits of Complex III; 3 proteins (COI, II, and III) of the 13 subunits of Complex IV; and 2 proteins (ATPase 6 and 8) of 16 subunits of Complex V. There are no proteins encoded by the mitochondrial genome for Complex II (Fig. 1-2).

The mitochondrial genome is very different from the nuclear genome. For example, the mitochondrial genome is circular and compact, containing one regulatory region called the D-loop (16,18,19). MtDNA consists of two strands; heavy and light strand. Each strand encodes genes, with expression regulated by their own promoter located in the D-loop region. The heavy strand encodes twelve mRNAs, two rRNAs (12S and 16S), and some of the mitochondrial tRNAs. The light strand encodes only one mRNA, ND6, and eight tRNAs (20). Some of mRNAs partially overlap with others, which mean two entirely different genes share the same nucleotide sequence. For example, overlapping genes have been found for ND4/ND4L and ATP6/ATP8 in the mitochondrial genome (16). The mitochondrial genome also does not have a complete set of universal codons (21). For example, UGA, which is a stop codon as a universal codon, can be recognized as tryptophan (Trp) in mitochondria (22). In addition, some of the mRNAs have AUU and AUA as the start codon instead of AUG (23). For example, ND1, ND3, and ND5 have AUU and ND2 has AUU as a start codon, respectively. Two
rRNAs (12S and 16S) are essential for the assembly of mitochondrial ribosome. The ribosome contains the small and large subunit rRNA, 12S rRNA and 16S rRNA, interacting with ribosomal proteins, respectively. The tRNAs carry the amino acids which correspond to the appropriate mRNA codon for the translation. There are 22 tRNAs encoded by the mitochondrial genome. Therefore, each amino acid has only one tRNA except for leucine and serine, each of which have two tRNAs (24).
Figure 1-2. Products of mtDNA and OXPHOS. Human mtDNA is a very compact and circular genome which encodes 7 (ND1, 2, 3, 4, 4L, 5, and 6) of the 43 subunits of Complex I (blue); 1 (cyt b) of the 11 subunits of Complex III (pink); 3 (COI, II, and III) of the 13 subunits of Complex IV (sky blue); and 2 (ATPase 6 and 8) of 16 subunits of Complex V (red). Human mtDNA also encodes 2 rRNAs (12S and 16S rRNAs) (green) and 22 tRNAs (yellow with black labeled letters), which indicates one letter nomenclature corresponding to amino acids. The control region (CR), also known as the D-loop, plays a role in regulation of transcription, but does not contain genes. The replication origin for the heavy strand is also located on CR. The illustration of OXPHOS complexes is generated by Protein Data Bank with accession number 2FUG (Thermus thermophilus Complex I), 2FYU (bovine Complex III), 1OCC (bovine Complex IV), and 1QO1 (yeast Complex V). Q and cyt c represent ubiquinone (also called coenzyme Q) and cytochrome c, respectively. Figure 1-3 is adapted from (25).
1.3 Mitochondrial ribosome

Synthesis of 13 mitochondrially encoded proteins of OXPHOS is carried out by an essential and specific protein synthesizing system, mitochondrial ribosomes, within this organelle. All of the mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and imported into mitochondria where they are assembled with the mitochondrially transcribed rRNAs into ribosomes. Figure 1-3A shows the cryo-EM structure of the mitochondrial ribosome (17). Briefly, a small subunit (28S) and a large subunit (39S) are the components of the 55S mammalian mitochondrial ribosome and each subunit consists of proteins and RNA molecules. The small subunit is composed of twenty-nine proteins and 12S rRNA while the large subunit consists of forty-eight proteins and 16S rRNA (26-28). Mammalian mitochondrial ribosomes (55S) differ from both bacterial ribosomes (70S) and mammalian cytoplasmic ribosomes (80S) in terms of size and RNA/protein content (17,29,30). However, mitochondrial ribosomes have similarities with bacterial ribosomes in terms of structure and mechanism of translation. Especially, most functional domains needed for translation are conserved in the mitochondrial ribosome. Many of these proteins have bacterial homologs with conserved functions in translation (TABLE 1-1) (27).
Figure 1-3A. Side view of mitochondrial ribosome by 3D cryo-EM reconstruction. The mitochondrial ribosome is assembled by nuclear encoded mitochondrial ribosomal proteins and mitochondrial encoded rRNAs (12S, 16S). The small subunit is 28S and shown in yellow and green. The large subunit is 39S and shown in sky blue and dark blue. The regions conserved in bacterial and mitochondrial ribosomes are shown in yellow and sky blue. The green and dark blue regions are the mitochondrial specific protein which have non-bacterial homologs. Details of functional domains shown in the figure are described in text. SRL: sarcin-ricin loop, CP: central protuberance and PTC: the peptidyl transferase site in the large subunit. This figure is adapted from (17).
TABLE 1-1 – List of mitochondrial ribosomal proteins with their bacterial homologs

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\(^a\) The proteins in red are only found in bacterial ribosomes.
1.3.1 Small subunit (28S) of the mitochondrial ribosome

The functionally conserved regions of the small subunit are the head, body, and platform regions. Especially the platform, which is located between the head and body of the small subunit, is responsible for the mRNA binding to the ribosome. In bacteria, S2, S11, S18, and S21 create the platform with the central domain of 16S rRNA interacting with Shine-Dalgarno sequences in the 5'-untranslated region of mRNA for initiation. Especially, the S2 and S18 play an important role in initiation of translation for mRNA movement on the ribosome (31). In mitochondria, MRPS6, MRPS11, MRPS15, MRPS18-1, MRPS18-2, MRPS18-3, and MRPS21 have been found in the platform region (28). It has been found that three variants of MRPS18 proteins (MRPS18-1, MRPS18-2, and MRPS18-3) are located in mitochondrial ribosomes based on our previous proteomics studies (28). These variants of MRPS18s are 25-30% identical to bacterial S18 and are conserved in the central region. N-terminal and C-terminal domains vary in the number of additional amino acids and are quite divergent among the different MRPS18s. In the head region, MRPS2, MRPS7, MRPS9, MRPS10, and MRPS14 are formed by interacting with the 3’ domain of the 12S rRNA (28). In mitochondria, the counterparts of S3, S13, and S19 are missing and mitochondrial specific proteins replace these proteins in the head region for proper function. Especially, S13 and S19 interact with the central protuberance (CP) of the large subunit (50S) of ribosome in bacteria (28). MRPS5, MRPS12, MRPS16, and MRPS17 are positioned in the body region with the 5’ domain of the 12S rRNA (28). The counterparts of S4 and S20 are missing in the body of mitochondrial ribosome and their binding sites in rRNA are also missing in the mitochondrial ribosome by comparison.
between bacterial (16S) and mitochondrial (12S) rRNA. In mitochondria, MRPS5 is larger than its bacterial counterpart, which could compensate for the loss of MRPS4 and MRPS8 (28).

1.3.2 Large subunit (39S) of the mitochondrial ribosome

The functionally conserved regions of the large subunit are L1 stalk, L7/L12 stalk, and CP regions. In the CP region, MRPL18 has been identified as a counterpart of L18, however, other counterparts of bacterial L5 and L25 are missing in mitochondria (27). In bacteria, L5 interacts with S13 and S19 to associate small and large subunit as described above. One of the functionally conserved regions in the large subunit is the L7/L12 stalk region, which is composed of L10, L11, and multiple copies of L7/L12 in bacteria with counterparts MRPL10, MRPL11, and MRPL12 in mitochondrial ribosomes, respectively (Fig. 1-3B). Since there is no report to indicate the N-terminal acetylation of MRPL12, which corresponds to L7 in bacterial L7/L12, we use the terminology MRPL12 instead of MRPL7/L12 in this study. The L7/L12 stalk plays a significant role in translation by interacting with several different translation factors, such as IF2, EF-Tu, EF-G, and RF3, which are referred to as GTPases, since they hydrolyze GTP to carry out their function in translation (32-34). The L7/L12 protein exists as multiple copies in the ribosome. In different bacteria, the ribosomal L7/L12 stalk consists of different numbers of L7/L12 dimers, which is determined by the length of the L10 helix $\alpha_8$ (35). In the case of *E. coli*, 4 copies of L7/L12 as two dimers are bound on the C-terminal domain of L10 (36). However, in *Thermatoga maritima*, 6 copies of L7/L12 as three dimers are localized on L10 (37). The number of copies of MRPL12 that could bind to
MRPL10 has not been determined in mitochondria. The L7/L12 protein has three functional domains: the N-terminal domain, which binds to the C-terminal helix of L10 and plays a role in dimerization; the flexible hinge region, which connects the N-terminal and C-terminal domains; and the C-terminal domain which binds to several different GTPases for translation (34,38-40). Recent NMR studies revealed that all the GTPases involved in translation such as IF2, EF-Tu, EF-G, and RF3 bind to the same region of several consensus amino acids (Lys70, Leu80, and Glu82) in the C-terminal domain of L7/L12 (34). Another significant difference between L7/L12 and other ribosomal proteins is the flexible binding on the ribosomes. The L7/L12 binds to ribosomes only by interacting with L10, without rRNA interactions. Therefore, the L10 protein acts as an anchor for L7/L12 on the ribosome. It has two functional domains: the N-terminal domain, which interacts with the ribosome; and the C-terminal domain, which interacts with dimers of L7/L12 proteins (41). Sarcin-ricin loop (SRL) is a highly conserved rRNA sequence (5’-AGUACGAGAGGA-3’) in the large subunit, which includes an important binding site for elongation factors within the ribosome. In mitochondria, MRPL3 and MRPL13 are located in the SRL region (27).
Figure 1-3B. Model for L7/L12 the stalk. The L7/L12 stalk is composed of L10 (green), L11 (yellow), and multiple copies of L7/L12 (pink). The L7/L12 protein has 3 functional domains; the N-terminal domain which binds to C-terminal helix of L10 and plays a role in dimerization; the flexible hinge region which connects the N-terminal and C-terminal domains; and the C-terminal domain which binds to several different GTPases for translation. SRL is the sarcin-ricin loop of 23S rRNA in bacterial ribosomes, which is known to be a translation factor binding site. This figure is adapted from (32) and generated by Protein Data Bank with accession number 1MMS (L11), 1RQU (L7/L12), and 1S72 (ribosome).
1.3.3 Additional function for mitochondrial ribosomal proteins

Although the primary function of the mitochondrial ribosome is translation of 13 proteins for the OXPHOS complexes, there is a growing evidence that suggests the involvement of mitochondrial ribosomal proteins in cancer, apoptosis and other metabolic diseases (42). Four different mitochondrial ribosomal proteins, MRPS29 (also called DAP3, death associated protein 3), MRPS30 (also called as PDCD9, programmed cell death protein 9), MRPL37, and MRPL41, which have no homologs in bacterial ribosomes, are known to be involved in apoptosis (43-47). Previous reports showed that expression of *Gallus gallus* MRPS30 in mouse fibroblasts induced apoptosis through activation of c-Jun N-terminal kinase 1 (JNK1) and upregulation of transcription factor, c-jun (48). In our proteomic analyses, we confirmed that homologs of MRPS30 were present in bovine as a mitochondrial ribosomal protein (43). MRPL37 has a homologous domain with MRPS30 and is believed to be involved in the process of apoptosis (47). MRPL41 plays an important role in p53 dependent apoptosis and cell cycle arrest by increasing the levels of p21 and p27 under serum starvation (44,49). MRPS29 was initially identified by transfecting HeLa cells with an anti-sense cDNA library followed by functional screening. In this functional knock-out screening, the anti-sense cDNA resulted in down-regulation of the protein expressed from the corresponding mRNA. After anti-sense cDNA library transfection, cells were exposed to interferon-γ (IFN-γ) to induce apoptosis and then isolated for analysis to identify genes involved in apoptosis (45). Cells containing anti-sense cDNAs for knock-out of pro-apoptotic proteins survived after the induction of apoptosis (45). MRPS29 is first isolated by this screening method and further studies have shown that it is an apoptotic
mediator of tumor necrosis factor-α (TNF-α), IFN-γ, and FAS induced apoptosis (45,46,50). In contrast, some reports showed that over-expression of MRPS29 protects Ataxia telangiectasia (genetic neurodegenerative disease) cells from UV and glioma cells from camptothecin, which is an inhibitor of DNA topoisomerase-induced apoptosis (51). MRPS29 also directly interacts with human nitric oxide associated protein 1 (hNOA1) (52). The mitochondrial morphology is affected by over-expression or knock-down of hNOA1. The hNOA1 directly interacts with Complex I and MRPS29 to regulate their functions. In contrast, knock-down of hNOA1 protects cells from apoptosis and makes cells more resistant to apoptosis induced by IFN-γ and staurosporine, a non-selective protein kinase inhibitor, supporting a role for MRPS29 (52). Even though MRPS29 is a mitochondrial ribosomal protein and ubiquitously expressed in normal cells, its involvement in both induction of apoptosis and resistance to apoptosis is very intriguing (53). Interestingly, the over-expression of MRPS29 is also found to be involved in resistance to apoptosis in glioma and Ataxia telangiectasia cells in the presence of various apoptotic stimuli such as streptonigrin, ionizing radiation, and camptothecin.

Other than the pro-apoptotic proteins of mitochondrial ribosomes, there are also several mitochondrial ribosomal proteins, MRPS23, MRPL11, and MRPL28, and their mRNAs which are expressed differentially in tumor cells or tissues (54,55). Several different mechanisms have also been shown to regulate the expression of mammalian mitochondrial ribosomal proteins, such as MRPS12, MRPL11, and MRPL12, by different splice variants or uORF containing variants to support protein synthesis in different physiological conditions (56,57). Therefore, it is possible that the changes in expression of mitochondrial ribosomal proteins essential for mitochondrial protein synthesis/function
might influence the balance between apoptosis and tumor formation due to the Warburg effect in mitochondrial energy production. However, the regulatory mechanism behind the role of MRPS29 in apoptosis is still not fully understood. Therefore, studies to understand the translational regulation of MRPS29 in regards to apoptosis are necessary. In this study, we will discuss the regulation of MRPS29 expression by an uORF in the 5′-UTR for the regulation of MRPS29-induced apoptosis (Chapter 2).
1.4 Mitochondrial translation

Translation is the process of converting genetic information (mRNA) to a specific sequence of amino acids (a polypeptide). Ribosomes are factories involved in this process. Although there are subtle differences, the mechanism of translation in mitochondria has many similarities with that in bacteria (58). For example, not only are there structural similarities between bacterial and mitochondrial ribosomes but also functional similarities to cause similar antibiotic sensitivity (59). Both bacterial and mitochondrial ribosomes use formyl-methionyl tRNA (fMet-tRNA) for initiation instead of Met-tRNA (60). Mitochondrial translation requires specific initiation factors, elongation factors, and release factors, which have a homolog in bacteria for each step of translation. Especially, the mammalian mitochondrial elongation factors can function on bacterial ribosomes. However, some aspects of mitochondrial translation are also unique. Mitochondrial mRNA does not contain 5’-untranslated region (UTR) and 3’-UTRs other than a short poly A tail. It does not require a Shine-Dalgarno sequence as in prokaryotes, or a 5’-cap as in eukaryotes, for initiation of translation (15,19).

Translation proceeds in three phases: initiation, elongation, and termination (Fig 1-4A). Each specific step will be described below.

1.4.1 Initiation

Initiation is the first step in translation. In prokaryotic cells, translation is initiated by formation of an initiation complex consisting of the small subunit of the ribosome (30S), fMet-tRNA, mRNA, and initiation factors (IF1, IF2, and IF3). The fMet-tRNA is specifically used for translation initiation in bacteria and mitochondria but
not in cytoplasmic translation in eukaryotes and archaea (60). The small subunit of the ribosome binds to the mRNA with IF1 and IF3 at the start codon (AUG) that is recognized by fMet-tRNA with IF2 and then the large subunit of the ribosome (50S) joins the initiation complex forming the 70S ribosome. The 70S ribosomes are ready for the synthesis of polypeptides and there are three sites on the 70S ribosome to which tRNA can bind. One is called the peptidyl site (P site) and another is called the aminoacyl or acceptor site (A site) and a third is called the exit site (E site) where tRNAs are released. IF1 is a small protein which plays a crucial role in the fidelity of the initiation process by associating with the small subunit in A site to prevent non-specific binding of aminoacyl-tRNA in P site. IF1 also helps to prevent assembling of 70S before initiating tRNA (fMet-tRNA) is occupied on the P site (61). IF3 is another small protein, which contains two structural domains connected by a flexible linker. The C-terminal domain contains the RNA binding motif to achieve the first interaction with the small subunit (30S) of the ribosome, whereas the N-terminal domain stabilizes the interaction between IF3 and the small subunit (30S) afterwards (62,63). Therefore, IF3 binds to the small subunit (30S) and inhibits the association with the large subunit (50S) to prevent formation of 70S ribosome (64). IF3 also accelerates the codon-anticodon pairing for the fMet-tRNA to the P site to ensure efficiency and fidelity of translation (63). IF2, which is 78–97 kDa in size, is a monomeric guanosine triphosphatase (GTPase) and binds to fMet-tRNA to control entering and transferring fMet-tRNA to the P site for initiation. The initiation factors are released after assembly of the 70S ribosome on the mRNA by using GTP hydrolysis. In mitochondria, only IF2_{mt} and IF3_{mt} have been identified (65-67). The smallest initiation factor, IF1_{mt}, has not been identified in mammalian mitochondria,
which implies that IF$_{2\text{mt}}$ may compensate for the lack of IF$_{1\text{mt}}$ by insertion of additional 37 amino acids between functional conserved domains V and VI (68). IF$_{3\text{mt}}$ is less conserved compared to IF$_{2\text{mt}}$. It has only 20-30% identity even in the central region whereas IF$_{2\text{mt}}$ has 69% identity. IF$_{3\text{mt}}$ plays a role in dissociating the intact 55S ribosome into small subunit (28S) and large subunit (39S) for IF$_{2\text{mt}}$ binding (65). IF$_{3\text{mt}}$ also has an extension of approximately 30 amino acids at both N-terminal and C-terminal domains compared to its bacterial counterpart (65,69). Especially, the C-terminal extension has been found to be important for IF$_{3\text{mt}}$ to promote the dissociation of the bound initiator tRNA from the small subunit (28S) in the absence of the mRNA (69).

1.4.2 Elongation

The elongation step follows the initiation step. Elongation is the most rapid step in translation. In prokaryotes, approximately 15-20 amino acids per second can be added on the elongated polypeptides during the translation. Elongation factors play a crucial role in this rapid and accurate process. Recently the crystal structures of ribosome complex with EF-Tu (Fig. 1-4B) and EF-G (Fig. 1-4C) have been revealed (70,71). When the initiating fMet-tRNA binds to the P site, another aminoacyl-tRNA (aa-tRNA) comes to the next codon on the ribosome and binds at the A site. This step is mediated by elongation factor-Tu (EF-Tu), a small GTPase (Fig. 1-4B) (70). The ternary complex formed by aa-tRNA·EF-Tu·GTP promotes binding of the aa-tRNA to the A site. Therefore, the P site contains the first amino acid, formyl-methionine (fMet), and the A site contains the next incoming amino acid. Once locked into the A site by aa-tRNA, the GTP in the ternary complex is hydrolyzed and the ternary complex is released
from the ribosome. A peptide bond is formed between fMet and incoming amino acid. The A site contains the newly formed peptide, while the P site has a tRNA without an amino acid. Following the release from the ribosome as an EF-Tu·GDP, the elongation factor Ts (EF-Ts) catalyzes the conversion of the inactive form, EF-Tu·GDP, to the active form, EF-Tu·GTP, for the next round of elongation.

The final stage of elongation is called translocation, in which the ribosome moves downstream on the mRNA. This process requires another small GTPase, elongation factor G (EF-G) (Fig. 1-4C) (71). Sequential steps of elongation add amino acids to the growing polypeptide. The growing polypeptide exits the ribosome through the exit tunnel in the large subunit. The ribosome continues to translate until the ribosome meets a stop codon (UAA, UGA, or UAG) on the mRNA.

Mitochondria have their own elongation factors (EF-G_{mt} and EF-Tu_{mt}) and mitochondrial elongation factors are structurally and functionally similar to their counterparts in bacteria (72,73).

1.4. 3 Termination

Termination is the final step in translation, in which the complete polypeptide is released from the ribosome. Termination occurs when the ribosome meets a stop codon. There are no tRNAs with anti-codons for the three stop codons (UAA, UAG, and UGA). However, release factors recognize stop codons when they reach the A site. The small GTPase, RF1, recognizes UAA and UAG codons as signals to stop translation and another GTPase, RF2, recognizes UAA and UGA as stop codons. These release factors trigger hydrolysis by using GTP, causing the release of the newly synthesized
polypeptides from the ribosome. To be a functional protein, synthesized polypeptides must be folded into tertiary structures and/or regulated by post-translational modifications (PTMs) after translation.

A third release factor, RF3, catalyzes the release of RF1 and RF2 at the end of the termination process. After this step, the ribosome dissociates into small and large subunits and later the dissociated ribosomes are reassembled for the next round of translation. In mitochondria, several different families of release factors have been identified. RF1\textsubscript{mt} recognizes and terminates translation at UAA/UAG codons and acts as a major release factor (74). Recently, immature colon carcinoma transcript-1 (ICT1) has been found as another family of release factors, which acts as a ribosome-dependent peptidyl-tRNA hydrolase (75).
Figure 1-4A. Schematic diagram of translation in mitochondria. Details of each step are given in the text. The large subunit and small subunit of mitochondrial ribosomes are shown in 39S and 28S, respectively. The mitochondrial initiation factors, IF2mt and IF3mt are shown in red and green, respectively. The mitochondrial elongation factors, EF-Tumt and EF-Gmt are shown in brown and red, respectively. The release factor, RFmt is shown in yellow. The initiating tRNA (fMet-tRNA) and elongated aminoacyl-tRNA (aa-tRNA) are shown in light green and blue, respectively. The A and P sites correspond to aminoacyl and peptidyl sites on the ribosome.
Figure 1-4B. Schematic diagram of overall view of ribosome with EF-Tu during elongation step. Details of each step are given in the text. The large subunit (50S) and small subunits (30S) of bacterial ribosomes (*Thermus thermophilus*) are shown in brown and blue color, respectively. PTC and DC represented peptidyl transferase center and decoding center, respectively (70). The A/T, P, and E sites correspond to the transient state with the partially bound aminoacyl-tRNA on the A site, peptidyl, and exit sites on the ribosome, respectively. When the initiating tRNA which carries fMet-tRNA binds to the P site, another aminoacyl-tRNA comes to the next codon on the ribosome and binds at the A site. This step is mediated by EF-Tu, a small GTPase, by hydrolysis of GTP. Reprint from Science, 326, Schmeing et al. *The Crystal Structure of the Ribosome Bound to EF-Tu and aminoacyl-tRNA*, 688-694, copyright © 2009, with permission from Science.
Figure 1-4C. Schematic diagram of overall view of ribosome with EF-G during elongation step. Details of each step are given in the text. The large subunit (50S) and small subunits (30S) of bacterial ribosomes (*Thermus thermophilus*) are shown in brown and blue color, respectively. PTC and DC represented peptidyl transferase center and decoding center, respectively (71). The P and E sites correspond to peptidyl and exit sites on the ribosome, respectively. The final stage of elongation is called translocation, in which the ribosome moves downstream on the mRNA exposing a new codon at the A site. This process requires a small GTPase, EF-G, by hydrolysis of GTP. Reprint from *Science*, 326, Gao et al. The Structure of the Ribosome with Elongation Factor G Trapped in the Posttranslocational State, 694-699, copyright © 2009, with permission from Science
1.5 Apoptosis

Mitochondria play a crucial role in several major pathways for programmed cell death, apoptosis, in mammalian cells, in addition to their role in energy production and intermediary metabolism (76,77). This process is tightly regulated, therefore, malfunction of apoptotic regulation in human leads to severe pathogenesis. Abnormally high levels of apoptosis are associated with degenerative diseases. In contrast, suppression of apoptosis is involved in carcinogenesis and autoimmune diseases (78). Figure 1.5 illustrates the intrinsic pathway of apoptosis induced by different stimuli.

