Published online 05 March 2014 *Nucleic Acids Research, 2014, Vol. 42, No. 9* ***5483–5494***
doi: 10.1093/nar/gku179

A bifunctional protein regulates mitochondrial protein synthesis

Tara R. Richman[1], Stefan M.K. Davies[1], Anne-Marie J. Shearwood[1], Judith A. Ermer[1], Louis H. Scott[1], Moira E. Hibbs[1], Oliver Rackham[1,2] and Aleksandra Filipovska[1,2,*]

[1]Harry Perkins Institute of Medical Research and Centre for Medical Research, The University of Western Australia, Nedlands, Western Australia 6009, Australia and [2]School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia 6009, Australia

Received September 26, 2013; Revised February 12, 2014; Accepted February 12, 2014

ABSTRACT

Mitochondrial gene expression is predominantly regulated at the post-transcriptional level and mitochondrial ribonucleic acid (RNA)-binding proteins play a key role in RNA metabolism and protein synthesis. The AU-binding homolog of enoyl-coenzyme A (CoA) hydratase (AUH) is a bifunctional protein with RNA-binding activity and a role in leucine catabolism. AUH has a mitochondrial targeting sequence, however, its role in mitochondrial function has not been investigated. Here, we found that AUH localizes to the inner mitochondrial membrane and matrix where it associates with mitochondrial ribosomes and regulates protein synthesis. Decrease or overexpression of the AUH protein in cells causes defects in mitochondrial translation that lead to changes in mitochondrial morphology, decreased mitochondrial RNA stability, biogenesis and respiratory function. Because of its role in leucine metabolism, we investigated the importance of the catalytic activity of AUH and found that it affects the regulation of mitochondrial translation and biogenesis in response to leucine.

INTRODUCTION

Mitochondria are ubiquitous organelles within eukaryotic cells that are essential for energy production by oxidative phosphorylation (OXPHOS) and the regulation of cell death. The biogenesis of mitochondria requires cooperative interactions between the mitochondrial and nuclear genomes. The mitochondrial genome contains 37 genes that encode 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 11 messenger RNAs (mRNAs), which produce essential components of the mitochondrial respiratory complexes (1). The 11 mRNAs are translated on mitochondrial ribosomes that are located in the mitochondrial matrix and associated with the inner membrane (2,3). The remaining mitochondrial proteins are encoded by the nuclear genome, translated on cytoplasmic ribosomes and transported into mitochondria post-translationally via specific protein complexes known as translocases, located in the outer and inner mitochondrial membranes (4,5). Although the import of nuclear encoded proteins into the mitochondria is well understood, little is known about the coordination of cytoplasmic and mitochondrial protein synthesis and assembly of membrane-bound protein complexes within mitochondria (6,7).

Recently, a number of mitochondrial proteins containing ribonucleic acid (RNA)-binding domains have been shown to be important for the regulation of post-transcriptional gene expression in mitochondria (reviewed in (8)). A search for cytoplasmic AU-rich element (ARE)-binding proteins that might direct rapid RNA degradation through mRNA deadenylation identified a 32 kDa protein known as the AU-binding homolog of enoyl-coenzyme A (CoA) hydratase (AUH) (9). The RNA-binding site of AUH was found to be unique and distinct compared to previously described motifs of known AU-binding proteins (10). Because of its RNA-binding ability and striking homology to the enoyl-CoA hydratase enzyme, AUH was suggested to have bifunctional activity in cells (9). AUH was subsequently identified as a 3-methylglutaconyl-CoA hydratase that catalyzes the conversion of 3-methylglutaconyl-CoA to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) in leucine catabolism (11). Mutations in the AUH gene have been shown to cause the metabolic disorder, 3-methylglutaconic aciduria type I (MGA I) that results in 3-methylglutaconyl-CoA deficiency (12,13). Although AUH belongs to the enoyl-CoA hydratase/isomerase superfamily, it is the only homolog that has RNA-binding activity, likely as a result of positive charges that coat the surface of the protein (14), and are required for RNA-binding but not its catalytic activity (10).

Analysis of the mouse AUH gene and the encoded protein revealed that AUH was present in mitochondria and its distribution enriched in organs with higher energy demands including skeletal muscle, heart and brain (15), however, the

*To whom correspondence should be addressed. Tel: +61 8 6151 0736; Fax: +61 8 6151 0736; Email: aleksandra.filipovska@uwa.edu.au

role of AUH in mitochondria has not been investigated. Here, we show that AUH is localized to the mitochondrial matrix and the inner mitochondrial membrane through its association with mitochondrial ribosomes. AUH regulates mitochondrial protein synthesis and is important for mitochondrial RNA metabolism, biogenesis, morphology and function. Finally, we show that changes in AUH levels affect translation in response to leucine levels and mitochondrial morphology in cells.

MATERIALS AND METHODS

Expression plasmids

Transient expression vectors were based on pcDNA3 (Invitrogen). Human AUH (NCBI accession number NM_001698) was expressed fused to a C-terminal tandem affinity purification (TAP, derived from pCeMM-CTAP(SG), obtained from EUROSCARF, ABO76910) tag, or EGFP (enhanced green fluorescent protein) (BD Biosciences) at the C-terminus, or with its natural stop codon to produce untagged AUH. To make stable cell lines, AUH-CTAP was subcloned into the pEF plasmid (16). The A240V and E209A mutations were introduced by QuikChange mutagenesis (Stratagene). MTERF-TAP (NM_006980) was fused to the TAP tag as a control plasmid. Plasmids were tested for expression by transfection and immunoblotting.

Cell culture

143B cells were cultured at 37°C under humidified 95% air/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies) containing glucose (4.5 g l^{-1}), 1 mM pyruvate, 2 mM glutamine, penicillin (100 U ml^{-1}), streptomycin sulfate (100 μg ml^{-1}) and 10% fetal bovine serum (FBS).

Mitochondrial isolation and subfractionation

Mitochondria were prepared from 10^7 cells grown overnight in 15 cm^2 dishes and isolated as described previously (17) with some modifications. Mitochondria were lysed for 30 min in buffer containing 250 mM sucrose, 100 mM KCl, 20 mM magnesium acetate, 10 mM Tris–HCl pH 7.5, 0.5% Triton X-100 and ethylenediaminetetraacetic acid (EDTA)-free Complete protease inhibitor cocktail (Roche). The clarified mitochondrial lysates were loaded on continuous sucrose gradients prepared in the same buffer without detergent. Subfractionation was performed as described previously (17), 20 μg of protein were resolved by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes and analyzed by immunoblotting.

Transient and stable cell transfections

143B cells were plated at 60% confluence in six-well plates, 10 or 15 cm dishes and transfected with annealed small interfering RNAs (siRNAs) or mammalian expression plasmids in OptiMEM media (Gibco, Life Technologies). 125 nM (for six-well plates) or 145 nM (for 10 cm dishes) of AUH or control, off-target siRNAs (Dharmacon) were transfected using Lipofectamine 2000 (Invitrogen, Life Technologies). 158 ng/cm^2 of AUH or control EGFP plasmid DNA was transfected using Fugene HD (Roche). Cell incubations were carried out for up to 72 h following transfection. For 6- or 9-day transfections cells were re-seeded and re-transfected every 72 h. AUH expression was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and by immunoblotting.

Stable expression of plasmids encoding AUH, EGFP-TAP, AUH E209A or AUH A240V was established by transfecting 0.5×10^6 143B cells with 1.5 μg of pEF plasmids in OptiMEM media (Invitrogen) using Fugene HD (Roche). After 48 h, the transfection media was replaced with DMEM containing glucose (4.5 g l^{-1}), 1 mM pyruvate, 2 mM glutamine, penicillin (100 U ml^{-1}), streptomycin sulfate (100 μg ml^{-1}), 10% FBS, 25 μg/ml uridine and 1 μg/ml puromycin. Human AUH-GIPZ lentiviral shRNAmir (clone ID V2LHS_132885, Gene Targeting Sequence CTCGCTATAAAGGAGAATA) viral particles (ThermoScientific) were used to stably knockdown AUH in cells according to the manufacturer's instructions. Non-silencing-GIPZ lentiviral shRNAmir control viral particles were used as a non-targeting control. Clonal selection was carried out to expand cells that stably expressed AUH or EGFP proteins or stably knocked down AUH compared to cells transfected with control shRNAs. The expression levels of the AUH mRNA and protein relative to controls were determined by qRT-PCR and immunoblotting, respectively.

