Cell. Mol. Life Sci. 66 (2009) 721 – 730
1420-682X/09/040721-10
DOI 10.1007/s00018-009-8687-4

Cellular and Molecular Life Sciences

Research Article

Transient and constitutive repression of cytoplasmic translation signaling in cells with mtDNA mutation

G. M. C. Janssen*, P. Schwertman, T. A. T. Wanga, R. S. Jahangir Tafrechi, P. J. A. van den Broek and A. K. Raap

Department of Molecular Cell Biology, Leiden University Medical Center, Post Zone S1-P, Einthovenweg 20, PO Box 9600, 2300RC Leiden (The Netherlands), Fax: +31-71-526-8270, e-mail: G.M.C.Janssen@lumc.nl

Received 29 October 2008; received after revision 11 December 2008; accepted 16 December 2008
Online First 14 January 2009

Abstract. Cytoplasmic translation is under sophisticated control but how cells adapt its rate to constitutive loss of mitochondrial oxidative phosphorylation is unknown. Here we show that translation is repressed in cells with the pathogenic A3243G mtDNA mutation or in mtDNA-less ρ^0 cells by at least two distinct pathways, one transiently targeting elongation factor eEF-2 and the other initiation factor eIF-2α constitutively. Under conditions of exponential cell growth and mammalian target of rapamycin (mTOR) activation, eEF-2 becomes transiently phosphorylated by an AMP-activated protein kinase (AMPK)-dependent pathway, especially high in mutant cells. Independent of AMPK and mTOR, eIF-2α is constitutively phosphorylated in mutant cells, likely a signature of endoplasmic reticulum (ER)-stress response induced by the loss of oxidative phosphorylation. While the AMPK/eEF-2K/eEF-2 pathway appears to function in adaptation to physiological fluctuations in ATP levels in the mutant cells, the ER stress signified by constitutive protein synthesis inhibition through eIF-2α-mediated repression of translation initiation may have pathobiochemical consequences.

Keywords. mtDNA disease, elongation, initiation, cytoplasmic translation, diabetes.

Introduction

The main site of ATP synthesis (>90%) is the mitochondrion, which produces ATP by oxidative phosphorylation of ADP (OXPHOS). The OXPHOS system comprises five complexes embedded in the inner mitochondrial membrane and is composed of ~90 protein subunits. Mitochondria contain their own circular genome, the mtDNA, which encodes a subset of 13 of these OXPHOS subunits together with the 22 tRNAs and 2 rRNAs required for mitochondrial translation within the matrix. Maternally inherited mutations in mtDNA in general and nucleotide substitutions in the mitochondrial tRNA genes in particular lead to altered mitochondrial translation, decreased OXPHOS activity and respiration, and are important factors in the pathogenesis of several multisystem disorders including maternally inherited diabetes and deafness (MIDD; OMIM 520000) and mitochondrial encephalomyopathies such as mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS; OMIM 540000) and myoclonic epilepsy associated with ragged red fibers (MERRF; OMIM 545000) [1–11]. Also clonal accumulation of acquired mtDNA mutation and OXPHOS failure has been implicated in aging diseases such as sarcopenia and Parkinson's disease [12–14]. Next to a role in degenerative diseases, acquired mtDNA mutations have been increasingly implicated

* Corresponding author.

in tumor progression by conferring tumor cells with growth advantages and metastatic potential [15–17]. With the equivalent of more than four ATPs invested in each peptide bond, cytoplasmic translation is highly demanding on ATP supply in any cell type. Following cell division the complete proteome has to be duplicated in a relative short period of time, making cell growth and proliferation highly energy demanding processes. In exponentially growing cells, cytoplasmic protein synthesis is high but drops considerably when the culture reaches confluency and cells enter the stationary phase ([18] *cf.* [19]). Also non-proliferative cells that function to produce large amounts of secretory proteins have high energy demands, for instance insulin synthesis in pancreatic β cells.

Protein synthesis can be conveniently divided into three stages: initiation, elongation and termination of mRNA translation. Especially at the initiation and elongation level elaborate control systems have evolved to regulate protein synthesis rates [20, 21]. They use the energy sensing mechanism of AMP-activated protein kinase (AMPK) [22] as well as nutrient availability cues and growth and proliferation signals centering around the mammalian target of rapamycin (mTOR), thus regulating initiation factor 4E binding protein (4E-BP) and elongation factor 2 (eEF-2). AMPK activates tuberous sclerosis complex 1/2 (TSC1/2) [23] leading to reduced mTOR signaling and thereby reduction of inactivating phosphorylation of eEF-2 kinase (eEF-2K) at Ser366 by ribosomal protein S6 kinase (S6K) [24], as well as Ser78 and Ser359 by unknown kinases (for a review see [25]). Ultimately, this pathway through mTOR inactivation leads to eEF-2 phosphorylation and consequently inhibition of elongation [24, 26].

However, a direct route from AMPK to eEF-2K and translation regulation has also emerged [27, 28]. In this mTOR-independent pathway, AMPK directly phosphorylates eEF-2K at Ser398 leading to its activation [29]. Finally, AMPK is also implicated in inhibition of the initiation phase of translation through mTOR and initiation factor 4E-BP1 dephosphorylation [30]. A second initiation factor, eIF-2α, is regulated independently of AMPK and mTOR, but acts in response to a variety of cellular stresses like unfolded proteins in the endoplasmic reticulum (ER) through PKR-like ER kinase (PERK) or amino acid deprivation through general control non-repressible 2 (GCN2) [31–34]. While phosphorylation of eIF-2α serves to impair general protein synthesis, it also causes up-regulation of the translation of certain specific mRNAs that encode transcription factors like activating transcription factor 4 (ATF4), thereby launching an integrated stress response [35].

When cells are temporarily challenged by diminution of ATP production by glucose deprivation, hypoxia or inhibitors of OXPHOS, they respond to these conditions of acute energy stress by down-regulating ATP-consuming processes and activating protective pathways until the stress is corrected or removed [28, 34, 36–38]. However, in the case of a constitutive loss of mitochondrial energy supply by mtDNA mutation, the energy crisis is sustained, and to maintain viability and functional integrity they have to adapt to the condition that substrate level ADP phosphorylation during glycolysis has become the only ATP source. Using an A3243G cybrid cell and a mtDNA-less cell model, we investigated the role of key regulators of translation in this process to gain clues to the underlying adaptive mechanisms. We found that these cells have engaged a constitutive repression of translation initiation by phosphorylation of eIF-2α and that they transiently repress energy-consuming elongation only under conditions of rapid cell growth by phosphorylation of eEF-2.

