

C7orf30 is necessary for biogenesis of the large subunit of the mitochondrial ribosome

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ABSTRACT

Defects of the translation apparatus in human mitochondria are known to cause disease, yet details of how protein synthesis is regulated in this organelle remain to be unveiled. Here, we characterize a novel human protein, C7orf30 that contributes critically to mitochondrial translation and specifically associates with the large subunit of the mitochondrial ribosome (mt-LSU). Inactivation of C7orf30 in human cells by RNA interference results in respiratory incompetence owing to reduced mitochondrial translation rates without any appreciable effects on the steady-state levels of mitochondrial mRNAs and rRNAs. Ineffective translation in C7orf30-depleted cells or cells overexpressing a dominant-negative mutant of the protein results from aberrant assembly of mt-LSU and consequently reduced formation of the monosome. These findings lead us to propose that C7orf30 is a human assembly and/or stability factor involved in the biogenesis of the large subunit of the mitochondrial ribosome.

INTRODUCTION

It is estimated that 3–5% of all human proteins are localized to mitochondria (1), vital organelles that supply energy to the cell through the process of oxidative phosphorylation (OXPHOS). Approximately 90 proteins form the five enzyme complexes that perform OXPHOS and 13 of those are encoded in the mitochondrial genome (mtDNA). For this reason, mitochondria contain a separate translation apparatus, necessary for the synthesis of the mtDNA-encoded polypeptides of the OXPHOS system. The RNA components (2 rRNAs and 22 tRNAs) of the mitochondrial translation machinery are encoded by mtDNA, while all protein components including the mitochondrial ribosomal subunits, translation factors, tRNA synthetases, tRNA-modifying enzymes, and other auxiliary factors are encoded by

nuclear genes and imported to mitochondria from the cytosol. Human mitoribosomes consist of a large (mt-LSU) and small subunit (mt-SSU) of 39S and 28S, respectively, and the entire ribosome has a sedimentation coefficient of 55S (2).

Many genetic defects can lead to perturbations of the OXPHOS system and result in multi-system, often fatal, human diseases. These genetic defects can be located within nuclear DNA (nDNA) or mtDNA. Isolated OXPHOS deficiencies, that affect a specific biochemical activity of the OXPHOS system, are usually caused by mutations in structural genes (coding for a specific component of the OXPHOS machinery) or in genes encoding proteins responsible for the assembly of a particular respiratory complex. In contrast, inherited pathological mutations, affecting either mitochondrially or nuclearly encoded components of the mitochondrial translation machinery, are associated with combined OXPHOS deficiencies affecting multiple enzymes involved in cellular respiration. Many mutations in mtDNA-encoded genes for the RNA components of the mitochondrial translation apparatus (mt-rRNAs and mt-tRNAs) have been identified, and detection of yet uncharacterized mutations is relatively straightforward. On the contrary, a group of mutations in nuclear gene products involved in mitochondrial translation linked to human disease has emerged only in recent years (3). This group currently contains <20 genes including those that encode: translation elongation factors, proteins of the mitoribosomal subunits, several tRNA synthetases, tRNA-modifying enzymes [for recent review see (4)] and also other translation auxiliary proteins, such as TACO1 (CCDC44) (5) or c12orf65 (6). Given the complexity of the mitochondrial translation system, this list is unlikely to be complete. By analogy to other translation systems one would, for example, anticipate that mitoribosome assembly factors would be present within the above inventory. However, to date only paraplegin, a conserved subunit of the ATP-dependent m-AAA protease, links the regulation of mitoribosome assembly with human mitochondrial disease based on studies of the paraplegin homologue in yeast mitochondria and a mouse model (7,8). Therefore, it

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is vital to identify and characterize novel proteins involved in the regulation of mitochondrial translation, in particular those involved in the assembly of mitochondrial ribosomes.

Systematic identification of genes that are found in organisms from several phylogenetic lineages, but remain largely uncharacterized ('conserved hypothetical' proteins) facilitates prediction of human homologues with important functions. In this context, we analysed a family of well-conserved proteins harbouring the YbeB/iojap domain (also known as DUF143). For two members of this family, YbeB from *Escherichia coli* (9) and iojap from *Zea mays*, a role in translation regulation has been suggested (10,11). We identified a human protein that shares sequence homology with YbeB/iojap—C7orf30. Published proteomics data (12,13) and our *in silico* analysis predicted mitochondrial localization. Therefore, we hypothesized that C7orf30 is involved in translation in human mitochondria. Here we show that C7orf30 is indeed a mitochondrial protein that specifically associates with the large 39S subunit of the mitochondrial ribosome (mt-LSU). We depleted C7orf30 using RNAi and analysed the mitochondrial ribosomal subunits by sucrose gradient sedimentation and affinity purification methods. Inactivation of C7orf30 leads to instability of mt-LSU and a consequent loss of fully assembled ribosomes that is accompanied by reduced mitochondrial translation and respiratory deficiency. We propose that C7orf30 participates in the assembly and/or regulation of the stability of the large subunit of the mitochondrial ribosome.

MATERIALS AND METHODS

Plasmids

In order to construct the pcDNA5-C7orf30.FLAG.STREP2 plasmid used to generate an inducible Flp-In T-RexTM human embryonic kidney 293T (HEK293T) cell line, the cDNA encoding C7orf30 (cDNA clone MGC:20424, IMAGE:4646294) was modified by PCR to introduce unique KpnI (5') and AgeI (3') sites and the resulting fragment was cloned into pcDNA5-FST2 constructed as described in (14) using the above restriction sites. The D178A and H185A mutants were generated using PCR-based mutagenesis and the resulting cDNA was cloned into pcDNA5-FST2 as above.

Maintenance and transfection of mammalian cell lines

Human HeLa cells that are routinely used by us for immunofluorescence localization of mitochondrial proteins were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (Invitrogen) with 10% FCS (PAA Laboratories). For immunofluorescence experiments HeLa cells were transfected with pcDNA5-C7orf30.FLAG.STREP2 using Lipofectamine (Invitrogen) according to manufacturer's instructions.

The Flp-In T-RexTM human embryonic kidney 293T (HEK293T) cell line (Invitrogen), which allows for the generation of stable doxycycline-inducible expression of transgenes by FLP recombinase-mediated integration,

was used to express C7orf30.FLAG.STREP2 and the conserved residue mutants. HEK293T cells were grown in DMEM containing 2 mM L-glutamine (Invitrogen), 10% tetracycline free FCS (Autogen Bioclear) and supplemented with 100 µg/ml Zeocin (Invitrogen) and 15 µg/ml Blasticidin (Invivogen). Cells were transfected using Lipofectamine according to the manufacturer's instruction. Twenty-four hours after transfection the selective antibiotics hygromycin (100 µg/ml, Invivogen) and blasticidin (15 µg/ml) were added with selective media replaced every 3–4 days.

Cell fractionation

Cell fractionation was performed as described in (14) without the sucrose gradient step.

FLAG-tag affinity purification

Mitochondria were isolated from HEK293T cells overexpressing ICT1.FLAG, MRPS27.FLAG or PDE12.FLAG essentially as described in (15). Pelleted mitochondria were resuspended in lysis buffer and immunoprecipitation (IP) was performed with a FLAG M2 affinity gel (Sigma) following manufacturer's recommendations. Elution was effected with the FLAG peptide.

Immunodetection of proteins

The localization of proteins by immunofluorescence in fixed HeLa cells was performed as described previously (16). The following antibodies were used: mouse anti-Flag IgG (Sigma, 1:200), FITC-conjugated anti-mouse IgG (Sigma, 1:200). Immunofluorescence images were captured using a Zeiss LSM 510 META confocal microscope.

For immunoblot analysis equal amounts of protein corresponding to total cell lysates or protein fractions were subjected to SDS-PAGE, semi-dry transferred to nitrocellulose membranes, blocked in 5% non-fat milk (Marvel) in PBS for 1 h and incubated with specific primary antibodies in 5% non-fat milk in PBS for 1 h or overnight. The blots were further incubated with HRP-conjugated secondary antibodies in 5% non-fat milk in PBS for 1 h and visualized using ECL+ (Amersham).

