Glucose-Regulated Protein 78 Controls Cross-talk between Apoptosis and Autophagy to Determine Antiestrogen Responsiveness

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Abstract

While more than 70% of breast cancers express estrogen receptor-α (ER+), endocrine therapies targeting these receptors often fail. The molecular mechanisms that underlie treatment resistance remain unclear. We investigated the potential role of glucose-regulated protein 78 (GRP78) in mediating estrogen resistance. Human breast tumors showed increased GRP78 expression when compared with normal breast tissues. However, GRP78 expression was reduced in ER+ breast tumors compared with HER2-amplifed or triple-negative breast tumors. ER+ antiestrogen-resistant cells and ER+ tumors with an acquired resistant antiestrogen phenotype were both shown to overexpress GRP78, which was not observed in cases of *de novo* resistance. Knockdown of GRP78 restored antiestrogen sensitivity in resistant cells, and overexpression of GRP78 promoted resistance in sensitive cells. Mechanistically, GRP78 integrated multiple cellular signaling pathways to inhibit apoptosis and stimulate prosurvival autophagy, which was dependent on TSC2/AMPK-mediated mTOR inhibition but not on beclin-1. Inhibition of autophagy prevented GRP78-mediated endocrine resistance, whereas caspase inhibition abrogated the resensitization that resulted from GRP78 loss. Simultaneous knockdown of GRP78 and beclin-1 synergistically restored antiestrogen sensitivity in resistant cells. Together, our findings reveal a novel role for GRP78 in the integration of cellular signaling pathways including the unfolded protein response, apoptosis, and autophagy to determine cell fate in response to antiestrogen therapy. *Cancer Res; 72(13); 3337–49. ©2012 AACR.*

Introduction

More than 200,000 American women are diagnosed with breast cancer annually in the United States. (1). Approximately 70% of these cancers express estrogen receptor-α (ER+, ESR1) and are potentially responsive to a therapy targeting this receptor (2). Such therapies include treatment with the selective ER modulator tamoxifen, the selective ER downregulator fulvestrant (Faslodex; ICI 182,780; ICI), or an aromatase inhibitor that blocks the production of 17β-estradiol (3, 4). While these interventions increase overall survival for some women, their curative potential is limited by either *de novo* (intrinsic) or acquired resistance. Unfortunately, recurrent ER+ breast cancer remains an incurable disease for most women. A better understanding of the molecular mechanisms of resistance is urgently needed.

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Recent studies implicate a complex interaction between prosurvival and prodeath signaling in determining the cell fate outcome in response to endocrine and other therapies. Apoptosis is widely described as one cell death pathway activated in sensitive cells; the prodeath and/or prosurvival function of autophagy has also been recently implicated. Autophagy is a cellular process whereby cells cannibalize their proteins and organelles to recover nutrients and restore metabolic homeostasis (5). This process involves the formation of a double-membrane vesicle to isolate cellular cargo, catabolism of the cargo, and the release of nutrients from the autophagosome to fuel cellular metabolism (6). In response to antiestrogen therapy, stimulation of autophagy is associated with increased breast cancer cell survival, suggesting a role for a prosurvival autophagy in resistance (7–9). How autophagy is induced or maintained and how the balance between its prosurvival and prodeath activities is affected remain unclear.

One potential regulator of autophagy is activation of the unfolded protein response (UPR), an endoplasmic reticulum stress pathway (10, 11). UPR activation occurs when unfolded proteins accumulate within the endoplasmic reticulum, resulting in the protein chaperone glucose-regulated protein 78 (GRP78, also known as BiP or HSPA5) being released from either PKR-like endoplasmic reticulum kinase (PERK, EIF2AK3), inositol requiring enzyme 1 (IRE1, ERN1), and/or activating transcription factor 6 (ATF6; ref. 12). A key upstream activator of the UPR GRP78 participates in regulating protein

folding, assembly and degradation, endoplasmic reticulum stress sensing, and cellular calcium homeostasis. GRP78 upregulation is reported in breast cancer cells lines and in malignant but not benign breast lesions (13, 14). A role for GRP78 has been proposed in responsiveness to cytotoxic drugs and histone deacetylase (HDAC) inhibitors (15–17) and in affecting response to estrogen deprivation (a model somewhat representative of aromatase inhibitor resistance, but different from antiestrogen therapy resistance; refs. 18, 19). How GRP78 regulates these processes and whether activation of the UPR, apoptosis, and autophagy are central determinants of its action are unknown.

Focusing on the clinically relevant problem of antiestrogen resistance, we hypothesized that the UPR can use GRP78 to coordinate prodeath and prosurvival activities and activate a prosurvival autophagy in endocrine resistance. Because these functions may be affected by cellular context, we used several isogenic models of endocrine resistance to study the role of GRP78: MCF7 (ER+, estrogen-dependent, tamoxifen- and ICI-sensitive) and MCF7-RR [ER+, estrogen-independent, tamoxifen-resistant, ICI-sensitive derived from MCF7 cells selected against low serum and tamoxifen (refs. 20, 21)], and MCF7/LCC1 [ER+, estrogen-independent, tamoxifen- and ICI-sensitive model derived by *in vivo* selection of MCF7 cells (ref. 22)], and MCF7/LCC9 [ER+, estrogen-independent, ICI-resistant, tamoxifen cross-resistant derived from MCF7/LCC1 cells by selection against ICI (ref. 23)]. Our studies show that GRP78 directly modulates antiestrogen responsiveness by integrating UPR, apoptosis, and apoptosis though mTOR, tuberous sclerosis 2 (TSC2), AMP-activated protein kinase (AMPK, PRKAA1), p62 (SQSTM1), LC3 (MAP1LC3A), and caspase-7 (CASP7) to determine cell fate. These observations on the integration of signaling to regulate cell fate decisions are likely to be applicable beyond the cellular context of breast cancer resistance to endocrine therapies.

