Retraction

Retraction Notice

***Experimental Biology and Medicine* 2022; X: 1. DOI: 10.1177/15353702221123254**

Zhao P, Xiao XY, Kim AS, Leite MF, Xu JX, Zhu XL, Ren J, Li J. c-Jun inhibits thapsigargin-induced ER stress through up-regulation of DSCR1/Adapt78. *Exp Biol Med (Maywood)* 2008; **233**:1289-1300. https://doi.org/10.3181/0803-RM-84.

The University of Wyoming has asked for a retraction of this article due to apparent data irregularities and image reuse in Figures 1, 3, and 4 that significantly affect the results and conclusions reported in the manuscript. Specifically, the following irregularities were found:

1. Reuse of multiple Western blot bands representing different experimental conditions in Figure 3a. Bands appear identical within CnA and Adapt78.
2. Tubulin loading control blots between Figures 3A and 4C appear to be identical despite representing different experimental conditions, and the bands in Figure 3A appear to be vertically stretched. In addition, tubulin blots appear identical to bands representing eEF2, Figure 2D, in *J. Cell. Mol. Med. Vol 13, No 8A, 2009 pp. 1513–1525*.
3. Tubulin loading control blots between Figure 1B and 4C appear identical and display unreported alterations in contrast and/or brightness.

ISSN 1535-3702

RETRACTED: c-Jun Inhibits Thapsigargin-Induced ER Stress Through Up-Regulation of DSCR1/Adapt78

Peng Zhao,*,† Xiaoyan Xiao,‡ Agnes S. Kim,§ M. Fatima Leite,|| Jinxia Xu,† Xinglei Zhu,* Jun Ren,† and Ji Li†,§,[1]

*Department of Cardiology, Shandong Provincial Hospital, Shandong University, Jinan, Shandong 250021, China; †Department of Pharmaceutical Sciences & Center for Cardiovascular Research and Alternative Medicine, Dept 3375, School of Pharmacy, University of Wyoming, Laramie, Wyoming 82071; ‡Department of Endocrinology, Qilu Hospital, Shandong University, Jinan, Shandong 250012, China; §Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520; and ||Department of Physiology and Biophysics, Universidade Federal de Minas Gerais, CEP 30310-100, Belo Horizonte, Brazil

The endoplasmic reticulum (ER) is exquisitely sensitive to changes in its internal environment. Various conditions, collectively termed "ER stress", can perturb ER function, leading to the activation of a complex response known as the unfolded protein response (UPR). Although c-Jun N-terminal kinase (JNK) activation is nearly always associated with cell death by various stimuli, the functional role of JNK in ER stress-induced cell death remains unclear. JNK regulates gene expression through the phosphorylation and activation of transcription factors, such as c-Jun. Here, we investigated the role of c-Jun in the regulation of ER stress-related genes. c-Jun expression levels determined the response of mouse fibroblasts to ER stress induced by thapsigargin (TG, an inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase). c-jun$^{-/-}$ mouse fibroblast cells were more sensitive to TG-induced cell death compared to wild-type mouse fibroblasts, while reconstitution of c-Jun expression in c-jun$^{-/-}$ cells (c-Jun Re) enhanced resistance to TG-induced cell death. The expression levels of ER chaperones Grp78 and Gadd153 induced by TG were lower in c-Jun Re than in c-jun$^{-/-}$ cells. Moreover, TG treatment significantly increased calcineurin activity in c-jun$^{-/-}$ cells, but not in c-Jun Re cells. In c-Jun Re cells, TG induced the expression of Adapt78, also known as the Down syndrome critical region 1 (DSCR1), which is known to block calcineurin activity. Taken together, our findings suggest that c-Jun, a transcription factor downstream of the JNK signaling pathway, up-regulates Adapt78 expression in response to TG-induced ER stress and contributes to protection against TG-induced cell death. Exp Biol Med 233:1289–1300, 2008

Key words: c-Jun; thapsigargin; ER stress; calcineurin; Adapt78; apoptosis; caspase-12

Introduction

c-Jun was originally identified as the cellular counterpart of a viral oncogenic protein (v-Jun) encoded by a chicken retrovirus (ASV17) (1). The 39-kDa c-Jun protein comprises a C-terminal basic leucine zipper (B-ZIP) DNA binding domain and an N-terminal transcriptional activation domain. c-Jun is the prototype of B-ZIP proteins, which form homo- and hetero-dimeric complexes capable of binding *TPA response elements* (TRE) (consensus 5′-TGAC/GTCA-3′) (2). Dimeric complexes that bind TRE can consist of the Jun, Fos, ATF/CREB, and Maf families of B-ZIP proteins, and these complexes collectively give rise to the generic transcription factor "activator protein 1" (AP-1) (3). Although the composition of AP-1 varies among different cell types, c-Jun is the main component of AP-1 in many cell types (4).

The endoplasmic reticulum (ER) requires a specialized environment to fold, modify, and assemble newly synthesized proteins destined for secretion or insertion into cellular membranes. Molecular chaperones in the ER provide local environments favorable for protein folding. The dominant ER chaperone BiP/GRP78 (binding protein/glucose-regulated 78 kDa protein) plays an important role in the storage

This work was supported in part by grants from National Institute of Health, University of Wyoming Northern Rockies Regional INBRE (5P20RR016474), and the Claude D. Pepper Older American Independence Center at Yale (T30AG21342).

[1] To whom correspondence should be addressed at School of Pharmacy, University of Wyoming, 1000 E. University Ave., Dept 3375, Laramie, WY 82071. E-mail: jli6@uwyo.edu

Received March 11, 2008.
Accepted May 13, 2008.

DOI: 10.3181/0803-RM-84
1535-3702/08/23310-1289$15.00

of ER Ca^{2+} with a stoichiometry of 1–2 moles of Ca^{2+} per mole of BiP/GRP78 (5). BiP/GRP78 also acts as the principal regulator of three ER resident transmembrane proteins which play key roles in the unfolded protein response (UPR): PERK (double-stranded RNA-activated protein kinase-like ER kinase), IRE-1 (inositol requiring-1), and ATF6 (activating transcription factor 6) (6–9). A decrease in ER [Ca^{2+}] results in the dissociation of BiP/GRP78 from PERK and IRE1 resulting in their activation (10, 11). Similarly, the dissociation of BiP/GRP78 from ATF6 leads to its translocation to the Golgi and its subsequent activation (12, 13).

Various pathological conditions and pharmacological reagents that alter Ca^{2+} levels, oxidizing environment, or glycosylation machinery in the ER can trigger the unfolded protein response (UPR) (14–16). Stimuli that perturb ER Ca^{2+} homeostasis are particularly potent elicitors of the UPR. Conditions which trigger ER stress compromise folding reactions and result in protein aggregation (14). Persistent protein aggregation signals apoptotic cell death via the ER resident caspase-12 (17). In addition to eliciting the UPR, perturbations in calcium homeostasis in the ER, a major storage organelle for calcium in the cell, impacts on a variety of calcium-sensitive signal transduction pathways in the cytoplasm and the nucleus (18–20).

During the unfolded protein response, general protein synthesis is attenuated by PERK-mediated eIF-2α phosphorylation in an effort to reduce ER client protein load, and the expression of certain UPR target genes, such as the pro-apoptotic gene *gadd153,* is selectively promoted through up-regulation of the transcription factor ATF4 (21). Signal transduction from the ER to the nucleus is mediated by signal cascades that are similar to those initiated by the plasma membrane. For example, ER stress is coupled to the activation of the JNK signaling pathway (22). In as much as the ER resident transmembrane proteins play a major role in the adaptation of the ER, their activities may also contribute to the broader cellular adaptation to ER stress via the JNK pathway which controls cell viability and proliferation.

Recently, Adapt78 protein has been found to bind to and inhibit intracellular calcineurin, a protein phosphatase that mediates diverse cellular responses to calcium. Calcineurin is involved in numerous cellular functions, including T-cell activation, cytokine gene transcription, apoptosis, neurite extension, long-term memory formation, neurotransmitter release, and myocyte and adipocyte growth and differentiation (23–26). Its abnormal expression is associated with multiple pathologies (27–29). The inhibition of calcineurin by Adapt78 establishes an important functional role for Adapt78.

Here, we investigated the response of three cell lines: c-jun knock-out mouse embryo fibroblasts (c-$jun^{-/-}$), reconstitution of c-Jun expression in c-$jun^{-/-}$ cells (c-Jun Re), and wild-type mouse fibroblast NIH 3T3 (c-$jun^{+/+}$) to thapsigargin (TG, an inhibitor of sarco/endoplasmic reticulum Ca^{2+}/ATPase) which induces ER stress. The results demonstrate that TG treatment leads to the activation of caspase-12 and apoptosis in c-$jun^{-/-}$ cells, while c-Jun Re cells are relatively resistant to TG-induced ER stress. The up-regulation of Adapt78, which blocks the activity of calcineurin, in c-Jun Re cells may underlie their resistance to TG-induced ER stress.

