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Pentatricopeptide repeat domain protein 3 associates with the mitochondrial small ribosomal subunit and regulates translation

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ABSTRACT

The basic components and mechanisms of mitochondrial transcription in mammals have been described, however, the components involved in mRNA processing, translation and stability remain largely unknown. In plants, pentatricopeptide domain RNA-binding proteins regulate the stability, expression and translation of mitochondrial transcripts. Here, we investigated the role of an uncharacterized mammalian pentatricopeptide domain protein, pentatricopeptide repeat domain protein 3 (PTCD3), and showed that it is a mitochondrial protein that associates with the small subunit of mitochondrial ribosomes. PTCD3 knockdown and over expression did not affect mitochondrial mRNA levels, suggesting that PTCD3 is not involved in RNA processing and stability. However, lowering PTCD3 in 143B osteosarcoma cells decreased mitochondrial protein synthesis, mitochondrial respiration and the activity of Complexes III and IV, suggesting that PTCD3 has a role in mitochondrial translation.

Structured summary:
MINT-7033995: *PTCD3* (uniprotkb:Q96EY7) *associates* (MI:0914) with *MRPS15* (uniprotkb:P82914) by *tandem affinity purification* (MI:0676)

1. Introduction

Mammalian mitochondrial DNA (mtDNA) is a circular, double stranded, minimal genome that encodes 22 tRNAs, two rRNAs, and 13 protein subunits of the electron transport chain complexes [1]. Consequently, human mtDNA is dependent on nuclear encoded proteins for replication, repair, transcription and translation. This dependency is evident in several human diseases that are caused by defects of mtDNA maintenance and expression [2]. Very little is known about the regulation of mitochondrial gene expression and how it affects mitochondrial function in health and disease [3,4]. This is particularly important since tissue-, cell- and disease-specific variations have been observed in the levels of different mitochondrial RNAs and the proteins they encode, but cannot be explained at present [5–8]. Mitochondrial RNAs are transcribed as long polycistronic, precursor transcripts that generally encompass the entire mtDNA and are processed by removing interspersed tRNAs to release the individual mRNAs and rRNAs [9]. The levels of the 13 individual mRNAs and their proteins must be controlled at a post-transcriptional level, although little is known about how these RNAs are regulated in mammalian mitochondria.

Pentatricopeptide repeat (PPR) domain proteins were identified first in plants, where they constitute a large family of mitochondrial and plastid proteins involved in transcript processing, editing and translation [10]. In mammals, there are seven identified mitochondrial PPR domain proteins, the mitochondrial RNA polymerase (POLRMT), the leucine-rich PPR cassette (LRPPRC) protein, mitochondrial ribosomal protein of the small subunit 27 (MRPS27), PPR domain proteins (PTCD) 1, 2, and 3 and mitochondrial RNase P protein 3 (MRPP3), which is a recently identified subunit of the mammalian mitochondrial RNase P complex [11,12]. Mutation of the LRPPRC gene has been shown to decrease cytochrome oxidase (COX) I and III mRNAs [13], and is the cause for the rare French–Canadian variant of Leigh syndrome, a debilitating neurodegenerative condition resulting from mitochondrial cytochrome *c* oxidase deficiency [14]. Recently, the PPR domain protein PTCD2 was

Abbreviations: PPR, pentatricopeptide repeat; PTCD3, pentatricopeptide repeat domain protein 3; TAP, tandem affinity purification

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shown to regulate cytochrome *b* RNA processing in mice [15]. Here we have investigated the role of the previously uncharacterized mammalian pentatricopeptide repeat domain protein 3 (PTCD3) and shown that it associates with the small subunit of the mitochondrial ribosome and is important for mitochondrial translation. We show that by decreasing PTCD3, we lower the translation of mitochondrial encoded proteins and consequently observe a decreased activity of Complexes III and IV and decreased mitochondrial respiration.

2. Materials and methods

2.1. Plasmid expression vectors

All expression vectors were based on pcDNA3 (Invitrogen). Full length human PTCD3 (NCBI accession number NP_060422) was expressed fused to a C-terminal tandem affinity purification tag (tandem affinity purification (TAP), derived from pCeMM-CTAP(SG), obtained from EUROSCARF, ABO76910) [16], or EYFP (BD Biosciences) at the C-terminus. Plasmids were tested for expression by transfection and immunoblotting.

