Herp Is Dually Regulated by Both the Endoplasmic Reticulum Stress-specific Branch of the Unfolded Protein Response and a Branch That Is Shared with Other Cellular Stress Pathways*

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The mammalian unfolded protein response (UPR) includes two major branches: one(s) specific to ER stress (Ire1/XBP-1 and ATF6-dependent), and one(s) shared by other cellular stresses (PERK/eIF-2α phosphorylation-dependent). Here, we demonstrate that the ER-localized protein Herp represents a second target, in addition to CHOP, that is dually regulated by both the shared and the ER stress-specific branches during UPR activation. For the first time, we are able to assess the contribution of each branch of the UPR in the induction of these targets. We demonstrate that activation of the shared branch of the UPR alone was sufficient to induce Herp and CHOP. ATF4 was not required during ER stress when both branches were used but did contribute significantly to their induction. Conversely, stresses that activated only the shared branch of the UPR were completely dependent on ATF4 for CHOP and Herp induction. Thus, the shared and the ER stress-specific branches of the UPR diverge to regulate two groups of targets, one that is ATF6 and Ire1/XBP-1-dependent, which includes BiP and XBP-1, and another that is eIF-2α kinase-dependent, which includes ATF4 and GADD34. The two branches also converge to maximally up-regulate targets like Herp and CHOP. Finally, our studies reveal that a PERK-dependent target other than ATF4 is contributing to the cross-talk between the two branches of the UPR that has previously been demonstrated.

The accumulation of malformed proteins in the endoplasmic reticulum (ER) activates a cytoprotective signaling cascade termed the unfolded protein response (UPR). Proximal transducers of the mammalian UPR, which include PERK, Ire1, and ATF6, are all ER-localized transmembrane proteins (1–5). They are kept in an inactive monomeric state under non-stressed conditions by binding to the ER chaperone BiP (GRP78). During ER stress, malformed proteins accumulate in the ER, and BiP is dissociated from the luminal domains of these sensor proteins, leading to their activation (6, 7). For ATF6, dissociation from BiP allows its translocation to the Golgi, where its cytosolic domain encoding a transcription factor, is cleaved by the site 1 (S1P) and site 2 (S2P) proteases (8). The liberated ATF6 then migrates to the nucleus, where it directly binds to and activates the consensus ER stress response element (ERSE) found in the promoters of various UPR targets including transcription factors XBP-1 and CHOP, and ER chaperones like BiP and GRP94 (9, 10). For Ire1p, dissociation from BiP leads to its dimerization and activation of the cytosolic kinase domain, which in turns stimulates an endoribonuclease activity located at its C terminus. Activated Ire1 recognizes and cleaves two specific stem loop sequences in the XBP-1 mRNA, which are then religated, resulting in a larger XBP-1 protein (spXBP-1) due to a resulting frameshift (11–13). Spliced XBP-1 encodes a transcription factor that binds and transactivates the same ERSE site in vitro as ATF6 (13). However, recent data suggest that not all ATF6 and XBP-1 targets are shared (14, 15). In the case of PERK, BiP dissociation during UPR activation leads to oligomerization and activation of its cytosolic kinase domain. Activated PERK phosphorylates eIF-2α, leading to an immediate, yet transient protein synthesis inhibition (16). Unlike ATF6 and Ire1 activation, which are specific to ER stress, the downstream consequences of PERK activation are shared with other cellular stress responses including amino acid deprivation, infection with double-stranded RNA viruses, heme deficiency and oxidative stress (16–21). These cellular stresses all activate specific eIF-2α kinases that also cause a transient protein synthesis inhibition. Paradoxically, any condition that causes eIF-2α phosphorylation and the resulting protein synthesis inhibition, will induce ATF4 at the translational level (16), leading to activation of its downstream targets (22, 23). Therefore, the mammalian ER stress response can be divided into two main branches: one that is downstream of both ATF6 and Ire1p/XBP-1 and is specific to ER stress, and another that is downstream of PERK and is shared by other cellular stresses that activate eIF-2α kinases (24).

