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Ribosome Reinitiation Directs Gene-specific Translation and Regulates the Integrated Stress Response*

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Background: eIF2α-P induced GADD34 and constitutively expressed CReP target PP1c to dephosphorylate eIF2α-P to dictate translation control of the ISR.
Results: Differential expression of GADD34 and CReP is regulated by upstream ORF (uORF)-mediated ribosome reinitiation.
Conclusion: uORFs regulate differential expression of GADD34 and CReP and are important for cell adaptation to stress.
Significance: Regulation of eIF2α-P is central for protein homeostasis and cell viability.

In the integrated stress response, phosphorylation of eIF2α (eIF2α-P) reduces protein synthesis to conserve resources and facilitate preferential translation of transcripts that promote stress adaptation. Preferentially translated GADD34 (PPP1R15A) and constitutively expressed CReP (PPP1R15B) function to dephosphorylate eIF2α-P and restore protein synthesis. The 5′-leaders of *GADD34* and *CReP* contain two upstream ORFs (uORFs). Using biochemical and genetic approaches we show that features of these uORFs are central for their differential expression. In the absence of stress, translation of an inhibitory uORF in *GADD34* acts as a barrier that prevents reinitiation at the *GADD34* coding region. Enhanced eIF2α-P during stress directs ribosome bypass of the uORF, facilitating translation of the *GADD34* coding region. *CReP* expression occurs independent of eIF2α-P via an uORF that allows for translation reinitiation at the *CReP* coding region independent of stress. Importantly, alterations in the *GADD34* uORF affect the status of eIF2α-P, translational control, and cell adaptation to stress. These results show that properties of uORFs that permit ribosome reinitiation are critical for directing gene-specific translational control in the integrated stress response.

Protein synthesis is dynamic and is modulated in response to a variety of environmental stresses. An important mechanism regulating translation involves phosphorylation of eukaryotic initiation factor 2 (1). During the initiation phase of translation, eIF2 associates with initiator Met-tRNA$_i^{Met}$, GTP, and ultimately the 40S ribosomal subunit to facilitate start codon selection. Phosphorylation of the α subunit of eIF2 at serine 51 (eIF2α-P) inhibits the exchange of eIF2-GDP for eIF2-GTP, blocking delivery of the initiator tRNA that triggers a global reduction in initiation of protein synthesis (2). Reduced protein synthesis serves to conserve energy and resources and allows cells to reconfigure gene expression to alleviate stress damage. Facilitating the reprogramming of gene expression, eIF2α-P also leads to the preferential translation of specific transcripts that facilitate adaptation to a specific stress condition. Because there are multiple mammalian eIF2 kinases, each directing translational control in response to different stress arrangements, this pathway has been referred to as the integrated stress response (ISR)[2] (2).

The 5′-leader of mRNAs containing upstream ORFs (uORFs) that precede the coding sequence (CDS) is critical for translational control in response to eIF2α-P. Among the ISR gene transcripts that are subject to preferential translation are those encoding transcription factors ATF4 (CREB2) and CHOP (DDIT3/GADD153) that serve to direct the transcriptome to address cellular stress, and GADD34 (PPP1R15A) that combines with the catalytic subunit of protein phosphatase 1 (PP1c) to target dephosphorylation of eIF2α-P and restore protein synthesis (3–6). GADD34 functions in combination with a constitutively expressed PP1c-targeting subunit CReP (PPP1R15B) (7), and together their expression, along with activities of eIF2 kinases, dictate the amount of eIF2α-P and degree of translational control. Emphasizing the importance of the GADD34 and CReP in the ISR, pharmacological agents have been reported that inhibit their functions and have significant medical implications (8–10). Preferential translation of *ATF4*, *CHOP*, and *GADD34* ensure that the levels of these short-lived regulatory proteins in the ISR are tightly linked to the levels of eIF2α-P. Additionally, each of these key ISR regulatory genes are transcriptionally induced in response to stress, ensuring the availability of mRNAs for enhanced translation.

The uORFs in the ISR gene transcripts perform specific roles in preferential translation in response to eIF2α-P. For example, two uORFs in *ATF4* convey translational control (3). In the "delayed translation reinitiation" model, the short 5′-proximal uORF1 of the *ATF4* mRNA serves as a positive-acting element that during eIF2α-P allows for scanning ribosomes to surpass an inhibitory uORF2 that overlaps out-of-frame with the CDS

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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; ER, endoplasmic reticulum; TK, thymidine kinase; FRT, Flp recombination target; qPCR, quantitative PCR.

and instead translate the *ATF4* polypeptide. This model has features that are conserved with *GCN4* translational control in yeast (11). Preferential translation of *CHOP* during eIF2α-P occurs via a bypass mechanism, where a single inhibitory uORF is bypassed due in part to a less than optimal start codon context (4). Of interest, translation of the *CHOP* uORF is suggested to trigger an elongation pause, ensuring no translation reinitiation at the downstream CDS.

Although uORFs are central for preferential translation of the ISR genes, the presence of uORFs alone are not sufficient to ensure enhanced translation in response to eIF2α-P. Approximately 40% of mammalian mRNAs contain uORFs, and genome-wide analyses of changes in translation in response to eIF2α-P suggest that uORFs are equally present among those gene transcripts whose translation are enhanced, repressed, or resistant to eIF2α-P (12). These findings suggest that there are specific properties for each uORF that delineate whether the 5′-leader of the mRNA directs activation or repression of translation in response to eIF2α-P. These uORF properties could include the sequences of the uORF coding and/or flanking regions, the length of the uORF, and the proximity of the uORF to the CDS of the transcript and with other uORFs.

In this study, we addressed the nature of uORFs that facilitate preferential translation in response to eIF2α-P. Translation of *GADD34* mRNA is enhanced in response to eIF2α-P and serves a central role for feedback regulation of the ISR (5, 13), whereas the related CReP (PPP1R15B) is suggested to be expressed independently of eIF2α-P and functions to target PP1c for dephosphorylation of eIF2α-P under basal conditions (7, 14). Both *GADD34* and *CReP* mRNAs contain two uORFs, and using biochemical and genetic approaches we define the central regulatory features that direct translational control of the two paralogs. We also show that alteration of these regulatory features in *GADD34* alter the dynamics of the induction of the ISR, which has significant effects on cell adaptation to stress. This study not only provides a mechanistic understanding of translational control of key ISR genes during eIF2α-P, but also provides for rules to help predict the effects of uORFs in translational control and for new insight into the utility of therapeutic approaches to modulating levels of eIF2α-P and the ISR.

Experimental Procedures

Cell Culture and Generation of Stable Cell Lines—WT and A/A mouse embryonic fibroblast (MEF) cells, which express a WT version of eIF2α and eIF2α-S51A, were cultured in Dulbecco's modified Eagle's medium (DMEM) as previously described (15). GADD34ΔC/ΔC MEF cells were kindly provided by David Ron (University of Cambridge, UK) and were previously described (16). Stable Flp-In GADD34ΔC/ΔC cells lines were generated by using the Flp-In System (Invitrogen) and full-length *GADD34* cDNAs including 1-kb of the *GADD34* promoter and mutant versions of the *GADD34* 5′-leader that were integrated into the genome following the manufacturer's instructions. GADD34ΔC/ΔC FRT, GADD34-WT2, GADD34-OPT2, GADD34-AAA2, and GADD34-Δ2 MEFs were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1× nonessential amino acids, and 55 μM β-mercaptoethanol.

TABLE 1
Description of primers used for qPCR in this study

Primer name	Primer sequence
GADD34: forward primer	5′-AGGACCCCGAGATTCCTCT-3′
GADD34: reverse primer	5′-CCTGGAATCAGGGGTAAGGT-3′
CReP: forward primer	5′-GGCTACAGTGGCCTTCTCTG-3′
CReP: reverse primer	5′-CATCCATCCCTTGCAAATTC-3′
CHOP: forward primer	5′-CGGAACCTGAGGAGAGAG-3′
CHOP: reverse primer	5′-CGTTTCCTGGGGATGAGATA-3′
β-Actin: forward primer	5′-TGTTACCAACTGGGACGACA-3′
β-Actin: reverse primer	5′-GGGGTGTTGAAGGTCTCAAA-3′
Firefly luciferase: forward primer	5′-CCAGGGATTTCAGTCGATGT-3′
Firefly luciferase: reverse primer	5′-AATCTCACGCAGGCAGTTCT-3′

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 (17). Quantification of immunoblots was conducted using ImageJ software. Antibodies used for the immunoblot analyses include: GADD34 (Proteintech catalog number 10449-1-AP), CReP (Proteintech catalog number 14634-1-AP), eIF2α-P (Abcam catalog number ab32157), CHOP (Santa Cruz Biotechnology catalog number sc-7351), and β-actin (Sigma catalog number A5441). Monoclonal antibody measuring total eIF2α was kindly provided by Dr. Scott Kimball (Pennsylvania State University College of Medicine, Hershey, PA).

mRNA Measurement by qRT-PCR—RNA was isolated from MEF cells and polysome 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 qPCR using SYBR Green (Applied Biosystems) on a Realplex2 Master Cycler (Eppendorf). Primers used for measuring transcripts are listed in Table 1.

Polysome Profiling and Sucrose Gradient Ultracentrifugation—MEF cells were left untreated or treated with 1 μM thapsigargin for 6 h. Cells were incubated in culture media containing 50 μg/ml of cycloheximide just prior to lysate collection. Lysates were collected, sheared, and layered on top of 10–50% sucrose gradients followed by ultracentrifugation as previously described (12, 18). Sucrose gradients were fractionated and whole cell lysate polysome profiles were collected using a Piston Gradient Fractionator (BioComp) and a 254-nm UV monitor with Data Quest Software.

Following fractionation, 10 ng/ml of firefly luciferase control RNA (Promega) was spiked into each pooled sample to generate polysome shifts for specific transcripts normalized to an exogenous RNA control (12, 18). Samples were mixed with 750 μl of TRIzol, and RNA isolation and cDNA generation was performed as described above. Calculations for % total gene transcript and % transcript shifts are as described previously (12). Whole cell lysate polysome profiles and mRNA polysome shifts are representative of three independent biological experiments.

