Biochemistry and Biophysics Reports 24 (2020) 100834

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Characterization of the 5′-flanking region of the human and mouse CHAC1 genes

Yuki Nomura [a,1], Charity F. Sylvester [b,1], Lisa O. Nguyen [b], Mahmoud Kandeel [c,d], Yoko Hirata [a,e], Imran N. Mungrue [b,**], Kentaro Oh-hashi [a,e,*]

[a] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan
[b] Department of Pharmacology and Experimental Therapeutics, LSU Health Sciences Center, 1901, Perdido St, New Orleans, LA, USA
[c] Department of Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, King Faisal University, Hofuf, Alahsa, 31982, Saudi Arabia
[d] Department of Pharmacology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt
[e] Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan

ARTICLE INFO

Keywords:
ATF4
ATF/CRE
CHAC1
DDIT3

ABSTRACT

The **U**nfolded **P**rotein **R**esponse pathway is a conserved signaling mechanism having important roles in cellular physiology and is perturbed accompanying disease. We previously identified the novel UPR target gene CHAC1, a direct target of ATF4, downstream of PERK-EIF2A and activated by the UPR pathway. CHAC1 enzyme directs catalysis of γ-linked glutamate bonds within specific molecular targets. CHAC1 is the first enzyme characterized that can catalyze intracellular glutathione degradation in eukaryotes, having implications for regulation of oxidative stress. DDIT3 (CHOP) is a terminal UPR transcription factor, regulated by ATF4 and an output promoting cell death signaling. Herein we examine the relationship of CHOP controlling CHAC1 transcription in humans and mice. We note parallel induction of CHOP and CHAC1 in human cells after agonist induced UPR. Expanding upon previous reports, we define transcriptional induction of CHAC1 in humans and mice driven by ATF4 through a synergistic relationship with conserved ATF/CRE and CARE DNA sequences of the CHAC1 promoter. Using this system, we also tested effects of CHOP on CHAC1 transcription, and binding at the CHAC1 ATF/CRE using IM-EMSA. These data indicate a novel inhibitory effect of CHOP on CHAC1 transcription, which was ablated in the absence of the ATF/CRE control element. While direct binding of ATF4 to CHAC1 promoter sequences was confirmed, binding of CHOP to the CHAC1 ATF/CRE was not evident at baseline or after UPR induction. These data reveal CHAC1 as a novel CHOP inhibited target gene, acting through an upstream ATF/CRE motif via an indirect mechanism.

1. Introduction

The UPR signaling pathway monitors integrity of the **E**ndoplasmic **R**eticulum (ER) compartment [1–3]. The UPR functions to maintain cellular homeostasis accompanying ER-stress, and aberrations in UPR signaling are implicated in a widening set of disease states [4,5]. Since errant UPR signaling might have a causal contribution to common disease, modulation of this pathway is an active area of study and represents an attractive target for therapeutics [4,6–8].

Proximal molecular sensors: IRE1, ATF6 and PERK reside on the ER-membrane and transmit specific activation signals to induce the UPR pathway [7]. After sensing stress within the ER, these molecules generate active transcription factors, which initiate a robust transcriptional response [8,9]. Induction of downstream molecular targets correct initiating ER deficits, or alternatively signal cell death if the stimulus cannot be corrected [10,11]. Although proximal molecular mechanisms of UPR control have been delineated, downstream biological effects after UPR activation remain poorly defined. Identification of novel UPR end-effector genes, like CHAC1, will have positive impacts on understanding the pathway revealing novel molecular functions and regulation. In addition, growing evidence defines UPR cross-talk leading to complex context-dependent connections to other important cellular

* Corresponding author. Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan.
** Corresponding author.
E-mail addresses: imungr@lsuhsc.edu (I.N. Mungrue), oohashi@gifu-u.ac.jp (K. Oh-hashi).
[1] Yuki Nomura and Charity F. Sylvester equally contributed to this study.

https://doi.org/10.1016/j.bbrep.2020.100834
Received 12 May 2020; Accepted 9 October 2020
Available online 17 October 2020

signaling pathways: oxidative stress, autophagy, apoptosis, amino acid transport, cell death, cholesterol transport, mTOR and ferroptosis [12–18].

DDIT3 (aka. CHOP, GADD153, CHOP10) was first identified as a DNA-damage induced transcript that promotes cell death [19,20]. Subsequently, UPR-activation of CHOP was defined, linking UPR inputs to cell death outputs under select conditions [11,21,22]. Additionally, CHOP promotes oxidative stress [23,24]. Roles for CHOP in homeostasis and disease have been identified in cellular and whole-animal mouse models. CHOP–KO mice were produced with expected frequencies, and female CHOP–KO mice were obese compared to controls [25]. While the absence of a dramatic baseline phenotype for CHOP–KO mice suggested a dispensable function, subsequent studies revealed critical roles for CHOP in cardiovascular disease [26].

CHOP–KO mice had reduced atherosclerosis querying in two mouse models: high fat diet fed ApoE-KO or LDLr-KO strains [27]. Roles for CHOP in mediating cholesterol induced macrophage cell death [28], or alternatively modulation of arterial smooth muscle proliferation [29] provides mechanisms for these phenotypes. CHOP and UPR are induced in myocardium of failing human heart tissue and in mice after aortic constriction induced heart failure [30]. In a mouse model of heart failure, CHOP–KO mice revealed a protective effect preserving cardiac structure and function [31]. These data imply that CHOP inhibition could have benefit for treatments that block initiation of cardiovascular disease and cardiac cell dysfunction accompanying heart failure.

We previously queried a cellular atherosclerosis model: human primary aortic endothelial cells activated by oxidized LDL derivatives. Systems genetics predictions based on its central location within a UPR enriched mRNA co-expression network [32], identified CHAC1 as a putative UPR induced pro-death signaling gene [33,34]. CHAC1 was subsequently confirmed as a UPR induced gene [35]. Using *in vitro* biological model systems, we also examined the transcriptional regulation of CHAC1 in humans and mice. We delineated CHAC1 transcriptional induction by the UPR pathway, specifically mediated by actions of the transcription factors ATF3, CHOP, C/EBPβ and ATF4 [36,37]. Additionally, CHAC1 is controlled by post-translational regulation, including the proteasome and lysine independent ubiquitination [38]. The study herein further expands on the transcriptional regulation for CHAC1 in humans and mice using promoter-reporter and DNA binding assays. We identify a novel synergistic relationship between upstream ATF/CRE and CARE elements of the CHAC1 promoter in regulation of transcription in humans and mice. ATF4 directs CHAC1 induction via these ATF/CRE and CARE DNA control elements. Moreover, we define an inhibitory effect for the transcription factor CHOP, on CHAC1 mRNA transcription. Although the inhibitory effect of CHOP on CHAC1 transcription is mediated via a core ATF/CRE DNA control element, no binding of CHOP to ATF/CRE sequences of the CHAC1 promoter was identified. These data reveal a novel cell death signaling input within the UPR, whereby CHOP paradoxically inhibits the transcription of CHAC1. Previous reports reveal actions for CHOP as a transcription factor decoy inhibiting downstream target gene expression. The inhibitory effect of CHOP on CHAC1 transcription does not appear dependent on direct DNA binding, but might be mediated through effects on ATF4 and C/EBPβ at the ATF/CRE element of the CHAC1 promoter.