Materials and methods

Cell culture. The mitochondrial transformants (cybrids) used in this study were generated earlier by the transfer of mitochondria from fibroblasts from an A3243G MIDD patient to mitochondrial DNA-less 143B ρ^0 cells [6, 39, 40]. They contained either the wild-type (VW6 and VW7) or the A3243G mutant-type (VM48, VM50 and VM50rev) $tRNA^{Leu(UUR)}$ gene. Cybrids and ρ^0 cells were characterized with respect to mtDNA genotype, respiration and population doubling time (Table 1). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing high glucose (4.5 mg/ml) and 110 μg/ml pyruvate supplemented with 50 μg/ml uridine and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and subcultured twice a week *via* trypsin treatment to a cell plating density of 10–15% confluency. The A3243G mutation level was determined before each experiment using a PCR-RFLP assay [3]. Absence of detectable mtDNA in ρ^0 cells was confirmed by lack of the expected band after the mtDNA PCR [3]. mtDNA copy numbers of the cybrids were comparable (~2000) as determined by the ΔCt method [41].

Inhibitors. Antimycin A, oligomycin and rapamycin were from Sigma-Aldrich. Rotenone was from BDH. AMPK inhibitor compound C was from Calbiochem.

Protein synthesis. Cytoplasmic protein synthesis rate was estimated from the incorporation of L-

Table 1. Genotype and phenotype of cell lines.

Cell type	mtDNA genotype	Respiration (fmol O_2/cell/h)[a]	Doubling time (h)	ATP (pmol/μg protein)
VW6	wt	~78	23 ± 3	n.d.[b]
VW7	wt	82 ± 20	23 ± 5	15.8 ± 2.2
VM48	98% A3243G	~9	29 ± 1	n.d.
VM50	>98% A3243G	7.1 ± 5.0	27 ± 4	11.9 ± 2.6
VM50rev	~70% A3243G	70 ± 21	n.d.	n.d.
ρ^0	no mtDNA	< 0.5	27 ± 2	12.4 ± 2.5

[a] Values ± SD ($n \geq 3$)
[b] n.d., not determined

[4,5-^{3}H]leucine into trichloroacetic acid-precipitable material essentially as described [19, 40]. Cells were seeded at different densities in six-well plates (day 1) and allowed to proliferate for 2 days on high glucose DMEM with one refreshment at day 2 so as to prevent acidification and to compensate for excessive glucose consumption by the mtDNA mutant cells. At day 3 the cells were again refreshed but this time using DMEM with low glucose (1 mg/ml) and without serum but 2.5 mg/ml bovine serum albumin to prevent induction of serum-stimulated pathways just before experiments were performed. The low glucose served as an additional energy challenge, but was not essential for observing translation repression and phosphorylation of eEF-2 and S6. The cells were allowed to starve in the serum-free, low-glucose medium for 3 h before [^{3}H]leucine incorporation was performed. Cells were washed three times with PBS and incubated for 90 min at 37°C in 0.75 ml leucine-free DMEM/F12 medium containing 10 μCi L-[4,5-^{3}H]leucine (Amersham/GE healthcare) and 10 μM unlabeled leucine. Cells were then again washed three times with PBS and thoroughly dissolved in 1 ml 0.2 M NaOH. Aliquots of 100 μl were precipitated by the addition of 100 μl 20% trichloroacetic acid, and assayed for total protein content by a bicinchoninic acid-based protein assay (Pierce, USA) or for the incorporation of [^{3}H]leucine into protein using a GF/C filter assay [40]. The rate of protein synthesis is expressed as the amount of [^{3}H]leucine incorporated per μg protein per hour. Protein concentration was also used as an estimate for cell density, 30–35 μg/cm^2 protein corresponding to confluency (i.e., all area just covered with cells) for all cells, whereas maximum densities were estimated as ~80, ~60 and ~45 μg/cm^2 for VW7, VM50 and ρ^0 cells, respectively.

ATP determination. ATP levels were determined from perchloric acid extracts using an ATP bioluminescence assay (Roche). Briefly, cells at ~90% confluency (~30 μg protein/cm^2) were deprived of serum and glucose as described in the "protein synthesis" section, washed with PBS and scraped in 8% perchloric acid. After centrifugation the supernatant was neutralized with 3 M K_2CO_3 and used for ATP determination. The precipitate was dissolved in 0.2 M NaOH and used for protein determination, which served for normalization of ATP values.

Preparation of extracts and *in vitro* translation. *In vitro* translation experiments were performed with cytosolic and salt-washed (poly)ribosomal fractions both freed from the endogenous amino acid and nucleotide pools using the method essentially as described [42]. Post-mitochondrial supernatant was prepared from the cells of ten 14-cm plates of VW7, VM50 or ρ^0 as described in [6] up to and including centrifugation for 45 min at 10 000 *g* to remove the mitochondria. The post-mitochondrial supernatant was passed through G-25 to remove small molecular weight material and processed by centrifugation to obtain the cytosolic and salt-washed (poly)ribosomal fractions [42]. *In vitro* translation was assessed from [^{3}H]leucine incorporation in a 40-μl assay containing 25 μg cytosolic protein and 25 μg (poly)ribosomal protein [42]. Reactions were performed in the presence of added ATP and GTP (200 μM each), and a NTP-regenerating system consisting of 10 mM phosphocreatine and creatine phosphokinase (Roche).

Western blotting. For Western blotting, series of VM50, VW7 and ρ^0 cells in 9-cm dishes were treated identically and in parallel with the cells for [^{3}H]leucine incorporation in 6-well plates up to and including the starvation period. Then cells were washed three times with PBS and thoroughly dissolved by shearing in 2% SDS, 50 mM Tris-HCl pH 6.8. After determination of protein content, samples were diluted to obtain 3.0 mg/ml protein in SDS-PAGE sample buffer. To ensure direct comparison of phosphorylation and protein level between wild-type and mtDNA mutant cells, the six different samples from both cell types were always analyzed on the same Western blot. Blots were probed with antibodies from Cell Signaling

