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https://www.sciencedirect.com/science/article/abs/pii/S1874939914002624?via%3Dihub

https://doi.org/10.1016/j.bbagrm.2014.10.004

Damiano F, Tocci R, Gnoni GV, Siculella L. Expression of citrate carrier gene is activated by ER stress effectors XBP1 and ATF6α, binding to an UPRE in its promoter. Biochim Biophys Acta. 2015 Jan;1849(1):23-31

Highlights

1) Expression of CiC is activated in cells during ER stress induced by tunicamycin.

2) A functional Unfolded Protein Response Element has been found in CiC promoter.

3) ER stress activation of CiC is under the ATF6α and XBP1 branches of UPR pathway.

4) Increase of ER proteins acetylation upon ER stress is dependent on the CiC expression.

Expression of citrate carrier gene is activated by ER stress effectors XBP1 and ATF6α, binding to an UPRE in its promoter.

Authors: Fabrizio Damiano*, Romina Tocci, Gabriele Vincenzo Gnoni, Luisa Siculella

Laboratory of Biochemistry and Molecular Biology, Department of Biological and Environmental Sciences and Technologies, University of Salento, Via Prov. le Lecce-Monteroni, Lecce 73100, Italy.

*Address correspondence to: Fabrizio Damiano, Laboratorio di Biochimica e Biologia Molecolare, Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Via Prov.le Lecce-Monteroni, Lecce 73100, Italy. E-mail: fabrizio.damiano@unisalento.it; Telephone:0832298698 ; Fax: 0832298626

Abstract

The Unfolded Protein Response (UPR) is an intracellular signalling pathway which is activated when unfolded or misfolded proteins accumulate in the Endoplasmic Reticulum (ER), a condition commonly referred to as ER stress. It has been shown that lipid biosynthesis is increased in ER-stressed cells. The N^{ε}-lysine acetylation of ER-resident proteins, including chaperones and enzymes involved in the post-translational protein modification and folding, occurs upon UPR activation. In both ER proteins acetylation and lipid synthesis, acetyl-CoA is the donor of acetyl group and it is transported from the cytosol into the ER. The cytosolic pool of acetyl-CoA is mainly derived from the activity of mitochondrial citrate carrier (CiC). Here, we have demonstrated that expression of CiC is activated in human HepG2 and rat BRL-3A cells during tunicamycin-induced ER stress. This occurs through the involvement of an ER stress responsive region identified within the human and rat CiC proximal promoter. A functional Unfolded Protein Response Element (UPRE) confers responsiveness to the promoter activation by UPR transducers ATF6α and XBP1. Overall, our data demonstrate that CiC expression is activated during ER stress through the binding of ATF6α and XBP1 to an UPRE element located in the proximal promoter of *Cic* gene. The role of ER stress-mediated induction of CiC expression has been discussed.

1. Introduction

An essential function of the Endoplasmic Reticulum (ER) is to ensure the correct folding of proteins residing within and transiting along the secretory pathway [1,2]. When the ER protein-folding capacity falls, a condition of ER-stress is established and leads to the activation of an evolutionarily conserved Unfolded Protein Response (UPR) signalling pathway in order to restore the ER homeostasis [3,4].

In mammalian cells, UPR signalling pathway consists of three main branches carried out by the UPR transducers Inositol Requiring Protein-1/X-box-Binding Protein-1 (IRE1/XBP1), the Activating Transcription Factor-6 (ATF6), and the Protein Kinase RNA (PKR)-like ER Kinase (PERK), respectively. IRE1, ATF6 and PERK are transmembrane ER proteins, with their N-terminus located in the lumen of the ER and the C-terminus in the cytosol [3,4]. Both IRE1 and PERK contain an unfolded protein-sensing domain in their N-terminus. In the direct recognition model of UPR activation, accumulating unfolded proteins directly interact with the lumenal domain of IRE1 and PERK, causing their oligomerization-induced activation. In the alternative indirect recognition model, the ER resident chaperone 78-kDa Glucose-Regulated Protein (GRP78, also known as immunoglobulin heavy chain binding protein, BiP) binds the N-terminus of IRE1, ATF6 and PERK, preventing their activation under normal conditions. When ER stress occurs, GRP78 binds unfolded proteins and releases IRE1, PERK, and ATF6, allowing them to transduce the UPR signal [5-9].

Disruption of ER homeostasis and the consequent activation of UPR have been observed in liver and adipose tissue of humans with nonalcoholic fatty liver disease (NAFLD) and/or obesity [10]. NAFLD is characterized by fatty infiltration (steatosis) of the liver in the absence of chronic alcohol consumption or other liver diseases. Sources of hepatic lipids in NAFLD include dietary chylomicron remnants, free fatty acids released from adipose tissue triglycerides, and the *de novo* lipogenesis in which acetyl-CoA is converted in fatty acids [10].

UPR activation is also accompanied by an increase of the status of N^{ε}-lysine acetylation of ER-resident proteins [11], most of them are chaperones and enzymes involved in the post-translational protein modification and folding, including GRP78 [11]. Recent studies reported that inactivation of some histone deacetylases through gene knockdown or treatment with specific inhibitors triggers the hyperacetylation of GRP78 and the activation of the UPR transducers PERK and ATF6 [12,13]. In the reaction of N^{ε}-lysine acetylation, the donor of the acetyl group is represented by acetyl-CoA, which is transported from the cytosol to the ER lumen by the membrane transporter SLC33A1/AT-1 [14,15].

The cytosolic pool of acetyl-CoA is mainly derived from glucose or aminoacids metabolism through the shuttle citrate/malate catalyzed by citrate carrier (CiC), also known as tricarboxylate carrier, which transports citrate from the mitochondrion to the cytosol [16].

CiC is an integral protein of the mitochondrial inner membrane, which catalyzes the efflux of citrate in exchange for tricarboxylates, dicarboxylate (malate) or phosphoenolpyruvate from the mitochondrial matrix to the cytosol. Here, by the action of ATP-citrate lyase, citrate provides the carbon units for fatty acids and cholesterol biosynthesis [16]. This carrier plays a pivotal role in

intermediary metabolism by connecting carbohydrate with lipid metabolism, supplying to cytosol acetyl-CoA, in the form of citrate.

It has been reported that CiC activity is under hormonal [17,18] and nutritional control [19-22]. Structural and functional studies of the rat *Cic* gene promoter led to the characterization of binding sites for transcription factors such as Sterol Regulatory Element-Binding Protein-1 (SREBP-1) and Peroxisomal Proliferator-Activated Receptors (PPARs), both involved in the control of lipid homeostasis [20,23]. It has also been shown that transport of citrate (acetyl-CoA) from mitochondria to the cytosol is essential for important functions other than fatty acids and cholesterol syntheses, such as the adipogenesis [20], inflammation response [24], maintaining of chromosomes integrity [25], insulin secretion [26], and cancer [27].

Given the importance of CiC as a key protein in lipogenesis and in aforementioned functions, and due to the poor knowledge about the ER stress-mediated activation of lipogenic genes expression, we investigated the modulation of CiC expression during the ER stress and the molecular mechanism underlying this regulation. To this aim, the characterization of the human and rat *Cic* gene promoter was carried out in order to locate the putative region involved in the activation of *Cic* gene expression in response to ER stress. Experiments were performed to demonstrate the activation of CiC promoter through the binding of ATF6α and XBP1 to an Unfolded Protein Response Element (UPRE), upon ER stress induction. Finally, results showed that the acetylation status of ER resident proteins was affected by CiC expression. On the basis of these results, a potential role of CiC activation by ER stress was proposed.

2. Materials and methods

2.1. Plasmid and reporter vector construction

Four DNA fragments of human CiC promoter (NCBI accession number BC008061.2), sized from -819 to +29, -413 to +29, -144 to +29, and -60 to +29 bp, were obtained by PCR using genomic DNA from HepG2 cells as template and the forward primers hCICfor(-819) (5’ AAGCTTGGTACCGGTACCGGACCTCATAAAAG-3’), hCICfor(-413) (5’-AAGCTTGGTACCGCTAGCCCCACGTGTTTTCG-3’), hCICfor(-144) (5’-AAGCTTGGTACCAGGCCTCAGTTTCCCGGCCC-3’), hCICfor(-60) (5’-AAGCTTGGTACCGGCCGCCCCGCCCCTGGGAC-3’). The common reverse primer was hCICrev(+29) (5’-GAATTCAAGCTTGTGGCGGCTTCGGGTCC-3’).

