RNA-binding proteins regulate cell respiration and coenzyme Q biosynthesis by post-transcriptional regulation of COQ7

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ABSTRACT

Coenzyme Q (CoQ) is a key component of the mitochondrial respiratory chain carrying electrons from complexes I and II to complex III and it is an intrinsic component of the respirasome. CoQ concentration is highly regulated in cells in order to adapt the metabolism of the cell to challenges of nutrient availability and stress stimuli. At least 10 proteins have been shown to be required for CoQ biosynthesis in a multi-peptide complex and COQ7 is a central regulatory factor of this pathway. We found that the first 765 bp of the 3′ untranslated region (UTR) of COQ7 mRNA contains cis-acting elements of interaction with RNA-binding proteins (RBPs) HuR and hnRNP C1/C2. Binding of hnRNP C1/C2 to COQ7 mRNA was found to require the presence of HuR, and hnRNP C1/C2 silencing appeared to stabilize COQ7 mRNA modestly. By contrast, lowering HuR levels by silencing or depriving cells of serum destabilized and reduced the half-life of COQ7 mRNA, thereby reducing COQ7 protein and CoQ biosynthesis rate. Accordingly, HuR knockdown decreased oxygen consumption rate and mitochondrial production of ATP, and increased lactate levels. Taken together, our results indicate that a reduction in COQ7 mRNA levels by HuR depletion causes mitochondrial dysfunction and a switch toward an enhanced aerobic glycolysis, the characteristic phenotype exhibited by primary deficiency of CoQ10. Thus HuR contributes to efficient oxidative phosphorylation by regulating of CoQ10 biosynthesis.

KEYWORDS

COQ7; coenzyme Q10; hnRNP C1/C2; HuR; mitochondrial respiration; post-transcriptional regulation; RNA-binding proteins

Introduction

Coenzyme Q (CoQ) or ubiquinone is a lipid-soluble molecule synthesized and mainly concentrated in the mitochondrial inner membrane of all eukaryotic cells. CoQ is an electron carrier from Complexes I and II to Complex III of the mitochondrial respiratory chain, it has antioxidant properties and regulates permeability transition pore, pyrimidine biosynthesis, inner mitochondrial respiratory chain, and it has antioxidant properties and regulates permeability transition pore, pyrimidine biosynthesis.

CoQ is not only a component of complex III in which involves the CoQ cycle, but it also contributes to its stability. Recently, we demonstrated that CoQ is a key component of the respiratory chain superassembly complex, which contributes to selecting internal electrons routes and to regulating the production of reactive oxygen species in the oxidative phosphorylation pathway.

CoQ is composed by a benzoquinone ring synthesized from tyrosine, attached to a poly-isoprenoid side chain derived from the mevalonate pathway. Benzoquinone ring final modifications take place in mitochondria by a pathway that involves at least 8 genes (COQ3-COQ9, ADCK4), whose products appear to form a multi-enzyme complex. CoQ homeostasis is essential for mitochondria efficiency, and thus its biosynthesis is highly regulated. Disruption of CoQ homeostasis can induce its deficiency caused by either mutations in any of the genes involved in its biosynthesis (primary deficiency), which leads to a rare disorder with variable clinical phenotypes exhibiting major effects on the neuromuscular axis and nephrotic syndrome, or as an adaptive response to dysfunctional mitochondria. On the contrary, healthy aging interventions such as calorie restriction and treatment with resveratrol induce mitochondrial efficiency, activating diverse bioenergetics pathways including CoQ biosynthesis. Additionally, it has been demonstrated that the levels of CoQ are essential for regulating longevity of invertebrate models. Therefore, CoQ levels in mitochondria must be tightly regulated to balance oxidative phosphorylation and to adapt to changes in nutritional and/or physiological conditions.

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Published with license by Taylor & Francis Group, LLC © María V. Cascajo, Kotb Abdelmohsen, Ji Heon Noh, Daniel J.M. Fernández-Ayala, Imke M. Willers, Gloria Brea, Guillermo López-Lluch, Marina Valenzuela-Villatoro, José M. Cuezva, Myriam Gorospe, Emilio Siendones, and Plácido Navas This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.
Regulatory mechanisms of CoQ biosynthesis include the transcriptional regulation of enzymes in the mevalonate pathway via the transcription factor PPARα, and the transcriptional induction of COQ7 expression by NF-κB in response to oxidative stress. Furthermore, CoQ biosynthesis is also regulated by post-translational modification of COQ7 by either a nutrient-dependent phosphorylation-dephosphorylation cycle in yeast or by binding to COQ9.