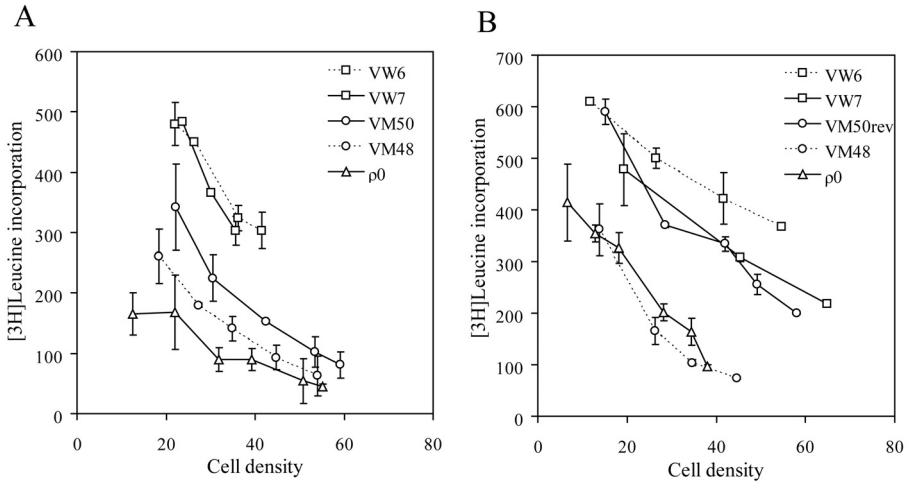

Figure 1. The cytoplasmic translation rate decreases at high cell density and is repressed in mitochondrial DNA (mtDNA) mutant cells. (*A*) [^{3}H]Leucine incorporation at different densities of wild-type (VW6, VW7), A3243G mutant (VM48, VM50) and ρ^0 cells is shown. Translation rate is expressed as cpm of [^{3}H]leucine per μg of protein ± SD (n=4), whereas the amount of cell protein present on the dish is taken as a measure of cell density (μg of protein per cm^2 culture area). Although [^{3}H]leucine incorporation assays suffer somewhat from intra-experimental variation (see also Fig. 2A), mtDNA mutant cells repeatedly showed reduced translation compared to wild-type cells in replicate experiments (n=7), including A3243G cybrids from a different donor (not shown). (*B*) The homoplasmic mutant VM50 cells from (A) turned out to be slowly shifting to wild type after prolonged continuous culture. After about 30 weeks they proved to be ~70 % mutant (below the critical threshold) and respiring. The translation rate of these cells (VM50rev in B) was similar to the wild-type rates. Presence of mitochondrial translation inhibitor chloramphenicol did not have a significant effect on [^{3}H]leucine incorporation (not shown).

Technology [eEF-2, p-Thr56-eEF-2, S6, p-Ser235/236-S6, eIF-2α, p-Ser51-eIF-2α, 4E-BP1, p-Thr37/46–4E-BP1, p-Ser65–4E-BP1, p-Thr70–4E-BP1, mTOR, p-Ser2448-mTOR, AMPKα, p-Thr172-AMPKα, acetyl-CoA carboxylase (ACC), p-Ser79-ACC] and Santa Cruz (p-Thr981-PERK) in the dilution suggested by the manufacturer. Phosphorylation of ACC was used as read-out for AMPK activity and that of S6 for mTOR activity.

Results

In cultures of human cells, the rate of protein synthesis per cell is progressively down regulated as the culture becomes more confluent [18]. Therefore, we compared the different cell types (Table 1) at increasing densities [19]. The rate of cytoplasmic translation is high in exponentially growing cells and gradually decreases as the cell cultures become denser and reach stationary phase (Fig. 1A). All cell clones investigated, whether near homoplasmic A3243G mutant, wild type or mtDNA-less (ρ^0), showed this density dependence. However, compared to wild-type cells, cybrid cells harboring the A3243G mutation and ρ^0 cells showed a large repression in translation at all densities, with ρ^0 cells often showing the largest decrease. As can be seen in Table 1, A3243G and ρ^0 cells have elevated population doubling times compared with wild-type cells, likely a direct consequence of the reduced translational rates. One of the near-homoplasmic cell lines, VM50 (>98 % A3243G), was kept in continuous culture for 30 weeks and 'shifted' to ~70 % mutation load. This 'revertant' cybrid cell line, denoted VM50rev, regained mitochondrial respiration (70±21 fmol O_2/cell/h) and also recovered from repression of translation (Fig. 1B). Thus, mtDNA mutations above the critical threshold cause reduced mitochondrial respiration and repression of cytoplasmic translation.

To rule out the trivial explanation that a dramatic drop in ATP level leads to repression of translation by direct ATP depletion, cellular ATP content was measured and found to be only ~25 % decreased in A3243G and ρ^0 cells (Table 1). Although ATP synthesis in ρ^0 and A3243G cells relies only on glycolysis, the total amount of cellular ATP obviously remains at a comparable level under our experimental translation conditions. However, when glucose is fully withdrawn from the culture medium, wild-type cells retain their ATP level for up to 3 h, but mutant cells show a large additional drop (>50 %) in cellular ATP level (not shown). A ~25 % reduction in ATP can hardly explain the large two- to fourfold repression of translation rates in mutant cells. To support this notion we performed *in vitro* translation experiments under conditions of equal and fixed ATP concentration and again found strong repression of translation in the extracts of mtDNA mutant cells (Fig. 2). Therefore, it is likely that the protein synthesis machinery senses

mitochondrial dysfunction through a signaling cascade that ultimately affects one or more translation factors regulating protein synthesis.

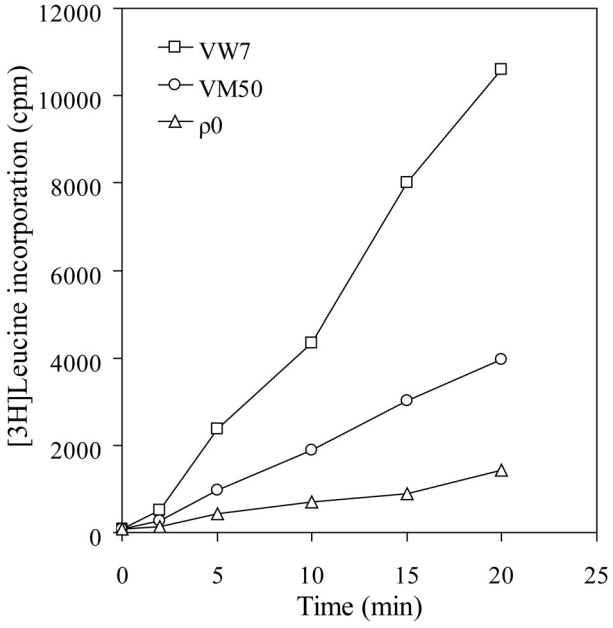

Figure 2. Repression of *in vitro* translation in the presence of excess ATP. Post-mitochondrial fractions from wild-type, A3243G and ρ^0 cells were freed from their endogenous nucleotide and amino acid pool as described in "Experimental procedures". *In vitro* translation was tested by [^{3}H]leucine incorporation into protein in the presence of added ATP, GTP and a NTP regenerating system. The incorporation of [^{3}H]leucine was reproducibly low in VM50 (n=2) and ρ^0 (n=2) compared to VW7 cell extracts (n=3). A representative experiment (with n=1 per time point) is shown. Note that the repression of *in vitro* translation is observed in the presence of added (thus equal) amino acid levels and equal specific radioactivity of the label.

