Accepted Manuscript

The balance between adaptive and apoptotic unfolded protein responses regulates β-cell death under ER stress conditions through XBP1, CHOP and JNK

Jeng Yie Chan, Jude Luzuriaga, Emma L. Maxwell, Phillip K. West, Mohammed Bensellam, D.Ross Laybutt

PII: S0303-7207(15)30004-6

DOI: 10.1016/j.mce.2015.06.025

Reference: MCE 9197

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 11 February 2015

Revised Date: 12 June 2015

Accepted Date: 24 June 2015

Please cite this article as: Chan, J.Y., Luzuriaga, J., Maxwell, E.L., West, P.K., Bensellam, M., Laybutt, D.R., The balance between adaptive and apoptotic unfolded protein responses regulates β-cell death under ER stress conditions through XBP1, CHOP and JNK, *Molecular and Cellular Endocrinology* (2015), doi: 10.1016/j.mce.2015.06.025.

The balance between adaptive and apoptotic unfolded protein responses regulates β-cell death under ER stress conditions through XBP1, CHOP and JNK

Jeng Yie Chan, Jude Luzuriaga, Emma L. Maxwell, Phillip K. West, Mohammed Bensellam and D. Ross Laybutt

Garvan Institute of Medical Research, St Vincent's Hospital, UNSW Australia, Sydney, NSW, Australia

Corresponding author:

D. Ross Laybutt, Ph.D.

Garvan Institute of Medical Research

384 Victoria St, Darlinghurst, NSW 2010, Australia.

Tel: 61-2-9295-8228

Fax: 61-2-9295-8201

Email: r.laybutt@garvan.org.au

Abstract

Endoplasmic reticulum (ER) stress and the subsequent unfolded protein response (UPR) have been implicated in β-cell death in type 1 and type 2 diabetes. However, the UPR is also a fundamental mechanism required for β-cell adaptation and survival. The mechanisms regulating the transition from adaptive to apoptotic UPR remain to be clarified. Here, we investigated the relationships between XBP1, CHOP and JNK in the transition from adaptive to apoptotic UPR and β-cell death in models of type 1 and type 2 diabetes. XBP1 inhibition potentiated cell death induced by pro-inflammatory cytokines or the saturated fatty acid palmitate in MIN6 β-cells. This response was prevented by CHOP inhibition. IRE1/XBP1 inhibition led to alterations in islets from diabetes-resistant *ob/ob* mice that resemble those found in diabetes, including increases in cell death and inflammation and antioxidant gene expression. Similarly, IRE1/XBP1 inhibition increased cell death in islets from NOD mice. On the other hand, JNK inhibition: 1) increased adaptive UPR and reduced cell death in islets from diabetic *db/db* mice, and 2) restored adaptive UPR while protecting against apoptotic UPR gene expression and β-cell death and dysfunction following cytokine exposure. These findings suggest that the balance between XBP1-mediated adaptive and CHOP-dependent apoptotic UPR is critically important for β-cell survival during ER stress. JNK activation regulates the transition from adaptive to apoptotic UPR, thus providing a mechanism for β-cell propensity to cell death rather than ER stress adaptation in type 1 and type 2 diabetes.

Highlights

Balance between adaptive and apoptotic UPR regulates β-cell survival during ER stress

XBP1 inhibition potentiates cytokine- and palmitate-induced β-cell death

JNK regulates the transition from adaptive UPR to β-cell death in diabetes

Keywords: β-cell; Diabetes; Endoplasmic reticulum stress; Islets; Unfolded protein response

Abbreviations

ATF4: activating transcription factor 4. ATF6: activating transcription factor 6. CHOP: C/EBP homologous protein. Edem1: ER degradation-enhancing alpha-mannosidase-like 1. eIF2α: eukaryotic translation initiation factor 2α. ER: endoplasmic reticulum. ERAD: ER-associated degradation. Erp72: endoplasmic reticulum protein 72. Fkbp11: FK506 binding protein 11. Grp94: 94 kDa glucose-regulated protein. IFN-γ: interferon-gamma. IL-1β: interleukin-1β. IRE1: inositol requiring enzyme 1. JNK: Jun N-terminal kinase. NOD: Nonobese diabetic. Orp150: 150 kDa oxygen-regulated protein. p58: protein kinase inhibitor of 58 kDa. PERK: PKR-like ER kinase. TNF-α: tumour necrosis factor-alpha. Xbp1: X-box binding protein 1

1. Introduction

The loss of β-cell mass is critical to the development of both type 1 and type 2 diabetes (Matveyenko and Butler, 2008). However, the triggering stimuli and the cellular mechanisms involved are poorly understood. Elevated plasma levels of pro-inflammatory cytokines and free fatty acids have been observed in type 1 and type 2 diabetes, and have been proposed as key contributors to β-cell apoptosis (Biden et al, 2014, Cnop et al, 2005, Donath and Shoelson, 2011). Indeed, exposure of β-cells to cytokines or fatty acids *in vitro* induces apoptosis along with the activation of multiple signaling networks, including endoplasmic reticulum (ER) stress (Cardozo et al, 2005, Cunha et al, 2008, Laybutt et al, 2007).

β-cells depend heavily on an efficient ER function due to their high rate of insulin synthesis and secretion. Cytokines and fatty acids have been shown to impair ER homeostasis by inducing depletion of ER Ca^{2+} and alteration of chaperone function, proinsulin processing and ER-Golgi trafficking, triggering accumulation of misfolded proteins and ER stress (Baldwin et al, 2012, Cardozo et al, 2005, Hara et al, 2014, Jeffrey et al, 2008, Oyadomari et al, 2001, Preston et al, 2009). ER stress and its ensuing unfolded protein response (UPR) have been implicated in β-cell pathophysiology in both forms of diabetes (Laybutt et al, 2007, Marhfour et al, 2012). However, the UPR can also serve an adaptive function to restore ER homeostasis via the ER stress sensors PKR-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and activating transcription factor (ATF) 6 (Fonseca et al, 2011, Scheuner and Kaufman, 2008). Activation of PERK and subsequent phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) leads to a rapid and transient reduction of global protein translation to reduce ER load, while increasing translation of the transcription factor ATF4. Activation of the endoribonuclease IRE1 results in

the unconventional splicing of *Xbp1* mRNA and increased translation of active XBP1 transcription factor. ATF6 is activated by proteolytic cleavage in the Golgi to release an active transcription factor. These transcription factors stimulate the expression of chaperones, protein folding enzymes and ER-associated degradation (ERAD) machinery to enhance ER folding capacity and clearance of misfolded proteins. Under prolonged or irresolvable ER stress, the UPR switches from an adaptive to an apoptotic role. The most widely implicated pro-apoptotic UPR effector is the transcription factor C/EBP Homologous Protein (CHOP) (Akerfeldt et al, 2008, Allagnat et al, 2012, Cardozo et al, 2005, Karaskov et al, 2006, Laybutt et al, 2007, Song et al, 2008). However, the mechanisms regulating the transition from adaptive to apoptotic UPR remain to be clarified.

Here, we used *in vitro* physiological inducers of ER stress (cytokines and saturated fatty acid, palmitate) and *in vivo* models of type 1 [Nonobese diabetic (NOD) mice] and type 2 diabetes (C57BL/KsJ *db/db* mice) as well as β-cell compensation (C57BL/6J *ob/ob* mice) to show that the balance between XBP1-mediated adaptive UPR and CHOP is critically important in the regulation of β-cell survival during ER stress. Furthermore, our studies reveal that JNK activation is a key factor that regulates the transition from adaptive to apoptotic UPR in β-cells. These findings uncover novel interrelationships between XBP1, CHOP and JNK that are crucial for β-cell survival in type 1 and type 2 diabetes.

2. Materials and Methods

2.1. Cell culture

MIN6 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 25 mM glucose, 10 mM HEPES, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Either 2 x 10^5 or 8 x 10^5 cells were seeded per well in a 24-well or 6-well plate, respectively. Cells were treated with 100 U/ml IL-1β, 250 U/ml IFN-γ and 100 U/ml TNF-α (R&D Systems, Minneapolis, MN, USA); 0.92% BSA or 0.4 mM palmitate coupled to 0.92% BSA. 4µ8c (15 µM; Tocris Biosciences, Bristol, UK), and JNK inhibitor II (JNKi, 20 µM SP600125; Merck, Kilsyth, VIC, Australia) were used to inhibit IRE1/XBP1 and JNK activations, respectively. 4µ8c is a selective inhibitor of IRE1 ribonuclease activity that prevents splicing (activation) of *Xbp1* mRNA in response to ER stress (Cross et al, 2012). For siRNA transfection, MIN6 cells were transfected with XBP1 and/or CHOP ON-TARGET*plus* SMARTpool siRNA or Non-Targeting siRNA using DharmaFECT 3 (Dharmacon, Thermo Scientific, Scoresby, VIC, Australia) according to the manufacturer's instruction. For insulin secretion assay, cells were incubated for 1 h at 37°C in Krebs-Ringer HEPES buffer containing 2.8 or 25 mM glucose. Insulin was measured in an aliquot of the buffer by radioimmunoassay (Millipore, Billerica, MA, USA)

2.2. Islet isolation and culture

Female Nonobese diabetic (NOD) and Balb/c mice, and male C57BL/KsJ *db/db* and C57BL/6J *ob/ob* mice and their respective lean control mice (C57BL/KsJ or C57BL/6J) were taken from the Garvan Institute breeding colonies (Australian BioResources, Moss Vale, NSW, Australia). $Xbp1^{flox/flox}$ mice (kindly provided by L.H. Glimcher and A.H. Lee, Weill Cornell Medical College, New York, NY, USA) were crossed with Pdx1-Cre^{ER} mice to generate $Xbp1^{flox/flox}$ Pdx1-Cre^{ER} and control ($Xbp1^{+/+}$ Pdx1-Cre^{ER}) mice. Pancreatic islets were isolated by liberase digestion (Roche Diagnostics, Castle Hill, Australia), gradient centrifugation (Ficoll-Paque

PLUS gradient, GE Healthcare Bio-Sciences, Uppsala, Sweden) and handpicking under a stereomicroscope. Procedures were approved by the Garvan Institute/St.Vincent's Hospital Animal Experimentation Ethics Committee, following guidelines issued by the National Health and Medical Research Council of Australia. Islets were cultured at 37 °C in RPMI 1640 medium supplemented with 0.2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Islets from control and $Xbp1^{flox/flox}$ $Pdx1\text{-}Cre^{ER}$ mice were treated with 100 nM 4-hydroxytamoxifen (Sigma-Aldrich, St. Louis, MO, USA). Islets were treated with 100 U/ml IL-1β, 250 U/ml IFN-γ and 100 U/ml TNF-α (R&D Systems) for 24 hours. 4µ8c (15 µM) and JNKi (20 µM) were used to inhibit IRE1/XBP1 and JNK activations, respectively.

2.3. Cell death assay

Cell death was determined with the use of a Cell Death Detection ELISA (Roche Diagnostics) (Chan et al, 2011), which measures cytoplasmic histone-associated-DNA-fragments. Cells or islets were lysed in either 0.5 or 0.2 ml of the supplied lysis buffer, incubated for 30 min at room temperature, and the lysate was spun at 200xg for 10 min. The ELISA assay was then performed according to manufacturers' instructions and the results were corrected for DNA content.

2.4. Cell viability (MTT) assay

Cultured cells in 96-well plates were washed twice with 200 µL Krebs-Ringer bicarbonate buffer containing 16.8 mM glucose and 0.1% BSA and then incubated with 100 µL of the same buffer containing 1.2 mM MTT (Molecular Probes, Eugene, OR, USA) for 4 h at 37°C. Then 100 µL of

10% sodium dodecyl sulphate in 0.01M hydrochloric acid was added and incubated at 37°C for 4 h. Absorbance was measured in a plate reader at 570 nm.

2.5. Western blotting

Western blotting was performed as previously described (Chan et al, 2011). The following antibodies were used (1:1000 dilution unless otherwise indicated): CHOP (sc-575) and XBP1 (sc-7160) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-c-JUN (9164), phospho-JNK (Thr183/Tyr185, 9251), and total JNK (9252) (Cell Signaling Technology, Danvers, MA, USA); β-actin and α-tubulin (1:5000; Sigma-Aldrich, St. Louis, MO, USA).

2.6. RNA analysis

cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Qiagen, Victoria, Australia) according to manufacturer's instructions. Real-time PCR was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7900HT Real-Time PCR System (Applied Biosystems) (Chan et al, 2013). Primer sequences are provided in Supplementary Table 1. The value obtained for each specific product was normalized to a control gene (cyclophilin A) and expressed as a fold-change of the value in control extracts.

2.7. *Xbp1* splicing

Xbp1 cDNA was amplified by PCR and digested with *Pst*I (New England Biolabs, Ipswitch, MA, USA), which cuts unprocessed *Xbp1* cDNA into fragments. Processed (activated) *Xbp1* cDNA lacks the restriction site and remains intact. Processed (intact) and unprocessed (cut) *Xbp1* were separated using agarose gels and quantified by densitometry. The value obtained for processed

Xbp1 was expressed as a ratio of the total (processed + unprocessed) *Xbp1* mRNA level for each sample. These ratios are expressed as fold-change of the ratio compared to control.

