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Published in:
Mitochondrion

DOI:
[10.1016/j.mito.2015.01.005](https://doi.org/10.1016/j.mito.2015.01.005)

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for pulished version (HARVARD):

Michel, S, Canonne, M, Arnould, T & Renard, P 2015, 'Inhibition of mitochondrial genome expression triggers the activation of CHOP-10 by a cell signaling dependent on the integrated stress response but not the mitochondrial unfolded protein response', *Mitochondrion*, vol. 21, pp. 58-68.
<https://doi.org/10.1016/j.mito.2015.01.005>

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Inhibition of mitochondrial genome expression triggers the activation of CHOP-10 by a cell signaling dependent on the integrated stress response but not the mitochondrial unfolded protein response

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ARTICLE INFO

Article history:

Received 1 September 2014

Received in revised form 10 January 2015

Accepted 20 January 2015

Available online 30 January 2015

Keywords:

Mitochondrial dysfunction

Mitochondria unfolded protein response (mtUPR)

Integrated stress response (ISR)

C/EBP homologous protein 10 (CHOP-10)

mtDNA depletion

Doxycycline

ABSTRACT

Mitochondria-to-nucleus communication, known as retrograde signaling, is important to adjust the nuclear gene expression in response to organelle dysfunction. Among the transcription factors described to respond to mitochondrial stress, CHOP-10 is activated by respiratory chain inhibition, mitochondrial accumulation of unfolded proteins and mtDNA mutations. In this study, we show that altered/impaired expression of mtDNA induces CHOP-10 expression in a signaling pathway that depends on the eIF2 α /ATF4 axis of the integrated stress response rather than on the mitochondrial unfolded protein response.

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1. Introduction

Vestige of its α -proteobacterial origin, mitochondria keep DNA (mtDNA) encoding 13 polypeptides, all subunits of the electron transport chain (ETC), as well as 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) that are required for their translation. Still, this only represents 1% of the mitochondrial proteome, estimated at up to 1500 proteins that are therefore predominantly encoded by the nuclear DNA (Calvo and Mootha, 2010). Altered expression of mitochondrial proteins is increasingly associated with many and various human

diseases (Boczonadi and Horvath, 2014). If mutations within genes encoding components or assembly factors of the ETC are the most obvious cause of mitochondrial dysfunction and pathology, the better understanding of mtDNA maintenance and expression has shed light on new mutations associated with mitochondrial diseases. Indeed, those not only arise from alterations in the mtDNA but also in nuclear genes encoding proteins required for regulation of mtDNA expression. On one hand, mutations or deletions within one of the 2 rRNAs or 22 tRNAs encoding genes will affect the translation of the 13 mtDNA-encoded subunits of the ETC. Such alterations lead to mitochondrial diseases such as MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes), MERRF (Myoclonic Epilepsy with Ragged Red Fibers) and KSS (Kearns–Sayre Syndrome) mainly associated with mutations within tRNA^{Leu} (A3243G), tRNA^{lys} (A8344G) or large-scale mtDNA deletion, respectively (Goto et al., 1990; Maceluch and Niedziela, 2006; Shoffner et al., 1990). On the other hand, the proteins required to support mtDNA expression are encoded by the nuclear genome (Vafai and Mootha, 2012). Therefore, it is not surprising that an increasing number of human mitochondrial diseases have been linked with nuclear genes encoding proteins involved in mitochondrial protein synthesis such as tRNA-modifying enzymes, aminoacyl-tRNA synthetases, ribosomal proteins, elongation, mRNA stability and termination factors as well as translation activators (Pearce et al., 2013). Altered expression of mtDNA results in mitochondrial dysfunction and perturbs cellular homeostasis as mitochondria is important not only for ATP production by oxidative phosphorylation but also for lipid

Abbreviations: AARE, amino acid responsive element; ATF4, activating transcription factor 4; CHOP-10, C/EBP homologous protein 10; CK2, casein kinase 2; CREB, cAMP-responsive element binding protein; COX, cytochrome C oxidase; eIF2 α , eukaryotic translation initiation factor 2A; ERAD, endoplasmic-reticulum-associated protein degradation; ERSE, endoplasmic reticulum stress element; erUPR, unfolded protein response of the endoplasmic reticulum; ETC, electron transport chain; HERP, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1; EtBr, ethidium bromide; FCS, fetal calf serum; GCN2, general control non-derepressible-2; HRI, heme-regulated inhibitor; HSPD1, heat shock 60 kDa protein 1; ISR, integrated stress response; JNK, c-Jun N-terminal Kinase; KSS, Kearns–Sayre Syndrome; MELAS, Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes; MERRF, Myoclonic Epilepsy with Ragged Red Fibers; mtUPR, mitochondrial unfolded protein response; mtDNA, mitochondrial DNA; NFAT, nuclear factor of activated T cells; NF κ B, Nuclear Factor kappa B; OTC, ornithine transcarbamylase; PERK, PKR-like ER-kinase; PKR, protein kinase double-stranded RNA-dependent; TOM40, translocase of the outer membrane; Trib3, tribble 3.

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metabolism, synthesis of iron–sulfur cluster, calcium and ROS signaling as well as regulation of programmed cell death. Hence, general feature of mitochondrial diseases include reduced ATP production, decreased membrane potential, altered calcium homeostasis and ROS production (James et al., 1996; Lenaz et al., 2004; Moudy et al., 1995; Vives-Bauza et al., 2006).

Coordinated expression of both genomes and communication between mitochondria and nucleus is thus critical to maintain proper cell function. Transcriptional regulators are key players in mitochondria-to-nucleus communication as they orchestrate the expression of nuclear genes in response to both external challenges and organelle dysfunction to regulate processes such as mitochondrial biogenesis, cell proliferation, metabolism and apoptosis, as reviewed in Ryan and Hoogenraad, (2007). Some of them have very well described signaling pathways activated in response to mitochondrial stress such as among other NFAT (nuclear factor of activated T cells), NF- κ B (nuclear factor kappa B) and CREB (cAMP-responsive element binding protein) triggered by increased cytosolic calcium concentration because of altered mitochondrial buffering function (Arnould et al., 2002; Biswas et al., 1999, 2003). First and mainly reported in the context of endoplasmic reticulum stress, the transcription factor CHOP-10 (C/EBP homologous protein 10, also known as DDIT3 or GADD153) has also been reported to respond to mitochondrial dysfunction. Indeed, increased expression of the transcription factor has been demonstrated in the context of disrupted mitochondrial protein homeostasis (proteostasis) (Moisoi et al., 2009; Zhao et al., 2002), inhibition of mitochondrial ETC (Ishikawa et al., 2009; Vankoningsloo et al., 2006) and mutation within mtDNA (Cortopassi et al., 2006; Fujita et al., 2007). However, events and stress signaling pathways associated with mitochondrial dysfunction-induced CHOP-10 overexpression are not clear. Hoogenraad and colleagues have demonstrated that CHOP-10 is regulated by AP-1 in the context of the mitochondrial unfolded protein response (mtUPR), a specific quality control process that aims to resolve proteotoxic stress by coordinating expression of mitochondrial chaperones and proteases such as HSPD1 (also known as chaperonin 60 or HSP60) and ClpP, respectively (Horibe and Hoogenraad, 2007). Briefly, in this model, overexpression of a truncated version of the enzyme ornithine transcarbamylase (OTC Δ) results in its accumulation as an unfolded proteins within mitochondrial matrix that leads to JNK (c-Jun N-terminal Kinase) activation and phosphorylation of the AP-1 family member c-Jun. Once translocated into the nucleus, this transcription factor increases CHOP-10 expression that in turn triggers expression of stress responsive genes (Horibe and Hoogenraad, 2007). Moreover, the impairment of the stoichiometric equilibrium between nuclear and mtDNA-encoded proteins is another condition known to induce mtUPR as suggested in rho⁰ cells and demonstrated for murine cells incubated in the presence of doxycycline, an antibiotic of the tetracycline family that inhibits mitochondrial translation (Houtkooper et al., 2013; Martinus et al., 1996). So far, the global mechanism of mtUPR is still missing in mammals as cell signaling activated by accumulation of unfolded proteins has been mainly described in *Caenorhabditis elegans* (Pellegrino et al., 2013).