Key regulators/markers of translation, elongation factor eEF-2, ribosomal protein S6 and initiation factors eIF-2α were initially analyzed at different densities of wild-type VW7 and ρ^0 cells. Interestingly, they all showed a distinct phosphorylation pattern. The phosphorylation of eEF-2 was highly elevated in ρ^0 cells compared to VW7 cells and showed a strong density-dependent response in both cell types (Fig. 3). VW7 cells exhibited a comparable but less vigorous response to low densities. Phosphorylation of S6 also showed a clear density-dependent response, but in contrast to eEF-2, there was no large difference between wild-type and ρ^0 cells. The phosphorylation of eIF-2α was clearly elevated in ρ^0 cells but with no pronounced effect of cell density in either cell type. These experiments were replicated and extended with VM50 cybrid cells harboring the A3243G mutation (Fig. 4). Similar phosphorylation patterns were observed for eEF-2, S6 and eIF-2α in VM50 cells: eEF-2

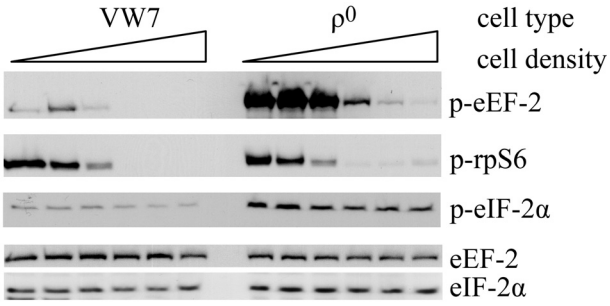

Figure 3. Transient and constitutive repression mechanisms control cytoplasmic translation factors. VW7 and ρ^0 cells were grown at six different densities and equal amounts of protein subjected to Western blot analysis. Total eEF-2 and eIF-2α are shown as equal load controls of this series of samples.

phosphorylation was elevated at low density in all cell types, but with a much more pronounced effect in the A3243 mtDNA mutant and ρ^0 cells. S6 phosphorylation was also enhanced at low densities, but with no strong effect of mtDNA mutation or absence of mtDNA. In contrast, eIF-2α phosphorylation was clearly elevated in A3243G and the ρ^0 cells but without strong cell density dependence. In summary, elongation factor eEF-2 responded cell density-dependently with the stronger effect in the mtDNA mutant cells, whereas initiation factors eIF-2α showed constitutively increased phosphorylation that was largely independent of cell density.

Reduced ATP synthesis leads to accumulation of AMP, a major end product of elongation, and by dismutation of ADP by the action of adenylate kinase [43]. Increased AMP/ATP ratios are sensed by AMPK, which signals to downstream translation factors. The activation of the energy sensor AMPK in mutant cells correlated well with the phosphorylation of eEF-2 and of its read-out ACC (Fig. 4). This strongly suggests the involvement of AMPK in inactivating eEF-2. In addition, compound C, an ATP-competitive inhibitor of AMPK, reduces AMPK phosphorylation and activity (as inferred from ACC phosphorylation) in ρ^0 cells with a concomitant decrease of eEF-2 phosphorylation (Fig. 5). Furthermore, when energy stress was induced in wild-type cells by treatment with inhibitors of OXPHOS, both AMPK and eEF-2 responded concomitantly to a level nearly equal to that seen in untreated ρ^0 cells (Fig. 5). Thus, eEF-2 becomes inactivated under conditions of diminished energy supply, whether induced by the mutant mtDNA genome or chemically. AMPK activates TSC1/2 [23] leading to reduced mTOR signaling, activation of eEF-2K by reduced phosphorylation at Ser366 by S6K [24, 25] and, ultimately, phosphorylation and inactivation of eEF-2 [24, 26]. The phosphorylation of mTOR, and its

