nature cell biology

DOI: 10.1038/ncb2738

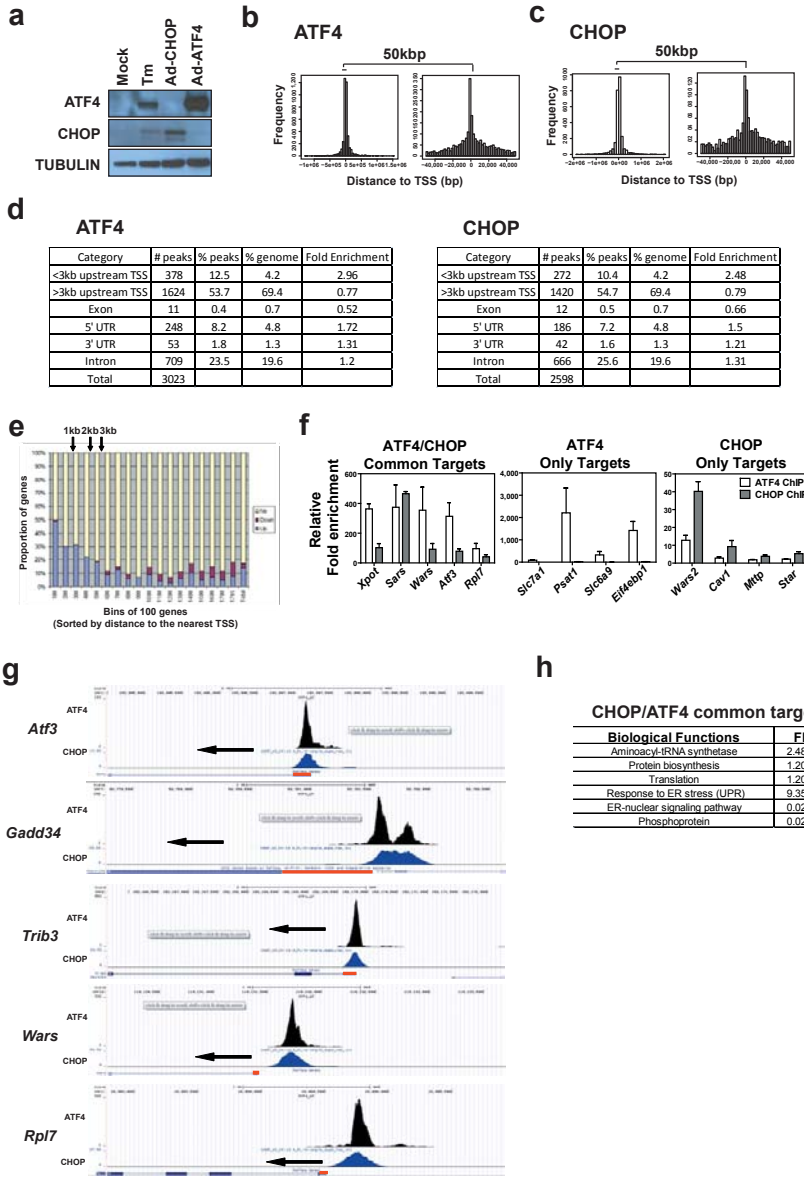


Figure S1 ChIP-seq analysis for ATF4 and CHOP. (a) Recombinant adenoviruses expressing CHOP or ATF4 were infected into MEFs (MOI 100) for 24 hours. As an endogenous control, MEFs were treated with tunicamycin (Tm)(2 mg/ml) for 8 hours. Cell lysates were collected for Western blot analysis. *Chop+/+, Chop-/-, Atf4+/+* and *Atf4-/-* MEFs were treated with Tm (2 mg/ml) (Sigma) for 10 hr, followed by cross-linking with 1% formaldehyde for 10 minutes and subsequent chromatin immunoprecipitation as described in Methods. (b, c) Histogram showing the distribution of DNA sequence reads from ATF4 ChIP-Seq (b) and CHOP ChIP-Seq (c) data with respect to the distance from the transcription start site (TSS) from the nearest gene. Distribution of whole peaks across the genome is shown in the left panel, while distribution of peaks within 50kbp from the TSS is shown in right panel. (d) Distribution of CHOP and ATF4 binding sites from ChIP-Seq data across the genome. The peaks were classified as; within introns (Intron), within coding sequences (Exon), <3 kb from the TSS, >3 kb from the TSS, in the intergenic region, within 5' untranslated regions (UTRs) and within 3' UTRs. (e) Correlation between DNA binding and distance to TSSs. Peaks for ATF4 were sorted by distance to the nearest TSS and then binned into groups of 100 genes. The proportion of significantly up-regulated (> 2 fold and FDR< 0.05, blue) and significantly down-regulated (< 2 fold and FDR<0.05, red) for each group are illustrated. As shown, the correlation with differential expression does not approach baseline until DNA binding is > 3kb from a TSS. Distances from TSS are indicated as arrows above the graph.(f) Validation of ChIP-Seq by conventional ChIP followed by qPCR. MEFs of the indicated genotypes were treated with Tm (2mg/ml) for 10hr, followed by conventional ChIP using anti-CHOP or anti-ATF4 antibodies. Relative fold enrichments (wild-type versus knock-out) were measured by qPCR with specific primers listed in Supplementary Table 5. Data are presented as means ± SEM (n=3 independent experiments). (g) Genomic distribution of binding profiles of common target genes of ATF4 and CHOP (*Atf3, Gadd34*, *Trb3*, *Wars* and *Rpl7*). The peaks represent sequences bound by ATF4 and CHOP identified by ChIP-Seq. Red lines indicate 5' UTRs of each gene. Arrows indicate the direction of transcription. (h) Functional enrichment analysis of ATF4 and CHOP common target genes identified by ChIP-Seq.

