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Ribosome Elongation Stall Directs Gene-specific Translation in the Integrated Stress Response*

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Upon exposure to environmental stress, phosphorylation of the α subunit of eIF2 (eIF2α-P) represses global protein synthesis, coincident with preferential translation of gene transcripts that mitigate stress damage or alternatively trigger apoptosis. Because there are multiple mammalian eIF2 kinases, each responding to different stress arrangements, this translational control scheme is referred to as the integrated stress response (ISR). Included among the preferentially translated mRNAs induced by eIF2α-P is that encoding the transcription factor CHOP (DDIT3/GADD153). Enhanced levels of CHOP promote cell death when ISR signaling is insufficient to restore cell homeostasis. Preferential translation of *CHOP* mRNA occurs by a mechanism involving ribosome bypass of an inhibitory upstream ORF (uORF) situated in the 5′-leader of the *CHOP* mRNA. In this study, we used biochemical and genetic approaches to define the inhibitory features of the *CHOP* uORF and the biological consequences of loss of the *CHOP* uORF on *CHOP* expression during stress. We discovered that specific sequences within the *CHOP* uORF serve to stall elongating ribosomes and prevent ribosome reinitiation at the downstream *CHOP* coding sequence. As a consequence, deletion of the *CHOP* uORF substantially increases the levels and modifies the pattern of induction of CHOP expression in the ISR. Enhanced CHOP expression leads to increased expression of key CHOP target genes, culminating in increased cell death in response to stress.

In response to a variety of environmental stresses, protein synthesis is modulated to facilitate reprogramming of gene expression to ameliorate stress damage. A key mechanism in the regulation of translation initiation is phosphorylation of serine 51 of the α subunit of eIF2 (eIF2α-P) (1). During translation initiation, eIF2 combines with initiator Met-tRNA$_i^{Met}$, GTP, and the 40S ribosomal subunit and, when associated with the eIF4 proteins on the mRNA, forms the 48S complex that facilitates start codon selection. eIF2α-P inhibits the exchange of eIF2-GDP for eIF2-GTP, thereby blocking delivery of the initiator tRNA and triggering a global reduction in translation initiation (2). Reduced protein synthesis serves to lower consumption of resources and facilitates reprogramming of gene expression to alleviate stress damage. To direct changes in gene expression, eIF2α-P also enhances the translation of a select collection of mRNAs encoding proteins that facilitate adaptation to a specific stress condition or alternatively trigger apoptosis if the stress damage is too great to be overcome. Because there are multiple mammalian eIF2 kinases that are alternatively activated by different stress arrangements, this pathway is referred to as the integrated stress response (ISR)[2] (2).

The ISR features many preferentially translated genes, including those encoding transcription factors ATF4 (CREB2) and CHOP (DDIT3/GADD153), which serve to reprogram the transcriptome to respond to cellular stress, and GADD34 (PPP1R15A), which interacts with the catalytic subunit of protein phosphatase 1 to target eIF2α-P for dephosphorylation and restore protein synthesis (3–6). Preferential translation of *ATF4*, *CHOP*, and *GADD34* ensures that expression of these short-lived proteins in the ISR are tightly linked to the degree of eIF2α-P and stress. The extent and duration of induced *CHOP* expression during stress is suggested to trigger programmed cell death when the ISR signaling is unable to restore cellular homeostasis (7–9). CHOP can induce apoptosis in response to stress by enhancing the transcription of select target genes, including BIM, a BCL2 protein family member, and ATF5, which is an additional transcriptional regulator subject to preferential translation that can promote apoptosis (10–12).

Central to the mechanisms of preferential translation in response to increased eIF2α-P are upstream ORFs (uORFs) that precede coding sequences (CDS) in the target mRNAs. For example, two uORFs in the *ATF4* mRNA confer translation control by a mechanism of "delayed translation reinitiation." In this model, a short 5′-proximal uORF1 in the *ATF4* mRNA serves as a positive-acting element that allows for translation reinitiation at subsequent ORFs in the transcript. In response to stress and eIF2α-P, the eIF2-GTP levels are reduced, delaying delivery of the initiator tRNA to reinitiating ribosomes. As a consequence, ribosomes scan past the inhibitory uORF2 start codon and instead reinitiate translation at the subsequent *ATF4* CDS start codon (3). This *ATF4* translation model thus shares features with *GCN4* translation control in yeast (13).

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[2] The abbreviations used are: ISR, integrated stress response; uORF, upstream ORF; CDS, coding sequence; MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FRT, Flp recombination target; qRT-PCR, quantitative RT-PCR.

Preferential translation of *CHOP* and *GADD34* are suggested to occur via a "bypass mechanism," in which a single inhibitory uORF is bypassed during eIF2α-P by a process involving, in part, a less than optimal start codon context (4, 5). Although uORFs are key components that promote preferential translation of ISR transcripts, the presence of an uORF alone is insufficient to render a transcript preferentially translated in response to eIF2α-P. Genome-wide analyses of changes in translation in response to eIF2α-P recently indicated that ~40% of mammalian mRNAs that contain uORFs are distributed equally among those transcripts whose translation are either enhanced, repressed, or resistant to eIF2α-P (14). This finding suggests that there are specific properties of each uORF in mRNAs that determine the mechanism by which translation initiation at the CDS is regulated and that uORFs can serve to either activate or repress downstream translation.

In this study, we addressed the nature of uORFs that serve to repress downstream translation during basal conditions and facilitate preferential translation in response to eIF2α-P. Both transcription and translation of *CHOP* is enhanced in response to eIF2α-P, and accumulation of CHOP serves as a tipping point from stress remediation to programmed cell death when stress damage is insufficiently cleared (4, 7–9). Using biochemical and genetic approaches, we show that specific sequences within the uORF in the *CHOP* mRNA serve to stall translation elongation, which culminates in lowered reinitiation of translation at the *CHOP* CDS. Deletion of the *CHOP* uORF significantly alters the dynamics of induced *CHOP* expression, which leads to increased sensitivity of cells to stress. Together, this study illuminates key features of uORFs that direct preferential mRNA translation in the ISR and the roles that these translational control mechanisms play in restoring cell homeostasis in response to environmental stresses.

Experimental Procedures

Cell Culture and Generation of Stable Cell Lines—WT mouse embryonic fibroblast (MEF) cells were cultured in DMEM as previously described (15). *CHOP*$^{-/-}$ MEF cells were provided by David Ron (University of Cambridge, Cambridge, UK) and were previously described (8). Stable Flp-In *CHOP*$^{-/-}$ cells lines were generated by using the Flp-In system (Invitrogen) and full-length *CHOP* cDNAs, including 1 kb of the *CHOP* promoter and either a WT version of the *CHOP* 5′-leader or one with mutated *CHOP* uORF initiation codons, which were integrated into the genome of the *CHOP*$^{-/-}$ cells following the manufacturer's instructions. The *CHOP* uORF has two in-frame initiation codons at codons 1 and 4, with the second ATG being the primary site for translation initiation (4). In the mutant *CHOP* uORF, both of these ATG codons were substituted to AGG, thus eliminating translation of the uORF. The resulting WT-uORF *CHOP* and ΔuORF *CHOP* MEF cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM nonessential amino acids. ER stress was induced by the addition of 1 μM thapsigargin, as indicated.

Immunoblot Analyses—MEF cells were treated with 1 μM thapsigargin for up to 6 h or left untreated. Protein lysates were collected and quantitated, and immunoblot analyses were carried out as previously described (10). Antibodies used for the immunoblot analyses include: CHOP (Santa Cruz Biotechnology; catalog no. sc-7351), eIF2α-P (Abcam; catalog no. ab32157), and β-actin (Sigma; catalog no. A5441). Monoclonal antibody measuring total eIF2α was provided by Dr. Scott Kimball (Pennsylvania State University College of Medicine, Hershey, PA).

mRNA Measurement by Quantitative PCR—RNA was isolated from MEF cells and sucrose gradient fractions using TRIzol reagent (Invitrogen), and single-strand cDNA synthesis was performed using TaqMan reverse transcriptase kit (Applied Biosystems) according to the manufacturer's instructions. Transcript levels were measured by quantitative PCR using SYBR Green (Applied Biosystems) on a Realplex2 Master Cycler (Eppendorf). Primers used for quantitative PCR analysis include: *CHOP* forward, 5′-CCTAGCTTGGCTGACAGAGG-3′, and reverse, 5′-CTGCTCCTTCTCCTTCATGC-3′; *ATF5* forward, 5′-GGCTGGCTCGTAGACTATGG-3′, and reverse, 5′-CCAGAGGAACCAGAGCTGTG-3′; *BIM* forward, 5′-TTTGACACAGACAGGAGCCC-3′, and reverse, 5′-CAGCTCCTGTGCAATCCGTA-3′; *Firefly luciferase* forward, 5′-CTCACTGAGACTACATCAGC-3′, and reverse, 5′-TCCAGATCCACAACCTTCGC-3′; and *β-actin* forward, 5′-TGTTACCAACTGGGACGACA-3′, and reverse, 5′-GGGGTGTTGAAGGTCTCAA-3′.

Polysome Profiling and Sucrose Gradient Ultracentrifugation—MEF cells were exposed to 1 μM thapsigargin for 6 h or left untreated. Cells were then treated with 50 μg/ml cycloheximide just prior to lysate collection. Lysates were collected, sheared with a sterile syringe and 23-gauge needle, and layered on top of 10–50% sucrose gradients, followed by ultracentrifugation as previously described (14, 16). Sucrose gradients were fractionated using a piston gradient fractionator (BioComp) and a 254-nm UV monitor, and Data Quest Software was used to monitor distributions of free and translating ribosomes.

