Transient protein synthesis inhibition is an important protective mechanism used by cells during various stress conditions including endoplasmic reticulum (ER) stress. This response centers on the phosphorylation state of eukaryotic initiation factor (eIF)-2α, which is induced by kinases like protein kinase R-like ER kinase (PERK) and GCN2 to suppress translation and is later reversed so translation resumes. GADD34 was recently identified as the factor that activates the type 1 protein serine/threonine phosphatase (PP1), which dephosphorylates eIF-2α during cellular stresses. Our study delineates a negative feedback regulatory loop in which the eIF-2α-controlled inhibition of protein translation leads to GADD34 induction, which promotes translational recovery. We show that activating transcription factor 4 (ATF4), which is paradoxically translated during the eIF-2α-mediated translational block, is required for the transactivation of the GADD34 promoter in response to ER stress and amino acid deprivation. ATF4 directly binds to and trans-activates a conserved ATF site in the GADD34 promoter during ER stress. Examination of ATF4−/− MEFs revealed an absence of GADD34 induction, prolonged eIF-2α phosphorylation, delayed protein synthesis recovery, and diminished translational up-regulation of BiP during ER stress. These studies demonstrate the essential role of GADD34 in the resumption of protein synthesis, define the pathway for its induction, and reveal that cytoprotective unfolded protein response targets like BiP are sensitive to the eIF-2α-mediated block in translation.

When mammalian cells encounter physiological or chemical stresses that impinge upon the normal folding of proteins in the endoplasmic reticulum (ER), 1 rapid and complex signal transduction cascades, known collectively as the unfolded protein response (UPR), are put into play that are responsible for a number of cytoprotective measures (1–4). If homeostasis of the ER cannot be re-established, apoptotic pathways are activated to destroy the damaged cell in order to ultimately protect the organism (5). The protective components of the UPR include the transcriptional up-regulation of ER chaperones and folding enzymes. These proteins bind to unfolded proteins that begin accumulating in the ER and prevent their aggregation. In addition, the increased expression of the chaperones allows for the rapid refolding of nascent secretory pathway proteins once homeostasis has been re-established. A second protective response is the transient block in protein synthesis (6). This limits the load of proteins being introduced into a hostile environment where they would be unable to fold. The inhibition of protein synthesis is not limited to ER proteins and is responsible for a third aspect of the response, which is the arrest of cells in the G1 phase of cell cycle. The inhibition of the synthesis of the short lived cyclin D1 protein results in a rapid depletion of this protein and a concomitant cell cycle arrest (7). Finally, the up-regulation of proteins associated with proteasomal degradation helps to dispose of the accumulated unfolded proteins, further reducing the load on the ER (8, 9).

In the past 5 years, many components of the signaling pathways controlling these cytoprotective responses have been described. The ER-localized transmembrane protein, ATF6, encodes a cytosolic transcription factor tethered to an ER luminal “stress sensing” domain (10). ER stress allows ATF6 to travel to the Golgi where it is cleaved by the S1P and S2P proteases, thus liberating the cytosolic transcription factor domain (11, 12). Cleaved ATF6 binds to ER stress response elements in the chaperone promoters and up-regulates their transcription. In addition, three transmembrane ER kinases have been identified that also possess luminal “sensing” domains in addition to their cytosolic kinase domains. Two of the kinases, Ire1α and β (13, 14), are orthologues of the sole ER stress transducer in yeast, Ire1p. Like yeast Ire1p, these proteins possess a unique C-terminal endonuclease domain, which upon activation of the kinase domains targets a specific RNA sequence. Most recently, the XBP-1 transcription factor was shown to be the target of endonuclease activity of the mammalian Ire1 (15–17). The Ire1-catalyzed removal of 26 nucleotides from XBP-1 mRNA followed by a religation reaction alters the XBP-1 reading frame. The remodeled XBP-1 protein encodes a novel transcription factor with the original DNA binding domain tethered to a new transactivation domain. The targets of spliced XBP-1 are not clear but may be involved in ER-associated degradation (18). The ATF6 transcription factor up-regulates XBP-1 mRNA providing a convergence of the Ire1-ATF6 pathways.

