Estrogen-mediated regulation of mitochondrial gene expression

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Abstract

Estrogens, in particular 17β-estradiol, are well known regulators of essential cellular functions; however, discrepancies remain over the mechanisms by which they act on mitochondria. Here, we propose a novel mechanism for direct regulation of mitochondrial gene expression by estrogen under metabolic stress. We show that in serum-depleted medium estrogen stimulates a rapid relocation of estrogen receptor alpha (ERα) to mitochondria, where it elicits a cellular response resulting in an increase in mitochondrial RNA abundance. Mitochondrial RNA levels are regulated through the association of ERα with hydroxysteroid (17-β) dehydrogenase 10 (HSD17B10), a multifunctional protein involved in steroid metabolism that is also a core subunit of the mitochondrial RNase P complex responsible for the cleavage of mitochondrial polycistronic transcripts. Processing of mitochondrial transcripts affects mitochondrial gene expression by controlling the levels of mature RNAs available for translation. This work provides the first mechanism linking RNA processing and estrogen activation in mitochondrial gene expression and underscores the coordinated response between the nucleus and mitochondria in response to stress.
Introduction

Estrogens, in particular 17β-estradiol (E2), are responsible for the regulation of key cellular processes such as proliferation and apoptosis, inducing changes in nuclear gene expression or by activating cell signaling pathways (1,2). Estrogen receptors (ERs) can function as receptors and transcription factors upon E2 exposure in the nucleus and as signaling molecules in the plasma membrane (3). In the nucleus, ERs regulate the expression of E2-responsive genes through binding to estrogen-responsive elements and inducing changes in transcription (4). In the plasma membrane ERs participate in rapid signal transduction mechanisms and activate G proteins, leading to calcium and cyclic adenosine monophosphate generation and the activation of proximal and distal kinases, which results in the phosphorylation of proteins involved in the modulation of cell migration, survival and proliferation (4,5).

More recently, research has focused on the effects of estrogen on mitochondrial function, as mitochondria are involved in crucial cellular processes in health and in disease. It has been reported that E2 treatment increases the abundance of mitochondrially encoded RNAs that results in elevated respiration (6,7), indicating that E2 plays a role in the modulation of mitochondrial gene expression. It has been suggested that E2 effects on mitochondrial function are indirectly mediated by transcription factors acting on nuclear genes that regulate mitochondrial gene expression (7). In particular, it has been suggested that E2 stimulates the nuclear respiratory factor 1 (NRF-1), promoting the transcription of mitochondrial transcription factor A (TFAM), which subsequently increases mitochondrial DNA (mtDNA) transcription (7,8). The localization of ERs to mitochondria in cells has initiated interest in their specific roles within these organelles. ERs have been localized to mitochondria of several cell lines including the breast cancer-derived MCF-7, and hepatoma-derived HepG2 cells (9-11), where they have been suggested to act as transcription factors affecting mitochondrial gene expression directly (11-13). Other studies have suggested that the interaction between ERs and putative estrogen-responsive elements in mitochondrial DNA is responsible for the induction of mitochondrial transcription by E2 (14,15). There are different modes suggested by which
estrogens exert their effects on the mitochondrial genome; however a general agreement over the mechanism by which ERs may affect mitochondrial gene expression is lacking.

Most of the genetic information necessary for mitochondrial function is encoded by the nuclear genome whereas mitochondrial DNA encodes 11 mRNAs, 2 rRNAs and 22 tRNAs that are transcribed as long polycistronic precursor transcripts from both DNA strands (16). Despite their common polycistronic origin, there is wide variation in the abundance of individual mitochondrial tRNAs, mRNAs, and rRNAs in different tissues and cell types (17). This highlights the importance of post-transcriptional processing mechanisms in the regulation of mitochondrial gene expression. RNA processing is a crucial stage in the regulation of mitochondrial RNA metabolism and consequently mitochondrial function (18-20). Two nuclear DNA-encoded protein complexes account for the majority of the cleavage events involved in mitochondrial RNA processing, the mitochondrial RNase P complex that cleaves the 5' end of tRNAs and (18) the mitochondrial RNase Z, encoded by the ELAC2 gene, which cleaves the 3' ends of tRNAs (19,20).

The mitochondrial RNase P complex is composed of three proteins: tRNA methyltransferase 10 C (TRMT10C), (17-β) dehydrogenase 10 (HSD17B10) and proteinaceous RNase P (PRORP) that are all necessary for efficient cleavage of the 5' tRNA ends. TRMT10C and HSD17B10 have additional functions suggesting that recruitment into the RNase P complex is an additional role for these proteins (20-22). Recently it was shown that TRMT10C, also known as mitochondrial RNase P protein 1 (MRPP1), is a methyltransferase responsible for a common methylation modification of tRNAs and it may be responsible for RNA-recognition for RNase P cleavage (20,21). HSD17B10 is a member of the short-chain dehydrogenase/reductase family known under a range of names including MRPP2, ABAD, ERAB, HADH2, MHBD or SCHAD (Table 1). It has been described as a multifunctional protein that has a NAD^+-binding domain, although this is not required for RNase P activity (21). In addition, reports suggest that HSD17B10 participates in the degradation of the amino acid isoleucine via its 2-methyl-3-hydroxybutyryl-CoA dehydrogenase activity (23,24). PRORP, also known as mitochondrial RNase P protein 3 (MRPP3), belongs to the mammalian pentatricopeptide repeat (PPR) domain protein family (25). PRORP is composed of a PPR domain and a putative metallocnuclease
domain that is hypothesized to harbor the catalytic site of the RNase P complex. In mammalian
mitochondria it was shown that ELAC2 is the mitochondrial RNase Z responsible for processing the
3' end of tRNAs and that it associates with PTCD1, another PPR protein that also acts as a negative
regulator of leucine tRNAs (20,26,27).

Interestingly, HSD17B10 catalyzes the conversion of 17β-estradiol to the less potent estrogen
metabolite estrone in mitochondria (28). It has previously been reported that the estrogen receptor
alpha (ERα) interacts with HSD17B10 in a bacterial two-hybrid screen (29), however, no further
investigation into the relevance of this interaction has been carried out. Therefore we investigated the
biological significance of the interaction between HSD17B10 and ERα in mitochondria of estrogen-
responsive breast adenocarcinoma MCF-7 cells and how this affects mitochondrial gene expression.