Fluorescence cell microscopy

143B cells were plated onto 13 mm diameter glass coverslips and allowed to attach overnight. Cells were transfected with pAUH-EGFP for 48 h, and at the end of the incubation treated with 50 nM Mitotracker Orange for 15 min then washed with Tris buffered saline (5 mM Tris–HCl, (pH 7.4), 20 mM NaCl, TBS). Cells were mounted in 1, 4-Diazabicyclo-octane (DABCO)/polyvinyl alcohol (PVA) medium. Images were acquired using a Nikon Ti Eclipse inverted microscope using a Nikon ×60 objective.

Affinity purification and RNA isolation

143B cells were lysed in 50 mM Tris–HCl (pH 7.5), 125 mM NaCl, 5% glycerol, 1% Igepal CA-630, 1.5 mM $MgCl_2$, 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1× Complete protease inhibitors (Roche), 200 U ml^{-1} RNaseOUT (Invitrogen, Life Technologies) at 4°C. The lysate was cleared by centrifugation and incubated with rabbit-IgG agarose (Sigma) at 4°C for 2 h. The agarose was washed with lysis buffer and then with cleavage buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1% Igepal CA-630, 200 U ml^{-1} RNaseOUT) and protein was eluted by addition of 0.25 U μl^{-1} of AcTEV protease (Invitrogen, Life Technologies, room temperature (RT) for 1 h). The MRPS27 and MRPL11 proteins associated with AUH were detected by immunoblotting. RNA was isolated using the miRNeasy Mini kit (Qiagen) incorporating an on-column RNase-free DNase digestion to remove all deoxyribonucleic acid (DNA) and qRT-PCR was performed as below.

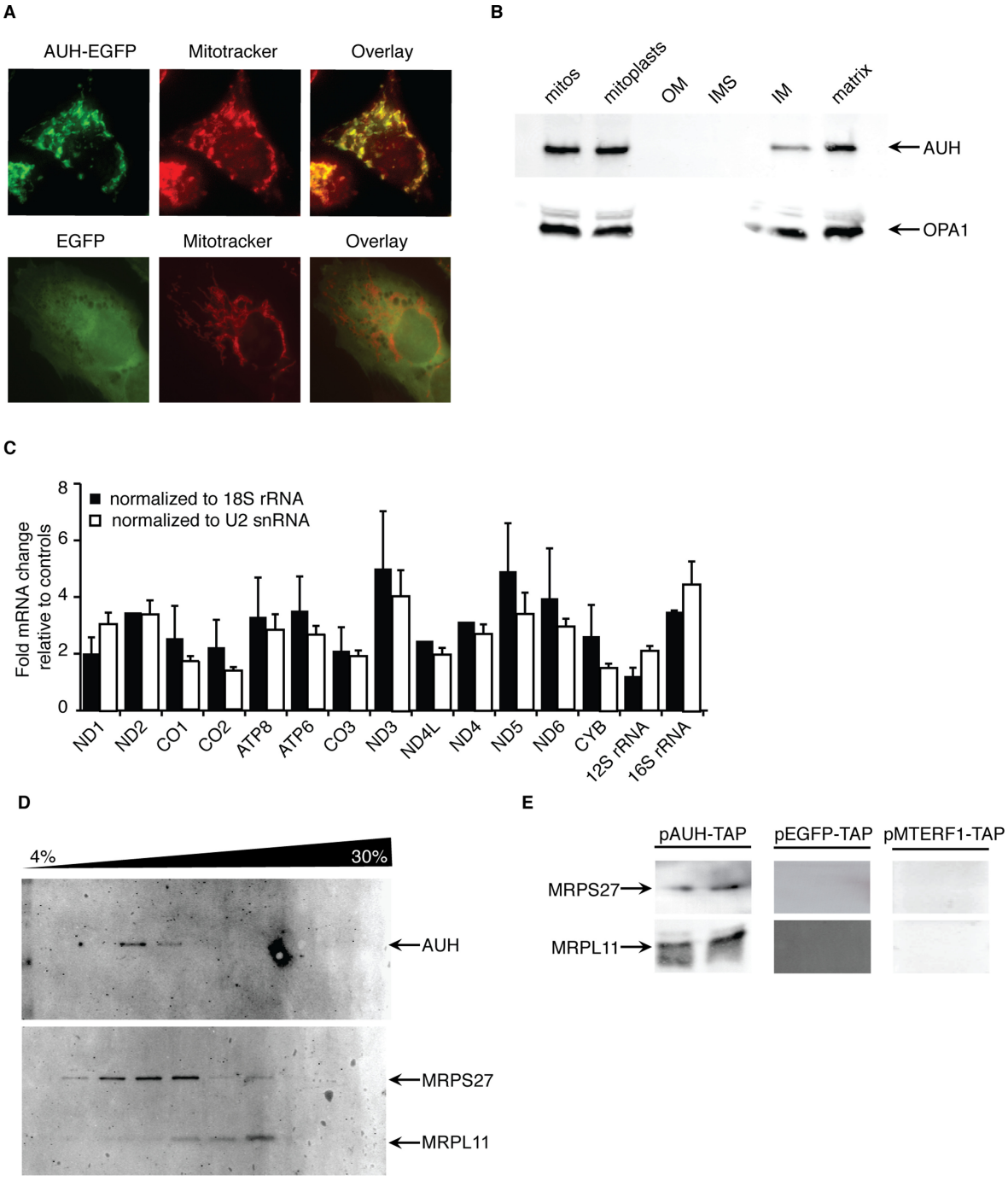

Figure 1. AUH is a matrix and inner membrane mitochondrial protein. (**A**) 143B cells were transiently transfected with pAUH-EGFP or pEGFP and incubated with 50 nM Mitotracker Orange and fixed. AUH-EGFP (green) was co-localized to mitochondria stained with Mitotracker Orange (red) directly by fluorescence microscopy. In the overlaid images, yellow indicates co-localization of AUH with mitochondria. (**B**) Isolated mitochondria were fractionated into their four compartments, proteins from each fraction were resolved by SDS-PAGE and marker proteins for each mitochondrial compartment were detected by immunoblotting. AUH associated predominantly with the mitochondrial inner membrane and matrix. The data in (A) and (B) are representative of results obtained from four to six biologically independent experiments. (**C**) AUH-TAP protein expressed in 143B cells was purified using rabbit IgG agarose and eluted by tobacco etch virus (TEV) protease cleavage. RNA associated with the eluted AUH-TAP was analyzed by qRT-PCR. Values are expressed as a ratio of AUH-TAP compared to purification from cells expressing EGFP-TAP protein as control. Data normalized to 18S rRNA and U2 small nuclear RNA (RNU2) are means ± standard deviation of six and three independent biological experiments, respectively. Student's 2-tailed *t* test for paired data was carried out and showed that all mitochondrially encoded mRNAs were enriched relative to the controls, $P < 0.05$, with the exception of the 12S rRNA when normalized to 18S rRNA. (**D**) A continuous sucrose gradient was used to resolve mitochondrial proteins from cells expressing AUH-TAP. Fractions were removed and used to determine protein distribution relative to mitochondrial ribosomal protein markers of the small (MRPS27) and large (MRPL11) ribosomal subunits by immunoblotting with specific antibodies. (**E**) AUH, MTERF1 and EGFP were purified using TAP tags and the association with the mitochondrial ribosomal subunit was assessed by immunoblotting using MRPS27 and MRPL11 antibodies. The data in (D) and (E) are representative of results obtained from four biologically independent experiments.

