The role of *de novo* protein synthesis and SIRT1 in ER stress-induced *Atf4* and *Chop* mRNA expression in mammalian cells

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Running title: ATF4 and ER stress

Abstract

Endoplasmic reticulum (ER) stress and the activation of the unfolded protein response (UPR) have been implicated in the pathogenesis of many common human diseases. Integral to the UPR and an important determinant in cell fate is the expression of the pro-apoptotic transcription factor C/EBP homologous protein (CHOP). This is promoted by activating transcription factor 4 (ATF4) whose expression is rapidly up-regulated in response to ER stress through an eIF2α phosphorylation-dependent increase in protein synthesis. Our data demonstrates that this ER stress-induced increase in ATF4 and CHOP expression is initiated by an increase in *Atf4* and *Chop* mRNA, which is also dependent upon eIF2α phosphorylation. Despite being dependent on eIF2α phosphorylation, we provide evidence that these increases in *Atf4* and *Chop* mRNA expression may occur independently of *de novo* protein synthesis. Moreover, we show that ER stress-induced *Chop* mRNA expression is exacerbated by Sirtuin-1 (SIRT1) inhibition indicating that changes in the energy status of the cell may play an important role in its regulation. This work highlights and extends previous findings, and provides important new insights into the mechanism of ER stress-induced expression of *Atf4* and *Chop* mRNA that clearly warrants further investigation.

Highlights

- An increase in *Atf4* mRNA is a prerequisite for ER stress-induced ATF4 expression
- PERK activation is required for ER stress-induced *Atf4* and *Chop* mRNA.
- eIF2α phosphorylation is required for ER stress-induced *Atf4* and *Chop* mRNA.
- Increased *Atf4* and *Chop* mRNA expression occur independently of *de novo* protein synthesis
- SIRT1 represses *Chop* mRNA expression but enhances G*add*34 and *Ero1l* expression

Keywords: UPR, ER stress, eIF2α, ATF4, CHOP, PERK, SIRT1, Transcription, mRNA

Abbreviations: ER (endoplasmic reticulum), UPR (unfolded protein response), PERK (PKR-like ER kinase), CHOP (C/EBP Homologous Protein), ATF4 (activating transcription factor 4), IRE1 (inositol requiring enzyme 1), SIRT 1 (Sirtuin 1)

Introduction

The endoplasmic reticulum (ER) serves as a site for the synthesis of almost all secreted and membrane proteins. Perturbations in ER homeostasis that result in the accumulation of mis-folded proteins within the ER leads to 'ER stress' [1,2]. This is sensed by ER-transmembrane proteins whose activation initiates the unfolded protein response (UPR), an adaptive response intended to restore ER homeostasis and alleviate ER stress. If ER homeostasis is not restored, the UPR switches from a cell protective to a pro-apoptotic program in order to clear the mal-functioning cell from the organism. Activating transcription factor 4 (ATF4) ATF4 plays a critical role in this as it drives the transcription of a large number of genes, including that encoding the pro-apoptotic protein C/EBP homologous protein (CHOP) also known as DNA damage-inducible transcript 3 (DDIT3), an important determinant in cell fate [1,3–6].

The UPR is triggered by the activation of ER transmembrane proteins including: PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). When activated PERK phosphorylates the translation initiation factor eIF2 on its alpha subunit (eIF2α) resulting in the attenuation of global protein synthesis [7,8]. However, it also enhances the rate of protein synthesis from several mRNAs which encode upstream open reading frames (uORFs) within their 5'untranslated region (5'UTR), including that

encoding activating transcription factor 4 (ATF4) [3,9–11]. This increase in the rate of ATF4 protein synthesis is often reported and/or generally inferred to be the initiating step in the up-regulation of ATF4 expression. However, ER stress also promotes an increase in *Atf4* mRNA expression [3,10,12]. The mechanism by which ER stress induces an increase in ATF4 mRNA is poorly understood. Although it is significantly reduced in both eIF2α S51A knock-in mouse embryonic fibroblasts (MEF) or PERK knock-out MEF [9,13–15].

In this report we investigate the role of transcription in ER stress-induced ATF4 and CHOP expression in mammalian cells and endeavour to gain mechanistic insights into how ER stress promotes increased *Atf4* and *Chop* mRNA expression.

Methods

General Reagents and Materials. GSK2606414 and thapsigargin were purchased from Merck. 4μ8C and EX527 were purchased from Tocris Bioscience. All other chemicals and reagents were purchase from Fisher Scientific, Sigma-Aldrich or VWR unless otherwise specified.

Cell Culture. Wild type and PERK knock out mouse embryonic fibroblasts (MEF) [13] were cultured in DMEM media containing 25mM glucose supplemented with 10% heat-inactivated FBS, 100μg/ml streptomycin, 100units/ml penicillin, 100units/ml neomycin and maintained at 37ºC and 5% CO_2. Mouse Insulinoma 6 (MIN6) cells [16] were used between passages 25 and 35 at ~80% confluence and cultured as previously described [17].

SDS-Polyacrylamide Gel Electrophoresis and Immunohistochemistry. SDS-PAGE and western blotting were performed as previously described [18] using antibodies to: BiP (BD Transduction Laboratories, USA), phospho-IRE1α (Ser 724; Abcam, USA), phospho-PERK

(Thr 980), phospho-eIF2α (Ser 51), phospho-c-jun (Ser 63), CHOP, ATF4, rpS6 and GAPDH (Cell Signaling Technology, USA). Immuno-labeled bands were quantified by densitometry (Image Lab, Bio-Rad Inc., USA).

RNA isolation and qPCR analyses Total RNA was extracted from tissue using the ReliaPrep™ RNA Cell Miniprep System (Promega, USA) according to manufacturer's instructions. Reverse transcription was carried out using 0.2 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative PCR was carried out using the SYBR® Green PCR Master Mix (Applied Biosystems, UK). The gene expression from each sample was either analysed in duplicate or triplicate and normalized against 18S ribosomal RNA. All reactions were performed on the Rotor-Gene Q (Qiagen, USA) using cycling conditions recommended by the manufacturer. The results are expressed as relative gene expression using the ΔCt method [19].

Infection of cell lines with recombinant adenoviruses. Adenovirus expressing GADD34ΔN were generated as previously described [17]. Prior to experimentation, cells were transduced by incubation for 96h with high titre virus. Adenoviruses transduction efficiency was determined by monitoring GFP expression using a Nikon fluorescence microscope fitted with a mercury lamp.

Statistical analysis Data are expressed as mean ± SEM. Unless otherwise stated data were analysed by one-way ANOVA followed by Tukey's post-hoc test for multiple comparison between means using Prism 6 (GraphPad Software, USA).

Results

ER stress-induced Atf4 and Chop mRNA expression precedes an increase in ATF4 and CHOP protein expression. To investigate the temporal relationship between changes in ER stress-induced *Atf4* and *Chop* mRNA expression and ATF4 and CHOP protein expression, mouse embryonic fibroblasts (MEF) were treated with thapsigargin, a classic pharmacological inducer of ER stress, for up to 6h (Figure 1). Induction of ER stress and the activation of the UPR were determined by measuring changes in the phosphorylation status of PERK, IRE1 and eIF2α by Western blot analysis (Figure 1a). Changes in ATF4 and CHOP protein and mRNA expression were determined by Western blot and qPCR analysis respectively (Figure 1). Thapsigargin treatment led to a rapid increase (within 15 minutes) in the phosphorylation of IRE1, PERK and its downstream target, eIF2α (Figure 1a). Increased ATF4 protein expression was detected after 1h, whereas CHOP protein expression was detected after 2h (Figure 1a). The expression of *Atf4* mRNA was detected after 30 min of treatment whereas an increase in *Chop* mRNA was detected after 1h (Figure 1b). Therefore, in response to ER stress, an increase in *Atf4* and *Chop* mRNA expression precedes an increase in ATF4 and CHOP protein expression. Moreover, an increase in ATF4 expression precedes an increase in the expression of CHOP.