The C/EBP homologous transcription factor CHOP (GADD153) is the only UPR target that has been shown to be dually regulated by both the ER stress-specific and the shared branches of the UPR (10, 22, 25). The CHOP promoter contains both an ERSE and a C/EBP-ATF composite site, through which these two branches act. Deletion of either element from a CHOP promoter construct leads to a decrease in its transcriptional activity (22). The ATF6 and XBP-1 transcription factors can bind to ERSEs in vitro, as demonstrated by gel shift assays.
Fig. 1. Identification of elements in the Herp promoter that could lead to dual regulation during ER stress. The mouse and human Herp promoter sequences are compared. The C/EBP-ATF composite, the ERSE I, and the ERSE II sites are indicated with boxes and the TATA box underlined. The conserved C/EBP-ATF composite site present in the human and hamster CHOP promoters is boxed in the bottom panel.

(13), and ATF6 overexpression leads to CHOP induction in cells (10). The ATF4 transcription factor binds to the C/EBP-ATF composite site in vitro in an ER stress-dependent manner (22). However, the relative contributions of these two branches in regulating CHOP during different cellular stresses have not been determined. In PERK-null and eIF-2αS51A knock-in cells, where eIF-2α phosphorylation is blocked during ER stress, the induction of CHOP and XBP-1 is completely lost and the up-regulation of BiP is reduced (11, 16, 26). These data support the existence of a cross-talk between the two pathways, where a PERK-dependent target is facilitating the activation of the ATF6 and Ire1/XBP-1-dependent branches of the UPR. Even though the molecular pathway for this cross-talk has not been established, ATF4 was implicated, as it is the only known direct target of eIF-2α phosphorylation during ER stress. Here, we identified Herp, an ER localized protein with an N-terminal ubiquitin-like domain (27, 28), to be another UPR target that is dually regulated by both the shared and the ER stress-specific branches of the UPR pathway. Using ATF4-null MEFs, we analyzed the relative contributions of the different pathways in controlling CHOP and Herp transcription during ER and other cellular stresses. Our data reveal that although the ATF4-dependent pathway is not required for the induction of these genes during UPR activation, it is essential in response to cellular stresses that activate only the shared branch of the UPR. Furthermore, our data excluded ATF4 as the molecule that is responsible for the cross-talk between the shared and ER stress-specific branches of the UPR. In toto, our results enable us to separate UPR targets into three sets (Fig. 7), those that are specific to the UPR and evidently downstream of ATF6 and Ire1/XBP-1, those that are downstream of PERK and are shared by other cellular stresses, and those that are dually regulated by both pathways. The latter group is likely to be induced by all cellular stresses involving activation of an eIF-2α kinase with maximal levels of induction occurring during UPR activation.

EXPERIMENTAL PROCEDURES
DNA Constructs and Chloramphenicol Acetyl Transferase (CAT) Assay—S94 to +56 bp of the mouse Herp promoter was cloned immediately upstream of the CAT reporter gene via a PCR-based method (22) using primer pair 5′-CTCCTCATACCTCTCGTACG and 5′-CTCGTGTGGCGGCTTC. The C/EBP-ATF composite site (–183 to –176bp) was deleted in the mutant promoter construct. 1 μg of the wild-type or mutant Herp promoter construct was transfected into COS-1 cells, and cell lysates were analyzed for CAT activity as detailed previously (22). Each experimental group was analyzed in triplicate, normalized against protein concentration, and the experiment was repeated twice. The error bars shown in Fig. 2A represent the standard deviations for each set of triplicates.

A wild-type eIF-2α cDNA was kindly provided by Dr. John W. B. Hershey (University of California, Davis). A point mutant mimicking the phosphorylated form of eIF-2α (S51D), therefore constitutively blocking protein translation, was made with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and sequenced before subcloning both constructs into the pCMV5 vector.

Cell Culture, Transfection, and Stress Induction—Primary wild-type and ATF4−/− MEFs were harvested and propagated in tissue culture as described (23). Passage 5 through 7 MEFs were plated and left untreated (control), treated with thapsigargin (Tg, 1 μM), or cultured in media that lacked leucine (amino acid deprivation) for the indicated periods of time. Components of the leucine-free media are based on Dulbecco’s modified Eagle’s medium (cat. no. 11960), except that leucine is omitted. 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, and 5 mg/liter Fungizone (Mediatech, Herdon, VA). 293T cells were transfected with the indicated vectors using the calcium precipitation method, and cell lysates were harvested 24 h later as described.