Following fractionation, 10 ng/ml firefly luciferase control RNA (Promega) was spiked into each sample to measure shifts of *CHOP* transcript in the sucrose fractions that were normalized to an exogenous RNA control (14, 16). Samples were mixed with TRIzol, and RNA isolation and cDNA generation were performed as described (14). Calculations for the percentage of total gene transcript and the percentage of transcript shifts are as described previously (14). mRNA polysome shifts are representative of three independent biological experiments.

Plasmid Constructions and Luciferase Assays—The cDNA segments encoding the 5′-leader of *CHOP* were inserted between HindIII and NcoI restriction sites situated between the TK promoter and firefly luciferase CDS in a derivative of plasmid pGL3 (3). The resulting reporter plasmid CHOP-Luc contains the mouse *CHOP* 5′-leader and the start codon for the *CHOP* CDS fused to a luciferase reporter. Site-directed mutagenesis or DNA directly synthesized with the desired *CHOP* 5′-leader sequences were used to generate the mutant versions of CHOP-Luc highlighted in Table 1. Each of the reporter plasmids were sequenced to verify the desired nucleotide substitutions.

The full-length *CHOP* uORF was fused in-frame to the luciferase CDS, which was transcriptionally expressed from the TK

TABLE 1
Description of *CHOP* mutations used in this study

Gene construct	Description of mutation
CHOP uORF ΔATG	Mutation of ATGTTGAAGATG to AGGTTGAAGAGG
CHOP uORF TGA to GGA	Mutation of CACACCTGAAAGCAG to CACACCGGAAAGCAG
CHOP uORF stong start codon Kozak context	Mutation of TATATCATGTTGAAGATGA to GCCACCATGGCCACCATGG
CHOP uORF ΔAA 14–34	Deletion of ACCAGCCGGAACCTGAGGAGAGAGTGTTCCAGAAGGAAGTGCATCTTCATACACCACCACACC
CHOP uORF ΔAA 2–23	Deletion of TTGAAGATGAGCGGGTGGCAGCGACAGAGCCAGAATAACAGCCGGAACCTGAGGAGAGAGTGTTCC
CHOP uORF frameshift	Deletion of C 3′ of GTGTTC and insertion of C 3′ of CACACC
CHOP uORF RRK to AAA	Mutation of AGAAGGAAG to GCAGCGGCG
CHOP uORF IFI to AAA	Mutation of ATCTTCATA to GCCGCCGCA
CHOP uORF HHH to AAA	Mutation of CACCACCAC to GCCGCCGCC
CHOP uORF C27 to A	Mutation of TGC to GCC
CHOP uORF I28 to A	Mutation of ATC to GCC
CHOP uORF F29 to A	Mutation of TTC to GCC
CHOP uORF I30 to A	Mutation of ATA to GCA
CHOP uORF AGA to TGA	Mutation of AGA to TGA
CHOP uORF strong start codon Kozak context with AGA to TGA	Mutation of TATATCATGTTGAAGATGA to GCCACCATGGCCACCATGG and mutation of AGA to TGA

promoter, generating uORF-Luc. Site-directed mutagenesis was performed following the manufacturer's instructions (Stratagene), and all mutant uORF-Luc constructs listed in Table 1 were sequenced to verify the desired nucleotide residue substitutions. CHOP-Luc and CHOP uORF-Luc constructs were transiently co-transfected with a *Renilla* reporter plasmid into WT MEF cells for 24 h followed by either no treatment or a 6 h exposure to 0.1 μM thapsigargin. Lysates were collected, and firefly and *Renilla* luciferase activities were measured as described previously (3). At least three independent biological experiments were conducted for each luciferase measurement, and relative values are represented with S.D. values indicated.

We generated a three-part CDS fusion construct that featured the *Renilla* luciferase CDS fused in-frame to the last 30 nucleotides of the *CHOP* uORF, followed by the firefly luciferase CDS. We began by inserting DNA encoding the *Renilla* CDS between HindIII and NcoI restriction sites that were downstream of a TK promoter and upstream of the firefly CDS in a derivative of plasmid pGL3. Annealed oligonucleotide cloning was then used to insert WT and mutant versions of the *CHOP* uORF sequences into the AatII and NarI sites situated between the *Renilla* and firefly luciferase CDS. All constructs were characterized by restriction mapping and sequenced to verify the desired recombinant DNA ligations and nucleotide substitutions. *Renilla*-uORF-Luc constructs were transiently transfected into WT MEF cells for 24 h, followed by either no treatment or a 6-h exposure to 0.1 μM thapsigargin. Lysates were collected, and firefly and *Renilla* luciferase activities were measured as described previously (3).

We created plasmids used for expression of mRNAs in toeprint assays following a previously described strategy (17). For expression of the fusion transcript *α-globin-CHOP-Luc*, we first inserted the T7 promoter containing sequence TAATACGACTCACTATAGGGAGA between SacI and MluI restriction sites in the pGL3 luciferase vector (Promega). Next the cDNA sequences encoding rabbit α-globin fused in-frame to last 30 nucleotides of the *CHOP* uORF were inserted between HindIII and NarI sites of the pGL3-derived plasmid. This plasmid yielded a CDS encoding a fusion polypeptide featuring α-globin, the last 10 amino acid residues encoded in the *CHOP* uORF, and firefly luciferase. The α-globin-CHOP-Luc plasmid was then used for the synthesis of mRNAs to be used in toeprinting assays described below. The WT *CHOP* uORF sequence inserted into the α-globin-CHOP-Luc plasmid was designated p1335. A frameshift mutant of the *CHOP* uORF sequence was designated p1336, mutation of the Ile-Phe-Ile codons to Ala-Ala-Ala was p1337, and insertion of a stop codon just following the *CHOP* uORF sequence was p1338. Sequencing was used to verify the plasmid constructs and desired base substitutions.

In Vitro Transcription and Translation Assays—Capped and polyadenylated RNAs were synthesized with T7 RNA polymerase using mMESSAGE mMACHINE T7 Ultra (Ambion). Primer extension inhibition (toeprint) assays used cell-free translation extracts derived from *Neurospora crassa* that were prepared as described (18) and treated with cycloheximide upon addition of the *α-globin-CHOP-Luc* mRNA to measure initiating ribosomes (time 0), 15 min after addition of the transcript to measure ribosomal localization during steady-state translation (time 15), or left untreated to measure prominent ribosomal stalls. Toeprint assays were conducted using primer ZW4 (5′-TCCAGGAACCAGGGCGTA-3′) as previously described (19).

Cell Number and Viability Assays—MTT and caspase 3/7 assays were carried out by seeding cells at 5,000 cells/well in a 96-well plate. The cells were cultured for 24 h and treated with either 25 nM thapsigargin or 0.5 μM tunicamycin for up to an additional 24 h, and MTT and caspase 3/7 activity was measured using a CellTiter 96-well nonradioactive cell proliferation assay and Caspase-Glo 3/7 assay system (Promega).

Statistical Analyses—The values indicate the means ± standard deviation and represent at least three independent experiments. Statistical significance was calculated using the two-tailed Student's *t* test. Differences between multiple groups were analyzed using a one-way analysis of variance, followed by a post hoc Tukey HSD test. *p* values less than 0.05 were considered statistically significant and are indicated by *asterisks*, and treatment groups considered statistically significant from WT control are indicated by a *pound sign*.

Results

The Inhibitory Function of CHOP uORF Is Reliant upon an Ile-Phe-Ile Sequence—The uORF in the *CHOP* mRNA serves as a barrier that prevents downstream translation at the *CHOP* coding region during basal conditions. However, upon stress and increased eIF2α-P, the inhibitory effects of the uORF can