2. Materials and methods

2.1. Materials

Thapsigargin (Tg), Tunicamycin (Tm) and Brefeldin A (BFA) were obtained from Sigma-Aldrich. Antibodies against ATF4 (sc-200), CHOP (sc-793), C/EBPβ (sc-150) were purchased from Santa Cruz Biotechnology and actin (CP01) was purchased from Calbiochem.

2.2. Construction of plasmids

For generating of the human CHAC1 promoter reporter construct, genomic DNA from HEK293 cells was extracted, and the human CHAC1 promoter (−265/+77) was amplified using polymerase chain reaction (PCR). The PCR product was then cloned into the pGL3-Basic (pGL3b) vector (Promega). Other constructs containing either deleted or mutated human CHAC1 promoters were also prepared by PCR and sequence specific mutagenesis. The promoter region was defined using the NCBI Reference Sequence file NM_024111, and our prior reports [36,39]. For generating mouse CHOP, cDNA was amplified from C/57BL6 mouse by reverse transcriptional-polymerase chain reaction (RT-PCR) and then cloned into the pcDNA3.1 vector (Life Technologies). Reporter constructs for the mouse CHAC1 promoter and the ATF4 expression vector were previously described [37].

2.3. Cell culture and treatment

The human embryonic kidney cell line, HEK293, was maintained in Dulbecco's modified eagle's minimum essential medium (DMEM) containing 5% fetal bovine serum. The transfection of each plasmid DNA vector was performed using polyethylenimine "Max" (Polysciences, U.S. A.), in accordance with the manufacturer's instructions. For ER-stress stimulations, HEK293 cells were treated with Tg (0.1 μM), Tm (2 μg/ml), BFA (BFA) (5 μg/ml) or serum starvation using serum free media (SF) for the indicated time.

2.4. RT-PCR

To estimate the mRNA expression level of each gene, we used RT-PCR. Total RNA was extracted from cells with Trizol (Life Technologies) [37]. After incubation of total RNA for 10 min at 72 °C, cDNA was generated by reverse transcription per the manufacturer's instructions using the prime superscript III RNase-free reverse transcriptase (Life Technologies, 18080–051). In detail, total RNA (0.5 μg/10 μl), DTT (10 mM), dNTP (0.5 mM), random nucleotide nonamers (0.05 μg/10 μl), RNaseOUT (2 U/10 μl) (Life Technologies) and prime superscript III RNase-free reverse transcriptase (RT) (25 U/10 μl) (Life Technologies) were incubated for 60 min at 42 °C. To quantify specific transcripts, cDNAs were mixed and amplified using a PCR reaction mixture containing the indicated primer pair (1 μM), in addition to dNTP (0.1 mM), rTaq (0.25 U/10 μl) (Taq PCR kit, Takara). The following RT-PCR primers were used in this study:

GRP78 sense primer 5′-ATGAAAGAAACCGCTGAGGCTT-3′, GRP78 antisense primer 5′-ATGTTCTTCTCCCCCTCCCTCT-3′, C/EBPβ sense primer 5′-ACTTTGGCACTGGGGCACTT-3′, C/EBPβ antisense primer 5′-GCAACAAGCCCGTAGGAACA-3′, CHOP sense primer 5′-GATGAAAATGGGGGCACCTA-3′, CHOP antisense primer 5′-TGTTTCCGTTTCCTAGTTCT-3′, CHAC1 sense primer, 5′-CATAGGGGCAGCGACAAGATG-3′, CHAC1 antisense primer 5′-CTGTGTGGCAATGACCTCTTC-3′, G3PDH sense primer, 5′-ACCACAGTCCATGCCATCAC-3′ and G3PDH antisense primer, 5′-TCCACCACCCTGTTGCTGTA-3′.

Thermal cycling reaction conditions were 30 s at 96 °C, 30 s at 58 °C and 30 s at 72 °C. The results represent 20–35 cycles of amplification. The cDNA amplification products were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide.

2.5. Reporter assay

Each plasmid reporter construct (0.05 μg) was transfected into HEK293 cells in a 48-well plates. For all examinations, the constitutive pGL4.70 renilla-luciferase (Rluc) (0.02 μg) expression vector (Promega) was included in transfections, to serve as a normalization control for luciferase assays. Twenty-four hours after transfection, cells were treated with Tm (2 μg/ml) or vehicle for 24 h. To determine the effect of

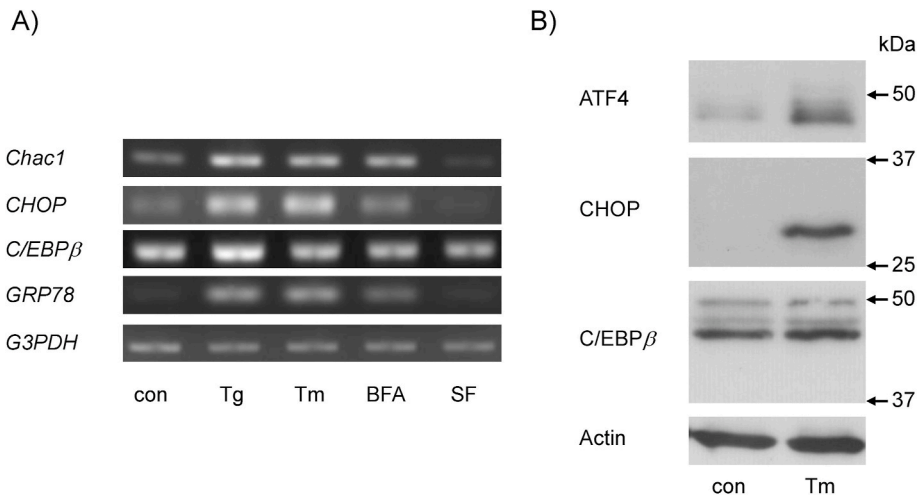

Fig. 1. CHAC1 is a UPR induced gene. Examinations of A) mRNA expressions of CHAC1, CHOP, C/EBPβ, GRP78 and control G3PDH in HEK293 cell extracts after treatments with UPR agonists. Treatments: Thapsigargin (Tg), Tunicamycin (Tm), Brefeldin A (BFA) or serum starvation (SF). B) Western blots to define protein expression of ATF4, CHOP and C/EBPβ or Acitin (loading control) in HEK293 cells after activation of the UPR using Tunicamycin treatment (Tm), versus control treated.

ATF4 or CHOP overexpression on the reporter activity, plasmids coding ATF4, CHOP or empty vector (mock) (0.05 μg), were co-transfected with the CHAC1 promoter-reporter constructs into HEK293 cells. Cells were then cultured for 48 h to allow ATF4 and CHOP induction. For quantitative promoter-reporter assays, we scored luciferase activities accompanying stated conditions. Cells were lysed with 1x passive lysis buffer (Promega) for 15 min at room temperature and briefly centrifuged. Luciferase activity in each lysate was measured using the Dual-Luciferase assay system (Promega). The luciferase reporter activity in each lysate was normalized to the co-transfected Renilla luciferase activity, dividing the raw luciferase by renilla-luciferase values. In all figures, we report relative luciferase activity, which is the renilla-normalized luciferase values.