Materials and Methods

Materials

The following materials were obtained as indicated: 4-hydroxytamoxifen (Sigma-Aldrich); ICI 182,780 (Tocris Bioscience); penicillin and Improved Minimal Essential Medium (IMEM; Gibco Invitrogen BRL); FBS and bovine calf charcoal-stripped serum (CCS; Equitech-Bio Inc.); Lipofectamine RNAiMAX reagent (Invitrogen); GRP78 and AMPK siRNA (On-Target plus SMART pool; consisting of 3 different siRNA for same target; ThermoScientific Dharmacon); GRP78 plasmid (HSPA5 Trueclone cDNA; OriGene); ATG5 and TSC2 siRNA (Cell Signaling Technology); mouse IgG negative control antibody (Dako); crystal violet (Fisher Scientific); and caspase inhibitor Z-VAD-FMK (Tocris Bioscience). Antibodies were obtained from the following sources: GRP78, GRP94, LC3B, p62, BECN1, ATG5, phospho-AMPK (Thr172), phospho-TSC2 (Ser1254), TSC1, mTOR, phospho-mTOR (Ser2448), TORC1, PARP, and cleaved caspase-7 (Cell Signaling Technology); Annexin V (Enzo Life Sciences); Atg9A (Novus); ATF6 (Sigma-Aldrich); IRE1 (ThermoScientific); XBP1-S (Genway); PERK (Abcam); and ER-α, β-actin, GRP78 [for immunohistochemistry (IHC) and used as a blocking antibody] and polyclonal and horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology).

Human breast tumors and corresponding normal breast tissue

Human breast tumors were surgically removed, fixed in neutral-buffered formalin, and processed using routine histologic methods. Histologic grade, ER, progesterone receptor, and HER2 levels were previously determined using IHC.

Orthotopic xenografts in athymic mice

Five-week-old ovariectomized athymic nude mice (Harlan Laboratories) were injected orthotopically with 0.5×10^6 LCC1 or LCC9 cells in Matrigel into the mammary fat pads and implanted s.c. with a 17β-estradiol pellet (0.72 mg, 60-day release; Innovative Research of America). Mice were sacrificed after 9 weeks, and tumors removed at necropsy, fixed in neutral-buffered formalin, and processed using routine histologic methods.

Carcinogen-induced mammary tumors in rats

Fifteen 50-day-old intact female Sprague-Dawley (Harlan Laboratories) were gavaged *per os* with 10.0 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA; Sigma Chemical Co.) in 1 mL of corn oil to induce mammary tumors. When a tumor reached 15 ± 3 mm in its longest axis, the rat was switched to AIN-93G diet containing 337 ppm tamoxifen citrate (Harlan-Teklad) that provides a dose of approximately 15 mg/kg/d tamoxifen. Tumors were classified by their growth responsiveness: those in the control group (nontreated) were classified as growing tumors. Tumors in the tamoxifen-treated rats were classified as exhibiting complete response—these tumors became nonmeasurable and remained so for 3 consecutive weeks; acquired resistant—tumors that regrow after $\geq$4 weeks of complete response; and *De Novo* resistant—new tumors that started to grow during tamoxifen treatment. Animals were euthanized 37 to 38 weeks after tumor induction. Tumors were fixed in neutral-buffered formalin and processed with routine histologic methods; tumors used in this study were confirmed as mammary adenocarcinomas by histopathologic evaluation.

Cell culture

MCF7 breast carcinoma cells and MCF7-RR breast carcinoma cells were grown in IMEM containing 5% FBS and 100 μg/mL penicillin. MCF7/LCC1 (LCC1) and MCF7/LCC9 (LCC9) breast carcinoma cells were grown in phenol-red–free IMEM containing 5% CCS and 100 μg/mL penicillin. Cells were grown at 37°C in a humidified, 5% CO_2:95% air atmosphere.

Cell proliferation

Human breast cancer cells (3×10^3 cells/mL) in IMEM containing 5% FBS or CCS were plated in 24-well tissue culture plates. On day 1 after plating, and every 72 hours thereafter, cells were treated with varying doses (10–1,000 nmol/L) of either tamoxifen or fulvestrant. On day 6, media were aspirated and cells were stained with crystal violet. Cells were permeabilized using citrate buffer and absorbance was read at 660 nm

on a plate reader. For studying cell surface–localized GRP78 effects on antiestrogen resistance, LCC9 breast cancer cells were plated in a 24-well tissue culture plate and treated with 1 μg/mL GRP78 or goat IgG control antibody and treated with varying doses (10–1,000 nmol/L) of fulvestrant; cell density was measured by the crystal violet assay.

Western blot hybridization

Treated cell monolayers were solubilized in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 mmol/L sodium orthovanadate, 1× Roche complete mini protease inhibitor cocktail) and protein was measured using a standard bicinchoninic acid assay. Proteins were size-fractionated by PAGE and transferred to nitrocellulose membrane. Nonspecific binding was blocked by incubation with Blotto (TBS with 5% powdered milk and 1% Triton X-100). Membranes were incubated overnight at 4°C with primary antibodies followed by incubation with polyclonal HRP-conjugated secondary antibodies (1:2,000) for 1 hour at room temperature. Immunoreactive products were visualized by chemiluminescence (SuperSignal Femto West; Pierce Biotechnology) and quantified by densitometry using the ImageJ digital densitometry software (http://rsbweb.nih.gov/ij/). Protein loading was visualized by incubation of stripped membranes with a monoclonal antibody to β-actin (1:1,000).

Immunohistochemistry

Tumors were fixed in 10% formalin for 24 hours before embedding in paraffin. Embedded tumors were cut into 5-μm thick sections and stained with hematoxylin and eosin to determine histopathology. Immunostaining was conducted with an antibody to GRP78 (1:100) or a nonspecific negative control antibody using the streptavidin–biotin method. Stained sections were visualized and photographed.

Apoptosis and autophagy

LCC9 and MCF7-RR cells were transfected with control (sequence-specific scrambled oligonucleotide) or GRP78 siRNAs and LCC1 and MCF7 cells were transfected with control pcDNA or GRP78(+) and treated with ICI or tamoxifen (100 nmol/L) for 6 days. To measure apoptosis, cells were stained as described in the Annexin V-FITC Apoptosis Detection Kit (Enzo Life Sciences) and counted by flow cytometry (LCCC Flow Cytometry Shared Resources). LCC9 cells were transfected with GFP-LC3B (Addgene) and control or GRP78 siRNA and LCC1 cells were transfected with GFP-LC3B and control pcDNA or GRP78(+), and then treated with 0.1% v/v ethanol vehicle or 500 nmol/L ICI for 72 hours. LC3II-GFP–positive punctate pattern was observed by confocal microscopy. Confocal microscopy was conducted using an Olympus IX-70 confocal microscope with 405- and 488-nm excitation lasers.

Statistics

All data are presented as the mean ± SEM. Statistical differences were evaluated by Student *t* test (single pairwise comparison) or one-way ANOVA followed by Dunnett (multiple comparisons to the same control) or Bonferroni (multiple comparisons) *post hoc* tests. The criterion for statistical significance was set at $P < 0.05$. Drug synergy was defined as R index (RI) [(survival A × survival B)/(survival A+B)] > 2.0 (24).