Materials and Methods

Plasmid Constructs. The segment of intron 3 from human MCIP1 (DSCR1) gene was isolated by polymerase chain reaction (PCR) using human genomic DNA as template and primers based on sequence information from human chromosome 21 data bank (30). This ~900-bp fragment was subcloned into a pGL3 luciferase reporter vector (gift from Dr. B. Rothermel (31)). Constitutively active forms of calcineurin A (pCMV-CnA) have been described elsewhere (32). Expression vector for hemagglutinin (HA) epitope-tagged wild-type c-Jun (pSRα-HA-c-Jun) was a gift from Dr. F. Claret (33).

Tissue Culture, Cell Transfection, and Reporter Gene Assays. Mouse fibroblast cells were grown and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Reporter gene assays were performed on fibroblast cells plated on 60 mm culture dishes transfected with 8 μg of each plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were harvested ~40 h after transfection in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay System (Promega) (34). β-galactosidase activity was measured using the Galacto-light system (Tropix, Inc.) and used to normalize the reporter assay results (32). All transfection data are presented as means ± SE for two or three separate transfection experiments, each of which was done in triplicate.

Calcineurin Activity Assays. Calcineurin assays were performed according to the manufacturer's instructions (Promega, Madison, WI) as described (35). Briefly, cells were homogenized in lysis buffer (50 mM Tris, pH 7.4/5 mM ascorbic acid/protease inhibitors, Roche, IN) followed by further disruption by sonication. Lysates were then centrifuged at 21,000 *g* at 4°C for 30 min to remove particulate matter. Free phosphate was removed by passing the lysate through Sephadex® G25 spin column. Calcineurin activity was measured by using the Ser/Thr phosphopeptide. The amount of free phosphate released was measured with molybdate dye solution. Calcineurin-specific phosphatase activity was measured as the amount inhibited by cyclosporin A. Western blots to detect calcineurin protein content were performed on the above lysates using an antibody specific for calcineurin (BD Biosciences, Palo Alto, CA).

Cell Culture, Treatments, and Viability Assay. c-jun knock-out mouse embryo fibroblast (c-$jun^{-/-}$) and reconstitution of c-Jun expression in c-$jun^{-/-}$ cells (c-Jun Re) were obtained from Dr. Michael Karin's lab (36), and wild-type cells (c-$jun^{+/+}$) from American Type Culture

Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone). To assess the cells' sensitivity to thapsigargin (TG), cell viability was evaluated by trypan blue dye exclusion (22). Briefly, cell suspensions were mixed with an equal volume of 0.4% trypan blue, and the percentage of viable cells (those excluding trypan blue) relative to the total number of cells present was determined using a hemacytometer.

Analysis of Apoptosis by Fluorescence Activated Cell Sorting (FACS) and DNA Staining. Mouse fibroblast cells were harvested from 60-mm tissue culture dishes 24 h after treatment with TG. Supernatants were collected in 15 ml conical tubes, and adherent cells were loosened by incubation in 0.05% trypsin for 10 min, followed by inactivation with serum. Both components were combined and centrifuged at 2,500 rpm for 5 min. Pellets were resuspended, fixed for 30 min in 4 ml 1% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C, centrifuged again, and resuspended in 1 ml PBS. Cells were counted with a hemacytometer, and for each subsequent step, an exact volume of 1 ml of solution per 1×10^6 cells was used. Cells were permeabilized in 0.1% Triton X-100 in PBS for 3 min at 4°C. After another centrifugation, the cells were resuspended in a DNA staining solution (50 mg/ml PI) for 30 min. Cell-cycle analysis was performed at the Yale Analytical Cytology Core Facility on a Becton–Dickinson cell counter (FACScan). Apoptotic cells appeared as a hypodiploid peak due to nuclear fragmentation and loss of DNA.

For detection of apoptosis by DNA staining with DAPI, cells were washed with phosphate-buffered saline (PBS) and harvested with trypsin–EDTA. Cells (50,000) were resuspended in 50 μl PBS, and 100 μl of 22% bovine serum albumin (BSA) was added to the sample. This suspension was added to the bottom of a cytofunnel mounted with a microscope slide into the cyto-centrifuge and spun at 500 rpm for 5 min. Slides were air-dried for 30 min at room temperature and washed with PBS. DAPI (200 μl solution containing 2.5 μg/ml in PBS) was applied for 30 min at room temperature. After washing with PBS, samples were stored in the dark at 4°C and viewed under the fluorescence microscope.

Antibodies and Immunoblotting. Polyclonal anti-c-Jun antibody and polyclonal anti-phospho-c-Jun (Ser73) antibody were purchased from Cell Signaling (Beverly, MA). Polyclonal anti-phospho-JNK antibody was from Promega (Madison, WI). Polyclonal anti-JNK antibody, polyclonal anti-Grp78 antibody, polyclonal anti-Gadd153 antibody, and polyclonal anti-αtubulin antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-calcineurin antibody was from BD Biosciences (Palo Alto, CA). Monoclonal anti-caspase-12 antibody was a kind gift from Dr. J. Yuan (17). Polyclonal anti-Adapt78 antibody was obtained from Dr. K. J. Davies (18). Immunoblotting was performed as described previously (32). Briefly, mouse fibroblast cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (1% Triton X-100, 20 mM HEPES (pH 7.4), 50 mM β-glycerol phosphate, 2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, and protease inhibitor cocktail). The cell lysates were clarified by centrifugation at 14,000 rpm for 10 min. Samples normalized for total protein content were resolved by electrophoresis through either straight or gradient NuPAGE Bis–Tris gels (10 or 4–12%) (Invitrogen, Carlsbad, CA) and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ) was used for the detection of the immunoreactive bands. For reprobing, blots were stripped with a buffer containing 50 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 0.1 M β-mercaptoethanol.

Northern Blot Analysis. Total RNA was isolated with STAT-60 (Tel-Test B, Friendwood, TX). Northern blot analysis was performed as described (22). Hybridizations were carried out using radiolabeled cDNA probes specific for *adapt78* (kind gift from Dr. D. R. Crawford (37)).

Cytosolic Ca^{2+} Measurements. Intracellular Ca^{2+} changes were evaluated in c-jun$^{-/-}$ and c-Jun Re cells using time-lapse confocal microscopy (38). Cells were plated onto glass coverslips and confocal imaging was performed 48 h later. Briefly, cells were loaded with 6 μM fluo-4/AM (fluo-4 acetomethyl ester; Molecular Probes) for 30 min at 37°C. Coverslips containing the cells were transferred to a custom-built perfusion chamber on the stage of a Bio-Rad MRC-1024 microscope (Hercules, CA) and observed using a 63×, 1.4N.A. objective. The 488 nm line of a krypton/argon laser was used to excite the dye, and emission signals between 505 and 550 nm were collected. Cells were stimulated by perfusion with TG and were observed at a rate of 5 frames/sec. Changes in fluorescence intensity were calculated by dividing the measured fluorescence intensity during application of TG by the measured average baseline fluorescence intensity (F0). In order to determine intracellular Ca^{2+} concentration, cells were loaded with 5 μM fura-2/AM for 1 h at 37°C (39, 40). Cell suspensions loaded with fura-2 were then transferred to a cuvette to monitor changes in the baseline of fura-2 fluorescence ratio using a Hitachi F-2000 spectrofluorometer (Danbury, CT).

Staining of the Endoplasmic Reticulum. Cells were loaded with 100 nM of ER-Tracker dye (Invitrogen, Carlsbad, CA) for 30 min. Coverslips containing the cells were transferred to a custom-built perfusion chamber on the stage of a BioRad MRC-1024 confocal microscope (Hercules, CA) equipped with a Spectra-Physics Ti:Sapphire laser and a Millenia X pump laser (Mountain View, CA) for two photon excitation, and observed using a 63×, 1.4 N.A. objective lens. ER-Tracker was excited at 790 nm by two photon excitation and fluorescence observed at 500–540 nm using custom-built external detectors (38).

Statistical Analysis. All data are reported as means

± SE. The number of experiments in each group is presented in the figure legend. Data were analyzed by two-tailed, unpaired Student's t test. Differences were considered significant when $P < 0.05$.

Results

c-Jun Expression Alters the Sensitivity of the Cell to ER Stress. A protective role for c-Jun in the cellular response to DNA damage has previously been reported (41). To investigate the importance of c-Jun in the adaptive response of the cell to ER stress, three cell lines were compared for their sensitivity to a classic ER stress-inducing agent, thapsigargin (TG): c-jun knock-out mouse embryo fibroblasts (c-jun$^{-/-}$), c-jun$^{-/-}$ cells in which c-Jun expression is restored and expressed at high levels constitutively (c-Jun Re), and wild-type mouse fibroblast NIH 3T3 (c-jun$^{+/+}$) obtained from ATCC. TG, an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity, results in major perturbations in calcium homeostasis. The three cell lines exhibited dramatic differences in sensitivity to TG (Fig. 1A). c-jun$^{-/-}$ cells were the most sensitive, while c-Jun Re cells were relatively resistant, to TG-induced cell death.