However, others have shown that increased expression of CHOP-10 associated with mitochondrial respiration defect relies more on the integrated stress response (ISR) than mtUPR (Cortopassi et al., 2006; Fujita et al., 2007; Ishikawa et al., 2009; Moisoi et al., 2009). ISR is a signaling pathway converging to eIF2 α phosphorylation and characterized by a global reduction in protein synthesis, together with a selective overexpression of stress-responsive genes (Fujita et al., 2007; Silva et al., 2009). ISR sensors consist of a set of four different kinases: PERK (PKR-like ER-kinase), GCN2 (general control non-derepressible-2), HRI (heme-regulated inhibitor) and PKR (protein kinase double-stranded RNA-dependent). These four kinases are activated in response to various cues such as endoplasmic reticulum stress, amino acid depletion, viral infection, oxidative stress, heme deprivation, UV irradiation and proteasome inhibition (Donnelly et al., 2013; Harding et al., 2000, 2003). Even if eIF2 α phosphorylation reduces cytosolic protein

synthesis, mRNAs containing short upstream open reading frames are preferentially translated, such as ATF4, ATF3 and CHOP-10 (Wek et al., 2006). The latter is a pleiotropic transcription factor that is associated with cell death/survival under ER stress as it can either trigger or prevent apoptosis (Fujihara et al., 2009; Zinszner et al., 1998). In addition to its expression level, CHOP-10 can be regulated by post-transcriptional modifications such as phosphorylation by p38 or CK2 (casein kinase 2), resulting in an enhanced or attenuated transcriptional activity, respectively (Ubeda and Habener, 2003; Wang and Ron, 1996). Together with other transcription factors of the bZIP family, ATF4 and CHOP-10 regulate the expression of specific stress genes such as the ERAD (endoplasmic-reticulum-associated protein degradation)-associated protein HERP (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1) and the pseudo-kinase Trib3 (tribble 3) (Harding et al., 2003; Kilberg et al., 2009).

In this study, as CHOP-10 can be activated by both mtUPR and ISR, we analyzed the regulation of CHOP-10 expression in different cell models in which mtDNA expression is impaired, such as mitochondrial genome depletion and translation inhibition, to better understand signaling pathways triggered by mitochondrial dysfunction that could converge to the activation of this transcription factor. The investigation of these stress signaling pathways might help to better characterize mitochondrial diseases associated with mtDNA depletion or impaired organelle translation.

2. Materials and methods

2.1. Cell culture, transfection and reagents

The HeLa adenocarcinoma and Hep3B hepatocellular carcinoma cell lines were purchased from ATCC (CCL-2 and HB-8064, respectively). HeLa rho⁰ cells were a kind gift of Professor R. Wiesner (University of Cologne, Germany). HeLa cells were grown in Minimum Essential Media (MEM, Gibco) supplemented with pyruvate (11360-039, Gibco), non-essential amino acid (11140-035, Gibco) and 10% of fetal calf serum (FCS) (Gibco). HeLa rho⁰ cells were grown in 4.5 g/l glucose-containing Dulbecco's modified Eagle Medium (DMEM, Gibco) supplemented with pyruvate, non-essential amino acid, 10% of fetal calf serum and 50 μ g/ml uridine. Hep3B cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640 Invitrogen, 21875) containing 10% of FCS. Fresh human hepatocytes (HEP220) were obtained from Biopredic international and kept in medium for long-term culture (MIL600C) supplemented with additive (ADD27111C). All cell lines were kept at 37 °C under a humidified atmosphere containing 5% CO₂. Stock solution of doxycycline (Sigma, D9891-1G) was prepared in deionized water at a concentration of 15 mg/ml, filter sterilized and used at a final concentration of 15 or 30 μ g/ml as indicated.

For reporter gene experiments, HeLa cells have been transfected with SuperFect transfection reagent from Qiagen (301305). Briefly, cells were seeded one day before transfection in 12-well plates (50,000 cells/well). Cells were transiently transfected with 0.6 μ g of a Firefly luciferase reporter plasmid and 0.4 μ g of a plasmid containing a cDNA encoding Renilla luciferase under the control of the TK promoter (pRenillaLuc-TK), used for normalization. The DNA/SuperFect ratio was 1:2 and cells were incubated with complexes for 3 h in the absence of serum before refreshing with new medium, containing doxycycline or not, for 48 h.

For silencing experiments, On-target plus siRNA smartpools purchased from Dharmacon were used: ATF4 (L-005125-00-0005), PERK (L-004883-00-0005), GCN2 (L-005314-00-0005), HRI (L-005007-00-0005), PKR (L-003527-00-0005). Non-targeting On-target plus siRNA (D-001810-10-05) have been used as negative control. 200,000 cells were seeded in 6-well plates in complete growth medium. The next day, cells were transfected with 50 nM siRNA diluted in opti-MEM (Gibco, 31985-070) and using Dharmafect 1 (T-2001-01) as transfection reagent, following the manufacturer's recommendation. After 24 h of

incubation with the complexes under standard culture condition, the medium was replaced with complete culture medium for another 24 h. Cells were then incubated with doxycycline or control medium for the indicated time.

2.2. Luciferase reporter assay

The pCHOP-10_{Luc} constructs, a kind gift of Dr. Vaandrager (Utrecht University, Nederland's), consist of the pGL3 basic plasmid with the –442 to +91 human CHOP-10 promoter region fused to the luciferase gene. The reporter plasmids contain either wild-type or mutated sequences within the AARE (amino acid responsive element), AP-1 or ERSE (endoplasmic reticulum stress element) binding sites as fully described in [van der Sanden et al. \(2004\)](#). Reporter gene assay was performed using the dual-luciferase reporter assay from Promega (E1910) and following manufacturer's recommendation. Luminescence was measured using Lucetta luminometer (Lonza, AAL-1001) and Firefly activity was normalized to Renilla activity.

2.3. Real-time RT-qPCR

Cells were scraped in RLT lysis buffer and total RNA was prepared using the RNeasy mini kit from Qiagen (74104). An optional on-column DNase (79254, Qiagen) digestion of 15 min incubation step was performed to prevent any co-purification of DNA along with RNA. Complementary DNA was synthesized from 2 µg of total RNA and with oligoT primers using the Transcriptor First Strand cDNA Synthesis kit from Roche (04896866001). Real-time PCR analysis was performed on the StepOneplus system from Applied Biosystem using FastStart Universal SYBR Green Master mix (04913914001, Roche) and specific primers at a final concentration of 300 nM in a volume of 25 µl. Primers were designed, using the online primer blast software, to evaluate mRNA abundance of the following targets: CHOP-10 forward: 5'-GCAAGAGGTCCTGTCTTCAGATG-3' and reverse: 5'-CTCAGTCAGCCAAGCCAGAGA-3'; HSPD1 forward: 5'-TGATGCTATGGCTGGAGATTT-3' and reverse: 5'-ACACCAGCAGCATCCAATAA-3'; ClpP forward: 5'-GTTGCCAGCCTTGTTATCG-3' and reverse: 5'-TGCATCGTGTCTAGATGG-3'; Trib3 forward: 5'-CGTGATCTCAAGCTGTGTCG-3' and reverse: 5'-AGCTTCTTCTCTCACGGTC-3'; ATF3 forward: 5'-GGAGCCTGGAGCAAAATGATG-3' and reverse: 5'-AGGGCGTCAGGTTAGCAAAA-3'; HERP forward: 5'-ATTTAGACCGAGGCCGGTTC-3' and reverse: 5'-CAGGATCAGTCGCTTCTGTGA-3'; PPIE forward: 5'-TCATGCTGCGTTCATTCCTT-3' and reverse: 5'-GCTCAGATTCATTATGTTGTCG-3'. PCR program started with 5 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A melt curve was added at the end of the PCR to check intended product amplification. PPIE was used as a reference gene for normalization and mRNA expression level was quantified using the threshold cycle method as described in [Livak and Schmittgen, 2001](#).

2.4. Western blotting analysis

For protein extraction, cells were kept on ice and scraped in lysis buffer (20 mM Tris, pH 7.4; 150 mM KCl, 1 mM EDTA and 1% Triton X-100) complemented with protease inhibitor cocktail (complete from Roche) and phosphatase inhibitor mixture (1 mM NaVO₃, 10 mM p-nitrophenyl phosphate, 10 mM β-glycerophosphate and 5 mM NaF). Clear lysates were retrieved from the supernatant of cell lysates after a 10 min centrifugation at 13,000 rpm at 4 °C and protein concentration was determined using the Pierce 660 nm Protein Assay (Thermo scientific, 22660).