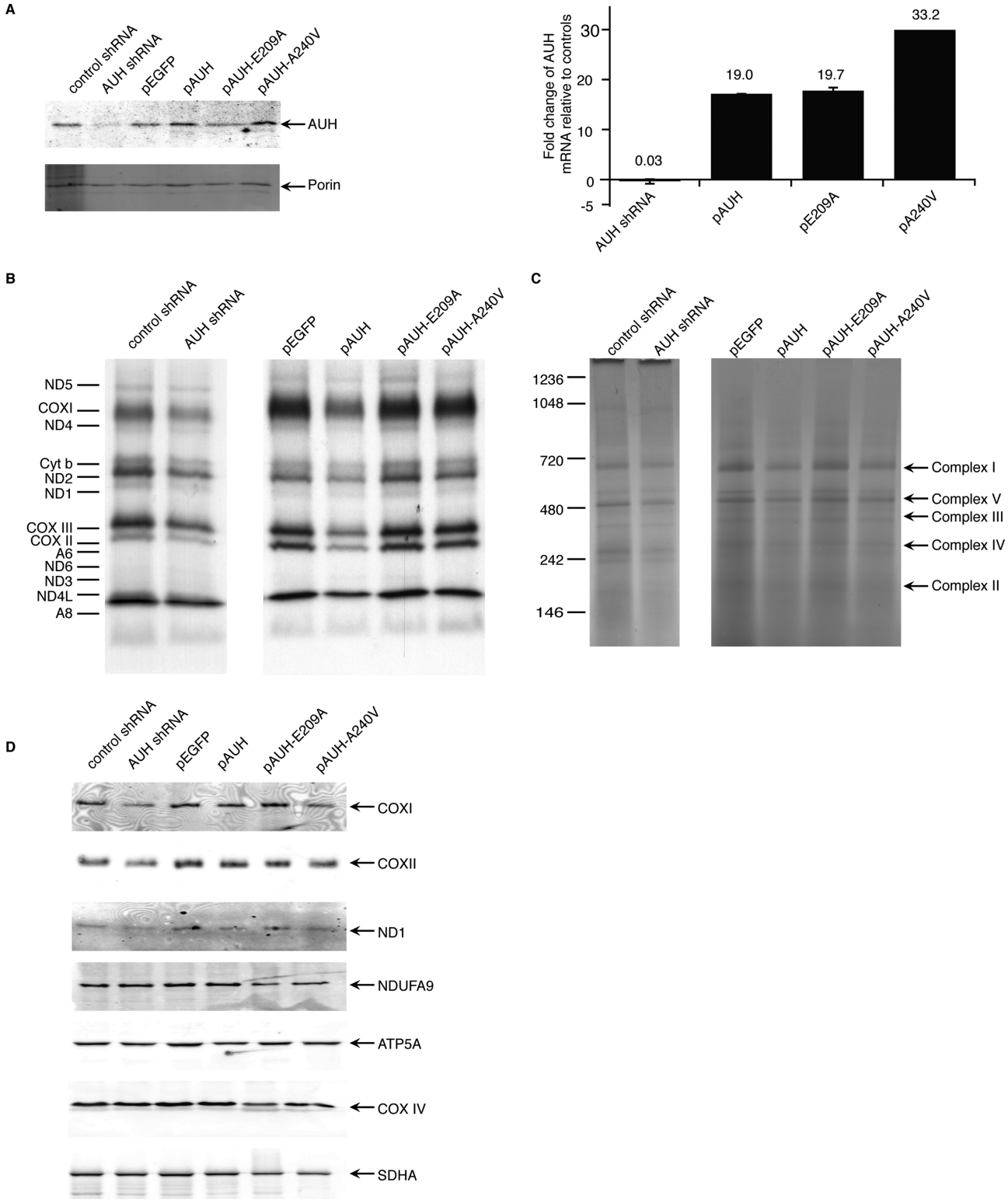

Figure 2. AUH affects mitochondrial protein synthesis. (**A**) AUH protein and mRNA levels after knockdown and overexpression of AUH and overexpression of the mutant AUH-E209A and AUH-A240V proteins relative to non-targeted shRNA or EGFP-TAP controls, measured by immunoblotting and qRT-PCR, respectively. Porin was used as a loading control. (**B**) AUH knockdown and overexpression lowers mitochondrial translation in cells. Protein synthesis was measured by pulse incorporation of 35S-labeled methionine and cysteine. Equal amounts of cell lysate protein (20 μg) were separated by SDS-PAGE and visualized by autoradiography. The gels were stained with Coomassie to confirm equal loading (Supplementary Figure S1A). (**C**) BN-PAGE of mitochondrial respiratory complexes following AUH knockdown and overexpression. Equal amount of mitochondrial lysates (85 μg) was loaded per lane. (**D**) Protein expression of mitochondrial and nuclear encoded proteins following knockdown and overexpression of AUH and the mutant AUH-E209A and AUH-A240V proteins in cells was detected by immunoblotting using antibodies against subunits of Complex I (ND1 and NDUFA9), Complex II (SDHA), Complex IV (COXI, COXII and COXIV) and Complex V (the alpha subunit of the ATP synthase, ATP5A). The data shown are typical of results obtained from at least four biologically independent experiments.

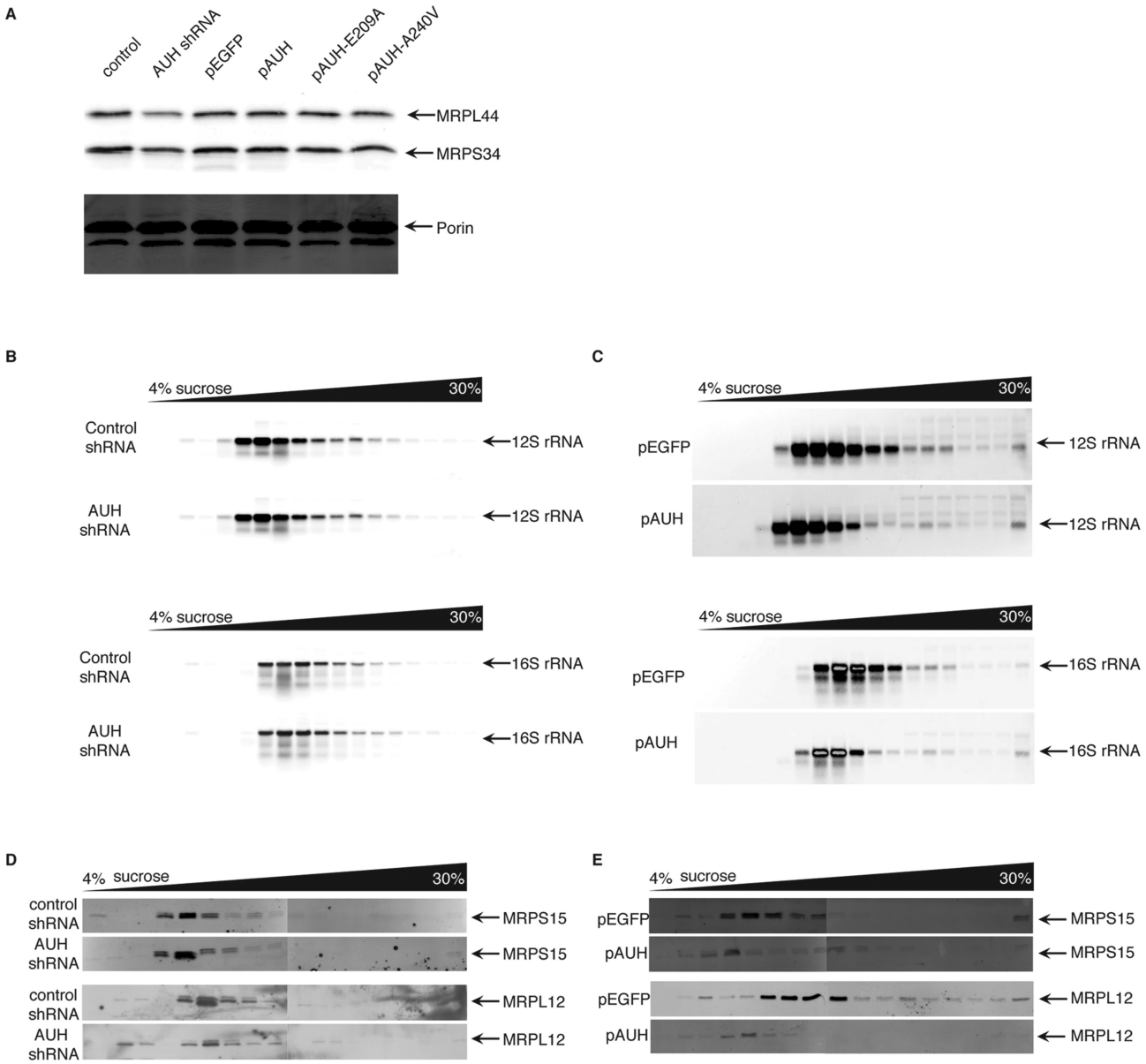

Figure 3. AUH affects ribosome stability and polysome formation. (**A**) Abundance of mitochondrial ribosomal proteins detected by immunoblotting. Quantification of three independent biological experiments is provided in Supplementary Figure S2. (**B**) Distribution of rRNAs following AUH knockdown, analyzed by fractionating ribosomes on sucrose gradients and northern blotting against these transcripts. (**C**) Distribution of rRNAs following AUH overexpression compared to control. (**D**) Distribution of ribosomal proteins analyzed by immunoblotting against ribosomal subunit marker antibodies following AUH knockdown. (**E**) Distribution of ribosomal proteins analyzed by immunoblotting following AUH overexpression. The data are typical of results from three independent biological experiments.