The ER participates in the initiation of apoptosis by at least two different mechanisms: UPR and Ca^{2+} signaling (14, 42). An ER-associated caspase, caspase-12, is implicated in the local regulation of apoptosis (17). TG treatment led to the activation of caspase-12, as indicated by the cleavage of procaspase-12, in c-jun$^{-/-}$ cells in a time-dependent manner (Fig. 1B and 1C). In contrast, caspase-12 activation was significantly attenuated in wild-type fibroblast cells and abrogated in c-Jun Re cells in response to TG treatment (Fig. 1B and 1C).

The Protective Role of c-Jun in the Cellular Response to ER Stress. As depicted by our data, c-jun$^{-/-}$ cells exhibited a large degree of apoptosis after treatment with TG, while c-Jun Re cells demonstrated relative resistance to TG-induced apoptosis (Fig. 2A and 2B). To determine whether TG caused a different degree of ER stress in c-jun$^{-/-}$ versus c-Jun Re cells, we examined the expression of two markers of ER stress, the ER resident chaperones Grp78 and Gadd153. Interestingly, c-Jun Re cells had dramatically lower Grp78 and Gadd153 expression in response to TG treatment compared to c-jun$^{-/-}$ cells (Fig. 2C). These results indicate that constitutive reexpression of c-Jun can increase the cell's tolerance to TG-induced ER stress.

Thapsigargin's principal mechanism of action is inhibition of SERCA activity (43), which causes calcium to leak out of the ER into the cytoplasm. The rise in free intracellular [calcium] activates calcium/calmodulin-dependent signaling molecules, including the protein phosphatase calcineurin (44). The activity of calcineurin is modified by a protein recently identified by Ermak *et al.* (18), which is encoded by DSCR1 (Down syndrome candidate region 1). This protein known as calsipressin 1 (or Adapt78) can bind to calcineurin and inhibit its activity (18). Adapt78 has been demonstrated to protect cells against oxidative stress by down-regulating the activity of calcineurin. Because TG perturbs calcium homeostasis in a similar manner as oxidative stress, we sought to determine whether Adapt78 expression is associated with greater protection against TG-induced cell death. Intriguingly, Adapt78 expression was up-regulated in response to TG treatment in c-Jun Re cells but not in c-jun$^{-/-}$ cells (Fig. 2C).

Calcineurin Is Involved in TG-induced Apoptosis. Calcineurin is a heterodimer composed of a catalytic A subunit (CnA) and a calcium-binding regulatory B subunit (CnB). Calcineurin activation induces expression of *adapt78* transcripts (31), and the induction is conferred by a cluster of 15 NFAT binding sites immediately upstream of exon 4 (31). Exogenous expression of calcineurin via pCMV-CnA led to increased Adapt78 expression in both c-jun$^{-/-}$ and c-Jun Re cells (Fig. 3A), although TG treatment induced Adapt78 expression only in c-Jun Re cells. Interestingly, Adapt78 expression induced by pCMV-CnA in c-jun$^{-/-}$ cells was associated with protection against TG-induced apoptosis (Fig. 3A and 3B).

Calcineurin has been implicated in the signal transduction pathway leading to Ca^{2+}-dependent apoptosis (44). Therefore, we examined the level of calcineurin activity in our model system. We observed a higher level of calcineurin activity in c-jun$^{-/-}$ cells compared with c-Jun Re cells (Fig. 4A), and TG treatment led to significantly increased calcineurin activity in c-jun$^{-/-}$ cells but not in c-Jun Re cells (Fig. 4A). The changes in calcineurin activity were not due to changes in calcineurin protein levels (Fig. 4B). These findings suggest that Adapt78 is an endogenous negative feedback regulator of calcineurin, a key mediator of TG-induced apoptosis. Adapt78 is a target of positive transcriptional regulation by calcineurin probably as a direct consequence of NFAT binding to a calcineurin-responsive region in proximity to exon 4 of the *adapt78* gene (31). We observed that there are 5 consensus binding sites for AP-1 transcription factors in the nucleotide sequence flanking exon 4 (Fig. 5A), which may contribute to the increase in Adapt78 expression in response to TG treatment.

The calcineurin specific inhibitor, cyclosporin A (CsA), attenuated TG-induced caspase-12 activation in c-jun$^{-/-}$ cells (Fig. 4C). Interestingly, CsA also blocked TG-induced Adapt78 expression in c-Jun Re cells (Fig. 4C). These results indicate that TG-induced calcineurin activation contribute to caspase-12 activation in c-jun$^{-/-}$ cells. The up-regulation of Adapt78 expression in response to TG treatment in c-Jun Re cells is also related to calcineurin activity, because CsA blocked TG-induced Adapt78 expression in c-Jun Re cells as well (Fig. 4C).

Transcriptional Induction of *adapt78* in c-jun$^{-/-}$ and c-Jun Re Cells. *adapt78* mRNA expression in c-jun$^{-/-}$ and c-Jun Re cells following treatment with TG was

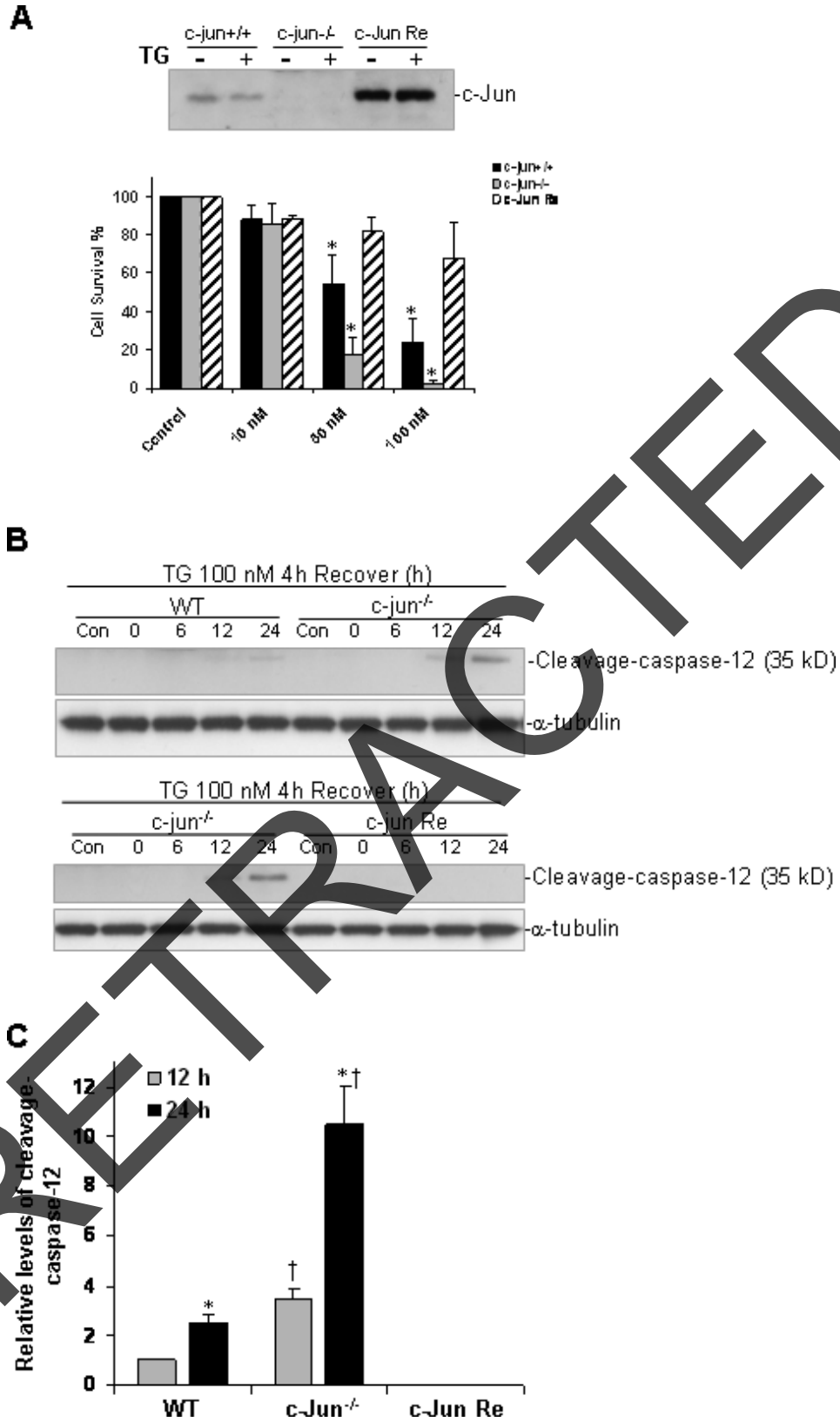

Figure 1. c-Jun protects against cell death caused by TG-induced ER stress. (A) Wild-type (c-jun$^{+/+}$), c-jun$^{-/-}$ and c-Jun Re cells were treated with the indicated concentrations of thapsigargin (TG) for 4 h. They were then placed in fresh medium lacking TG and allowed to incubate for an additional 24 h, after which cell viability was assessed using trypan blue exclusion as described in Experimental Procedures. Results shown are mean ± SE from four independent experiments. * $P < 0.05$ vs. controls. (B) Cells were treated with 100 nM TG for 4 h, then incubated in fresh medium. After the indicated time interval, cells were lysed as described in Experimental Procedures for immunoblotting with the specific caspase-12 antibody. (C) The bar graph quantifies the relative cleavage caspase-12 levels expressed as means ± SE for three independent experiments. * $P < 0.05$ vs.12 h group; † $P < 0.05$ vs. WT 12 h.