For western blotting analysis, 15 to 20 µg of proteins were loaded on 4–15% Mini-PROTEAN TGX precast gel (Bio-rad #456-1086) or 3–8% Tris–Acetate gel (NuPAGE, Life technologies #EA0375BOX) and resolved by electrophoresis in Tris–Glycine or Tris–Acetate running buffer, respectively. Proteins were transferred onto a low-fluorescence PVDF membrane (Immobilon FL, Merck-Millipore), blocked in Li-cor Odyssey

blocking buffer (927–40,000) diluted in PBS and incubated with appropriate primary and secondary antibodies diluted in Odyssey blocking buffer supplemented with 1% Tween. Proteins were revealed using the infrared fluorescence Odyssey scanner (Li-cor). Protein abundance was evaluated using the following primary antibodies: COXI (Ab14705, Abcam), COXII (AbAA0258, Abcam), COXIV (A21348, Life Technologies), CHOP-10 (ALX-804-551-C100, Enzo Life Sciences), P-eIF2α Ser51 (3795S, Cell signaling), eIF2α (9722S, Cell signaling), HSPD1 (SAB1405973-50UG, Sigma), TOM40 (sc-11414, Santa-cruz), α-tubulin (T5168, Sigma) and corresponding secondary antibodies coupled to infrared dyes (IRDye, Li-cor).

2.5. Statistical analysis

Data from at least three independent experiments are presented as means ± S.D. and have been analyzed using the Sigma-stat software with the appropriate statistical test such as ANOVA-I or ANOVA-II followed by a Holm–Sidak post-test. Differences between means were only considered as statistically significant when $p < 0.05$ or less. For some experiments, when normality test failed, data have been transformed into logarithmic values.

3. Results

3.1. Mitochondrial dysfunction triggers CHOP-10 overexpression

As few studies have described the induction of CHOP-10 upon different kinds of mitochondrial dysfunction ([Cortopassi et al., 2006](#); [Fujita et al., 2007](#); [Ishikawa et al., 2009](#); [Moisoi et al., 2009](#)), we first assessed CHOP-10 mRNA abundance in two different models of impaired mtDNA expression. First, we used rho⁰ cells, an extensively described and useful model to study OXPHOS defect and retrograde signaling induced by mtDNA depletion ([Chandel and Schumacker, 1999](#); [Mercy et al., 2005](#); [Mineri et al., 2009](#); [Miranda et al., 1999](#)). The mRNA abundance of CHOP-10 assessed by RT-qPCR indicates that the abundance of CHOP-10 mRNA is significantly increased in rho⁰ HeLa cells when compared with their wild-type counterpart ([Fig. 1A](#)). This result has been confirmed in HeLa cells incubated with ethidium bromide (EtBr), an intercalating agent known to inhibit mtDNA transcription and replication and to be responsible for a progressive dilution and depletion of mitochondrial genome as cells divide ([Seidel-Rogol and Shadel, 2002](#)). A 15 day-treatment with 50 ng/ml ethidium bromide strongly decreases the relative copy number of mtDNA ([Fig. S1A](#)) that is associated with an increase in CHOP-10 mRNA abundance suggesting that CHOP-10 induction in mtDNA-depleted cells is not just a clone effect or a result of long term cell adjustment but an early response observed in cells facing a reduction of mtDNA content ([Fig. S1B](#)).

As a second cell model of impaired mitochondrial genome expression, we used doxycycline. In bacteria, doxycycline is well known to interfere with the binding of the amino-acylated tRNA to the mRNA, thus inhibiting translation ([Chopra et al., 1992](#)). In line with the α-proteobacteria origin of mitochondria, such antibiotic has been described to inhibit mitochondrial translation ([van den Bogert et al., 1986](#)). As decreased abundance of mtDNA-encoded proteins is therefore expected in doxycycline-treated cells, we first assessed the steady state level of COXI and COXII, by western blot, in HeLa cells incubated for 24, 48, or 72 h in the presence of 15 µg/ml of the antibiotic. A decrease in the abundance of COXI and COXII is observed as early as after 24 h of incubation, and is even more pronounced after 48 and 72 h, while the level of the nuclear-encoded protein TOM40 stays unchanged ([Fig. 1B](#)). As for the rho⁰ cell model, the mitochondrial impairment provoked by doxycycline is accompanied by a time-dependent increase in CHOP-10 mRNA abundance that reaches a 20 fold-induction after 72 h of treatment ([Fig. 1C](#)). The induction of CHOP-10 expression in response to doxycycline was reflected at the protein level as illustrated at [Fig. 1D](#). To rule out unspecific effect of doxycycline,

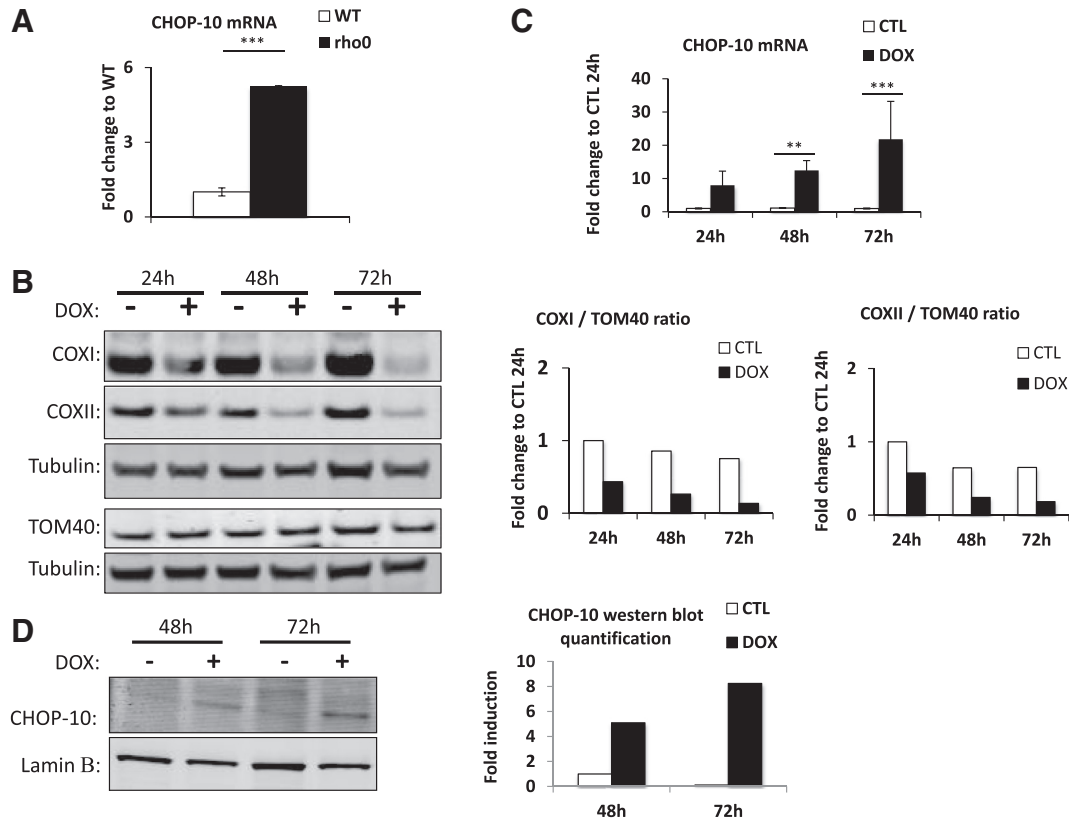


Fig. 1. Disruption of mtDNA expression increases CHOP-10 expression in HeLa cells. CHOP-10 has been studied in 2 different models of impaired protein synthesis within mitochondria. (A) CHOP-10 mRNA abundance has been evaluated by RT-qPCR in HeLa cells totally depleted of mtDNA (rho⁰) and compared to wild-type (WT) cells. (B) Doxycycline-mediated inhibition of mitochondrial translation efficiency was monitored by western blot analysis of the abundance of COXI and COXII, 2 mtDNA-encoded proteins, and TOM40 used as a control for nuclear-encoded proteins. HeLa cells have been incubated with control or 15 µg/ml doxycycline (DOX) containing medium during 24, 48 or 72 h. Immunodetection of tubulin was used as loading control. To highlight the mito-nuclear protein imbalance triggered by doxycycline, on the right of the graph is presented the western blot quantifications and expressed as the ratio of COXI or COXII to TOM40, all normalized to their respective tubulin. (C) CHOP-10 mRNA abundance has been studied by RT-qPCR in HeLa cells incubated with control medium (CTL) or with medium containing 15 µg/ml doxycycline (DOX) for 24, 48 or 72 h as well as (D) at the protein level by western blot analysis for the last two time points. Western blot quantification using the Odyssey software is represented at the right for CHOP-10 abundance normalized to lamin β used as the loading control. Results represent means ± 1 SD for 3 independent experiments and are expressed as fold change compared to WT (A) and CTL 24 h (C). **: p < 0.01 and ***: p < 0.001.

we also assessed CHOP-10 mRNA abundance in HeLa cells incubated for 24 h with chloramphenicol, another antibiotic described to inhibit mitochondrial translation in mammals (Balbi, 2004). A strong CHOP-10 induction was also obtained for cells incubated with the antibiotic (Fig. S2A).

