Published in final edited form as:

Biochim Biophys Acta. 2011 December ; 1814(12): 1779–1784. doi:10.1016/j.bbapap.2011.09.013.

Contacts between mammalian mitochondrial translational initiation factor 3 and ribosomal proteins in the small subunit

Md. Emdadul Haque^{1,*}, Hasan Koc², Huseyin Cimen², Emine C. Koc^{2,#,^}, and Linda L. Spremulli^{1,#}

¹Department of Chemistry, Campus Box 3290, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3290

²Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802

Abstract

Mammalian mitochondrial translational initiation factor 3 (IF3_{mt}) binds to the small subunit of the ribosome displacing the large subunit during the initiation of protein biosynthesis. About half of the proteins in mitochondrial ribosomes have homologs in bacteria while the remainder are unique to the mitochondrion. To obtain information on the ribosomal proteins located near the IF3_{mt} binding site, cross-linking studies were carried out followed by identification of the cross-linked proteins by mass spectrometry. IF3_{mt} cross-links to mammalian mitochondrial homologs of the bacterial ribosomal proteins S5, S9, S10, and S18-2 and to unique mitochondrial ribosomal proteins MRPS29, MRPS32, MRPS36 and PTCD3 (Pet309) which has now been identified as a small subunit ribosomal protein. IF3_{mt} has extensions on both the N- and C-termini compared to the bacterial factors. Cross-linking of a truncated derivative lacking these extensions gives the same hits as the full length IF3_{mt} except that no cross-links were observed to MRPS36. IF3 consists of two domains separated by a flexible linker. Cross-linking of the isolated N- and C-domains was observed to a range of ribosomal proteins particularly with the C-domain carrying the linker which showed significant cross-linking to several ribosomal proteins not found in prokaryotes.

Keywords

Initiation factor 3; mitochondria; Cross-linking; ribosome; ribosomal protein; protein synthesis; mammal

1. Introduction

Mammalian mitochondria synthesize thirteen polypeptides encoded by the mitochondrial genome. All of these proteins are subunits of the oligomeric complexes required for

© 2011 Elsevier B.V. All rights reserved.

[#]To whom correspondence should be addressed. Tel: 1-919-966-1567, Fax: 1-919-962-2388, lls@email.unc.edu, Tel: 1-814-865-8300; Fax: 1-814-863-7024; koco@marshall.edu.

^{*}Present Address, Department of Ophthalmology, Eye Care Center, Duke University, Durham NC

[^]Present Address, Marshall University Joan C. Edwards School of Medicine, Department of Biochemistry and Microbiology, One John Marshall Drive, Huntington, WV 25755

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

oxidative phosphorylation. The synthesis of these proteins is carried out by specialized ribosomes that associate with the inner membrane of the mitochondrion [1]. Mammalian mitochondrial ribosomes are 55S particles and consist of 28S small subunits (SSU) and 39S large subunits (LSU) [2]. The protein and RNA content of these particles is quite different from that of bacterial ribosomes. The 28S subunit contains approximately 29 proteins of which 14 are homologs of bacterial ribosomal proteins while 15 are classified as mitochondrial specific ribosomal proteins [3]. The 39S subunit contains about 50 proteins of which 28 are homologs of bacterial ribosomal proteins and 22 of which do not have bacterial homologs. The rRNAs in the mammalian mitochondrial ribosome are considerably shorter than bacterial rRNAs and have little primary sequence conservation with other rRNAs. Cryo-electron microscopy indicates that the core of the mitochondrial ribosome is decorated with many proteins that have no bacterial homologs [4]. Therefore, the interactions of the ribosome with translational factors are expected to show novel differences compared to those occurring in bacterial systems.

Two translational initiation factors have been identified in mammalian mitochondria. Initiation factor 2 (IF2_{mt}) stimulates the binding of fMet-tRNA to the P-site of the small subunit [5,6]. Mitochondrial initiation factor 3 (IF3_{mt}) promotes the dissociation of the mitochondrial ribosome [7] and reduces the binding of fMet-tRNA to the small subunit in the absence of mRNA [8]. No factor equivalent to IF1 has been observed in mammalian mitochondria and a short segment in IF2_{mt} is thought to play the role of IF1 in this system [9,10].

IF3_{mt} has a central region with homology to the bacterial factors with additional N-terminal and C-terminal extensions (Figure 1). Like *Escherichia coli* IF3, the homology domain is divided into two segments (N-terminal domain and C-terminal domain) separated by a flexible linker [11]. The roles of IF3_{mt} and its terminal extensions and domains in initiation complex formation have been investigated [7,8,11–13]. The three-dimensional structure of IF3_{mt} is still unknown but the N-domain has been modeled based on the structure of the N-domain of *Bacillus stearothermophilus* of IF3 and the NMR structure of the C-domain of mouse IF3_{mt} without the C-terminal extension has been reported. The mature form of IF3_{mt} (after removal of the predicted mitochondrial import signal) binds to the 28S subunit tightly with a K_d of about 30 nM. The C-terminal region binds almost as tightly to the small subunit with a K_d of about 60 nM [13]. This interaction is considerably stronger than that observed with the isolated C-domain of bacterial IF3 [14]. Unlike bacterial IF3, the N-terminal region of IF3_{mt} can also bind independently to the 28S subunit with a K_d of 240 nM.

