Inhibition of Foxo1 Protects Pancreatic Islet β-Cells Against Fatty Acid and Endoplasmic Reticulum Stress–Induced Apoptosis

Sara C. Martinez,[1] Katsuya Tanabe,[1] Corentin Cras-Méneur,[1] Nada A. Abumrad,[2] Ernesto Bernal-Mizrachi,[1] and M. Alan Permutt[1]

OBJECTIVE—β-Cells are particularly susceptible to fatty acid–induced apoptosis associated with decreased insulin receptor/phosphatidylinositol-3 kinase/Akt signaling and the activation of stress kinases. We examined the mechanism of fatty acid–induced apoptosis of mouse β-cells especially as related to the role played by endoplasmic reticulum (ER) stress–induced Foxo1 activation and whether decreasing Foxo1 activity could enhance cell survival.

RESEARCH DESIGN AND METHODS—Mouse insulinoma (MIN6) cells were administered with fatty acids, and the role of Foxo1 in mediating effects on signaling pathways and apoptosis was examined by measuring Foxo1 activity and using dominant-negative Foxo1.

RESULTS—Increasing fatty acid concentrations (100–400 μmol/l palmitate or oleate) led to early Jun NH_2-terminal kinase (JNK) activation that preceded induction of ER stress markers and apoptosis. Foxo1 activity was increased with fatty acid administration and by pharmacological inducers of ER stress, and this increase was prevented by JNK inhibition. Fatty acids induced nuclear localization of Foxo1 at 4 h when Akt activity was increased, indicating that FoxO1 activation was not mediated by JNK inhibition of Akt. In contrast, fatty acid administration for 24 h was associated with decreased insulin signaling. A dominant-negative Foxo1 adenovirus (Adv-DNFoxo) conferred cells with protection from ER stress and fatty acid–mediated apoptosis. Microarray analysis revealed that fatty acid induction of gene expression was in most cases reversed by Adv-DNFoxo, including the proapoptotic transcription factor CHOP (C/EBP [CCAAT/enhancer binding protein] homologous protein).

CONCLUSIONS—Early induction of JNK and Foxo1 activation plays an important role in fatty acid–induced apoptosis. Expressing a dominant-negative allele of Foxo1 reduces expression of apoptotic and ER stress markers and promotes β-cell survival from fatty acid and ER stress, identifying a potential therapeutic target for preserving β-cells in type 2 diabetes. ***Diabetes*** **57: 846–859, 2008**

Insulin-resistant states are characterized by an initial adaptive expansion of β-cell mass to increase insulin production for maintaining euglycemia. However, a significant number of insulin-resistant individuals develop impaired insulin secretion associated with a reduction in β-cell mass that subsequently results in hyperglycemia and overt diabetes (1). A common feature of insulin-resistant states is high serum fatty acids (2). In addition to reducing insulin sensitivity in peripheral tissues, fatty acids also impact islet β-cell function. Whereas short-term fatty acid exposure (hours) augments insulin secretion, chronic exposure (days) results in decreased secretion in rodent and human islets and in insulinoma cells in culture (3–5). Chronic elevation of fatty acids has also been shown to be associated with increased β-cell apoptosis in rodents and has been implicated in the etiology of the reduced β-cell mass of type 2 diabetes (6,7).

Oleate administration of insulinoma cells induced apoptosis that was dependent on decreased protein kinase B/Akt phosphorylation (8). Palmitate- and oleate-induced apoptosis was associated with expression of endoplasmic reticulum (ER) stress markers such as immunoglobulin heavy-chain binding protein (BiP) and C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP) (7). Pharmacological induction of ER stress within the β-cell was associated with decreased Akt phosphorylation and with activation of c-Jun NH_2-terminal kinase (JNK) (9). In the liver, high fatty acids promote an ER stress response associated with JNK activation (10). In adipocytes, fatty acid reduction of insulin signaling is associated with JNK activation and is reversed by JNK inhibition (11). JNK phosphorylates an inhibitory serine residue (serine 307) on the insulin receptor substrate-1 protein, which results in downregulation of insulin signaling (12). JNK also activates Foxo1 by phosphorylation at sites independent of those phosphorylated by Akt (threonine 447 and threonine 451) (13,14). Deletion of JNK in mice reduces obesity-associated insulin resistance (15). In summary, fatty acid administration of β-cells has been shown in separate studies to activate JNK, promote ER stress, and decrease insulin signaling. How these pathways interact and the sequence of events involved in fatty acid–induced apoptosis of β-cells remain unknown. A prime candidate

From the [1]Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, St. Louis, Missouri; and the [2]Department of Medicine, Division of Nutritional Science, Washington University School of Medicine, St. Louis, Missouri.

Address correspondence and reprint requests to M. Alan Permutt, MD, Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8127, St. Louis, MO 63110. E-mail: apermutt@im.wustl.edu.

Received for publication 30 April 2007 and accepted in revised form 27 December 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 15 January 2008. DOI: 10.2337/db07-0595.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0595.

BiP, immunoglobulin heavy-chain binding protein; CHOP, C/EBP (CCAAT/enhancer binding protein) homologous protein; DAPI, 4′6-diamidino-2-phenylindole; eIF2α, eukaryotic initiation factor-2α; Ddit3, DNA damage–inducible transcript 3; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; GFP, green fluorescent protein; Gsk3β, glycogen synthase kinase 3β; IGFBP, IGF-binding protein; JNK, Jun NH_2-terminal kinase; MOI, multiplicity of infection; Pdx, pancreatic and duodenal homeobox; SCD1, stearoyl CoA desaturase 1; TUDCA, tauroursodeoxycholic acid; TUNEL, transferase-mediated dUTP nick-end labeling.

for mediating fatty acid–induced toxicity downstream of decreased insulin signaling is the proapoptotic antiproliferative transcription factor Foxo1, which is negatively regulated by insulin/phosphatidylinositol-3 kinase/Akt activation. Phosphorylation of Foxo1 by Akt promotes its nuclear exclusion and inhibits its function (16). Glucose stimulation of β-cells resulted in Akt phosphorylation with inhibition of Foxo1 nuclear activity by an autocrine/paracrine effect of released insulin on its receptor (17). In contrast, fatty acid administration of insulinoma β-cells decreased phosphorylation of Akt and Foxo1 (8). Oxidative stress resulted in nuclear to cytoplasmic translocation of pancreatic and duodenal homeobox 1 (Pdx1), and this was blocked by dominant-negative Foxo (18). However, none of the previous studies evaluated the role of Foxo1 activation in fatty acid–induced β-cell death. We here demonstrate an essential role of Foxo1 activation in β-cell apoptosis. We show that fatty acids as well as pharmacological ER stress–inducing agents rapidly induce JNK, the ER stress response, and increase Foxo1 nuclear localization and activity at a time when Akt phosphorylation is increased. Foxo1 activation is blocked by JNK inhibition. Expressing a dominant-negative allele of Foxo1 reduces expression of apoptotic and ER stress markers and promotes β-cell survival from fatty acid and ER stress. A potential mechanism for the proapoptotic action of Foxo1 is provided by documenting its transcriptional regulation of CHOP.

RESEARCH DESIGN AND METHODS

Thapsigargin, tunicamycin, oleic acid, palmitic acid, formalin, 4′6-diamidino-2-phenylindole (DAPI), Hoechst dye, and propidium iodide were purchased from Sigma (Saint Louis, MO). SP610025 was from Tocris Bioscience (Ellisville, MO). Antibodies used were anti–α-tubulin (monoclonal; Sigma), anti-BiP, anti-Gadd153/CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), anti–phospho-eukaryotic initiation factor-2α (eIF2α), anti–phospho-JNK1/2, anti–total JNK1/2, anti–phospho-c-Jun, anti–cleaved caspase 3, anti-Foxo1, anti–phospho-Ser473-Akt, anti–phospho-Thr308-Akt (Cell Signaling Technology, Beverley, MA). The anti-hemaglutinin antibody is a mouse monoclonal from Covance Research Products (Cumberland, VA). All primary antibodies were used at a 1:1,000 dilution in 5% BSA/Tris-buffered saline with Tween/0.1% NaN_3, with the exception of the CHOP antibody, which was used at a 1:200 dilution in 5% milk/Tris-buffered saline with Tween.

Cell culture of mouse insulinoma cells. MIN6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose, with 15% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml L-glutamine, and 5 μl/l β-mercaptoethanol in humidified 5% CO_2, 95% air at 37°C (19). MIN6 cells used were between passages 21 and 31.

Islet isolation and culture. All procedures were performed in accordance with Washington University's animal studies committee. Animals were killed in a carbon dioxide chamber. Islets of 8- to 10-week-old wild-type male C57BL/6 mice were isolated by ductal collagenase distension/digestion of the pancreas (20), followed by filtering and washing through a 70-μmol/l nylon cell strainer (BD Biosciences, San Jose, CA). Isolated islets were then maintained in RPMI medium containing 11 mmol/l glucose, 10% FBS, 200 units/ml penicillin, and 200 μg/ml streptomycin in humidified 5% CO_2, 95% air at 37°C.

Fatty acid administration of MIN6 cells and islets. MIN6 cells were incubated in modified DMEM media with 0.5% (wt/vol) BSA alone or 0.2, 0.3, or 0.4 mmol/l palmitate or 0.4 mmol/l oleate complexed to 0.5% (wt/vol) BSA for 24 h. Preparation of the 0.4 mmol/l fatty acid media was carried out as previously described (8). Briefly, a 20 mmol/l solution of the fatty acid in 0.01 mol/l NaOH was incubated at 70°C for 30 min. Then, 330 μl of 30% BSA and 200, 300, or 400 μl of the free fatty acid/NaOH mixture was mixed together and filter-sterilized with 20 ml of either the DMEM or RPMI media. These conditions were chosen because we originally showed that 25 versus 5 mmol/l glucose inhibited Foxo1 activation (17) and was protective of apoptosis (74). In the current studies, we chose standard culture media (25 mmol/l) to begin the experiments with Foxo1 activity and apoptosis at a minimum to optimize the effects of FFA. The approximate molar ratio of fatty acids to BSA is 6:1 with 0.4 mmol/l palmitate. The addition of BSA or a fatty acid/BSA mixture has not been shown to affect the pH of the media. For transferase-mediated dUTP nick-end labeling (TUNEL) staining, cells were kept in culture in Lab-Tek II, CC^2-administered chamber slides (Nunc, Rochester, NY).

Propidium iodide/DAPI cell death assay. MIN6 cells were grown on glass coverslips within the wells of a 6-well plate and incubated with either normal media, thapsigargin, BSA alone, or 0.2, 0.3, or 0.4 mmol/l palmitate complexed with BSA for 24 h. For the last hour of incubation, 10 μg/ml of propidium iodide and 20 μg/ml DAPI were added directly to the media. After this incubation, the MIN6 cells were washed 3× with PBS and fixed with 3.7% formalin for 15 min at 4°C. After fixation, the MIN6 cells were washed again 3 times with PBS and then mounted with antifading gel mounting medium (Biomeda, Foster City, CA) onto glass slides. Each condition reported represents >600 cells counted by randomized field selection. The percentage of cell-death is reported as the number of propidium iodide–stained nuclei over the total number of nuclei stained by DAPI, as quantitated by ImageJ version 1.3.8s (National Institutes of Health) (21). TUNEL staining was performed using a rhodamine ApopTAG kit (Amersham BioSciences, Piscataway, NJ) according to the manufacturer's instructions.