2.2. Cell culture

143B Osteosarcoma cells were cultured at 37 °C under humidified 95% air/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) 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).

2.3. Mitochondrial isolation

Mitochondria were prepared from 10^7 cells grown overnight in 15 cm dishes and isolated as described previously [17].

2.4. Transfections

143B Cells were plated at 60% confluence in six-well plates or 10 cm dishes and transfected with annealed siRNAs or mammalian expression plasmids in OptiMEM media (Invitrogen). 125 nM (for six-well plates) or 145 nM (for 10 cm dishes) of PTCD3 or control, non-targeting siRNAs (Dharmacon) were transfected using Lipofectamine 2000 (Invitrogen). 158 ng/cm^2 of PTCD3 or control EYFP plasmid DNA was transfected using Fugene HD (Roche). Cell incubations were carried out for up to 72 h following transfection or cells were re-seeded and re-transfected for another 72 h for 6-day transfections. PTCD3 expression was measured by quantitative reverse transcriptase (RT)-PCR.

2.5. Fluorescence cell microscopy

For immunocytochemistry 143B cells were plated onto 13 mm diameter glass coverslips and allowed to attach overnight. Cells were transfected with pPTCD3-EYFP for 48 h, and at the end of the incubation treated with 100 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 DABCO/PVA medium. Images were acquired using an Olympus DP70 fluorescent inverted microscope using a Nikon 40× objective.

2.6. Affinity purification and RNA isolation

143B Cells (2.5×10^7) 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) 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, RT for 1 h). RNA was isolated using the miRNeasy Mini kit (Qiagen) incorporating an on-column RNase-free DNase digestion to remove all DNA. For protein isolation 5×10^7 143B cells were used and the PTCD3 was isolated and eluted as described above, then bound to Ultralink Immobilized Streptavidin Plus beads (Pierce), washed with cleavage buffer and eluted in cleavage buffer containing 1 mM D-biotin. The MRPS15 protein associated with PTCD3 was detected by immunoblotting.

2.7. Tiling array analysis of mitochondrial RNA

Microarray analysis was performed using Affymetrix GeneChip Human Mitochondrial Resequencing arrays, version 2.0. cDNA was prepared from the entire sample of total RNA purified from TEV eluted PTCD3 or EYFP as described in the Affymetrix GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual. Array washing, staining, and scanning was performed as described in the Affymetrix GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual, using an Affymetrix Hybridization Oven 640, an Affymetrix Fluidics Station 450 and an Affymetrix GeneChip Scanner 3000 7G. All .CEL files were analysed using the Affymetrix GSeq 4.1 software. As expected, the background fluorescence and the TaglQ-EX controls were found to be comparable between PTCD3 and EYFP experiments. The data are presented as a ratio of fluorescence detected in the PTCD3 associated RNA sample to that detected in the EYFP sample.

2.8. Quantitative RT-PCR

The abundance of mitochondrial transcripts was measured on RNA isolated from 2×10^6 143B cells using the miRNeasy RNA extraction kit (Qiagen). Levels of PTCD3 mRNA were measured from RNA isolated from normal human tissues and pooled (Ambion). Each pool is comprised of RNA from at least three tissue donors and the integrity of the RNA was verified by capillary electrophoresis using an Agilent 2100 bioanalyzer and quantitated using a NanoDrop spectrophotometer. cDNA was prepared from 2 μg of RNA using ThermoScript reverse transcriptase (Invitrogen) and random hexamers and used as a template in the subsequent PCR that was performed using a Corbett Rotorgene 3000 using Platinum UDG SYBR Green mastermix (Invitrogen) and normalized to 18S ribosomal RNA.

2.9. Sucrose gradient centrifugation

Mitochondria isolated from 143B cells (0.25 mg) were lysed in a buffer as described in [18], 20 mM Tris, 10 mM magnesium acetate, pH 7.5, supplemented with 1 mM PMSF, 0.1% SDS and 1.6% Triton-X 100 for 30 min, the lysate centrifuged at 10 000×*g* for 10 min, the clarified lysate was loaded on a 3.5 mL continuous 15–30% sucrose gradient (in 20 mM Tris, 10 mM magnesium acetate, pH 7.5, supplemented with 1 mM PMSF) and centrifuged at 100 000×*g* for 16 h using a SW60 Ti rotor in an Optima Beckman Coulter preparative ultracentrifuge. Fractions were collected and precipitated with 40% trichloroacetic acid, washed in acetone, and the entire fraction was resolved by SDS–PAGE. The ribosomal subunits and PTCD3-TAP were detected by immunoblotting.