Post-transcriptional control of gene expression includes the formation of ribonucleoprotein (RNP) complexes that can regulate mRNA translation. COQ7 mRNA was identified as a putative target of HuR by ribonucleoprotein immunoprecipitation (RIP) followed by microarray detection of target mRNAs using arrays. HuR is a ubiquitously expressed RNA-binding protein (RBP) member of ELAVL protein family that can regulate the stability and translation of hundreds mRNA involved in cell division, carcinogenesis, muscle cell differentiation, replicative senescence, immune cell activation, and stress response. HuR and several members of the hnRNP family as hnRNP D (also known as AUF1) are able to bind specifically to AU-rich elements (AREs) in the 3′UTR of target mRNAs, in cooperative or competitive manners to regulate the translation of shared target mRNAs.

We show here that HuR and hnRNP C1/C2 proteins, both of which bind to the COQ7 3′UTR, determine the rates of CoQ10 biosynthesis. On the one hand, HuR enhances COQ7 mRNA stability and helps maintain physiological COQ7 mRNA, COQ7 protein levels, and CoQ10 biosynthesis, while hnRNP C1/C2 binds to COQ7 mRNA by interacting with HuR and triggers its degradation. The stability of COQ7 mRNA is decreased by serum withdrawal leading to an enhanced aerobic glycolysis. We propose that these 2 proteins are required to maintain the aerobic respiratory metabolism through the post-transcriptional regulation of CoQ10 level.

**Results**

3′UTR of COQ7 mRNA is involved in regulating its translation

The COQ7 3′UTR (1.9 kb) contains a U/AU-rich segment that includes 3 AUUUUA repeats in the proximal 765 base pairs of the 3′UTR (Fig. 1A). We assessed the involvement of COQ7 3′UTR in the translational regulation of coding region (CR), by using different constructs spanning the COQ7 mRNA (Fig. 1B). The translational efficiency of each in vitro-synthesized RNA was assayed in a rabbit reticulocyte lysate system. All COQ7 transcripts were translated into a single 25-kDa polypeptide, in...
agreement with the size of COQ7 (Fig. 1B). Interestingly, deletion of the entire 3′UTR increased COQ7 protein production compared to the full-length transcript, while deletion of the 5′UTR decreased it. COQ7 production was moderately reduced by the removal of the distal 1172 bp of the 3′UTR (fragment Δ3′UTR BC, bearing the 3′UTR proximal 765 bp), but expression was restored to full-length levels after additional segments spanning all but the distal 690 bp (fragment Δ3′UTR C) was tested. These findings suggested that the first 765-bp segment of the COQ7 3′UTR contains cis-acting elements involved in controlling the stability and/or translation of COQ7 mRNA.

**HuR and hnRNP C1/C2 bind COQ7 mRNA**

Stability and translation of mRNA are governed robustly by binding of RBPs to 3′UTRs, and thus we explored the interaction of COQ7 mRNA with RBPs by synthesising several RNA probes spanning the entire COQ7 transcript by *in vitro* transcription (Fig. 2A, upper panel). UV-crosslinking assays after incubation with a protein extract from HeLa cells revealed the existence of protein complexes interacting with the first 765 bp of 3′UTR of the COQ7 mRNA (Fig. 2A, bottom panel).

In order to identify what RBPs might associate with COQ7 mRNA, we studied the interaction between the biotinylated partial COQ7 transcripts and RBPs from HeLa cells. The COQ7 mRNA-RBP complexes (mRNPs) were further tested by biotin pulldown followed western blot analysis using antibodies against 10 different RBPs (Materials and Methods) of sizes in the range of molecular weights of the signals detected in Fig. 2A. A biotinylated GAPDH 3′UTR RNA was used as a negative control. Two out of the 10 proteins assayed, HuR and hnRNP C1/C2,
appeared to bind to a common region inside of the first 765 bp of 3′UTR (fragment A) of the COQ7 mRNA (Fig. 2B). No other RBPs appeared to bind using this assay.

