Molecular Cell, Volume *29*

# Supplemental Data

# A Single Mammalian Mitochondrial

# Translation Initiation Factor

# Functionally Replaces Two Bacterial Factors

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## SUPPLEMENTAL RESULTS

**Homology modeling of $IF2_{mt}$:** A homology model of $IF2_{mt}$ (Fig. 7) shows an overall similarity with the X-ray crystallographic structure of *Methanobacterium thermoautotrophicum* aIF5B (Roll-Mecak et al., 2000). This model is also very similar to the model that was derived by docking a homology model of *E. coli* IF2 (Fig. 7A) into the cryo-EM map of the 70S-initiation factor complex (PDB ID 1ZO1; Allen et al., 2005). However, the $IF2_{mt}$ model revealed that the conserved 37 amino acid insertion in $IF2_{mt}$ forms a helix that projects out from the main body of the molecule. We fitted the $IF2_{mt}$ model into the density corresponding to IF2 within the cryo-EM map of the *E. coli* initiation complex (Fig. 7B), using structurally conserved domains between the bacterial IF2 and $IF2_{mt}$ (excluding the insertion domain) as the main guide. A rigid-body fitting orients the conserved 37 amino acid insertion of $IF2_{mt}$ toward the small (30S) ribosomal subunit (Fig. 7B), such that a portion of the insert would partially overlap with the IF1 molecule in an X-ray crystallographic structure of the *Thermus thermophilus* 30S-IF1 complex (Carter et al., 2001). However, the contact sites of IF1 and the insertion domain of $IF2_{mt}$ on the 30S subunit appear to be different. IF1 interacts with three of the 30S-subunit components, helix 18, and the tip of helix 44 of the 16S rRNA, and protein S12 (Carter et al., 2001), which are

located on the inter-subunit side, the side that faces with 50S subunit in the 70S ribosome. In the case of $IF2_{mt}$, we find that the insertion sequence interacts only with S12. Helix 18 and helix 44 lie within ~7.5 Å and ~9.5 Å, respectively, from the position of the insert. Considering that the insertion domain is linked to the rest of the $IF2_{mt}$ molecule through unstructured flexible loops, the possibility of an interaction between $IF2_{mt,}$ and rRNA helices 18 and 44 cannot be ruled out in the ribosome-bound state. Nevertheless, the observed partial overlap in our fittings of atomic models into the cryo-EM map suggests that the $IF2_{mt}$ insert can partially act as a structural complement of the bacterial IF1.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Cloning the IF1 gene from *E. coli* and *M. tuberculosis*:** *E. coli infA* was amplified from *E. coli* HB101 genomic DNA by *Pfu* DNA polymerase using forward primer *Eco*IF1-FP and reverse primer *Eco*IF1-RP (Table 2) and cloned between the NcoI and PstI sites of pTrc99C using standard procedures (Sambrook et al., 1989). Similarly, *M. tuberculosis infA* was amplified from the *M. tuberculosis* H37Rv genome by *Pfu* DNA polymerase using the forward primer *Mtu*IF1-FP and the reverse primer *Mtu*IF1-RP and cloned into the NcoI and KpnI sites of pTrc99C.

**Generation of IF2 variants:** The plasmid pTrc-$IF2_{mt}$ comprises the predicted mature form of $IF2_{mt}$ (beginning at amino acid 78 which follows the mitochondrial localization signal) cloned from the original pET21c(+) (Spencer and Spremulli, 2005) into pTrc99C using the NcoI and XbaI sites. An alanine residue was incorporated at the N-terminus after the initial Met to maintain the sequence in-frame. The $IF2_{mt}$ was excised from pTrc-$IF2_{mt}$ using NcoI and HindIII and cloned into the same sites of pACDH to yield pACDH-$IF2_{mt}$. The plasmid pACDH-*Eco*IF2 harbors *Eco*IF2.