Amplimers were digested with *Kpn*I and *Hind*III, then subcloned into the same sites of pGL3 basic vector (Promega), obtaining the phCiC819, phCiC413, phCiC144 and phCiC60 constructs. The construct phCiC144(UPREm) containing the mutated UPRE at −66 bp of the human CiC promoter

was created by site-directed mutagenesis, by using the phCiC144 as template for PCR reaction. The pairs of the complementary mutagenic primers were forward UPREmFor (5'-TGGAGCTCTGCAGGCCGCCCCGCCCCT-3') and reverse UPREmRev 5'-GGCGGCCTGCAGAGCTCCAGGTCCCGC-3'. Plasmids UPREhCiC3X and UPREhCiCm3X were obtained by annealing the two couples of primers 5'-CGGAGCTGACGCGGCCGCCCTCGAGGAGCTGACGCGGCCGCCCTCGAGGAGCTGACGCGGCCGCCCA-3' with 5'-GATCTGGGCGGCCGCGTCAGCTCCTCGAGGGCGGCCGCGTCAGCTCCTCGAGGGCGGCCGCGTCAGCTCCGGTAC-3' and 5'-CGGAGCTCTGCAGGCCGCCCTCGAGGAGCTCTGCAGGCCGCCCTCGAGGAGCTCTGCAGGCCGCCCA-3 with 5'-GATCTGGGCGGCCTGCAGAGCTCCTCGAGGGCGGCCTGCAGAGCTCCTCGAGGGCGGCCTGCAGAGCTCCGGTAC -3', and by cloning the oligonucleotide dimers into the *Kpn*I and *Bgl*II sites of pGL3prom. PcDNA-ATF6α (373) and pcDNA-XBP1s, harboring the cDNA for the active form of ATF6α (1-373 aa) and XBP1, respectively, were kindly provided by Dr. K. Mori (Department of Biophysics, Kyoto University) [28,29]. The cDNAs for the active form of XBP1 and ATF6α (373) were cloned into the pcDNA3 expression vector containing an in-frame HA epitope at the 5′ end, to facilitate the immunodetection of the recombinant proteins.

2.2. Cell culture and transient transfection assay

Human hepatoma HepG2 and Buffalo rat liver BRL-3A cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml) and with 10% (v/v) or 5% (v/v) FBS (fetal bovine serum), respectively, at 37°C under 5% CO_2 atmosphere. For luciferase activity assay, cells (2×10^5) were plated onto 6-well cell culture plates. After 48 h, cells were co-transfected with one of the CiC promoter–luciferase reporter vectors (500 ng/well), and a *Renilla* luciferase reference plasmid, pGL4.73 (20 ng/well), a control for transfection efficiency, by using Metafectene transfection reagent (Biontex). Following an 8 h transfection period, the medium was changed to fresh DMEM supplemented with FBS, and cells were incubated for 24 h. Cells were then lysed and luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega). The effect of tunicamycin, an ER stressor, on human or rat CiC promoter activity was determined incubating the cells in DMEM medium supplemented with 1μg/ml tunicamycin or with the vehicle alone for 12 h. For transcriptional activation by XBP1s and ATF6α (373), HepG2 cells were transiently co-transfected with 500 ng of phCiC144 or the mutant phCIC144(UPREm) and 20 ng of pGL4.73 reference plasmid, together with 50 ng of either pcDNA3-

HA-XBP1s or pcDNA3-HA-ATF6α (373), or an empty control vector (pcDNA3). After transfection the cells were incubated in DMEM with 10% (v/v) of FBS, for 24 h.

2.3. Isolation of RNA from cultured-cells and Real-Time qPCR analysis

Total RNA from HepG2 and BRL-3A cells was isolated using the SV Total RNA Isolation System kit (Promega), following manufacturer's instructions. The RT (reverse transcriptase) reaction (20 μl) was carried out using 5 μg of total RNA, 100 ng of random hexamers and 200 units of SuperScriptTM III RNase H-Reverse transcriptase (Life Technologies).

Quantitative gene expression analysis was performed (SmartCycler System, Cepheid) using SYBR Green technology (FluoCycle, Euroclone) and 18S rRNA for normalization. The primers used for real-time PCR analysis were the following: hCiCfor (5'-GAAGTTCATCCACGACCAGAC-3'); hCiCrev (5'-TCGGTACCAGTTGCGCAGG-3'); rCiCfor (5'-GCCTCAGCTCCTTGCTCTA-3'); rCiCrev (5'-ACTACCACTGCCTCTGCCA-3').

2.4. ChIP (chromatin immunoprecipitation) assay

The ChIP assay was performed essentially as in [30]. After centrifugation to remove cell debris, 5% of the sample was kept as DNA input. Chromatin complex was immunoprecipitated for 12–18 h at 4°C with 2 μg of ATF6 antibody (sc-22799, Santa Cruz Biotechnology), or XBP1 (sc-7160, Santa Cruz Biotechnology), or rabbit IgG overnight at 4°C on a rotating wheel. After immunoprecipitation with the antibodies, quantitative Real Time PCR was conducted using primers hCIC for (-144) 5'-GCTAGCCCCACGTGTTTTCG-3', and hCICrev (+29) 5'-GTGGCGGCTTCGGGTCC-3', designed to amplify a 173 bp fragment (−144 bp to +29 bp) of the proximal promoter region in the human *Cic* gene. The PCR reaction was performed with 2 μl of immunoprecipitate, in a final volume of 25 μl using SYBR Green technology (FluoCycle, Euroclone). Samples were incubated for an initial denaturation at 94°C for 60 s, followed by 30 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s. The PCR amplimers were sequenced to confirm the accuracy of ChIP experiments, by using BigDyeTM Terminator cycle sequencing kit.

2.5. Preparation of highly purified ER fraction and Western blotting analysis

Preparation of highly purified ER fraction (more than 90%) was carried out as previously described, with minor modifications [11]. Briefly, confluent cells were washed with ice-cold phosphate-buffered saline and homogenized in homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) plus a protease inhibitor cocktail (Sigma) using a 25-gauge needle and a tight-pestle Dounce homogenizer. The homogenate was centrifuged at

1500×*g* for 15 min at 4°C and the resulting supernatant was centrifuged at 12000×*g* for 15 min at 4°C. The supernatant was then centrifuged at 100000×*g* in a Ti75 rotor for 30 min at 4°C. Membrane pellet was resuspended in 5% Nycodenz solution (10 mM Tris-pH 7.4, 0.75% NaCl, 3 mM KCl, and 1 mM EDTA) and layered on top of a step gradient consisting of 24, 19.33, 14.66, and 10% Nycodenz solution, and centrifuged at 100000×*g* in a SW41 rotor for 18 h at 4°C. Fractions (0.5 ml) were collected from the top to the bottom of the gradient and further concentrated by centrifugation at 100000×*g* for 15 min. Fractions were analysed by Western blotting with antibodies against GRP78, Site 2 Protease (S2P), and Early Endosome Antigen 1 (EEA1), used as makers for the ER, Golgi apparatus, and endosomes, respectively.

Cell protein extract and nuclear protein extract for Western-blot analysis were obtained essentially as previously described [31]. 50μg proteins were dissolved in sodium dodecyl sulfate (SDS) sample buffer and separated on 10% (w/v) SDS gels. Separated proteins were transferred electrophoretically onto nitrocellulose membrane (Pall, East Hills, NY). Equal protein loading was confirmed by Ponceau S staining. The filter was blocked with 5% (w/v) non-fat dried milk in buffered saline. Blots were incubated with specific primary antibodies directed against XBP1, ATF6α, CiC (sc-7160, sc-22799, sc-86392, Santa Cruz Biotechnology). The immune complexes were detected using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent detection reagent (GE healthcare). Densitometric analysis was carried out on the Western-blots using the NIH Image 1.62 software (National Institutes of Health, Bethesda, MD), normalizing to β-actin or Lamin B used as standard controls.

2.6. RNA interference analysis

In HepG2 cells gene silencing was performed by RNA interference, by using synthetic short hairpin RNA (shRNA). ShRNA expression vectors, targeting human CiC, XBP1 and ATF6α RNA, were generated by annealing complementary oligonucleotides corresponding to the respective target sequence (for CiC: 5′-GGAGATTGTGCGGGAACAA-3′; for ATF6α: 5'-GCAGCAACCAATTATCAGT-3'; for XBP1: 5'-GGGTCATTAGACAAATGTC-3') and ligating the fragments into *Bgl*II/*Hind*III-digested pSuperior vector (OligoEngine). A shRNA expression vector with a scrambled sequence of each target sequence was used as a negative control. The expression of CiC was rescued by co-transfecting HepG2 cells with pcDNA-rCiC, which harbours the rat CiC cDNA lacking the target of CiC shRNA. Transfection was performed for 48 h by using Metafectene Pro (Biontex), according to the manufacturer's recommendations.