Here we show that upon exposure to E2, ERα rapidly localizes to mitochondria, where it interacts
with HSD17B10 to modulate the expression of mitochondrial RNA transcripts. We show that changes
in the expression of proteins involved in the processing of mitochondrial transcripts can have
profound effects on mitochondrial gene expression, by affecting the levels of mature species, the final
processing of the different RNAs, and overall protein synthesis in the presence of estrogen.
**Materials and Methods**

**Cell culture and estrogen treatment**

Estrogen-responsive MCF-7, and ER-negative MDA-468 and MDA-231 human breast adenocarcinoma cells were grown at 37°C under humidified 95% air/5% CO₂ in Dulbecco’s phenol red-free modified Eagle medium (DMEM, Invitrogen) supplemented with 25 mM glucose, 1mM pyruvate, 2 mM glutamine, uridine (0.05 g.l⁻¹), penicillin (0.1 U.l⁻¹), streptomycin sulphate (0.1 g.l⁻¹) and 10% fetal bovine serum (FBS). To evaluate the effects of 17β-estradiol (E2), cells were cultured in serum-depleted medium containing 5% charcoal-stripped FBS for 6 days prior to treatment with 100 nM estradiol in ethanol (Sigma-Aldrich).

**Mammalian expression plasmids**

All expression vectors were based on pcDNA3 (Invitrogen). Full length human TRMT10C (NCBI accession number NP_060289.2), HSD17B10 (NCBI accession number NP_0044841), PRORP (NCBI accession number NP_055487.2), PTCD1 (NCBI accession number NP_056360.2), ELAC2 (NCBI accession number NP_060597.4) or ERα (NCBI accession number NP_000116.2) were expressed with their native termination codon or fused to a tandem affinity purification tag (TAP tag, ABO76910) or EGFP (BD Biosciences) at the C-terminus. All plasmids were tested for expression by transfection and immunoblotting.

**siRNA and plasmid transfections**

MCF-7 cells were plated at 60% confluence in 6-well plates or 10 cm dishes and transfected with annealed siRNAs in OptiMEM medium (Invitrogen) according to manufacturer's instructions. 125 nM (for 6-well plates) or 145 nM (for 10 cm dishes) of TRMT10C, HSD17B10, PRORP, PTCD1, ELAC2 or non-targeting (NT) control siRNA (Thermo Scientific) was transfected using Lipofectamine 2000 (Invitrogen). Cells were incubated 72 h following transfection and then re-transfected for an additional 72 h to enhance knockdown efficiency, followed by a further incubation in the absence or presence of 100 nM E2 for 18 h and then harvested.
MCF-7 cells were plated at 60% confluence in 10 cm dishes in serum-depleted medium and transfected with mammalian expression plasmids in OptiMEM medium (Invitrogen) containing puromycin (1µg.ml⁻¹) to maintain plasmid expression. 158 ng/cm² of TRMT10C-TAP, HSD17B10-TAP, PRORP-TAP, PTCD1-TAP, ELAC2-TAP, ERα-TAP or control EGFP-TAP plasmid DNA was transfected using Fugene HD (Roche). Cells were incubated for 48 h following transfection to allow integration and expression of the plasmid, in serum-depleted medium containing puromycin (1µg.ml⁻¹) to maintain plasmid expression. This was followed by a further incubation in the absence or presence of 100 nM E2 for 18 h before cells were harvested.

**Mitochondrial isolation**

Mitochondria were prepared from 2 x 10⁷ MCF-7 cells grown in basal, non serum-depleted or serum-depleted medium or transfected with siRNA and treated with E2 (as described above). Cells in PBS were collected by centrifugation at 150 g for 5 min at 4°C and the pellet was suspended in 4 ml of ice-cold 10 mM NaCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl, pH 7.5. Cells were allowed to swell for 5 min on ice and briefly homogenized with a 7 ml glass homogenizer. The nuclei from this suspension were sedimented at 1300 g for 3 min at 4°C, and this centrifugation step was repeated for the supernatant once more. The mitochondria from this suspension were sedimented (10,000 g for 10 min at 4°C) and suspended in 120 mM KCl, 10 mM HEPES, 1mM EGTA. Protein concentration was determined by the bicinchoninic acid assay as described previously (30).

**Mitochondrial subfractionation**

Mitochondria were prepared from MCF-7 cells grown in serum-depleted medium and treated with E2 for 18 h as described above. Protein concentration was normalized to 100 mg.ml⁻¹ in STE containing 0.1% (w/v) fat-free BSA (STE/BSA) and determined by the bicinchoninic acid assay. The mitochondrial outer membrane was solubilized by adding 0.2 mg digitonin (12.5 mg.ml⁻¹ in STE) /mg protein to the mitochondrial preparation followed by incubation for 15 min at 4°C. Three volumes STE/BSA were added to each sample, mixed and gently homogenized using micro pestles (Ambion). Mitoplasts were sedimented by centrifugation (10,000 g for 10 min at 4°C) and the pellet was
suspended in 20 µl STE/BSA, while the supernatant was retained for further fractionation. The mitochondrial outer membrane fraction was collected as a pellet following centrifugation (144,000 g for 20 min at 4°C) and suspended in 70 µl STE containing 1% lauryl maltoside representing the membrane, insoluble fraction. The supernatant from this centrifugation step contained the soluble fraction.

**Immunoblotting**

Protein expression was detected by immunoblotting using Sigma-Aldrich antibodies according to manufacturer’s recommendations: TRMT10C (Cat. No. HPA036671), HSD17B10 (Cat. No. HPA001432), PRORP (Cat. No. HPA020459), PTCD1 (Cat. No. HPA020106), ELAC2 (Cat. No. HPA019535), ERα (Cat. No. HPA000450). Specific mitochondrial proteins were detected using MitoSciences antibodies: COX1 (Cat. No. MS404), COX2 (Cat. No. MS405) and complex II subunit 70 KDa, SDHA (Cat. No. MS204). Porin (Abcam, Cat. No. ab15895) was used as a control to confirm normalized protein loading. The primary antibodies were detected using the Odyssey Infrared IRDye 680RD goat anti-rabbit or IRDye 800CW goat anti-mouse secondary antibodies (Li-Cor Biosciences) and visualized using the Odyssey imaging system (Li-Cor Biosciences).

**RNA harvesting and northern blotting**

RNA (5 µg) was extracted from siRNA-transfected MCF-7 cells, MDA-468 or MDA-231, followed by E2 treatment as described above, using the miRNeasy RNA extraction kit (Qiagen) with a DNase I on-column digestion step included to remove DNA, according to manufacturer’s instructions. Northern blotting was carried out as described in (31). The signal was detected using IRDye labelled streptavidin (Li-Cor Biosciences) diluted 1:10000 in 3x SSC, 5% SDS, 25 mM Na2HPO4, pH 7.5 and visualized using the Odyssey (Li-Cor Biosciences) imaging system.

**Quantitative RT-PCR**

The transcript abundance of mRNAs of interest was measured on RNA isolated from MCF-7 cells following siRNA treatment. cDNA was prepared 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 rRNA.

**Tandem affinity purification**

Tandem affinity purification was carried out from plasmid-transfected MCF-7 cell lysates previously treated with E2 as described previously (27,31).

