

# The Apoptosis Inhibitor ARC Alleviates the ER Stress Response to Promote $\beta$ -Cell Survival

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Type 2 diabetes involves insulin resistance and  $\beta$ -cell failure leading to inadequate insulin secretion. An important component of  $\beta$ -cell failure is cell loss by apoptosis. Apoptosis repressor with caspase recruitment domain (ARC) is an inhibitor of apoptosis that is expressed in cardiac and skeletal myocytes and neurons. ARC possesses the unusual property of antagonizing both the extrinsic (death receptor) and intrinsic (mitochondria/endoplasmic reticulum [ER]) cell death pathways. Here we report that ARC protein is abundant in cells of the endocrine pancreas, including >99.5% of mouse and 73% of human  $\beta$ -cells. Using genetic gain- and loss-of-function approaches, our data demonstrate that ARC inhibits  $\beta$ -cell apoptosis elicited by multiple inducers of cell death, including ER stressors tunicamycin, thapsigargin, and physiological concentrations of palmitate. Unexpectedly, ARC diminishes the ER stress response, acting distal to protein kinase RNA-like ER kinase (PERK) and inositol-requiring protein 1 $\alpha$ , to suppress C/EBP homologous protein (CHOP) induction. Depletion of ARC in isolated islets augments palmitate-induced apoptosis, which is dramatically rescued by deletion of CHOP. These data demonstrate that ARC is a previously unrecognized inhibitor of apoptosis in  $\beta$ -cells and that its protective effects are mediated through suppression of the ER stress response pathway. *Diabetes* 62:183–193, 2013

**H**yperglycemia in type 2 diabetes is mediated by insulin resistance and  $\beta$ -cell failure, the latter leading to inadequate insulin secretion relative to the degree of insulin resistance.  $\beta$ -Cell failure results from dysfunction of these cells and decreases in their numbers, a significant portion of which is attributable to cell death (reviewed in 1,2) (3). Multiple studies have demonstrated a strong correlation between  $\beta$ -cell apoptosis and type 2 diabetes in humans (4,5).

Apoptosis is mediated by an extrinsic pathway that uses cell surface receptors and an intrinsic pathway involving the mitochondria and endoplasmic reticulum (ER) (reviewed in 6,7). The extrinsic pathway is triggered by

specialized death ligands that stimulate the assembly of a multiprotein complex termed the death inducing signaling complex (DISC). The intrinsic pathway is activated by a wider spectrum of stimuli, including metabolic, oxidative, and proteotoxic stress, and triggers permeabilization of the outer mitochondrial membrane, an event regulated by Bcl-2 proteins. Extrinsic and intrinsic pathways converge to activate caspases, a class of cysteinyl proteases, which cleave multiple cellular proteins to kill the cell.

Apoptosis in type 2 diabetes was first demonstrated in islets from Zucker diabetic rats, and treatment of those islets with free fatty acids exacerbated cell death (8). Free fatty acids also induced cell death in nondiabetic human islets, which was inhibited by broad-spectrum caspase inhibitors (4). Postmortem human pancreata exhibited a threefold increase in islet cell apoptosis associated with a 63% reduction in  $\beta$ -cell volume in obese patients with type 2 diabetes compared with obese nondiabetic control subjects (5).

Involvement of the extrinsic pathway was demonstrated by  $\beta$ -cell-specific deletion of procaspase-8, which protected mice from diet-induced islet-cell apoptosis, hyperglycemia, and impaired glucose tolerance (9). The intrinsic pathway also plays an important role, as evidenced by multiple studies showing that overexpression of anti-apoptotic Bcl-2 proteins in INS1E  $\beta$ -cells, isolated islets, and  $\beta$ -cells of transgenic mice inhibited apoptosis elicited by the free fatty acid palmitate and the ER stressor thapsigargin (10,11). Although these studies establish a role for apoptosis in the pathogenesis of type 2 diabetes, the underlying pathways remain incompletely understood.

Apoptosis repressor with caspase recruitment domain (ARC) is a cell death inhibitor that is expressed in cardiac and skeletal myocytes and some neurons (12,13). ARC is unusual in its antagonism of both intrinsic and extrinsic death pathways (14). The extrinsic pathway is inhibited through direct interactions of ARC with components of the DISC that prevent DISC assembly (13,14). ARC inhibits the intrinsic pathway by direct binding to Bax, a proapoptotic Bcl-2 protein, preventing Bax conformational activation and translocation to the mitochondria (14,15).

In this study, we discovered that abundant ARC resides in the mouse and human endocrine pancreas and protects  $\beta$ -cells against stresses relevant to type 2 diabetes. Surprisingly, inhibition of  $\beta$ -cell death in this context involves a novel effect of ARC on the ER stress response pathway.

## RESEARCH DESIGN AND METHODS

**Cell culture and treatments.** Mouse insulinoma MIN6 cells (provided by Dr. Peter Arvan, University of Michigan, Ann Arbor, MI) were cultured as described (16), except for the addition of 140  $\mu$ mol/L  $\beta$ -mercaptoethanol to the media.  $\beta$ TC-tet cells (provided by Dr. Norman Fleischer, Albert Einstein College of Medicine, Bronx, NY) were used as described (17). MIN6 cells with stable expression of ARC were generated by transduction with a retrovirus encoding

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human ARC containing a 3' HA-tag (18). Empty vector was used as the corresponding control. Stable knockdown of ARC in MIN6 cells was carried out using lentiviruses encoding short hairpin (sh)RNAs corresponding to the coding region or 3' untranslated region of mouse ARC from Sigma-Aldrich (St. Louis, MO). Numbers of the shRNAs in Fig. 4 correspond to the last two digits of The RNAi Consortium (TRC) number. Scrambled shRNA was used as the control. In all experiments, populations of stable transductants were studied. Cells were plated at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> and treated with the specified concentrations of thapsigargin, tunicamycin, staurosporine, or palmitate for the specified times. Palmitate was dissolved in culture media that had been supplemented with physiologic levels of fatty acid-free BSA (600  $\mu$ mol/L) at 60°C overnight, and was filtered before use in treatments.