To further determine if COQ7 mRNA is a direct target of HuR and hnRNP C1/C2, we performed RIP analysis from cytoplasmic extract of HeLa cells, using anti-HuR and anti-hnRNP C1/C2 antibodies under conditions that prevented

Figure 3. HuR silencing, but not hnRNP C1/C2, reduces COQ7 expression. (A) After IP of RNA-protein complexes from HeLa cell lysates using anti-HuR, anti-hnRNP C1/C2 or control IgG antibodies, RNA was isolated and used in RT reactions. Fold differences in transcript abundance in HuR and in hnRNP C1/C2 IP samples compared with IgG IP samples were measured by RT-qPCR analysis. VHL mRNA and GAPDH mRNA were measured in the IP samples as positive and loading controls, respectively. (B) IP reactions using anti-hnRNP or anti-HuR antibodies were carried out using lysates from cells expressing normal or reduced (silenced) HuR or hnRNP, respectively, and COQ7 mRNA was quantified by RT-qPCR reactions. Data represent the means ± SD from 3 independent experiments. siRNA control vs. siRNA HuR. *P < 0.05. (C) The expression of HuR, hnRNP C1/C2, COQ7, and loading controls β-ACTIN and GAPDH, after silencing of HuR or hnRNP C1/C2, were monitored by western blot analysis.
the dissociation of the RNPs. After the RNA associated with HuR or hnRNP C1/C2 was isolated, reverse transcription (RT) and real-time quantitative (q)PCR analysis was used to determine the degree of enrichment of COQ7 mRNA in each RIP sample. These results show that both anti-HuR and anti-hnRNP C1/C2 IP materials were enriched in COQ7 PCR product compared with control IgG IP samples (Fig. 3A). Von Hippel-Lindau (VHL) mRNA and GAPDH mRNA were included as positive and negative controls, respectively.24

Given that the 2 RBPs associate with the same RNA region, we wondered whether the interaction between HuR and hnRNP C1/C2 with COQ7 mRNA might be competitive or cooperative. To test this possibility, we performed HuR RIP analysis under conditions of anti-hnRNP C1/C2 silencing, and conversely performed hnRNP C1/C2 RIP analysis under conditions of HuR silencing. We found that the reduced levels of hnRNP C1/C2 did not affect HuR binding. However, COQ7 mRNA enrichment in hnRNP C1/C2-IP fraction decreased significantly inHuR-silenced cells compared with control IgG IP samples (Fig. 3B). This result suggests that HuR promotes the binding of hnRNP C1/C2 to COQ7 mRNA.

To ascertain the role of HuR and hnRNP C1/C2 on COQ7 protein production, MRC-5 cells were transfected with small interfering RNA (siRNA) to reduce the levels of these RBPs. Fig. 3C illustrates the levels of COQ7 protein after siRNA transfection. This intervention caused a decrease in COQ7 protein levels (50%) in the absence of HuR but not after hnRNP C1/C2 silencing. Given that HuR promotes the stability of many target mRNAs, we set out to investigate if it also enhanced COQ7 mRNA half-life. We were unable to examine directly if COQ7 mRNA translation was affected by analyzing polysomes because the levels of COQ7 mRNA on each polysome fraction was too low (not shown).

**HuR affects mitochondrial respiration**

As CoQ is an electron carrier of the mitochondrial respiratory chain, it is a key component of the respiratory chain superassembly complex.4 Thus, the reduced CoQ10 biosynthesis capacity caused by reducing HuR might disrupt mitochondrial metabolism in a way similar to what is seen in primary CoQ10 deficiency. To test this hypothesis, we analyzed mitochondrial oxygen consumption rate (OCR) in MRC5 human cells expressing low HuR levels after transfection of HuR shRNA using a Seahorse Bioscience XF analyzer. In parallel, we performed the same assay in cells expressing reduced COQ7 mRNA levels (from transfection with COQ7 shRNA), included as a positive control. The OCR data in Fig. 5A shows the mitochondrial respiratory response before and after sequential addition of oligomycin (OL) and 2,4 dinitrophenol (DNP) to HuR shRNA HuR and control shRNA cells. Basal mitochondrial respiration (BR) was reduced in HuR-silenced cells compared with control counterparts. However, unlike COQ7 shRNA cells, maximal mitochondrial respiration (MR) and spare mitochondrial respiration capacity (SRC) were not significantly reduced (Fig. 5A). Furthermore, total ATP levels were decreased after HuR silencing while lactate production was higher than in control cells (Fig. 5B). The enhanced production of lactate in both shRNA- HuR and shRNA- COQ7 silenced cells indicates a stimulation of aerobic glycolysis, likely due to reduced ATP production in mitochondria by oxidative phosphorylation.