2.10. Immunoblotting

Specific proteins were detected using rabbit αMRPL11 (NEB, diluted 1:1000) and αMRPS15 antibodies (Abcam, diluted 1:1000)

and mouse αmyc antibody (Upstate Biotechnology, diluted 1:1000) in 5% skim milk powder in phosphate-buffered saline (4.3 mM sodium phosphate, dibasic, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.4 mM potassium phosphate, monobasic, PBS), 0.05% Tween-20. Control immunoblots for MnSOD in the fractions from the sucrose gradient were detected using a mouse antibody (BD Transduction Laboratories, diluted 1:2000). The primary antibodies were detected using goat anti-mouse or goat anti-rabbit horse radish peroxidase (Biorad, diluted 1:10 000). PTCD3-TAP was detected using goat anti-mouse HRP.

2.11. Mitochondrial protein synthesis

143B Cells were grown in six-well plates until 60% confluent, transfected and 3 days later de novo protein synthesis was analyzed. The growth medium was replaced with methionine and cysteine free medium containing 10% dialysed FBS for 30 min before addition of 100 μg ml^{-1} emetine for 5 min. Next, 200 μCi Expres35S Protein Labeling Mix [^{35}S] (14 mCi, Perkin–Elmer) was added and incubated at 37 °C for 1 h, then washed in PBS and centrifuged. The cells were suspended in PBS and 20 μg of proteins were separated on 12.5% SDS–PAGE and the radiolabeled proteins were visualized on film.

2.12. Respiration

State 3 respiration using 0.5 mM TMPD and 2 mM ascorbate was measured in permeabilized cells according to Kuznetsov et al. [19].

2.13. Complex enzyme assays

Enzyme assays of citrate synthase, complex III 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 before [20].

3. Results

3.1. PTCD3 is a mitochondrial protein with nine PPR domains

The most distinctive features of PTCD3 are its nine PPR domains and an N-terminal mitochondrial targeting sequence (Fig. 1A). We measured the mRNA levels of PTCD3 in human tissues and found that it is most abundant in testes, skeletal muscle and heart tissue (Fig. 1B). We fused the C-terminus of the protein to EYFP and showed that PTCD3 localizes specifically to mitochondria in 143B cells (Fig. 1C).

3.2. PTCD3 is a mitochondrial RNA-binding protein

We fused the C-terminus of PTCD3 to a tandem affinity purification (TAP) tag and expressed this protein in 143B osteosarcoma cells. We used the TAP tag to isolate the PTCD3-TAP protein from cells and measured the enrichment of mitochondrial mRNA transcripts using a human mtDNA re-sequencing array. We adapted the re-sequencing array for use as a single nucleotide tiling array of the complete mitochondrial genome to detect RNAs that were associated with PTCD3 compared to a control purification of EYFP. The fluorescence was strikingly higher for the 930 probes that tile the 12S rRNA region (Fig. 2A).

To confirm the RNA targets of PTCD3, quantitative reverse transcriptase PCR (Q RT-PCR) was carried out on RNA pulled down from the TAP. There was an over 25-fold enrichment observed only for the 12S rRNA transcript and not the remaining mitochondrial transcripts compared to the control EYFP-TAP protein (Fig. 2B). Because many plant PPR domain proteins are involved in processing of organelle transcripts [10], we investigated if PTCD3 bound the junction between the 12S rRNA and the 16S rRNA. We found that there was no significant enrichment observed of this junction transcript compared to the control EYFP-TAP protein (Fig. 2B).