The third ER stress-transducing kinase is pancreatic eIF-2α kinase (PEK/PERK (19, 20), which shares homology with the luminal domains of Ire1α and -β but does not encode an endonuclease domain. The target of the kinase activity of PERK is
the eukaryotic translation initiation factor, eIF-2α. Phosphorylation of eIF-2α during ER stress interferes with the assembly of the translation initiation complex, thereby dramatically diminishing translation (21, 22). This aspect of the UPR is shared by a number of other cellular stresses, which activate different eIF-2α kinases. Interestingly, although most cellular protein synthesis is shut down, translation of the ATF4 transcription factor is specifically induced. ATF4 is constitutively transcribed in non-stressed cells, even though its transcripts cannot be efficiently translated due to the presence of short open reading frames (5′-ORF) in its 5′-untranslated region (5′-UTR) that interfere with initiation at the proper start codon. During ER stress, these short ORFs are no longer utilized which leads to a dramatic increase in ATF4 protein synthesis (23). One target of the ATF4 transcription factor is the CHOP promoter (24, 25), which appears to play a role in the induction of apoptosis during ER stress. Unlike the up-regulation of ER chaperones, the inhibition of protein synthesis during the UPR and other cellular stresses is transient. This is accomplished by inhibition of the PERK kinase itself and dephosphorylation of eIF-2α. Very recently, it was demonstrated that a constitutively expressed double-stranded RNA-activated protein kinase (PKR) inhibitor, p58IPK, is induced during ER stress and plays an important role in down-regulating PERK phosphorylation later during ER stress (26, 27). GADD34 was originally identified in a screen for genes induced during growth arrest (26) and are not immune to the eIF-2α phosphorylation and are not presently known.

In this study, we demonstrate that the translational induc- tion of ATF4 in response to eIF-2α phosphorylation is immediately upstream of GADD34 induction. ATF4 null MEFs are unable to induce GADD34 mRNA during ER or other cellular stresses, which leads to prolonged eIF-2α phosphorylation and diminished protein synthesis recovery. ATF4 is able to bind specifically to and trans-activate a conserved ATF site in the GADD34 promoter in a stress-dependent manner. Through the chromatin immunoprecipitation assay, we further demonstrate that ATF4 directly binds to the GADD34 promoter region encompassing the ATF site in an ER stress-dependent manner. Together, our study delineates an autoregulatory loop in which PERK (or presumably other stress-specific eIF-2α kinases) phosphorylates eIF-2α, in turn inhibits protein synthesis, and allows ATF4 translation. This then leads to GADD34 induction, eIF-2α dephosphorylation, and finally the resumption of protein synthesis. Furthermore, our data revealed that BiP up-regulation at the translational level is significantly diminished in ATF4 null cells where protein synthesis inhibition persists, demonstrating that the cytoprotective UPR targets are not immune to the eIF-2α-dependent translational inhibition and thus underscore the importance of translational recovery early in the response.

**EXPERIMENTAL PROCEDURES**

**Isolation, Genotyping, Culture, and Treatment of WT and ATF4 Null Mouse Embryonic Fibroblasts**—Our ATF4 heterozygous mice were generated by Tanaka et al. (32) and were further maintained by Dr. Adrian F. Gombart, Cedars-Sinai Medical Center, who kindly provided our breeding pairs. In keeping with previous reports, homozygous ATF4 null mice are born at significantly less than expected Mendelian ratios and are blind due to the lack of lens development (32, 33). Mouse embryos were explanted at day 13.5–14.5 of gestation from ATF4+/lo f/lo f mice.
**RESULTS**