**Serum withdrawal reduces levels of HuR, COQ7 protein and CoQ10 biosynthesis rate**

Serum administration is required to boost mitochondrial respiratory capacity in cultured cells,34 and serum withdrawal induces aerobic glycolysis in fibroblasts with increasing lactate production,35 similarly to what is seen in primary CoQ10-deficient human fibroblasts.36 As indicated by Atasoy et al (1998),26 HuR expression decreased in NIH-3T3 cells in serum limiting condition. Thus, we hypothesized that the decreased expression of HuR in serum-deprived cells should lead to a decrease in levels of COQ7 and CoQ10 biosynthesis, playing a role in the switch of the 2 main pathways involved in energy provision, i.e., from oxidative phosphorylation to an enhanced aerobic glycolysis. To ascertain the role of serum on the functional consequences of HuR-COQ7 binding, we monitored the cytoplasmic levels of HuR and COQ7 by western blotting (Fig. 6A, left). The levels of these proteins were decreased in MRC5 cells after 48 h in the absence of serum. CoQ10 biosynthesis rate assayed by incorporation of 14C-pHB, was also decreased by 50% in MRC5 grown without serum (Fig. 6A, right). The stability of COQ7 mRNA was also significantly decreased in serum-deprived MRC5 cells (Fig. 6B).

**Discussion**

CoQ10 biosynthesis is a complex process involving the products of at least 10 nuclear genes, although additional genes have been identified recently in yeast.37 Both CoQ levels and COQ genes expression in mammals are tissue-specific and depend on nutritional conditions and the age of the organism.13,38
Figure 4. HuR silencing, but not hnRNP C1/C2 silencing, regulates COQ7 mRNA stability and CoQ10 biosynthesis. (A) The half-lives of COQ7 and GAPDH mRNAs after silencing HuR (upper panel) or hnRNP C1/C2 (bottom panel) were measured by incubating cells with actinomycin D, extracting total RNA at the times shown, and measuring mRNA levels by RT-qPCR analysis. The data were normalized to 18S rRNA levels and represented as a percentage of the mRNA levels measured at time 0, before adding actinomycin D, using a semilogarithmic scale. mRNA half-life was calculated as the time required for each mRNA decrease to 50% of its initial abundance (discontinuous horizontal line). (B) MRC-5 cells were transfected with a siRNA targeting the HuR 3’UTR (siHuR), or a control siRNA (siControl), along with either TAP- or HuR-TAP-expressing vectors. HuR-TAP is a chimeric protein expressed from a cDNA that lacks the HuR 3’UTR; the levels of endogenous (HuR) or ectopic (HuR-TAP) HuR, COQ7, and loading control β-actin were tested by Western blot analysis. (C) CoQ10 biosynthesis rate. HuR-silenced and hnRNP-silenced cells were incubated (24 h) with radiolabeled CoQ10 precursor (pHB). Cells were subjected to a lipid extraction and separation by HPLC coupled to radioactivity detector to quantify 14C CoQ10. All data represent the means ± SD of 3 independent experiments. siControl vs. siHuR ** p < 0.01, *** p < 0.001.
which suggests the existence of highly conserved regulatory mechanisms to support cellular metabolism. To-date, transcriptional regulation has been demonstrated to involve PPARα and NF-κB transcription factors mainly in response to either nutrient or stress conditions. Additionally, recent literature highlights the importance of the post-transcriptional control of COQ proteins and the structure of the complex in the regulation of CoQ10 levels. Specifically, COQ7 is regulated by phosphorylation depending on nutritional conditions and is regulated in part by the PTC7 mitochondrial phosphatase. COQ7 protein also requires COQ9 encoded peptide interaction mechanisms to support cellular metabolism. To-date, transcriptome analysis of many species revealed that COQ7 mRNA interaction with RBPs as a novel post-transcriptional regulatory mechanism of CoQ biosynthesis. Over the past decade, ARE-binding RBPs have been described to regulate the turnover and/or translation of target RNAs, including mitochondrial RNAs. In this work, we show evidence that COQ7 mRNA forms an RNP complex that regulates COQ7 expression post-transcriptionally. The elements regulating the translational efficiency tend to be located at 5′UTR of the transcripts, although it was also described that the 3′UTR of β-F1 ATPase mRNA promoted the translation initiation, and 3 clustered AUUUA motifs in the 3′UTR of the interleukin-1α (IL-1α) caused the instability to the transcript. In an in vitro assay to measure protein production, deletion of 5′UTR, a region essential to initiate translation, reduced the translation of COQ7 mRNA. However, deletion of the proximal 3′UTR segment, which included 3 repeats of the AUUUA hexanucleotide and also contained the binding sites for HuR and hnRNPs C1/C2, decreased in vitro COQ7 production. The explanation for this effect is not clear, but perhaps the regulatory elements identified in the proximal segment were not accessible to positive regulators such as HuR to form a functional RNP complex. Whether other positive or negative regulators (e.g., other RBPs or possibly microRNAs) become available to this truncated RNA remains to be examined.