2.6. Western blot analysis

Cells were lysed with homogenate buffer (20 mM Tris–HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% TritonX-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) [37]. After the protein concentration was determined, using the Bradford assay, each cell lysate was diluted into sodium dodecyl sulfate (SDS)-Laemmli sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS and 10% glycerol), and denatured by hearting at 100 °C for 3 min. Equal amounts of cell lysate were separated on 10 or 12.5% SDS–polyacrylamide electrophoresis gels, immunoblotted onto polyvinylidene difluoride (PVDF) membrane (Merck Millipore) and specific signals identified using ECL Detection System (GE Healthcare Bioscience) or Western Blotting Substrate Plus (Thermo Fisher Scientific). We used the following concentrations of antibodies ATF4 (1: 1000), CHOP (1: 1000), C/EBPβ (1: 1000) and actin (1: 5000).

2.7. IM-EMSA

We queried binding of transcription factors ATF4 and CHOP to elements of the CHAC1 promoter using the Immunoblot-Electrophoretic Mobility Shift Assay (IM-EMSA) method, as previously described [36]. HEK293 cells were grown in 100 mm dishes and harvested in 1 ml lysis buffer: 10 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1% Igepal-CA630 and protease inhibitors (Sigma P8340). Nuclei were pelleted and the nuclear extracts collected in 20 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 25% glycerol, 420 mM $NaCl_2$, 0.4 mM EDTA, 1 mM DTT and protease inhibitors. Nuclear extracts were incubated at 4 °C for 30 min in binding buffer (20 mM Hepes pH 8.0, 50 mM KCl, 0.1 mM EDTA, 1 mM $MgCl_2$, poly dIdC 0.1 mg/ml, and 0.1% NP40) with selected unlabeled oligonucleotides. Samples were resolved on 6% DNA-retardation gels (Invitrogen EC6365) in 0.5x TBE running buffer (Invitrogen) and transferred to 0.5x TBE Hybond N+ (GE Life Sciences RPM203B) at 4 °C. Next, the membranes were dried, UV-crosslinked, and blocked for 30 min in 5% Milk/1x TBS-T. The blots were incubated in blocking buffer containing the indicated antibodies for 1 h, washed three times in TBS-T and then incubated with secondary antibody diluted in blocking buffer. The blots were washed and developed using ECL-prime and ECL-film (GE Life Sciences). We used the following antibodies from Santa Cruz biotech, ATF4 (sc-200X) and CHOP (sc-575X).

2.8. Statistical analysis

All results are expressed as mean ± SD of the indicated number. Statistical analysis was carried out using one way-ANOVA followed by Tukey's post-test, where appropriate. $*p < 0.01$, $**p < 0.001$ are presented as statistically significant.

3. Results and discussion

We previously identified CHAC1 was identified as a pro-apoptotic factor using a systems genetic screen [33,35,40]. CHAC1 is induced after treatment with the pro-atherosclerotic oxidized phospholipid: oxidized 1-palmitoyl-2-arachidonyl-*sn*-3-glycero-phosphorylcholine (ox-PAPC) in primary aortic endothelial cells (HAEC) from a human population [40]. Subsequently, we delineated ER stress regulation of CHAC1 mRNA [35,36]. Human CHAC1 transcription is activated by ATF4, and acts at 5' flanking regions of the CHAC1 gene [36]. In this manuscript, we further elucidate salient aspects of CHAC1 regulation in humans and mice. We characterize effects of UPR and ATF4 on CHAC1 expression using pharmacology and reporter plasmids *in vitro*. Querying action of UPR and ATF4 transcription factor, we note a synergistic action through upstream ATF/CRE and CARE elements of the CHAC1 promoter. Since DDIT3 (CHOP) is a well-characterized pro-apoptotic ATF4 target gene, also activated by UPR, we examined the relationship between CHOP and CHAC1 in humans and mice.

We first examined the induction of UPR mRNAs and proteins after chemical agonist stimulation of the UPR using Thapsigargin, Tunicamycin and Brefeldin A, versus control or serum starvation. Consistent with other reports, CHAC1 mRNA was robustly induced after stimuli triggered ER stress in HEK293 cells, but not serum starvation or compared to control. UPR-agonist induction of CHAC1 mRNA occurred in parallel with other canonical ER stress inducible genes, GRP78 and CHOP (Fig. 1A). We confirmed that treatment with Tunicamycin induced robust ATF4 and CHOP protein expression in HEK293 cells (Fig. 1B), while the induction of C/EBPβ was negligible.

Nucleotide sequences of human and mouse CHAC1 promoters (Fig. 2A) are well conserved implying commonality in regulation.