There are various changes in the nucleus during apoptosis, such as nuclear condensation and DNA fragmentation (79). In addition to the involvement of the nucleus in extrinsic pathways of apoptosis, mitochondria are also important organelles in promoting intrinsic pathway of apoptosis (76). In particular, a change in the permeability of the mitochondrial membrane and release of cytochrome c (cyt c) are key steps in the intrinsic pathway for apoptosis. Mitochondria also contain many other pro-apoptotic proteins, such as apoptosis inducing factor (AIF), endonuclease G (Endo G), second mitochondrial-derived activator of caspase (Smac)/ direct inhibitor of apoptosis protein-binding protein with low pI (Diablo). The voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT), and cyclophilin D (CD) creates the permeability transition pore (PTP) in the mitochondrial outer and inner membrane to release apoptotic factors such as AIF, cyt c, Endo G, and Smac/Diablo. The released cyt c from mitochondria activates caspase 9, which is a member of the cysteine protease family, and leads to the formation of the apoptosome with apoptosis protease activating factor 1 (Apaf1). Activated caspase 9 activates downstream caspases such as caspase 3...
and caspase 7, which are responsible for cell destruction in apoptosis (80). The released Smac/Diablo binds to inhibitor of apoptosis proteins (IAP) to promote apoptosis by inhibition of IAP (81,82). AIF induces apoptosis by a caspase-independent manner. The released AIF translocates into the nucleus and induces chromatin condensation and DNA fragmentation without activation of caspases (83).

Released factors such as cyt c, AIF, and Smac/Diablo from mitochondria are followed by the formation of a pore in the mitochondrial membrane called the permeability transition pore (PTP) as described above. Members of the Bcl-2 family are involved in forming PTP leading to release of apoptotic factors. The Bcl-2 family can be divided into pro-apoptotic (Bad, Bak, Bax, or Bid) and anti-apoptotic (Bcl-2 or Bcl-xL) proteins depending on their function in apoptosis (84). The intrinsic pathway is regulated by the balance of activity between pro- and anti-apoptotic members of the Bcl-2 family. The breakdown of the balance between anti-apoptotic and pro-apoptotic proteins in mitochondria also changes the sensitivity of cells to apoptotic stimuli (85).
Figure 1-5. Involvement of mitochondria in apoptosis. Voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT), and cyclophilin D (CD) creates the permeability transition pore (PTP) in the mitochondrial outer and inner membrane to release apoptotic factors such as cytochrome c (cyt c), apoptosis inducing factor (AIF), and endonuclease G (Endo G). The Bcl-2 family regulates PTP for release of apoptotic factors. The Bcl-2 family is divided into pro-apoptotic (Bad, Bak, Bax, or Bid) and anti-apoptotic (Bcl2 or Bcl-xL) proteins. The anti-apoptotic proteins, which are shown in yellow, locate in the outer membrane and inhibit cyt c release. The pro-apoptotic proteins, which are shown in orange, reside in the cytoplasm and translocate to mitochondria following upstream signaling for release of cyt c. Bad translocates to mitochondria and forms a pro-apoptotic complex with Bcl-xL. This translocation is inhibited by some of growth factors and cytokines mediated by PKA and Akt signaling by phosphorylation of Bad. Bid is cleaved by caspase-8 following Fas activation and fragmented Bid (tBid) translocates to mitochondria to induce activation of pro-apoptotic proteins such as Bax and Bak. Bax translocates to mitochondria response to death stimuli mediated by JNK. Released cyt c binds to Apaf-1 and forms an activation complex with caspases for apoptosis.
1.6 Post-translational modifications in mitochondria

Many mitochondrial processes, such as oxidative phosphorylation, ATP synthesis, transport, metabolic enzymes and programmed cell death, have been known to be regulated by post-translational modifications (PTMs) such as phosphorylation and acetylation. Phosphorylation is one of the most important PTMs to regulate mitochondrial functions involved in disease (86-89). At least 27 different kinases and phosphatases have been identified to be involved in phosphorylation/dephosphorylation reactions in the mitochondria (90).

In addition to phosphorylation, acetylation also plays an important role in regulation of energy metabolism (Fig. 1-6A) (91). Almost 30% of mitochondrial proteins have been identified as acetylated at their ε-amino groups Lys residues by proteomics approaches (92,93). There is substantial evidence explaining why reversible acetylation could be an important PTM and a common regulatory mechanism in mitochondria. First of all, the acetyl group donor for acetylation reactions, acetyl-CoA, is generated by oxidation of glucose and fatty acids in mitochondria. Secondly, although there are no known protein acetyl transferarases identified in mitochondria, three different NAD⁺-dependent deacetylases, sirtuin family (SIRT3, SIRT4, and SIRT5), are localized to the mitochondria (92,94,95). The deacetylation reaction catalyzed by NAD⁺-dependent deacetylases, sirtuins, is shown in Figure 1-6B. Nicotinamide adenine dinucleotide (NAD⁺) generates nicotinamide (NAM) and 2′-O-Acetyl-ADP-ribose by removing the acetyl group from Lys residues. For this reason, NAM and 2′-hydroxyl of NAD⁺ induce chemical competition between base exchange and deacetylation on the ribose moiety, which leads to inhibition of deacetylation on ε-amino group of Lys by
sirtuins (Fig. 1-6B) (96).

As described in the previous sections, mitochondrial ribosomes are responsible for the synthesis of essential proteins involved in energy generation, the regulation of mitochondrial translation by PTMs of ribosomal proteins would also be mitochondrial function/dysfunction. To test this hypothesis, our laboratory has been investigating the role of ribosomal protein phosphorylation and acetylation in regulation of mitochondrial translation and apoptosis (97-99). To date, we have demonstrated that phosphorylation of mitochondrial ribosomal proteins is critical for induction of apoptosis and regulation of translation (97). We have also identified phosphorylated and acetylated proteins of mitochondrial ribosomal proteins at steady-state levels using mass spectrometry-based proteomics (97,98). Phosphorylation and acetylation cause an 80 Da and a 42 Da increase in molecular weight amino acid, respectively, which can be easily detected by mass spectrometry. We applied a capillary liquid chromatography - nanoelectrospray ionization - tandem mass spectrometry (LC-MS/MS) technique to identify acetylated mitochondrial ribosomal proteins involved in translation (Chapter 3).
1-6A. ε–amino group of Lys acetylation. The ε-amino group of Lys acetylation is an important reversible regulation, which occurs at the side chain of Lys residue. Acetyl-coA is used as acetyl donor in acetylation. Details of mechanisms and functions are described in the text. The illustration of acetylation is generated with Acetyl-coA synthetase (Protein Data Bank with accession number 1PG3).
Figure 1-6B. Overall reaction of deacetylation mediated by sirtuins. The reaction of deacetylation on ε-amino group of Lys by NAD$^+$-dependent deacetylases, sirtuins. Nicotinamide adenine dinucleotide (NAD) generates nicotinamide and 2′-O-Acetyl-ADP-ribose by removing acetyl group from Lys.
1.7 Sirtuins, NAD⁺-dependent deacetylase/ADP-ribosyltransferases

Sirtuins are a family of NAD⁺-dependent deacetylases and ADP-ribosyltransferases which are homologs of the yeast silent mating type information regulator 2 (SIR2). In yeast, SIR2 is associated with extension of life span by calorie restriction. Sirtuins are very conserved proteins from bacteria to humans and their function of regulating life span is also similar among the different species (100). In humans, 7 different sirtuins (SIRT1-SIRT7, also known as class III histone deacetylases) have been identified (101) (Fig. 1-7). SIRT1 is responsible for the change in nutrient availability and is involved in increasing cell survival by deacetylation of histones and p53. SIRT1 expression is also increased in response to calorie restriction to be involved in extension of life span (101). SIRT2 is mainly localized in the cytoplasm and deacetylates α-tubulin (102). However, some reports show that SIRT2 could translocate into the nucleus, deacetylating histone H4 to regulate mitotic checkpoints during mitosis (103). SIRT3, SIRT4, and SIRT5 are localized in the mitochondria, mostly in the matrix. As described above, almost 30% of mitochondrial proteins are acetylated and the regulation of longevity and energy metabolism in mitochondria are regulated by reversible acetylation (92,101). For example, SIRT3 deacetylates and activates acetyl-coA synthetase 2 (AceCS2) to enhance acetyl-coA production (104). In addition to metabolic enzymes, nuclear encoded subunits of the electron transport chain complexes were found to be acetylated (92). For example, Complex I subunit, NDUFA9, is a SIRT3 substrate and acetylation/deacetylation of Complex I is proposed to regulate and maintain basal ATP levels in mammalian mitochondria (94). Similarly, we discovered the regulation of Complex II activity by reversible acetylation of succinate
dehydrogenase flavoprotein (SdhA) by SIRT3 (105). Interestingly, SIRT4 does not have deacetylase activity and no substrate for deacetylation has been found. However, SIRT4 acts as an ADP-ribosyltransferase in mitochondria. SIRT4 inhibits glutamate dehydrogenase (GDH) activity, which is involved in insulin signaling (106). Recently, carbamoyl phosphate synthetase 1 (CPS1) has been identified as a substrate of SIRT5. SIRT5 deacetylates CPS1 and up-regulates activity of CPS1 to regulate the urea cycle involved in ammonia detoxification (107). SIRT6 is localized in the nucleus, but its substrate has not been identified; however, the function of SIRT6 has been identified. SIRT6 plays a role in DNA repair and genomic stability by acting as an ADP-ribosyltransferase (108,109). SIRT7 is localized in the nucleolus and interacts with RNA polymerase I to regulate rRNA transcription (110). There are no reports to date of SIRT7 acting as a deacetylase or ADP-ribosyltransferase.
Figure 1-7. Sirtuins. Localization and substrates of each sirtuin are shown. Details of functions are described in the text. SIRT1 and SIRT6 are localized in the nucleus. There is no known target for SIRT6. SIRT2 is localized mainly in the cytoplasm and is involved in deacetylation of microtubules. However, SIRT2 also translocates into the nucleus during mitosis to regulate a mitotic checkpoint. SIRT3, SIRT4, and SIRT5 are localized in mitochondria. They primarily regulate energy metabolism. SIRT7 is localized in the nucleolus and interacts with RNA polymerase I to regulate rRNA transcription. There is no report demonstrating deacetylase or ADP-ribosylase activity for SIRT7. SIRT7 is not shown in this figure. The sirtuins and their substrates are shown in orange and yellow, respectively. Unidentified acetyl transferase (AT) are shown in blue. This figure is adapted from (101).
References


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Chapter 2

Regulation of mitochondrial ribosomal protein S29 (MRPS29) expression by a 5’-upstream open reading frame

Rationale

Mitochondrial ribosomal protein S29 (MRPS29) is a mitochondrial pro-apoptotic protein also known as death associated protein 3 (DAP3). Over-expression of MRPS29 has been shown to induce apoptosis through a mechanism that involves activation of caspases and mitochondrial fragmentation. Interestingly, the over-expression of MRPS29 is also found to be involved in resistance to apoptosis in glioma and Ataxia telangiectasia cells in the presence of various apoptotic stimuli such as streptonigrin, ionizing radiation, and camptothecin. These two contradictory reports led us to investigate the MRPS29-induced apoptosis further. In our EST database searches, we have discovered at least two different splice variants of MRPS29 mRNA in human. Among the splice variants, one contains an upstream open reading frame (uORF) in 5′-untranslated region (UTR). Therefore, our objective is to determine novel regulatory mechanism(s) involved in MRPS29 expression during progression of apoptosis in human cell lines.

The presence of the uORF in human cells was confirmed by real-time reverse transcriptase polymerase chain reaction (RT-PCR). We propose that the presence of the upstream AUG codon in the MRPS29 mRNA is likely to regulate the expression levels of the full length MRPS29. Several different approaches were employed to investigate our hypothesis in human cells. Specifically, luciferase assay and green fluorescent protein were used to show the recruitment of ribosome on the uORF for translation of MRPS29. The functional studies were performed by cell viability assay and immunoblot analyses to detect apoptosis as we propose uORF could be responsible for regulation of MRPS29 expression involved in apoptosis. Our findings suggest that the expression of the
MRPS29 could be translationally controlled by the uORF found in the 5'-UTR in this study. Finally, we found that ectopically expressed MRPS29 was not incorporated into mitochondrial ribosomes even though it was translocated into the mitochondria. This implies that apoptosis induced by over-expression of MRPS29 is probably due to alterations in protein composition of the mitochondria but not specifically by the mitochondrial ribosome.
Introduction

Mitochondria contain their own 16.5 kb circular DNA (mtDNA) and a unique translational machinery responsible for the synthesis of 13 mitochondrially encoded proteins. These proteins are all essential components of the respiratory chain complexes involved in the production of adenosine triphosphate (ATP) by oxidative phosphorylation (1-3). Although the mitochondrial translational machinery has some similarities to that of bacteria, mammalian mitochondrial 55S ribosome differs from its bacterial counterpart in terms of size, protein composition, and rRNAs (4-6). Mitochondrial ribosome is estimated to have about 80 proteins and our current data suggest that about half of these proteins have homologs in bacterial ribosomes while the remainder represents new classes of ribosomal proteins (5,6).

We have recently discovered the post-translational modifications of mitochondrial ribosomal proteins by phosphorylation and acetylation and their possible roles in regulation of mitochondrial translation (7-9). However, there is growing evidence that suggest the involvement of mitochondrial ribosomal proteins in cancer, apoptosis and other metabolic diseases (8,10-15). For example, four different mitochondrial ribosomal proteins such as MRPS29, MRPS30, MRPL37, and MRPL41 have been reported to play a role in apoptosis (12,13,16-18). We had identified two of these apoptotic proteins, MRPS29 and MRPS30 in our proteomics studies and mapped the critical phosphorylation sites found in MRPS29 by tandem mass spectrometry (8). Moreover, we determined the essential serine (Ser) and threonine (Thr) residues needed to be phosphorylated for the induction of apoptosis (8). In fact, phosphorylation of MRPS29 by Akt kinase and LKB1, a Ser/Thr kinase, was reported to be important for suppression of anoikis and pro-
apoptotic function of MRPS29 in osteosarcoma cells (19,20). Other than the pro-
apoptotic proteins of mitochondrial ribosomes, there are also several mitochondrial
ribosomal proteins and their mRNAs which are expressed differentially such as MRPS23,
MRPL11, and MRPL28 in tumor cells or tissues (10,21). Several different mechanisms
are also shown to regulate the expression of mammalian mitochondrial ribosomal
proteins such as MRPS12, MRPL11, and MRPL12 by different splice or uORF
containing variants to support protein synthesis in different physiological conditions
(22,23). Therefore, it is possible that the changes in expression of mitochondrial
ribosomal proteins essential for mitochondrial protein synthesis/function might influence
the balance between apoptosis and tumor formation due to the Warburg effect in
mitochondrial energy production.

One such well-known mitochondrial ribosomal protein is MRPS29, a GTP-
binding protein, found in the matrix and inner membrane of mitochondria as part of the
small subunit of the mammalian and yeast mitochondrial ribosomes (17,24,25).
MRPS29 was initially identified as a pro-apoptotic protein in functional knock-out
studies using anti-sense cDNAs (26). Further studies have shown that MRPS29 is an
apoptotic mediator of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and FAS-
induced apoptosis by activating caspases such as caspase-8 and by mitochondrial
fragmentation in mammalian cells (12,19,27,28). Recent knock-out studies also
revealed that MRPS29 is an essential gene and its deletion is lethal in embryos (29).
MRPS29 is known as a positive mediator of apoptosis; however, its over-expression in
different cells lines and tumors such as thyroid and leukemia is not completely
understood yet (30-32). In addition, over-expression of MRPS29 conferred resistance to
the induction of apoptosis in *Ataxia telangiectasia* (AT) cells treated by streptonigrin and ionizing radiation, and in invasive glioblastoma and glioma cells treated by camptothecin (16,33).

These two contradictory reports led us to investigate the regulation of MRPS29 expression further in human cell lines. In this study, we are reporting the presence of different splice variants of *MRPS29* discovered through screening of human ESTs. One of these splice variants contains an uORF in the 5'-UTR, which suggests that this uORF possibly blocks and decreases the expression of full length MRPS29. This could be a possible explanation for the regulation of this protein in different cell and cancer types.
Materials and Methods

Cells and reagents

Jurkat (human T cell leukemia) and K562 (human chronic myelogenous leukemia) cells were grown in RPMI-1640 media with 10% (v/v) fetal calf serum (FCS) and 1% penicillin/streptomycin at 37 °C with 5% CO₂ in a humidified incubator. HeLa (human cervical cancer), CV-1 (african green monkey kidney), and HEK293T (human embryonic kidney) cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) under the same conditions as Jurkat and K562 cells, and cells were routinely subcultured in the semi-confluent state. RPMI-1640, DMEM, FCS, trypsin, and penicillin/streptomycin were purchased from Mediatech, Inc. (Herndon, USA).

Generation of plasmid DNA constructs

cDNAs containing the human MRPS29 variants were purchased from ATCC (Manassas, USA). GenBank accession numbers for MRPS29 and uORF-MRPS29 are NM_004632 and NM_033657, respectively. The mammalian expression vector, pcDNA 3.1(+) (Invitrogen, Carlsbad, USA), was used to subclone various MRPS29 constructs for overexpression of MRPS29 in mammalian cells. PCR products for MRPS29 constructs were digested with KpnI and XhoI and subcloned into the KpnI/XhoI site of pcDNA 3.1(+) vector. Constructs were subcloned into mammalian expression vectors containing Kozak sequences either from the vector or native sequences from MRPS29 mRNAs. Especially, uORF-MRPS29 constructs contained native Kozak sequence GAGGGAaugG. The enhanced green fluorescent protein vector pEGFP-N1 (Clontech, Mountain View, USA) was used to determine the location of MRPS29. The full length
MRPS29, partial MRPS29, and the uORF constructs were cloned into the XhoI/KpnI sites of pEGFP-N1 after digestion with appropriate restriction enzymes for the GFP constructs. The firefly luciferase vector pGL3-Control and the Renilla luciferase vectors pRL-TK and pRL-SV40 (Promega, Madison, USA) were utilized for luciferase reporter assays.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from various cell lines using RNeasy Mini Kit (QIAGEN, Valencia, USA) and cDNA was synthesized with ThermoScript™ RT-PCR system (Invitrogen). Primers used were as follows:

GAPDH: forward 5’-gtcttcaccaccatggagaagg-3’
   reverse 5’-atgaggtccaccacccctgttgc-3’

MRPS29: forward 5’-atgatgctgaaaggaataacaagg-3’
   reverse 5’-tgcagaagatcccgacaatttttcaccca-3’

uORF-MRPS29: forward 5’-atggaccgacacgggtattgtaccgctga-3’
   reverse 5’-tgcagaagatcccgacaatttttaccca-3’

Quantitative real-time PCR

Quantification of the MRPS29 mRNA levels was performed by ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) using 200 ng of total RNA extracted from each cell line. The housekeeping gene cyclophilin A was used
as an internal control to normalize mRNA levels in different cell lines. For the quantification, the cycle threshold (Ct) values for MRPS29, uORF-MRPS29, and cyclophilin A mRNA were measured according to the previously described method (34). The relative amounts of MRPS29 mRNA were calculated as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = [(Ct_{MRPS29 \text{ or } uORF-MRPS29 \text{ level of cell line}} - Ct_{Cyclophilin}) - (Ct_{MRPS29 \text{ or } uORF-MRPS29 \text{ level of HeLa}} - Ct_{Cyclophilin})]$. To determine the ratio of MRPS29 to uORF-MRPS29, amplification efficiency was verified using the known copy number of MRPS29 and uORF-MRPS29 plasmids in PCR reactions. MRPS29 and uORF-MRPS29 amounts in each cell lines were calculated as relative folds of HeLa MRPS29 and uORF-MRPS29 levels. The following primer and probe sequences were used for real-time PCR designed by software supported by Applied Biosystems:

MRPS29: forward 5`-ccccaggattggagactgtatt-3`
   reverse 5`-gaatgtetctcactgtcaca-3`
   probe 5`-ccccatggccttcctcctcgc-3`

uORF-MRPS29: forward 5`-ggtcgcctagtgctggagaactagt-3`
   reverse 5`-ggagtcggcggccttccttc-3`
   probe 5`-agggagtggacgacacggttattgac-3`

Cytotoxicity assays

Cell cytotoxicity was measured using WST-1 colorimetric cell proliferation assays (Roche, Basel, Switzerland) based on the cleavage of tetrazolium salt, MTS, by
mitochondrial dehydrogenases in viable cells. Approximately $5 \times 10^3$ cells were seeded in 96-well plates without antibiotics. Transfections were performed after cells attached to the plates. For the transient transfection, the cells underwent a further incubation period (48 h) and then $10 \mu l/well$ of WST-1 solution were added for cell viability assays. The absorbance was measured at 450 nm versus a 650 nm reference using a Microplate Reader (Molecular Devices, Sunnyvale USA). Results were expressed as mean ± SD and analyzed using the ANOVA's t test. Values of *$P < 0.05$* were considered statistically significant.

**Luciferase reporter assays**

The pGL3-control vector (Promega, Madison USA) was utilized to subclone 5′-UTRs from MRPS29 cDNAs for firefly luciferase assays. After amplification and digestion of 5′-UTR containing uORF of MRPS29 with HindIII and NcoI, the PCR product was cloned upstream of the luciferase gene in the pGL3-Control vector. The primers 5′-AAAAAAAAGCTTTTCAGGACGGGCGCTTTGGA-3′ (forward), 5′-AAAAACCATGGGCATCTTGCACCTCCTGGA-3′ (reverse) were utilized for the PCR amplification. The pRL-TK and pRL-SV40 vectors were used for Renilla luciferase constructs. For luciferase assays, approximately $5 \times 10^4$ of HeLa and CV-1 cells were seeded in a 24-well plate in antibiotic-free media. After attachment, cells were transfected with luciferase constructs using Lipofectamine 2000 (Invitrogen) and then cells were lysed in passive lysis buffer (Promega) at 48 h post-transfection. Firefly and Renilla luciferase signals were measured by Dual-luciferase reporter assay system (Promega) in a Junior LB 9509 luminometer (Berthold Technologies, Oak Ridge, USA).
Results were expressed as mean ± SD and analyzed using the ANOVA’s t test. Values of *P < 0.05 were considered statistically significant.