Among the proteins targeting the proximal 3′UTR segment of COQ7 mRNA, we identified HuR and hnRNP C1/C2, which do not have competitive effects on mRNA stability. Interestingly, HuR promoted the formation of hnRNP C1/C2-COQ7 mRNA complex, indicating a functional association between both RBPs and a modest cooperative impact on binding. In contrast, hnRNP C1/C2 was not required for HuR binding to COQ7 mRNA. HuR induced the stability of COQ7 mRNA and consequently the rate of CoQ biosynthesis. The decrease of HuR led to CoQ deficiency and reduced oxidative phosphorylation, which depleted ATP and increased lactate levels. Similarly, primary deficiency of CoQ10 led to a decrease of mitochondrial respiratory chain activities affecting oxidative phosphorylation and forcing cells toward enhanced glycolysis.

The functional association between HuR and other RBPs, such as AUFI, has been widely described, and the interactions between different RBPs may be cooperative or competitive depending on cell type, growth conditions, RBP concentration, cellular compartment and target mRNA. Cooperative hnRNP C1 and HuR interaction to modulate alternative Fas splicing has been also described.

HuR and hnRNP C1/C2 are predominantly located in the nucleus, but these proteins can shuttle between the nucleus and the cytoplasm in different cellular conditions, such as oxidative stress, UV radiation exposure or genotoxic stress. The relation of HuR with mitochondria is described throughout its association with β-F1-ATPase mRNA and the regulation of antiapoptotic proteins such as Bcl2 or BCL-xL, and here we show the direct interaction of both HuR and hnRNP C1/C2 to COQ7 mRNA, the key enzyme of CoQ biosynthesis pathway.

The CoQ10 is synthesized in mammals in quantities that vary widely among tissues and among pathophysiological conditions, such as aging, dietary intake and mitochondrial diseases. The dynamic and composition of the COQ7 mRNA-RBPs complex could depend on the specific tissue but also on metabolic and stress conditions. For example, expression of target mRNAs encoding proteins implicated in cellular proliferation and replicative senescence may be linked to the decreased levels of HuR with senescence, and HuR expression is reduced in NIH-3T3 fibroblasts in the absence of growth factors after serum withdrawal. We observed that the levels of cytoplasmic HuR decreased in serum-deprived MRC-5 cells in parallel to the reduction of COQ7 levels and CoQ10 biosynthesis, in agreement to the increase of aerobic glycolysis described in serum-deprived cells. Importantly, deprivation of serum of 3T6 mouse cells triggered an arrest of the cell cycle and a decrease of CoQ10 biosynthesis independent of the mevalonate pathway. Our results suggest that serum withdrawal affects CoQ biosynthesis by reducing levels of COQ7 mRNA through a decline in HuR levels.

In summary, we have identified a new mechanism for the regulation of CoQ levels that could correspond to specific requirements between long-term transcriptional regulation and the transient modulation of biosynthesis of certain enzymes in the complex. Furthermore, an increasing number of patients display CoQ10 deficiency syndrome; for some of them, mutations in CoQ biosynthesis genes are responsible for the deficiency, but for many others the molecular pathogenic causes are unknown. We hypothesize that alterations in regulatory sequences in the UTRs of COQ genes or in the levels or activities of specific RBPs might contribute to the CoQ10 deficiency in patients with unknown genetic defects.