**Isolation and Analysis of ATF4 Null Mouse Embryonic Fibroblasts**—Recently, the stress-induced GADD34 protein was shown to bind to the PP1 phosphatase and activate the dephosphorylation of eIF-2α, thereby triggering the resumption of protein translation during cellular stresses (29–31). Induction of GADD34 during ER stress and amino acid deprivation stress did not occur in PERK−/− or GCN2−/− cells, respectively, which are defective in the initial block in protein synthesis during ER stress or amino acid deprivation (29). This suggests the presence of a feedback regulatory loop, in which the eIF-2α kinases block protein synthesis and by an unknown mechanism induce GADD34, which then allows translation to resume. Ron and co-workers (29) suggested that this might occur either via a mechanism similar to the induction of ATF4 where small 5′-ORFs that interfere with correct translation initiation are suppressed by the inhibition of protein synthesis thus allowing initiation at the correct site or, conversely, that GADD34 might be a target of the ATF4 transcription factor. To address the role of ATF4 in the activation of GADD34 directly, we isolated WT and ATF4−/− null mouse embryonic fibroblasts (MEFs) by mating ATF4−/− mice. To confirm the lack of ATF4 transcription and translation in these cells, cytoplasmic mRNA and whole cell lysates were harvested from WT and ATF4−/− cells that were left untreated or treated with Tg to induce ER stress. The ATF4 transcript was detected using Northern blotting (Fig. 1B), and ATF4 protein expression was detected via Western blotting (Fig. 1A). ATF4 message was detected only in the WT MEFs but not in the ATF4−/− cells (Fig. 1B). Consistent with published data, ATF4 was constitutively transcribed but not translated in WT cells under non-stressed conditions. ATF4 protein, as detected by Western blotting, was induced in WT MEFs as early as 2 h after Tg treatment but could not be detected even after 8 h of UPR activation in ATF4−/− null cells (Fig. 1A). When nuclear extracts harvested from either WT or ATF4−/− cells were added in gel shift assays, ATF4 binding was detected only with those from WT cells (Fig. 6C). These
data confirm the lack of ATF4 transcripts and protein in our null MEFs both before and after ER stress. The targeted disruption of the ATF4 allele was generated by replacing the second exon of ATF4 gene containing the basic leucine zipper domain, which is essential for the dimerization of ATF4 and DNA binding activity, with the neomycin resistance gene (32). Thus, even if a partial ATF4 protein was expressed that was not recognized by our antibody, it would not be functional due to the lack of the leucine zipper domain.

**Induction of BiP Transcription Was Intact in ATF4−/− Cells**—To begin characterizing the ER stress response in the ATF4 null cells, we first analyzed the transcriptional up-regulation of the ER chaperone BiP. BiP transcription is a target of the ATF6 transcription factor (38) and may be partially regulated by Ire1-altered XBP-1 (15, 39) and thus represents a read-out of these arms of the UPR. We harvested cytoplasmic mRNA from WT as well as ATF4−/− cells that had been treated with Tg for the indicated times, and we performed both Northern blotting and semi-quantitative reverse transcriptase-PCRs to detect the BiP transcript (Fig. 1, B–D). Results from three independent reverse transcriptase-PCRs were quantified, normalized to the actin signal, and summarized in Fig. 1D, demonstrating that ATF4 null MEFs responded to Tg treatment by induc-}

**Prolonged eIF-2α Phosphorylation in ATF4−/− Cells**—To determine whether the PERK activation signal was intact in these cells, we monitored the phosphorylation status of its target protein, eIF-2α, during the course of ER stress using an antiserum that is specific for the phospho-form. WT and ATF4−/− cells were treated with Tg for the indicated periods of time, and protein blots were analyzed (Fig. 2A). The level of total eIF-2α was also measured as a loading control. Phosphorylation of eIF-2α peaked from 0.5 to 1.5 h after Tg treatment in both WT and ATF4−/− cells, although that in the null cells was higher (Fig. 2B). As expected in the WT MEFs, eIF-2α phosphorylation started to decline 2–4 h after Tg treatment until it reached near basal levels. However, the level of eIF-2α phosphorylation in ATF4 null MEFs underwent a slower decline and remained comparable with the maximal level in WT cells until 8 h after ER stress. Together, our data demonstrated that even though the UPR-activated eIF-2α phosphorylation signal was intact in ATF4 null cells, the eIF-2α dephosphorylation signal was diminished. This suggested that ATF4 was required for the dephosphorylation of eIF-2α later in the ER stress.