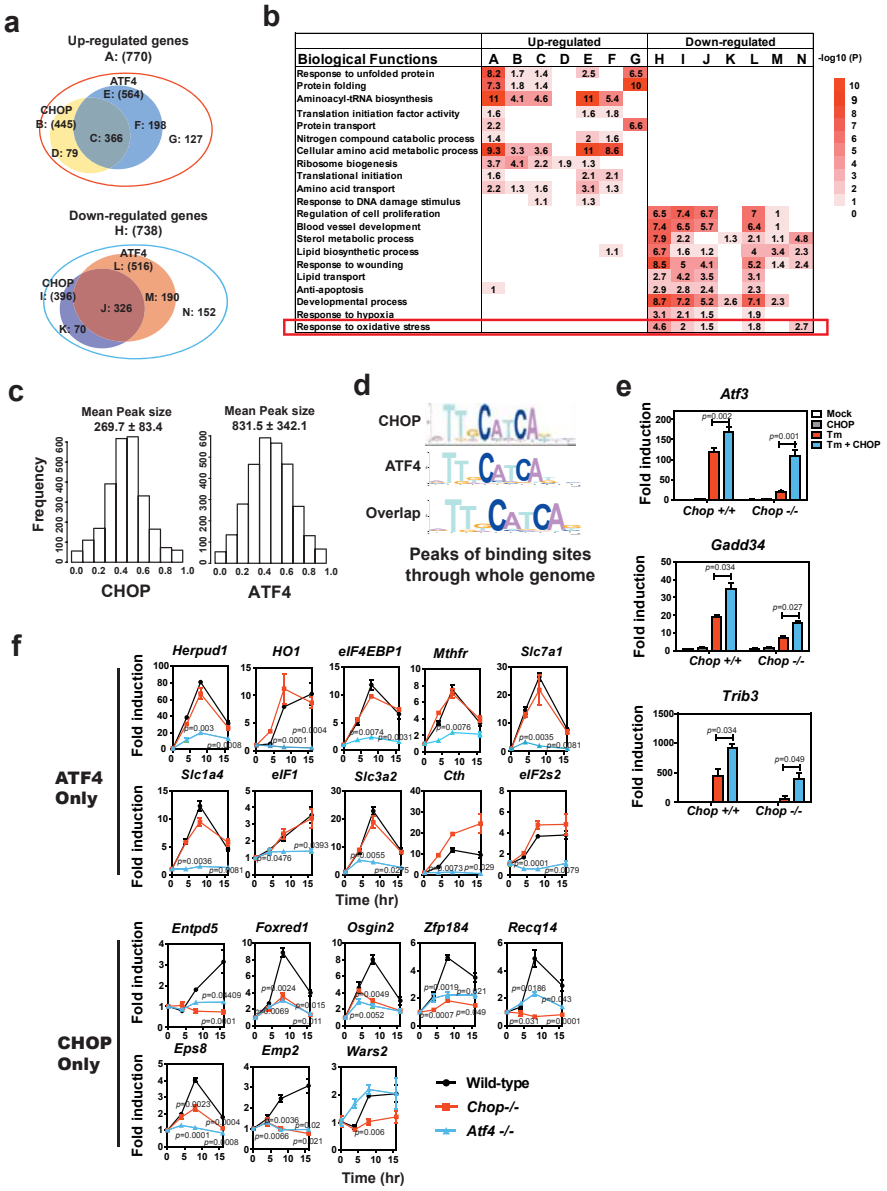


Figure S2 Validation and analysis of mRNA-Seq analysis and Functional regulation of common target genes through ATF4 interaction with CHOP. (a) Venn diagram illustrating genes that are up-regulated (> 2 fold) or down-regulated (< 2 fold) in response to Tm (2mg/ml) treatment for 10hr in wild-type MEFs without (G and N) or with attenuated induction levels in *Atf4-/-* and/or *Chop-/-* MEFs (B-F, I-M). A-G show up-regulated genes. H-N show down-regulated genes. A and H show up- and down-regulated genes in response to Tm treatment, respectively. B and I show up- and down-regulated genes that require CHOP for regulation, i.e., attenuated regulation in *Chop-/-* MEFs. C and J show up- and down-regulated genes, respectively, with attenuated regulation in both *Chop-/-* and *Atf4-/-* MEFs. D and K show up- and down-regulated genes, respectively, with reduced regulation in *Chop-/-*, but not *Atf4-/-* MEFs. E and L show up- and down-regulated genes, respectively with reduced regulation in *Atf4-/-* MEFs. F and M show up- and down-regulated genes, respectively, with reduced regulation in *Atf4-/-*, but not *Chop-/-* MEFs. G and N show up- and down-regulated genes that are not affected by deletion of *Atf4* or *Chop*. The genes in each subgroup are listed in Supplementary Table S3. (b) Functional enrichment analysis of each group of genes in Fig. S4a. The EASE scores (p-value) of DAVID functional enrichment of selected GO terms are represented in a heat map with $-\log_{10}$(EASE score) as a color index. White corresponds to an EASE score of 1.0 with no statistical significance, while the darkest red corresponds to an EASE score of $1.0e^{-10}$ or lower. The functional category of "Response to oxidative stress" is highlighted by a red box. (c) Histogram showing distribution of core motifs in sequences identified by ChIP-Seq. The x-axis represents the length of peak, where the two ends (0.0 and 1.0) represent the two ends of the peak (0.5 is the center of the peak). The y-axis is the number of occurrences of the motif at given x coordinates within a peak. The lengths for peaks within 3kb of a TSS (mean ± s.d.) were 269.7 ± 83.4 (CHOP) and 831.5 ± 342.1 (ATF4). (d) Motif analysis of CHOP and ATF4 binding sites. Sequences from peaks of CHOP and ATF4 binding sites through the whole genome identified in ChIP-Seq were analyzed. Significantly over-represented motifs from each set of genes are shown. (e) CHOP requirement for maximal induction of target genes. In *Chop+/+* or *Chop-/-* MEFs, CHOP was over-expressed in the absence (CHOP) or presence of Tm (2mg/ml) (Tm+CHOP). Total RNAs were purified and subjected to qRT-PCR with primers listed in Supplementary Table S5 (n=3 independent experiments). (f) Gene expression profile during Tm treatment. MEFs were treated with Tm (2mg/ml) for the indicated times and total RNA isolated for qRT-PCR analysis. The results show the expression of genes dependent on only ATF4 (ATF4 only) or on only CHOP (CHOP only) (n=3 independent experiments). Data are presented as means ± SEM.