A number of biochemical and biophysical studies suggest that *E. coli* IF3 interacts with 30S subunits in the region of the platform, cleft, and the head, facing the 50S subunit [15–19]. The C-domain of IF3 is predicted to bind on the platform side of the 30S subunit near G700 in the 16S rRNA [20]. The platform region of the small subunit where the C-domain binds is one of the highly conserved regions between bacterial and mitochondrial ribosomes [4,21]. However, the edges of the platform do contain proteins that are specific to the mitochondrial ribosome, and it is possible that IF3_{mt} makes contacts with these proteins. The N-domain may interact with the A790 region in close proximity to the P-site close to the head of the SSU although no consensus exists for the placement of the N-domain on the small subunit [16,20]. The head of the 28S subunit lacks a number of the bacterial ribosomal protein homologs and appears to contain several proteins specific to mitochondrial ribosomes. In the present study we have examined the nearest neighbor proteins of IF3_{mt} on the 28S subunit using chemical cross-linking followed by mass spectrometry.

2. Materials and Methods

2.1 Materials

High purity grade chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Dimethyl suberimidate (DMS) was purchased from Pierce. Bovine mitochondria and mitochondrial ribosomes (55S) and yeast [^{35}S]fMet-tRNA were prepared as described [22–24]. In order to purify small ribosomal subunit (28S), 55S ribosomes were prepared from crude mitochondrial ribosomes following the standard sucrose density gradient procedure [25]. The purified 55S ribosomes were dissociated into 28S and 39S subunits in buffer containing low Mg^{2+} concentrations. The subunits were then separated on linear sucrose gradients as described [25]. Fractions corresponding to the 28S subunits were combined and collected by high speed centrifugation. These preparations were adjusted to 20 mM Mg^{2+} to convert any contaminating 39S subunits into 55S monosomes. The 28S subunits were then purified on a further linear sucrose gradient at 20 mM Mg^{2+} and again collected by high speed centrifugation. The 28S subunit preparations were greater than 95 % pure having only about 2–5 % contamination of 39S subunits.

Full length mature human IF3_{mt}, the C-domain with the linker region of IF3_{mt} (IF3_{mt}(C+L)), the N-domain IF3_{mt} with the linker (IF3_{mt}(N+L)), and a derivative of IF3_{mt} lacking the N- and C-terminal extensions IF3_{mt}(IF3_{mt}ΔNC) (Fig. 1) were cloned, expressed, and purified as described previously [8,12,13]. All of these derivatives carry a C-terminal His₆-tag.

2.2 Identification of ribosomal proteins near IF3_{mt} and its domains by dimethyl suberimidate (DMS) cross-linking followed by mass spectrometry

A sample of 28S ribosomal subunits (20 pmol in 100 μL final volume) was mixed with 10-fold excess of IF3_{mt} (2 μM) or with one of the IF3 derivatives at the following levels; a 10-fold excess of IF3_{mt}ΔNC (2 μM), a 15-fold molar excess (3 μM) IF3_{mt}(C+L) or a 20-fold excess (4 μM) IF3_{mt}(N+L) in cross-linking buffer (20 mM HEPES-KOH, pH 7.6, 50 mM KCl, 10 mM MgCl_2 , and 1 mM Tris(2-carboxyethyl) phosphine hydrochloride, TCEP) and incubated for 20 min at room temperature. A 50-fold molar excess of DMS with respect to protein concentration was added to the above reaction mixtures and incubation was continued for 1 h at room temperature. The reaction was stopped by the addition of 5 μL of 100 mM Tris-HCl, pH 7.6. The cross-linked products were separated from free IF3_{mt} by layering the reaction mixture on a 30% sucrose cushion (7 mL) in buffer containing 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 5 mM MgCl_2 , and 1 mM TCEP. The centrifuge tube was then filled with approximately 2 mL of the buffer above. Samples were subjected to centrifugation at 42,000 rpm for 12 h in a Beckman Ti70.1 rotor. The pellet was dissolved in 100 μL of Buffer A (20 mM HEPES-KOH, pH 7.6, 500 mM NH_4Cl , 1 mM TCEP and 8 M urea) and incubated for 30 min at room temperature. After incubation, 20 μL Ni-NTA resin in Buffer A was added to the solution followed by rocking for 1 h at 4 °C. The ribosomal proteins cross-linked to IF3_{mt} or its domains bound to the Ni-NTA and unbound proteins were removed by three washes of 400 μL each with Buffer A and two washes of 400 μL each with Buffer B (20 mM HEPES-KOH, pH 7.6, 500 mM NH_4Cl , and 1 mM TCEP). The bound proteins were eluted with two aliquots of 50 μL each of Buffer C (20 mM HEPES-KOH, pH 7.6 and 250 mM imidazole) which were combined for subsequent analysis. A control sample for the identification of ribosomal proteins bound nonspecifically to the Ni-NTA resin was prepared in an identical manner except that IF3_{mt} was omitted from the incubation mixture.

The cross-linked proteins from both the control sample and the IF3_{mt} cross-linked samples were digested with 50 ng trypsin overnight at 37 °C [26]. The digested samples were lyophilized using a speed vacuum concentrator. The peptides were dissolved in 20 μL of 5%

acetonitrile and 0.1% formic acid solution and then subjected to tandem mass spectrometry analysis as described previously [26,27]. Tandem MS spectra obtained from peptide fragmentation 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 spectra (peaks) were analyzed using a site-licensed Mascot database searching program. Individual ion scores of greater than 35 indicate identity or extensive sequence homology ($p < 0.05$) when MS/MS data are queried against a database consisting of mitochondrial ribosomal protein sequences [28]. A cut-off score of about 50 was used in the current analysis to ensure rigor in the identification of the cross-linked proteins.