Nuclear and cytoplasmic fractionation. MIN6 cells were plated on 10-cm dishes and allowed to grow to 60–70% confluence. Media was then exchanged for 0.5% BSA or 0.4 mmol/l palmitate/0.5% BSA or 0.4 mmol/l oleate/0.5% BSA media for the times indicated. Cells were trypsinized and fractionated according to a previously published protocol evaluating Foxo1 localization (18).

Western blotting. Protein was extracted with a cell lysis buffer (diluted from 10× cell lysis buffer from Cell Signaling Technology and an additional protease cocktail tablet from Roche at one tablet/10 ml final buffer volume). Protein samples (30 μg) were separated by SDS-electrophoresis through either 4–15% gradient or 15% polyacrylamide gels (BioRad, Hercules, CA) and transferred to nitrocellulose membranes, followed by immunoblotting using all primary antibodies according to the manufacturer's instructions. Immunodetection was developed with ECL Advance (Amersham Biosciences, Buckinghamshire, U.K.) and imaged with a charge-coupled device camera (Alpha Innotech, San Leandro, CA).

Plasmid constructs. Hemaglutinin-tagged Δ256-Foxo1 in the pCMV5 vector (DNFoxo) was a gift from D. Accili (Columbia University, New York). The IGF-binding protein-1 (IGFBP-1) promoter/luciferase gene construct (p925GL3) was a gift from M. Rechler (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). The CHOP promoter/luciferase construct (pGL2/3) was a gift from the lab of D. Ron (Skirball Institute, New York University, New York). The pRL-TK control vector contains the thymidine kinase promoter of the herpes simplex virus upstream of the *Renilla* luciferase (Promega, Madison, WI).

Luciferase assay. MIN6 cells were plated in 12-well plates 2 days before transfection. At ~60–70% confluence, each well was transfected with 100 ng IGFBP-1/luciferase or CHOP/luciferase plasmid, 20 ng pRL-TK control vector, and either 100 ng of the pCMV5-Δ256-Foxo1 or an empty pCMV5 vector in 2 μl lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 100 μl of OptiMem. For cell lysis, 200 μl of passive lysis buffer (Promega) was used. The firefly and *Renilla* luciferase activities were measured after the indicated hours of incubation with BSA or 0.4 mmol/l palmitate in a Monolight 3010 luminometer (BD Biosciences) using the dual-luciferase reporter assay system (Promega).

Adenovirus infection. The hemaglutinin-tagged dominant-negative Foxo1 adenovirus (AdV-Δ256-Foxo1) and the hemaglutinin-tagged wild-type Foxo1 adenovirus were generous gifts from D. Accili (22). The green fluorescent protein (GFP) adenovirus was a gift from D. Kelly (Washington University, St. Louis, MO). Infection of the MIN6 cells was carried out at the indicated multiplicity of infection (MOI) for 1 h in serum-free media. The MIN6 cells were then washed in PBS and maintained in the DMEM/15% FBS media, and then experiments were carried out 24 h after infection.

Generation of β-cell–specific dominant-negative Foxo transgenic mice. The truncated and hemaglutinin-tagged dominant-negative Foxo1 allele (DN-Foxo) (23) was a generous gift from D. Accili (Columbia University, New York, NY). It was inserted into a RIP-I/β-globin expression vector (24,25), sequenced, and microinjected into fertilized eggs of C57BL/6 × CBA mice according to standard protocol of the Mouse Genetics Core of the Washington University School of Medicine. Nine founders were obtained, of which five passed the transgene through germline transmission, as evidenced by genotyping and backcrossing to C57BL/6J mice (The Jackson Laboratory). Of the five that transmitted, one line maintained consistent and strong hemaglutinin staining within pancreatic islets, designated as RIP-DNFoxo. Transgenic and nontransgenic littermate male (designated as wild type) mice from C57BL/6J-backcrossed F4-F7 generations of this one line, known as RIP-DNFoxo, were used in all experiments in accordance with Washington University's animal studies committee. These mice are currently being phenotyped.

Fatty acid administration of primary islets and MIN6 cells. Primary islets from wild-type or RIP-DNFoxo mice were pooled (three per genotype) and divided to be incubated in modified RPMI media with 0.5% (wt/vol) BSA alone or with 0.4 mmol/l palmitate complexed to 0.5% (wt/vol) BSA for 24 h. Preparation of the 0.4 mmol/l free fatty acid media was carried out as previously described (8). Briefly, a 20 mmol/l solution of palmitic acid (Sigma) in 0.01 mol/l NaOH was incubated at 70°C for 30 min. Then, 330 μl of 30% BSA and 400 μl of the palmitic acid/NaOH mixture was mixed together and filter-sterilized with 20 ml of the RPMI media. The approximate molar ratio of fatty acids to BSA is 6:1 with 0.4 mmol/l palmitate. MIN6 cells were incubated in modified DMEM media with 0.5% (wt/vol) BSA alone or with 0.4 mmol/l palmitate plus 0.5% (wt/vol) BSA for 24 h.

Thapsigargin administration of islets. After isolation and a 6-h recovery period after isolation in RPMI/10% FBS media, primary islets from wild-type or RIP-DNFoxo mice were pooled and divided into 12-well plates with 1 ml of RPMI/10% FBS media to be administered with either 10 μmol/l thapsigargin (10 μl of a 1 mmol/l stock; Sigma) or vehicle (10 μl of ethanol) alone for 48 h.

Statistical analysis. Western blot quantitation consisted of acquiring a per-lane ratio of the chemiluminescent signal intensities from phospho-Ser (256)-Foxo1 or Gadd153 to its respective α-tubulin using ImageJ (21) and then normalizing each signal to the first condition of the starved state in each respective blot. The Western blot figures indicate concentrations for which there is a significant difference ($P < 0.05$) from the starved state and a significant difference ($P < 0.05$) from the intensity of the glucose phosphorylation of Foxo1. For the luciferase assays, ratios of luciferase to *Renilla* were generated with standard deviations and error. Error was propagated for fold calculations between different conditions. Student's two-tailed t test for independent samples was used in significance calculations. The P value for significance is mentioned in the figure legends.

RNA isolation from MIN6 cells. MIN6 cells infected with either a GFP or DNFoxo adenovirus were maintained in triplicate samples within 6-well plates in DMEM with either 0.5% BSA or 0.5% BSA plus 0.4 mmol/l palmitate for 24 h. Each well was then aspirated and rinsed once with PBS. Then, 1 ml of ice-cold Trizol (Invitrogen) was added to each well. The protocol was followed according to manufacturer's instructions until the aqueous phase was isolated. RNase-free 70% ethanol was added (twice the aqueous volume), and the mixture was run through a purification column within the RNeasy mini-kit (Qiagen, Valencia, CA). Purification of total RNA was completed using the kit and eluted with RNase-free water. RNA quality was assessed with gel electrophoresis and an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarray experiment. We used a MouseRef-8 Expression BeadChip (Illumina, San Diego, CA), which allows for the processing of eight samples to be analyzed simultaneously for the quantitation of absolute expression of up to 16,435 genes and controls with a 30-fold redundancy of 50-mer oligos per gene. For control samples, four independent RNA preparations from the AdV-GFP BSA were pooled together, as were four independent RNA samples from the AdV-DNFoxo–infected samples administered with BSA and without palmitate. These two control groups occupied two arrays. For the experimental condition, administered with 0.4 mmol/l palmitate, three independent RNA samples for each of the GFP and DNFoxo virally infected samples occupied the other six gene expression arrays on the chip. For each gene, the Illumina software calculated the P value of the reliability of the spot. Only spots that had a significant P value were kept for further analysis. For each gene, the per-chip variance was integrated across the 30 repeats per gene on each array and the array variance. For each ratio (GFP Palm–to–BSA, DNFoxo Palm–to–BSA), 95% confidence intervals were calculated taking into account internal repeats (~30) on multiple gene arrays. The cutoff fold change was arbitrarily chosen at 2.0: genes up- or downregulated more than twofold and with a 95% confidence interval not overlapping 0 (in Log_{10} scale) were considered for further analysis. Annotation for the microarray platform was collected through the SOURCE repository (26). All transcripts significantly regulated by fatty acids were then clustered according to their Gene Ontology (27) functions using Genesis 1.7.2 (28).

The normalized unprocessed data are currently available online at http://drtc.im.wustl.edu/files/apermutt/microarray/FA/normalized_data.csv and will be made available in the MIAME (Minimum Information About a Microarray Experiment) standard on the NCBI (National Center for Biotechnology Information) Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/.

The microarray data were validated by real-time PCR on selected genes. cDNA was generated (Superscript III; Invitrogen) and amplified by real-time PCR (Power SYBR Green Master Mix; Applied Biosystems) on an ABI 7500 real-time PCR system (Applied Biosystems). Primers are shown in Supplemental Table S2 (available in an online appendix at http://dx.doi.org/10.2337/db07-0595). Results were normalized to the acidic ribosomal protein 36B4 and quantified using the comparative Ct method (29).

Promoter analysis. For each gene analyzed, 2,500 bp were collected from the NCBI Entrez databases (http://www.ncbi.nlm.nih.gov/) using EZ-Retrieve (http://siriusb.umdnj.edu:18080/EZRetrieve/). Perl global regular expression patterns were then used in BBEdit 8.6.1 (Bare Bones Software, Bedford, MA) to find all consensus sequences or their reverse complements in the sequences.

RESULTS

Fatty acids induce dose-dependent apoptosis in MIN6 cells. Glucose-responsive MIN6 insulinoma cells were assessed for apoptosis at 24 h after administration with a saturated fatty acid, palmitate (16:0), or a monounsaturated fatty acid oleate (18:1). Effects of palmitate versus oleate were compared because of the known different potencies of saturated versus monounsaturated fatty acids in inducing cell apoptosis (6,30,31) and in contributing to symptoms of diabetes and cardiovascular disease (32). Cells were incubated at a constant 0.5% BSA (wt/vol) and at concentrations of palmitate or oleate of 0.2, 0.3, and 0.4 mmol/l. Apoptosis, characterized by propidium iodide staining, normalized with DAPI staining, was visibly enhanced with increasing concentrations of fatty acids, as illustrated with palmitate in Fig. 1*A*. A dose response was observed in cell death rates reaching 11.6 and 3.4%, respectively, for administration with 0.4 mmol/l palmitate and oleate (Fig. 1*B*). That apoptosis contributes at least partly to palmitate-induced cell death in MIN6 insulinoma cells was evidenced by increased TUNEL staining (Supplemental Fig. S1).

Fatty acids induce JNK activation, a dose-dependent reduction in insulin signaling, and reciprocal increased expression of apoptotic markers and decreased Pdx1 expression. Several recent studies in peripheral insulin target tissues have documented the relationship between fatty acid administration and the activation of the stress-activated kinases known as JNK1/2 in conjunction with decreased insulin signaling (10,11,15). We confirmed these observations using a 24-h administration of MIN6 cells with palmitate and oleate. As fatty acid concentration was increased, an apparent dose-response increase in JNK1/2 activation was observed, as evidenced by the two bands representing increased JNK1/2 phosphorylation and concomitant phosphorylation of its substrate target, c-Jun (Fig. 2*A*). This treatment did not affect the total amount of the JNK proteins. The effects on JNK activation appeared to be greater for palmitate than for oleate. A decrease in Akt phosphorylation, most evident with 0.4 mmol/l palmitate or oleate, was noted (Fig. 2*B*), consistent with previous findings (8).

Pdx1 is a transcription factor known to be involved in the regulation of insulin production and protection of pancreatic islet β-cells from apoptosis (33). A recent report observed that palmitate administration of rat islets resulted in decreased insulin gene expression that was associated with a nuclear to cytoplasmic translocation of Pdx1 without a change in total Pdx1 protein (34). In our studies a dose-dependent decrease in Pdx1 protein expression was observed with fatty acid administration (Fig. 2*C*). This was associated with dose-dependent increases in expression of the proapoptotic transcription factor CHOP. Cleaved and activated caspase 3 also increased, consistent with its role as a major participant in the apoptotic cell death pathway.