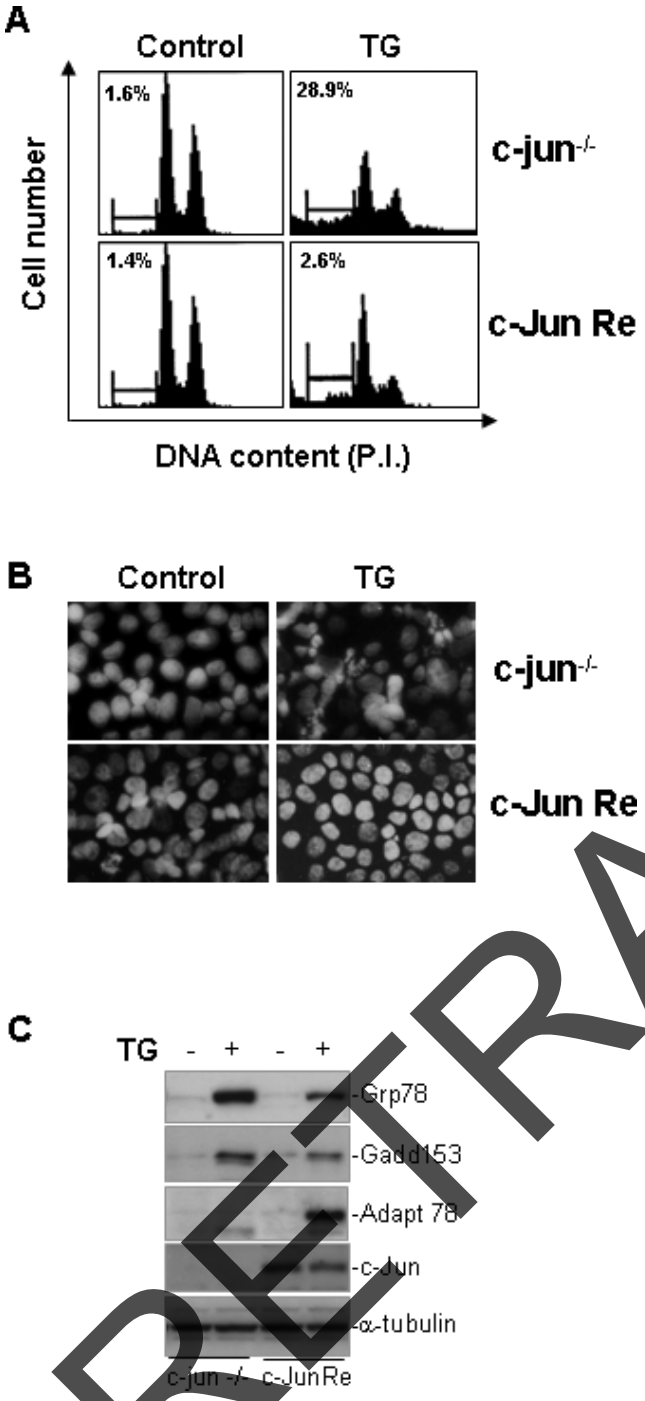

Figure 2. c-jun$^{-/-}$ cells challenged with TG have greater apoptosis and loss of up-regulation of Adapt78. Extent of apoptosis was detected by analysis of DNA content by FACS of fixed, propidium iodide-stained mouse fibroblast cells (A) and DNA staining with DAPI (B) in cells treated with TG (100 nM) for 4 h, followed by an additional 24 h growth in fresh medium. Note the presence of a population of cells with hypodiploid DNA content typical of apoptosis (A). (C) c-jun$^{-/-}$ and c-Jun Re cells were treated with TG (100 nM) for 4 h, then incubated in fresh medium for additional 24 h, then immunoblotted with specific antibodies recognizing Grp78, Gadd153, Adapt78, c-Jun or tubulin.

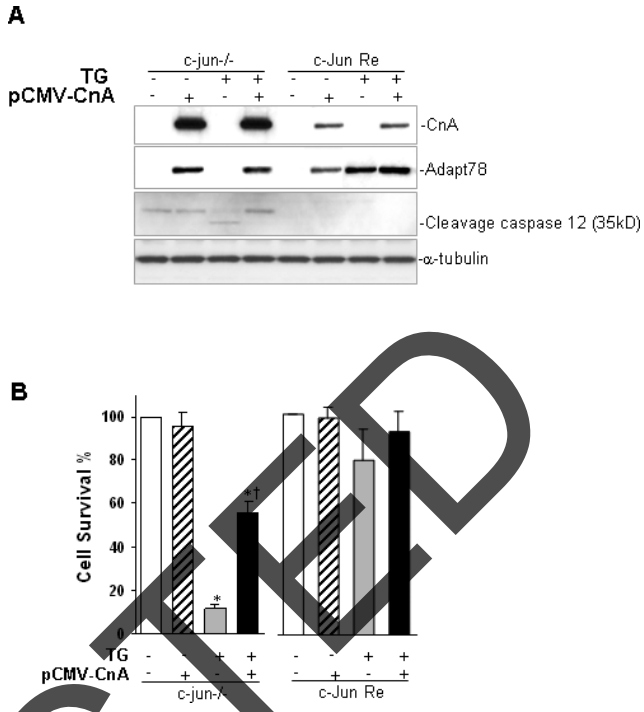

Figure 3. Up-regulation of Adapt78 in c-jun$^{-/-}$ cells by introduction of a constitutively active form of calcineurin inhibits TG-induced cell death. (A) Presence of a constitutively active calcineurin up-regulates Adapt78 expression and inhibits caspase-12 cleavage caused by TG (4 h treatment followed by incubation in fresh medium for 24 h). (B) Control cells or cells transfected with pCMV-CnA were treated with TG (100 nM) for 4 h. They were placed in fresh medium lacking TG and allowed to incubate for an additional 24 h, after which cell viability was assessed by trypan blue exclusion as described in Experimental Procedures. Results shown are means ± SE for four independent experiments. * $P < 0.05$ vs. untreated controls; † $P < 0.05$ vs. TG treatment alone.

investigated by Northern blotting. While basal *adapt78* mRNA levels were very low in both c-jun$^{-/-}$ cells and c-Jun Re cells, TG treatment resulted in a potent increase in *adapt78* mRNA abundance in c-Jun Re cells but not in c-jun$^{-/-}$ cells (Fig. 5B). The induction of *adapt78* mRNA by TG was consistent with the up-regulation of Adapt78 protein expression by TG (Fig. 2C).

To examine whether *adapt78* induction was mediated through enhanced transcription, a luciferase reporter construct (25), which was generated using a human *adapt78* genomic DNA fragment spanning nucleotides –874 to +30 relative to the first nucleotide of exon 4, was utilized (Fig. 5A and 5C). This reporter was transiently transfected into c-jun$^{-/-}$ and c-Jun Re cells, and luciferase activity was assayed after treatment of cells with TG. As shown in Figure 5C, no activation of the promoter occurred in c-jun$^{-/-}$ cells. In contrast, a dramatic increase in *adapt78* promoter activity was seen following TG treatment of c-Jun Re cells.

Because the promoter region (–874 to +30) of exon 4 was found to contain a remarkably dense cluster of consensus AP-1 binding motifs (TGAC/GTCA), we examined c-Jun N-terminal protein kinase (JNK) signaling pathway in response to TG treatment. TG treatment leads

to strong JNK activation in c-Jun Re cells but not in c-jun$^{-/-}$ cells (Fig. 5D). Furthermore, phosphorylation of c-Jun, one target of JNK, increased in response to TG treatment in c-Jun Re cells (Fig. 5D).