Sucrose gradient centrifugation

Mitochondria isolated from 143B cells (0.25 mg) untreated, treated with viral particles to knockdown AUH or plasmids for AUH overexpression were lysed in 30 mM Tris–HCl, 150 mM NaCl, 1.5% n-Dodecyl β-D-maltoside (DDM) for 30 min, the lysate centrifuged at 10,000 *g* for 10 min, the clarified lysate was loaded on a 3.5 ml continuous 4–30% sucrose gradient (in 20 mM Tris, 10 mM magnesium acetate, pH 7.5, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF)) and centrifuged at 100 000 g for 3 h using a SW60 Ti rotor in an Optima Beckman Coulter preparative ultracentrifuge. Fractions were collected and precipitated with 30% trichloroacetic acid, washed in acetone, and the entire fraction was resolved by SDS-PAGE. The ribosomal subunits and AUH-TAP were detected by immunoblotting. Alternatively, RNA was isolated from the collected fractions and northern blotting was carried out as described below.

Immunoblotting

Specific proteins were detected using rabbit polyclonal antibodies against: MRPL11, MRPS15, porin, NDUFA9 (a subunit of Complex I), Complex II (SDHA), COX1, COXII, COXIV and Complex V subunit α (Abcam di-

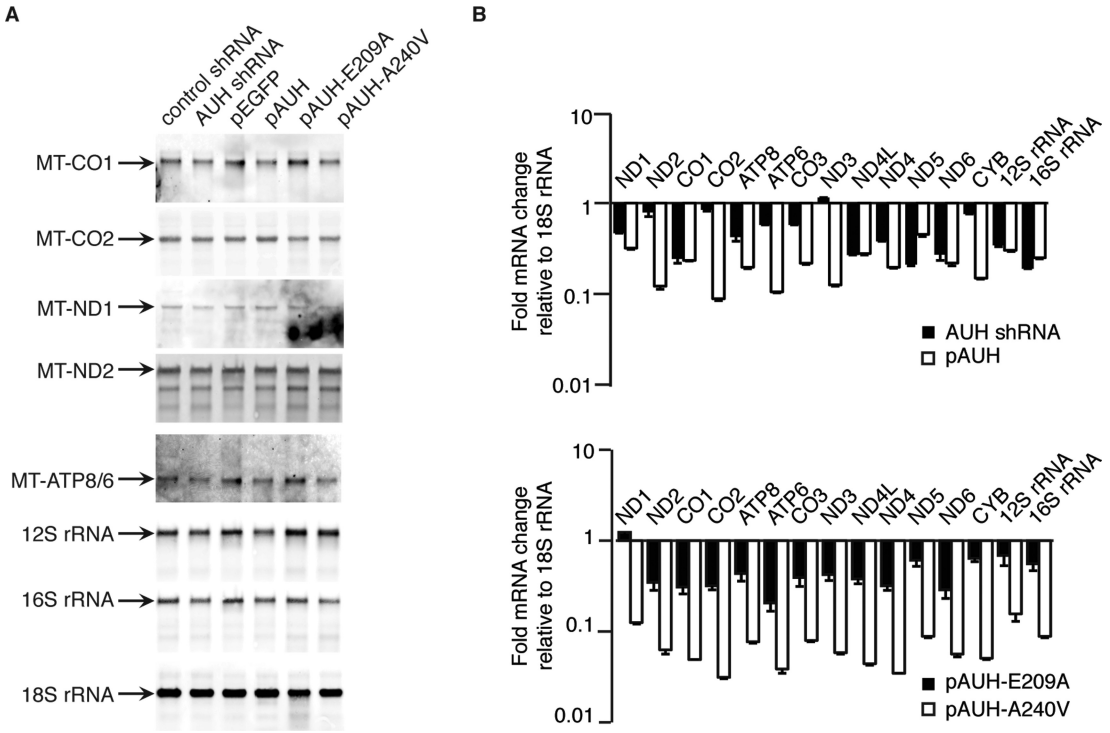

Figure 4. AUH affects mitochondrial RNA metabolism. (**A**) The abundance of mature mitochondrial transcripts was analyzed by northern blotting. The data are typical of results repeated on three separate RNA preparations. (**B**) RNA isolated from cells with AUH knockdown, overexpression or mutation was analyzed by qRT-PCR. The data is expressed as a ratio of transcripts from experimental samples compared to control samples. Amplification of mature mitochondrial mRNA transcripts is shown. Data normalized to 18S rRNA are means ± standard deviation of four independent biological experiments, respectively. Similar effects were observed when qRT-PCR results were normalized to the U2 snRNA (Supplementary Figure S3). Student's 2-tailed *t* test for paired data was carried out and showed that mitochondrial mRNAs were decreased relative to the controls, $P < 0.05$. There was no significant change in MT-ND3 upon AUH knockdown and MT-ND1 upon AUH-E209A overexpression.

luted 1:1000), AUH (Sigma, diluted 1:500) and MRPS27 (Proteintech, diluted 1:500) and mouse monoclonal antibodies against: MRPS35 (Sigma, diluted 1:1000), MRPL44 (Proteintech, diluted 1:1000), MRPL12 (Abnova, diluted 1:1000) in Odyssey Blocking Buffer (Li-Cor). IR Dye 800CW Goat Anti-Rabbit IgG or IRDye 680LT Goat Anti-Mouse IgG (Li-Cor) secondary antibodies were used and the immunoblots were visualized using an Odyssey Infrared Imaging System (Li-Cor).

Northern blotting

RNA (5 μg) was resolved on 1.2% agarose formaldehyde gels, then transferred to 0.45 μm Hybond-N^+ nitrocellulose membrane (GE Lifesciences) and hybridized with biotinylated oligonucleotide probes specific to mitochondrial tRNAs. The hybridizations were carried out overnight at 50°C in 5× SSC, 20 mM Na_2HPO_4, 7% SDS and 100 μg ml^{-1} heparin, followed by washing. The signal was detected using either streptavidin-linked horseradish peroxidase or streptavidin-linked infrared antibody (diluted 1:2000 in 3× SSC, 5% SDS, 25 mM Na_2HPO_4, pH 7.5) by enhanced chemiluminescence (GE Lifesciences) or using an Odyssey Infrared Imaging System.

Quantitative RT-PCR

The transcript abundance of mitochondrial genes and preprocessed junctions was measured on RNA isolated from cells using the miRNeasy RNA extraction kit (Qiagen). Levels of AUH mRNA were measured from RNA isolated from cells or purified mitochondria. Complementary DNA (cDNA) was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) and used as a template in the subsequent PCR that was performed using a Corbett Rotorgene 6000 using SensiMix SYBR mix (Bioline) and normalized to 18S rRNA, U2 snRNA and HPRT1 mRNA.

Mitochondrial protein synthesis

Cells were grown in six-well plates until 60% confluent, transfected for 3 or 9 days or stably transfected cells were used and *de novo* protein synthesis was analyzed, as described previously (18).

Blue native page electrophoresis

BN-PAGE was carried out using isolated mitochondria from stably transfected cells where AUH was knocked down or overexpressed wild-type AUH, or AUH with either the E209A or A240V mutation and their respective controls, as described previously (19).