Plasmid Constructions and Luciferase Assays—A 5′-rapid amplification of cDNA ends (5′-RACE; FirstChoice Ambion) was performed using RNA lysates collected from WT MEF cells treated with 1 μM thapsigargin for 6 h, or left untreated, to

TABLE 2
Description of *GADD34* and *CReP* mutations used in this study

Gene construct	Description of mutation
GADD34 stem loop insertion	Insertion of CTGCAGCCACCACGGCCCCCAAGCTTGGGCCG-TGGTGGCTGCAG 3′ of GCTCTGAGT
GADD34 uORF1 ΔATG	ATG to AGG
GADD34 uORF2 ΔATG	ATG to ATA
GADD34 uORF2 strong Kozak Context	GGCGACATGA to GCCACCATGA
GADD34 uORF2 extension	TGA to GGA
GADD34 uORF2 ΔAA 5–25	Deletion of CTTCGCGAGCAGTCCGGACCCACGATCGCTTTTGGCAACCAGAACCGG CGCTTCAGCCCCCGGGG
GADD34 uORF2 ΔAA 15–25	Deletion of TTTGGCAACCAGAACCGGCGCTTCAGCCCCCGGGG
GADD34 uORF2 frameshift	Deletion of A 3′ of ATGA and insertion of A 5′ of GGTGA
GADD34 uORF2 PPG to AAA	Mutation of CCCCCGGGGTGA to GCCGCGGCGTGA
GADD34 uORF2 insert AAA	Insertion of GCCGCCGCC 3′ of CCCCCGGGG
CReP stem loop insertion	Insertion of CTGCAGCCACCACGGCCCCCAAGCTTGGGCCGTGGTGGCTGCAG 3′ of GCGGCCATT
CReP uORF1 ΔATG	ATG to AGG
CReP uORF2 ΔATG	ATG to AGG
CReP uORF2 extension	TAG to GGA
CReP uORF2 replaced with GADD34 uORF2	Replacement of ATGGACCTAACTGTGTAG with ATGAACCCGCCCCCGGGGTGA
CReP 21 nucleotides surrounding uORF2 start codon replaced with GADD34 21-nucleotide surrounding uORF2 start codon	Replacement of CTAGCCGGGATGGACCTAACC with GAGGGCGACATGAACCCGCTG
CReP 21 nucleotides surrounding uORF2 stop codon replaced with GADD34 21-nucleotide surrounding uORF2 stop codon	Replacement of GAGACTGTGTAGACCTCGGAT with CCCCCGGGGTGACGTGCAGCC
CReP 9 nucleotides 5′ of uORF2 stop codon replaced with GADD34 9 nucleotides 5′ of uORF2 stop codon (encoding PPG)	Replacement of GAGACTGTG with CCCCCGGGG
CReP 9 nt 3′ of uORF2 stop codon replaced with GADD34 9 nucleotides 3′ of uORF2 stop codon	Replacement of ACCTCGGAT with CGTGCAGCC

determine the transcriptional start sites for *GADD34* and *CReP*. The cDNA segments encoding the 5′-leader of *GADD34* and *CReP* were inserted between HindIII and NcoI between the TK-promoter and firefly luciferase CDS in a derivative of plasmid pGL3 (3). The resulting P_{TK}-GADD34-Luc and P_{TK}-CReP-Luc contain the mouse *GADD34* and *CReP* 5′-leaders and the start codon for each CDS fused to a luciferase reporter. Site-directed mutagenesis and subcloning of synthesized cDNAs were used to generate mutant P_{TK}-GADD34-Luc and P_{TK}-CReP-Luc constructs (Table 2) that were sequenced to verify nucleotide substitutions. P_{TK}-GADD34-Luc and P_{TK}-CReP-Luc constructs were transiently co-transfected with a *Renilla* reporter plasmid into WT or A/A MEF cells for 24 h followed by a 6-h 0.1 μM thapsigargin treatment. 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. indicated.

The T7 promoter of sequence, TAATACGACTCACTATAGGGAGA, and the *GADD34* 5′-leader containing the start codon for the *GADD34* CDS were inserted between HindIII and NcoI in the pGL3 basic luciferase vector (Promega) for generation of P_{T7}-GADD34-Luc constructs for *in vitro* translation assays. Sequencing was used to verify nucleotide substitutions and *in vitro* assays were conducted as described below.

In Vitro Transcription and Translation Assays—Capped and polyadenylated RNA was synthesized with T7 RNA polymerase using mMESSAGE mMACHINE T7 Ultra (Ambion) from P_{T7}-GADD34-Luc constructs. Synthesized *GADD34-Luc* mRNA was added to rabbit reticulocyte lysate (Promega) per the manufacturers' instructions. For luciferase assays, *in vitro* translation reactions with *GADD34-Luc* mRNA were carried out for 20 min at 30 °C, and firefly luciferase activity was measured.

For primer extension inhibition (Toeprint) assays, reticulocyte lysates were treated with cycloheximide upon addition of the *GADD34-Luc* mRNA to measure initiating ribosomes (time 0) or 5 min after addition of the transcript to measure ribosomal localization during steady-state translation (time 5). Toeprint assays were conducted as previously described and using primers: 5′-TGAAGCGCCGGTTCTGGTTG-3′ (Fig. 4*D*) and ZW4, 5′-TCCAGGAACCAGGGCGTA-3′ (Fig. 4*E*) (19).

Cell Number and Viability Assays—For cell proliferation assays, GADD34-WT2, GADD34-OPT2, GADD34-AAA2, and GADD34-Δ2 MEFs were seeded at 5,000 cells/well in a 96-well plate. Cells were fixed (3.7% formalin) and stained (10 μg/ml Hoechst) immediately following seeding, or 24 and 48 h after seeding, and fluorescence was measured on a Synergy H1 Microplate reader (BioTek).

MTT assays were carried out by seeding cells at 5,000 cells/well in a 96-well plate. Cells were cultured for 24 h, and MTT activity was measured using CellTiter 96-well Non-radioactive Cell Proliferation Assay (Promega). For measurements of MTT activity after ER stress treatment, cells were seeded, allowed to grow for 24 h, and treated with 0.4 μM thapsigargin with or without 1 μM guanabenz, or left untreated for an additional 24 h.

Statistical Analyses—Values represent the mean ± S.D. and were derived from 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 "*", and treatment groups considered statistically significant from WT control are indicated by a "#" sign.

Results

eIF2α-P Is Required for GADD34 Transcription and Translation, but CReP Expression Occurs Independent of eIF2α-P—GADD34 expression is enhanced during eIF2α-P, whereas CReP levels are suggested to be independent of eIF2α-P (5, 7). To further explore the role that eIF2α-P and translational control play in the differential expression of GADD34 and CReP, we measured changes in their mRNA and protein levels in wild-

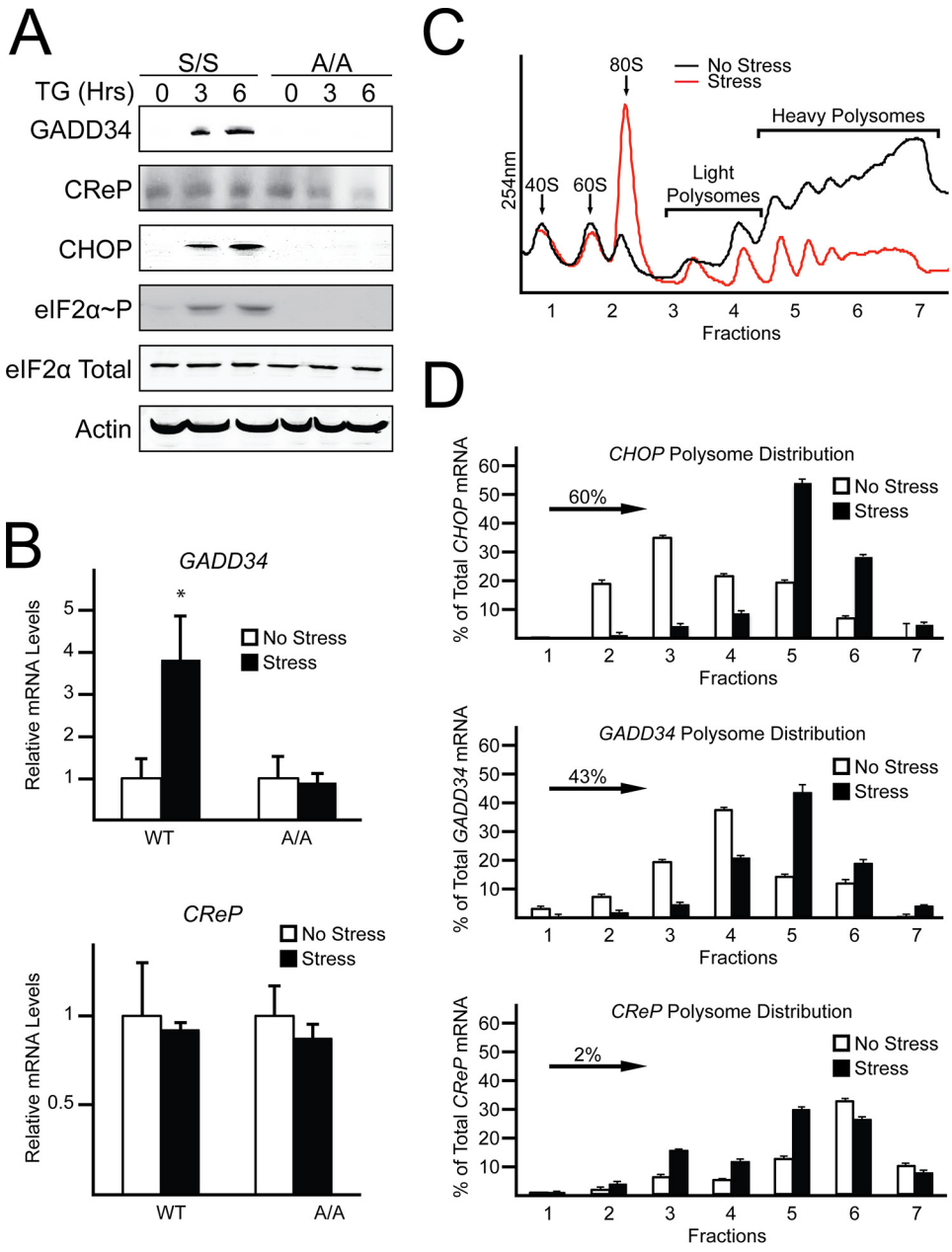

FIGURE 1. **eIF2α-P is required for induced *GADD34* translation, but CReP expression occurs independent of eIF2α-P.** *A*, WT and A/A MEF cells were treated with thapsigargin, for up to 6 h or left untreated. Lysates were processed and levels of GADD34, CReP, CHOP, eIF2α-P, eIF2α total, and β-actin were measured by immunoblot. *B*, total RNA was collected from WT and A/A MEFs treated with thapsigargin for 6 h or left untreated and relative levels of *GADD34* and *CReP* mRNA were measured by qRT-PCR. *C*, WT MEF 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. *D*, total RNA was isolated from sucrose fractions and the percentage of total *CHOP*, *GADD34*, and *CReP* mRNAs was determined by qRT-PCR. *Panels C* and *D* are representative of three independent biological experiments.

type (WT) MEF cells and mutant MEF cells (A/A) expressing eIF2α-S51A that cannot be phosphorylated. eIF2α-P was induced only in WT cells by treatment with thapsigargin, a potent trigger of endoplasmic reticulum (ER) stress (Fig. 1*A*). Both *GADD34* mRNA and protein levels were increased in WT MEF cells, whereas there was no change in *GADD34* mRNA and minimal protein expression in A/A cells (Fig. 1, *A* and *B*). By contrast, there was no change in the amount of *CReP* mRNA and protein in WT cells upon ER stress. Of interest, whereas the levels of *CReP* mRNAs were similar between WT and A/A cells, there was reduced CReP protein in A/A cells during ER stress (Fig. 1, *A* and *B*).