Active transcription is required for ER stress-induced increased ATF4 protein expression. To determine whether ER stress-induced *Atf4* mRNA expression is a prerequisite for the rapid increase in ATF4 protein, MEFs and the pancreatic beta-cell line MIN6 were treated with thapsigargin in the presence or absence of the transcriptional inhibitor actinomycin-D for the times indicated (Figure 2). In both MEFs and MIN6 cells thapsigargin lead to a robust and rapid increase in both *Atf4* mRNA and protein expression as determined using qPCR and Western blot analysis (Figure 2). *Chop* mRNA expression also increased rapidly in response to ER stress (Figure 2b and d). As expected actinomycin-D inhibited ER stress-induced

increases in *Atf4* and *Chop* mRNA (Figures 2b and d). Importantly, actinomycin-D also inhibited ER stress-induced increase in ATF4 and CHOP protein expression (Figures 2a and c). Of note, actinomycin-D increased eIF2α phosphorylation in both MEFs and MIN6 cells yet no increase in ATF4 protein expression was detected (Figure 2a and c). Therefore, based on these results, *Atf4* mRNA transcription is a prerequisite for ER stress-induced increases in ATF4 protein expression in both MIN6 cells and MEFs and that eIF2α phosphorylation alone is insufficient to promote ATF4 protein expression in the absence of an increase in ATF4 mRNA. This conclusion is supported by previous findings showing that actinomycin D inhibits ER stress-induced ATF4 protein expression in MEFs [3,20].

PERK is required for ER stress-induced Atf4 and Chop mRNA expression. To investigate how ER stress stimulates an increase in ATF4 transcription, MEFs were treated with thapsigargin for 2 or 6 h in the presence or absence of either an inhibitor of PERK (GSK2606414) or IRE1 (4μ8C) (Figure 3a). As anticipated, thapsigargin-induced expression of both ATF4 and CHOP were inhibited by GSK2606414. Importantly, GSK2606414 also inhibited or significantly reduced ER stress-induced increases in *Atf4* and *Chop* mRNA expression. In contrast, the inhibition of IRE1 had no significant effect on either *Atf4* or *Chop* mRNA or protein expression.

To investigate the critical role of PERK in *Atf4* and *Chop* transcription in an alternative cell type, MIN6 cells were also treated with thapsigargin for 2 and 6 h in the presence or absence of GSK2606414 (Figure 3b). Thapsigargin caused an increase in PERK and eIF2α phosphorylation as well as an increase in the expression of CHOP and ATF4 protein which was co-incident with an increase in both *Atf4* and *Chop* mRNA expression (Figure 3b). In the presence of GSK2606414, thapsigargin-induced increases in eIF2α phosphorylation was significantly reduced at 2h but not by 6h indicating that in these cells there may be a compensatory increase in the activity of another eIF2α kinase, most likely PKR [21]. Despite

this both and ATF4 and CHOP protein expression was ablated at both 2 and 6h (Figure 3b). Importantly, as observed in MEFs, GSK2606414 also inhibited thapsigargin-induced increases in both *Atf4* and *Chop* mRNA expression (Figure 3b).

To confirm the role of PERK in ER stressed-induced *Atf4* and *Chop* mRNA expression we compared changes in the expression of *Atf4* and *Chop* mRNA and protein in response to thapsigargin in wild-type (WT) versus PERK 'knock-out' MEFs ($PERK^{-/-}$) (Figure 4). Thapsigargin caused an increase in the expression of both *Atf4* and *Chop* mRNA and protein in WT MEF but not in $PERK^{-/-}$ cells (Figure 4a and b).

Taken together, these results provide strong evidence that PERK activation is required for ER stress-induced increases in *Atf4* and *Chop* mRNA expression in mammalian cells.

eIF2α phosphorylation is required for increased Atf4 and Chop mRNA expression. There has been some contradictory findings regarding the role of eIF2α in ER stress-induced ATF4 expression using eIF2α ser51ala knock in MEFs [9,15]. To reinvestigate the role of eIF2α phosphorylation in the up-regulation of *Atf4* and *Chop* mRNA expression using an alternative approach, MEFs were transduced with adenovirus expressing an N-terminal deletion mutant of GADD34 (GADD34ΔN), which directs protein phosphatase 1 to eIF2α resulting in its constitutive de-phosphorylation [14,22]. Cells were then treated with thapsigargin to induce ER stress and effects on eIF2α phosphorylation and *Atf4* and *Chop* mRNA expression determined (Figure 5). As anticipated, thapsigargin increased eIF2α phosphorylation and *Atf4* and *Chop* mRNA and protein expression. Importantly, in cells expressing GADD34ΔN thapsigargin-induced eIF2α phosphorylation and ATF4 and CHOP protein and mRNA expression were effectively blocked. Therefore, in MEFs increased *Atf4* and *Chop* mRNA expression in response to ER stress are dependent upon eIF2α phosphorylation**.**

The role of de-novo protein synthesis in ER stress-induced increases in Atf4 and Chop mRNA expression. As eIF2α phosphorylation is required for increased *Atf4* mRNA expression (Figures 3, 4 and 5) and the only known role for eIF2α phosphorylation is in regulating protein synthesis, we figured that eIF2α phosphorylation likely impacts on *Atf4* mRNA expression by increasing the protein synthesis of a yet unidentified transcription factor. To investigate this possibility we treated MEFs with thapsigargin in the presence or absence of cycloheximide, an inhibitor of protein synthesis (Figure 6). Thapsigargin caused the phosphorylation of eIF2α and an increase in ATF4 and CHOP mRNA and protein expression (Figure 6a). As anticipated, in the presence of cycloheximide, the synthesis of ATF4 and CHOP was inhibited (Figure 6a). Surprisingly, in the presence of cycloheximide, thapsigargin was still able to promote an increase in *Atf4* and *Chop* mRNA expression (Figure 6b). These novel results demonstrate that an increase in *Atf4* and *Chop* transcription can occur independently of *de novo* protein synthesis and that an increase in ER stress-induced *Chop* mRNA can occur independently of ATF4. However, cycloheximide alone promoted an increase in eIF2α phosphorylation as well as an increase in *Atf4* and *Chop* mRNA expression (Figure 6b). Whether these increases were mediated by an eIF2α-dependent mechanism akin to what happens in ER stress is unknown. To investigate this, MEFs or MEFs transduced with adenovirus expressing GADD34ΔN were treated with thapsigargin, cycloheximide or cycloheximide and thapsigargin and the effects on *Atf4* and *Chop* mRNA expression investigated (Figure 6c). As previously shown, thapsigargin-induced *Atf4* and *Chop* mRNA expression was inhibited in GADD34ΔN expressing cells. However, cycloheximide or cycloheximide plus thapsigargin-induced *Atf4* mRNA expression was only partially inhibited in GADD34ΔC expressing cells, indicating that an increase in *Atf4* mRNA transcription can occur independently of eIF2α phosphorylation and that cycloheximide

promotes an increase in *Atf4* transcription via a mechanism distinct from ER stress induced *Atf4* expression. In contrast, cycloheximide-induced *Chop* mRNA expression in the presence or absence of thapsigargin was inhibited in the presence of GADD34ΔN indicating that eIF2α phosphorylation is required for *Chop* mRNA expression but that *de novo* protein synthesis is not. It should be noted that the expression of 18S RNA was unaffected by either thapsigargin or cycloheximide treatment over the time course of these experiments (results not shown).