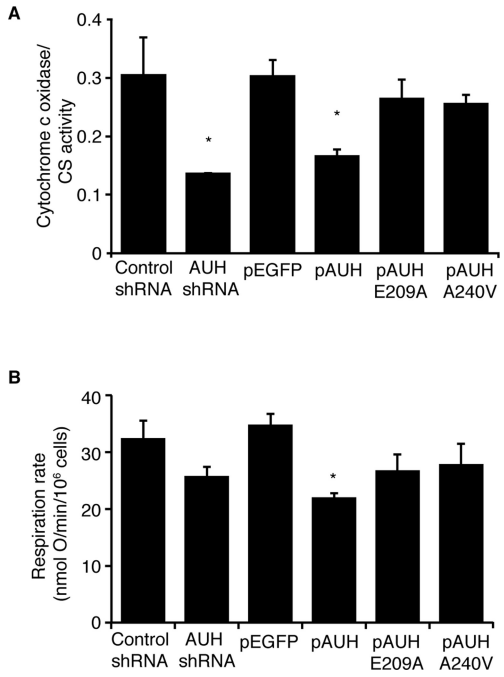

Figure 5. Knockdown and overexpression of AUH decrease mitochondrial respiratory function. (**A**) The enzyme activity of cytochrome oxidase was measured spectrophotometrically and normalized to citrate synthase activity. (**B**) State 4 respiration on glutamate/malate was measured in digitonin-permeabilized 143B cells where endogenous levels of AUH were stably knocked down or in cells overexpressing wild type AUH or mutant AUH-E209A and AUH-A240V proteins using an OROBOROS oxygen electrode. Data are means ± standard error of the mean of three to four separate experiments; $*P < 0.05$ compared with control treatments by a 2-tailed paired Student's *t* test.

Respiration

State 3 respiration using 0.5 mM N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and 2 mM ascorbate was measured in permeabilized cells according to (20).

Complex enzyme assays

Enzyme assays of citrate synthase and Complex IV were carried out in a 1 ml cuvette at 30°C using a Perkin Elmer lambda 35 dual beam spectrophotometer, as described previously (21).

Leucine treatment of cells

Stably transfected 143B cells were grown in six-well dishes with DMEM containing 10% FBS for 48 h. Media was replaced with DMEM containing 0.5% FBS and after 18 h, the cells were deprived of amino acids by incubation in Hank's balanced salt solution (HBSS, Life Technologies) supplemented with a vitamin mixture (Life Technologies) for 3 h. The cells were analyzed for *de novo* protein synthesis as described above with a minor modification that they were incubated in the presence or absence of 10 mM leucine with emetine for 15 min before the labeling commenced.

RESULTS

AUH is localized to the mitochondrial matrix where it associates with mitochondrial ribosomes

To investigate the localization of AUH, we fused it to EGFP at the C-terminus and visualized its cellular distribution by fluorescence microscopy. AUH localizes to mitochondria in cells confirmed by its co-localization with the mitochondria specific marker, Mitotracker Orange (Figure 1A). To determine where AUH resides within mitochondria we prepared mitoplasts (mitochondria devoid of their outer membrane) as well as fractionated purified mitochondria in their four compartments, outer membrane, inner membrane space, inner mitochondrial membrane and matrix. We analyzed the distribution of AUH within these fractions and found it enriched in mitoplasts suggesting that the protein was not associated with the outer mitochondrial membrane. AUH was found predominantly in the matrix and at the inner mitochondrial membrane (Figure 1B). We found that the distribution of AUH within mitochondria is similar to that for the OPA1 protein that is involved in inner mitochondrial membrane fusion (22).

To determine the RNA targets of AUH we fused the C-terminus of AUH to a TAP tag and expressed this protein in 143B cells. We isolated the AUH-TAP protein by affinity purification from cells and measured the enrichment of mitochondrial encoded mRNAs and rRNAs by qRT-PCR. We found that most of the mitochondria encoded transcripts were enriched compared to the control RNAs (Figure 1C), suggesting that AUH has a role in mitochondrial post-transcriptional regulation.

Next we analyzed if AUH associates with mitochondrial ribosomes, because of its localization at the inner mitochondrial membrane and association with most of the mitochondrial transcripts, by separating mitochondrial lysates on a 4–30% continuous sucrose gradient and immunoblotting for marker proteins of the small and large subunits of mitochondrial ribosomes. AUH co-migrated predominantly with the small ribosomal subunits, indicating that it associates with the mitochondrial translation machinery (Figure 1D). To validate this association of AUH with mitochondrial ribosomes, we isolated AUH from cells using the TAP tag and used immunoblotting to detect association of AUH with protein markers of the small and large ribosomal subunits. We found that both MRPS27 and MRPL11 were enriched with AUH but not with the mitochondrial transcritption termination factor 1 (MTERF1) protein or the control EGFP, suggesting that AUH associates with mitochondrial ribosomes (Figure 1E).

AUH is involved in mitochondrial translation

Because of its association with mitochondrial ribosomes we investigated the effects of modulating levels of AUH on mitochondrial translation. We used cells that were stably expressing AUH shRNAs or non-targeting shRNAs as a control, as well as cells stably over expressing the wild-type AUH protein or EGFP as a control. In addition, we overexpressed AUH that contained the E209A mutation, which abolishes the 3-MG-CoA hydratase activity of AUH to determine if this activity is required for protein synthesis. Fur-

thermore, we overexpressed AUH that contains an A240V mutation that has been found to significantly lower the enzyme activity of AUH and cause MGA I in patients (12). We observed a significant decrease in AUH mRNA and protein levels in stable shRNA expressing 143B cells (Figure 2A). Also we found that stable transfection of the AUH plasmids resulted in an increase of AUH mRNA and protein expression (Figure 2A).

Next we investigated the effects of AUH knockdown or overexpression of wild type and mutated AUH proteins relative to their respective controls on *de novo* mitochondrial protein synthesis in cells. We observed a significant overall decrease of mitochondrial protein synthesis in cells where AUH was knocked down compared to cells that were stably transfected with control non-targeting shRNAs, suggesting that AUH is required for translation (Figure 2B). Overexpression of the wild-type AUH protein had a dominant negative effect in cells, causing reduced mitochondrial protein synthesis. However, the overexpression of AUH that contained the catalytically inactive E209A mutation or the pathogenic A240V mutation did not affect mitochondrial translation significantly, suggesting that the catalytic activity of the protein is necessary for its role in mitochondrial protein synthesis (Figure 2B).

Next we investigated the effects of stable AUH knockdown or overexpression on the abundance of the mitochondrial respiratory complexes by blue native polyacrylamide gel electrophoresis (BN-PAGE). We observed decreased abundance of respiratory complexes following knockdown of AUH. Furthermore, we found that the overexpression of the wild-type AUH protein caused a significant reduction in the complexes compared to the overexpression of mutated AUH and the EGFP control (Figure 2C). The catalytically inactive E209A mutation did not affect the abundance of respiratory complexes. However, the pathogenic A240V mutation, which unlike the E209A mutation only decreases the enzyme activity of AUH, caused a slight decrease in respiratory complex abundance relative to controls (Figure 2C).

We analyzed the effects of AUH knockdown and overexpression on the steady-state abundance of mitochondrial proteins by immunoblotting. We show that the levels of the mitochondrially encoded COXI, COXII and ND1 were reduced in cells where AUH was stably knocked down or overexpressed (Figure 2D). The catalytically inactive E209A mutation does not affect the steady-state abundance of mitochondrial proteins, although the A240V mutation causes a subtle decrease in their abundance (Figure 2D), consistent with the effects found with the BN-PAGE experiments. This confirms that AUH affects the steady-state levels of mitochondrially encoded proteins.

AUH affects ribosomal protein abundance and assembly

The decrease of core mitochondrial ribosomal proteins has been shown to cause a reduction in the abundance or stability of other ribosomal proteins (23). Therefore, we investigated the effects of AUH knockdown or overexpression on the abundance of mitochondrial ribosomal proteins from the large and small subunit. Immunoblotting showed that the abundance of MRPS34 and MRPL44, as markers of the small and large ribosomal subunits respectively, were reduced in cells where AUH was knocked down (Figure 3A and Supplementary Figure S2). This indicates that AUH is required for the steady-state levels of specific ribosomal proteins. Overexpression of the AUH proteins did not affect the stability of the ribosomal proteins significantly indicating that increased abundance of AUH does not affect the stability of ribosomal proteins. These results suggest that knocking down or overexpressing AUH causes decreased mitochondrial protein synthesis by different means.