2.7. Statistical analysis

All data are presented as means ± S.D. for the number of experiments indicated in each case. Statistical analysis was performed using one-way ANOVA, followed by a post hoc Tukey's B test. Values sharing a different letter differ significantly. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Role of ER stress on CiC expression in HepG2 and BRL-3A cells

To investigate the effect of ER stress on *Cic* expression, the abundance of CiC mRNA was analyzed by real-time qPCR in human HepG2 and in rat BRL-3A cells, both cultured without (control) or with 1μg/ml tunicamycin. Quantitation of CiC mRNA abundance showed that tunicamycin caused a time-dependent increase of *Cic* gene expression in ER-stressed HepG2 and BRL-3A cells, reaching maximum levels at 24 h and 12 h, respectively (Fig. 1A). The increase of the abundance of CiC mRNA, induced by ER stressor, was accompanied by an increment of CiC protein level, as confirmed by Western blotting experiments (Fig. 1B). In agreement with previous studies [32], treatment with 1 μg/ml tunicamycin induced the expression of the spliced form of XBP1, a classical index of the UPR (data not shown).

3.2. Effect of ER stress on the human and rat CiC promoter activity

We then evaluated the effect of ER stressor on the CiC promoter activity in HepG2 and BRL-3A cells. To this aim, the construct phCiC819 containing the promoter region between -819 and +29 of the human *Cic* gene, fused to the firefly luciferase reporter gene, was synthesized. The construct prCiC1484 containing the rat CiC promoter [23] was also used. HepG2 and BRL-3A cells were transiently transfected with phCiC819 and prCiC1484, respectively, together with the *Renilla* luciferase reference plasmid, pGL4.73, used as a control for the transfection efficiency. Incubation of cultured cells with tunicamycin enhanced luciferase expression from both the phCiC819 and prCiC1484 constructs with respect to the untreated control cells (Fig. 2A).

To define DNA sequences responsible for the up-regulation of the *Cic* gene by the ER stressor, a series of nested deletion constructs within the 5'-flanking region of both the human and rat *Cic* gene fused to the luciferase (Luc) gene was used. Cells were transfected with each construct and then incubated without (control) or with 1 μg/ml tunicamycin for 12h.

When HepG2 cells were transiently transfected with constructs containing progressive 5' deletions of sequences from -819 to -144 of the human CiC promoter, treatment with tunicamycin enhanced luciferase activity with respect to the control (Fig. 2B, upper panel). By contrast, no increase of the luciferase activity was observed in cells transfected with phCiC60 or empty pGL3basic vector (Fig.

2B, upper panel). Analogously, treatment with the ER stressor caused an increase of the activity of rat CiC promoter in BRL-3A cells transfected with plasmids prCiC1484, prCiC1114, prCiC469 or prCiC147, but not with prCiC42 (Fig. 2B, lower panel). These results suggested that a putative ER stress responsive element that mediates the transactivation of CiC promoter in ER-stressed cells may be located in the -144/-60 bp and -147/-42 bp regions of the human and rat CiC promoter, respectively.

Pairwise sequence alignment showed a significant similarity (approx. 80%) between ER stress responsive region of rat CiC promoter and the corresponding region of human CiC promoter (Fig. 3A). *In silico* analysis of both minimal region responsive to ER stress by Match and P-Match programs (http://www.generegulation.com) identified a putative Unfolded Protein Response Element (UPRE) at -66 bp and at -73 bp in human and in rat CiC promoter, respectively (Fig. 3A). The comparison of the UPRE found in the promoter of human *Cic* gene with the UPRE consensus sequence (TGACGTGAG/A) showed a similarity of 7 out of 8 bases. The highest similarity (100%) was found when UPRE identified in rat CiC promoter was compared with UPRE of mouse CiC promoter or with UPRE consensus sequence (Fig. 3B).

3.3. Transcriptional activation of Cic gene by ATF6α and XBP1

The transcription factor XBP1 binds the UPRE as homodimer or as heterodimer together with ATF6α [33]. To investigate whether ATF6α or XBP1 are implicated in the expression of the *Cic* gene, real-time PCR analysis was carried out on total RNA extracted from HepG2 or BRL-3A cells transfected with pcDNA-HA-XBP1s or/and with pcDNA-HA-ATF6α (373), carrying the cDNA for the active form of XBP1 (XBP1s) and ATF6α (373), respectively. Transfection experiment with the empty pcDNA3 vector was performed as a control. 24 h after transfection, XBP1s and ATF6α (373) overexpression caused an increase of both CiC mRNA abundance and protein level when compared to the control, in HepG2 (Fig. 4A and Fig. 4B) and in BRL-3A cells (data not shown). In the transfected cells, the expression levels of HA-tagged recombinant XBP1s and ATF6α (373) were similar.

The involvement of XBP1 and ATF6α in the regulation of CiC expression was also evaluated through gene silencing by RNA interference. HepG2 cells were transfected with pSUPshATF6α, or pSUPshXBP1 containing the sequence for ATF6α and XBP1 RNA targeting, respectively, or pSuperior vector containing the corresponding scrambled sequence. After 48 h transfection, cells were treated with 1 μg/ml tunicamycin for 12 h. The control was represented by untreated cells transfected with scramble shRNA. Expression of CiC was induced in tunicamycin-treated cells with respect to control cells at both mRNA and protein level (Fig. 4C and Fig. 4D). Unexpectedly, both

CiC mRNA abundance (Fig. 4C) and protein content (Fig. 4D) increased in *Xbp1*- or in *Atf6α*-knockdown cells with respect to tunicamycin-treated cells transfected with scramble shRNA. In order to explain the increment of CiC expression, the nuclear levels of XBP1 and ATF6α were analyzed in nuclei from each sample. As expected, the levels of XBP1 and ATF6α detected in stressed-cells transfected with scramble shRNA were higher than those observed in untreated control cells. Furthermore, XBP1 and ATF6α were absent in the nuclei from *Xbp1*- and *Atf6α*-knockdown cells, respectively (Fig. 4D). Conversely, when compared to tunicamycin-treated cells transfected with scramble shRNA the levels of XBP1 were augmented in *Atf6α*-knockdown cells, whereas the levels of ATF6α were augmented in *Xbp1*-knockdown cells (Fig. 4D). When both *Xbp1* and *Atf6α* genes were silenced, the expression of CiC were reduced with respect to tunicamycin-treated cells transfected with scramble shRNA, even though it persisted at higher level compared to control unstressed cells (Fig. 4D).

To assess whether ATF6α or XBP1 could induce the transcriptional activity of CiC promoter, transient transfections of HepG2 cells with the phCiC144 or phCiC42 promoter-reporter constructs or with the empty pGL3basic vector, together with increasing concentrations of the pcDNA-HA-XBP1s or pcDNA-HA-ATF6α (373) plasmids were carried out. While phCiC42 (Fig. 5A) as well as the pGL3basic vector (data not shown) were unresponsive to the overexpression of XBP1s or ATF6α (373), the promoter activity of phCiC144 was activated in a dose-dependent manner by both XBP1s or ATF6α (373) in HepG2 cells (Fig. 5A). A marked activation of promoter activity, following XBP1s and ATF6α (373) overexpression, was also observed in BRL-3A cells transfected with prCiC147 (data not shown). The involvement of XBP1 and ATF6α on CiC promoter activity was also analyzed by gene knockdown experiments (Fig. 5B). In *Xbp1*- or *Atf6α*-knockdown cells treated with tunicamycin, CiC promoter activity was increased with respect to the tunicamycin-treated cells transfected with the scramble shRNA. Conversely, in HepG2 cells transfected with both ATF6α- and XBP1-shRNA, CiC promoter activity was reduced when compared to the tunicamycin-treated cells transfected with scramble shRNA. However, after ATF6α- and XBP1-knockdown, CiC promoter activity measured in tunicamycin-treated cells was higher with respect to the untreated cells (Fig. 5B).

Mutagenesis of UPRE at -66 bp abolished the induction of human CiC promoter by ATF6α (373) or XBP1s overexpression (Fig. 5C). Three copies in tandem of the wild type or mutated sequence of human UPRE(−66) were inserted upstream the SV40 promoter into a pGL3Prom vector, obtaining the UPREhCiC3X and UPREhCiCm3X constructs, respectively. Transfection experiments performed with UPRECiC3X and UPREmCiC3X constructs showed that the UPRE(−66) conferred

responsiveness to ATF6α (373) and XBP1s when inserted upstream the unresponsive SV40 promoter (Fig. 5C), indicating that this sequence was able to mediate the response to the UPR effectors.

To investigate whether ATF6α and XBP1 are able to bind *in vivo* the proximal promoter of the endogenous *Cic* gene, ChIP assay was performed. Chromatin was extracted from HepG2 treated with 1μg/ml tunicamycin for 12 h or from untreated-cells used as control. After immunoprecipitation with antibodies against ATF6α or XBP1, or with non-specific IgGs, the region of the *Cic* promoter containing the putative UPRE was amplified by PCR. The amplimer was detected in input and in anti-ATF6α or anti-XBP1-immunoprecipitated chromatin from tunicamycin treated-cells. Conversely, no amplification was obtained in ATF6α- or XBP1-immunoprecipitated chromatin from control cells or in samples treated without antibody or immunoprecipitated with IgGs, used as negative controls (Fig. 5D).