**Materials and methods**

**Cell culture**

Human cervical carcinoma HeLa (ATCC) and primary human fibroblasts MRC5 cells (CCL-171, ATCC, Manassas, USA) were...
cultured in Dulbecco’s modified essential medium (DMEM, Gibco) with 4.5 g/L glucose and 1 g/L glucose respectively, supplemented with 10% bovine serum and antibiotics (FBS, Sigma). Cells were cultured in a humidified incubator at 37°C and 5% CO2, and detached by trypsinization. Serum withdrawal condition was established by culturing cells 48 h without FBS.

**Immunoprecipitation (IP) of RNP complexes (RIP analysis)**

IP of endogenous RNA-protein complexes was performed using 400 µg of cytoplasmic extract (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, 100 U de RNase-OUT) prepared from HeLa cells. Previously, Protein A Sepharose-beads (Sigma) were pre-coated with 30 µg (overnight at 4°C) of either anti-IgG1 (BD PharMingen), anti-HuR or anti-hnRNP C1/C2 (Santa Cruz Biotech.). Next, lysates were incubated with beads (50% v/v) for 2 h at 4°C, and washed using NT2 (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.05% Nonidet P-40). To isolate and analyze the RNA in the IP fraction, the beads were incubated with NT2 buffer containing 30 µg of RNA and washed with NT2 buffer. Finally, complexes were incubated with 0.1% SDS and 0.5 mg/ml proteinase K (15 min at 55°C) in NT2 buffer. RNA was isolated from the supernatant by phenol-chloroform extraction and measured by real-time qPCR analysis.

**Analysis of binding of RBPs to biotinylated RNA**

The MAXIscript T7 kit (Ambion) was used to synthesize the biotinylated transcripts, including the modified nucleotide biotin-CPT (Enzo Life Sciences AG), following the manufacturer’s instructions. Biotinylated transcripts were purified with NucAway Spin Columns (Ambion). DNA templates for the production of biotinylated transcripts were synthesized using the following oligonucleotides (sense and antisense): for coding region: 5’-(T7)ACGAAGTGGT TGCTTTTTTAG-3’ and 5’-(T7)ACGAAGTGGTTGCTT TTGTTTAG-3’; for 5’UTR: 5’-(T7)GTCGGAGCCAAGG G-CACTA-3’ and 5’-TGCTCCATATTCGCCCTGA-3’; for 3’UTR A: 5’-(T7)GGCTCAGTGACCTCCCG-3’ and 5’-TAGGAGAAAATGGGCCTGG-3’; for 3’UTR B: 5’-(T7)GGCTCAGTGATCCCTCCG-3’ and 5’-TTGGGGATT TTTGGG-3’; for 3’UTR C: 5’-(T7)AGTGTGATTATTT GTGAAATTTGGCCTGG-3’ and 5’-TTGGGGATT TTTGGG-3’; for 3’UTR GAPDH: 5’-(T7)CCTCAACGAC CTTTTGTCA-3’ and 5’-GGTTGAGCACAGGGTACTT TATT-3’. Biotin pulldown assays were carried out by incubating either whole-cell lysates (HeLa) with purified biotinylated transcripts (40 µg lysate, 1 µg RNA) for 2 h at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway) as previously described.24,68 Proteins bound in the pulldown

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*Figure 5. HuR regulates mitochondrial respiration.* Oxygen consumption rate (A) and ATP levels and lactate production (B) were measured in HuR-silenced and in COQ7-silenced cells. Mitochondrial oxygen consumption rates (Mit. OCR) following the sequential addition of oligomycin A (OL), 2,5-dinitrophenol (DNP) to MRC-5 cells stably transfected with HuR or COQ7 shRNA (B, left panel). Right panel: quantification of the mitochondrial basal respiration (BR), maximal respiration (MR), and spare respiration capacity (SRC). These parameters were calculated as described in the Materials and Methods section. Values are the means ± SD of 5 independent experiments. control shRNA vs. HuR shRNA or COQ7 shRNA. *P < 0.05, **P < 0.01, ***P < 0.001.
material were analyzed by Western blotting using specific antibodies against HuR, hnRNP C1/C2, nucleolin, TIAR, FMR, TIA-1, TTP (Santa Cruz Biotech.), NF90 (BD-biosciences), AUF1 (Millipore), PTBP1 and hnRNP K (Abcam).