Calcium Signal Levels in c-jun$^{-/-}$ and c-Jun Re Cells. Thapsigargin is an inhibitor of SERCA (43), which causes calcium release from the ER into the cytoplasm, leading to perturbations in calcium homeostasis and inducing ER stress. Calcium signaling is likely an important factor contributing to TG-induced ER stress. To investigate the cytosolic [calcium] changes of c-jun$^{-/-}$ and c-Jun Re cells in response to TG stimulation, we used time-lapse confocal microscopy to determine the calcium signal (38). We found that TG triggers a much stronger intracellular calcium signal in c-Jun Re cells than in c-jun$^{-/-}$ cells relative to their respective baseline (Fig. 6A). However, c-jun$^{-/-}$ cells seemed to load 'bethel' higher, so we used spectrofluorometry to measure the basal intracellular free calcium of c-jun$^{-/-}$ and c-Jun Re cells. Intriguingly, the basal intracellular [calcium] of c-jun$^{-/-}$ cells was indeed higher than that of c-Jun Re cells by about 2.5-fold (Fig. 6B), suggesting that some calcium has already entered in the cytoplasm in c-jun$^{-/-}$ cells. Because the relatively higher intracellular [calcium] could induce ER structural changes (45), we used the ER-tracker to examine the ER structure of c-jun$^{-/-}$ and c-Jun Re cells and found different patterns in the ER structure between c-jun$^{-/-}$ and c-Jun Re cells (Fig. 6C).

Exogenous Expression of c-Jun in c-jun$^{-/-}$ Cells Restores TG-Induced Adapt78 Expression and Inhibits TG-Induced Cell Death. To further confirm the role of c-Jun in regulating Adapt78 expression in response to ER stress, we transfected the SRα-HA-c-Jun construct into c-jun$^{-/-}$ cells to determine the effect of exogenous c-Jun on sensitivity to TG treatment and Adapt78 expression levels. Interestingly, overexpression of exogenous c-Jun in the c-jun$^{-/-}$ cells caused both an autophosphorylation of c-Jun in the target cells (Fig. 7A) and induced up-regulation of Adapt78 expression (Fig. 7B). Moreover, the exogenous expression of c-Jun recovered thapsigargin-induced Adapt78 up-regulation (Fig. 7A) and inhibited TG-induced cell death (Fig. 7C).

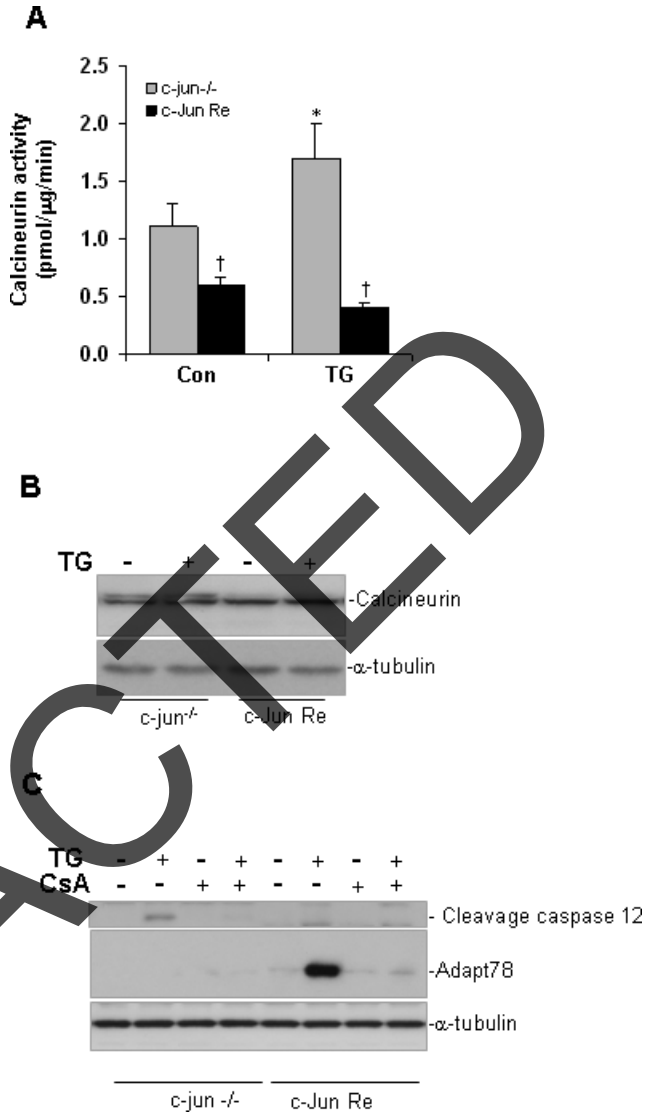

Figure 4. Calcineurin activation induced by TG contributes to apoptosis in c-jun$^{-/-}$ cells. (A) TG treatment induces activation of calcineurin in c-jun$^{-/-}$ cells, but not in c-Jun Re cells. Calcineurin activity was assayed according to the manufacturer's instruction as described in Experimental Procedures. * $P < 0.05$ vs. untreated control; † $P < 0.05$ vs. c-jun$^{-/-}$. (B) TG treatment does not affect calcineurin protein expression in either c-jun$^{-/-}$ or c-Jun Re cells. (C) Calcineurin inhibitor cyclosporin A (CsA) blocks TG-induced apoptosis in c-jun$^{-/-}$ cells. Calcineurin inhibition with CsA abrogates TG-stimulated Adapt78 expression in c-Jun Re cells. c-jun$^{-/-}$ and c-Jun Re cells were treated with TG (100 nM) for 4 h and incubated for an additional 24 h in fresh medium. The cells were then lysed, and extracted proteins were immunoblotted for calcineurin or tubulin (B), and caspase-12 or Adapt78 (C).

Discussion

The unfolded protein response (UPR) stimulates the expression of ER resident chaperones to adapt to the increased demand for protein folding in the ER. The ER plays a central role in the function and viability of the cell, and perturbations in ER function impact on a diverse array of important cellular functions. Underlying the general cellular response to ER stress are stress-activated kinase pathways, including the JNK signaling pathway. c-Jun, a downstream target of JNK, is expressed in many different cell types at low levels, and its expression is enhanced in response to many stimuli including PMA, growth factors, UV irradiation and cytokines (46). The DNA binding and transcriptional activities of c-Jun are regulated by several mechanisms. c-Jun interacts with numerous other transcription factors such as the NFAT (nuclear factor of activated T-cells) family of proteins (47). The NFAT proteins are critical in the regulation of cytokines and other immune response genes. Many of these genes contain composite NFAT-AP-1 binding sites in their regulatory regions to which Jun/Fos dimer and NFAT bind coopera-