4. Discussion

Citrate carrier has so far been studied mainly as a protein which provides the cytosol of acetyl-CoA, precursor of the *de novo* fatty acids and cholesterol syntheses. It represents, indeed, the link between the glycolytic and lipogenic pathways [16].

In recent years, new insights are highlighting the role of the citrate carrier in important cellular functions other than lipid synthesis, such as the adipogenesis [20], maintaining of chromosomes integrity [25], inflammation response [24], insulin secretion [26], and cancer [27]. In this work we reported lines of evidence on the activation of CiC expression in response to ER stress.

In the ER lumen, proteins destined for secretion or insertion into membranes undergo to a controlled folding process which proceeds through several post-translational modifications, such as glycosylation and disulfide bond formation [1,2]. This is accomplished by molecular chaperones and enzymes involved in protein folding and maturation [1,2]. ER stress occurs in cells with diminished ER protein-folding capacity and leads to the activation of an evolutionarily conserved UPR signalling system, in order to monitor and respond to the accumulation of improperly folded proteins in the ER lumen [3,4]. The presence of ER stress has been observed in various tissues from obese mice and humans [34]. In these tissues, besides promoting the ER protein folding process, ER stress and UPR activation can regulate lipid metabolism [34]. In fact, accumulating lines of evidence suggest that activation of the UPR triggers the transcriptional regulation of lipogenic genes. As a consequence of UPR activation, an increased *de novo* lipogenesis and tryglicerides accumulation occur in adipose tissue concurring in the development of obesity [34]. Furthermore, enhanced *de novo* fatty acid synthesis triggered by UPR significantly contributes to hepatic lipid accumulation in nonalcoholic fatty liver disease (NAFLD) [10]. The role of ER stress and UPR pathways in the induction of *de*

novo lipogenesis has been under intense investigation. However, the molecular mechanisms by which ER stress and UPR pathways activate the expression of lipogenic genes are poorly understood. On the basis of these observations, we decided to evaluate whether the expression of CiC is activated by ER stress and, if so, to investigate the molecular mechanism underlying the regulation of CiC mediated by ER stress. We demonstrated that the treatment with an ER stressor as tunicamycin induced CiC expression both in HepG2 and BRL-3A cells (Fig. 1), owing to the activation of *Cic* promoter activity (Fig. 2). Deletional analysis of *Cic* promoter revealed an ER responsive region within -144/-60 and -147/-42 sequences of the human and rat proximal promoter region, respectively (Fig. 2). By *in silico* analysis a putative Unfolded Protein Response Element (UPRE) having high similarity with the UPRE consensus sequence (TGACGTGAG/A) has been identified in both human and rat ER stress responsive region (Fig. 3). The UPRE represents one of the three *cis*-acting elements known to respond to ER stress in mammals, together with ERSE (ER stress Response Element, consensus sequence CCAAT-N9-CCACG), and ERSE II (consensus sequence ATTGG-N1-CCACGT) [35]. UPRE was initially selected through artificial binding site selection experiments using the transcription factor ATF6α [36], which is one of the UPR effectors. Moreover, the transcription factor XBP1, another UPR effector, binds the UPRE as homodimer or heterodimer with ATF6α [33]. Thus, XBP1 and ATF6α may be involved in the tunicamycin-mediated activation of CiC expression. Indeed, overexpression of these transcription factors induced both CiC expression (Fig. 4A and B) and CiC promoter activity (Fig. 5A). Transactivation of CiC promoter by XBP1 and ATF6α was dependent on the UPRE as it was abolished when this element was altered (Fig. 5C). Contrary to what it was expected, both CiC expression (Fig. 4C and D) and CiC promoter activity (Fig. 5B) were increased in *Atf6α*- or in *Xbp1*-knockdown cells with respect to the ER stressed cells transfected with scramble shRNA. In order to explain the discrepancy between the results from gene silencing experiments and those obtained from the overexpression experiments, the content of XBP1 and ATF6α protein was analyzed in *Atf6α*- or in *Xbp1*-knockdown HepG2 cells. When compared to tunicamycin-treated cells transfected with scramble shRNA, an increase in the expression of XBP1 and ATF6α was observed in tunicamycin-treated cells transfected with ATF6α and XBP1 shRNA, respectively (Fig. 4D). These findings are in agreement with previous results [15], according to which a compensatory mechanism occurs in cells where a UPR-branch is up-regulated as a consequence of the down-regulation of the other UPR-branch, in order to sustain the UPR. Conversely, in *Atf6α*- and *Xbp1*-knockdown cells both CiC expression (Fig. 4C and D) and CiC promoter activity (Fig. 5B) were reduced with respect to stressed cells transfected with scramble shRNA. However, in *Atf6α*- and *Xbp1*-knockdown tunicamycin-treated cells the CiC expression (Fig. 4C and D) and the CiC promoter activity (Fig. 5B) were higher than those observed in unstressed cells (control). This residual

tunicamycin-mediated induction of CiC expression upon ATF6α-/XBP1-knockdown suggests that other regulatory elements and/or transcription factors could participate in the induction of CiC expression triggered by tunicamycin.

Results from ChIP assay demonstrated that an increase of the binding of XBP1 and ATF6α to the CiC promoter was observed in tunicamycin-treated HepG2 cells with respect to untreated control cells (Fig. 5D).

Our data demonstrated that ER stress induces CiC expression through CiC promoter transactivation by XBP1 and ATF6α binding to an UPRE site. Previously, the presence of UPRE-like elements has been postulated in the promoter of genes coding for ER-Associated Degradation (ERAD) components. Even though the putative UPRE-like site has not been finely characterized in ERAD genes promoter, its responsiveness has been hypothesized observing that ER stress-induced transactivation of ERAD genes through this element is abolished in IRE1- or XBP1-knockout cells [37] as well as in ATF6α-knockout cells [33].

What is the significance of CiC activation induced by ER stress? Induction of the *de novo* lipogenesis has been observed in ER stressed cells, depending on the activation of UPR branch IRE1/XBP1, even though the molecular mechanisms are not fully clarified [38]. XBP1 plays an important role in the ER membrane phospholipid synthesis which allows the ER biogenesis and expansion under ER stress conditions [39]. It is noteworthy that the chronic activation of the UPR branch IRE1/XBP1 can determine dysregulation of lipogenic genes expression and lipid accumulation in adipose tissue and liver [40-42]. Thus, the activation of CiC by XBP1 is consistent with the involvement of XBP1 in the control of the *de novo* lipogenesis in ER stressed cells. Conversely, the role of ATF6α in the regulation of CiC is less clear. While XBP1 induces lipogenic genes expression, ATF6α, which shares DNA binding site with XBP1, plays a protective role against hepatic lipid accumulation [43]. Our data showed that overexpression of ATF6α (373) triggers both CiC expression and CiC promoter activity. However, ATF6α heterodimerizes with XBP1 [33], and the ATF6α-XBP1 heterodimer binds to the UPRE with an affinity higher than XBP1 homodimer [33]. In the light of these observations, induction of CiC expression in cells upon ER stress might be involved in the ER biogenesis and in the lipid accumulation in the cells. Moreover, the induction of CiC expression, mediated by ER stress, is under the control of XBP1 and ATF6α UPR branches.

The ER stress-induced transport of citrate, and thus of acetyl-CoA, by CiC can support cellular processes other than lipid synthesis. Previous findings showed that several ER-resident proteins are acetylated at the ε-amino group of lysine residues [11], and the status of acetylation of ER-resident proteins increases upon UPR induction [15]. By proteomic approach it has been shown that N^{ε}-lysine acetylated ER-resident proteins are mostly chaperones and enzymes involved in post-translational

modification and folding, including the "master" effector of the UPR pathway GRP78 [15]. The source of acetyl group for the N^{ε}-lysine acetylation is represented by acetyl-CoA, which enters into the ER lumen through the ER membrane transporter SLC33A1/AT1 [14,13]. In agreement with previous results [15] the acetylation status of ER proteins increased in tunicamycin-treated HepG2 cells (Supplementary Fig. 1). However, this increment did not occur in *Cic*-knockdown cells but, in these cells, induction of the acetylation status of ER proteins by tunicamycin was rescued upon overexpression of rat *Cic* (Supplementary Fig. 1, left panel) or addition of 5 mM citrate in the medium (Supplementary Fig. 1, right panel). Recent studies have shown that induction of UPR pathway and hyperacetylation of GRP78 have been observed in cells upon inactivation of specific histone deacetylases, by their inhibitors or knockdown of the corresponding genes [12,13]. The increment of acetylated GRP78 determines the activation of ATF6α [12] and PERK [13]. Thus, besides overload of unfolded proteins into the ER lumen, acetylation of GRP78 seems to be a prerequisite for the induction of UPR. On the basis of these considerations, it can be hypothesized that the activation of CiC expression, upon ER stress induction, provides an adequate cytosolic pool of acetyl-CoA necessary for the acetylation of ER resident chaperones and enzymes involved in post-translational modification and folding [15]. It is noteworthy that the induction of CiC expression in the ER stress response is consistent with the increase of influx of acetyl-CoA into the ER lumen by up-regulation of the ER transporter AT-1 in ER stressed cells [15].