UV cross-linking assays

The MAXIscript T7 kit (Ambion) was used to synthesize the radiolabeled riboprobes, including 20 μCi of the nucleotide α-(P32)-UTP (GE Healthcare), following the manufacturer’s instructions. DNA templates for the production of biotinylated transcripts were synthesized using the following oligonucleotides (sense and antisense): for the coding region: 5'-(T7)ACGAAGTTGAGTTGGCTTTTTTAG-3' and 5'-(T7)ACGAAGTTGAGTTGGCTTTTTTAG-3'; for 5'UTR: 5'-(T7)GTCCGAGCCAAGGGCACTA-3' and 5'-(T7)GTCCGAGCCAAGGGCACTA-3'; for 3'UTR A: 5'-(T7)GTCCGAGCCAAGGGCACTA-3' and 5'-(T7)GTCCGAGCCAAGGGCACTA-3'; for 3'UTR B: 5'-(T7)GTCCGAGCCAAGGGCACTA-3' and 5'-(T7)GTCCGAGCCAAGGGCACTA-3'; for 3'UTR C: 5'-(T7)GTCCGAGCCAAGGGCACTA-3' and 5'-(T7)GTCCGAGCCAAGGGCACTA-3'; for 3'UTR D: 5'-(T7)GTCCGAGCCAAGGGCACTA-3'. Whole-cell lysate of HeLa cells (30 μg) was incubated with radiolabeled probes (1×105 c.p.m.), 10 min at 30°C, in RLP buffer (NaPHO4- Na2PO4 50 mM pH 7.4, NaCl 140 mM, MgCl2 1.5 mM, DTT 1 mM), containing 4 μg of Escherichia coli ARNt. Next, 20 U of RNase T1 (Boehringer Mannheim) was added (30 min at 37°C). The UV cross-linking assay was carried out by exposition of the reaction mixtures to 254 nm of UV light (Stratalinker 1800; Stratagene) for 6 min. in ice. For competition studies, an excess of unlabeled RNA was added 10 min before the addition of the radiolabeled RNA to liver extracts. The RNA-protein complexes were resolved by SDS–12% PAGE. After electrophoresis, the gels were vacuum dried and the 32P-labeled complexes were visualized by exposure of the gels to X-ray films.

In vitro translation of mRNAs

In vitro-synthesized mRNAs (100 ng), derived from the corresponding plasmids, were utilized as templates for protein synthesis in a nuclease-treated rabbit reticulocyte lysate (Amersham). DNA templates for the production of the transcripts were synthesized using the following oligonucleotides (sense and antisense): for COQ7 full length: 5'-(T7)GTCCGAGCCAAGGGCACTA-3' and 5'-(T7)GTCCGAGCCAAGGGCACTA-3'; for Δ3'UTR: 5'-(T7)GTCCGAGCCAAGGGCACTA-3' and 5'-(T7)GTCCGAGCCAAGGGCACTA-3'.
AGGGCACTA-3’ and 5’-AACGATAACTGTACACCTGT TCTC-3’; for ΔBC: 5’-(T7)GTCCGAGCCAAGGGCACTA-3’ and 5’-TAGGAGAAAAATGGGCTTGG-3’: for ΔC: 5’-(T7) GTCCGAGCCAAGGGCACTA-3’ and 5’-TTGGGGGATTT TTTTGGG-3’. The riboprobes were quantified by spectrophotometry at 260 nm and the quality was monitored by A260/A280 and A260/A230 ratios. The reactions were performed in the presence of 40 mM of L-[35S]methionine (1,000 Ci/mM) and 40 U of RNase A. At different reaction times (up to 1 h), the products were analyzed by SDS-PAGE.

**Determination of mitochondrial oxygen consumption rate**

Cellular oxygen consumption rates (OCR) were determined in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were plated on XF24 microplates at 15,000 cells/well in supplemented medium and incubated at 37°C and 5% CO2 for 24 h. After measuring of basal respiration rate, 6 μM oligomycin was added to inhibit complex V, next 0.75 mM 2,4-dinitrophenol was added to uncouple respiration. Finally, complex I and III were inhibited by addition of 1 μM rotenone and 1 μM antimycin A, respectively. OCR was determined by subtracting the ‘non mitochondrial OCR’ after treatment with rotenone+actinomycin A. Mitochondrial basal respiration was determined from mitochondrial OCR before administration of oligomycin. Mitochondrial maximal respiration was mitochondrial OCR after administration of 2,4-dinitrophenol. Spare respiration capacity was mitochondrial maximal respiration minus mitochondrial basal respiration.