2.8. Statistical analysis. All results are presented as means±SEM. Statistical analyses were performed using ANOVA with Bonferroni *post hoc* tests.

3. Results

3.1. IRE1/XBP1 inhibition potentiates cytokine-induced β-cell death

We first investigated the influence of IRE1/XBP1 signaling on the ER stress response induced by cytokines. In MIN6 cells, exposure to the combination of cytokines (IL-1β + TNF-α + IFN-γ) for 24 h led to an atypical pattern of UPR activation. Expression of adaptive UPR genes including *Xbp1* (Fig. 1A), *Bip* (Fig. 1C), *Erp72* (Fig. 1D), *Fkbp11* (Fig. 1E), *Grp94* (Fig. 1F) and *Edem1* (Fig. 1G) were reduced after cytokine exposure, whereas expression of pro-apoptotic UPR genes, *Chop* (Fig. 1H) and *Trib3* (Fig. 1I) were increased. In addition, XBP1 activation, as measured by the level of its splicing, was significantly reduced by cytokines (Fig. 1B). Treatment of MIN6 cells with IRE1/XBP1 inhibitor 4μ8c lowered expression of adaptive UPR genes and *Xbp1* splicing (Fig. 1A-G), but did not alter the expression of *Chop* (Fig. 1H) and *Trib3* (Fig. 1I). The decline in the adaptive UPR induced by 4μ8c treatment was associated with increased cell death (Fig. 1J) and reduced cell viability (Fig. 1K) in both control- and cytokine-treated cells. These results demonstrate the importance of XBP1 and the adaptive UPR in β-cell survival under conditions of cytokine-induced ER stress. In agreement with our findings in MIN6 cells, cytokine-induced cell death was potentiated in mouse islets that were treated with cytokines + 4μ8c as compared to islets treated with cytokines alone (Fig. 1L). Furthermore, β-cell-specific

Xbp1 deficiency (Fig. 2A) in islets from Xbp1$^{flox/flox}$ Pdx1-Cre^{ER} mice was associated with reduced adaptive UPR gene expression (Fig. 2B-F) and potentiation of cytokine-induced cell death (Fig. 2I).

3.2. The balance between XBP1-mediated adaptive UPR and CHOP regulates cytokine-induced β-cell death

We investigated the mechanisms whereby inhibition of XBP1 potentiates cytokine-induced β-cell death using siRNA-mediated knockdown of XBP1 and CHOP in MIN6 cells. In both control- and cytokine-treated cells, siXBP1 transfection significantly reduced XBP1 protein (Fig. 3A and Supplementary material Figs. S1) and mRNA (Fig. 3B) levels. This was associated with the widespread downregulation of adaptive UPR genes (Fig. 3C-G), whereas CHOP levels were similar to control (Fig. 3A, H and Figs. S1). *Trib3* (Fig. 3I) mRNA levels were slightly reduced by siXBP1 in cytokine-treated cells. XBP1 knockdown was associated with increased cell death in control- and cytokine-treated MIN6 cells (Fig. 3J). This contrasts with previous findings (Allagnat et al, 2010), and suggests that XBP1 activity is crucial for β-cell survival during ER stress induced by cytokines. The siXBP1-mediated increase in cell death was completely abolished when cells were co-transfected with siCHOP (Fig. 3J). Co-transfection with siCHOP significantly reduced the expression of CHOP (Fig. 3A, H and Figs. S1) without affecting the expression of XBP1 (Fig. 3A, B and Figs. S1) and other adaptive UPR genes (Fig. 3C-G) or JNK phosphorylation (Fig. 3A and Figs. S1). These data indicate that CHOP expression is required for the siXBP1-mediated increase in both control and cytokine-induced β-cell death. Interestingly, this occurs despite unchanged CHOP levels in XBP1-deficient cells (Fig. 3A, H

and Figs. S1), suggesting that an integrated XBP1-CHOP response regulates cell death during ER stress induced by cytokines.

3.3. The balance between XBP1-mediated adaptive UPR and CHOP regulates palmitate-induced β-cell death

We investigated the influence of XBP1 and CHOP on MIN6 cell death in the context of lipotoxic ER stress induced by the saturated fatty acid palmitate. MIN6 cells transfected with siXBP1 displayed reduced levels of XBP1 protein (Fig. 4A and Figs. S2) and mRNA (Fig. 4B), accompanied by decreases in the adaptive UPR genes (Fig. 4C-G). *Chop* (Fig. 4H) and *Trib3* (Fig. 4I) mRNA levels were slightly reduced by XBP1 silencing following palmitate exposure. XBP1 knockdown significantly increased cell death in both control- and palmitate-treated MIN6 cells (Fig. 4J). This increase in cell death was completely prevented by siCHOP co-transfection (Fig. 4J). siCHOP co-transfection lowered CHOP protein and mRNA levels (Fig. 4A, H and Figs. S2), but did not affect other UPR genes (Fig. 4C-G). These results suggest that the maintenance of the adaptive UPR by XBP1 inhibits CHOP-dependent cell death under conditions of palmitate-induced ER stress. Together, the findings suggest that the balance between XBP1 and CHOP, rather than their expression levels *per se*, is crucial for β-cell survival during ER stress induced by both fatty acids and cytokines.

3.4. IRE1/XBP1 inhibition increases cell death in islets from NOD mice

Recent studies in NOD mice suggest that type 1 diabetes is preceded by the presence of ER stress in β-cells at 5-6 weeks of age (Engin et al, 2013, Tersey et al, 2012), and that progressive loss of adaptive UPR mediators, including XBP1, occurs between pre-diabetic and diabetic

stages (Akerfeldt et al, 2008, Engin et al, 2013). To assess the influence of XBP1 expression on β-cell survival in pre-diabetic NOD mice, islets from 5-6 week-old mice were treated with IRE1/XBP1 inhibitor, 4μ8c. Treatment of NOD islets with 4μ8c led to significant decreases in *Xbp1* expression (Fig. 5A) and splicing (Fig. 5B). Along with a widespread downregulation of adaptive UPR genes (Fig. 5C-G), *Chop* and *Trib3* mRNA levels (Fig. 5H, I) were increased. This altered pattern of UPR gene expression was associated with increased cell death in 4μ8c-treated NOD islets compared to control NOD islets (Fig. 5J). These results suggest that an XBP1-mediated adaptive UPR protects against terminal UPR gene expression (*Chop* and *Trib3*) and cell death in NOD islets prior to the onset of type 1 diabetes.

3.5. IRE1/XBP1 inhibition increases oxidative stress, inflammation and apoptosis in islets from obese diabetes-resistant *ob/ob* mice

The C57BL/KsJ *db/db* and C57BL/6J *ob/ob* mouse models of obesity exhibit opposing disposition to diabetes development (Chan et al, 2013). The C57BL/6J *ob/ob* mouse displays resistance to diabetes due to successful β-cell compensation, whereas the C57BL/KsJ *db/db* mouse displays time-dependent progression to diabetes due to the failure of β-cell compensation. We previously reported that the mRNA levels of adaptive UPR genes were progressively increased in islets of diabetes-resistant *ob/ob* mice, whereas they declined in diabetic *db/db* mice (Chan et al, 2013). Here, we show that the status of the adaptive UPR in these models correlates with *Xbp1* expression; *Xbp1* mRNA expression displayed a time-dependent increase in islets of *ob/ob* mice, whereas it declined in diabetic *db/db* islets (Figs. S3).

We investigated the role of *Xbp1* expression in islets of diabetes-resistant *ob/ob* mice using *ex vivo* treatment with 4μ8c. In comparison to untreated *ob/ob* islets, 4μ8c treatment markedly reduced the levels of *Xbp1* mRNA and splicing (Fig. 6A, B). mRNA levels of other adaptive UPR genes were downregulated (Fig. 6C-G), whereas *Chop* tended to be increased (Fig. 6H) and *Trib3* was significantly increased (Fig. 6I), suggesting that XBP1 protects against deleterious UPR signaling in the *ob/ob* mouse model of β-cell compensation.

A notable difference in the gene expression profiles of C57BL/KsJ *db/db* and C57BL/6J *ob/ob* islets is the preferential upregulation of antioxidant and inflammatory genes in islets of diabetes-prone *db/db* mice (Chan et al, 2013), supporting the notion that β-cell failure is accompanied by worsening oxidative stress and inflammation (Cnop et al, 2005, Del Guerra et al, 2005, Donath and Shoelson, 2011, Song et al, 2008). Here, we assessed the influence of IRE1/XBP1 activity on antioxidant and inflammatory gene expression in *ob/ob* islets. Catalase (Fig. 6J), heme oxygenase-1 (*HO-1*) (Fig. 6K), *IL-1β* (Fig. 6M), and *Cxcl1* (Fig. 6N) mRNA levels were slightly increased in islets from *ob/ob* mice compared to control islets, whereas glutathione peroxidase (*GPx*) (Fig. 6L), death receptor *Fas* (Fig. 6O), *IL-6* (Fig. 6P) and *Ccl2* (Fig. 6Q) mRNA levels were unchanged. Interestingly, inhibition of IRE1/XBP1 with 4μ8c treatment led to significant increases in the expression of each of these antioxidant and inflammation genes in *ob/ob* islets (Fig. 6K-Q). Moreover, treatment of *ob/ob* islets with 4μ8c led to increased cell death compared to control-treated *ob/ob* islets (Fig. 6R). These results suggest for the first time that the IRE1/XBP1-mediated adaptive UPR protects against islet inflammation, oxidative stress and cell death in the *ob/ob* mouse model of β-cell compensation. Taken together with previous findings (Chan et al, 2013), our results suggest that IRE1/XBP1 deficiency leads to alterations in islets

from diabetes-resistant *ob/ob* mice that resemble those found in diabetes-prone *db/db* mice, namely declining adaptive UPR gene expression, increased cell death and marked upregulation of antioxidant and inflammatory genes. Thus, our studies suggest that the maintenance (or loss) of IRE1/XBP1-mediated adaptive UPR contributes to β-cell compensation (or failure) in obese mice.

3.6. JNK regulates the transition from adaptive to apoptotic UPR in cytokine-treated β-cells

Having established that the balance between UPR pathways is crucial for β-cell survival, we next investigated mechanisms for the transition from adaptive to apoptotic UPR during ER stress induced by cytokines. Previous studies implicate JNK activation in cytokine-mediated apoptosis (Bonny et al, 2001, Brozzi et al, 2014, Marroqui et al, 2014) and suggest that JNK activity is required for *Chop* promoter activation (Pirot et al, 2007) and expression (Chan et al, 2011). We therefore tested whether JNK activity influences the overall profile of adaptive and apoptotic UPR gene expression following cytokine exposure. In cells treated with JNKi, cytokine-mediated JNK and c-JUN phosphorylation were reduced (Fig. 7A and Figs. S4). Strikingly, inhibition of JNK activity completely protected against the cytokine-mediated reductions in adaptive UPR gene expression (Fig 7B-H and Figs. S4). Furthermore, the cytokine-mediated increases in CHOP (Fig. 7 A, I and Figs. S4), *Trib3* (Fig. 7J) and β-cell death (Fig. 7K) were partially prevented by JNK inhibition. Moreover, the cytokine-mediated impairment of glucose-stimulated insulin secretion was partially prevented by JNK inhibition (Fig. 7L). These data indicate that JNK signaling is necessary for alterations in the general pattern of UPR gene expression following cytokine exposure. JNK activation contributes to the downregulation of

XBP1 and adaptive UPR while promoting apoptotic UPR gene expression in cytokine-stimulated β-cells. Thus, our findings indicate that JNK is a key factor regulating the transition from adaptive to apoptotic UPR under conditions of immune-mediated ER stress.

3.7. JNK inhibition increases adaptive UPR gene expression and reduces cell death in *db/db* mouse islets

Studies in mouse models of type 2 diabetes have implicated JNK activation in β-cell dysfunction (Matsuoka et al, 2010), but are yet to demonstrate that JNK makes a necessary contribution to β-cell death. Here we assessed the influence of JNK activation on UPR gene expression and cell death in islets of diabetic *db/db* mice. Inhibition of JNK significantly increased both the expression and splicing of *Xbp1* in *db/db* islets (Fig. 8A, B). Furthermore, JNK inhibition increased the mRNA levels of adaptive UPR genes (Fig. 8C-G), whereas *Chop* (Fig. 8H) and *Trib3* (Fig. 8I) mRNA levels were similar amongst the groups. JNK inhibition significantly reduced cell death in *db/db* islets compared to control-treated *db/db* islets (Fig. 8J). These data are the first indication that JNK signaling is required for both the decline of adaptive UPR gene expression and the increase in cell death in islets of the *db/db* mouse model of type 2 diabetes. Overall, our studies suggest that JNK inhibition would be beneficial for preserving functional β-cell mass during the pathogenesis of diabetes.