To investigate how AUH may affect ribosome assembly and stability we analyzed the effect of AUH knockdown and overexpression on the mitochondrial ribosome profile of the 12S and 16S rRNA or ribosomal protein markers of the large and small subunit by fractionating ribosomes on sucrose gradients followed by northern blotting or immunoblotting, respectively. We show that overexpression of AUH causes a change in the distribution of the mitochondrial rRNAs, however AUH knockdown does not seem to affect the distribution of the rRNAs significantly (Figure 3B and C). Similarly, we observed that mitochondrial ribosomal proteins of the large and small subunits were re-distributed to less dense fractions of the gradient when AUH was overexpressed in cells, however ribosomal assembly was not affected when AUH was knocked down in cells (Figure 3D and E). These data indicate that although decrease or overexpression of AUH in cells affects the efficiency of translation, only reduction of AUH destabilizes mitochondrial ribosome assembly, while overexpression of AUH affects the ability of mitochondrial ribosomes to form polysomes.

Decreased protein synthesis affects mitochondrial RNA stability

We analyzed the effects of AUH knockdown and overexpression on mitochondrial RNA stability in 143B cells by qRT-PCR and northern blotting. Although transient knockdown and overexpression of AUH does not affect RNA stability (data not shown), stable knockdown or overexpression of AUH causes a decrease in mitochondrial mRNAs, including MT-CO1, MT-CO2, MT-ND1, MT-ND5, MT-CYB and MT-ATP8/6, and rRNAs (Figure 4A). We confirmed that these RNAs were decreased by qRT-PCR in cells where AUH was either knocked down or overexpressed (Figure 4B and Supplementary Figure S3). These data suggest that prolonged decrease in mitochondrial protein synthesis as a result of AUH knockdown or overexpression affects the stability of the mature transcripts encoded by the mitochondrial genome.

AUH knockdown and overexpression decrease mitochondrial respiratory function and alter mitochondrial morphology

To determine if AUH overexpression or knockdown causes mitochondrial dysfunction, we measured cytochrome c oxidase activity normalized to citrate synthase (Figure 5A) as well as Complex IV activity by respiration of digitonin-permeabilized cells on ascorbate/TMPD (Figure 5B) following knockdown and overexpression of AUH. We found that AUH knockdown or overexpression caused decreased

cytochrome c oxidase activity relative to control treatments (Figure 5A). Furthermore, we observed lowered mitochondrial respiration in cells where AUH was overexpressed or knocked down compared to controls (Figure 5B). Decrease in protein synthesis and the consequent lower abundance of respiratory complexes as a result of changes in AUH abundance in cells negatively affects mitochondrial respiration and Complex IV activity.

Mitochondrial proteins associated with the inner membrane and mitochondrial ribosomes can affect the shape and distribution of mitochondria in cells (24–26). Therefore, we investigated the effects of AUH knock down or overexpression on the morphology of mitochondria in cells. AUH knockdown in cells leads to disruption of the tubular network, typical of healthy mitochondria, and the appearance of individual punctate and rounded mitochondria (Figure 6A). Similarly, the transition from tubular to punctiform mitochondria was observed in cells stably overexpressing AUH (Figure 6A). We observed the same changes in mitochondrial morphology in cells expressing AUH fused to EGFP when we stained mitochondria with Mitotracker Orange (Figure 6B). These findings indicate that physiological levels of AUH are required for normal inner mitochondrial membrane structure and maintenance of a mitochondrial reticular network.

AUH modulates mitochondrial translation in response to leucine

The amino acid leucine has been shown to stimulate protein synthesis by cytoplasmic ribosomes (27). Since AUH has been shown to catalyze the conversion of an intermediate substrate in the leucine degradation pathway (28), we investigated the effect of leucine on protein synthesis in mitochondria and if the role of AUH in leucine catabolism affects this process. We analyzed *de novo* mitochondrial protein synthesis in cells where AUH was knocked down, or where mutant or wild-type AUH were overexpressed, in the presence of normal or excess leucine by 35S-labeled incorporation of methionine and cysteine in the 13 proteins encoded by the mitochondrial genome. We found that leucine treatment alone decreased mitochondrial translation, unlike the stimulatory effect that leucine has on cytoplasmic translation (Figure 7) (27). Moreover, we observed a significant overall decrease of mitochondrial protein synthesis when AUH was overexpressed or knocked down in cells compared to controls as seen previously (Figure 2B), and this decrease was more pronounced in the presence of leucine (Figure 7A and B). Protein synthesis was not further reduced by the expression of the catalytically inactive AUH or AUH containing the pathogenic A240V mutation in the presence of leucine. This suggests that catalytic activity of AUH may be linked to its effect on mitochondrial protein synthesis in the presence of leucine.

DISCUSSION

The human AUH protein was originally discovered as a RNA binding protein that has *in vitro* binding activity for AREs of mRNAs, that are present in the cytoplasm (9). The discovery that AUH shared sequence homology to enoyl-CoA hydratases indicated that this protein may have an additional role in cellular metabolism and led to the suggestion that AUH is a bifunctional protein (14). Mutations in the AUH gene were found to be involved in the metabolic disorder MGA1 caused by deficiency of 3-methylglutaconyl-CoA hydratase and the finding that it may be involved in leucine catabolism (12) further emphasized the importance of the enzymatic function of this protein in cells in addition to its RNA-binding activity.

Although AUH was found associated with mitochondria (15) its function inside these organelles had not been investigated. Here, we have shown that AUH is most abundant in the matrix and to a lesser degree the inner membrane of mitochondria where it associates with mitochondrial ribosomes and regulates protein synthesis. In addition, prolonged defects in mitochondrial translation in cells where AUH is stably knocked down or overexpressed affected mitochondrial mRNA and rRNA stability and mitochondrial morphology within cells. Interestingly, the catalytic activity of AUH is required for its function in mitochondrial translation and biogenesis, since we do not observe changes in mitochondrial translation when we overexpress a catalytically inactive AUH protein in cells. Overexpression of AUH carrying the pathogenic mutation A240V that has residual enzyme activity was sufficient to cause subtle decreases in mitochondrial protein abundance, further indicating that the enzyme activity is required for protein synthesis. Although the overexpression of AUH has a dominant negative effect on mitochondrial protein synthesis, its mode of action may be different to that observed when AUH is knocked down in cells. Decreased levels of AUH affected the abundance of mitochondrial ribosomal proteins and the efficiency of translation. However, overexpression of AUH led to re-distribution of rRNAs and mitochondrial ribosomal proteins suggesting that the assembly of mitochondrial ribosomes on mRNAs was compromised. Increased abundance of ribosomal factors can inhibit translation (29) and it may be that overexpression of AUH hinders mitochondrial translation in a similar manner.

In mammalian cells, mitochondrial biogenesis involves a cooperative effort of both mitochondrial and nuclear genetic systems (30). Nuclear encoded proteins are imported into the mitochondria by translocases (5,31), while the mitochondrially encoded proteins are synthesized within the organelle by mitochondrial ribosomes and inserted into the inner membrane (2,32). In addition, eukaryotic cells have a quality control system monitoring their mitochondria (24,33). Fusion and fission events, regulated by specific proteins, ensure a functioning mitochondrial network is maintained while misfolded or aggregated proteins are destroyed by proteases (24,33). Such events cause morphology changes and it has been previously noted that a decrease in connectivity and formation of short, rounded mitochondria is indicative of compromised mitochondrial function (24,26,34,35). Mitochondrial ribosomes and thereby protein synthesis of mitochondrially encoded proteins are intimately associated with the inner mitochondrial membrane (2,36). Through its localization at the inner mitochondrial membrane in association with mitochondrial ribosomes and its role in translation AUH affects the integrity of the membrane and the morphology of mitochondria.