The importance of CiC activation during ER stress is further highlighted by recent findings which support the idea that the ER-based acetylation machinery is intimately linked to ERAD(II). ERAD(II) is a cellular process under the control of UPR, in which large unfolded or misfolded protein aggregates, accumulated in the ER, are mostly dealt with by expanding the ER and activating autophagy. The regulation of ERAD(II) involves acetylation of the ER-resident autophagy protein Atg9A [15].

Overall, the data here presented highlight the up-regulation of CiC expression in response to the ER stress. Indeed, this carrier could provide an adequate cytosolic pool of acetyl-CoA necessary for the ER homeostasis. Fig. 6 shows a graphical representation of data here reported and the proposed mechanism for the CiC activation by ER stress.

ACKNOWLEDGEMENTS

We thank prof. Kazutoshi Mori (Department of Biophysics, Kyoto University) for providing the pcDNA3-ATF6 (373) and pcDNA3-XBP1s and prof. Bruno Di Jeso (Department of Biological and Environmental and Sciences and Technologies, University of Salento) for providing the antibody against GRP78.

FUNDING

The research discussed in the present study was supported by the Ministero dell'Università e Ricerca (MIUR).

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Figure Legends

Fig. 1. Effect of ER stress on CiC expression in cultured cells.

(A) The histograms represent CiC mRNA abundance determined using RT-qPCR and expressed as relative amounts (18S rRNA as a reference) in HepG2 or BRL-3A cells, treated with 1μg/ml tunicamycin for the indicated times or in untreated human or rat cells (controls). Values are means ± S.D. of triplicate samples from each of four independent experiments. (B) HepG2 or BRL-3A cells were incubated in DMEM without (control) or with 1μg/ml tunicamycin for the indicated times. Cells were then harvested for preparation of total protein extract. Proteins (50 μg) were separated by SDS/PAGE and immunoblotted with antibody against CiC. The content of CiC in stressed cells was analysed by Western blotting, quantified by densitometric analysis and expressed as the fold change relative to CiC content in control cells. Values are means ± S.D. Statistical analysis was carried out within each experimental group, marked by different letters (a, b, etc.) or by different letters with the same superscript (a', b', etc.). Within the same group, samples bearing different letters differ significantly ($P < 0.05$, $n = 4$).

Fig. 2. Activation of the CiC promoter activity by tunicamycin-induced ER stress.

(A) HepG2 and BRL-3A cells were transiently co-transfected with phCiC819 and prCiC1484, respectively, together with pGL4.73 control plasmid. After transfection, cells were incubated with 1 μg tunicamycin for the indicated times. Normalized luciferase activity was expressed as percentage of value obtained in the control cells (time 0). Values are means ± S.D. Within the same group, samples bearing different letters differ significantly ($P < 0.05$, $n = 6$).

(B) HepG2 and BRL-3A cells were transiently co-transfected with the CiC promoter-luciferase constructs or the empty pGL3basic vector shown in the figure, together with *Renilla* luciferase reference plasmid pGL4.73. After 12 h with 1 μg tunicamycin, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity and to protein concentration. For each construct, the induction of promoter activity by ER stressor was expressed as fold change relative to the untreated cells. Values are means ± S.D. Groups bearing different letters differ significantly ($P < 0.05$, $n = 4$).

Fig. 3. Identification of putative Unfolded Protein Responsive Element (UPRE) in the promoter region of the rat and human *Cic* gene.

(A) The ER stress responsive region of about 120 pb from human CiC promoter was aligned with the corresponding region from rat CiC promoter. Four sites for Sp1 transcription factor and the UPRE are boxed.

(B) Comparison of the human CiC UPRE (*H.s.* CiC) with the corresponding sequence from rat (*R.n.* CiC) and mouse (*M.m.* CiC) CiC promoter and with UPRE consensus. Underlined is the nucleotide in the UPRE that differs from the consensus sequence.

Fig. 4. Effect of overexpression or knockdown of XBP1 and ATF6α on CiC expression in HepG2.

(A) The histograms represent CiC mRNA abundance determined using RT-qPCR and expressed as relative amounts (18S rRNA as a reference) in HepG2 cells transfected with pcDNA-HA-XBP1s or with pcDNA-HA-ATF6α (373). Values were expressed as percentage of the mRNA abundance in control cells transfected with the empty vector pcDNA3. Values are means ± S.D. Within the same group, samples bearing different letters differ significantly ($P < 0.05$, $n = 4$).

(B) HepG2 cells were transfected with pcDNA-HA-XBP1s or with pcDNA-HA-ATF6α (373). After 24 h, cells were lysed and CiC protein content was determined by Western blotting. CiC protein content was normalized with respect to β-actin used as a control. The normalized level of CiC protein in cells transfected with pcDNA3 empty vector was set to 1. The blot shown in the figure is from a representative experiment and similar results were obtained in four independent experiments. Values are means ± S.D. Within the same group, samples bearing different letters differ significantly ($P < 0.05$, $n = 4$).

(C) HepG2 cells were transfected with pSUPshATF6α, or pSUPshXBP1 containing the sequence of ATF6α and XBP1 RNA target or pSuperior vector containing the respective scrambled sequence. After 48 h transfection, cells were treated with 1 μg/ml tunicamycin for 12 h. CiC mRNA abundance was determined as previously described and values were expressed as percentage of the mRNA abundance in untreated cells transfected with the scrambled shRNA (control). Within the same group, samples bearing different letters differ significantly ($P < 0.05$, $n = 4$).

(D) HepG2 cells were transfected as described in Fig. 4C. Total protein extracts (50 μg) were fractionated by 10% SDS-PAGE and immunodecorated with the antibody against CiC. β-actin was used as a standard control. Nuclear protein extracts (50 μg) were fractionated as above and immunodecorated with the antibody against ATF6α or XBP1. Lamin B was used as a standard control. The blots shown in the figure are from a representative experiment and similar results were obtained in three independent experiments. The content of CiC protein was reported in histograms as

described in Fig. 1B and values are means ± S.D. Within the same group, samples bearing different letters differ significantly ($P < 0.05$, $n = 4$).

Fig. 5. Effect of overexpression or knockdown of XBP1 and ATF6α on CiC promoter activity

(A) HepG2 cells were transiently co-transfected with the 500 ng CiC promoter-luciferase constructs together with 20 ng *Renilla* luciferase reference plasmid pGL4.73, and with increasing amounts of pcDNA3-HA-XBP1s or pcDNA3-HA-ATF6α (373) as indicated. Cells were harvested after 24 h and the luciferase activity was normalized to *Renilla* luciferase activity. Results are expressed as fold induction of the luciferase activity by XBP1s or ATF6α (373), with respect to control, transfected with empty pcDNA3 vector. Values are means ± S.D. Within the same experiment, groups bearing different letters differ significantly ($P < 0.05$, $n = 4$).

(B) HepG2 were transiently co-transfected with one of the CiC promoter-luciferase constructs or the empty pGL3basic vector, together with pSUPshATF6α, or pSUPshXBP1, or pSuperior vector containing the ATF6α or XBP1 scrambled sequence, as shown in the figure. After 12 h incubation with 1 μg tunicamycin, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity and to protein concentration. For each construct, promoter activity was expressed as fold change relative to untreated cells transfected with scramble shRNA. Values are means ± S.D. Within the same experiment, groups bearing different letters differ significantly ($P < 0.05$, $n = 4$).

(C) In the upper panel, 500 ng pGL3Basic vector, phCiC144, or the mutant phCiC144(UPREm), in which the UPRE has been mutated, were transfected into HepG2 cells together with 50 ng pcDNA-HA-ATF6α (373), pcDNA-HA-XBP1s or with 50 ng empty pcDNA3vector. In the lower panel, HepG2 cells were transfected with 500 ng pGL3Prom, or with 500 ng UPREhCiC3X or UPREhCiCm3X, the last two constructs containing three copies of the wild type CiC UPRE or of the corresponding mutant, respectively, in the presence or in the absence of 50 ng pcDNA-HA-ATF6α (373) or pcDNA-HA-XBP1s. Firefly luciferase activity measurements and statistical analysis were carried out as described above ($P < 0.05$, $n = 4$). Values are means ± S.D.