4. Discussion

ER stress has been implicated in β-cell death in type 1 (Marhfour et al, 2012, Tersey et al, 2012) and type 2 diabetes (Laybutt et al, 2007, Oyadomari et al, 2002, Scheuner and Kaufman, 2008, Song et al, 2008). However, an intact UPR is also required for physiological maintenance of β-

cell mass (Fonseca et al, 2011, Scheuner and Kaufman, 2008). Our study examines relationships between XBP1, CHOP and JNK in the transition from adaptive to apoptotic UPR during ER stress in models of type 1 and type 2 diabetes. The main findings of this study are that: 1) IRE1/XBP1-mediated adaptive UPR regulates cytokine-induced β-cell death; 2) the balance between XBP1 and CHOP is crucial for β-cell survival during ER stress induced by cytokines and saturated fatty acid palmitate; 3) IRE1/XBP1-mediated adaptive UPR protects against apoptotic UPR gene expression and cell death in NOD mouse islets prior to the onset of type 1 diabetes; 4) IRE1/XBP1-mediated adaptive UPR protects against islet inflammation, oxidative stress, apoptotic UPR and cell death in the C57BL/6J *ob/ob* mouse model of β-cell compensation; 5) JNK is a key factor regulating the transition from adaptive to apoptotic UPR during ER stress induced by cytokines; and 6) JNK activation is required for both the decline of adaptive UPR gene expression and the increase in cell death in islets from the C57BL/KsJ *db/db* mouse model of β-cell failure and type 2 diabetes. Our findings strongly implicate a role for the loss of optimal XBP1:CHOP balance in the induction of β-cell death in type 1 and type 2 diabetes, as well as providing JNK activation as a mechanism for the transition from adaptive to apoptotic UPR.

The contribution of the PERK-ATF4-CHOP arm of the UPR in cytokine-induced β-cell death has received much attention (Akerfeldt et al, 2008, Allagnat et al, 2012, Cardozo et al, 2005, Oyadomari et al, 2001, Pirot et al, 2007). Our studies suggest that CHOP induction alone is not sufficient to trigger MIN6 cell death following cytokine exposure ((Akerfeldt et al, 2008) and Fig. 3). Instead, our findings introduce a novel concept to the field suggesting that optimal XBP1:CHOP balance is crucial for β-cell survival, as evidenced by the CHOP-dependent potentiation of basal and cytokine-induced cell death in XBP1-deficient cells. This increase

occurred in the absence of CHOP upregulation, highlighting the importance of the relative XBP1:CHOP expression in cell survival (Fig. 3), which is also fundamental in the context of lipotoxic ER stress (Fig. 4).

The observation that XBP1 inhibition leads to increased β-cell death in both our models of lipid- and cytokine-induced ER stress contrasts with previous reports (Allagnat et al, 2010, Cunha et al, 2014, Zhang et al, 2014). These differences may be attributed to the use of different experimental models, species and timing of experiments. Furthermore, our studies uncover the importance of XBP1:CHOP balance, which was not investigated in past studies. Interestingly, our findings suggest that siXBP1 alone is sufficient to promote cell death in MIN6 cells (Fig. 3J and 4J), whereas XBP1 deficiency in islets potentiates cell death only following cytokine stimulation (Fig. 1L and 2I). The enhanced sensitivity of the cell line likely reflects the greater demand for protein required for cell replication, which undoubtedly places a burden on the ER. The deleterious impact of XBP1 deficiency in primary β-cells is only apparent in chronic (Lee et al, 2011) or ER stress conditions.

The results of our studies using islets from mouse models of obesity and diabetes reinforce the importance of the balance between XBP1 and CHOP in the regulation of β-cell survival during ER stress. Inhibition of IRE1/XBP1 in islets from pre-diabetic NOD and diabetes-resistant C57BL/6J *ob/ob* mice led to reductions of chaperone, protein folding and ERAD gene expression, while *Chop* and *Trib3* mRNA levels were increased. These changes were accompanied by increased cell death. Furthermore, inhibition of IRE1/XBP1 in *ob/ob* islets augmented oxidative stress and inflammation, suggesting for the first time that IRE1/XBP1 inhibition in islets of a

model of successful β-cell compensation is sufficient to trigger at least some of the defects associated with β-cell decompensation and type 2 diabetes (Chan et al, 2013, Cnop et al, 2005, Del Guerra et al, 2005, Donath and Shoelson, 2011, Song et al, 2008). Interestingly, this also indicates that oxidative stress and inflammation are not necessarily direct consequences of hyperglycaemia (Bensellam et al, 2012, Donath and Shoelson, 2011, Poitout and Robertson, 2008). Together, the data support the notion that ER stress may be an early event that precedes oxidative stress and inflammation in the progression to β-cell failure (Back et al, 2009, Kaufman et al, 2010, Oslowski et al, 2012, Tang et al, 2012).

Growing evidence links downregulation of the adaptive UPR with β-cell failure in type 1 and type 2 diabetes (Akerfeldt et al, 2008, Chan et al, 2013, Engin and Hotamisligil, 2010, Engin et al, 2013, Engin et al, 2014, Tersey et al, 2012). Our studies implicate inflammation as a potential triggering factor for downregulation of the adaptive UPR. In MIN6 cells, cytokines concomitantly reduce adaptive UPR while increasing apoptotic UPR in a time-dependent manner (Figs. S5). Notably, an adaptive response was never observed following cytokine exposure (Figs. S5).

IRE1 has been implicated in ER stress-induced β-cell apoptosis and loss of differentiation via activation of JNK and degradation of ER-associated mRNAs, including proinsulin mRNA (Han et al, 2009, Lee et al, 2011, Lipson et al, 2008, Pirot et al, 2007, Urano et al, 2000). IRE1-independent activation of JNK by cytokines has been implicated in β-cell apoptosis via yet to be fully-identified mechanisms (Bonny et al, 2001, Brozzi et al, 2014, Chan et al, 2011, Marroqui et al, 2014). Importantly, the expression of DP5, a protein implicated in cytokine- and lipid-

mediated β-cell death during ER stress, is regulated by JNK-dependent transcription factor c-JUN (Cunha et al, 2012, Gurzov et al, 2009). JNK also plays an important role in the activation of CHOP downstream of nitric oxide (Chan et al, 2011, Pirot et al, 2007). Our current study is the first to show that JNK acts as a master regulator of UPR gene expression, controlling the transition from adaptive to apoptotic UPR, and thereby β-cell death. Therefore, JNK is a potential target to restore XBP1:CHOP balance and improve β-cell survival in response to inflammatory- and obesity-associated ER stress.

5. Conclusion

Our studies show for the first time that: 1) β-cell survival during cytokine-mediated and lipotoxic ER stress is regulated by the balance between XBP1 and CHOP; 2) the IRE1/XBP1-mediated adaptive UPR protects against hallmarks of β-cell failure associated with type 1 and type 2 diabetes; and 3) JNK is a crucial regulator of the transition from adaptive to apoptotic UPR during ER stress. These observations reveal important molecular mechanisms governing β-cell survival during ER stress and support the targeting of ER function for the treatment of both type 1 and type 2 diabetes (Engin and Hotamisligil, 2010, Engin et al, 2013, Urano, 2014, Xiao et al, 2011).

Acknowledgements

This work was supported by a grant from the National Health and Medical Research Council (NHMRC) of Australia. D.R.L. is supported by an Australian Research Council (ARC) Future Fellowship.

References

Akerfeldt, M.C., Howes, J., Chan, J.Y., Stevens, V.A., Boubenna, N., McGuire, H.M., King, C., Biden, T.J. and Laybutt, D.R., 2008. Cytokine-induced beta-cell death is independent of endoplasmic reticulum stress signaling. Diabetes. 57, 3034-44.

Allagnat, F., Christulia, F., Ortis, F., Pirot, P., Lortz, S., Lenzen, S., Eizirik, D.L. and Cardozo, A.K., 2010. Sustained production of spliced X-box binding protein 1 (XBP1) induces pancreatic beta cell dysfunction and apoptosis. Diabetologia. 53, 1120-30.

Allagnat, F., Fukaya, M., Nogueira, T.C., Delaroche, D., Welsh, N., Marselli, L., Marchetti, P., Haefliger, J.A., Eizirik, D.L. and Cardozo, A.K., 2012. C/EBP homologous protein contributes to cytokine-induced pro-inflammatory responses and apoptosis in beta-cells. Cell Death Differ. 19, 1836-46.

Back, S.H., Scheuner, D., Han, J., Song, B., Ribick, M., Wang, J., Gildersleeve, R.D., Pennathur, S. and Kaufman, R.J., 2009. Translation attenuation through eIF2alpha phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells. Cell Metab. 10, 13-26.

Baldwin, A.C., Green, C.D., Olson, L.K., Moxley, M.A. and Corbett, J.A., 2012. A role for aberrant protein palmitoylation in FFA-induced ER stress and beta-cell death. Am J Physiol Endocrinol Metab. 302, E1390-8.

Bensellam, M., Laybutt, D.R. and Jonas, J.C., 2012. The molecular mechanisms of pancreatic beta-cell glucotoxicity: Recent findings and future research directions. Mol Cell Endocrinol. 364, 1-27.

Biden, T.J., Boslem, E., Chu, K.Y. and Sue, N., 2014. Lipotoxic endoplasmic reticulum stress, beta cell failure, and type 2 diabetes mellitus. Trends Endocrinol Metab. 25, 389-398.

Bonny, C., Oberson, A., Negri, S., Sauser, C. and Schorderet, D.F., 2001. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. Diabetes. 50, 77-82.

Brozzi, F., Gerlo, S., Grieco, F.A., Nardelli, T.R., Lievens, S., Gysemans, C., Marselli, L., Marchetti, P., Mathieu, C., Tavernier, J. and Eizirik, D.L., 2014. A combined "omics" approach identifies N-Myc Interactor as a novel cytokine-induced regulator of IRE1alpha protein and c-Jun N-terminal Kinase in pancreatic beta cells. J Biol Chem. 289, 20677-20693.

Cardozo, A.K., Ortis, F., Storling, J., Feng, Y.M., Rasschaert, J., Tonnesen, M., Van Eylen, F., Mandrup-Poulsen, T., Herchuelz, A. and Eizirik, D.L., 2005. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca2+ ATPase 2b and deplete endoplasmic reticulum Ca2+, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. Diabetes. 54, 452-461.

Chan, J.Y., Cooney, G.J., Biden, T.J. and Laybutt, D.R., 2011. Differential regulation of adaptive and apoptotic unfolded protein response signalling by cytokine-induced nitric oxide production in mouse pancreatic beta cells. Diabetologia. 54, 1766-76.

Chan, J.Y., Luzuriaga, J., Bensellam, M., Biden, T.J. and Laybutt, D.R., 2013. Failure of the adaptive unfolded protein response in islets of obese mice is linked with abnormalities in beta-cell gene expression and progression to diabetes. Diabetes. 62, 1557-68.

Cnop, M., Welsh, N., Jonas, J.C., Jorns, A., Lenzen, S. and Eizirik, D.L., 2005. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes. 54, S97-S107.

Cross, B.C., Bond, P.J., Sadowski, P.G., Jha, B.K., Zak, J., Goodman, J.M., Silverman, R.H., Neubert, T.A., Baxendale, I.R., Ron, D. and Harding, H.P., 2012. The molecular basis for

selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. Proc Natl Acad Sci U S A. 109, E869-78.

Cunha, D.A., Hekerman, P., Ladriere, L., Bazarra-Castro, A., Ortis, F., Wakeham, M.C., Moore, F., Rasschaert, J., Cardozo, A.K., Bellomo, E., Overbergh, L., Mathieu, C., Lupi, R., Hai, T., Herchuelz, A., Marchetti, P., Rutter, G.A., Eizirik, D.L. and Cnop, M., 2008. Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J Cell Sci. 121, 2308-18.

Cunha, D.A., Igoillo-Esteve, M., Gurzov, E.N., Germano, C.M., Naamane, N., Marhfour, I., Fukaya, M., Vanderwinden, J.M., Gysemans, C., Mathieu, C., Marselli, L., Marchetti, P., Harding, H.P., Ron, D., Eizirik, D.L. and Cnop, M., 2012. Death protein 5 and p53-upregulated modulator of apoptosis mediate the endoplasmic reticulum stress-mitochondrial dialog triggering lipotoxic rodent and human beta-cell apoptosis. Diabetes. 61, 2763-75.

Cunha, D.A., Gurzov, E.N., Naamane, N., Ortis, F., Cardozo, A.K., Bugliani, M., Marchetti, P., Eizirik, D.L. and Cnop, M., 2014. JunB protects beta-cells from lipotoxicity via the XBP1-AKT pathway. Cell Death Differ. 21, 1313-24.

Del Guerra, S., Lupi, R., Marselli, L., Masini, M., Bugliani, M., Sbrana, S., Torri, S., Pollera, M., Boggi, U., Mosca, F., Del Prato, S. and Marchetti, P., 2005. Functional and molecular defects of pancreatic islets in human type 2 diabetes. Diabetes. 54, 727-735.

Donath, M.Y. and Shoelson, S.E., 2011. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol. 11, 98-107.

Engin, F. and Hotamisligil, G.S., 2010. Restoring endoplasmic reticulum function by chemical chaperones: an emerging therapeutic approach for metabolic diseases. Diabetes Obes Metab. 12 Suppl 2, 108-15.