JNK activation by fatty acids precedes markers of ER-stress and caspase 3 activation. To define the sequence of molecular events that occur in β-cells on fatty acid exposure, MIN6 cells were incubated with either 0.4

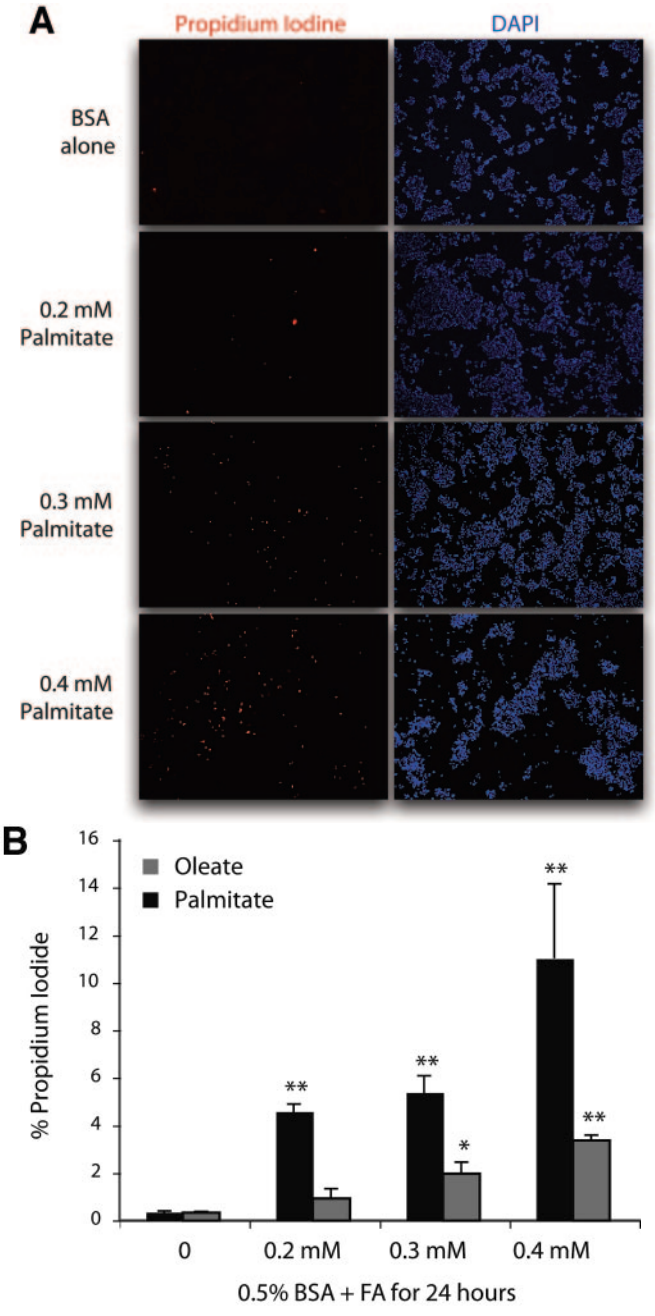

FIG. 1. Fatty acids result in a dose-dependent increase in MIN6 cell death. Propidium iodide and DAPI staining of MIN6 cells grown on coverslips and administered with the indicated concentrations of palmitate and 0.5% BSA for 24 h. *B*: Quantitation of the percentage of propidium iodide–stained nuclei of MIN6 cells administered with the indicated concentrations of palmitate or oleate and 0.5% BSA for 24 h. **P* < 0.05, ***P* < 0.01, respectively, for the fatty acid (FA) exposure compared with the administration with 0.5% BSA alone.

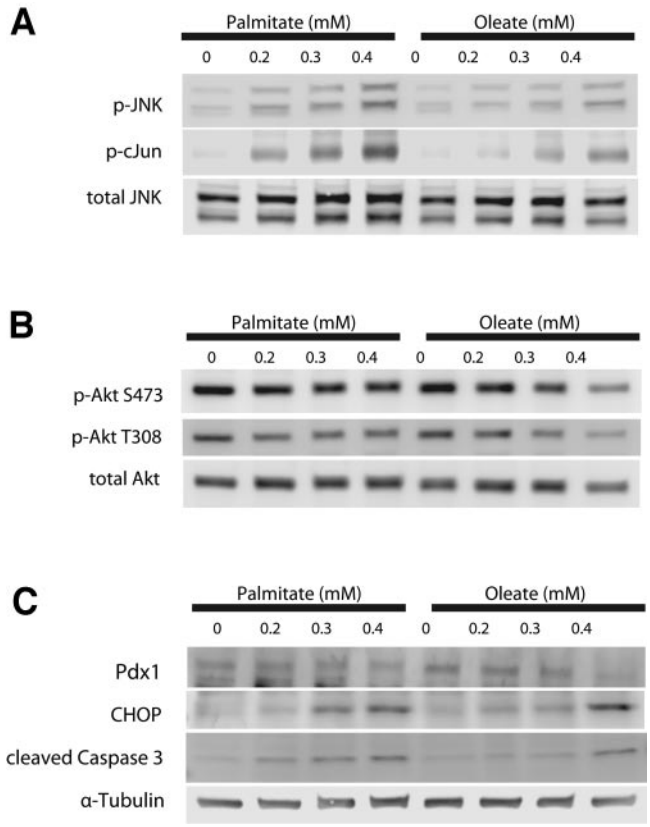

FIG. 2. Intracellular signaling with various concentrations of palmitate and oleate. MIN6 cells were grown at the indicated concentrations of 0.5% BSA alone or with additional palmitate or oleate, and on 24 h of administration, whole-cell lysates were evaluated for protein expression. *A*: Western blot of increasing phosphorylation of JNK and its substrate c-Jun with increasing concentrations of fatty acids. Total JNK was used as a loading control. *B*: Western blot of Akt phosphorylation and total Akt as a loading control. *C*: Western blot of decreasing Pdx1 expression with increasing concentrations of palmitate or oleate, correlating with increased CHOP and cleaved caspase 3 expression. α-Tubulin was used as a loading control.

mmol/l palmitate or oleate and analyzed over an 8-h period (Fig. 3*A*). JNK activation, as evidenced by phosphorylation of what appeared to be three isoforms, was noted as early as 1 h after administration with either fatty acid. The lowest phosphorylated JNK band, possibly JNK3, which has been shown to be expressed primarily in neurons (35), demonstrated transient expression. Activated JNK1/2 persisted over the 8-h incubation, as further evidenced by phosphorylation of the JNK substrate c-Jun.

The temporal relationship between fatty acid–induced ER stress and JNK activation was evaluated next. Whereas fatty acid administration resulted in insignificant changes in BiP (HSPA5, GPR78) protein, a major chaperone of the ER lumen (36), there was a progressive increase in phosphorylation of eIF2α, a protein that attenuates translation and initiates a program of apoptosis with continued stress exposure (37). This was most apparent at 4 h (Fig. 3*B*) and was followed by increased expression of CHOP and cleaved caspase 3. These data showed that JNK activation preceded induction of ER stress markers that in turn preceded induction of apoptotic markers.

To confirm the role of ER stress in fatty acid–induced apoptosis, cells were examined 24 h after palmitate administration in the presence and absence of the ER stress inhibitor tauroursodeoxycholic acid (TUDCA), a chemical chaperone (38). Addition of TUDCA reduced the levels of cleaved caspase 3, phospho-eIF2α, and CHOP protein expression by Western analysis (Fig. 3*C*), although incomplete inhibition suggests the possibility of other mechanisms contributing to fatty acid–induced cell death. TUDCA is not a selective inhibitor of ER stress and also inhibits Bax translocation and mitochondrial stress.

Fatty acid administration and activators of ER stress in MIN6 cells result in an increase in Foxo1 nuclear localization and transcriptional activation. To examine the relationship between fatty acid administration, ER stress, and Foxo1 activity, MIN6 cells were administered

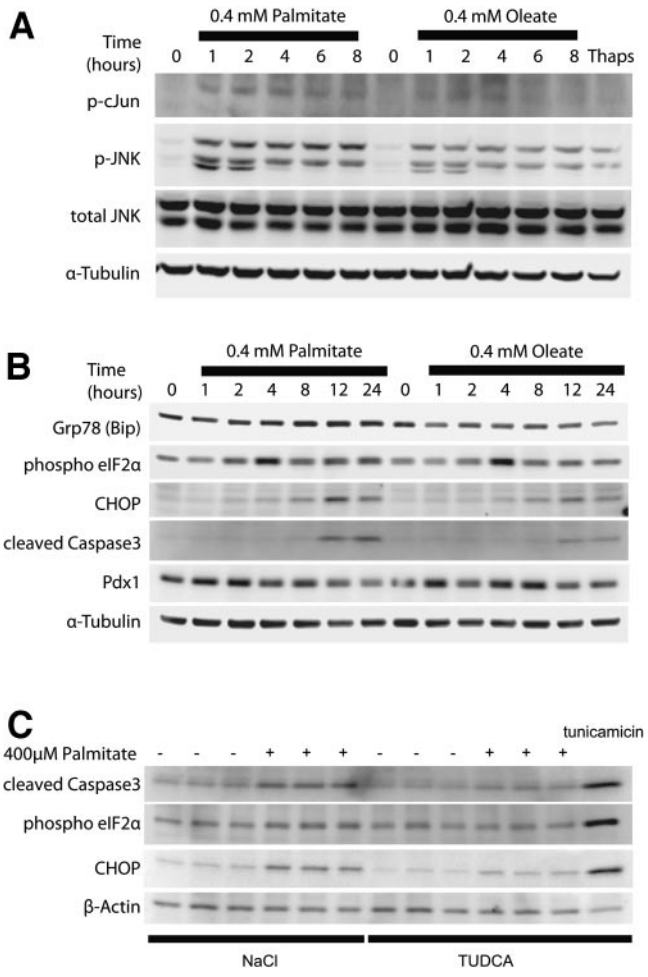

FIG. 3. Time course of JNK and ER stress activation with palmitate and oleate administration of MIN6 cells. Cells were administered with palmitate (0.4 mmol/l) or oleate (0.4 mmol/l) for the indicated time points and harvested for whole-cell lysates to be used in Western blots. *A*: A time course of JNK activation with fatty acid administration, indicated by Western blotting for JNK phosphorylation and its target, c-Jun. Total JNK was probed as well. Thapsigargin administration for 8 h (300 nmol/l) was used as a positive control for JNK activation. *B*: BiP protein, phosphorylation of eIF2α, CHOP protein, Pdx1, and cleaved caspase 3 were assessed by Western blot during the time course of administration indicated. α-Tubulin was probed as a loading control. *C*: The effects of the addition of the ER stress inhibitor TUDCA on expression of cleaved caspase 3, phospho eIF2α, and CHOP 24 h after the addition of palmitate.