3.2. Impairment of mtDNA expression does not trigger the mitochondrial unfolded protein response but activates the integrated stress response

CHOP-10 is known to be associated with the unfolded protein response of the endoplasmic reticulum (erUPR) (Schroder and Kaufman, 2005). However, more than 10 years ago, Hoogenraad and colleagues already reported that CHOP-10 is also activated in a mitochondria-specific unfolded protein response (mtUPR) (Zhao et al., 2002). Still, if some mechanisms of mtUPR have been recently described in *C. elegans* (Pellegrino et al., 2013), the mammalian counterpart is less defined. In addition, among the different gene markers that are potentially overexpressed in response to mtUPR in mammals such as the protease YME1L1, MPPβ and the endonuclease G (Aldridge et al., 2007), only genes encoding ClpP and HSPD1 have been consistently assessed in subsequent studies dealing with mito-nuclear protein imbalance (Houtkooper et al., 2013; Mouchiroud et al., 2013). As disrupting mtDNA expression leads to depletion of proteins encoded by the mitochondrial genome, we thus studied the putative activation of mtUPR by assessing the relative mRNA abundance of ClpP and HSPD1 using RT-qPCR. Unexpectedly, ClpP expression is strongly repressed in rho⁰ HeLa cells when compared with wild-type counterpart while there is

no significant difference for HSPD1 between mtDNA-depleted and wild-type HeLa cell lines (Fig. 2A). Similarly, there is no induction of the mtUPR-responsive ClpP and HSPD1 genes in EtBr-treated HeLa cells (Fig. S1C). The abundance of those two mtUPR markers has also been assessed in doxycycline- (Fig. 2B and C) and chloramphenicol- (Fig. S2B) treated HeLa cells, but there is neither induction of the mitochondrial protease nor the chaperone at the transcript level. Results obtained at the mRNA level have been confirmed at the protein level for HSPD1 abundance but no change was observed in doxycycline-treated HeLa cells when compared with untreated control cells (Fig. 2D).

Based on the two main markers used to characterize mtUPR activation, the data shows that CHOP-10 induction is not associated with this specific stress pathway in the different models of impaired mitochondrial DNA expression tested, corresponding to mtDNA depletion or inhibition of mitochondrial translation. As previously mentioned, CHOP-10 is also induced during the ISR, thus, to answer the question whether impaired mtDNA expression could trigger this stress response, the mRNA abundance of Trib3, ATF3 and HSPD1 was assessed by RT-qPCR (Harding et al., 2003; Kilberg et al., 2009). As illustrated in Fig. 3A, the transcript abundance of these three ISR markers is increased in rho⁰ when compared with wild-type HeLa cells (Fig. 3A) and in EtBr-treated HeLa cells (Fig. S1D). Trib3 and ATF3 are also overexpressed in HeLa cells incubated with doxycycline in a time-dependent manner and HSPD1 mRNA is also significantly more abundant in cells treated with doxycycline for the three time points studied (Fig. 3B). As for CHOP-10, the mRNA abundance of these three genes is also increased in HeLa cells incubated for 24 h with chloramphenicol (Fig. S2C).

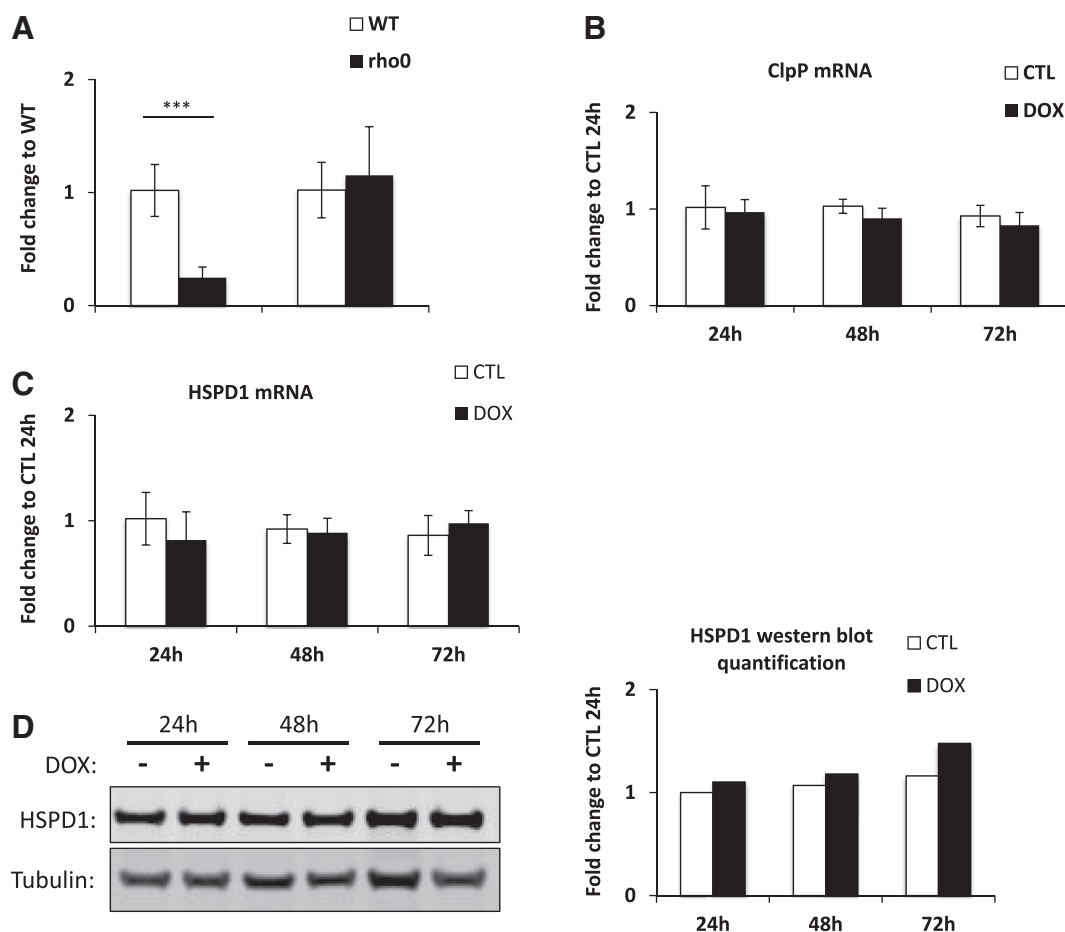


Fig. 2. Impairing mitochondrial protein synthesis in HeLa cells does not increase expression of genes associated with the mitochondrial unfolded protein response. (A) mRNA abundance of the two mtUPR-associated protein markers ClpP and HSPD1 was evaluated by RT-qPCR in ρ^0 HeLa cells compared to wild-type counterpart. The abundance of ClpP (B) and HSPD1 (C) mRNA was assessed by RT-qPCR in HeLa cells incubated in control medium (CTL) or in the presence of 15 $\mu\text{g}/\text{ml}$ doxycycline (DOX) for 24, 48 or 72 h. Results are presented as means \pm 1 SD for three independent experiments and are expressed as fold change to WT (A) or CTL 24 h (B, C) and ***: $p < 0.001$. (D) Protein level of HSPD1 was determined by western blot analysis in HeLa cells incubated with or without 15 $\mu\text{g}/\text{ml}$ doxycycline for 24, 48 or 72 h. Tubulin is used as a loading control. The quantification of HSPD1 protein abundance normalized to tubulin abundance is shown at the right of the chart.