Results

GRP78 upregulation in acquired resistance

Immunohistochemical analysis of human triple-negative (ER^-, PR^-, HER2 normal), HER2-overexpressing, and ER+ breast tumors stained for GRP78 shows elevated expression of GRP78 in the tumors when compared with the normal surrounding breast tissue (Fig. 1A). Moreover, quantification of GRP78 expression in the malignant tissue shows reduced levels of GRP78 in untreated ER+ breast tumors when compared with either the triple-negative or HER2-amplified breast cancers (Fig. 1B). Higher levels of GRP78 are also observed in the normal tissue surrounding either triple-negative or HER2-amplified breast tumors when compared with the normal breast tissues surrounding ER+ breast tumors. GRP78 protein levels were measured in MCF7, MCF7-RR, LCC1, and LCC9 cells. Increased GRP78 expression was observed in the antiestrogen-resistant cell lines when compared with their respective controls. Moreover, immunohistochemical analysis of untreated orthotopic LCC1 and LCC9 xenografts also show increased expression of GRP78 in antiestrogen-resistant tumors (Fig. 1C). In a carcinogen-induced rat mammary tumor model that includes the spectrum of tamoxifen responses seen in patients (complete response, partial response, *de novo* resistance, acquired resistance) GRP78 expression measured by IHC is increased in the acquired resistant mammary tumors when compared with untreated, complete response, and *de novo* resistant mammary tumors (Fig. 1D). These data strongly suggest that changes in GRP78 expression reflect an adaptive response to the stress of antiestrogenic intervention.

Modulation of GRP78 affects endocrine responsiveness

Silencing GRP78 using a fixed dose of siRNA shifts the dose–response of the resistant LCC9 cells to both ICI and tamoxifen (Fig. 2A and B). Similarly, inhibition of GRP78 resensitizes MCF7-RR cells to tamoxifen (Fig. 2C). Tamoxifen and ICI treatment of LCC1 and MCF7 exhibit their established dose-dependent decrease in relative cell density, whereas overexpression of GRP78 in both cell lines significantly reduces antiestrogen sensitivity. Unlike the resistant models, this overexpression in LCC1 and MCF7 cells also reduces proliferation by approximately 25% in the absence of antiestrogen treatment. Thus, GRP78 may have some basal growth-inhibitory functions in sensitive cells, this function being lost in resistant cells (Fig. 2D and E).

Along with being present in the endoplasmic reticulum, recent studies showed the presence of cell surface GRP78 localization mediating pro-oncogenic Cripto signaling resulting in Src activation and cell survival (25, 26). To study whether or not this phenomenon plays a role in GRP78-mediated antiestrogen resistance, we pretreated LCC9 breast cancer cells with a GRP78-blocking antibody (previously shown to inhibit cell surface GRP78 signaling function; refs. 27, 28) or control antibody and treated with ICI. As shown in