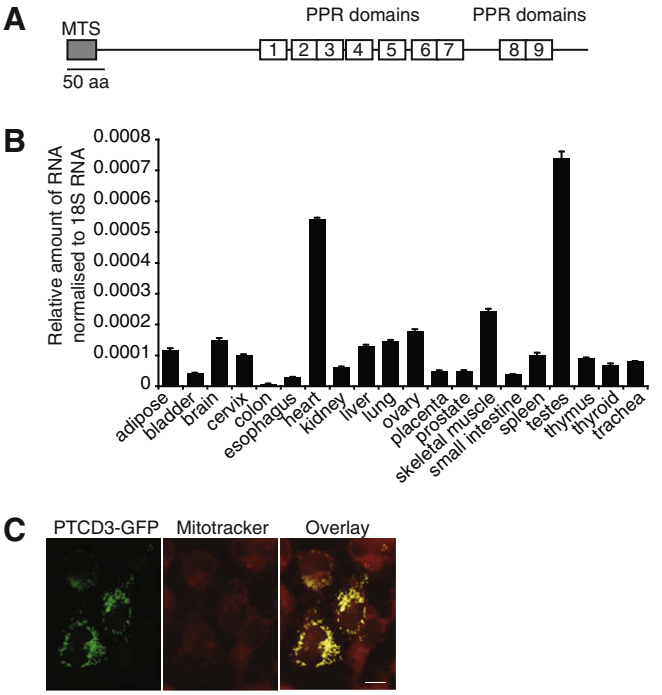

Fig. 1. PTCD3 is a mitochondrial protein with 9 PPR domains. (A) Gene structure of PTCD3. (B) mRNA distribution in normal human tissues analysed by Q RT-PCR. (**C**) 143B cells transfected with pPTCD3-EYFP. PTCD3-EYFP (green) was co-localized to mitochondria stained with Mitotracker orange (red) directly by fluorescence microscopy and the overlaid image (yellow) indicates co-localization of PTCD3 with mitochondria.

We analysed if PTCD3 associated with mitochondrial ribosomes by separating mitochondrial lysates on a 15–30% continuous sucrose gradient and immunoblotting against protein markers of the small and large subunits of the ribosome. PTCD3 co-migrated predominantly with the small ribosomal subunit protein 15 (MRSP15) suggesting that PTCD3 associates with the small subunit of mitochondrial ribosomes (Fig. 2C). In addition, we isolated PTCD3 that was expressed in 143B cells using the TAP tag and by immunoblotting found that MRSP15 was enriched with PTCD3 but not with the control EYFP isolation (Fig. 2D), suggesting further that PTCD3 associates with the mitochondrial small ribosomal subunit.

3.3. PTCD3 has a role in mitochondrial translation but not in RNA metabolism

We investigated the effects of PTCD3 knockdown and overexpression in 143B cells. We found that the PTCD3 directed siRNA duplex significantly lowered the mRNA levels of PTCD3 (Fig. 3A inset) but did not affect the abundance of the mature mitochondrial RNA transcripts (Fig. 3A). Overexpression of PTCD3 increased its mRNA abundance over 170-fold, however, it did not change the levels of the mitochondrial RNAs. The junction between the 12S rRNA and the 16S rRNA was not affected by PTCD3 knockdown and overexpression (Fig. 3A). This suggested that PTCD3 does not affect the abundance or stability of precursor and mature mitochondrial rRNAs.

We investigated changes in de novo mitochondrial protein synthesis in cells where PTCD3 was knocked down or overexpressed