Engin, F., Yermalovich, A., Nguyen, T., Hummasti, S., Fu, W., Eizirik, D.L., Mathis, D. and Hotamisligil, G.S., 2013. Restoration of the unfolded protein response in pancreatic beta cells protects mice against type 1 diabetes. Sci Transl Med. 5, 211ra156.

Engin, F., Nguyen, T., Yermalovich, A. and Hotamisligil, G.S., 2014. Aberrant islet unfolded protein response in type 2 diabetes. Sci Rep. 4, 4054.

Fonseca, S.G., Gromada, J. and Urano, F., 2011. Endoplasmic reticulum stress and pancreatic beta-cell death. Trends Endocrinol Metab. 22, 266-74.

Gurzov, E.N., Ortis, F., Cunha, D.A., Gosset, G., Li, M., Cardozo, A.K. and Eizirik, D.L., 2009. Signaling by IL-1beta+IFN-gamma and ER stress converge on DP5/Hrk activation: a novel mechanism for pancreatic beta-cell apoptosis. Cell Death Differ. 16, 1539-50.

Han, D., Lerner, A.G., Vande Walle, L., Upton, J.P., Xu, W., Hagen, A., Backes, B.J., Oakes, S.A. and Papa, F.R., 2009. IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. Cell. 138, 562-75.

Hara, T., Mahadevan, J., Kanekura, K., Hara, M., Lu, S. and Urano, F., 2014. Calcium efflux from the endoplasmic reticulum leads to beta-cell death. Endocrinology. 155, 758-68.

Jeffrey, K.D., Alejandro, E.U., Luciani, D.S., Kalynyak, T.B., Hu, X., Li, H., Lin, Y., Townsend, R.R., Polonsky, K.S. and Johnson, J.D., 2008. Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis. Proc Natl Acad Sci U S A. 105, 8452-7.

Karaskov, E., Scott, C., Zhang, L., Teodoro, T., Ravazzola, M. and Volchuk, A., 2006. Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. Endocrinology. 147, 3398-3407.

Kaufman, R.J., Back, S.H., Song, B., Han, J. and Hassler, J., 2010. The unfolded protein response is required to maintain the integrity of the endoplasmic reticulum, prevent oxidative stress and preserve differentiation in beta-cells. Diabetes Obes Metab. 12 Suppl 2, 99-107.

Laybutt, D.R., Preston, A.M., Akerfeldt, M.C., Kench, J.G., Busch, A.K., Biankin, A.V. and Biden, T.J., 2007. Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia. 50, 752-63.

Lee, A.H., Heidtman, K., Hotamisligil, G.S. and Glimcher, L.H., 2011. Dual and opposing roles of the unfolded protein response regulated by IRE1alpha and XBP1 in proinsulin processing and insulin secretion. Proc Natl Acad Sci U S A. 108, 8885-90.

Lipson, K.L., Ghosh, R. and Urano, F., 2008. The role of IRE1alpha in the degradation of insulin mRNA in pancreatic beta-cells. PLoS ONE. 3, e1648.

Marhfour, I., Lopez, X.M., Lefkaditis, D., Salmon, I., Allagnat, F., Richardson, S.J., Morgan, N.G. and Eizirik, D.L., 2012. Expression of endoplasmic reticulum stress markers in the islets of patients with type 1 diabetes. Diabetologia. 55, 2417-20.

Marroqui, L., Santin, I., Dos Santos, R.S., Marselli, L., Marchetti, P. and Eizirik, D.L., 2014. BACH2, a candidate risk gene for type 1 diabetes, regulates apoptosis in pancreatic beta-cells via JNK1 modulation and crosstalk with the candidate gene PTPN2. Diabetes. 63, 2516-27.

Matsuoka, T.A., Kaneto, H., Miyatsuka, T., Yamamoto, T., Yamamoto, K., Kato, K., Shimomura, I., Stein, R. and Matsuhisa, M., 2010. Regulation of MafA expression in pancreatic beta-cells in db/db mice with diabetes. Diabetes. 59, 1709-20.

Matveyenko, A.V. and Butler, P.C., 2008. Relationship between beta-cell mass and diabetes onset. Diabetes Obes Metab. 10 Suppl 4, 23-31.

Oslowski, C.M., Hara, T., O'Sullivan-Murphy, B., Kanekura, K., Lu, S., Hara, M., Ishigaki, S., Zhu, L.J., Hayashi, E., Hui, S.T., Greiner, D., Kaufman, R.J., Bortell, R. and Urano, F., 2012. Thioredoxin-interacting protein mediates ER stress-induced beta cell death through initiation of the inflammasome. Cell Metab. 16, 265-73.

Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E. and Mori, M., 2001. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. Proc Natl Acad Sci U S A. 98, 10845-10850.

Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E. and Mori, M., 2002. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest. 109, 525-532.

Pirot, P., Naamane, N., Libert, F., Magnusson, N.E., Orntoft, T.F., Cardozo, A.K. and Eizirik, D.L., 2007. Global profiling of genes modified by endoplasmic reticulum stress in pancreatic beta cells reveals the early degradation of insulin mRNAs. Diabetologia. 50, 1006-14.

Pirot, P., Ortis, F., Cnop, M., Ma, Y., Hendershot, L.M., Eizirik, D.L. and Cardozo, A.K., 2007. Transcriptional regulation of the endoplasmic reticulum stress gene chop in pancreatic insulin-producing cells. Diabetes. 56, 1069-77.

Poitout, V. and Robertson, R.P., 2008. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev. 29, 351-66.

Preston, A.M., Gurisik, E., Bartley, C., Laybutt, D.R. and Biden, T.J., 2009. Reduced endoplasmic reticulum (ER)-to-Golgi protein trafficking contributes to ER stress in lipotoxic mouse beta cells by promoting protein overload. Diabetologia. 52, 2369-73.

Scheuner, D. and Kaufman, R.J., 2008. The unfolded protein response: a pathway that links insulin demand with beta-cell failure and diabetes. Endocr Rev. 29, 317-33.

Song, B., Scheuner, D., Ron, D., Pennathur, S. and Kaufman, R.J., 2008. Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. J Clin Invest. 118, 3378-89.

Tang, C., Koulajian, K., Schuiki, I., Zhang, L., Desai, T., Ivovic, A., Wang, P., Robson-Doucette, C., Wheeler, M.B., Minassian, B., Volchuk, A. and Giacca, A., 2012. Glucose-induced beta cell dysfunction in vivo in rats: link between oxidative stress and endoplasmic reticulum stress. Diabetologia. 55, 1366-79.

Tersey, S.A., Nishiki, Y., Templin, A.T., Cabrera, S.M., Stull, N.D., Colvin, S.C., Evans-Molina, C., Rickus, J.L., Maier, B. and Mirmira, R.G., 2012. Islet beta-cell endoplasmic reticulum stress precedes the onset of type 1 diabetes in the nonobese diabetic mouse model. Diabetes. 61, 818-27.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H.P. and Ron, D., 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science. 287, 664-666.

Urano, F., 2014. Diabetes: Targeting endoplasmic reticulum to combat juvenile diabetes. Nat Rev Endocrinol. 10, 129-30.

Xiao, C., Giacca, A. and Lewis, G.F., 2011. Sodium phenylbutyrate, a drug with known capacity to reduce endoplasmic reticulum stress, partially alleviates lipid-induced insulin resistance and beta-cell dysfunction in humans. Diabetes. 60, 918-24.

Zhang, L., Nosak, C., Sollazzo, P., Odisho, T. and Volchuk, A., 2014. IRE1 inhibition perturbs the unfolded protein response in a pancreatic beta-cell line expressing mutant proinsulin, but does not sensitize the cells to apoptosis. BMC Cell Biol. 15, 29.

Figure Legends

Figure 1. Effect of IRE1/XBP1 inhibition on cytokine-mediated changes in gene expression and β-cell death. MIN6 cells (n=6) or mouse islets (n=5) were incubated in the absence *(white bars)* or presence *(black bars)* of cytokines with or without 4μ8c for 24 h. Changes in mRNA expression (A, C-I), *Xbp1* splicing (B), cell death (J) and cell viability (K) in MIN6 cells. Changes in cell death in islets (L). **p*<0.05, ***p*<0.01, ****p*<0.001 cytokine effect in each group; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 4μ8c effect in each treatment group.

Figure 2. XBP1 deficiency in islets potentiates cytokine-induced cell death. 4-Hydroxytamoxifen -treated islets from control and $Xbp1^{flox/flox}$ $Pdx1\text{-}Cre^{ER}$ mice were incubated in the absence *(white bars)* or presence *(black bars)* of cytokines for 24 h. Changes in mRNA expression (A-H) and cell death (I). n=4-7. **p*<0.05, ***p*<0.01, ****p*<0.001 cytokine effect in each group; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 genotype effect in each treatment group.

Figure 3. Effect of siRNA-mediated silencing of XBP1 and CHOP on cytokine-mediated changes in gene expression and cell death. MIN6 cells transfected with either negative control non-targeting siRNA (*white bars*) or siRNA against XBP1 (*black bars*), CHOP (*light grey bars*) or both XBP1 and CHOP (*dark grey bars*) were cultured in the absence or presence of cytokines for 6 h. Changes in protein levels (A; n=3-5), mRNA expression (B-I; n=4-6) and cell death (J; n=4-6). *p<0.05, **p<0.01, ***p<0.001 versus siControl; †p<0.05, ††p<0.01, †††p<0.001 versus siXBP1.

Figure 4. Effect of siRNA-mediated silencing of XBP1 and CHOP on palmitate-mediated changes in gene expression and cell death. MIN6 cells transfected with either negative control non-targeting siRNA (*white bars*) or siRNA against XBP1 (*black bars*), CHOP (*light grey bars*) or both XBP1 and CHOP (*dark grey bars*) were cultured in the absence or presence of palmitate for 24 h. Changes in protein levels (A; n=3-4), mRNA expression (B-I; n=5) and cell death (J; n=5). *p<0.05, **p<0.01, ***p<0.001 versus siControl; †p<0.05, ††p<0.01, †††p<0.001 versus siXBP1.

Figure 5. Effect of IRE1/XBP1 inhibition on the changes in UPR gene expression and cell death in NOD mouse islets. Islets from control and NOD mice at 5-6 weeks of age were cultured in the absence (control, *white bars*; and NOD, *black bars*) or presence (NOD, *striped bars*) of IRE1/XBP1 inhibitor (4μ8c) for 40 h. Changes in mRNA expression (A, C-I; n=7-8), *Xbp1* splicing (B; n=7-8) and cell death (J; n=10-11). *p<0.05, **p<0.01, ***p<0.001 genotype effect; †p<0.05, ††p<0.01, †††p<0.001 4μ8c treatment effect.

Figure 6. Effect of IRE1/XBP1 inhibition on the changes in UPR, antioxidant and inflammation gene expression and cell death in *ob/ob* mouse islets. Islets from C57BL/6J control and *ob/ob* mice were cultured in the absence (control, *white bars*; and *ob/ob*, *black bars*) or presence (*ob/ob*, *striped bars*) of IRE1/XBP1 inhibitor (4μ8c) for 40 h. Changes in mRNA expression (A, C-Q; n=7-8), *Xbp1* splicing (B; n=7-8) and cell death (R; n=7). ***p*<0.05, ***p*<0.01, ****p*<0.001 genotype effect; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 4μ8c treatment effect.

Figure 7. Effect of JNK inhibition on cytokine-mediated changes in gene expression and cell death. MIN6 cells were incubated in the absence *(white bars)* or presence *(black bars)* of cytokines with JNKi or negative control for 24 h. Changes in protein levels (A), mRNA expression (B, D-J), *Xbp1* splicing (C) and cell death (K). Changes in insulin secretion at 2.8 mM *(white bars)* and 25 mM *(black bars)* glucose (L). n=3-5; **p*<0.05, ***p*<0.01, ****p*<0.001 cytokine effect in each group; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 JNKi treatment effect.

Figure 8. Effect of JNK inhibition on the changes in UPR gene expression and cell death in *db/db* mouse islets. Islets from C57BL/KsJ control and *db/db* mice at 16 weeks of age were cultured in negative control (control, *white bars*, n=3-10; and *db/db*, *black bars*, n=3-5) or JNKi (*db/db*, *striped bars*, n=3-5) for 24 h. Changes in mRNA expression (A, C-I), *Xbp1* splicing (B) and cell death (J). **p*<0.05, ***p*<0.01, ****p*<0.001 genotype effect; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 JNKi treatment effect.

Supplementary Material Figure Legends

Figs. S1. Effect of siRNA-mediated silencing of XBP1 and CHOP on cytokine-mediated changes in protein levels. MIN6 cells transfected with either negative control non-targeting siRNA (*white bars*) or siRNA against XBP1 (*black bars*), CHOP (*light grey bars*) or both XBP1 and CHOP (*dark grey bars*) were cultured in the absence or presence of cytokines for 6 h. XBP1 (A), CHOP (B), pJNK1 (C) and pJNK2 (D) were quantified by densitometry. n=3-5. *p<0.05, ***p<0.001 versus siControl; †p<0.05 versus siXBP1

Figs. S2. Effect of siRNA-mediated silencing of XBP1 and CHOP on palmitate-mediated changes in protein levels. MIN6 cells transfected with either negative control non-targeting siRNA (*white bars*) or siRNA against XBP1 (*black bars*), CHOP (*light grey bars*) or both XBP1 and CHOP (*dark grey bars*) were cultured in the absence or presence of palmitate for 24 h. XBP1 (A), CHOP (B), pJNK1 (C) and pJNK2 (D) were quantified by densitometry. n=3-4. **p<0.01, ***p<0.001 versus siControl; ††p<0.01, †††p<0.001 versus siXBP1.

