Cellular Signalling 28 (2016) 1262–1269

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

A novel role of c-FLIP protein in regulation of ER stress response

Silvia Conti [a], Simonetta Petrungaro [a], Elettra Sara Marini [b], Silvia Masciarelli [a], Luana Tomaipitinca [a], Antonio Filippini [a], Claudia Giampietri [a,1], Elio Ziparo [a,*,1]

[a] Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Sapienza University of Rome, 00161 Rome, Italy
[b] Department of Biosciences, Centre for Immune Regulation, University of Oslo, Blindernveien, 0371 Oslo, Norway

ARTICLE INFO

Article history:
Received 2 November 2015
Received in revised form 30 May 2016
Accepted 2 June 2016
Available online 3 June 2016

Keywords:
Mouse embryonic fibroblast
Endoplasmic reticulum stress
UPR
Apoptosis
c-FLIP

ABSTRACT

Cellular-Flice-like inhibitory protein (c-FLIP) is an apoptosis modulator known to inhibit the extrinsic apoptotic pathway thus blocking Caspase-8 processing in the Death Inducing Signalling Complex (DISC). We previously demonstrated that c-FLIP localizes at the endoplasmic reticulum (ER) and that c-FLIP-deficient mouse embryonic fibroblasts (MEFs) display an enlarged ER morphology. In the present study, we have addressed the consequences of c-FLIP ablation in the ER stress response by investigating the effects of pharmacologically-induced ER stress in Wild Type (WT) and c-FLIP −/− MEFs. Surprisingly, c-FLIP −/− MEFs were found to be strikingly more resistant than WT MEFs to ER stress-mediated apoptosis. Analysis of Unfolded Protein Response (UPR) pathways revealed that Pancreatic ER Kinase (PERK) and Inositol-Requiring Enzyme 1 (IRE1) branch signalling is compromised in c-FLIP −/− cells when compared with WT cells. We found that c-FLIP modulates the PERK pathway by interfering with the activity of the serine threonine kinase AKT. Indeed, c-FLIP −/− MEFs display higher levels of active AKT than WT MEFs upon ER stress, while treatment with a specific AKT inhibitor of c-FLIP −/− MEFs subjected to ER stress restores the PERK but not the IRE1 pathway. Importantly, the AKT inhibitor or dominant negative AKT transfection sensitizes c-FLIP −/− cells to ER stress-induced cell death while the expression of a constitutively active AKT reduces WT cells sensitivity to ER stress-induced death. Thus, our results demonstrate that c-FLIP modulation of AKT activity is crucial in controlling PERK signalling and sensitivity to ER stress, and highlight c-FLIP as a novel molecular player in PERK and IRE1-mediated ER stress response.

1. Introduction

Disruption of endoplasmic reticulum (ER) homeostasis interferes with protein folding and leads to the accumulation of unfolded and misfolded proteins in the ER lumen. This condition, defined as "ER stress", triggers a series of pathways collectively known as the Unfolded Protein Response (UPR), which is an important adaptive response to ER stress. The UPR is designed to re-establish homeostasis by increasing the ER folding capacity and reducing the ER client load. Indeed, UPR activation results in increased ER size, expression of genes involved in protein folding and in the ER-associated protein degradation, and in a temporary reduction in global protein synthesis. However, if homeostasis cannot be restored, the UPR leads cells to apoptosis [1]. The UPR is triggered by three ER-transmembrane proteins that detect perturbations in the ER and start a signalling cascade in the cytosol: Activating Transcription Factor 6 (ATF6), Pancreatic ER Kinase (PERK), Inositol-Requiring Enzyme 1 (IRE1). Although the mechanisms of activation underlying these three sensors have yet to be fully elucidated, they clearly involve the ER molecular chaperone Glucose Regulated Protein 78 (also known as BIP). Under normal conditions, BIP binds to the ER luminal domain of the UPR sensor proteins and inactivates their signalling; in response to accumulation of unfolded proteins in the ER lumen, BIP dissociates from these sensors, thereby allowing the activation of their proper signalling [2]. After onset of ER stress, ATF6 migrates from the ER to the Golgi apparatus, undergoing a proteolytic cleavage that releases a cytosolic domain that moves to the nucleus and acts as a transcription factor promoting the transcription of UPR target genes mainly involved in protein folding [3]. After BIP dissociation, PERK oligomerization and activation determine phosphorylation of the eukaryotic translation initiation factor-2 (eIF2α) which, in turn, inhibits mRNA translation and reduces the load on the ER. Furthermore, phosphorylated eIF2α triggers the translation of the mRNA of the Activating Transcription Factor 4 (ATF4). The ATF4 targets include Growth Arrest and DNA-Damage inducible protein-34 (GADD34), the UPR-inducible regulatory subunit of the Protein Phosphatase 1 (PP1C) that de-phosphorylates eIF2α and removes the translational arrest [4]. Another ATF4 target is CHOP, a transcription factor that moves to the nucleus and up-regulates pro-apoptotic genes, thereby leading to programmed cell

* Corresponding author at: Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Section of Histology and Medical Embryology, Sapienza University of Rome, Via A. Scarpa, 14 00161 Rome, Italy.
E-mail address: elio.ziparo@uniroma1.it (E. Ziparo).
[1] These authors equally contributed to the work.

http://dx.doi.org/10.1016/j.cellsig.2016.06.003

death [5,6]. Taken together, these molecular events allow the PERK pathway to protect cells from mild or short-lasting ER stress by reducing the load on the ER through translation attenuation, whereas when ER stress is marked or prolonged it does not offer any protection and leads to apoptosis via expression of the pro-apoptotic CHOP. The cytosolic domain of activated IRE1 possesses ribonucleolytic activity that processes the mRNA of the transcription factor XBP1 through an atypical splicing mechanism that removes 22 nucleotides to produce the spliced, active form XBP1s. XBP1s then migrates to the nucleus and controls the transcription of genes encoding proteins involved in protein folding, ER-associated protein degradation (ERAD) and enzymes of the lipid biosynthesis pathways. XBP1s plays a critical role in the ER increase in size upon ER stress [7]. However, sustained IRE-1 activation can trigger apoptosis through its ability to activate BCL-2 family members and caspases [8].