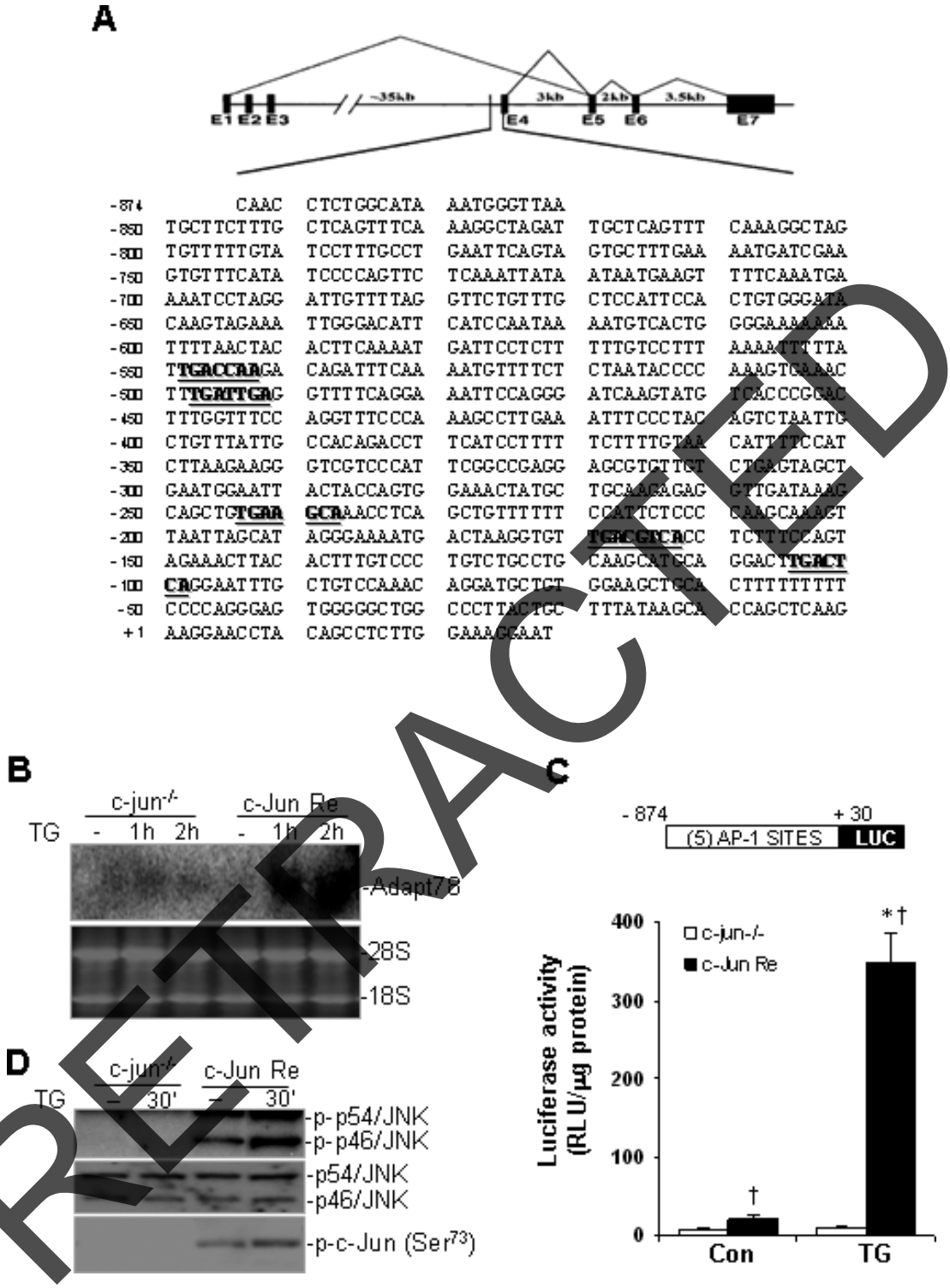

Figure 5. An intragenic calcineurin response element from the adapt78 gene. (A) Schematic representation of the organization of the human adapt78 (DSCR1) gene, indicating four alternative initial exons (E1 through E4) and three exons common to all forms of adapt78 mRNA (E5 through E7) (58). The nucleotide sequence proximal to exon 4 contains five consensus binding sites for AP-1 transcription factors. The first nucleotide of exon 4 is designated as +1. (B) Total RNA was extracted from c-jun$^{-/-}$ and c-Jun Re cells, and Northern blot analysis was performed to assess adapt78 expression in response to TG treatment. (C) Transient transfection assays of adapt78-luciferase reporter plasmids in c-jun$^{-/-}$ and c-Jun Re cells. adapt78-luciferase reporter plasmids were constructed to link-defined genomic segments proximal to exon 4 of the human adapt78 gene to luciferase reporter gene. Values are expressed in relative light units (RLU) and represent means ± SE for three independent transfections of c-jun$^{-/-}$ or c-Jun Re cells. Results were corrected for variation in transfection efficiency by normalizing to the expression of a cotransfected pSV-β-galactosidase plasmid. * $P < 0.01$ vs. control; † $P < 0.01$ vs. c-jun$^{-/-}$. (D) TG treatment induces phosphorylation of JNK and c-Jun in c-Jun Re cells but not in c-jun$^{-/-}$ cells. Protein extracts were immunoblotted with antibodies specific for phospho-JNK (Thr^{183}/Tyr^{185}), JNK or phospho-c-Jun (Ser^{73}).

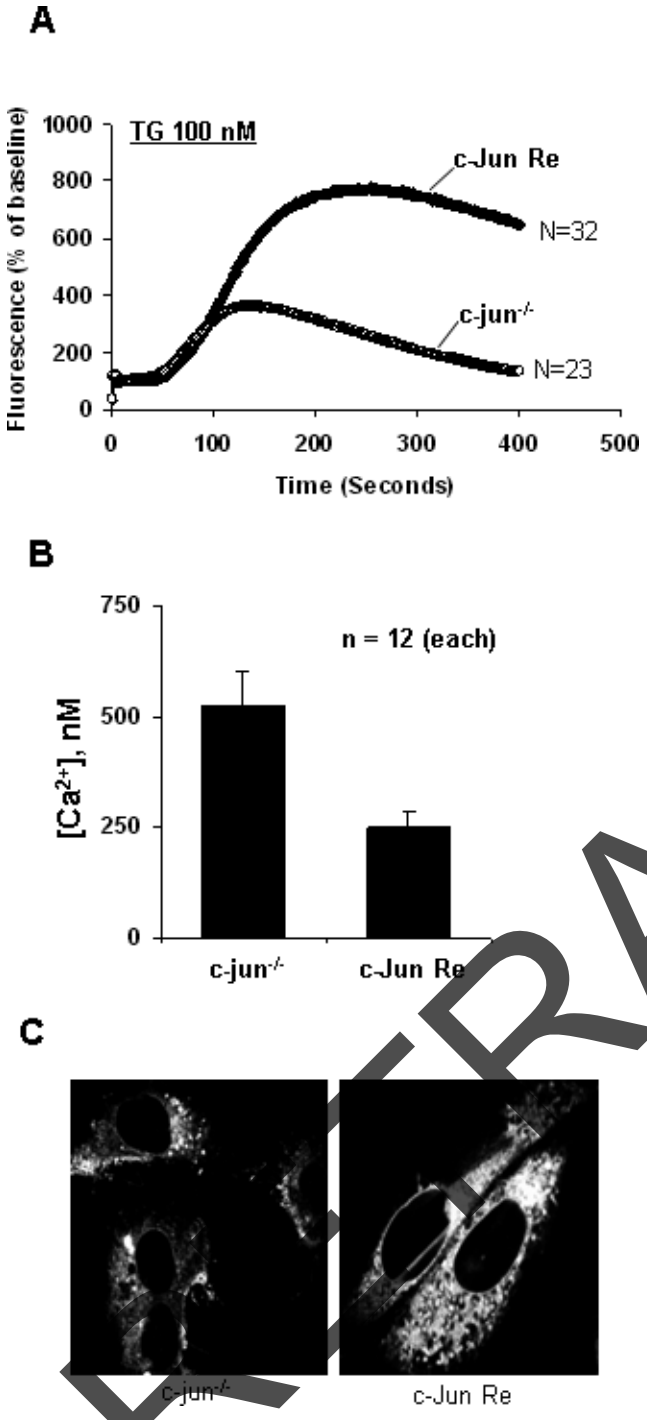

Figure 6. TG induces a rise in intracellular Ca^{2+} levels in c-jun$^{-/-}$ and c-Jun Re cells. (A) Tracing of fluo-4 fluorescence in cells stimulated with 100 nM TG over time. Intracellular [Ca^{2+}] increases in c-Jun Re cells in response to TG treatment starting at 100 seconds and persists after 400 seconds. An increase in intracellular [Ca^{2+}] is also observed in c-jun$^{-/-}$ cells but at smaller amplitude. The graph is representative of that seen in 32 cells for c-Jun Re and 23 cells for c-jun$^{-/-}$. (B) Magnitude of free Ca^{2+} concentration in cells loaded with the ratiometric Ca^{2+} dye fura-2. Ratio measurements of fura-2 loaded cells demonstrates that the baseline free Ca^{2+} concentration in c-jun$^{-/-}$ is about twice as big as that observed in c-Jun Re cells ($n = 12$, $P < 0.01$ relative to either group of cells). (C) Confocal images of cells loaded with the ER-Tracker and visualized by two-photon microscopy. The ER extends throughout the cells in a reticulated pattern. The ER structures are more abundant and widespread in c-Jun Re compared to c-jun$^{-/-}$ cells.

tively (47). The promoter region of *adapt78* contains both NFAT and AP-1 binding motifs, but this study did not provide direct evidence that the motifs are functionally active. Both reconstitution of c-Jun expression in c-jun$^{-/-}$ cells and the constitutively active calcineurin expression resulted in c-Jun and NFAT translocation to the nucleus, leading to the up-regulation of Adapt78 expression in response to TG treatment. TG induced JNK activation and downstream c-Jun phosphorylation in c-Jun Re cells, associated with an up-regulation of Adapt78 expression, but not in c-Jun deficient cells.

We further examined the responsiveness of c-Jun deficient cells to other stress stimuli known to activate JNK pathway; i.e. oxidative stress and cytokines. We found that H_2O_2 and TNFα treatment failed to activate JNK signaling pathway in a manner reminiscent of TG treatment (data not shown). We hypothesize that the senescent phenotype of c-Jun deficient cells (48) might decrease the sensitivity of JNK to stress stimuli. The greater sensitivity of c-jun$^{-/-}$ cells to TG-induced apoptosis may result from a rise in intracellular calcium levels and a consequent increase in calcineurin activity caused by TG treatment. In contrast, the up-regulation of Adapt78 in response to TG-induced ER stress in c-Jun Re cells led to an inhibition of calcineurin activity and resistance to TG-induced apoptosis.

It has been shown that four ER transmembrane proteins PERK, IRE1α, IRE1β and ATF6 act in concert to mediate the UPR. The ER resident BiP/Grp78 negatively regulates each of these four proteins; its dissociation results in their activation (10, 12, 13). It is suggested that these four regulatory proteins can be activated simultaneously to initiate the cascade of events comprising the UPR. One or more of these ER transmembrane proteins may functionally interact with JNK to mediate specific aspects of the UPR. For example, IRE1 has been shown to be required for the activation of JNK in mouse embryonic fibroblasts (MEFs) upon treatment with TG (49). We show in the present study that TG induces JNK activation in c-Jun Re and WT MEF (data not shown) cells, but not in c-jun$^{-/-}$ MEF cells. This finding suggests that c-Jun, a downstream target of JNK, has a feedback effect on JNK activation in response to TG-induced ER stress.