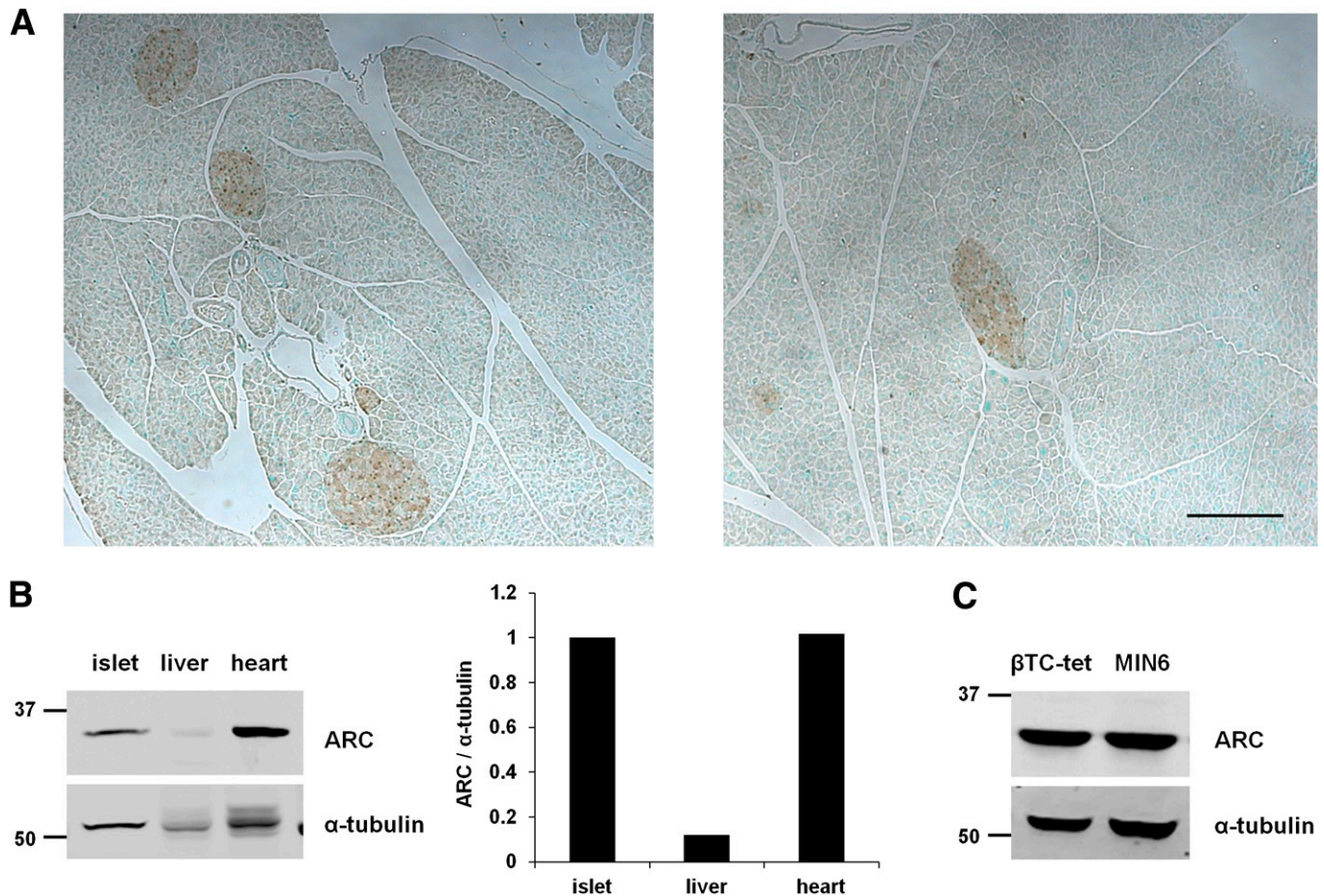
**Islet isolation.** Isolated mouse islets were collected as previously described (19). Briefly, islets were handpicked after mouse pancreata were digested with collagenase and were separated via gradient centrifugation using Histopaque from Sigma-Aldrich (St. Louis, MO). Islets were placed on matrigel-coated plates and allowed to recover in RPMI media (Invitrogen, Grand Island, NY) supplemented with 5.5 mmol/L glucose and 10% (v/v) FBS for 18 h. After 2-h serum starvation, islets were transduced with adenovirus encoding scrambled shRNA or ARC shRNA (20) at  $4 \times 10^6$  pfu/islet. Palmitate treatment was initiated 24 h after addition of the adenovirus.

**Mice.** These studies used male 8–12-week-old wild-type C57Bl/6 mice and C/EBP homologous protein knockout (CHOP<sup>-/-</sup>) mice (B6.129S-Ddit3<sup>tm1Dron/J</sup>), back-bred at least five generations onto a C57Bl/6 background from Jackson Laboratories (Bar Harbor, ME). Mouse maintenance and all experimental procedures were approved by the Albert Einstein College of Medicine Institute for Animal Studies.

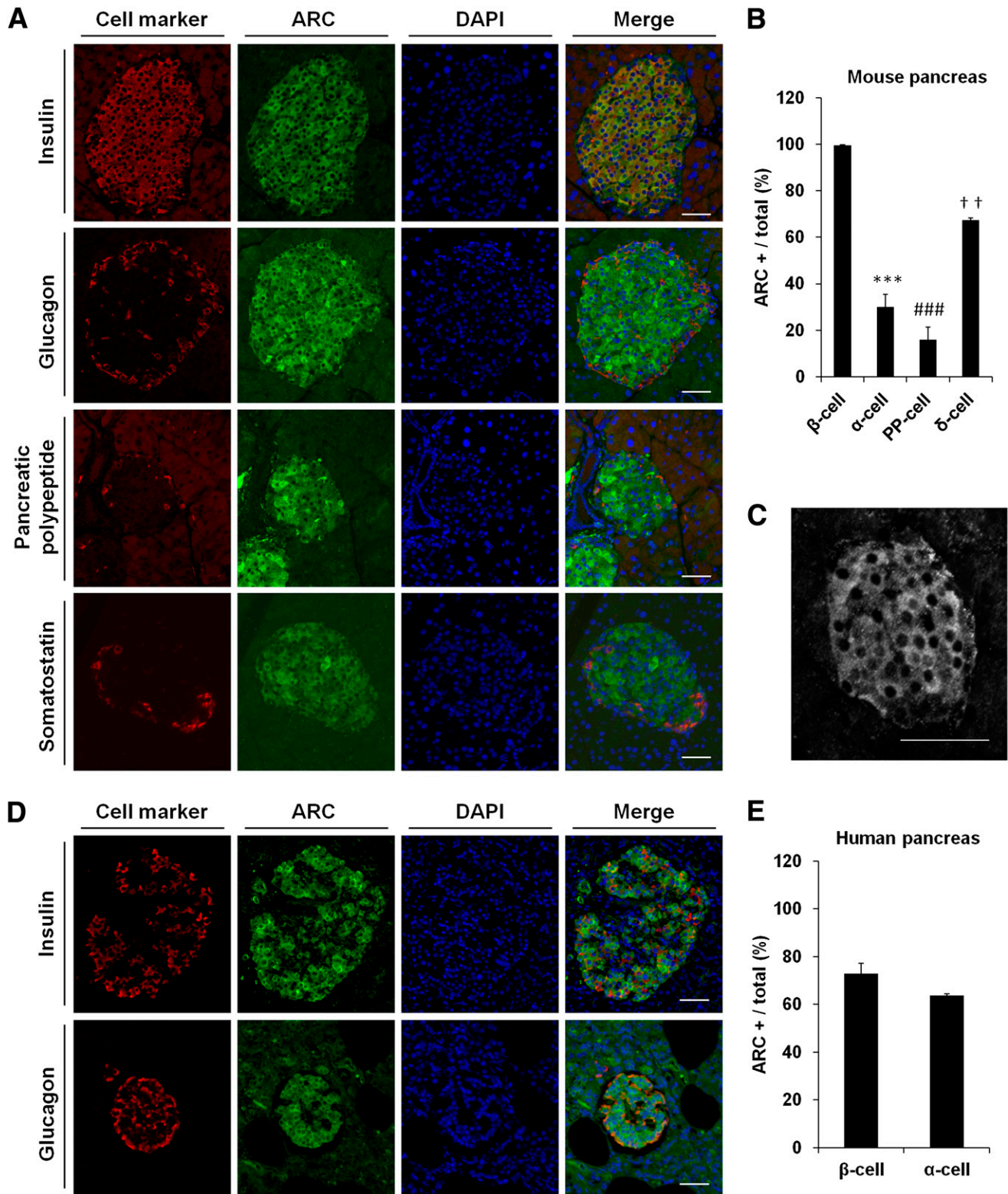
**Immunoblotting.** Cultured cells and isolated islets were lysed in 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% (v/v) Triton X-100, 100  $\mu$ g/mL phenylmethanesulfonyl fluoride, and 1  $\mu$ g/mL aprotinin, after which cells were