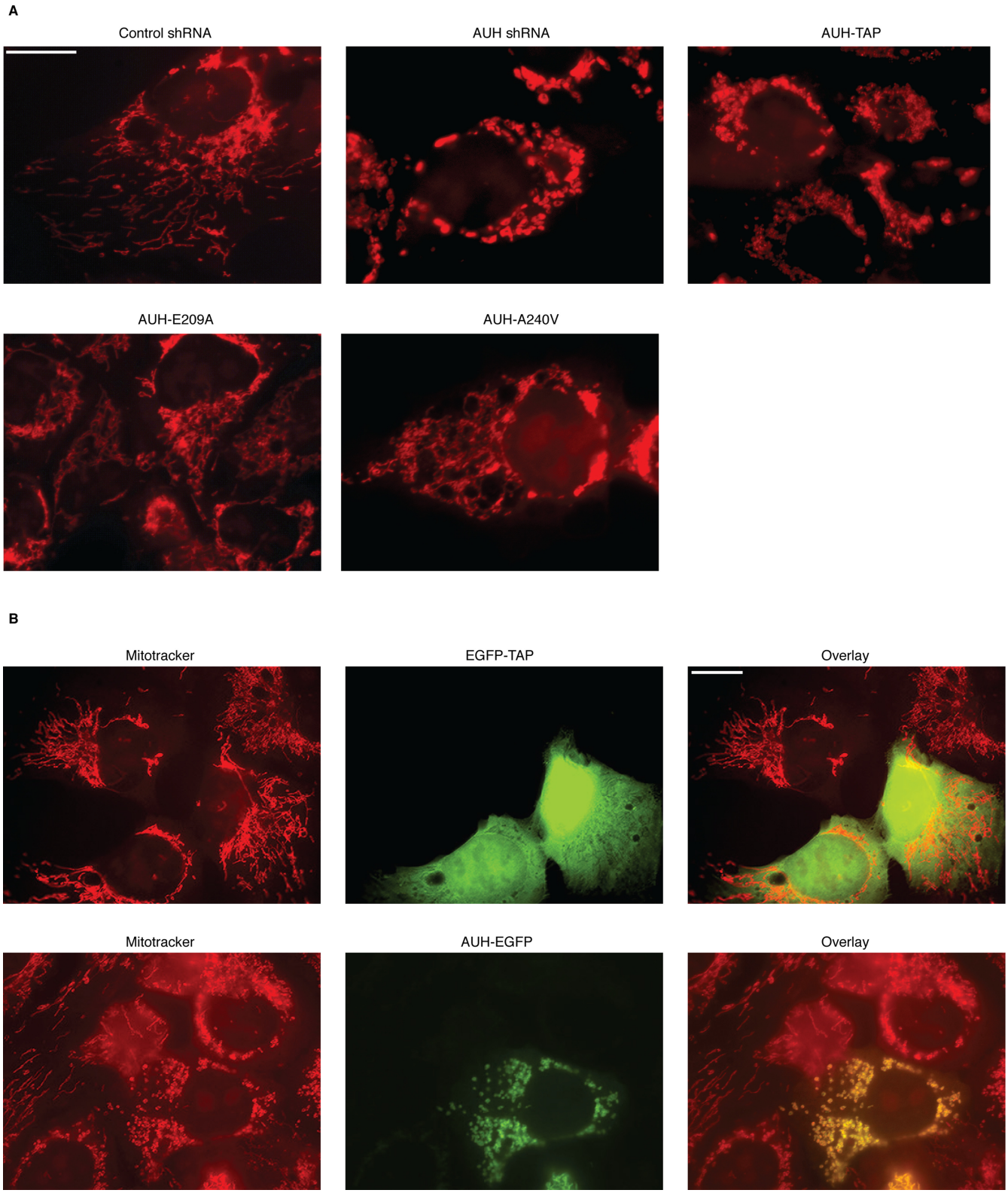

Figure 6. AUH affects mitochondrial morphology in cells. (**A**) Cells where AUH is knocked down or the wild type or mutant AUH-E209A and AUH-A240V proteins are overexpressed were incubated with 50 nM Mitotracker Orange, fixed and visualized by fluorescent microscopy. (**B**) Cells expressing AUH-EGFP were incubated with 50 nM Mitotracker Orange, fixed and visualized by fluorescent microscopy. The data are representative of results from three independent biological experiments. Additional images were provided in Supplementary Figure S4.

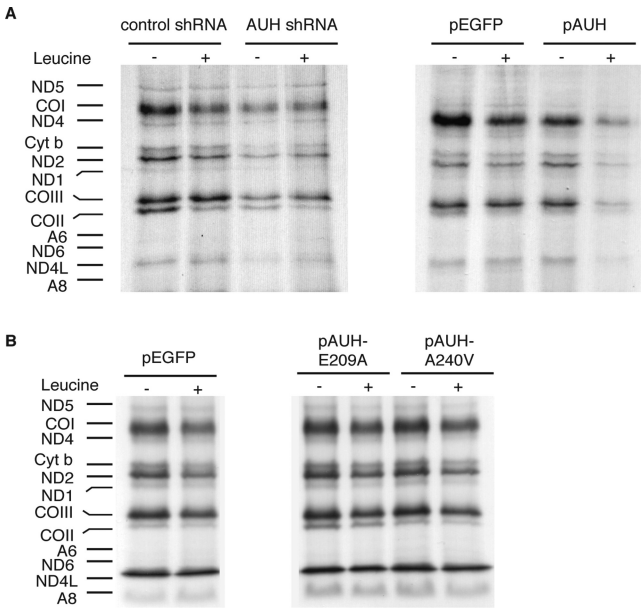

Figure 7. AUH affects translation in response to leucine levels. Protein synthesis was measured by pulse incorporation of 35S-labeled methionine and cysteine in cells where endogenous AUH levels are knocked down (**A**) or overexpressed (**B**) in the presence of 10 mM leucine. Equal protein amounts (20 μg) were resolved by SDS-PAGE and the gels were stained with Coomassie to confirm equal protein loading (Supplementary Figure S5). The data are typical of results from three independent biological experiments.

Therefore, AUH contributes to mitochondrial membrane integrity and the tubular morphology of mitochondria in cells. Recently, it was shown that the AFG3L2 protein that is part of the *m*-AAA protease complex is required for mitochondrial ribosomal assembly, protein synthesis and the tubular structure of mitochondria (24). This suggests that the association of mitochondrial ribosomes with proteins at the inner membrane can affect the morphology of mitochondria and impact on protein synthesis and mitochondrial function.

Regulation of leucine catabolism is important to conserve sufficient leucine levels for protein synthesis in the cytoplasm and to provide anaplerotic substrates to the citrate cycle within the mitochondrial matrix (27). The 3-methylglutaconyl-CoA hydratase activity in leucine catabolism of AUH (11) and its association with the mitochondrial ribosomes led us to investigate how increased levels of leucine affected protein synthesis in the presence and absence of AUH. Our findings suggest that the role of AUH in leucine catabolism may be linked to its effect on protein synthesis in mitochondria; this is particularly evident since protein synthesis was not decreased to the same extent by the catalytically inactive forms of AUH compared to those seen when the wild-type AUH was overexpressed or knocked down. Furthermore, we found that decreased AUH levels lead to slower cell proliferation compared with control cells (data not shown), as previously observed for the mitochondrial MCL-1 protein (35), supporting the role of AUH in fine-tuning mitochondrial biogenesis and therefore cell growth.

The mitochondrion is a hub of cellular metabolism and plays critical roles in both energy production and biosynthesis. Therefore, it is crucial for the cell to regulate the biogenesis and maintenance of mitochondria in response to changes in cellular metabolism. The coordinated control of cytoplasmic and mitochondrial protein synthesis is critical for the production of mitochondrial protein complexes. The bifunctional protein AUH, which can modulate mitochondrial translation and metabolize leucine, provides a potential link between mitochondrial metabolism and gene regulation. Future studies of regulators of mitochondrial protein synthesis should provide new insights into the control of mitochondrial metabolism and function.

ACCESSION NUMBERS

NM_001698 and NM_006980.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

Funding for open access charge: Medical Health Research Infrastructure Funding of Western Australia; National Health and Medical Research Council [APP1058442, APP1045677, APP1041582, APP1023460, APP1005030 to A.F. and O.R.]; Australian Research Council [FT0991008, FT0991113, DP140104111]; Dora Lush scholarship from the National Health and Medical Research Council [APP1039101 to T.R.].
Conflict of interest statement. None declared.