**Microscopy**

MCF-7 cells were plated onto 13 mm diameter glass coverslips and allowed to attach overnight. Before transfection, the cells were placed in serum-depleted medium as described above. Cells were transfected with ERα-EGFP or HSD17B10-EGFP plasmid for 48 h, followed by incubation with 50 nM MitoTracker Orange in PBS for 15 min. In experiments examining the effects of E2, the cells were cultured in serum-depleted medium for 6 days prior to E2 treatment. After washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 30 min and washed again with PBS. Cells were mounted in DABCO/PVA medium and images were acquired using a Nikon Ellipse Ti fluorescent inverted microscope using a Nikon 60x or 100x objective.

**Mitochondrial protein synthesis**

MCF-7 cells were grown in six-well plates, transfected, incubated in the presence or absence of E2 and de novo protein synthesis was analyzed as described in (32).

**Mitochondrial respiration**

Mitochondrial respiration using 0.5 mM N,N,N’,N’-Tetramethyl- p-phenylenediamine dihydrochloride (TMPD) and 2 mM ascorbate was measured in permeabilized cells as described in (20).

**Native PAGE**
Mitochondria were prepared from MCF-7 cells grown in serum-depleted medium and incubated in the presence or absence of 100 nM E2 for 18 h. Mitochondrial proteins were solubilized in sample buffer containing 1% dodecyl maltoside for 15 min followed by centrifugation (20 000 g, 30 min at 4˚C) and the supernatant resolved by one-dimensional native PAGE in the NativePAGE Novex Bis-Tris Gel System (Life Technologies). Electrophoresis was carried out in an XCell SureLock Mini-cell (Life Technologies) and followed by transfer to PVDF membrane (Immobilon FL, Millipore) using a Mini-Protean Trans blot system (Bio-Rad). Transfer was followed by incubation in Odyssey Blocking buffer (Li-Cor Biosciences) for 1 h, followed by overnight incubation with primary antibodies at 4˚C.

The primary antibodies were detected using the Odyssey Infrared IRDye 680RD goat anti-rabbit or IRDye 800CW goat anti-mouse secondary antibodies and visualized using the Odyssey imaging system (Li-Cor Biosciences).

**Immunoblot densitometry and statistical analysis**

Band intensity from electronic (tiff) images of immunoblots was calculated by densitometry using Image J freeware (rsb.info.nih.gov/ij). Data are presented as the mean and standard deviation. The statistical significance was examined using a two-tailed Student’s t-test.
Results

**HSD17B10 and ERα expression in MCF-7 cells**

Previous studies have indicated that the E2 effects on mitochondria are mediated by ERα (33), since ERα has been found associated with mitochondria in cells (9-11). To determine if there is an E2-dependent effect in the abundance of ERα the endogenous distribution of ERα was assessed by immunoblotting experiments carried out on mitochondria isolated from MCF-7 cells grown in basal, non serum-depleted medium, compared with mitochondria from cells grown in serum-depleted medium, followed by E2 treatment for 18h (Fig. 1A). We found ERα associated with mitochondria of MCF-7 cells independent of E2 treatment, however ERα is significantly enriched in mitochondria from cells grown in serum-depleted medium. This suggests that the serum-depletion may enhance the localization of ERα to mitochondria.

Mitochondria contain the HSD17B10 enzyme that is involved in E2 degradation and this protein has been suggested to interact with ERα in a bacterial two-hybrid system (29). The levels of this protein are not affected by growth in basal or serum-depleted medium (Fig. 1A). However, we wanted to gain further insight into the distribution of HSD17B10 and ERα in mitochondria and in response to E2. We carried out cell and mitochondrial subfractionation experiments to analyze the distribution of ERα and HSD17B10 in mitochondria from MCF-7 cells grown in basal (Fig. 1B) and serum-depleted medium (Fig. 1C), followed by incubation in the presence or absence of 100 nM E2 for 18h. Enriched fractions were obtained containing whole cell lysates, intact mitochondria, mitoplasts (mitochondria devoid of outer membrane), mitochondrial insoluble fraction (membranes) and mitochondrial soluble fraction. Immunoblotting for ERα and HSD17B10 indicates that both proteins are enriched in mitoplasts under normal and metabolic stress conditions, however, ERα has a significantly stronger association with mitochondria following serum starvation.

**HSD17B10 interacts with ERα in MCF-7 cells**

Next we investigated possible association of ERα with HSD17B10 within mitochondria of MCF-7 cells. The C-terminus of HSD17B10 was fused to a tandem affinity purification (TAP) tag to isolate it.
and determine if it associates with ERα by immunoblotting (Fig. 2). We found that endogenous ERα associated with HSD17B10-TAP, in the presence or absence of E2 (Fig. 2A), although the presence of E2 this association was weaker as suggested previously (29). These interactions were further assessed by reverse TAP, in which the C-terminus of ERα was fused to a TAP tag to determine if it interacts with HSD17B10 (Fig. 2B). Endogenous HSD17B10 was detected in association with ERα-TAP, and this association was weaker in the presence of E2. These results indicate that ERα associates with HSD17B10 and that this association can be affected by E2, validating a previous report of this interaction identified in a bacterial two-hybrid screen, hereby establishing the relevance of this interaction in mammalian cells. EGFP was used as a control to show specific antibody detection and that neither HSD17B10 (Fig. 2C) nor ERα (Fig. 2D) associate with EGFP, indicating the specificity of the interactions between ERα and HSD17B10.

**HSD17B10 and ERα co-localize in mitochondria of MCF-7 cells**

The localization of HSD17B10 in mitochondria has been found in rat cardiac myocytes (29), brain vascular smooth muscle cells (34) and monkey kidney fibroblast-like COS-7 cells (35), whereas ERα has been reported to be present in the nucleus and mitochondria of MCF-7 cells (10). Confocal fluorescence microscopy analyses were carried out to determine the specific localization of HSD17B10 and ERα in MCF-7 cells grown in serum-depleted medium, where we found ERα enriched in our immunoblotting experiments (Fig. 1). We transfected plasmids expressing either ERα or HSD17B10 fused to EGFP at the C-terminus and incubated the cells with 100 nM E2 for 5 min, 3 h or 18 h to investigate possible changes in their intracellular localization relative to control, untreated MCF-7 cells. We used MitoTracker Orange to specifically stain mitochondria within the cells. In the absence of E2, ERα is found predominantly in the nuclei of MCF-7 cells, although we also observed it associated with mitochondria (Fig. 3A). Next we observed the distribution of ERα following the addition of 100 nM E2 after 5 minutes and found that ERα is rapidly enriched within mitochondria (Fig. 3A, 5 min E2 treatment). ERα is found localized in the nucleus after prolonged, 3 and 18 h treatments with E2 (Fig. 3A, 3 h and 18 h E2 treatment). This suggests that E2 has a rapid effect on mitochondrial function via the recruitment of ERα to mitochondria. Next, we investigated the
localization of HSD17B10 in MCF-7 cells to determine if its distribution follows a similar pattern to ERα, since these two proteins associate with each other (Fig. 2). HSD17B10 was found in the cytoplasm and in specific puncta within mitochondria of MCF-7 cells, however its distribution is not changed as a result of E2 treatment (Fig. 3B), confirming our immunoblot experiment (Fig. 1A). Together with our immunoblotting and TAP experiments, these results show that ERα is rapidly recruited to mitochondria where it associates with HSD17B10 and this association is affected by E2.