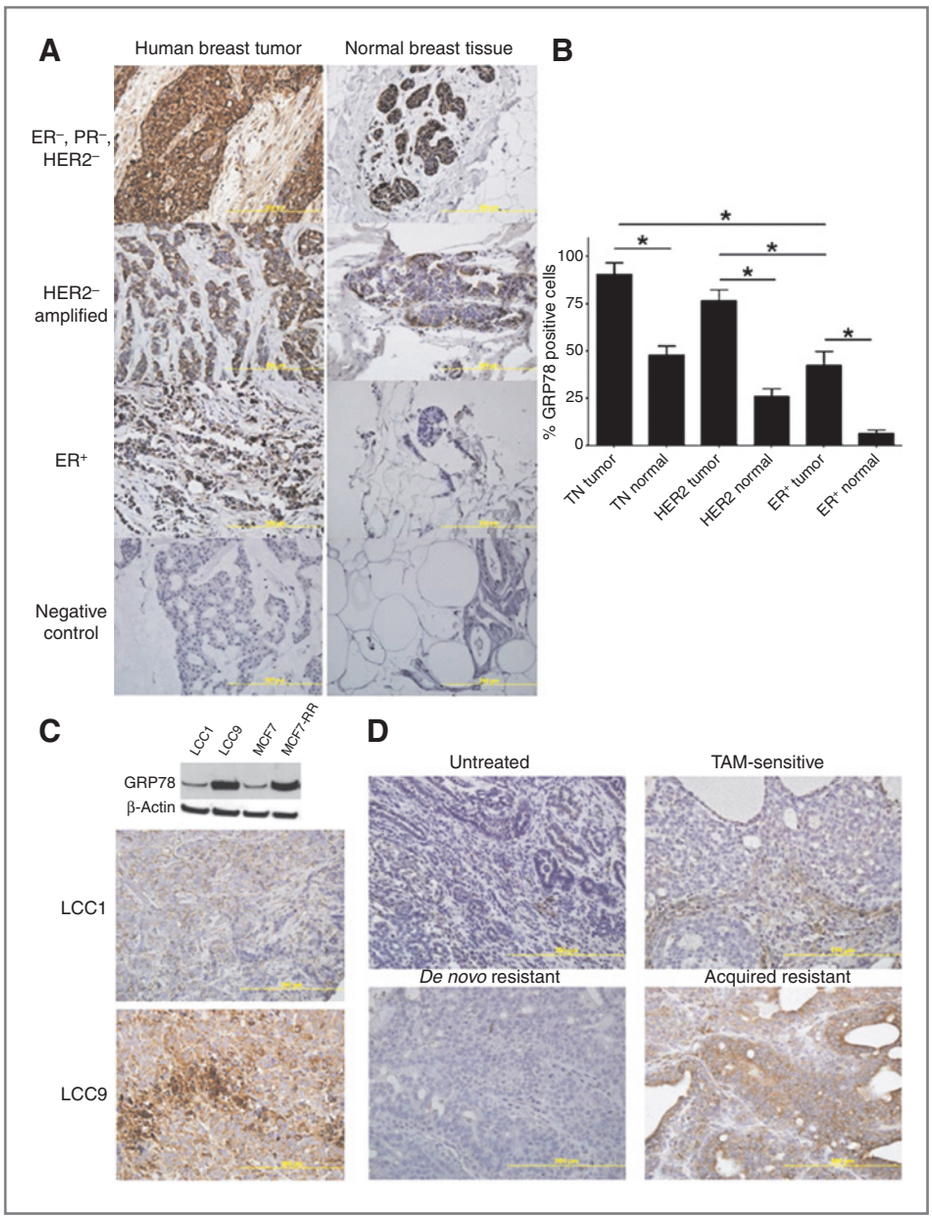

Figure 1. GRP78 is elevated in antiestrogen-resistant breast cancer. A, immunohistochemical staining of human ER+, triple-negative, and HER2-amplified breast tumors and their surrounding normal tissue. Sections were stained using a GRP78-specific antibody; tissue sections incubated with a nonspecific mouse IgG were used as a negative control. B, quantification of GRP78 expression in human ER+, triple-negative (TN), and HER2-amplified breast tumors. *, $P < 0.05$; $n = 2$–4, average of 3 fields for each section was quantified. C, proteins were isolated from LCC1, LCC9, MCF7, and MCF7-RR human breast cancer cell lines and Western blotting hybridization conducted to measure GRP78 protein expression. Immunohistochemical staining of LCC1 and LCC9 orthotopic tumor sections using a GRP78-specific antibody, $n = 4$. D, representative tumor sections from tamoxifen (TAM)-treated and control rat mammary tumors stained with GRP78-specific antibody.

Supplementary Fig. S1, treatment of LCC9 ICI-resistant breast cancer cells with GRP78-blocking antibody has no significant effect on the restoration of endocrine responsiveness as we observed with GRP78 siRNA knockdown (Fig. 2A), implying that cell surface–localized GRP78 expression does not mediate antiestrogen resistance.

GRP78 controls a phenotypic switch

GRP78 overexpression in antiestrogen-sensitive cells reduces expression of the UPR sensors PERK and IRE1 (Table 1). Establishing relevance, endogenous expression of the downstream effectors CCAAT/enhancer-binding protein homologous protein (CHOP, DDIT3; downstream of PERK) and XBP1-S (downstream of IRE1) is also reduced. Decreased phospho-mTOR:mTOR ratio and TOR complex 1 (TORC1, CRTC1) and increased ATG9 expression were also detected, suggesting an induction of autophagy. GRP78 overexpression increases several antiapoptotic B-cell lymphoma-2 (BCL2) family members including BCL2, BCL2L1 (Bcl-X_L), and BCL2L2 (BCL-W), implying an inhibition of apoptosis in sensitive cells. Thus, antiestrogen-resistant cells may respond in an opposite manner than in sensitive cells when GRP78 expression is inhibited. Indeed, GRP78 knockdown by RNA interference (RNAi) increases expression of PERK and IRE1 and their corresponding downstream effectors CHOP and XBP1-S in antiestrogen-resistant cells, whereas Bcl-X_L and Bcl-W expression is