Calcineurin is a calcium/calmodulin-activated serine/threonine phosphatase. The most thoroughly characterized calcineurin substrate proteins are members of the nuclear factor of activated T-cells (NFAT) family of transcription factors (50). Calcineurin dephosphorylates multiple residues within the regulatory domain of NFAT, leading to its nuclear translocation and activation of target genes through cooperation with multiple partners including AP-1 (47), MEF2 (51), and GATA (52) proteins. The calcineurin/NFAT pathway was first described in the activation of T-cells upon antigen presentation and binding to the T-cell receptor (53). Calcineurin has also been reported to play an important role in numerous other physiologic processes that include memory formation, neuronal apoptosis (54), differ-

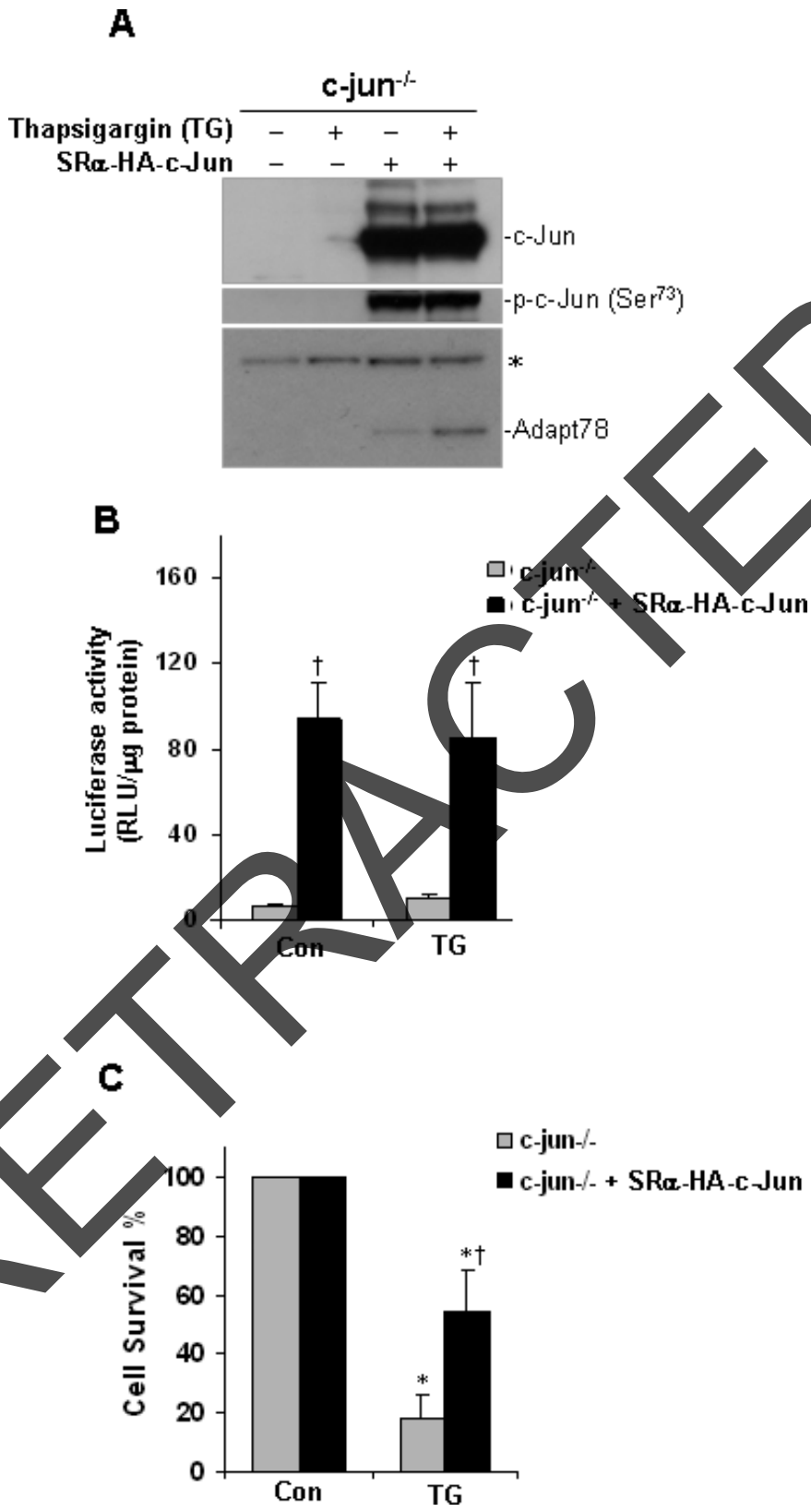

Figure 7. Exogenous, constitutive expression of c-Jun restores TG-induced up-regulation of Adapt78 in c-jun$^{-/-}$ cells. (A) Overexpression of SRα-HA-c-Jun in c-jun$^{-/-}$ cells causes autophosphorylation of c-Jun and restores the up-regulation of Adapt78 in response to TG (100 nM). Proteins extracts were immunoblotted for c-Jun, phospho-c-Jun (Ser^{73}) and Adapt78. * Non specific protein as a loading control. (B) Transient transfection assays of adapt78-luciferase reporter plasmids in c-jun$^{-/-}$ and c-jun$^{-/-}$ + SRα-HA-c-Jun cells. The adapt78-luciferase reporter plasmid is the same as that described in Figure 5. Luciferase activity was normalized to β-galactosidase values and expressed in relative light

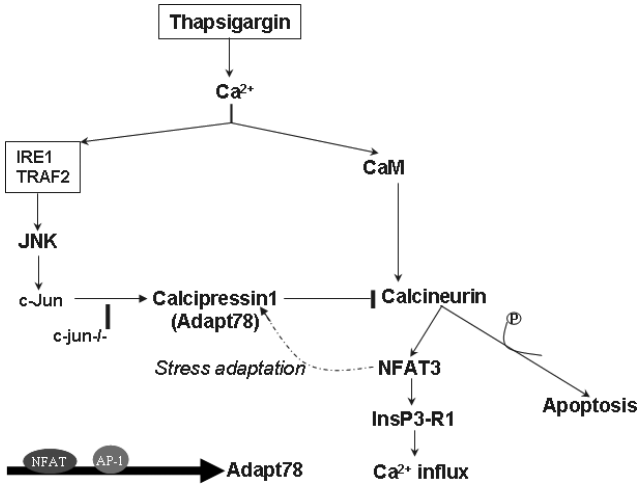

Figure 8. Schematic diagram illustrating the up-regulation of Adapt78 (Calcipressin1) by c-Jun in response to thapsigargin (TG) treatment, leading to adaptive protection against apoptosis. Evidence presented in the current study identifies Adapt78 as a target for positive transcriptional regulation by c-Jun, as well as, by calcineurin, probably as a direct consequence of AP-1 and NFAT binding to the calcineurin-responsive region near exon 4 of the adapt78 gene. Adapt78, in turn, is capable of inhibiting the activity of calcineurin (31). This regulatory circuit likely serves as an adaptive mechanism to inhibit calcineurin activity and protect against cell death during TG-induced ER stress.

entiation and remodeling of skeletal and cardiac myocytes (51, 52). Here, we found that c-jun$^{-/-}$ cells have higher basal calcineurin activity compared to c-Jun Re cells. TG treatment significantly increased calcineurin activity in c-jun$^{-/-}$ cells but not in c-Jun Re cells, likely as a result of Adapt78 up-regulation in c-Jun Re cells which blocks calcineurin activity in response to TG-induced ER stress. Adapt78, previously referred to as DSCR1 or MCIP1, is an important intracellular phosphatase that mediates many cellular responses to calcium. Adapt78 was first identified as a Down syndrome critical region-localized gene on human chromosome 21 (55). Adapt78 protein has been shown to bind to calcineurin and inhibit its intracellular activity (56, 57), which may be the mechanism for the resistance of c-Jun Re cells to TG-induced ER stress, as illustrated in our study.

In conclusion, the results suggest that c-Jun acts as a positive transcriptional regulator of Adapt78 in response to TG-induced ER stress, and Adapt78, in turn, blocks the activity of calcineurin and contributes to the protection against TG-induced cell death (Fig. 8).

We greatly appreciate the gift of c-jun$^{-/-}$ and c-Jun Re cells lines from Dr. Michael Karin (Laboratory of Gene Regulation and Signal Transduction, School of Medicine, University of California at San Diego, La Jolla, CA), and the gift of adapt78 antibody and adapt78 cDNA from Dr. Kelvin Davies (Ethel Percy Andrus Gerontology Center, The University of Southern California, Los Angeles, CA). We would like to give our thanks to Dr. Nikki Holbrook for the advice obtained in numerous discussions of these experiments.