Other mechanisms besides BIP-mediated-activation of PERK and IRE1 are known be involved in UPR regulation [9–16]. In particular Z. Mounir et al. [15] recently reported a link between AKT and the PERK pathway. They demonstrated that AKT inhibits PERK activation through phosphorylation at T799, which blocks PERK and impairs its autophosphorylation at T980. The AKT interactors include the Cellular-Flice-like inhibitory protein (c-FLIP), which is an anti-apoptotic protein that prevents procaspase-8 processing at DISC and inhibits Death Receptor (DR)-mediated apoptosis [17]. All three splicing variants, named c-FLIP$_L$, c-FLIP$_R$ and c-FLIP$_s$, contain two tandem Death Effector Domains (DEDs) at their N-terminal, which is structurally similar to the N-terminal part of procaspase-8. The two DED motifs of c-FLIP$_R$ and c-FLIP$_s$ are followed by a short and varying stretch of amino acids at the C-terminal. By contrast, c-FLIP$_L$ contains a longer C-terminal caspase-like domain, which is not active [18,19]. The structural differences between the c-FLIP isoforms correlate with a different cellular localization and function; indeed, c-FLIP$_{S,R}$ are found in the cytosol alone, whereas c-FLIP$_L$ localizes in the nucleus, cytoplasm, ER and Mitochondria-Associated Membranes (MAMs) [20–22]. Moreover, whereas the anti-apoptotic role of c-FLIP$_{S,R}$ has been widely demonstrated, c-FLIP$_L$ has been reported to exert the opposite effect on apoptosis regulation; c-FLIP$_L$ has been shown to block Fas-induced Caspase-8 activation when expressed at high levels, but to act as a pro-apoptotic molecule when its expression level is moderate [23–25]. In addition to its involvement in apoptosis modulation, c-FLIP plays important roles in controlling proliferation [26] and cardiac hypertrophy after pressure overload [27]. Furthermore, c-FLIP can activate several pro-survival signalling pathways by regulating proteins such as NF-κB, ERK and AKT [28–30]. More recently, we demonstrated that c-FLIP −/− mouse embryonic fibroblasts (MEFs) display an enlarged ER structure and strong lipid accumulation [22,31]. In the present study, we investigated ER stress response in c-FLIP −/− MEFs. We were surprised to find that c-FLIP ablation protects cells from ER stress-mediated apoptosis because of a reduced induction of UPR signalling. Our findings indicate that c-FLIP plays an important role in PERK and IRE1-mediated ER stress response.

2. Materials and methods

2.1. Cell cultures and reagents

Wild type (WT) and c-FLIP −/− MEFs were obtained and cultured as previously described [22].

Brefeldin A (Bref) (400 ng/ml), Thapsigargin (Tg) (1 μM) and Tunicamycin (Tu) (1 μg/ml) were purchased from Sigma-Aldrich (St Louis, MO, USA).

The following plasmids were used: 294 c-FLIP$_L$, HA-E40K-AKT, HA-AKT-K179M and the corresponding control vectors. Plasmid transfections were performed on subconfluent cells using lipofectamine 2000 reagent (Invitrogen, San Giuliano Milanese, Italy) according to manufacturer's instructions. 24 h after transfection, cells were treated with Tu (1 μg/ml) for 16 h.

The AKT inhibitor VIII (20 μM) was purchased from Calbiochem (San Diego, CA, USA). The vehicle DMSO was used as a control. Cells were photographed on the selected hours of treatment by microscopy (Axioskop 2 plus; Carl Zeiss Microimaging, Inc.). Images were obtained at room temperature using an AxioCamHRC camera (Carl Zeiss Microimaging, Inc., Milan, Italy) by Axiovision 3.1 software and assembled in panels using Photoshop 7.0 (Adobe, Waltham, MA, USA).

2.2. Western blotting

Western blot analysis was carried out as previously described [31]. The sources of the primary antibodies were: anti-BIP/GRP78 from BD Transduction Laboratories™ (USA); anti-β-actin from Sigma-Aldrich (St. Louis, MO); anti-HA, anti-Cleaved Caspase-3, anti-PARP, anti-LC3, anti-CHOP, anti-Phospho-eIF2α Ser51, anti-eIF2α, anti-Phospho-AKT Ser 473 and anti-AKT from Cell Signaling Technology (Danvers, MA); anti-Phospho-IRE1α Ser724 and anti-IRE1α from Novus Biologicals (Littleton, CO); anti-CREB-2 and anti-XBP1s from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (Bio-Rad, Hercules, CA).

2.3. Immunofluorescence

Immunofluorescence was performed as previously described [31]. To analyze the accumulation of LC3 II on autophagic vesicles 24 h after plating, MEFs were treated with Bref (400 ng/ml) for 16 h before being incubated overnight with the rabbit anti-LC3 (Cell Signaling Danvers, MA); secondary goat anti-rabbit IgG-FITC (Sigma-Aldrich St. Louis, MO) was then added to the samples for 1 h. Immunofluorescence was visualized using an AxioCamHRC camera by Axiovision 3.1 software and images were assembled in panels using Photoshop 7.0.

2.4. Apoptosis assay

Apoptosis was evaluated by propidium iodide (PI) staining, as previously described [32].

2.5. Statistical analysis

Values are expressed as mean ± standard error (s.e.m.). The statistical analyses were performed by Student's *t*-test; a value of $P \le 0.05$ and $P \le 0.01$ was considered statistically significant.

3. Results

3.1. c-FLIP −/− MEFs are resistant to ER stress-induced apoptosis

We recently demonstrated that c-FLIP −/− cells display an enlarged ER morphology [22]. Since this morphological modification may be associated with functional alterations [16], we decided to examine the effect of ER stress in WT and c-FLIP −/− MEFs. To this end, we cultured WT and c-FLIP −/− cells in the presence of three distinct drugs that induce ER stress: Thapsigargin (Tg), an inhibitor of the sarcoplasmic reticulum and ER Ca^{2+}-ATPase that reduces ER calcium levels by affecting chaperone activity, Tunicamycin (Tu), which blocks glycosylation of newly synthesized proteins thereby causing misfolding, and Brefeldin A (Bref), which blocks forward transport between the ER and the Golgi apparatus. After 16 h of treatment with each drug, we found that c-FLIP −/− but not WT MEFs were resistant to ER stress-induced cell death, as shown by bright-field microscopic images (Fig. 1A). Interestingly, propidium iodide staining and flow cytometry revealed that a significant number of WT cells, though not of c-FLIP −/− cells, died after induction of ER stress (Fig. 1B). Previous studies have demonstrated that prolonged ER stress activates apoptosis [33]. To determine whether cell death, observed in WT cells, was consequent to an