**Lack of GADD34 Induction in ATF4−/− Cells during Both ER Stress and Amino Acid Deprivation**—Recently, Novoa et al. (29, 31) demonstrated that GADD34, also known as Myd116 in mouse, promotes the dephosphorylation of eIF-2α and is induced by both ER stress and amino acid deprivation. Because eIF-2α phosphorylation is prolonged in the absence of ATF4 synthesis, we determined if ATF4 played a role in the regulation of GADD34 transcription. We harvested cytoplasmic mRNA from cells treated with Tg for the indicated periods of time and then performed Northern blot to measure the induction of GADD34 mRNA in WT and ATF4−/− cells (Fig. 2C). Similar experiments were also performed using cells deprived of leucine for the indicated times (Fig. 2D). In both cases, the induction of GADD34 transcripts was readily detected in the WT MEFs. However, treatment of the ATF4−/− MEFs with either stress-inducing regimen induced GADD34 mRNA to a

**Fig. 2.** **ATF4 was required for the dephosphorylation of eIF-2α and the induction of GADD34 during cellular stresses.** A, cell lysates from WT and ATF4 null MEFs treated with Tg for the indicated times were run on a denaturing SDS gel. Phosphorylated (top panels) and total eIF-2α (bottom panels) were detected with respective antibodies. The amount of eIF-2α was quantified, normalized to that of the total eIF-2α, and summarized in B. C and D, mRNA was isolated from WT or ATF4−/− cells that had been treated with Tg (B) or deprived of leucine (C) for the indicated times and run on a Northern blot to detect the levels of GADD34 and GAPDH transcripts.
minimal level. Hence, ATF4 is required for the up-regulation of GADD34 during both ER and amino acid deprivation stresses. This presumably explains the persistence of eIF-2α phosphorylation in the ATF4 null MEFs during ER stress (Fig. 2B).

The Resumption of Translation after ER Stress Was Delayed and Incomplete in ATF4−/− MEFs—To determine the consequences of prolonged eIF-2α phosphorylation in the ATF4−/− MEFs, we examined its effect on total protein synthesis. Cells were pulse-labeled with [35S]methionine at different time points after Tg treatment as indicated in Fig. 3A. Total cell lysates were directly run on SDS gels, Coomassie-stained, and exposed to film as shown in A. The total 35S signal for each lane was quantified and summarized in B with that for the untreated WT MEFs set as 1. C, WT and ATF4−/− cells were treated as indicated and labeled with [35S]methionine as in A. The lysates were then immunoprecipitated with a polyclonal antiserum specific for rodent BiP, separated by denaturing SDS-PAGE and detected by autoradiography. An equal amount of total cell lysates for each experimental group was run on a separate SDS gel and Coomassie-stained as loading control. The 35S signals for the immunoprecipitated BiP protein at different time points after ER stress were then quantified and summarized in D.

Fig. 3 Persistent eIF-2α phosphorylation during the course of ER stress in ATF4 null cells blocked translation recovery and BiP induction. A and B, WT and ATF4−/− MEFs were treated with Tg for the indicated times and then pulse-labeled with [35S]methionine. Lysates were prepared, and a portion of each was run on SDS gels, Coomassie-stained, and exposed to film as shown in A. The total 35S signal for each lane was quantified and summarized in B with that for the untreated WT MEFs set as 1. C, WT and ATF4−/− cells were treated as indicated and labeled with [35S]methionine as in A. The lysates were then immunoprecipitated with a polyclonal antiserum specific for rodent BiP, separated by denaturing SDS-PAGE and detected by autoradiography. An equal amount of total cell lysates for each experimental group was run on a separate SDS gel and Coomassie-stained as loading control. The 35S signals for the immunoprecipitated BiP protein at different time points after ER stress were then quantified and summarized in D.

ATF4 Transactivates GADD34 during ER Stress

Identification of a Potential ATF4-binding Site That Is Conserved in the Mouse and Human GADD34 Promoter—The ATF4-dependent up-regulation of GADD34 transcription during ER and other cellular stresses could occur either directly via the binding of ATF4 to the GADD34 promoter or indirectly as a consequence of the induction of other ATF4 target genes. Because a full-length GADD34 promoter sequence has not been reported and only ~200 bp of human (U33982) and mouse (U33984) GADD34 promoter sequences had been entered in the EST data base, we utilized the published human GADD34
cDNA (NM014330) and mouse Myd116 cDNA (NM008654) sequences to search the Ensembl genomic sequence data base at www.ensembl.org. We were able to identify the putative mouse and human GADD34 promoter sequences immediately upstream of the respective exon 1 sequences located on chromosome 7 in mouse and chromosome 19 in human. Both sequences contained the respective −200-bp promoter sequences entered in the EST data base. When the first 1-kb upstream sequences of both promoters were aligned, we observed stretches of homology across the whole sequence (−74%). However, if more stringent parameters were used in the comparison, only about 210 bp of the promoter sequences immediately upstream of the transcription start site can be aligned, revealing a higher homology in this region.