sonicated and isolated islets rotated for 30 min at 4°C. Mouse heart and liver tissue were flash-frozen in liquid nitrogen, lysed in RIPA buffer, and homogenized. Immunoblotting was performed with rabbit polyclonal antisera against ARC (1:4,000, Cayman Chemical, Ann Arbor, MI), active caspase-3 (1:500, Cell Signaling Technology, Danvers, MA), poly(ADP-ribose) polymerase (PARP; 1:1,000, Cell Signaling Technology), caspase-8 (1:1,000, Cell Signaling Technology), eukaryotic initiation factor 2  $\alpha$ -subunit (eIF2 $\alpha$ ; 1:1,000, Cell Signaling Technology), p-eIF2 $\alpha$  (1:1,000, Cell Signaling Technology), and protein kinase RNA-like ER kinase (PERK; 1:1,000, Cell Signaling Technology), GADD 153 (CHOP; 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal antibody against  $\alpha$ -tubulin (1:20,000, Sigma-Aldrich). Primary antibodies were incubated overnight at 4°C in 5% milk and 0.1% (v/v) Tween-20 in PBS. IRDye 800 and 680 secondary antibodies (1:4,000, LI-COR, Lincoln, NE) of the corresponding species were used for detection of the primary antibodies using LI-COR Odyssey and quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

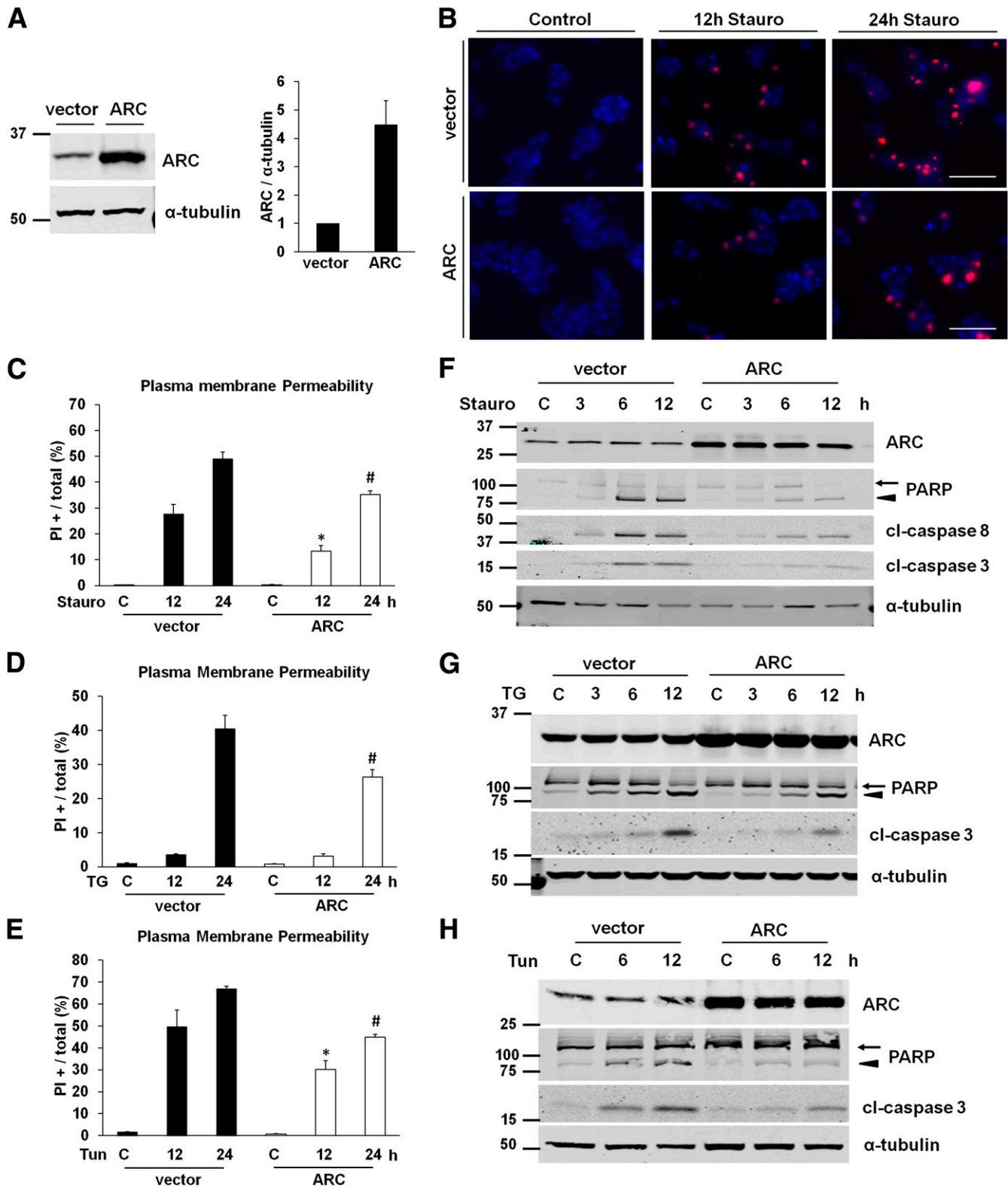
**Immunofluorescence.** Mouse pancreatic tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue was sectioned at 5  $\mu$ m, deparaffinized, and antigen retrieval performed using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Islets were fixed in 4% paraformaldehyde, embedded in Tissue Tek OCT compound (Electron Microscopy Sciences, Hatfield, PA), flash-frozen on dry ice, and sectioned at 5  $\mu$ m. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% (v/v) Triton X-100. After blocking for 1 h in 10% goat or donkey serum, samples were incubated with primary antibodies diluted in blocking solution. Primary antibodies used were active caspase-3 (1:200, Cell Signaling Technology), ARC (1:400, Cayman Chemical), insulin (1:100, Abcam, Cambridge, MA), glucagon (1:2,000, Abcam), somatostatin (1:50, Santa Cruz Biotechnology), and pancreatic polypeptide (1:200, Millipore, Billerica, MA). For immunofluorescence, primary antibodies were detected using Alexa Fluor 488, 568, and 647 secondary