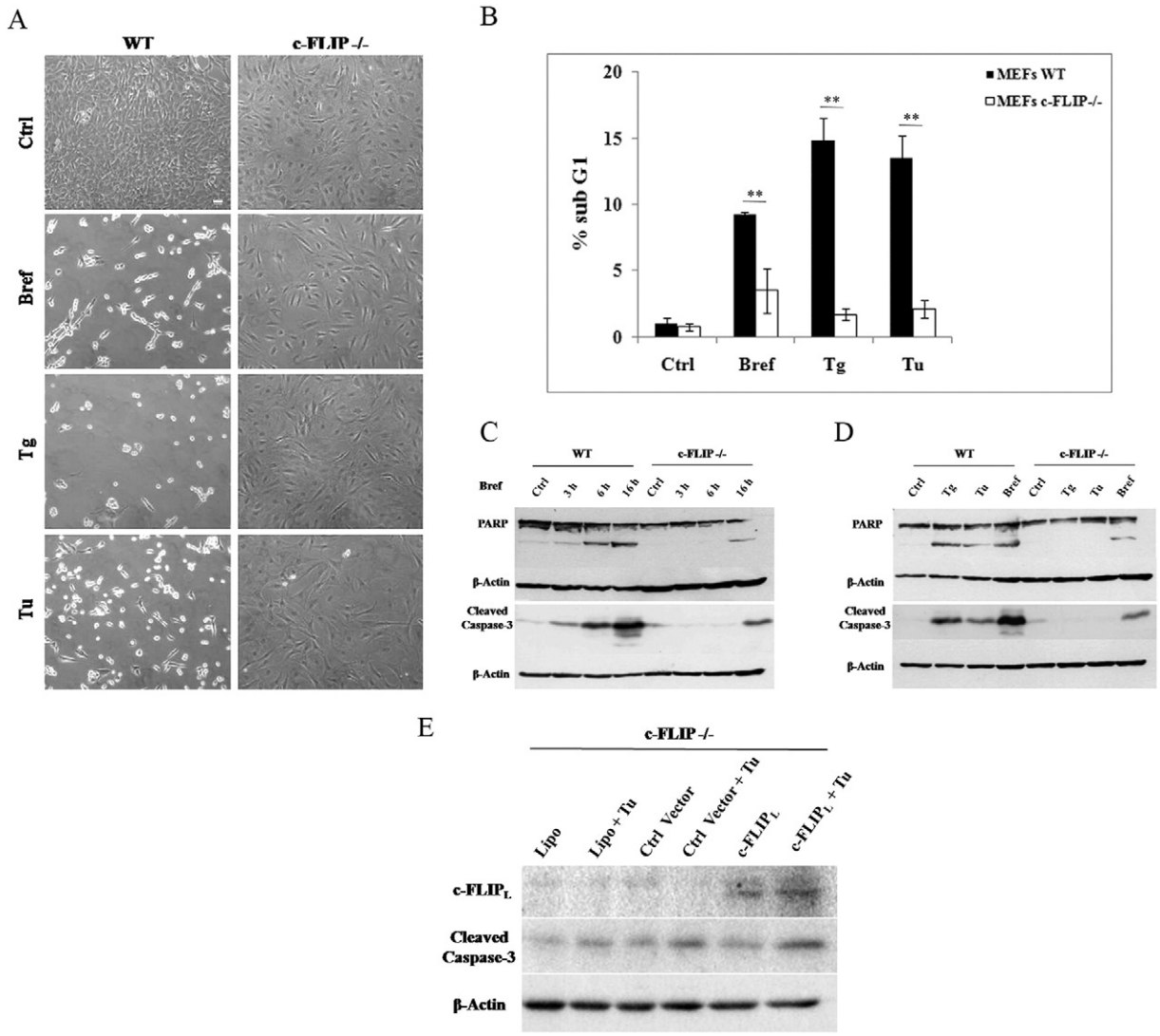

Fig. 1. c-FLIP −/− MEFs are resistant to ER stress-induced apoptosis. (A) WT and c-FLIP −/− MEFs were treated with Brefeldin A (Bref) (400 ng/ml), Thapsigargin (Tg) (1 μM) and Tunicamycin (Tu) (1 μg/ml) for 16 h and cell death was observed by bright-field microscopic images. The images shown are representative of three independent experiments. (B) Cell death was assessed by evaluating the percentage of cells with a subG1 DNA content by flow-cytometry analysis of cells fixed in ethanol and stained with Propidium Iodide. WT and c-FLIP −/− cells were analyzed after incubation with Tg, Tu and Bref for 30 h. The data shown are the mean ± s.e.m. of three independent experiments. **P ≤ 0.01 WT versus c-FLIP MEFs. Upon treatment with Bref for 3, 6 and 16 h (C) and with Tg, Tu and Bref for 16 h (D), WT and c-FLIP −/− cells were lysed, and protein simples were subjected to Western blot analyses to detect cleavage of Caspase-3 and PARP. β-Actin was used as a loading control. Data shown are representative of three independent experiments. E: c-FLIP −/− cells were transfected with a c-FLIP$_L$ vector, a control vector or mock-transfected. 24 h after transfection cells were treated with Tu for 16 h. Lysates were subjected to Western blot analyses to detect c-FLIP$_L$ and Caspase-3 cleavage. β-Actin was used as a loading control. Data shown are representative of three independent experiments.

apoptotic process, Western blot analyses were carried out to assess the cleavage of Caspase 3 and PARP upon treatment with Bref for 3, 6 and 16 h. In keeping with the increased cell death of WT cells, the activation of Caspase-3 and PARP was already evident after 6 h, whereas in c-FLIP −/− cells it was only observed after 16 h of treatment (Fig. 1C). Reduced sensitivity to ER-stress induced apoptosis in c-FLIP −/− MEFs was also observed after treatment with Tg and Tu for 16 h (Fig. 1D). As shown in Fig. 1E, when we transfected c-FLIP −/− MEFs with a c-FLIP$_L$ plasmid we observed higher Caspase-3 cleavage after 16 h Tu treatment as compared to Tu-treated control vector or mock transfected cells (69.6 ± 5 vs 51.1 ± 3.8 or 40.9 ± 2.7 A.U.). This result demonstrates that the specific isoform c-FLIP$_L$ is sufficient to make c-FLIP −/− MEFs more sensitive to ER stress-induced apoptosis. We and others previously showed that TNFα and staurosporine cause apoptosis in c-FLIP −/− cells at levels that are respectively higher than and comparable to those of WT MEFs [22,34]. These data demonstrate that both extrinsic and intrinsic apoptotic pathways are intact in c-FLIP −/− MEFs, thus suggesting that the higher resistance to ER stress observed in c-FLIP −/− cells may be due to differences in how UPR is activated in c-FLIP −/− and WT cells.

3.2. ER stress weakly activates autophagy in WT and c-FLIP −/− cells

It has been demonstrated that ER stress can also induce autophagy [35]. To investigate whether the difference in sensitivity displayed by our cells to ER stress was associated with different autophagy levels, WT and c-FLIP −/− cells were treated with Bref, which was the strongest inducer of cell death even in c-FLIP −/− cells (Fig. 1), and autophagy was assayed at different times by immunodetection of the membrane-bound form of LC3 (LC3 II). Bref caused a comparable increase in LC3 II levels in WT and c-FLIP −/− cells (Fig. 2A). These results are in agreement with the formation of LC3 II punctate spots, which represent the accumulation of LC3 II on autophagic vesicles, observed in both cell lines (Fig. 2B). These data suggest that ER stress weakly activates autophagy in WT and c-FLIP −/− MEFs to a similar extent, thereby