ATF and cAMP-response element-binding protein family members form homodimers or heterodimers to bind to a conserved ATF/cAMP-response element motif, TGACGTCA (40). Only one completely conserved ATF-binding site was identified in the mouse and human sequences. The ATF site is located in a stretch of highly conserved nucleotides and is just 20 bp upstream of the TATA box (−67 bp to −60 bp in mouse GADD34 promoter and −58 bp to −51 bp in human GADD34 promoter, Fig. 4). There is a stretch of 14 nucleotides just upstream of the GADD34-ATF site that is also completely conserved between the mouse and human GADD34 promoter (underlined in Fig. 4). This is the longest completely conserved sequence between the two GADD34 promoters, suggesting that it might also contain regulatory motif that is important for GADD34 transcriptional regulation during cellular stress conditions. Closer inspection of this motif revealed potential binding sites for both CHOP and NF-κB, which are underlined.

**Fig. 4. Identification of potential ATF4-binding sites that are conserved between human and mouse GADD34 promoters.** Promoter sequences for human and mouse GADD34 were obtained from ensemble genomic sequence data base and aligned to identify regions of homology. The transcription initiation site and the TATA box are identified. The conserved ATF/cAMP-response element-binding site (GADD34-ATF) is boxed and indicated. A highly conserved stretch of nucleotides upstream of the GADD34-ATF site contains potential binding sites for CHOP and/or NF-κB, which are underlined.

ATF4 bound to the conserved GADD34-ATF site specifically in an ER stress-dependent manner—Next we wished to directly test the binding of ATF4 to the conserved GADD34 promoter using in vitro electrophoretic mobility shift assays (EMSA). We synthesized a 29-bp probe encompassing the most highly conserved region (210 bp) between the human and mouse GADD34 promoters (Fig. 5A). The second target sequence, region B, was −1.2 kb upstream of the ATF site (Fig. 5A). Activation of the UPR induced ATF4 binding to region A in the GADD34 promoter, whereas binding of ATF4 to the B region was below the level of detection. Antibodies to YY-1 did not immunoprecipitate either region (Fig. 5C). Therefore, our data demonstrate that ATF4 directly binds to the GADD34 promoter in an ER stress-dependent manner and suggests that this binding occurs through a region encompassing the conserved ATF site.

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ATF4 bound to the conserved GADD34-ATF site specifically in an ER stress-dependent manner (24). The YY-1 antibody that recognizes YY-1 binding to the BiP ER stress response element site (data not shown) was used as a negative control. Cross-linked chromatin-protein complexes prepared from untreated or Tg-treated NIH3T3 cells were fragmented to an average of 600−700 bp (Fig. 5B). One target sequence designated region A encompasses the ATF site that is immediately upstream of the TATA box and contains the most highly conserved region (210 bp) between the human and mouse GADD34 promoters (Fig. 5A). Activation of the UPR induced ATF4 binding to region A in the GADD34 promoter, whereas binding of ATF4 to the B region was below the level of detection. Antibodies to YY-1 did not immunoprecipitate either region (Fig. 5C). Therefore, our data demonstrate that ATF4 directly binds to the GADD34 promoter in an ER stress-dependent manner and suggests that this binding occurs through a region encompassing the conserved ATF site.

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ATF4 transactivates GADD34 during ER stress—To examine whether ATF4 directly binds to the GADD34 promoter upon ER stress, we performed CHIP assays using an ATF4 antibody that recognizes epitopes that are exposed while ATF4 binds to its target sequence in gel shift assays (24). The YY-1 antibody that recognizes YY-1 binding to the BiP ER stress response element site (data not shown) was used as a negative control. Cross-linked chromatin-protein complexes prepared from untreated or Tg-treated NIH3T3 cells were fragmented to an average of 600−700 bp (Fig. 5B). One target sequence designated region A encompasses the ATF site that is immediately upstream of the TATA box and contains the most highly conserved region (210 bp) between the human and mouse GADD34 promoters (Fig. 5A). The second target sequence, region B, was −1.2 kb upstream of the ATF site (Fig. 5A). Activation of the UPR induced ATF4 binding to region A in the GADD34 promoter, whereas binding of ATF4 to the B region was below the level of detection. Antibodies to YY-1 did not immunoprecipitate either region (Fig. 5C). Therefore, our data demonstrate that ATF4 directly binds to the GADD34 promoter in an ER stress-dependent manner and suggests that this binding occurs through a region encompassing the conserved ATF site.