Role of SIRT1 in Atf4 and Chop mRNA expression. Protein synthesis is a major consumer of energy and thus the inhibition of protein synthesis by phosphorylating eIF2α will cause a change in the energy status of the cell. Thus it is possible that eIF2α phosphorylation increases *Atf4* and *Chop* mRNA transcription by indirectly changing the activity of an intracellular energy sensor. Sirtuin 1 (SIRT1) is a highly conserved mammalian NAD^+-dependent protein deacetylase which regulates gene expression and is inactivated in response to an increase in energy status [23]. Therefore, we reasoned whether increased *Atf4* or *Chop* mRNA expression were mediated by the inactivation of SIRT1. To investigate this, MEFs were treated with thapsigargin in the presence or absence of EX527 and sirtinol, selective inhibitors of SIRT1 and 2 (Figure 7) (n.b.EX527 has IC50 values are 0.1–1 and 20–33 µM whereas sirtinol has IC50 values of 37.6–131 38–58 for SIRT1 and SIRT2 respectively [24]). As a control, the levels of acetylation in whole cell extracts was also determined using an anti-acetyl lysine antibody (Figure 7a and b). As anticipated, EX527 and sirtinol promoted increased acetylation demonstrating their effectiveness. However, thapsigargin had no global effect on acetylation (Figure 7a and b). EX527 or sirtinol had no effect on thapsigargin-induced ATF4 protein expression (Figure 7a and b). However, thapsigargin induced *Chop* mRNA expression was significantly increased in the presence of these inhibitors (Figure 7c and d) or in the presence of an alternative SIRT1 inhibitor suramin (IC50 0.3–2.6 and 1.1–20 µM for SIRT 1 and SIRT2 respectively [24]) (Figure 7d). Thus SIRT may act as a repressor

of ER stress-induced *Chop* mRNA expression. To investigate whether these inhibitors affected the mRNA expression of other ER stress markers, changes in *Atf4*, *Bi*p *Gadd34*, and *Ero1l* mRNA were also quantified. None of the inhibitors affected ER stress-induced *Bip* or *Atf4* mRNA expression (Figure 7c, d and e) but interestingly EX527 and suramin significantly inhibited *Gadd34* and *Ero1l* mRNA expression. As these inhibitors are more specific for SIRT1 it is likely that these effects are mediated through the inhibition of SIRT1 rather than SIRT2.

Discussion

The classical and often cited mechanism by which ER stress increases ATF4 expression is through an increase in the rate of ATF4 translation mediated by the PERK-dependent phosphorylation of eIF2α. What is often overlooked is the role of *Atf4* mRNA transcription. Our results show that ER stress induced increases in *Atf4* mRNA transcription is a prerequisite for increased ATF4 protein expression. Moreover, we show that this increase in *Atf4* mRNA is dependent upon both PERK activation and eIF2α phosphorylation. These results confirm and extend previous findings [3,15,20,25]. The only known role of eIF2α is in regulating protein synthesis and therefore the most plausible explanation for the role of eIF2α phosphorylation in *Atf4* mRNA transcription is that it promotes a rapid increase in the synthesis of a yet unidentified transcription factor or factors that stimulates an increase in ATF4 transcription. This factor is unlikely to be ATF4 as overexpression of ATF4 is unable to induce ATF4 expression and ATF4 does not bind to the ATF4 promoter based on genome-wide chromatin immunoprecipitation and RNA sequencing analysis [26]. However, we show that ER stress induced *Atf4* mRNA expression is increased in the presence of cycloheximide, indicating that increased transcription of ATF4 can occur independently of *de novo* protein synthesis. However, given the effects of cycloheximide alone on *Atf4* mRNA expression, it is

difficult to confirm that ER stress-induced *Atf4* mRNA expression also occurs independently of *de novo* protein synthesis.

We also show that *Chop* mRNA expression is dependent upon PERK activation and eIF2α phosphorylation, thus supporting and extending previous findings [15,20]. As ATF4 acts as a transcriptional activator of CHOP, and ATF4 expression requires PERK/eIF2α phosphorylation, the most plausible explanation for the effect of PERK inhibition or the forced dephosphorylation of eIF2α on *Chop* mRNA expression is through the inhibition of ATF4 protein expression. However, *Chop* mRNA expression is increased in the presence of cycloheximide or thapsigargin plus cycloheximide demonstrating that, at least under these conditions, ATF4 is not a prerequisite for increased *Chop* mRNA expression. As cycloheximide promotes an increase in eIF2α phosphorylation and inhibition of eIF2α phosphorylation represses cycloheximide-induced *Chop* mRNA expression, it seems likely that cycloheximide and ER stress-induced *Chop* mRNA induction is mediated by similar mechanisms. This supports the idea that cycloheximide and ER stress-induced *Chop* mRNA expression does not require *de novo* protein synthesis despite requiring eIF2α phosphorylation.

Protein synthesis is a major energy consuming process. Thus inhibition of protein synthesis, either by phosphorylating eIF2α or through the use of protein synthesis inhibitors, can lead to a rise in the energy status of the cell. Therefore it is possible that cycloheximide or agents that induce eIF2α phosphorylation increase *Atf4* and *Chop* mRNA transcription via a signalling pathway that senses the intracellular energy status of the cell. SIRT1, an NAD^+-dependent protein deacetylase which modulates gene expression, is inactivated with increased energy status [23]. Interestingly, SIRT inhibition by three distinct SIRT inhibitors, EX527, sirtinol and suramin, promotes thapsigargin-induced expression of *Chop* mRNA indicating that acetylation represses *Chop* mRNA transcription. In contrast, thapsigargin induced expression

of *EroR1* and *Gadd34* mRNAs were strongly inhibited by EX527 and suramin but not by Sirtinol. This likely reflects the selectivity of these SIRT inhibitors for particular SIRT isoform. EX527 and suramin having a higher affinity for SIRT1 than SIRT2 [24]. Therefore, SIRT1 activation may promote *Ero1l and GADD34* expression. There is evidence that SIRT1 can protect cells from ER stress [27]. Therefore it is possible that this protective effect is mediated, at least in part, by a repression of CHOP transcription and an increase in *Gadd34.*

In summary, this work highlights the fact that ER stress-induced transcription of *Atf4* and *Chop* requires PERK activation and eIF2α phosphorylation and that this increase in mRNA is a prerequisite for increased ATF4 and CHOP protein expression. We also present evidence that an increase in *Atf4* and *Chop* mRNA can occur independently of *de novo* synthesis. However, the mechanism by which this occurs is unknown. Further investigation is clearly warranted as this initiating step in the UPR may be an important pharmacological target for modulating ER stress.

Acknowledgments

SMHC, CR and TPH were supported by the School of Health and Biomedical Sciences, RMIT University. AE was funded by a Libyan Government Scholarship.

Duality of interest

The authors declare that they have no duality of interest associated with this manuscript.

Author contributions

SMHC made substantial contributions in the acquisition, analysis and interpretation of data, helped in drafting the article, and had final approval of the version of the article to be published. XZ, AE, and CR, made contributions to the acquisition of data and had final approval of the version to be published. TPH made substantial contributions to conception and design, acquisition of data, and analysis and the interpretation of the data. TPH also drafted the article and had final approval of the version to be published.

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Figures legends

Figure 1. ER stress-induced *Atf4* and *Chop* mRNA expression precedes an increase in ATF4 and CHOP protein expression. MEF were treated for up to 6 hours in the presence of thapsigargin (Tg 1µM). (**a**) Protein samples were resolved by SDS-PAGE and Western blotted using ATF4, CHOP and the phosphorylated form of PERK (p-PERK) and eIF2α (p-eIF2α). GAPDH was used as a loading control. Representative blots are shown and densitometric analyses of ATF4 and CHOP are presented below. (**b**) Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. The results are expressed as mean +/- S.E.M of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs time zero.