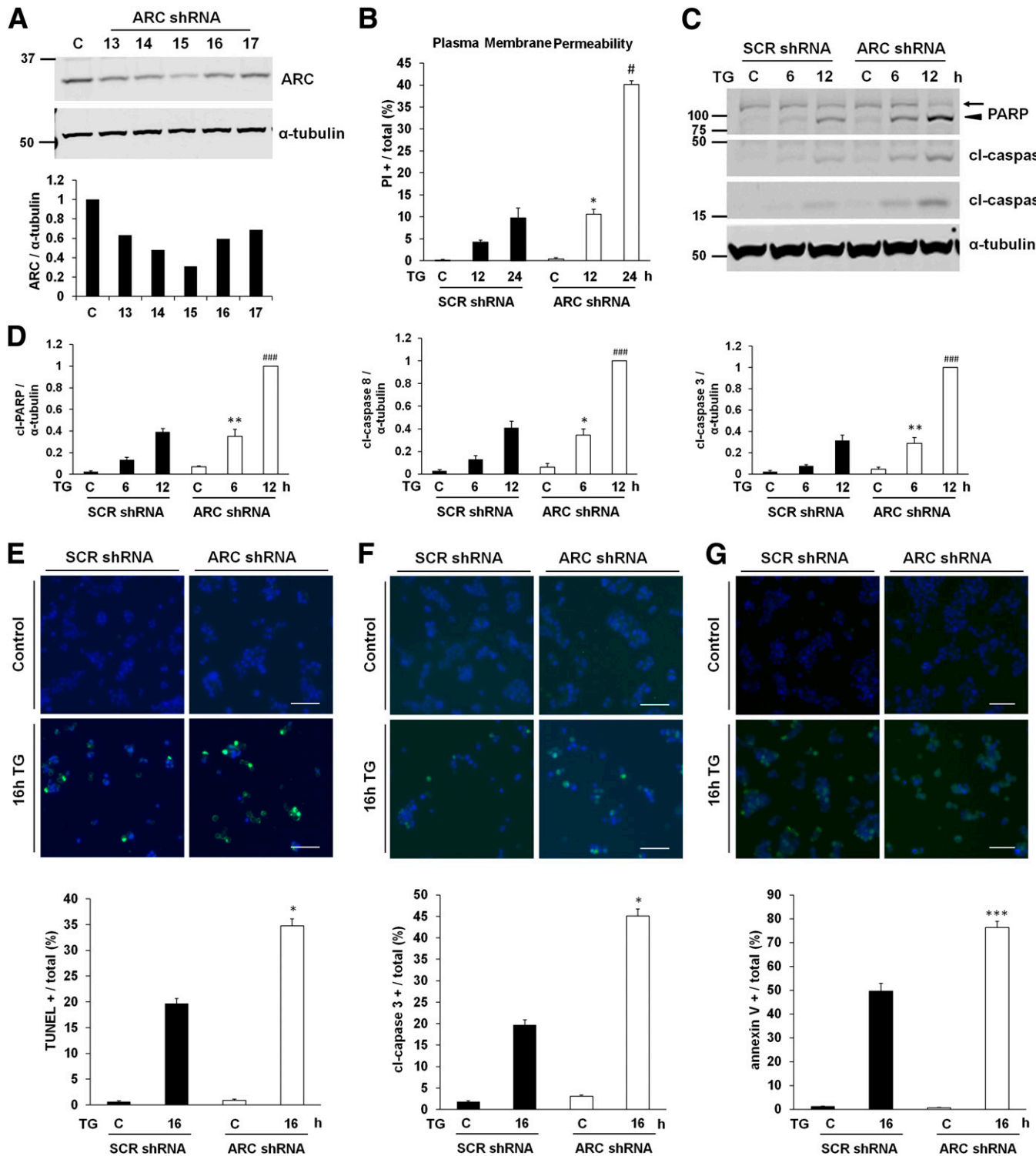
**FIG. 1.** ARC protein in mouse pancreas. **A:** Immunohistochemistry of pancreas stained for ARC (brown). Nuclei counterstained with methyl green. Two fields are shown. Scale bar: 100  $\mu$ m. Representative of three independent experiments. **B:** Immunoblot for ARC in the indicated tissues with quantification. Representative of two experiments using tissue from different mice. **C:** Immunoblot for ARC in the indicated  $\beta$ -cell lines. Representative of three independent experiments.



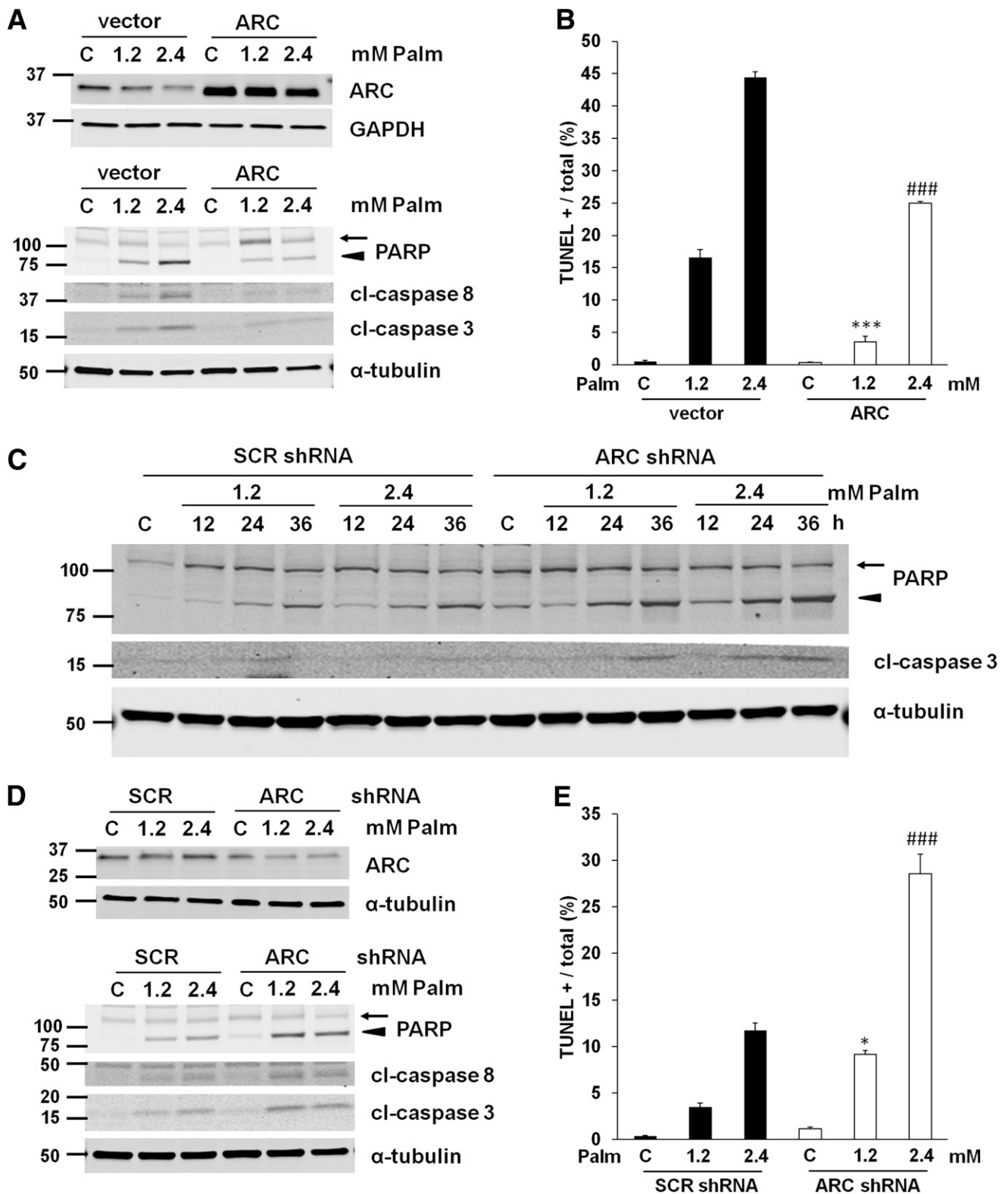
**FIG. 2.** Mouse and human pancreatic tissue demonstrating ARC protein in islet cell types. **A:** Immunostaining for ARC and cell type-specific markers in mouse with  $\beta$ -cells,  $\alpha$ -cells, pancreatic polypeptide (PP) cells, and  $\delta$ -cells indicated by insulin, glucagon, PP, and somatostatin respectively. **B:** Respective quantification. Cells in 5–10 islets in one pancreas section  $\times$  three mice were scored (2,000 cells). Scoring repeated by a second individual blinded to the results. **C:** High-magnification immunostaining for ARC in mouse. **D** and **E:** Analysis of ARC expression with cell type-specific markers in human. Cells in three islets in one pancreas section of one human sample were scored (500 cells). Scale bar: 50  $\mu$ m. Comparisons made to  $\beta$ -cells only: \*\*\* $P$  < 0.001 for  $\alpha$ -cells, ### $P$  < 0.001 for PP cells, †† $P$  < 0.01 for  $\delta$ -cells.