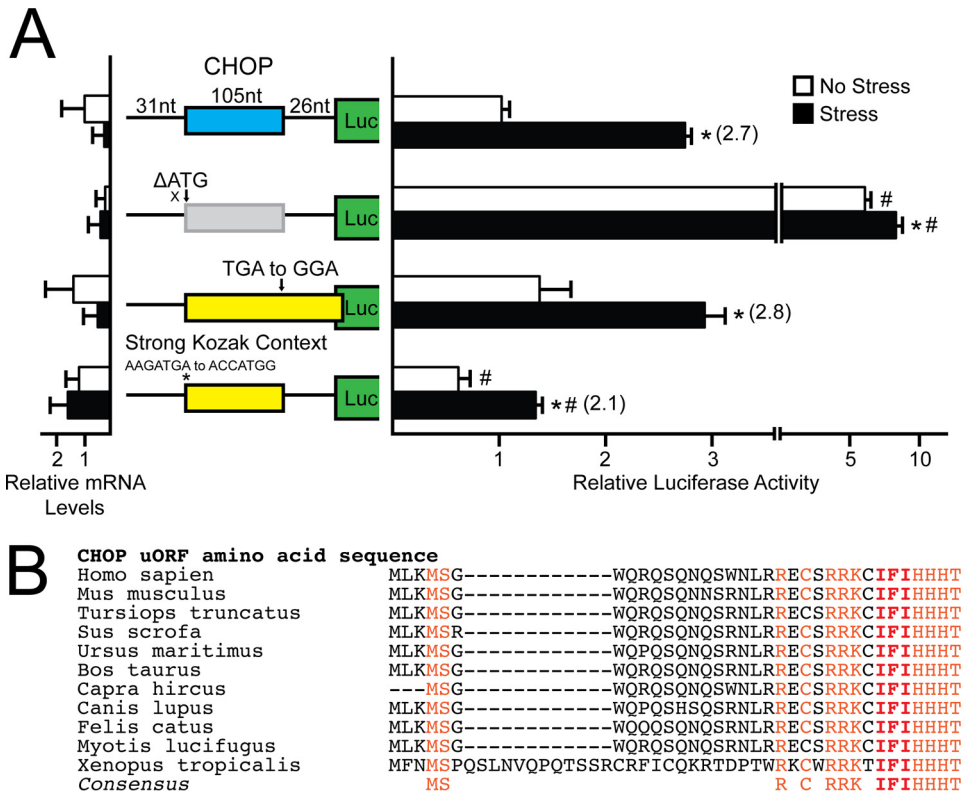

FIGURE 1. ***CHOP* translation control involves bypass of an inhibitory uORF due in part to poor start codon context.** *A*, WT and mutant versions of CHOP-Luc and a *Renilla* luciferase reporter were co-transfected into MEF cells and treated for 6 h with thapsigargin or left untreated. *CHOP* translation control was measured via a Dual-Luciferase assay and corresponding *CHOP-Luc* mRNA was measured by qRT-PCR. The CHOP-Luc construct contains the cDNA sequence corresponding to the *CHOP* 5′-leader fused to the luciferase reporter gene with the *CHOP* uORF and CDS of the CHOP-Luc fusion indicated with *colored boxes*. Mutant versions of CHOP-Luc include substitution of the *CHOP* uORF ATG start codons to AGG, mutation of the *CHOP* uORF stop codon from TGA to GGA, and optimization of the *CHOP* uORF start codons to the Kozak consensus sequence. Relative values are represented as histograms for each, with the S.D. indicated. The following values represent firefly luciferase activity normalized for mRNAs expressed from the indicated WT and mutant versions of CHOP-Luc reporters. These values feature the no stress values, stress values, and then in parentheses the induction ratios: WT, 1, 10.2 (10.2); ATG to AGG, 32.4, 24.1 (0.75); TGA to GGA, 0.9, 5.6 (6); and strong Kozak consensus, 0.5, 0.8 (1.6). *B*, polypeptide sequence encoded by the *CHOP* uORF from different vertebrates. The uORF polypeptide sequences were from cDNAs derived from *CHOP* orthologs, including *Homo sapiens* (BC003637), *Mus musculus* (BC013718), *Tursiops truncates* (XM_004316348), *Sus scrofa* (AK346731), *Ursus maritimus* (GW278660), *Bos taurus* (BC122721), *Capra hircus* (NM_001287231), *Canis lupus* (DN431044), *Felis catus* (XM_006933848), *Myotis lucifugus* (XM_006093575), and *Xenopus tropicalis* (BC153679). Residues conserved between the uORF sequences are listed in the consensus and are highlighted.

be bypassed to increase translation of the *CHOP* CDS. This is recapitulated by using luciferase reporter assays, where a cDNA segment encoding the 168-nucleotide sequence of the 5′-leader of *CHOP* was inserted between a minimal TK promoter and the firefly luciferase reporter CDS, generating CHOP-Luc. This luciferase reporter contains the initiation codon of the *CHOP* CDS fused in-frame to the luciferase CDS. Expression of CHOP-Luc was increased almost 3-fold in MEF cells treated with 6 h of thapsigargin, a potent inducer of endoplasmic reticulum stress and eIF2α-P (Fig. 1*A*). Deletion of the uORF by mutation of two ATG start codons at codon positions 1 and 4 in the uORF to AGG led to constitutively high levels of luciferase activity, which emphasizes the inhibitory function of the uORF. Furthermore, mutation of the stop codon of the uORF from TGA to GGA extended the uORF so that it now overlapped out of frame with the CDS (Fig. 1*A*). In this case, there was a similar induction of CHOP-Luc as observed for the WT version of the uORF, consistent with the idea that eIF2α-P facilitates bypass of the uORF as opposed to changing translation reinitiation. Finally, substitution of the initiation codon context for both start codons in the uORF to an optimal Kozak consensus sequence lowered luciferase expression and induction upon ER stress (Fig. 1*A*). This finding indicates that a less than optimal initiation context contributes in part to the ribosomal bypass of the inhibitory uORF upon eIF2α-P. Normalization of luciferase activity to *CHOP-Luc* mRNA levels among these reporter constructs resulted in similar trends as luciferase activity alone, supporting the idea that the observed changes in luciferase activity are the result of translational control (Fig. 1*A*). Together, these results support the key tenets of the bypass model described for *CHOP* translational control (4, 20).

The coding sequence of the *CHOP* uORF shares many conserved features among vertebrates (Fig. 1*B*). To explore the basis of the inhibitory nature of the *CHOP* uORF sequences during basal conditions, we generated an in-frame fusion of the *CHOP* uORF with the firefly luciferase CDS downstream of a minimal TK promoter, creating uORF-Luc (Fig. 2). This luciferase reporter lacks the luciferase start codon, ensuring that any measurable luciferase activity is a product of the uORF-luciferase fusion polypeptide. Basal expression of uORF-Luc (construct 1) was minimal in MEF cells, suggesting that the *CHOP* uORF coding sequence reduced translation of the uORF-luciferase fusion polypeptide (Fig. 2, *A* and *B*). In this reporter construct and those that follow, there was no significant difference