Isolation of Cytosolic mRNA, Northern Blotting, and Semi-quantitative RT-PCR—Cytosolic mRNA was harvested from wild-type and ATF4−/− MEFs, and 293T cells using the Qiagen RNeasy kit (Valencia, CA). 20 μg of cytoplasmic mRNA from each experimental group was electrophoresed, analyzed by Northern blotting as described (29). The human GAPDH probe was purchased from Clontech (Palo Alto, CA). The hamster BiP and mouse ATF4 probes were prepared as described (23). The mouse CHOP probe was amplified with the primer pair 5′-GGAGACGTCAGAAGCTCTGGAT and 5′-TGGCAAGGCATACGTGCT-GCC, and the human Herp probe was produced using the primer pair...
5’-CCAGGCGCGCGGGCTGTGAGGTGACGAGGCGGGCGGGCCCTGAGGTTGCATCAGCCCGTGA-3’
Total XBP-1 mRNA was detected with a mouse probe amplified with the
primer pair 5’-CCGCGCTGTGAGGTGACGAGGCGGGCGGGCCCTGAGGTTGCATCAGCCCGTGA-3’
and 5’-ACGAAAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA

To determine if the regulation of CHOP
was synthesized and la-

tion but did significantly decrease its transcriptional activity

construction of CHOP
in the Herp Promoter
after ER stress (Fig. 2

tion sites and tethered it to a CAT reporter. The wild-type
promoter (28, 30) and is

with Molecular Dynamics Phosphorimager (Amersham Biosciences).

To quantitate specific mRNAs by RT-PCR, 1 μg of RNA was used to
first generate 50 μl of cDNA using a poly(dT) primer (1 nm).

molar oligonucleotides encompassing the composite site, which
are the same length as those synthesized for Herp, were used
as a positive control. As expected, ATF4 bound to the CHOP
probe in an ER stress-dependent manner when nuclear ex-
tracts from wild-type MEFs were used (Fig. 2B, lane 3) and
could be supershifted with an antiserum to ATF4 (lane 4).

Identification of a functional C/EBP-ATF composite site in the Herp promoter.
A, left panel, wild-type and C/EBP-ATF composite site mutant Herp promoter constructs driving CAT expression
were used to transfect COS-1 cells. 40 h after transfection, COS-1 cells were left untreated (white bar) or treated with Tg for 14 h (gray bar), and cell lysates were prepared and analyzed for CAT activity.

The level of untreated wild-type promoter activity was set to 1, and the values for the other cell lysates were set relative to this. The values for each lane are the average of a set of three.

B, electrophoretic mobility shift assays were performed using either the CHOP or the Herp C/EBP-ATF composite site probe as indicated. 10 μg of nuclear extract from wild-type or ATF4-null MEFs that were left untreated (−) or treated with Tg for 4 h was used for each reaction. BSA or ATF4 supershift antibody was added where indicated. The unshifted (a) and supershifted (b) ATF4 binding complexes are indicated by arrows. Two ATF4-containing complexes seen only with the Herp probe are indicated with asterisks.

RESULTS

Identification of a Conserved C/EBP-ATF Composite Site in the Herp Promoter—To determine if the regulation of CHOP
was unique among UPR targets, we searched the promoters of other genes up-regulated during ER stress for elements that
might suggest they were also dually regulated. We identified
the presence of a potential C/EBP-ATF composite site in the
promoter of an ER-localized protein, Herp (Fig. 1).

This site is located just upstream of the ERSE I and II sites that
were previously characterized in the Herp promoter (28, 30) and
is completely conserved between the human and mouse genes.

To determine if the C/EBP-ATF composite site contributed to the
transcriptional up-regulation of Herp during ER stress, we iso-
lated a 930-bp fragment of the murine Herp promoter con-
taining ERSE I, ERSE II, and the potential C/EBP-ATF com-
posite sites and tethered it to a CAT reporter. The wild-type
promoter showed low basal activity and was induced ∼12-fold
when the cells were treated with UPR inducing agents, dem-
onstrating that this DNA fragment contained the necessary
elements for responding to ER stress. Mutation of the com-
posite site alone in the context of the murine Herp promoter
construct did not dramatically alter the basal level of transcript-
ion but did significantly decrease its transcriptional activity
after ER stress (Fig. 2A).