The primary antibodies used were: rabbit anti-C7orf30 (Sigma, 1:1000), mouse anti-FLAG IgG (Sigma, 1:5000), mouse anti-β-actin IgG (Sigma, 1:7000), mouse anti-CO2 IgG (Abcam, 1:5000), rabbit anti-SSB1 IgG (kindly donated by Prof. D. Kang, 1:4000), mouse anti-GAPDH IgG (Abcam, 1:10 000), mouse anti-complex I subunit NDUFB8 (MitoSciences, 1:2000), mouse anti-complex II subunit 30 kDa (MitoSciences, 1:2000), mouse anti-complex III subunit core 2 (MitoSciences, 1:2000), mouse anti-DAP3/MRPS29 IgG (Abcam, 1:1000) and goat anti-MRPL3 (Abcam, 1:1000), mouse anti-MRPL12 (Abcam, 1:1000), rabbit anti-MRPS18B [Proteintech group (PTG), 1:1000].

Secondary antibodies were: anti-rabbit IgG-HRP (Promega, 1:2000), anti-mouse IgG-HRP (Promega, 1:2000) and anti-goat IgG-HRP (Sigma, 1:1000)

siRNA gene silencing

Stealth™ siRNAs were obtained from Invitrogen and delivered to cells using Lipofectamine RNAiMAX. Stealth™ RNAi duplexes were generated using the following sequences: C7orf30 siRNA1: UCGAUAUGAUG GUUUCACUUCUGAG and C7orf30 siRNA2: CGACA CUUACAUGCCAUGGCCUUCU.

Measurement of mitochondrial respiration

HeLa cells transfected with siRNA against C7orf30 were seeded at 3×10^4 cells/well in 200 μ l growth medium in XF 24-well cell culture microplates (Seahorse Bioscience) and incubated at 37°C in 5% CO₂ for 36–40 h. One hour before the assay growth medium was removed and replaced with assay medium (low buffered DMEM, 10 mM L-glutamine, 1 mM sodium pyruvate, 2 mM glucose), with one wash of assay medium, and left to stabilize in a 37°C non-CO₂ incubator. Analysis was performed in quadruplicate using a XF24 Extracellular Flux Analyzer (Seahorse Bioscience). The wells were sequentially injected with 20 mM 2-deoxyglucose (2-DG) to inhibit glycolysis, 100 nM oligomycin to inhibit ATP-synthase, 500–1000 nM carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP) to uncouple the respiratory chain, and 200 nM rotenone to inhibit complex I. Oxygen consumption rate (OCR) was measured for each well every 5 min. before and after each injection. Test compounds: 2-DG, oligomycin, FCCP and rotenone were all obtained from Sigma.

³⁵S-methionine metabolic labelling of mitochondrial proteins

HeLa cells transfected with siRNA against C7orf30 or stably transfected HEK293T cells expressing C7orf30. FLAG.STREP2 or mutants thereof were induced with doxycycline (50 ng/ml) for 3 days prior to ³⁵S labelling. Growth medium was replaced with methionine/cysteine-free DMEM (Sigma) supplemented with 2 mM L-glutamine, 48 μ g/ml cysteine and 50 μ g/ml uridine. The cells were incubated for 2 \times 10 min in this medium, then transferred to methionine/cysteine-free DMEM medium containing 10% (v/v) dialysed FCS and emetine dihydrochloride (100 μ g/ml). Cells were incubated for 10 min before addition of 120 μ Ci/ml of [³⁵S]-methionine. Labelling was performed for 15 min and the cells were washed twice with standard growth medium. Protein samples (30 μ g) were separated on 4–12% SDS-PAGE gels, with products visualized and quantified using a PhosphorImager system in ImageQuant software (Molecular Dynamics, GE Healthcare).

Analysis of mitochondrial ribosome profile on density gradients

Total cell lysates (0.7 mg) were loaded on a linear sucrose gradient [2 ml 10–30% (v/v)] in 50 mM Tris-HCl (pH 7.2), 10 mM Mg(OAc)₂, 80 mM NH₄Cl, 0.1 M KCl, 1 mM PMSF and centrifuged for 2 h 15 min at 100 000g_{max} at 4°C (39 000 rpm, Beckman Coulter TLS-55 rotor). Twenty fractions (100 μ l) were collected and 10- μ l aliquots were analysed directly by western blotting.

RNA isolation and northern blotting

Total RNA from HeLa cells was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. For northern blots, RNA was resolved on 1% agarose gels containing 0.7 M formaldehyde in 1 \times MOPS buffer, transferred to a nylon membrane in 2 \times SSC and hybridized with radioactively labelled PCR fragments corresponding to appropriate regions of mtDNA or 28S rDNA.

RESULTS

Identification of C7orf30 and *in silico* analysis of proteins containing the DUF143 domain

Analysis of the primary structure of C7orf30 by pairwise alignment revealed an evolutionarily well-conserved domain of unknown function named DUF143. The DUF143 domain of C7orf30 (residues 93–194) shares 33% sequence identity with YbeB of *E. coli* (residues 6–105), which has been reported to associate with the large subunit (50S) of the bacterial ribosome (9,17) and 29% sequence identity with iojap of *Zea mays* (residues 116–213), a protein which is involved in the regulation of chloroplast ribosome stability (10,11). Additionally, 24% of residues within the DUF143 domain of C7orf30 are identical to the N-terminal domain of *Saccharomyces cerevisiae* ATP25 protein (residues 104–246), which is required for the expression and assembly of the Atp9p subunit of the mitochondrial ATPase (18) (Figure 1A and B). Bacterial YbeB and plant iojap share a similar domain architecture to C7orf30, with the DUF143 domain located at the C-terminus of these proteins. In ATP25, however, the DUF143 domain is located within the N-terminal portion of the protein and the C-terminus shares weak sequence homology with the metallo-beta-lactamase superfamily (Figure 1B). Three dimensional structures of two proteins containing the DUF143 domain have been solved: uncharacterized protein CV0518 from *Chromobacterium violaceum* (PDB: 2id1) and uncharacterized protein BH1328 from *Bacillus halodurans* (PDB: 2o5a). On the basis of structural features, DUF143 has been assigned to the superfamily of nucleotidyltransferases (NTases) (19), enzymes that transfer nucleoside monophosphate (NMP) from nucleoside triphosphate (NTP) to an acceptor hydroxyl group belonging to a protein, nucleic acid or small molecule. NTases are characterized by the presence of a common minimal core of α - β - α - β - α topology (19) (Figure 1). NTases contain the following common sequence motifs which include conserved active site residues involved in the coordination of divalent ions: (i) [DE]h[DE]h (h indicates a hydrophobic amino acid) located on the second core β -strand and (ii) h[DE]h placed on the third β -strand (Figure 1C and D, see hmtCCase). Our 3D homology modelling of C7orf30 based on the crystal structure of 2id1 indicated that none of the three active site aspartate/glutamate residues are conserved in C7orf30 suggesting that the protein is not an active NTase.