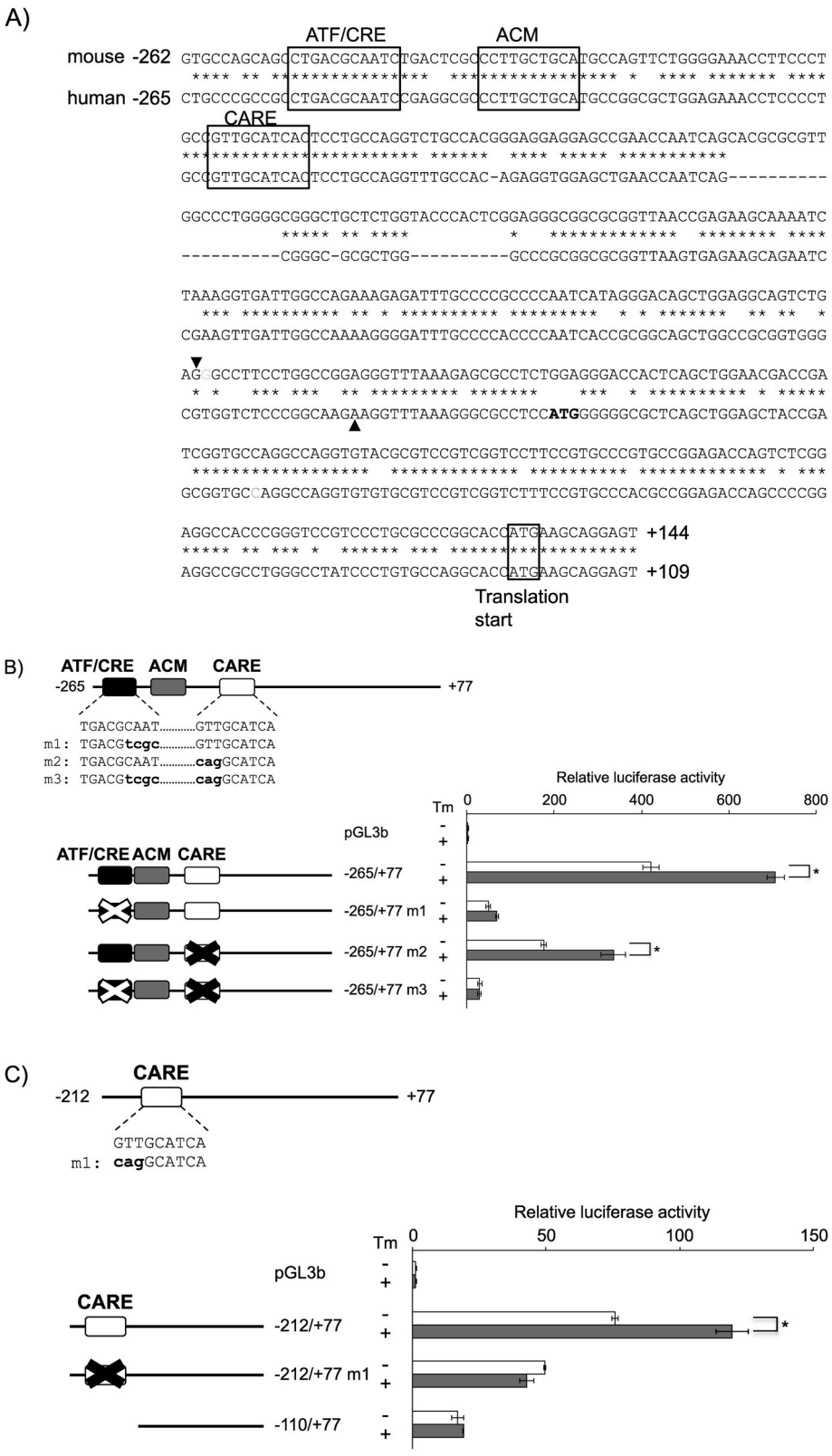

Fig. 2. A) Bioinformatics analysis of the mouse (top) and human (bottom) CHAC1 promoter sequences. Nucleotide sequences conserved between the 5′-flanking region of the mouse and human Chac1 genes are indicated with *asterisks*. Locations of critical cis DNA elements are highlighted by boxes, including the ATF/CRE, ACM, and CARE. Translation start: ATG codon is also highlighted. Putative transcriptional start sites of the mouse and human Chac1 gene are shown with arrows. **B) The UPR agonist Tunicamycin activates CHAC1 expression, owing to actions at cis ATF/CRE and CARE DNA elements**. Indicated derivatives of the CHAC1 promoter were linked to a luciferase reporter system for quantitative determination of transcriptional induction after agonist stimulation with Tunicamycin (Tm). Site directed mutagenesis was used to scramble important elements of the CHAC1 promoter, m1: ATF/CRE, m2: CARE, or m3: ATF/CRE + CARE. **C) The CARE element within the human CHAC1 promoter participates in UPR induction of CHAC1.** The conserved cis CARE element of the CHAC1 promoter was used for transcriptional assays, using the dual-luciferase system for quantification, in the absence of upstream ATF/CRE and ACM sequences. Constructs containing the CHAC1 promoter CARE, versus a scrambled mutant, or downstream deleted mutant, were examined in their ability to induce CHAC1 transcriptional activity, in control or after Tunicamycin treatment. Values represent the mean ± SD from three independent cultures and are expressed relative to the basal activity of the pGL3-Basic vector. Data were analyzed by one way-ANOVA followed by Tukey's post test. The values marked with an asterisk highlight a statistical difference between the groups compared ($p < 0.01$).

Upstream ATF/CRE, ACM and CARE DNA elements are identical, and are similarly located in humans and mice relative to transcription start. Of note, we previously defined the translation start codon for the human CHAC1 gene [36], which was distinct from prior database annotations. This start codon sequence is exactly conserved in mice (Fig. 2A). To compare transcriptional regulation of CHAC1 in humans and mice, we constructed several CHAC1 gene reporters based on our previous studies to evaluate function in more detail. Founded upon our previous publications, we generated mutations of the a) ATF/CRE, b) CARE and c) both ATF/CRE and CARE; and compared activities in humans and mice CHAC1 promoters. These constructs were used for *in vitro* evaluation of luciferase activities in the presence or absence of the chemical UPR

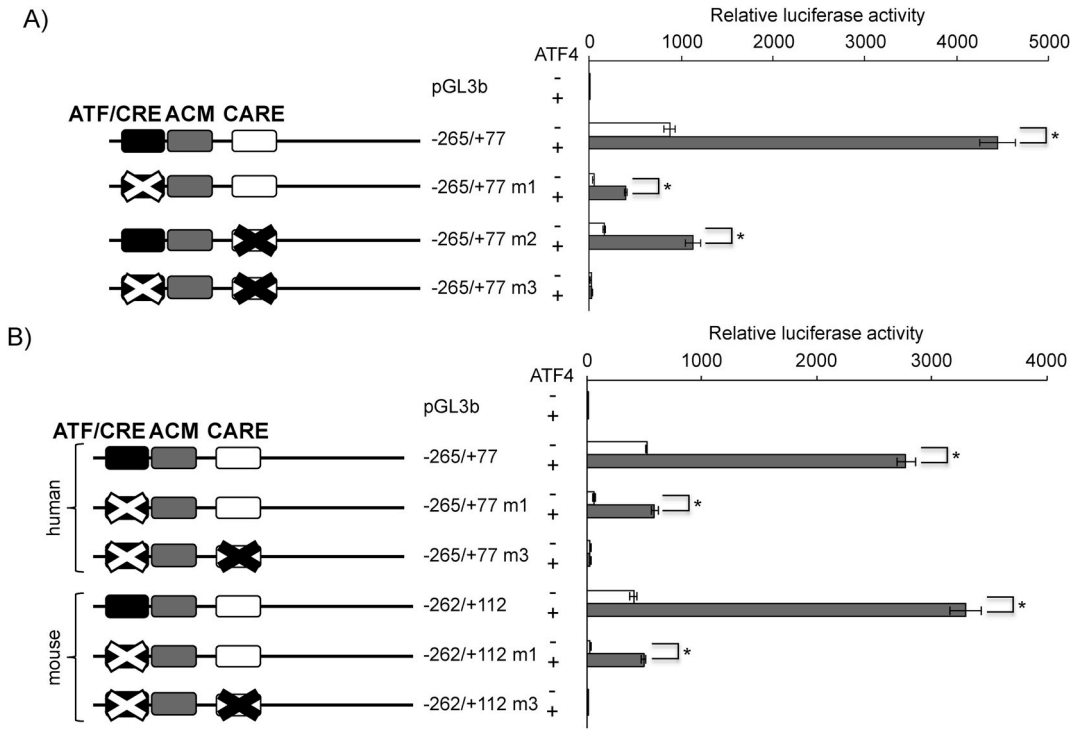

Fig. 3. A) ATF4 activates the CHAC1 promoter acting synergistically at upstream ATF/CRE and CARE elements. Indicated derivatives of the CHAC1 promoter were linked to a luciferase reporter system for quantitative determination of transcriptional induction accompanying co-transfection of a mature ATF4 expressing plasmid or control. Comparison of ATF4 effects on CHAC1 transcription are also examined after mutations of the m1: ATF/CRE, m2: CARE, or m3: ATF/CRE + CARE. **B) Synergistic actions of ATF4 on CHAC1 promoter ATF/CRE and CARE in mice and humans.** Indicated derivatives of the CHAC1 promoter were linked to a luciferase reporter system for quantitative determination of transcriptional induction accompanying co-transfection of a mature ATF4 expressing plasmid or control. Comparison of ATF4 effects on CHAC1 transcription accompanying mutation of the m1: ATF/CRE, or m3: ATF/CRE + CARE. Values represent the mean ± SD from three independent cultures and are expressed relative to the basal activity of the pGL3-Basic vector. Data were analyzed by one way-ANOVA followed by Tukey's post test. The values marked with an asterisk highlight a statistical difference between the groups compared ($p < 0.01$).