Figure 2. Active transcription is required for ER stress-induced increased ATF4 protein expression. MEF (**a** and **b**) and MIN6 (**c** and **d**) cells were treated with thapsigargin (1µM) in the presence or absence of actinomycin D (ActD) for 2h or 6h. (**a** and **c**) Changes in ATF4, P-eIF2α and CHOP were determined by Western Blot analysis. GAPDH was used as a loading control. Representative blots are shown and densitometric analyses of ATF4 is presented below. (**b** and **d**) Changes in the expression of *Atf4* and *Chop* mRNA were determined by qPCR analysis. The results are expressed as mean +/- S.E.M of at least three independent experiments. * $p<0.05$, ** $p<0.01$vs time zero; †† $p<0.01$ between the relevant groups.

Figure 3. Inhibition of PERK prevents ER stress-induced *Atf4* and *Chop* mRNA expression. (**a**) MEF cells were treated with thapsigargin (Th, 1µM) in the presence or

absence of vehicle (DMSO), 0.5μM GSK2606414 (PERK inhibitor = PERKi) or 30 μM 4μ8C (IRE1 inhibitor = IRE1i) for 2h or 6h. Protein samples were resolved by SDS-PAGE and Western blotted using ATF4, CHOP and the phosphorylated form of PERK (p-PERK) and eIF2α (p-eIF2α). GAPDH was used as a loading control. Representative blots for and the corresponding densitometric analyses are shown. Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. The results are expressed as mean +/- S.E.M of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs control; † $p<0.05$, †† $p<0.01$ for the compared groups. (**b**) MIN6 cells were treated with thapsigargin (Th, 1μM) in the presence or absence of vehicle (DMSO) or 0.5μM GSK2606414 (PERK inhibitor = PERKi) for 2h or 6h. Protein samples were resolved by SDS-PAGE and Western blotted using ATF4, CHOP and the phosphorylated form of PERK (p-PERK) IRE1 (p-IRE1) and eIF2α (p-eIF2α). GAPDH was used as a loading control. Representative blots and the corresponding densitometric analyses are shown. Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. The results are expressed as mean +/- S.E.M of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs control; †† $p<0.01$ for the compared groups.

Figure 4. PERK is required for ER stress-induced *Atf4* and *Chop* mRNA expression.MEF wildtype and PERK -/- cells were incubated in presence of 1μM thapsigargin for 0, 2, 6, 12 and 24 hours. (**a**) Protein samples were resolved by SDS-PAGE and Western blotted using ATF4, CHOP and the phosphorylated form of eIF2α (p-eIF2α). rpS6 was used as a loading control. Representative blots and the corresponding densitometric analyses are shown below. (**b**) Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. All results are expressed as mean +/- S.E.M of at least

three independent experiments. Unless otherwise indicated, * $p<0.05$, ** $p<0.01$ vs control; † $p<0.05$, †† $p<0.01$ for the compared groups.

Figure 5. ER stress induced ATF4 and CHOP induction is dependent on PERK-eIF2α activation in MEF cells. MEFs mock infected or infected with adenovirus expressing GADD34ΔN were incubated in the absence or presence of 1μM thapsigargin for 2h. Cell lysate were analysed by western blot and probe for antibodies against P-eIF2α, ATF4, CHOP and GAPDH was used as a loading control. Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. The results are expressed as mean +/- S.E.M of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs control; † $p<0.05$, †† $p<0.01$ for the compared groups.

Figure 6. Role of de-novo protein synthesis in stress induced increases in *Atf4* and *Chop* mRNA. MEF was treated with cycloheximide (CHX or X, 50μg/ml) for 2 or 6 hours in the presence or absence of thapsigargin (Thaps or T 1μM). (**a**) Protein samples were resolved by SDS-PAGE and Western blotted using antibodies against ATF4, CHOP and the phosphorylated form of PERK (P-PERK), eIF2α (P-eIF2α) and P-c-Jun. GAPDH was used as a loading control. Representative blots are shown and densitometric analyses of ATF4, CHOP and eIF2α are presented below. (**b**) Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. (**c**) Wildtype and adeno-virus transduced GADD34 constitutively active MEF cells were exposed to cycloheximide in the absence or presence of 1μM thapsigargin for 6h. Total RNA was isolated for qPCR analyses and the expression of *Atf4* and *Chop* were determined. The results are expressed as mean +/- S.E.M

of at least three experiments. * $p<0.05$, ** $p<0.01$ vs control; †† $p<0.01$ for the compared groups.

Figure 7. Role of SIRT1 in *Atf4* and *Chop* mRNA expression. MEF was exposed to EX527 (SIRT1i, 10µM) (a and c) or sirtinol (20 or 100µM as indicated) (b and d) or suramin (2µM) (e) for 6 hours in the presence or absence of thapsigargin (Thaps, 1µM). (a and b) Protein samples were resolved by SDS-PAGE and Western blotted using antibodies against BiP, ATF4, CHOP, pan-acetylated lysine and the phosphorylated form of PERK (p-PERK), IRE1α (P-IRE1α) and eIF2α (P-eIF2α). GAPDH was used as a loading control. Representative blots are shown. (c, d and e) Total RNA was isolated for qPCR analyses and the expression of *Atf4, Chop, BiP, Ero1l* and *Gadd34* were determined. The results are expressed as mean +/- S.E.M of three to four independent experiments. ** $p<0.01$ vs control; †† $p<0.01$ for the compared groups.

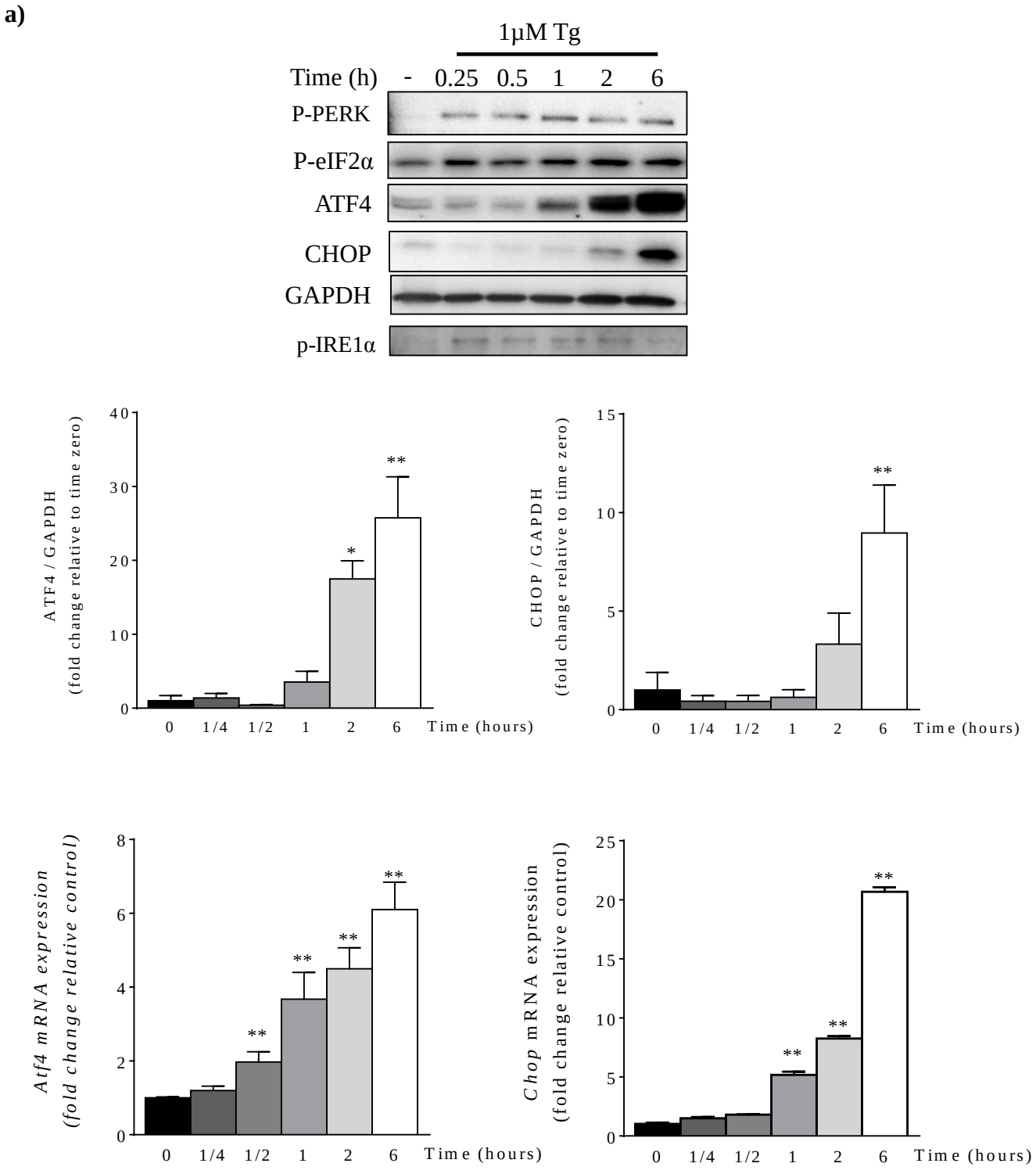
a)
1μM Tg
Time (h) - 0.25 0.5 1 2 6
P-PERK
P-eIF2α
ATF4
CHOP
GAPDH
p-IRE1α
ATF4 / GAPDH (fold change relative to time zero)
CHOP / GAPDH (fold change relative to time zero)
0 1/4 1/2 1 2 6 Time (hours)
b)
Atf4 mRNA expression (fold change relative control)
Chop mRNA expression (fold change relative control)
0 1/4 1/2 1 2 6 Time (hours)