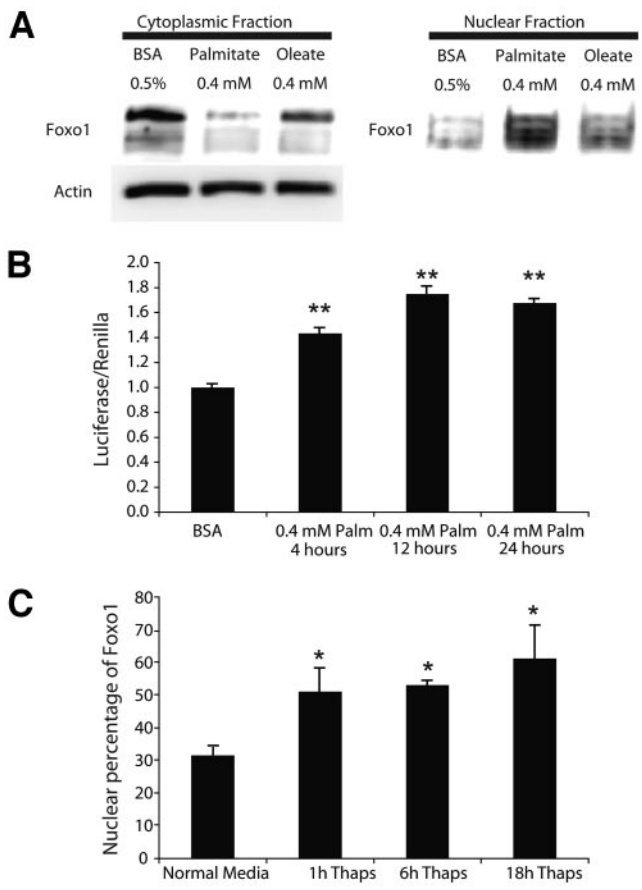

FIG. 4. Fatty acid administration increased Foxo1 nuclear localization and transcriptional activity at a time when Akt phosphorylation was increased. *A*: Western blot for total Foxo1 in cytoplasmic and nuclear protein fractions of MIN6 cells incubated for 4 h in 0.5% BSA alone or BSA with 0.4 mmol/l palmitate or oleate. *B*: Foxo1 activity and palmitate (Palm) administration. MIN6 cells cotransfected with the IGFBP1 promoter-luciferase and *Renilla* vectors were exposed to 0.4 mmol/l palmitate/0.5% BSA for the indicated times. **$P < 0.01$ compared with incubation in 0.5% BSA administration alone. *C*: Intracellular localization of GFP-tagged Foxo1 expressed in MIN6 cells is represented in a graph of the percentage of nuclear localization with administration of 1 μmol/l thapsigargin (Thaps) at the indicated hours of exposure. *$P < 0.05$.

with palmitate or oleate for 4 h, and the intracellular localization of Foxo1 was examined. As shown, fatty acids induced a shift of Foxo1 from the cytoplasm to the nucleus (Fig. 4*A*). Consistent with the increased effect of palmitate on apoptosis and intracellular signaling, palmitate administration induced more Foxo1 translocation than oleate administration. Using an IGFBP-1 promoter-luciferase construct containing a Foxo1 binding site (39), palmitate administration resulted in a sustained increase in endogenous Foxo1 transcriptional activity from 4 to 24 h of incubation (Fig. 4*B*). Next, the relationship between ER stress and Foxo1 transcriptional activity in insulinoma cells was assessed. We previously demonstrated that the ER stress agent thapsigargin reduced Akt phosphorylation (9). As shown in Fig. 4*C*, the addition of thapsigargin (1 μmol/l) to MIN6 cells resulted in significant cytosolic to nuclear translocation of GFP-tagged Foxo1 within 1 h of administration that was sustained up to 18 h. Using an IGFBP-1 promoter-luciferase construct containing a Foxo1 binding site (39), we assessed Foxo1 activity of MIN6 cells after 12 h of administration with two ER stress–inducing agents, thapsigargin (1 μmol/l) and tunicamycin (2 μg/ml). Consistent with an increase in Foxo1 nuclear translocation, a significant increase in Foxo1 activity was observed (data not shown). The results of these studies indicated that the effect of fatty acid administration to induce ER stress could contribute to activation of Foxo1.

JNK activity contributes to Foxo1 localization and apoptosis in palmitate-administered MIN6 cells. The data in Fig. 3 indicated that JNK activation is observed early after fatty acid administration. To test JNK's contribution to the action of fatty acid to induce Foxo1 and apoptotic markers, we used SP600125, a JNK-specific inhibitor (40). The addition of SP600125 resulted in a dose-dependent decrease of palmitate-induced phosphorylation of c-Jun along with expression of cleaved caspase 3 (Fig. 5*A*). Administration with 0.4 mmol/l palmitate resulted in an increase of nuclear Foxo1 at 4 h (Fig. 5*B*). A 1-h preincubation and continued incubation with 300

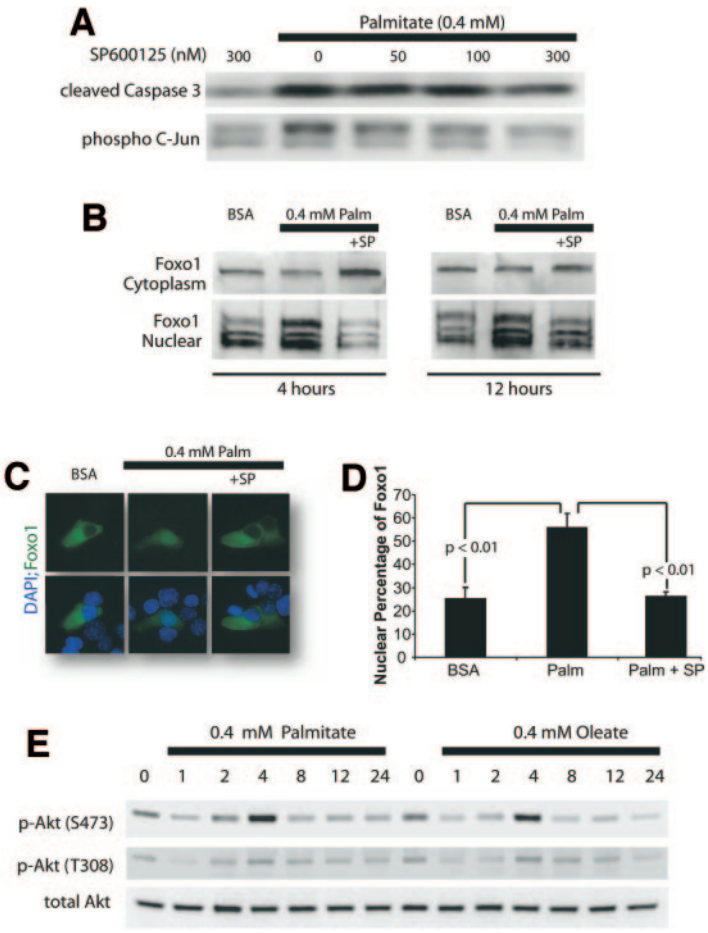

FIG. 5. JNK inhibition decreased nuclear Foxo1 localization and apoptosis with palmitate administration. *A*: Western blot of cleaved caspase 3 and phospho–c-Jun from MIN6 cells incubated in BSA, BSA and palmitate, and the indicated concentrations of SP600125 for 24 h. *B*: Western blot of total Foxo1 of nuclear and cytoplasmic protein extractions from MIN6 cells incubated for 4 and 12 h in either 0.5% BSA alone or BSA and 0.4 mmol/l palmitate (Palm) with or without the addition of 300 nmol/l SP610025 (SP), a concentration that provided JNK inhibition. *C*: Intracellular quantitation of GFP-tagged Foxo1 in MIN6 cells incubated for 4 h with BSA, palmitate, or palmitate and 300 nmol/l of SP600125. *D*: Cellular localization of GFP-tagged Foxo1 and DAPI staining by immunofluorescence. *E*: Akt phosphorylation was assessed by Western blot during the time course of administration with palmitate (0.4 mmol/l) or oleate (0.4 mmol/l).

nmol/l of SP600125 resulted in maintenance of Foxo1 in the cytoplasm and decreased Foxo1 in the nucleus, despite the presence of 0.4 mmol/l palmitate. A repeat of this experiment with a 12-h incubation yielded similar findings. Because cell fractionation can result in cross-contamination of nuclear and cytoplasmic proteins, to confirm these findings, the experiment was repeated by direct visualization with GFP-tagged Foxo1, showing significant cytosolic to nuclear translocation after 4-h administration with palmitate and a reduction after JNK inhibition with SP600125 (Fig. 5*C* and *D*).

These results demonstrated that JNK activation contributes an important part of fatty acid–induced cytoplasmic to nuclear translocation of Foxo1. JNK activation enhances transcriptional activity of Foxo1 via direct binding and active phosphorylation of Foxo1 (14). However, JNK could also activate Foxo1 indirectly through inhibition of insulin signaling (41). The latter mechanism was previously suggested because decreased Akt activity was measured 24 h after oleate administration of insulinoma cells, and overexpression of a constitutively active Akt prevented oleate-induced apoptosis (8). We confirmed a decreased Akt phosphorylation 24 h after fatty acid administration (Fig. 2*B*). However, the time course of Akt phosphorylation after fatty acid administration shown in Fig. 5*E* was complex. An early decrease at 1 h after fatty acid administration was consistently followed by increased phosphorylation at 4 h when Foxo1 activity was increased. Thus, the JNK-dependent Foxo1 activation (Fig. 5*B*) appeared to reflect a direct action of JNK rather than JNK effects to decrease insulin signaling. We have observed a time course for glycogen synthase kinase 3β (Gsk3β) phosphorylation that is very similar to that of Akt phosphorylation (data not shown). This observation supports our interpretation that Akt is phosphorylated/activated after its early transient dephosphorylation and that this does not coincide with FoxO retention in the cytoplasm. The progressive decline in Akt phosphorylation after 4 h (Fig. 5*E*) suggested that decreased insulin signaling likely contributes additionally to JNK-induced Foxo1 activation at later time points.

Inhibiting Foxo1 activity reduces ER stress and palmitate-induced apoptosis in MIN6 cells. To directly examine the contribution of Foxo1 to fatty acid–induced ER stress and cell death, we used an adenoviral construct of an hemaglutinin-tagged dominant-negative Foxo (AdV-DNFoxo) and evaluated its effects on ER stress– and fatty acid–induced apoptosis in MIN6 cells. This DNFoxo is a truncated allele of Foxo1 that retains the DNA-binding domain (amino acid residues 1–256) but lacks the transactivation domain (23). This construct was created to examine the contribution of the three Foxo proteins expressed in β-cells under normal conditions, and with loss of β-cell mass in insulin-resistant models. This allele has been demonstrated to function as a dominant-negative inhibitor of endogenous Foxo activity in the liver (42), in myoblasts (43), and in the β-cell (17,18). MIN6 cells transfected with 200 MOI of the DNFoxo adenovirus exhibited a nearly 60% reduction in propidium iodide staining and cell death when exposed to 100 nmol/l thapsigargin, compared with that in control GFP adenovirus (AdV-GFP)-transfected cells (Fig. 6*A* and *B*). Similar protection against cell death was also observed with palmitate administration of MIN6 cells (Fig. 6*C* and *D*). The effects of adenoviral administration after 24 h of palmitate were further examined by Western blot analysis of protein extracts (Fig. 6*E*). Increasing the MOI of the adenovirus resulted in augmented expression of the hemaglutinin-tagged DNFoxo protein, as indicated by Western blot using a hemaglutinin antibody. Increasing titers of the AdV-DNFoxo in cells exposed to 0.4 mmol/l palmitate for 24 h resulted in decreased expression of CHOP, along with a significant reduction in cleaved caspase 3 compared with cells similarly administered with palmitate but transfected with AdV-GFP. These results correlate with the observation of DNFoxo reducing the percentage of propidium iodide–positive cells (Fig. 6*D*). They support the interpretation that Foxo1 activation is important in mediating cell death after ER stress and fatty acid administration.