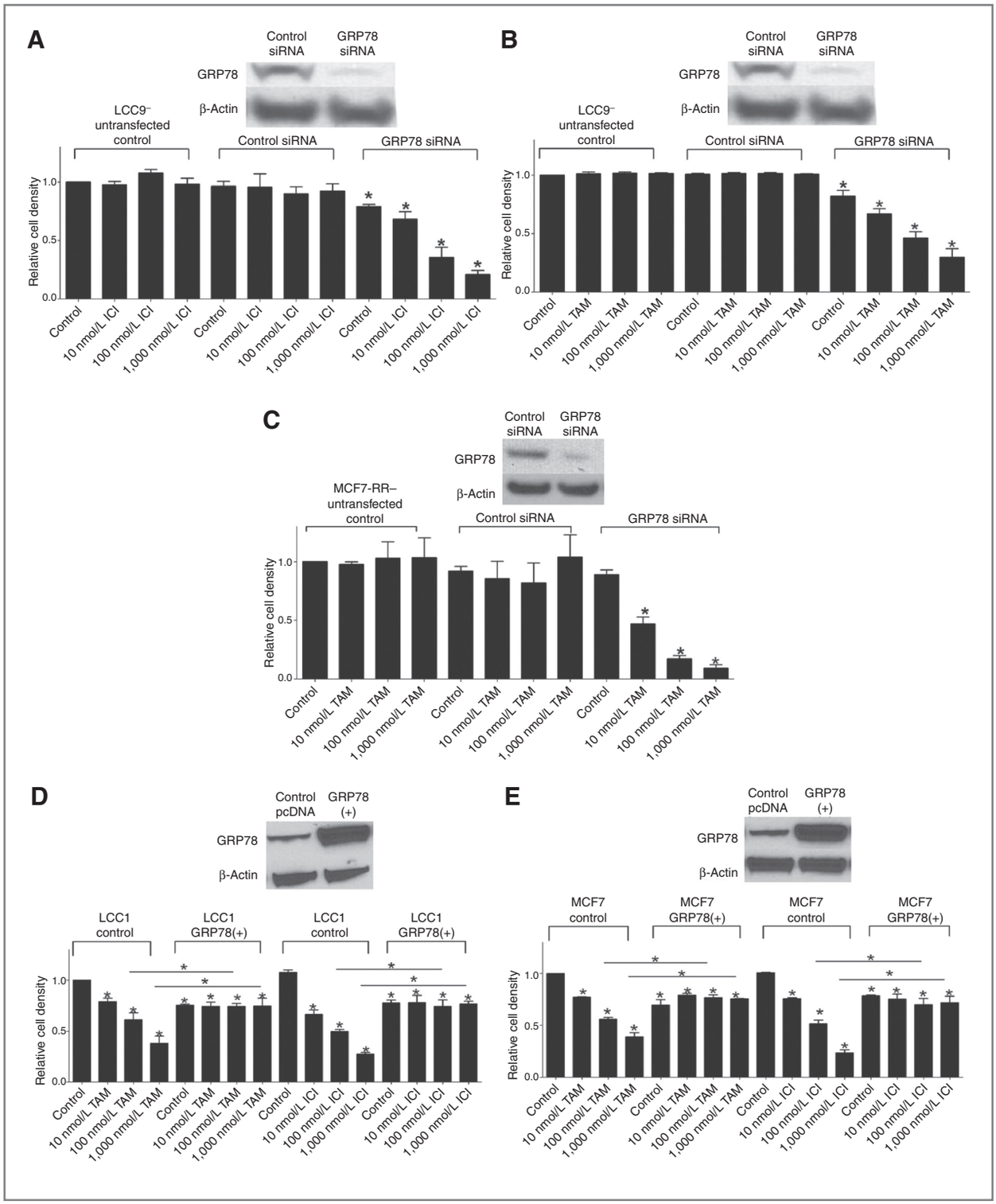

Figure 2. Modulation of GRP78 in human breast cancer cells alters antiestrogen responsiveness. LCC9 and MCF7-RR cells were transfected with control or GRP78 siRNA, and LCC1 and MCF7 cells were transfected with control pcDNA or GRP78(+); protein homogenates were isolated to determine GRP78 protein knockdown by Western blotting hybridization. LCC9 transfected with control or GRP78 siRNA, treated with ICI (A) or tamoxifen (TAM; B; 0.1% v/v ethanol vehicle, 10, 100, 1,000 nmol/L for 6 days), and cell density measured by crystal violet. C, MCF7-RR cells transfected with either control or GRP78 siRNA, treated with TAM (0.1% v/v ethanol vehicle, 10, 100, 1,000 nmol/L for 6 days), and cell density measured by crystal violet. LCC1 (D) or MCF7 (E) cells transfected with either control pcDNA or GRP78(+), treated with ICI or TAM (0.1% v/v ethanol vehicle, 10, 100, 1,000 nmol/L for 6 days), and cell density measure following crystal violet staining ($n = 3$–4; one-way ANOVA with Dunnett *post hoc* analysis; *, $P < 0.05$ compared with vehicle-treated control).

Table 1. GRP78 alteration results in phenotypic cellular switch

Protein	HUGO gene symbol	GO physiologic role	Effect of GRP78 overexpression (fold change)	Effect of GRP78 knockdown (fold change)
GRP94	*HSP90B1*	EnR HSP90 chaperone involved in EnR stress response	↔	↑ 3.31
ER-α	*ESR1*	Estrogen receptor-α, growth and proliferation	↔	↓ 1.76
HSC70	*HSPA8*	ATP-binding protein in the HSP70 family	↔	↓ 2.94
NF-κB (p65)	*RELA*	Transcription factor	↔	↔
TORC1	*CRTC1*	mTOR complex 1, glucose-mediated growth and inhibits autophagy	↓ 6.89	↔
Phospho-mTOR/ mTOR	*MTOR*	Glucose-mediated growth and inhibits autophagy	↓ 2.47	↔
IRE1	*ERN1*	UPR sensor	↓ 1.50	↑ 2.29
XBP1-S	*XBP1*	UPR effector, unconventionally spliced by activated IRE1	↓ 1.88	↑ 2.11
CHOP	*DDIT3*	UPR effector, proapoptotic	↓ 3.04	↑ 2.40
PERK	*EIF2AK3*	UPR sensor	↓ 2.50	↑ 2.34
ATF6	*ATF6*	UPR sensor	↔	↔
BCL-2	*BCL2*	Antiapoptotic, prosurvival	↑ 2.62	↔
BCL-W	*BCL2L2*	Antiapoptotic (BCL2 family member)	↑ 1.87	↓ 2.17
BCL-X_L	*BCL2L1*	Antiapoptotic (BCL2 family member)	↑ 4.69	↓ 1.71
Beclin-1	*BECN1*	Autophagy regulator	↔	↔
ATG9	*ATG9A*	Integral membrane protein found in autophagosomes	↑ 3.08	↔

NOTE: GRP78 was overexpressed in LCC1 cells (low-endogenous GRP78 expression) and knocked down in LCC9 cells (high-endogenous GRP78 expression), proteins were isolated, and protein expression of GRP94, ER-α, HSC70, NF-κB (p65), phospho-mTOR, mTOR, TORC1, PERK, ATF6, IRE1, XBP1-S, CHOP, BCL2, BCL-W, BCL-X_L, BECN1, and ATG9 were investigated by Western blot hybridization.

Abbreviations: GO, Gene Ontology (http://www.geneontology.org/); HUGO, human genome organization (http://bioportal.bioontology.org/ontologies/44453); EnR, endoplasmic reticulum.

decreased. Unlike the sensitive cells, no effect of GRP78 knockdown on BCL2, ATG9, phospho-mTOR:mTOR, and TORC1 is detected, although both ER-α and HSC70 (HSPA8) expression is decreased. Perhaps as an attempted compensatory mechanism, inhibition of GRP78 potently induced GRP94, a protein chaperone also involved in the endoplasmic reticulum stress response. Neither overexpression nor reduction of GRP78 expression affects ATF6, NF-κB (RELA), or beclin-1 (BECN1) protein levels (Table 1); representative Western blotting images are shown in Supplementary Fig. S2.

GRP78 affects both apoptosis and autophagy

Inhibition of GRP78 in LCC9 and MCF7-RR cells significantly increases the levels of cleaved caspase-7, cleaved PARP, PARP1 (Fig. 3A and B) and Annexin V–stained positive cells (Fig. 3C) when treated with an antiestrogen. Conversely, overexpression of GRP78 in LCC1 and MCF7 cells [LCC1-GRP78(+) and MCF7-GRP78(+)] potently inhibits cleaved caspase-7 and cleaved PARP expression (Fig. 3D and E) and reduces the percentage of Annexin V–stained positive cells following antiestrogen treatment (Fig. 3F). Thus, GRP78 plays a central role in the regulation of apoptosis, consistent with the changes observed in the expression of BCL2 family members (5).

With antiestrogen therapy, inhibition of GRP78 in LCC9 and MCF7-RR decreases autophagy (LC3-II and p62 expression; Fig. 4A and B); GRP78 knockdown alone has no effect. Conversely, overexpressing GRP78 in LCC1 and MCF7 cells markedly increases LC3-II protein (Fig. 4D) and decreases p62 protein levels (Fig. 4E), indicating an increase in autophagy. p62 labels cargo for autophagosome degradation, therefore decreased p62 levels are indicative of increased autophagy. Confocal microscopy showed an increase in LC3-GFP–positive puncta formation, indicative of autophagosome formation, in LCC1 cells treated with ICI when compared with controls; a more pronounced response was observed with GRP78 overexpression (Fig. 4F). LCC9 cells treated with ICI showed LC3-GFP–positive puncta expression when compared with vehicle controls; ICI did not induce a LC3-GFP–positive puncta pattern in LCC9 cells when GRP78 expression was inhibited by RNAi (Fig. 4C). Thus, GRP78 also plays a central role in the regulation of autophagy initiation.