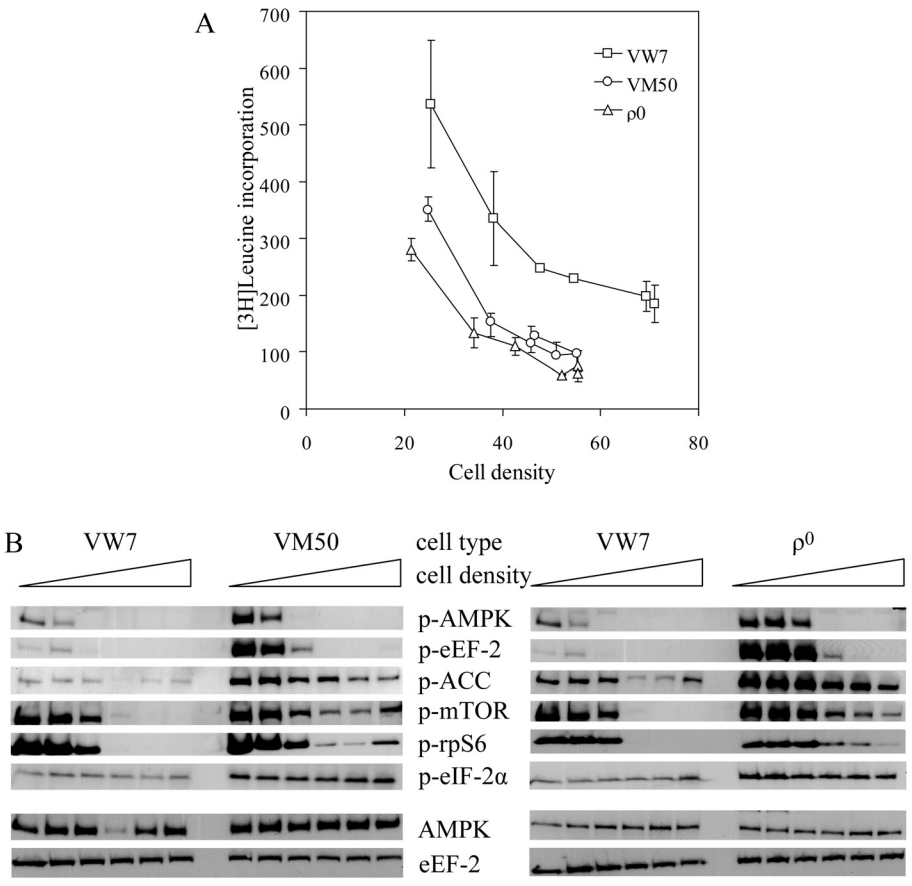

Figure 4. Altered AMP-activated protein kinase (AMPK) signal transduction in A3243G cybrid cells and ρ^0 cells. (*A*) Wild-type (VW7), A3243G type (VM50) cybrid cells and ρ^0 cells at six different densities were tested for translation.(*B*) The same series of samples was also analyzed on Western blots. AMPK and eEF-2 protein were probed for equal load of this set of samples. The duplicate patterns for VW7 (left and right panel) were from a single series of six lysates, which were analyzed twice, together with VM50 on the blot on the left and with ρ^0 on the blot on the right, so as to enable direct comparison of wild type and mtDNA mutant cells. Note that the sixth density sample of the VM50 series in fact is at a lower density than intended (see A). Results have been replicated for each individual protein with at least two different sets of samples.

downstream target S6, was high at low density and decreased when cells reached higher densities, irrespective of the cell type, wild type or mtDNA mutant (Fig. 4). Thus, all three cell types show the higher phosphorylation of AMPK, ACC, mTOR, S6 and eEF-2 at low cell density when proliferation and ATP consumption are high. However, there is no clear effect of mtDNA mutation or absence of mtDNA on mTOR and S6 phosphorylation. This indicates that the elevated phosphorylation of eEF-2 in mtDNA mutant cells is not mediated by mTOR under our experimental conditions.

Additional support for the independence of eEF-2 phosphorylation of mTOR activation comes from inhibition of mTOR with rapamycin. The phosphorylation pattern of eEF-2 in ρ^0 cells remained unchanged after treatment with rapamycin while phosphorylation of mTOR and S6 were severely decreased (Fig. 6). Furthermore, chemical inhibitors of oxidative phosphorylation evoked phosphorylation of AMPK, eEF-2, and ACC in wild-type cell similar to the levels of ρ^0 cells, but without a clear effect on mTOR and S6 phosphorylation (Fig. 5), suggesting also the absence of AMPK signaling to mTOR.

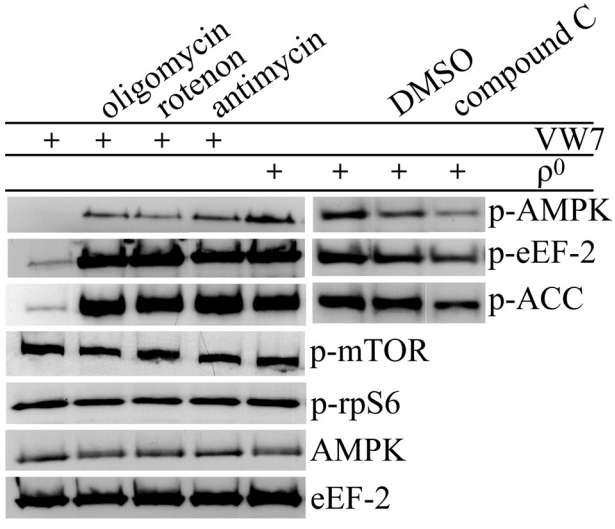

Figure 5. Stimulation and inhibition of AMPK-activated pathways. Energy stress was chemically induced in wild-type cells at high density (~50 μg) by treatment (30 min) with inhibitors of oxidative phosphorylation (OXPHOS), 10 μM rotenone (complex I), 1 μM antimycin A (complex III) and 0.5 μM oligomycin (complex V). This response was nearly equal to that seen in untreated ρ^0 cells at low density (~25 μg). Inhibition of AMPK phosphorylation by incubation (5 h) with 0.25% DMSO alone or 20 μM compound C (dissolved in DMSO) leads to a concomitant decrease of the phosphorylation of eEF-2 and ACC in ρ^0 cells.

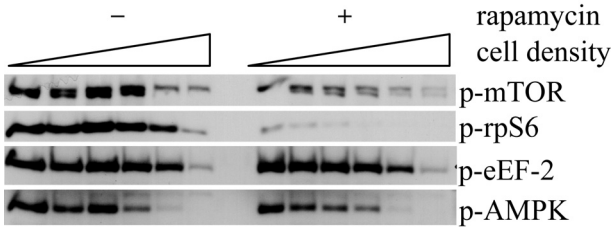

Figure 6. Signaling to eEF-2 is not mediated by mammalian target of rapamycin (mTOR). ρ^0 cells at different densities were treated for 30 min with 100 nM rapamycin or left untreated.

Compared to wild-type cells, the level of phosphorylation of the initiation factor eIF-2α was found to be systematically higher in the mtDNA mutant cells and largely independent of cell density (Figs. 3 and 4), indicating a constitutively active upstream pathway. Four different kinases are known to regulate eIF-2α. Three of them are unlikely to be involved under the conditions used here (see discussion) and we considered PERK as the most likely candidate. PERK phosphorylation was slightly but significantly elevated in VM50 A3243G cybrid cells (Fig. 7) and in ρ^0 cells (not shown) and, similar to eIF-2α phosphorylation, largely independent of cell density.

Discussion

Translation of mRNA is a basic cellular process that is under sophisticated control. Nutrient and energy availability, growth and proliferation signals as well as a variety of cellular stresses exert global and mRNA-specific translation control *via* different pathways. Here we show that cells with constitutive loss of OXPHOS due to mtDNA mutations repress translation and activate two independent signaling pathways, one targeting eEF-2 in a transient way and one targeting eIF-2α in a constitutive way. Repression mechanisms other than those mediated by phosphorylation of eEF-2 or eIF-2α can, however, not be excluded at present. Note that phosphorylation of most of the proteins analyzed here strictly depended on cell density. Density dependent analyses thus permitted distinction between transient and constitutive phosphorylation mechanisms. Figure 8 illustrates the working model used to explain cell-density and mtDNA mutant effects on translation.