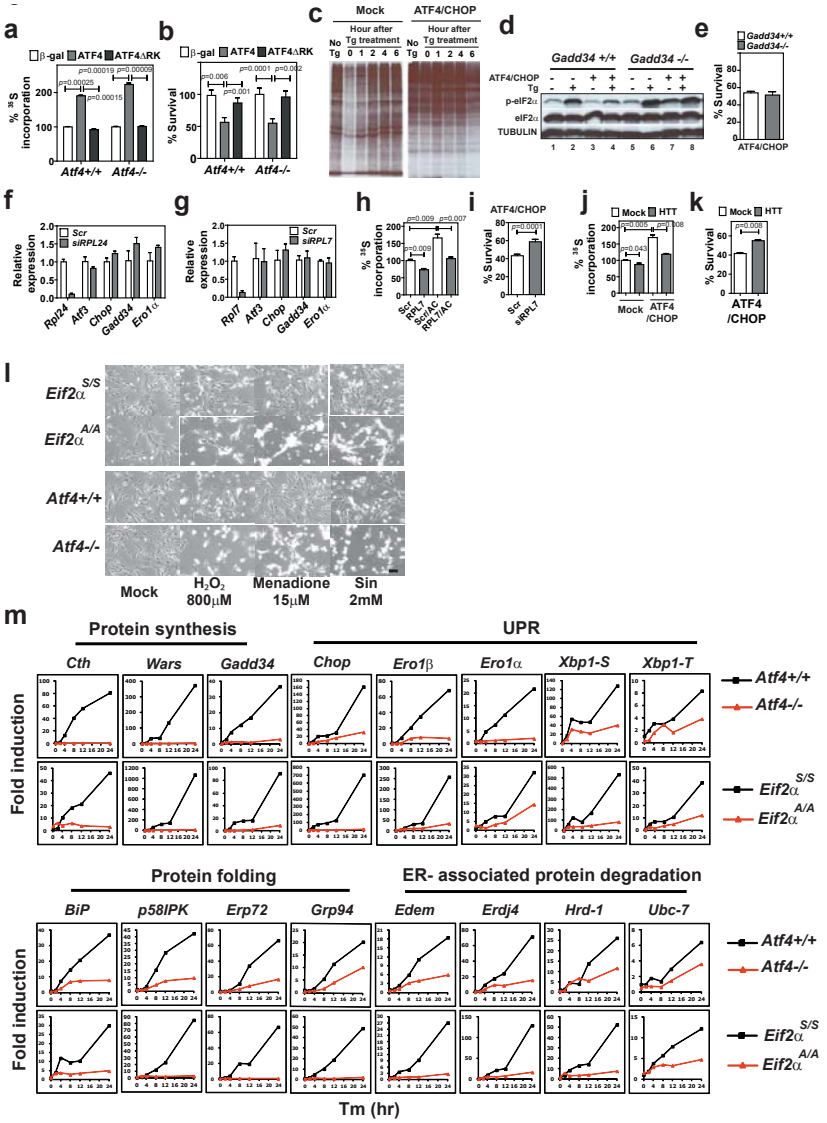


Figure S3 Tight control of protein synthesis is required for cell survival in response to ER stress. (a, b) Effect of ATF4 mutant lacking DNA binding activity (ATF4DRK) on protein synthesis and cell viability. Cells were infected with adenoviruses expressing b-gal, ATF4, and ATF4DRK for 24 hr followed by (a) metabolic labeling with [^{35}S]-methionine and cysteine for 15 min (n=3 independent experiments) and (b) analysis of cell viability at 48 hr after infection (n=3 independent experiments). (c) Effect of ATF4 and CHOP over-expression on translation attenuation. At 24 hr after adenovirus-mediated delivery of ATF4 and CHOP, MEFs were treated with 1mM thapsigargin (Tg) for one hour, followed by replacement with fresh medium. Metabolic labeling with [^{35}S]-methionine and cysteine for 15 min was performed at the indicated time of recovery. Cell lysates were collected at the indicated times and analyzed by SDS-PAGE. No Tg indicates vehicle treatment. 0 hr indicates immediately after one hour Tg treatment. (d) Western blot analysis for ATF4 and CHOP over-expression in *Gadd34+/+* and *Gadd34-/-* MEFs. Cell lysates were collected at 24hr after over-expression of ATF4 and CHOP in *Gadd34+/+* or *Gadd34-/-* MEFs. MEFs were treated with Tg (1 mM) or vehicle for one hour prior to harvesting cell lysates. (e) Effect of Gadd34 on ATF4 and CHOP-mediated cell death. *Gadd34+/+* and *Gadd34-/-* MEFs were infected with adenoviruses expressing ATF4 and CHOP and cell viability was measured at 48 hr after infection (n=3 independent experiments). (f) Knock-down efficiency of *siRpl24*. Wild-type MEFs were transfected with scrambled siRNA (Scr) or siRNA against *Rpl24*, and incubated for 48 hr. Total RNAs were isolated for qRT-PCR (n=3 independent experiments). (g-i) Effect of *Rpl7* knock-down on protein synthesis and cell viability. (g) Knock-down efficiency of *siRpl7*. Wild-type MEFs were transfected with scrambled siRNA (Scr) or siRNA against *Rpl7*, and incubated for 48 hr. Total RNAs were isolated for qRT-PCR (n=3 independent experiments). Effect of *siRpl7*-silencing on (h) protein synthesis at 24 hr (n=3 independent experiments) and (i) ATF4/CHOP-mediated cell death at 48hr were measured (n=3 independent experiments). (j-k) Effect of homoharringtonine (HTT) on protein synthesis and cell viability. HTT inhibits protein synthesis by acting on the first cycle of the elongation phase of translation by preventing substrate binding to the acceptor site on the eukaryotic 60S ribosome subunit, therefore blocking aminoacyl-tRNA binding and peptide bond formation[1]. Wild-type MEFs were treated with HTT (50 mM) one day prior to infection with Ad-ATF4 and Ad-CHOP. At 24 hr after infection protein synthesis (j) and cell viability (k) were measured at 48 hr after infection or at 24 hr after treatment with Tm (2 mg/ml) in the absence (Mock) or presence of HTT (HTT) (n=3 independent experiments). (l) Sensitivity of cells to oxidative stress. MEFs were treated with the inducers of reactive oxygen species (ROS) H_2O_2, Menadione, and 3-Morpholinosydnonimine (Sin) at the indicated concentrations for 2-6 hr in b-mercaptoethanol-lacking growth media, and cell morphology was examined. Representative images from each condition are shown. Scale bar represents 100 mm. (m) mRNA expression in *Eif2α$^{A/A}$* and *Atf4 -/-* MEFs in response to Tm. MEFs were treated with Tm (2 mg/ml) for the indicated times and total RNAs were isolated for qRT-PCR for genes with functions in protein synthesis, the UPR, protein folding, and ER-associated protein degradation (n=3 independent experiments). Data are presented as means ± SEM.