**FIG. 3.** ARC overexpression protects  $\beta$ -cells. **A:** ARC after stable retroviral transduction of MIN6 cells with the indicated plasmids. Control is empty vector. Quantification represents three independent experiments. **B:** Representative images of PI-stained (red) unfixed ARC-overexpressing cells after treatment with staurosporine (Stauro, 1  $\mu$ mol/L). Nuclei counterstained with DAPI (blue). **C-E:** Quantification of PI staining of ARC-overexpressing cells after treatment with Stauro, thapsigargin (TG, 1  $\mu$ mol/L), and tunicamycin (Tun, 10 ng/ $\mu$ L), respectively. Three fields were scored for each condition. ARC vs. vector: \* $P < 0.05$  at 12 h, # $P < 0.05$  at 24 h. **F-H:** Immunoblot analysis of the indicated proteins after treatment of ARC-overexpressing cells with Stauro, TG, and Tun. The arrow denotes full-length PARP and the arrowhead denotes cleaved (cl) PARP. Representative of three independent experiments for Stauro and TG and of two experiments for Tun.



**FIG. 4.** ARC knockdown potentiates cell death. **A:** ARC levels after stable lentiviral transduction of MIN6 cells with the indicated shRNAs with corresponding quantification. The control scrambled (SCR) shRNA is represented by the letter C. **B:** Quantification of PI staining of unfixed ARC-knockdown cells after thapsigargin (TG, 1  $\mu$ M) treatment. Three fields were scored for each condition. ARC shRNA vs. SCR shRNA: \* $P < 0.05$  at 12 h, # $P < 0.05$  at 24 h. **C and D:** Immunoblot analysis of the indicated proteins in ARC-knockdown cells treated with TG and corresponding quantification. The arrow denotes full-length PARP and the arrowhead denotes cleaved (cl)-PARP. Representative of three independent experiments. ARC shRNA vs. SCR shRNA: \* $P < 0.05$  at 6 h, \*\* $P < 0.01$  at 6 h, ### $P < 0.001$  at 12 h. **E:** TUNEL (green) in ARC-knockdown cells treated with TG with quantification. **F:** Immunofluorescence for cl (active) caspase-3 (green) in ARC-knockdown cells treated with TG with quantification. **G:** Immunofluorescence for annexin V (green) in ARC-knockdown cells treated with TG with quantification. Nuclei counterstained with DAPI (blue). Scale bar: 100  $\mu$ m. Six fields scored for each condition. \* $P < 0.05$  for ARC shRNA vs. SCR shRNA at 16 h. \*\*\* $P < 0.001$  for ARC shRNA vs. SCR shRNA at 16 h.



**FIG. 5.** ARC inhibits palmitate-induced cell death. **A:** Immunoblot of the indicated proteins in ARC-overexpressing cells treated with the indicated concentrations of palmitate (Palm) for 3 days (cl, cleaved). Quantification is available in Supplementary Fig. 2 (omitted because of space constraints). **B:** TUNEL analysis of ARC-overexpressing cells treated with Palm at 3 days. Six fields were scored for each condition. ARC vs. vector: \*\*\* $P < 0.001$  at 1.2 mmol/L Palm, ### $P < 0.001$  at 2.4 mmol/L Palm. **C:** PARP cleavage and active caspase-3 in ARC-knockdown cells treated with Palm at the indicated concentrations and times. **D:** Immunoblot of the indicated proteins in ARC-knockdown cells treated with the indicated concentrations of Palm for 2 days. **E:** TUNEL analysis of ARC-knockdown cells treated with Palm for 2 days. Six fields were scored for each condition. ARC shRNA vs. scrambled (SCR) shRNA: \* $P < 0.05$  at 1.2 mmol/L Palm, ### $P < 0.001$  at 2.4 mmol/L Palm. The *arrow* denotes full-length PARP and the *arrowhead* denotes cl-PARP. Immunoblots in A, C, and D are representative of three independent experiments.

antibodies (1:1,000, Invitrogen, Grand Island, NY) of the corresponding species. Slides were coverslipped using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories) to counterstain nuclei. For immunohistochemistry, nuclei were counterstained with methyl green as described (21). All images were collected using an Axio Observer .Z1 microscope (Zeiss, Thornwood, NY).