3. Results

3.1. IF3_{mt} derivatives used

As indicated above (Fig. 1) mammalian IF3_{mt} contains a central region homologous to the bacterial factors. This region is divided into N- and C-domains connected by a linker region. The homology domain is preceded by an N-terminal extension of about 30 amino acids and followed by a C-terminal extension which is also about 30 residues long. The N- and C-terminal domains allow the protein to achieve an extended conformation spanning as much as 95Å (Fig. 1). In order to explore the ribosomal proteins at or near the IF3_{mt} binding site on the mitochondrial 28S subunit, four different IF3_{mt} derivatives were used; full length IF3_{mt} (lacking only the predicted mitochondrial import signal), a derivative lacking the N- and C-terminal extensions (IF3_{mt}ΔNC) and, therefore, consisting of the region homologous to the bacterial factors, the N-domain with the linker (IF3_{mt}(N+L)) or the C-domain with linker (IF3_{mt}(C+L)) (Fig. 1). The linker was included in the N- and C-domain constructs since it improves the binding of these regions to the small subunit [13].

3.2. Identification of 28S ribosomal proteins located at or near the binding site for IF3_{mt}

To identify ribosomal proteins near the IF3_{mt} binding site, a cross-linking strategy was used (Fig. 2). In this approach, IF3_{mt} or its derivatives was incubated with 28S subunits under conditions in which the majority of the subunits would have IF3_{mt} bound [12]. IF3_{mt} binds to the 28S subunit with a K_d of 20–30 nM. Removal of the N- and C-terminal extensions has no effect on the strength of this interaction [12]. IF3_{mt} or its derivatives were cross-linked to the small subunit using DMS. The cross-linked complexes were separated from unbound IF3_{mt} by sedimentation through a sucrose cushion. The complexes were solubilized using buffer containing 8M urea and IF3_{mt} and ribosomal proteins cross-linked to IF3_{mt} were selectively retained on Ni-NTA resins through the His₆-tag on IF3_{mt}. The proteins eluted from the Ni-NTA resin were digested with trypsin and identified by LC/MS/MS. The cross-linking experiment was repeated five independent times with different preparations of material to ensure the accurate identification of the proteins present. A summary of the results is presented in Table 1 with more detail provided in Tables 2 and 3.

Full-length IF3_{mt} and the ΔNC derivative were cross-linked to a number of small subunit ribosomal proteins (Table 2). There were strong cross-links to 4 proteins with homologs in bacterial systems (MRPS5, MRPS9, MRPS10 and MRPS18-2). Peptides from MRPS18-2 were occasionally observed in control samples analyzed in the absence of IF3_{mt} (Table 2) although many more peptides were consistently observed in samples cross-linked in the presence of IF3_{mt}. Hence, we believe that the cross-links to MRPS18-2 represent a meaningful location of this protein close to the IF3_{mt} binding site. Four proteins without homologs in bacterial systems (MRPS29, MRPS32, MRPS36, and PTCD3) are also convincingly cross-linked to IF3_{mt} (Table 2). Of these, only PTCD3 gave two peptides in

control samples (Table 2) and these were observed in only a few analyses. Hence, we believe that PTCD3 is located on the small ribosomal subunit near the binding site for IF3_{mt}. It should be noted that PTCD3 was originally identified as a pentatricopeptide repeat domain protein associated with the small subunit of the mammalian mitochondrial ribosome [29]. Decreasing the levels of PTCD3 reduces mitochondrial protein synthesis without effecting mRNA levels. The current work suggests that this protein is an integral component of the 28S subunit which may be located close to the head and platform of the subunit.

To determine which proteins on the 28S subunit are close to the binding region of the N- and C-domains of IF3_{mt}, cross-linking studies were carried out with the individual domains (Fig. 1). The linker improves the strength of the interaction between these domains and the small subunit and was retained in both of the constructs used. The C-domain with the linker (C+L construct) binds to the 28S subunit with a K_d of about 60 nM while the N+L binds with a K_d of 240 nM [13]. As indicated in Table 3, little cross-linking was observed to the N-terminal region. One peptide was observed from MRPS10 which was also observed cross-linking to full-length IF3_{mt}. The low level of cross-linking with this sample probably reflects its weaker binding to the 28S subunit. The C-domain with the linker was cross-linked to several ribosomal proteins with bacterial homologs including MRPS5, MRPS9, MRPS10, and MRPS18-2. All of these proteins were observed to be cross-linked to IF3_{mt} or the ΔNC derivative. In addition, IF3_{mt} (C+L) was cross-linked to several proteins which do not have homologs in bacterial systems including MRPS29, MRPS32, MRPS36, and PTCD3.

The specificity of the cross-linking observed was evaluated by examining the MS data for other ribosomal proteins (Supplementary Table S1). Of the approximately 30 proteins in the 28S subunit, no peptides were observed for 15 of them. Peptides were detected from six ribosomal proteins not listed in Table 1 but cross-linking to these proteins was not observed with any consistency and generally gave peptides with poor ion scores. Thus, we believe that the cross-linked proteins observed here represent proteins at or near the IF3_{mt} binding site in the 28S subunit.