To determine if ATF4 could bind directly to the Herp C/EBP-
ATF composite site upon UPR activation, we synthesized wild-
type and mutant oligonucleotides encompassing the composite site and performed electrophoretic mobility shift assays (EMSA) with nuclear extracts harvested from both wild-type and ATF4-null primary MEFs that were left untreated or
incubated with thapsigargin to induce ER stress (Fig. 2B). Oli-

Protein extracts were incubated with the CHOP probe. Although trace amounts of this complex formed in the absence of ER stress (lane 5), the signal dramatically increased after ER stress (lane 7), and in both cases it was
supershifted with the ATF4 antibody (lanes 6 and 8). A fainter,
faster migrating complex (†), which also contained ATF4 bind-
ing activity, was observed with the Herp (lanes 7 and 8), but not
the CHOP probe. This difference could either be due to the fainter signal obtained with the CHOP probe, or it is possible
that it is specific to Herp. When nuclear extracts harvested from
ATF4-null MEFs were used in the gel shift reactions (lanes 9–16), no binding activity was detected with either probe
that could be supershifted with the ATF4 antibody, further

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demonstrating that the binding activity detected in the wild-type nuclear extracts was dependent on the presence of ATF4. Hence, we conclude that Herp, like CHOP, represents another UPR target that is dually regulated by both the shared cellular stress response and the UPR-specific pathways during ER stress.

**eIF-2α Phosphorylation Alone Was Sufficient to Induce Herp and CHOP, but Not BiP**—To further determine if Herp was a direct target of the PERK/eIF-2α/ATF4-dependent pathway and could be induced independent of ATF6 and XBP-1 activation, we transfected cells with GFP, wild-type eIF-2α, or the eIF-2α S51D mutant, which conformationally and functionally imitates the phosphorylated form of eIF-2α and as a result inhibits protein synthesis (31). Cells expressing GFP alone served as a control for basal and stress-induced expression of UPR targets (Fig. 3). The transfection itself did not induce ER stress, and the cells were still capable of inducing UPR targets like ATF4 and CHOP when treated with thapsigargin (Fig. 3, A and B). When cells transfected with the eIF-2α constructs were similarly examined, we found that expression of the constitutively active eIF-2α S51D mutant led to the robust induction of ATF4 and CHOP proteins, whereas expression of the wild-type eIF-2α construct caused only a very slight induction of these targets. To more closely examine the induction of UPR targets, mRNA was harvested from these cells and Northern blot analyses were performed (Fig. 3B). In keeping with the Western blot data, expression of the eIF-2α S51D mutant, but not the wild-type eIF-2α construct, resulted in Herp and CHOP induction, although to a level significantly lower than that obtained with thapsigargin treatment. Transcription of BiP, another UPR target, was not significantly altered by either wild-type or mutant eIF-2α expression, demonstrating that the ATF6 and Ire1/XBP-1 arms of the UPR were not affected.

**ATF4 Was Not Essential for Herp and CHOP Induction during ER Stress, but Was Required for Their Optimal Activation**—We wished to measure the relative contribution of the ER-specific and the shared UPR pathways in the induction of both Herp and CHOP. First, we compared the mRNA levels of these two genes in the wild-type and ATF4-null cells during UPR activation, where both the shared (PERK-dependent) and the ER stress-specific (ATF6 and XBP-1-dependent) arms are activated. The thapsigargin-mediated induction of both CHOP and Herp transcripts in ATF4-null cells was reduced to ~35% of that observed in wild-type cells, but was still very much apparent (Fig. 4A). The accumulation of CHOP protein in response to ER stress was also decreased in the ATF4-null cells, but could still be easily detected (Fig. 4B), demonstrating that although ATF4 clearly contributes to the transcription of these two genes during UPR activation, it is not completely essential. This result is different from a previous Western blot study where CHOP protein induction during ER stress was completely abolished in their ATF4-null MEFs (32). The phenotypes of the two ATF4-null mice are very similar with both showing lens development defect and with the majority of

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Fig. 3. Overexpression of the constitutively active eIF-2α mutant (S51D) was sufficient to induce ATF4, CHOP, and Herp, but not BiP. A. 293T cells were transfected with a vector containing GFP, wild-type (WT) eIF-2α, or the eIF-2α S51D mutant. GFP-transfected cells were left untreated or treated with Tg as indicated. Cell lysates were prepared and separated on a SDS protein gel and blotted for total eIF-2α, ATF4, CHOP, and Hsc70. B. RNA was harvested from cells transfected and treated as described above and subjected to Northern blotting to detect the levels of Herp, CHOP, BiP, and GAPDH transcripts.