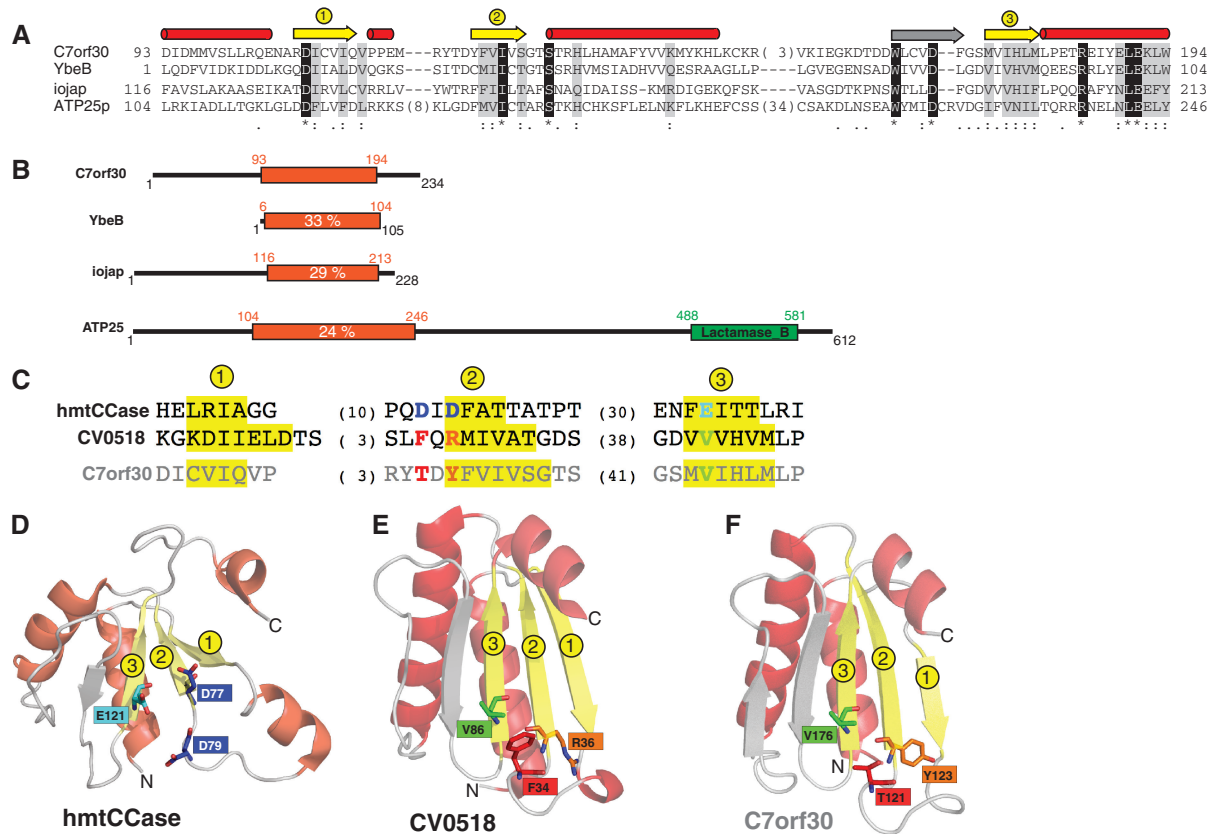


Figure 1. Properties of the NTase-like fold of C7orf30. (A) Multiple protein sequence alignment of the DUF143 domain. Protein sequence of the DUF143 domain (PFAM: PF02410) of *E. coli* YbeB (Genbank Accession No. NP_752658), *Z. mays* iojap (Genbank Accession No. NP_001105495), *S. cerevisiae* Atp25p (Genbank Accession No. NP_013816) and human C7orf30 (Genbank Accession No. NP_612455) were extracted from the PFAM database (42) and aligned using CustalW2. The boundaries of the sequence segment used in the alignment are indicated. Colons and dots denote chemical similarity between the sequences, whereas asterisks indicate identical residues. The secondary structure based on the resolved structure of the bacterial DUF143 protein CV0518 (pdb: 2id1) is indicated above the alignment [helix (cylinders), strand (arrow)]. Three β -sheets (numbered 1–3) and four α -helices of NTases α/β -fold are presented in red and yellow, respectively. (B) Domain architecture of proteins comprising the DUF143 domain. Functional domains of proteins containing DUF143 are schematically represented. The position of the DUF143 domain is indicated and represented in each protein by an orange box containing the percentage of identical residues between this domain and that of C7orf30. The green box represents a partial metallo-beta-lactamase domain (PFAM: PF00753) found in Atp25p using PSI-BLAST searches. (C) A comparison of C7orf30 NTase-like fold with that of a known NTase. Protein sequence alignment of the three core β -strands of the NTase fold (yellow) (43) of the human mitochondrial CCA tRNA nucleotidyltransferase 1 (hmtCCase) (PDB: 2ou5), bacterial YbeB-type protein from *C. violaceum* (CV0518) (PDB: 2id1) and predicted core β -strands of the homology model of C7orf30 constructed with the reference to the crystal structure of CV0518. The two conserved aspartates (D) and a glutamate (E) involved in coordination of divalent ions by hmtCCase are indicated in blue and cyan, respectively. Corresponding residues in the CV0518 structure and the C7orf30 model are also coloured. (D–F) Ribbon diagrams indicating the key secondary structure elements of the NTase fold. The structures of hmtCCase (D), CV0518 (E) and the homology model of C7orf30 (F) are presented. The three-stranded, mixed β -sheets (yellow) and four α -helices (red) of the common core of the minimal fold are presented. The conserved aspartate/glutamate residues and the corresponding residues in CV0518 and the C7orf30 model are shown as sticks and colour coded as presented in C.

C7orf30 is localized to human mitochondria

Several computer programmes that analyse the N-terminal region of proteins for the presence of a potential mitochondrial targeting sequence (MTS) indicated a high probability of a MTS in C7orf30 (MitoProt II 98%, MultiLoc 95%, TargetP 84%, Predotar 67%, PSORT (mitochondrial matrix) 50%, PSORT II 45%). Moreover, C7orf30 was reported to be present in the mitochondrial proteome of a motor neuron-like cell line, NSC34 (12). In order to study the cellular localization of C7orf30 we transiently expressed the corresponding cDNA with a FLAG tag in HeLa cells. Immunofluorescence analysis revealed that the recombinant protein co-localizes with mitochondria (Figure 2A).

Cell fractionation experiments confirmed the mitochondrial localization of the protein, as endogenous C7orf30 co-fractionated with the well-characterized mitochondrial matrix protein, mtSSB1, and was resistant to proteinase K treatment. In similar conditions the outer membrane protein TOM22 was truncated by proteinase K (Figure 2B). These results suggest that C7orf30 is localized within the mitochondria of human cells.

C7orf30 associates with the large subunit of the mitochondrial ribosome

Since C7orf30 shares sequence homology with the YbeB protein that associates with the large subunit of the bacterial ribosome, we set out to determine whether the

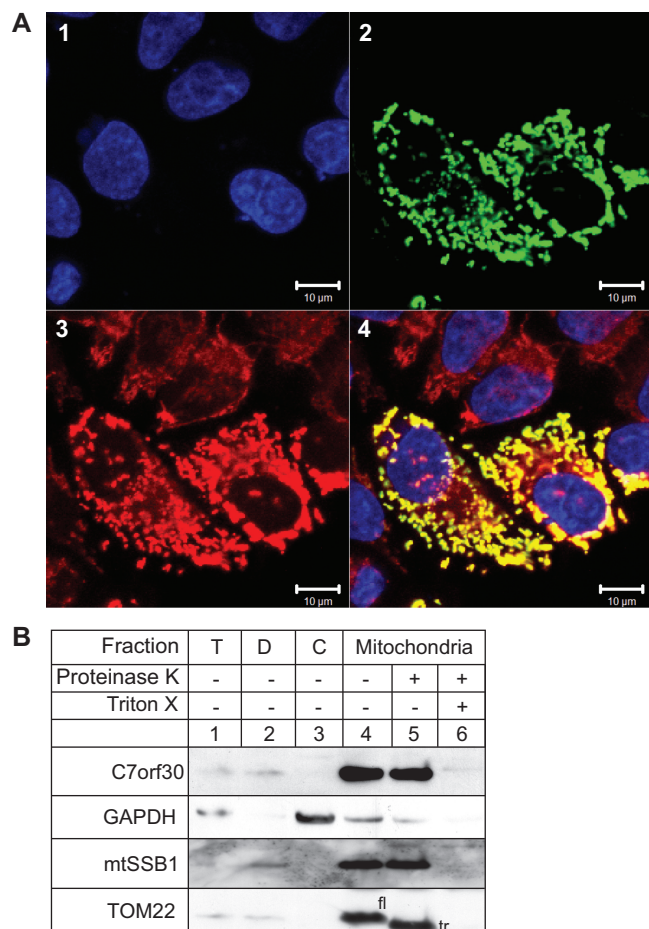


Figure 2. Mitochondrial localization of C7orf30. (A) The intra-cellular localization of C7orf30 by immunofluorescence. The cDNA encoding the STREP2- and FLAG-tagged variant of C7orf30 (C7orf30.FLAG.STREP2) was transiently transfected into HeLa cells. Cell nuclei were stained with DAPI (blue, 1). C7orf30.FLAG.STREP2 was detected by anti-Flag antibody and visualised by secondary antibodies conjugated with FITC (green, 2). Mitochondria were stained with MitoTracker Red CMXRos (red, 3). Co-localization of the mitochondria-specific red signal and C7orf30-specific green signal appears yellow on digitally merged images (4). (B) Location of C7orf30 in sub-cellular fractions. The HeLa cells were fractionated into debris (D, lane 2), cytosol (C, lane 3) and mitochondria (lanes 4–6). The mitochondrial fraction was treated with 25 μ g/ml proteinase K in the absence (lane 5) or presence of 1% Triton X-100 (lane 6). ‘T’ indicates the total cell lysate. The fractions were analysed by western blotting using antibodies to C7orf30. The location of C7orf30 was compared with that of the following marker proteins: mtSSB1 (mitochondrial matrix side), TOM22 (mitochondrial outer membrane) and GAPDH (cytosol). ‘fl’ means TOM22 of full length, ‘tr’ means truncated TOM22.