4. Discussion

Cross-linking studies using *E. coli* IF3 indicate that this factor is located close to a number of ribosomal proteins including S12 in the upper portion of the body, S2, S3, S7, S13 and S19 in the head and S11, S18 and S21 in the platform [30,31]. Dallas and Noller [16] using RNA cleavage by Fe(II)-derivatized IF3 have shown that the C-domain of this factor is located on the platform. They also suggest that the N-domain may be wedged between the platform and head close to the E-site.

No cross-links between IF3_{mt} and ribosomal protein S12 located in the body of the SSU were observed despite this protein being a major cross-linking partner of bacterial IF3. This difference could be due to the size and high Lys and Arg content of the mitochondrial S12 which would make its detection by LC/MS/MS challenging. Rather, cross-links were observed to MRPS5 in the body of the small subunit. This protein forms part of the mRNA tunnel in the small subunit. Mitochondrial MRPS5 is about twice the size of its bacterial counterpart and may have regions exposed on the interface side of the small subunit.

A number of cross-links between bacterial IF3 and small subunit are observed to proteins located in the head of the small subunit. The contacts between IF3 and the head to the 30S subunit appear to be enriched in protein-protein interactions while considerable contacts between the C-domain of IF3 and the platform are mediated through RNA-protein interactions [16]. The head and the neck of the SSU have eight proteins S2, S3, S7, S9, S10, S13, S14 and S19 in the bacterial ribosome, and five (MRPS2, MRPS7, MRPS9, MRPS10

and MRPS14) of these have been retained in the mammalian mitochondrial ribosome. *E. coli* IF3 can be cross-linked to S2, S3, S7, S13 and S19 in the head region (Fig. 3A). Of these proteins, S2 is located on the back of the head close to the platform; S7 is found in the head just above the platform; S3 is largely on the back of the head and S13 and S19 are on the interface side of the head. It should be noted that a number of these proteins have finger-like extensions that penetrate a considerable distance into the structure of the subunit. Of the proteins found in the head of the small subunit that cross-link to bacterial IF3, S3, S13 and S19 are absent in the mammalian mitochondrial ribosome. Further, no cross-links are observed to S2, S3 or S7. These observations indicate that the contacts between bacterial IF3 and proteins of the small subunit are quite different than those observed with the mitochondrial factor.

In the case of IF3_{mt}, cross-links are observed with MRPS9 and MRPS10 in the head (Fig. 3B). Both MRPS9 and MRPS10 are about twice the size of their bacterial homologs. MRPS9 is located largely on the solvent side of the head but has a long extension that approaches the P-site. MRPS10 is also located primarily on the back of the head; however, the larger size of the mitochondrial homolog and the truncation of rRNA in the small subunit may allow this protein to interact with IF3_{mt} if the factor is bound on the interface side of the small subunit (Fig. 3B). It should be noted the MRPS10 was the only ribosomal protein providing a clear cross-link to the N-domain of IF3_{mt} suggesting that the N-terminal domain of this factor is located in contact with the head of the small subunit. This observation is in agreement with the proximity of *E. coli* IF3 near nucleotide 790 close to the decoding site on the small subunit [20].

The platform of the small subunit of bacterial ribosomes contains six proteins (S6, S8, S11, S15, S18, and S21). *E. coli* IF3 can be cross-linked to three of the platform proteins, S11, S18, and S21 (Fig. 3A). The platform of the mammalian mitochondrial ribosome is the most conserved region of the small subunit and has homologs of all of these proteins except S8 [3,21]. CryoEM studies [4] indicate that edges of the platform are decorated with ribosomal proteins specific for the mitochondrial ribosome. IF3_{mt} and the C-terminal domain cross-link strongly to MRPS18-2 in the platform. Surprisingly, no cross-links are observed to other platform proteins. The lack of cross-linking to MRPS6 and MRPS21 could be due to the low molecular weights and high Lys and Arg contents of these proteins leading in peptides difficult to be detected by mass spectrometry after trypsin digestion. However, cross-links to MRPS11 and MRPS15 would, in principle have been readily detected if they had occurred. This observation and the lack of conserved cross-links to proteins in the head suggest that the contacts between IF3_{mt} and the 28S subunit of the mitochondrial ribosome are quite different from the contacts between bacterial and the 30S subunit of the prokaryotic ribosome.

A number of cross-links were obtained between IF3_{mt} and proteins that do not have homologs in bacterial ribosomes. The C-domain and full length IF3_{mt} cross-linked to non-homolog proteins MRPS32 and MRPS36, therefore, these proteins may be located at the edge of the platform. Cross-links were also obtained with MRPS29. MRPS29 is also referred to as DAP3 and is believed to play a role in mitochondrially-mediated apoptosis [32–35]. This cross-link was somewhat surprising since immune electron microscopy has suggested that MRPS29 is located on the solvent side of the small subunit. MRPS29 is large for a ribosomal protein (nearly 44 kDa) and may have extensions that penetrate a considerable distance from the body of the protein.