The chimeric constructs $IF2_{mt}\Delta37$ and *Eco*IF2::37 were generated by PCR using *Pfu* DNA polymerase. For pACDH-$IF2_{mt}\Delta37$, the first round of PCR was done with pACDH-*Eco*IF2 as a template and a forward ($IF2_{mt}$Del-FP) and reverse primer ($IF2_{mt}$Del-RP). This PCR generated a ~130 bp megaprimer that was used for an inverse PCR with $pTrcIF2_{mt}$ as a template. The StuI-HindIII fragment from the resulting plasmid contained the chimeric region and was moved into StuI-HindIII sites of pACDH-$IF2_{mt}$ to generate pACDH-$IF2_{mt}\Delta37$. The construct *Eco*IF2::37 was generated by a similar approach. PCR using a forward primer (*Eco*IF2Del-FP) and a reverse primer (*Eco*IF2Del-RP) with pET21-$IF2_{mt}$ as a template generated a PCR product that introduced silent mutations resulting in PmlI and BamHI restriction sites at the 5' and 3' ends of the primer, respectively. The pET21-*Eco*IF2 gene was also doubly mutated using *Eco*IF2PmlI-FP/RP and *Eco*IF2BamHI-FP/RP to create PmlI and BamHI silent restriction sites and the PCR product was subsequently digested and ligated into the gene to yield pET21 *Eco*IF2::37. The *Eco*IF2::37 insert was subcloned into NcoI-HindIII sites of pACDH to yield pACDH*Eco*IF2::37. The chimeric nature of the constructs was confirmed by DNA sequencing.

**Generation and analysis of gene knockouts in *E. coli* TG1:** Gene knockouts were generated in *E. coli* TG1 as described by Datsenko and Wanner, 2000 using a one-step homologous recombination approach to create the deletions. To generate ΔIF2, the *infB* gene was replaced with $Kan^R$. *Pfu* DNA polymerase was used with forward (IF2-KO-FP) and reverse (IF2-KO-RP) primers (whose initial 36 nucleotides are homologous to 5′ and 3′ ends of *infB*, respectively; and the remaining sequences are complementary to the flanking regions of $Kan^R$ marker in pKD4) to amplify a $Kan^R$ cassette from pKD4. The PCR product was purified by gel elution (Bio101 Geneclean II™) and electroporated into *E. coli* TG1 harboring pKD46 (expresses λ Red recombinase) and pACDH-*Eco*IF2. The transformants were selected on kanamycin (25 μg/ml)

and the allelic exchange (*infB*::*Kan*$^R$) was confirmed by PCR using indicated primers (Fig. 2). In the confirmed knockouts, pKD46 (Amp$^R$) was cured by growth at 37 $^oC$. To remove the genomic *Kan*$^R$, pCP20 (Amp$^R$) was transformed into the Δ*infB*::*Kan*$^R$ strain and the transformants were selected at 34 $^oC$ and further grown at 37 $^oC$ (Datsenko and Wanner, 2000). The plasmid pCP20 was cured during growth at 37 $^oC$ and the *E. coli* ΔIF2 strain thus generated was sensitive to kanamycin and ampicillin. This strain was used as the recipient to generate *E. coli* ΔIF1ΔIF2.

To generate ΔIF1, the *infA* gene was replaced by a *Chl*$^R$ gene in *E. coli* DY330, a strain that harbors genes for λ Red recombinase in its genome (Lee et al., 2001). The *Chl*$^R$ gene was amplified from the pKD3 template by *Pfu* DNA polymerase with forward (IF1-KO-FP) and reverse (IF1-KO-RP) primers whose initial 36 nucleotides are homologous to 5′ and 3′ ends of *infA*, respectively and the remaining sequences are complementary to the flanking sequences of *Chl*$^R$ marker in pKD3. The PCR product was purified by gel elution (Bio101 Geneclean II™) and electroporated into *E. coli* DY330 harboring pTrc-*Mtu*IF1, followed by recovery in 1 ml LB broth at 30 $^oC$ for 10 h. The cells were plated on LB agar containing chloramphenicol (30 μg/ml), with or without 1 mM IPTG and incubated at 30 $^oC$. After two days of incubation, the plates were shifted to 37 $^oC$ and the colonies obtained after a day were analyzed by PCR using upstream (*Eco*IF1up-FP) and downstream (*Eco*IF1dn-RP) primers (Fig. 4).