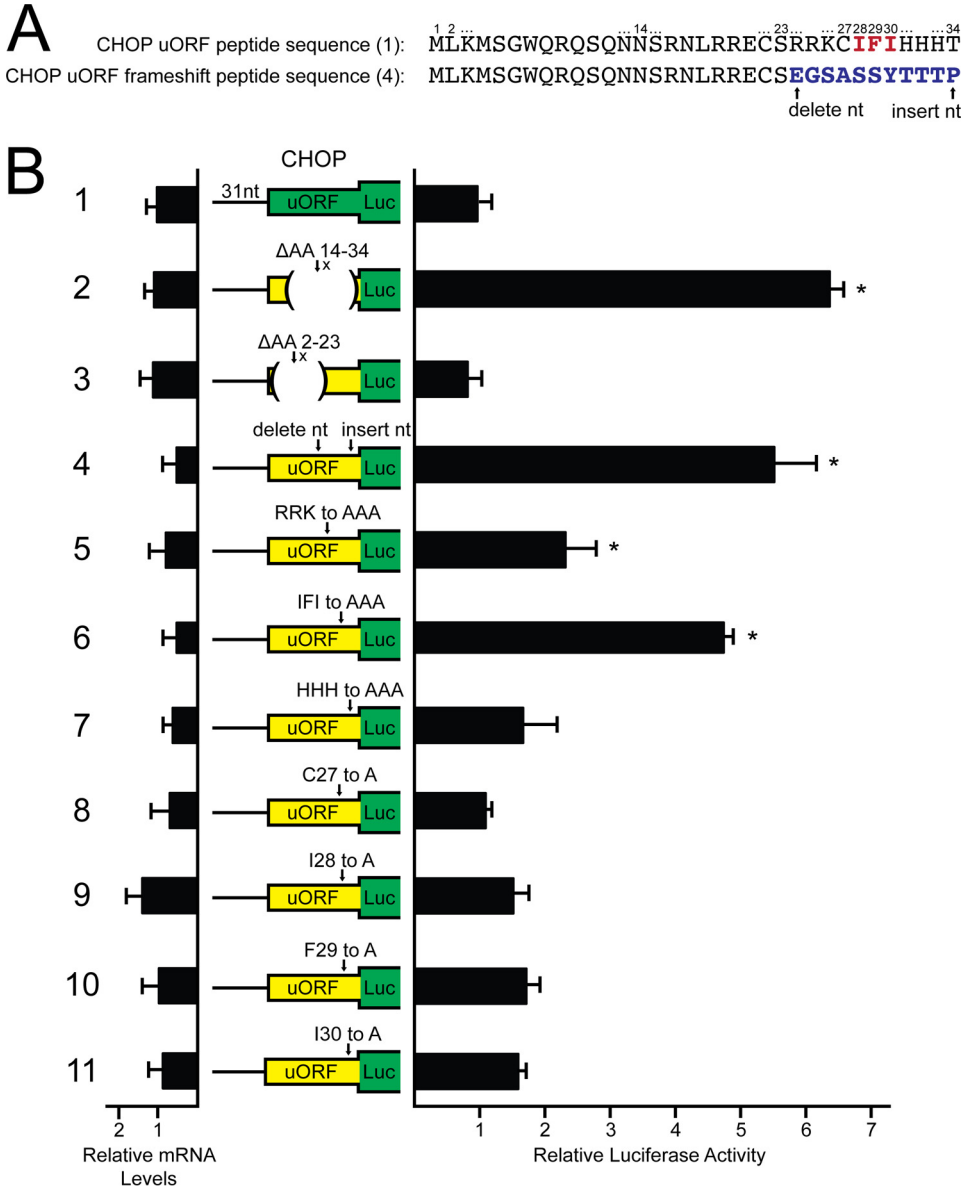

FIGURE 2. ***CHOP* translation control involves an inhibitory uORF that relies on an encoded Ile-Phe-Ile sequence.** *A*, representation of the *CHOP* uORF amino acid sequence in the wild-type context (construct 1) and the frameshift peptide sequence in which a nucleotide was deleted just after amino acid 23 and inserted following amino acid 34 (construct 4). The amino acid sequences in the uORF polypeptide are listed, with corresponding positions. The inhibitory Ile-Phe-Ile sequence is highlighted in *red* in the WT *CHOP* uORF peptide sequence. The last 10 amino acid residues in the *CHOP* uORF were altered as a consequence of the uORF frameshift construct and are highlighted in *blue*. *B*, the WT and mutant versions of the uORF-Luc constructs and a *Renilla* luciferase reporter were co-transfected into MEF cells. 24 h later, *CHOP* uORF translation control was measured via Dual-Luciferase assay, and the corresponding *CHOP-Luc* mRNAs were measured by qRT-PCR. The uORF-Luc constructs contain the *CHOP* uORF fused in-frame to the luciferase reporter gene, with the ATG start codon of luciferase deleted. The *CHOP* uORF sequence and luciferase CDS are indicated by the *colored boxes*. The *green CHOP* uORF *box* represents the wild-type *CHOP* uORF sequence. *Yellow CHOP* uORF *boxes* represent mutant constructs in which a change was made to the *CHOP* uORF sequence. Mutant versions of uORF-Luc include an in-frame deletion of uORF codons 14–34 or 2–23, frameshift in the last 10 *CHOP* uORF codons, substitution of *CHOP* uORF codons Arg-Arg-Lys to Ala-Ala-Ala, change of Ile-Phe-Ile to Ala-Ala-Ala, mutation of His-His-His to Ala-Ala-Ala, and alanine substitutions for Cys-27, Ile-28, Phe-29, and Ile-30. Relative values are represented as histograms for each, with the S.D. indicated. The following values represent firefly luciferase activity normalized for mRNA for the WT and mutant versions of the uORF-Luc reporters. The luciferase activity to mRNA ratios are: *construct 1*, 1; *construct 2*, 6.1; *construct 3*, 0.8; *construct 4*, 10.9; *construct 5*, 3; *construct 6*, 9.3; *construct 7*, 2.8; *construct 8*, 1.6; *construct 9*, 1.1; *construct 10*, 1.8; and *construct 11*, 1.9.

in the *uORF-Luc* mRNA, and normalization of luciferase activity to *uORF-Luc* mRNA levels resulted in similar trends as luciferase activity alone, supporting the idea that the observed changes in luciferase activity are the result of translation control.

To determine which portion of the *CHOP* uORF is critical for inhibition, in-frame deletions of codons 14–34 and 2–23 were next analyzed in the uORF-Luc (*constructs 2* and *3* in Fig. 2*B*). Deletion of codons 2–23 led to no change in luciferase activity compared with the WT construct, whereas deletion of codons 14–34 increased luciferase expression 6-fold, suggesting that the repressing function of *CHOP* uORF lies within the carboxyl-terminal coding sequence. To investigate whether the *CHOP* uORF RNA sequence contributes to the inhibitory function of this uORF, a single nucleotide was deleted just after codon 23, and a single nucleotide was inserted following codon 34. The

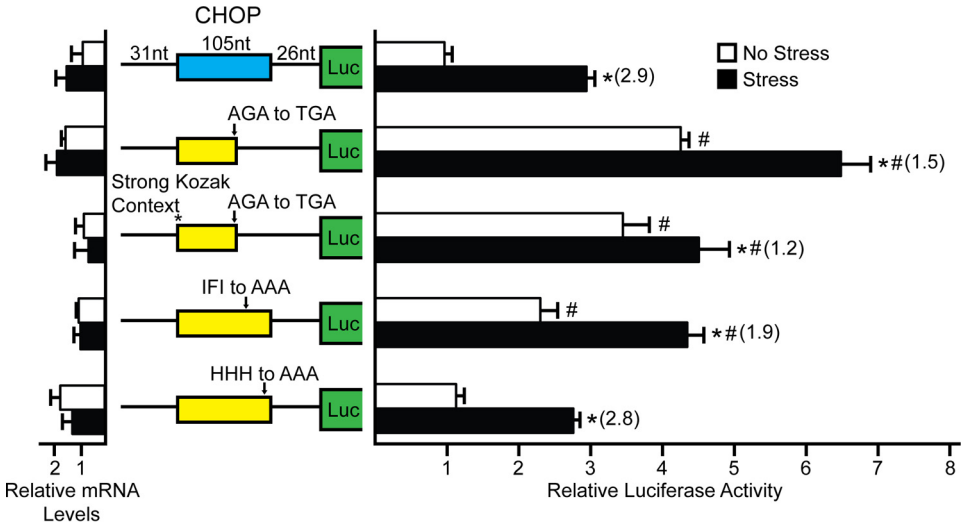

FIGURE 3. ***CHOP* translation control involves bypass of an inhibitory uORF.** WT and mutant versions of CHOP-Luc and a *Renilla* luciferase reporter were co-transfected into MEF cells and treated for 6 h with thapsigargin or left untreated. *CHOP* translation control was measured via a Dual-Luciferase assay, and corresponding *CHOP-Luc* mRNA was measured by qRT-PCR. The CHOP-Luc construct contains the cDNA sequence corresponding to the *CHOP* 5′-leader fused to the luciferase reporter gene with the *CHOP* uORF and CDS of the CHOP-Luc fusion indicated with *colored boxes*. Mutant versions of CHOP-Luc include mutation of the AGA codon of Arg-24 to TGA, simultaneous mutation of the *CHOP* uORF start codon to Kozak consensus sequence with the AGA to TGA mutation, change of the Ile-Phe-Ile codons to those encoding Ala-Ala-Ala, and mutation of the His-His-His codons to those encoding Ala-Ala-Ala. Relative values are represented as histograms for each, with the S.D. indicated. The following values represent firefly luciferase activity normalized for mRNA from the WT and mutant versions of CHOP-Luc reporters. The following features the no stress values, stress values, and then in parentheses the induction ratios: WT, 1, 1.8 (1.8); AGA to TGA, 2.5, 3.1 (1.2); optimized Kozak context with AGA to TGA, 3.8, 6.5 (1.7); IFI to AAA, 2.1, 4.1 (2); and HHH to AAA, 0.6, 2 (3.4).

resulting frameshift thereby largely retains the uORF nucleotide sequence, but the uORF now encodes a polypeptide of different sequence for the last 10 amino acids in the carboxyl-terminal region of the *CHOP* uORF (Fig. 2*A* and *construct 4* in Fig. 2*B*). Luciferase activity for this CHOP uORF-Luc frameshift reporter was increased to the same extent as deletion of codons 14–34, suggesting that the encoded carboxyl-terminal polypeptide sequence is responsible for the inhibitory nature of the *CHOP* uORF.

Phylogenetic analysis of the *CHOP* uORF polypeptide sequence among vertebrates indicates that there are several conserved amino acid residues in the carboxyl-terminal region of the *CHOP* uORF (Fig. 1*B*). Single amino acid substitutions, including Cys-27, Ile-28, Phe-29, and Ile-30, to alanine resulted in no significant change in luciferase activity (*constructs 8–11* in Fig. 2*B*). Furthermore, substitution of consecutive residues His-His-His to Ala-Ala-Ala resulted in no change in luciferase activity. By comparison, Ala substitutions for the consecutive Arg-Arg-Lys and Ile-Phe-Ile sequences resulted in 2.4- and 4.9-fold increases in luciferase activity, respectively (*constructs 5–7* in Fig. 2*B*). In fact, the fold induction observed for the Ile-Phe-Ile substitution (construct 6) was similar to that measured for the carboxyl-terminal uORF-Luc amino acid frameshift reporter (construct 4), suggesting that the Ile-Phe-Ile sequence plays a dominant role in the repressive function of the *CHOP* uORF.

Next we wished to establish whether the activity of the inhibitory amino acid sequences identified in the uORF-luciferase fusion were conserved in translational control directed by the endogenous *CHOP* 5′-leader. As noted earlier, there was almost a 3-fold induction of CHOP-Luc expression in the reporter that features the full *CHOP* 5′-leader inserted between a minimal TK promoter and the firefly luciferase CDS (Figs. 1*A* and 3). In this reporter and those that follow, there was no significant difference in the *CHOP-Luc* mRNA upon stress treatment, supporting the idea that the observed changes in luciferase activity are the result of translation control. The Arg-24 codon (encoded by AGA) was mutated to a TGA stop codon in CHOP-Luc, generating a *CHOP* uORF that lacks the last 10 amino acid residues (Fig. 3). Removal of these carboxyl-terminal residues from the *CHOP* uORF resulted in a 4-fold increase in basal luciferase activity, which was further induced upon thapsigargin treatment. Combined mutation of the initiation codon context for each start codon in the uORF to the Kozak consensus sequence and stop codon insertion at codon 24 resulted in a similar basal level of luciferase activity as the stop codon insertion construct alone but was even less inducible (1.2-fold). These results indicate that the 10 amino acid residues in the carboxyl terminus of the *CHOP* uORF thwart reinitiation of ribosomes at the downstream CDS and are thus critical for its inhibition of translation of the downstream CDS during basal conditions. Furthermore, bypass of the *CHOP* uORF is required for maximal *CHOP* expression during cellular stress.

Next Ile-Phe-Ile and His-His-His sequences were each substituted to Ala-Ala-Ala in the CHOP-Luc reporter. Substitution of the Ile-Phe-Ile sequence resulted in a basal increase in luciferase activity that was further induced with thapsigargin treatment, whereas mutation of the His-His-His sequence resulted in no significant difference from the WT CHOP-Luc (Fig. 3). These results further support the idea that the carboxyl-terminal Ile-Phe-Ile residues are a major reason for the repressing function of the *CHOP* uORF and are critical for maintaining low levels of *CHOP* expression during basal conditions.