Of considerable interest are the strong cross-links between IF3_{mt} and its C-domain to the protein designated PTCD3. This pentatricopeptide domain protein is known to associate with the small subunit of mitochondrial ribosomes [29]. Lowering PTCD3 levels in

osteosarcoma cells has been shown to decrease mitochondrial protein synthesis. Our recent analysis has shown that PTCD3 is a small subunit ribosomal protein in mammals (E. Koc, *manuscript in preparation*). The location of this protein in the mitochondrial small subunit is not known. However, its extensive cross-linking to IF3_{mt} and mitochondrial mRNAs (*unpublished observations*) suggest that it may be at least partially located at the interface side of the 28S subunit.

Highlights

- Thirteen proteins are synthesized by a specialized protein biosynthetic system in mitochondria.
- Mitochondrial ribosomes have many proteins with no homologs in other systems.
- Ribosomal proteins at the binding site of initiation factor 3 were identified.
- IF3_{mt} has very different contacts with the ribosome than observed with bacterial IF3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health (Grant GM 32734 to LLS and GM071034 to ECK). These funding sources had no direct role in the conduct of the research or in preparation of the manuscript.

Reference List

1. Liu M, Spremulli LL. Interaction of mammalian mitochondrial ribosomes with the inner membrane. *J. Biol. Chem.* 2000; 275:29400–29406. [PubMed: 10887179]
2. O'Brien, TW.; Denslow, ND.; Faunce, W.; Anders, J.; Liu, J.; O'Brien, B. Structure and function of mammalian mitochondrial ribosomes. In: Nierhaus, K.; Franceschi, F.; Subramanian, A.; Erdmann, V.; Wittmann-Liebold, B., editors. *The translational apparatus: Structure, function regulation and evolution*. New York: Plenum Press; 1993. p. 575-586.
3. Koc EC, Haque MdE, Spremulli LL. Current views of the structure of the mammalian mitochondrial ribosome. *Isr. J. Chem.* 2010; 50:45–59.
4. Sharma MR, Koc EC, Datta PP, Booth TM, Spremulli LL, Agrawal RK. Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. *Cell.* 2003; 115:97–108. [PubMed: 14532006]
5. Liao H-X, Spremulli LL. Initiation of protein synthesis in animal mitochondria: Purification and characterization of translational initiation factor 2. *J. Biol. Chem.* 1991; 266:20714–20719. [PubMed: 1939122]
6. Liao H-X, Spremulli LL. Identification and initial characterization of translational initiation factor 2 from bovine mitochondria. *J. Biol. Chem.* 1990; 265:13618–13622. [PubMed: 2380177]
7. Christian B, Spremulli L. Evidence for an Active Role of IF3_{mt} in initiation of translation in mammalian mitochondria. *Biochem.* 2009; 48:3269–3278. [PubMed: 19239245]
8. Bhargava K, Spremulli LL. Role of the N- and C-terminal extensions on the activity of mammalian mitochondrial translational initiation factor 3. *Nuc. Acids Res.* 2005; 33:7011–7018.
9. Gaur R, Grasso D, Datta PP, Krishna PDV, Das G, Spencer A, Agrawal RK, Spremulli L, Varshney U. A single mammalian mitochondrial translation initiation factor functionally replaces two bacterial factors. *Mol. Cell.* 2008; 29:180–190. [PubMed: 18243113]