RIP-DNFoxo islets resist ex vivo stimuli of apoptosis. The results obtained with MIN6 cells expressing DNFoxo were confirmed with primary islets, as shown in Fig. 7. Islets were isolated from transgenic RIP-DNFoxo mice expressing the dominant-negative Foxo driven by the rat insulin promoter and were cultured in the presence of 10

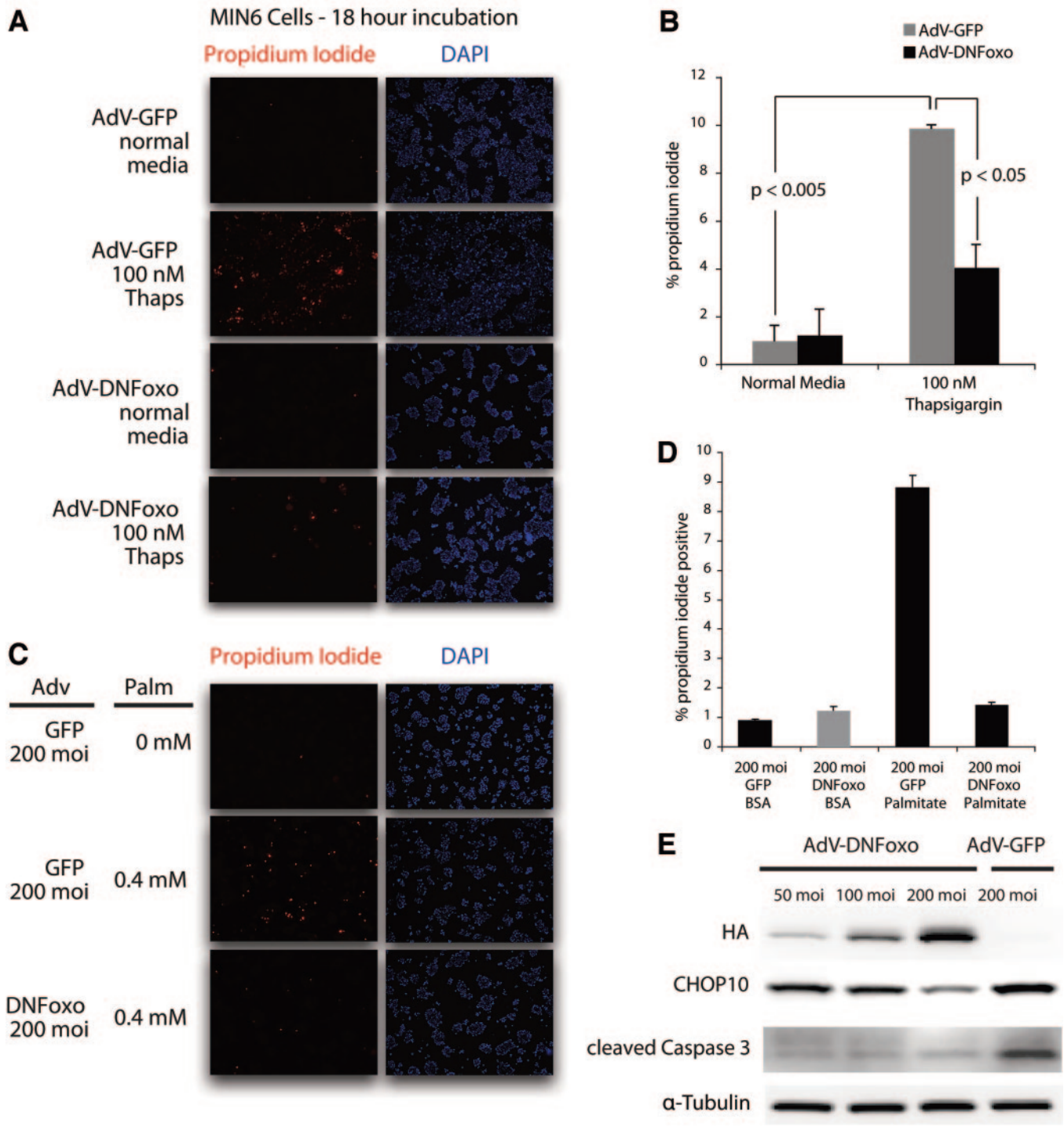

FIG. 6. Dominant-negative Foxo1 expression decreased thapsigargin and palmitate-induced cell death. *A*: Propidium iodide and DAPI staining of MIN6 cells infected with either GFP or DNFoxo adenovirus (Adv-GFP and Adv-DNF, respectively) and exposed for 18 h in either normal culture media or media plus the addition of 100 nmol/l thapsigargin. *B*: Cell death from thapsigargin as quantitated by the percentage of nuclei staining for propidium iodide and DAPI in three independent experiments. *C*: Propidium iodide and DAPI staining of cells infected with either GFP or DNFoxo adenovirus and incubation for 24 h in either 0.5% BSA alone or BSA and 0.4 mmol/l palmitate. *D*: Quantitation of cell death by 0.4 mmol/l palmitate in GFP or DNFoxo virally infected MIN6 cells from three independent experiments. Significance is indicated within the figures of quantitation. *E*: Western blot of cleaved caspase 3, CHOP, and hemaglutinin expression of MIN6 cells infected with the GFP adenovirus and increasing titers of DNFoxo adenovirus and incubated in 0.4 mmol/l palmitate and 0.5% BSA for 24 h.

μmol/l thapsigargin for 48 h to induce ER stress. This resulted in cleaved caspase 3 expression only in wild-type islets (Fig. 7*A*). The resistance to apoptosis in RIP-DNFoxo islets also applied to fatty acid administration. Isolated islets from wild-type and RIP-DNFoxo mice cultured in the presence of 0.4 mmol/l palmitate for 24 h exhibited similar JNK phosphorylation without changes in total JNK protein. (Fig. 7*B*). However, protein expression of the proapoptotic transcription factor CHOP was only observed in wild-type islets. RIP-DNFoxo islets also resisted caspase 3 activation by palmitate, which was reproduced with wild-type primary islets. Additionally, the abundance

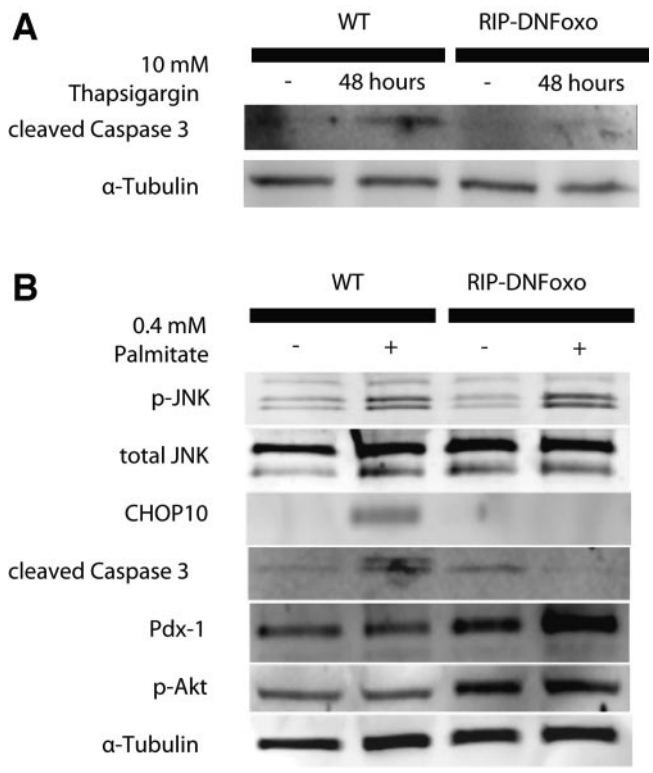

FIG. 7. Ex vivo administration of wild-type (WT) and RIP-DNFoxo islets with thapsigargin and palmitate. *A*: Western blot for cleaved caspase 3 expression and α-tubulin expression as a loading control of pooled isolated islets from each genotype, which were divided into two culture conditions with and without 10 μmol/l thapsigargin for 48 h. *B*: Immunoblotting for JNK activation, caspase activation, CHOP, phosphorylated-Akt, and Pdx1 in pooled islets from wild-type and RIP-DNFoxo mice cultured for 24 h in RPMI containing 0.5% BSA with or without 0.4 mmol/l palmitate

of phosphorylated Akt and the transcription factor Pdx1 were elevated in RIP-DNFoxo islets compared with similarly administered wild-type islets.

Microarray analysis reveals that most of the genes regulated by palmitate administration are Foxo dependent. To define the contribution of Foxo activation to fatty acid regulation of gene expression relevant to apoptosis, we performed two parallel microarray experiments. The first experiment compared expression profiles in cells administered or not administered with fatty acid and infected with Adv-GFP. The second experiment further evaluated the gene expression profiles of MIN6 cells in fatty acid–administered and unadministered cells but infected with AdV-DNFoxo. This latter experiment permitted comparison of the effects of fatty acids in the presence and absence of Foxo signaling. Expression profiles of ~16,000 genes were assessed. After 24-h administration with 0.4 mmol/l palmitate and AdV-GFP, 124 genes were significantly regulated (see RESEARCH DESIGN AND METHODS) more than twofold (91 genes upregulated and 33 downregulated). The Foxo-dependent fatty acid–regulated genes were clustered according to their ontology physiological processes (27,44). The degree of activation of the individual regulated genes and the effects of administration with AdV-DNFoxo are illustrated in Fig. 8. There was a pronounced participation of genes involved primarily in cellular metabolism, cellular physiology, and transport. These included a number of genes that could influence fatty acid–induced apoptosis. Immediately apparent was that the overwhelming majority of genes that were regulated by palmitate in the control condition were not regulated to the same extent when Foxo was inhibited, suggesting that the fatty acid–induced transcriptional regulation of these genes is predominantly Foxo dependent (Supplemental Fig. S2). The magnitude of expression of the genes up- and downregulated by palmitate in control conditions (AdV-GFP) and in the presence of AdV-DNFoxo are further illustrated in Supplemental Figs. S2*A* and *B*. These results were validated by an independent assessment of changes in mRNA by quantitative RT-PCR on selected transcripts (Supplemental Fig. S3).

Foxo1 regulates ER stress– and fatty acid–induced CHOP (DNA damage–inducible transcript 3) expression. One of the genes that contained multiple putative conserved Foxo binding sites whose transcription was activated by fatty acid administration was CHOP (DNA damage–inducible transcript 3 [Ddit3]) (Fig. 8). Previous studies had noted an association between fatty acid–induced ER stress and CHOP expression in insulinoma cells (7,30). We previously studied genes activated in MIN6 insulinoma cells on nutrient withdrawal by microarray analysis and noted that the most highly expressed gene was Ddit3 (CHOP, Gadd153). Observing a conserved canonical Foxo binding site in the CHOP promoter, we demonstrated with a CHOP-luciferase assay increased transcription of CHOP with nutrient withdrawal that was inhibited by coexpression with a plasmid expressing DN-Foxo (17). This established that CHOP-induced expression with nutrient withdrawal was Foxo dependent. In the current studies, we sought to determine how fatty acid– and ER stress–induced CHOP expression and Foxo activation were related. MIN6 cells were transfected with a luciferase construct driven by 10 kb of the CHOP promoter (45), either an empty pCMV vector or pCMV-DNFoxo, and a thymidine kinase promoter driving *Renilla* expression as a control vector. CHOP promoter activity was induced in cells administered with 0.4 mmol/l palmitate for 24 h, as shown in Fig. 9. Cotransfection of the DNFoxo plasmid significantly reduced CHOP promoter activity in a dose-dependent manner. Nonspecific effects of pCMV-DNFoxo on CHOP-luciferase were corrected by normalization with inclusion of pTK-*Renilla*. These results clearly demonstrated that ER stress–induced CHOP expression is regulated at least in part by Foxo activation. In the absence of mutation of the Foxo binding sites in the CHOP promoter, however, these results are consistent with an indirect effect of Foxo activation on CHOP expression.