Figs. S3. Changes in *Xbp1* mRNA expression in islets of diabetes-prone *db/db* and diabetes-resistant *ob/ob* mice at 6 and 16 weeks of age. Islets were isolated from 10 C57BL/KsJ control and 8 pre-diabetic *db/db* mice at 6 weeks of age, 15 C57BL/KsJ control and 12 diabetic *db/db* mice at 16 weeks of age, 6 C57BL/6J control and 5 *ob/ob* mice at 6 weeks of age, and 5 C57BL/6J control and 7 *ob/ob* mice at 16 weeks of age. mRNA levels were expressed as fold-change of the levels in respective age-matched controls (represented by the dashed line). Shown are changes in islets of *db/db* and *ob/ob* mice at 6 (*white bars*) and 16 (*black bars*) weeks of age. *p<0.05, **p<0.01, ***p<0.001 genotype effect in each age group

Figs. S4. Effect of JNK inhibition on cytokine-mediated changes in protein levels. MIN6 cells were incubated in the absence *(white bars)* or presence *(black bars)* of cytokines with JNKi or negative control for 24 h. pJNK1 (A), pJNK2 (B), p c-JUN (C), XBP1 (D) and CHOP (E) were quantified by densitometry. n=3-5; **p*<0.05, ***p*<0.01, ****p*<0.001 cytokine effect in each group; ††*p*<0.01, †††*p*<0.001 JNKi treatment effect.

Figs. S5. Time-dependent changes in mRNA expression of UPR genes after cytokine stimulation. MIN6 cells were incubated in the absence *(white bars)* or presence *(black bars)* of cytokines for 3, 6 or 24 h. n=3-12. **p*<0.05, ***p*<0.01, ****p*<0.001 cytokine effect at the same time point.

Figure 1

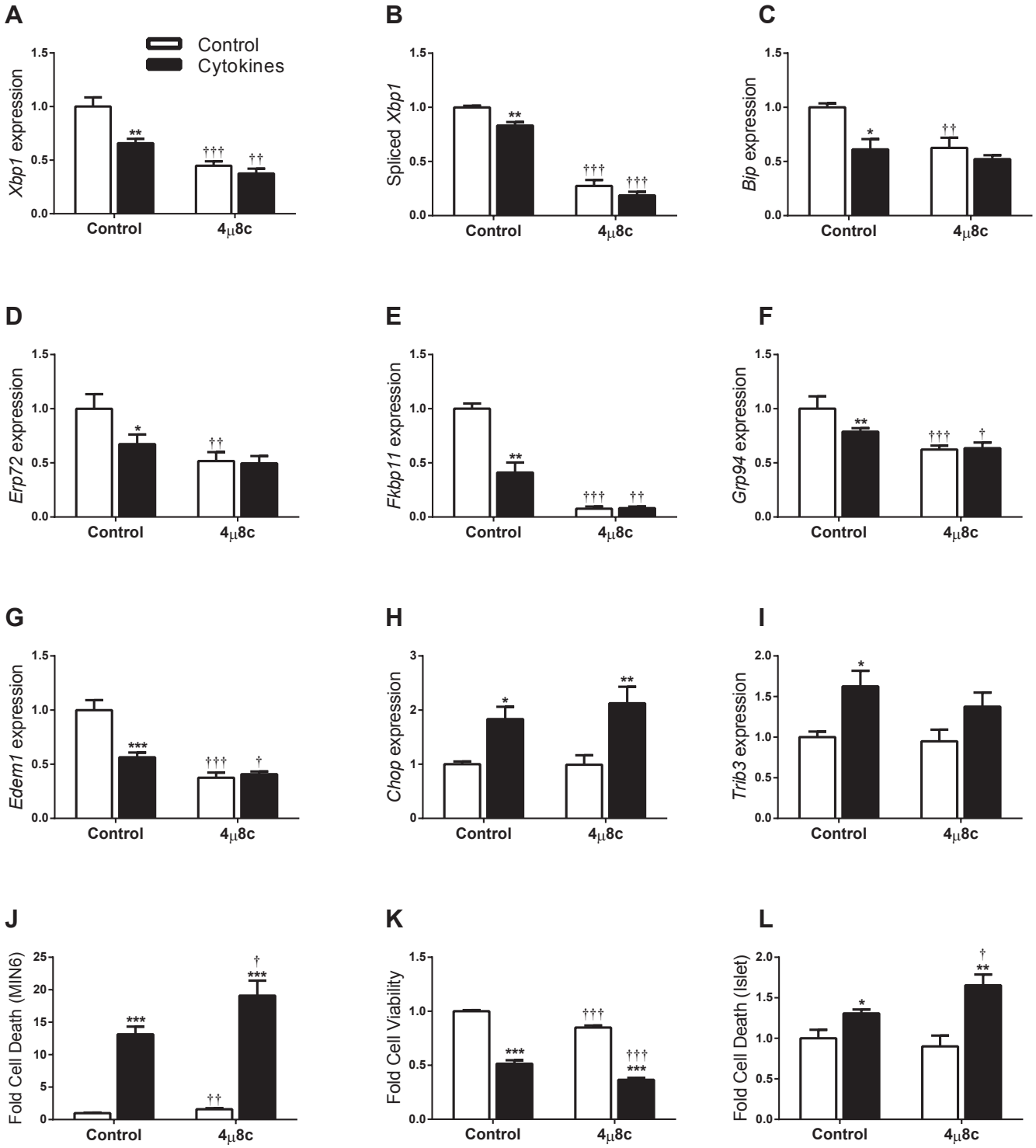

A
Control
Cytokines
Xbp1 expression
B
Spliced Xbp1
C
Bip expression
D
Erp72 expression
E
Fkbp11 expression
F
Grp94 expression
G
Edem1 expression
H
Chop expression
I
Trib3 expression
J
Fold Cell Death (MIN6)
K
Fold Cell Viability
L
Fold Cell Death (Islet)
Control
4μ8c