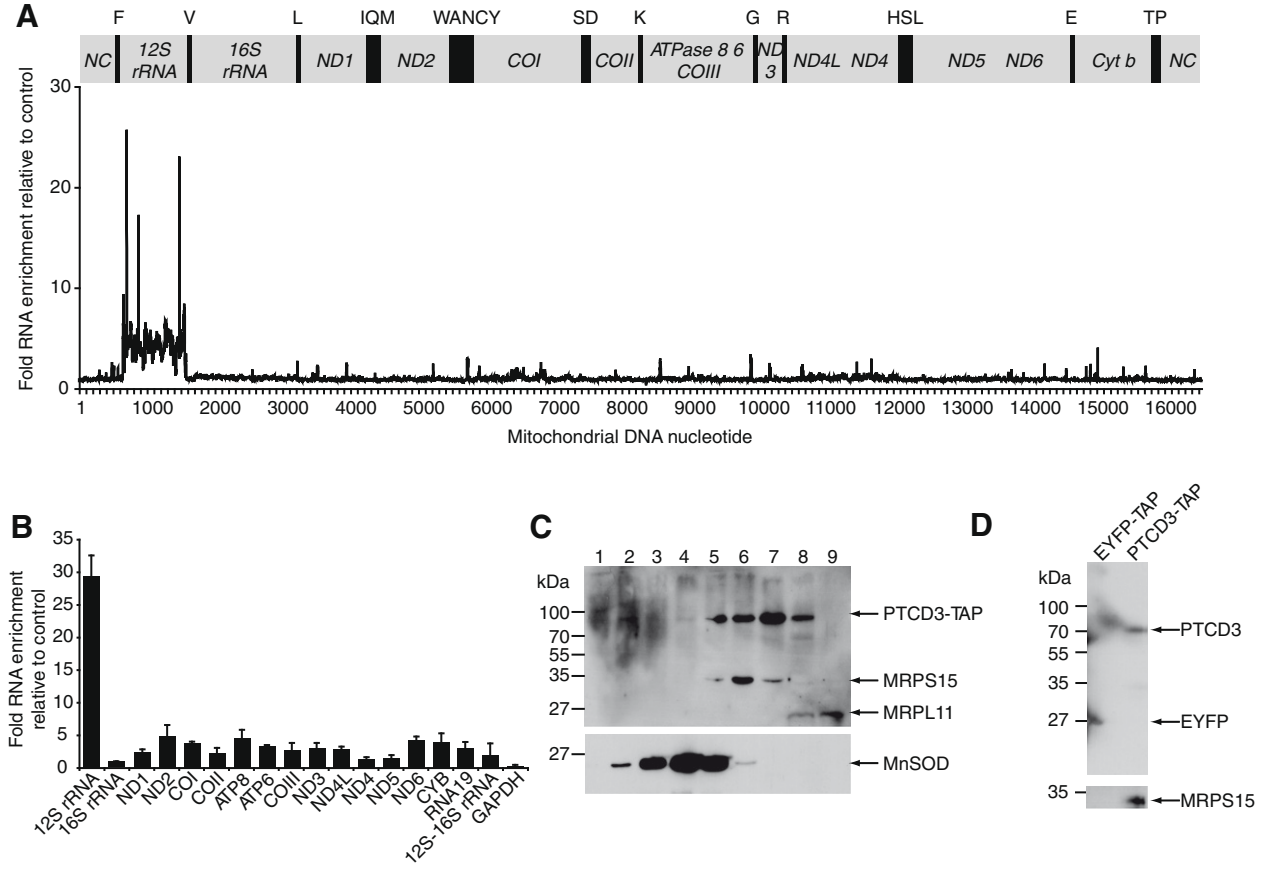

Fig. 2. PTCD3 is a mitochondrial RNA-binding protein that associates with the 12S rRNA. PTCD3-TAP protein expressed in 143B cells was purified using rabbit-IgG agarose and eluted by tobacco etch virus (TEV) protease cleavage. RNA associated with the eluted PTCD3-TAP was reverse transcribed and analysed by tiling microarray and by Q-PCR analysis. Values are expressed as a ratio of PTCD3-TAP compared to a purification from cells that expressed a control, EYFP-TAP protein. (A) Ratio of fluorescence from cDNA hybridized to a mitochondrial DNA tiling array. (B) Ratio of mitochondrial mRNA transcripts detected by Q RT-PCR. Data are means ± S.E.M. of three separate experiments. (**C**) Mitochondria from cells transfected with PTCD3-TAP were lysed and resolved on 15–30% continuous sucrose gradients to determine the protein distribution relative to mitochondrial ribosomal proteins that are markers of the mitochondrial large (MRPL11) and small subunit (MRPS15). The blot was re-probed for MnSOD as a control. (D) PTCD3 and EYFP were purified using the TAP tag and the association with the mitochondrial small subunit was assessed by immunoblotting using an αMRPS15 antibody. The presence of PTCD3 or EYFP in the eluates was determined using an αmyc antibody.

by ^{35}S-labeled incorporation of methionine and cysteine in mitochondrial proteins. We observed a significant overall decrease of mitochondrial protein synthesis when PTCD3 was knocked down in cells compared to non-targeting siRNAs and non transfected cells (Fig. 3B). This suggests that PTCD3 has a role in mitochondrial translation.