To generate *E. coli* ΔIF1ΔIF2, P1 phage raised on *E. coli* DY330 Δ*infA*::*Chl*$^R$ + pTrc-*Mtu*IF1 was used to deliver the *infA*::*Chl*$^R$ locus into *E. coli* ΔIF2 harboring pACDH-IF2$_{mt}$. The recipient strain did not contain *infB* or *Kan*$^R$ as the *Kan*$^R$ marker originally used to disrupt *infB* was removed by introducing pCP20 (mentioned above). Thus, the resulting *E. coli* ΔIF1ΔIF2 strain is resistant to chloramphenicol and tetracycline (5 μg ml$^{-1}$) but sensitive to Kan.

**Genomic and RT-PCR for *infA*:** To analyse for replacement of *infA*, the flanking upstream (*Eco*IF1up-FP) and downstream (*Eco*IF1dn-RP) primers were used. To amplify a 160 bp region of *infA* ORF, internal forward (IF1-RT-FP) and reverse (IF1-RT-RP) primers were used along with Taq DNA polymerase and the genomic DNA (~100 ng) of various strains as template. For detection of *infA* mRNA, total RNA from various strains was prepared using hot-phenol method (Sarmientos et al 1983), and treated with RNase free DNaseI. Reverse transcriptase-PCR was carried out using iScript$^{TM}$ one step RT-PCR kit (BioRad). Briefly, total RNA (200 ng) was used to make cDNA against IF1 mRNA to serve as template for PCR using forward (IF1-RT-FP) and reverse (IF1-RT-RP) primers. The PCR involved synthesis of DNA template at 50 $^{o}$C for 10 min followed by heat inactivation of the reverse transcriptase at 95 $^{o}$C for 10 min which also activated the hot start Taq DNA polymerase. To spike the DNA/RNA preparations, 50 ng DNA or 50 ng total RNA from the wild type strain was used. In both the genomic and RT-PCR reactions, as an internal control, for ORF specific primers for *ung* (*Ecoung*Seq-FP and *Ecoung*-RT-RP) were also included to amplify a 137 bp fragment. The PCR products were analyzed by 8% PAGE using TBE buffer system and visualized by ethidium bromide staining (Sambrook et al., 1989).

***In vitro* assay of IF2 activity:** The activity of the factors in initiation complex formation was tested in reaction mixtures (0.1 ml) that contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 80 mM KCl, 0.25 mM GTP, 1.25 mM phospho(enol)pyruvate, 0.1 unit pyruvate kinase, 1 mM dithiothreitol (DTT), 12.5 µg poly(A,U,G), 0.06 µM (6 pmol) [$^{35}$S]fMet-tRNA$^{fMet}$ (approximately 70,000 cpm/pmol), 0.2 µM *E. coli* initiation factor 3 (IF3), 0.23 µM *E. coli* 70S ribosomes and the indicated amounts of IF1 and of the various IF2s. Samples were incubated at 37 °C for 15 min. After incubation, each sample was rapidly diluted with 3-4 ml of cold Buffer

Wash (50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 80 mM KCl) and filtered through a nitrocellulose membrane (Millipore Corporation) that had been wetted with cold Buffer Wash. The filters were washed with the 3 aliquots (3-4 ml each) of cold Buffer Wash. Filters were dried for 5-7 min at 100 °C and counted in 5 ml toluene containing 5 g/l 2, 4 diphenyloxazole (PPO) scintillation cocktail. A blank representing the amount of [$^{35}$S]fMet-tRNA$^{fMet}$ retained on the filter in the absence of IF2 (0.05 pmol) has been subtracted from each value.