10. Yassin AS, Haque ME, Datta PP, Elmore K, Banavali NK, Spremulli LL, Agrawal RK. Insertion domain within mammalian mitochondrial translation initiation factor 2 serves the role of eubacterial initiation factor 1. *Proc. Natl. Acad. Sci U. S. A.* 2011; 108:3918–3923. [PubMed: 21368145]
11. Koc EC, Spremulli LL. Identification of mammalian mitochondrial translational initiation factor 3 and examination of its role in initiation complex formation with natural mRNAs. *J. Biol. Chem.* 2002; 277:35541–35549. [PubMed: 12095986]
12. Haque ME, Grasso D, Spremulli LL. The interaction of mammalian mitochondrial translational initiation factor 3 with ribosomes: evolution of terminal extensions in IF3_{mt}. *Nucleic Acids Res.* 2008; 36:589–597. [PubMed: 18056078]
13. Haque M, Spremulli LL. Roles of the N- and C-Terminal Domains of Mammalian Mitochondrial Initiation Factor 3 in protein biosynthesis. *J. Mol. Biol.* 2008; 384:929–940. [PubMed: 18930736]
14. Petrelli D, LaTeana A, Garofalo C, Spurio R, Pon CL, Gualerzi CO. Translation initiation factor IF3: two domains, five functions, one mechanism? *EMBO J.* 2001; 20:4560–4569. [PubMed: 11500382]
15. McCutcheon J, Agrawal R, Philips SM, Grassucci R, Gerchman S, Clemons WM, Ramakrishnan V, Frank J. Location of translational initiation factor IF3 on the small ribosomal subunit. *Proc. Natl. Acad. Sci U. S. A.* 1999; 96:4301–4306. [PubMed: 10200257]
16. Dallas A, Noller HF. Interaction of translation initiation factor 3 with the 30S ribosomal subunit. *Mol. Cell.* 2001; 8:855–864. [PubMed: 11684020]
17. Pioletti M, Schlunzen F, Harms J, Zarivach R, Gluhmann M, Avila H, Bashan A, Bartels H, Auerbach T, Jacobi C, Hartsch T, Yonath A, Franceschi F. Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. *EMBO J.* 2001; 20:1829–1839. [PubMed: 11296217]
18. Moazed D, Samaha RR, Gualerzi C, Noller HF. Specific protection of 16 S rRNA by translational initiation factors. *J. Mol. Biol.* 1995; 248:207–210. [PubMed: 7739034]
19. Ehresmann C, Moine H, Mougél M, Dondon J, Grunberg-Manago M, Ebel JP, Ehresmann B. Cross-linking of initiation factor IF3 to *Escherichia coli* 30S ribosomal subunit by trans-diamminedichloroplatinum(II): characterization of two cross-linking sites in 16S rRNA; a possible way of functioning for IF3. *Nuc. Acids Res.* 1986; 14:4803–4821.
20. Fabbretti A, Pon CL, Hennelly SP, Hill W, Lodmell J, Gualerzi CO. The real-time path of translation factor IF3 onto and off the ribosome. *Molecular Cell.* 2007; 25:285–296. [PubMed: 17244535]
21. Koc EC, Burkhart W, Blackburn K, Moseley A, Spremulli LL. The small subunit of the mammalian mitochondrial ribosome: Identification of the full complement of ribosomal proteins present. *J. Biol. Chem.* 2001; 276:19363–19374. [PubMed: 11279123]
22. Ma J, Spremulli LL. Expression, purification and mechanistic studies of bovine mitochondrial translational initiation factor 2. *J. Biol. Chem.* 1996; 271:5805–5811. [PubMed: 8621449]
23. Graves M, Spremulli LL. Activity of *Euglena gracilis* chloroplast ribosomes with prokaryotic and eukaryotic initiation factors. *Arch. Biochem. Biophys.* 1983; 222:192–199. [PubMed: 6404223]
24. Grasso DG, Christian BE, Spencer AC, Spremulli LL. Over-expression and purification of mitochondrial translational initiation factor 2 and initiation factor 3, *Methods Enzymology. Translation Initiation: Reconstituted Systems and Biophysical Methods.* 2007:59–78.
25. Spremulli, LL. Large-scale Isolation of Mitochondrial Ribosomes from Mammalian Tissues. In: Leister, D.; Herrmann, J., editors. *Mitochondria: Practical Protocols.* Vol. 372. Totowa, NJ: Humana Press; 2007. p. 265–275.
26. Miller JL, Koc H, Koc EC. Identification of phosphorylation sites in mammalian mitochondrial ribosomal protein DAP3. *Protein Sci.* 2008; 17:251–260. [PubMed: 18227431]
27. Miller JL, Cimen H, Koc H, Koc EC. Phosphorylated proteins of the mammalian mitochondrial ribosome: implications in protein synthesis. *J. Proteome. Res.* 2009; 8:4789–4798. [PubMed: 19702336]
28. Haque ME, Elmore KB, Tripathy A, Koc H, Koc EC, Spremulli LL. Properties of the C-terminal tail of human mitochondrial inner membrane protein Oxa1L and its interaction with mammalian mitochondrial ribosomes. *J. Biol. Chem.* 2010:28353–28362. [PubMed: 20601428]

29. Davies SM, Rackham O, Shearwood AM, Hamilton KL, Narsai R, Whelan J, Filipovska A. Pentatricopeptide repeat domain protein 3 associates with the mitochondrial small ribosomal subunit and regulates translation. *FEBS Lett.* 2009; 583:1853–1858. [PubMed: 19427859]
30. Cooperman BS, Expert-Bezancon A, Kahan L, Dondon J, Grunberg-Manago M. IF-3 crosslinking to *Escherichia coli* ribosomal 30 S subunits by three different light-dependent procedures: identification of 30 S proteins crosslinked to IF-3--utilization of a new two-stage crosslinking reagent, p-nitrobenzylmaleimide. *Arch. Biochem. Biophys.* 1981; 208:554–562. [PubMed: 7020604]
31. MacKeen LA, Kahan L, Wahba AJ, Schwartz I. Photochemical cross-linking of initiation factor-3 to *Escherichia coli* 30 S ribosomal subunits. *J. Biol. Chem.* 1980; 255:10526–10531. [PubMed: 7000779]
32. Berger T, Brigl M, Herrmann JM, Vielhauer V, Luckow B, Schlondorff D, Kretzler M. The apoptosis mediator mDAP-3 is a novel member of a conserved family of mitochondrial proteins. *J. Cell Sci.* 2000; 113:3603–3612. [PubMed: 11017876]
33. Kissil JL, Cohen O, Raveh T, Kimchi A. Structure-function analysis of an evolutionary conserved protein, DAP3, which mediates TNF-alpha- and Fas-induced cell death. *EMBO J.* 1999; 18:353–362. [PubMed: 9889192]
34. Koc EC, Ranasinghe A, Burkhart W, Blackburn K, Koc H, Moseley A, Spremulli LL. A new face on apoptosis: Death-associated protein 3 and PDCD9 are mitochondrial ribosomal proteins. *FEBS Lett.* 2001; 492:166–170. [PubMed: 11248257]
35. Han MJ, Chiu DT, Koc EC. Regulation of mitochondrial ribosomal protein S29 (MRPS29) expression by a 5'-upstream open reading frame. *Mitochondrion.* 2010; 10:274–283. [PubMed: 20079882]

Figure 1. Domain organization of bacterial IF3 and human IF3_{mt} and its truncated C- and N-domains

A three dimensional model of IF3_{mt} was developed [7] based on the structure of the N-domain of *Bacillus stearothermophilus* of IF3 (PDB coordinates 1TIG) and the NMR structure of the C-domain of mouse IF3_{mt} without the C-terminal extension (PDB coordinates 2CRQ). The figure shown represents the predicted structure of IF3_{mt}ΔNC (residues 61–245) since the N- and C-terminal extensions could not be modeled. The regions included in the domains are indicated above and below the model. Again these constructs contain the N- and C-terminal extensions which are not shown. The N-terminal domain contains residues 32–157 (including the linker) beginning with the first amino acid of the predicted mature form of the protein. The C-terminal construct contains residues 131–278 (including the linker) of IF3_{mt}. The dashed lines indicate the extensions which are not modeled.