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The UPR-induced translational arrest is essential for the survival of cells that are experiencing ER stress. Animals that are nullizygous for PERK (22) or in which a non-phosphorylated form of eIF-2α has been inserted in place of the wild type gene (21) are unable to down-regulate protein synthesis during ER stress and, as a result, experience severe difficulties in handling the accumulation of unfolded proteins which leads to apoptotic cell death. The block in protein synthesis is not unique to ER stress but is also elicited by a number of cellular stresses including amino acid deprivation and heme deficiency (6). Even though each of these stresses activates a unique eIF-2α kinase (GCN2 and HRI, respectively) that causes the block in translation, many aspects of the downstream signaling pathways are shared. First, the translational arrests elicited by these stresses are reversible (29, 41, 42). Recently, Ron and co-workers (29, 31) identified GADD34 as a component of the PP1-phosphatase complex that is responsible for dephosphorylating eIF-2α and allowing translation to resume. Their study revealed that GADD34 induction is downstream of the eIF-2α kinase PERK and GCN2, suggesting the existence of a negative regulatory feedback loop. A second shared aspect of the cellular stress responses is the induction of the ATF4 transcription factor (23), which is largely regulated at the translational level. Many cells possess ATF4 transcripts but do not produce the protein under normal physiological conditions due to the presence of several small ORFs in the 5’-untranslated region (5’-UTR) of the transcript. Only after the phosphorylation of eIF-2α is the correct start site used to translate the ATF4 protein. We showed that ATF4 might contribute to CHOP induction during ER stress (24), and Holbrook and co-workers (25) has demonstrated its involvement in CHOP induction during arsenite poisoning. Most recently, the study by Harding et al. (43) revealed that ATF4 mediates an integrated stress response pathway, which protects the cells from oxidative stress.

Our data showing the absence of GADD34 transcript induction in response to either UPR-inducing agents or amino acid deprivation in ATF4+/- cells demonstrated that ATF4 is upstream of and required for GADD34 induction during both stress conditions. Absence of GADD34 induction in ATF4 null cells resulted in persistent eIF-2α phosphorylation, delayed translation recovery, and diminished induction of UPR target genes like BIP. Next, combined with the CHIP assay data showing that endogenous ATF4 was able to bind to the GADD34 promoter directly in a stress-dependent manner, we conclude that GADD34 is a direct target of ATF4 during various cellular stresses. Finally, we demonstrated that ATF4 acts through a conserved ATF site, which is important for the activity of the GADD34 promoter during ER stress.

Thus, we have delineated a negative regulatory feedback

DISCUSSION

Fig. 5. CHIP assay demonstrated that ATF4 directly bound to the GADD34 promoter in an ER stress-dependent manner. A. Relative positions of the targeting sequences designated regions A and B are indicated. The location of the ATF site within region A is highlighted. B. Cross-linked chromatin used for CHIP assay in C were fragmented and run on a DNA-agarose gel and stained with ethidium bromide. C. Cross-linked chromatin from untreated or Tg-treated (6 h) NIH3T3 fibroblasts was immunoprecipitated with either anti-ATF4 or anti-YY-1 antibody as negative control. 1% of total input before immunoprecipitation was used as positive control. Immunoprecipitated chromatin fragments were then amplified by PCR methods to detect ATF4 binding to the two regions of GADD34 promoter as described in A. and 12). We were unable to observe the original ATF4-binding complex before supershifting with the ATF4 antisera. There was a relatively large diffuse complex (complex A) that formed with the GADD34-ATF probe, which might mask the unsheilded ATF4-binding complex. When a control EMSA was performed in parallel using the CHOP C/EBP-ATF composite site as the probe (Fig. 6B, lanes 7-9) to reproduce the ATF4 and C/EBP-β-binding complexes to the CHOP composite site as we demonstrated previously (24), the diffuse complex (complex A) binding to GADD34-ATF probe migrated at a similar rate as the ATF4-binding complex to the CHOP composite site probe, as pointed out by the arrow in Fig. 6B (lanes 7 and 9). Because the two radioactively labeled probes are at the same length (29 bp), this is consistent with the possibility that the unsheilded ATF4-binding complex observed with the GADD34-ATF probe is masked by the diffuse complex (complex A).