human homologue might bind to the mitoribosome. To investigate this, we carried out sucrose gradient sedimentation of human HeLa cell extracts followed by detection of C7orf30 and the components mt-SSU (28S) and mt-LSU (39S) by western blotting of gradient fractions. We used MRPS18B or DAP3/MRPS29 as markers for mt-SSU and MRPL3 or MRPL12 as markers for mt-LSU. Analyses of the sedimentation of mitoribosomal subunits revealed that C7orf30 is located in fractions positive for the mt-LSU markers (Figure 3A,

fractions 9–12) and low molecular weight fractions (Figure 3A, fractions 1–6). These results indicate that a part of the mitochondrial pool of C7orf30 associates with mt-LSU. A proportion of C7orf30 that is found at the top of the gradient likely represents free protein or protein in a low molecular weight complex, similarly to the MRPL12 protein (Figure 3A, fractions 1–6). A very similar sedimentation pattern has been obtained for YbeB, the bacterial homologue of C7orf30 (9).

In order to gain further insights into the interaction of C7orf30 with the mitoribosome, we performed pull-down experiments in Flp-In T-RexTM Human embryonic kidney (HEK293T) cell lines inducibly expressing either FLAG-tagged MRPS27, a component of mt-SSU, or FLAG-tagged ICT1, a component of mt-LSU (15). It has been reported previously that MRPS27.FLAG and ICT1.FLAG are able to pull-down components of mt-SSU or mt-LSU, respectively, with each of these proteins also capable of pulling down the entire monosome (15). We have performed IP with anti-FLAG antibodies after inducing MRPS27.FLAG or ICT1.FLAG for 3 days with 50 ng/ml doxycycline and analysed the eluates by western blotting. The C7orf30 protein co-purified with ICT1.FLAG (Figure 3B, lane 2) consistent with the interaction of C7orf30 with mt-LSU. The lack of other highly abundant mitochondrial proteins, such as subunit 2 of complex IV (CO2), in the elution fraction implies specific interaction (Figure 3B). In contrast, C7orf30 was absent from the elution fraction of the MRPS27.FLAG IP suggesting that the C7orf30 protein does not interact with mt-SSU (Figure 3B, lane 4). Proteins of mt-LSU (MRPL3 and MRPL12) were present in the elution fraction of the MRPS27.FLAG IP, consistent with co-purification of the intact mitochondrial monosome (Figure 3B, lane 4). However, the lack of C7orf30 in this fraction suggests that the protein does not bind to the fully assembled mitoribosome. Finally, the specificity of C7orf30 association with mt-LSU was further confirmed in an IP experiment with a FLAG-tagged version of the PDE12 protein (PDE12.FLAG) that according our previous studies is a mitochondrial RNase not associated with the mitoribosome (20). There was no C7orf30 nor any mitoribosomal proteins readily detected in the IP experiment with PDE12.FLAG (Figure 3B, lane 5). Taken together, the results of sedimentation of C7orf30 on sucrose gradients and the IP experiments indicate that the C7orf30 protein associates exclusively with the large subunit of the mitochondrial ribosome.

C7orf30 is necessary for OXPHOS function

In order to assess the role of C7orf30 in OXPHOS, we silenced the expression of the gene by siRNA. We identified two siRNAs that depleted the protein in HeLa cells as verified by western analysis (Figure 4A), however, ‘siRNA 1’ was more effective as compared to ‘siRNA 2’, which was particularly apparent after 3 days of siRNA treatment (Supplementary Figure S1). Upon inactivation of C7orf30 a reduction in the steady-state levels of the respiratory chain subunits was observed, which correlated

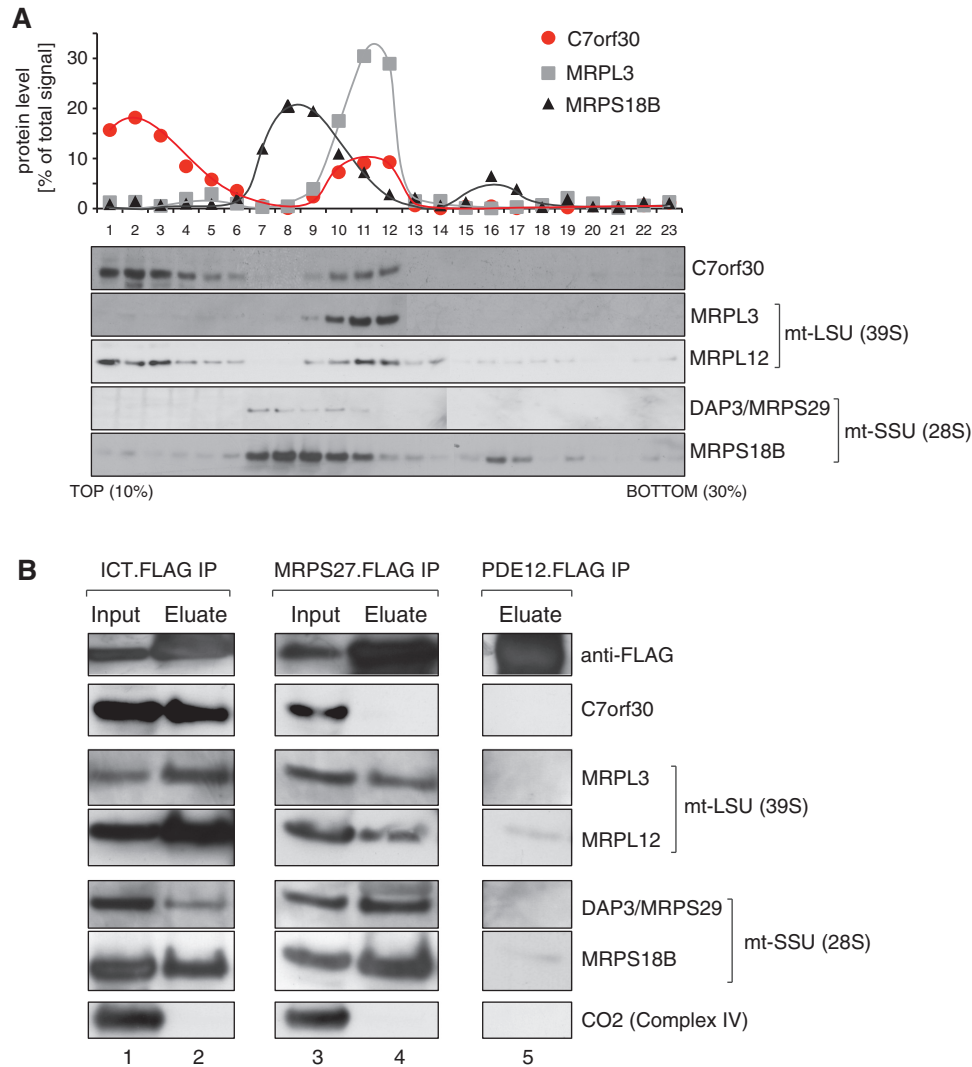


Figure 3. Interaction of C7orf30 with the mitochondrial ribosome. **(A)** Endogenous C7orf30 co-sediments with the large subunit of the mitochondrial ribosome. Total lysate of HeLa cells was separated through a 10–30% (v:v) isokinetic sucrose gradient and fractionated. The fractions were analysed by western blotting with antibodies specific for C7orf30, components of mt-LSU (MRPL3 and MRPL12) and components of mt-SSU (DAP3/MRPS29 and MRPS18B). Western blot signal was quantified using ImageQuant software and presented in the graph above. **(B)** IP of the endogenous C7orf30 with the mitochondrial ribosome. Cells expressing MRPS27.FLAG (a component of mt-SSU), ICT1.FLAG (a component of mt-LSU) or PDE12.FLAG (mitochondrial RNase that does not associate with the mitoribosome) were lysed and IP was performed using anti-FLAG antibodies. The lysates ('Input') and eluates were analysed by western blot with the antibodies as indicated in A. CO2—antibody against subunit 2 of mitochondrial complex IV.