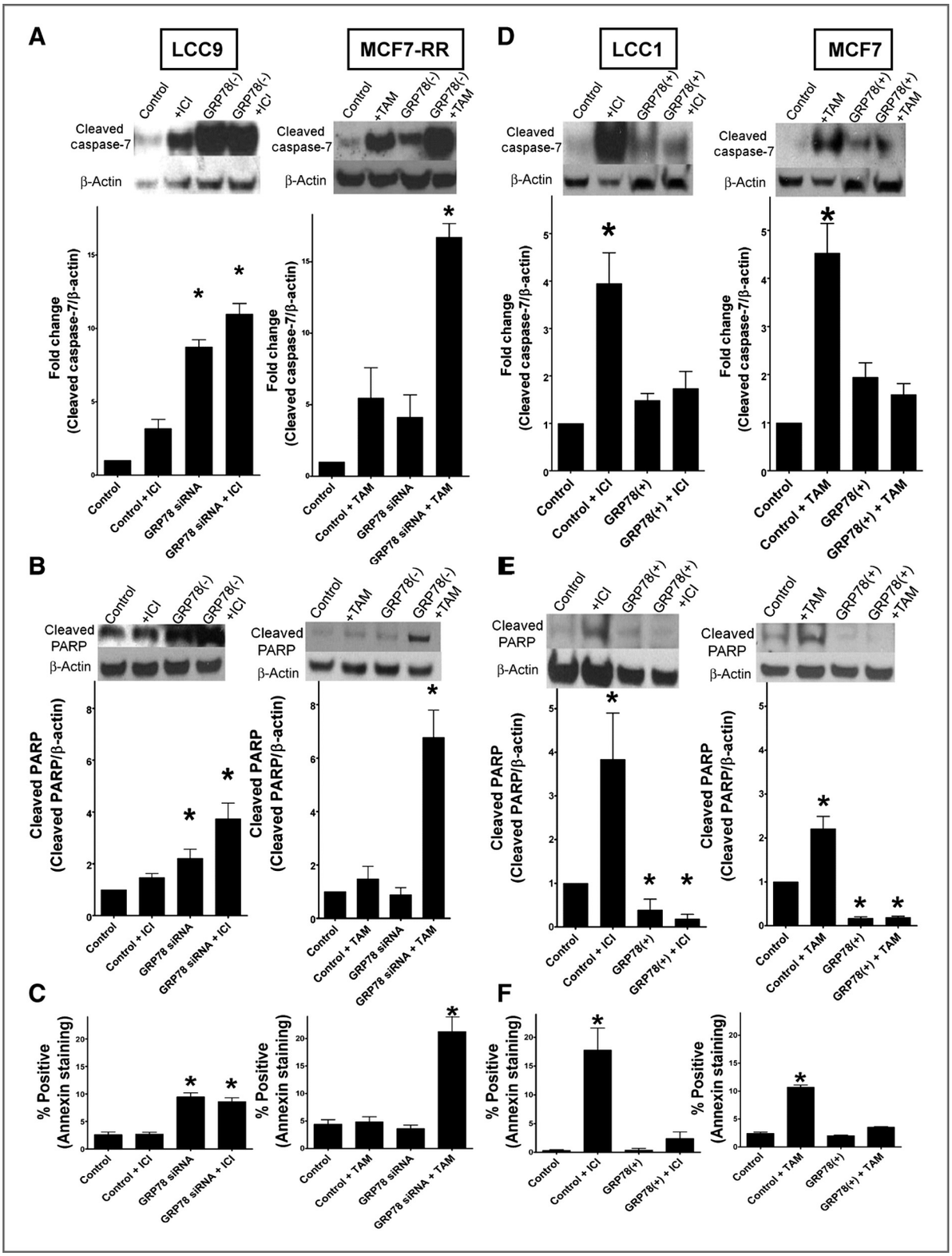

Figure 3. GRP78 regulates apoptosis. LCC9 and MCF7-RR breast cancer cells were transfected with control or GRP78 siRNA, and LCC1 and MCF7 human breast cancer cells were transfected with control pcDNA or GRP78 expression vector and treated with either 0.1% v/v ethanol vehicle, 100 nmol/L ICI (LCC9/LCC1), or 100 nmol/L tamoxifen (TAM; MCF7-RR/MCF7) for 6 days. Western blotting hybridization of protein homogenates was used to measure cleaved caspase-7 (A + D) or cleaved PARP (B + E) levels. C + F, Annexin V-FITC–stained cells counted using flow cytometry. All studies, $n = 3$–5; one-way ANOVA with Dunnett *post hoc* analysis; *, $P < 0.05$ compared with vehicle control. FITC, fluorescein isothiocyanate.