agonist Tunicamycin. The intact CHAC1 promoter containing both the ATF/CRE and CARE elements (−265/+77) had high basal activity and was robustly induced after Tunicamycin-treatment, compared to the promoter-less control (Fig. 2B). On the other hand, mutations in either the ATF/CRE or CARE reduced basal promoter activity. Notably, the ATF/CRE-CARE double mutant completely ablated basal promoter activity and abolished responsiveness to the UPR agonist Tunicamycin. These inhibitory effects of the ATF/CRE and CARE mutations were noted despite maintaining the ACM, revealing minimal impact for the ACM regulating CHAC1 under these conditions. Comparing roles in regulating human CHAC1 promoter, mutation of the ATF/CRE had a more profound effect, whereas mutation of the CARE had a lessor impact on transcriptional activity. Since the double ATF/CRE-CARE mutant lost all responsiveness to chemical UPR activation, whereas the UPR agonist Tunicamycin activated transcription in the presence of eithers single mutant, synergistic action through these sites is revealed. To refine the role of the CHAC1 CARE in UPR responsiveness, we constructed shorter reporters lacking upstream ATF/CRE and ACM elements. The CARE alone could activate CHAC1 transcription (Fig. 2C) after UPR agonist treatment in HEK293 cells, whereas mutation of the CARE in this abbreviated context ablated Tunicamycin induction of CHAC1. Whereas the ATF/CRE is the primary driver of basal expression, deletion of both ATF/CRE and CARE is necessary to completely abolish UPR activation of CHAC1 after Tunicamycin treatment. Collectively, these data define a synergistic interaction between ATF/CRE and CARE elements in UPR-induction of CHAC1 after Tunicamycin treatment.

Since we previously defined roles for ATF4 in regulation of CHAC1, we next transfected a plasmid encoding mature ATF4 together with the indicated luciferase reporter constructs to query relative roles of the ATF/CRE and CARE. Consistent with previous data, ATF4 co-transfection activated CHAC1 transcription in the full-length promoter, and mutation of either the ATF/CRE or CARE reduced this effect (Fig. 3A). Interestingly, in the double ATF/CRE-CARE mutant no remaining effect of ATF4 co-transfection was evident. Considering previous differences between human and mice CHAC1 promoters, we tested whether human and mouse CHAC1 reporters behaved similarly after deleting both the ATF/CRE and CARE elements. CHAC1 transcriptional activation was parallel in the human and mice contexts, and the ATF/CRE-CARE double mutant ablated ATF4 responsiveness in both species (Fig. 3B). These results and previous studies on CHAC1 promoter, indicate functional commonality for the ATF/CRE and CARE within the 5' flanking region of human and mouse CHAC1 genes. As previously demonstrated using mouse CHAC1 promoters, the CARE is recognized by ATF4, but its contribution to basal and ER stress-induced promoter activities was less than the ATF/CRE. In the ATF/CRE-CARE dual mutant, a synergistic function is revealed, identifying dual direct actions for ATF4. In contrast, other regions of the promoter, including the ACM, are dispensable for controlling CHAC1 activity under basal and the ATF4 mediated, Tunicamycin-induced ER stress condition. These experiments precisely define the cis regulatory elements that control CHAC1 transcription in humans and mice. These data form the basis for definition of commonality between human and mouse CHAC1 regulation, and future studies examining surrogate mice models to study human physiology and disease.

We next queried effects of CHOP on transcriptional activity of CHAC1 using promoter-reporter constructs (Fig. 4A), comparing basal activity versus co-transfection with a CHOP over-expression plasmid. In contrast to CHAC1 stimulation by ATF4, CHOP significantly reduced CHAC1 promoter activity in the full-length context. Using mutants of the CHAC1 a) ATF/CRE, b) CARE or c) ATF/CRE-CARE we next dissected