In addition to its role in estrogen degradation, HSD17B10 is an essential subunit of the mitochondrial RNase P complex that cleaves the 5’ ends of tRNAs. Since ERα translocates to mitochondria in response to E2, where it interacts with HSD17B10, we investigated whether E2 treatment led to a differential recruitment of HSD17B10 that could affect the formation or stability of the mitochondrial RNase P complex. To maintain mitochondrial proteins in their native complexes we resolved mitochondrial lysates from MCF-7 cells by native electrophoresis, followed by immunoblotting to detect the three protein subunits of the RNase P complex, TRMT10C, HSD17B10 or PRORP (Supplementary Fig. 1). We found that E2 treatment did not affect the stability of the multimeric conformations of the components of the RNase P complex.

Estrogen-dependent regulation of mitochondrial transcripts by RNA processing proteins

Since HSD17B10 is part of the RNase P complex and RNA processing enzymes regulate mitochondrial gene expression (20,36) we sought to identify the role of these proteins upon E2 treatment. To examine the role of the RNA processing proteins TRMT10C, HSD17B10, PRORP, PTCD1 or ELAC2 on RNA metabolism in MCF-7 cells, we assessed the effects of the siRNA-mediated knockdown of these proteins in the presence of E2. We carried out qRT-PCR to measure the efficiency of knockdown and found that the siRNAs significantly decreased the mRNA levels of each RNA processing protein (Fig 4A). In addition, treatment of MCF-7 cells with siRNAs against each of the mitochondrial RNA processing enzymes leads to a significant decrease in their protein abundance compared to cells treated with control siRNAs in the presence and absence of E2 (Fig. 4B and C). Interestingly, knockdown of HSD17B10 also leads to a decrease in TRMT10C, suggesting
that the stability of TRMT10C depends on the presence of HSD17B10. These two proteins have been shown to form a stable sub-complex of the mitochondrial RNase P enzyme previously (18,21). In addition, knockdown of HSD17B10 also leads to a subtle decrease of PRORP compared to control indicating the association of these proteins as part of the RNase P complex. Furthermore we observed that knockdown of ELAC2 leads to an increase in PTCD1 abundance in MCF-7 cells (Fig 4B). We have shown that PTCD1 interacts with ELAC2 and affects the 3’ end processing of mitochondrial tRNAs as a negative regulator (20,27) and this increase may represent a compensatory response.

Next we investigated the effects of knocking down TRMT10C, HSD17B10, PRORP, PTCD1 or ELAC2, relative to controls, on the steady state levels of mitochondrial RNA transcripts by northern blotting in the presence or absence of E2 (Fig. 5A and B). We observed an increase in many mitochondrial RNAs in response to E2 treatment in the control (NT) siRNA-treated cells (Supplementary Fig. 2). In contrast, mitochondrially encoded cytochrome c oxidase subunit 1 (MT-CO1) mRNA abundance was decreased upon E2 treatment, and the levels of mitochondrially encoded cytochrome b (MT-CYB) and the 12S rRNA were not significantly affected (Supplementary Fig. 2).

Knockdown of TRMT10C or ELAC2 led to a dramatic reduction of most RNA transcripts (Fig. 5B and Supplementary Fig. 2) and E2 treatment only subtly increased their abundance as in the control samples, providing further evidence that these two enzymes are required for RNA processing in mitochondria. The significant reduction of mature mitochondrial transcripts upon TRMT10C or ELAC2 knockdown is consistent with that observed in HeLa cells (20). In contrast, tRNA^{Trp} steady state levels in MCF-7 cells were not reduced by the knockdown of any of the mitochondrial RNA processing enzymes (Fig. 5B and Supplementary Fig. 2).

Knockdown of HSD17B10 did not result in significant changes in the levels of mitochondrial RNAs (Fig. 5D), however upon addition of E2 we observed a significant increase in the abundance of a particular subset of mitochondrial RNAs (Fig. 5B and Supplementary Fig. 2). In the absence of E2 treatment, PRORP silencing resulted in the up-regulation of some mitochondrial tRNAs and mRNAs, including tRNA^{Glu}, tRNA^{His} and tRNA^{SerAGY}, compared to NT siRNA control (Fig. 5D). Knockdown of PTCD1 resulted in an overall increase in the steady state levels of RNA transcripts compared to NT
siRNA control upon estrogen treatment (Fig. 5E). These findings indicate that the levels of many mitochondrial RNA transcripts are affected by both the hormonal status of the cellular environment and the levels of the RNA processing proteins.

Mutations in the gene encoding HSD17B10 cause 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency, an X-linked neurodegenerative inborn error of isoleucine metabolism which mimics the symptoms of mitochondrial disease (23,37). This indicates that HSD17B10 has an additional role to mitochondrial RNA processing, in the regulation of isoleucine metabolism. Therefore, we investigated the steady state levels of mitochondrial tRNA\textsuperscript{ile} in MCF-7 cells compared to cells treated with TRMT10C, HSD17B10, PRORP or NT siRNAs in the absence or presence of E2 (Fig. 5B).

TRMT10C knockdown led to dramatically decreased tRNA\textsuperscript{ile} levels, similar to the changes observed in other RNAs upon knockdown of this protein (Fig. 5B). Interestingly, we observed E2-dependent changes in tRNA\textsuperscript{ile} abundance upon knockdown of HSD17B10, that were not observed for any other tRNA investigated in this study. In the absence of E2 treatment, we observed increased levels of tRNA\textsuperscript{ile} upon HSD17B10 knockdown compared to NT siRNA control and PRORP knockdown, whereas E2 treatment led to a significant decrease in the steady state levels of tRNA\textsuperscript{ile} compared to control and PRORP knockdown (Fig 5F). This pattern was not observed for other mitochondrial RNA transcripts investigated, which indicates that HSD17B10 may play a specific role in the regulation of the tRNA\textsuperscript{ile} transcript in an estrogen-dependent manner.