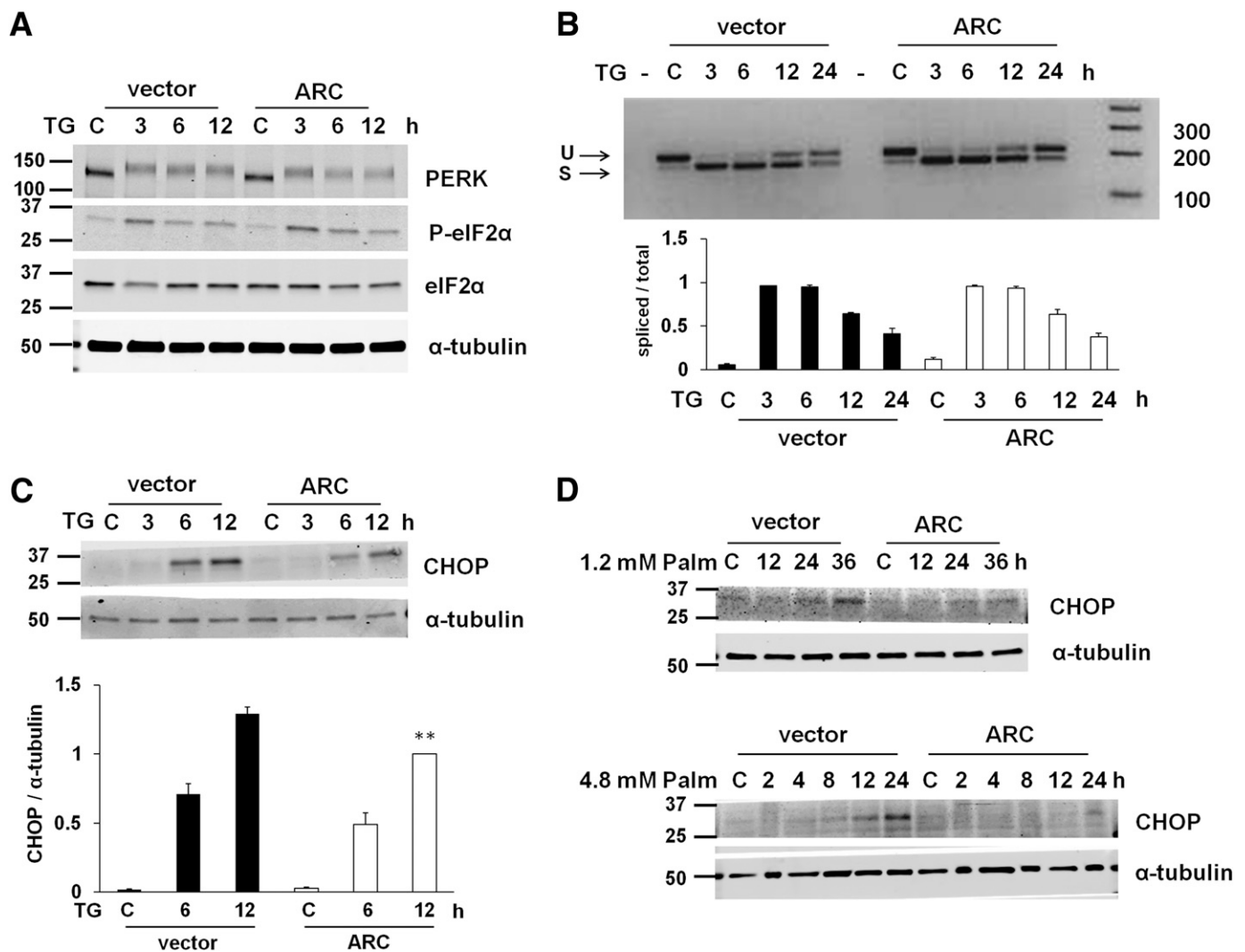
**Cell death assays.** For the live/dead assay, propidium iodide (PI) and Hoechst 33342 (Invitrogen) were directly added to media. Cells were incubated for 5 min in the dark at 37°C and immediately imaged. Three random fields (1,000–2,000 cells) were scored. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was performed using the Fluorescein or TMR red In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's protocol. Six random fields (1,000–4,000 cells) were scored. When TMR red was used, it was displayed as pseudocolor green. Annexin V staining was performed using the Annexin V-FTIC Apoptosis Detection Kit (Abcam) according to manufacturer's protocol. Three random fields (1,000–3,000 cells) were scored.

**X-box binding protein 1 (XBP1) splicing assay.** RNA was isolated from cells using TRIzol reagent (Ambion, Grand Island, NY). Oligo(dT)20 primers were used to produce cDNA with SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR was performed using the following primer pairs: mouse XBP1 sense 5'-GAA CCA GGA GTT AAG AAC ACG-3' and mouse XBP1 antisense 5'-AGG CAA CAG TGT CAG AGT CC-3'. Samples were then separated on a 3% (w/v) agarose gel.

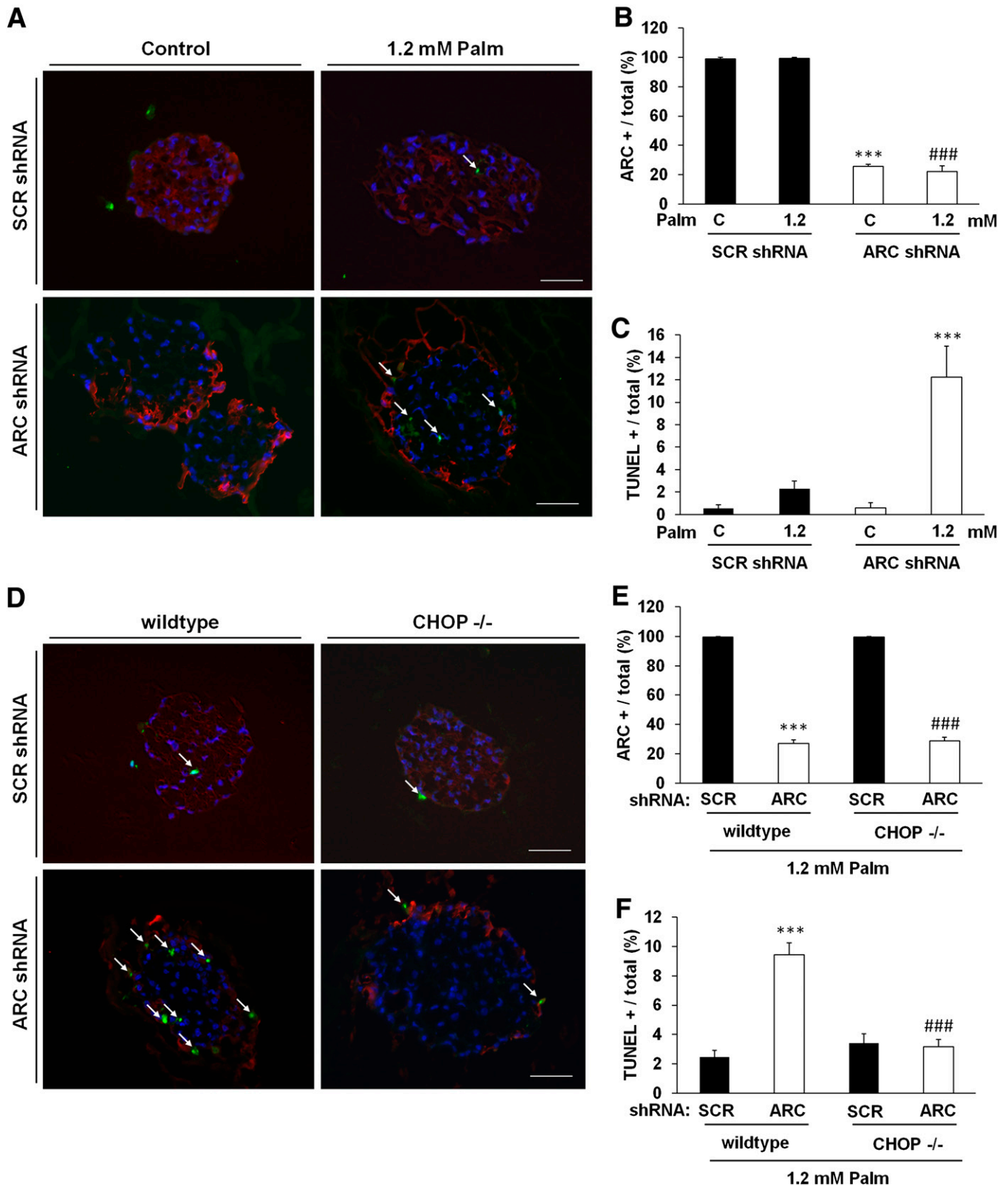
**Statistics.** Data are presented as mean  $\pm$  SEM. The two-tailed Student *t* test was used to compare two groups. ANOVA, followed by a Tukey post hoc test, was used for multiple comparisons. GraphPad Prism 5 software (GraphPad, La Jolla, CA) was used to calculate statistics.  $P < 0.05$  was considered significant.