Figure 2

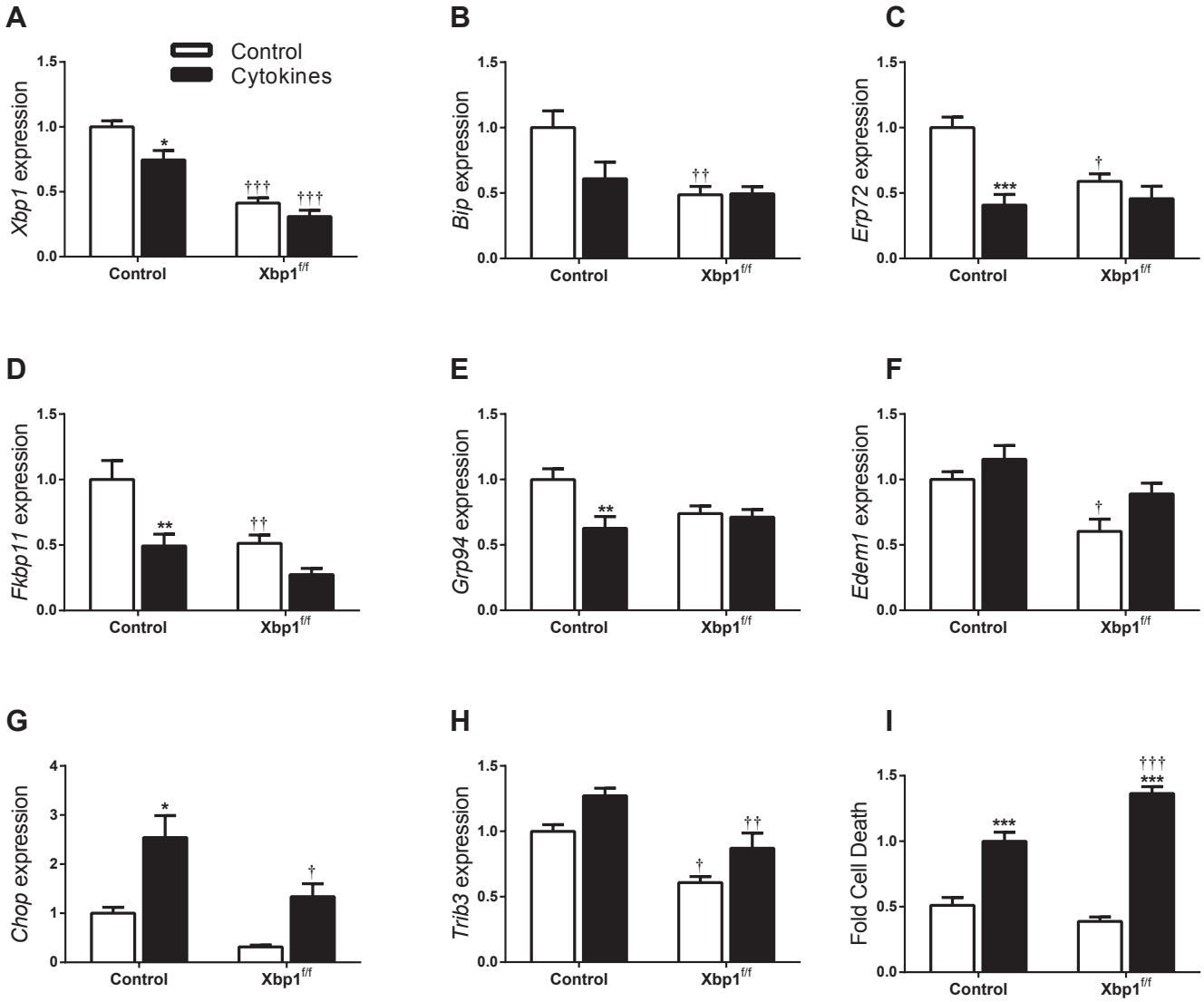


Figure 3

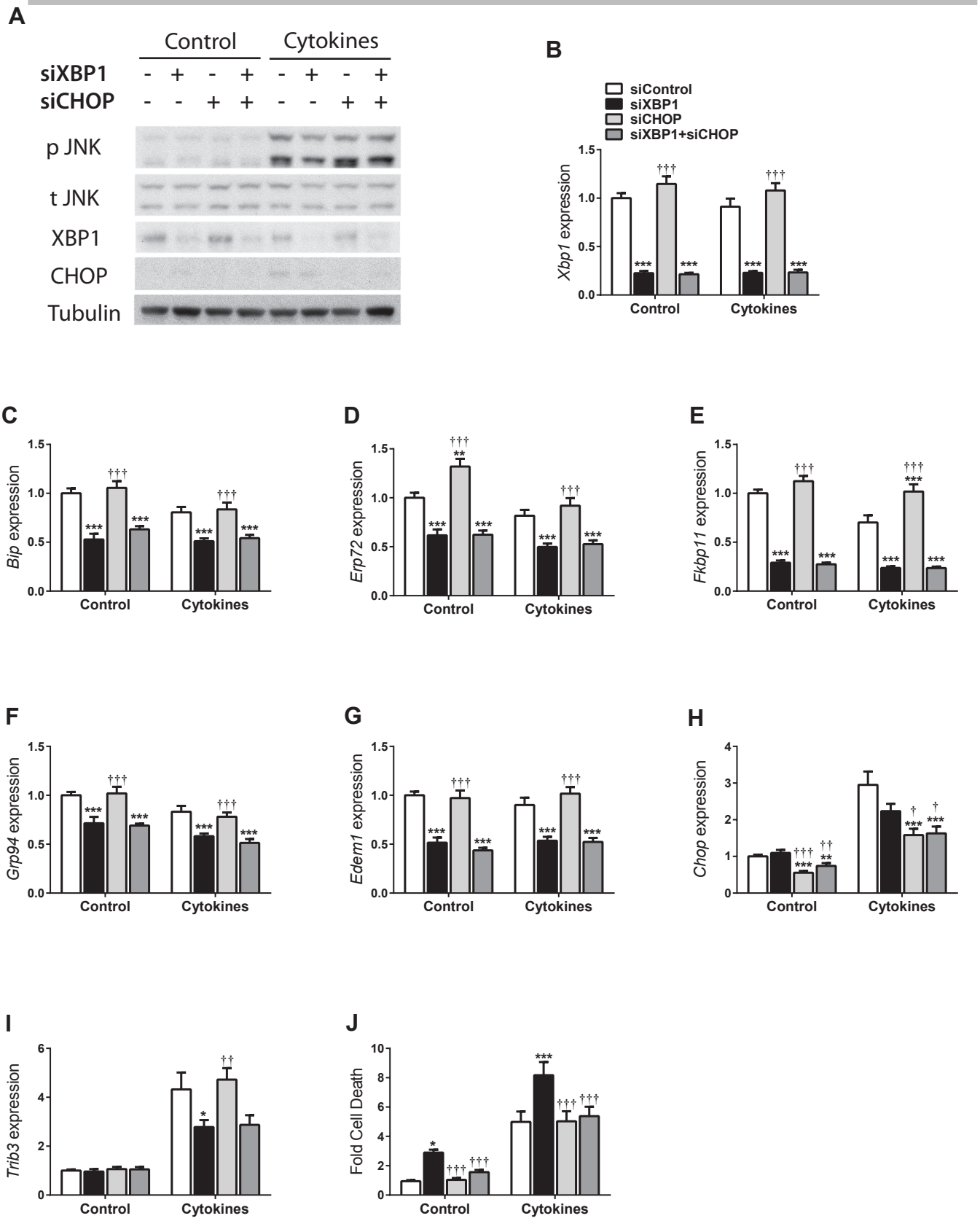


Figure 4

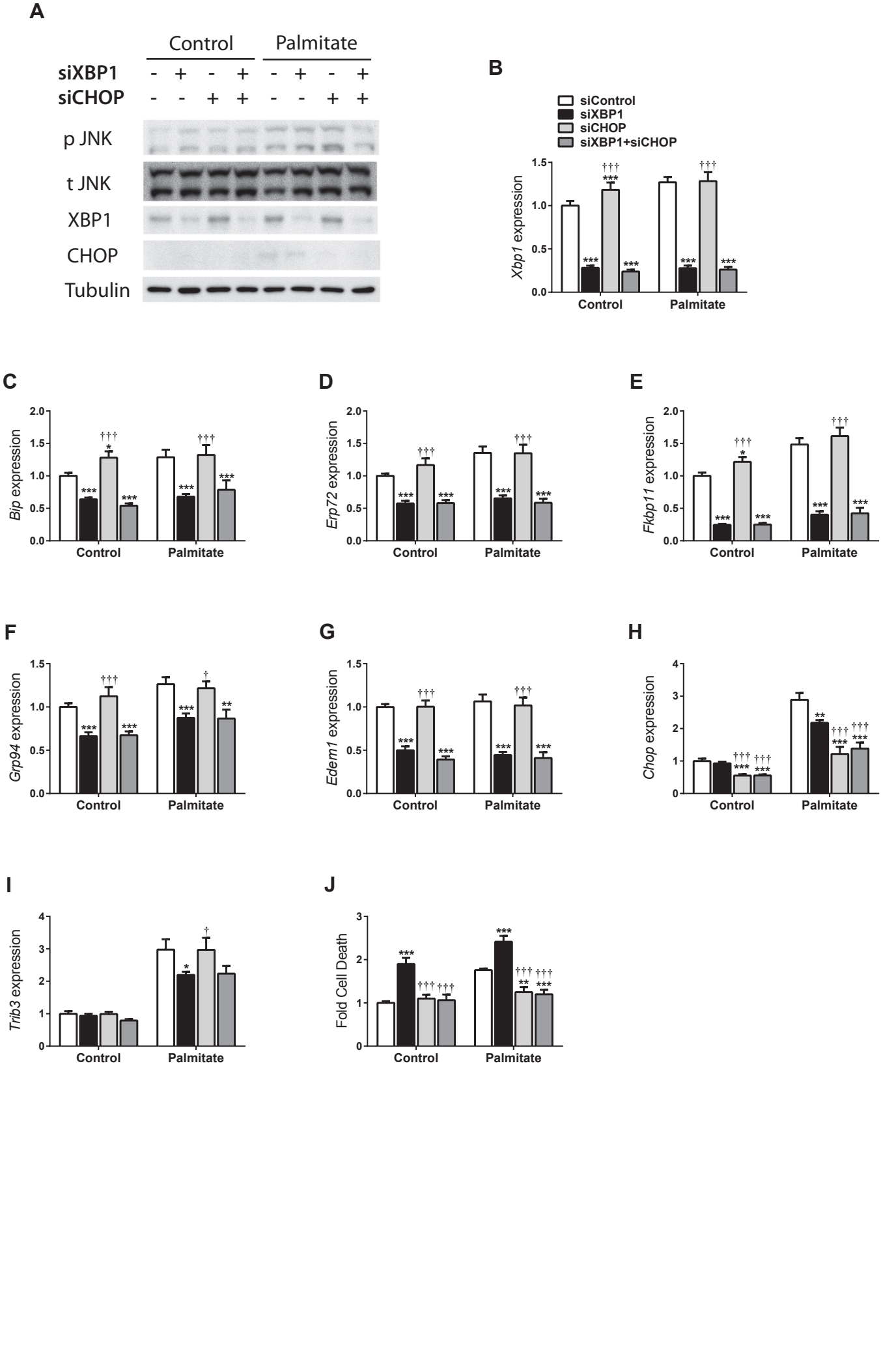


Figure 5

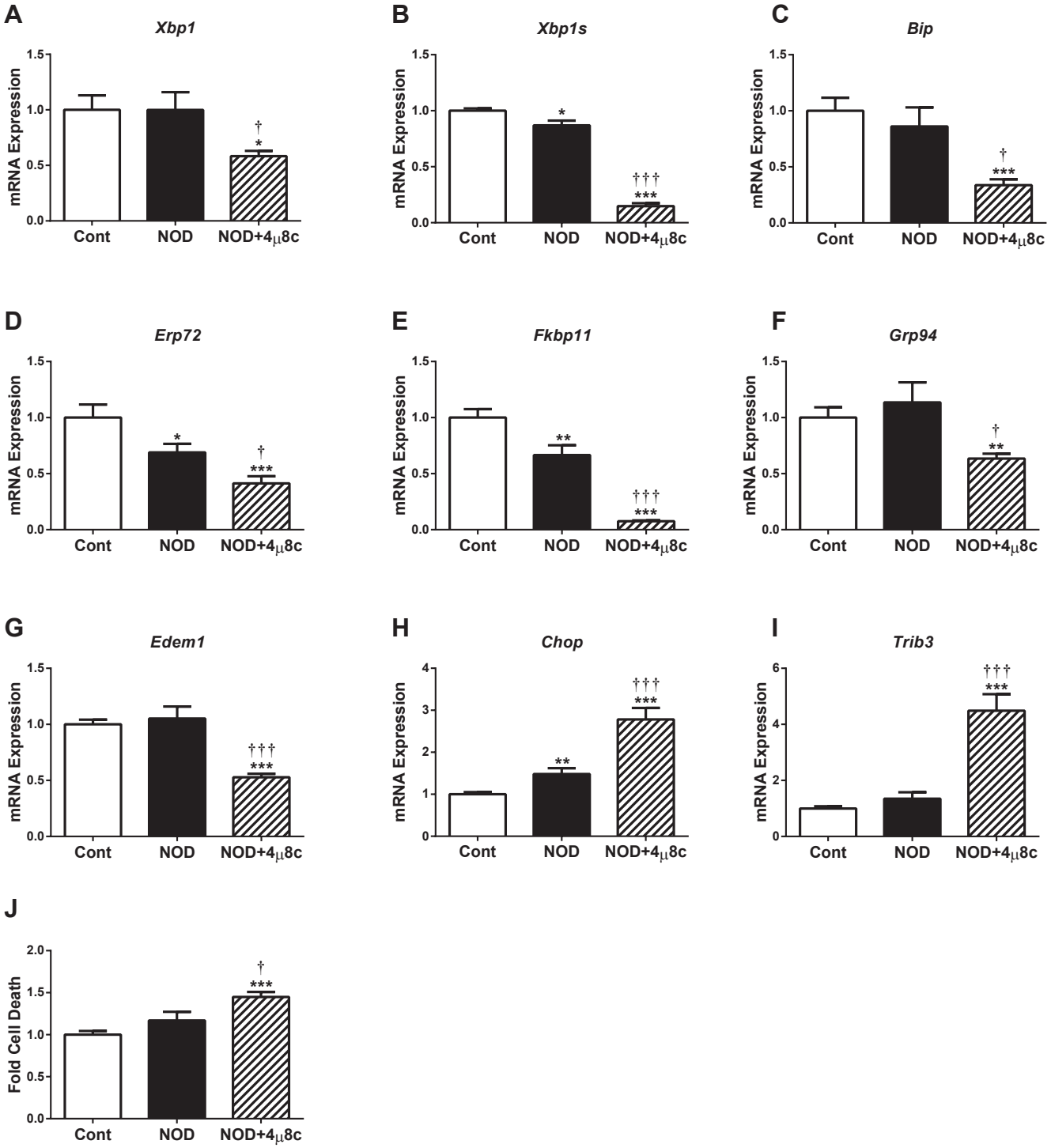

A
Xbp1
mRNA Expression
1.5
1.0
0.5
0.0
†
*
Cont
NOD
NOD+4μ8c
B
Xbp1s
mRNA Expression
1.5
1.0
0.5
0.0
*
†††
***
Cont
NOD
NOD+4μ8c
C
Bip
mRNA Expression
1.5
1.0
0.5
0.0
†
***
Cont
NOD
NOD+4μ8c
D
Erp72
mRNA Expression
1.5
1.0
0.5
0.0
*
†
***
Cont
NOD
NOD+4μ8c
E
Fkbp11
mRNA Expression
1.5
1.0
0.5
0.0
**
†††
***
Cont
NOD
NOD+4μ8c
F
Grp94
mRNA Expression
1.5
1.0
0.5
0.0
†
**
Cont
NOD
NOD+4μ8c
G
Edem1
mRNA Expression
1.5
1.0
0.5
0.0
†††
***
Cont
NOD
NOD+4μ8c
H
Chop
mRNA Expression
4
3
2
1
0
**
†††
***
Cont
NOD
NOD+4μ8c
I
Trib3
mRNA Expression
6
4
2
0
†††
***
Cont
NOD
NOD+4μ8c
J
Fold Cell Death
2.0
1.5
1.0
0.5
0.0
†
***
Cont
NOD
NOD+4μ8c