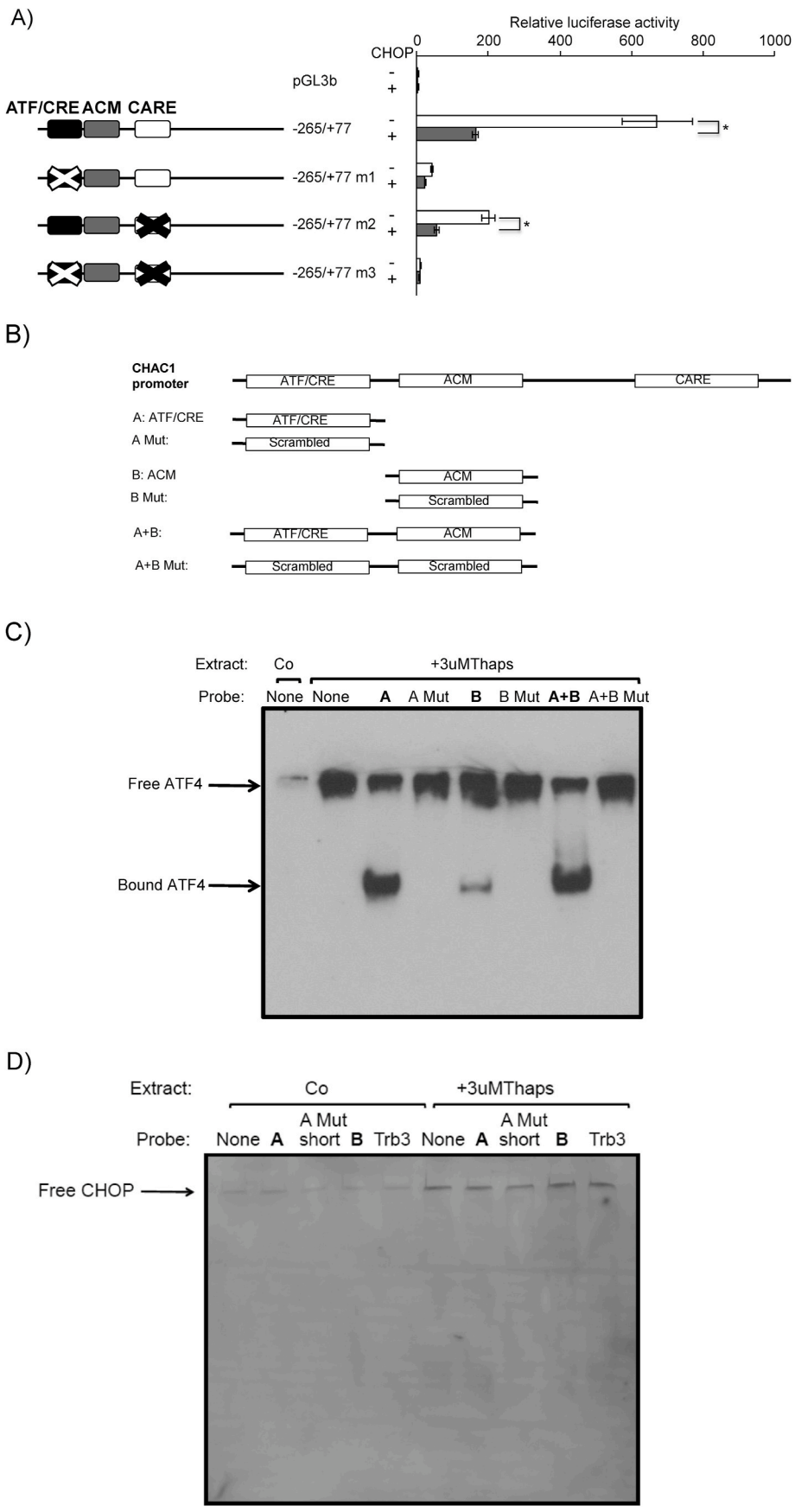

Fig. 4. A) CHOP co-transfection inhibits expression of CHAC1, via the cis ATF/CRE element. Indicated derivatives of the CHAC1 promoter were linked to a luciferase reporter system for quantitative determination of transcriptional induction accompanying co-transfection of a mature CHOP expressing plasmid or control. Comparison of CHOP effects on CHAC1 transcription accompanying mutation of the m1: ATF/CRE, m2: CARE, or m3: ATF/CRE + CARE are examined. Values represent the mean ± SD from three independent cultures and are expressed relative to the basal activity of the pGL3-Basic vector. Data were analyzed by one way-ANOVA followed by Tukey's post test. The values marked with an asterisk highlight a statistical difference between the groups compared ($p < 0.01$). B**) Schematic of important cis elements of the CHAC1 promoter,** and makeup of probes used for IM-EMSA to probe transcription factor binding to DNA sequences. **C) ATF4 binds to the CHAC1 ATF/CRE after induction of the UPR**. Examinations of ATF4 protein expression in native nuclear extracts after control (co) or induction of the UPR with 3 uM Thapsigargin for 24 h, and binding to probes corresponding to cis elements of the CHAC1 promoter. The respective probe added to each lane is listed at the top of the well. Slow mobility unbound ATF4 is shown at the top of the blot, and the presence of an increased mobility DNA-bound ATF4 is shown with an arrow. **D) CHOP does not bind to the CHAC1 ATF/CRE, at baseline or after induction of the UPR.** IM-EMSA examinations of CHOP protein expression in native nuclear extracts after control (co) or induction of the UPR with 3 uM Thapsigargin for 24 h, and binding to probes corresponding to cis elements of the CHAC1 promoter. The respective probe added to each lane is listed at the top of the well. A control CARE element from the TRIB3 gene (Trb3) is also examined. Slow mobility of free CHOP is shown at the top of the blot, and no indication of any increased mobility DNA-bound CHOP was detected in this assay.

relative contributions of these elements on upstream inhibitory action by CHOP. The negative effect of CHOP was abrogated by mutation of the ATF/CRE within the CHAC1 promoter, revealing action through this site. In contrast, querying the CARE mutant revealed that the negative effect of CHOP on CHAC1 transcription remained. Collectively, these data reveal CHAC1 as a novel target of the UPR activated transcription factor CHOP, via action at the CHAC1-ATF/CRE. Importantly, CHOP is a negative regulator of CHAC1, and CHAC1 might be an important downstream target of CHOP.

We next examined direct binding of ATF4 and CHOP to promoter elements using the IM-EMSA strategy (Fig. 4BCD). This method assays transcription factor binding to synthesized oligonucleotides, using antibody based detection run on a native polyacrylamide gel [36]. Consistent with our previous work, we identified a strong signal using antibodies to detect the transcription factor ATF4, which was increased after UPR activation. We also noted an ATF4 shift having increased mobility, in the presence of sequences corresponding to the CHAC1 ATF/CRE, versus a weak signal using oligonucleotides containing the CHAC1 ACM (Fig. 4BC). This result supports a central role for ATF4 in the regulation of CHAC1, through binding ATF/CRE sequences, and that the ACM plays a minor role in regulation of CHAC1 transcription after ER stress. These data contrast with results obtained querying actions of CHOP at the CHAC1 ATF/CRE, using IM-EMSA. In the absence of any oligonucleotide sequence, we noted native CHOP was detected by our specific antibody, and was increased after UPR activation. After the addition of DNA oligonucleotides corresponding the CHAC1 ATF/CRE, we did not detect any specific shift in mobility of the CHOP band (Fig. 4BD). These data agree with the body of functional CHOP literature, with scant evidence of actions through direct DNA binding.

While our data do not provide a direct explanation for the effect of CHOP on regulation of CHAC1 promoter activity, our evidence demonstrates requirement for the ATF/CRE DNA element of the CHAC1 promoter. Since CHOP does not directly bind to this component of the CHAC1 promoter, we posit that CHOP might act via formation of a complex with another transcription factor, with ATF4 being a likely candidate [41]. Previous reports have defined numerous transcription factors such as ATF3, ATF5 and C/EBP family that are also regulated by ER stress, and might be targets modulated by CHOP. While CHOP was originally postulated to function as a transcriptional decoy, forming inhibitory complexes with other bZIP transcription factors, subsequent work identified direct action of CHOP at specific DNA elements, for example in activating the expression of TRIB3, as well as other genes [42,43]. Our results do not exclude action of CHOP that results in the binding of alternate regions of DNA, and indirect CHAC1 regulation via other candidate intermediaries.

The sequelae of functional effects for CHAC1 induction are governed by the enzymatic activity of the CHAC1 protein. CHAC1 generates an enzyme of ~26 kDa that directly cleaves bonds between γ-carboxyl linkages of glutamate. The γ-carboxyl linkage of glutamate is a non-standard peptidyl like linkage, seen for example in the tripeptide glutathione. CHAC1 cleavage of the γ-linked glutamate in glutathione: γ-Glu-Cys-Gly, is the first example of an enzyme in the cell that can mediate glutathione destruction within the cell. The action of CHAC1 on glutathione might define an important role for CHAC1 in regulation of oxidative stress, and provide a mechanistic molecular link bridging the UPR. More broadly, catalysis of intracellular glutathione suggests that ER stress inputs affect oxidative stress, with importance for the onset and progression of various diseases, including neurodegenerative diseases, ischemia and inflammation. Both CHOP and CHAC1 activate cell death signaling when over-expressed. Importantly, since CHOP inhibits CHAC1 transcription, these data suggest that the cell death signals mediated by these two genes act through distinct pathways.

Independently, a γ-glutamate #1669 linkage of the Notch receptor to a glycine residue has been previously reported as another substrate of CHAC1 enzyme [44,45]. Removal of this glycine linked to the γ-carboxyl bond of glutamate #1669, by the CHAC1 enzyme blocked maturation of the notch receptor at the earliest maturation step prior to furin cleavage in the Golgi. These data identify roles for CHAC1 in the regulation of notch signaling. Further studies examining roles for CHAC1 *in vitro* and *in vivo* could be informative to understand novel effects of the Notch pathway and regulation of downstream pathophysiology.