well with the effectiveness of the specific siRNA (Figure 4A). Although almost complete knockdown of C7orf30 was observed after 6 days for the two siRNAs (Figure 4A and Supplementary Figure S1), it is expected that differences in the reduction of steady-state levels of the OXPHOS components between the two siRNAs result from an unequal rate of gene inactivation.

Inhibition of C7orf30 expression by 'siRNA 1' over 6 days reduced the abundance of complexes I and IV subunits by 70 and 65%, respectively (Figure 4B). The steady-state levels of a component of complex III was also down-regulated, though to a lesser extent—by 30% after 6 days of siRNA treatment (Figure 4B). There was no marked reduction in the steady-state levels of complex

II, containing only nuclearly encoded subunits, suggesting that the C7orf30 depletion interferes only with mitochondrial gene expression. The observed decrease in respiratory chain components was accompanied by a reduced cellular OCR of <50% (Figure 4C). Quantification of the steady-state levels of mitochondrial mRNAs and rRNAs in cells subjected to C7orf30 RNAi did not reveal any marked reductions (Figure 4D). These results indicate that C7orf30 plays an important role in mitochondrial energy production. Given its association with the mitoribosome, and lack of observed reduction in the steady-state levels of mitochondrial transcripts upon gene inactivation we hypothesized that this role is fulfilled via contributions to mitochondrial translation.

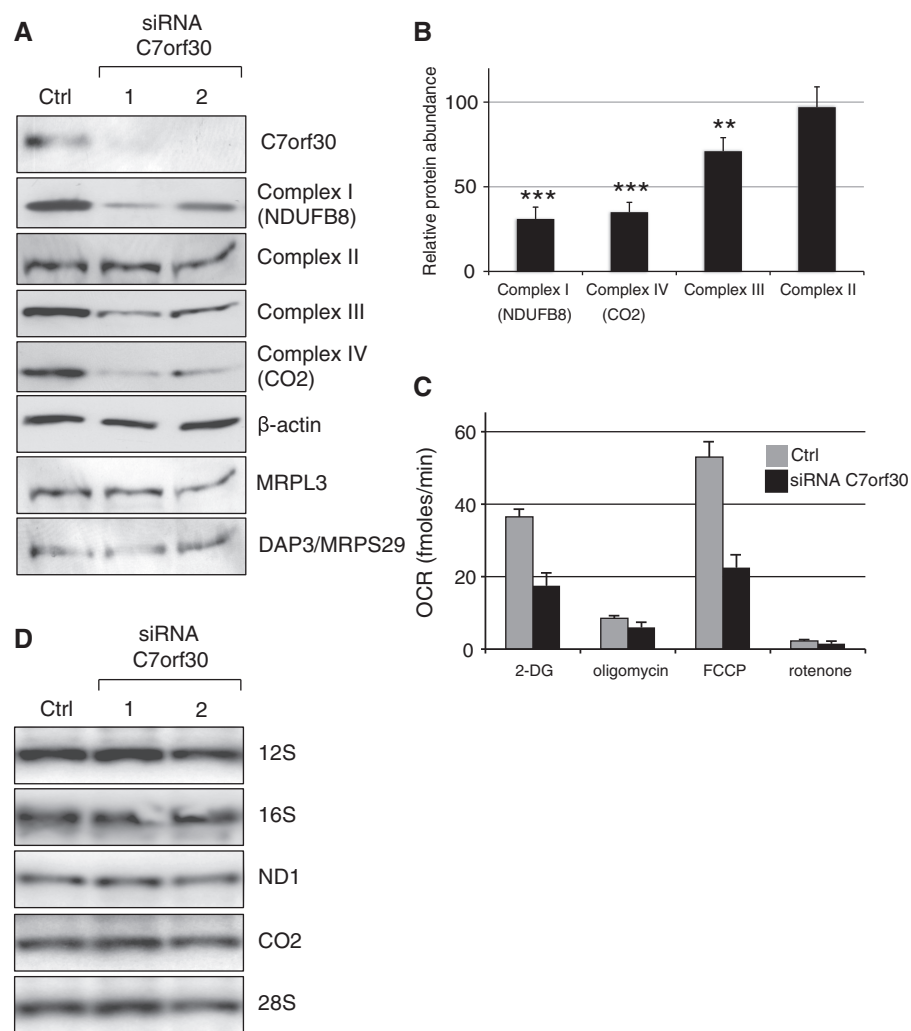


Figure 4. Inactivation of *C7orf30* perturbs OXPHOS function without affecting mitochondrial transcripts. (A) Steady-state level of the OXPHOS subunits in cells transfected with siRNA to *C7orf30*. Steady-state protein levels of the endogenous *C7orf30* protein, subunits of respiratory chain complexes and components of the mitoribosome (DAP3/MRPS29 and MRPL3) were analysed by western blotting in control cells transfected with an unrelated siRNA (Ctrl) or cells transfected with two siRNAs specific for *C7orf30* for 6 days. β -actin was used as a loading control. (B) Quantification of steady-state levels of OXPHOS components in *C7orf30*-depleted cells. Western blot signals from the experiments as per (A) for 'siRNA 1' were quantified for *C7orf30*-depleted cells using ImageQuant software. Relative abundance is presented as a percentage of the steady-state level for control cells transfected with an unrelated siRNA. $n = 3$, $**P < 0.01$, $***P < 0.001$; two-tailed unpaired Student's *t*-test error bars = 1 SD. (C) OCR in *C7orf30*-depleted cells. OCR measured in an extracellular flux Seahorse instrument in control cells transfected with an unrelated siRNA or cells treated with siRNA to *C7orf30* for 6 days. The wells containing cells were sequentially injected with 20mM 2-DG to inhibit glycolysis, 100 nM oligomycin to inhibit ATP-synthase, 1 μ M FCCP to uncouple the respiratory chain and 200 nM rotenone to inhibit complex I. $n = 6$, error bars = 1 SD. (D) Steady-state levels of mitochondrial transcripts upon inactivation of *C7orf30*. Total RNA from control cells (Ctrl) and cells transfected with two siRNAs specific for *C7orf30* were analysed by northern blots using radioactive probes specific for the indicated mitochondrial transcripts. Nuclear-encoded 28S rRNA was used as a loading control.

***C7orf30* gene silencing inhibits mitochondrial translation, alters the profile of mt-LSU and results in reduction of monosome formation**

In order to establish whether OXPHOS defects observed upon inactivation of *C7orf30* (Figure 4) were a result of impaired mitochondrial protein synthesis, we inhibited cytosolic translation and labelled *de novo* synthesized mitochondrially encoded subunits of the electron transport chain with ^{35}S -Met. The incorporation of radioactive ^{35}S -Met into mitochondrial proteins was analysed 6 days after the HeLa cells were transfected with *C7orf30* siRNA. Translation of all mitochondrially encoded respiratory

chain subunits was compromised upon *C7orf30* gene silencing, with incorporation of ^{35}S -Met reduced by <60% (for the more potent *C7orf30* siRNA oligo 1) as compared to controls transfected with an unrelated siRNA (Figure 5A). In order to investigate whether inactivation of *C7orf30* has an effect on the integrity of the mitochondrial ribosome, we carried out gradient sedimentation analyses of lysates from HeLa cells transfected with *C7orf30* siRNA for 6 days and control cells transfected with an unrelated siRNA (Figure 5B and Supplementary Figure S2). Analysis of the sedimentation of mitoribosomal subunits upon silencing of *C7orf30* revealed anomalous sedimentation of mt-LSU.