Figure 1

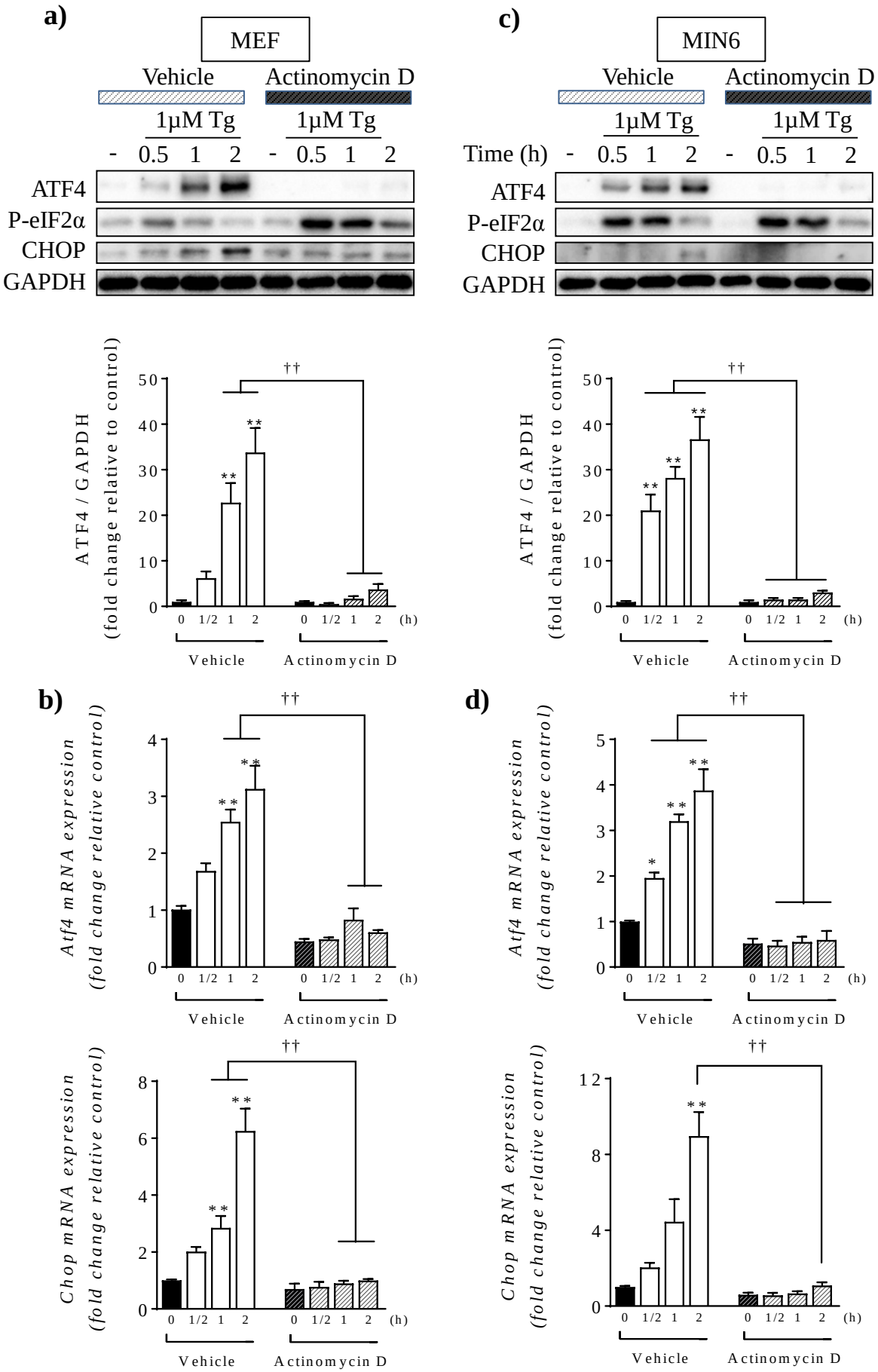
a)
MEF
Vehicle
Actinomycin D
1μM Tg
1μM Tg
- 0.5 1 2 - 0.5 1 2
ATF4
P-eIF2α
CHOP
GAPDH
c)
MIN6
Vehicle
Actinomycin D
1μM Tg
1μM Tg
Time (h) - 0.5 1 2 - 0.5 1 2
ATF4
P-eIF2α
CHOP
GAPDH
ATF4 / GAPDH
(fold change relative to control)
0 1/2 1 2 0 1/2 1 2 (h)
Vehicle
Actinomycin D
b)
Atf4 mRNA expression
(fold change relative control)
Chop mRNA expression
(fold change relative control)
d)
Atf4 mRNA expression
(fold change relative control)
Chop mRNA expression
(fold change relative control)