To explore the role of translational control in the differential expression of GADD34 and CReP, WT MEF cells were subjected to thapsigargin treatment, and lysates were prepared and analyzed by polysome profiling using sucrose density ultracentrifugation. As expected, polysome profiling revealed that ER stress led to reduced global translation initiation as viewed by a decrease in polysomes coincident with increased monosomes (Fig. 1*C*). *GADD34* and *CReP* mRNAs, along with *CHOP* mRNA, which is known to be subject to preferential translation, were then measured by qRT-PCR in the polysome fractions. Both *GADD34* and *CHOP* transcripts were predominantly associated with monosomes and disomes in the absence of

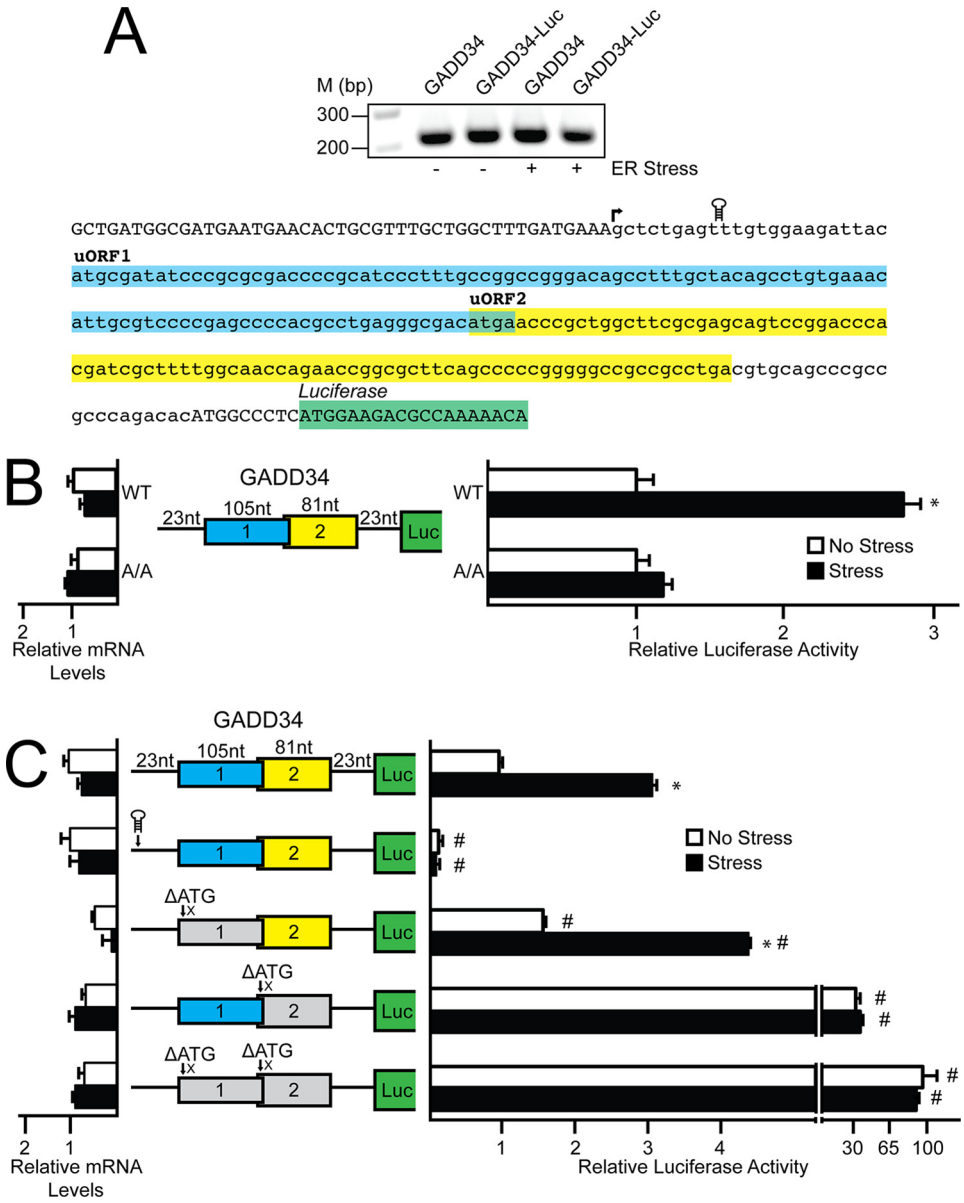

FIGURE 2. **Preferential translation of *GADD34* features an inhibitory uORF.** *A*, *top panel*, 5′-RACE was carried out for *GADD34* using WT MEFs treated with thapsigargin for 6 h or left untreated; total RNA was prepared and DNA products were separated by gel electrophoresis, with markers of the indicated base pair sizes illustrated on the *left*. *A*, *bottom panel*, Representation of *GADD34* 5′-leader in *lowercase letters*, with *uppercase letters* representing the 5′-linker added during the 5′-RACE procedure and the beginning of the CDS of the GADD34-Luc fusion. *Colored boxes* represent the *GADD34* uORFs and the coding region of the GADD34-Luc fusion. The transcription start site is indicated with an *arrow*, and location of the stem loop insertion is illustrated. *B*, the P_{TK}-GADD34-Luc construct and a *Renilla* luciferase reporter were co-transfected into WT or A/A MEF cells and treated for 6 h with thapsigargin or left untreated. *GADD34* translation control was measured via dual luciferase assay and corresponding *GADD34-Luc* mRNA was measured by qRT-PCR. The P_{TK}-GADD34-Luc construct contains the cDNA sequence corresponding to the *GADD34* 5-leader fused to the luciferase reporter gene with both *GADD34* uORFs and the CDS of the GADD34-Luc fusion indicated with *colored boxes*. *C*, WT and mutant versions of P_{TK}-GADD34-Luc were transfected into WT MEFs, treated for 6 h or left untreated, and measured using a dual luciferase assay and qRT-PCR. Mutant versions of P_{TK}-GADD34-Luc include a stem loop insertion and mutation of the initiation codons for uORFs individually or together, as represented by ΔATG. Relative values are represented as histograms for each with the S.D. indicated.

stress. However, upon thapsigargin treatment and eIF2α-P, there was a significant shift of the *GADD34* and *CHOP* transcripts to heavy polysomes (Fig. 1*D*). By contrast, *CReP* mRNA was associated with heavy polysomes in both thapsigargin-treated cells and those not subjected to stress. Together these results suggest that in addition to transcriptional induction, *GADD34* is preferentially translated upon stress and eIF2α-P, whereas *CReP* is largely translated independent of the stress conditions. These results are consistent with earlier reports that indicated that expression of GADD34 is induced upon stress as part of a feedback control of the ISR and CReP is constitutively present (5, 7).

Preferential Translation of GADD34 Features an Inhibitory uORF—Next we carried out a 5′-RACE to define the transcriptional start site in mouse of the *GADD34* gene (Fig. 2*A*). A cDNA segment encoding the 228-nucleotide sequence of the 5′-leader of *GADD34* was then inserted between a minimal TK promoter and the firefly luciferase reporter CDS, generating P_{TK}-GADD34-Luc. This luciferase reporter featured the initiation codon of the *GADD34* CDS fused in-frame to the luciferase

CDS. Expression of GADD34-Luc was increased 3-fold in WT MEF cells treated with thapsigargin as compared with no change in luciferase activity in A/A cells (Fig. 2*B*). In these reporter measurements, and subsequent ones discussed below, there was no significant change in the luciferase mRNA. These results indicate that the 5′-leader of *GADD34* directs preferential translation in response to eIF2α-P.

To determine whether enhanced *GADD34* translation occurs via ribosome scanning, a palindromic sequence with a predicted free energy of $\Delta G = -41$ kcal/mol was inserted 10 nucleotides downstream of the 5′ cap of the *GADD34-Luc* mRNA (Fig. 2*A*). Addition of this stem-loop to the *GADD34-Luc* transcript significantly decreased luciferase activity independent of stress, indicating that preferential translation mediated by the 5′-leader of *GADD34* occurs by ribosome scanning (Fig. 2*C*). Ribosomes scanning the 5′-leader of *GADD34* encounter two uORFs before reaching the start codon for the *GADD34* CDS. To determine the contribution of the two uORFs to *GADD34* translation regulation, the uORF start codons were mutated from ATG to an AGG or ATA, as indicated by the ΔATG in Fig. 2*C*. Deletion of uORF1 alone led to a small increase, albeit significant, in the basal luciferase expression, with an induction upon ER stress that was similar to the reporter with the WT version of the *GADD34* 5′-leader. This result suggests that uORF1 serves to modestly dampen downstream translation regardless of stress. By comparison, deletion of uORF2 led to a 30-fold increase in luciferase activity independent of ER stress, indicating that uORF2 is inhibitory to downstream translation and is the dominant regulatory uORF in the *GADD34* 5′-leader, a finding consistent with Lee *et al.* (13). Combined deletion of uORF1 and uORF2 led to an additional increase in luciferase activity, further supporting the roles of uORF1 and uORF2 as repressing elements in *GADD34* translational expression (Fig. 2*C*).

GADD34 Translation Control Involves Bypass of an Inhibitory uORF—The initiation codon context for the *GADD34* uORF2 (GGCGACAUGU) is less than optimal compared with the Kozak consensus sequence (GCC(A/G)CCAUGG), a feature similar to the single uORF present in the *CHOP* mRNA that is subject to the bypass model of translational control (4). To determine whether context of the start codon plays a role in uORF2-mediated regulation of *GADD34* translation, the poor start codon context of uORF2 was mutated to the optimal Kozak consensus. Mutation of uORF2 to the strong Kozak context reduced luciferase expression basally and decreased stress-induced luciferase activity to 2.7-fold as compared with a 3.3-fold induction for the WT GADD34-Luc reporter (Fig. 3*A*). This finding suggests that uORF2 can be bypassed during eIF2α-P in part due to its poor initiation codon context, thereby enhancing translation of the downstream *GADD34* CDS.