Fig. 4. ATF4 was important for the optimal induction of CHOP and Herp during ER stress, but was not required. A. RNA from wild-type (WT) and ATF4+/− MEFs left untreated or treated with Tg for the indicated periods of time was harvested and analyzed by Northern blotting to detect the levels of ATF4, Herp, CHOP, and GAPDH mRNAs. B, cell lysates harvested from wild-type and ATF4-null cells left untreated or treated with Tg as in A were separated and analyzed by Western blotting for CHOP and Hsc70 protein.
homozygotes dying between E14.5 and birth (our ATF4−/− mice birth rate: 6.5% versus Dr. J. M. Leiden, 7.5%) (23, 33, 34). Combined with the facts that in our ATF4−/− MEFs, 58% of the coding region of ATF4 including the entire basic DNA binding domain and the leucine-zipper dimerization domain was substituted with a neomycin resistance gene, no ATF4 transcript was detected by Northern blotting (Fig. 4A), and no ATF4 protein was detected by either Western blotting (23) or gel shift assays (Fig. 2B), we believe that the difference between the two experiments could be a result of differences in the sensitivities of the two CHOP antisera.

We next determined if the ATF4 effects we observed on the transcription of these two genes was exerted entirely through the ATF composite site, or if ATF4 could be the PERK-dependent target that facilitates the activation of the Ire1/XBP-1 and ATF6-dependent branches of the UPR as previously suggested (11, 16, 26). We attempted to transfect both wild-type and ATF4-null MEFs with a CHOP promoter construct that contains the ERSEs, but not the ATF composite site, tethered to a reporter gene (22). Unfortunately, we were unable to achieve a high enough transfection efficiency to get a measurable signal in either of the cells. As an alternative approach, we treated

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**Fig. 5. The ATF6 and Ire1/XBP-1-dependent pathway is intact in ATF4-null cells.**

A, RNA was isolated from wild-type and ATF4−/− MEFs left untreated or treated with Tg for the indicated periods of time and analyzed by Northern blot to detect the levels of BiP, tXBPs, ERdj4, and GAPDH mRNA. B, RNA isolated from wild type and ATF4-null cells treated with Tg for the indicated periods of time was used to synthesize cDNA. Semi-quantitative PCRs were then performed with these cDNAs using oligonucleotides that specifically amplify only the spliced form of XBP-1 mRNA and ones that were specific for actin. C, level of spliced XBP-1 mRNA (spXBPs) present in each sample was quantified from three independent experiments, normalized to the actin signal, and set relative to the untreated sample.
wild-type and ATF4-null MEFs with thapsigargin to induce ER stress and compared the induction of BiP and total XBP-1 transcripts, which are downstream of ATF6 (9, 10). We found no evidence to suggest that the induction of BiP was reduced in the ATF4-null cells, but instead found that the total XBP-1 transcripts were actually slightly increased (Fig. 6A). We then designed PCR oligonucleotides that specifically amplified only the spliced form of the XBP-1 transcript, and semi-quantitative RT-PCR reactions were performed to measure XBP-1 splicing. We found that XBP-1 splicing, which is mediated by the activated Ire1-1 endoribonuclease domain, was also not inhibited in the absence of ATF4 (Fig. 5B). A comparison of quantitative data from three different experiments showed that again there was a slight increase in the activity of this arm of the UPR in the ATF4-null cells (Fig. 5C). Finally, we accessed the induction of ERdj4 (35), a recently identified DnaJ homologue that is regulated by XBP-1, not ATF6 (15). In keeping with the slightly higher levels of total XBP-1 transcripts and the increased splicing of XBP-1 in the ATF4-null cells, our data showed that the induction of ERdj4 was also somewhat higher in the absence of ATF4 (Fig. 5A).

The kinetics of ERdj4 induction are somewhat delayed compared with XBP-1 induction and splicing, which is consistent with it being an XBP-1 target (15). Therefore, during ER stress, the attenuated CHOP and Herp induction in ATF4-null MEFs must be due to a loss of activation at the composite site and not to changes in the ATF6 and Ire1/XBP-1 pathways that converge at the ERSE site. Our data reveal that these pathways are completely intact, and even a bit more vigorous, in the ATF4-null animals.