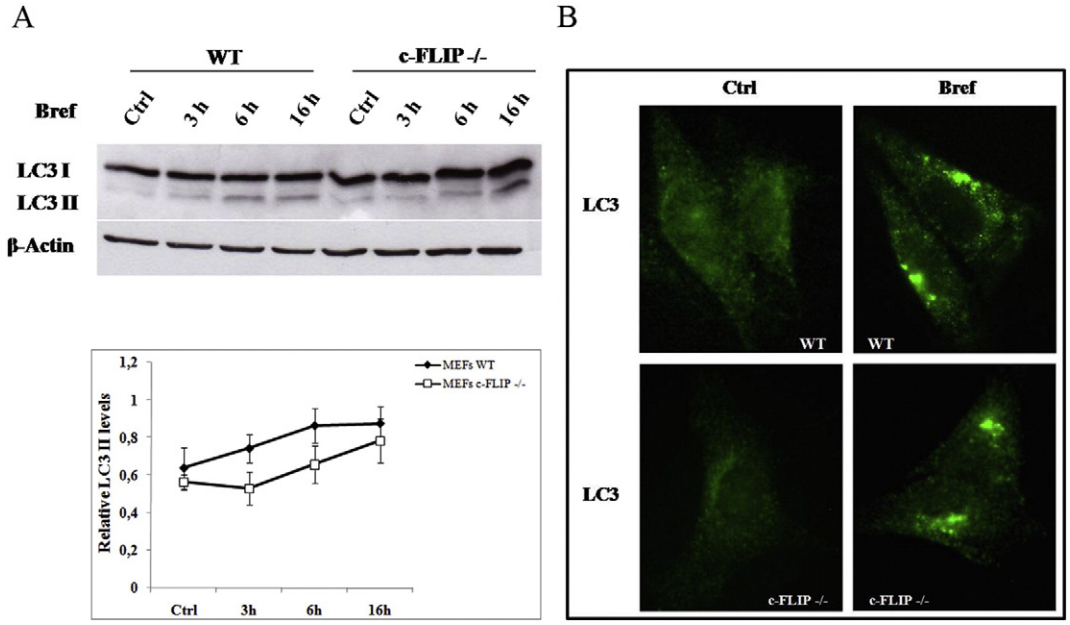

Fig. 2. ER stress activates autophagy to a slight and similar extent in WT and c-FLIP −/− cells. WT and c-FLIP −/− MEFs were treated with Bref for 3 to 16 h and cell lysates were used to analyze LC3 I to LC3 II conversion by Western blotting (upper panel). The graph represents the densitometric values of LC3 II relative to β-actin protein levels induced by Bref (lower panel). The data shown are the mean ± s.e.m. of three independent experiments. (B) Representative images of fluorescence microscopy show LC3 II accumulation in WT and c-FLIP −/− cells treated with Bref for 16 h and stained with anti-LC3 antibody (green). The images shown are representative of three independent experiments. Magnification: 400×.

demonstrating that c-FLIP ablation does not alter the autophagic process as a consequence of ER stress onset.

3.3. c-FLIP −/− cells display compromised PERK and IRE1 signalling upon ER stress induction

To investigate possible differences in UPR activation that might determine the differences in sensitivity to ER stress between WT and c-FLIP −/− cells, we assessed the modulation of key players of the UPR. We first analyzed the expression of one of the main ER chaperones, BIP, which is up-regulated upon ER stress induction. Treatments with Bref, Tg and Tu in both WT and c-FLIP −/− MEFs induced a rapid and comparable increase in BIP expression at the reported time points, thereby demonstrating that these drugs activate an ER stress response in both cell lines (Fig. 3A).

Since we did not find any significant difference in ATF6 activation between WT and c-FLIP −/− MEFs (data not shown), we focused on the PERK and IRE1 branches of the UPR, which have been shown to

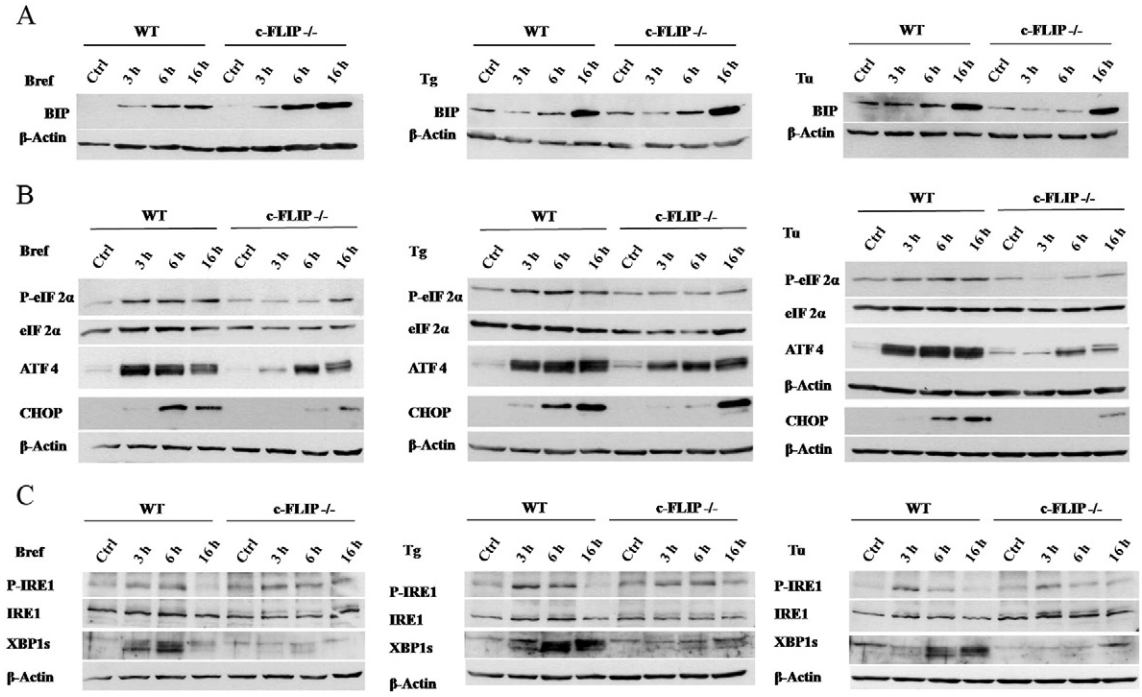

Fig. 3. c-FLIP −/− cells display compromised PERK and IRE1 UPR branch signalling under ER stress induction. WT and c-FLIP −/− MEFs were treated with Bref, Tg and Tu for 3, 6 and 16 h and cell lysates were analyzed by Western blotting to examine BIP (A), P-eIF2α, eIF2α, ATF4 and CHOP (B) and P-IRE1, IRE1 and XBP1s levels (C). β-Actin was used as a loading control. The data shown are representative of three independent experiments.

play a critical role in activating apoptosis upon ER stress [1]. We observed that upon Bref, Tg and Tu treatments P-eIF2α, ATF4 and CHOP induction was compromised in c-FLIP −/− as compared with WT MEFs (Fig. 3B), thus demonstrating that the PERK pathway was less active in c-FLIP −/− cells.

Interestingly, although we observed similar IRE1 phosphorylation in both cell lines after ER stress induction, the expression of its target XBP1s was drastically reduced in c-FLIP −/− MEFs alone (Fig. 3C). These data reveal that c-FLIP ablation dramatically disrupts PERK and IRE1 signalling in response to ER stress, hence suggesting that the resistance to ER stress-induced apoptosis observed in c-FLIP −/− MEFs may be a consequence of these UPR alterations.

3.4. c-FLIP −/− cells display greater AKT activation than WT

As c-FLIP has no catalytic activity, it is unlikely to directly regulate the UPR components, though it may take part in a regulatory complex. C. Quintavalle et al. [30] demonstrated that c-FLIP interacts with AKT and inhibits its ability to bind to and regulate its substrate GSK3β, probably by sequestering AKT and limiting its access to the target protein. Interestingly, we have previously demonstrated that a pool of c-FLIP proteins localizes at ER [22]. There is also a considerable amount of evidence showing that AKT, localized at ER, is phosphorylated in response to ER stress [36], thereby allowing it to promote cell survival [11] and control the PERK branch. Since PERK and IRE1 are ER resident proteins, we hypothesized that the proximity of all these proteins may regulate PERK and IRE1 signalling. To test whether c-FLIP, by binding to AKT, modulates the UPR response, we first analyzed P-AKT induction in WT and c-FLIP −/− cells treated with the three ER stress inducers. As shown in Fig. 4, c-FLIP −/− MEFs displayed higher levels of AKT phosphorylation at serine 473 than WT cells, even in basal conditions. In addition, such AKT phosphorylation in c-FLIP −/− MEFs increased in a time-dependent manner after ER stress induction, decreasing only after 16 h of treatment. Remarkably, c-FLIP −/− cells started dying after 16 h (Fig. 1D).Conversely, no remarkable differences in AKT phosphorylation at threonine 308 and mTOR phosphorylation at serine 2448 were found between WT and c-FLIP −/− MEFs upon Tu treatment (Supplementary figure).