Translation of an Ile-Phe-Ile Sequence in the CHOP uORF Results in an Elongation Stall—An *in vitro* translation assay using selected *CHOP* uORF mutants followed by toeprinting analysis was carried out to map the position of ribosomes potentially stalled at the inhibitory *CHOP* uORF sequence. The *CHOP* uORF carboxyl-terminal sequence was fused in-frame between a rabbit α-globin domain and the firefly luciferase CDS to determine whether the inhibitory uORF amino acid sequence could regulate translation when placed internally as a part of a polypeptide of heterologous sequence (Fig. 4*A*) (17). The *α-globin-CHOP-Luc* reporters were constructed with a WT portion of the *CHOP* uORF (WT), a frameshift version with single nucleotide deleted after codon 23 and an inserted nucleotide following codon 34 (FS), a substitution of Ala residues for Ile-Phe-Ile (IFI), and a version containing a stop codon following the inserted *CHOP* uORF sequence (STOP). T7 RNA polymerase was used to synthesize the WT and mutant versions of the *α-globin-CHOP-Luc* mRNAs, which were then introduced into cell-free translation extracts for toeprint mapping of translating ribosomes. Cell-free translation extracts were treated with cycloheximide simultaneous to addition of *α-globin-CHOP-Luc* mRNA to measure translation initiation events (time 0) or 15 min after introduction of *α-globin-CHOP-Luc* mRNA to map the position of ribosomes during steady-state translation and polypeptide synthesis (time 15). Alternatively, cycloheximide was not added to the *in vitro* translation reactions after addition of *α-globin-CHOP-Luc* mRNA to map any ribosome stalls strong enough to result in detectable toeprint signals without the addition of an elongation inhibitor (time -).

Initiation at the AUG start codon was observed for the *α-globin-CHOP-Luc* mRNA (*green star*) without cycloheximide treatment and at time 15 but was strongest at time 0, indicative of efficient translation initiation at this codon (Fig. 4*B*). Toeprints were also observed with the second Ile codon of the repressing Ile-Phe-Ile sequence in the ribosomal P site both without cycloheximide addition and with greater intensity at time 15 (*yellow star*), but not at time 0. Modest toeprints were additionally present at the Phe codon of the same amino acid sequence in the ribosomal P site, suggesting that the repressing capability of the *CHOP* uORF is due to an elongation stall at the encoded Ile-Phe-Ile sequence of the *CHOP* uORF. Importantly these toeprint patterns suggest that this *CHOP* uORF sequence can sustain the same capacity for translation inhibition when transferred to an internal position of a coding sequence in a heterologous polypeptide.

Introduction of a single nucleotide just prior to the *CHOP* portion of the *α-globin-CHOP-Luc* mRNA and deletion of a single nucleotide just after the *CHOP* sequence significantly reduced the toeprint signals for stalled ribosomes at the *CHOP* uORF sequence. This finding provides additional evidence that the inhibitory nature of the *CHOP* uORF is predominantly caused by a specific encoded amino acid sequence rather than RNA sequence *per se*. Alanine substitutions for the Ile-Phe-Ile *CHOP* uORF sequence resulted in a similar reduction in toeprint signals for a stalled elongating ribosome, suggesting that Ile-Phe-Ile in the *CHOP* uORF can serve as a barrier to downstream translation. Finally, introduction of a TGA stop codon just following the *CHOP* portion of the *α-globin-CHOP-Luc* mRNA resulted in a strong toeprint signal at both the termination codon (red octagon) and the Ile-Phe-Ile sequence (now shifted up three nucleotides in the sequencing gel; blue star). These results indicate that the Ile-Phe-Ile sequence has the capacity to stall elongating ribosomes in the *CHOP* uORF (Fig. 4, *A* and *B*).

To address whether the capacity of the Ile-Phe-Ile sequence to stall elongating ribosomes is regulated in a stress-dependent manner, we generated an in-frame fusion of the *CHOP* uORF carboxyl-terminal sequence in between the *Renilla* CDS and the firefly luciferase CDS (Fig. 4*C*). This luciferase reporter lacks the firefly luciferase start codon, ensuring that any measurable luciferase activity is a product of the *Renilla*-uORF-Luc fusion polypeptide. Basal firefly luciferase activity of *Renilla*-uORF-Luc was minimal in MEF cells, consistent with reduced translation of the fusion polypeptide because of the placement of the *CHOP* uORF coding sequence between the *Renilla* and firefly luciferase CDSs (Fig. 4*C*). Thapsigargin treatment resulted in no difference in luciferase activity as compared with no stress, indicating that the inhibitory nature of the *CHOP* uORF is not regulated in stress-dependent manner. In this reporter and those that follow, there was no significant difference in the *Renilla-uORF-Luc* mRNA upon stress treatment, supporting the idea that the observed changes in luciferase activity are the result of translation control.

Next a single nucleotide was deleted just after codon 23, and a single nucleotide was inserted following codon 34 of the *CHOP* uORF, generating a *Renilla*-uORF-Luc fusion polypeptide of different sequence encoded in the *CHOP* uORF region. Introduction of the frameshift resulted in almost a 3-fold increase in luciferase activity independent of stress, emphasizing that the encoded carboxyl-terminal polypeptide sequence is responsible for the inhibitory nature of the *CHOP* uORF (Fig. 4*C*). Mutation of the nucleotides encoding Ile-Phe-Ile to those for Ala-Ala-Ala also led to a 2-fold increase luciferase activity in both stress and nonstressed conditions, illustrating that the Ile-Phe-Ile sequence is critical for the ribosomal elongation stall. Finally, addition of a stop codon just following the *CHOP* uORF sequence resulted in almost no luciferase activity as expected for a termination event prior to the firefly luciferase CDS. These results further support the idea that the carboxyl-terminal Ile-Phe-Ile residues are a major reason for the repressing function of the *CHOP* uORF and that the nature of the inhibitory activity of the Ile-Phe-Ile is not regulated in a stress-dependent manner. A model for *CHOP* translational control and its broader implications will be highlighted under "Discussion."

Alterations in CHOP uORF Translation Control Change the Dynamics of CHOP Expression—We posit that increased translation of *CHOP* during eIF2α-P is central for determining expression of CHOP and its downstream transcriptional activity, which are suggested to be critical for stress-induced apoptosis when ISR signaling is insufficient to alleviate stress damage and restore cellular homeostasis (7–9). To address this idea, we engineered *CHOP*$^{-/-}$ MEF cells to stably express *CHOP* with either the WT uORF or an uORF with mutant initiation codons (ΔuORF CHOP) (Fig. 5*A*). To generate these CHOP-expressing cell lines, a Flp recombination target (FRT) site was integrated in the genome of *CHOP*$^{-/-}$ cells in a single location (CHOP$^{-/-}$