Figure 2. Strategy used to identify ribosomal proteins near the IF3_{mt} binding site
IF3_{mt} and its domains were incubated with 28S subunits and cross-linked to nearby proteins using DMS as described in Materials and Methods. Cross-linked complexes were purified by centrifugation through a sucrose cushion to remove excess free IF3_{mt}. The ribosomes were then denatured and ribosomal proteins cross-linked to IF3_{mt} and its domains were recovered on Ni-NTA resin. Cross-linked proteins were digested with trypsin and identified by LC/MS/MS.

Figure 3. Binding site of IF3 on the small ribosomal subunit

(A) Model of the 30S ribosomal subunit (PDB 3R8O) with proteins cross-linking to *E. coli* IF3 shown as space-filled while other ribosomal proteins are shown as pink ribbons. The rRNA is shown as blue. (B) Representation of the bacterial 30S subunit showing the proteins cross-linking to IF3_{mt} that have homologs in the mammalian mitochondrial 28S subunit. The regions of the rRNA missing in the mammalian mitochondrial ribosome have been manually removed from the coordinates of the subunit. Proteins cross-linking to IF3_{mt} are space filled while other ribosomal proteins are shown as pink ribbons.

Table 1Summary of the ribosomal proteins cross-linked to IF3_{mt} or its derivatives

IF3 _{mt} Derivative	Cross-linking ribosomal proteins	Bacterial homolog
Full length	MRPS10	S10
	MRPS18-2	S18
	MRPS29	None
	MRPS32	None
	MRPS36	None
	PTCD3	None
ΔNC	MRPS5	S5
	MRPS9	S9
	MRPS10	S10
	MRPS18-2	S18
	MRPS29	None
	MRPS32	None
N-domain	PTCD3	None
	MRPS10	S10
C-domain	MRPS5	S5
	MRPS9	S9
	MRPS10	S10
	MRPS18-2	S18
	MRPS29	None
	MRPS32	None
	MRPS36	None
	PTCD3	None

Table 2Ribosomal proteins cross-linking to full-length and Δ NC IF3_{mt}*

<u>Full-length IF3_{mt}</u>				
Protein	Sequence	Score	m/z	Mr (expt)
MRPS10	NLPEGVAMEVTK	65	644.7	1287.4
	PVWETTPEEK	58	672.5	1342.9
	KPVWETTPEEKGDSKS	84	909.3	1816.6
	AVLDSYEYAFVLAALK	110	830.6	1659.1
	DLTKPTITISDEPDLYK	115	1026.3	2050.7
	DLTKPTITISDEPDLYKR	113	736.1	2205.4
MRPS18-2	EESGPPPEMPK	64	643.1	1172.5
	VPLTAPTEATSTEQAGPQSAL	115	1035.7	2069.4
	YLDSEEHNR	66	662.8	1323.7
	DHGLLSYHIPQVEPR	87	881.3	1760.7
	LYQGHLREESGPPPEMPK	80	1077.0	2152.1
	ARDHGLLSYHIPQVEPR	58	664.2	1989.7
MRPS29	NATDAVGIVLK	59	550.5	1098.9
	KAYLPQELLGK	62	630.7	1259.4
	KPALELLHYLK	56	442.0	1323.1
	GSPLAEVVEQGIMR	73	751.1	1500.2
	LLVAVDGVNALWGR	57	743.2	1484.5
MRPS32	VELALTSAR	60	539.0	1076.0
MRPS36	SAGLPSHTSSISQHSK	80	812.8	1623.7
	LVSQEEIEFIQR	78	746.2	1490.3
	KLVSQEEIEFIQR	92	810.5	1619.0
	DNPKNVSEVLR	61	684.7	1367.4
PTCD3	ASSSPAQAVEVVK	76	636.8	1271.6
	VAVLQALASTVHR	70	682.8	1363.6
	LTADFTLSQEQK	79	690.8	1379.6
	NELLNEFMDSAK	55	705.7	1409.4
	DPDDDMFFQSAMR	92	787.4	1572.8
	DEGADIAGTEEVVIPK	101	822.2	1642.5
	LTADFTLSQEQK	64	690.6	1379.2
<u>ΔNC IF3_{mt}</u>				
Protein	Sequence	Score	m/z	Mr (expt)
MRPS5	VSGSVNMLSLTR	93	633.1	1264.1
	KDPEPEDEVDPDIKLDWDDVK	53	794.5	2380.4
MRPS9	LSDQDYAQFIR	79	678.1	1354.3
	TANAEAVVYGHGSGK	68	730.6	1459.2
	HLANMMGEDPETFTQEDVDR	55	778.6	2332.8
MRPS10	NLPEGVAMEVTK	60	645.0	1288.0
	KPVWETTPEEK	57	672.1	1342.2