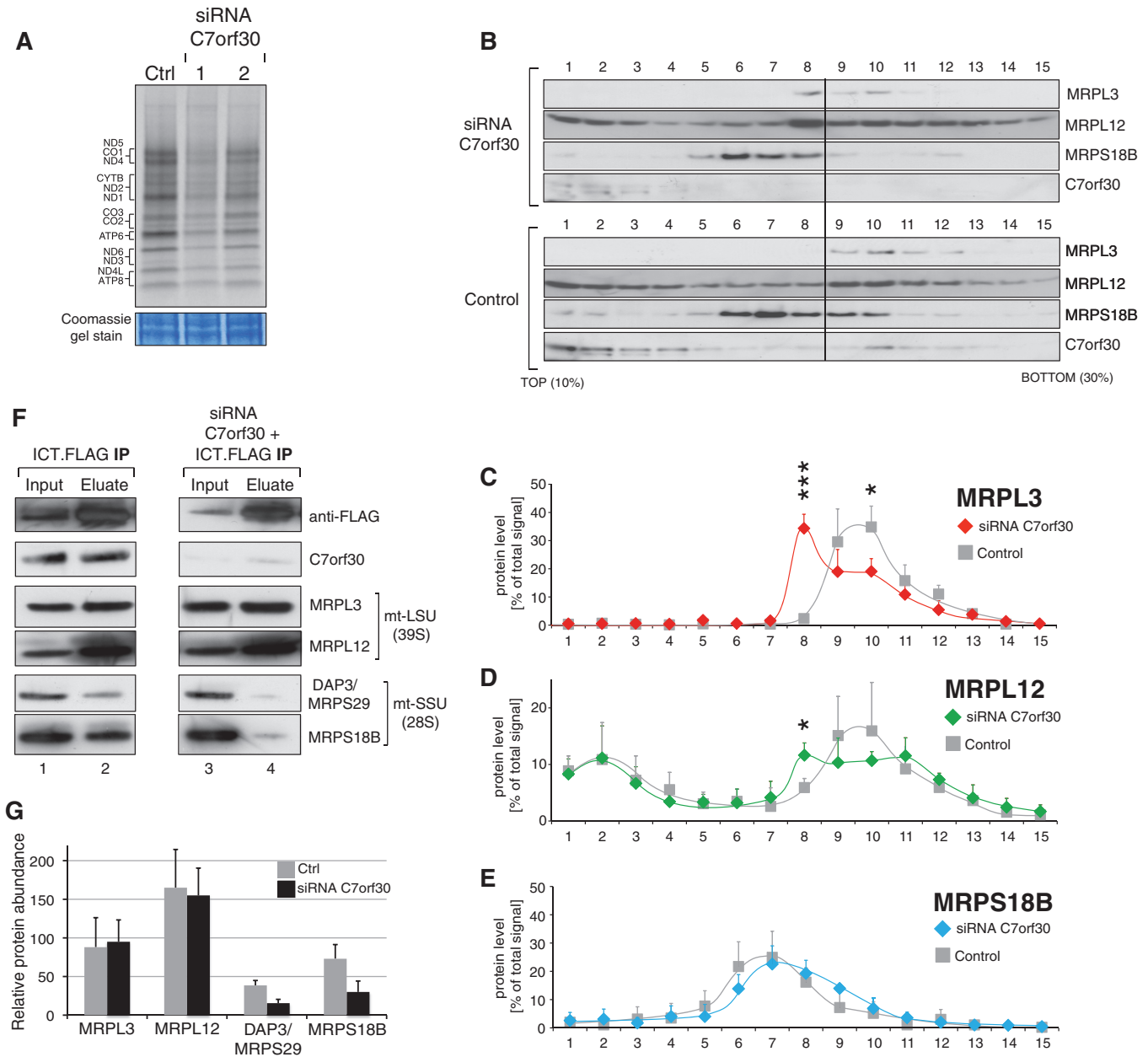


Figure 5. Mitochondrial translation and mitoribosome integrity upon inactivation of C7orf30. (A) Mitochondrial translation in cells treated with siRNA to C7orf30. Products of mitochondrial translation were labelled with ^{35}S methionine in control cells transfected with an unrelated siRNA (Ctrl) or cells transfected for 6 days with two siRNAs specific for C7orf30. Mitochondrial proteins were separated by 4–12% gradient SDS–PAGE and visualized by autoradiography. To validate equal protein loading, a small section of the gel was stained with Coomassie dye. (B) The mitochondrial ribosome profile in C7orf30-depleted cells. Total cell lysates of control HeLa cells transfected with an unrelated siRNA or HeLa cells transfected with siRNA to C7orf30 were separated on a 10–30% (v:v) isokinetic sucrose gradient. Fractions obtained for control and RNAi-treated cells were analysed by western blotting with antibodies to C7orf30, mt-LSU (MRPL3, MRPL12) and mt-SSU (MRPS18B). (C–E) Quantification of the gradient distribution of mitoribosomal components upon C7orf30 silencing. Western blot signal for MRPL3 (C), MRPL12 (D) and MRPS18B (E) for control and C7orf30-depleted cells was quantified using ImageQuant software. MRPL3 $n = 3$, MRPL12 $n = 3$, MRPS18B $n = 4$. All significant value changes are indicated as $*P < 0.05$, $***P < 0.001$; two-tailed unpaired Student's t -test. Error bars = 1 SD. The remaining western blots used for quantification are shown in Supplementary Figure S2. (F) IP of the mitochondrial ribosome from C7orf30-depleted cells. Cells expressing ICT1.FLAG (a component of mt-LSU) transfected with an unrelated siRNA or siRNA to C7orf30 were lysed and IP was performed using anti-FLAG antibodies. The lysates ('Input') and eluates were analysed by western blotting with antibodies against the FLAG tag, C7orf30, components of mt-LSU (MRPL3 and MRPL12) and mt-SSU (DAP3/MRPS29 and MRPS18B). (G) The abundance of mitoribosomal components in IP fractions of C7orf30-depleted cells. Western blot signal from the experiments as per (F) were quantified for control and C7orf30-depleted cells using ImageQuant software. Relative abundance is presented as a percentage of input, $n = 2$, Error bars = 1 SD. The remaining western blots used for quantification are shown in Supplementary Figure S3.