4. Conclusions

In this manuscript we expand upon functional molecular definitions that explain regulation of UPR induction directing CHAC1 in humans and mice. We note that regulation of CHAC1 is controlled by two cis DNA elements that have synergistic action, the ATF/CRE and CARE. These ATF/CRE and CARE transcriptional control elements are exactly conserved between mice and humans, and function similarly querying *in vitro* dual luciferase reporter assays, after Tunicamycin induction of the UPR. After deletion of both ATF/CRE and CARE elements, Tunicamycin induction of CHAC1 transcription was ablated. These data enhance important aspects of transcriptional control for CHAC1 in humans and mice.

Overexpression of the mature transcription factor ATF4 lead to robust induction of CHAC1 transcription, in parallel to effects of the UPR agonist Tunicamycin noted above. ATF4 actions on new CHAC1 transcription is dependent on the ATF/CRE and CARE elements within the CHAC1 promoter. Deletion of both the ATF/CRE and CARE within the CHAC1 promoter rendered the remaining reporter unresponsive to ATF4 co-transfection. These data define synergistic roles for ATF4 acting at the ATF/CRE and CARE elements of the CHAC1 promoter, that reflect downstream sequelae of UPR induction.

In contrary to the actions of ATF4 and UPR on CHAC1 induction, we noted that CHOP overexpression on a plasmid did not activate CHAC1. Instead, the putative UPR induced transcription factor, CHOP, inhibited expression of the CHAC1 promoter-reporter system. Using a set of deletion constructs we noted that the effects of CHOP were abrogated after deletion of the CHAC1 ATF/CRE element, but deletion of the CARE revealed it was dispensable. Using IM-EMSA, our recently established method for querying sequence specific DNA binding of transcription factors, we confirmed ATF4 binding at the CHAC1 ATF/CRE. We did not detect any parallel binding of CHOP to the CHAC1 ATF/CRE, using IM-EMSA. These data identify CHAC1 as a novel target gene that is inhibited by CHOP, after induction of the UPR, likely dependent of the relative amounts of ATF4, C/EBPβ and CHOP. Since CHAC1 and CHOP are both UPR induced genes, and promote cell-death when over-expressed, this inhibitory relationship was not expected. These data might reveal CHAC1 as a novel target gene of the UPR transcription factor CHOP, or a parallel UPR output that promotes cell death.

Declaration of competing interest

There was no conflict of interest in this study.

Acknowledgements

INM was funded by grants from the LSU Research and Technology Foundation- LIFT15A3 NHLBI- HL094709 and AHA- 16IRG27330013. KO was funded by Grant-in-aid from the Japan Society for the Promotion of Science (JSPS, Japan, KAKENHI, 19H04030) and a grant from the OGAWA Science and Technology Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100834.

Author contributions

YN, INM and KO discussed and designed the research; YN, CFS and

LON performed experiments; KO, MK, INM and YH wrote the manuscript.