Translation initiation downstream of uORFs can also be dependent on translation reinitiation (2, 11). To determine the contribution of post-uORF2 translation reinitiation in *GADD34* translation regulation, the stop codon of uORF2 was mutated from TGA to GGA, resulting in an uORF that overlaps out-of-frame with the luciferase CDS (Fig. 3*A*). There was no statistically significant difference between the WT P_{TK}-GADD34-Luc and the reporter with the uORF2 overlapping the CDS. This finding argues against significant ribosome reinitiation at the *GADD34* CDS following synthesis of the uORF2 polypeptide, and instead supports the idea that preferential translation of *GADD34* CDS relies on ribosomal bypass of the inhibitory uORF2.

Inhibitory Function of GADD34 uORF2 Is Reliant on Pro-Pro-Gly Juxtaposed to the uORF2 Stop Codon—Many features of uORFs, including length and coding sequences, can promote the repressing functions of uORFs. To investigate the inhibitory nature of the *GADD34* uORF2, in-frame deletions from codons 5–25 and 15–25 were analyzed in the GADD34-Luc reporter. Both deletions in the uORF2 coding sequence increased luciferase expression independent of stress, suggesting that the repressing function of uORF2 lies at least in part within its coding sequence (Fig. 3*C*).

To address whether the RNA sequence in uORF2 per se contributes to the repressing functions of this uORF, a single nucleotide was deleted just after the ATG start codon in uORF2 and a single nucleotide was inserted just prior to the TGA termination codon. The resulting frameshift thus maintains the uORF2 nucleotide sequence and length, but the uORF now encodes a different polypeptide. Luciferase activity of this frameshift reporter was increased in the presence or absence of stress, consistent with the hypothesis that the encoded uORF2 polypeptide sequence is responsible for the inhibitory function of uORF2 in translational control (Fig. 3*C*).

A comparison of the uORF2-encoding polypeptide sequences among mammals revealed several conserved segments, including a carboxyl-terminal Pro-Pro-Gly sequence (Fig. 3*B*). Contiguous prolines and Pro-Pro-Gly sequences have been suggested to be problematic for translation elongation and require eIF5A for efficient protein synthesis (20). Substitution of the uORF2 codons encoding the Pro-Pro-Gly sequence with codons encoding Ala-Ala-Ala resulted in a 5-fold increase in luciferase activity basally, while retaining a modest induction during ER stress (Fig. 3*C*). Alteration of the uORF2 start codon to an optimal Kozak sequence in the presence of the Pro-Pro-Gly to Ala-Ala-Ala substitution also led to elevated luciferase activity in the absence of stress, along with a modest increase (1.3-fold) upon thapsigargin treatment. These results suggest that bypass of the uORF2 during stress is required for maximal induction of *GADD34* translation, because translation of the codons encoding the uORF2 Pro-Pro-Gly residues precludes the ribosome from efficiently reinitiating at the downstream CDS. Insertion of three alanine residues between the Pro-Pro-Gly sequence and the uORF2 termination codon also led to similar increases in luciferase activity in the presence or absence of stress (Fig. 3*C*), indicating that the ability of the Pro-Pro-Gly sequence in uORF2 to inhibit translation reinitiation involves its juxtaposition to a termination codon.

Translation of the *CHOP* inhibitory uORF is suggested to trigger an elongation pause that is responsible for lowering downstream translation reinitiation (4). This can be visualized by low expression of an in-frame fusion of the *CHOP* uORF with the luciferase CDS. Luciferase activity of the *CHOP* uORF-Luc fusion protein is significantly enhanced upon deletion of its critical inhibitory sequences and alleviation of the elongation pause (Fig. 4*A*). We therefore asked if the Pro-Pro-Gly

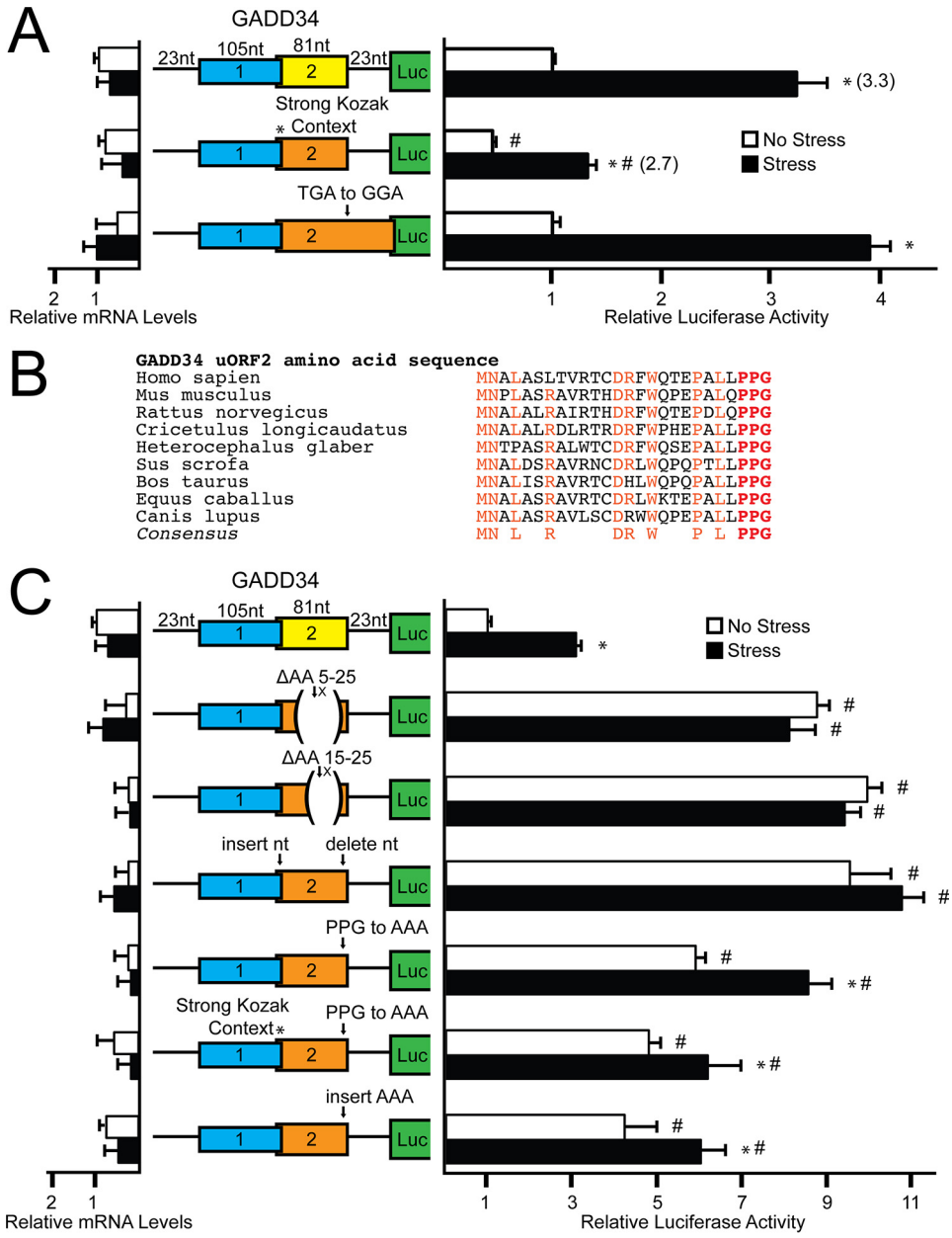

FIGURE 3. ***GADD34* translation control involves bypass of an inhibitory uORF that relies on a Pro-Pro-Gly juxtaposed to the uORF2 stop codon.** *A*, WT and mutant versions of P_{TK}-GADD34-Luc were transfected into WT MEFs, treated for 6 h or left untreated, and measured using a dual luciferase assay and qRT-PCR. Mutant versions of P_{TK}-GADD34-Luc include mutation of the *GADD34* uORF2 poor start codon context to "*, strong Kozak context," and mutation of the stop codon of uORF2 to generate and overlapping out-of-frame uORF (TGA to GGA). Relative values are represented as histograms for each with the S.D. indicated. *B*, polypeptide sequence encoded by *GADD34* uORF2 from different vertebrates. The uORF2 polypeptide sequences were from cDNAs derived from *GADD34* orthologs, including human (GenBank™ accession number NM_014330), mouse (NM_008654), rat (NM_133546), hamster (L28147), naked mole rat (XM_004889808), pig (XM_003127275), cow (NM_001046178), horse (XM_001489532), and dog (XM_533626). Residues conserved between the uORF sequences are listed in the consensus and are highlighted. *C*, WT and mutant versions of P_{TK}-GADD34-Luc were transfected into WT MEFs, treated for 6 h or left untreated, and measured using a dual luciferase assay and qRT-PCR. Mutant versions of P_{TK}-GADD34-Luc include deletion of codons 5–25 or 15–25 (ΔAA 5–25 or ΔAA 15–25) and a frameshift construct in which a nucleotide was inserted just following the uORF2 ATG start codon and deleted just prior to the uORF2 stop codon. Additional constructs included mutation of the codons encoding Pro-Pro-Gly to codons for Ala-Ala-Ala (PPG to AAA), simultaneous mutation of the uORF2 start codon to Kozak consensus sequence with the Pro-Pro-Gly to Ala-Ala-Ala mutation (* Strong Kozak Context, PPG to AAA), and insertion of codons encoding Ala-Ala-Ala just prior to the uORF2 stop codon (insert AAA). Relative values are represented as histograms for each with the S.D. indicated.

sequence in *GADD34* uORF2 results in an elongation pause by making a similar in-frame fusion between *GADD34* uORF2 and the luciferase CDS. There was elevated expression of the *GADD34* uORF-Luc fusion, suggesting that the *GADD34* Pro-Pro-Gly sequence does not facilitate a pause in ribosomal elongation (Fig. 4*A*).