**Immunoblot analysis**

Protein samples for immunoblot analyses were prepared from cells lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.1% SDS, and a protease inhibitor cocktail (Sigma, St. Louis, USA). After incubation of the whole cell lysates for 10 min on ice, soluble protein fractions were collected by centrifugation for 15 min at 14,000 × g at 4 °C. Protein concentrations were determined by BCA assays (Pierce, Rockford, USA), using bovine serum albumin (BSA) as a calibration standard. Approximately, 20 μg of protein lysates were electrophoresed on a 10-16% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Richmond, USA). Membranes were blocked for 3 h in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) dry skim milk powder. Blotted membranes were washed with TBST (TBS containing 0.1% Tween-20) three times and incubated at 4 °C with primary and secondary antibodies. The bound antibody signal was detected by chemiluminescence SuperSignal® West Pico kit (Pierce). Immunoblot analyses were performed with anti-β-actin (Abcam, Cambridge, USA), anti-cleaved-PARP (Cell Signaling, Danvers, USA), anti-His-Tag (MBL, Woburn, USA), anti-MRPS29, and anti-HSP60 (BD Biosciences, San Jose, USA) antibodies.

**Confocal microscopy**

HeLa and CV-1 cells transfected with GFP fusion constructs were seeded in glass slides
and stained with MitoTracker Red CMXRos (Molecular probes, Eugene, USA) and
DraQ5 (Biostatus Limited, Leicestershire, UK) for mitochondrial and nuclear localization
signals, respectively. Images were examined using a confocal microscope (Olympus
Fluoview 300 Confocal Laser Scanning Microscope, Melville, USA).

Analysis of MRPS29 incorporation into mitochondrial ribosomes

For analysis of ribosomal incorporation of endogenous and ectopically expressed
MRPS29 in human cell lines, approximately 6X10^6 HEK293T cells were transfected with
MRPS29 constructs. The whole cell pellets were suspended in a mild lysis buffer
containing 15 mM Tris-HCl, pH 7.6, 0.26 M sucrose, 40 mM KCl, 20 mM MgCl₂, 0.8
mM EDTA, 6 mM β-mercaptoethanol, 0.05 mM spermine, 0.05 mM spermidine,
proteinase inhibitor cocktail, and 1.6% Triton X-100) to keep the mitochondrial
ribosomes intact. Lysates were then loaded on 10-30% linear sucrose gradients and
centrifuged in a SW28 rotor (Beckman Coulter Inc., Fullerton, USA) at 22,000 rpm for
16 h. Sucrose gradients were formed and fractionated using a Model 160 gradient
former equipped with a UV detector and Foxy Jr. fraction collector (Teledyne Isco Inc.,
Lincoln, USA). Fractions were analyzed by immunoblotting probed with anti-MRPS29,
anti-MRPS18-2 and anti-HSP60 antibodies.
Results and Discussion

Presence of the uORF-MRPS29 in human cell lines

We discovered at least two different splice variants of MRPS29 mRNA (NM_004632 and NM_033657) through human EST database searching. Partial sequence alignment for two of these MRPS29 splice variants, one of which contained an uORF in 5'-UTR, was shown in Fig. 2-1A. Only about 30% of the ESTs available in the public databases contained the uORF in their 5'-UTRs. Moreover, genomic DNA analysis by UCSC Genome browser (http://genome.ucsc.edu/) revealed an alternative splice junction in the intron region of the MRPS29 gene. To confirm the presence of these uORF containing MRPS29 mRNAs (uORF-MRPS29), RT-PCR analysis was performed using total RNA isolated from a monkey and several different human cell lines. Our results from the RT-PCR analysis demonstrated the presence of the uORF-MRPS29 in several human cell lines, but not in the CV-1 (african green monkey kidney) cells (Fig. 2-1B). This observation was in agreement with the sequence databases as the uORF-containing ESTs were only found in human cell lines such as the Jurkat (human T cell leukemia cell) and HeLa (human cervical cancer cell) and tissues.
Figure 2-1A. Presence of uORF in human MRPS29 transcripts. Alignment of 5’-end sequences of alternatively spliced cDNAs obtained from human EST database searching. cDNAs for MRPS29 and uORF containing MRPS29 were shown as MRPS29 and uORF-MRPS29, respectively. Protein products possibly encoded from MRPS29 and uORF MRPS29 sequences were indicated above and below cDNA sequences, respectively. (*) represented the stop codons found at the 3’ end of the uORF.
Figure 2-1B. RT-PCR analyses of MRPS29 and uORF-MRPS29 transcripts in human cells. cDNAs for HeLa (H), Jurkat (J) and CV-1 (C) were synthesized using 200 ng of total RNA extracted from each cell line. PCR products obtained for MRPS29, uORF-MRPS29 and GAPDH were resolved on a 1.5% agarose gel with a size marker (S). PCR with water (W) was also resolved on the gel for a negative control. The gel was stained with ethidium bromide to visualize products.
For quantitation of MRPS29 and uORF-MRPS29 expression, we performed real-time PCR analyses in HeLa, K562 (human chronic myelogenous leukemia), Jurkat, and HEK293T (human embryonic kidney) cells. Changes in expression levels of MRPS29 and uORF-MRPS29 mRNAs in different cell lines were reported as fold of the mRNA levels in HeLa cell lines after normalizing the values against one of the housekeeping genes, cyclophilin A (Fig. 2-1C). Interestingly, expression of both mRNAs changed cell line to cell line; however, relative changes in both MRPS29 and uORF-MRPS29 levels were consistent in all cell lines (Fig. 2-1D). This observation coincides with the microarray data available for DAP3 in public gene expression databases (http://wombat.gnf.org/index.html) (35). The ratio between the MRPS29 and uORF-MRPS29 mRNAs was calculated as the percentage of relative fold. For example, the total MRPS29 level was 4.4 and the contribution from uORF-MRPS29 was 1.1 in HeLa cells; therefore, percentages of uORF-MRPS29 and MRPS29 were calculated as 20.1% and 79.9%, respectively, in these cells (Fig. 2-1D).

Changes in expression of the MRPS29 transcript in the real-time PCR analyses were also supported by variation in protein expression levels in different cell lines. As shown in Fig. 2-1E, the amount of MRPS29 protein expressed in the same cell lines was also in agreement with the mRNA expression obtained from the real-time PCR analyses. Here, immunoblot analyses performed with the anti-HSP60 and anti-β-Actin antibodies served as equal loading controls (Fig. 2-1E). To confirm the linearity of the signal intensity obtained with these antibodies, different amounts of proteins were separated on SDS-PAGE and probed with the same antibodies (Fig. 2-1F).
Figure 2-1C. Quantitation of MRPS29 variants in human cell lines using real-time PCR. The cycle threshold (Ct) values for MRPS29 and uORF-MRPS29 mRNA were measured and the Ct value obtained for each gene was normalized by one of the housekeeping genes, cyclophilin A. The relative amounts of MRPS29 or uORF-MRPS29 mRNA were calculated as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = [(Ct_{MRPS29 \ or \ uORF-MRPS29 \ level \ of \ cell \ line} - Ct_{Cyclophilin}) - (Ct_{MRPS29 \ or \ uORF-MRPS29 \ level \ of \ HeLa} - Ct_{Cyclophilin})]$ and MRPS29 and uORF-MRPS29 amounts in each cell lines were shown as relative folds of HeLa MRPS29 and uORF-MRPS29 levels.
Figure 2-1D. Ratio of MRPS29 to uORF-MRPS29 in human cell lines. To determine the ratio of MRPS29 to uORF-MRPS29, amplification efficiency was verified using the known copy number of MRPS29 and uORF-MRPS29 plasmids in PCR reactions. After confirming the same amplification efficiency, uORF-MRPS29 and MRPS29 levels in different cell line were normalized using uORF-MRPS29 levels in HeLa cells. The ratio between the MRPS29 and uORF-MRPS29 mRNAs in each cell line was calculated as the relative fold and compared to the uORF-MRPS29 mRNA level in HeLa cells. Relative ratio of MRPS29 and uORF-MRPS29 transcripts was shown with solid and open bars, respectively.
Figure 2-1E. Relative amount of MRPS29 protein expression in human cell lines. Approximately 20 µg of cell lysate from each cell line were loaded onto a 12% SDS-PAGE. Immunoblot analyses were performed with anti-MRPS29, anti-HSP60, and anti-β-Actin antibodies.
Figure 2-1F. Confirmation of the linear signal intensity obtained with several different antibodies used in this study. To confirm the MRPS29 expression in different cell lines, different amounts of protein loading showed a linear signal intensity for MRPS29, HSP60, and β-Actin antibodies which confirmed the differently expressed MRPS29 in different cell lines. Approximately 2-40 µg of cell lysates from K562 cells were loaded onto a 12% SDS-PAGE. Immunoblot analyses were performed with anti-MRPS29, anti-HSP60, and anti-β-Actin antibodies.
Regulation of MRPS29 translation by the 5′- uORF

To assess the role of the uORF found in the 5′-UTR of the uORF-MRPS29 mRNA, luciferase reporter assays were performed using CV-1 and HeLa cells. In these assays, 5′-UTR of the uORF-MRPS29 and an AUG→UUG mutant created at the AUG codon of the uORF-MRPS29 constructs were cloned into the pGL3-control vector upstream of the luciferase gene. The pGL3-control vector containing the luciferase gene was used as a positive control due to continuous production of firefly luciferase. The schematic diagrams of different constructs used in luciferase assays are shown in Fig. 2-2A. Here, reduced expression of the luciferase was anticipated if the initiation codon of the uORF in the 5′-UTR was recognized by ribosomes due to a frameshift generated in the coding sequence of luciferase. On the other hand, if the initiation codon in the uORF was not recognized by ribosomes, expression level of luciferase would be similar to its expression from the pGL3-control vector. Dual luciferase assays were performed in CV-1 cells transfected with firefly luciferase constructs and Renilla luciferase expressing vectors, pRL-TK and pRL-SV40, to normalize and reduce differences in transfection efficiency and variation in these experiments. As expected, the expression of luciferase decreased significantly in cells transfected with the uORF containing constructs (Fig. 2-2B). This finding clearly demonstrated the recognition of the initiation codon in the uORF of the MRPS29 5′-UTR rather than the initiation codon for the luciferase expression by cytoplasmic ribosomes. The HSV-thymidine kinase promoter (pRL-TK) is a relatively weak promoter compared to the early SV40 enhancer/promoter (pRL-SV40). For this reason, the relative luciferase activity (RLU) level, which was normalized by pRL-TK, has shown relatively high luciferase activity compared to the RLU level in pRL-SV40.
Mutation of the initiation codon in the uORF containing construct, however, reversed the effect of the uORF and the luciferase activity was similar to that of the pGL3-control vector in the same cell line (Fig. 2-2B).
Figure 2-2A. Schematic diagram of the constructs used in luciferase reporter assays.

The expected initiation codon recognition site and direction of translation were shown with arrows. The 5'-UTR region, which contained \textit{uORF-MRPS29}, and initiation codon for \textit{luciferase} were shown as striped box and closed box, respectively.
Figure 2-2B. Luciferase assays. CV-1 cells were transfected with pRL only (NO), pRL + pGL3-control (pGL3), pRL + pGL3-uORF (pGL3-uORF), and pRL + pGL3-mutant-uORF (pGL3-muORF) using Lipofectamine 2000. The pRL-TK and pRL-SV40 plasmids which produced Renilla luciferase were used for normalization to reduce differences in transfection efficiency and variations during experiments. Activity of luciferase was measured by a dual-luciferase assay system. The results were expressed as average luciferase value after normalization (the mean ± SD from three independent experiments). Values of *P < 0.05 were considered statistically significant. ANOVA’s t test was used to compare the significance of values.
Our findings suggest that the MRPS29 protein expression can be regulated by the uORF found in the 5'-UTR of the alternatively spliced MRPS29 mRNA. Above, we have verified ribosome recruitment at the first initiation codon in the 5'-UTR of MRPS29 using the luciferase reporter assays (Fig. 2-2B). We have also investigated a possible blockage of the translation of the full length MRPS29 in cells by transiently expressing uORF-MRPS29 to understand the role of uORF in down-regulation of MRPS29 protein expression. Especially in eukaryotes, optimal context sequence, also known as Kozak sequence, could play a significant role in ribosome recruiting and the binding of the initiation codon for translation to start (36). In a Kozak sequence, nucleotides at positions -3 and +4 are important bases for translation initiation. For a good Kozak sequence, these nucleotides should be purine and guanine bases, respectively. If these nucleotides are absent in the sequence, ribosomes can bypass the start codon due to a poor context. The Kozak sequence for uORF-MRPS29 (GAGGGAAugG) is in fact a better sequence compared to the sequence for MRPS29 (GCAAGGaugA). In this case, ribosomes would prefer to bind to the start codon in the uORF-MRPS29 rather than the first AUG in the coding sequence of MRPS29 for the initiation of translation, and therefore, the full length MRPS29 could not be translated efficiently in the presence of this uORF in the 5'-UTR. Moreover, the uORF ends with two consecutive UGA stop codons that overlap with the two possible initiation codons in the coding sequence of MRPS29 (Fig. 2-1A). To confirm this possibility, MRPS29 constructs containing the C-terminal His-tag were cloned into mammalian expression vector, pcDNA 3.1 (+) (Fig. 2-3A). As seen in Fig. 2-3B, increased MRPS29 levels were observed in cells expressing His-tagged MRPS29 constructs. However, expression of the uORF-MRPS29 containing
construct significantly reduced the expression level of His-tagged MRPS29 protein as probed with both anti-MRPS29 and anti-His-tag antibodies. Recombinant His-tagged MRPS29 protein purified from *Escherichia coli* (Rec-MRPS29) was used as a control for detection with anti-His-tag antibody. Membranes were also probed with anti-HSP60 antibodies to ensure the equal amount of protein was loaded. Theoretically, His-tagged MRPS29 protein would not be detected if the first initiation codon in the uORF was the only initiation codon, AUG, recognized by ribosomes in cells transfected with uORF-MRPS29 constructs. Due to leaky scanning of initiation codons by ribosomes or alternative translation initiation site(s), the full length MRPS29 could still be expressed at much lower levels in cells over-expressing uORF-MRPS29 (Fig. 2-3B). Therefore, our findings suggest that the presence of the uORF in the 5'-UTR of *MRPS29* translationally controls and reduces the expression of the full length protein by blocking the ribosome binding at the first AUG in the coding sequence of the *MRPS29* gene.

As shown in Fig. 2-1A, in the case of ribosome binding to the first AUG in uORF of *MRPS29*, translation could start from the uORF of *MRPS29*, which would synthesize a 23-amino acid (aa) residue peptide (approximately 2.4kDa). Attempts to detect expression of the 23-aa residue small peptide encoded from the *uORF-MRPS29* were also made. For this purpose, a Flag-tag was incorporated at the 5' end of the uORF used for the expression of MRPS29 with C-terminal His-Tag in pcDNA3.1(+) vector (Fig. 2-3A). To detect the expression of the Flag-tagged small peptide, either enzyme linked immunosorbent assays (ELISA) or immunoblot analysis were performed using anti-Flag antibody. Nevertheless, expression of the Flag-tagged small peptide was not detected with both assays (Fig. 2-3C and Fig. 2-3D ).
Figure 2-3A. Schematic diagram of the constructs in mammalian expression.

Design of the MRPS29 and uORF-MRPS29 constructs to detect expression of N-terminally Flag tagged uORF peptide and C-terminally His-tagged MRPS29. Each construct was cloned into mammalian expression vector, pcDNA3.1(+).
Figure 2-3B. Regulation of MRPS29 expression by the uORF. The HEK293T cells were transfected with 4 µg of plasmid DNA in a 6-well plate for 48 h. Protein samples (20 µg of each) obtained from lysing of transfected cells were loaded on a 12% SDS-PAGE. Immunoblot probing with anti-HSP60 antibody were employed to ensure equal loading amounts. Changes in MRPS29 expression were detected by anti-MRPS29 and anti-His-tag antibodies in HEK293T cells transfected with pcDNA 3.1 (+) (Control); His-tagged MRPS29 (MRPS29) and C-terminally His-tagged uORF-MRPS29 (uORF-MRPS29) constructs. Recombinant His-tagged MRPS29 protein purified from Escherichia coli (Rec-MRPS29) was used as a control for detection with anti-His-tag antibody.
Figure 2-3C. Absence of the small peptide expressed by the uORF detected by ELISA. The HEK293T cells were transfected with 4 µg of plasmid DNA in a 6-well plate for 48 h. Protein samples (10 µg of each) obtained from lysing of transfected cells and were used for ELISA with anti-Flag antibody. HEK293T cells transfected with pcDNA 3.1 (+) (Con); Flag tagged uORF-MRPS29 (uORF). Recombinant Flag-tagged BAP protein purified from *E. coli* was used as a standard for the quantitation.
Figure 2-3D. Absence of the small peptide expressed by the uORF detected by Immunoblot. The HEK293T cells were transfected with 4 µg of plasmid DNA in a 6-well plate for 48 h. Protein samples (20 µg of each) obtained from lysing of transfected cells were loaded on a 16% SDS-PAGE. Immunoblot probing with anti-Flag antibody were employed to detect small peptide expressed by the uORF-MRPS29. HEK293T cells transfected with pcDNA 3.1 (+) (Con); Flag tagged uORF-MRPS29 (uORF). Recombinant Flag-tagged BAP protein purified from *E. coli* (Flag) was used as a control for detection with anti-Flag antibody.
Role of the uORF in cellular localization of MRPS29 in mitochondria

The MRPS29 constructs described above, with and without an uORF, were subcloned into a mammalian enhanced GFP vector, pEGFP-N1, to study the effect of uORF in translocation of MRPS29 into mitochondria using confocal microscopy. The GFP tag was fused at the C-terminal end of MRPS29 constructs to maintain the native mitochondrial localization signal peptide at the N-terminus of the protein. For the cellular localization analysis of gene products, HeLa cells were transiently transfected with various GFP fusion constructs, MRPS29-GFP, uORF-MRPS29-GFP, and uORF-GFP, possibly expressing MRPS29 and 23 aa residue long peptide from the uORF (Fig. 2-4). After transfection with each construct, cells were stained with MitoTracker Red CMXRos and DRAQ5 to localize mitochondria and nuclei, respectively. Control cells containing the pEGFP-N1 vector were used to demonstrate non-specific and diffused localization of ubiquitously expressed GFP protein in cells (Fig. 2-4E). Fig. 2-4A showed images obtained from localization of the MRPS29-GFP fusion protein localized in the mitochondria as the fluorescent signal from the fusion protein overlapped with the red signal from MitoTracker. Even though mitochondrial localization of MRPS29 was clear in some cells, GFP-tagged MRPS29 protein was not expressed all possibly due to low transfection efficiency in HeLa cells (Fig. 2-4A). Next, we examined the effect of the uORF in cellular localization of MRPS29 and observed an overall decrease in the GFP signal in cells transfected with uORF-MRPS29-GFP (Fig. 2-4B). Here, some of the GFP-fusion protein(s) of the cells transfected with the uORF-MRPS29-GFP constructs appeared to express that was translocated into mitochondria (indicated by arrow heads), other cells ubiquitously expressed the GFP fusion protein giving a pattern similar to that
obtained from the pEGFP-N1 control cells (Fig. 2-4B). This observation could be because of the impaired expression of the MRPS29 coding region in cells transfected with the uORF-MRPS29, as described earlier (Fig. 2-1A). However, after recognition of the initiation codon in the uORF, ribosomes may continue to scan for additional in frame codons and translate N-terminally truncated MRPS29 proteins with the GFP tags by cap-independent manner or due to leaky scanning of initiation codons. To test this possibility, variety of MRPS29 constructs, including four of the in frame AUG codons, were cloned into mammalian enhanced GFP vector, pEGFP-N1. Each constructs was transfected into CV-1 cells to follow their localization in the cell (Fig. 2-4D). Surprisingly, some of the N-terminally truncated GFP constructs were localized to the mitochondria similar to location of the full length MRPS29 protein in to the mitochondria (Fig. 2-4E). For this reason, it is possible to observe translocation of the full length and/or some of the truncated protein(s) into the mitochondria when uORF-containing MRPS29-GFP construct is expressed in cells (Fig. 2-4B).
A  MRPS29-GFP  

GFP  DRAQ5+Mito  

GFP+Bright  Merged  

B  uORF-MRPS29-GFP  

GFP  DRAQ5+Mito  

GFP+Bright  Merged  

C  uORF-GFP  

GFP  DRAQ5+Mito  

GFP+Bright  Merged
Figure 2-4A, 2-4B, and 2-4C. Role of uORF in translocation of MRPS29 into the mitochondria. Localization of GFP fusion products expressed from MRPS29-GFP (2-4A), uORF-MRPS29-GFP (2-4B), and uORF-GFP (2-4C) constructs were visualized by confocal microscopy. Some of the cells transfected with the uORF-MRPS29-GFP constructs were translocated into mitochondria (indicated by arrow heads), while the others ubiquitously expressed the GFP fusion protein in the cytosol (2-4B). Similarly, the 23-residue long peptide expressed from the uORF-GFP construct did not specifically localize the GFP fusion protein into the mitochondria (2-4C). HeLa cells were transfected with GFP fusion constructs (green) and stained with MitoTracker Red CMXRos (red) and DRAQ5 (blue) to visualize localization of MRPS29-GFP products, mitochondria, and nuclei, respectively. Morphology of cells were imaged in the bright field and merged with the GFP images (GFP+Bright). Images were examined using Olympus FluoView 300 confocal laser scanning microscope and merged digitally (Merged).
Figure 2-4D. Schematic diagram of the GFP fusion constructs. Several GFP fusion constructs were cloned into mammalian enhanced GFP vector, pEGFP-N1. There were six in frame AUG codons in the coding sequence of MRPS29 and some of partial MRPS29 products which were translated from internal AUG in MRPS29 predicted to be exported into mitochondria by MitoProt. The predicted value for each truncated form P1, P2, P3, P4 is 0.5297, 0.4203, 0.2098, 0.075, respectively.
Figure 2-4E. The location of truncated form of MRPS29 into the mitochondria.