We have investigated the abundance of marker proteins for the small and large mitochondrial ribosomal subunits (MRPS15 and MRPL11) after siRNA knockdown of PTCD3 and control siRNA after 3 and 6 days, respectively, and found the levels of these proteins were unaffected by the reduction of PTCD3 (Fig. 3C). It has previously been shown that the loss of core mitochondrial small ribosomal subunit proteins causes the loss or reduction of other small ribosomal subunit proteins [21]. This data suggest that ribosome assembly is not affected by the decrease in PTCD3 levels.

3.4. PTCD3 knockdown leads to a decrease in mitochondrial respiration and activity of the respiratory complexes

Because we observed a significant decrease in the abundance of mitochondrial encoded proteins when PTCD3 was knocked down, we measured mitochondrial oxygen consumption and the respiratory complex activities in 143B cells where PTCD3 was knocked down or overexpressed. The respiration of mitochondria in digitonin-permeabilized cells was significantly lowered when PTCD3 was knocked down after 3 and 6 days but not when PTCD3 was overexpressed (Fig. 3D and E). PTCD3 knockdown led to decreased activity of Complex III after 3 days and this was even more pronounced after 6 days (Fig. 3F). Similarly, a decrease in Complex IV activity was observed after a 3-day knockdown of PTCD3 and this decrease was more dramatic after 6 days (Fig. 3G). Overexpression of PTCD3 did not affect the activity of these complexes.

4. Discussion

In this study, we show that PTCD3 is a mitochondrial protein that is more abundant in mitochondria-rich tissues. The predicted PPR domains may give PTCD3 RNA-binding affinity for the 12S rRNA that was detected in the tiling array. In addition, we found that PTCD3 associated with the small subunit of mitochondrial ribosomes. This corroborates a previous proteomic study of the 28S small ribosome subunit of bovine mitochondria that identified several proteins including a homolog of PTCD3 [22]. Overexpression and knockdown of PTCD3 in cells did not affect the abundance or processing of mitochondrial transcripts, however knockdown of PTCD3 significantly decreased protein synthesis of mitochondrial encoded polypeptides. Consequently, we observed lowered respiration and decreased activities of Complex III and Complex IV following a 3–6 day knockdown of PTCD3. Overexpression of PTCD3 did not affect mitochondrial de novo protein synthesis and mito-