Protein synthesis consumes a large fraction of cellular ATP production, making it subject to down regulation when ATP levels drop. A3243G and ρ^0 cells have decreased growth rates, likely a direct consequence of the reduced translational rates. The elongation phase uses almost all the energy required for translation and it makes sense to target this phase when ATP levels transiently drop during normal cellular functioning: polysomes remain preserved and cells can resume protein synthesis when the energy supply is restored [25]. For instance, when ATP synthesis is suddenly diminished through hypoxia, anoxia or inhibitors of OXPHOS, the cell rapidly responds (minutes to hours) to the altered condition by increasing phosphorylation of eEF-2 and decreasing translation [27, 28, 38]. How the cell will deal with long term absence or low mitochondrial ATP synthesis is unknown, but this is crucial for understanding the adaptive processes which leads from dysfunction to death, survival or (cancerous) growth of cells with an mtDNA mutation. We show that both mutant cell types, and to a much lesser extent the wild type cell, display a transient energy saving response through phosphorylation of eEF-2 at low cell density when growth and proliferation temporarily demand for increased amounts of ATP (Figs. 3 and 4). In a confluent culture, in the absence of high ATP demand, the three cell types display very low, if any, phosphorylation of eEF-2 or AMPK. Phosphorylation of eEF-2 therefore cannot play a role in density-dependent down regulation of translation but rather seems crucial for transient energy saving under temporary physiological and non-physiological ATP restrictions [27, 28, 38].

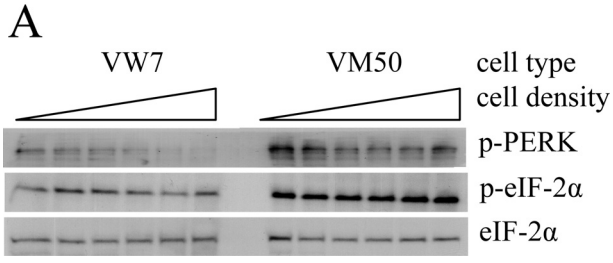

Figure 7. A3243G mutant cells show a constitutive elevated phosphorylation of PERK and eIF-2α. (*A*) Representative blots of PKR-like ER kinase (PERK) and eIF-2α phosphorylation.

mTOR has emerged as a critical growth control node integrating signals from growth factors, nutrient and energy availability [20, 21, 25, 44]. In particular mTOR regulates factors involved in mRNA translation like S6, 4E-BP1 and eEF-2. The mTOR and S6 phosphorylation paralleled each other as expected, as well as the density-dependent down regulation of translation. TSC1/2 mediates cellular energy response through mTOR when HEK 293 cells are ATP depleted by treatment with 2-deoxy glucose and mitochondrial inhibitors [23]. In our cells, which are adapted to constitutive loss of mitochondrial ATP synthesis, mTOR and S6 phosphorylation did not respond to mtDNA mutation or to chemical inhibition of OXPHOS complexes. Thus, mTOR activity closely

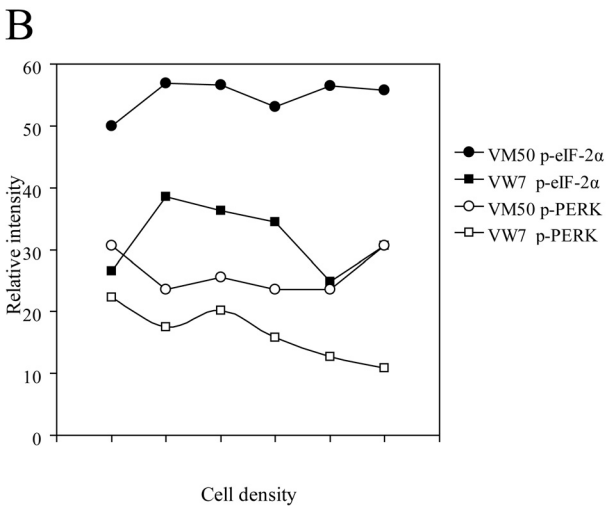

Figure 7. *(continued)* (*B*) graphical representation of the data in (*A*) after correction for total eIF-2α protein. VM50 cells show an increase in phosphorylation with $p<0.05$ for p-PERK and $p<0.01$ for p-eIF-2α.

correlates with growth and proliferation in all cell types, but presumably does not receive additional signals from activated AMPK due to energy shortage in mtDNA mutant cells ([28] *cf.* [23]). Interestingly, as shown here mTOR is activated at low cell densities in the absence of growth factors (Fig. 4).

Growth factor stimulated dephosphorylation of eEF-2 is under the control of mTOR and P70 S6kinase [24, 26]. In contrast, in the absence of growth factors but under conditions that activate AMPK, phosphorylation of eEF-2 is mediated independent of mTOR [27, 28, 38]. Interestingly, the inhibitor of mTOR rapamycin can inhibit insulin and TPA stimulated translation in HEK 293 cells and cardiomyocytes, but has little effect, if any, on basal (non-stimulated) translation itself [25]. In this mTOR-independent pathway, AMPK directly phosphorylates eEF-2K at Ser398 leading to its activation [29]. Therefore, it seems reasonable to assume that also in our cells this direct pathway is used to modulate protein synthesis rates in response to ATP fluctuations during normal cellular functioning, whether the cell relies only on glycolysis or full glucose oxidation. Part of the mtDNA mutation dependent repression of translation at the lower cell densities can thus be explained by suppression of elongation through the direct AMPK/eEF-2K/eEF-2 pathway involving the activating phosphorylation of eEF-2K at Ser398. This pathway, however, cannot explain the strong repression of translation that occurs at all densities under mtDNA induced dysfunction.

With eIF-2α being systematically at a higher phosphorylation level in mutant cells with marginal effects of cell density (Figs. 3, 4 and 7), it seems responsible for the mtDNA mutation-induced translational repression. eIF-2α appears at the end of a constitutively active signal transduction route originating from respiration-deficient mitochondria (Fig. 8). To adapt to a state of constitutive low energy production as is present in the glycolytic mtDNA mutant cells, it makes sense indeed to permanently down regulate protein synthesis rates by inhibition of initiation of translation.

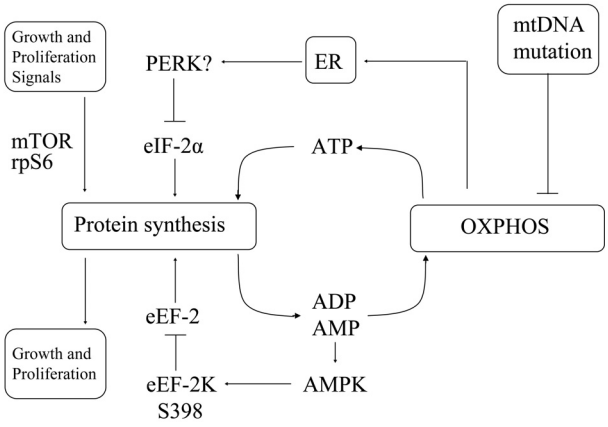

Figure 8. Scheme depicting an integrated model for transient and constitutive repression mechanisms of translation. In this model, under conditions of high ATP consumption such as protein synthesis in proliferative cells, the transient repression of eEF-2 is mediated by AMPK by direct phosphorylation of Ser398 of eEF-2K. The constitutive absence of OXPHOS indirectly underlies the constitutive repression of eIF-2α possibly *via* perturbed ER-mitochondrion interaction and PERK activation. Note that both translation factors are essential workhorses in translation and that their activity is inhibited by phosphorylation.