References

[1] M. Schroder, R.J. Kaufman, The mammalian unfolded protein response, Annu. Rev. Biochem. 74 (2005) 739–789.
[2] C.M. Federovitch, D. Ron, R.Y. Hampton, The dynamic ER: experimental approaches and current questions, Curr. Opin. Cell Biol. 17 (2005) 409–414.
[3] J. Hwang, L. Qi, Quality control in the endoplasmic reticulum: crosstalk between ERAD and UPR pathways, Trends Biochem. Sci. 43 (2018) 593–605.
[4] M. Wang, R.J. Kaufman, Protein misfolding in the endoplasmic reticulum as a conduit to human disease, Nature 529 (2016) 326–335.
[5] S.J. Marciniak, D. Ron, Endoplasmic reticulum stress signaling in disease, Physiol. Rev. 86 (2006) 1133–1149.
[6] M. Wang, R.J. Kaufman, The impact of the endoplasmic reticulum protein-folding environment on cancer development, Nat. Rev. Canc. 14 (2014) 581–597.
[7] S. Wang, R.J. Kaufman, The impact of the unfolded protein response on human disease, J. Cell Biol. 197 (2012) 857–867.
[8] R.J. Kaufman, Orchestrating the unfolded protein response in health and disease, J. Clin. Invest. 110 (2002) 1389–1398.
[9] S.N. Manie, J. Lebeau, E. Chevet, Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 3. Orchestrating the unfolded protein response in oncogenesis: an update, Am. J. Physiol. Cell Physiol. 307 (2014) C901–907.
[10] M. Brown, N. Strudwick, M. Suwara, L.K. Sutcliffe, A.D. Mihai, A.A. Ali, J. N. Watson, M. Schroder, An initial phase of JNK activation inhibits cell death early in the endoplasmic reticulum stress response, J. Cell Sci. 129 (2016) 2317–2328.
[11] A. Verkhratsky, O.H. Petersen, The endoplasmic reticulum as an integrating signalling organelle: from neuronal signalling to neuronal death, Eur. J. Pharmacol. 447 (2002) 141–154.
[12] K.M. Glover-Cutter, S. Lin, T.K. Blackwell, Integration of the unfolded protein and oxidative stress responses through SKN-1/Nrf, PLoS Genet. 9 (2013), e1003701.
[13] J. Han, B. Song, J. Kim, V.K. Kodali, A. Pottekat, M. Wang, J. Hassler, S. Wang, S. Pennathur, S.H. Back, M.G. Katze, R.J. Kaufman, Antioxidants complement the requirement for protein chaperone function to maintain beta-cell function and glucose homeostasis, Diabetes 64 (2015) 2892–2904.
[14] S.J. Dixon, D.N. Patel, M. Welsch, R. Skouta, E.D. Lee, M. Hayano, A.G. Thomas, C. E. Gleason, N.P. Tatonetti, B.S. Slusher, B.R. Stockwell, Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis, Elife 3 (2014), e02523.
[15] C. Appenzeller-Herzog, M.N. Hall, Bidirectional crosstalk between endoplasmic reticulum stress and mTOR signaling, Trends Cell Biol. 22 (2012) 274–282.
[16] J. Han, R.J. Kaufman, The role of ER stress in lipid metabolism and lipotoxicity, J. Lipid Res. 57 (2016) 1329–1338.
[17] J. Grootjans, A. Kaser, R.J. Kaufman, R.S. Blumberg, The unfolded protein response in immunity and inflammation, Nat. Rev. Immunol. 16 (2016) 469–484.
[18] R. Kim, M. Emi, K. Tanabe, S. Murakami, Role of the unfolded protein response in cell death, Apoptosis 11 (2006) 5–13.
[19] A.J. Fornace Jr., I. Alamo Jr., M.C. Hollander, DNA damage-inducible transcripts in mammalian cells, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 8800–8804.
[20] M.A. Papathanasiou, N.C. Kerr, J.H. Robbins, O.W. McBride, I. Alamo Jr., S. F. Barrett, I.D. Hickson, A.J. Fornace Jr., Induction by ionizing radiation of the gadd45 gene in cultured human cells: lack of mediation by protein kinase C, Mol. Cell Biol. 11 (1991) 1009–1016.
[21] X.Z. Wang, B. Lawson, J.W. Brewer, H. Zinszner, A. Sanjay, L.J. Mi, R. Boorstein, G. Kreibich, L.M. Hendershot, D. Ron, Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153), Mol. Cell Biol. 16 (1996) 4273–4280.
[22] H. Zinszner, M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J. L. Stevens, D. Ron, CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum, Genes Dev. 12 (1998) 982–995.
[23] J. Han, S.H. Back, J. Hur, Y.H. Lin, R. Gildersleeve, J. Shan, C.L. Yuan, D. Krokowski, S. Wang, M. Hatzoglou, M.S. Kilberg, M.A. Sartor, R.J. Kaufman, ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death, Nat. Cell Biol. 15 (2013) 481–490.
[24] S.J. Marciniak, C.Y. Yun, S. Oyadomari, I. Novoa, Y. Zhang, R. Jungreis, K. Nagata, H.P. Harding, D. Ron, CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum, Genes Dev. 18 (2004) 3066–3077.
[25] Y. Ariyama, H. Shimizu, T. Satoh, T. Tsuchiya, S. Okada, S. Oyadomari, M. Mori, M. Mori, Chop-deficient mice showed increased adiposity but no glucose intolerance, Obesity 15 (2007) 1647–1656.
[26] C.M. Scull, I. Tabas, Mechanisms of ER stress-induced apoptosis in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 2792–2797.
[27] E. Thorp, G. Li, T.A. Seimon, G. Kuriakose, D. Ron, I. Tabas, Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of Apoe-/- and Ldlr-/- mice lacking CHOP, Cell Metabol. 9 (2009) 474–481.
[28] B. Feng, P.M. Yao, Y. Li, C.M. Devlin, D. Zhang, H.P. Harding, M. Sweeney, J. X. Rong, G. Kuriakose, E.A. Fisher, A.R. Marks, D. Ron, I. Tabas, The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages, Nat. Cell Biol. 5 (2003) 781–792.
[29] A.X. Zhou, X. Wang, C.S. Lin, J. Han, J. Yong, M.J. Nadolski, J. Boren, R. J. Kaufman, I. Tabas, C/EBP-Homologous protein (CHOP) in vascular smooth muscle cells regulates their proliferation in aortic explants and atherosclerotic lesions, Circ. Res. 116 (2015) 1736–1743.
[30] K. Okada, T. Minamino, Y. Tsukamoto, Y. Liao, O. Tsukamoto, S. Takashima, A. Hirata, M. Fujita, Y. Nagamachi, T. Nakatani, C. Yutani, K. Ozawa, S. Ogawa, H. Tomoike, M. Hori, M. Kitakaze, Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis, Circulation 110 (2004) 705–712.
[31] H.Y. Fu, K. Okada, Y. Liao, O. Tsukamoto, T. Isomura, M. Asai, T. Sawada, K. Okuda, Y. Asano, S. Sanada, H. Asanuma, M. Asakura, S. Takashima, I. Komuro, M. Kitakaze, T. Minamino, Ablation of C/EBP homologous protein attenuates endoplasmic reticulum-mediated apoptosis and cardiac dysfunction induced by pressure overload, Circulation 122 (2010) 361–369.
[32] C.E. Romanoski, N. Che, F. Yin, N. Mai, D. Pouldar, M. Civelek, C. Pan, S. Lee, L. Vakili, W.P. Yang, P. Kayne, I.N. Mungrue, J.A. Araujo, J.A. Berliner, A.J. Lusis, Network for activation of human endothelial cells by oxidized phospholipids: a critical role of heme oxygenase 1, Circ. Res. 109 (2011) e27–41.
[33] P.S. Gargalovic, M. Imura, B. Zhang, N.M. Gharavi, M.J. Clark, J. Pagnon, W. P. Yang, A. He, A. Truong, S. Patel, S.F. Nelson, S. Horvath, J.A. Berliner, T. G. Kirchgessner, A.J. Lusis, Identification of inflammatory gene modules based on variations of human endothelial cell responses to oxidized lipids, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 12741–12746.
[34] P.S. Gargalovic, N.M. Gharavi, M.J. Clark, J. Pagnon, W.P. Yang, A. He, A. Truong, T. Baruch-Oren, J.A. Berliner, T.G. Kirchgessner, A.J. Lusis, The unfolded protein response is an important regulator of inflammatory genes in endothelial cells, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 2490–2496.
[35] I.N. Mungrue, J. Pagnon, O. Kohannim, P.S. Gargalovic, A.J. Lusis, CHAC1/MGC4504 is a novel proapoptotic component of the unfolded protein response, downstream of the ATF4-ATF3-CHOP cascade, J. Immunol. 182 (2009) 466–476.
[36] R.R. Crawford, E.T. Prescott, C.F. Sylvester, A.N. Higdon, J. Shan, M.S. Kilberg, I. N. Mungrue, Human CHAC1 protein degrades glutathione, and mRNA induction is regulated by the transcription factors ATF4 and ATF3 and a bipartite ATF/CRE regulatory element, J. Biol. Chem. 290 (2015) 15878–15891.
[37] K. Oh-Hashi, Y. Nomura, K. Shimada, H. Koga, Y. Hirata, K. Kiuchi, Transcriptional and post-translational regulation of mouse cation transport regulator homolog 1, Mol. Cell. Biochem. 380 (2013) 97–106.
[38] Y. Nomura, Y. Hirata, K. Kiuchi, K. Oh-Hashi, Translational and post-translational regulation of mouse cation transport regulator homolog 1, Sci. Rep. 6 (2016) 28016.
[39] R.R. Crawford, A.N. Higdon, D.B. Casey, D.E. Good, I.N. Mungrue, Multiple lithium-dependent Brugada syndrome unmasking events in a bipolar patient, Clin Case Rep 3 (2015) 14–18.
[40] C.E. Romanoski, S. Lee, M.J. Kim, L. Ingram-Drake, C.L. Plaisier, R. Yordanova, C. Tilford, B. Guan, A. He, P.S. Gargalovic, T.G. Kirchgessner, J.A. Berliner, A. J. Lusis, Systems genetics analysis of gene-by-environment interactions in human cells, Am. J. Hum. Genet. 86 (2010) 399–410.
[41] N. Su, M.S. Kilberg, C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively regulates the stress-dependent induction of the asparagine synthetase gene, J. Biol. Chem. 283 (2008) 35106–35117.
[42] N. Ohoka, S. Yoshii, T. Hattori, K. Onozaki, H. Hayashi, TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death, EMBO J. 24 (2005) 1243–1255.
[43] J. Sok, X.Z. Wang, N. Batchvarova, M. Kuroda, H. Harding, D. Ron, CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI, Mol. Cell Biol. 19 (1999) 495–504.
[44] Z. Chi, S.T. Byrne, A. Dolinko, M.M. Harraz, M.S. Kim, G. Umanah, J. Zhong, R. Chen, J. Zhang, J. Xu, L. Chen, A. Pandey, T.M. Dawson, V.L. Dawson, Botch is a gamma-glutamyl cyclotransferase that deglycinates and antagonizes Notch, Cell Rep. 7 (2014) 681–688.
[45] Z. Chi, J. Zhang, A. Tokunaga, M.M. Harraz, S.T. Byrne, A. Dolinko, J. Xu, S. Blackshaw, N. Gaiano, T.M. Dawson, V.L. Dawson, Botch promotes neurogenesis by antagonizing Notch, Dev. Cell 22 (2012) 707–720.