**Modeling of IF2$_{mt}$ on *E. coli* ribosomes/initiation complex:** Homology models of all IF2 sequences were obtained from the SWISS MODEL server (http://swissmodel.expasy.org/; Schwede *et al.,* 2003), using the X-ray crystallographic structure of *Methanobacterium thermoautotrophicum* aIF5B (Roll-Mecak et al., 2000) as a template structure. Atomic structure of the *Thermus thermophilus* 30S-IF1 complex (Carter et al., 2001; PDB ID 1HRO) was fitted into the small ribosomal subunit portion of the cryo-EM map of *E. coli* 70S-initiation complex (Allen et al., 2005; EMD ID 3523) using the 30S structure as the main guide. The molecular modeling package, O (Jones et al., 1991) was used for dockings. Visualization of the atomic structures and the cryo-EM density maps was done with Ribbons (Carson, 1991) and IRIS EXPLORER (Numerical Algorithms group, Inc. Downers Grove, IL), respectively.

**Table S1.** List of strains and plasmids used in this study.

| ***E. coli* strains** | **Relevant genotype/features** | **Reference** |
|---|---|---|
| ΔIF1 | Δ*infA*::*Chl*$^R$ | This study |
| ΔIF1ΔIF2 | Δ*infA*::*Chl*$^R$ Δ*infB* | This study |
| ΔIF2 | Δ*infB*::*Kan*$^R$, the *Kan*$^R$ was later removed from the genome | This study |
| DY330 | W3110 ΔlacU169 gal490 [lc1857Δ(cro-bioA)] | Lee et al., 2001 |
| HB101 | *supE*44 *hsdS*20 ($r_B^-$ $r_B^-$) *recA13 ara*-14 *proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | Boyer and Roulland-Dussoix, 1969 |
| TG1 | *supE hsd*Δ5 *thi* Δ(*lac-proAB*) F` [*traD*36 *proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15] | Sambrook et al., 1989 |
| **Plasmids** | | |
| pACDH | Tet$^R$, a medium copy number vector with pACYC origin of replication, compatible with ColE1 origin of replication | Mangroo and RajBhandary, 1995 |
| pACDH-*Eco*IF2 | pACDH harboring *Eco*IF2 | This study |
| pACDH-*Eco*IF2::37 | *Eco*IF2 containing the conserved 37 amino acid insert from $IF2_{mt}$ | This study |
| pACDH-$IF2_{mt}$ | $IF2_{mt}$ cloned into NcoI and HindIII sites of PACDH | This study |
| pACDH-$IF2_{mt}$Δ37 | $IF2_{mt}$ lacking the conserved 37 amino acid insertion | This study |
| pCP20 | Amp$^R$, expresses FLP recombinase, temperature sensitive replicon | Datsenko and Wanner, 2000 |
| pET21-*Eco*IF2 | *Eco*IF2 cloned between NdeI and NotI sites | This study |
| pET21-*Eco*IF2::37 | *Eco*IF2 containing the conserved 37 amino acid insert from $IF2_{mt}$ | This study |
| pET21-$IF2_{mt}$ | $IF2_{mt}$ cloned into NdeI and XhoI sites of pET21c | Spencer and Spremulli, 2005 |
| pET24b-$IF2_{mt}$Δ37 | $IF2_{mt}$ lacking the conserved 37 amino acid insertion, cloned between NdeI and NcoI sites | This study |
| pKD3 | Amp$^R$, Chl$^R$, *Chl*$^R$ is flanked by FRT sequences | Datsenko and Wanner, 2000 |
| pKD4 | Amp$^R$, Kan$^R$, *Kan*$^R$ is flanked by FRT sequences | Datsenko and Wanner, 2000 |
| pKD46 | Amp$^R$, harbors λ Red recombination genes (γ, β and *exo*), temperature sensitive replicon. | Datsenko and Wanner, 2000 |
| pTrc-*Eco*IF1 | *E. coli* IF1 cloned into NcoI and PstI sites of pTrc99C | This study |
| pTrc-$IF2_{mt}$ | Predicted mature form of $IF2_{mt}$ cloned between NcoI and HindIII sites | This study |
| pTrc-*Mtu*IF1 | *M. tuberculosis* IF1 cloned into NcoI and KpnI sites of pTrc99C | This study |