These data reveal that c-FLIP depletion increases AKT activation via phosphorylation at serine 473, and that this increase is even more evident after ER stress onset.

3.5. Activation of AKT is involved in apoptosis resistance in c-FLIP −/− cells upon ER stress induction

To investigate whether the P-AKT upregulation observed in c-FLIP −/− cells correlates with a higher degree of resistance to ER stress-induced apoptosis, we treated c-FLIP −/− MEFs with the AKT inhibitor VIII, a pharmacological inhibitor that targets the pleckstrin homology (PH) domain of AKT, thus completely blocking AKT activation. We assessed the effect of combined AKT inhibitor VIII and Tu treatment on cell viability and apoptotic response. As shown by bright-field microscopic images (Fig. 5A), while single AKT inhibitor VIII or Tu treatment had no effect on cell viability, the combined treatment significantly sensitized c-FLIP −/− cells to ER stress-induced death. Accordingly, we found higher Caspase-3 and PARP activation levels following combined treatment than single Tu or AKT inhibitor VIII treatment (Fig. 5B). To further confirm the role of AKT in modulating the resistance to ER stress-induced apoptosis, we transiently transfected WT MEFs with a plasmid expressing constitutively active AKT (HA-E40K-AKT) and c-FLIP −/− cells with dominant negative AKT (HA-AKT-K179M). Efficient plasmid transfection was revealed by both HA-AKT and P-AKT Western blot experiments, as shown in Fig. 5C and D. Transfected cells were treated with Tu and successively cell death was investigated. As shown in Fig. 5C, WT MEFs became less sensitive to Tu-induced cell death, evaluated by Caspase-3 cleavage, when previously transfected with HA-E40K-AKT plasmid as compared to control vector transfected cells. Conversely, c-FLIP −/− MEFs became more sensitive to Tu-induced cell death, evaluated by Caspase-3 cleavage, when previously transfected with HA-AKT-K179M plasmid as compared to control vector transfected cells (Fig. 5D).

Altogether these results demonstrate that the increased phosphorylation and activation of AKT in c-FLIP ablated cells promotes cell survival in response to ER stress.

3.6. Inhibition of AKT restores PERK but not IRE1 signalling pathway during ER stress

We subsequently assessed whether increased AKT activation was involved in the reduced activation of PERK and IRE1 pathways in c-FLIP −/− MEFs. To this end, we analyzed the expression levels of targets of these proteins upon the combined treatment with AKT inhibitor VIII and Tu. AKT inhibition did not enhance IRE1 activation in c-FLIP −/− cells upon Tu treatment, as demonstrated by comparable XBP1s induction in c-FLIP −/− cells treated with either Tu alone or with Tu plus AKT inhibitor VIII (Fig. 6A). In keeping with this finding, XBP1s expression was also lower in c-FLIP −/− cells that underwent combined treatment than in WT cells treated with Tu (Fig. 6B).

By contrast, when we investigated PERK signalling in c-FLIP −/− cells, we observed significantly higher ATF4 and CHOP expression upon combined treatment with AKT inhibitor VIII and Tu than upon Tu treatment alone (Fig. 6C). To ascertain whether AKT inhibition completely restores the PERK pathway in FLIP −/− cells, we compared the induction of ATF4 and CHOP expression in c-FLIP −/− upon combined treatment with AKT inhibitor VIII and Tu with that of WT cells treated with Tu alone. No differences were detected between the two cell lines in ATF4 and CHOP expression after 6 h of treatment (Fig. 6D), thus demonstrating that AKT inhibition alone is sufficient to restore the activation of the PERK pathway upon ER stress induction to levels comparable to those observed in WT cells. Thus AKT plays a critical role in inhibiting the PERK pathway in c-FLIP −/− cells.

4. Discussion

In this work, we provide evidence of a novel role of c-FLIP protein in the regulation of the UPR signalling pathway and ER stress mediated-apoptosis. For the first time, we demonstrate that c-FLIP −/− MEFs are resistant to apoptosis induced by ER stress and that this resistance correlates with compromised PERK and IRE1 UPR branch signalling. In

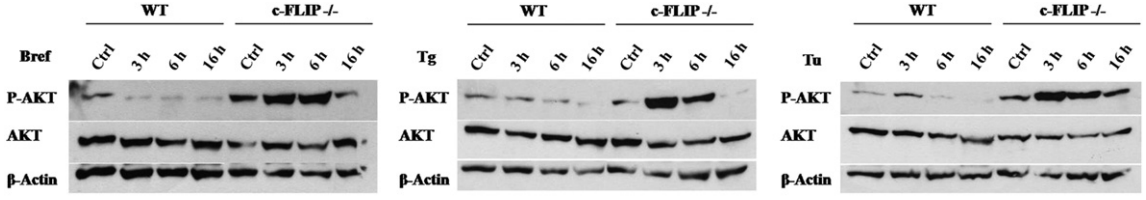

Fig. 4. c-FLIP −/− cells display increased AKT activation compared with WT cells. Western blot analyses for P-AKT at serine 473 and AKT of whole cell lysates obtained from WT and c-FLIP −/− cells treated with Bref, Tg and Tu for 3, 6 and 16 h. β-Actin was used as a loading control. Data shown are representative of three independent experiments.