MRPS18-2	DLTKPTITISDEPDTLYKR	75	735.6	2203.9
	EESGPPPESEMPK	51	642.6	1283.1
	YLDSEEHYHNR	72	662.8	1323.6
	VPLTAPTEATSTEQAGPQSAL	63	1034.9	2067.9
	LYQGHLREESGPPPESEMPK	53	718.0	2151.0
	RLYQGHLR	51	348.2	1041.6
MRPS29	DHGLLSYHIPQVEPR	51	880.7	1759.3
	APPEDDSLPIPVSPYEDEPWK	59	1240.1	2478.2
	NATDAVGIVLK	82	550.7	1099.3
	ESTKGSPLAEVVEQGIMR	50	687.1	2058.4
	KPALELLHYLK	56	442.3	1323.8
	GSPLAEVVEQGIMR	65	743.0	1484.0
MRPS32	VELALTSAR	68	537.7	1073.4
	SEHLEQGPMIEQLSK	94	863.1	1724.2
PTCD3	NELLNEFMDSAK	61	705.5	1409.1
	ASSSPAQAVEVVK	54	636.6	1271.1
	VAVLQALASTVHR	76	682.4	1362.7
	LTADFTLSQEQK	68	690.7	1379.5
	DPDDDMFFQSAMR	84	787.9	1573.8
	DEGADIAGTEEVVIPK	61	821.6	1641.3
<u>-IF3_{mt} control</u>				
Protein	Sequence	Score	m/z	Mr (expt)
MRPS18-2	EESGPPPESEMPK	60	643.1	1284.3
	YLDSEEHYHNR	65	663.5	1324.9
	DHGLLSYHIPQVEPR	85	881.4	1760.7
	VPLTAPTEATSTEQAGPQSAL	91	1035.6	2069.3
	APPEDDSLPIPVSPYEDEPWK	91	1239.8	2477.7
PTCD3	VAVLQALASTVHR	59	682.9	1363.8
	NELLNEFMDSAK	68	706.3	1410.6

* Many of the peptides listed in the sample cross-linked to IF3_{mt} were observed in multiple samples. They are listed only once for simplicity.

Table 3Cross-linking to the N- and C-terminal domains of IF3_{mt}.*

<u>N-domain + Linker</u>				
Protein	Sequence	Score	m/z	Mr(expt)
MRPS10	DLTKPTITISDEPDTLYKR	61	736.1	2205.2
<u>C-domain + Linker</u>				
Protein	Sequence	Score	m/z	Mr(expt)
MRPS5	LIGIKDMYAKVSGSVNMLSLTR	48	810.8	2429.3
	KDPEPEDEVDPDKLDWDDVK	63	794.2	2379.7
MRPS9	LSDQDYAQFIR	70	678.2	1354.3
	WLIKEELEEMLVEK	74	596.9	1787.7
	HLANMMGEDPETFTQEDVDR	63	778.8	2333.5
MRPS10	LSVLVK	51	329.5	657.0
	NLPEGVAMEVTK	67	644.8	1287.6
	AVLDSYEYFAVLA AK	86	830.6	1659.3
	DLTKPTITISDEPDTLYKR	92	735.7	2204.1
MRPS18-2	LEQLPEHIKKPVWETTPEEK	51	810.9	2429.8
	PLTAPTEATSTEQAGPQSAL	107	1035.6	2069.2
	YLDSEYHNR	60	662.9	1323.8
	DHGLLSYHIPQVEPR	72	880.6	1759.1
	VPLTAPTEATSTEQAGPQSAL	70	690.2	2067.6
MRPS29	APPEDDSLPPIPVSPYEDEPWK	61	827.9	2480.6
	KAYLPQELLGK	51	630.3	1258.5
	KPALELLHYLK	77	441.9	1322.6
	FDQPLEASIW LK	63	723.7	1445.3
	GSPLAEVVEQGIMR	60	743.0	1484.1
MRPS32	KGSP LAEVVEQGIMR	8	687.1	2058.3
	SEHLEQGP MIEQLSK	80	863.1	1724.2
MRPS36	SAGLP SHSSVISQHSK	37	542.0	1623.1
	LVSQEEIEFIQR	70	746.5	1491.0
	KLVSQEEIEFIQR	78	810.2	1618.4
PTCD3	SAGLP SHTSSISQHSK	45	812.8	1623.6
	PQIWK	50	579.2	1156.3
	VAVLQALASTVHR	71	682.7	1363.5
	LTADFTLSQEQK	69	690.5	1379.1
	NELLNEFMDSAK	62	705.8	1409.5
	SDLKEEILMLMAR	92	774.6	1547.3
	DPDDDMFFQSAMR	91	787.2	1572.4
	GSSLIHYDIMDEITGK	73	877.6	1753.3
	AHTQALSMYTELLNNR	84	931.6	1861.2
	DLELAYQVHGLLNTGDNR	95	1014.4	2026.7
	TFSPKDPDDDMFFQSAMR	68	1068.1	2134.2

Control for Domains

No peptides observed above the cut-off score set at 50

* Many of the peptides listed in the sample cross-linked to IF3_{mt} were observed in multiple samples. They are listed only once for simplicity.