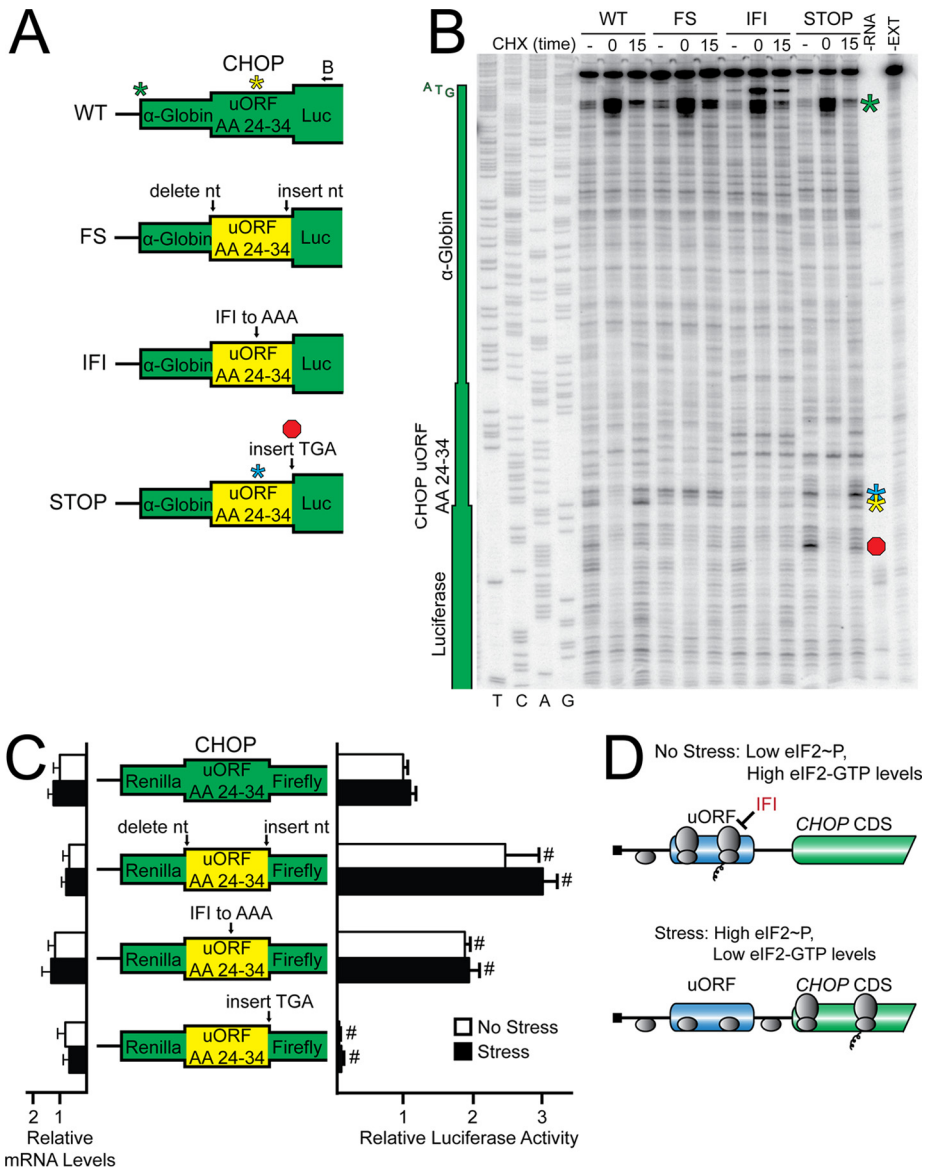

FIGURE 4. **Translation of the *CHOP* uORF results in a ribosome elongation stall that is dependent on an Ile-Phe-Ile sequence.** *A*, depiction of toeprint design using the last 30 nucleotides of the *CHOP* uORF inserted in-frame between the rabbit α-globin and luciferase coding sequences to generate *α-globin-CHOP-Luc* fusion mRNA. Mutant versions of *α-globin-CHOP-Luc* mRNA include frameshift of the 30 nucleotides corresponding to the *CHOP* uORF (*FS*), mutation of the Ile-Phe-Ile codons to those encoding Ala-Ala-Ala (*IFI*), and insertion of a TGA stop codon just following the 30 *CHOP* uORF nucleotides (*STOP*). The *black arrow* depicted above the WT *α-globin-CHOP-Luc* mRNA represents the location of the primer used in *B*. Toeprints corresponding to ribosome initiation at the start codon for the WT and mutant *α-globin-CHOP-Luc* mRNAs are represented by a *green star*. Toeprints corresponding to an ribosome elongation stall for the WT, FS, and IFI mRNAs are represented by a *yellow star*. Toeprints corresponding to an elongation stall and ribosome termination for the STOP mRNA are represented by a *blue star* and a *red octagon*, respectively. *B*, cell-free translation extracts were treated with cycloheximide upon addition of WT or mutant versions of the *α-globin-CHOP-Luc* mRNA to measure initiating ribosomes (time 0), 15 min after addition of the transcript to measure ribosome localization during steady-state translation (time 15 min), or left untreated to measure prolonged ribosomal stalls that present without the use of an elongation inhibitor (time -). Toeprint assays were conducted for each sample, and sequencing reactions can be read 5′ to 3′ from *top* to *bottom*. The nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is listed on the *left*, below the *first four lanes*. The products from control primer extension assays in the absence of RNA (−*RNA*) or in the absence of cell-free translation extracts are indicated on the *right*. The *green star* represents the toeprint corresponding to initiation at the *α-globin-CHOP-Luc* fusion, the *yellow* and *blue stars* represent prominent ribosome elongation stalls, and the *red octagon* represents the toeprint corresponding to termination at the introduced stop codon. The *green boxes* on the *left* span the sequences corresponding to the α-globin, *CHOP* uORF, and luciferase CDS and are comparable to the *α-globin-CHOP-Luc* schematic in *A*. Mutant constructs are the same listed in *A*, and the data are representative of three independent biological experiments. *C*, WT and mutant versions of *Renilla*-uORF-Luc were transfected into MEF cells and treated for 6 h with thapsigargin or left untreated. *CHOP* translation control was measured via a Dual-Luciferase assay and corresponding *CHOP-Luc* mRNA was measured by qRT-PCR. The *Renilla*-uORF-Luc construct includes the last 30 nucleotide residues of the *CHOP* uORF inserted in-frame between the *Renilla* and firefly luciferase coding sequences. Mutant versions of CHOP-Luc include frameshift of the 30 nucleotide segment corresponding to the *CHOP* uORF, mutation of the Ile-Phe-Ile codons to those encoding Ala-Ala-Ala, and insertion of a TGA stop codon just following the 30 nucleotide *CHOP* uORF segment. Relative values are represented as histograms for each, with the S.D. indicated. The following values represent firefly luciferase activity normalized for mRNA from the WT and mutant versions of CHOP-Luc reporters. The following features the no stress values, stress values, and then in parentheses the induction ratios: WT, 1, 0.9 (0.9); frameshift, 4, 4.1 (1); IFI to AAA, 1.6, 1.5 (0.9); and TGA insertion, 0.1, 0.1 (1). *D*, model for *CHOP* translation control. In the absence of stress, low eIF2α-P, and high eIF2-GTP, ribosomes scan the 5′-leader of the *CHOP* mRNA and initiate translation at the *CHOP* uORF. During translation of the uORF, elongating ribosomes are stalled at an Ile-Phe-Ile sequence, as depicted by the IFI sequence and *black bar* adjacent to the elongating ribosome in the uORF. The ribosome stall would preclude ribosome reinitiation downstream at the *CHOP* CDS. In the presence of stress and induced eIF2α-P, there would be lower levels of eIF2-GTP that would allow scanning ribosomes to bypass the *CHOP* uORF, in part because of its poor start codon context, and instead initiate translation at the *CHOP* CDS.