Figure 2

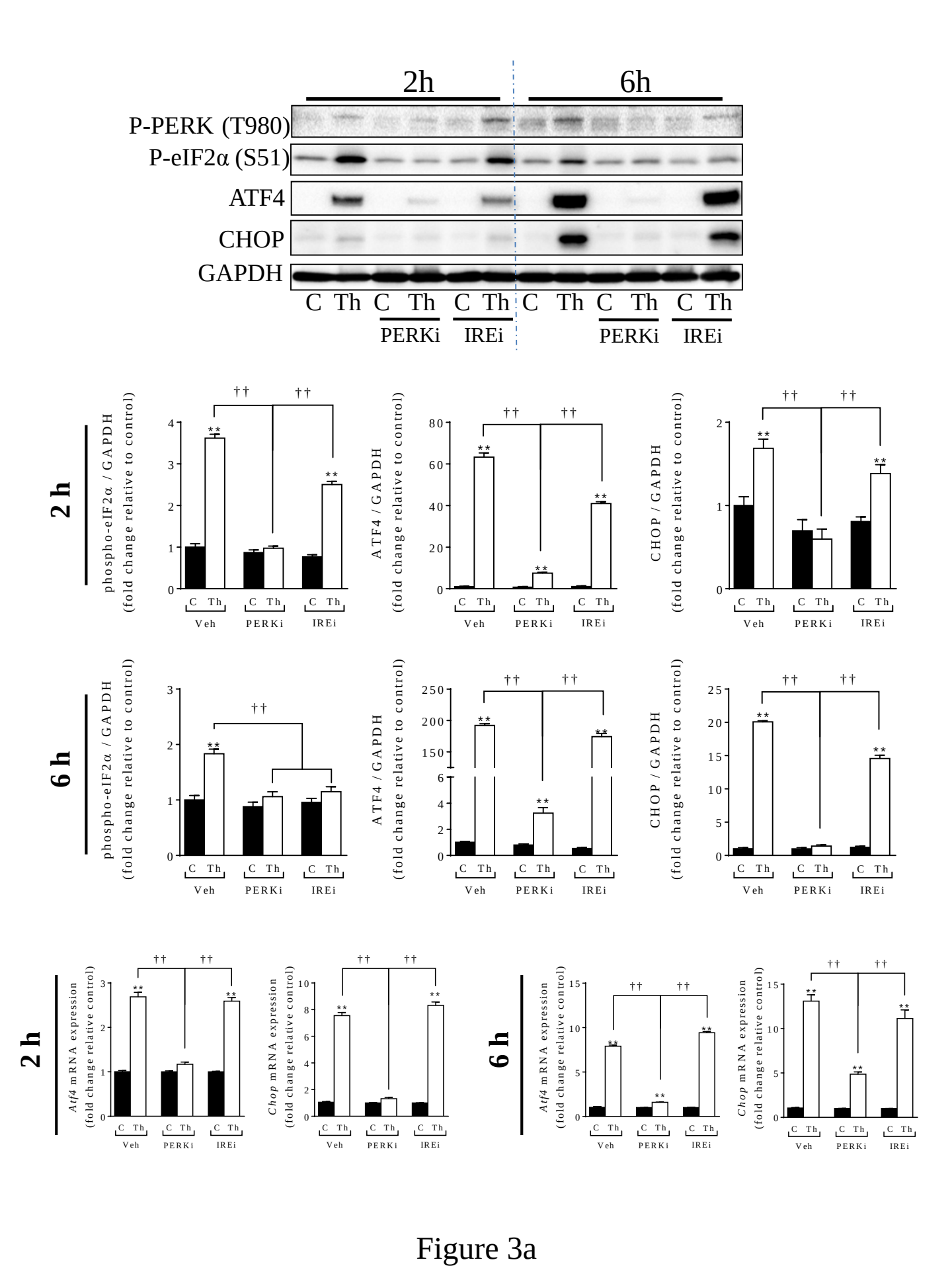
2h
6h
P-PERK (T980)
P-eIF2α (S51)
ATF4
CHOP
GAPDH
C Th C Th C Th C Th C Th C Th
PERKi
IREi
2 h
6 h
phospho-eIF2α / GAPDH
(fold change relative to control)
ATF4 / GAPDH
CHOP / GAPDH
Veh
PERKi
IREi
Atf4 mRNA expression
Chop mRNA expression

Figure 3a

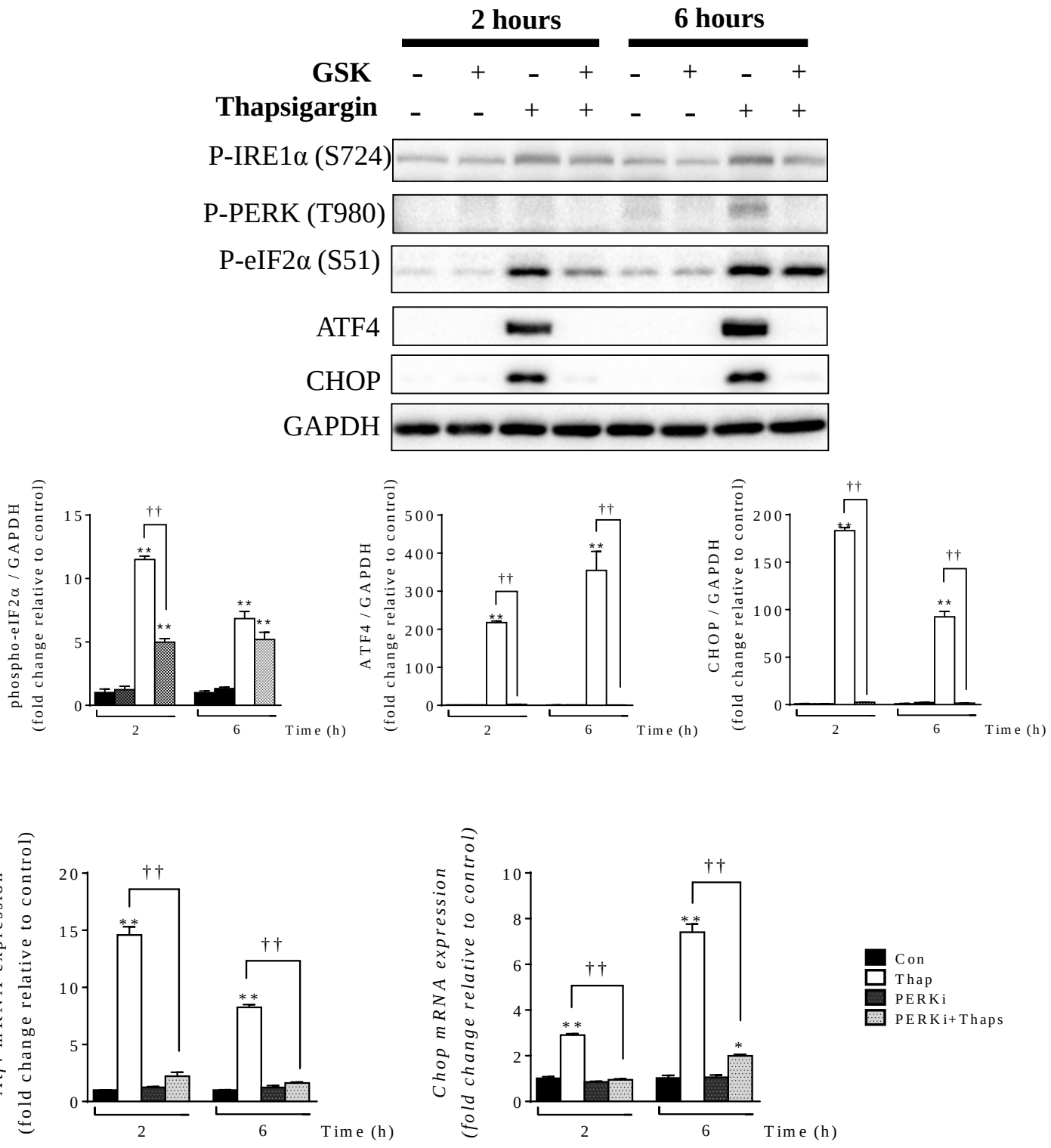
2 hours
6 hours
GSK
Thapsigargin
P-IRE1α (S724)
P-PERK (T980)
P-eIF2α (S51)
ATF4
CHOP
GAPDH
phospho-eIF2α / GAPDH (fold change relative to control)
ATF4 / GAPDH (fold change relative to control)
CHOP / GAPDH (fold change relative to control)
Atf4 mRNA expression (fold change relative to control)
Chop mRNA expression (fold change relative to control)
Time (h)
Con
Thap
PERKi
PERKi+Thaps