**ATF4 Was Required for the Activation of CHOP and Herp by Cytosolic Stresses That Activate Other eIF-2α Kinases like GCN2**—We next determined the role of ATF4 in the induction of Herp and CHOP by cellular stresses that activate other eIF-2α kinases. We incubated both wild-type and ATF4-null MEFs with medium lacking leucine to induce amino acid deprivation stress. This treatment does not activate the ATF6 and Ire1/XBP-1-dependent signaling pathways (36) but activates GCN2 (16), which in turn phosphorylates eIF-2α and induces a downstream signaling pathway that is shared by the PERK kinase-dependent branch of the UPR pathway. Consistent with published data, BiP and XBP-1, two UPR targets predominantly activated via ERSE-dependent mechanism, are not induced during amino acid deprivation stress (Fig. 6A). However, both CHOP and Herp are induced by these stress conditions in the wild-type MEFs. Together with our data showing that expression of the eIF-2α S51D mutant alone was sufficient to induce CHOP and Herp (Fig. 3, A and B), we conclude that activation of the eIF-2α-kinase-dependent pathway is sufficient to induce these dually regulated targets in the absence of ERSE activation, although the level of induction is not as robust as that occurring during ER stress when both arms of the UPR are utilized. CHOP and Herp induction during amino acid deprivation stress was completely abolished in ATF4−/− MEFs, demonstrating that ATF4 is required for the induction of both targets by eIF-2α kinases (Fig. 6A). Consistent with Northern blot data, CHOP induction at the protein level was also completely abolished in ATF4-null cells (Fig. 6B).

**DISCUSSION**

We demonstrated previously that the transcriptional induction of CHOP during ER stress is dually regulated by the PERK/ATF4-dependent pathway that acts through the C/EBP-ATF composite site and by the ATF6 and Ire1/XBP-1-dependent pathways that act through the ERSE in its promoter. Until now, CHOP was the only known target that these two branches of the UPR pathway converged upon (10, 22, 25). Here, we demonstrate that Herp represents a second gene that is also dually regulated during ER stress. ATF6 can transactivate the Herp gene through the ERSEI and II sites present in its promoter (30). Our data reveal that during both amino acid deprivation and ER stress, eIF-2α phosphorylation causes ATF4 induction, which then directly binds to and transactivates the composite site in the Herp promoter, similar to earlier data obtained for CHOP. We also show for the first time that although Herp is an ER localized protein, it can be induced by cellular stress conditions that are not thought to affect the ER. Together, our data demonstrate that the convergence of two signaling branches of the ER stress response upon a single target is a conserved feature of the UPR, which suggests there could be additional targets that are regulated this way.

The PERK-dependent and ATF6/XBP-1-dependent pathways would appear to be independent signaling cascades with each acting through its own cis-response element during ER stress. However, the transcriptional induction of CHOP during ER stress is completely abolished in both PERK-null and eIF-2αS51A mutant knock-in MEFs (16, 26). In addition, XBP-1 up-regulation is completely inhibited in PERK-null cells, and when eIF-2αS51A knock-in cells are examined, BiP transcripts induction is induced to 21%, ERp72 to 18%, and p58IPK to 19% of the levels obtained in wild-type MEFs upon UPR activation (11, 26). The only common characteristic of these UPR targets that has been identified is the existence of ERSEs in their promoters, which suggests that they should be regulated by...
ATF6 and/or XBP-1 (9, 10, 37–39). Therefore, these data suggest that a downstream target of PERK/eIF2α phosphorylation is playing an important role in regulating the activation of the ERSE site and has led to the speculation that the ATF4 transcription factor, which is a direct target of PERK-induced eIF-2α phosphorylation, might be required for the optimal activation of the ERSE site. Our data clearly provided a negative answer to this possibility. First, since ATF4 is required for the activation of the composite site in both the CHOP and Herp promoters, the 35% residual induction of both transcripts in response to UPR activation in ATF4-null cells directly proves that the ERSE site can be activated independent of ATF4. Second, the Ire1/XBP-1-dependent branch of the UPR is not down-regulated in ATF4-null cells, but instead is slightly up-regulated compared with wild-type MEFs, arguing that ATF4 has no negative effect upon this arm of the UPR. Last, the activation of ERSE sites in general by either an ATF6 or XBP-1-dependent mechanism is intact in ATF4-null cells as demonstrated by the normal induction of both BiP and xBP-1 transcripts. In conclusion, our data reveal for the first time that a target of PERK-dependent eIF-2α phosphorylation other than ATF4 is likely to play an important role in facilitating the activation of the ERSE site by ATF6 and/or spXB-1.