We also analyzed the effects of selected *GADD34* uORF2 mutations for translational expression by using T7 RNA polymerase to synthesize *GADD34-Luc* mRNAs that were introduced into *in vitro* translation assays using rabbit reticulocyte lysates. Consistent with our analysis of MEF cells, mutations of the initiation codon of uORF2 led to elevated luciferase activity, which was further enhanced by combined loss of uORF1 (Fig. 4*B*). Substitution of the Pro-Pro-Gly codons to Ala-Ala-Ala in uORF2 also led to significantly enhanced luciferase expression compared with WT. Finally, introduction of an optimized

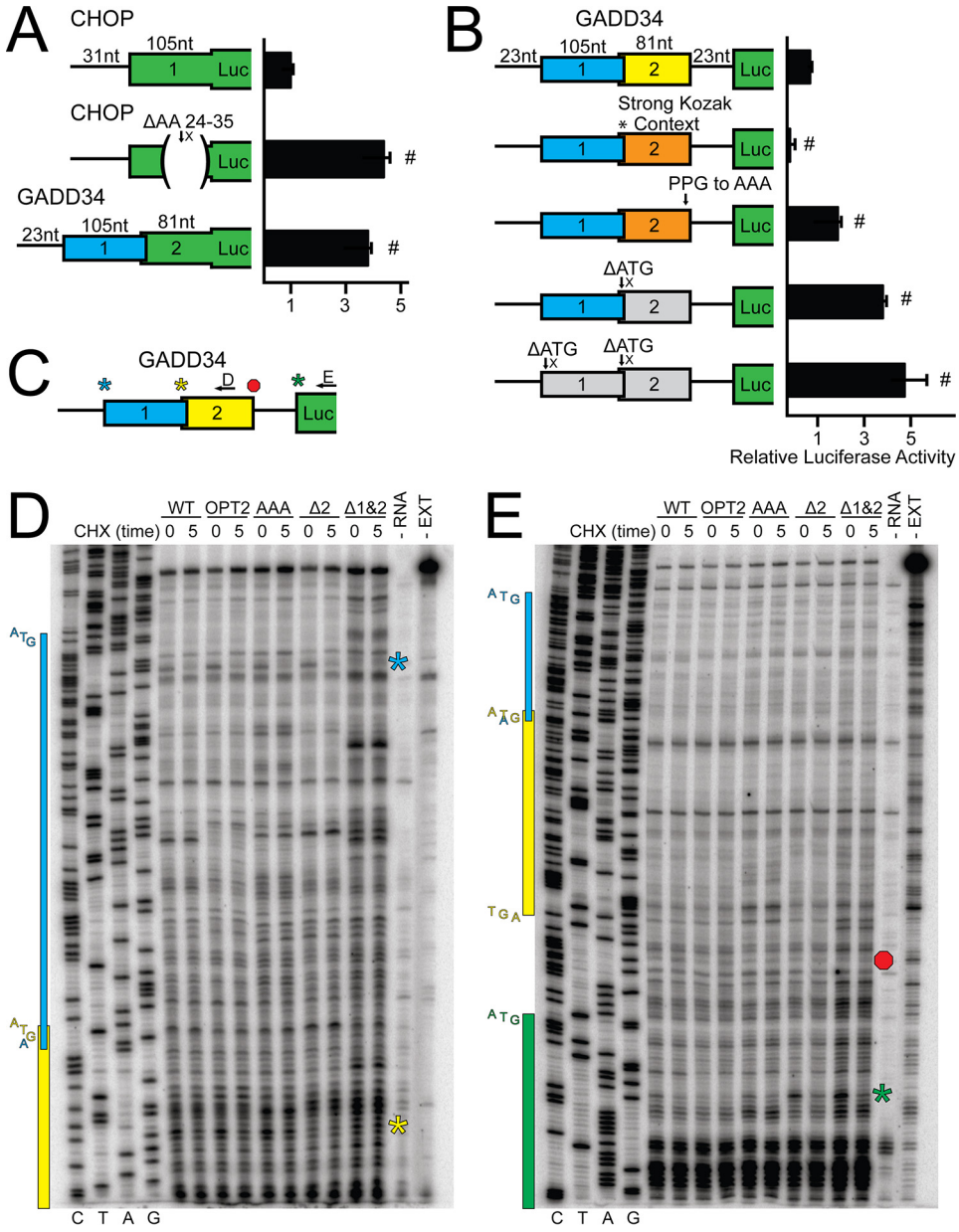

FIGURE 4. ***GADD34* translation control involves bypass of an inhibitory uORF.** *A*, in-frame fusions between firefly luciferase reporter with the WT *CHOP* uORF, *CHOP* uORF deleted for codons 24–35, or *GADD34* uORF2 and firefly luciferase, were transfected into WT MEF cells, treated for 6 h, or left untreated, and measured using a dual luciferase assay. Relative values are represented as histograms for each with the S.D. indicated. *B*, WT and mutant versions of *GADD34-Luc* mRNAs were added to *in vitro* translation reactions and expression was measured using a luciferase assay. Mutant versions of P_{T7}-GADD34-Luc included uORF2 start codon context mutated to the Kozak consensus sequence (*, strong Kozak context), substitution of the codons for Pro-Pro-Gly to codons for Ala-Ala-Ala (PPG to AAA), and deletion of uORF2 or uORF1&2 (ΔATG). Data are representative of three independent biological experiments. *C*, depiction of the toeprint design using the WT version of *GADD34-Luc* mRNA. *Black arrows* represent the location of primers used in *panels D* and *E*. Toeprints corresponding to initiation at uORF1, uORF2, or the luciferase CDS are indicated by *blue*, *yellow*, or *green stars*, respectively. Termination at uORF2 is indicated by a *red octagon*. *D* and *E*, reticulocyte lysates were treated with cycloheximide (*CHX*) upon addition of WT or mutant versions of *GADD34-Luc* mRNA to measure initiating ribosomes (time 0), or 5 min after addition of the transcript to measure ribosome localization during steady state translation (time 5). Toeprint assays were conducted for each sample and sequencing reactions can be read 5′ to 3′ from top to bottom. The nucleotide residue 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 absence of rabbit reticulocyte lysate translation mixture (−*EXT*) are indicated on the *right*. The *green star* represents the toeprint corresponding to initiation at the luciferase coding region and the *yellow star* and *red octagon* represent the toeprint corresponding to initiation and termination of the *GADD34* uORF2. Initiation at uORF1 is indicated by a *blue star*. The *blue*, *yellow*, and *green colored boxes* on the *left* span the sequences to corresponding to uORF1, uORF2, and the luciferase CDS, respectively, and are comparable with the *GADD34* 5′-leader schematic in *panel C*. The start and stop codons for each ORF are represented to the *left* of their corresponding colored box. Mutant constructs utilized are the same as listed in *panel B* and data are representative of three independent biological experiments.

Kozak context for the initiation codon of uORF2 sharply lowered luciferase activity (Fig. 4*B*). Together these results support the idea that the Pro-Pro-Gly codons are important for the uORF2 inhibition of downstream CDS translation.

As the *GADD34* uORF2 fusion construct from Fig. 4*A* does not take into account the dependence of the Pro-Pro-Gly sequence on juxtaposition to a termination codon for its inhibitory nature, toeprinting experiments using two different prim-

ers were used to map the positions of ribosomes along the 5′-leader of *GADD34* transcripts during *in vitro* translation (Fig. 4*C*). Reticulocyte lysates were treated with cycloheximide upon addition of the *GADD34-Luc* mRNA to measure where ribosomes first initiate translation (time 0). Alternatively, cycloheximide was added 5 min after addition of the transcript to measure ribosome positions during steady-state translation and active polypeptide synthesis (time 5). At time 0 there were toeprints measuring the ribosomes positioned at the initiation codons of uORF1 (*blue star*) and uORF2 (*yellow star*) and the luciferase CDS (*green star*) (Fig. 4, *D* and *E*). Mutation of the initiation codon of uORF2 individually or in combination with uORF1 resulted in lowered toeprint signals at the uORF2, with a similar corresponding increase in initiation at the luciferase CDS (Fig. 4, *D* and *E*). Initiation at the luciferase CDS is also observed at time 5 for these mutant transcripts, indicating that translation initiation at the dominant uORF2 precludes ribosome reinitiation at the downstream CDS during steady state translation (Fig. 4*E*).

Introduction of optimized Kozak context for the initiation codon of uORF2 significantly reduced translation initiation at the CDS at both time 0 and time 5, indicating increased ribosomal preference for the more 5′ optimized start codon of uORF2 largely precludes translation at the downstream CDS (Fig. 4*E*). A modest toeprint at the uORF2 termination codon is also observed (red hexagon) for both the WT and optimized uORF2 mRNAs at time 5. Substitution of the Pro-Pro-Gly codons to Ala-Ala-Ala resulted in a 32% reduction in the toeprint signal at the uORF2 termination codon as compared with the WT mRNA. Collectively these results indicate that there is not an extended ribosome pause at uORF2, but the Pro-Pro-Gly sequence can promote inefficient ribosome termination (Fig. 4*E*).

CReP Translation Is Dampened by an Inhibitory uORF in an eIF2α-P Independent Manner—CReP has two uORFs with similar spatial arrangements to the 5′-leader of *GADD34* mRNA, yet CReP expression appears to be unchanged in WT cells upon eIF2α-P and stress. We carried out a 5′-RACE to define the transcriptional start site for mouse *CReP*, and determined that the 5′-leader of the *CReP* mRNA is 421 nucleotides in length (Fig. 5*A*). The cDNA encoding the *CReP* 5′-leader was inserted between the TK promoter and luciferase reporter gene, generating P_{TK}-CReP-Luc. Transfection of P_{TK}-CReP-Luc into WT and A/A MEF cells resulted in similar levels of luciferase activity independent of stress treatment and eIF2α-P (Fig. 5*B*). Levels of *CReP-Luc* mRNA in these assays, as well as those described below, were similar, indicating that luciferase activity is a measure of translational expression. These results further support the idea that *CReP* mRNA is efficiently translated independent of eIF2α-P.

To determine whether *CReP* translation occurs by ribosome scanning, a stem-loop was inserted 10 nucleotides downstream of the 5′-end of the *CReP* mRNA (Fig. 5*C*). Insertion of the stem-loop structure sharply reduced luciferase activity in the presence or absence of ER stress, consistent with the requirement for ribosome scanning for *CReP* translation. As ribosomes scanning the 5′-leader of *CReP* would encounter two uORFs, the uORF start codons were changed from ATG to AGG individually or in combination, as indicated by ΔATG in Fig. 5*C*. Deletion of uORF1 led to a modest reduction in luciferase activity in the presence or absence of stress (Fig. 5*C*). By contrast, deletion of uORF2 led to a 7-fold increase in luciferase activity independent of stress, suggesting that uORF2 is the dominant repressing uORF in *CReP* translation, a feature shared with *GADD34*. Deletion of both uORF1 and uORF2 led to a further increase in luciferase activity.