Figure 3b

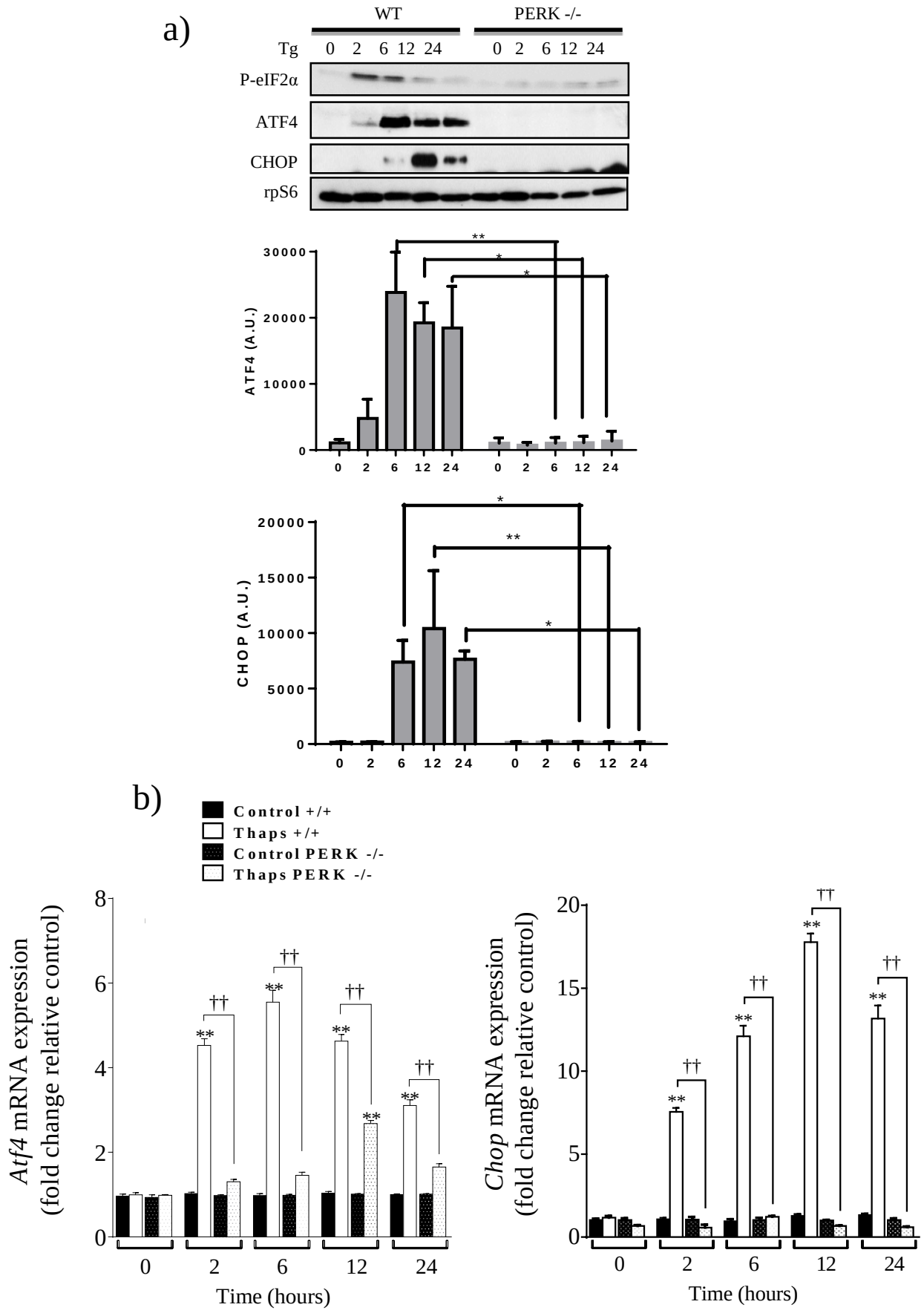
a)
WT
PERK -/-
Tg
0 2 6 12 24
0 2 6 12 24
P-eIF2α
ATF4
CHOP
rpS6
ATF4 (A.U.)
30000
20000
10000
0
CHOP (A.U.)
20000
15000
10000
5000
0
b)
Control +/+
Thaps +/+
Control PERK -/-
Thaps PERK -/-
Atf4 mRNA expression
(fold change relative control)
Chop mRNA expression
(fold change relative control)
Time (hours)
Time (hours)

Figure 4

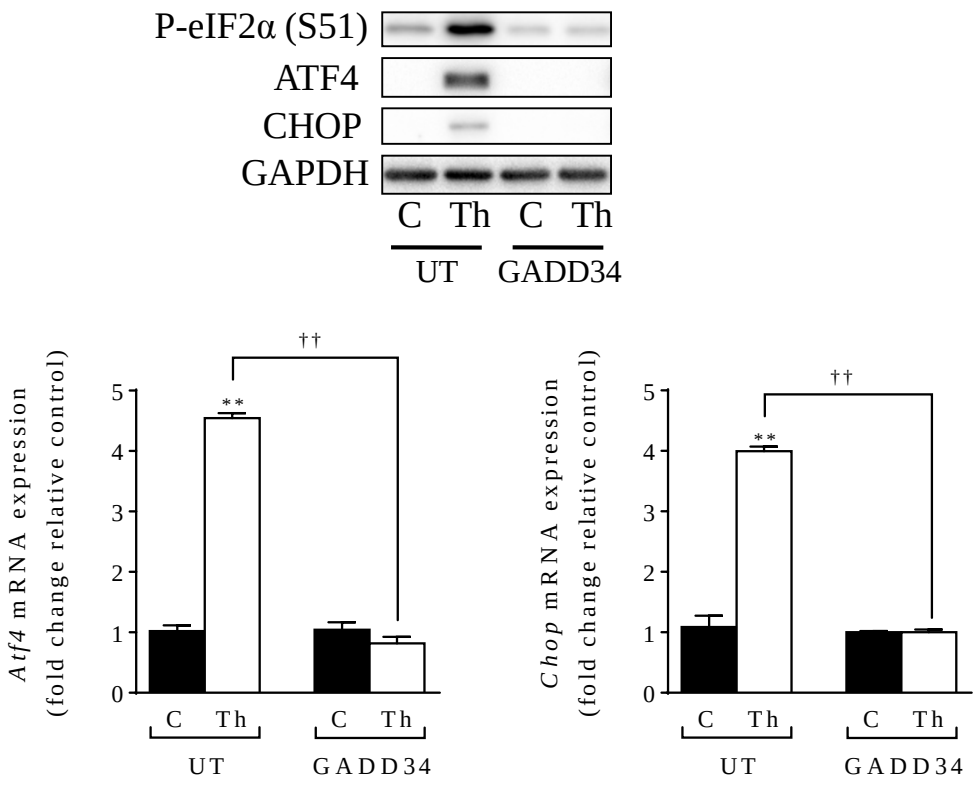
P-eIF2α (S51)
ATF4
CHOP
GAPDH
C Th C Th
UT GADD34
Atf4 mRNA expression
(fold change relative control)
Chop mRNA expression
(fold change relative control)
0
1
2
3
4
5
**
††
C Th C Th
UT GADD34