## RESULTS

**ARC is abundant in mouse and human pancreas.** The expression of ARC was originally reported to be restricted to cardiac and skeletal myocytes and some neurons (12,13), and more recent studies have uncovered that ARC is also highly induced in multiple cancers (18,22–25). Because tissues contain a heterogeneous mixture of cells, some of which perform unique functions, we hypothesized that ARC expression may have been overlooked in studies that examined homogenates of whole organs. Accordingly, we screened for ARC expression in subpopulations of cells thought not to express ARC. Unexpectedly, we found that cells of the mouse (Fig. 1A) and human (Fig. 2D) endocrine pancreas contain high levels of ARC. Moreover, ARC



**FIG. 6.** ARC alleviates ER stress. **A:** Markers of the unfolded protein response in ARC-overexpressing cells treated with thapsigargin (TG, 1  $\mu$ mol/L). Representative of four independent experiments. **B:** RT-PCR for XBP1 splicing in ARC-overexpressing cells treated with TG with quantification of spliced (S) XBP1 over total XBP1 (unspliced [U] + S). Representative of three independent experiments. **C:** CHOP levels in ARC-overexpressing cells treated with TG with quantification. Representative of five independent experiments. **\*\*** $P < 0.01$  for ARC vs. vector at 12 h. **D:** CHOP levels in ARC-overexpressing cells treated with palmitate (Palm) at the indicated concentrations and times. Representative of three independent experiments at each palmitate concentration.



**FIG. 7.** Deletion of CHOP inhibits augmentation of palmitate-induced cell death elicited by ARC knockdown in isolated islets. **A:** Palmitate (Palm)-induced apoptosis is augmented by ARC knockdown. TUNEL (green), ARC immunostaining (red), and DAPI counterstain (blue) in isolated islets transduced with adenovirus expressing scrambled (SCR) or ARC shRNA, with or without Palm treatment. The arrows denote TUNEL-positive cells within islets. Only cells clearly within islets were scored. **B:** Quantification of the percentage of ARC-positive cells in **A** shows that ARC knockdown is not affected by Palm treatment. All cells in 10 islets were scored for each condition (400–800 cells). ARC shRNA vs. SCR shRNA: \*\*\* $P < 0.001$  control condition, ### $P < 0.001$  Palm treatment. **C:** Quantification of TUNEL staining in **A**. ARC shRNA vs. SCR shRNA: \*\*\* $P < 0.001$  Palm treatment. **A–C** are representative of two independent experiments. **D:** Rescue of apoptosis by deletion of CHOP. TUNEL (green), ARC immunostaining (red), and DAPI counterstain (blue) in Palm-treated isolated islets from wild-type or CHOP<sup>-/-</sup> mice transduced with adenovirus expressing SCR or ARC shRNA. The arrows denote TUNEL-positive cells within islets. **E:** Quantification of percentage of ARC-positive cells in **D**



abundance in isolated islets is roughly equivalent to that in heart tissue (Fig. 1B). In contrast, ARC was not detectable in the exocrine pancreas (Fig. 1A), explaining previous studies reporting its absence in the pancreas (13). ARC levels in isolated islets from wild-type mice were similar to those from *ob/ob* mice (Supplementary Fig. 1D). These data demonstrate that ARC protein is abundant in cells of the islet.

To determine which cells within islets express ARC, we immunostained mouse and human pancreatic tissue. ARC is expressed primarily in  $\beta$ -cells and is present in >99.5% of these cells in the mouse (Fig. 2A and B). It is predominantly localized to the cytoplasm because ARC immunofluorescence overlaps with insulin but not with the nuclear DAPI staining (Fig. 2A and C). Abundant ARC was also detected in various mouse  $\beta$ -cell lines (Fig. 1C). In addition, ARC is found in 30% of mouse  $\alpha$ -cells, 16% of pancreatic polypeptide (PP) cells, and in 67% of  $\delta$ -cells, the latter sharing a common progenitor cell with  $\beta$ -cells (26) (Fig. 2B). Moreover, in the human, ARC is present in 73% of  $\beta$ -cells and in 64% of  $\alpha$ -cells (Fig. 2E). These data indicate that ARC is abundant in the endocrine pancreas, predominantly in  $\beta$ -cells.

**ARC regulates cell death in  $\beta$ -cells.** To determine whether ARC plays a functional role in  $\beta$ -cells, we used gain- and loss-of-function approaches. Stable retroviral transduction of ARC in MIN6 cells (Fig. 3A) inhibited cell death triggered by the general apoptosis inducer staurosporine. This was indicated by changes in the abundance of active caspases-3 and -8, cleavage of the caspase substrate PARP, and loss of plasma membrane integrity (PI staining), the latter a readout of overall death in cultured cells (Fig. 3B–C, F). Because of metabolic disturbances,  $Ca^{2+}$  abnormalities, and proteotoxic stress resulting from increased insulin production, ER stress is a key component of type 2 diabetes (27–31) and an initiator of  $\beta$ -cell death (32,33). Accordingly, we tested whether ARC inhibits ER stress-induced apoptosis in these cells. Cell death triggered by the ER stressors thapsigargin (an inhibitor of sarcoplasmic/endoplasmic reticulum  $Ca^{2+}$ ATPase [SERCA], which mediates  $Ca^{2+}$  reuptake into the ER) and tunicamycin (an inhibitor of N-linked glycosylation, which is required for proper folding of proteins) was suppressed by ARC overexpression (Fig. 3D, E, G, and H). Of note, levels of endogenous ARC were not affected by any of these death inducers (Supplementary Fig. 1A–C).

Conversely, knockdown of ARC was carried out using RNAi. Five independent shRNAs were tested for their abilities to knockdown ARC after stable transduction into MIN6 cells. Compared with scrambled shRNA, ARC shRNAs 14 and 15 were most effective, with 55% and 70% knockdown, respectively (Fig. 4A). To define the importance of endogenous levels of ARC in regulating ER stress-mediated cell death, we used shRNA 15. Depletion of ARC resulted in two- to fourfold increases in cell death induced by thapsigargin (Fig. 4). This was demonstrated by activation of caspases-3 and -8 and PARP cleavage (Fig. 4C and D). In addition, there were increases in the percentage of cells exhibiting TUNEL, active caspase-3

immunofluorescence, annexin V, and PI entry (Fig. 4B and E–G). These data demonstrate that endogenous levels of ARC protect  $\beta$ -cells against ER stress-induced cell death.

**ARC regulates cell death in a pathophysiological context.** ER stress is a critical component in the pathogenesis of type 2 diabetes (27,28). The free fatty acid palmitate, which models this stress, has been shown to induce  $\beta$ -cell death (34,35). Accordingly, we assessed whether ARC inhibits cell death in response to this pathophysiological stimulus. Palmitate at 1.2 and 2.4 mmol/L concentrations induced  $\beta$ -cell apoptosis, which was dramatically reduced by ARC overexpression (Fig. 5A and B, Supplementary Fig. 2). In addition, ARC overexpression continued to prevent cell death even at late time points (Supplementary Fig. 3). Conversely, knockdown of ARC potentiated palmitate-induced apoptosis in a concentration- and time-dependent manner (Fig. 5C–E). These data indicate that ARC regulates  $\beta$ -cell death elicited by a pathophysiologically relevant stimulus of ER stress.