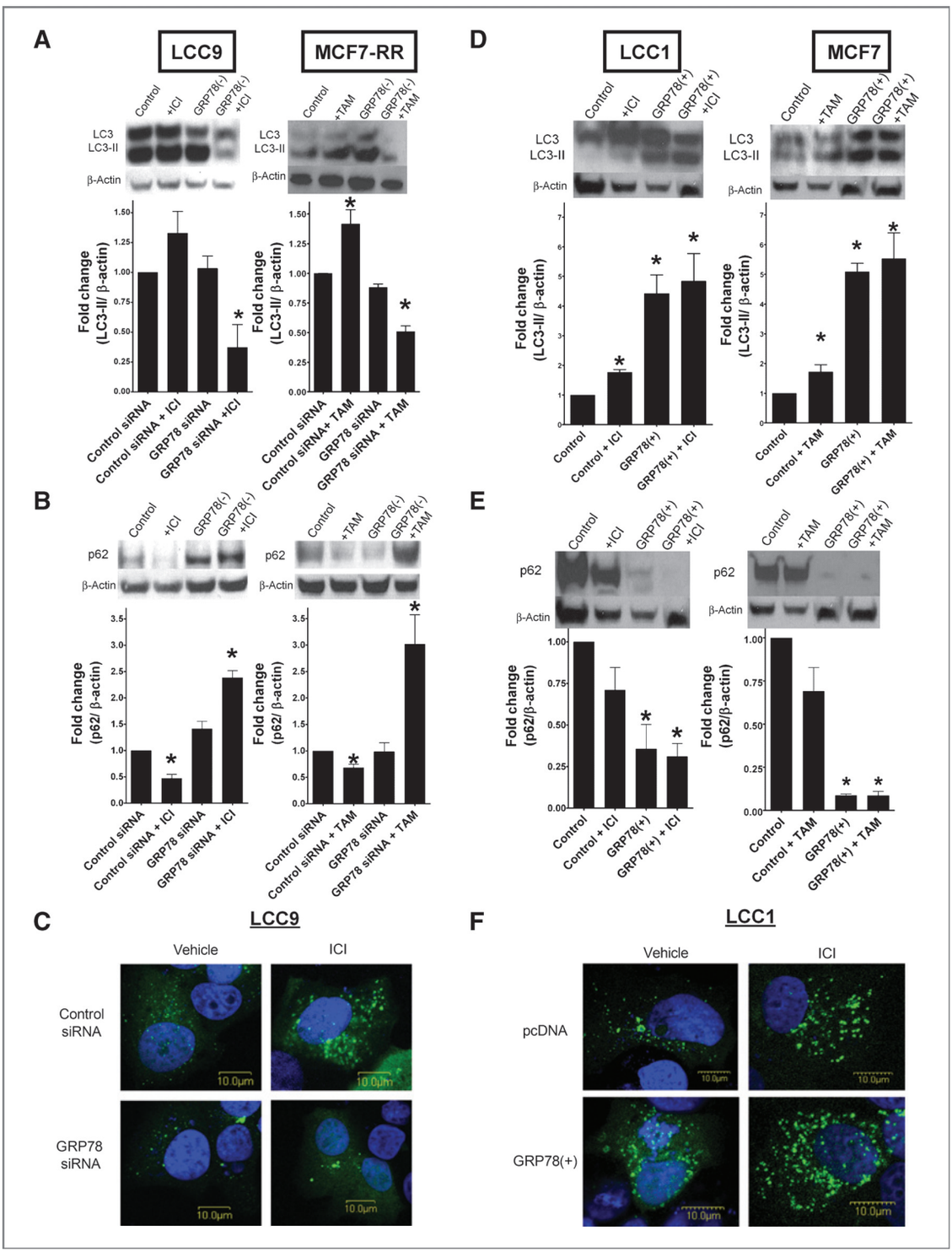

Figure 4. GRP78 regulates autophagy. LCC9 and MCF7-RR cells transfected with control or GRP78 siRNA and LCC1 and MCF7 cells transfected with control pcDNA or GRP78 expression vector and treated with 0.1% v/v ethanol vehicle, 100 nmol/L ICI (LCC9/LCC1), or 100 nmol/L tamoxifen (TAM; MCF7-RR/MCF7) for 6 days. Western blotting hybridization of protein homogenates was used to measure LC3-II (A + D) or p62 (B + E) protein expression. One-way ANOVA with Dunnett *post hoc* analysis; *, $P < 0.05$ compared with vehicle-treated controls. C, LCC9 cells transfected with GFP-LC3 and control or GRP78 siRNA and LCC1 cells were transfected with GFP-LC3 and control pcDNA or GRP78(+) (F), and treated with 0.1% v/v ethanol vehicle or 500 nmol/L ICI for 72 hours. LC3-positive punctate pattern was observed by confocal microscopy.

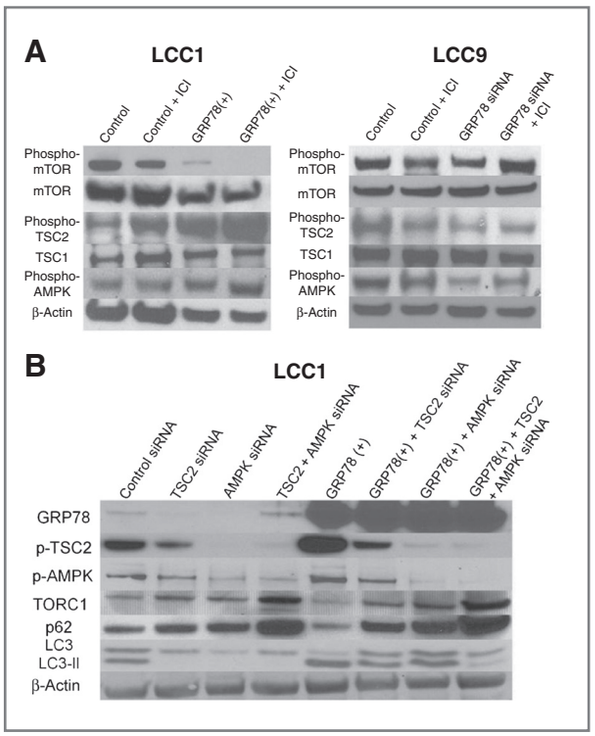

Figure 5. GRP78 modulates mTOR activity to promote autophagy. A, LCC1 cells transfected with GRP78(+) cDNA or control pcDNA, and LCC9 cells transfected with control or GRP78 siRNA and treated with either 0.1% v/v ethanol vehicle or 100 nmol/L ICI for 6 days. Protein homogenates were isolated and Western blotting hybridization used to measure phospho-mTOR, mTOR, phospho-TSC2, TSC1, phospho-AMPK, and β-actin expression. B, LCC1 cells transfected with control, TSC2, and/or AMPK siRNA and with control pcDNA or GRP78(+) cDNA. Protein homogenates were isolated and Western blotting hybridization was used to measure GRP78, phospho-TSC2, phospho-AMPK, TORC1, p62, LC3, and β-actin expression.

GRP78-mediated autophagy depends on mTOR suppression

Figure 5A shows that overexpression of GRP78 in LCC1 cells inhibits phospho-mTOR:mTOR protein ratio, with a corresponding increase in phospho-TSC2. There is no change in TSC1 protein and a slight increase in phospho-AMPK. Conversely, ICI treatment of LCC9 cells with inhibition of GRP78 by RNAi increases phospho-mTOR:mTOR protein ratio, accompanied by an inhibition of phospho-TSC2 and phospho-AMPK expression; no change was detected in TSC1 expression. Transfection with TSC2 siRNA and/or AMPK siRNA in control and GRP78-overexpressing LCC1 cells inhibits autophagy and increases TORC1 expression (Fig. 5B). Dual inhibition of both TSC2 and AMPK in LCC1-GRP78(+) produces a greater inhibition of autophagy (as determined by p62 levels) and increases the expression of TORC1 greater than either single target knockdown alone.

Inhibition of caspase activity and autophagosome formation block the effects of GRP78

LCC9 cells pretreated with 50 μmol/L of the pan-caspase inhibitor Z-VAD-FMK and then transfected with GRP78 siRNA lost the ability of GRP78 inhibition to restore a dose-dependent, antiestrogen-induced cell death (Fig. 6A and B). However, GRP78 RNAi treated cells retain the about 25% reduction in basal proliferation described above, even when pretreated with Z-VAD-FMK. These data suggest a caspase-independent mechanism of cell death, perhaps unrelated to antiestrogen-induced cell death mechanisms. Inhibition of caspase activity has no effect on the GRP78-mediated inhibition of autophagy in response to ICI. When autophagy is blocked by RNAi targeting ATG5, a protein necessary for the formation of the preautophagosomal structure, autophagy is inhibited in both LCC1 control and GRP78(+) cells. Inhibition of autophagy in control LCC1 cells potentiates the cell death response induced by ICI. Moreover, reduction of ATG5, and the consequent inhibition of autophagy in LCC1-GRP78(+) cells resensitizes these cells to ICI (Fig. 6C and D).