Four eIF-2α kinases are known [21, 33]. Protein kinase RNA activated (PKR) is involved in the dsRNA interferon response, heme-regulated eIF-2α kinase (HRI) in balancing globin synthesis to hemin availability in erythrocytes, GCN2 in sensing availability of amino acid and PERK in ER stress responses. From these, PERK is the most likely candidate for elevated eIF-2α phosphorylation because PERK phosphorylation was also slightly but significantly elevated in mtDNA mutant cells (Fig. 7). Furthermore, we did not introduce stimuli such as amino acid deprivation that might trigger GCN2 and cells were cultured under the same conditions. Although formally a contribution of PKR or HRI cannot be excluded, the activation of PERK strongly suggests that the mtDNA mutant cells have launched an ER stress response upon loss of OXPHOS.

Recently, evidence has accumulated that Ca^{2+} exchange occurs at close contacts points between the ER and mitochondria, the so-called mitochondria-associated ER membranes or MAMs [45–50]. Given the strong dependence of mitochondrial Ca^{2+} uptake on

the mitochondrial electrochemical proton gradient over the inner membrane, it is likely that cells with no OXPHOS have perturbed Ca^{2+} homeostasis. Indeed, fibroblasts of MELAS patients cannot properly sequester calcium influxes [51]. Also it has been shown that mitochondria from ρ^0 and MERRF cells have lowered ability to take up Ca^{2+} released from the ER [52, 53]. This would indicate that in cybrid cells loss of mitochondrial respiration perturbs Ca^{2+} signaling at the ER-mitochondrial contact points leading to an ER stress with PERK and eIF-2α phosphorylation and concomitant suppression of translation initiation (Fig. 7), and suggests that loss of OXPHOS *per se* rather than energy status, leads to ER stress, activation of PERK and enhanced eIF-2α phosphorylation.

Acknowledgements. We thank Ronny Kassub (University of Greifswald) and Marchien van de Sande (Leiden University Medical Center) for help with initial experiments. This work was supported in part by the Dutch Diabetes Research Foundation, grant 2005.11.012.

1 Goto, Y., Nonaka, I. and Horai, S. (1990) A mutation in the $tRNA^{Leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348, 651-653
2 Shoffner, J. M., Lott, M. T., Lezza, A. M., Seibel, P., Ballinger, S. W. and Wallace, D. C. (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA $tRNA^{Lys}$ mutation. Cell 61, 931–937
3 Van den Ouweland, J. M. W., Lemkes, H. H. P. J., Ruitenbeek, W., Sandkuijl, L. A., de Vijlder, M. F., Struijvenberg, P. A. A., van de Kamp, J. J. P. and Maassen, J. A. (1992) Mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. Nat. Genet. 1, 368-371
4 Larsson, N. G. and Clayton, D. A. (1995) Molecular genetic aspects of human mitochondrial disorders. Annu. Rev. Genet. 29, 151–178
5 Jacobs, H. T. and Holt, I. J. (2000) The np 3243 MELAS mutation: Damned if you aminoacylate, damned if you don't. Hum. Mol. Genet. 9, 463–465
6 Janssen, G. M., Hensbergen, P. J., van Bussel, F. J., Balog, C. I., Maassen, J. A., Deelder, A. M. and Raap, A. K. (2007) The A3243G $tRNA^{Leu(UUR)}$ mutation induces mitochondrial dysfunction and variable disease expression without dominant negative acting translational defects in complex IV subunits at UUR codons. Hum. Mol. Genet. 16, 2472–2481
7 Sasarman, F., Antonicka, H. and Shoubridge, E. A. (2008) The A3243G $tRNA^{Leu(UUR)}$ MELAS mutation causes amino acid misincorporation and a combined respiratory chain assembly defect that is partially suppressed by overexpression of the translation elongation factors EFTu and EFG2. Hum. Mol. Genet. 17, 3697–3707
8 Shoubridge, E. A. and Sasarman, F. (2007) Mitochondrial translation and human disease. In: *Translational control in biology and medicine*, pp. 775–801, Mathews, M. B., Sonenberg, N. and Hershey, J. W. B. (eds.), CSHL Press, NY
9 DiMauro, S. and Schon, E. A. (2003) Mitochondrial respiratory-chain diseases. N. Engl. J. Med. 348, 2656–2668
10 Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S. T., Nonaka, I., Angelini, C. and Attardi, G. (1992) MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. Proc. Natl. Acad. Sci. USA 89, 4221-4225
11 King, M. P., Koga, Y., Davidson, M. and Schon, E. A. (1992) Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the $tRNA^{Leu(UUR)}$ mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. Mol. Cell. Biol. 12, 480–490
12 Bua, E., Johnson, J., Herbst, A., Delong, B., McKenzie, D., Salamat, S. and Aiken, J. M. (2006) Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. Am. J. Hum. Genet. 79, 469–480
13 Bender, A., Krishnan, K. J., Morris, C. M., Taylor, G. A., Reeve, A. K., Perry, R. H., Jaros, E., Hersheson, J. S., Betts, J., Klopstock, T., Taylor, R. W. and Turnbull, D. M. (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat. Genet. 38, 515–517
14 Kraytsberg, Y., Kudryavtseva, E., McKee, A. C., Geula, C., Kowall, N. W. and Khrapko, K. (2006) Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat. Genet. 38, 518–520
15 Petros, J. A., Baumann, A. K., Ruiz-Pesini, E., Amin, M. B., Sun, C. Q., Hall, J., Lim, S., Issa, M. M., Flanders, W. D., Hosseini, S. H., Marshall, F. F. and Wallace, D. C. (2005) mtDNA mutations increase tumorigenicity in prostate cancer. Proc. Natl. Acad. Sci. USA 102, 719–724
16 16. Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., Nakada, K., Honma, Y. and Hayashi, J. (2008) ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science 320, 661–664
17 Dasgupta, S., Hoque, M. O., Upadhyay, S. and Sidransky, D. (2008) Mitochondrial cytochrome B gene mutation promotes tumor growth in bladder cancer. Cancer Res. 68, 700–706
18 Levine, E. M., Becker, Y., Boone, C. W. and Eagle, H. (1965) Contact inhibition, macromolecular synthesis, and polyribosomes in cultured human diploid fibroblasts. Proc. Natl. Acad. Sci. USA 53 350–356
19 Jahangir Tafrechi, R. S., Svensson, P. J., Janssen, G. M. C., Szuhai, K., Maassen, J. A. and Raap, A. K. (2005) Distinct nuclear gene expression profiles in cells with mtDNA depletion and homoplasmic A3243G mutation. Mutat. Res. 578, 43–52
20 Proud, C. G. (2007) Signalling to translation: How signal transduction pathways control the protein synthetic machinery. Biochem. J. 403, 217–234
21 Holcik, M. and Sonenberg, N. (2005) Translational control in stress and apoptosis. Nat. Rev. Mol. Cell Biol. 6, 318–327
22 Hardie, D. G. (2007) AMP-activated/SNF1 protein kinases: Conserved guardians of cellular energy. Nat. Rev. Mol. Cell Biol. 8, 774–785
23 Inoki, K., Zhu, T. and Guan, K. L. (2003) TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577–590
24 Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R. and Proud, C. G. (2001) Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J. 20, 4370–4379
25 Wang, X. and Proud, C. G. (2006) The mTOR pathway in the control of protein synthesis. Physiology 21, 362–369
26 Redpath, N. T., Foulstone, E. J. and Proud, C. G. (1996) Regulation of translation elongation factor-2 by insulin *via* a rapamycin-sensitive signalling pathway. EMBO J. 15, 2291–2297
27 McLeod, L. E. and Proud, C. G. (2002) ATP depletion increases phosphorylation of elongation factor eEF2 in adult cardiomyocytes independently of inhibition of mTOR signalling. FEBS Lett. 531, 448–452
28 Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C. and Rider, M. (2002) Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. Curr. Biol. 12, 1419–1423