DISCUSSION

This study was designed to examine the sequence of molecular signaling events in fatty acid–induced apoptosis in insulinoma cells and islets and to test the hypothesis that Foxo1 activation plays a significant role in this process. Fatty acids have been shown to contribute to β-cell apoptosis by decreasing insulin/Akt signaling (7,8). Only one study examined Foxo1 involvement in this process, measuring changes in phosphorylation of Foxo1 as an indicator of protein activity but not definitively or directly implicating Foxo in the process (8). In the current study, Foxo involvement is documented in a definitive way, showing changes in its nuclear localization and its role in transcriptional effects of fatty acids. In addition, these studies determined the earliest events in fatty acid–induced apoptosis and documented the involvement of

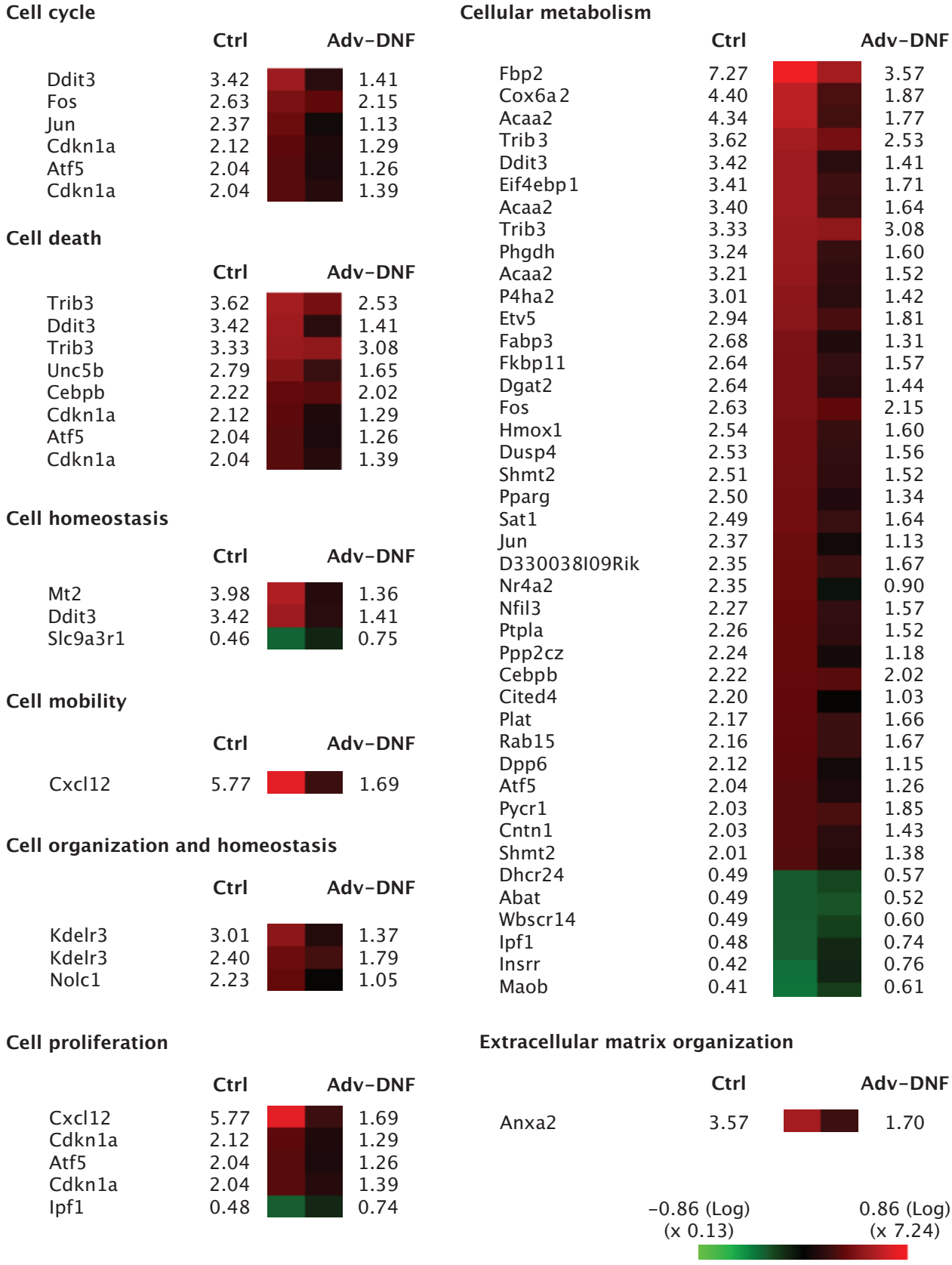

FIG. 8. Expression profiles of all genes regulated by fatty acids in control (Ctrl) conditions or with Adv-DNF. For each gene significantly regulated by fatty acids in the control (Adv-GFP) condition, the fold change is indicated in the fatty acids–control column and the corresponding regulation in the presence of the Adv-DNFoxo is indicated in the fatty acids-DNFoxo column. A heat-map provides a visual representation of the regulation in both conditions. Genes are clustered according to their gene ontology (cellular physiological processes) functions. Some of the genes belong to more than one category. A scale bar indicates the extent of the fold changes in the heat-map (in Log10 scale).

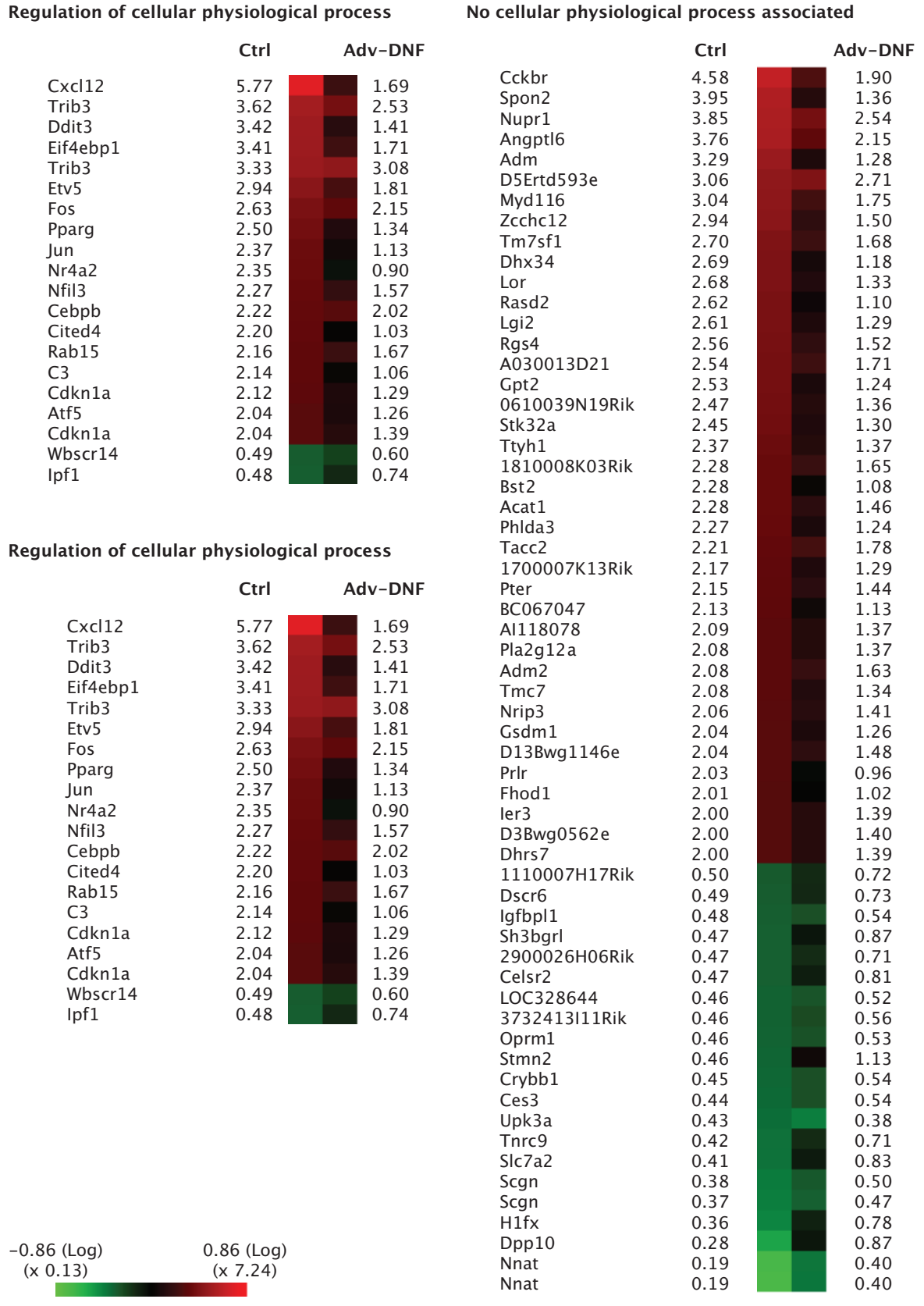

FIG. 8—Continued.

JNK (as opposed to Akt) in the early action of fatty acid to induce nuclear localization of Foxo1. Compelling evidence is presented to indicate that ER stress via activation of JNK and Foxo1 contributes to the apoptotic outcome after fatty acid administration. Finally, novel evidence shows that fatty acids alter expression of a large number of

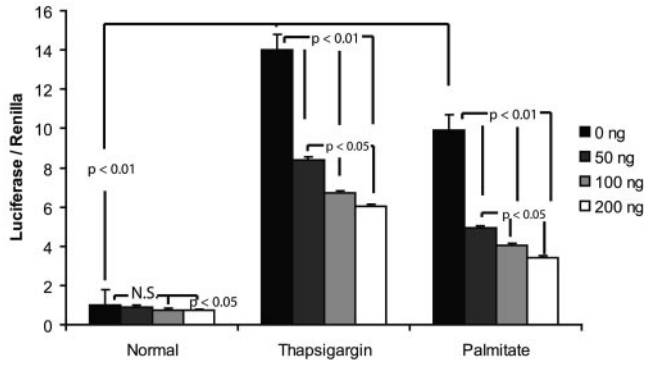

FIG. 9. Foxo1 activity regulates CHOP promoter activity. CHOP promoter activity at 24 h of thapsigargin (100 nmol/l) or palmitate (0.4 mmol/l with 0.5% BSA) administration with cotransfection of empty pCMV5 plasmid or pCMV5-DNFoxo (as indicated), as measured by a luciferase expression and normalized to TK-*Renilla* expression.

proapoptotic genes in islets and that many of these effects are mediated by Foxo1.

Administration of MIN6 cells with palmitate or oleate at various concentrations established a pattern of intracellular signaling that was common to both fatty acids. The data indicated that both fatty acids regulate the same signaling events, with palmitate being more potent possibly as a result of its less effective processing by esterification (6,30,31). In various mammalian cell lines including β-cells, lower fatty acid toxicity was associated with increased cellular capacity for triglyceride synthesis and with upregulation of stearoyl CoA desaturase 1 (SCD1) (31,46). SCD1 functions in fatty acid desaturation before incorporation into triglycerides, and its expression was shown to be downregulated by Foxo1 (47). This effect of Foxo1 could contribute to cell death indirectly by inhibiting fatty acid incorporation into the neutral triglyceride pool, consequently increasing available fatty acids and the potential for accumulation of toxic metabolites such as ceramides (48,49). Possibly, fatty acid activation of Foxo1 with subsequent inhibition of SCD1 would create a self-sustained cycle with deleterious cellular effects (31,46, 48,49). The relatively greater effects of the saturated fatty acid on Foxo1 activation and apoptosis may have relevance to nutritional treatment of patients with type 2 diabetes.