REFERENCES

1. Ojala,D., Montoya,J. and Attardi,G. (1981) tRNA punctuation model of RNA processing in human mitochondria. *Nature*, **290**, 470–474.
2. Agrawal,R.K. and Sharma,M.R. (2012) Structural aspects of mitochondrial translational apparatus. *Curr. Opin. Struct. Biol.*, **22**, 797–803.
3. Schieber,G. and O'Brien,T. (1985) Site of synthesis of the proteins of mammalian mitochondrial ribosomes. Evidence from cultured bovine cells. *J. Biol. Chem.*, **260**, 6367–6372.
4. Neupert,W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.*, **66**, 863–917.
5. Kutik,S., Guiard,B., Meyer,H.E., Wiedemann,N. and Pfanner,N. (2007) Cooperation of translocase complexes in mitochondrial protein import. *J. Cell Biol.*, **179**, 585–591.
6. Ghezzi,D. and Zeviani,M. (2012) Assembly factors of human mitochondrial respiratory chain complexes: physiology and pathophysiology. *Adv. Exp. Med. Biol.*, **748**, 65–106.
7. Kulawiak,B., Höpker,J., Gebert,M., Guiard,B., Wiedemann,N. and Gebert,N. (2013) The mitochondrial protein import machinery has multiple connections to the respiratory chain. *Biochim. Biophys. Acta*, **1827**, 612–626.
8. Rackham,O., Mercer,T.R. and Filipovska,A. (2012) The human mitochondrial transcriptome and the RNA-binding proteins that regulate its expression. *WIREs RNA*, **3**, 675–695.
9. Nakagawa,J., Waldner,H., Meyer-Monard,S., Hofsteenge,J., Jeno,P. and Moroni,C. (1995) AUH, a gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydratase activity. *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 2051–2055.
10. Nakagawa,J. and Moroni,C. (1997) A 20-amino-acid autonomous RNA-binding domain contained in an enoyl-CoA hydratase. *Eur. J. Biochem.*, **244**, 890–899.

11. Mack,M., Schniegler-Mattox,U., Peters,V., Hoffmann,G.F., Liesert,M., Buckel,W. and Zschocke,J. (2006) Biochemical characterization of human 3-methylglutaconyl-CoA hydratase and its role in leucine metabolism. *FEBS J.*, **273**, 2012–2022.
12. Ly,T.B.N., Peters,V., Gibson,K.M., Liesert,M., Buckel,W., Wilcken,B., Carpenter,K., Ensenauer,R., Hoffmann,G.F., Mack,M. *et al.* (2003) Mutations in the AUH gene cause 3-methylglutaconic aciduria type I. *Hum. Mutat.*, **21**, 401–407.
13. IJlst,L., Loupatty,F.J., Ruiter,J.P.N., Duran,M., Lehnert,W. and Wanders,R.J.A. (2002) 3-Methylglutaconic aciduria type I is caused by mutations in AUH. *Am. J. Hum. Genet.*, **71**, 1463–1466.
14. Kurimoto,K., Fukai,S., Nureki,O., Muto,Y. and Yokoyama,S. (2001) Crystal structure of human AUH protein, a single-stranded RNA binding homolog of enoyl-CoA hydratase. *Structure*, **9**, 1253–1263.
15. Brennan,L.E., Nakagawa,J., Egger,D., Bienz,K. and Moroni,C. (1999) Characterisation and mitochondrial localisation of AUH, an AU-specific RNA-binding enoyl-CoA hydratase. *Gene*, **228**, 85–91.
16. Ekert,P.G., Silke,J. and Vaux,D.L. (1999) Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. *EMBO J.*, **18**, 330–338.
17. Rackham,O., Davies,S.M.K., Shearwood,A.-M.J., Hamilton,K.L., Whelan,J. and Filipovska,A. (2009) Pentatricopeptide repeat domain protein 1 lowers the levels of mitochondrial leucine tRNAs in cells. *Nucleic Acids Res.*, **37**, 5859–5867.
18. Davies,S.M.K., Rackham,O., Shearwood,A.-M.J., Hamilton,K.L., Narsai,R., Whelan,J. and Filipovska,A. (2009) Pentatricopeptide repeat domain protein 3 associates with the mitochondrial small ribosomal subunit and regulates translation. *FEBS Lett.*, **583**, 1853–1858.
19. Wittig,I., Braun,H.-P. and Schägger,H. (2006) Blue native PAGE. *Nat. Protoc.*, **1**, 418–428.
20. Kuznetsov,A., Veksler,V., Gellerich,F., Saks,V., Margreiter,R. and Kunz,W. (2008) Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.*, **3**, 965–976.
21. Davies,S.M.K., Lopez Sanchez,M.I.G., Narsai,R., Shearwood,A.-M.J., Razif,M.F.M., Small,I.D., Whelan,J., Rackham,O. and Filipovska,A. (2012) MRPS27 is a pentatricopeptide repeat domain protein required for the translation of mitochondrially encoded proteins. *FEBS Lett.*, **586**, 3555–3561.
22. Delettre,C., Lenaers,G., Griffoin,J.M., Gigarel,N., Lorenzo,C., Belenguer,P., Pelloquin,L., Grosgeorge,J., Turc-Carel,C., Perret,E. *et al.* (2000) Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat. Genet.*, **26**, 207–210.
23. Emdadul Haque,M., Grasso,D., Miller,C., Spremulli,L.L. and Saada,A. (2008) The effect of mutated mitochondrial ribosomal proteins S16 and S22 on the assembly of the small and large ribosomal subunits in human mitochondria. *Mitochondrion*, **8**, 254–261.
24. Almajan,E.R., Richter,R., Paeger,L., Martinelli,P., Barth,E., Decker,T., Larsson,N.-G., Kloppenburg,P., Langer,T. and Rugarli,E.I. (2012) AFG3L2 supports mitochondrial protein synthesis and Purkinje cell survival. *J. Clin. Invest.*, **122**, 4048–4058.
25. Lenaers,G., Reynier,P., Elachouri,G., Soukkarieh,C., Olichon,A., Belenguer,P., Baricault,L., Ducommun,B., Hamel,C. and Delettre,C. (2009) OPA1 functions in mitochondria and dysfunctions in optic nerve. *Int. J. Biochem. Cell Biol.*, **41**, 1866–1874.
26. Ehses,S., Raschke,I., Mancuso,G., Bernacchia,A., Geimer,S., Tondera,D., Martinou,J.-C., Westermann,B., Rugarli,E.I. and Langer,T. (2009) Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. *J. Cell Biol.*, **187**, 1023–1036.
27. Schriever,S.C., Deutsch,M.J., Adamski,J., Roscher,A.A. and Ensenauer,R. (2012) Cellular signaling of amino acids towards mTORC1 activation in impaired human leucine catabolism. *J. Nutr. Biochem.*, **24**, 824–831.
28. IJlst,L., Loupatty,F.J., Ruiter,J.P.N., Duran,M., Lehnert,W. and Wanders,R.J.A. (2002) 3-Methylglutaconic aciduria type I is caused by mutations in AUH. *Am. J. Hum. Genet.*, **71**, 1463–1466.
29. Betney,R., de Silva,E., Krishnan,J. and Stansfield,I. (2010) Autoregulatory systems controlling translation factor expression: thermostat-like control of translational accuracy. *RNA*, **16**, 655–663.
30. Vafai,S.B. and Mootha,V.K. (2012) Mitochondrial disorders as windows into an ancient organelle. *Nature*, **491**, 374–383.
31. Neupert,W. and Herrmann,J.M. (2007) Translocation of proteins into mitochondria. *Annu. Rev. Biochem.*, **76**, 723–749.
32. Gruschke,S. and Ott,M. (2010) The polypeptide tunnel exit of the mitochondrial ribosome is tailored to meet the specific requirements of the organelle. *Bioessays*, **32**, 1050–1057.
33. Rugarli,E.I. and Langer,T. (2012) Mitochondrial quality control: a matter of life and death for neurons. *EMBO J.*, **31**, 1336–1349.
34. Nolden,M.M., Ehses,S.S., Koppen,M.M., Bernacchia,A.A., Rugarli,E.I.E. and Langer,T.T. (2005) The m-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria. *Cell*, **123**, 277–289.
35. Perciavalle,R.M., Stewart,D.P., Koss,B., Lynch,J., Milasta,S., Bathina,M., Temirov,J., Cleland,M.M., Pelletier,S., Schuetz,J.D. *et al.* (2012) Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat. Cell Biol.*, **14**, 575–583.
36. Christian,B.E. and Spremulli,L.L. (2012) Mechanism of protein biosynthesis in mammalian mitochondria. *BBA - Bioenergetics*, **1819**, 1035–1054.