Figure 7

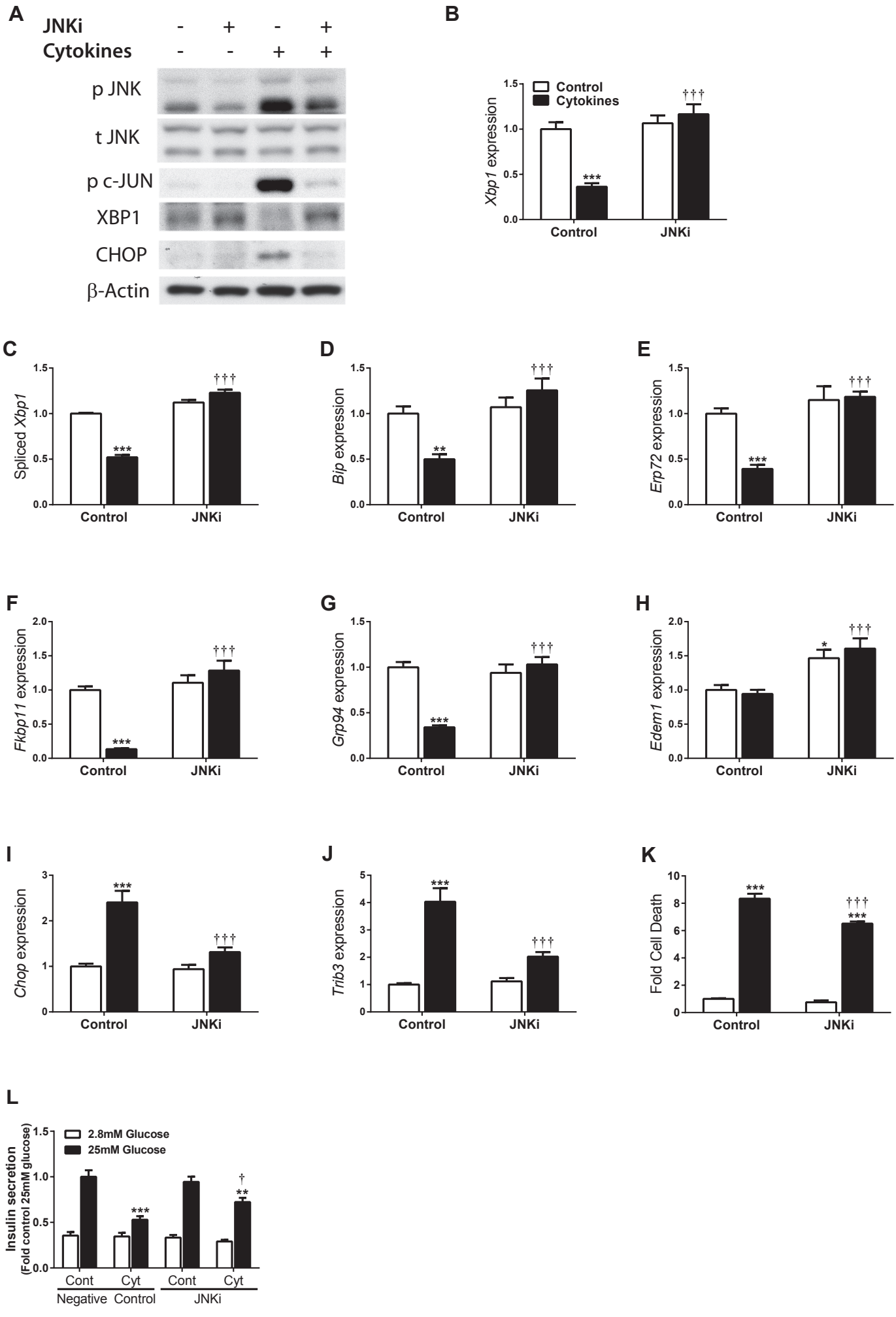


Figure 8

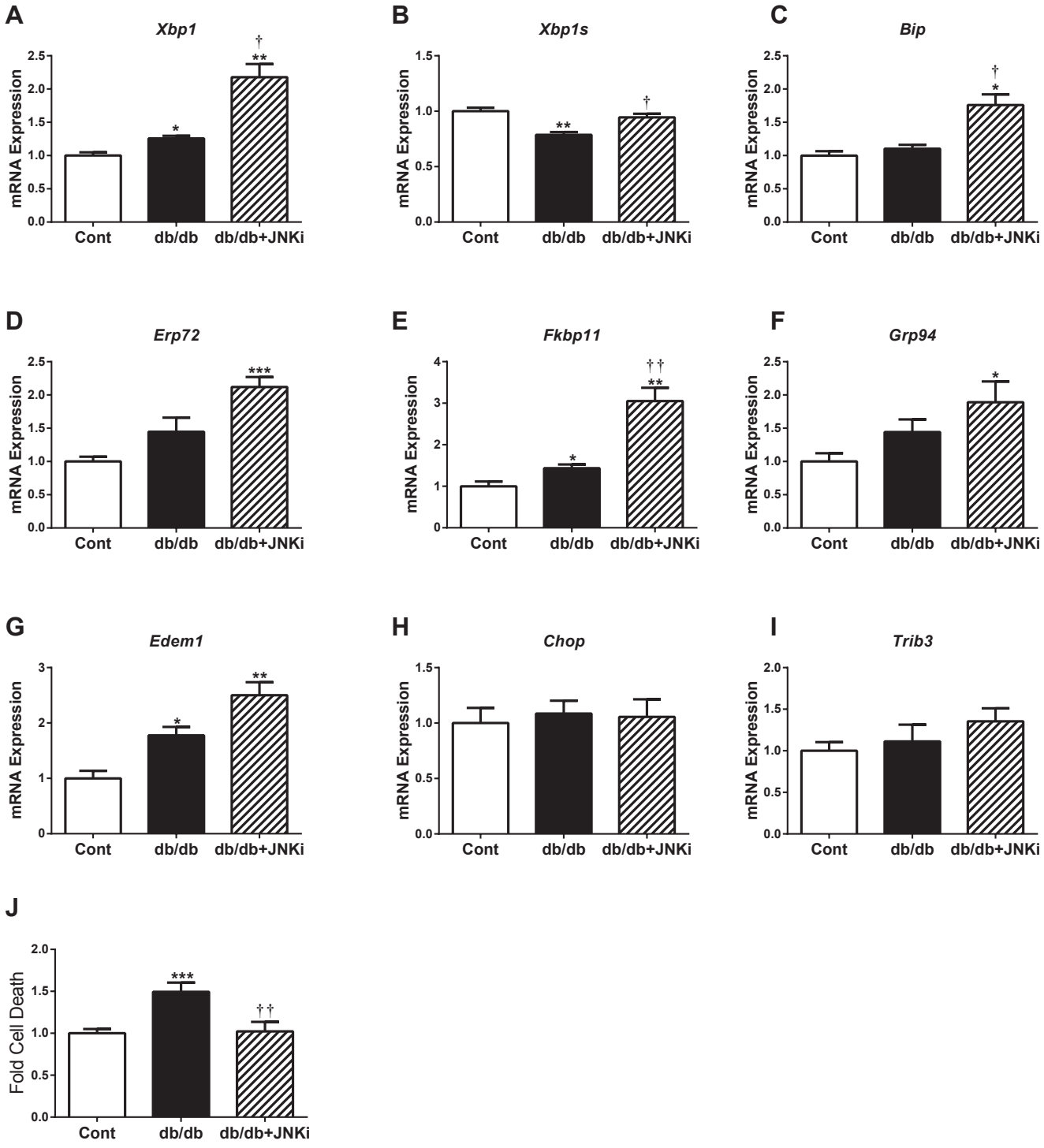

A
Xbp1
mRNA Expression
Cont
db/db
db/db+JNKi
B
Xbp1s
C
Bip
D
Erp72
E
Fkbp11
F
Grp94
G
Edem1
H
Chop
I
Trib3
J
Fold Cell Death

Figure 6

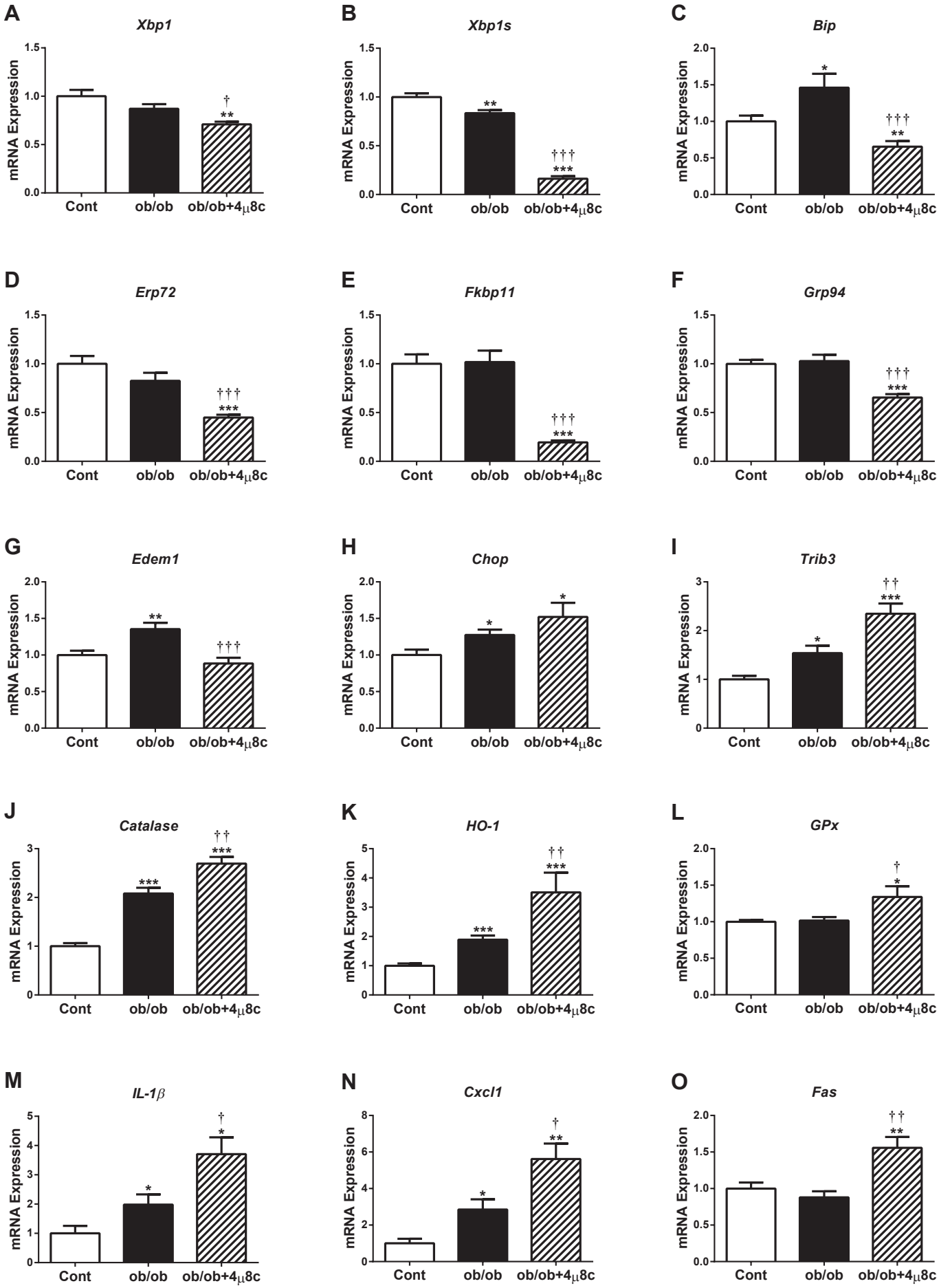
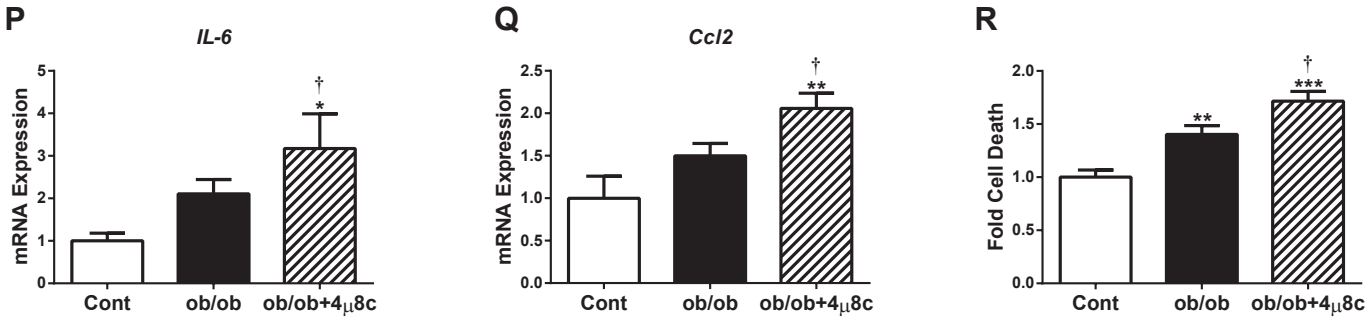
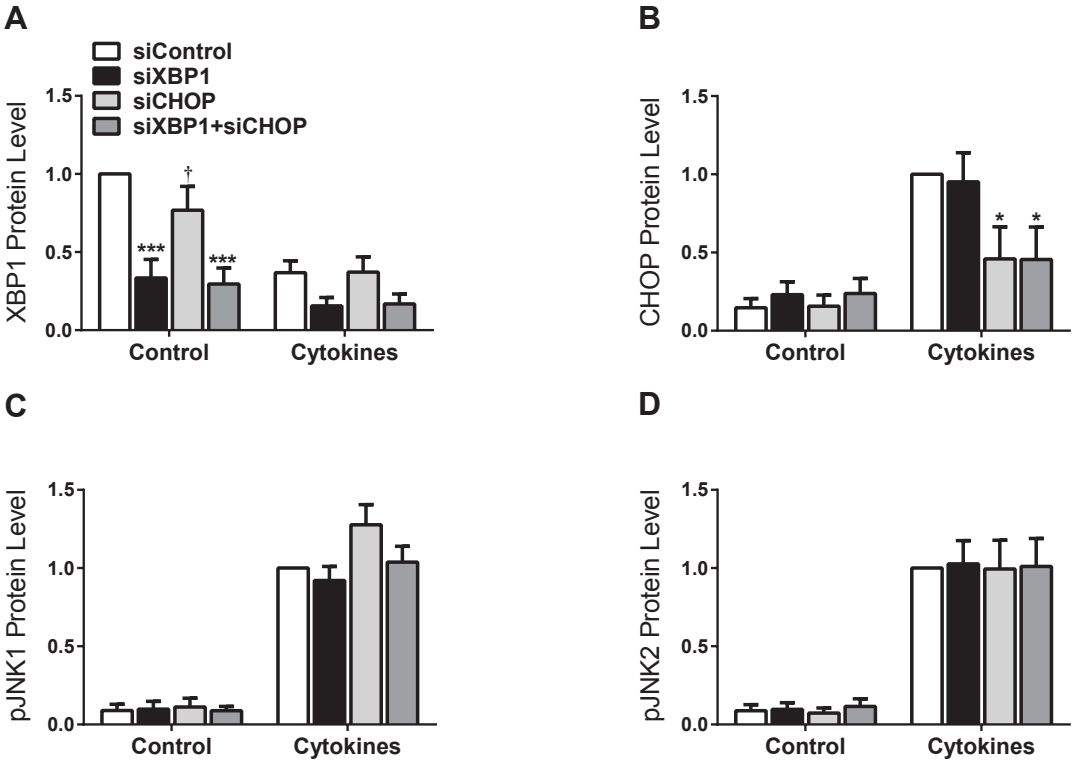

A
Xbp1
B
Xbp1s
C
Bip
D
Erp72
E
Fkbp11
F
Grp94
G
Edem1
H
Chop
I
Trib3
J
Catalase
K
HO-1
L
GPx
M
IL-1β
N
Cxcl1
O
Fas
mRNA Expression
Cont
ob/ob
ob/ob+4μ8c