A time course of administration with either palmitate or oleate in MIN6 cells suggested that JNK phosphorylation precedes markers of ER stress and apoptosis, including phosphorylation of eIF2α, expression of CHOP, and the eventual increase of cleaved caspase 3. In addition, JNK inhibition decreased nuclear Foxo1 along with the reduction in fatty acid–induced apoptosis. These findings position JNK as an important, early participant upstream of Foxo1 in fatty acid activation of the proapoptotic pathway. Previous studies on islets and insulinoma cells demonstrated JNK activation via oxidative stress, cytokines, and ER stress–inducing agents (9,18,50), but the underlying mechanisms remain unclear. A recent study demonstrated palmitate binding to the proinflammatory TLR2 (Toll-like receptor 2), leading to enhanced JNK activation and insulin resistance in murine myocytes (51). Another report documented dependence of JNK activation in macrophages on signaling via the membrane fatty acid translocase CD36 (75).

The important role of decreased insulin signaling and Akt phosphorylation in fatty acid–induced β-cell apoptosis has been reported previously (8). However, our data would indicate that early activation of Foxo1, which would set in motion the apoptotic program after fatty acids, is not dependent on Akt activity. The transient apparent increase in Akt activity at 4 h after fatty acid administration is unexplained, although it could be secondary to the acute stimulatory effect of fatty acids on insulin secretion (5). Alternatively, a recent report has described a complex interaction between the activities of Foxo and Akt (52). Administration of primary hepatocytes with either wild-type, constitutively nuclear, or dominant-negative Foxo adenovirus resulted in activation of protein kinase B pathways. In the current report, at 4 h after fatty acid administration, a time when Foxo1 was observed to be nuclear and transcriptionally active, we also observed increased phosphorylation of Akt (Fig. 3*B*). Possibly JNK-activated Foxo1 may have contributed to this transient increase in Akt phosphorylation. We repeatedly observed decreased Akt phosphorylation after 24 h of fatty acid administration, a finding consistent with that in another insulinoma cell line (8). Taken together, these results suggest that reduced Akt phosphorylation chronically contributes to maintenance of the early, direct activation of Foxo1 by JNK.

Whereas the current results show that fatty acid administration results in increased Foxo activity, previous studies (8) have shown that fatty acid administration results in reduced phosphorylation of another Akt substrate, Gsk3β, that could also contribute to apoptosis. In preliminary studies we have confirmed that palmitate and oleate administration of MIN6 cells results in reduced phosphorylation of Gsk3β, and we extended these studies to show that inhibitors of Gsk3β activity reduced fatty acid–induced apoptosis (76). These combined results thus indicate that reduced insulin signaling leads to activation of at least two proapoptotic proteins, Foxo and Gsk3β, that contribute to the deleterious effects of fatty acid on β-cells.

A total of 2,500 bp of the promoter regions upstream of the start codon for all of the fatty acid regulated genes were assessed in the mouse, rat, and human genomes for those that contained an evolutionarily conserved 7-bp sequence (T[A/G]TT[T/C][A/C]C) encoding Forkhead binding sites (53,54). By chance alone, the consensus binding site can be found once every 1,024 bp ($4^4 \times 2^3 \times 0.5$). The number of putative Forkhead binding sites for each gene is represented in Supplemental Table 1 by an asterisk appended to the symbol of the gene. Notable is that very few genes in this group had fewer than two potential Foxo binding sites. However, whether these genes are directly regulated by Foxo binding can only be determined by direct experimentation.

The fatty acid–induced genes that could be mediating apoptosis in β-cells were examined by a whole-genome expression study. We found that >100 genes exhibited a more than twofold up- or downregulation in expression after 24 h of administration with palmitate (Fig. 8). Validation of these expression results is suggested by the concomitant observation of fatty acid–reduced expression of Pdx1 and increased expression of CHOP proteins by Western analysis (Figs. 2*C* and 3*B*) and by RT-PCR of selected transcripts (Supplemental Fig. S3). A number of fatty acid–regulated genes that may contribute to reduce insulin signaling or secretion were noted. Trib3 (Trb3) is a known inhibitor of Akt activation and potentiator of apoptosis that has been incriminated in the insulin resis-

tance of type 2 diabetes (55). Its 3.6-fold induction by fatty acids suggests the involvement of Trib3 in fatty acid impairment of insulin signaling. Trib3 was also identified as a target of ER stress–induced apoptosis in human embryonic kidney (293) cells (56). Furthermore, it was shown that Trib3 was downstream of CHOP activation and that upregulation of Trib3 by ER stress was required for apoptosis. The hypothesis that Trib3 is downstream of fatty acid–induced ER stress– and CHOP-mediated apoptosis in insulinoma cells can now be tested.

Other fatty acid–regulated genes included Eif4ebp1, a protein that binds Eif4E to inhibit translation. Phosphorylation of Eif4ebp1 leading to its dissociation from Eif4E has been implicated at least in part in insulin-mediated enhancement of translation and cell growth (57). Similarly, cyclin-dependent kinase inhibitor 1A (Cdkn1a or p21) is a protein that binds to cyclin complexes and inhibits cell cycle progression (58), and its activation is associated with ER stress and apoptosis of β-cells (59). Peroxisome proliferator–activated receptor-γ is a transcription factor that senses and regulates lipid metabolism. Its induction in β-cells has been associated with increased fatty acid uptake, triglyceride synthesis, and inhibited insulin secretion (58,60–62).

Remarkably, regulation of >95% of the fatty acid–sensitive genes was blunted or completely reversed with concomitant overexpression of dominant-negative Foxo1. For example, a sixfold induction by fatty acids of Cxcl12 (chemokine [C-X-C motif] ligand 12), a proinflammatory cytokine responsive to lipopolysaccharides, tumor necrosis factor, or interleukin 1 (63), was blunted by 85%. The threefold upregulation of Eif4ebp1 was reduced by 70%. Genes upregulated by palmitate administration that exhibited blunted response in the presence of dominant-negative Foxo1 also included Dgat2 (diacylglycerol O-acyltransferase 2), responsible for conversion of diglycerides to triglycerides (64), and Acaa2 (acetyl-CoA acyltransferase 2), which catalyzes the last step of mitochondrial β-oxidation (62,65).

Among the genes downregulated by fatty acid administration that may contribute to β-cell apoptosis, Pdx1 is especially interesting. There was a twofold reduction in Pdx1 gene expression after palmitate administration that correlated with the decrease in Pdx1 protein (Fig. 2*C*). Fatty acid reduction of Pdx1 expression was blunted by 69% with ADv-DNFoxo administration, documenting Foxo dependence of the effect. Previous work had shown that insulin receptor substrate-2 deficiency was associated with Pdx deficiency, and in this model β-cell destruction was corrected by either Foxo haploinsufficiency or overexpression of Pdx1 (61). Together, these results emphasize the link between fatty acid/ER stress reduction of insulin signaling, Foxo activation, and subsequent Pdx regulation and apoptosis.

We examined potential mechanisms for the Foxo1-dependent induction or repression of genes by fatty acids. β-Cells have a highly developed and active ER to maintain insulin secretion, which translates to a high susceptibility to stress (66). Foxo1 has been shown to contribute to apoptosis through transcription of several proapoptotic genes, such as Bim, FasL, and TRAIL (54). Our study identifies a novel mechanism by which Foxo1 could promote β-cell apoptosis through its activation of CHOP (66). Conditions that increase CHOP expression have consistently been associated with ER stress, decreased insulin signaling, and apoptosis in β-cells (7,9). Disruption of the CHOP gene delayed but did not prevent the onset of hyperglycemia and β-cell death in an in vivo model of pancreatic β-cell ER stress, the Akita mouse, heterozygous for a point mutation in the insulin 2 gene (Ins2C96Y) (67). Thus, these results suggest redundancy in ER stress–mediated pathways leading to apoptosis, potentially exclusive of, or in addition to, Foxo1 and CHOP.

This study documented the role of FoxO activation by fatty acids or ER stress in mediating β-cell death. The data do not rule out effects of fatty acid and oxidative stress in inducing mitochondrial dysfunction that triggers programmed cell death, as reviewed recently (68,69). There is limited information related to Foxo proteins and mitochondrial stress, but a recent study suggested differential roles of Foxo1 and Foxo3a. Silencing of Foxo1 did not alter susceptibility of endometrial cells to oxidative cell death, whereas silencing of Foxo3a prevented apoptosis induced by hydrogen peroxide (70).

Our findings suggest that further in vivo characterization of the role of Foxo1 within β-cells is merited especially in the context of the β-cell failure associated with diabetes. Prolonged administration of islets or insulinoma cells with fatty acids results in decreased glucose-stimulated insulin secretion (5,71). Foxo1 may mediate some of this effect as it contributes to the phenotype of insulin resistance by increasing fatty acid utilization and decreasing glucose utilization in skeletal muscle and by increasing gluconeogenesis in the liver (22,72,73). Foxo1 activation in β-cells could likewise downregulate glucose metabolism and thus insulin secretion and synthesis. Nutrient withdrawal and decreased insulin signaling have previously been shown to activate Foxo1 in β-cells (17), and now we extend the agonists of Foxo1 to include fatty acids and pharmacological ER stress inducers. We were able to significantly decrease β-cell apoptosis from ER stress or fatty acids by overexpressing a dominant-negative Foxo1. Inhibiting Foxo1 could be clinically relevant for promoting the survival of β-cells in obesity and diabetes, conditions associated with insulin resistance, increased serum fatty acid levels, and ER stress. These results thus identify Foxo1 as a potential new therapeutic target for preservation of β-cell mass and function.

ACKNOWLEDGMENTS

This work was supported in part by an National Institutes of Health (NIH) grant (R37 DK16746, to M.A.P.), a Ruth L. Kirschstein Pre-Doctoral National Research Service Award (F32 DK06537, to S.C.M.), and a grant from the Philip Morris USA External Research Program. The Washington University Diabetes Research and Training Center (NIH P60 DK20579) is acknowledged for assistance from the Immunoassay, Morphology, and Transgenic Mouse Cores, and the Adipocyte Biology Core, Clinical Nutrition Research Unit (NIH DK56351).

The authors thank H. Harding and D. Ron (New York University) for reagents, D. Accili (Columbia) for insightful comments and reagents, K. Polonsky for helpful comments on the experiments, Cris M. Welling for lab assistance, and other members of the Permutt lab for helpful discussions.