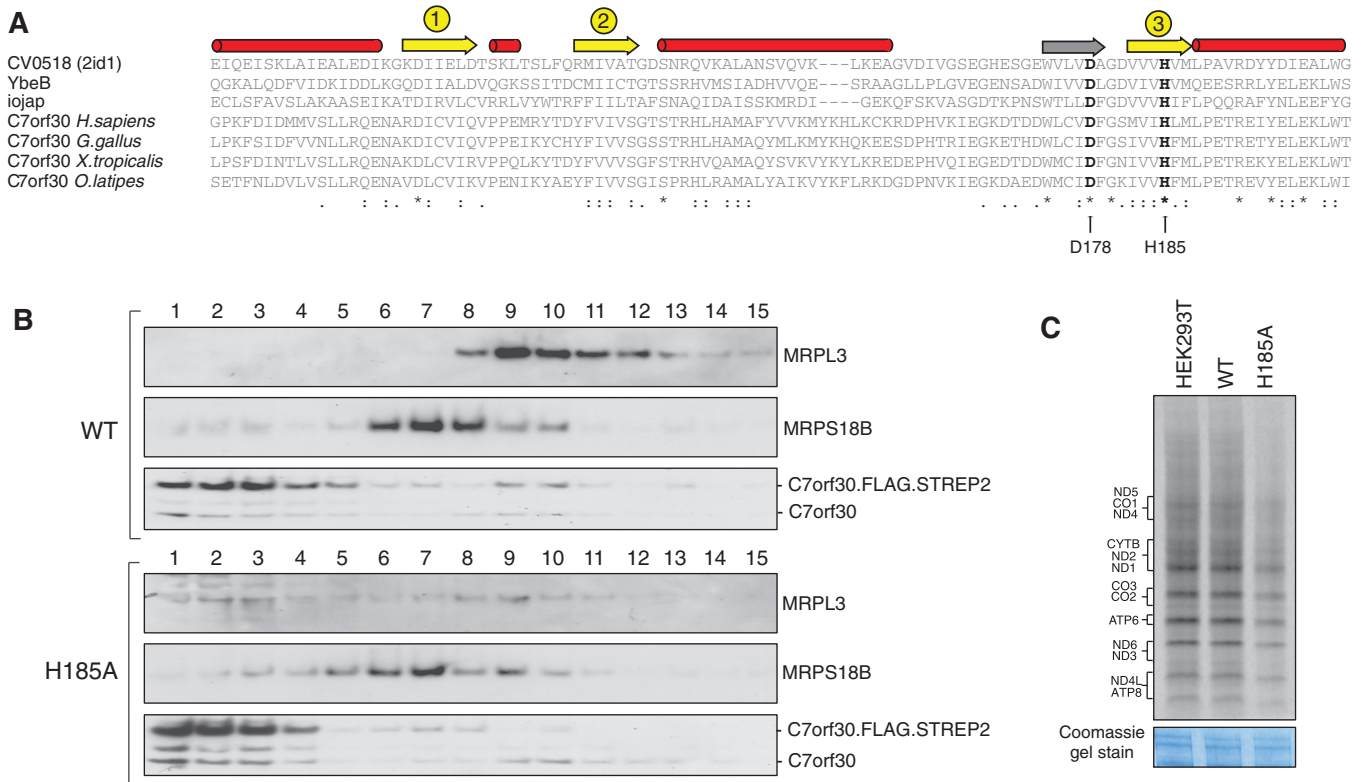


Figure 6. Effects of overexpression of C7orf30 mutants on mitochondrial translation and mitoribosome integrity. (A) Design of C7orf30 mutants. Protein sequence of *C. violaceum* CV0518, *E. coli* YbeB, *Z. mays* iojap and orthologues of C7orf30 were aligned using ClustalW2. Colons and dots denote chemical similarity between the sequences, whereas asterisks indicate identical residues. The secondary structure based on the resolved structure of CV0518 (pdb: 2id1) is indicated above the alignment [helix (cylinders), strand (arrow)]. Three β -sheets (numbered 1–3) and four α -helices of the NTase-type fold are presented in red and yellow, respectively. Two residues, D178 or H185 (numbering according to the human sequence) subjected to site directed mutagenesis are in bold. (B) Mitochondrial ribosome profile in cells overexpressing the H185A mutant of C7orf30. Cell lysates of HEK293T cells overexpressing wild-type C7orf30 or the H185A mutant for 3 days were separated on a 10–30% (v:v) isokinetic sucrose gradient. Gradient fractions were analysed by western blotting with antibodies to C7orf30, mt-LSU (MRPL3) and mt-SSU (MRPS18B). (C) Mitochondrial translation in cells overexpressing the H185A mutant of C7orf30. Products of mitochondrial translation were labelled with ^{35}S methionine in control untransfected HEK293T cells, cells overexpressing wild-type C7orf30 (WT) or the H185A mutant for 3 days. Mitochondrial proteins were separated by 4–12% gradient SDS–PAGE and visualized by autoradiography. To validate equal protein loading, a small section of the gel was stained with Coomassie dye.

A significant proportion of the mt-LSU markers (MRPL3 and MRPL12) relocated to less dense fractions as compared to control cells (Figure 5C and D, fraction 8). The profile of the mt-SSU marker MRPS18B was largely unchanged after C7orf30 depletion (Figure 5E). Of note, the steady-state levels of the component of mt-SSU and mt-LSU have not been altered upon silencing of C7orf30 (Figure 4A). Next we examined whether the alterations in mt-LSU sucrose gradient profile observed in C7orf30-depleted cells result in a concomitant reduction in the level of the intact monosome. We pulled-down mt-LSU and the entire monosome using a FLAG-tagged component of the large subunit, ICT1.FLAG, from control cells and cells treated with siRNA to C7orf30 for 6 days, then analysed the eluates by western blotting (Figure 5F and Supplementary Figure S3). The components of mt-LSU were equally well pulled-down for control and C7orf30-depleted cells (Figure 5F, lane 2 and Figure 5G). However, there was a 60% reduction in the levels of the mt-SSU proteins (DAP3/MRPS29 and MRPS18B) in the C7orf30-depleted cells (Figure 5F, lane 4 and Figure 5G), consistent with a decrease in monosome

formation, presumably due to alterations in mt-LSU integrity. These results, together with the observation that the steady-state levels of mitochondrial rRNAs were not changed (Figure 4D), suggest that the inhibition of mitochondrial translation upon inactivation of C7orf30 could be attributed mainly to instability and/or impaired assembly of the large mitoribosomal subunit, consequently interfering with formation of the mitochondrial monosome.

Overexpression of C7orf30 mutant alters the profile of mt-LSU

In order to further investigate how C7orf30 contributes to regulation of the biogenesis of mt-LSU, we overexpressed mutants of the protein in human mitochondria. A FLAG- and STREP2-tagged version of C7orf30 (C7orf30.FLAG.STREP2) was stably transfected into HEK293T cells. Similarly to the endogenous C7orf30, the recombinant protein was associated with mt-LSU as verified by sucrose gradient sedimentation of HEK293T cell extracts after induction of the transgene for 3 days with 50 ng/ml of doxycycline (Figure 6). Overexpression of

C7orf30.FLAG.STREP2 did not have any appreciable adverse effect on the mitoribosome profile (Figure 6). However, we reasoned that overexpressed mutants of C7orf30 might exhibit dominant-negative effects through, for example, outcompeting the endogenous protein for mitoribosome binding. We selected two residues conserved between C7orf30 orthologues, bacterial DUF143-containing proteins and *iojap*: D178 and H185 (Figure 6A). These residues were replaced with alanines via site-directed mutagenesis and cDNAs encoding the D178A or H185A mutants with the FLAG.STREP2 tag were stably integrated into the genome of HEK293T cells. Overexpression of the H185A mutant, but not D178A (Supplementary Figure S4) for 3 days with 50 ng/ml doxycycline resulted in a significant alteration to the profile of mt-LSU as assessed by sucrose gradient sedimentation and western blotting with specific antibodies. A significant proportion of the mt-LSU marker (MRPL3) migrated in less dense fractions typical for low molecular weight complexes as compared with control cells expressing wild-type C7orf30 (Figure 6B, fractions 1–4). The sedimentation of the H185A mutant (Figure 6B, fractions 7 and 8) was different from wild-type C7orf30, suggesting an interaction with sub-ribosomal complex(es) rather than the fully assembled mt-LSU. The profile of the mt-SSU marker, MRPS18B, was essentially unchanged after overexpression of the H185A mutant. The changes in the mitoribosomal profile in cells overexpressing the H185A mutant were accompanied by reduced mitochondrial protein synthesis as measured by incorporation of radioactive ³⁵S-Met into mtDNA-encoded proteins (Figure 6C). These results provide further evidence that C7orf30 is important for regulation of the stability and/or assembly of the large mitoribosomal subunit and suggest that β -sheet 3 of the conserved NTase-type fold is important for this process.

DISCUSSION

In all biological systems translation of the genetic information into proteins is carried out by the ribosome. Complex regulatory pathways have evolved to modulate ribosomal function in response to various stimuli. Many protein factors regulate ribosome synthesis including those involved in rRNA processing and modification, *trans*-acting assembly factors, chaperones and a variety of stress response proteins. We propose that the novel human protein C7orf30 participates in the biogenesis of the mitochondrial ribosome, in particular the biosynthesis of the large 39S mitoribosomal subunit.