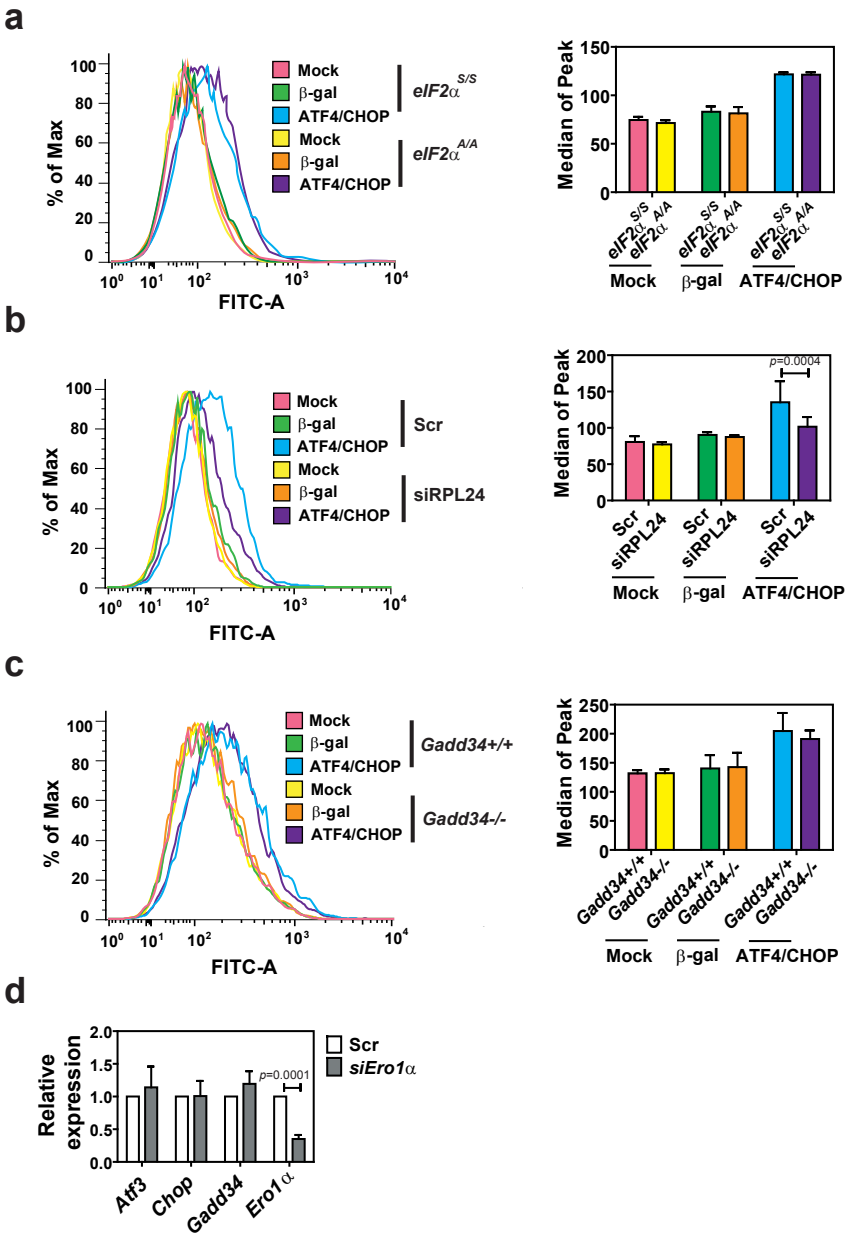


Figure S4 Oxidative stress upon ATF4/CHOP over-expression and Knock-down efficiency of *Ero1α*. *Eif2α*$^{A/A}$ and *Eif2α*$^{S/S}$ MEFs (n=3 independent experiments) (a), MEFs treated with scrambled siRNA (Scr) or siRNA for *Rpl24* (n=3 independent experiments) (b) and *Gadd34+/+* and *Gadd34-/-* MEFs (n=3 independent experiments) (c) were mock-treated or infected with Ad-ATF4 and Ad-CHOP for 24 hr and stained with chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H2DCFDA) for analysis of oxidative stress by flow cytometry (left). For a negative control, cells were infected with Ad-b-gal. Histograms for median peaks of cell populations are shown in right panels. (d) Knock-down efficiency of *Ero1α*. Wild-type MEFs were transfected with scrambled siRNA (Scr) or siRNA against *Ero1a*, and incubated for 48hr. Total RNAs were isolated for qRT-PCR (n=3 independent experiments). Data are presented as means ± SEM.

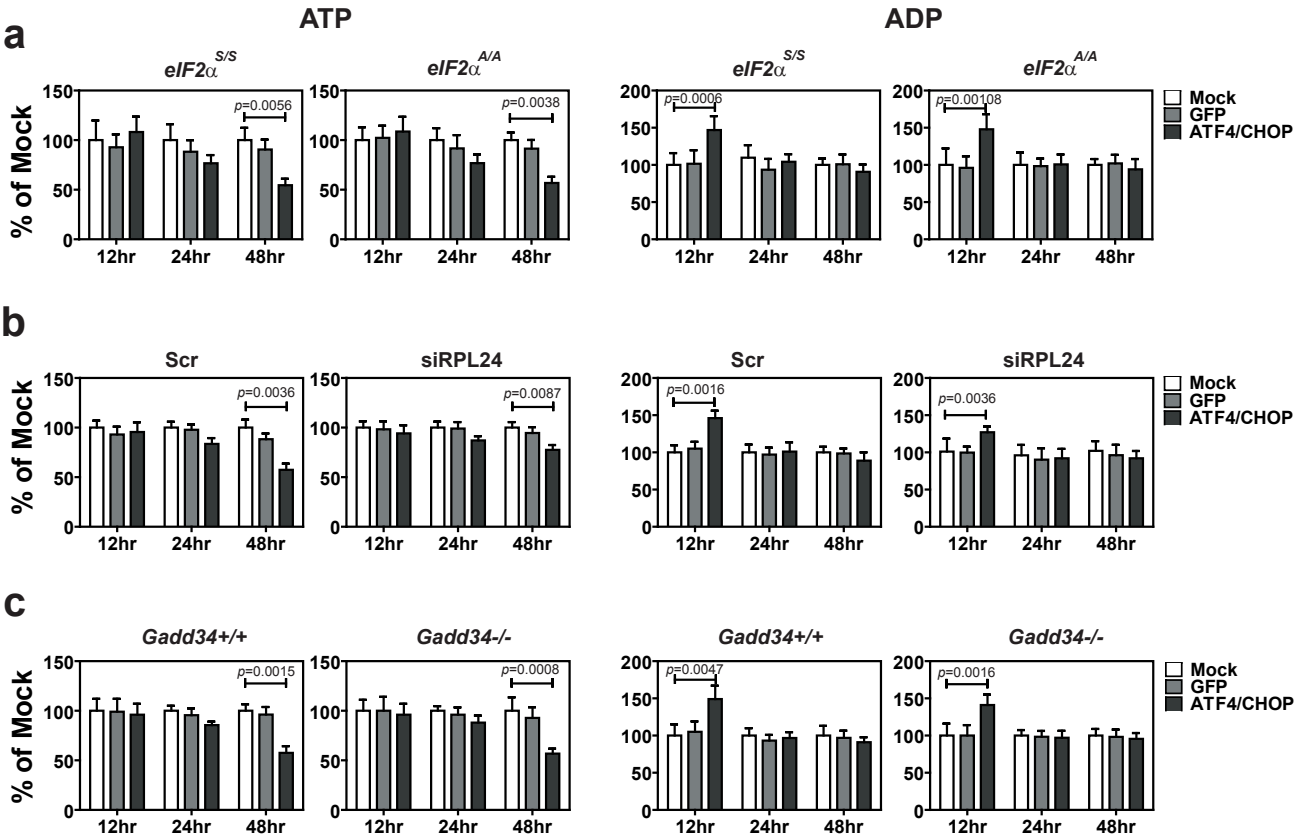


Figure S5 ATP and ADP levels during ATF4 and CHOP over-expression. (a-c) ATP and ADP levels measured at indicated time points after infection with Ad-ATF4 and Ad-CHOP or Ad-GFP as control in *Eif2α*$^{A/A}$ and *Eif2α*$^{S/S}$ MEFs (n=3 independent experiments) (a) and Scrambled siRNA (Scr) or siRpl24-treated MEFs (n=3 independent experiments) (b) and *Gadd34+/+* and *Gadd34-/-* MEFs (n=3 independent experiments) (c). Data are presented as means ± SEM.