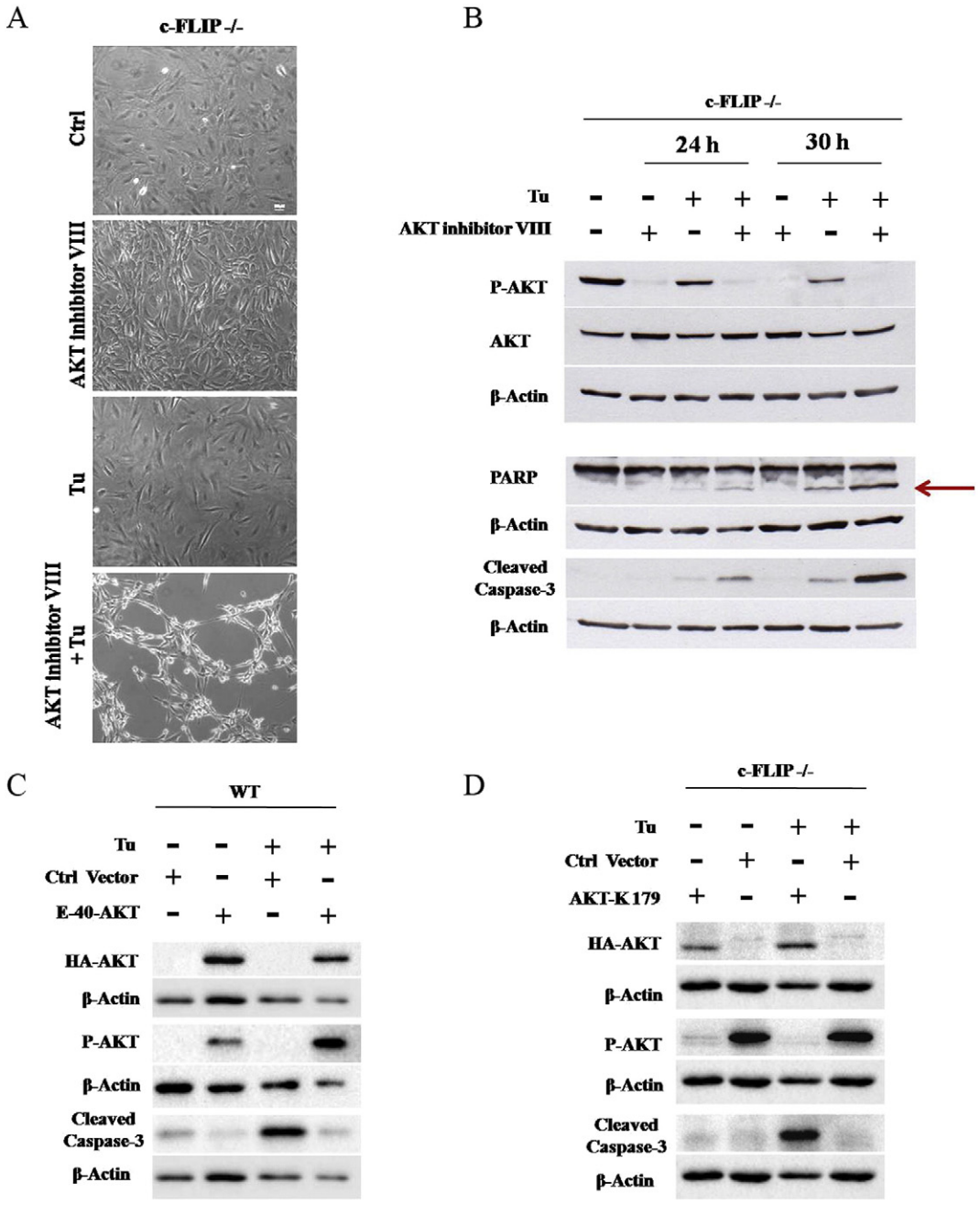

Fig. 5. Activation of AKT is involved in apoptosis resistance in c-FLIP −/− cells upon ER stress induction. (A) c-FLIP −/− MEFs were treated with Tu for 16 h with or without AKT inhibitor VIII (20 μM), added 1 h before Tu, and cell death was observed by bright-field microscopic images. The images shown are representative of three independent experiments. (B) Upon treatment with Tu for 24 and 30 h with or without AKT inhibitor VIII, c-FLIP −/− cells were lysed and protein samples were subjected to Western blot analyses to evaluate P-AKT, AKT, PARP and Cleaved Caspase-3 levels. The arrow indicates cleaved PARP. β-Actin was used as a loading control. The data shown are representative of three independent experiments. (C) WT MEFs were transfected with constitutively active AKT (HA-E40K-AKT) or control vector and 24 h after transfection cells were treated with Tu for 16 h. Lysates were subjected to Western blot analyses to evaluate P-AKT Ser 473, HA-AKT and Cleaved Caspase-3. β-Actin was used as a loading control. The data shown are representative of three independent experiments. (D) c-FLIP −/− MEFs were transfected with dominant negative AKT (HA-AKT-K179M) or control vector and 24 h after transfection cells were treated with Tu for 16 h. Lysates were subjected to Western blot analyses to evaluate P-AKT Ser 473, HA-AKT and Cleaved Caspase-3. β-Actin was used as a loading control. The data shown are representative of three independent experiments.

addition, our results show that PERK branch inhibition in FLIP −/− cells is due to higher AKT activation.

Besides its well-known role as an anti-apoptotic protein, c-FLIP has been shown to be involved in other cellular functions. We recently described its novel role as a modulator of ER morphology and ER-mitochondria crosstalk, showing that c-FLIP$_L$ localizes at ER and MAMs and that c-FLIP −/− MEFs display an enlarged ER structure [22]. Several proteins, localized as c-FLIP at ER and MAMs, are involved in the regulation of ER stress response [10,16,37,38]. These proteins include Mitofusin 2 (Mfn 2), which has been studied extensively. An altered ER morphology in Mfn2 −/− cells modifies the ER stress response. J.P. Munoz et al. [16] demonstrated that Mfn2 ablation causes a dramatic ER expansion, which increases upon ER stress. This morphological alteration modifies UPR activation and apoptosis sensitivity after ER stress induction. Here we demonstrate that c-FLIP, which has previously been shown to alter ER morphology [22], acts as a pro-apoptotic factor in response to ER stress. Indeed, c-FLIP −/− MEFs are more resistant than WT MEFs to apoptosis upon treatment with the three different