**ARC alleviates ER stress.** ER stress has been linked to  $\beta$ -cell death (32,33), but the precise functional and mechanistic relationships between these processes remain unclear. The major role of the ER stress response is to restore ER homeostasis, including refolding of misfolded proteins (reviewed in 36–38). Upstream mediators include the kinases PERK and inositol-requiring protein 1 $\alpha$  (IRE1 $\alpha$ ), the combined actions of which include attenuating translation and activating a complex transcriptional response that promotes protein refolding. However, when the ER stress response is prolonged, apoptosis can result (reviewed in 39,40). Although inhibition of cell death by ARC has traditionally been thought to be mediated solely through its antagonism of the central death machinery (14,41), we considered the possibility that ARC also regulates the ER stress response itself.

Using thapsigargin to stimulate the ER stress response, we observed that overexpression of ARC does not affect PERK activation (as indicated by phosphorylation of its downstream target eIF2 $\alpha$ ) or IRE1 $\alpha$  activation, as indicated by XBP1 splicing (Fig. 6A and B). In contrast, ARC overexpression inhibited upregulation of the transcription factor CHOP, an important initiator of apoptosis in response to ER stress that has been implicated in type 2 diabetes (42,43) (Fig. 6C). This effect was even more marked when CHOP induction was elicited by the physiological ER stressor palmitate (Fig. 6D). These data indicate that ARC acts distal to activation of PERK and IRE1 $\alpha$  to inhibit the ER stress response.

**Endogenous ARC suppresses apoptosis in isolated islets through CHOP.** To determine whether endogenous levels of ARC are important in suppressing palmitate-induced apoptosis in the context of islet tissue, we used isolated islets. Transduction of islets with adenovirus encoding ARC shRNA knocked down ARC levels in ~74% of cells (Fig. 7A and B). Although treatment with palmitate (1.2 mmol/L) did not result in significant cell death in islets transduced with scrambled shRNA, knockdown of ARC augmented apoptosis more than fivefold (Fig. 7A and C). Moreover, deletion of CHOP rescued the incremental cell

show ARC knockdown is not affected by deletion of CHOP. All cells in 20 islets were scored for each condition (900–2,200 cells). ARC shRNA vs. SCR shRNA: \*\*\* $P$  < 0.001 wild-type islets, ### $P$  < 0.001 CHOP<sup>-/-</sup> islets. F: Quantification of percentage of TUNEL-positive cells in D. ARC shRNA vs. SCR shRNA: \*\*\* $P$  < 0.001 wild-type. CHOP<sup>-/-</sup> vs. wild-type: ### $P$  < 0.001 ARC shRNA. D–F are representative of three independent experiments (two others shown in Supplementary Fig. 4). Palm treatment was for 4 days in all panels. Scale bar: 50  $\mu$ m.

death resulting from ARC knockdown (Fig. 7D and F, Supplementary Fig. 4) without influencing ARC levels (Fig. 7D and E, Supplementary Fig. 4). These data indicate that endogenous levels of ARC are critical in suppressing palmitate-induced cell death in islets and that this inhibition is mediated through CHOP.

## DISCUSSION

These experiments reveal the unexpected presence of ARC in pancreatic islets and a novel role for ARC in modulating the ER stress response and inhibiting  $\beta$ -cell death. Under normal conditions, the expression of ARC was previously thought to be restricted to cardiac and skeletal myocytes and neurons. Accordingly, ARC has never been studied in the context of diabetes. Surprisingly, we found that ARC is as abundant in the endocrine pancreas as it is in the heart. Although expressed in variable percentages of cell types in islets, almost all mouse  $\beta$ -cells and a high proportion of human  $\beta$ -cells contain ARC. On the basis of studies in other cell types (21,41,44,45), the abundance of ARC in the  $\beta$ -cell may be mediated by both transcriptional and posttranslational mechanisms.

Although ARC has been shown to be a potent inhibitor of apoptosis in other systems, cell death is often regulated in a cell type- and stimulus-specific manner; therefore, we investigated the role of ARC in  $\beta$ -cell apoptosis. Overexpression of ARC inhibited cell death elicited by generic cell death stimuli and ER stressors, including palmitate, a free fatty acid relevant to the pathogenesis of type 2 diabetes. Conversely, knockdown experiments demonstrated the importance of endogenous levels of ARC in protecting against these death signals in the  $\beta$ -cell. These effects were demonstrated using multiple parameters indicative of apoptosis assessed at a variety of time points.

In addition to its known effects on the two central apoptosis pathways, a new finding in this study is that ARC modulates cell death by alleviating the ER stress response. This pathway plays an important adaptive role in helping the cell to resolve various stresses. When insults are of overwhelming magnitude or prolonged duration, however, this pathway is no longer sufficient to compensate, sometimes triggering cell death. This duality of the ER stress response is attributable, in part, to effectors such as CHOP, which are capable of signaling cellular adaptation and also demise. Although the mechanisms that control the transition from adaptation to death are currently not understood, our data indicate that ARC acts downstream of PERK and IRE1 $\alpha$  and requires CHOP to ameliorate the ER stress response and inhibit  $\beta$ -cell death.

We observed that ER stress activates caspase-8 in MIN6 cells, an effect suppressed by ARC overexpression and exacerbated by ARC knockdown. These observations are consistent with a previous study in melanoma cells (46). It remains unclear, however, whether caspase-8 is modulated through known actions of ARC at the DISC or whether these effects of ARC involve a pool of caspase-8 known to reside at the ER (47). Interestingly,  $\beta$ -cell-specific deletion of procaspase-8 in mice ameliorates high fat diet-induced diabetes (9), a significant component of which involves ER stress.

This study raises a number of interesting avenues for future investigation. One relates to the mechanisms by which ARC suppresses CHOP. This potentially includes effects on CHOP transcription, mRNA or protein stability,

posttranslational modifications, and nuclear localization. Moreover, indirect mechanisms may also be involved. Second, given known effects of ARC in regulating mitochondrial events in cell death, another important question is whether bidirectional ER-mitochondrial crosstalk plays a role in effects of ARC on the ER stress response. Third, and perhaps most interesting, is the possibility that ARC functions as a survival factor for  $\beta$ -cells exposed to the noxious milieu of type 2 diabetes in vivo. Experiments using ARC gain- and loss-of-function mice will be needed to test this possibility. In addition, because ARC is also expressed in skeletal muscle and brain, there remains the possibility that ARC modulates other aspects in the pathogenesis of type 2 diabetes.

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W.M.M.<sup>c</sup>.K. conceived experiments, researched data, analyzed experiments, contributed to discussion, and wrote the manuscript. J.W. researched data and contributed to discussion. L.C. and M.Z. researched data. M.C.T. contributed reagents and reviewed and edited the manuscript. J.E.P. and S.C.C. conceived experiments, contributed to discussion, and reviewed and edited the manuscript. R.N.K. conceived and analyzed experiments, contributed to discussion, and wrote the manuscript. R.N.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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