(D) ChIP assay of the CiC promoter was carried out using HepG2 cells treated with 1 μg/ml tunicamycin for 12 h or untreated cells used as a control. Chromatin fragments immunoprecipitated with anti-XBP1 or anti-ATF6α antibodies were amplified by RT-qPCR with primers spanning the UPR region of the CiC promoter. Samples incubated with non-specific preimmune IgGs or no antibodies were used as negative controls.

Fig. 6. Proposed mechanism for the CiC activation by ER stress

In ER-stressed cells, accumulation of misfolded or unfolded proteins leads to the activation of ER transmembrane proteins PERK (not shown), IRE1 and ATF6α. Upon ER stress, the ER chaperone GRP78, which is normally bound to these ER stress sensors, dissociates from them in order to assist

with the folding of proteins in the ER lumen. Activated IRE1 catalyzes the unconventional splicing of XBP1 mRNA to give the XBP1s mRNA, encoding for a transcription factor involved in the UPR pathway. Activation of precursor ATF6α (pATF6α) leads to its translocation to the Golgi where the active N-terminal fragment (nATF6α) is generated upon proteolysis. The active nATF6α translocates to the nucleus inducing the expression of UPR genes. Among UPR genes activated by nATF6α and XBP1s is GRP78, which is required for the folding of proteins in the ER lumen. nATF6α and XBP1s activate CiC expression as well, through their binding to CiC promoter. Finally, the enhanced expression of CiC supports the increased demand of acetyl-CoA which can be directed to the lipogenesis and to ER-resident proteins acetylation, both activated in ER stressed-cells.

Supplementary Fig. 1. Effect of *Cic* gene knockdown on the acetylation status of ER resident proteins

A) In the left panel, HepG2 cells were co-transfected with pSUPshCiC or pSuperior vector containing the sequence of CiC RNA target or the scrambled sequence, respectively, together with the plasmid containing the rat CiC cDNA (pcDNA-rCiC) or with empty pcDNA3 vector for 48 h. After transfection, cells were treated with 1 μg/ml tunicamycin for 12 h. ER proteins (50 μg) were fractionated by 10% SDS-PAGE and the acetylated proteins were immunodecorated with the antibody against acetyl lysine. In the right panel, HepG2 cells were co-transfected with pSUPshCiC or pSuperior vector containing the sequence of CiC RNA target or the scrambled sequence, respectively, for 48 h. After transfection, cells were treated with 1 μg/ml tunicamycin and with 5mM citrate for 12 h. The level of acetylated proteins was analyzed as described above. Results are from a representative experiment and similar results were obtained in four independent experiments.

B) In the left panel, the fractionation of intracellular membranes was carried out by ultracentrifugation on a 10–24% discontinuous Nycodenz gradient. 30 μl from each fraction was fractionated by SDS-PAGE and probed by Western blotting with antibodies against GRP78 (ER), S2P (Golgi apparatus) and EEA1 (endosomes). The ER is found in fractions 18-24. In the right panel, 30 μg of ER-enriched proteins (ER) and of total membrane proteins (input) was fractionated and probed as described above, resulting in a good enrichment yield of ER membranes.

Figure 1

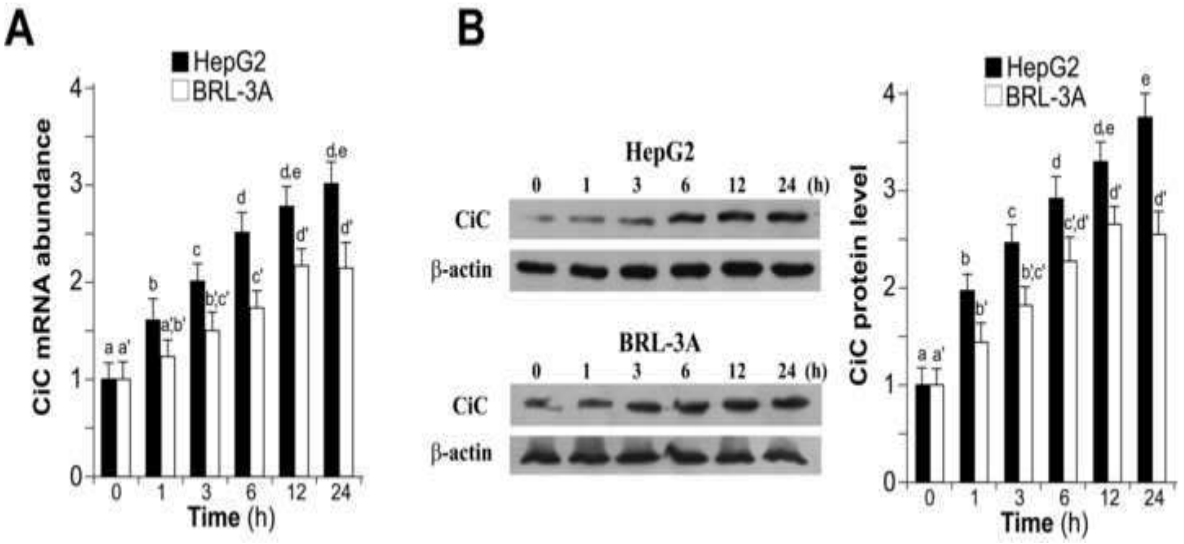
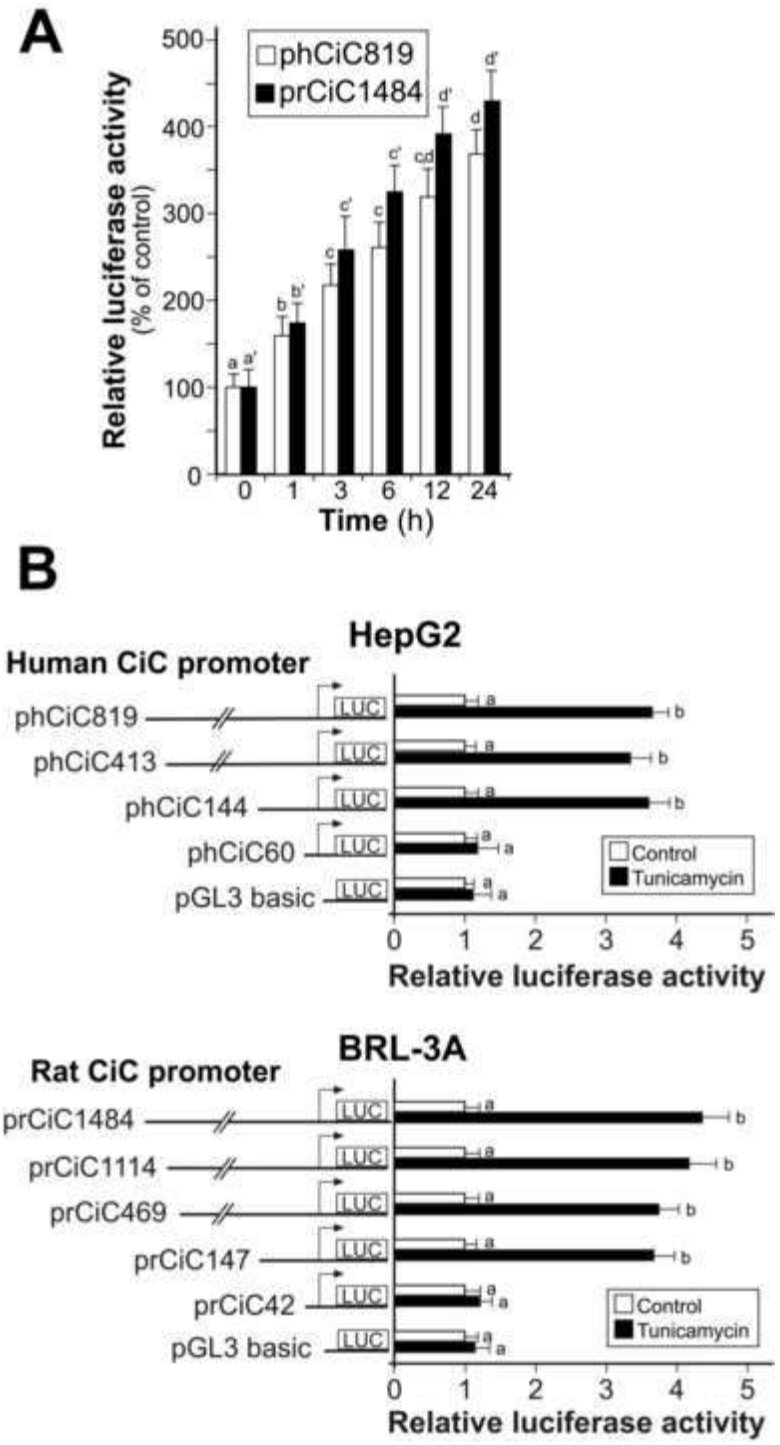