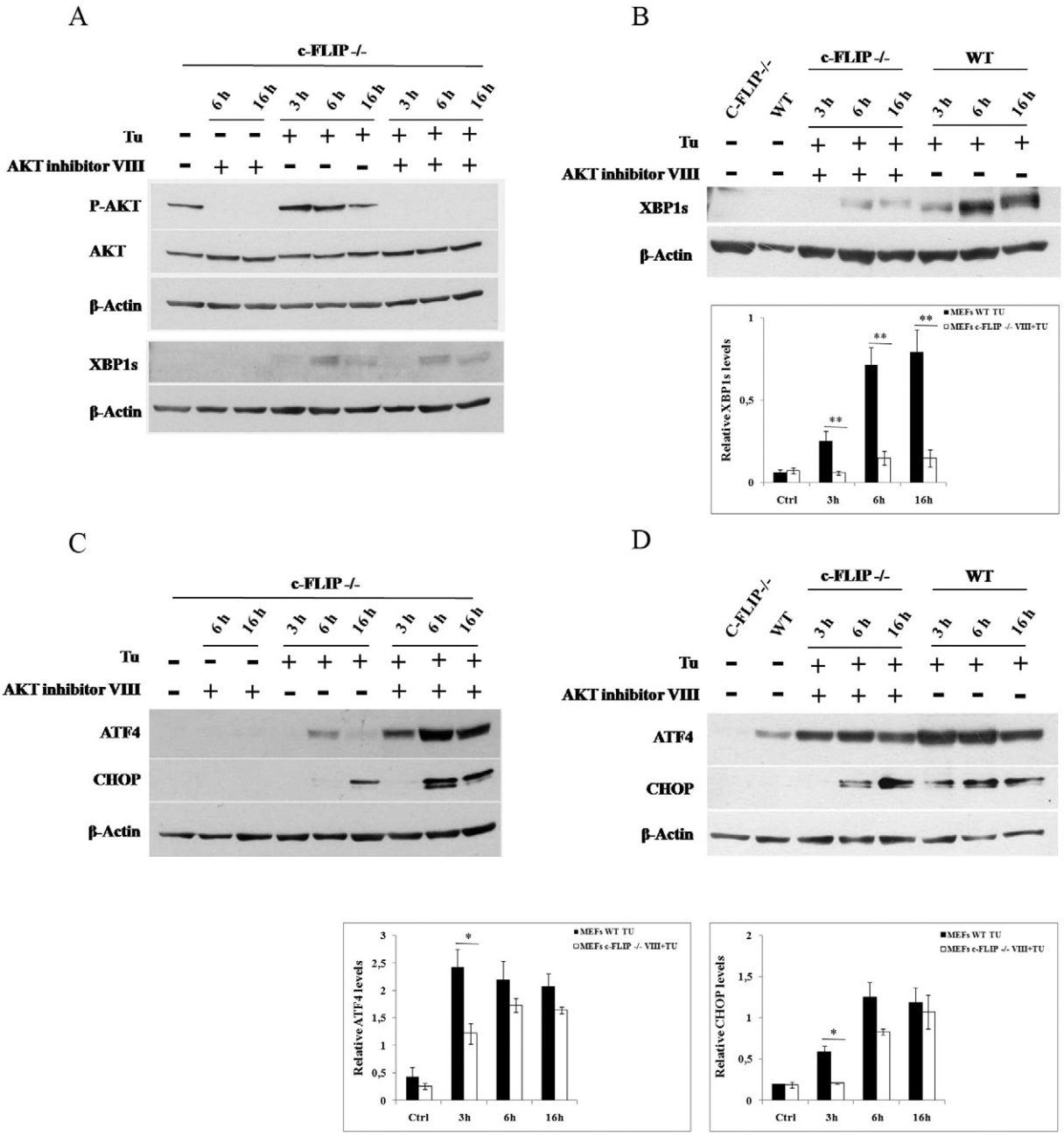

Fig. 6. Inhibition of AKT restores the PERK, but not IRE1, signalling pathway during ER stress. (A) Western blot analyses of P-AKT Ser 473, AKT, XBP1s and of ATF4 and CHOP (C) from whole cell lysates of c-FLIP −/− MEFs treated with Tu for 3, 6 and 16 h with or without AKT inhibitor VIII. β-Actin was used as a loading control. Data shown are representative of three independent experiments. XBP1s (B), ATF4 and CHOP (D) proteins were assessed by Western blotting to compare their expression levels in c-FLIP −/− cells treated with Tu plus AKT inhibitor VIII and WT cells treated with Tu alone for 3, 6 and 16 h (upper panel). The histograms (lower panels) show the densitometric analysis of XBP1s, ATF4 and CHOP levels relative to β-actin in both cell lines. Data shown are the mean ± s.e.m. of three independent experiments. *P ≤ 0.05 WT versus c-FLIP.

drugs that induce ER stress. As it is known that the autophagic process may have a cytoprotective effect upon ER stress induction [35], we analyzed the involvement of autophagy in the resistance that characterizes c-FLIP −/− cells. We observed a comparable time-dependent increase in LC3 II expression and punctate spot formation in both WT and c-FLIP −/− MEFs, thus demonstrating that autophagy levels are similar in both cell lines. This suggests that the reduced ER stress-induced apoptosis in c-FLIP −/− cells is not due to an altered autophagic response. In order to shed light on the molecular mechanism underlying the differences in the sensitivity displayed by WT and c-FLIP −/− MEFs to ER stress, we analyzed the activation of the three UPR branches. Upon treatment with Bref, Tg and Tu, we found no differences in ATF6 activation between WT and c-FLIP −/− MEFs. By contrast, we observed impaired PERK and IRE1 signalling in c-FLIP −/− cells. We hypothesized that the c-FLIP protein ER resident pool is able to regulate these ER resident kinases thanks to their shared cellular localization. Remarkably, the pathway initiated by ATF6, which is the only ER stress sensor that moves from ER to the Golgi apparatus in response to ER stress, is not altered in c-FLIP −/− cells. The pathways triggered by the ER membrane-located PERK and IRE1 are hindered. Since c-FLIP does not display any catalytic activity, we hypothesized that it may interact with a regulator of these sensors. In particular, we focused on AKT, a serine-threonine kinase that prevents apoptosis induced by various stress conditions by interacting with apoptotic modulators such as BAD [39], Caspase-9 [40], FOXO3 [41] and Bcl-w [42]. AKT has previously been shown to interact with c-FLIP$_L$, which inhibits its function and blocks its ability to

activate the substrate GSK3β [30]. Interestingly, several studies point to the involvement of AKT in ER stress response. It has been observed that the activation of ER-located AKT protects primary cultured glial cells [36] and breast, lung and prostate cancer cell lines [11] from ER stress-induced apoptosis. Z. Mounir et al. clarified the molecular mechanism underlying this process, demonstrating that activated AKT impairs PERK autophosphorylation [15]. We found that upon ER stress induction, c-FLIP −/− MEFs displayed higher levels of AKT phosphorylation at serine 473 than WT cells. When we interfered with AKT activity, using the AKT inhibitor VIII, the PERK pathway activation was restored and the resistance to ER stress-induced apoptosis in c-FLIP −/− cells was partially reduced. This demonstrates that the absence of c-FLIP results in AKT activation, which in turn inhibits PERK signalling and promotes cell survival in response to ER stress. C. Quintavalle and colleagues showed that c-FLIP over-expression in HeLa cells did not alter AKT phosphorylation levels in response to insulin but did inhibit the ability of AKT to bind to and phosphorylate GSK3β. In addition to this mechanism of AKT regulation, in the present manuscript we demonstrate that c-FLIP also impairs AKT function by inhibiting its phosphorylation in a different cellular model, i.e. that of MEF cells. It is not possible, on the basis of our data, to conclude that c-FLIP directly inhibits AKT phosphorylation: c-FLIP might interact with other elements that modulate this process. The findings shown in this work are in agreement with our previous reports [26,27], in which we demonstrated that AKT phosphorylation in muscle tissues of c-FLIP transgenic mice is lower than in that of control animals.

It is noteworthy that in the present manuscript we found that pharmacological inhibition of AKT completely rescued the PERK, though not the IRE1, signalling pathway. This suggests that c-FLIP may also act on elements other than AKT to modulate the activation of IRE1 cytosolic targets. Further studies are warranted to shed light on this aspect.

5. Conclusion

In conclusion our work provides evidence indicating that c-FLIP may, besides performing its well-known anti-apoptotic function, also promote apoptosis following ER stress-inducing stimuli. Indeed, we demonstrate that the absence of c-FLIP protects MEFs from cell death in response to ER stress by modifying PERK and IRE1 signalling transduction pathways. We thus show, for the first time, an important novel role of c-FLIP as a modulator of the UPR.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2016.06.003.

Acknowledgments

The authors thank Dr. Tak W. Mak for providing WT and c-FLIP-/- MEFs, Marcus Peter for providing c-FLIPL plasmid and the researchers from the “P. Valdoni” Department of Sapienza University for their technical support. The authors also wish to thank Drs. Sara Palchetti, Annarita Favia and Donatella Starace for their helpful suggestions. We also thank Prof. A. Musarò for providing P-AKT T308 and mTOR antibodies.

This work was supported by PRIN 2010–2011, Fondazione Roma 2013 and 2015 grants, “Ricerca Scientifica Sapienza 2014” and “Ricerca Scientifica Sapienza 2015”to EZ.

The authors declare that they have no conflicts of interest.