To address the ability of ribosomes to reinitiate downstream after translation of uORF2, the *CReP* uORF2 stop codon was mutated from a TGA to a GGA, generating an extended uORF that overlaps out-of-frame with the *CReP* CDS. The overlapping uORF2 resulted in a 5-fold reduction in basal luciferase activity, which was increased 2-fold upon ER stress (Fig. 6*A*). These results suggest that although a small portion of the scanning ribosomes can bypass *CReP* uORF2 and initiate downstream translation, the majority of ribosomes that initiate at the downstream *CReP* CDS are reinitiating ribosomes that have previously translated uORF2.

Regulatory Properties of GADD34 uORF2 Are Transferable to a Heterologous 5′-Leader Derived from CreP—We next determined if the regulatory properties of *GADD34* uORF2 could be transferred to a heterologous 5′-leader derived from *CReP*. We began by replacing the *CReP* uORF2 with the coding sequence of *GADD34* uORF2 (Fig. 6*A*). The proximity of the *GADD34* uORF2 in the context of the *CReP* uORF1 and CDS was also the same as that of WT *CReP*. Introduction of the *GADD34* uORF2 into the CReP-Luc reporter led to a significant reduction in luciferase activity in the absence of stress, which was induced 3.3-fold upon ER stress (Fig. 6*A*). This result indicates that uORF2 of *GADD34* is a transferable element that can direct preferential translation of a heterologous mRNA.

To define critical portions of the *GADD34* uORF2 that confer translational control, we introduced smaller portions of the *GADD34* uORF2 into the CReP-Luc reporter (Fig. 6*A*). Exchange of 21 nucleotides centered on the initiation codon of the *GADD34* uORF2 for the corresponding sequences in *CReP* resulted in no significant differences from the WT CReP-Luc reporter (Fig. 6*A*). We interpret this finding to suggest that the enhanced ability of *CReP* uORF2 to allow for reinitiation at the downstream CDS diminishes in part the translational control that can be imparted by bypass of the substituted *GADD34* start codon.

Exchange of a 21-nucleotide sequence that includes the Pro-Pro-Gly and *GADD34* uORF2 stop codon for the corresponding sequences in CReP-Luc resulted in a large decrease in luciferase activity in non-stressed conditions that was enhanced over 4-fold upon ER stress (Fig. 6*A*). This result indicates that the 3′-portion of the *GADD34* uORF2 is sufficient to confer significant preferential translation to *CReP* upon stress. To delineate further the contribution of this 21-nucleotide sequence for preferential translation, we exchanged only the 9 nucleotides encoding the Pro-Pro-Gly residues from *GADD34* uORF2 for the corresponding sequences in the *CReP* uORF2 in the CReP-Luc reporter (Fig. 6*A*). Introduction of the nucleotides encoding the Pro-Pro-Gly sequence led to a decrease in basal luciferase activity that was stress-inducible, similar to that observed for exchange of the entire *GADD34* uORF2 in *CReP*.

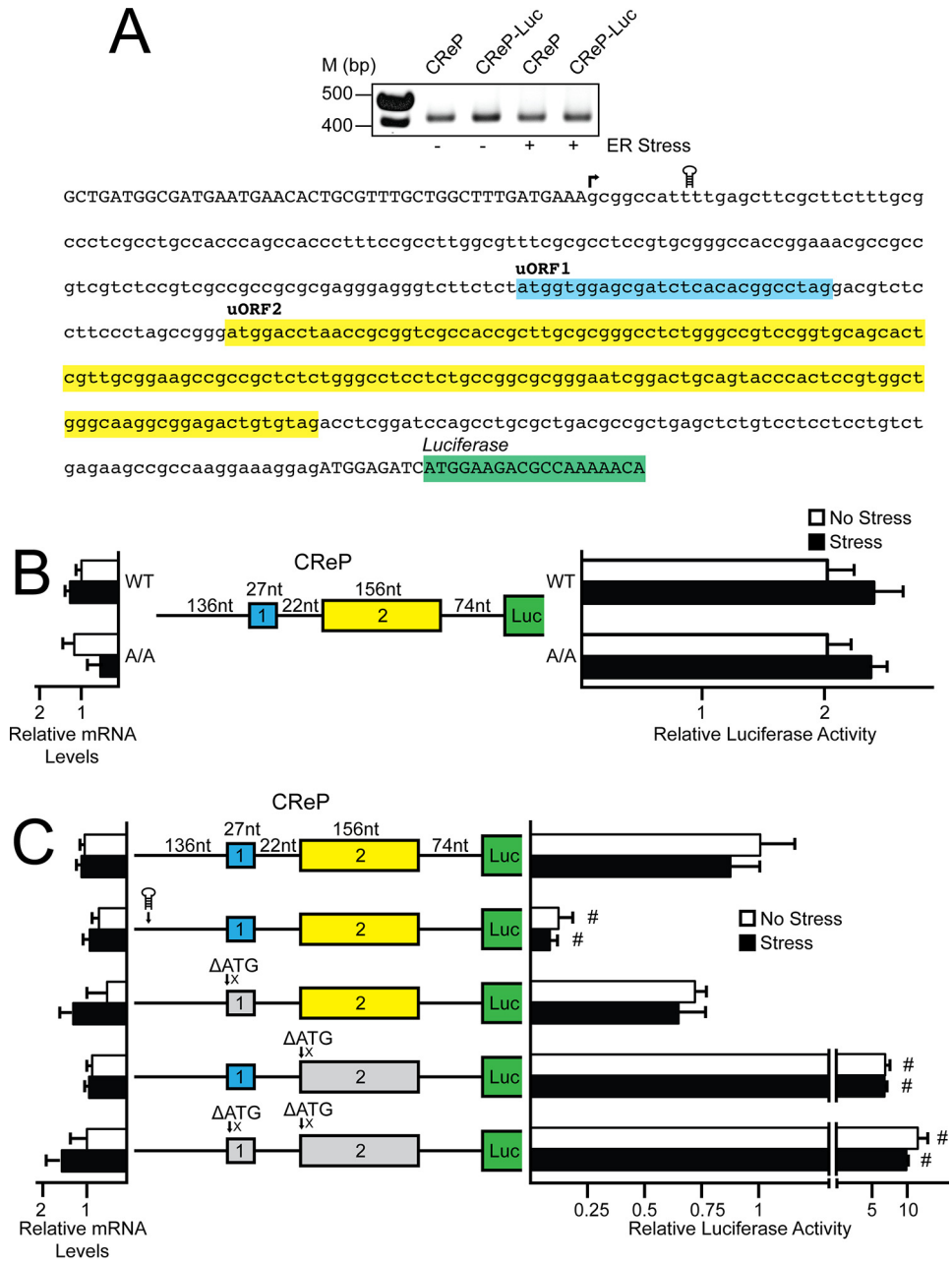

FIGURE 5. ***CReP* translation is dampened by an inhibitory uORF in an eIF2α-P independent manner.** *A*, *top panel*, a 5′-RACE was carried out for *CReP* using WT MEF cells either transfected with P_{TK}-CReP-Luc or left untransfected. MEFs were treated with thapsigargin for 6 h or left untreated and total RNA was prepared. DNA products were separated by gel electrophoresis and a DNA ladder with markers of the indicated base pair sizes is illustrated on the *left*. *A*, *bottom panel*, the sequence of the *CReP* 5′-leader is represented in *lowercase letters* with *uppercase letters* representing the 5′-linker added during the 5′-RACE procedure and the coding region of the CReP-Luc fusion. *Colored boxes* represent the two *CReP* uORFs and the coding region of the CReP-Luc fusion. The transcription start site is indicated with an *arrow* and the location of stem loop insertion is illustrated. *B* and *C*, WT and mutant versions of P_{TK}-CReP-Luc and a *Renilla* luciferase reporter were co-transfected into WT or A/A MEF cells, as indicated, and treated for 6 h with thapsigargin or left untreated. *CReP* translation control was measured by dual luciferase assay and corresponding *CReP-Luc* mRNA was measured by qRT-PCR. The P_{TK}-CReP-Luc construct contains the cDNA sequence corresponding to the *CReP* 5′-leader fused to the luciferase reporter gene with both *CReP* uORFs and the CDS of the CReP-Luc fusion indicated with *colored boxes*. Relative values are represented as histograms for each with the S.D. indicated. Mutant versions of P_{TK}-CReP-Luc include a stem loop insertion and deletion of both uORFs individually or together, as represented by ΔATG.

This result suggests that the *GADD34* uORF2 Pro-Pro-Gly sequence can serve to block translation reinitiation in the heterologous *CReP* 5′-leader. The exchange of the 9 nucleotides following the termination codon of the *GADD34* uORF2 for the corresponding region of the *CReP* 5′-leader resulted in luciferase activities similar to WT CReP-Luc, although there was some induction upon ER stress. These findings suggest that the Pro-Pro-Gly sequence encoded in *GADD34* uORF2 is the dominant regulator of downstream translation reinitiation and is central to preferential translation, with the 9 nucleotides following the uORF2 of *GADD34* playing a modest role in this regulation. Additionally, the proximity of the uORF to the CDS of the transcript does not appear to be a key feature of the regulation imparted by the *GADD34* Pro-Pro-Gly sequence due to its ability to regulate expression predictably in the *CReP* 5′-leader, even though *CReP* uORF2 is nearly 3 times further