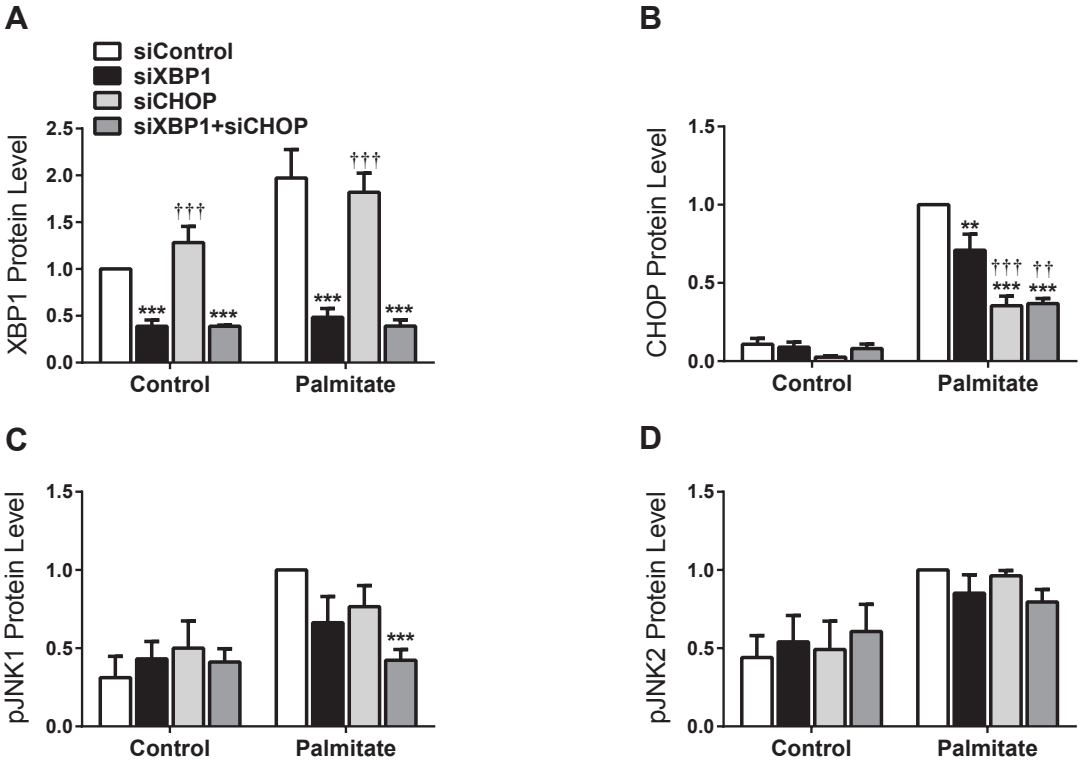
Figure 6



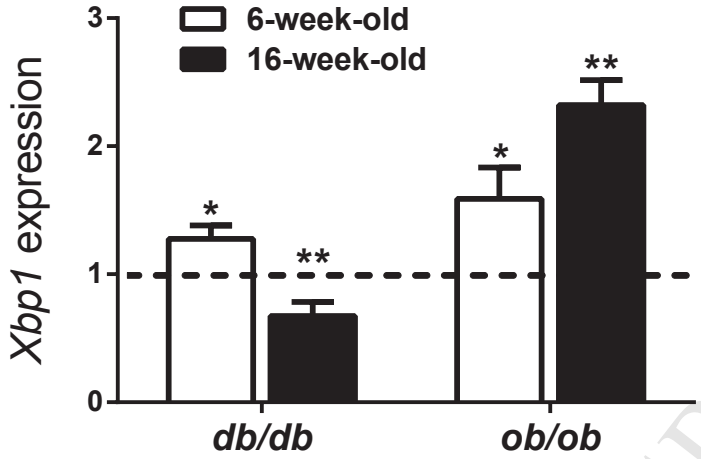
Supplementary Figure S1



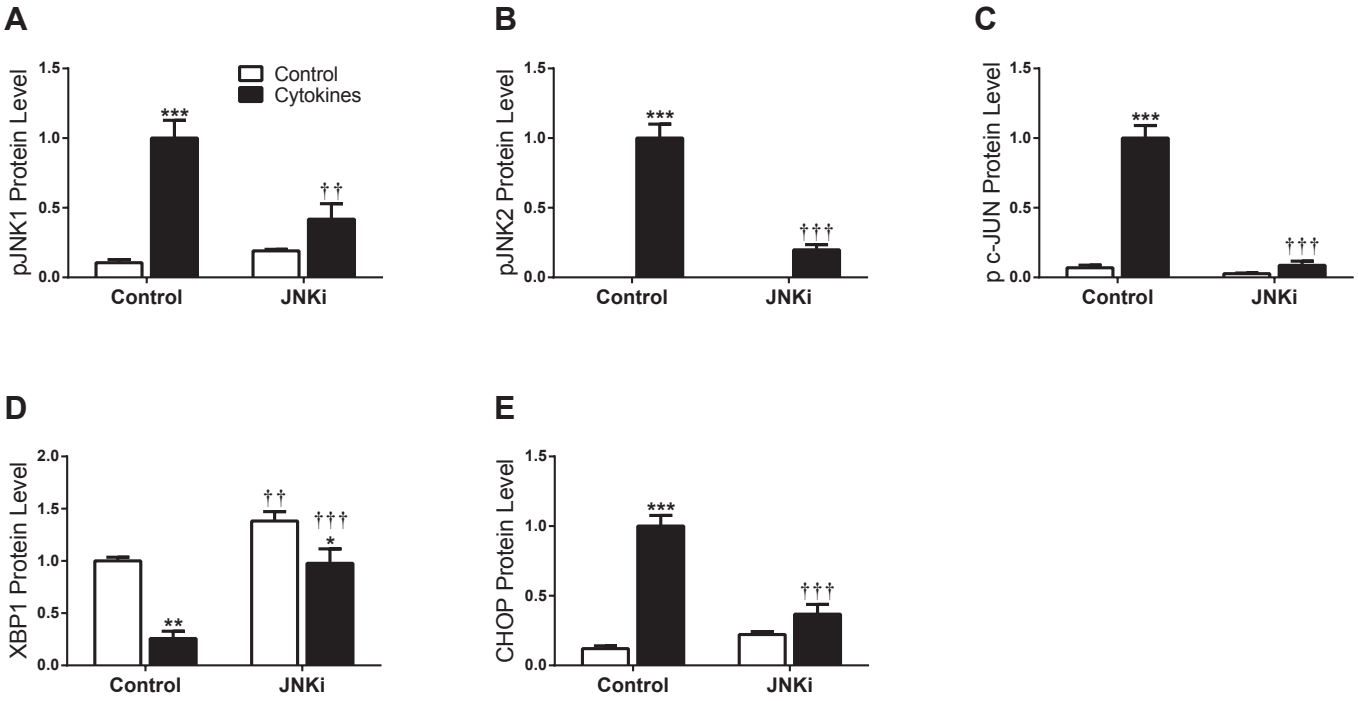
Supplementary Figure S2



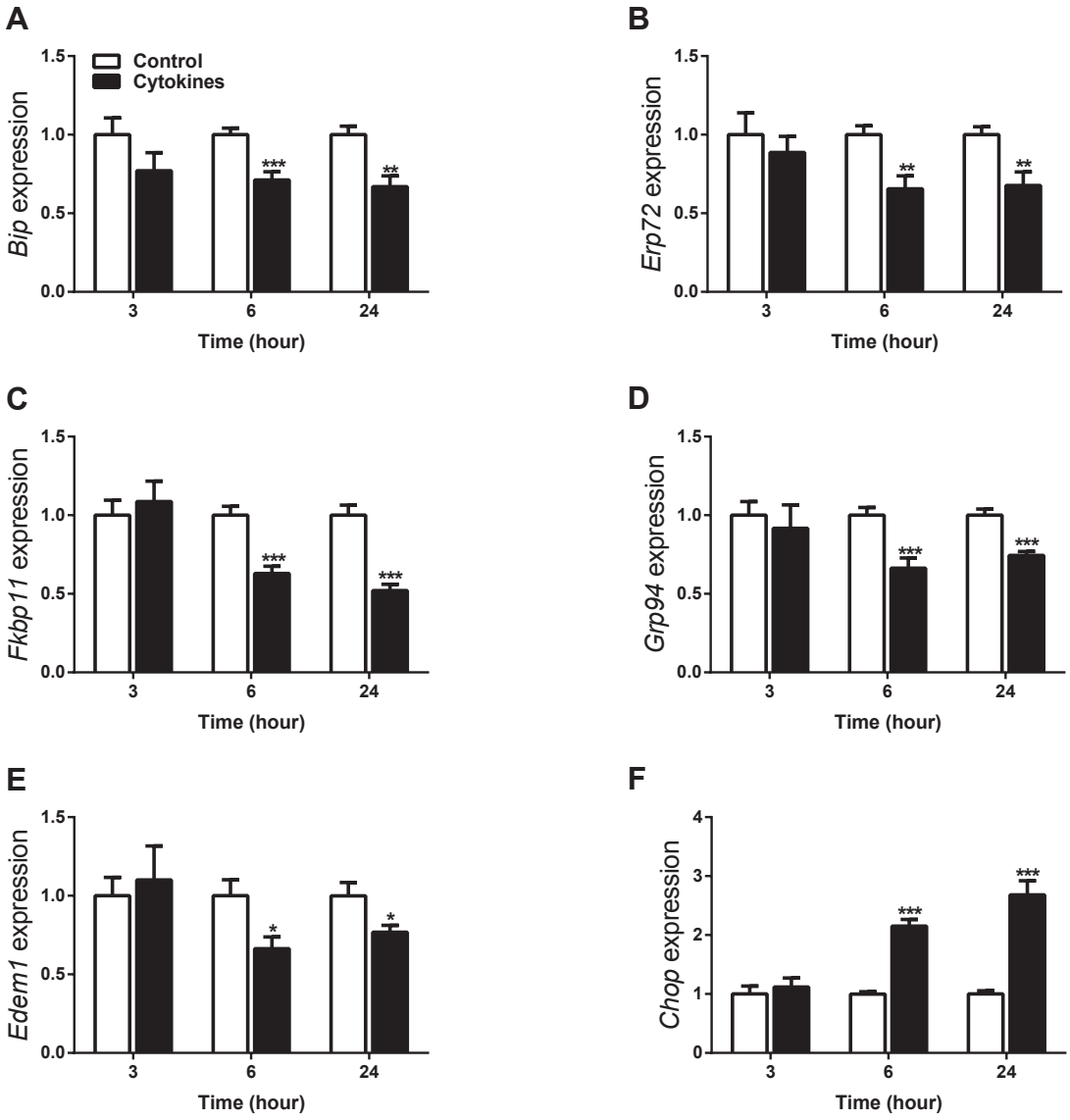
Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5



SUPPLEMENTARY DATA

Supplementary Table S1. Sequences of oligonucleotide primers.

Gene symbol	5' Oligonucleotide	3' Oligonucleotide
Bip (Hspa5, Grp78)	AGGACAAGAAGGAGGATGTGGG	ACCGAAGGGTCATTCCAAGTG
Catalase (Cat)	ATGAAGCAGTGGAAGGAGCAGC	CTGTCAAAGTGTGCCATCTCGTC
Ccl2 (MCP-1)	CCACTCACCTGCTGCTACTCATTC	TCTGGACCCATTCCTTCTTGG
Chop (Ddit3)	TTCACTACTCTTGACCCTGCGTC	CACTGACCACTCTGTTTCCGTTTC
Cxcl1 (Gro1)	CAAACCGAAGTCATAGCCACACTC	TTGTCAGAAGCCAGCGTTCAC
Cyclophilin A	TGTGCCAGGGTGGTGACTTTAC	TGGGAACCGTTTGTGTTTGG
Edem1	GCAATGAAGGAGAAGGAGACCC	TAGAAGGCGTGTAGGCAGATGG
Erp72 (Pdia4)	AGTCAAGGTGGTGGTGGGAAAG	TGGGAGCAAAATAGATGGTAGGG
Fas	AACCAGACTTCTACTGCGATTCTCC	CCTTTTCCAGCACTTTCTTTTCCG
Fkbp11	ACACGCTCCACATACACTACACGG	ATGACTGCTCTTCGCTTCTCTCCC
GPx (Gpx1)	ACAGTCCACCGTGTATGCCTTC	CTCTTCATTCTTGCCATTCTCCTG
Grp94 (Hsp90b1)	AAACGGCAACACTTCGGTCAG	GCATCCATCTCTTCTCCCTCATC
HO-1 (Hmox1)	CCACACAGCACTATGTAAAGCGTC	GTTCGGGAAGGTAAAAAAAGCC
IL-1β	TGTTCTTTGAAGTTGACGGACCC	CCACAGCCACAATGAGTGATACTG
IL-6	CAAGAGACTTCCATCCAGTTGCC	CATTTCCACGATTTCCCAGAGAAC
Trib3	TCTTCAGCAACTGTGAGAGGACG	TCCAGACATCAGCCGCTTTG
Xbp1	GCAGCAAGTGGTGGATTTGG	AGATGTTCTGGGGAGGTGACAAC
Xbp1 (Splicing)	AAACAGAGTAGCAGCGCAGACTGC	GGATCTCTAAAACTAGAGGCTTGGTG

Aliases of gene symbols given in parenthese