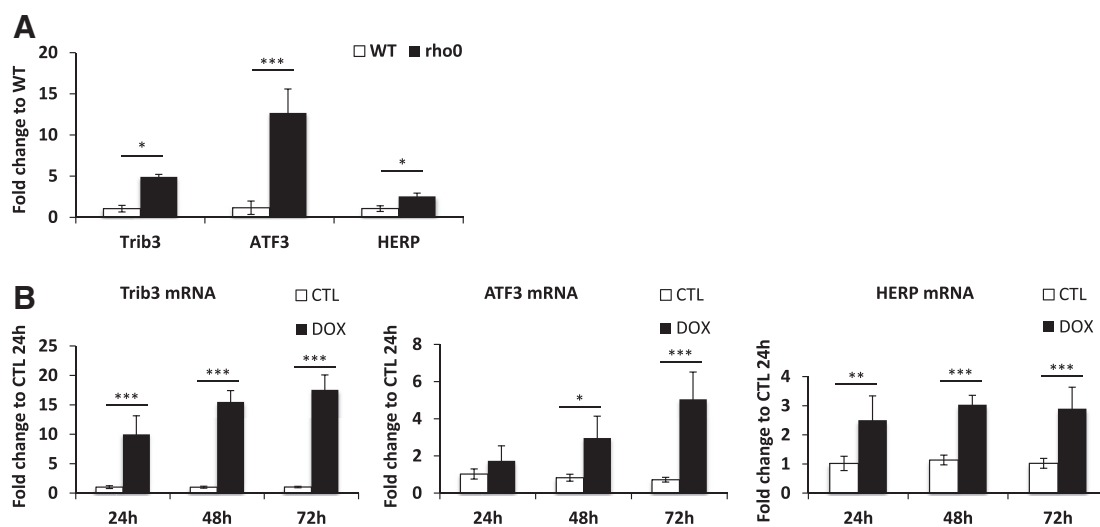


Fig. 3. Interfering with mtDNA expression in HeLa cells triggers the integrated stress response. mRNA abundance of ISR-related gene markers Trib3, ATF3 and HERP was assessed by RT-qPCR in HeLa cells totally depleted of mtDNA (A) and in HeLa cells incubated with control medium or supplemented with 15 $\mu\text{g}/\text{ml}$ doxycycline for 24, 48 and 72 h (B). Results are expressed as fold change to WT cells (A) or control 24 h (B) and represent means for 3 independent experiments. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

Despite the use of different models in which mtDNA expression is altered, we were thus unable to evidence an induction of mtUPR markers. In view of these unexpected results and even if they have also been confirmed in breast cancer MCF7 cells (Fig. S3A and B), we next questioned the cell models studied. Indeed, although cancer cell lines represent a very useful tool, their nuclear gene expression background is often altered when compared with their healthy counterpart. Thus, we next compared the effect of doxycycline treatment in transformed versus primary human hepatocytes to validate the results obtained. The hepatocellular carcinoma-derived Hep3B cells were incubated with 15 $\mu\text{g}/\text{ml}$ doxycycline for 24, 48 or 72 h. The doxycycline efficiency was first checked by western blot analysis of COXI and COXII protein abundance. In these conditions, comparable results to HeLa cells were obtained: the COXI/TOM40 and COXII/TOM40 ratio decreases after 24 h of incubation of Hep3B cells with the antibiotics (Fig. S4A). The mRNA abundance of CHOP-10, ClpP, HSPD1, Trib3, ATF3 and HERP was then assessed in the same experimental conditions (Fig. 4A). First, CHOP-10 expression is induced already after 24 h of treatment of Hep3B cells. Second, the levels of ClpP and HSPD1 mRNA were either unchanged or decreased regardless of the duration of incubation with

doxycycline. Third, the abundance of Trib3, ATF3 and HERP is increased in doxycycline-treated Hep3B cells after a 24, 48 or 72 h-treatment when compared with untreated cells. A comparable experiment was conducted in primary human hepatocytes that have been incubated with doxycycline at 15 or 30 $\mu\text{g}/\text{ml}$ during 24 or 48 h and results are illustrated in Fig. 4B. As for cancer cells lines, CHOP-10 mRNA abundance increases in a time and concentration-dependent manner in treated hepatocytes when compared with untreated cells. While no difference between control and doxycycline-treated hepatocytes is detectable regarding ClpP mRNA, there is a slight but time- and concentration-dependent increase in HSPD1 mRNA (up to 2 fold-increase in cells incubated for 48 h with doxycycline at 30 $\mu\text{g}/\text{ml}$). However, the up-regulation of HSPD1 could not be reflected at the protein level as illustrated at Fig. 4C. Finally, Trib3 and ATF3 mRNA are both induced in hepatocytes treated with doxycycline whereas HERP transcript abundance is only barely increased when compared with control cells. Even if the intensity of induction of the different ISR markers in response to doxycycline varies depending on the cell type, these results show that ISR activation in response to mitochondrial translation inhibition is not only restricted to cancer cells but is also observed in primary

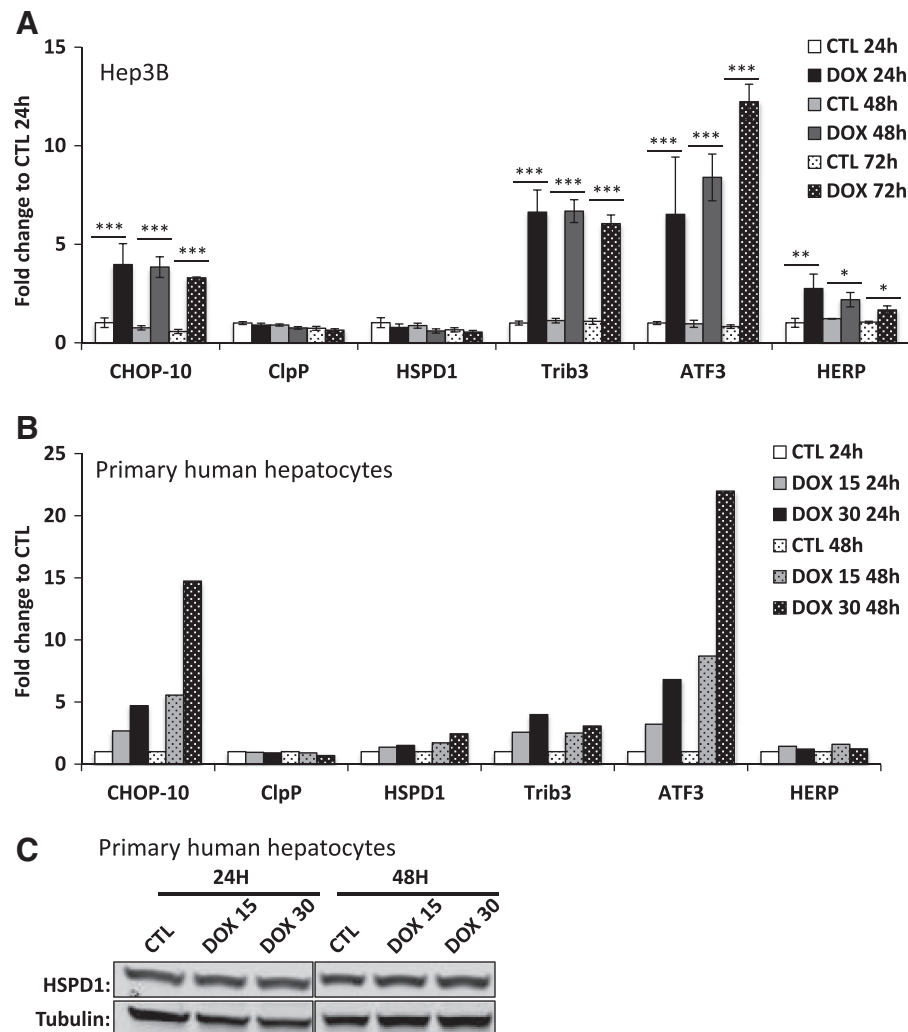


Fig. 4. Doxycycline triggers expression of genes associated with the integrated stress response but not the mitochondrial unfolded response in both cancer and primary hepatocytes. (A) mRNA abundance of CHOP-10, mtUPR-related ClpP and HSPD1 and ISR-induced Trib3, ATF3 and HERP was evaluated by RT-qPCR in Hep3B cells. Cells were incubated with control (CTL) or 15 $\mu\text{g}/\text{ml}$ doxycycline-supplemented (DOX) medium for 24, 48 or 72 h. Results are expressed as fold change to CTL 24 h as means \pm 1 SD for 3 independent experiments. *, **, ***: $p < 0.05$; 0.01; and 0.001, respectively. (B) Human primary hepatocytes were incubated 24 or 48 h with control (CTL) or doxycycline containing (15 or 30 $\mu\text{g}/\text{ml}$, DOX) medium. Results are expressed as fold change to corresponding CTL and represent means of 2 independent experiments. (C) HSPD1 protein abundance assessed by western blot in human primary hepatocytes incubated with doxycycline (15 or 30 $\mu\text{g}/\text{ml}$) during 24 or 48 h.

human cells such as hepatocytes. However, it is important to point out that COXI and COXII abundance is kept unchanged in doxycycline-treated hepatocytes (Fig. S4B), which could explain the non-induction of mtUPR markers in this cell type. Most importantly, this result questions the triggering event of ISR activation, since the latter is observed without mito-nuclear imbalance. It can be hypothesized that either the mito-nuclear protein imbalance is not at the origin of the stress response or it involves an alternative stress-signaling mechanism in primary hepatocytes that also leads to ISR activation. Altogether, the experiments performed in Hep3B cells and primary human hepatocytes incubated with doxycycline confirmed the results obtained with HeLa cells showing the induction of ISR-related targets such as CHOP-10, Trib3 and ATF3.