Figure 5

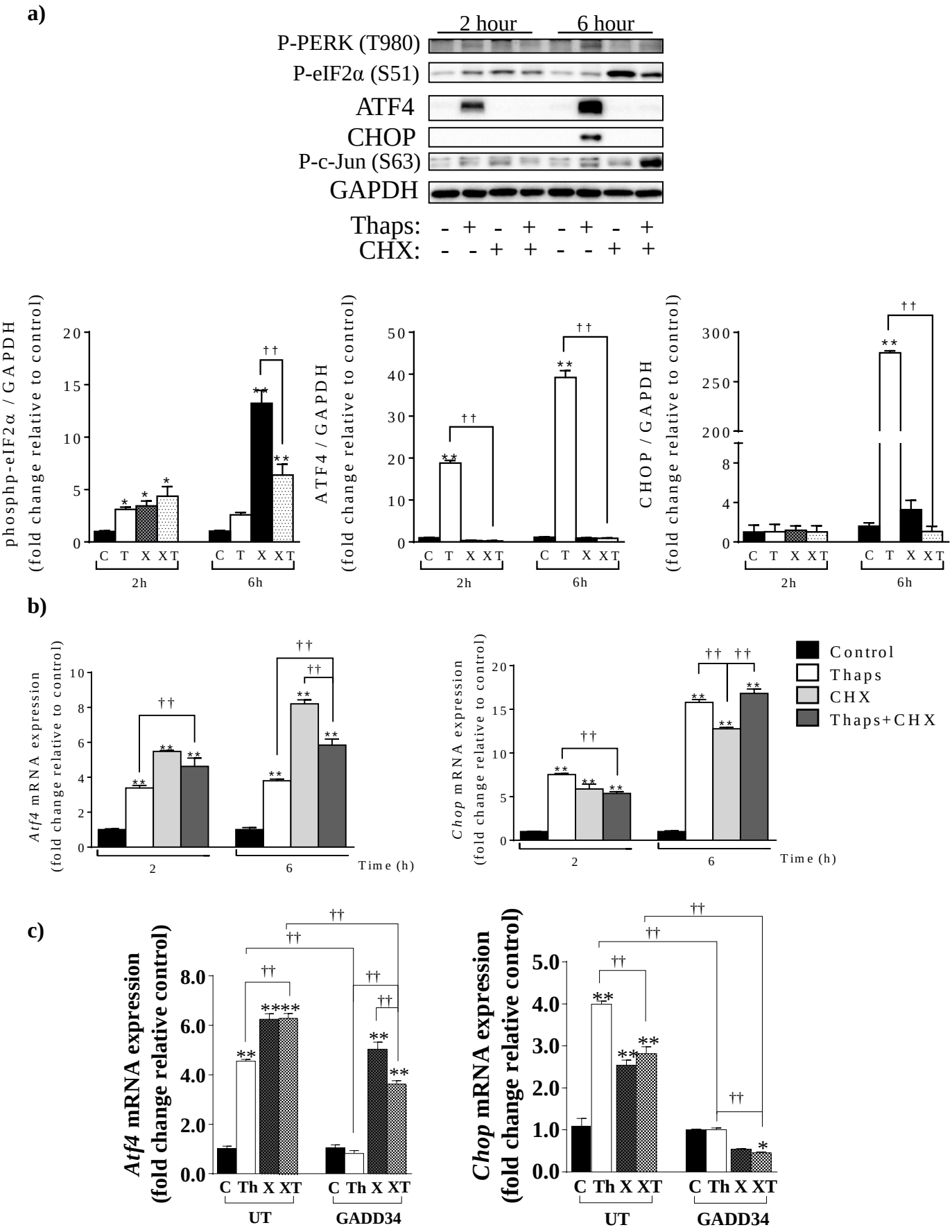
a)
2 hour
6 hour
P-PERK (T980)
P-eIF2α (S51)
ATF4
CHOP
P-c-Jun (S63)
GAPDH
Thaps: - + - + - + - +
CHX: - - + + - - + +
phosphp-eIF2α / GAPDH (fold change relative to control)
ATF4 / GAPDH (fold change relative to control)
CHOP / GAPDH (fold change relative to control)
C T X XT
2h
6h
b)
Atf4 mRNA expression (fold change relative to control)
Chop mRNA expression (fold change relative to control)
Time (h)
Control
Thaps
CHX
Thaps+CHX
c)
Atf4 mRNA expression (fold change relative control)
Chop mRNA expression (fold change relative control)
C Th X XT
UT
GADD34

Figure 6

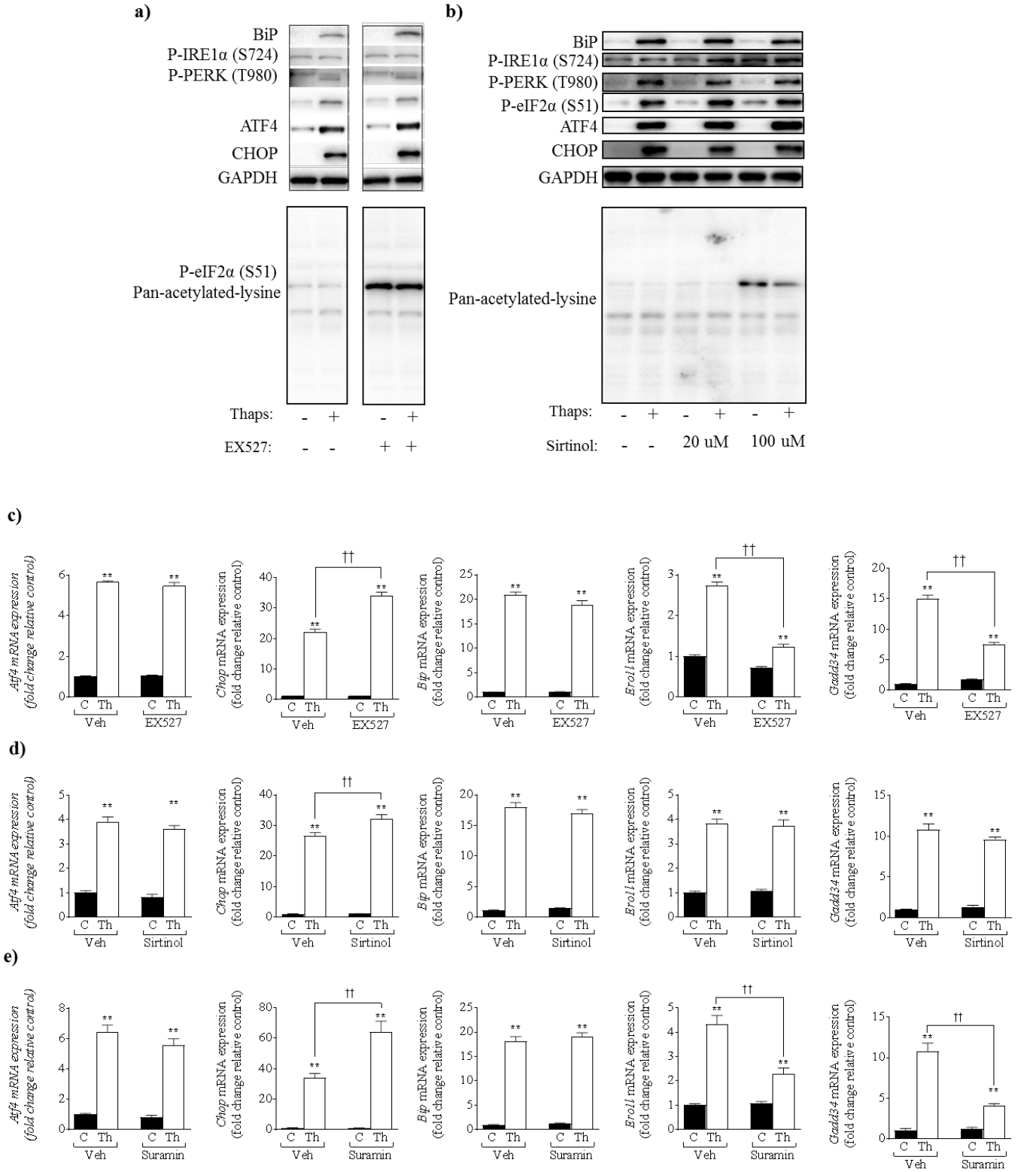
a)
BiP
P-IRE1α (S724)
P-PERK (T980)
ATF4
CHOP
GAPDH
P-eIF2α (S51)
Pan-acetylated-lysine
Thaps: - + - +
EX527: - - + +
b)
BiP
P-IRE1α (S724)
P-PERK (T980)
P-eIF2α (S51)
ATF4
CHOP
GAPDH
Pan-acetylated-lysine
Thaps: - + - + - +
Sirtinol: - - 20 uM 100 uM
c)
Atf4 mRNA expression (fold change relative control)
Chop mRNA expression (fold change relative control)
Bip mRNA expression (fold change relative control)
Erol1 mRNA expression (fold change relative control)
Gadd34 mRNA expression (fold change relative control)
C Th
Veh
EX527
d)
Sirtinol
e)
Suramin

Figure 7