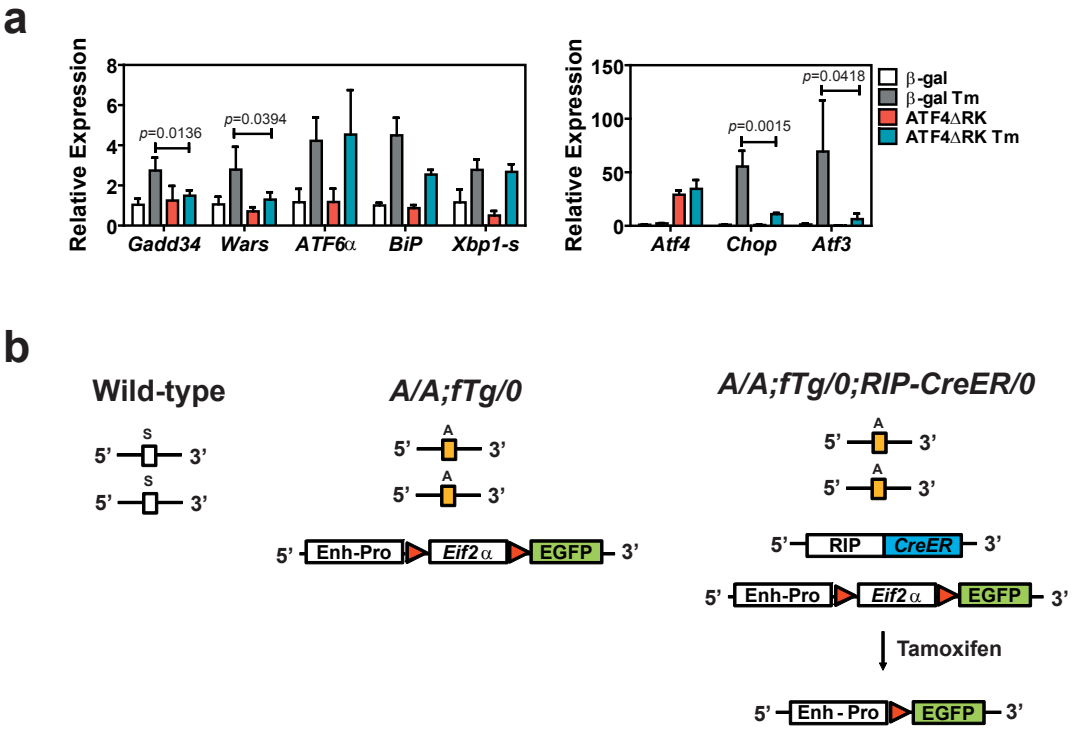


Figure S6 Effect of ATF4 on gene expression *in vivo.* (a) Effect of forced expression of ATF4DRK on UPR gene expression upon Tm treatment in the liver. At 3 days after infection with adenovirus expressing β-gal or ATF4DRK, mice were administered with Tm (2g/kg body weight) or vehicle for 8 hours and liver tissues were collected for RNA isolation and qRT-PCR. Data are presented as means ± SEM (n=3 independent experiments). (b) Diagram illustrating the genotypes of wild-type, *A/A;fTg/0* and *A/A;fTg/0;RIP-CreER/0 mice*. *A/A;fTg/0* mice harbor two Ser51Ala mutant *Eif2α* alleles (*A/A*) as well as a floxed wild-type *Eif2α* transgene (*fTg*) driven by the CMV enhancer and chicken β-actin promoter (middle panel). LoxP sequences (red arrowheads) allow excision of floxed wild-type *Eif2α* transgene and turn on EGFP expression. The tamoxifen-dependent CreER recombinase is driven by the rat insulin promoter 2 (*A/A;fTg/0;RIP-CreER/0*). After deletion of the wild-type *Eif2α* transgene (*fTg*), EGFP expression is expressed. S indicates endogenous wild-type *Eif2α* allele and A indicates the Ser51Ala mutant allele of *Eif2α*.