**Determination of lactate and ATP levels**

Cells were plated on 24-well microplates and cultured for 24 h. Medium was collected and lactate determined by using colorimetric lactate oxidase-peroxidase assay lactate kit (Spinreact, Spain) as instructed by the manufacturer. The concentration of lactate in the medium was calculated by comparison with the signal obtained from standard with known amount of lactate provided by the manufacturer. Signals determined in medium without cells were also analyzed and subtracted from the amount determined in the presence of cells. ATP levels in cells were determined by using the CellTiter-Glo Luminiscient assay (Promega, USA), following the instructions of the manufacturer. Briefly, the same amount of reactive solution was added to culture medium to produce cell lysis with gentle movement. The mixture was homogenized and transferred to a white polystyrene 96-well assay plate. A standard with known amounts of ATP were also added in the same plate and mixed with reactive solution in a 1:1 ratio. A POLAR Star Omega fluorimeter and Omega Data Analysis Software (BMG Labtech) was used to analyze luminiscence. ATP amount was referred to the total of cells counted by hemocytometer after trypsin detachment seeded in a 24 well plate seeded in parallel.

**Transfection and plasmids**

Cells were seeded in DMEM supplemented with 5% serum without antibiotics 24 h after transfection with lipofectamine 2000 reagent (Invitrogen) and 2 mg of TAP and HuR-TAP plasmids were diluted in Opti-MEN media (Gibco) and incubated 10 min at room temperature, separately. After 30 min of incubation of plasmid and lipofectamine at room temperature, the transfection complex was added to the cells and incubated for 6 h. Then, the medium was replaced with fresh medium for the ensuing 48 h.

**siRNA transfection and shRNA infection**

siRNAs targeting HuR or hnRNP C1/C2 and control siRNA were acquired from Qiagen. Cells were transfected with oligofectamine (Invitrogen) using the manufacturer’s conditions. Briefly, 24 h before transfection cells were incubated in DMEM 5% serum without antibiotics. Then, for each single transfection, siRNA (10 nM) and oligofectamine were diluted in Opti-MEM media (Gibco). After 30 min of incubation at room temperature the transfection complexes were added to the cells. Lentiviral particles and shRNA of HuR, COQ7 and control incorporated into lentivirus were acquired from Santa Cruz Biotechnology. Cells were plated 24 h before viral infection in supplemented medium. Medium was removed from the plate and replaced with supplemental medium with Polybrene (sc-134220) at final concentration of 10 mg/ml. Cells were infected by adding of shRNA lentiviral particles to the culture.

**Quantitative real-time PCR**

Total RNA was isolated with the TriPure Isolation Reagent (Roche, Mannheim, Germany), treated for DNA removal with DNase I (Sigma-Aldrich) and reverse transcribed by using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time qPCR was performed to measure the expression of select target genes. The reactions were carried out with iQ SYBR Green Supermix (Bio-Rad) and MyiQTM Single-Color Real-Time PCR Detection System (Bio-Rad) on a Bio-Rad conventional thermocycler. The primers were designed with the Beacon Designer software, and the primer pair sequences used in this study were as followed: human forward and reverse primers were GGACGCCATAGGCTGGAC and GATGGAGGAGGAC and AGGACGGCATAGGCTGGAC for COQ7, and TGACCACCACTGGCTTAGC and GCCATGGACTGCTGGTACG for GAPDH. The quality of the PCR reaction products was determined by melting curve analysis and visualization on agarose gels. Negative controls containing water only confirmed the absence of cross-contamination. Efficiency (E) of the PCR reaction was determined using a dilution series, and the data analyzed using the formula 

\[
E = \frac{\text{OD}_{\text{TARGET}} - \text{OD}_{\text{HOUSEKEEPING}}}{\text{CT}_{\text{TARGET}} - \text{CT}_{\text{HOUSEKEEPING}}}
\]

with GAPDH mRNA as loading control. Each PCR reaction was carried out with 3 or more biological replicates in each group.

**Statistical analysis**

Significant differences between the data groups were evaluated using a paired t test (2-tailed P values) for comparing 2 groups and by 2-way ANOVA for ≥2 groups using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons of ANOVA were followed with post hoc Bonferroni. Results are expressed as the means ± SD and were considered significant at P ≤ 0.05.
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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