Next we investigated the effects of E2 on the processing of mitochondrial RNAs by qRT-PCR (Fig. 6). Knockdown of the RNA processing proteins causes an accumulation of pre-processed mitochondrial transcripts (Fig. 6A and B) relative to controls, however E2 can decrease the abundance of specific pre-processed transcripts by affecting the RNase P complex (Fig. 6A), but not the 3' tRNA processing proteins ELAC2 and PTCD1 (Fig. 6B), suggesting that E2 stimulation can partially rescue processing defects, possibly by recruiting HSD17B10 into the RNAse P complex. Taken together these results show that ER\(\alpha\) is rapidly recruited to mitochondria upon E2 activation where it associates with HSD17B10 and stimulates mitochondrial RNA processing.
Since we observed E2-stimulated changes in mitochondrial transcripts when PTCD1 was knocked down we investigated if this protein associates with ER\(\alpha\) by TAP pull down (Fig. 7). We found that endogenous ER\(\alpha\) associated with PTCD1-TAP (Fig. 7A), however endogenous PTCD1 could not be detected in association with ER\(\alpha\)-TAP (Fig. 7B), suggesting that the TAP-tag may obscure the site through which it associates with PTCD1. Next we examined if PTCD1 and HSD1\(\beta\)10 associated with each other but we could not detect an association between them by TAP pull down, independent of E2 treatment (Supplementary Fig. 3A). This finding indicates that RNase P and ELAC2/PTCD1 complexes are distinct within mitochondria. Additional TAP pull down experiments confirmed the association between TRMT10C and HSD1\(\beta\)10, HSD1\(\beta\)10 and PRORP (Supplementary Fig. 3B) and between PTCD1 and ELAC2 (Supplementary Fig. 3C), independent of E2 treatment. These results confirm the co-existence of TRMT10C, HSD1\(\beta\)10 and PRORP in the RNase P complex in cells and confirm the association between PTCD1 and ELAC2, both originally observed in HeLa cells (18,20).

**Impaired processing of mitochondrial transcripts leads to decreased protein expression**

Changes in mitochondrial tRNA levels as a result of defects in RNA processing can affect the translation of mitochondrially-encoded mRNAs (20,38). To investigate the effects of TRMT10C, HSD1\(\beta\)10, PRORP, PTCD1 or ELAC2 knockdown on mitochondrial protein synthesis in the presence and absence of E2, we carried out a *de novo* incorporation assay using \(^{35}\)S methionine and cysteine pulse labeling (Fig. 8A and 8B). Coomassie staining of pulse labeling gels showing normalized protein loading is shown in Supplementary Fig. 4. As observed for mitochondrial RNA levels (Supplementary Figure 2), we also found a significant increase in mitochondrial protein synthesis in response to E2 treatment in the control cells (Fig. 7C and D). In contrast, we observed decreased mitochondrial protein synthesis when TRMT10C and ELAC2 were knocked down (Fig. 8C and D), consistent with the most significant decrease in mitochondrial RNAs observed by northern blotting (Fig. 5A). Knockdown of HSD1\(\beta\)10 or PRORP did not have a significant effect on mitochondrial protein synthesis (Fig 8C). We did not observe changes in mitochondrial protein synthesis upon knockdown of PTCD1, independent of E2 treatment (Fig. 8D). PTCD1 knockdown
increases the abundance of mature tRNAs that are further stimulated upon E2 treatment (Fig. 5E),
however this increase does not affect the rate of protein synthesis, suggesting that these tRNAs are not
normally present at rate-limiting amounts for mitochondrial protein synthesis.

Next we investigated the steady state levels of mitochondrial proteins by immunoblotting from MCF-7 cells grown in serum-depleted medium and transfected with TRMT10C, HSD17B10, PRORP, PTCD1 or ELAC2 siRNAs and control NT siRNAs in the presence and absence of E2. We observed a
decrease in the levels of mtDNA-encoded COX1 and COX2 cytochrome c oxidase polypeptides upon
TRMT10C and ELAC2 knockdown relative to controls, independent of E2 treatment (Fig. 8E and F),
consistent with our northern blots (Fig. 5A) and translation measurements (Fig. 8A and 8B). A
decrease was also observed in the expression of nuclear-encoded complex II subunit SDHA upon
TRMT10C knockdown, independent of E2 and likely as a response to feedback from the decrease in
mitochondrially-encoded polypeptides of the electron transport chain. Knockdown of HSD17B10,
PRORP or PTCD1 did not markedly affect the steady state levels of mitochondrial proteins
(Supplementary Figure 5). Together, the pulse labeling and immunoblotting experiments show a
significant decrease of mitochondrial protein expression upon TRMT10C or ELAC2 knockdown.
These decreases reflect their crucial role in the processing of mitochondrial transcripts as core
subunits of the RNase P and RNase Z complexes and their requirement for mitochondrial gene
expression. The dramatic effects of TRMT10C knockdown may be due to the dual role of this protein
as a methyltransferase responsible for a tRNA modification and as a component of the RNase P
complex (18,20,21). The E2-dependent nature of the changes observed upon HSD17B10 or PRORP
knockdown may reflect additional roles of these proteins in mitochondrial function, including
HSD17B10’s enzymatic activity in estrogen metabolism and as a subunit of the RNase P processing
complex.

We investigated the effects of E2 on mitochondrial oxygen consumption in MCF7 cells treated with
NT siRNAs and upon knockdown of HSD17B10. Mitochondrial respiration is increased upon E2
treatment, which correlates well with increased mitochondrial gene expression (Fig. 8G). Knockdown
of HSD17B10 led to decreased oxygen consumption, indicating the requirement of this protein for mitochondrial function, however the addition of E2 in cells where HSD17B10 was knocked down improved mitochondrial respiration (Fig. 8G). We carried out qRT-PCR to show that the increase in mitochondrial gene expression in the control MCF7 cells was not due to increased biogenesis driven by nuclear encoded master regulators such as TFAM, PGC-1 and PPARγ as their expression is not increased upon E2 treatment (Fig. 8H). Instead, we observe upregulated master regulators in E2-treated cells only when HSD17B10 is knocked down. Taken together these data suggest a possible mechanism by which HSD17B10 can be recruited into the RNase P complex upon E2 stimulation under stress to increase mitochondrial gene expression that consequently improves energy production. However if HSD17B10 is decreased or lost mitochondrial gene expression can be upregulated by increasing the levels of transcriptional activators of mitochondrial biogenesis in the nucleus.

**Discussion**

Traditionally, estrogens regulate mitochondrial function by modulating the transcription of nuclear genes involved in mitochondrial gene expression or by acting on receptor-dependent signaling cascades that might affect mitochondrial function. This work explored a new avenue for the regulation of mitochondrial gene expression upon estrogen activation. The results presented here reveal a role for direct action of E2 on mitochondrial RNA metabolism via HSD17B10 in association with ERα. A direct action would allow the hormone to induce rapid changes in mitochondrial gene expression, suggesting that E2 might directly influence energy production by the organelle, by regulating the availability of subunits of the respiratory chain. This would be of particular relevance under metabolic stress conditions, where changes in the energy demand might require a quick, adaptive response by the cell.