A
HepG2
BRL-3A
CiC mRNA abundance
Time (h)
B
HepG2
0 1 3 6 12 24 (h)
CiC
β-actin
BRL-3A
0 1 3 6 12 24 (h)
CiC
β-actin
HepG2
BRL-3A
CiC protein level
Time (h)

Figure 2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phCiC819
prCiC1484
Relative luciferase activity
(% of control)
500
400
300
200
100
0
0 1 3 6 12 24
Time (h)
B
HepG2
Human CiC promoter
phCiC819
phCiC413
phCiC144
phCiC60
pGL3 basic
LUC
Control
Tunicamycin
0 1 2 3 4 5
Relative luciferase activity
BRL-3A
Rat CiC promoter
prCiC1484
prCiC1114
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LUC
Control
Tunicamycin
0 1 2 3 4 5
Relative luciferase activity

Figure 3

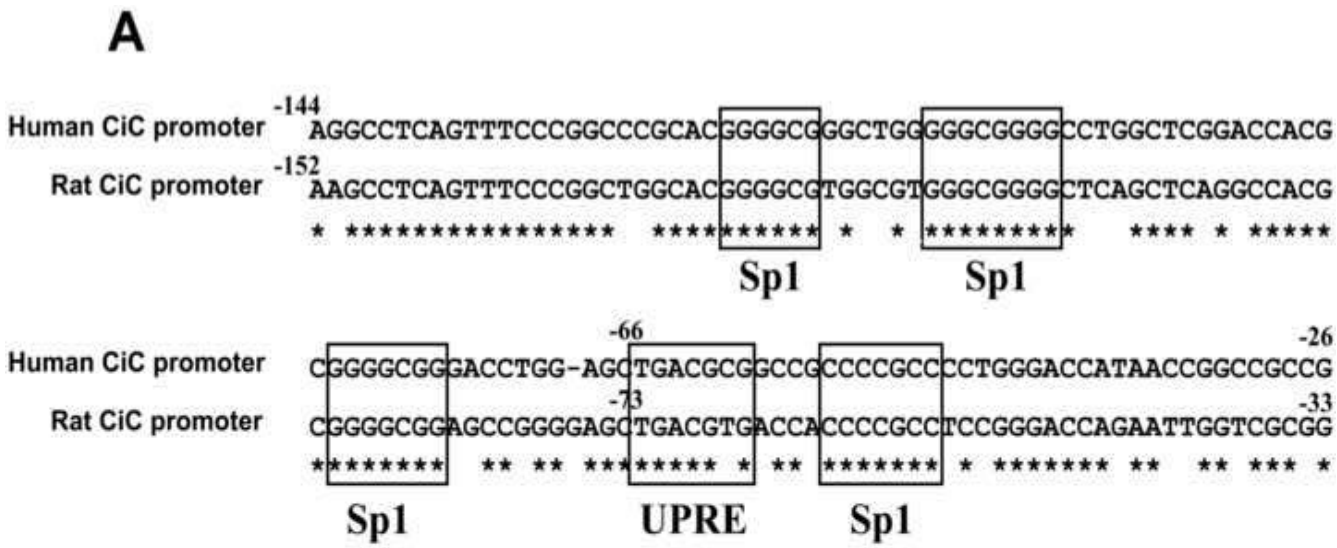

A
Human CiC promoter -144 AGGCCTCAGTTTCCCGGCCCGCACGGGGCGGGCTGGGGGCGGGGCCTGGCTCGGACCACG
Rat CiC promoter -152 AAGCCTCAGTTTCCCGGCTGGCACGGGGCGTGGCGTGGGCGGGGCTCAGCTCAGGCCACG
Sp1
Sp1
-66
-26
Human CiC promoter CGGGGCGGGACCTGG-AGCTGACGCGGCCGCCCCGCCCCTGGGACCATAACCGGCCGCCG
-73
-33
Rat CiC promoter CGGGGCGGAGCCGGGGAGCTGACGTGACCACCCCGCCTCCGGGACCAGAATTGGTCGCGG
Sp1
UPRE
Sp1
B
UPRE
H.s. TGACGCGG
R.n. TGACGTGA
M.m. TGACGTGA
Consensus TGACGTGG/A

Figure 4

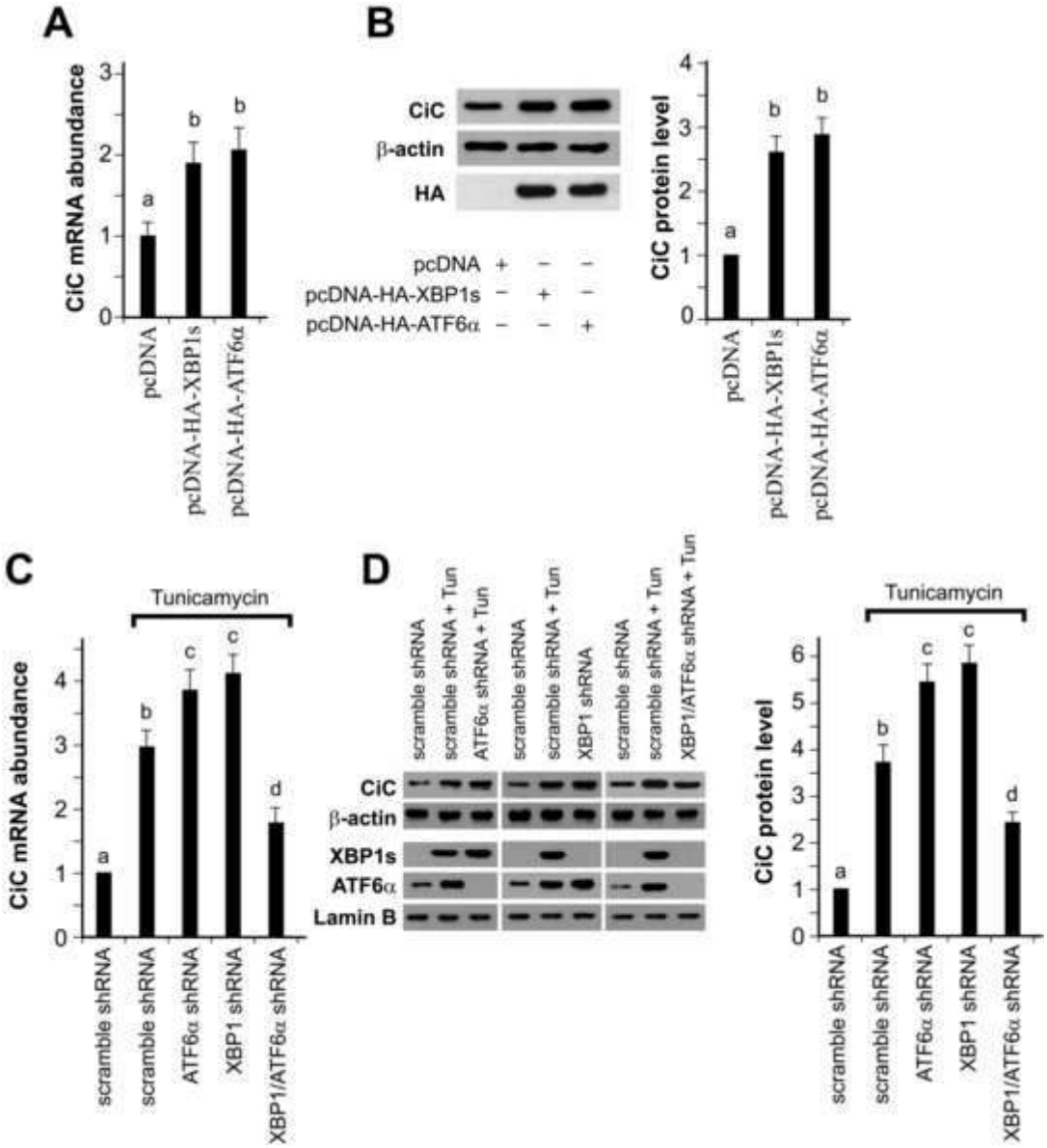

A
CiC mRNA abundance
pcDNA
pcDNA-HA-XBP1s
pcDNA-HA-ATF6α
B
CiC
β-actin
HA
pcDNA + − −
pcDNA-HA-XBP1s − + −
pcDNA-HA-ATF6α − − +
CiC protein level
C
Tunicamycin
CiC mRNA abundance
scramble shRNA
ATF6α shRNA
XBP1 shRNA
XBP1/ATF6α shRNA
D
scramble shRNA + Tun
ATF6α shRNA + Tun
XBP1 shRNA
XBP1/ATF6α shRNA + Tun
XBP1s
ATF6α
Lamin B
CiC protein level