The pEGFP was used as the control and showed GFP ubiquitously in the cells. In the case of P1-GFP and P4-GFP, the same pattern as pEGFP distribution was observed; however, P2-GFP and P3-GFP, showed translocation into mitochondria. The CV-1 cells were transfected with each GFP fusion constructs and fluorescent microscopy images for GFP fusion product(s) were shown by Adobe Photoshop.
Determine the expression of 23 residues long peptide possibly expressed from the uORF. The 23-residue long small peptide encoded from the uORF-MRPS29 was fused with the GFP and the expression of this fusion protein was detected by immunoblot analysis. HeLa cell were transfected with NO (no vector), pEGFP (GFP only) and pEGFP-ORF (uORF-GFP). Approximately, 20 µg of protein from each cell lysate was loaded onto a 12% SDS-PAGE. Immunoblot analyses were performed with anti-MRPS29 and anti-GFP antibodies.
Finally, to determine the location of the 23 residue long peptide possibly expressed from the uORF, uORF-GFP construct was transfected in to the HeLa cells (Fig. 2-4C). However, the uORF-GFP protein showed a diffused GFP expression similar to that of the GFP expression from the pEGFP-N1 vector (Fig. 2-4C). According to the immunoblot analysis of cells transfected with the uORF-GFP construct, the initiation codon in the uORF recognized by the ribosomes and the GFP tagged peptide is expressed in these cells as observed by an increased molecular weight obtained with the anti-GFP antibody (Fig. 2-4F). All these findings described above suggested that the uORF found in the 5'-UTR of the differentially spliced MRPS29 mRNA can regulate the expression of the full length MRPS29 protein and impair its import into the mitochondria.
Role of uORF-MRPS29 in MRPS29-induced apoptosis

To determine the role of uORF-MRPS29 in induction of apoptosis, constructs for MRPS29 and uORF-MRPS29 were cloned into the mammalian expression vector pcDNA3.1 (+) (Fig. 2-5A). Possible translation initiation sites were indicated with arrows. After the transient transfection of these constructs into HEK293T cells, WST-1 cell viability assays were performed. The WST-1 assay is a modified form of the MTT assay, which is based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases in viable cells. Over-expression of uORF-MRPS29 resulted in cell viability values similar to the control while the over-expression of MRPS29 reduced the cell viability by 30% in HEK293T cells (Fig. 2-5B). Previous reports showed that the presence of a mutation in one of the mitochondrial proteins could affect the mitochondrial mRNA translation severely (37,38). We also evaluated the effect of MRPS29 over-expression on mitochondrial proteins synthesis using $[^{35}\text{S}]$-Met pulse labeling of 13 mitochondrially-encoded proteins in the presence of cytoplasmic translation inhibitor, emetine (Fig. 2-5C). Consistent with the previous reports, over-expression of MRPS29 did not alter the protein synthesis in mammalian mitochondria (Fig. 2-5C).
Figure 2-5A. Schematic diagram of the MRPS29 constructs cloned into mammalian expression vector. Several MRPS29 constructs were cloned into mammalian expression vector, pcDNA3.1 (+), to perform cell viability assays and detection of PARP cleavage by immunoblot analysis as a measurement of apoptotic cell death. Possible translation initiation sites were indicated with arrows.
Figure 2-5B. Cell viability assays (WST-1). WST-1 assays were performed after transfection of 0.2 µg of each construct in 96-well plate with HEK293T cells. The results were expressed as percent of control (pcDNA) (the mean ± SD from four independent experiments). Values of *P < 0.05 were considered statistically significant between control and experiment group. ANOVA’s t test was used to compare the significance of values.
Figure 2-5C. *In vivo* labeling of mitochondrially encoded proteins with MRPS29 over-expression. To assess mitochondrial translation, pulse-labeling experiments with $^{[35S]}$-methionine was performed in HEK293T cells after transient transfection of MRPS29. About $3 \times 10^5$ MRPS29 over-expressed cells were treated in methionine-free DMEM media at 37 °C for 15 min in 6-well plates. Then cells were rinsed with PBS and incubated at 37 °C for 5 min in 100 μg/ml emetine containing methionine-free DMEM to inhibit cytosolic protein synthesis. Mitochondrially encoded proteins were labeled in the presence of 100 μCi of $^{[35S]}$-methionine/ml for 4 h at 37 °C. Approximately, 10 μg of mitochondrial lysate from each sample was separated on 14% SDS-PAGE. A representative electrophoretic pattern of newly synthesized translational products was presented. ND1, -2, -3, -4, -4L, -5, and -6, subunits of Complex I; Cytb, subunit of Complex III; COI, -II, and -III, subunits of Complex IV; ATP6 and ATP8, subunits of Complex V. Coomassie Blue staining gel showed same amount of proteins were loaded on the gel. Immunoblot analysis was performed with anti-MRPS29 antibody to show over-expression of MRPS29 in the cells.
To confirm the incorporation of over-expressed MRPS29 on the mitochondrial ribosome, HEK293T cells were transfected with pcDNA-MRPS29 construct and immunoblot analyses were performed with the sucrose gradient fractions to locate mitochondrial ribosomes and the MRPS29 pool(s). As shown in Fig. 2-5D, endogenous MRPS29 was sedimented in fractions 8-10 due to its association with the mitochondrial ribosomes in the sucrose gradients as detected by anti-MRPS29 and anti-MRPS18-2 antibodies. However, the ectopically expressed MRPS29 was not completely incorporated into mitochondrial ribosomes according to the immunoblot analyses performed with anti-MRPS29 and anti-MRPS18-2 antibodies (Fig. 2-5E). Over-expressed MRPS29 was detected in earlier fractions rather than in fractions 11-12 where the majority of mitochondrial ribosomes sedimented in the sucrose gradients. Its location was similar to that of the mitochondrial matrix protein HSP60. This data also supported our previous data shown in Fig. 2-5C. Mitochondrial ribosome required the incorporation of numerous ribosomal proteins in a stoichiometric ratio. Therefore, the upregulation of one of mitochondrial ribosomal proteins, MRPS29, could not increase the level of mitochondrial translation.
Figure 2-5D. The location of endogenous MRPS29. Mitochondrial lysates were prepared from HeLa cells and loaded on 10-30% linear sucrose gradients to determine co-sedimentation of endogenous MRPS29 with the 55S ribosome. After fractionation of each gradient prepared from mitochondrial lysates, 20 μL of each fraction was loaded on a 12% SDS-PAGE. Immunoblot analyses were performed with anti-MRPS29, anti-MRPS18-2, and anti-HSP60 antibodies.
**Figure 2-5E. The location of ectopically expressed MRPS29.** Mitochondrial lysates were prepared from HEK293T cells (6 X 10^6) transfected for 3 days with 4 µg of pcDNA-MRPS29 and loaded on 10-30% linear sucrose gradients to determine cosedimentation of endogenous and ectopically expressed MRPS29 with the 55S ribosome. After fractionation of each gradient prepared from mitochondrial lysates, 20 µL of each fraction was loaded on a 12% SDS-PAGE. Immunoblot analyses were performed with anti-MRPS29, anti-MRPS18-2, and anti-HSP60 antibodies.
Besides the cell viability assays, immunoblot analysis with anti-cleaved Poly(ADP-ribose) polymerase (PARP) antibodies was employed to detect induction of apoptosis in cell lines transiently expressing the MRPS29. The PARP is a nuclear DNA binding protein detected in DNA strand breaks and involved in base excision repair. Once PARP is cleaved by caspases, its DNA repair function is impaired and this phenomenon contributes to the destruction of cells. Therefore, cleaved PARP is used as an indicator of apoptosis (39). As shown in Fig. 2-5F, cleaved PARP detected in cells transiently expressing MRPS29; however, cleavage was not induced in cells transfected with uORF-MRPS29 containing construct.
Figure 2-5F. The induction of apoptosis by ectopically expressed MRPS29 but not uORF-MRPS29. HEK293T cells were transfected with no plasmid (293T); pcDNA 3.1 (+) (pcDNA); pcDNA-uORF-MRPS29 (uORF-MRPS29); pcDNA-MRPS29 (MRPS29); constructs. About 20 µg of cell lysates were loaded on a 10% SDS-PAGE. Changes in MRPS29 expression and cleaved PARP levels were detected by immunoblot analyses with anti-MRPS29 and anti-cleaved PARP antibodies. Immunoblot analyses were also performed with anti-β-actin and anti-HSP60 antibodies to ensure equal loading amounts were in the gel.
Conclusions and Future directions

In addition to their role in oxidative phosphorylation, mitochondria play a crucial role in several major signaling pathways involved in apoptosis. An elevated level of apoptosis is associated with degenerative diseases while the suppression of this process is involved in carcinogenesis and autoimmune diseases (40,41). For this reason, changes in expression levels and/or post-translational modifications of pro- and anti-apoptotic proteins are tightly regulated in cells. One of the pro-apoptotic mitochondrial ribosomal protein, MRPS29, has been known to induce apoptosis in a variety of cell lines (12,19,20,42). Besides its role in the induction of apoptosis, MRPS29 was also reported to be involved in resistance to apoptosis in invasive glioblastoma and Ataxia telangiectasia cells (16,33). Here, we report the presence of an alternative form of MRPS29 mRNA that has an uORF in the 5'-UTR (uORF-MRPS29) in a variety of human cell lines, and this uORF might regulate the expression of MRPS29 during progression of apoptosis in human cells. Interestingly, the relative level of uORF-MRPS29 mRNA was found to be 14-20% of the total MRPS29 mRNA as determined by quantitative real time RT-PCR analyses in the human cell lines used in this study. Presence of both MRPS29 and uORF-MRPS29 mRNAs might be a significant indication for the regulation of MRPS29 expression in response to a wide variety of apoptotic stimuli in different cell lines. When the initiation codon in this uORF is recognized by ribosomes for translation initiation, the first AUG of the MRPS29 coding sequence overlap with the stop codons found at the 3' end of the uORF. Recognition of the first AUG of the MRPS29 coding sequence is impaired in uORF-MRPS29; therefore, a decrease in expression of the MRPS29 occurs. Data presented in this study suggest that the
MRPS29 expression is impaired due to the uORF found in the 5'-UTR of *MRPS29* mRNA as shown by luciferase assays and immunoblot analyses. As previously observed, reduction in endogenous MRPS29 expression by siRNA treatment prevented HeLa and CHO cells to undergo STS-induced mitochondrial fragmentation and apoptosis (28). Therefore, reduction of MRPS29 expression by this uORF may cause resistance to MRPS29-induced apoptosis. In fact, this could be one of the possible explanations for resistance to streptonigrin- and ionization radiation-induced apoptosis in *Ataxia telangiectasia* cells when the uORF containing *MRPS29* mRNA (GenBank Acc. # U18321) is over-expressed in these cell lines (33). In the presence of these findings, we propose that due to the decrease in the expression of MRPS29 by this uORF, cells transfected with uORF-MRPS29 containing construct would not undergo MRPS29-induced apoptosis since MRPS29 is not exceedingly above the endogenous MRPS29 level. Therefore, additional experiments to confirm the level of *MRPS29* and uORF-MRPS29 in different tissues and cell lines including *Ataxia telangiectasia* cells may be necessary for the future.

There is also growing evidence that suggest the involvement of mitochondrial translation activity in apoptosis and cancer (12,13,15-18). Interestingly, our results have shown that the overall mitochondrial translation was not significantly affected by the over-expression of MRPS29 in human cell lines. Similar findings for the effect of MRPS29 over-expression and knock-down on mitochondrial protein synthesis were also reported by Kimchi *et al.* (28). In the immuno-EM studies, MRPS29 is found to be located towards the lower portion of the small subunit away from the substrate binding sites interacting with the human nitric oxide associated protein 1 (hNOA1) and Complex
I in the inner membrane of mitochondria was reported recently (43,44). All these observations suggest that the pro-apoptotic function of MRPS29 might not be related to its direct function in translation. This also could be explained by the nucleoribosomic nature of the mitochondrial ribosome, which requires the incorporation of numerous mitochondrial ribosomal proteins in a stoichiometric ratio. The upregulation of just one of these proteins could be expected not to upregulate the level of mitochondrial translation. This implies that unincorporated ribosomal protein, MRPS29, could play a role in apoptosis by changing the inner membrane composition and/or binding to other apoptotic proteins and factors. These findings strengthen the idea that over-expression of other bacterial non-homolog mitochondrial ribosomal proteins may have additional function(s), which may not be involved in translation. Finding binding partners of MRPS29 and its interactions with the inner membrane using proteomic approaches will lead us to unreveal the role of mitochondrial proteins in apoptosis and their specific functions in the future.

**Acknowledgements**

The authors thank Drs. Linda Spremulli, Joseph Reese, and Hasan Koc for the critical reading and review of the manuscript. I thank Drs. Elaine Kunze and Deborah Grove for their help with the FACS and quantitative RT-PCR analyses, respectively. I also thank Dr. Chun-Hyung Kim for providing pEGFP-N1, pRL-TK, pRL-SV40, and pGL3-Control vectors. This work was supported by the National Institute of Health grants GM071034 to E.C.K. and EB005197 to D.C. and E.C.K.
References

NAD⁺-dependent deacetylase SIRT3 regulates mitochondrial protein synthesis by deacetylation of the mitochondrial ribosomal protein MRPL10 and reduction of MRPL12 binding on the mitochondrial ribosome.


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Rationale

Almost 30% of proteins in mitochondria have been identified as acetylated proteins using proteomics studies and reversible acetylation has been well established as regulatory mechanism for many biological processes, such as longevity and energy metabolism in mitochondria, recently (1-4). It has been observed that resveratol, which is an activator of sirtuin derived from red wine, has been linked to longevity and cancer prevention among the different species (5). This observation could explain what is known as the “French paradox” and the importance of the acetylation in the regulation of mitochondrial function. A member of the sirtuin family of NAD\(^+\)-dependent deacetylases, SIRT3, is located in mammalian mitochondria. Therefore, acetylation could be one of the important PTMs (post-translational modifications) in mitochondria to regulate their functions. Previously, we proposed that phosphorylation of mitochondrial ribosomes could regulate their function in translation (6,7). However, regulation of mitochondrial ribosome by acetylation is not yet fully understood.

In this study, we investigated acetylation of mitochondrial ribosomal proteins involved in regulation of mitochondrial translation. Specifically, we identified mitochondrial ribosomal protein L10 (MRPL10) as an acetylated protein among the mitochondrial ribosomal proteins and ribosome associated SIRT3 was found to be responsible for the deacetylation of MRPL10 in a NAD\(^+\)-dependent manner using biochemical, molecular and cell biology techniques. We also mapped the acetylated Lys residues by tandem mass spectrometry and determined the role of these residues in the acetylation of MRPL10 via site-directed mutagenesis. The increased acetylation of
MRPL10 led to an increase in translational activity of mitochondrial ribosomes in SIRT3 knock-out (Sirt3−/−) mice. In contrast, ectopic expression of SIRT3 in mouse brown preadipocyte (HIB1B) cells resulted in the suppression of mitochondrial protein synthesis. To understand the mechanism of translation enhanced by acetylation, we also propose that flexible binding of mitochondrial ribosomal protein L12 (MRPL12) on the ribosome could be regulated by acetylation of MRPL10 to regulate mitochondrial translation, as a result, ATP synthesis in mitochondria.

Our findings constitute the first evidence for the regulation of mitochondrial protein synthesis by reversible acetylation of the mitochondrial ribosome, as well as propose a mechanism for the regulation of mitochondrial translation via MRPL12 binding on the mitochondrial ribosome.
Introduction

Mitochondria produce over 90% of the energy used by mammalian cells through the process of oxidative phosphorylation. Previous reports showed that reversible acetylation regulates many biological processes, including mitochondrial energy metabolism (1-4,8,9). In mitochondria, almost 30% of proteins are acetylated and reversible acetylation is well known as a regulator of many biological processes such as longevity and mitochondrial energy metabolism (1-4). Although the enzymes involved in the acetylation of mitochondrial proteins are not yet identified, members of the class III histone deacetylases (sirtuins), SIRT3, SIRT4, and SIRT5, have been found to reside in the mitochondria (3,10,11). Sirtuins are homologs of the yeast SIR2 (silent mating type information regulation 2) gene and use NAD$^+$ as a co-substrate (12-14). Both SIRT3 and SIRT4 are required in a NAD$^+$-dependent manner for the maintenance of cell survival after genotoxic stress (15,16). Genetic variations in the human Sirt3 gene have also been linked to longevity (15,16). Also, SIRT3 expression in adipose tissue is increased by caloric restriction and cold exposure (1,17). Mitochondrial acetyl-CoA synthetase 2 (ACS2) and glutamate dehydrogenase (GDH) are the two key metabolic enzymes regulated by SIRT3 mediated deacetylation (2,3,18). Thus, SIRT3 and SIRT4 modulate mitochondrial function in response to the [NADH]/[NAD$^+$] ratio by regulating the activity of key metabolic enzymes.

Mitochondria have their own 16.5 kb circular DNA (mtDNA) that encodes 2 rRNAs, 22 tRNAs, and 13 mRNAs (19). For translation, mitochondria use a mechanism that utilizes mitochondrial genes and ribosomes to encode 13 proteins. These proteins are the core components of oxidative phosphorylation, which is the
process by which cellular energy is generated in the form of adenosine triphosphate (ATP) within mammalian cells (20). The mitochondrial ribosome is similar to the bacterial ribosome both structurally and functionally. All ribosomes play an important role in the translation and synthesis of new proteins in living cells, from bacteria to eukaryotes. However, there is also a discrepancy between the bacterial and mitochondrial ribosome. Mainly, the mitochondrial ribosome has less rRNA and more proteins compared to bacterial ribosome. In brief, a small subunit (28S) and a large subunit (39S) are the components of the 55S mitochondrial ribosome, whereas the bacterial ribosome has a 30S as a small subunit and 50S as a large subunit that comprises the 70S ribosome. Each small and large subunit of the mitochondrial ribosome contains 12S rRNA and 16S rRNA, respectively (21). Notably, some mitochondrial ribosomes have been mapped to regions associated with disorders of mitochondrial energy metabolism (22). Previously, our lab has identified 77 mammalian mitochondrial ribosomal proteins, of which 29 are in the small subunit and 48 are in the large subunit (23-27). Approximately half of these proteins have homologs in bacterial ribosomes, while the remainders represent new classes of ribosomal proteins (25,27). Changes in expression levels and mutations in these ribosomal proteins affect mitochondrial protein synthesis, cell growth, and apoptosis (28-32). Some of the ribosomal proteins with bacterial homologs such as MRPS12, MRPS16, and MRPL12, have been shown to be essential in mitochondrial protein synthesis (28,33-35). The functional core of the mitochondrial ribosome, which is essential for proteins synthesis, is conserved in cryo-EM reconstruction studies (20). Particularly, the L7/L12 stalk region, which consists of L10, L11, and L7/L12, is highly conserved between species and even within the
mitochondrial ribosome. Furthermore, the L7/L12 stalk region recruits translation factors such as IF-2, EF-Tu, EF-G, and RF-3 that are able to function on the ribosome during translation (36). Previous studies also showed that depleted L7/L12 on the ribosome was impaired in the GTPase activation of both EF-Tu and EF-G (37). In contrast to other ribosomal proteins, L7/L12 exists in multi-copy form on the ribosome and interacts with L10. In case of *Escherichia coli* and *Bacillus subtilis*, there are 4 copies of L7/L12 bound to the C-terminus of L10 (38,39); however, in *Thermatoga maritima* and *Thermus thermophilus*, 6 copies of L7/L12 can bind on the L10, which implies there could be an additional binding site on the C-terminus of L10. Among these 4 different species, the sequence alignment of L10 shows that there are additional residues at the C-terminus in *Thermatoga maritima* and *Thermus thermophilus*, which could explain how additional copies of L7/L12 bind on the L10 (40). In mitochondria, the number of MRPL12 copies bound to MRPL10 has not yet been identified. Another significant difference of L12 as compared to other mitochondrial ribosomal proteins is its flexibility of binding on the ribosome. Also, L12 only has protein-protein interactions with L10 and does not interact with rRNA. This observation drove us to investigate the role of MRPL12 binding on the mitochondrial ribosome in the regulation of translation.

In this study, we propose that mitochondrial protein synthesis is regulated by reversible acetylation of MRPL10 and the NAD⁺-dependent deacetylase, SIRT3, which stimulates deacetylation of MRPL10 and consequently regulates protein synthesis in the mammalian mitochondria. To understand the role of acetylation in mitochondrial translation, we focus on the mechanism of translation by MRPL12 binding to the mitochondrial ribosome via acetylation. Acetylated MRPL10 may induce more binding
of MRPL12 on MRPL10 to recruit more elongation factors involved in translation. Therefore, SIRT3 over-expression reduces overall acetylation levels in the mitochondrial ribosome and decreases translational activity by removing MRPL12 from the mitochondrial ribosome. In contrast, more MRPL12 could bind on MRPL10 via acetylation to recruit more elongation factors, such as EF-G, and enhance translation in SIRT3 knock-out (Sirt3−/−) mice.
Materials and Methods

Plasmid constructs

The full length human MRPL10 and MRPL19 mRNA amplified from Jurkat cell lines. Primers used were as follows:

MRPL10: forward 5’- aaacggggtaccatatggctgcggccgtggcggggatgctg-3’
    reverse 5’- agatctctegagcagtgccggaacagtgcagggctg-3’

MRPL19: forward 5’-aaacggggatccatgcggcctgcattgcaacg-3’
    reverse 5’-agacggtcagactattctgtctgctcaatcagacgctgctgtaatcagaccttttggctcaat-3’

For transient expression, the DNA fragments from MRPL10 and MRPL19 were digested with KpnI-XhoI and BamHI-XhoI, respectively, and then inserted into the pcDNA3.1-MycHis and pcDNA-Flag vectors that were derived from pcDNA3.1(+) to yield C-terminal Flag-tagged constructs (Invitrogen, Carlsbad, USA). To generate stable cell lines over-expressing MRPL10, the MRPL10 cDNA was first cloned into pLNCX2 (Clontech, Mountain View, USA) by using BglII and Sal I. MRPL10 mutants were created by site-directed mutagenesis (QuikChange® II Site-Directed Mutagenesis Kits, Stratagene, La Jolla USA) at K124, K162, and K196. The primers for each mutation utilized for the PCR amplification were shown in below.

K124A (AAG to GCG) forward 5’- cacaagatctgatgGCggtcttcccaaccag -3’
    reverse 5’- ctgggtgggaagagaceGCcatcaggatctgtg -3’

K162A (AAG to GCG) forward 5’-gaagagcccaaggtcGCggagatggtacgg -3’
    reverse 5’- ccgtaccatctccGCgaccttggctcctc -3’

K196A (AAG to GCG) forward 5’- ggettttatcaactctccGCgctccccagctgccc -3’
    reverse 5’- gggcaggcgtgggagcGCGagtagttgataagcc -3’
Mutated sequences were capitalized.

Cell culture

The cervical cancer cell (HeLa), human embryonic kidney 293T (HEK293T), and RetroPack™PT67 cells were cultured in DMEM (Hyclone) supplemented with 10% bovine calf serum (Hyclone, Logan, Utah), 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 °C and 5% CO₂ in a humidified atmosphere and those cells were routinely subcultured in the semi-confluent state. Mouse brown preadipocyte (HIB1B) cells with retroviral stable expression of murine SIRT3 were previously described and were kindly provided by Qiang Tong (Baylor college of Medicine) (6,17). HIB1B cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% (v/v) FCS, puromycin (4 μg/mL) and 1% (v/v) penicillin/streptomycin at 37 °C with 5% CO₂ in a humidified atmosphere and those cells were routinely subcultured in the semi-confluent state. Stable expression of murine SIRT3 (amino acids 78-334) and an alternative transcript of murine SIRT3 expressing a longer form of murine SIRT3 (amino acids 1-334) were represented as tSIRT3 and fSIRT3, respectively in this study. The human chronic myelogenous leukaemia (K562) cells were grown in RPMI with 10% (v/v) FCS, and 1% (v/v) penicillin/streptomycin at 37 °C. DMEM, RPMI, FCS, puromycin, and penicillin/streptomycin were purchased from Mediatech, Inc. (Herndon USA).