3.3. Doxycycline-induced eIF2 α phosphorylation is mediated by GCN2

To confirm these results and the activation of ISR, we also analyzed the phosphorylation status of eIF2 α in a time-course experiment. As shown in Fig. 5A, eIF2 α is phosphorylated in cells incubated with doxycycline as soon as after 1 h of treatment and the post-translational modification is still observed for longer incubation time up to 18 h of treatment. We next aimed to identify the kinase upstream eIF2 α phosphorylation. So far, four serine-threonine kinases have been described to phosphorylate eIF2 α : PERK, GCN2, PKR and HRI (Donnelly et al., 2013). First, we checked the silencing efficiency of siRNA for the kinases. After 48 h of transfection, the protein level of the siRNA-targeted PERK, GCN2 and PKR in HeLa cells is reduced below 20% when compared to control cells, without affecting the level of the other kinases (Fig. S5A–B). Although, we were not able to assess the silencing efficiency of HRI at the protein level, due to the poor quality of the antibodies, a strong decrease (97% decrease) was observed on the abundance of the HRI transcript, a data compatible with a down-regulation of the kinase at the protein level (Fig. S5C). In these experimental conditions of silencing, the level of eIF2 α phosphorylation in doxycycline-treated HeLa cells was assessed by western blot (Fig. 5B). In the absence of doxycycline, the level of eIF2 α phosphorylation is quite comparable

between the different conditions, excepted in the presence of siRNA against GCN2 and HRI that reduces the phosphorylation of eIF2 α . In doxycycline-treated HeLa cells, the level of eIF2 α phosphorylation increases in all conditions except when GCN2 abundance is reduced, a result suggesting that the kinase that phosphorylates eIF2 α in cells treated with doxycycline is GCN2.

3.4. ATF4 triggers CHOP-10 induction in doxycycline-treated HeLa cells

To further understand CHOP-10 transcriptional regulation upon mitochondrial dysfunction, we next determined the putative contribution of three well-described cis-acting elements within its promoter: the ER stress responsive element (ERSE, –104/–75, (Ubeda and Habener, 2000)), the AP-1 binding site (–250/–225, (Guyton et al., 1996)) and the amino acid responsive element (AARE, –313/–295, (Bruhat et al., 1997)). A reporter plasmid containing the luciferase gene under the control of the CHOP-10 promoter region, spanning from nucleotides –442 to +91, that is either wild-type or mutated for one of these three regulatory sequences was used (van der Sanden et al., 2004) and is illustrated on the left of Fig. 6A. First, a 48 h-incubation of HeLa cells with doxycycline increases reporter activity in cells transiently transfected with the plasmid containing the wild-type sequence of the CHOP-10 promoter, a data in agreement with the results shown for the endogenous mRNA and protein levels (Fig. 1). Second, mutation of either the ERSE or the AP-1 binding site has no drastic effect on the reporter activity. By contrast, the luciferase activity induced in response to doxycycline is strongly reduced when the AARE binding site is mutated (Fig. 6A), suggesting the requirement for ATF4 activation in the doxycycline-induced CHOP-10 expression, as ATF4 is well known to bind to the AARE (Averous et al., 2004). In addition, increased ATF4 abundance is consistent with the doxycycline-induced phosphorylation of eIF2 α (Fig. 5) as eIF2 α is known to trigger preferential ATF4 translation (Vattem and Wek, 2004). Indeed, ATF4 protein abundance increases in a time-dependent manner in HeLa cells treated with 15 μ g/ml doxycycline while being undetectable in control cells (Fig. 6B).

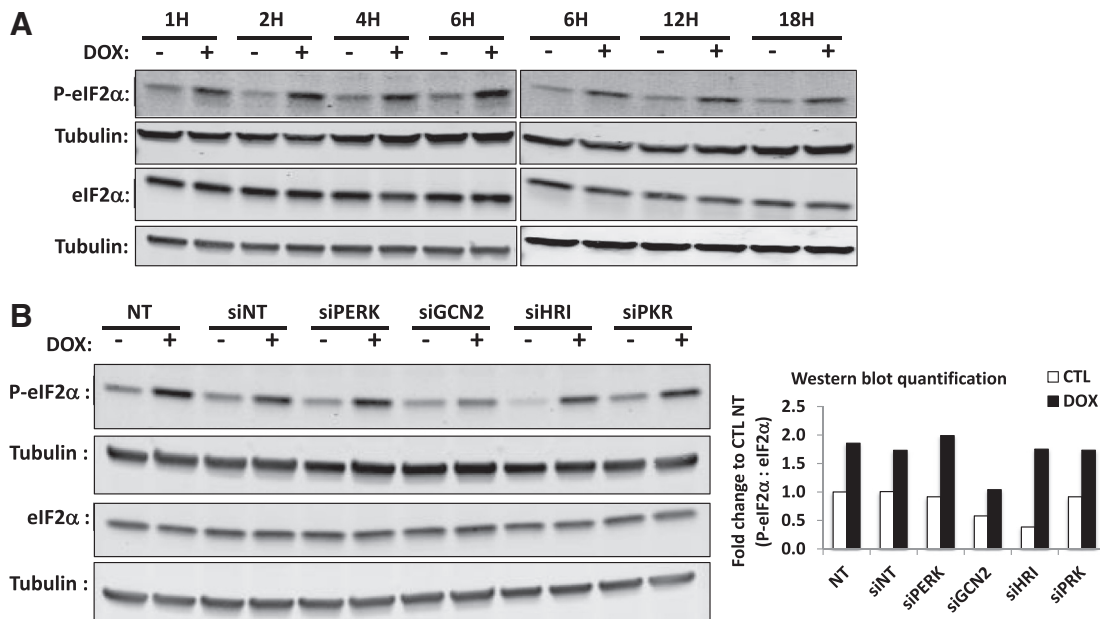


Fig. 5. Doxycycline-induced eIF2 α phosphorylation in HeLa cells is dependent on the GCN2 kinase. (A) The level of eIF2 α phosphorylation has been evaluated during a time-course experiment in HeLa cells incubated with or without doxycycline at 15 μ g/ml during 1 up to 18 h. Both phosphorylated and total eIF2 α abundance were assessed by western blot and immunodetection of tubulin was used as a loading control. (B) HeLa cells were not transfected (NT) or transfected with siRNA against each of the four kinases described to phosphorylate eIF2 α : PERK (siPERK), GCN2 (siGCN2), HRI (siHRI) and PKR (siPKR), as well as with non-targeting siRNA (siNT). After 48 h of transfection, cells were incubated with control or 15 μ g/ml doxycycline-containing medium for 1 h. Phosphorylated and total eIF2 α abundance was assessed by western blot and immunodetection of tubulin was used as a loading control. On the right is shown the quantification of P-eIF2 α represented as the ratio of P-eIF2 α to eIF2 α , both normalized to their respective loading control. Results are finally expressed as fold change to non-transfected HeLa cells incubated with control medium (CTL NT). The result is the representative of three independent experiments.