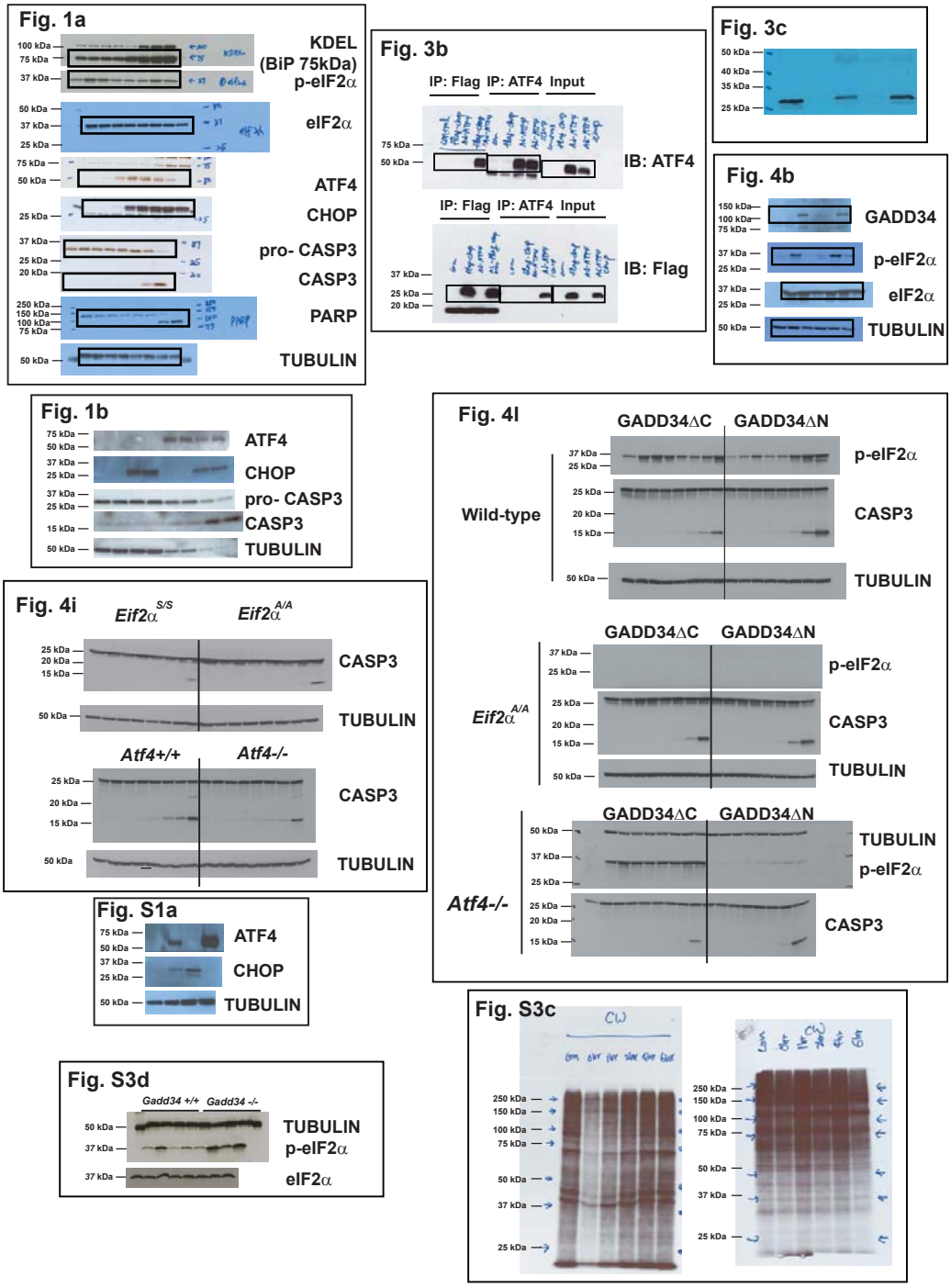


Figure S7 Uncropped Western blot images used in the figures

Legends for Supplementary Tables.

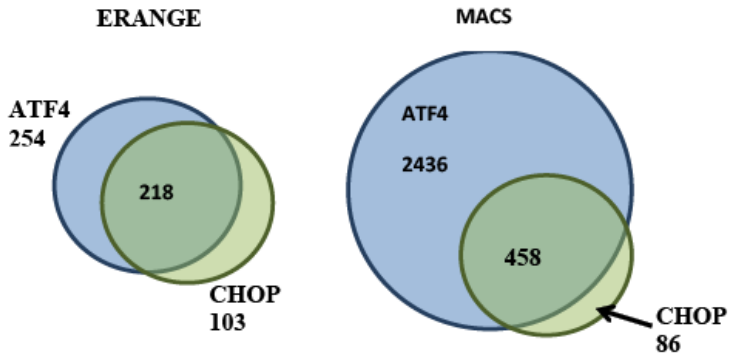
Table S1. The number of reads and % mapped reads in CHOP and ATF4 ChIP-seq data.
Table S2. List of ATF4 and CHOP target genes that have binding peaks within 3kb from TSSs of annotated gene. dTSS corresponds to the distance (bps) between the transcriptional start site (TSS) of the nearest annotated gene and peak start/end depending on the strand.
Table S3. Comparison of genes targeted by ATF4 and CHOP identified by ChIP-Seq. Genes were categorized as groups with similar functional properties using DAVID. Shaded box indicates targets of each transcription factor.
Table S4. Matrix of genes that have differential expression levels in response to ER stress. Group labels correspond to the set definition in Supplementary Figure S2b.
Table S5. List of genes that are directly targeted and regulated by ATF4 and CHOP. FC represents fold induction of each gene in wild-type MEFs in response to Tm treatment.
Table S6. Distance between ATF4 and CHOP peaks and motifs. Distances of peaks indicate distances between the middle position of ATF4 and CHOP peaks.
Table S7. Primer lists used for qRT-PCR, cloning and ChIP.

Supplemental References:

1. Fresno, M., Jimenez, A. & Vazquez, D. Inhibition of translation in eukaryotic systems by harringtonine. *Eur J Biochem* **72**, 323-330 (1977).

Analysis of ChIP-Seq data to identify binding motifs.

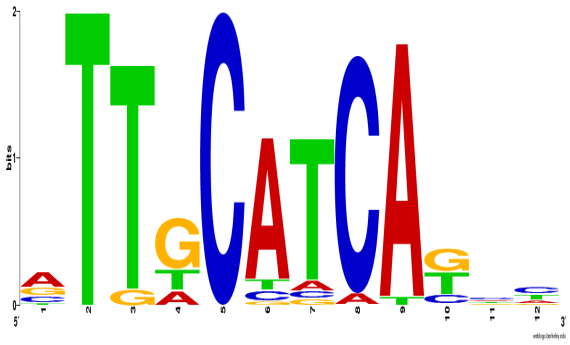
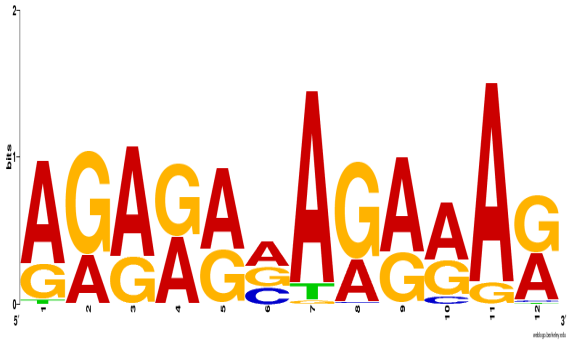
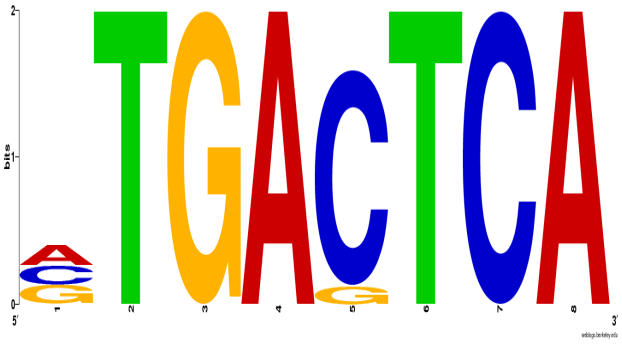
Using MACS (with a p-value cutoff of 1E-5), we identified a total of 32,656 peaks for ATF4 and 6,162 for CHOP. Approximately 81% of the CHOP peaks (4,972 out of 6,162) overlapped with the ATF4 peaks, however only 15% of the 32,656 ATF4 peaks overlapped with CHOP, mainly due to the extremely imbalanced number of peaks identified. From the identified peaks, 2,894 genes had a peak within 3kb of its TSS for ATF4, and 544 genes had a peak within 3kb of its TSS for CHOP. Of these, 458 genes had both factors bound (See figure below). Thus, compared to the targets identified by ERANGE, a higher percent of targets for CHOP and lower percent of targets for ATF4 were identified as overlapping. This can be explained by the excess of ATF4 peaks identified by MACS, i.e., increased sensitivity. Using the results for ATF4/CHOP from MACS, we analyzed for enriched GO terms using the program GREAT (Genomic Regions Enrichment of Annotations Tool) (http://great.stanford.edu/public/html/splash.php with default settings).