To confirm that the binding activity detected by the antisera was specific to ATF4, we performed gel shift assay with the WT GADD34-ATF probe using nuclear extracts from either WT or ATF4 null MEFs (Fig. 6C). Again, ATF4 binding was detected only after ER stress (4 h of Tg treatment) in the WT MEFs (lanes 1-4). Addition of ATF4 antibody to the binding reaction did not change the migration of any complex either before or after ER stress when nuclear extracts from ATF4 null cells were examined (lanes 5-8). Together, our EMSA data demonstrated that an ATF4-binding site exists in the GADD34 promoter region, which is conserved in both the mouse and human promoters and binds to ATF4 specifically in an ER stress-dependent manner.

Finally, to evaluate the role of the ATF site in the context of GADD34 promoter, we cloned both WT GADD34 promoter encompassing 700 bp upstream of the transcription start site or the same region with ATF site mutated. As shown in Fig. 6D, mutation of the ATF site resulted in a significant decrease in the activity of the promoter both before and after ER stress even though the fold inducibility of the promoter is only slightly decreased. This suggests that even though ATF4 plays an important role in transactivating the ATF site, other transcription factors binding to the site under non-stressed conditions may contribute to the basal activity of the GADD34 promoter in this assay. In conclusion, ATF4 directly binds to and transactivates the conserved ATF site of the GADD34 promoter in an ER stress-dependent manner.
loop that controls the phosphorylation state of eIF-2α during cellular stresses (Fig. 7A). UPR-induced activation of PERK (or other eIF-2α kinases activated by their respective stresses) leads to the phosphorylation of eIF-2α. This in turn inhibits much of the cellular translation and, ironically, allows the translation of ATF4. ATF4 in turn directly trans-activates the GADD34 promoter by binding to the ATF site. GADD34 translation then occurs via a mechanism that is likely to be eIF-2α-independent. GADD34 activates the PP1 phosphatase, which leads to eIF-2α dephosphorylation and the resumption of general translation (Fig. 7A). Therefore, phosphorylation of eIF-2α sets in place the pathway leading to its dephosphorylation, which ensures that the translation block will be reversed. The fact that GADD34 acts on the phospho-form of eIF-2α instead of PERK suggests that it should be able to alleviate the block in protein synthesis for many other cellular stresses that activate eIF-2α kinases. The kinetics of this loop or the activation of different downstream components may vary in different cells or under different physiological and pathological conditions. For instance, the phosphorylation of eIF-2α in response to double-stranded RNA-activated protein kinase activation by double-stranded RNA appears to be relatively long lived (44), although the mechanism for this difference is not currently understood.

In the absence of GADD34-dependent dephosphorylation, as occurred in the ATF4 null cells, continuous cellular stress resulted in prolonged eIF-2α phosphorylation and an extended suppression of protein synthesis. During ER stress, the significant loss of BiP protein induction in ATF4−/− MEFs due to persistent protein synthesis inhibition suggests that the quick translation recovery is essential for the up-regulation of cytoprotective mechanisms.

A similar feedback loop was hypothesized by the Ron and...
**ATF4 Transactivates GADD34 during ER Stress**

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A. **AA deprivation** or **ER stress** causes phosphorylation of **eIF-2α kinases** leading to **eIF-2α phosphorylation** (eIF-2α-P) and **PERK activation**. PERK activation results in **GADD34 translation** inhibition and **protein synthesis inhibition**.

B. Schematic illustration of the 5'-UTR of the mouse and human **GADD34** gene showing the position of two small ORFs that are relatively conserved in the mouse and human. The locations of the 5'-ORFs are indicated relative to the first nucleotide of the GADD34 initiating methionine (ATG), which is designated +1.