Transfection and stable cell lines

HEK293T cells (4 X 10⁵) were seeded on 6 well plate and grown to about 80% confluence in antibiotic-free DMEM supplemented with 10% FBS. 4 μg of plasmid DNA was used
each transient transfection experiment by using Lipofectamine 2000 (Invitrogen). After 2 days incubation with culture media, the transfected cells were lysed in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 0.5% NP-40) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). To generate stable cell, RetroPack™PT67 cells were transfected with a retro-viral expression vector, pLNCX2, by using Lipofectamine 2000 to get virus particles. After getting virus soup, HeLa cells were infected by using polybrene (8 μg/mL). To knock-down endogenous MRPL10, shRNA was designed at 3’-UTR to distinguish endogenous and ectopically expressed MRPL10. HEK293T cells were transfected with a lenti-vrial shRNA expression vector, pGIPZ (Oen Biosystems, Huntsville, USA), for MRPL10 by using Lipofectamine 2000 to get virus particles. The MRPL10 over-expressed stable HeLa cells were infected by collected virus particles to knock-down endogenous MRPL10.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from HIB1B cells by using RNeasy Mini Kit (QIAGEN, Valencia, USA) and cDNA was synthesized with ThermoScript™ RT-PCR system (Invitrogen). Primers used were as follows:

ND4L : forward 5’- atgccatctaccttcttcaa-3’
       reverse 5’- aaactaaggtgatggggatt-3’

COIII : forward 5’- aatccaagtccatgaccatt-3`
       reverse 5’- tgtgttggtacgaggctaga-3`

16S rRNA : forward 5’- acagctagaaaccccaac-3’
reverse 5’- aagataagacagttggac-3’

12S rRNA: forward 5’- accgceggtacgattaac-3’
reverse 5’- cccagtttgggtcttagctg-3’

MRPL12: forward 5’- gaagacgtccccaaacagaa-3’
reverse 5’- tccaaggtcgttgcgctct-3’

\[^{35}S\]-methionine pulse-labeling assays

Cells (HIB1B and K562) were seeded on 6 well plates (5 X 10^5). For the \[^{35}S\]-methionine incorporation, experiments were performed with dialysed serum (25mM Tris, pH7.4, 137mM NaCl, 10mM KCl) and minimum essential DMEM medium which had no methionine, glutamine, and cystine, after cells were attached (HIB1B) on the plates. Cells were incubated with emetine containing medium for 5 min to arrest cytoplasmic protein synthesis and add 0.2 mCi/ml of \[^{35}S\]-methionine containing medium onto cells to label mitochondrially synthesized 13 proteins. After 4 h incubation, whole cell lysates were collected as described earlier. Samples (10 μg) were electrophoresed in 12% SDS-PAGE and gel was dried into 3MM chromatography paper (Whatman, Maidstone, England). Gel was exposed onto phosphor screen overnight and read by typhoon scan (41).

Preparation of crude mitochondrial ribosomes from cell lines

For the detection of mitochondrial ribosome bound MRPL12 in HIB1B cells, approximately 4 X 10^7 cells were seeded on the several 100 mm cell culture dishes. After confluent growth of cells on the culture dishes, cells were washed with PBS and
trypsinized cells to detach from the culture dishes. The whole cell pellets were resuspended in a mild lysis buffer containing 50 mM Tris-HCl, pH 7.6, 0.26 M sucrose, 60 mM KCl, 20 mM MgCl₂, 0.8 mM EDTA, 2 mM DTT, 0.05 mM spermine, 0.05 mM spermidine, proteinase inhibitor cocktail (Sigma) by 20 G needle. Cells were lysed by Dounce-homogenizer and mitochondria were broken by adding 1.6% Triton X-100. To collect the crude mitochondrial ribosomes, cell lysates were loaded onto 34% sucrose cushion buffer (50 mM Tris-HCl, pH 7.6, 60 mM KCl, 20 mM MgCl₂, 6mM BME and 34% sucrose) and centrifuged in a type 40 rotor (Beckman Coulter Inc., Fullerton, USA) at 35,000 rpm for 10 h. After washed with resuspension buffer (50 mM Tris-HCl, pH 7.6, 60 mM KCl, 20 mM MgCl₂, 1 mM DTT and proteinase inhibitor cocktail), each mitochondrial ribosome was resuspended.

ATP determination assays

Cellular ATP concentration was measured by using ATP determination kit (Molecular Probe, Carlsbad, USA). HIB1B cell (control, SIRT3 over-expression) were washed with PBS three times and boiled for 5 min with water to lysis cell. Cell lysate were collected by centrifugation 15 min at 4 °C. The chemiluminescent detection was performed to measure ATP by using firefly luciferase and luciferin and measured by a Junitor LB 9509 Luminometer (Berthold Technologies, Oak Ridge, USA). Protein concentration of cell lysate was determined by BCA assay (Pierce) and RLU (relative luminescent unit) was normalized by protein concentration.

Complex I (NADH dehydrogenase) activity assays
Mitochondrial electron transport chain complex activity was measured by using whole cell lysate of HIB1B cells (control, SIRT3 over-expression). Cells were washed with PBS and cells were collected. Cells were lysed by n-dodecyl-β-maltoside (1.67 μg/μL) and sonication. The activity of Complex I was determined by monitoring the reduction of 2,6-dichloroindophenolate (DCIP) at 600 nm as described (42). In brief, the assay was performed in reaction buffer (25 mM potassium phosphate, pH 7.2, 5 mM MgCl₂, 2.5 mg/ml BSA, 2 mM KCN, 0.13 mM NADH, 60 μM DCIP, 65 μM decylubiquinone, and 2 μg/mL antimycin A. The reaction was initiated by adding 100 μg of cell lysate and reduction of DCIP was monitored for 10 min. After first reading, complex I inhibitor, rotenone (2 μg/mL), was added on the same sample and monitored another 10 min to show rotenone insensitive activity.

*Poly(U)-directed in vitro translation assay*

Poly(U)-directed *in vitro* translation assays were performed in 100 μl reactions containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 0.1 mM spermine, 40 mM KCl, 7.5 mM MgCl₂, 2.5 mM phosphoenolpyruvate, 0.18 U pyruvate kinase, 0.5 mM GTP, 50 U RNAsin Plus, 12.5 μg/mL poly(U), 20 pmol [¹⁴C]-Phe-tRNA, 0.15 μM EF-Tumt, 1 μg EF-Gmt, and varying amounts of mitochondrial ribosomes obtained from mitochondria. The EF-Tumt and EF-Gmt were prepared from the recombinant proteins. The reaction mixtures were incubated at 37 °C for 15 min and terminated by the addition of cold 5% trichloroacetic acid followed by incubation at 90 °C for 10 min. The *in vitro* translated [¹⁴C] labeled-poly(Phe) was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter. For the elongation factors binding assay, same
amount of GTP analog, GTPγS, was used instead of GTP for reaction.

*Bovine mitochondrial ribosome preparation*

Preparation of mitochondrial ribosomes from bovine liver was adapted from previously described methods (25,27,43,44). In order to preserve the phosphorylation and acetylation status of ribosomal proteins, phosphatase inhibitors (2 mM imidazole, 1 mM sodium orthovanadate, 1.15 mM sodium molybdate, 1 mM sodium fluoride, and 4 mM sodium tartrate dehydrate) and deacetylase inhibitor (1 mM sodium butyrate) were added during the homogenization process. Crude ribosomes prepared at 0.2, 0.4 and 1.6% Triton-X100 concentrations were loaded onto 10-30% sucrose gradients and fractionated to isolate mitochondrial 55S ribosomes (16,18,34). Purified ribosomes were sedimented by ultracentrifugation at 40,000 rpm for 5 h in a Beckman Type 40 rotor. Acetone pellets of ribosome preparations (approximately 1.8 A_{260} units) were resuspended in 2D-lysis buffer consisting of 9.8 M urea, 2% (w/v) NP-40, 2% ampholytes pI 3-10 and 8-10, and 100 mM DTT. The samples were loaded on NEPHGE (non-equilibrium pH gradient electrophoresis) tube gels and equilibrated in buffer containing 60 mM Tris-HCl pH 6.8, 2% SDS, 100 mM DTT, and 10% glycerol. The 14% second dimension gel was stained with Coomassie Blue and the protein spots corresponding to the acetylated ribosomal proteins were excised based on their locations determined by immunoblotting analysis. To complement this, in-gel and in-solution tryptic digestions were carried out using ribosomes prepared at different Triton X-100 concentrations and analyzed by LC-MS/MS to identify acetylated ribosomal proteins.
Identification of mitochondrial ribosomal proteins and mapping of PTM sites was achieved by database searching of tandem mass spectra of proteolytic peptides searched against protein database (7). Tandem MS spectra obtained by fragmenting a peptide by collision-induced dissociation (CID) were acquired using a capillary liquid chromatography - nanoelectrospray ionization - tandem mass spectrometry (LC-MS/MS) system that consisted of a Surveyor HPLC pump, a Surveyor Micro AS autosampler, and an LTQ linear ion trap mass spectrometer (ThermoFinnigan). The raw CID tandem MS spectra were converted to Mascot generic files (.mgf) using the extract msn software (ThermoFinnigan). Both, the .mgf and .raw files were submitted to site-licensed Mascot (version 2.2) and Sequest search engines, respectively, to search against in-house generated sequences of 55S proteins, all known human and bovine mitochondrial proteins, and proteins in the Swiss-Prot database. The variable modifications were methionine oxidation (+16 Da), acetylation of lysine residues (+42 Da) and phosphorylation (+80 Da) of Ser, Thr, and Tyr residues. Up to 2 missed cleavages were allowed for the protease of choice. Peptide mass tolerance and fragment mass tolerance were set to 3 and 2 Da, respectively. Tandem MS spectra were manually evaluated at the raw data level with the consideration of overall data quality, signal-to-noise of matched peaks, and the presence of dominant peaks that did not match to any theoretical m/z value.

Immunoprecipitation

For immunoprecipitation assays, lysates of HEK293 cells expressing proteins by transient transfection were incubated with anti-Flag-agarose beads (Sigma, St. Louis, MO) at 4 °C
overnight or with Talon Metal Affinity Resin (BD, Biosciences) at 4 °C for 2 h. Beads were washed three times with lysis buffer, and proteins were released from beads in SDS-sample buffer and analyzed by immunoblot analysis.

**Immunoblot analysis**

Protein samples for immunoblot analysis were prepared from cells which were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% SDS supplemented with protease inhibitor cocktail. After incubating the whole cell lysates on the ice for 10 min, soluble protein fractions were collected by centrifugation for 15 min at 14,000 X g at 4 °C. Protein concentrations were determined by BCA assay (Pierce, Rockford, USA) using bovine serum albumin (BSA) as a standard. Samples were separated in 10-16% SDS-PAGE and transfer onto PVDF membranes (Bio-Rad, Richmond, USA). After the transfer, membranes were blocked for 3 h in 5% (w/v) dry skim milk powder dissolved in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST). The membranes were washed with TBST three times and then incubated overnight at 4 °C with primary antibody. Following the primary antibody incubation and 2 h incubation with appropriate secondary antibody (Pierce), bound antibody signal was detected by chemiluminescence, SuperSignal® West Pico or femto kit (Pierce) according to the protocol. The primary antibodies were used as bellows. A monoclonal MRPS29 antibody at a 1:5000 dilution (Transduction Laboratories), a monoclonal Hsp60 antibody at a 1:5000 dilution (Transduction Laboratories), polyclonal mouse MRPL10 antibodies at 1:3000 dilution, a polyclonal anti-SIRT3 antiserum (against C-terminal domain of murine SIRT3) at 1:3000, a mouse monoclonal anti-Flag
M2-Peroxidase at 1:3000 (Sigma, St. Louis, MO), a monoclonal anti-acetylated-lysine antibody at 1:1000 (Cell Signalling Technology Inc.), or a rabbit polyclonal anti-Myc and anti 6XHis antibody at 1:1000 (Clontech, BD, Biosciences) were used. Total OXPHOS Human Antibody Cocktail and SdhA (MitoSciences Inc, Eugene, OR) were used at 1:5000 dilution. The polyclonal human MRPL40, MRPL41, MRPL47, MRPL10, and MRPL12 antibodies were generated in rabbits by COVANCE (Denver, USA) and used at 1:3000 dilution except MRPL12 which was used at 1:500 dilution. The secondary antibody was ImmunoPure Antibody Goat Anti Mouse IgG (Pierce Biochemicals Inc.) at a 1:5000 dilution or Goat Anti Rabbit IgG at a 1:1000 dilution or Affinipure Rabbit Anti-Mouse IgG, Rabbit Anti-Goat IgG, or Goat Anti-Rabbit IgG (Jackson Immuno Research) all at a 1:10,000 dilution, followed by development with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals Inc.)

In vitro deacetylation assays

Deacetylation of ribosomal proteins was performed in the presence of NAD$^+$ as a substrate for the deacetylase using previously described methods (14,45). For this reaction, 0.1 or 0.2 A$_{260}$ units of sucrose gradient purified or crude 55S ribosomes, respectively, prepared in 0.2% Triton-X100 were incubated with 3 mM NAD$^+$ for 30 min at 37 °C in the presence and absence of 0.25 g of recombinant mouse SIRT3. Acetylated/deactylated ribosome samples were analyzed by immunoblotting using rabbit polyclonal anti-Acetyl-Lys antibody.

Mouse mitochondrial ribosome isolation
Liver tissues obtained from $Sirt3^{+/+}$, $Sirt3^{+/}$, and $Sirt3^{-/-}$ mice were kindly provided by Qiang Tong (Baylor College of Medicine). The frozen liver tissues were resuspended in an isotonic mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES-KOH, pH 7.5), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 50 µg/ml leupeptin), and then homogenized in a Dounce homogenizer on ice. The suspension was centrifuged at 400 X g at 4 °C. This procedure was repeated twice, and supernatants were centrifuged at 10,000 X g at 4 °C for 10 min to pellet mitochondria. Mitochondria were lysed in a buffer containing 0.26 M sucrose, 20 mM Tris-HCl, pH 7.6, 40 mM KCl, 20 mM MgCl$_2$, 0.8 mM EDTA, 0.05 mM spermine, 0.05 mM spermidine, 6 mM β-mercaptoethanol, and 1.6% Triton X-100, and the lysates were loaded onto 34% sucrose cushions and centrifuged at 100,000 X g at 4 °C for 16 h. The crude ribosome pellets were resuspended in 20 mM Tris-HCl, pH 7.6, 40 mM KCl, 20 mM MgCl$_2$ and 1 mM dithiothreitol.
Results and Discussion

The Mitochondrial ribosomal protein MRPL10 is acetylated and SIRT3 is responsible for its deacetylation

A combination of two-dimensional gel separation and capillary LC-MS/MS analyses has been successfully used by our laboratory for the identification of ribosomal proteins and their post-translational modifications (7,32,46-48). Acetylation of several components of the translational machinery, either at N-terminal amino groups or at ε-amino groups of Lys residues in bacteria, has been reported previously (49). To determine the acetylated proteins of mammalian mitochondrial ribosomes, 55S ribosomes were purified from the bovine liver using previously described methods (43,44). Ribosomal proteins were then separated by two-dimensional gel electrophoresis, and acetylated ribosomal proteins were identified by immunoblot analysis with anti-Acetyl-Lys antibody, which detects protein only when it has been post-translationally modified by acetylation on the ε-amino groups of Lys residues (Fig. 3-1A). Protein bands corresponding to acetylated proteins detected in the gel were excised, digested with trypsin, and analyzed by capillary LC-MS/MS for identification as described in Materials and Methods. The mass spectrometric analyses of the two-dimensional gel spots revealed the presence of the two mitochondrial ribosomal proteins, MRPL10 (29.3 kDa) and MRPL19 (33.3 kDa) (Fig. 3-1A, TABLE 2-1). Since they were detected in the same gel spot in the two-dimensional gel analysis, these two proteins have almost the same pI and molecular weight values after cleavage of the N-terminal signal sequences during their translocation into mitochondria (Fig. 3-1A).
Figure 3-1A. Detection of acetylated mitochondrial ribosomal proteins. Acetylated proteins found in ribosomes purified from bovine mitochondria were detected by immunoblot analysis with anti-Acetyl-Lys antibody. Approximately 1.8 $A_{260}$ units of purified bovine 55S ribosomes were separated on two dimensional non-equilibrium pH gradient electrophoresis (2D-NPHGE) gels and acetylated proteins were detected with anti-Acetyl-Lys antibody. Protein spots corresponding to the acetylated ribosomal proteins were identified by LC-MS/MS. (Courtesy of Dr. Koc and Cimen, H.)
TABLE 2-1

Peptides detected from tryptic digests of acetylated band detected in mammalian mitochondrial ribosomal proteins by LC-MS/MS analysis

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>m/z</th>
<th>Mr(expt)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRPL10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QKLoMAVTEYIAMPKPVVNPR</td>
<td>725.1</td>
<td>2172.3</td>
<td>39</td>
</tr>
<tr>
<td>QacKLoMAVTEYIAMPKPVVNPR</td>
<td>738.8</td>
<td>2213.3</td>
<td>22</td>
</tr>
<tr>
<td>LoMAVTEYIAMPKPVVNPR</td>
<td>958.5</td>
<td>1915.1</td>
<td>72</td>
</tr>
<tr>
<td>oMIAVCQNVAoMSAEDK</td>
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<td>1642.9</td>
<td>96</td>
</tr>
<tr>
<td>VFPNQILKPFLEDSK</td>
<td>888.4</td>
<td>1774.8</td>
<td>69</td>
</tr>
<tr>
<td>YQNLPLFVGHNLLLVSEEPK</td>
<td>1213.1</td>
<td>2424.1</td>
<td>96</td>
</tr>
<tr>
<td>VKEoMVRILK</td>
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<td>1132.3</td>
<td>21</td>
</tr>
<tr>
<td>VacKEoMVRILK</td>
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<td>1173.5</td>
<td>15</td>
</tr>
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<td>ILacKpSVPFLPLLGCCIDDTILSR</td>
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<td>2571.0</td>
<td>47</td>
</tr>
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<td>QGFINYpSacKLPpSLALAQGELVGGGLpTLLTAR</td>
<td>1104.6</td>
<td>3310.9</td>
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<td>MRPL19</td>
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<tr>
<td>FLSPEFIPPR</td>
<td>602.4</td>
<td>1202.8</td>
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<td>ILHIPEFYVGSIILR</td>
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<td>LDDSLLYLRL</td>
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<td>WSQPWLEFDOMMR</td>
<td>822.1</td>
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<tr>
<td>IEAAIWEIEEASK</td>
<td>737.7</td>
<td>1473.5</td>
<td>91</td>
</tr>
</tbody>
</table>

CID spectra of peptides were searched by MASCOT as described. Modifications of peptides by acetylation (ac), phosphorylation (p) and oxidation (o) were shown for each peptide.
To confirm the acetylation status of these two mitochondrial ribosomal proteins, another approach was taken. First, coding sequences for MRPL10 and MRPL19 were inserted into pcDNA3-MycHis and pcDNA3-Flag vectors, respectively, and were expressed in HEK293T cells by transient transfection. After enriching the target proteins by His-tag affinity chromatography and immunoprecipitation using anti-Flag antibody, the acetylation status of these proteins was assessed by immunoblot analysis with anti-Acetyl-Lys antibody. No significant acetylation of MRPL19 was detected (Fig. 3-1B), whereas MRPL10 was found to be acetylated in a parallel experiment (Fig. 3-1C). To confirm the incorporation of MRPL10 on the mitochondrial ribosome, stably expressed MRPL10 in HeLa cells was generated using a retro-virus system. A crude mixture of mitochondrial and cytoplasmic ribosomes was prepared using discontinuous sucrose gradients. The level of acetylation and incorporation of MRPL10 on the ribosomes was analyzed by anti-Acetyl-Lys and anti-His-tag antibodies, respectively, after the ribosomes were isolated from the cells (Fig. 3-1D).

Overall, these observations allowed us to conclude that the acetylated protein spots detected in the immunoblot analysis of the mitochondrial ribosome preparations were primarily from acetylated MRPL10 and possibly its proteolytic products. For this reason, MRPL10 has been designated as the major acetylated protein of the 55S ribosome, leading to speculation that its reversible acetylation may play a role in the regulation of mitochondrial translation.
Figure 3-1B. Detection of MRPL19 as an unacetylated protein. HEK293T cells were transfected with Flag-tagged MRPL19. The tagged proteins were enriched by immunoprecipitation (IP) and MRPL19 acetylation levels were detected by immunoblot analysis (IB) using anti-Acetyl-Lys antibody. Expression of MRPL19 was confirmed by immunoblot using anti-Flag antibody.
Figure 3-1C. Detection of MRPL10 acetylation. HEK293T cells were transfected with Myc/His-tagged MRPL10. The tagged proteins were enriched by His-tag affinity chromatography and MRPL10 acetylation levels were detected by immunoblot analysis (IB) with anti-Acetyl-Lys antibody. Expression of MRPL10 was confirmed by immunoblot using anti-His-tag antibody.
Figure 3-1D. Detection of acetylated ribosome associated MRPL10. HeLa stable cells were generated using His-tagged MRPL10. Mitochondrial crude ribosomes were isolated from each HeLa and MRPL10 stable cells. 0.1 A\textsubscript{260} of each ribosome preparation was loaded onto a 12% SDS-PAGE. Acetylation levels of MRPL10 were detected by immunoblot analysis (IB) using anti-acetyl-Lys antibody. Anti-MRPL10 and anti-His-tag were used to show the location of MRPL10 on the mitochondrial ribosome. Two bands were detected by immunoblot analysis with anti-MRPL10 antibody. The lower and the upper bands indicated endogenous MRPL10 and ectopically expressed His-tagged MRPL10, respectively.
In addition to confirming the acetylation of MRPL10, we also performed LC-MS/MS analysis to map the acetylated Lys residues from the tryptic digests of acetylated MRPL10 fractions. This analysis enabled us to map several highly conserved acetylated Lys residues (Lys 46, 124, 162, 169, and 196) in bovine 55S ribosomes (Table 2-1). The majority of these Lys residues are highly conserved in human, bovine and mouse MRPL10 proteins (Fig. 3-2A). To investigate the contribution of highly conserved Lys residues in the acetylation of MRPL10, wild type, double (Lys162Ala and Lys196Ala), or triple (Lys124Ala, Lys162Ala, and Lys196Ala) mutants of MRPL10 were stably expressed in HeLa cells. Acetylation and expression of these MRPL10 proteins were detected by anti-Acetyl-Lys and anti-His-tag antibodies, respectively, after enriching the proteins using His-tag affinity chromatography (Fig. 3-2B). As confirmed by immunoblot analysis, over-expressed MRPL10 is acetylated, and the acetylation status of MRPL10 gradually declined in both double and triple Lys to Ala mutants (Fig. 3-2B). In the triple mutant, only three of the four acetylated lysines detected by mass spectrometry were mutated to Ala; therefore, it is possible that another acetylated lysine residue(s) contributes to the residual acetylation signal observed in the immunoblot analysis (Fig. 3-2B).
**Figure 3-2A. Alignment of MRPL10 and expected acetylated Lys residues.** Primary sequence alignment of MRPL10 homologs from different species. The bovine (XP_592952), human (NP_660298) and mouse (NP_080430) mitochondrial ribosomal MRPL10 proteins were aligned using *Thermotoga maritima* (*Thermotoga*) (NP_228266) and *E. coli* (AAC43083) L10 proteins. (*) denotes the acetylated Lys residues detected in the LC-MS/MS analysis. The alignment was created using the CLUSTALW program in Biology Workbench and is displayed in BOXSHADE.
Figure 3-2B. Role of Lys124, Lys162, and Lys196 in acetylation of MRPL10.