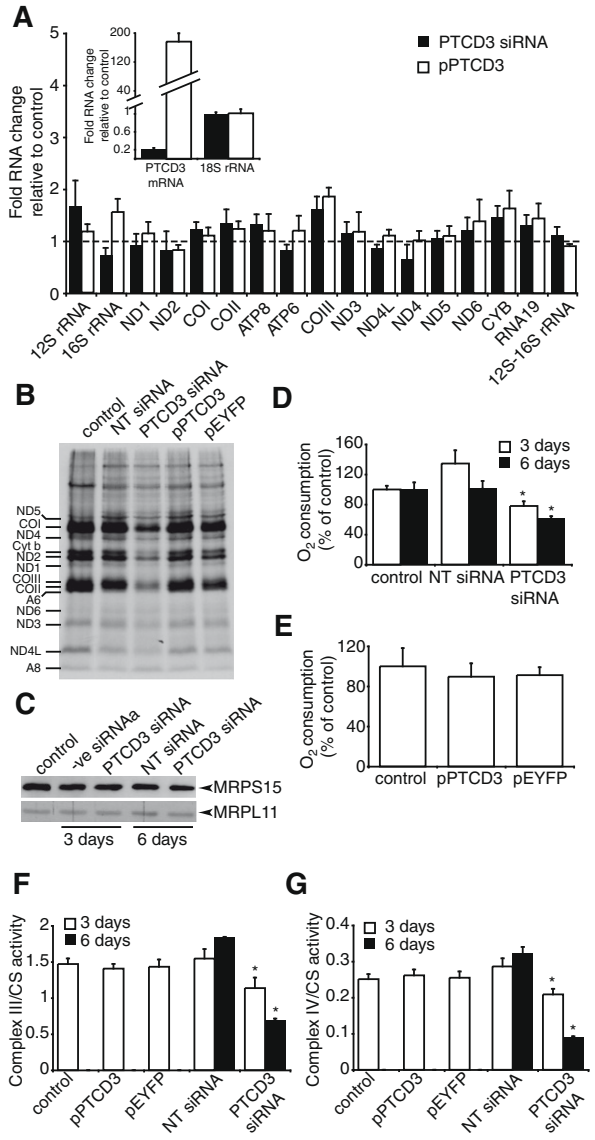

Fig. 3. PTCD3 affects mitochondrial protein synthesis and consequently mitochondrial function, but not mitochondrial transcript levels and processing. (A) RNA isolated from cells transfected with PTCD3 siRNA, non-targeting siRNA, pPTCD3-TAP, pEYFP-TAP was analysed by Q RT-PCR. The data is expressed as a ratio of transcript abundance from experimental samples compared to control samples. Amplification of mitochondrial mRNA transcripts is shown. Data are means ± S.E.M. of three separate experiments. (B) Mitochondrial protein synthesis is lowered when PTCD3 abundance is decreased in cells. The protein synthesis of cytoplasmic proteins was inhibited with emetine, allowing the incorporation of ^{35}S-labeled methionine and cysteine in the 13 mitochondrial proteins synthesized by the mitochondrial ribosomes. 143B Cells were transfected with siRNAs targeting PTCD3, non-targeted siRNAs as control, PTCD3-TAP and EYFP-TAP as control. Equal amounts of cell lysate protein (20 μg) were separated on 12.5% SDS–polyacrylamide gels and visualized on film. Data are representative of six independent experiments with the same outcome. (C) 143B Cells were transfected with siRNAs targeting PTCD3 and non-targeted siRNAs as controls for 3 and 6 days, mitochondria were isolated and the abundance of protein markers for the mitochondrial small and large ribosomal subunit were detected by immunoblotting using antibodies for the MRSP15 and MRLP11 proteins. (D and E) State 3 respiration on ascorbate/TMPD was measured in digitonin-permeabilized 143B cells using an oxygen electrode. Oxygen consumption is shown as percent of the control treatment with transfection reagent only and the decrease in respiration is significant compared to the control and non-targeting siRNA treatment after 3 and 6 days, respectively. Enzyme activities of Complex III (F) and Complex IV (G) of mitochondria isolated from 143B cells transfected with PTCD3 siRNA, non-targeting siRNA, PTCD3-TAP and EYFP-TAP were measured spectrophotometrically. Enzyme activity is shown as ratios of complex activity compared to citrate synthase activity. Data are means ± S.E.M. of three–five separate experiments; $^{*}P < 0.05$ compared with control treatments by a two-tailed paired Student's *t*-test.

chondrial function suggesting that PTCD3 is present in stoichiometric amounts to mitochondrial ribosomes and increasing the amount of PTCD3 cannot further stimulate mitochondrial translation. This indicates that PTCD3 is necessary for efficient mitochondrial translation, however its exact role is yet to be elucidated. The association of PTCD3 with the 12S rRNA and the small subunit of mitochondrial ribosomes raises the question of whether PTCD3 is an RNA-binding protein of the 12S rRNA and as such is a *bona fide* ribosomal protein, or if it is a transiently associated small subunit translational regulator that may be involved in the recognition or decoding of mRNA transcripts.

In yeast, mitochondrial transcripts have 5′ untranslated regions that bind translational activators, however, these are not conserved in mammals [23]. Because mammalian mitochondrial mRNAs begin at the start codon and lack 5′ untranslated regions or Shine-Dalgarno sequences [22], mitochondrial ribosomes must have evolved an alternative way to regulate translation initiation and to ensure accurate start codon recognition. Comparative studies of the mammalian mitochondrial ribosome with bacterial and cytoplasmic ribosomes of eukaryotes have identified large variations in the composition and number of mitochondrial ribosomal subunits [24,25] and the number of additional ribosomal subunits may increase to include previously uncharacterized proteins such as PTCD3. Core mitochondrial ribosomal proteins have been identified that are essential for the assembly of ribosomes in addition to proteins that are not essential for ribosome assembly but are required for ribosomal function [21]. It may be that PTCD3 is a mitochondrial ribosome protein that is required for protein synthesis but not for ribosome assembly. As part of the small ribosomal subunit, PTCD3 may be important for structural integrity or it could have a role in translation initiation and decoding. Further investigation of the role of PTCD3 in mitochondrial ribosomes will provide insights into the regulation of mitochondrial translation.

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