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**Fig. 7.** **ATF4 is required for the up-regulation of GADD34 during cellular stresses like amino acid deprivation and ER stress, thus forming a phospho-eIF-2α-dependent negative feedback regulatory loop.** A. When eIF-2α kinases like GCN2 and PERK are activated during various cellular stress conditions, the resultant protein synthesis inhibition allows enhanced ATF4 translation, which in turn binds to the ATF site in the promoter of GADD34, directly resulting in the up-regulation of GADD34 transcription and translation. GADD34 then binds to and enhances the activity of the PP1 phosphatase, which dephosphorylates eIF-2α and finally relieves the translation inhibition in the stressed cells. B. Schematic illustration of the 5'-UTR of the mouse and human GADD34 gene showing the position of two small ORFs that are relatively conserved in the mouse and human. The locations of the 5'-ORFs are indicated relative to the first nucleotide of the GADD34 initiating methionine (ATG), which is designated +1.

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co-workers (29), along with a second possibility in which GADD34 translation might be regulated via the presence of ORFs in the 5'-UTR similar to ATF4. When the 5'-UTR of the mouse and human GADD34 transcript were examined, we did locate two small ORFs that are relatively conserved (Fig. 7B). Therefore, it is possible that GADD34 is regulated at both transcriptional and translational levels like ATF4. However, neither the human nor the mouse GADD34 mRNA 5'-small ORFs (Fig. 7B) overlaps with the correct translation initiation site as is observed with the human and mouse ATF4 transcripts (23). More importantly, the stop codon of the first 5'-ORF (untranslated ORF1) in the mouse GADD34 transcript overlaps with the start codon of the second 5'-ORF (untranslated ORF2), thus potentially diminishing the use of the second ORF and, therefore, its effect on interfering with the correct translation start site (45). In addition, unlike ATF4, we see no evidence of GADD34 transcripts present in unstressed MEFs (Fig. 2, C and D).

Interestingly, not all transcripts appear to be equally affected by the phospho-eIF-2α-mediated block in translation, nor does the resumption of protein synthesis occur equally for all. After 2 h of Tg treatment, ATF4 protein was detected at near maximal level in the wild type MEFs (Fig. 1A), even though eIF-2α phosphorylation levels were still elevated (Fig. 2A) and total protein synthesis was very much decreased (Fig. 3B). In other studies, it appears that the CHOP transcription factor may also be fairly resistant to the eIF-2α-mediated protein synthesis inhibition, as its translation is detectable almost as early as the ATF4 protein (24). GADD34 may represent a third member of this class of proteins, because its biosynthesis is required to alleviate the translation block. Perhaps because these proteins play critical roles in regulating early components of the stress response, their messages have evolved to escape this translation block. Conversely, synthesis of the BiP chaperone is clearly affected early in the response and in ATF4−/− cells. In WT MEFs, increased transcription of the BiP gene was detectable 2 h after ER stress (Figs. 1B and 2). However, BiP translation diminished initially and was just returning to basal rates between 1 and 2 h after activating the UPR (Fig. 3D). Because BiP levels control the activation of UPR signaling molecules (Ire1, PERK, and ATF6) by binding to their luminal domains and keeping them in an inactive form (11, 46), it is plausible that an increase in the level of this chaperone too early in the response could shut it down prematurely. An internal ribosome entry site has been identified in the 5'-UTR of the BiP mRNA, whose activity does not change after ER stress or amino acid deprivation (44, 47). It is unclear whether the internal ribosome entry site-mediated eIF-2α-independent BiP translation plays an important role in the overall synthesis of the BiP protein. Our data demonstrated that BiP translation is down-regulated just like other genes when eIF-2α is phosphorylated during the 1st h of ER stress, and BiP translation does not recover even long after eIF-2α is dephosphorylated and general protein synthesis has resumed (7). Because cyclins control entry of the cells into the G1 phase of cell cycle, it is possible that this protein remains affected to ensure that cells experiencing stress do not propagate. How many proteins are represented in the various classes and how these differences in susceptibility to translation inhibition are achieved are not known.

We conclude that an eIF-2α-dependent feedback regulatory loop exists to ensure the transient nature of protein synthesis inhibition during the mammalian UPR. It is initiated by ATF4, a product of eIF-2α phosphorylation, which directly induces GADD34 transcription, a component of the phosphatase complex that dephosphorylates eIF-2α, thereby allowing translation to resume. ATF4 null mice cannot induce GADD34, and as a result, show prolonged eIF-2α phosphorylation and protein synthesis inhibition.

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