The function of HSD17B10 as a hydroxysteroid dehydrogenase involved in the regulation of estrogen levels is well established (39,40). The association of HSD17B10 with ERα and their co-localization in mitochondria of MCF-7 cells in the presence of estrogen suggests that this hormone affects mitochondrial gene expression via RNA processing. The estrogen response was rapid and stimulated
the distribution of ERα to mitochondria, suggesting that the cellular stress response in MCF-7 cells is mediated by ERα through its association with HSD17B10. The interaction of HSD17B10 with ERα was initially found in a bacterial two-hybrid screen (29). In that study, a putative mechanism for the interaction between ERα and HSD17B10 was proposed, in which, under low E2 conditions, ERα’s binding to HSD17B10 inhibits its catalytic activity in estradiol degradation. In contrast, in the presence of high concentrations of estradiol, HSD17B10 is proposed to dissociate from the HSD17B10-ERα complex and metabolize estradiol to estrone. Although HSD17B10’s binding to ERα could indeed lead to its inactivation in its role in estradiol degradation, this model does not take into account HSD17B10’s fundamental role in mitochondrial RNA processing, as a core subunit of the RNase P processing enzyme. It is reasonable to hypothesize that the recruitment of HSD17B10 by the RNase P complex or its association with ERα upon E2 activation, may underlie the changes observed in this work on mitochondrial RNA transcript levels. In an E2-responsive cellular context, such as that of MCF-7 cells, HSD17B10’s role in estradiol degradation or as a component of the RNase P might result in changes in RNase P activity that in turn lead to variations in mitochondrial transcript abundance.

We have investigated the roles of TRMT10C, HSD17B10, PRORP, PTCD1 and ELAC2 in the regulation of mitochondrial gene expression in MCF-7 cells and showed that mitochondrial RNA processing can be affected by E2 treatment in cells expressing ERα. Some of the changes observed upon knockdown of these proteins on steady state RNA levels indicate that there are discrete, cell type-specific variations in their action on mitochondrial RNA processing. For instance, in contrast with previous studies in HeLa cells, tRNA^Trp was not affected by the knockdown of TRMT10C or ELAC2 in MCF-7 cells (20) and it remains to be determined how the levels of tRNA^Trp are controlled in MCF-7 cells. Knockdown of TRMT10C decreased the levels of most mitochondrial RNAs dramatically, consistent with previous reports that suggest this may be due to its dual role as a tRNA-methyltransferase and as part of the mitochondrial RNase P complex in RNA processing (18,20,21). It has been shown that all three components of the mammalian mitochondrial RNase P complex are required for its activity and here we observe that knockdown of each component leads to a decrease of
mitochondrial RNAs, albeit at different levels. The metallocnuclease domain of PRORP provides the
catalytic component of the RNase P complex; however, the effects of its knockdown on mitochondrial
RNA abundance are not particularly dramatic. With the exception of tRNA^leu^, E2 treatment of cells
where HSD17B10 was knocked down did not result in significant changes in mitochondrial RNAs.
Control cells showed an increase in many mitochondrial RNAs in response to E2 treatment;
interestingly, in the absence of E2 treatment, MCF-7 cells where PRORP was knocked down
increased mitochondrial RNA abundance. The estrogen-dependent changes observed upon PRORP
knockdown may reflect the dual role of HSD17B10, as a subunit of the RNase P enzyme and as a
binding partner of ERα. In the presence of estrogen, HSD17B10 may be preferentially recruited to the
steroid metabolism pathway rather than its role as a core subunit of the RNase P complex. Hence, in
the absence of E2, mitochondrial transcript levels may be recovered as the protein is available to join
the RNase P complex and carry out the processing activity that results in increased mature transcript
levels. The changes observed at the RNA level were consistent with changes in mitochondrial protein
translation, with decreases in mitochondrial protein synthesis upon TRMT10C or ELAC2 knockdown.
The increase in PTCD1 abundance in response to ELAC2 knockdown suggests that the two proteins
have opposing roles in tRNA processing, whereby decreased abundance of PTCD1 and consequent
increase in ELAC2 should result in increased abundance of mature mitochondrial transcripts.
Alternatively, it could be that the association of the two proteins is required for 3’ tRNA processing
and the increase in ELAC2 abundance is a compensatory response to decreased PTCD1 levels. The
association of ERα with both HSD17B10 and PTCD1 provides the means to affect both 5’ and 3’
mitochondrial RNA processing in response to E2 and enables coordinated control of mitochondrial
gene expression. The effects on mitochondrial gene expression by E2 and ERα under stress growth
conditions are specific, since no significant changes in mitochondrial gene expression were observed
in cells lacking ERα that were grown in basal and serum depleted medium (Supplementary Fig. 6).
Since its identification as a component of the mitochondrial RNase P complex, the role of HSD17B10
in RNA processing has remained obscure. The association between HSD17B10 and TRMT10C into a
subcomplex is required for TRMT10C to carry out an important nucleotide modification of
mitochondrial tRNAs (18,20,21). Initially, it was suggested that the NAD+ binding site present in
HSD17B10 was necessary for the processing activity of RNase P. However, recent studies showed
that both HSD17B10’s dehydrogenase activity and NAD+ binding site are not necessary for RNase P
activity (21). HSD17B10’s role in the conversion of 17β-estradiol to estrone within mitochondria
suggests that mitochondria may be able to directly regulate the intracellular levels of the hormone.
This is of particular importance given estrogen’s role in cellular proliferation and tumor progression
in cancer (41,42). Furthermore, a number of mutations in the gene encoding HSD17B10 have been
associated with disease (43). For instance, patients carrying mutations in HSD17B10 present with
neurological abnormalities and neuromotor disorders associated with defects in the metabolism of
isoleucine. Accordingly, our work shows a specific role for HSD17B10 in tRNA\text{\textsuperscript{Ile}} transcript
abundance. The role of HSD17B10 on isoleucine availability may explain the effect of HSD17B10
knockdown on tRNA\text{\textsuperscript{Ile}} abundance. HSD17B10’s role in isoleucine degradation represents yet another
function of this versatile, multifunctional protein that requires further investigation.
Mitochondria play a range of key roles in the cell, including the integration of extracellular signals,
such as hormones, and the execution of intracellular responses. There is increasing evidence
suggesting a crosstalk between mitochondria and the nucleus, which is directly related to cellular
function and reveals the existence of signaling pathways that may provide new therapeutic targets in
disease. Estrogen mediated carcinogenesis caused by spontaneous mutations that lead to enhanced cell
proliferation has been associated with increased levels of estrogen and long-term stimulation of
estrogen signaling pathways (44). Although the exact mechanisms underlying these signaling
pathways are not fully understood, the results presented here indicate that increased estrogen levels
affect mitochondrial gene expression by modulating the activities of mitochondrial RNA processing
proteins via ER\text{\textalpha}, further supported by the evidence that the master regulators of mitochondrial
biogenesis (TFAM, PGC-1 and PPAR\text{\gamma}) are not upregulated with ER\text{\textalpha} treatment. Instead these factors
are upregulated when the levels of HSD17B10 are compromised, suggesting that there is a secondary
mechanism that can compensate for decreased mitochondrial function. These findings illustrate the
existence of feedback mechanisms between the nucleus and mitochondria directly modulating
mitochondrial gene expression. Here, HSD17B10 is described as a novel target for the regulation of mitochondrial gene expression by E2 and a clear example of a moonlighting enzyme, a multifunctional protein performing multiple, unrelated functions and involved in different aspects of mitochondrial metabolism.