Approximately, 20 μg of affinity-enriched lysates obtained from HeLa cells stably expressing His-tagged wild type, double (K162A and K196A) and triple (K124A, K162A and K196A) mutants were loaded onto a 12% SDS-PAGE and probed with anti-Acetyl-Lys and anti-His-tag antibodies.
NAD⁺-dependent deacetylase-SIRT3 is associated with the 55S mitochondrial ribosome and interacts directly with MRPL10

Although there are no known protein acetyl transferases in mitochondria, three NAD⁺-dependent deacetylases, SIRT3, SIRT4 and SIRT5, are localized in the mitochondria (6-8). SIRT3 possesses NAD⁺-dependent deacetylase activity, and SIRT3-dependent deacetylation of several metabolic enzymes as well as the complex I subunit, NDUFA9, has been demonstrated in the mammalian mitochondria (3,18,50). Given that the SIRT3 has a known protein deacetylase activity, Flag-tagged SIRT3 was also co-transfected with MRPL10 in HEK293 cells. This co-expression reduced the acetylation of MRPL10 significantly (6). The presence of acetylated MRPL10 in the mitochondrial ribosome (Fig. 3-1C and 3-1D) and the deacetylation of ectopically expressed MRPL10 by SIRT3 (6) prompted us to define the enzymes responsible for this reversible post-translational modification. To determine whether any of the deacetylases mentioned above are associated with ribosomes, crude mitochondrial ribosomes were isolated at different salt and non-ionic detergent concentrations to preserve their interactions with associated proteins and were then fractionated on 10-30% linear sucrose gradients (Fig. 3-3A). To determine the location of ribosomes and ribosome-associated proteins, immunoblot analyses of the sucrose gradient fractions were probed with antibodies to Acetyl-Lys, MRPL41, and SIRT3 (Fig. 3-3A). The SIRT3 containing fractions (12-18) overlapped with the acetylated-MRPL10 and another large subunit ribosomal protein MRPL41 (Fig. 3-3A).
Figure 3-3A. Interactions between SIRT3 and mitochondrial 55S ribosomes. Crude mitochondrial ribosomes were loaded on to 10-30% linear sucrose gradients to sediment 55S ribosomes. To demonstrate the co-sedimentation of SIRT3 with the 55S ribosome, immunoblot analyses (IB) were performed using anti-Acetyl-Lys antibody for the detection of acetylated MRPL10, as well as anti-SIRT3 and anti-MRPL41 antibodies, after separating 30 μl of each fraction onto a 12% SDS-PAGE. (Courtesy of Cimen, H.)
In our analysis, we have found SIRT3 association with the mitochondrial ribosome only at low detergent and ionic conditions, implying a possibly transient interaction. In addition to the immunoblot analysis, in-gel proteolytic digestion and mass spectrometric analysis of the protein band detected with the anti-SIRT3 antibody confirmed the association of SIRT3 with the ribosome. Peptide sequence matches obtained from the LC-MS/MS analysis of the in-gel digested SIRT3 bands are presented in TABLE 2-2. Furthermore, the other mitochondrial deacetylases, SIRT4 and SIRT5, were not detected by the immunoblot analysis performed with anti-SIRT4 and anti-SIRT5 antibodies or in LC-MS/MS analysis of ribosome preparations; therefore, we concluded that SIRT3 was the only mitochondrial deacetylase associated with the ribosome. Our findings are also in agreement with a recent report that deficiency of SIRT3, but not SIRT4 or SIRT5, affects mitochondrial protein deacetylation (18).
<table>
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<th>Mr(expt)</th>
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<td>1015.7</td>
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The SIRT3 and MRPL10 interaction domains were also evaluated using a structural model based on the coordinates of human SIRT3 and the *Thermotoga maritima* L10-L7/L12 complex (Fig. 3-4A and 3-4B) (51). As illustrated in Fig. 3-4B, the region within the first 148 residues of MRPL10 (marked in pink) required for SIRT3 binding is probably the most accessible region for this interaction since MRPL10 also interacts with MRPL11 and MRPL12 to form the mitochondrial L7/L12 stalk. The C-terminal helix 8 of *Thermotoga* L10 binds three L7/L12 dimers (two L7/L12 dimers in *E. coli*) and the N-terminal globular domain interacts with the N-terminal domain of L11 and SIRT3 (Fig. 3-4B). Regions of MRPL10 and SIRT3 responsible for binding to each other, and their interactions with the rest of the ribosome, are in agreement with the idea of a transient interaction between SIRT3 and the mitochondrial ribosome. Moreover, acetylated Lys residues in MRPL10 are located near the SIRT3 binding domain and reversible acetylation/deacetylation of these residues could be involved in the regulation of ribosomal function by NAD$^+$-dependent SIRT3 (Fig. 3-4B and 3-4C). The L7/L12 stalk is known to be essential as well as the most highly regulated and flexible region of the large subunit due to its interaction with elongation, initiation and release factors during different stages of translation (52-54). For this reason, it is plausible to suggest that the mitochondrial translational machinery might be regulated by reversible acetylation of the L7/L12 stalk protein, MRPL10.
Figure 3-4. Structural model of the SIRT3 and MRPL10 interactions in the L7/L12 stalk. A) Crystal structure model of the human SIRT3 was generated by Protein Data Bank with accession number 3GLU. The SIRT3 represented the MRP-L10 interaction site (green surface) and the acetyl-CoA synthetase2 (ACS2) peptide at the active site (red). B) Structure of the L10-L7/L12 complex from Thermotoga maritima was generated by Protein Data Bank with accession number 1ZAX and was used to model L7/L12 stalk in mitochondria. In the model, MRPL10 was colored in pink (to represent SIRT3 binding site) and green and the conserved Lys residues found to be acetylated in bovine MRPL10 (shown by asterisks in Fig. 3-2A) were colored in red. The three L7/L12 dimers (DI, DII, DIII) were colored in yellow. The structural model was generated using PyMol software (DeLano Scientific LLC).
Figure 3-4C. Structural model of the SIRT3 and MRPL10 interactions in the ribosomal L7/L12 stalk. Models of the human SIRT3 and *T. maritima* L10-L7/L12 complex were used to represent their possible interactions with 55S mitochondrial ribosomes using coordinates from the *E. coli* 50S subunit. The structure model was generated by Protein Data Bank with accession number 2AW4 (50S subunit) and 3GLU (SIRT3). The 50S ribosomal rRNAs, L10, L11, and SIRT3 were colored in blue, green, yellow, and pink, respectively. The other functional regions such as peptidyl transferase center (PTC), central protuberance (CP), sarcin-ricin loop (SRL), L1, and L7/L12 stalks of the large subunit and ribosomal proteins (salmon) were labeled in the model. The structural model was generated using PyMol software.
To confirm the effect of SIRT3 on MRPL10, *in vitro* deacetylation assays were performed using recombinant SIRT3 and MRPL10. The NAD\(^+\)-dependent deacetylation of ribosome-associated MRPL10 by recombinant SIRT3 was detected by immunoblot analysis with anti-Acetyl-Lys antibody (Fig. 3-5A). In the presence of NAD\(^+\), the acetylation level of the protein band containing MRPL10 significantly decreased without the addition of recombinant SIRT3, presumably due to the catalytic activity of the ribosome-associated endogenous SIRT3. This observation also confirmed the presence of ribosome-associated SIRT3 in the mitochondrial ribosome preparations, as detected by immunoblot analysis and mass spectrometric analysis (Fig. 3-3A and Table 2-2). Furthermore, the addition of recombinant SIRT3 to the *in vitro* deacetylation assay further decreased the acetylation level of MRPL10. In contrast, acetylated Hsp70, which is approximately 75 kDa and co-sedimented with the ribosome, was neither deacetylated by an endogenous deacetylase nor by the recombinant SIRT3 *in vitro*.

To evaluate whether SIRT3 was the major deacetylase responsible for the deacetylation of MRPL10, we also evaluated the acetylation level of MRPL10 in a SIRT3 knock-out (*Sirt3\(^{-/-}\)*) mouse. Analysis of mitochondrial ribosomes isolated from SIRT3 knock-out (*Sirt3\(^{-/-}\)*), wild-type (*Sirt3\(^{+/+}\)*), and heterozygote (*Sirt3\(^{+/-}\)*) mice revealed that the MRPL10 in the Sirt3\(^{-/-}\) mice was more heavily acetylated than in either of the other two more strains (Fig. 3-5B). Immunoblottings were also probed with mouse anti-MRPL10 and MRPS29 antibodies to ensure equal loading of mitochondrial ribosomal proteins (Fig. 3-5B). In agreement with Lombard et al., we also detected hyperacetylation of
mitochondrial glutamate dehydrogenase (GDH) using 2D-gel electrophoresis and capLC-MS/MS analysis in the mitochondria of Sirt3⁻/⁻ mice (Fig. 3-5B) (18). Data obtained from the deacetylation of ectopically expressed and endogenous ribosome-associated MRPL10 strongly suggest that the mitochondrial SIRT3 is associated with the mitochondrial ribosome and also involved in the specific deacetylation of MRPL10.

We also performed poly(U)-directed in vitro translation assays to monitor mitochondrial ribosome activity as a function of the acetylation status of MRPL10. This assay is one of the primary techniques in assessing the interactions of mitochondrial elongation factors with the L7/L12 stalk of the ribosome (55,56). To test the role of reversible acetylation in mitochondrial ribosome activity using this in vitro assay, we first isolated mitochondrial ribosomes from SIRT3 knock-out (Sirt3⁻/⁻), wild-type (Sirt3⁺/⁺) and heterozygote (Sirt3⁺/-) mouse liver mitochondria and performed poly(U)-directed poly(phenylalanine) synthesis in the presence of [¹⁴C]-tRNA_Phe and the mammalian mitochondrial elongation factors EF-Tu mt and EF-G1 mt (56). To ensure that equal amounts of mitochondrial ribosomes were used in each in vitro translation assay, ribosomes were quantified using A₂₆₀ measurements and immunoblot analysis was performed with anti-MRPS29 antibody as an equal loading control (Fig. 3-5B). In these assays, mitochondrial ribosomes isolated from Sirt3⁻/⁻ mice had a 50-60% higher translational activity compared to those from Sirt3⁺/⁺ or Sirt3⁺/- mice (Fig. 3-6B).
**Figure 3-5A. Deacetylation of MRPL10 by the NAD$^+$-dependent deacetylase, SIRT3.**

*In vitro* deacetylation reactions using approximately 0.1 $A_{260}$ units of 55S bovine mitochondrial ribosomes were performed in the presence of 3 mM NAD$^+$ and 0.2 μg of recombinant SIRT3 as labeled and immunoblot analysis (IB) was performed using anti-Acetyl-Lys antibody to detect acetylated MRPL10. Arrows indicated the specific deacetylation of MRPL10, but not the acetylated Hsp70, sedimented with ribosomes by endogenous and recombinant SIRT3 in the presence of 3 mM NAD$^+$. (Courtesy of Cimen, H.)
Figure 3-5B. Deacetylation of MRPL10 by NAD⁺-dependent deacetylase, SIRT3.

Mitochondrial ribosomes were prepared as described in the Materials and Methods from Sirt3⁺/-, Sirt3⁺/+ and Sirt3⁺/+ mice liver and the acetylation of ribosomal protein MRPL10 was detected by immunoblot analysis (IB) with anti-Acetyl-Lys antibody. As a control for acetylation of GDH in the absence of SIRT3 and an equal loading, immunoblots were probed with anti-Acetyl-Lys, mouse anti-MRPL10 and anti-MRPS29 antibodies. (Courtesy of Cimen, H.)
Figure 3-5C. Role of ribosome acetylation/deacetylation by SIRT3 in mitochondrial translation. Acetylated ribosomes promote mitochondrial protein synthesis in vitro. Mitochondrial ribosomes (0.05-0.1 A_{260} units) isolated from Sirt3^{+/+}, Sirt3^{+/-}, and Sirt3^{-/-} mice liver mitochondria were used in the poly(U)-directed in vitro translation assays described in the Materials and Methods. The results were expressed as the percent translation activity of the control (Sirt3^{+/+}) mice. The mean ± SD was calculated from three independent experiments. Asterisks denote p<0.05, which were considered statistically significant. (Courtesy of Cimen, H.)
Over-expression of SIRT3 regulates mitochondrial protein synthesis in HIB1B cells

The mitochondrial translation machinery is responsible for the synthesis of 13 proteins essential for oxidative phosphorylation: subunits of Complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), Complex III (Cyt b), Complex IV (COI, COII, and COIII), and Complex V (ATP6 and ATP8). Using a well-established in vivo labeling method (57), we examined the role of reversible acetylation on mitochondrial protein synthesis in cells ectopically expressing SIRT3. Stable expression of murine SIRT3 (amino acids 78-334) and an alternative transcript of murine SIRT3 expressing a longer form of this protein (amino acids 1-334) were represented as tSIRT3 and fSIRT3, respectively, in this study. In these assays, only de novo synthesized mitochondrial proteins were labeled with $[^{35}\text{S}]$-methionine in the presence of emetine, which is an inhibitor of cytoplasmic protein synthesis. In HIB1B cells expressing fSIRT3, incorporation of $[^{35}\text{S}]$-methionine into 13 mitochondrially-encoded proteins was significantly decreased by 50-60% compared to control cells (Fig. 3-6A). Since fSIRT3 showed more deacetylase and a greater reduction in translation activity (Fig. 3-6A), the rest of our experiments were performed using fSIRT3 cells. These observations suggest that the expression of SIRT3 down-regulates mitochondrial protein synthesis possibly by deacetylation of MRPL10 and/or other ribosomal proteins.
Figure 3-6A. Role of ribosome deacetylation by over-expressing SIRT3 in mitochondrial translation. SIRT3 expression decreased mitochondrial protein synthesis in HIB1B cells. Stable expression of murine SIRT3 (amino acids 78-334) and an alternative transcript of murine SIRT3 expressing a longer form of murine SIRT3 (amino acids 1-334) were represented as tSIRT3 and fSIRT3, respectively. Mitochondrially-encoded proteins were labeled in the presence of 100 μCi of [35S]-methionine/ml for 4 h at 37 °C. Approximately, 10 μg of cell lysates from each sample was separated on 14% SDS-PAGE. Gel was fixed, dried and autoradiographed. The signal intensities were determined by Typhoon. Gel was stained with Coomassie Blue to show same amount of protein loading on the SDS-PAGE. A representative electrophoretic pattern of newly synthesized translational products was presented. ND1, -2, -3, -4, -4L, -5, and -6, subunits of Complex I; Cytb, subunit of Complex III; COI, -II, and -III, subunits of Complex IV; A6 and A8, subunits of Complex V. The combined intensities of mitochondrially-encoded proteins from each lane were used as an indicator of relative mitochondrial protein synthesis.
Previously, we showed that MRPL10 was one of the substrates for the NAD$^+$-dependent deacetylase, SIRT3, and mitochondrial protein synthesis was stimulated in SIRT3 knock-out mice (6). In agreement with SIRT3 knock-out mice data, a reduction in the synthesis of 13 mitochondrially-encoded proteins was observed in SIRT3 over-expressing HIB1B cells (Fig. 3-6A). To understand the mechanism involved in regulation of translation by acetylation of MRPL10, we focused our efforts on MRPL12 which is responsible for the recruitment of translational factors on the ribosome. The mitochondrial L7/L12 stalk is composed of MRPL10, MRPL11 and multiple copies of MRPL12. However, MRPL12 binds to the ribosome by interacting only with MRPL10, and MRPL10 acts as an anchor for MRPL12 binding to the ribosome. Therefore, it is possible that acetylation of MRPL10 could cause a change in the L7/L12 stalk composition to regulate mitochondrial translation. Interestingly, a change in MRPL12 incorporation into the mitochondrial ribosome was observed in SIRT3 knock-out mice mitochondria without changing expression of MRPL12, supporting our hypothesis (unpublished data Cimen, H.).

To examine the role of SIRT3 over-expression on MRPL12 binding to the mitochondrial ribosome, we performed immunoblot analyses to check the expression of MRPL12 and incorporation of MRPL12 on the mitochondrial ribosome in control and SIRT3 over-expressing HIB1B cells. We isolated mitochondria from each cell line and checked expression level of MRPL12. To confirm the role of SIRT3 as deacetylase, mitochondria from corresponding cells were prepared and overall acetylation levels were analysed by immunoblotting probed with anti-Acetyl-Lys antibody. As shown in Fig. 3-
7A, overall acetylation levels were significantly reduced in SIRT3 over-expressing cells. To show the over-expression of SIRT3, Flag-tagged ectopically expressed SIRT3 was detected using anti-Flag antibody. Immunoblot analyses with anti-MRPL10, anti-MRPL47, anti-SdhA, and anti-HSP60 antibodies were performed to ensure equal loading amounts (Fig. 3-7A). However, no significant change was detected in expression level of MRPL12 from both whole cell and mitochondrial lysates (Fig. 3-7A) between HIB1B and SIRT3 over-expressing cells, as we expected. In bacteria, multiple copies of L7/L12 form a very flexible stalk along with L10 to recruit elongation factors during translation (53,58,59). Previous reports showed that the bacterial homolog of MRPL12 plays a role in the recruitment of several different GTPases, such as IF2, EF-Tu, RF-G, and RF3, that function in translation (36,53,60,61). This observation led us to further investigate role of MRPL12 binding on the mitochondrial ribosome during translation. To investigate the possible flexible binding of MRPL12 on the mitochondrial ribosome, immunoblot analyses were performed using ribosomes enriched from HIB1B and SIRT3 over-expressing cells. These results showed a significant reduction in MRPL12 binding on the ribosome (Fig. 3-7B). Furthermore, the blots probed with anti-Acetyl-Lys antibody showed reduced acetylation of ribosomal proteins in SIRT3 over-expressing cells. Immunoblot analyses with anti-MRPL10, anti-MRPS29, and anti-MRPL47 antibodies were also used to ensure equal loading amounts (Fig. 3-7B).
Figure 3-7A. Role of SIRT3 over-expression on MRPL12 binding to ribosome in HIB1B cells. Mitochondrial lysates were prepared from HIB1B (Con) and over-expressing SIRT3 (SIRT3) cells. Approximately 10 µg of mitochondrial lysate from each cell lines was loaded on to a 12% SDS-PAGE. Immunoblot analyses (IB) were performed using anti-Acetyl-Lys, anti-Flag, anti-HSP60, anti-SdhA, anti-MRPL10, anti-MRPL47, and anti-MRPL12 antibodies. (Cimen, H. contributed to this experiment)
Figure 3-7B. Role of SIRT3 over-expression on MRPL12 binding to ribosome in HIB1B cells. Mitochondrial ribosomes were enriched from control and SIRT3 over-expressing (SIRT3) HIB1B cells. Approximately 0.05 A$_{260}$ units of mitochondrial ribosomes from each cell lines were loaded on to a 12% SDS-PAGE. Immunoblot analyses (IB) were performed with anti-Acetyl-Lys, anti-MRPS29, anti-MRPL10, anti-MRPL47, and anti-MRPL12 antibodies. (Cimen, H. contributed to this experiment)

To confirm the MRPL12 expression level, RT-PCR was performed to examine the transcript level of MRPL12 in SIRT3 over-expressing cells. As shown in Fig. 3-7C, the transcript level of MRPL12 was not changed. ND4L and COIII, which are
mitochondrially-encoded mRNA genes, as well as 12S rRNA and 16S rRNA, which are mitochondrial encoded rRNA genes, were also examined to ensure equal loading amounts. As shown in Fig. 3-6A, although newly synthesized ND4L and COIII proteins were significantly reduced in SIRT3 over-expressing cells, transcript levels were not changed either in control or SIRT3 over-expressing HIB1B cells (Fig. 3-7C). Altogether, these observations allowed us to conclude that MRPL12 expression was not changed transcriptionally or translationally; however, ribosome bound form of MRPL12 was reduced in SIRT3 over-expressing cells. The functional changes in the translational activity of mitochondrial ribosomes were also checked. Mitochondrial ribosomes only translate 13 proteins, which are the components of electron transport chain complexes and ATP synthase, therefore, decreased translation of the complexes could reduce their activity and production of ATP. As shown in Fig. 3-7D, reduction in Complex I activity was in agreement with the decline in protein synthesis in SIRT3 over-expressing cells. Not only was the activity of complex I reduced in SIRT3 over-expression cells (Fig. 3-7D), but also ATP production (Fig. 3-7E). We observed over 50% of reduction in 13 mitochondrially-encoded proteins in SIRT3 over-expressing HIB1B cells (Fig. 3-6A); however, the ATP was reduced only about 20-30% compared to control cells (Fig. 3-7E). This discrepancy could be explained by direct regulation of OXPHOS complexes by SIRT3-dependent deacetylation. Previous reports showed that activities of Complex I and II are stimulated by SIRT3-deacetylation of their nuclear encoded subunits (50,62). This implies that activities of OXPHOS complexes and ATP production could be regulated by both acetylation and translation of their components in a SIRT3-dependent manner to maintain basal ATP level (6,50,62).
Figure 3-7C. Transcript level of mitochondrial ribosomal proteins in HIB1B and SIRT3 over-expressing cells. Total RNA was extracted from HIB1B (Con), over-expressing SIRT3 (SIRT3) and cDNA was synthesized using RT-PCR (reverse transcription polymerase chain reaction). PCR was performed with the synthesized cDNA and specific primers for each gene. Mitochondrially-encoded mRNA; ND4L and CO III, mitochondrially encoded rRNA; 12S rRNA and 16S rRNA, nucleic encoded mRNA; MRPL10, MRPL47, MRPL12, and GAPDH.
Figure 3-7D. Complex I activity in SIRT3 over-expressing HIB1B cells. Whole cell lysates were prepared from HIB1B (Con) and SIRT3 over-expressing (SIRT3) cells. Cells were lysed by n-dodecyl-β-maltoside and sonication in ice cold water. The activity of Complex I was determined by monitoring the reduction of 2,6-dichloroindophenolate (DCIP) at 600 nm as previously described in Materials and Methods. The reaction was initiated by adding 100 μg of cell lysate, and the reduction of DCIP was monitored for 10 min at 600 nm. After adding Complex I inhibitor, rotenone, samples were monitored for an additional 10 min. Only the rotenone sensitive Complex I activity was presented. The results were expressed as the percent of control. The mean ± SD was calculated from three independent experiments. Values of *P < 0.05 were considered statistically significant. ANOVA's t test was used to compare the significance of values.
Figure 3-7E. ATP production in SIRT3 over-expressing HIB1B cells. Whole cell lysates were prepared from HIB1B (Con), over-expressing SIRT3 (SIRT3). The chemiluminescent production using firefly luciferase and luciferin reaction was performed to measure ATP. Reaction was measured using a luminometer. Relative luminescent unit (RLU) was normalized by the protein concentration of each lysate. The results were expressed as the percent of control. The mean ± SD was calculated from three independent experiments. Values of *$P < 0.05$ were considered statistically significant. ANOVA’s t test was used to compare the significance of values.
Modulation of SIRT3 expression on MRPL12 binding on the ribosome in human cell lines

We showed that changes in acetylation status of mitochondrial proteins by SIRT3 affected MRPL12 binding on the mitochondrial ribosome possibly to regulate mitochondrial translation using mice model. Regulation of SIRT3 expression and activity by kaempferol (KP) and nicotinamide (NAM) was reported previously (3,62,63). NAM is a well-known inhibitor for sirtuins, and it has shown to inhibit SIRT3 activity in various cell lines (45,62). On the other hand, KP enhances SIRT3 expression in K562 (human chronic myelogenous leukaemia) cells (62,63). We have previously used NAM and KP to show their effect on regulation of Complex II activity by SIRT3-dependent deacetylation of SdhA subunit (62). Here, we investigated the role of NAM and KP treatments on mitochondrial translation. For this purpose, K562 cells were treated with 10 mM of NAM and 50 μM of KP for 48 h. As confirmed by immunoblotting analysis, SIRT3 level was increased in KP treated cells, while NAM treatment did not change SIRT3 expression level significantly (Fig. 3-8A). In Fig. 8A, acetylation level in mitochondria was altered by NAM and KP treatments. In particular, NAM enhanced the overall acetylation of mitochondrial proteins in K562 cells. Conversely, KP treatment reduced the acetylation of mitochondrial proteins. Overall, mitochondrial proteins were more acetylated as compared to control cells treated with NAM, though the SIRT3 expression level was not reduced by NAM treatment. These results showed that the activity of SIRT3 was inhibited by NAM even though the SIRT3 expression level was not changed by NAM. HSP60 and SdhA were used as loading controls to show equal loading on the gel. Mitochondrial ribosomal protein levels such as MRPL10, MRPL12, and MRPL47 were changed by neither NAM nor KP treatments, which was in agreement
with our previous experiments (Fig. 3-7A). We also performed $^{35}$S-methionine labeling assays to show the effects of KP and NAM treatments on mitochondrial translation in vivo. Interestingly, the $^{35}$S-methionine labeling assays showed fewer mitochondrial encoded proteins were synthesized in SIRT3 over-expressing cells treated with KP (Fig. 3-8B).