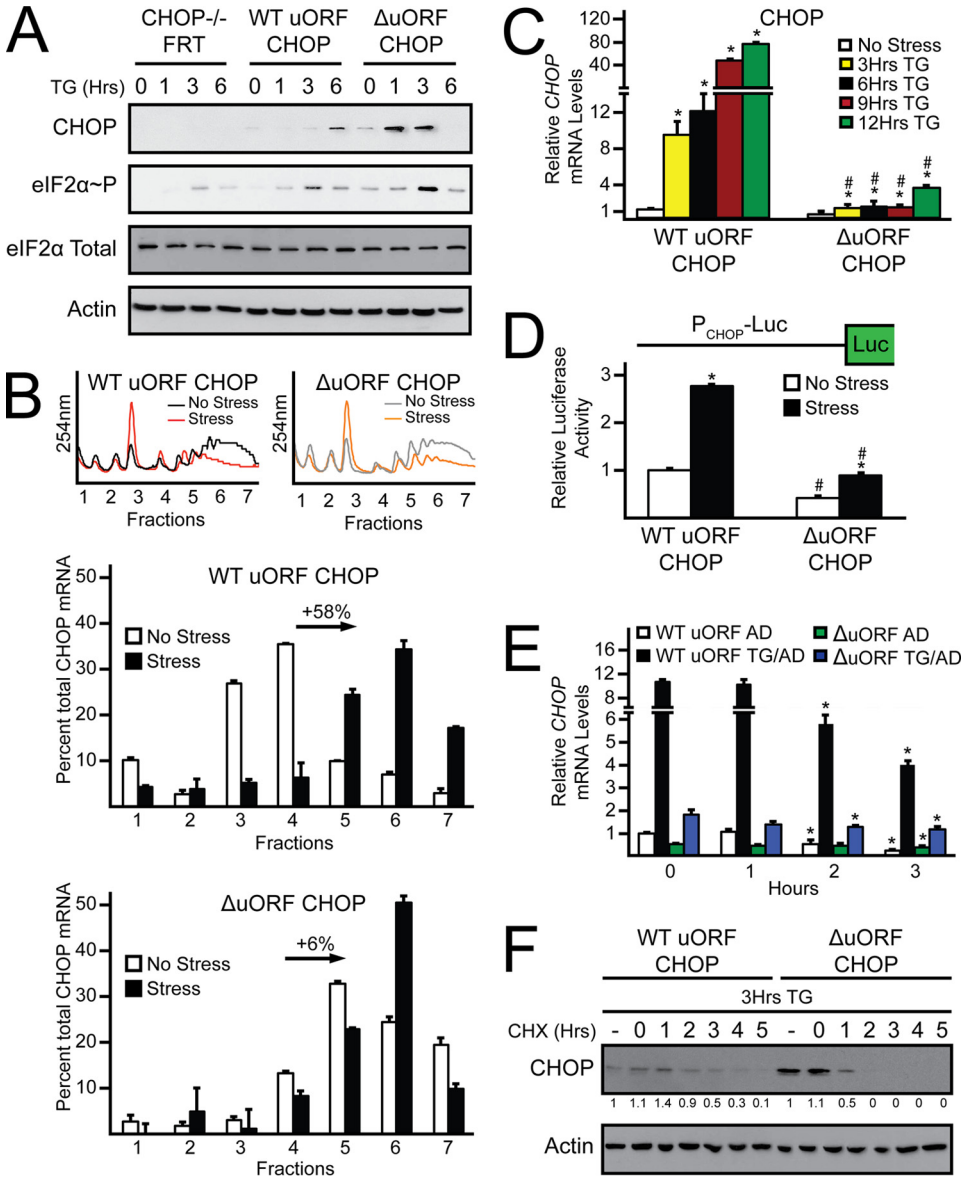

FIGURE 5. **Alterations in *CHOP* uORF translation control change the dynamics of *CHOP* expression.** *A*, MEF cells deleted for *CHOP* were stably selected to express WT *CHOP* (WT uORF CHOP) and *CHOP* with its uORF deleted (ΔuORF CHOP) and treated with the ER stress agent thapsigargin for up to 6 h or left untreated. The levels of CHOP, eIF2α-P, eIF2α total, and β-actin in these cultured cells were measured by immunoblot analyses. *B*, WT uORF CHOP and ΔuORF CHOP cells were treated with thapsigargin for 6 h or left untreated. Lysates were collected and layered on top of 10–50% sucrose gradients, followed by ultracentrifugation, and analysis of whole lysate polysome profiles at 254 nm. Sucrose gradients were fractionated simultaneous to analysis of polysome profiles at 254 nm. Total RNA was isolated from sucrose fractions and the percentage of total *CHOP* mRNA was determined by qRT-PCR. *B* is representative of three independent biological experiments. *C*, total RNA was collected from WT uORF CHOP and ΔuORF CHOP cells cultured in the presence or absence of thapsigargin and relative levels of *CHOP* mRNA were measured by qRT-PCR. *D*, fusion of 1 kb of the *CHOP* promoter (P_{CHOP}-Luc) and a *Renilla* luciferase reporter were co-transfected into MEF cells, treated for 6 h or left untreated, and measured using a Dual-Luciferase assay. Relative values are represented as histograms, and the S.D. is indicated. *E*, total RNA was collected from WT uORF CHOP and ΔuORF CHOP cells cultured in the presence or absence of thapsigargin for 3 h followed by 0, 1, 2, or 3 h of treatment with actinomycin D. Relative levels of *CHOP* mRNA were measured by qRT-PCR. *F*, WT uORF CHOP and ΔuORF CHOP cells were treated with thapsigargin for 3 h, washed, and lysed (*CHX −*) or washed and treated with cycloheximide for up to 5 h (*CHX 0*, *1*, *2*, *3*, *4*, and *5*). Levels of CHOP and β-actin in the cultured cells were measured by immunoblot analyses. Quantification of changes in CHOP protein expression are depicted under the CHOP immunoblot panel and are normalized to the no cycloheximide treatment for both WT uORF CHOP and ΔuORF CHOP cells.

FRT), followed by clonal isolation. Integration of the FRT site was followed by insertion of full-length *CHOP* cDNAs including the WT or ΔuORF *CHOP* 5′-leader under control of 1 kb of the *CHOP* promoter to ensure its proper transcriptional regulation in response to ER stress (9, 21). These isogenic CHOP-expressing cells were then assayed for changes in *CHOP* expression and cell viability in the presence or absence of thapsigargin. Measurements of CHOP protein levels showed the expected pattern of CHOP expression in WT uORF CHOP MEF cells with low basal levels of CHOP expression that increased in response to thapsigargin treatment (Fig. 5*A*). ΔuORF CHOP MEF cells presented with sharply elevated levels of CHOP protein in the absence of stress that was increased further after 1 and 3 h of stress, and reduced by 6 h. We suggest that the reduction of CHOP protein levels in the ΔuORF CHOP cells after 3 h of stress could be a consequence of feedback regulation, and we will address this feature of CHOP expression below. Phosphorylation of eIF2α after thapsigargin treatment

followed similar patterns, peaking at 3 h of treatment, with the highest levels of eIF2α-P being observed in the ΔuORF CHOP cells. In each of the CHOP-derived cells, eIF2α-P was reduced by 6 h, which is consistent with the ISR feedback control directed by GADD34 (22).

Polysome analysis of cells expressing either WT uORF or ΔuORF CHOP supported the translation control changes predicted based on our analysis of endogenous CHOP and CHOP-Luc reporters (Figs. 1*A* and 5*B*) (4). For this analysis, lysates were prepared from WT and ΔuORF CHOP cells that were subjected to thapsigargin or no ER stress. These lysates were then separated by sucrose gradient ultracentrifugation (Fig. 5*B*, *top panels*). Consistent with lowered global translation initiation in response to stress and eIF2α-P, both cell lines displayed lowered polysome levels coincident with increased monosomes after thapsigargin treatment. To assess the efficiency of translation of *CHOP* mRNA, we next measured *CHOP* transcript levels among the sucrose fractions. In the WT uORF CHOP cell line, *CHOP* mRNA was largely associated with light polysomes in the absence of stress, with a 58% shift of transcript to heavy polysomes with thapsigargin treatment (Fig. 5*B*). This is consistent with preferential translation in response to eIF2α-P. In the ΔuORF CHOP cells, *CHOP* transcripts were associated with increased polysome levels compared with the WT uORF CHOP cells in both nonstressed and ER stress conditions. Overall, these findings suggest that *CHOP* transcript is robustly translated for both the WT and ΔuORF CHOP cells after 6 h of thapsigargin treatment, the recovery phase of eIF2α-P. Furthermore, deletion of the *CHOP* uORF is suggested to result in resistance to translation repression in the presence of eIF2α-P, resulting in a constitutively translated transcript. These results suggest that CHOP protein abundance is tightly regulated through an uORF-mediated mechanism of translational control.

It is also known that *CHOP* expression is regulated by transcription through the activity of ISR-induced ATF4 and C/EBPβ (21, 23). Time course analysis of *CHOP* mRNA levels for up to 12 h after thapsigargin treatment of the CHOP-expressing cells lines revealed that *CHOP* mRNA expression is substantially reduced both basally and with thapsigargin treatment in ΔuORF CHOP as compared with WT uORF CHOP (Fig. 5*C*). Transfection of the WT uORF and ΔuORF CHOP cell lines with a luciferase reporter under the control of 1 kb of the *CHOP* promoter (P_{CHOP}-Luc) and measurement of luciferase activity resulted in a similar trend in expression as observed for the endogenous *CHOP* mRNA levels, with low levels of luciferase activity in the ΔuORF CHOP cell line (Fig. 5*D*). Combined, these results suggest that *CHOP* transcriptional regulation is altered in the ΔuORF CHOP cell line, resulting in lowered *CHOP* mRNA levels.

To determine whether changes in *CHOP* mRNA turnover also contribute to the differences observed in *CHOP* transcript abundance, we subjected WT uORF and ΔuORF CHOP cells to thapsigargin treatment or no stress treatment for 3 h, followed by actinomycin D treatment for up to an additional 3 h. *CHOP* mRNAs from the WT uORF cells presented with a half-life of ~2 h, consistent with an earlier report (9). Interestingly, deletion of the *CHOP* uORF increased the half-life of *CHOP* transcript to ~4.5 h. *CHOP* was previously identified in a genome-wide screen as a target of the nonsense-mediated mRNA decay pathway (24), and these findings suggest that deletion of the *CHOP* uORF thwarts the decay machinery to detect and lower the abundance of *CHOP* mRNA. Given that levels of the more stable ΔuORF *CHOP* mRNA were significantly less than WT, these results emphasize that decreased abundance of the ΔuORF CHOP is due to substantial reductions in *CHOP* transcription. Despite the lowered *CHOP* mRNA in the ΔuORF CHOP cells, there was a marked increase in basal and induced CHOP protein (Fig. 5*A*), which reinforces the idea that translational expression of *CHOP* is a major feature in its regulated expression. As will be highlighted further under "Discussion," a likely mechanism contributing to this difference in *CHOP* mRNA expression is direct or indirect feedback regulation by CHOP (25, 26).

CHOP is a short-lived protein with a half-life of less than 4 h (9). To determine whether CHOP protein turnover was differentially regulated in the WT uORF and ΔuORF CHOP cells, both CHOP-expressing cells lines were pretreated with thapsigargin for 3 h followed by either no cycloheximide treatment or cycloheximide treatment for up to 5 h (Fig. 5*E*). WT uORF CHOP cells presented with a CHOP protein half-life of ~3 h, similar to endogenous CHOP in WT MEF cells (9). However, CHOP protein half-life in the ΔuORF CHOP cells was decreased to ~1 h. This protein destabilization correlates with the sharp reduction in CHOP protein expression that is observed after 6 h of thapsigargin treatment in the ΔuORF CHOP cells. Overall, these results suggest that increased CHOP synthesis resulting from altered translation regulation elicits multiple compensating mechanisms targeted to lower CHOP protein expression.

Alterations in CHOP uORF Translation Control Affect Cell Viability—CHOP regulates the transcription of multiple genes controlling apoptosis via CHOP homodimerization and heterodimerization with additional factors (26, 27). Two of the pro-apoptotic genes that are known to be transcriptionally upregulated through CHOP activity are *ATF5* and *BIM* (10, 12). Analysis of *ATF5* and *BIM* mRNAs in both $CHOP^{-/-}$ and WT CHOP FRT cell lines revealed that CHOP serves to enhance expression of these two genes in response to ER stress (Fig. 6*A*). Of importance, basal and stress-induced levels of both *ATF5* and *BIM* mRNAs were sharply increased in the ΔuORF CHOP cells, coincident with the enhanced CHOP protein expression (Figs. 5*A* and 6*A*). These results suggest that disruption of *CHOP* translation control results in a CHOP-dependent increase in *ATF5* and *BIM* transcripts that requires stress for maximal expression.