C7orf30 does not regulate mitochondrial translation at RNA level

In yeast mitochondria a group of regulatory proteins exists to optimize the translation of individual mRNAs. The regulatory role of these proteins can be exerted through regulation of the stability of specific transcripts or by binding to the 5'-UTRs of mRNAs and assisting in translation initiation (21). Such translation regulatory

proteins have also been reported in mammalian mitochondria implying that, despite significant differences in mitochondrial mRNA architecture (for example: the length of UTRs or RNA polyadenylation), the requirement for precise regulation of the translation of individual transcripts is evolutionarily conserved. The TACO1 protein (CCDC44) has been identified as a specific translational activator of CO1—lack of TACO1 results in significantly reduced synthesis of the CO1 protein despite normal steady-state levels of the CO1 mRNA (5). Several human proteins of the pentatricopeptide (PPR) family have been proposed to play a regulatory role in mitochondrial translation. The PPR family of proteins is characterized by the presence of a tandemly repeated, 35-amino acid motif. Most studies of plant PPR proteins thus far have indicated various roles in RNA turnover, processing or translational regulation in mitochondria or chloroplasts (22). Mutations in human leucine-rich PPR-motif containing protein, LRPPRC (or LRP130) cause the French Canadian variant of Leigh syndrome (LSFC) (23), with proposed functions in positive translational control of mitochondrially encoded subunits of COX (24,25). Other human mitochondrial PPR proteins involved in the regulation of specific events in RNA metabolism or translational control include: (i) PTC1, a negative regulator of translation acting by modulating the abundance of the leucine tRNAs (26), PTC2, a regulator of cytochrome B mRNA maturation (27) and PTC3, which modulates mitochondrial protein synthesis via binding to 12S rRNA (28).

Given the structural homology of C7orf30 to NTases, which are mostly nucleic acid modifying enzymes, one of our initial hypotheses for the function of the protein in mitochondria considered its involvement in translation regulation at the RNA level. This position was initially supported by the observation that C7orf30 shares sequence homology with the yeast mitochondrial ATP25 protein (Figure 1), required for the processing and stability of ATP9 mRNA (18). Zeng *et al.* showed that mutations in ATP25 result in decreased levels of the ATP9 mRNA and its translation product thereby precluding assembly of the functional F₀ subunit of ATP synthase. However, the same study has shown that only the C-terminal fragment of Atp25p is sufficient to stabilize the ATP9 mRNA and restore synthesis of Atp9p (18). Our analysis revealed that the C-terminal section of Atp25p shares sequence homology with the metallo-beta-lactamase domain (Figure 1B), present in many RNA processing enzymes, such as mitochondrial RNase Z (29). As an equivalent of the Atp25p C-terminal domain is absent from C7orf30, it is likely that the human protein is not involved in regulating RNA processing and/or stability. Consistent with this conclusion is the absence of key catalytic residues of the NTase fold (Figure 1) suggesting that C7orf30 is not an active nucleic acid modifying enzyme. Also, our results of downregulation and overexpression (Joanna Rorbach and Payam Gammage, unpublished work) of C7orf30 showed no appreciable changes in the steady-state levels of any of the mitochondrial RNAs analysed (Figure 4D).

C7orf30 as a mitoribosome assembly/stability factor

A complex, multi-step pathway precedes ribosome production in all organisms studied thus far. In the yeast cytoplasm, approximately 200 proteins and 70 small nucleolar RNAs have been reported to participate in ribosome biosynthesis and at least half of these proteins are believed to function directly in the assembly and/or transport of pre-ribosomal complexes from the nucleolus to the cytoplasm (30). *Trans*-acting assembly factors are also involved in biosynthesis of the bacterial ribosome. However, owing to the lack of compartmentalization of ribosome synthesis steps fewer proteins are involved in this process. In contrast to the relatively well-studied ribosomal assembly pathway in bacteria and eukaryotic cytoplasm, assembly of the mammalian mitoribosome remains to be described.

Only two proteins playing a role in the biogenesis of mammalian mt-SSU have been identified. A homologue of bacterial Era protein, ERAL1, a member of the conserved family of GTP-binding proteins with RNA-binding activity, has been demonstrated to play a role in the formation mt-SSU (31,32). It has been suggested that ERAL1 functions as a mitochondrial RNA chaperone to protect 12S rRNA on mt-SSU during assembly (31). TFB1M (transcription factor B1, mitochondrial) is responsible for adenine dimethylation of 12S rRNA and its knock-out in mice causes impaired assembly of the mitochondrial ribosome (33). *Trans*-acting, non-ribosomal factors such as protein chaperones involved in the assembly of mt-SSU are yet to be found.

The complement of proteins comprising mt-LSU has been described by Spemulli and co-workers a decade ago (34), however, there is very limited published data on protein factors involved in assembly of this subunit. Multiple GTPases participate in the assembly of LSU in bacteria and eukaryotes (30). The yeast Mtg1p protein, a homologue of bacterial Y1qF GTPase (also known as RbgA) (35), has been suggested to function in the assembly of the ribosome in yeast mitochondria without a role in transcription or processing of mt-rRNAs (36). The evidence that a human orthologue of Mtg1p participates in an analogous process within mitochondria is circumstantial, though. Human Mtg1 is capable of partial rescue of respiratory deficiency in a yeast mtg1 mutant and it has been localized to mitochondria in human cells (36). Knock-out of NOA1 (c4orf14, mAtNOS1)—a member of the circularly permuted GTPase (cpGTPase) family—in mouse cells led to impaired protein synthesis without loss of mtDNA and/or transcription. It has been suggested NOA1 plays a role in the assembly of mt-LSU (37). hMTERF4, a member of the family of mitochondrial proteins involved in transcription regulation (38), forms a complex with a putative RNA m5C methyltransferase NSUN4. The hMTERF4-NSUN4 complex co-migrates with mt-LSU and participates in the assembly of the monosome (39). Lastly, another non-ribosomal protein involved mitoribosomal biogenesis is mitochondrial AAA (m-AAA) protease that processes MRPL32 at the late stages of the assembly (8).

Our data indicate that C7orf30 is important for the assembly and/or stability of the mitochondrial ribosome,

in particular the large 39S subunit. Consistent with the role of the DUF143 domain in ribosome assembly and/or stability is the phenotype of homozygous mutants of *iojap* in *Zea mays*; the plant homologue of C7orf30 (Figure 1A and B). Maize *iojap* is localized to plastids, and mutations in the coding gene result in depletion of chloroplast ribosomes and defects in translation. It has been previously suggested that the *iojap* phenotype results from impaired plastid ribosome assembly (10,11). Our results clearly show that RNAi-mediated depletion of C7orf30 or overexpression of a mutant of the conserved H185 residue compromised the integrity of mt-LSU. Our data also suggest that C7orf30 participates in late stages of assembly as relatively high molecular weight assemblies are still present after C7orf30 depletion. Consistent with this are the results of affinity purification of the large subunit using ICT1.FLAG. C7orf30 was clearly enriched with ICT1.FLAG, and recently published data confirmed that ICT1 is an important component of the complete monosome, not assembly intermediates. Also, the bacterial homologue of C7orf30, YbeB, purifies with the large ribosomal subunit (9), but not the 50S* particle (17), which constitutes a penultimate pre-ribosomal complex in the bacterial ribosome assembly pathway (40). Furthermore, in bacteria YbeB exists in an operon together with ORFs encoding proteins involved in late large subunit biogenesis (41). We could speculate that the DUF143 domain evolved to promote interactions between subunits of large complexes such as the ribosome or ATP synthase. The N-terminal fragment of Atp25p, the yeast homolog of C7orf30, contains the DUF143 domain. This N-terminal fragment plays a role in the oligomerization of Atp9p, a subunit of the F₀ portion of the yeast mitochondrial ATP synthase, into a correct size ring structure (18). An analogous role could be played by C7orf30 in assembly of the mitoribosomal large subunit.

In conclusion, C7orf30 is a novel human gene indispensable for mitochondrial translation, most probably via its contribution to the biogenesis of mt-LSU. The function of C7orf30 in mt-LSU is also supported by the study of Wanschers *et al.* (41). In this context C7orf30 could be prioritized for analysis as a candidate disease gene in patients with combined OXPHOS deficiencies. Further studies of this protein, for example by cross-link IP approach, should provide new insights into the largely unknown area of mitochondrial ribosome biogenesis pathway and could be helpful in identifying other protein factors involved in this process.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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