1. Maki Y, Bos TJ, Davis C, Starbuck M,Vogt PK. Avian sarcoma virus 17 carries the jun oncogene. Proc Natl Acad Sci U S A 84:2848–2852, 1987.
2. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49:729–739, 1987.
3. Chinenov Y, Kerppola TK. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. Oncogene 20:2438–2452, 2001.
4. Dunn C, Wiltshire C, MacLaren A, Gillespie DA. Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. Cell Signal 14:585–593, 2002.
5. Lievremont JP, Rizzuto R, Hendershot L, Meldolesi J. BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+. J Biol Chem 272:30873–30879, 1997.
6. Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. Mol Cell Biol 18:7499–7509, 1998.
7. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271–274, 1999.
8. Tirasophon W, Welihinda AA, Kaufman RJ. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. Genes Dev 12:1812–1824, 1998.
9. Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D. Cloning of mammalian Ire1 reveals diversity in the ER stress responses. Embo J 17:5708–5717, 1998.
10. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2:326–332, 2000.
11. Ma K, Vattem KM, Wek RC. Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress. J Biol Chem 277: 18728–18735, 2002.
12. Shen J, Chen X, Hendershot L, Prywes R. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev Cell 3:99–111, 2002.
13. Shen J, Snapp EL, Lippincott-Schwartz J, Prywes R. Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. Mol Cell Biol 25:921–932, 2005.
14. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13:1211–1233, 1999.
15. Niwa M, Walter P. Pausing to decide. Proc Natl Acad Sci U S A 97: 12396–12397, 2000.
16. Ma Y, Hendershot LM. The unfolding tale of the unfolded protein response. Cell 107:827–830, 2001.
17. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403:98–103, 2000.

←
units (RLU). Data represent means ± SE values of three independent transfections of c-jun$^{-/-}$ or c-jun$^{-/-}$ + SRα-HA-c-Jun cells. † $P < 0.01$ vs. c-jun$^{-/-}$. (C) c-jun$^{-/-}$ and c-jun$^{-/-}$ + SRα-HA-c-Jun cells were treated with TG (100 nM) for 4 h, then placed in fresh medium lacking TG and allowed to incubate for an additional 24 h, after which cell viability was assessed by trypan blue exclusion as described in experimental procedures. Results shown are mean ± SE for three independent experiments. * $P < 0.05$ vs. controls; † $P < 0.05$ vs. c-jun$^{-/-}$.

18. Ermak G, Davies KJ. Calcium and oxidative stress: from cell signaling to cell death. Mol Immunol 38:713–721, 2002.
19. Paschen W. Dependence of vital cell function on endoplasmic reticulum calcium levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. Cell Calcium 29:1–11, 2001.
20. Verkhratsky A, Petersen OH. The endoplasmic reticulum as an integrating signalling organelle: from neuronal signalling to neuronal death. Eur J Pharmacol 447:141–154, 2002.
21. van Huizen R, Martindale JL, Gorospe M, Holbrook NJ. P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling. J Biol Chem 278:15558–15564, 2003.
22. Li J, Holbrook NJ. Elevated gadd153/chop expression and enhanced c-Jun N-terminal protein kinase activation sensitizes aged cells to ER stress. Exp Gerontol 39:735–744, 2004.
23. Gorlach J, Fox DS, Cutler NS, Cox GM, Perfect JR, Heitman J. Identification and characterization of a highly conserved calcineurin binding protein, CBP1/calcipressin, in Cryptococcus neoformans. Embo J 19:3618–3629, 2000.
24. Kingsbury TJ, Cunningham KW. A conserved family of calcineurin regulators. Genes Dev 14:1595–1604, 2000.
25. Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN, Williams RS. Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A 98:3328–3333, 2001.
26. Rusnak F, Mertz P. Calcineurin: form and function. Physiol Rev 80: 1483–1521, 2000.
27. Shibasaki F, Hallin U, Uchino H. Calcineurin as a multifunctional regulator. J Biochem (Tokyo) 131:1–15, 2002.
28. Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, Colbert MC, Gualberto A, Wieczorek DF, Molkentin JD. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science 281: 1690–1693, 1998.
29. Zhuo M, Zhang W, Son H, Mansuy I, Sobel RA, Seidman J, Kandel ER. A selective role of calcineurin aalpha in synaptic depotentiation in hippocampus. Proc Natl Acad Sci U S A 96:4650–4655, 1999.
30. Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park HS, Toyoda A, Ishii K, Totoki Y, Choi DK, *et al*. The DNA sequence of human chromosome 21. Nature 405:311–319, 2000.
31. Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, Bassel-Duby R, Williams RS. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res 87:E61–E68, 2000.
32. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, *et al*. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108:1167–1174, 2001.
33. Mansouri A, Ridgway LD, Korapati AL, Zhang Q, Tian L, Wang Y, Siddik ZH, Mills GB, Claret FX. Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to Fas ligand induction and cell death in ovarian carcinoma cells. J Biol Chem 278:19245–19256, 2003.
34. Li J, Gorospe M, Hutter D, Barnes J, Keyse SM, Liu Y. Transcriptional induction of MKP-1 in response to stress is associated with histone H3 phosphorylation-acetylation. Mol Cell Biol 21:8213–8224, 2001.
35. Zhang ZY, Thieme-Sefler AM, Maclean D, McNamara DJ, Dobrusin EM, Sawyer TK, Dixon JE. Substrate specificity of the protein tyrosine phosphatases. Proc Natl Acad Sci U S A 90:4446–4450, 1993.
36. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 4:E131–E136, 2002.
37. Leahy KP, Davies KJ, Dull M, Kort JJ, Lawrence KW, Crawford DR. adapt78, a stress-inducible mRNA, is related to the glucose-regulated protein family of genes. Arch Biochem Biophys 368:67–74, 1999.
38. Leite MF, Thrower EC, Echevarria W, Koulen P, Hirata K, Bennett AM, Ehrlich BE, Nathanson MH. Nuclear and cytosolic calcium are regulated independently. Proc Natl Acad Sci U S A 100:2975–2980, 2003.
39. Leite MF, Burgstahler AD, Nathanson MH. Ca2+ waves require sequential activation of inositol trisphosphate receptors and ryanodine receptors in pancreatic acini. Gastroenterology 122:415–427, 2002.
40. Hagar RE, Burgstahler AD, Nathanson MH, Ehrlich BE. Type III InsP3 receptor channel stays open in the presence of increased calcium. Nature 396:81–84, 1998.
41. Potapova O, Basu S, Mercola D, Holbrook NJ. Protective role for c-Jun in the cellular response to DNA damage. J Biol Chem 276:28546–28553, 2001.
42. Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr Opin Cell Biol 13:349–355, 2001.
43. Xu C, Ma H, Inesi G, Al-Shawi MK, Toyoshima C. Specific structural requirements for the inhibitory effect of thapsigargin on the Ca2+ ATPase SERCA. J Biol Chem 279:17973–17979, 2004.
44. Mukerjee N, McGinnis KM, Park YH, Gnegy ME, Wang KK. Caspase-mediated proteolytic activation of calcineurin in thapsigargin-mediated apoptosis in SH-SY5Y neuroblastoma cells. Arch Biochem Biophys 379:337–343, 2000.
45. Toyoshima C, Nomura H. Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 418:605–611, 2002.
46. Mechta-Grigoriou F, Gerald D, Yaniv M. The mammalian Jun proteins: redundancy and specificity. Oncogene 20:2378–2389, 2001.
47. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15:707–747, 1997.
48. MacLaren A, Black EJ, Clark W, Gillespie AF. c-Jun-deficient cells undergo premature senescence as a result of spontaneous DNA damage accumulation. Mol Cell Biol 24:9006–9018, 2004.
49. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287:664–666, 2000.
50. Crabtree GR. Calcium, calcineurin, and the control of transcription. J Biol Chem 276:2313–2316, 2001.
51. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. Embo J 20:6414–6423, 2001.
52. Musaro A, McCullagh KJ, Naya FJ, Olson EN, Rosenthal N. IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. Nature 400:581–585, 1999.
53. Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB, Schreiber SL. Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. Biochemistry 31:3896–3901, 1992.
54. Malleret G, Haditsch U, Genoux D, Jones MW, Bliss TV, Vanhoose AM, Weitlauf C, Kandel ER, Winder DG, Mansuy IM. Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin. Cell 104:675–686, 2001.
55. Michtalik HJ, Narayan AV, Bhatt N, Lin HY, Mulligan MT, Zhang SL, Crawford DR. Multiple oxidative stress-response members of the Adapt78 family. Free Radic Biol Med 37:454–462, 2004.
56. Fuentes JJ, Genesca L, Kingsbury TJ, Cunningham KW, Perez-Riba M, Estivill X, de la Luna S. DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum Mol Genet 9:1681–1690, 2000.
57. Vega RB, Yang J, Rothermel BA, Bassel-Duby R, Williams RS. Multiple domains of MCIP1 contribute to inhibition of calcineurin activity. J Biol Chem 277:30401–30407, 2002.
58. Fuentes JJ, Pritchard MA, Estivill X. Genomic organization, alternative splicing, and expression patterns of the DSCR1 (Down syndrome candidate region 1) gene. Genomics 44:358–361, 1997.