Next we determined the consequences of the ΔuORF and enhanced CHOP protein levels on cell viability. The WT and ΔuORF CHOP-expressing cells were treated with thapsigargin or tunicamycin for up to 12 h, and MTT activity was measured. Tunicamycin blocks *N*-glycosylation and is also a potent inducer of ER stress. In both ER stress conditions, ΔuORF CHOP cells presented with decreased MTT activity compared with the cells expressing WT uORF CHOP (Fig. 6*B*). There was a 15% decrease in MTT activity in CHOP ΔuORF cells after 18 h of treatment with thapsigargin and nearly 20% lowered MTT

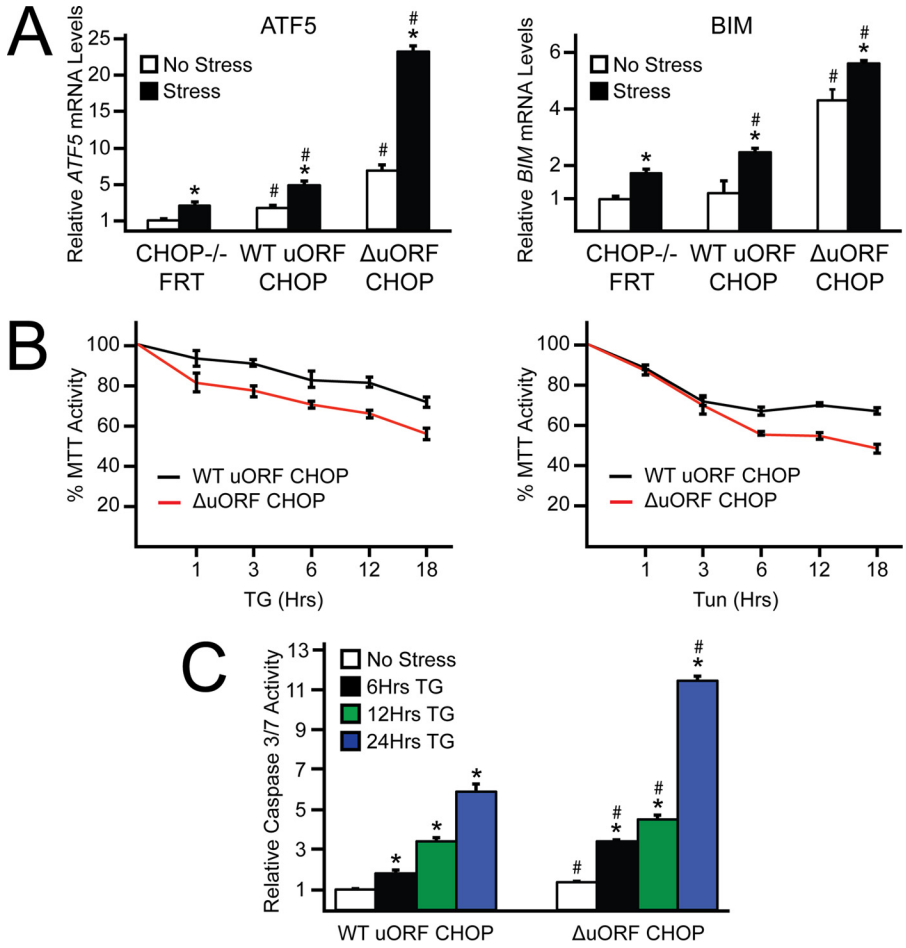

FIGURE 6. **Alterations in *CHOP* uORF translation control lower cell viability during stress.** *A*, total RNA was collected from WT uORF CHOP and ΔuORF CHOP cells cultured in the presence or absence of thapsigargin. Relative levels of *ATF5* and *BIM* mRNAs were measured in the cultured cells by qRT-PCR. *B*, equal numbers of WT uORF CHOP and ΔuORF CHOP cells were seeded in 96-well plates, cultured for 24 h, followed by treatment with thapsigargin or tunicamycin, as indicated, for up to an additional 18 h. MTT activity was measured by conversion of tetrazolium to formazan. *C*, equal numbers of WT uORF CHOP and ΔuORF CHOP cells were seeded in 96-well plates, cultured for 24 h, followed by treatment with or without thapsigargin for up to an additional 24 h. Caspase 3/7 activity was measured by cleavage of a proluminescent caspase-3/7 DEVD-aminoluciferin substrate.

activity in response to tunicamycin. Caspase 3/7 activity was also significantly increased in the ΔuORF cells following up to 24 h of exposure to thapsigargin (Fig. 6*C*), suggesting that increased apoptosis occurs in response to ER stress with the sharply enhanced CHOP protein levels. Collectively, these results suggest that disruption of *CHOP* uORF-mediated translation regulation decreases cell viability upon exposure to ER stress.

Discussion

CHOP expression is suggested to be critical for transitioning the transcriptome from a stress alleviation program to one of programmed cell death (7–10, 28, 29). The 5′-leader of *CHOP* mRNA contains an inhibitory uORF that is suggested to contribute to *CHOP* translation control through a Bypass mechanism (4, 20). In this study, we characterized the features of the *CHOP* uORF that serve to repress downstream translation during basal conditions and facilitate preferential translation in response to eIF2α-P and the role that these regulatory elements play in cell viability. As illustrated in the model presented in Fig. 4*C*, the *CHOP* uORF functions to block downstream CDS translation in basal conditions. Central to this low level of downstream translation is an inhibitory Ile-Phe-Ile sequence that efficiently stalls elongating ribosomes, thus promoting low levels of translation reinitiation at the CDS. During ER stress, however, eIF2α-P facilitates a ribosomal bypass, in part because of its poor start codon context, and allows for ribosome initiation at the downstream *CHOP* CDS (Fig. 4*C*).

Although the Ile-Phe-Ile sequence of the *CHOP* uORF appears to be important for the stall of elongating ribosomes during translation of the *CHOP* uORF, we do not yet fully understand the molecular basis underlying this ribosomal stall. The codon encoding the second Ile in the Ile-Phe-Ile sequence is a less frequently used codon, but individual alanine substitutions at each of the Ile-Phe-Ile positions were not sufficient to alleviate the elongation stall. Rather, simultaneous substitution of each of the codon positions to alanine reduced the inhibitory elongation stall. Ribosome profiling analysis of ribosome stalls genome-wide indicates that the mere presence of an Ile-Phe-Ile sequence does not appear to be sufficient for stalling ribosomes (30). However, in-frame fusions of a portion of the *CHOP* uORF containing the Ile-Phe-Ile sequence are suggested to be sufficient to stall ribosomes even when embedded into larger coding sequences, suggesting that short uORFs or placement of the

Ile-Phe-Ile sequence in the 5′-leader of the mRNA are not obligate. These findings suggest that other features of the *CHOP* uORF can also be contributors, such as additional RNA sequences or the encoded polypeptide residues flanking the Ile-Phe-Ile encoded in the *CHOP* uORF.

Role of uORFs in Regulating Cell Viability—Induced CHOP expression during eIF2α-P serves to promote programmed cell death when ISR signaling slated to alleviate stress damage is insufficient to restore cellular homeostasis. We show that loss of the *CHOP* uORF-mediated translation control results in a significant increase in CHOP levels, along with lowered cell viability upon exposure to ER stress. With the enhanced levels of CHOP protein, the CHOP ΔuORF cells displayed a different pattern of CHOP expression during a time course of thapsigargin treatment (Fig. 5). There was higher basal expression of CHOP protein in the CHOP ΔuORF cells that was accompanied by enhanced translation as judged by the association of *CHOP* mRNA with heavy polysomes (Figs. 1*A* and 5*B*). Of note, overexpression of CHOP resulted in decreased induction of *CHOP* gene transcription upon ER stress (Fig. 5, *C* and *D*) and overexpressed CHOP displayed increased turnover (Fig. 5*E*). As a consequence, both the transcriptional down-regulation and protein destabilization would contribute to the decrease in CHOP protein levels detected in the CHOP ΔuORF cells following 6 h of thapsigargin treatment (Fig. 5*A*). Previous reports have suggested that CHOP can serve to autorepress its own transcription by CHOP heterodimerization with and inhibition of the positive transcriptional activity of C/EBPβ (25). This suggests that in addition to ISR feedback dephosphorylation of eIF2α-P by GADD34 (22), additional autoregulatory mechanisms also serve to control CHOP levels and activity as a part of the ISR. It should be emphasized that even with the lowered *CHOP* mRNA and increased CHOP protein turnover, there was a significant enhancement of CHOP protein both basally and with the addition of ER stress in the ΔuORF CHOP cells. This finding highlights the fact that translational expression of CHOP is a major feature in its regulated expression.

Elevated translational expression of CHOP in the ΔuORF CHOP cells resulted in significant mRNA increases from the CHOP target genes *ATF5* and *BIM* (Fig. 6*A*). However, only upon addition of ER stress were there significant differences in cell viability between those cells expressing WT levels of CHOP and overexpressed CHOP (Fig. 6*B*). This is consistent with an earlier report that forced expression of CHOP up-regulated mRNA expression of downstream target genes but required an ER stress stimulus to induce apoptosis (31). Interestingly, there was substantial *BIM* expression independent of stress in the ΔuORF CHOP cells, whereas *ATF5* is largely induced by stress. The presence of both CHOP and ATF4 has been previously shown to be required for maximal *ATF5* expression, and both CHOP and ATF4 bind to the *ATF5* promoter (10, 11, 31). This argues for the requirement of additional stress-induced transcription factors to promote maximal expression of pro-apoptotic genes such as *ATF5* and that *ATF5*, and its pattern of expression is paramount in the observed stress-induced cell death. These findings indicate that misregulation of *CHOP* expression does not cause a substantial increase in apoptosis in unstressed cells but rather preprograms the transcriptome to alter the timing and magnitude of the change in cell fate to apoptosis after stress and the induction of the ISR. It is noted that the levels of CHOP protein levels expressed in ΔuORF CHOP cells were highly elevated at 1 and 3 h of ER stress but became diminished at 6 h. The consequential reduced MTT activity and increased caspase 3/7 activity of ΔuORF CHOP cells was readily detectable by 6 h of ER stress. These findings suggest that during early exposure to ER stress, CHOP protein levels achieved a critical level and duration that triggered a program of gene expression directing substantial death of the ΔuORF CHOP cells.

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