In our previous study of CHOP regulation during ER stress, we were unable to separate the relative contribution of the C/EBP-ATF-dependent and the ERSE-dependent arms of the UPR by promoter studies. Since ATF4 is required for the activation of the composite site and there are now two dually regulated targets, we were able to separate and compare the effects of the two arms by studying their induction in ATF4-null MEFs. Our results demonstrate that although activation of the eIF-2α kinase/ATF4-dependent pathway alone enhanced both promoter activities, the level is much weaker than that achieved by complete activation of the UPR. Because the activation of stress-specific eIF-2α kinases and their common downstream target ATF4 is well-conserved among various cellular stress conditions (Fig. 7), the ability of this pathway to transactivate the CHOP and Herp promoters ensures that these genes are also induced during other stresses that do not involve ATF6 cleavage, XBP-1 splicing, and ERSE activation. Our experiments provide further support for other studies and our own unpublished observations showing that dually regulated targets like CHOP are activated to a much higher level during ER stress than with other cytosolic stresses like hypoxia (40), amino acid deprivation, oxidative stress (16), and MMS treatment.

Why does ER stress require significantly higher levels of these target genes than other stress conditions, and why should they have evolved to be induced by cytosolic as well as ER stress? Herp is an ER-localized protein that is induced by UPR activation (28, 30). Unlike other UPR-induced ER proteins, the majority of Herp, including its N and C termini, faces the cytosol suggesting its major function may be on the cytosolic side of the ER membrane. The function of Herp is not currently known, but the presence of a type 2 ubiquitin-like domain in its N terminus is consistent with Herp playing some role in the degradation of ER proteins (41). We would speculate that, if indeed it plays a role in the degradation of ER proteins, its activity might be required for a variety of cellular stresses as well. It is reasonable to assume that cytosolic stresses could compromise the folding of portions of transmembrane proteins that reside in the cytosol, requiring that they be detected and targeted for degradation. On the other hand, conditions that alter the ER environment would affect the folding of the lumi-
nal domains of these same transmembrane proteins as well as that of soluble ER luminal proteins, all of which would need to be retrotranslocated and targeted for degradation by the 26 S proteasome. This would explain the need for Herp-independent induction during cytotoxic stress as well as the requirement for higher levels of this protein during ER stress when the demands for degradation would be greater. For CHOP, it is harder to speculate. Although there are a fair number of studies on CHOP (42), it is still not entirely clear what role(s) it plays during UPR activation. Studies have shown that CHOP contributes to apoptosis later in the ER stress response by decreasing the level of the anti-apoptotic molecule Bcl-2 and increasing the level of reactive oxygen species (ROS) in the mitochondria of certain cell types (43). However, it is induced very early in the ER stress response, well before it appears that pro-apoptotic decisions are being made (22). There are also data to show that CHOP can play an anti-apoptotic role in the oligodendrocytes of the central nervous system when they are experiencing ER stress (44). Studies have revealed that CHOP can either homodimerize or heterodimerize with different partners belonging to the C/EBP or ATF family of transcription factors and alter their transactivation targets (45–49). Thus, differences in CHOP protein levels may well affect the transcriptional programs it will either negatively or positively affect.

In conclusion, the data presented here demonstrate that UPR targets can be divided into three general groups (Fig. 7). The first includes those that are only downstream of the shared (eIF-2α kinase/ATF4-dependent) branch of the stress responses like GADD34, the second includes those that are only downstream of the ER stress-specific (ATF6 and Ire1/XBP-1-dependent) branch of the UPR like XBP-1, ERδ4b, BiP and other ER luminal chaperones, and the final group includes those that are dually regulated by both branches of the UPR, like CHOP and Herp. Thus, these two main branches can act independently and together to regulate downstream effectors of the UPR.

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Herp Is Dually Regulated by Both the Endoplasmic Reticulum Stress-specific Branch of the Unfolded Protein Response and a Branch That Is Shared with Other Cellular Stress Pathways

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