Figure 5

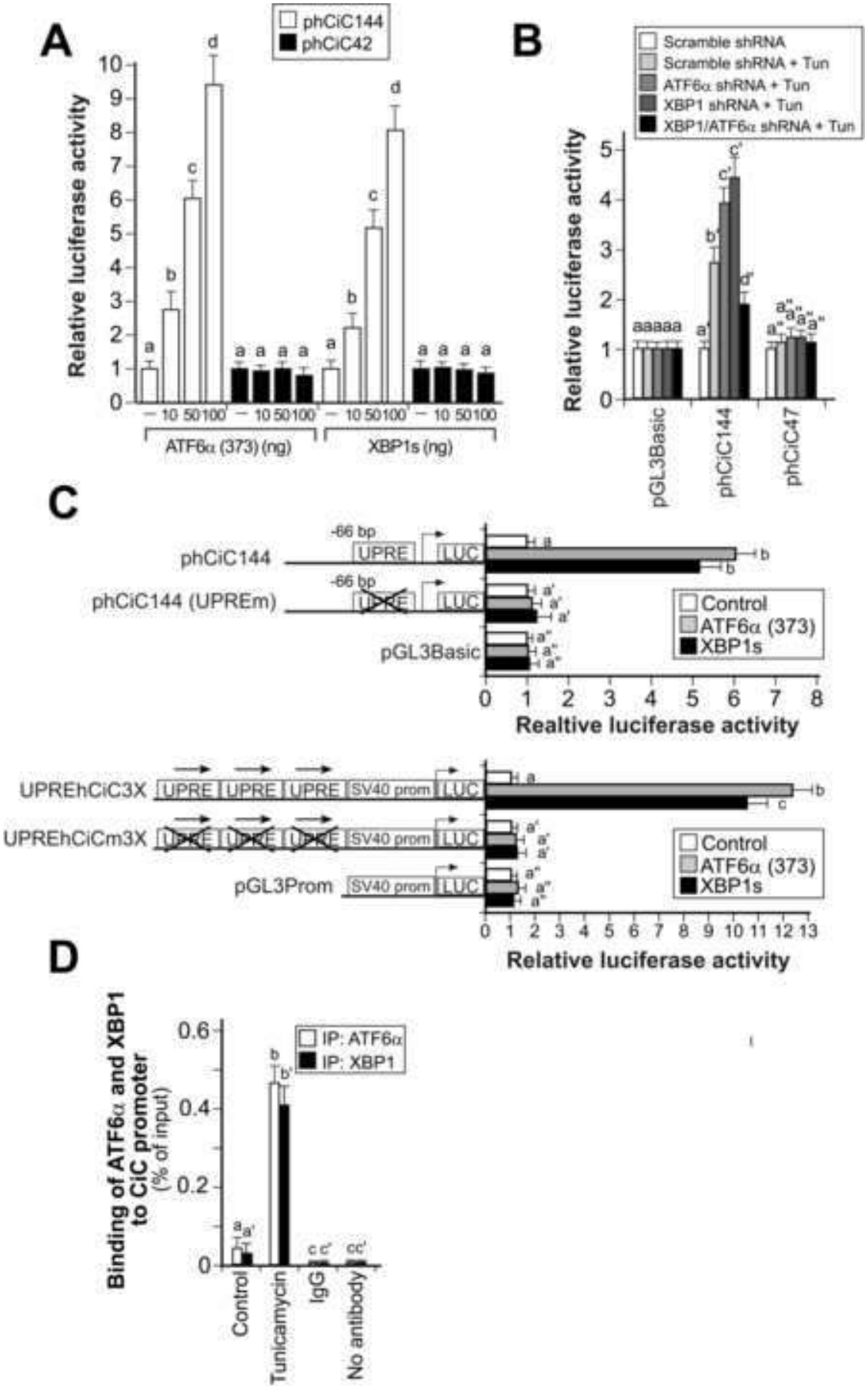
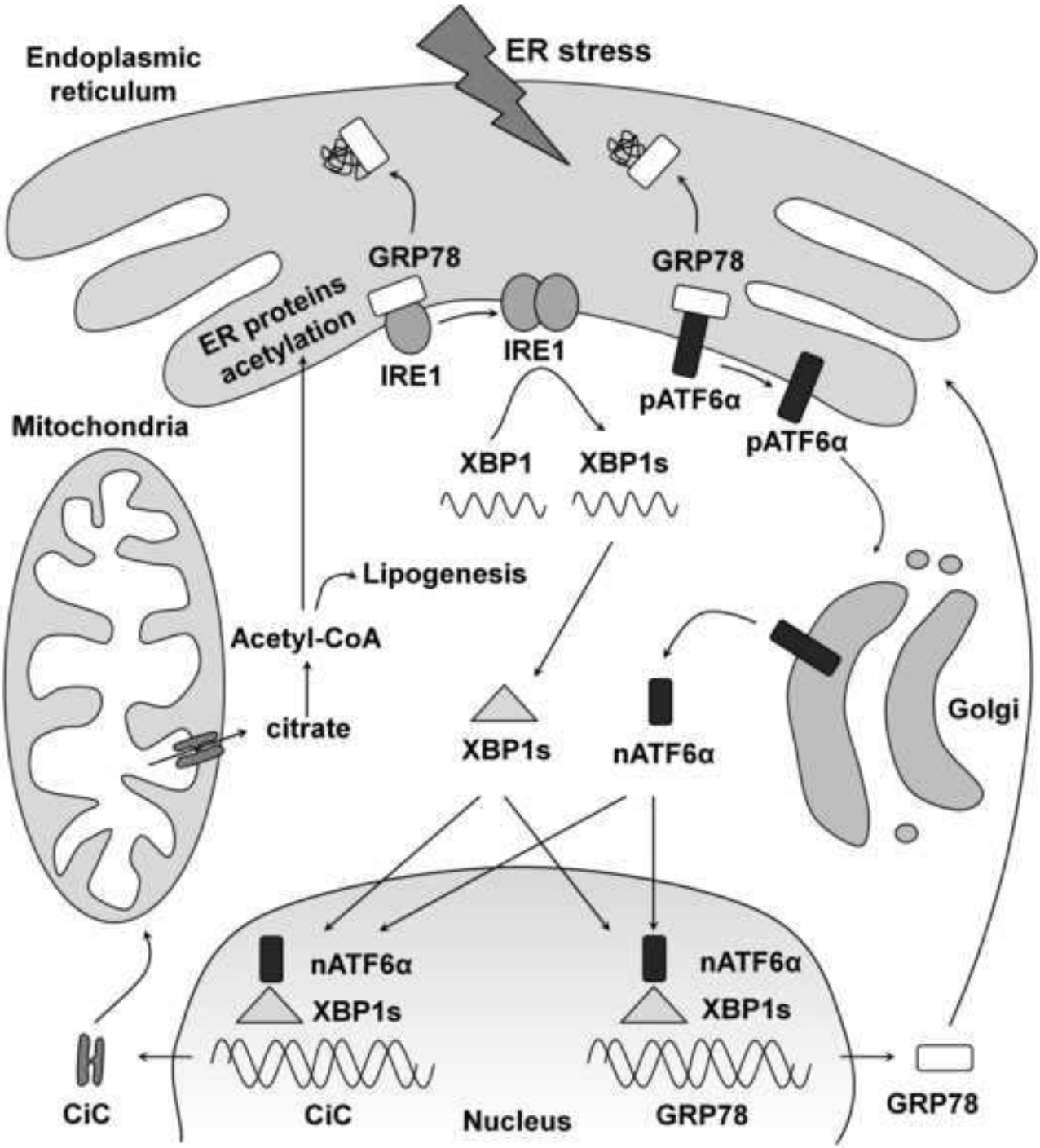

A
phCiC144
phCiC42
Relative luciferase activity
ATF6α (373) (ng)
XBP1s (ng)
B
Scramble shRNA
Scramble shRNA + Tun
ATF6α shRNA + Tun
XBP1 shRNA + Tun
XBP1/ATF6α shRNA + Tun
Relative luciferase activity
pGL3Basic
phCiC144
phCiC47
C
-66 bp
UPRE
LUC
phCiC144
phCiC144 (UPREm)
pGL3Basic
Control
ATF6α (373)
XBP1s
Realtive luciferase activity
UPREhCiC3X
UPREhCiCm3X
pGL3Prom
SV40 prom
Relative luciferase activity
D
Binding of ATF6α and XBP1 to CiC promoter (% of input)
IP: ATF6α
IP: XBP1
Control
Tunicamycin
IgG
No antibody

Figure 6


Endoplasmic reticulum
ER stress
GRP78
GRP78
ER proteins acetylation
IRE1
IRE1
pATF6α
pATF6α
Mitochondria
XBP1
XBP1s
Lipogenesis
Acetyl-CoA
citrate
XBP1s
nATF6α
Golgi
nATF6α
XBP1s
nATF6α
XBP1s
CiC
CiC
Nucleus
GRP78
GRP78

Supplementary Figure 1