**Table S2.** List of oligonucleotides used in this study. The sequence is 5′ to 3′ from left to right. Restriction enzyme sites are denoted in italics.

| | |
|---|---|
| *Eco*IF1-FP | GATT*CCATGG*CCAAAGAAGACA |
| *Eco*IF1-RP | CGCC*CTGCAG*GCGGTAAAACAA |
| *Mtu*IF1-FP | AAAG*CCATGG*CCAAGAAGGACG |
| *Mtu*IF1-RP | TG*GGTACC*TCGTTACTTGTACCGGT |
| IF1-KO-FP | GGTTCAAATTACGGTAGTGATACCCCAGAGGATTAGGTGT AGGCTGGAGCTGCTTCG |
| IF1-KO-RP | TCGTTCTTTCTCTTCGCCCATCAGGCGGTAAAACAACATA TGAATATCCTCCTTA |
| *Eco*IF1up-FP | GGCGCTTCTGGTATTCTG |
| *Eco*IF1dn-RP | GAGTTCACTGCCGTACAG |
| IF1-RT-FP | GACAATATTGAAATGCAAGG |
| IF1-RT-RP | GTCAGTTCAACAGTCACTTTG |
| IF2-KO-FP | GGTTCGGTGACGAAGCGTAATAAACTGTAGCAGGAGTGTA GGCTGGAGCTGCTTCG |
| IF2-KO-RP | AGGTGGAAGGGCTGTTCACGTTGACCTGATAAGACCATAT GAATATCCTCCTTA |
| IF2-FP5 | GCAAGGCTTCCAGAAGCC |
| *rbfA*-RP | ACGGAATTCCAGACAGAGAC |
| IF2-FP3 | GCTGAACGTGAGGCCGCA |
| IF2-RP3 | ACTTCGTCAGTAGACAGT |
| $IF2_{mt}$FP | CATGCCATGGCCCAGAAATCTCCATTACCTTC |
| $IF2_{mt}$RP | CTAGTCTAGAATTATTAAAATCCTGGATCCCAAGAAG |
| $IF2_{mt}$Del-FP | CCAAGGGCACGTGAAGTTGCACTCTAT |
| $IF2_{mt}$Del-RP | AAAATGGCTTCAACAGAACCCTGTACGTC |
| *Eco*IF2Del-FP | GAAGAAAGCACGTGAAGTTGTTGACTGGAGAAAGTATGAG |
| *Eco*IF2Del-RP | CGCTTCGACGGATCCCTGAACATCACCTTTAACAATTACA |
| *Eco*EFG-FP | ATGGCTCGTACAACACCCATC |
| *Eco*EFG-RP | GTAT*GGATCC*TTAGGCTTATTTACC |
| *Eco*IF2PmlI-FP | CGTGACGAGAAGAAAGCACGTGAAGTTGCACTCTATCG |
| *Eco*IF2PmlI-RP | CGATAGAGTGCAACTTCACGTGCTTTCTTCTCGTCACG |
| *Eco*IF2BamHI-FP | GGCAGACGTACAGGGATCCGTCGAAGCGATCTCCG |
| *Eco*IF2BamHI-RP | CGGAGATCGCTTCGACGGATCCCTGTACGTCTGCC |
| *Ecoung*RT-RP | CTGGCATCCAGTCAATCGGC |
| *Ecoung*Seq-FP | AGCAACGCCATCATGTAC |

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