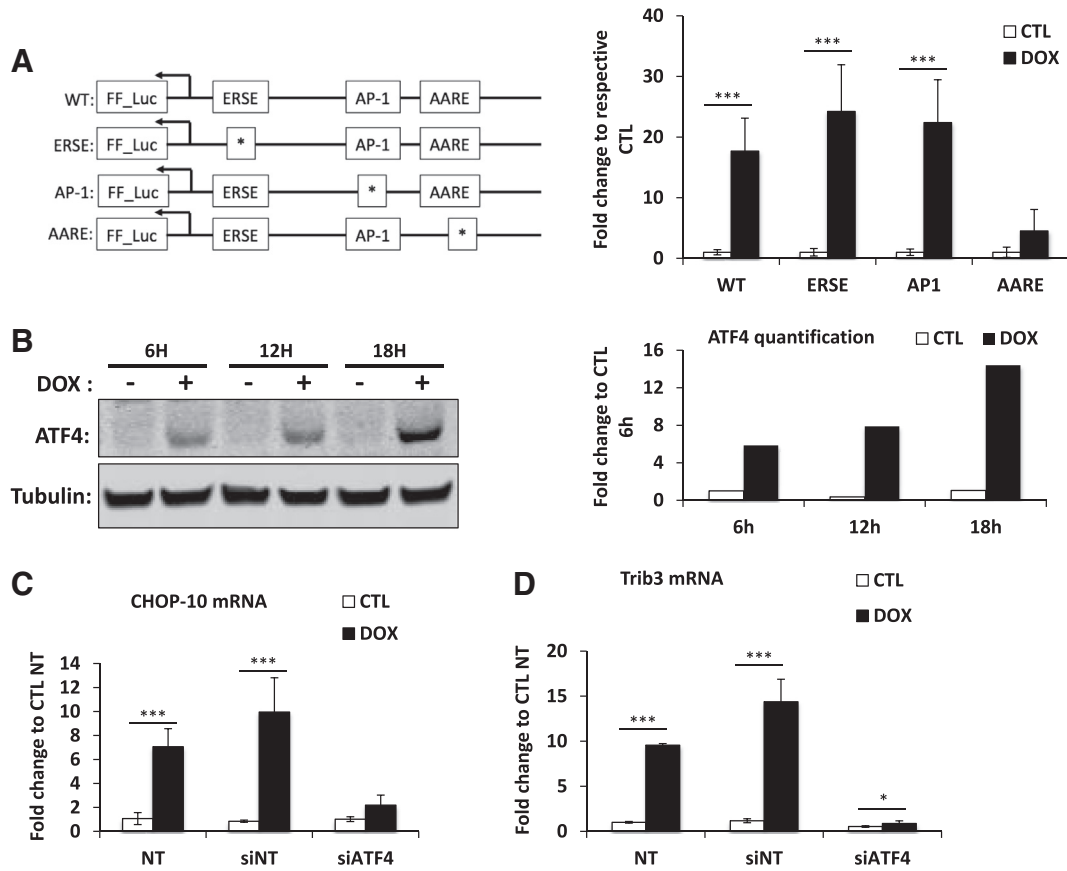


Fig. 6. ATF4 triggers CHOP-10 induction in doxycycline-treated HeLa cells. (A) Reporter plasmid assay using a fragment of the CHOP-10 promoter sequence (−442 to +91) upstream of the Firefly luciferase gene. 24 h post-transfection with the reporter and normalization plasmids, HeLa cells were incubated with or without (control: CTL) 15 µg/ml doxycycline containing medium (DOX) for 48 h before assessing reporter activity. Promoter sequence of the reporter plasmid contains either wild-type (WT) or mutated sequence for one of the three following cis-acting elements: ER stress response element (ERSE), AP-1 binding site (AP-1) or the amino acid responsive element (AARE) site as illustrated on the left panel. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as fold change to respective control. (B) ATF4 protein level evaluated by western blot. HeLa cells were incubated with medium containing or not with 15 µg/ml doxycycline (DOX) for 6, 12 or 18 h. Immunodetection of tubulin was used as the loading control. The graph on the right represents ATF4 abundance quantification normalized to tubulin and expressed as fold change compared to HeLa cells incubated for 6 h in control medium. (C) Impact of ATF4 silencing on increased doxycycline-induced CHOP-10 and Trib3 mRNA abundance. HeLa cells were either not transfected (NT), transfected with non-targeting siRNA (siNT) or siRNA against ATF4 (siATF4) during 24 h before medium renewal and incubation for 48 h with control (CTL) or 15 µg/ml doxycycline supplemented medium (DOX). Results are expressed as fold change to no-transfected (NT) CTL and represent means \pm 1 S.D. of three independent experiments. *: $p < 0.05$ and ***: $p < 0.001$.

The functional involvement of ATF4 in doxycycline-induced CHOP-10 expression was finally confirmed thanks to ATF4 silencing by siRNA. While transfection with non-targeting siRNA slightly increases CHOP-10 expression, silencing of ATF4 strongly decreases the abundance of CHOP-10 in HeLa cells treated for 24 h with doxycycline (Fig. 6C). We further checked the involvement of ATF4 in the expression of other ISR target genes such as Trib3. As shown in Fig. 6D, Trib3 induction is completely prevented when cells are transfected with siRNA against ATF4 while still robustly overexpressed in the non-targeting siRNA condition.

4. Discussion

The integrated stress response (ISR) is a stress response pathway based on four kinases that regulate eIF2 α phosphorylation and gene expression in response to broad types of cues, such as extensively described for endoplasmic reticulum stress and amino acid starvation (Donnelly et al., 2013). In this study, we have shown that disrupting mtDNA expression activates the ISR (Fig. 7). Indeed, either depletion or inhibition of mtDNA expression in HeLa cells increases expression of ISR-responsive genes such as the transcription factors CHOP-10 and ATF3, the pseudo kinase Trib3 and the ERAD-associated protein HERP (Figs. 1A, C, D, 3A and B). CHOP-10 and Trib3 inductions are dependent on the central transcriptional regulator ATF4 as demonstrated by

silencing experiments (Fig. 6C and D). Consistent with the observed eIF2 α phosphorylation, the protein abundance of ATF4 is increased when cells are incubated with doxycycline, an antibiotic that inhibits mitochondrial translation (Figs. 5A and 6B). Increased expression of these targets has also been confirmed in human primary hepatocytes (Fig. 4B) as well as in Hep3B (Fig. 4A) and MCF7 cells (Fig. S3).

Surprisingly, in doxycycline-treated hepatocytes, the protein abundance of COXI and COXII (Fig. S4B), both mtDNA-encoded proteins, is not decreased as observed in HeLa (Fig. 1B) and Hep3B (Fig. S4A) cells, suggesting that depletion of mtDNA-encoded ETC subunits is not the triggering event of ISR activation per se. A very recent study showed that ATF4, ATF3 and CHOP-10 are overexpressed in cells incubated with tetracyclines (Bruning et al., 2014). Doxycycline belongs to this class of antibiotics that, in addition to mitochondrial translation inhibitor, are also known as bivalent ions chelators. Bruning and colleagues attributed some of the observed effects to magnesium depletion (Bruning et al., 2014). However, mitochondrial translation impairment with other classes of antibiotics such as actinonin, the peptide deformylase inhibitor, in mouse embryonic fibroblasts (MEFs) also rapidly (and in a sustained manner) induces CHOP-10, Trib3 and ATF3 overexpression (Richter et al., 2013). Together with the results obtained in rho⁰ cells (Figs. 1A and 3A) as well as for cells treated with chloramphenicol (Fig. S2) or ethidium bromide (Fig. S1), these results suggest that the ISR is triggered by the doxycycline-induced disrupted mtDNA expression and not

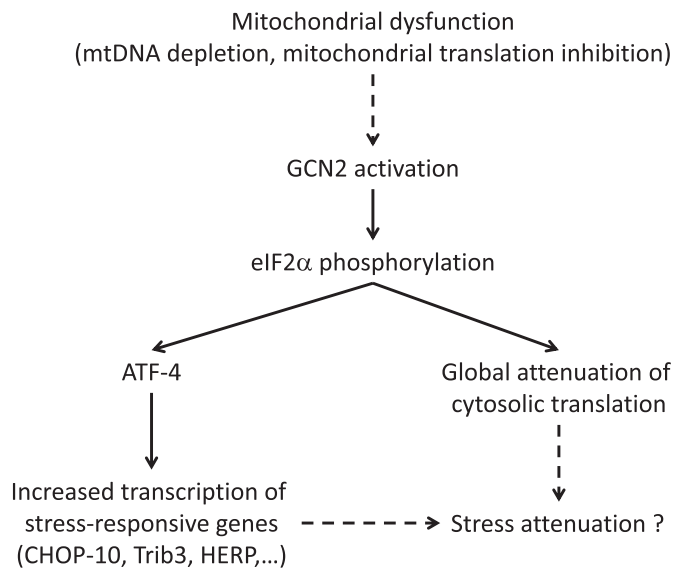


Fig. 7. Hypothetical model for stress response to mitochondrial dysfunction. In response to mitochondrial dysfunction such as mtDNA depletion and inhibition of mitochondrial translation, the GCN2 kinase is activated. In turn, the kinase phosphorylates eIF2 α that has at least 2 consequences. While the cytosolic translation is globally attenuated, mRNAs containing an uORF are preferentially translated such as the transcription factors ATF4 and CHOP-10. The profit of this GCN2-eIF2 α -ATF4 pathway might be stress attenuation by relieving the load of protein imported into mitochondria but also by increasing the expression of stress responsive genes. Dashed lines represent a hypothetical link.