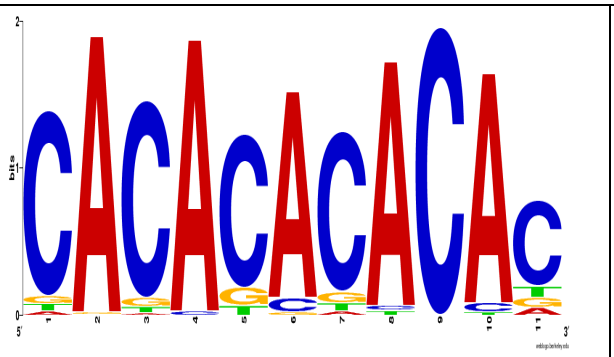


The targete for ATF4 nor the CHOP identified by MACS were not enriched in the GO term of apoptosis. Additionally, we analyzed enrichment for the ATF4 genes with a peak <3kb from their TSS using ConceptGen (http://conceptgen.ncibi.org); again, apoptosis was not identified. Although apoptosis was not over-represented among the genes with peaks overall, there were several genes identified that are possibly involved in apoptosis, namely *Trib3*, *ppp1r15a*, *Sqstm1*, and *Dnaja3*. However, direct roles for these gene products in apoptosis have not been established.

DNA motif search.

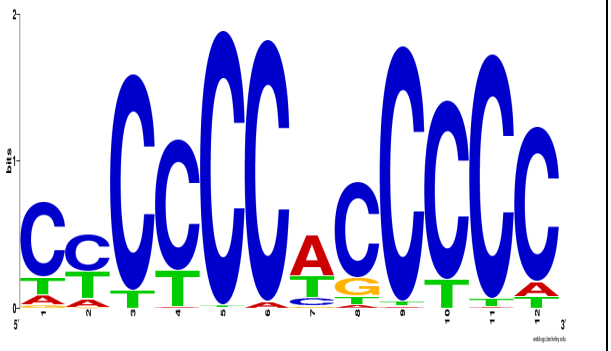
MEME analysis (see methods) identified TTGCATCAG as the most highly represented motif for both ATF4 and CHOP. There were no other *independent* motifs other than degenerate repeated sequences, which were only present in a minority of the peaks. Below are the MEME results for the top 5 motifs. Note that motif 2 for CHOP is very similar to the top ranked motif, but with the middle "CA" flipped, thus we considered this a (much less common) variant of the top motif. In the table, "llr" = log- likelihood ratio.

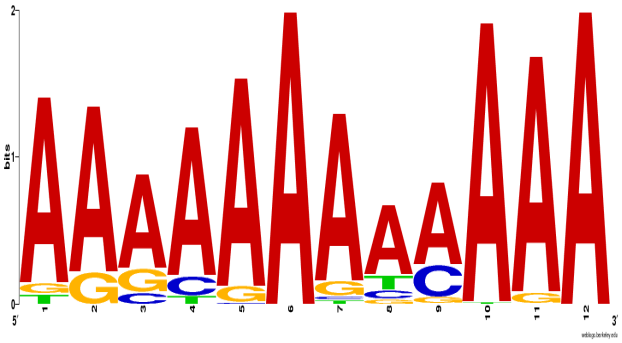
ATF4	CHOP
MOTIF 1 width = 12; sites = 3022; llr = 26415; E-value = 3.6e-1714 	MOTIF 1 width = 12; sites = 2598; llr = 20292; E-value = 6.5e-1622 
MOTIF 2 width = 12; sites = 3023; llr = 24411; E-value = 8.3e-834 	MOTIF 2 width = 8; sites = 580; llr = 5620; E-value = 2.2e-143 
MOTIF 3 width = 11; sites = 781; llr = 9156; E-value = 3.5e-530	MOTIF 3 width = 12; sites = 649; llr = 6083; E-value = 2.3e-086



MOTIF 4 width = 12; sites = 793; llr = 8918; E-value = 3.3e-375

MOTIF 4 width = 12; sites = 319; llr = 3395; E-value = 1.3e-081





MOTIF 5 width = 12; sites = 3013; llr = 23090; E-value = 1.3e-262

MOTIF 5 width = 12; sites = 136; llr = 1683; E-value = 3.6e-063