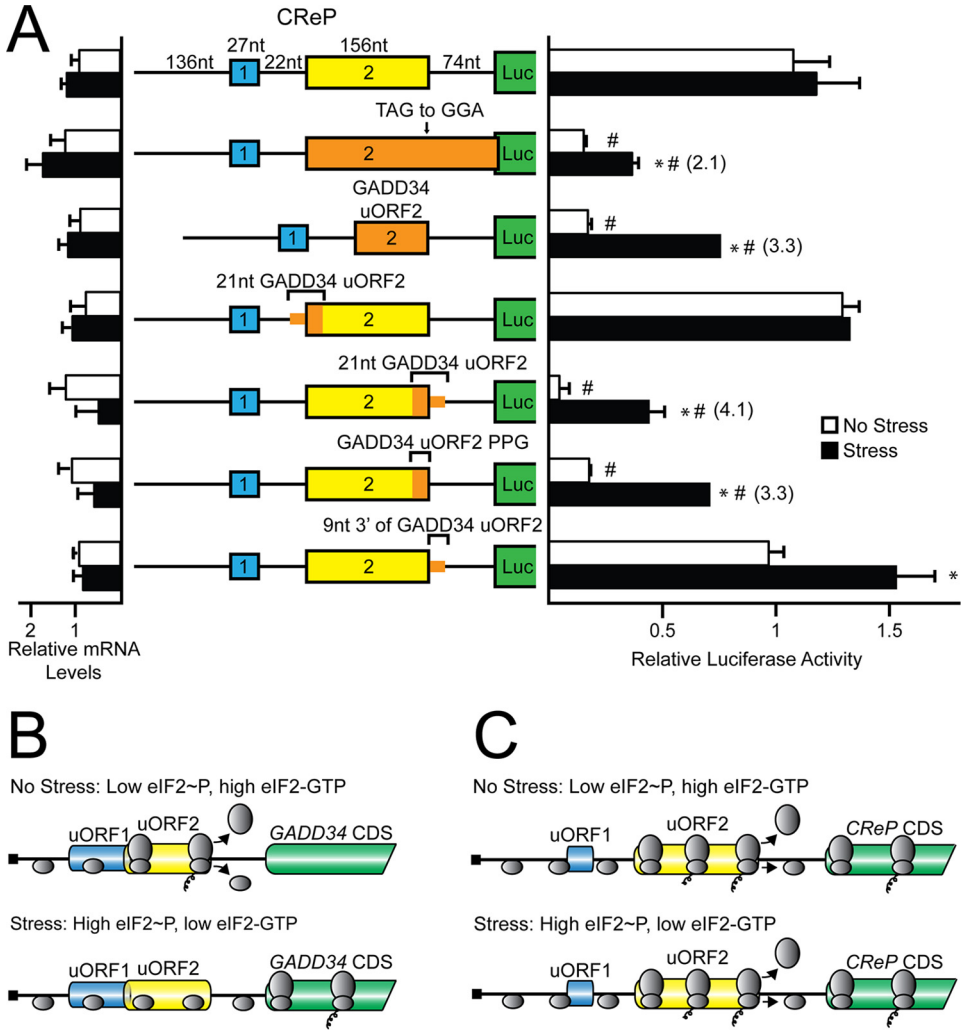

FIGURE 6. **Regulatory properties of *GADD34* uORF2 are transferable to a heterologous 5′-leader derived from *CReP*.** *A*, WT and mutant versions of P_{TK}-CReP-Luc were transfected into WT MEFs, treated for 6 h or left untreated, and measured using a dual luciferase assay and qRT-PCR. Mutant versions of P_{TK}-CReP-Luc include mutation of the uORF2 stop codon from TAG to GGA to generate an overlapping out-of-frame uORF (*TAG to GGA*), insertion of *GADD34* uORF2 in place of *CReP* uORF2 (*GADD34 uORF2*), insertion of the 21 nucleotides surrounding *GADD34* uORF2 start codon in place of the corresponding *CReP* uORF2 sequence (*21nt GADD34 uORF2*), insertion of the 21 nucleotides surrounding *GADD34* uORF2 stop codon in place of the corresponding *CReP* uORF2 sequence (*21nt GADD34 uORF2*), insertion of the codons encoding *GADD34* uORF2 Pro-Pro-Gly sequence in place of the corresponding *CReP* uORF2 sequence, and insertion of the 9 nucleotides 3′ of *GADD34* uORF2 in place of the corresponding *CReP* sequence. Relative values are represented as histograms for each with the S.D. indicated. *B*, model for *GADD34* translational control. In the absence of stress, low eIF2α-P, and high eIF2-GTP, ribosomes scan the 5′-leader of the *GADD34* mRNA and initiate translation at *GADD34* uORF2. After translation of uORF2, terminating ribosomes are precluded from translation reinitiation downstream and are suggested to dissociate from the mRNA. In the presence of stress, eIF2α-P and low eIF2-GTP levels allow for some scanning ribosomes to bypass the *GADD34* uORF2 in part due to poor start codon context, and instead initiate translation at the *GADD34* CDS. *C*, model for *CReP* translational control. In the presence or absence of stress, ribosomes scan the 5′-leader of the *CReP* mRNA and initiate translation at the *CReP* uORF2. After translation of uORF2, a portion of the terminating ribosomes resume scanning and initiate translation downstream at the *CReP* CDS. It is noted that during stress and high eIF2α-P, a small portion of ribosomes can bypass the uORF2 and initiate translation at *CReP* CDS. Together these processes are suggested to lead to *CReP* translation independent of eIF2α-P.

from the CDS as is found for *GADD34* uORF2. Fig. 6, *B* and *C*, illustrate models for *GADD34* and *CReP*, highlighting the differential abilities of the uORF2 from each to allow for translation reinitiation. The translation models for *GADD34* and *CReP* and their broader implications will be further highlighted under "Discussion."

Alterations in the Regulatory Features of GADD34 uORF2 Affect Cell Viability during ER Stress—GADD34 is central for determining the appropriate levels of eIF2α-P in the ISR during transitions from basal to stress conditions, and vice versa. In turn, the amounts of eIF2α-P can dictate the levels of global and gene-specific translation that determine protein homeostasis and the health of the cell. To determine the role that *GADD34* translational control by the uORF2 has on eIF2α-P and cellular adaptation to ER stress, MEF cells were engineered such that they stably expressed *GADD34* with WT or selected mutant versions of uORF2. Initially, a Flp recombination target (FRT) site was integrated in a single location in the genome of GADD34 functional knock-out MEF cells (GADD34ΔC/ΔC). Integration of the FRT site was then followed by the insertion and clonal isolation of cells expressing full-length *GADD34* cDNAs under the control of 1 kb of the *GADD34* promoter, which ensures its proper transcriptional induction in response to ER stress (21).

Four different versions of the GADD34 expressing cells were generated using the FRT strategy, including MEF cells express-

ing *GADD34* with a WT uORF2 (WT2), an uORF2 with a mutant initiation codon (Δ2), an uORF2 with an initiation codon with optimal Kozak consensus (OPT2), or an uORF2 with Pro-Pro-Gly substituted to Ala-Ala-Ala (AAA2). This isogenic collection of GADD34-expressing cells was then cultured in the presence or absence of thapsigargin. Measurements of GADD34 protein revealed the predicted pattern of expression based on our analyses of endogenous GADD34 and GADD34-Luc reporters (Fig. 7*A*). For each, there was a significant increase in *GADD34* mRNA upon ER stress, indicating that transcription induction was retained for each version of *GADD34* (Fig. 7*B*). Cells expressing GADD34-Δ2 displayed sharply elevated GADD34 protein in the absence of stress, which was induced 57-fold upon thapsigargin treatment as compared with GADD34-WT2 (Fig. 7*A*). The GADD34-AAA2 cells presented with GADD34 protein that was expressed independent of stress, which were much lower than that measured in GADD34-Δ2 cells, but greater than that expressed in cells with GADD34-WT2. Finally, GADD34-OPT2 displayed minimal detectable GADD34 protein even during ER stress.

Expression of these *GADD34* uORF variants led to significant changes in the levels of eIF2α-P during stress treatment. Of note, the sharply elevated GADD34 expression in GADD34-Δ2 cells led to a decrease in eIF2α-P in response to ER stress as compared with cells containing the GADD34-WT2 (Fig. 7*A*). GADD34-AAA2 cells presented with a partial lowering of induced eIF2α-P. Polysome analyses of cells expressing GADD34-WT2 or GADD34-Δ2 supported the translational control effects predicted from the patterns of induced eIF2α-P (Fig. 7*C*). In both non-stressed and ER stress conditions, the GADD34-Δ2 cells displayed increased polysome levels compared with GADD34-WT2. These results suggest that *GADD34* expression is tightly regulated through uORF2-mediated translational control to allow for the optimal amounts of eIF2α-P during stress. It is also noted that whereas *CReP* mRNA and protein levels are considered to be constitutively expressed independent of ER stress, that there were significant differences in *CReP* expression among the cells containing the selected uORF2 versions of *GADD34*. The most dramatic changes were found in cells expressing GADD34-Δ2, where coincident with increased GADD34 protein levels there was a sharp reduction in *CReP* mRNA and protein levels upon ER stress (Fig. 7, *A* and *B*). Furthermore, in GADD34-OPT2 cells there was a 2-fold increase in *CReP* mRNA upon thapsigargin treatment, although the CReP protein levels appeared to be unchanged. These results suggest that *CReP* mRNA levels can be modulated depending on the nature of *GADD34* translational expression.

To determine how the status of *GADD34* translational control by uORF2 affects cell homeostasis, cell nuclei were stained with Hoechst fluorescent dye, and MTT activity was measured to assess cell number and vitality. Both measures were significantly increased in GADD34-Δ2 and GADD34-AAA2 expressing cells compared with GADD34-WT2 (Fig. 7, *D* and *E*). GADD34-OPT2 cells were significantly reduced for MTT activity, and trended lower for Hoechst staining, although without significance. These results suggest that the status of *GADD34* translational expression can affect cell homeostasis.

Next the collection of GADD34-expressing cells were treated with thapsigargin to determine how the status of the uORF2 and *GADD34* translational expression can affect their ability to adapt to ER stress. Although each of the cells showed reduced MTT activity upon ER stress, the GADD34-Δ2 that expressed the highest levels of GADD34 fared the most poorly, whereas the GADD34-OPT2 with the lowest levels of GADD34 protein expression showed the most resistance (Fig. 7*F*). These results suggest that enhanced GADD34 expression can render cells more sensitive to stress. Supporting this idea, the addition of guanabenz, a potent inhibitor of GADD34 targeting of PP1c dephosphorylation of eIF2α-P (8) to the GADD34-Δ2 cells substantially alleviated its sensitivity to thapsigargin treatment (Fig. 7*G*).

Discussion

In this study, we address the nature of uORFs that facilitate preferential translation in response to eIF2α-P and the roles that these regulatory elements play in cell adaptation to stress. Levels of GADD34 and CReP expression are critical for determining the amounts of eIF2α-P and expression of the two paralogs has previously been shown to be differentially regulated in response to ER stress (5, 7, 12). The 5′-leaders of *GADD34* and *CReP* mRNAs contain two uORFs, with uORF2 in each serving as the dominant inhibitory element that is suggested to contribute to translational control (13, 14). We define here the central regulatory features by which each of the uORF2 sequences direct translational control of *GADD34* and *CReP*. As illustrated in a model presented in Fig. 6*B*, *GADD34* uORF2 serves as an efficient barrier to downstream CDS translation in basal conditions. Central to this low level of downstream translation reinitiation is an inhibitory Pro-Pro-Gly sequence juxtaposed to the termination codon in *GADD34* uORF2. However, during ER stress, eIF2α-P facilitates a bypass of *GADD34* uORF2 due, in part, to a poor start codon context, allowing for ribosome initiation at the *GADD34* CDS (Fig. 6*B*). It is important to note that only a small portion of ribosomes bypass the *GADD34* uORF2 during ER stress, as deletion of the uORF2 led to over 10 times more luciferase activity as compared with the WT during thapsigargin treatment (Fig. 2*C*). This level of bypass ensures that there is appropriate expression of GADD34 protein during feedback control of the ISR, which protects against premature restoration of translation during periods of ER stress.