an unspecific effect of the antibiotic. We have also shown that the GCN2 kinase is required for eIF2 α phosphorylation in response to mitochondrial translation inhibition in HeLa cells incubated with doxycycline (Fig. 5B). The mechanism of GCN2 activation in response to impaired mitochondrial translation is still unknown but, as suggested by results in primary human hepatocytes, it is unlikely to be triggered by the mito-nuclear protein imbalance. This idea is reinforced by the fact that eIF2 α phosphorylation is rapidly observed in response to doxycycline treatment (after 1 h of doxycycline incubation) while the mito-nuclear imbalance observed in HeLa cells requires at least 24 h of incubation with doxycycline (data not shown). The best-described mechanism of GCN2 activation is related to amino acid depletion and requires the binding of an unloaded tRNA to the His-tRNA synthetase-like and CTD (C-terminal dimerization and ribosome binding site) domains of the kinase (Castilho et al., 2014). On the other hand, although the molecular mechanism is missing, GCN2 activation is also reported under proteasome inhibition in several studies (Acharya et al., 2009; Jiang and Wek, 2005; Neznanov et al., 2008). Interestingly, the concentration of 15 amino acids and the proteasomal activity are both decreased in 143B cybrid cells harboring a 7.5 kb mtDNA deletion and in lymphoblasts from patients carrying a 5 kb mtDNA deletion (Alemi et al., 2007). In addition, in the yeast *Saccharomyces cerevisiae*, oxidative stress, such as incubation with peroxide hydrogen, also activates GCN2 by a still unclear mechanism requiring the His-RS-like domain of the kinase (Mascarenhas et al., 2008). Besides, induction of the GCN2-eIF2 α axis is associated with mitochondrial ETC defect in the *C. elegans* clk-1 (required for ubiquinone synthesis) mutant strain, which can be prevented by NAC treatment (Baker et al., 2012). Thus, it would be interesting to further investigate both the involvement of amino acid depletion, proteasome inhibition, as well as ROS production in GCN2 activation upon doxycycline treatment.

In mammals, the mitochondria-specific unfolded protein response (mtUPR) is currently accepted as a quality control relying on a retrograde signaling to coordinate expression of specific genes, such as the chaperone HSPD1 and the protease ClpP, to resolve organelle proteotoxic stress (Pellegrino et al., 2013). Different cell models have been described to induce mtUPR: either the accumulation of protein aggregates in the

mitochondrial matrix, caused by the OTCA overexpression (Rath et al., 2012; Zhao et al., 2002), or an imbalance between mitochondrial and nuclear-encoded proteins, as shown in mouse hepatocytes treated with doxycycline (Houtkooper et al., 2013) or in mtDNA depleted (ρ^0) rat hepatoma cells (Martinus et al., 1996). Even if it is known that sensitivity and response to drugs might differ between rodent and human hepatocytes (Black et al., 2012; Kotokorpi et al., 2007; Seok et al., 2013), in our hands, similar stresses increase neither HSPD1 nor ClpP expression despite testing four different models, of which one in three cancer cell lines as well as in primary human hepatocytes (Figs. 2A, B, C, D, S1C, S2B). However, in the later case, the non-induction of mtUPR markers might be the result of the absence of mito-nuclear protein imbalance as suggested by the steady-state protein level of COXI and COXII (Fig. S4B).

In the mtUPR context provoked by OTCA overexpression, CHOP-10 induction would be regulated by the AP-1 transcription factor after c-jun phosphorylation by JNK and/or PKR (Horibe and Hoogenraad, 2007; Rath et al., 2012). In the present study, even if CHOP-10 is overexpressed, reporter plasmid experiments clearly demonstrate that, when mitochondrial translation is inhibited by doxycycline, the AARE binding site is required for increased transcription of the luciferase reporter gene under the control of a region of the CHOP-10 promoter whereas mutation within the AP-1 binding site has no effect (Fig. 6A). In addition, we confirmed ATF4 requirement as silencing of this transcription factor prevents CHOP-10 induction by doxycycline.

Actually, a careful analysis of the literature reveals previous reports in line with our results showing no induction of mtUPR upon mitochondrial dysfunction. First, a study from Piechota and colleagues also analyzed the impact of mitochondrial genome expression inhibitors in HeLa cells and showed that a six-day-treatment with ethidium bromide or thiamphenicol (a derivative of chloramphenicol) does not increase HSPD1 abundance (Piechota et al., 2006). Second, a recent *in vivo* study conducted on mice KO for DARS2 (mitochondrial aspartyl-tRNA synthetase) in cardiac and skeletal muscle tissues highlights a tissue-specific response to mitochondrial translation impairment: no mtUPR marker is observed in skeletal muscle, while a weak induction of HSP60 and no modification of ClpP abundance are observed in cardiomyocytes (Dogan et al., 2014). Third, in the extensive expression profiling by Cortopassi and colleagues of mitochondrial diseases associated with mtDNA mutations/deletions, ClpP is not induced and is even repressed in two conditions of mtDNA deletion when compared with their respective controls (Cortopassi et al., 2006). Yet, among shared overexpressed genes between the different models of mitochondrial dysfunction, these authors highlighted ISR targets such as CHOP-10, ATF4, ATF3 and Trib3. Four, in addition to organelle dysfunction associated with altered mtDNA expression, mitochondrial proteotoxic stress in mice knock out for the mitochondrial intermembrane space protease HtrA2 also points CHOP-10, ATF3 and HERP as overexpressed in brain cells, whereas ClpP and HSPD1 expression stays unchanged (Moisoi et al., 2009). Finally, a transcriptomic analysis following the selective inhibition of heat shock protein-90 (Hsp90) in mitochondria by Gamitrinib^{TPP} (GA mitochondrial matrix inhibitor G-TPP) revealed increased mRNA abundance of CHOP-10, Trib3, ATF3 and HERP together with phosphorylation of eIF2 α (Siegelin et al., 2011). Altogether these results strongly suggest that ISR might be a general response to mitochondrial dysfunction while mtUPR would be restricted to more specific conditions affecting mitochondrial function.

Coordination between nuclear and mitochondrial genomes is necessary to ensure proper organelle function and cell integrity. The central ISR event eIF2 α phosphorylation might be crucial for such coordination in response to mitochondrial dysfunction (Fig. 7). Interestingly, in *C. elegans* the GCN2-eIF2 α axis acts in parallel with mtUPR to keep mitochondrial proteostasis and function. Indeed, GCN2 silencing increases mitochondrial stress and transcriptional activity of the HSP60-GFP reporter plasmid (Baker et al., 2012). Global attenuation of protein synthesis might also prevent excessive importation and accumulation

of unfolded proteins within mitochondria and thus detrimental overwhelming of resident chaperones and proteases.

This scenario is already well described for the endoplasmic reticulum. Indeed, it has been shown that eIF2 α phosphorylation and protein synthesis shutdown reduce ER-stress, promoting cell survival (Han et al., 2013; Harding et al., 2000). Likewise, reduced protein translation in mouse β -cells is required to keep organelle in shape as cells lacking eIF2 α have swollen mitochondria with disrupted cristae (Back et al., 2009). Reduced cytosolic protein synthesis also prevents mitochondrial degeneration in a yeast model of aging-related degenerative disease (Wang et al., 2008). PINK1 is a serine/threonine kinase involved in the mitochondrial quality control mitophagy. In a *Drosophila melanogaster* model of familial, early onset of Parkinson's disease, reduction of protein translation suppresses PINK1 mutant phenotype whereas increased translation through S6K activation worsen it (Liu and Lu, 2010). Finally, phosphorylation of eIF2 α also protects from oxidative stress triggered by the mitochondrial complex I inhibitor paraquat, both in *C. elegans* and mammalian cells (Rainbolt et al., 2013).

The phosphorylation of eIF2 α and global attenuation of protein synthesis represent thus an interesting mechanism to prevent/attenuate mitochondrial damage and dysfunction. Knowing that impaired mitochondrial translation is increasingly associated with mitochondrial diseases such as Leigh syndrome and Perrault syndrome (Boczonadi and Horvath, 2014; Pearce et al., 2013), further investigation of the functional impact of the GCN2/P-eIF2 α /ATF4 axis is now necessary to better understand the biological processes controlled by this cell signaling pathway associated with mitochondria dysfunction and related pathologies.

Acknowledgments

S. Michel is recipient of a doctoral fellowship from the Fonds pour la Recherche dans l'Industrie et l'Agriculture (FRIA, Belgium). This work was supported by the Belgian Association for Muscular Diseases (ABMM, Belgium, grant 2013–08). The authors especially thank Prof. Wiesner for the gift of the mtDNA-depleted HeLa rho⁰ cells.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mito.2015.01.005>.

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