REFERENCES

1. Marchetti P, Del Prato S, Lupi R, Del Guerra S: The pancreatic beta-cell in human type 2 diabetes. *Nutr Metab Cardiovasc Dis* 16 (Suppl. 1):S3–S6, 2006
2. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid

cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785–789, 1963
3. Newsholme P, Keane D, Welters HJ, Morgan NG: Life and death decisions of the pancreatic beta-cell: the role of fatty acids. *Clin Sci* (*Lond*) 112:27–42, 2007
4. Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J Clin Invest* 93:870–876, 1994
5. Noushmehr H, D'Amico E, Farilla L, Hui H, Wawrowsky KA, Mlynarski W, Doria A, Abumrad NA, Perfetti R: Fatty acid translocase (FAT/CD36) is localized on insulin-containing granules in human pancreatic beta-cells and mediates fatty acid effects on insulin secretion. *Diabetes* 54:472–481, 2005
6. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY: Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* 50:69–76, 2001
7. Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL: Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology* 145:5087–5096, 2004
8. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ: Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* 277:49676–49684, 2002
9. Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Bernal-Mizrachi E, Permutt MA: Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3{beta} in mouse insulinoma cells. *Diabetes* 54:968–975, 2005
10. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306:457–461, 2004
11. Nguyen MT, Satoh H, Favelyukis S, Babendure JL, Imamura T, Sbodio JI, Zalevsky J, Dahiyat BI, Chi NW, Olefsky JM: JNK and tumor necrosis factor-alpha mediate free fatty acid-induced insulin resistance in 3T3–L1 adipocytes. *J Biol Chem* 280:35361–35371, 2005
12. Aguirre V, Uchida T, Yenush L, Davis R, White MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275: 9047–9054, 2000
13. Matsumoto M, Accili D: All roads lead to FoxO. *Cell Metab* 1:215–216, 2005
14. Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, Burgering BM: FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *Embo J* 23:4802–4812, 2004
15. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333–336, 2002
16. Biggs WH 3rd, Meisenhelder J, Hunter T, Cavenee WK, Arden KC: Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* 96:7421–7426, 1999
17. Martinez SC, Cras-Meneur C, Bernal-Mizrachi E, Permutt MA: Glucose regulates Foxo1 through insulin receptor signaling in the pancreatic islet β-cell. *Diabetes* 55:1581–1591, 2006
18. Kawamori D, Kaneto H, Nakatani Y, Matsuoka TA, Matsuhisa M, Hori M, Yamasaki Y: The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. *J Biol Chem* 281:1091–1098, 2006
19. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K: Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
20. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA: Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest* 108:1631–1638, 2001
21. Girish V, Vijayalakshmi A: Affordable image analysis using NIH Image/ImageJ. *Indian J Cancer* 41:47, 2004
22. Nakae J, Kitamura T, Silver DL, Accili D: The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* 108:1359–1367, 2001
23. Nakae J, Barr V, Accili D: Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR. *Embo J* 19:989–996, 2000
24. Dandoy-Dron F, Itier JM, Monthioux E, Bucchini D, Jami J: Tissue-specific expression of the rat insulin 1 gene in vivo requires both the enhancer and promoter regions. *Differentiation* 58:291–295, 1995
25. Dandoy-Dron F, Monthioux E, Jami J, Bucchini D: Regulatory regions of rat insulin I gene necessary for expression in transgenic mice. *Nucleic Acid Res* 19:4925–4930, 1991
26. Diehn M, Sherlock G, Binkley G, Jin H, Matese JC, Hernandez-Boussard T, Rees CA, Cherry JM, Botstein D, Brown PO, Alizadeh AA: SOURCE: a unified genomic resource of functional annotations, ontologies, and gene expression data. *Nucleic Acid Res* 31:219–223, 2003
27. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G: Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 25:25–29, 2000
28. Sturn A, Quackenbush J, Trajanoski Z: Genesis: cluster analysis of microarray data. *Bioinformatics* 18:207–208, 2002
29. Mancuso DJ, Sims HF, Han X, Jenkins CM, Guan SP, Yang K, Moon SH, Pietka T, Abumrad NA, Schlesinger PH, Gross RW: Genetic ablation of calcium-independent phospholipase A2gamma leads to alterations in mitochondrial lipid metabolism and function resulting in a deficient mitochondrial bioenergetic phenotype. *J Biol Chem* 282:34611–34622, 2007
30. Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M, Volchuk A: Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. *Endocrinology* 147:3398–3407, 2006
31. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV Jr, Ory DS, Schaffer JE: Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A* 100:3077–3082, 2003
32. Corcoran MP, Lamon-Fava S, Fielding RA: Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. *Am J Clin Nutr* 85:662–677, 2007
33. Johnson JD, Ahmed NT, Luciani DS, Han Z, Tran H, Fujita J, Misler S, Edlund H, Polonsky KS: Increased islet apoptosis in Pdx1+/- mice. *J Clin Invest* 111:1147–1160, 2003
34. Hagman DK, Hays LB, Parazzoli SD, Poitout V: Palmitate inhibits insulin gene expression by altering PDX-1 nuclear localization and reducing MafA expression in isolated rat islets of Langerhans. *J Biol Chem* 280:32413–32418, 2005
35. Keramaris E, Vanderluit JL, Bahadori M, Mousavi K, Davis RJ, Flavell R, Slack RS, Park DS: c-Jun N-terminal kinase 3 deficiency protects neurons from axotomy-induced death in vivo through mechanisms independent of c-Jun phosphorylation. *J Biol Chem* 280:1132–1141, 2005
36. Urano F, Bertolotti A, Ron D: IRE1 and efferent signaling from the endoplasmic reticulum. *J Cell Sci* 113 (Pt. 21):3697–3702, 2000
37. Holcik M, Sonenberg N: Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* 6:318–327, 2005
38. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS: Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 313:1137–1140, 2006
39. Ooi GT, Tseng LY, Tran MQ, Rechler MM: Insulin rapidly decreases insulin-like growth factor-binding protein-1 gene transcription in streptozotocin-diabetic rats. *Mol Endocrinol* 6:2219–2228, 1992
40. Bogoyevitch MA, Boehm I, Oakley A, Ketterman AJ, Barr RK: Targeting the JNK MAPK cascade for inhibition: basic science and therapeutic potential. *Biochim Biophys Acta* 1697:89–101, 2004
41. Lee YH, Giraud J, Davis RJ, White MF: c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. *J Biol Chem* 278:2896–2902, 2003
42. Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, Meseck M, Accili D, Dong H: Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. *Am J Physiol Endocrinol Metab* 285:E718–E728, 2003
43. Hribal ML, Nakae J, Kitamura T, Shutter JR, Accili D: Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* 162:535–541, 2003
44. Lambrix P, Habbouche M, Perez M: Evaluation of ontology development tools for bioinformatics. *Bioinformatics* 19:1564–1571, 2003
45. Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J: A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 307:935–939, 2005
46. Busch AK, Gurisik E, Cordery DV, Sudlow M, Denyer GS, Laybutt DR, Hughes WE, Biden TJ: Increased fatty acid desaturation and enhanced expression of stearoyl coenzyme A desaturase protects pancreatic beta-cells from lipoapoptosis. *Diabetes* 54:2917–2924, 2005
47. Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R,

Xiao X, Franks R, Heidenreich KA, Sajan MP, Farese RV, Stolz DB, Tso P, Koo SH, Montminy M, Unterman TG: FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. *J Biol Chem* 281:10105–10117, 2006
48. Kelpe CL, Moore PC, Parazzoli SD, Wicksteed B, Rhodes CJ, Poitout V: Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. *J Biol Chem* 278:30015–30021, 2003
49. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY: Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* 52:726–733, 2003
50. Storling J, Binzer J, Andersson AK, Zullig RA, Tonnesen M, Lehmann R, Spinas GA, Sandler S, Billestrup N, Mandrup-Poulsen T: Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt. *Diabetologia* 48:2039–2050, 2005
51. Senn JJ: Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. *J Biol Chem* 281:26865–26875, 2006
52. Naimi M, Gautier N, Chaussade C, Valverde AM, Accili D, Van Obberghen E: Nuclear forkhead box O1 controls and integrates key signaling pathways in hepatocytes. *Endocrinology* 148:2424–2434, 2007
53. O'Brien RM, Streeper RS, Ayala JE, Stadelmaier BT, Hornbuckle LA: Insulin-regulated gene expression. *Biochem Soc Trans* 29:552–558, 2001
54. Greer EL, Brunet A: FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 24:7410–7425, 2005
55. Du K, Herzig S, Kulkarni RN, Montminy M: TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* 300:1574–1577, 2003
56. Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H: TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *Embo J* 24:1243–1255, 2005
57. Shen WH, Boyle DW, Wisniowski P, Bade A, Liechty EA: Insulin and IGF-I stimulate the formation of the eukaryotic initiation factor 4F complex and protein synthesis in C2C12 myotubes independent of availability of external amino acids. *J Endocrinol* 185:275–289, 2005
58. Rowland BD, Peeper DS: KLF4, p21 and context-dependent opposing forces in cancer. *Nat Rev Cancer* 6:11–23, 2006
59. Yamada T, Ishihara H, Tamura A, Takahashi R, Yamaguchi S, Takei D, Tokita A, Satake C, Tashiro F, Katagiri H, Aburatani H, Miyazaki J, Oka Y: WFS1-deficiency increases endoplasmic reticulum stress, impairs cell cycle progression and triggers the apoptotic pathway specifically in pancreatic beta-cells. *Hum Mol Genet* 15:1600–1609, 2006
60. Parton LE, Diraison F, Neill SE, Ghosh SK, Rubino MA, Bisi JE, Briscoe CP, Rutter GA: Impact of PPARgamma overexpression and activation on pancreatic islet gene expression profile analyzed with oligonucleotide microarrays. *Am J Physiol Endocrinol Metab* 287:E390–E404, 2004
61. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH 3rd, Wright CV, White MF, Arden KC, Accili D: The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest* 110:1839–1847, 2002
62. Drucker DJ, Nauck MA: The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 368:1696–1705, 2006
63. Zlotnik A: Chemokines and cancer. *Int J Cancer* 119:2026–2029, 2006
64. Millar JS, Stone SJ, Tietge UJ, Tow B, Billheimer JT, Wong JS, Hamilton RL, Farese RV Jr, Rader DJ: Short-term overexpression of DGAT1 or DGAT2 increases hepatic triglyceride but not VLDL triglyceride or apoB production. *J Lipid Res* 47:2297–2305, 2006
65. Kim JJ, Battaile KP: Burning fat: the structural basis of fatty acid beta-oxidation. *Curr Opin Struct Biol* 12:721–728, 2002
66. Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR: The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* 22:3864–3874, 2002
67. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M: Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 109:525–532, 2002
68. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B: Mitochondria, oxidative stress and cell death. *Apoptosis* 12:913–922, 2007
69. Riedl SJ, Salvesen GS: The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol* 8:405–413, 2007
70. Kajihara T, Jones M, Fusi L, Takano M, Feroze-Zaidi F, Pirianov G, Mehmet H, Ishihara O, Higham JM, Lam EW, Brosens JJ: Differential expression of FOXO1 and FOXO3a confers resistance to oxidative cell death upon endometrial decidualization. *Mol Endocrinol* 20:2444–2455, 2006
71. Milburn JL Jr, Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, BeltrandelRio H, Newgard CB, Johnson JH, Unger RH: Pancreatic beta-cells in obesity: evidence for induction of functional, morphologic, and metabolic abnormalities by increased long chain fatty acids. *J Biol Chem* 270:1295–1299, 1995
72. Bastie CC, Nahle Z, McLoughlin T, Esser K, Zhang W, Unterman T, Abumrad NA: FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. *J Biol Chem* 280:14222–14229, 2005
73. Nakae J, Kitamura T, Kitamura Y, Biggs WH 3rd, Arden KC, Accili D: The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev Cell* 4:119–129, 2003
74. Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA: Glucose promotes pancreatic islet beta-cell survival through a PI3-kinase/Akt-signaling pathway. *Am J Physiol Endocrinol Metab* 283:784–793, 2002
75. Rahaman SO, Lennon DJ, Febbraio M, Podrez EA, Hazen SL, Silverstein RL: A CD36-dependent signaling cascade is necessary for macrophage foam cell formation. *Cell Metab* 4:211–221, 2006
76. Martinez SC, Tanabe K, Cras-Méneur C, Abumrad N, Bernal-Mizrachi E, Permutt MA: Foxo1 activation plays a major role in ER-stress and fatty acid induced apoptosis in pancreatic islet beta-cells (Abstract). *Diabetes* 56 (Suppl. 1):A414, 2007