To confirm the acetylation/SIRT3-dependent changes in MRPL12 binding to the ribosome, mitochondrial ribosomes were separated using a 10-30% sucrose gradient. As shown in Fig. 3-8C, the amount of bound MRPL12 on the mitochondrial ribosome was significantly reduced by KP treatment, which was in agreement with SIRT3 over-expressing HIB1B cells (Fig. 3-7B). This observation confirmed that the SIRT3-dependent reversible acetylation expression could regulate mitochondrial translation by modulating MRPL12 binding on the mitochondrial ribosome in human cells. Therefore, human SIRT3 also plays a role in regulation of mitochondrial translation by enhancing binding of MRPL12 on mitochondrial ribosome in human cells as well as mice.
Figure 3-8A. Role of NAM and KP treatments on SIRT3 expression and acetylation of mitochondrial proteins in K562 cells. Human chronic myelogenous leukemia (K562) cells were treated with 10 mM of nicotinamide (NAM) and 50 μM of kaempferol (KP) for 48 h. Mitochondrial lysates were prepared from K562 (Con), nicotinamide (NAM), and kaempferol (KP). Approximately 10 μg of mitochondrial lysate from each treatment was separated by 12% SDS-PAGE. Immunoblot analyses (IB) were performed with anti-Acetyl-Lys, anti-SdhA, anti-HSP60, anti-SIRT3, anti-MRPL10, anti-MRPL12, and anti-MRPL47 antibodies.
Figure 3-8B. Role of NAM and KP treatments on mitochondrial translation in K562 cells. K562 cells were treated with 10 mM of nicotinamide (NAM) and 50 μM of kaempferol (KP) for 48 h. Cells were labeled with $[^{35}\text{S}]$-methionine and whole cell lysates were prepared from K562 (Con), nicotinamide (NAM), and kaempferol (KP) treated cells. Mitochondrially-encoded proteins were labeled in the presence of 100 μCi of $[^{35}\text{S}]$-methionine/ml for 4 h at 37 °C. Approximately, 10 μg of cell lysates from each sample was separated on 14% SDS-PAGE. Gel was fixed, dried and autoradiographed. The signal intensities were determined by Typhoon. ND1-6 showed, subunits of Complex I; COX I-III, subunits of Complex IV; cyt b, subunit of Complex III; ATP 6,8, subunits of Complex V, respectively. Gel was stained with Coomassie Blue to show equal protein loading.
Figure 3-8C. Role of reversible acetylation on MRPL12 binding to ribosome in K562 cells. K562 cells were treated with 10mM of nicotinamide (NAM) and 50 μM of kaempferol (KP) for 24 h. Whole cell lysates were loaded onto a 10-30% linear sucrose gradients to sediment mitochondrial ribosomes. Equal amounts of sucrose gradient fractions were separated on 12% SDS-PAGE. Immunoblot analyses (IB) were performed with anti-MRPS29, anti-MRPL10, anti-MRPL40, anti-MRPL47, and anti-MRPL12 antibodies.
Stimulation of elongation factor recruitment in NAM-treated ribosomes

Since nicotinamide (NAM) is a general inhibitor of sirtuins, including SIRT3, we incubated freshly isolated bovine mitochondria in 10 mM NAM for 16h. Mitochondrial ribosomes were prepared using NAM-treated mitochondria by discontinuous sucrose gradients (DG) as described in the Materials and Methods. To assess the effect of NAM treatment, we first verified the increased acetylation of MRPL10 (Fig. 3-9A), and translation activity of ribosomes prepared from NAM-treated mitochondria in poly (U)-directed \textit{in vitro} translation assays in the presence and absence of cytoplasmic translation inhibitor, emetine (Fig. 3-9B). In agreement with the increased activity obtained in SIRT3 knock-out (\textit{Sirt3}^{-/-}) mice (Fig. 3-5C), the translation activity of mitochondrial ribosomes from NAM-treated bovine mitochondria was also significantly increased (Fig. 3-9B). The increased acetylation and protein synthesis activity of ribosomes isolated from SIRT3 knock-out (\textit{Sirt3}^{-/-}) mice and NAM-treated bovine mitochondria indicate that ribosomes containing acetylated MRPL10 are more active in the synthesis of 13 mitochondrially-encoded proteins.

As shown in Fig. 3-9A and 3-9B, bovine mitochondria showed enhanced MRPL10 acetylation levels in ribosome and translational activity when treated with NAM. Furthermore, we observed an increase in MRPL12 binding to ribosome in NAM-treated mitochondria, which was in agreement with our previous results (Fig. 3-8C and unpublished data Cimen, H.). To investigate whether acetylated MRPL10/ribosomes were critical for elongation factor recruitment, the same amount of ribosomes (3 A_{260} of either control or NAM treated ribosomes) were used in a poly(U)-directed \textit{in vitro} translation assay and the reaction mixtures were loaded onto 10-30%
linear sucrose gradients. Immunoblotting analyses carried out with sucrose gradient fractions clearly showed an increase in EF-G recruitment to the NAM-treated ribosomes, especially in fractions 4 and 5 (Fig. 3-9C). Interestingly, MRPL12 signal was also higher in the NAM-treated ribosomes compared to the control. This result is not surprising as the bacterial homologs of MRPL12 is an essential ribosomal protein required for translocation of tRNA catalyzed by EF-G-dependent GTP hydrolysis (60,64). The acetylation level of MRPL10 was also confirmed by immunoblotting probing with anti-Acetyl-Lys antibody. Blots were also probed with anti-MRPS29, anti-MRPL40, and anti-MRPL47 to ensure equal ribosome loading.

In conclusion, we suggest that the mitochondrial protein synthesis could be stimulated by the stabilization of MRPL12 binding to acetylated MRPL10 and/or ribosome rather than the increased affinity of elongation factor binding to the acetylated MRPL10 and/or ribosome.
**Figure 3-9A. Role of nicotinamide (NAM) treatment on acetylation of mitochondrial ribosomal proteins.** Immunoblot analysis (IB) of mitochondrial ribosomes (0.2 A₂₆₀) prepared in the absence (Con) and presence of 10 mM nicotinamide (NAM) from bovine liver mitochondria probed with anti-Acetyl-Lys, anti-MRPL10 and anti-MRPS29 antibodies. (Cimen, H. contributed to this experiment)
Figure 3-9B. Role of nicotinamide (NAM) and emetine on mitochondrial protein synthesis. Mitochondrial ribosomes (0.1-0.2 A$_{260}$ units) isolated from control and NAM-treated bovine liver mitochondria were used in the poly(U)-directed in vitro translation assays described in the Materials and Methods. The poly(U)-directed in vitro translation assays using NAM-treated ribosomes were also repeated with the addition of 1 mM emetine to inhibit translation activity of cytoplasmic ribosomes. The results were expressed as the percent of the control. The mean ± SD was calculated from three independent experiments. (Cimen, H. contributed to this experiment)
Figure 3-9C. Role of increased MRPL10 acetylation on MRPL12 binding to ribosome in bovine mitochondria. The poly(U)-directed *in vitro* translation assay was performed with DG ribosomes isolated from control (Con) and 10 mM NAM-treated bovine mitochondria. Assay contents were loaded onto 10-30% sucrose gradients to probe for elongation factor binding on the mitochondrial ribosome. To block the GTP-hydrolysis by elongation factors, a non-hydrolyzable GTP analog, GTPγS, was used instead of GTP. Immunoblotting analyses were performed with anti-Acetyl-Lys, anti-His-tag, anti-MRPS29, anti-MRPL40, anti-MRPL47, anti-MRPL10, and anti-MRPL12 antibodies.

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Conclusions and Future directions

Mitochondria are essential for the production of 90% of the ATP required by eukaryotic cells. However, slower metabolic rates, a reduction in oxidative phosphorylation, and ATP synthesis have been associated with slower aging and an extended lifespan. In fact, reduced expression of oxidative phosphorylation components using RNAi in *C. elegans* decreases ATP production and oxygen consumption (65). Furthermore, reduction in caloric intake induces the expression of mitochondrial SIRT3 and causes similar effects by deacetylating mitochondrial proteins in a NAD$^+$-dependent manners. (16). Similarly, the specific deacetylation of MRPL10 by the ribosome-associated deacetylase, SIRT3, may play a pivotal role in coordinating the activity of mitochondrial protein synthesis machinery to the [NADH]/[NAD$^+$] ratio in the mammalian mitochondria. This idea is supported by our findings and suggests that acetylated MRPL10 and/or ribosomes from SIRT3 knock-out mice are more active in protein synthesis, leading to an increase in the expression of mitochondrially-encoded components of the oxidative phosphorylation. In this study, we demonstrate for the first time that the acetylation of a mitochondrial ribosomal protein, MRPL10, as well as its deacetylation by the NAD$^+$-dependent deacetylase SIRT3 to regulate mitochondrial translation. Deacetylation of MRPL10 and/or ribosomes may impair protein synthesis and reduce the rate of oxidative phosphorylation by lowering the expression of essential proteins involved in oxidative phosphorylation.

Particularly, the acetylation of mitochondrial ribosomes could enhance the translational activity of ribosomes and MRPL10 could be a substrate for the SIRT3 involved in the translational regulation by acetylation (6). We also propose that the
mechanism by which translation is regulated by acetylation. The L7/L12 stalk in mitochondria, which is composed of MRPL10, MRPL11, and multiple copies of MRPL12, is essential for translation. Specifically, MRPL12 plays a significant role in translation by recruiting initiation, elongation, and release factors to the ribosome. Acetylation of MRPL10 enhanced MRPL12 binding to the ribosome, and therefore recruitment of elongation factors to increase translation. On the other hand, SIRT3 over-expression reduced translation and MRPL12 binding to ribosomes possibly by the deacetylation of MRPL10.

**Acknowledgements**

I would like to thank Margaret Nguyen for technical assistance and Eric Verdin for providing human SIRT3-Flag clone. I thank Robert Schlegel and Joseph Reese for critical reading of the manuscript. This work was supported by a NIH grant to Y.B. (R01AG025223), Q.T (R01DK075978) and E.C.K (R01GM071034).
References


Chapter 4

Purification of functional human mitochondrial ribosomal protein L10 (MRPL10) with MRPL12 by duet expression in *Escherichia coli*
Rationale

Mitochondrial ribosomal protein L10 (MRPL10) is one of the components of L7/L12 stalk, which plays a significant role in translation by interacting with several different translation factors. Functionally and structurally, the MRPL10 acts as an anchor for MRPL12 in the ribosome to recruit translational factors; the N-terminal domain of MRPL10 interacts with the ribosome and the C-terminal domain of MRPL10 interacts with the dimers of MRPL12. Therefore, it is important to understand the role of MRPL10-MRPL12 interactions in regulation of mitochondrial translation. To investigate the interaction between MRPL10 and MRPL12 in vitro, we over-expressed components of the mitochondrial L7/L12 stalk proteins. However, purification of recombinant MRPL10 was not successful. Here, we designed a construct to co-express MRPL10 and MRPL12 using a duet expression system in *Escherichia coli* (*E. coli*) to make a soluble complex of MRPL10 and MRPL12. The goal is to use reconstituted hybrid ribosomes containing mitochondrial L7/L12 stalk and bacterial ribosomes for in vitro translation assay as supporting evidence for the effect of MRPL10-MRPL12 interaction on mitochondrial translation.
Introduction

The mitochondrial translation system is used to translate 13 proteins of the core components of the oxidative phosphorylation (OXPHOS) using their own DNA (mtDNA) to generate cellular energy, adenosine triphosphate (ATP), in eukaryotic cells (1). The mechanism of mitochondrial translation is very similar to the mechanism of bacterial translation as we described above (2,3).

The L7/L12 stalk is one of the most conserved functional regions on the ribosome among different species and plays a significant role in translation due to the interaction with initiation factor 2 (IF2), elongation factors EF-G and EF-Tu, and release factor 3 (RF3) on the ribosome (4-6). Those factors directly bind to the C-terminal domain of L12. Mutation in this region reduces the binding of ternary complex forming with aminoacyl-tRNA·EF-Tu·GTP to the ribosome (7). The mitochondrial L7/L12 stalk, which is composed of MRPL10, MRPL11 and multiple copies of MRPL12, is structurally and functionally very similar to its bacterial homolog (1,3). In bacteria, two different forms of L7/L12 exist. The N-terminal acetylated form is called L7 and unacetylated form is called L12. N-terminal acetylation increases binding between L10 and L12 by promoting helical folding of L12 structure (8). Since there is no report to indicate the N-terminal acetylation of MRPL12, which corresponds to L7 in bacterial L7/L12, we use the terminology MRPL12 instead of MRPL7/L12 in this study. A previous report showed that MRPL12 could be involved in mitochondrial transcription by interacting with the mitochondrial RNA polymerase (9). However, the primary function of MRPL12 is in translation (5,10).

A significant difference of L7/L12 compared to other ribosomal proteins is that
multiple copies of L7/L12 exist in the ribosome. Among different bacteria, it is found that the ribosomal L7/L12 stalk consists of different numbers of L7/L12 dimers which are determined by the length of the L10 helix $\alpha_8$ (11-13). For example in *E. coli*, 4 copies of L7/L12 are found as two dimers bound to the C-terminal domain of L10. However, in *Thermatoga maritima*, 6 copies of L7/L12 are found as three dimers bound to L10. In mitochondria, the number of copies of MRPL12 bound to MRPL10 is not determined yet. Another significant difference between L7/L12 and other ribosomal proteins is that L7/L12 binds to the ribosome by interacting with L10 only, without any protein-rRNA interaction(s). Therefore, L10 acts as an anchor for L7/L12 on the ribosome.

Our previous studies show that acetylation of MRPL10 can be regulated by NAD$^+$-dependent deacetylase, SIRT3, (14) and acetylation also regulates mitochondrial translation and MRPL12 binding on the ribosome (unpublished data, Chapter 3). Therefore, MRPL10 can play an important role in regulating translation by binding of differing numbers of MRPL12 dimers on the ribosome. Mitochondrial L7/L12 stalk function can be studied by using hybrid ribosomes, which are reconstituted by stripped *E. coli* ribosomes in the presence of purified mitochondrial ribosomal L7/L12 stalk proteins (10).

In this study, we expressed and purified MRPL10-MRPL12 complex using the duet expression system. We also confirmed that the purified MRPL10-MRPL12 complex was functional in reconstituted hybrid ribosomes to support translation *in vitro*.
**Materials and Methods**

*Plasmid DNA constructs*

The gene encoding human MRPL10, MRPL11, and MRPL12 were amplified by polymerase chain reaction (PCR) with a cDNA library from HeLa cells. Amplified MRPL10, MRPL11, and MRPL12 were cloned into *E. coli* expression vectors for this study. For the expression and purification in *E. coli*, MRPL10 was cloned into pET26 vector by using Ndel/XhoI (Novagen, Madison, USA). MRPL11 and MRPL12 were cloned into pET28 vector by using Ndel/XhoI (Novagen) (contributed by H. Cimen and J. Miller). To increase the level of expression and incorporation into bacterial ribosomes, truncated forms of MRPL10 (MRPL10-NT1) and MRPL12 (MRPL12-NT1) were generated by removing the mitochondrial targeting sequence predicted by MITOPROT (15). MRPL10 contained a 6xHistidine-tag (His-tag) at the C-terminus, while MRPL11 and MRPL12 contained the tag at the N-terminus. The primer sequences for PCR are indicated below.

MRPL10-NT1: Forward 5’-aaacggggtaccatgggctccaaggctgttacccgc-3’,

Reverse 5’-agatctctcgagcgagtccggaacagtgtcaggatc-3’.

MRPL12-NT1: Forward 5’- aaacggccatgggtgcacccctggataacgcc-3’,

Reverse 5’- gcgggtctcgagttactccagaaccaeggtgcc-3’.

MRPL11: Forward 5’-aaacggggtaccatgtcaaagctcggc-3’,

Reverse 5’- agatctctcgagtcactttggtg-3’.

*Expression and purification of human MRPL10 under denaturing conditions*

Human MRPL10-NT1 protein was expressed in *E. coli* (BL21-RIPL) by induction with
100 μM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h at 18 °C and harvested. The *E. coli* cell pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaH$_2$PO$_4$, 8 M Urea) and incubated for 60 min at room temperature by gently vortexing. Cell lysate was centrifuged at 10,000 X g for 30 min at room temperature and the cleared supernatant was applied to a Ni-NTA affinity column. Unbound proteins were washed with wash buffer (10 mM Tris-HCl, pH 6.3, 100 mM NaH$_2$PO$_4$, 8 M Urea). Proteins retained on the Ni-NTA beads were eluted with elution buffer (10 mM Tris-HCl, pH 4.5, 100 mM NaH$_2$PO$_4$, 8 M Urea). Eluted proteins were dialyzed by using SpectraPor dialysis tubing in dialysis buffer (50 mM Tris, pH 8.0, 200 mM KCl, 1 mM DTT, 10% glycerol) to remove urea.

*Expression and purification of human MRPL11 and MRPL12*

For the purification, a His-tag at the C-terminus of MRPL11 and a His-tag at the N-terminus of the truncated form of MRPL12-NT1 were generated, respectively. Human MRPL11 and MRPL12 proteins were expressed in *E. coli* (BL21-RIPL) by induction with 100 μM of IPTG for 5 h at 25 °C. The harvested cell pellet was broken by sonication and the cell lysate was centrifuged at 10,000 X g for 30 min at 4 °C. Cleared supernatant was applied to a Ni-NTA affinity column. After Ni-NTA purification, eluates were collected and dialyzed by using SpectraPor dialysis tubing in dialysis buffer (50 mM Tris, pH 8.0, 200 mM KCl, 10% glycerol, 1 mM DTT) for 8 h to remove imidazole.

*Purification of human MRPL10 using the duet expression system*
To overcome the low solubility of MRPL10, a truncated form of MRPL10 (MRPL10-NT1) and MRPL12 (MRPL12-NT1) were cloned into the pETDuet™-1 for coexpression in *E. coli* by using Ncol/SalI and NdeI/XhoI, respectively. To distinguish overexpressed MRPL10 which was in a complex with MRPL12, only MRPL10 had a His-tag at the C-terminus. The primer sequences for PCR are indicated below. The sequences for His-tag are in bold.

MRPL10-NT1: Forward 5’-aaacgggggtaccatatgggctccaaggctgttacccgc-3’,

Reverse 5’-gcgggtaccctcgagttacta atggtgatggtgatgatg cgagtccggaacagtgtcagg-3’;

MRPL12-NT1: Forward 5’-aaacggccat(630,338),(880,503)

Reverse 5’-gcgggtctcaggtactcctgtaacctccagaaccacggtgcc-3’.

For the purification, MRPL10-NT1 and MRPL12-NT1 were over-expressed by induction with IPTG (100 μM) for 5 h at 25 °C and harvested. The harvested cell pellet was broken by sonication and the cell lysate was centrifuged at 10,000 X g for 30 min at 4 °C. Cleared supernatant was applied to a Ni-NTA affinity column. After Ni-NTA purification, eluates were collected and dialyzed by using SpectraPor dialysis tubing in dialysis buffer (50 mM Tris, pH 8.0, 200 mM KCl, 10% glycerol, 1 mM DTT) for 8 h to remove imidazole. The dialyzed sample was then applied to a strong cation exchange column (SCX, SP Sepharose Fast Flow column, GE healthcare, Sweden) for further purification. The SCX column was eluted using a linear (50 mM –1 M KCl) salt gradient and eluted fractions were monitored by UV detection. The fractions containing the MRPL10-MRPL12 complex were collected. Combined fractions were dialyzed by using SpectraPor dialysis tubing in a dialysis buffer (50 mM Tris, pH 8.0, 200 mM KCl, 10% glycerol, 1 mM DTT) for 8 h.
Reconstitution of ribosome and poly(U)-directed in vitro translation assays

Poly(U)-directed in vitro translation assays adapted from previously described methods were performed using a reconstituted bacterial ribosome with purified MRPL10, MRPL11, and MRPL12 (10,16,17). For the reconstitution of ribosome, the L7/L12 stalk was removed by incubating the bacterial 70S ribosomes in extraction buffer (40 mM Tris-HCl, pH 7.5, 1 M NH₄Cl, 20 mM MgCl₂, 10 mM β-mercaptoethanol) at 30 °C for 5 min. 1 mL of pre-warmed ethanol at 30 °C was added and the mixture was incubated at 30 °C for 5 min, this was repeated twice. Stripped ribosomes were collected by ultracentrifugation at 40,000 rpm for 5 h at 4 °C and resuspended in reconstitution buffer (20 mM Tris-HCl, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 2 mM DTT). 35 pmol of stripped ribosomes were incubated with purified MPRL10 (70 pmol), MRPL11 (70 pmol), and MRPL12 (280 pmol) at 37 °C for 15 min. The poly(U)-directed in vitro translation assay reactions were performed (50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 0.1 mM spermine, 80 mM KCl, 6 mM MgCl₂, 2.5 mM phosphoenolpyruvate, 0.18 U pyruvate kinase, 0.5 mM GTP, 50 U RNasin Plus, 12.5 μg/mL poly(U), 20 pmol [¹⁴C]-Phe-tRNA, 0.15 μM EF-Tuₘᵦ, 1 μg EF-Gₘᵦ) at 37 °C for 30 min followed by 5% trichloroacetic acid treatment and incubated on ice for 5 min. Precipitated ribosome complex was boiled at 90 °C for 10 min. The in vitro translated [¹⁴C] labeled-poly-Phe was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter (18,19).
Results and Discussion

Expression and purification of human MRPL10 under denaturing conditions

Human MRPL10 was cloned into pET26 vector and expressed in BL21-RIPL cells by IPTG induction. However, due to low expression level and insolubility of full length MRPL10, several truncated forms of MRPL10 were generated by removing the mitochondrial targeting sequence predicted by MITOPROT (Fig. 4-1A). The expression level of MRPL10 was improved by truncation of the N-terminus; however, purification of MRPL10 was still not successful due to the insolubility of the protein. To overcome the low solubility of MRPL10, purification was performed under denaturing conditions. As shown in Fig. 4-1B, MRPL10 was successfully purified under denaturing conditions using 8 M urea. Dialysis was performed to remove urea and help refolding of the protein after purification. The majority of purified MRPL10 precipitated during dialysis when urea was removed. The precipitated MRPL10 was removed by centrifugation for 10 min at 14,000 rpm and the soluble portion was collected.
**Figure 4-1A. Primary sequence of the human MRPL10.** Mitochondrial targeting sequence of the human MRPL10 predicted by MITOPROT was shown in red (GenBank Acc. #AB051618). Truncated form of MRPL10 shown as NT-1, NT-2, NT-3, and NT-4 were generated and their start position is shown in blue, respectively.
Figure 4-1B. Expression and purification of human MRPL10 under denaturing conditions. MRPL10-NT1 protein was expressed in *E. coli* (BL21-RIPL) by induction with 100 μM of IPTG for 5 h at 18 °C. Cell pellet was resuspended under denaturing conditions for lysis. Expression of MRPL10 was confirmed by using total cell lysate. Uninduced (UI): No induction; Induced (I): 5 h induction with IPTG. Cell lysate was centrifuged at 10,000 X g for 30 min at room temperature and cleared supernatant was applied to a Ni-NTA affinity column. The column was washed and His-tagged MRPL10 was eluted. Lysate: Ly; Flow-Thru: F/T; Wash: W; Eluted fraction: E. 5 μL of each fraction was collected and loaded onto 12% SDS-PAGE. Proteins were visualized by Coomassie Blue staining.
To check the ribosomal activity, we generated a reconstituted hybrid ribosome with the bacterial 70S ribosome and purified mitochondrial proteins as described in materials and methods. In brief, bacterial lysates were separated on a 10 to 30% sucrose gradient and fractions containing 70S ribosomes were collected by ultracentrifugation. The L7/L12 stalk region of 70S ribosomes was stripped by incubation with high salt and ethanol as described in the materials and methods (10). Hybrid ribosomes were reconstituted with stripped bacterial ribosomes in the presence of purified MRPL10 and MRPL12 to examine translational activity. As shown in Fig. 4-1C, several ribosomal proteins were removed. Extracted ribosomal proteins from bacterial 70S ribosomes, which occurred during the stripping process were collected in supernatant and identified as L10, L11, and L7/L12 by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Previous reports showed that *E. coli* EF-G does not function on the mitochondrial ribosome during translocation, which is part of the elongation process; however, mitochondrial EF-G\textsubscript{mt} and EF-Tu\textsubscript{mt} could act as elongation factors on the *E. coli* ribosome (10,20,21). Therefore, mitochondrial elongation factors (EF-G\textsubscript{mt} and EF-Tu\textsubscript{mt}) were used in poly(U)-directed *in vitro* translation assays to check their ribosomal activity on bacterial 70S ribosomes. Reconstituted 70S ribosomes with purified bacterial L10 and L7/L12 proteins were as active as the 70S intact ribosome (Fig. 4-1D). However, reconstituted hybrid ribosomes with purified mitochondrial MRPL10 and MRPL12 proteins were not active in translation assays. One of the biggest issues is the loss of activity of the purified protein, when the protein is purified under denaturing conditions. Because protein lose their tertiary stucture under denaturing conditions and the loss of
tertiary structure may affect the activity of the protein. Even though the soluble portion of purified MRPL10 was used for the poly(U)-directed \textit{in vitro} translation assay, MRPL10 was not functional on the ribosome for translation (Fig. 4-1E).