References

[1] I. Tabas, D. Ron, Nat. Cell Biol. 13 (2011) 184–190.
[2] P. Walter, D. Ron, Science 334 (2011) 1081–1086.
[3] K. Haze, H. Yoshida, H. Yanagi, T. Yura, K. Mori, Mol. Biol. Cell 10 (1999) 3787–3799.
[4] P. Tsaytler, H.P. Harding, D. Ron, A. Bertolotti, Science 332 (2011) 91–94.
[5] H.P. Harding, Y. Zhang, D. Ron, Nature 397 (1999) 271–274.
[6] S.J. Marciniak, C.Y. Yun, S. Oyadomari, I. Novoa, Y. Zhang, R. Jungreis, K. Nagata, H.P. Harding, D. Ron, Genes Dev. 18 (2004) 3066–3077.
[7] A.L. Shaffer, M. Shapiro-Shelef, N.N. Iwakoshi, A.H. Lee, S.B. Qian, H. Zhao, X. Yu, L. Yang, B.K. Tan, A. Rosenwald, E.M. Hurt, E. Petroulakis, N. Sonenberg, J.W. Yewdell, K. Calame, L.H. Glimcher, L.M. Staudt, Immunity 21 (2004) 81–93.
[8] J.S. Cox, C.E. Shamu, P. Walter, Cell 73 (1993) 1197–1206.
[9] J.J. Credle, J.S. Finer-Moore, F.R. Papa, R.M. Stroud, P. Walter, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 18773–18784.
[10] C. Hetz, P. Bernasconi, J. Fisher, A.H. Lee, M.C. Bassik, B. Antonsson, G.S. Brandt, N.N. Iwakoshi, A. Schinzel, L.H. Glimcher, S.J. Korsmeyer, Science 312 (2006) 572–576.
[11] P. Hu, Z. Han, A.D. Couvillon, J.H. Exton, J. Biol. Chem. 279 (2004) 49420–49429.
[12] Y. Kimata, D. Oikawa, Y. Shimizu, Y. Ishiwata-Kimata, K. Kohno, J. Cell Biol. 167 (2004) 445–456.
[13] K.L. Lipson, S.G. Fonseca, S. Ishigaki, L.X. Nguyen, E. Foss, R. Bortell, A.A. Rossini, F. Urano, Cell Metab. 4 (2006) 245–254.
[14] D. Luo, Y. He, H. Zhang, L. Yu, H. Chen, Z. Xu, S. Tang, F. Urano, W. Min, J. Biol. Chem. 283 (2008) 11905–11912.
[15] Z. Mounir, J.L. Krishnamoorthy, S. Wang, B. Papadopoulou, S. Campbell, W.J. Muller, M. Hatzoglou, A.E. Koromilas, Sci. Signal. 4 (2011) ra62, http://dx.doi.org/10.1126/scisignal.
[16] J.P. Munoz, S. Ivanova, J. Sanchez-Wandelmer, P. Martinez-Cristobal, E. Noguera, A. Sancho, A. Az-Ramos, M.I. Hernandez-Alvarez, D. Sebastian, C. Mauvezin, M. Palacin, A. Zorzano, EMBO J. 32 (2013) 2348–2361.
[17] M. Irmler, M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J.L. Bodmer, M. Schroter, K. Burns, C. Mattmann, D. Rimoldi, L.E. French, J. Tschopp, Nature 388 (1997) 190–195.
[18] C. Scaffidi, I. Schmitz, P.H. Krammer, M.E. Peter, J. Biol. Chem. 274 (1999) 1541–1548.
[19] A. Golks, D. Brenner, C. Fritsch, P.H. Krammer, I.N. Lavrik, J. Biol. Chem. 280 (2005) 14507–14513.
[20] R. Katayama, T. Ishioka, S. Takada, R. Takada, N. Fujita, T. Tsuruo, M. Naito, J. Cell Sci. 123 (2010) 23–28.
[21] J. Zhang, Y. Chen, Q. Huang, W. Cheng, Y. Kang, L. Shu, W. Yin, Z.C. Hua, Int. J. Biochem. Cell Biol. 41 (2009) 1678–1684.
[22] E.S. Marini, C. Giampietri, S. Petrungaro, S. Conti, A. Filippini, L. Scorrano, E. Ziparo, Cell Death Differ. 22 (2015) 1131–1143.
[23] M.E. Peter, Biochem. J. 382 (2004) e1–e3, http://dx.doi.org/10.1042/BJ20041143.
[24] D.W. Chang, Z. Xing, V.L. Capacio, M.E. Peter, X. Yang, EMBO J. 22 (2003) 4132–4142.
[25] C. Giampietri, S. Petrungaro, P. Coluccia, A. D'Alessio, D. Starace, A. Riccioli, F. Padula, S.M. Srinivasula, E. Alnemri, F. Palombi, A. Filippini, E. Ziparo, P. De Cesaris, Cell Death Differ. 10 (2003) 175–184.
[26] C. Giampietri, S. Petrungaro, P. Coluccia, F. Antonangeli, K. Giannakakis, T. Faraggiana, A. Filippini, G. Cossu, E. Ziparo, Cell Death Dis. 1 (2010) e38, http://dx.doi.org/10.1038/cddis.
[27] C. Giampietri, S. Petrungaro, M. Musumeci, P. Coluccia, F. Antonangeli, P. De Cesaris, A. Filippini, G. Marano, E. Ziparo, J. Hypertens. 26 (2008) 1008–1016.
[28] P.M. Chaudhary, M.T. Eby, A. Jasmin, A. Kumar, L. Liu, L. Hood, Oncogene 19 (2000) 4451–4460.
[29] A. Koenig, I.A. Buskiewicz, K.A. Fortner, J.Q. Russell, T. Asaoka, Y.W. He, R. Hakem, J.E. Eriksson, R.C. Budd, J. Biol. Chem. 289 (2014) 1183–1191.
[30] C. Quintavalle, M. Incoronato, L. Puca, M. Acunzo, C. Zanca, G. Romano, M. Garofalo, M. Iaboni, C.M. Croce, G. Condorelli, Cell Death Differ. 17 (2010) 1908–1916.
[31] C. Giampietri, S. Petrungaro, S. Conti, A. Facchiano, A. Filippini, E. Ziparo, Biochim. Biophys. Acta 1851 (2015) 929–936.
[32] C. Giampietri, S. Petrungaro, F. Padula, A. D'Alessio, E.S. Marini, A. Facchiano, A. Filippini, E. Ziparo, Apoptosis 17 (2012) 1210–1222.
[33] C. Hetz, E. Chevet, S.A. Oakes, Nat. Cell Biol. 17 (2015) 829–838.
[34] W.C. Yeh, A. Itie, A.J. Elia, M. Ng, H.B. Shu, A. Wakeham, C. Mirtsos, N. Suzuki, M. Bonnard, D.V. Goeddel, T.W. Mak, Immunity 12 (2000) 633–642.
[35] M. Ogata, S. Hino, A. Saito, K. Morikawa, S. Kondo, S. Kanemoto, T. Murakami, M. Taniguchi, I. Tanii, K. Yoshinaga, S. Shiosaka, J.A. Hammarback, F. Urano, K. Imaizumi, Mol. Cell. Biol. 26 (2006) 9220–9231.
[36] T. Hosoi, K. Hyoda, Y. Okuma, Y. Nomura, K. Ozawa, Brain Res. 1152 (2007) 27–31.
[37] G.A. Ngoh, K.N. Papanicolaou, K. Walsh, J. Biol. Chem. 287 (2012) 20321–20332.
[38] T. Verfaillie, N. Rubio, A.D. Garg, G. Bultynck, R. Rizzuto, J.P. Decuypere, J. Piette, C. Linehan, S. Gupta, A. Samali, P. Agostinis, Cell Death Differ. 19 (2012) 1880–1891.
[39] S.R. Datta, H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, M.E. Greenberg, Cell 91 (1997) 231–241.
[40] M.H. Cardone, N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, J.C. Reed, Science 282 (1998) 1318–1321.
[41] A. Brunet, A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, M.E. Greenberg, Cell 96 (1999) 857–868.
[42] M. Garofalo, C. Quintavalle, C. Zanca, R.A. De, G. Romano, M. Acunzo, L. Puca, M. Incoronato, C.M. Croce, G. Condorelli, PLoS ONE 3 (2008) e4070, http://dx.doi.org/10.1371/journal.pone.0004070.