Acknowledgements

The authors thank Moira Hibbs for technical assistance and Jacky Bentel for providing MCF-7 cells and critical review of the manuscript.


44. Feigelson HS, Henderson BE. Estrogens and breast cancer. Carcinogenesis.
Table and Figure legends

Table 1. Nomenclature and synonyms of the studied proteins

Nuclear encoded proteins used in this work are listed with their UniProtKB identifiers a, gene symbol according to HUGO Nomenclature Committee b, common synonyms c and their function d.

Figure 1. HSD17B10 and ERα expression in MCF-7 cells

A. ERα was detected by immunoblotting of mitochondrial lysates from MCF-7 cells grown in basal, non serum-depleted medium and cells grown in serum-depleted medium, followed by incubation with 100 nM E2 for 18 h. Porin was used as a control to confirm normalized protein loading. Bars represent mean ± SD of relative mitochondrial ERα levels or HSD17B10 in serum-depleted cells, normalized to porin and compared with cells grown in basal, non-depleted medium in the presence or absence of E2. Statistical significance was examined using a two-tailed Student’s t-test, * p < 0.05, n=3.

ERα and HSD17B10 cellular distribution was assessed by immunoblotting from cell lysates and mitochondrial fractions isolated from MCF-7 cells grown in basal medium (B) or serum-depleted medium (C), followed by incubation with 100 nM E2 for 18 h.

Figure 2. HSD17B10 interacts with ERα

A. Endogenous ERα was isolated from MCF-7 cells grown in serum-depleted medium, expressing HSD17B10-TAP (48 kDa) in the presence or absence of 100 nM estradiol (E2) for 18 h, using IgG agarose beads, followed by TEV protease cleavage.

B. TAP analyses show endogenous HSD17B10 (27 kDa) isolated from cells expressing ERα-TAP (88 kDa). IgG heavy (50 kDa) and light chains (23 kDa) can be seen on the immunoblots and are marked with asterisks.

C and D. EGFP was used as a control to show the specificity of the TAP procedure.

Figure 3. HSD17B10 and ERα co-localize in mitochondria of MCF-7 cells
A. ERα is predominantly nuclear but is rapidly recruited to mitochondria of MCF-7 cells upon E2 treatment. Cells grown in serum-depleted medium were transfected with a plasmid expressing ERα fused to EGFP at the C-terminus, followed by incubation with 100 nM E2 for 5 min, 3 h or 18 h. Cells were then incubated with MitoTracker Orange (MT Orange) to stain mitochondria in live cells and fixed. ERα-EGFP (green) can be seen in the nucleus and also in mitochondria stained with MT Orange (red) in the overlaid images (yellow). Scale bar is 10 µm.

B. HSD17B10 is present in the cytoplasm and in puncta within mitochondria of MCF-7 cells. Cells grown in serum-depleted medium were transfected with a plasmid expressing HSD17B10 fused to EGFP at the C-terminus, followed by incubation with 100 nM E2 for 5 min, 3 h or 18 h. Cells were then incubated with MitoTracker Orange (MT Orange) to stain mitochondria in live cells and fixed. HSD17B10-EGFP (green) can be seen in the cytoplasm and also in mitochondria stained with MT Orange (red) in the overlaid images (yellow). Scale bar is 10 µm.

Figure 4. Knockdown of mitochondrial RNA processing proteins

A. The mRNA knockdown levels of the mitochondrial RNA processing proteins in MCF-7 cells were determined by qRT-PCR relative to control non-targeting (NT) siRNA-treated cells and normalized to 18S rRNA. Bars represent mean ± SD. Student's 2-tailed t test for paired data was carried out and showed that mRNAs were significantly decreased relative to controls, p < 0.05 n=3.

B. Immunoblotting of mitochondria isolated from MCF-7 cells transfected with control non-targeting (NT), TRMT10C, HSD17B10, PRORP, PTCD1 or ELAC2 siRNAs for 72 h in serum-depleted medium, followed by an additional re-transfection for 72 h to enhance knockdown efficiency, and in the presence or absence of 100 nM estradiol (E2) for 18 h. Antibodies were used to detect the expression levels of TRMT10C, HSD17B10, PRORP, PTCD1 or ELAC2 upon siRNA-mediated knockdown. Porin was used as a control to confirm equal protein loading. Blots are representative of 4 independent biological experiments.

C. Densitometry analysis of immunoblotting experiments show that siRNA treatment significantly decreases protein abundance compared to control cells, in the presence and absence of E2. Graph
shows relative protein levels normalized to porin and compared to non-targeting (NT) control. Bars
represent relative mean ± SD. Student's 2-tailed t test for paired data was carried out and showed that
protein levels were significantly decreased relative to controls, p < 0.05.

**Figure 5. E2-dependent effects of RNA processing proteins on mitochondrial RNA levels**

A. RNA was isolated from MCF-7 cells transfected with control non-targeting (NT), TRMT10C,
HSD17B10, PRORP, PTCD1 or ELAC2 siRNA, in the presence or absence of 100 nM estradiol (E2)
for 18 h, to determine steady state levels of mitochondrial transcripts by northern blotting. Nuclear
18S rRNA abundance was used as an internal control for total RNA loading. Data shown are typical
results repeated on three independent RNA preparations. Each blot was probed for the different RNA
species by stripping and re-probing.

B. Northern blot showing the effects of RNA processing protein knockdown on the steady state levels
of tRNA\textsubscript{Ile} in the presence and absence of 100 nM E2.

**Figure 6. E2-dependent effects on mitochondrial RNA processing**

RNA was isolated from MCF-7 cells transfected with control non-targeting (NT), TRMT10C,
HSD17B10, PRORP (A) PTCD1 or ELAC2 (B) siRNA, in the presence or absence of 100 nM
estradiol (E2) for 18 h, to determine the effects on mitochondrial RNA processing by qRT-PCR
relative to control NT siRNA-treated cells and normalized to 18S rRNA. Data shown are typical
results repeated on three independent RNA preparations. Data are means ± S.D. of three separate
experiments, p < 0.05 is marked with an asterisk.

**Figure 7. PTCD1 associates with ERα**

A. Endogenous ERα (67 kDa) was isolated from MCF-7 cells grown in serum-depleted medium,
expressing PTCD1-TAP (98 kDa) in the presence or absence of 100 nM estradiol (E2) for 18 h, using
IgG agarose beads, followed by TEV protease cleavage.
B. Reverse TAP analyses were carried out to detect endogenous PTCD1 (77 kDa) isolated from cells expressing ERα-TAP (88 kDa) using a PTCD1 specific monoclonal antibody.