29 Browne, G. J., Finn, S. G. and Proud, C. G. (2004) Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. J. Biol. Chem. 279, 12220–12231
30 Richter, J. D. and Sonenberg, N. (2005) Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature 433, 477–480
31 Ron, D. and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. 8, 519–529
32 van den Beucken, T., Koritzinsky, M. and Wouters, B. G. (2006) Translational control of gene expression during hypoxia. Cancer Biol. Ther. 5, 749–755
33 Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M. and Ron, D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099–1108
34 Gomez, E., Powell, M. L., Bevington, A. and Herbert, T. P. (2008) A decrease in cellular energy status stimulates PERK-dependent eIF2α phosphorylation and regulates protein synthesis in pancreatic beta-cells. Biochem. J. 410, 485–493
35 Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M. and Ron, D. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell 11, 619–633
36 Koumenis, C., Naczki, C., Koritzinsky, M., Rastani, S., Diehl, A., Sonenberg, N., Koromilas, A. and Wouters, B. G. (2002) Regulation of protein synthesis by hypoxia *via* activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2α. Mol. Cell. Biol. 22, 7405–7416
37 Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J. and Thompson, C. B. (2005) AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol. Cell 18, 283–293
38 Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B. and Simon, M. C. (2006) Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol. Cell 21, 521–531
39 van den Ouweland, J. M. W., Maechler, P., Wolheim, C. B., Attardi, G. and Maassen, J. A. (1999) Functional and morphological abnormalities of mitochondria harboring the $tRNA^{Leu(UUR)}$ mutation in mitochondrial DNA derived from patients with maternally inherited diabetes and deafness (MIDD) and progressive kidney disease. Diabetologia 42, 485–492
40 Janssen, G. M. C., Maassen, J. A. and van den Ouweland, J. M. W. (1999) The diabetes-associated 3243 mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene causes severe mitochondrial dysfunction without a strong decrease in protein synthesis rate. J. Biol. Chem. 274, 29744–29748
41 Szuhai, K., van den Ouweland, J. M. W., Dirks, R., Lemaître, M., Truffert, J., Janssen, G. M. C, Tanke, H., Holme, E., Maassen, J. A. and Raap, A. K. (2001) Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in myoclonus epilepsy and ragged-red fibers (MERRF) syndrome by a multiplex molecular beacon based real-time fluorescence PCR. Nucleic Acids Res. 29, E13
42 Brandsma, M., Janssen, G. M. C. and Möller W. (1997) Termination of quiescence in crustacea. The role of transfer RNA aminoacylation in the brine shrimp Artemia. J. Biol. Chem. 272, 28912–28917
43 Hardie, D. G. and Hawley, S. A. (2001) AMP-activated protein kinase: The energy charge hypothesis revisited. Bioessays 23, 1112–1119
44 Shaw, R. J. and Cantley, L. C. (2006) Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 441, 424–430
45 Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. and Pozzan, T. (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science 280, 1763–1766
46 Mendes, C. C., Gomes, D. A., Thompson, M., Souto, N. C., Goes, T. S., Goes, A. M., Rodrigues, M. A., Gomez, M. V., Nathanson, M. H. and Leite, M. F. (2005) The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic Ca^{2+} signals into mitochondria. J. Biol. Chem. 280, 40892–40900
47 Csordás, G., Renken, C., Várnai, P., Walter, L., Weaver, D., Buttle, K. F., Balla, T., Mannella, C. A. and Hajnóczky, G. (2006) Structural and functional features and significance of the physical linkage between ER and mitochondria. J. Cell Biol. 174, 915–921
48 Simmen, T., Aslan, J. E., Blagoveshchenskaya, A. D., Thomas, L., Wan, L., Xiang, Y., Feliciangeli, S. F., Hung, C. H., Crump, C. M. and Thomas, G. (2005) PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis. EMBO J. 24, 717–729
49 Hayashi, T. and Su, T. P. (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca^{2+}signaling and cell survival. Cell 131, 596–610
50 Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. and Ron, D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat. Cell Biol. 2, 326–332
51 Moudy, A. M., Handran, S. D., Goldberg, M. P., Ruffin, N., Karl, I., Kranz-Eble, P., DeVivo, D. C. and Rothman, S. M. (1995) Abnormal calcium homeostasis and mitochondrial polarization in a human encephalomyopathy. Proc. Natl. Acad. Sci. USA 92, 729–733
52 Kwong, J. Q., Henning, M. S., Starkov, A. A. and Manfredi, G. (2007) The mitochondrial respiratory chain is a modulator of apoptosis. J. Cell Biol. 179, 1163–1177
53 Brini, M., Pinton, P., King, M. P., Davidson, M., Schon, E. A. and Rizzuto, R. (1999) A calcium signaling defect in the pathogenesis of a mitochondrial DNA inherited oxidative phosphorylation deficiency. Nat. Med. 5, 951–954