Whereas the uORFs in *CReP* have some physical and functional similarities with *GADD34*, there are also several significant differences. Regarding similarities, both *GADD34* and *CReP* have two uORFs of comparable spatial arrangements, with uORF2 having a major repressing function on downstream CDS translation and uORF1 displaying a modest dampening role (Fig. 5*D*). Furthermore, ribosomes are suggested to bypass uORF2 in both *CReP* and *GADD34*, although the bypass occurs to a greater degree in *GADD34* (Fig. 6, *B* and *C*). The critical difference between *GADD34* and *CReP* lies in the ability of *CReP* uORF2 to facilitate more ribosome reinitiation at the downstream CDS. By comparing expression of CReP-Luc between WT and ΔuORF2 constructs in the absence of stress (Fig. 4*C*), we estimate that upwards of 12% of the ribosomes that translate uORF2 reinitiate at the *CReP* CDS. Using a similar

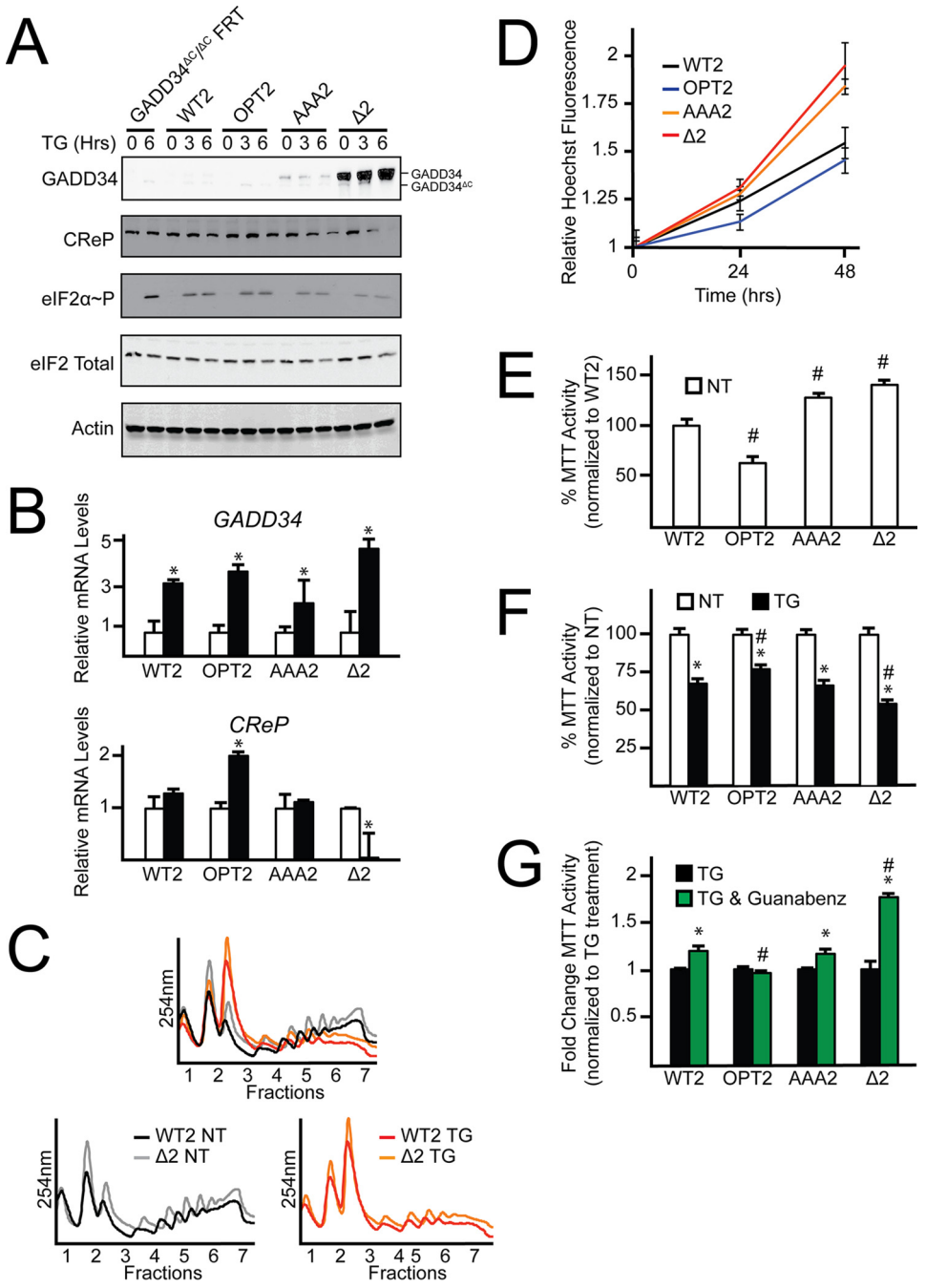

FIGURE 7. **Alterations in the regulatory features of *GADD34* uORF2 affect cell viability during ER stress.** *A*, MEF cells functionally deleted for *GADD34* via deletion of the GADD34 C-terminal PP1c interacting domain were stably selected to express WT *GADD34* (*WT2*), *GADD34* with an optimized uORF2 (*OPT2*), *GADD34* with uORF2 codons encoding Pro-Pro-Gly mutated to Ala-Ala-Ala (*AAA2*), and *GADD34* with uORF2 deleted (*Δ2*) and treated with ER stress agent, thapsigargin, for up to 6 h or left untreated. Full-length GADD34 is labeled as *GADD34*. Truncated GADD34 lacking the C-terminal PP1c interacting domain is labeled as *GADD34*$^{\Delta C}$. Lysates were processed and levels of GADD34, CReP, eIF2α-P, eIF2α total, and β-actin were measured by immunoblot. *B*, total RNA was collected from WT2, OPT2, AAA2, and Δ2 MEF cells cultured in the presence or absence of thapsigargin and relative levels of *GADD34* and *CReP* mRNA were measured by qRT-PCR. *C*, WT2 and Δ2 MEF 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 centrifugation and analysis of whole lysate polysome profiles at 254 nm. *D*, equal numbers of WT2, OPT2, AAA2, and Δ2 MEF cells were seeded in 96-well plates, cultured for 0, 24, or 48 h, and then fixed using 3.7% formalin with Hoechst stain. Relative values for Hoechst fluorescence are represented with the S.D. indicated. *E*, equal numbers of WT2, OPT2, AAA2, and Δ2 MEF cells were seeded in 96-well plates, cultured and allowed to grow for 24 h, and then MTT activity was measured. Relative values are represented as histograms for each with the S.D. indicated. *F*, the WT and mutant GADD34 cells were seeded in 96-well plates, cultured for 24 h, followed by treatment with or without thapsigargin for an addition 24 h. MTT activity was measured by the conversion of tetrazolium to formazan. *G*, the collection of GADD34 MEF cells were seeded in 96-well plates, cultured for 24 h, followed by treatment with thapsigargin with or without guanabenz for an additional 24 h. MTT activity was measured by the conversion of tetrazolium to formazan.

comparison for GADD34-Luc (Fig. 2*C*), it is estimated that less than 3% of ribosomes translating uORF2 reinitiate at the *GADD34* CDS. Together the modest bypass of uORF2 during ER stress and efficient ribosome reinitiation allow for constitutive ribosome translation of the *CReP* CDS. It is also of note that efficient reinitiation at the *CReP* CDS occurs with an uORF2 of longer length, 52 codons, which appears to differ with the suggested models whereby uORFs only a few codons in length are

necessary for appreciable ribosome reinitiation at a downstream CDS (22).

Roles of uORFs in Regulating the ISR and Cellular Resistance to Stress—Both GADD34 and CReP are responsible for directing PP1c to dephosphorylate eIF2α-P. As the amount of eIF2α-P can dictate the levels of global and gene-specific translation, regulation of GADD34 and CReP expression is central for maintaining protein homeostasis and health of the cell. We showed that alteration of the regulatory features in *GADD34* uORF2 results in significant changes in protein synthesis and cell vitality both basally and during ER stress (Fig. 7). Of note, deletion of *GADD34* uORF2 resulted in a dramatic increase in GADD34 expression, which then lowered levels of both eIF2α-P and translational control that coincided with increased sensitivity of the cells to ER stress. These results suggest that aberrant regulation of GADD34 expression alters the dynamics of the ISR, which would not allow sufficient time for stressed cells to induce ISR-target genes to alleviate stress damage before resumption of global translation. Paradoxically, functional deletion of GADD34 and chronically low levels of global translation have been previously shown to also result in increased sensitivity of cells to ER stress (23), which further emphasizes the importance of the mechanisms regulating GADD34 and CReP expression in the timing and magnitude of ISR induction. Interestingly, mice deleted for *GADD34* are resistant to renal toxicity upon ER stress treatment, suggesting that in tissues there are further complexities to the dynamics of the ISR (24). The mechanisms underlying differential regulation of *GADD34* and *CReP* translation also have implications for the utility of emerging drugs to modulate the ISR and its control of cell adaptation to intracellular and extracellular stresses (8–10).

uORFs Have Different Functions in Translational Control—Although uORFs are central for the preferential translation of key ISR genes, including *ATF4*, *CHOP*, and *GADD34*, the mere presence of an uORF is not sufficient to confer preferential translation in response to eIF2α-P. In fact, uORFs are suggested to be prevalent among gene transcripts whose translation is enhanced, repressed, or resistant to eIF2α-P. These findings suggest that specific features of each uORF delineate its ability to activate or repress downstream translation. This study suggests that the repressing function of *GADD34* uORF2 is dependent on low levels of ribosome reinitiation due to an inhibitory Pro-Pro-Gly sequence juxtaposed to a termination codon. There are different strategies for thwarting reinitiation, with the *ATF4* uORF2 overlapping out-of-frame with the downstream CDS and translation of the *CHOP* uORF leading to an elongation pause (3, 4). Interestingly, the sole *GADD34/CReP* paralog in *Drosophila melanogaster* has a single uORF that overlaps out-of-frame with the CDS (25), which suggests that this model organism has established an inhibitory uORF in a bypass mechanism through an alternative strategy from its *GADD34* mammalian counterpart. The rules for regulation of ribosome reinitiation examined here for *GADD34* and *CReP* provide new insight into uORF-mediated differences in expression in response to eIF2α-P. This has exciting implications for genome-wide assessments of translation, where the features of uORFs can be used to predict the translation control properties for a given mRNA during basal conditions and those inducing eIF2α-P.

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