In bacteria, L7/L12 binds to the ribosome by interacting with L10 only and without a rRNA interaction. Therefore, L10 protein acts as an anchor for L7/L12, which plays a role in the recruitment of several different GTPases such as IF2, EF-Tu, RF-G, and RF3 on the ribosome for translation (5). Protein sequence alignment among the different species also indicate that MRPL10 and MRPL12 are highly conserved among the different species, which implies the function of mitochondrial MRPL10 could be similar to L10 in bacteria. As shown in Fig. 4-1E, the stripped ribosome lost its translational activity. Even though MRPL12 was added on the stripped ribosome, reconstituted ribosome without MRPL10 still did not function in translation. Therefore, we concluded that MRPL10 is one of the essential components on the ribosome for translation.
**Figure 4-1C. Purification of bacterial ribosomes.** Bacterial ribosomes were purified by a 10-30% sucrose gradient and the 70S fractions were collected by ultracentrifugation. The L7/L12 stalk proteins of bacterial 70S ribosomes were removed by incubation at 30 °C for 5 min with high salt (1 M NH₄Cl) and additional incubation was done by adding 50% ethanol. Approximately, 0.1 A₂₆₀ of 70S intact ribosomes and stripped ribosomes were loaded onto 12% SDS-PAGE to show release of the stalk proteins. Ribosomal proteins were visualized by Coomassie Blue staining.
Figure 4-1D. Poly(U)-directed *in vitro* translation assay with reconstituted hybrid ribosome and purified bacterial L10 and L7/L12. Reconstituted hybrid ribosome with purified bacterial L10 and L7/L12 proteins were active. Stripped ribosomes (35 pmol) were incubated with purified L10 (70 pmol) and L7/L12 (280 pmol) at 37 °C for 15 min for hybrid ribosome reconstitution. The poly(U)-directed *in vitro* translation assay reactions were performed at 37 °C for 30 min followed by 5% trichloroacetic acid treatment and incubated on ice for 5 min. Precipitated ribosome complex was boiled at 90 °C for 10 min. The *in vitro* translated [\(^{14}\)C] labeled-poly-Phe was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter. The results were expressed as the relative percent of synthesized poly-Phe compared to the control (70S). The mean ± SD was calculated from three independent experiments. Values of # P < 0.05 were considered statistically significant between stripped ribosome and reconstituted ribosome. ANOVA’s t test was used to compare the significance of values.
Figure 4-1E. Poly(U)-directed \textit{in vitro} translation assay with reconstituted hybrid ribosome and purified MRPL10 by denaturing conditions. Reconstituted hybrid ribosome with mitochondrial MRPL10 purified under denaturing conditions did not activate the ribosomes. Stripped ribosomes (35 pmol) were incubated with purified MRPL10 (70 pmol) and MRPL12 (280 pmol) at 37 °C for 15 min for hybrid ribosome reconstitution. The poly(U)-directed \textit{in vitro} translation assay reactions were performed at 37 °C for 30 min followed by 5% trichloroacetic acid treatment and incubated on ice for 5 min. Precipitated ribosome complex was boiled at 90 °C for 10 min. The \textit{in vitro} translated \textsuperscript{14}C labeled-poly-Phe was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter. The results were expressed as the relative percent of synthesized poly-Phe compared to the control (70S). The mean ± SD was calculated from three independent experiments.
Expression and purification of human MRPL11 and MRPL12

For the MRPL11 purification, we generated His-tagged MRPL11 at the C-terminus, which was soluble and purification was done by using a Ni-NTA affinity column (Fig. 4-2A and 4-2C). For the MRPL12 purification, we generated a His-tag at the N-terminus with a truncated form of MRPL12-NT1, which was soluble and purification was done by using a Ni-NTA affinity column (Fig. 4-2B and 4-2C).
Figure 4-2A. Primary sequence of human MRPL11. Primary amino acid sequence of human MRPL11 was shown (GenBank Acc. #AB051338). Mitochondrial targeting sequence predicted by MITOPROT is shown in red. Full length MRPL11 was generated and its start position is shown in blue.

Figure 4-2B. Primary sequence of human MRPL12. Primary amino acid sequence of human MRPL12 was shown (GenBank Acc. #X79865). Mitochondrial targeting sequence predicted by MITOPROT was shown in red. Truncated form of MRPL12 shown as MRPL12-NT-1 was generated and its start position is shown in blue.
Figure 4-2C. Purification of human MRPL11 and MRPL12. Mitochondrial ribosomal protein MRPL11 (L11) and truncated form of MRPL12 (L12) were purified by Ni-NTA affinity column. Both His-tagged recombinant MRPL11 and MRPL12 were over-expressed in *E. coli* (BL21-RIPL) and purified by Ni-NTA affinity column. The recombinant proteins (2 μl) were loaded onto 12% SDS-PAGE. Proteins were visualized by Coomassie Blue staining. (contributed by H. Cimen for MRPL11 and J. Miller for MRPL12)
Expression and purification of human MRPL10 and MRPL12 using duet expression system

MRPL10 was insoluble and formed inclusion bodies when over-expressed in *E. coli*. Previous reports showed that over-expressed L10, which makes a complex with L7/L12 could remain in solution in *B. stearothermophilus* (13). By making a complex with L7/L12, the solubility of L10 was enhanced. These reports encouraged us to investigate the possibility that the solubility of MRPL10 could be increased by co-expressing MRPL12 in *E. coli*. To overcome the low expression of MRPL10, truncated forms of MRPL10 (MRPL10-NT1) and MRPL12 (MRPL12-NT1), where the mitochondrial targeting signals were removed, were cloned into pETDuet™-1 to co-express in BL21-RIPL (Fig. 4-1A and 4-2B). As shown in Fig. 4-2D, truncated forms of MRPL10 (MRPL10-NT1) and MRPL12 (MRPL12-NT1) were successfully co-expressed in *E. coli*. To distinguish over-expressed MRPL10, which was in complex with MRPL12, only MRPL10 had a His-tag at C-terminus. The MRPL10 and MRPL12 complex was purified by Ni-NTA affinity purification. Identification of purified proteins was analyzed by LC-MS/MS with database searching of proteolytic peptides by trypsin digestion (Fig. 4-2D). Two major bands located around 28kDa and 20kDa were identified as MRPL10 and MRPL12, respectively by LC-MS/MS analysis. Two major contaminants were found between 55kDa and 72kDa and identified as 60kDa chaperonin protein (groEL protein) and chaperon protein dnaK (heat shock protein 70). Another contaminant was found around 26 kDa, which was identified as *E. coli* ribosomal protein L4 and/or elongation factor P (EF-P) according to our LC-MS/MS analysis.
Figure 4-2D. Purification of human MRPL10 by duet expression with MRPL12.
The truncated forms of MRPL10 and MRPL12 were cloned into pETDuet™-1. For the purification, MRPL10-NT1 and MRPL12-NT1 were over-expressed by induction of IPTG. Uninduced (UI): No induction; Induced (I): 5 h induction with IPTG. The harvested cell lysates were applied to a Ni-NTA affinity column for purification. Ly and E represented the whole cell lysate and the elution fraction of Ni-NTA purification, respectively. Unknown contaminants between 26kDa, 55kDa, and 72kDa were identified as *E. coli* ribosomal protein L4 and/or EF-P (26kDa), 60kDa chaperonin protein (groEL protein), Chaperon protein dnaK (heat shock protein 70) by mass spectrometric analysis.
As shown in Fig. 4-2D, purification of MRPL10 by duet expression with MRPL12 by Ni-NTA affinity column contained some contaminants. Especially, EF-P has been identified as an essential protein for cell viability to stimulate of peptidyl transferase activity in bacterial ribosomes and also require for protein synthesis. Therefore, this contaminants affect on the poly(U)-directed in vitro translation assay. To remove these contaminants, SCX (strong cation exchange) column chromatography was performed after the Ni-NTA purification (Fig. 4-2E). For SCX, a continuous linear (50 mM –1 M KCl) salt gradient was applied and eluted fractions were monitored by UV detection at 260 nm. After SCX purification, no E. coli ribosomal protein L4 and/or EF-P which were located around 26kDa remained in fraction #10-15. Other contaminants found between 55kDa (groEL) and 72kDa (dnaK) were still eluted with MRPL10-MRPL12 complex. There was no direct evidence to show that bacterial chaperon involved in translation. Therefore, fractions #10-11 were combined and dialysis was performed in 50 mM Tris, pH 8.0, 200 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol for the poly(U)-directed in vitro translation assay.
Figure 4-2E. Purification of human MRPL10 by duet expression with MRPL12.
To remove contaminants, SCX (strong cation exchange) chromatography was performed after Ni-NTA purification. For SCX, a continuous linear (50 mM –1 M KCl) salt gradient was applied and eluted fractions were monitored by UV detector at 260 nm to trace proteins. E, F/T, #5-20 represented the elution fraction of Ni-NTA purification, flow through of SCX column, and #5-20 elution fraction of SCX purification, respectively.
Reconstitution of Functional hybrid ribosome using recombinant MRPL10-MRPL12 complex

Poly(U)-directed *in vitro* translation assays were performed with reconstituted hybrid ribosomes to check activation of translation by purified MRPL10-MRPL12 complex using duet expression system. As shown in Fig. 4-3A, stripped bacterial ribosomes lacking the L7/L12 stalk lost their translational activity by almost 80% compared to 70S intact ribosomes. Reconstituted ribosomes with only MRPL12 or MRPL10 could not restore translation activity (Fig. 4-3B). However, reconstituted ribosomes with purified MRPL10, MRPL11, and MRPL12 could recover translational activity up to 50% compared to the activity obtained with 70S ribosomes (Fig. 4-3A). This indicates that both MRPL10 and MRPL12 are essential components of the ribosome needed for the translation. Poly(U)-directed *in vitro* translation assay was performed with or without additional MRPL11 for the reconstitution to check its role in translation. However, no significant difference in translation was observed with additional MRPL11 (Fig. 4-3B). One explanation for this is that the L11 may not efficiently be stripped from the bacterial ribosome. Therefore, additional MRPL11 may not be required for the reconstitution to make a functional and active hybrid ribosome.
Figure 4-3A. Poly(U)-directed *in vitro* translation assay with reconstituted hybrid ribosome containing MRPL10-MRPL12 complex. Stripped ribosomes (35 pmol) were incubated with purified MRPL10-MRPL12 complex (70 pmol), MRPL11 (70 pmol), and additional MRPL12 (280 pmol) at 37 °C for 15 min for reconstitution. The poly(U)-directed *in vitro* translation assay reactions were performed at 37 °C for 30 min followed by 5% trichloroacetic acid treatment and incubated on ice for 5 min. Precipitated ribosome complex was boiled at 90 °C for 10 min. The *in vitro* translated [¹⁴C] labeled-poly-Phe was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter. The results were expressed as the relative percent of synthesized poly-Phe compared to the control (70S). The mean ± SD was calculated from three independent experiments. Values of # *P* < 0.05 were considered statistically significant between stripped ribosome and reconstituted ribosome. ANOVA’s t test was used to compare the significance of values.
Figure 4-3B. Poly(U)-directed *in vitro* translation assay with reconstituted hybrid ribosome and purified MRPL10. 35 pmol of stripped ribosomes were incubated with purified MRPL10 (70 pmol), MRPL11 (70 pmol), and MRPL12 (280 pmol) at 37 °C for 15 min for reconstitution. The poly(U)-directed *in vitro* translation assay reactions were performed at 37 °C for 30 min followed by 5% trichloroacetic acid treatment and incubated on ice for 5 min. Precipitated ribosome complex was boiled at 90 °C for 10 min. The *in vitro* translated [14C] labeled-poly-Phe was collected on nitrocellulose filter membranes and quantified using a liquid scintillation counter. The results were expressed as the relative percent of synthesized poly-Phe compared to the control (70S). The mean ± SD was calculated from three independent experiments. Values of # P < 0.05 were considered statistically significant between stripped ribosome and reconstituted ribosome. ANOVA’s t test was used to compare the significance of values.
Conclusions and Future directions

Here, we showed successful purification of functional human MRPL10 in *E. coli* by making a soluble MRPL10-MRPL12 complex. Previously we identified several acetylated Lys residues (Lys124, Lys162, Lys169, and Lys196) in human MRPL10 and the binding affinity of MRPL12 on MRPL10 also changed by acetylation to regulated translation (Chapter 3). To confirm the binding affinity of MRPL12 by changes in acetylation status of MRPL10, mutant forms of MRPL10 will be generated. Some of Lys mutants can mimic unacetylation form, such as Lys to arginine (Arg) and Lys to glutamine (Gln) mutants. In contrast, Lys to alanine (Ala) mutants can mimic the acetylated form of MRPL10. It would be relevant to determine the acetylation status of MRPL10 in regulation of translation by generating hybrid reconstituted ribosome with MRPL10 mutants to test in *in vitro* biochemical studies. In addition, this strategy will also allow us to determine number of MRPL12 copies functioning in mitochondrial ribosomes as well as the structure of the mitochondrial L7/L12 stalk using X-ray crystallography and/or cryo-electron microscopy in the future.
References

Chapter 5

Concluding remarks and Future directions

Recently, mitochondria have received much interest mainly due to their implications for many diseases and abnormalities. Mitochondria play a major role in various aspects of cell homeostasis by generating energy and regulating programmed cell death. However, a comprehensive understanding of the diverse functions of mitochondria does not yet exist. One of the significant differences between mitochondria and other organelles is that mitochondria have their own genome and ribosome, which are responsible for production of 13 proteins for OXPHOS complexes (1,2). The OXPHOS is a process of electron flow through the electron transport chain to generate ATP. Therefore, mutations in OXPHOS complexes contribute to many diseases. In addition, mitochondria also play a crucial role in several major pathways for programmed cell death (apoptosis) in mammalian cells (3,4) and malfunction of apoptotic regulation leads to severe pathogenesis. Abnormally high levels of apoptosis are associated with degenerative diseases. In contrast, suppression of apoptosis is involved in carcinogenesis and autoimmune diseases (3). Some of mitochondrial ribosomal proteins such as MRPS29, MRPS30, MRPL37, and MRPL41 are also involved in regulation of apoptosis, suggesting that the mitochondrial ribosome plays a role in delicate balance between energy production and apoptosis. By using proteomic approaches, our laboratory has been investigating the mitochondrial ribosome (5,6) and functional changes introduced by PTMs, such as phosphorylation (7,8) and acetylation (9,10). We have also been investigating the functional changes in mitochondrial ribosomal proteins by splice variants and isoforms (11).
Here, we propose two regulatory mechanisms involved in mitochondrial functions; apoptosis and energy production by OXPHOS. First, we reported that the presence of different splice variants of \textit{MRPS29}, which contained an uORF in the 5'-UTR in human (Chapter 2). Data presented in this study suggest that the MRPS29 expression is impaired due to the uORF found in the 5'-UTR of \textit{MRPS29} mRNA and reduction of MRPS29 expression by uORF may cause resistance to MRPS29-induced apoptosis. Presence of both \textit{MRPS29} and \textit{uORF-MRPS29} mRNAs in human might be a significant indication to answer the question how the same apoptotic stimuli react differently in different human cells. In this study, all the human cells that we analyzed showed the same ratio of mRNAs of \textit{MRPS29} to \textit{uORF-MRPS29}. Therefore, additional experiments are needed to confirm the levels of \textit{MRPS29} and \textit{uORF-MRPS29} in different tissues. Cell lines such as \textit{Ataxia telangiectasia} cells may be need to be studied in the future.

In addition, we demonstrated regulation of mitochondrial translation by acetylation of mitochondrial ribosomal proteins (10). More specifically, MRPL10 was identified as a SIRT3 substrate. The effects of SIRT3-dependent deacetylation on protein composition of mitochondrial ribosome were investigated (Chapter 3). Particularly, the acetylation of mitochondrial ribosomes could enhance the translational activity of ribosomes. We also propose that the mechanism by which the mitochondrial translation is regulated by reversible acetylation. The L7/L12 stalk of mitochondrial ribosomes, which is composed of MRPL10, MRPL11, and multiple copies of MRPL12, is essential for translation. Specifically, MRPL12 plays a significant role in translation by recruiting initiation, elongation, and release factors to the ribosome. Increased
acetylation status of mitochondrial ribosome in SIRT3 knock-out mice enhanced MRPL12 binding to the ribosome and recruitment of elongation factors to increase translation. On the other hand, SIRT3 over-expression reduced the translation and MRPL12 binding to ribosomes by deacetylation of the mitochondrial ribosome. Therefore, acetylation of MRPL10 due to inhibition of SIRT3 could enhance its interaction with MRPL12, resulting in increased MRPL12 binding on the ribosome and the recruitment of elongation factors during translation. We have previously identified several acetylated Lys residues in human MRPL10 and the binding affinity of MRPL12 on MRPL10 also changed by acetylation to regulate translation (Chapter 3). We also proposed a model to explain how acetylation of MRPL10 could regulate mitochondrial translation (Fig. 5-1). In bacteria, there are four copies of L12 (two dimers) bound to the C-terminal α-helix of the L10 and copies of L12 are found to be in extended and folded confirmations in each dimer. The location of acetylated Lys residues on the MRPL10 is at the N-terminal globular domain where one of the C-terminal domains of the folded L7/L12 copy or elongation factors could be interacting with the stalk as modeled in Fig. 5-1. When all the copies of L12 present on the ribosome, it is possible that one of the folded copies of L12 CTD could be interacting with the lysine-rich surface of the N-terminal globular domain of the L10 (Fig. 5-1). The bacterial and mitochondrial L12 homologs have a highly conserved lysine rich CTDs; therefore, packing this lysine-rich domain into a lysine-rich L10 surface would not be possible due to charge repulsion. In this scenario, having a neutralized L10 surface by acetylation would stabilize the lysine-rich CTD of L12 in between N- and C-terminal domains of L10 and L11, respectively (Fig. 5-1). Clearly, having increased MRPL12 binding to the
mitochondrial ribosome stimulated translation both \textit{in vitro} and \textit{in vivo} in our studies (Chapter 3). We therefore propose that the SIRT3-dependent reversible acetylation of MRPL10 may cause changes in MRPL12 binding to the ribosome by neutralizing the positively charged lysine residues and regulating mitochondrial translation (Fig. 5-1).
Figure 5-1. Proposed model for the role of MRPL10 acetylation on MRPL12 binding to ribosome. Crystal structure of the *T. thermophilus* L7/L12 stalk region in the EF-G bound confirmation (PDB# 2WRL) and solution structure of the *E. coli* L7/L12 dimers (PDB# 1RQU) were used to illustrate proposed model for accommodation of multiple copies of MRPL12 on the ribosome. The location of mitochondrial homologs of L7/L12 stalk proteins L10, L11 and multiple copies of L7/L12 were colored in green, yellow and two different shades of blue in the model, respectively. Acetylated lysine residues located at the N-terminal domain of MRPL10 and lysine residues in MRPL12 were shaded in red. The 50S ribosomal 23S and 5S rRNA, and ribosomal proteins were colored in cyan and pink, respectively. Structural models were generated by PyMol software (DeLano Scientific LLC).
In Chapter 4, we reported purification of functional human MRPL10 in *E. coli* as a soluble MRPL10-MRPL12 complex for *in vitro* biochemical studies. The goal was to use reconstituted hybrid ribosomes containing the mitochondrial L7/L12 stalk and bacterial ribosomes for poly(U)-directed *in vitro* translation assay as supporting evidence for the effect of MRPL10-MRPL12 interaction on mitochondrial translation. To confirm the binding affinity of MRPL12 due to changes in acetylation status of MRPL10, mutant forms of MRPL10 will be generated and tested in poly(U)-directed *in vitro* translation assays. Some of the Lys mutants such as Lys to arginine (Arg) and Lys to glutamine (Gln) mutants will be utilized to mimic the unacetylated and acetylated forms of Lys, respectively. In contrast, Lys to alanine (Ala) mutants will mimic the acetylated form.

Three different NAD⁺-dependent deacetylases, sirtuin family (SIRT3, SIRT4, and SIRT5), have been reported to be localized in mitochondria (12,13). Even though we identified acetylated mitochondrial ribosomal proteins, the specific acetyl transferase has not been identified in mitochondria yet. To confirm the role of acetylation on MRPL10 involved in translation, identification and characterization of the mitochondrial acetyl transferase which is responsible for the acetylation of MRPL10 are necessary. *In vitro* acetylation assay performed with fractions of bovine mitochondrial lysates hinted the existence of a protein acetyl transferase activity in mitochondria (unpublished data). Identification of acetyl transferases which are interacting with MRPL10 and/or mitochondrial ribosomal proteins will strengthen our findings on regulation of mitochondrial translation.

Our proteomics studies revealed all the protein components of mitochondrial
ribosome; however, location of the ribosomal proteins with no bacterial homologs is not known. These proteins with no bacterial homologs might have additional functions in translation as well as the other functions of mitochondria such as apoptosis. Immuno-electron microscopy (EM) studies using specific antibodies for mitochondrial ribosomal proteins along with cross-linking studies will provide evidence for the localization of those proteins on the ribosome and understanding their functions. Follow-up knock-out and/or over-expression studies of mitochondrial ribosomal proteins will also be necessary to confirm their functions.
References

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