Figure 8. Impaired processing of mitochondrial transcripts leads to decreased protein expression

Mitochondrial protein synthesis was analyzed by the incorporation of $^{35}$S-labeled methionine and cysteine in the 13 proteins synthesized by the mitochondrial translation machinery. MCF-7 cells were grown in serum-depleted medium and transfected with siRNAs targeting TRMT10C, HSD17B10, PRORP or NT control (A) and siRNAs targeting PTCD1, ELAC2 or NT control (B) in serum-depleted medium, followed by treatment with 100 nM E2 for 18 h. The protein synthesis of cytoplasmic proteins was inhibited with emetine. Coomassie stained gels showing equal loading of the cell lysates are shown in Supplementary Fig. 4A.

C. Knockdown of TRMT10C, in the presence of estrogen, decreases mitochondrial DNA-encoded protein synthesis. Graph shows relative protein levels normalized to porin. Bars represent relative mean ± SD. Statistical significance was examined using a two-tailed Student’s t-test, and indicated by * (* p < 0.05), n=3.

D. Knockdown of ELAC2 decreases mitochondrial DNA-encoded protein synthesis. Graph shows relative protein levels normalized to porin. Bars represent relative mean ± SD. Statistical significance was examined using a two-tailed Student’s t-test, and indicated by * (* p < 0.05), n=3.

Immunoblotting analyses of mitochondrial proteins isolated from MCF-7 cells grown in serum-depleted medium and transfected with siRNAs targeting TRMT10C, HSD17B10, PRORP or NT control (E) and siRNAs targeting PTCD1, ELAC2 or NT control (F), followed by treatment with 100 nM E2 for 18 h. Antibodies were used to detect the expression levels of mtDNA-encoded cytochrome c oxidase subunits COX1 and COX2 and nuclear DNA-encoded complex II SDHA subunit. Porin was used as a control to confirm normalized protein loading.
G. Mitochondrial oxygen consumption was measured in MCF-7 cells grown in serum-depleted medium, in the presence or absence of 100 nM E2 for 18 h. Data are means ± S.D. of three separate experiments, p < 0.05, n=3.

H. The mRNA levels of TFAM, PGC-1 and PPARγ were measured in MCF-7 cells treated with HSD17B10 or NT control siRNAs in the presence or absence of 100 nM E2 by qRT-PCR. The fold change in expression upon addition of E2 compared to absence of E2 is shown. Data were normalized to 18S rRNA and bars represent mean ± SD. Student's 2-tailed t test for paired data was carried out and showed that mRNAs were significantly changed relative to controls, p < 0.05, n=3.

Supplementary Figure 1. E2 treatment does not affect the stability of the RNase P complex

Mitochondria were prepared from MCF-7 cells grown in serum-depleted medium and incubated in the presence or absence of 100 nM E2 for 18 h. Mitochondrial proteins were then solubilized with 1% dodecyl maltoside, sedimented and the supernatant resolved by native PAGE. Immunoblotting was carried to detect TRMT10C, HSD17B10 or PRORP with antibodies specific to these proteins. Probing with a monoclonal TRMT10C antibody revealed the presence of two bands at ~240 kDa and ~300 kDa. The same two bands were observed after probing an independent blot with PRORP antibody. In contrast, a band of ~150 kDa was detected using a monoclonal antibody against HSD17B10. Proteins detected in native PAGE experiments have a size estimation error of ±15%. The theoretical, combined molecular weight of the protein subunits of RNase P is ~140 kDa (TRMT10C has a molecular weight of 47 kDa, HSD17B10 27 kDa and PRORP 67 kDa). It has been reported that HSD17B10 exists as a 108 kDa homotetramer (He, 2001), which might correspond to the band detected by native PAGE using a monoclonal antibody specific to HSD17B10, taking into account the size estimation error. The higher molecular weight bands observed with TRMT10C or PRORP antibodies might represent multimeric associations between the RNA processing subunits.

Supplementary Figure 2. E2 effects on steady-state levels of mitochondrial RNAs in control, and gene-specific siRNA treated cells.
A. Effects of knockdown of TRMT10C, HSD17B10 and PRORP on the steady-state levels of mitochondrial RNA transcripts in the presence and absence of E2.

B. Effects of knockdown of ELAC2 and PTCD1 on the steady-state levels of mitochondrial RNA transcripts in the presence and absence of E2.

The data are expressed relative to transcripts from control samples. Bars represent relative mean ± SD. Student's 2-tailed t test for paired data was carried out, p < 0.05.

C. HSD17B10 knockdown results in E2-dependent changes in tRNA Ile abundance. Data are expressed as the ratio of tRNA Ile transcript normalized to 18S rRNA. Bars represent relative mean ± SD. Statistical significance was examined using a two-tailed Student’s t-test, and indicated by * (* p < 0.05).

Supplementary Figure 3. Additional protein interactions detected by TAP

A. PTCD1 and HSD17B10 do not interact, as assessed by TAP followed by immunoblotting.

B. Endogenous HSD17B10 was isolated from MCF-7 cells expressing TRMT10C-TAP or PRORP-TAP, independent of E2 treatment.

C. Endogenous ELAC2 or PTCD1 isolated from MCF-7 cells expressing PTCD1-TAP or ELAC2-TAP respectively, independent of E2 treatment.

Supplementary Figure 4. Normalized protein loading of pulse labeling gels

A and B. Blue Coomassie staining of pulse labeling gels showing normalized protein loading.

Supplementary Figure 5. Knockdown of tRNA processing proteins and their effects on the steady state levels of mitochondrial proteins in MCF7 cells

Bars represent mean ± SD of relative protein expression, normalized to porin and compared with NT controls.
Supplementary Figure 6. The levels of mitochondrial RNA are not affected by E2 in ERα negative cells

RNA was isolated from MDA-468 and MDA-231 grown in basal or serum depleted medium in the presence or absence of 100 nM estradiol (E2) for 18 h, to determine steady state levels of mitochondrial transcripts by northern blotting. Nuclear 18S rRNA abundance was used as an internal control for total RNA loading. Data shown are typical results repeated on three independent RNA preparations. Each blot was probed for the different RNA species by stripping and re-probing and the signal was quantified from three independent experiments.
Figure 1
Figure 2

Click here to download Figure: Figure 2.eps
Figure 3

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Click here to download Figure: Figure 3.eps
**Figure 4**

(A) Fold RNA change relative to control.

(B) Normalized protein expression relative to control.

(C) Fold RNA change relative to control.
Figure 6

**A**

Fold RNA change relative to control

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**B**

Fold RNA change relative to control

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Figure 6
Figure 7

Click here to download Figure: Figure 7.eps
Table 1. Nomenclature and synonyms of nuclear encoded mitochondrial proteins investigated in this study

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