Dual inhibition of BECN1 and GRP78 synergistically increases cell death

Control and LCC9 cells stably expressing BECN1 short hairpin RNA (shRNA) were transfected with control or GRP78 siRNA. As observed in Fig. 6E and F, GRP78 silencing with concurrent antiestrogen treatment produces a dose-dependent inhibition of proliferation in LCC9-control/BECN1–knockdown cells. Inhibition of BECN1 in LCC9 reduces proliferation in response to the highest dose of ICI (1 μmol/L) by 20%, consistent with a prior report (29). However, concurrent knockdown of GRP78 and BECN1 potentiates the cell death response to ICI greater than single target knockdown alone. As defined by RI (24), dual knockdown of GRP78 and BECN1 results in a synergistic inhibition of cell proliferation at both 100 nmol/L (RI = 2.0) and 1,000 nmol/L ICI (RI = 2.5).

Discussion

Resistance to endocrine therapies in ER+ breast cancer remains a major clinical problem, partly because of limitations in current understanding of the resistant phenotype. Using multiple cell line models and different endocrine therapies, we now establish a central role for the UPR sensor GRP78, in which breast cancer cells use UPR-initiated signaling to integrate their responses to antiestrogens. In resistant cells, these responses include a coordinated suppression of proapoptotic activities and induction of prosurvival autophagy. In a rat DMBA mammary carcinogenesis model, the highest level of GRP78 was observed in the tamoxifen-acquired resistant tumors, consistent with the acquired resistant phenotype of the human breast cancer cell lines. No significant change in GRP78 expression was seen in ER+ mammary tumors that did not respond to tamoxifen or mammary tumors that appeared during tamoxifen treatment (*de novo* resistance), suggesting that GRP78 induction is an adaptive response to endocrine therapy. Moreover, these data indicate that acquired and *de novo* resistance mechanisms in ER+ breast tumors do not necessarily arise from the same mechanism. Both the epithelial and stromal components of the tumors and the surrounding normal tissues were GRP78-positive, suggesting activities both within the cancer cells and within the tumor

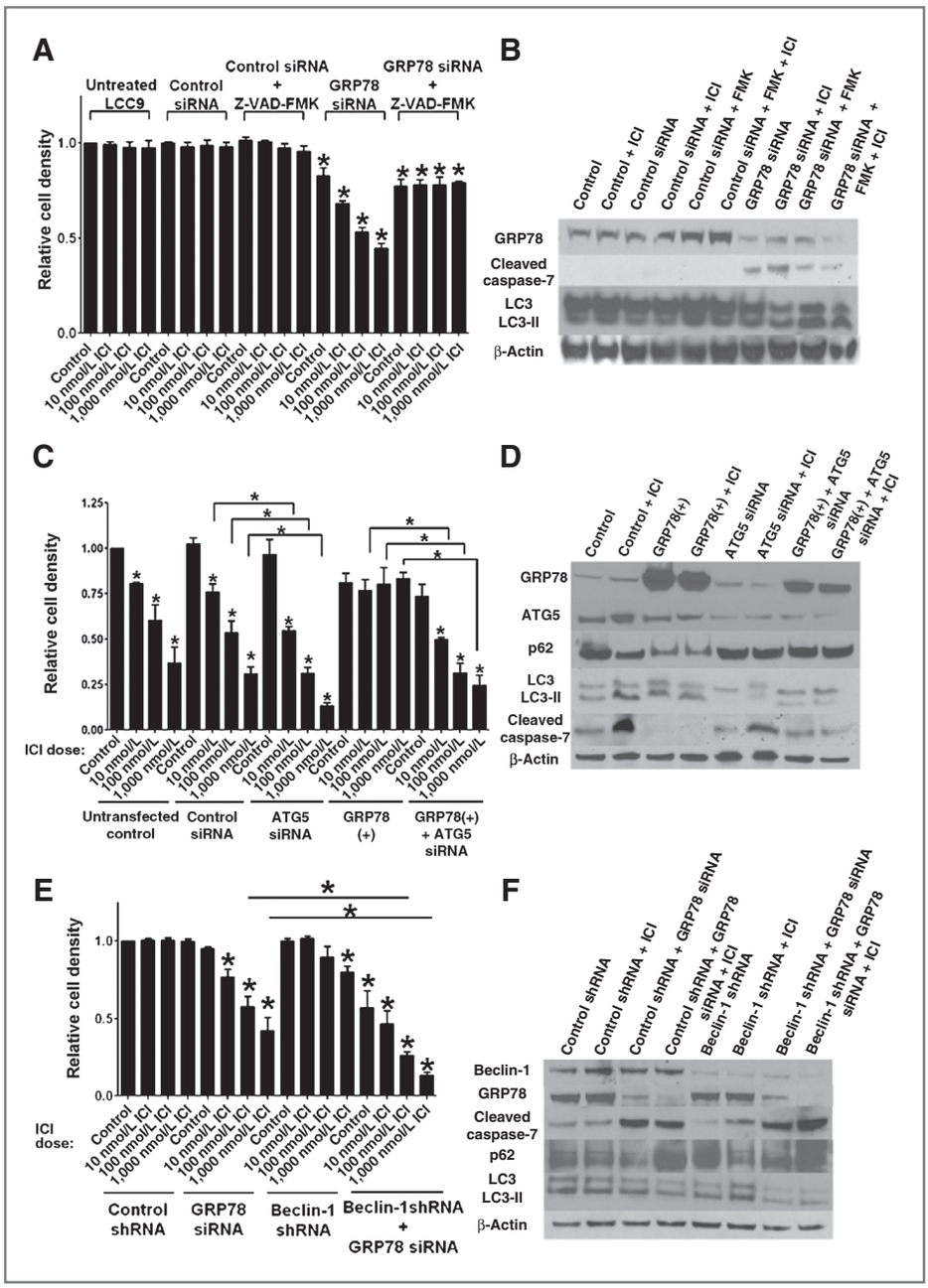

Figure 6. GRP78 facilitates cross-talk between apoptosis and autophagy to affect cell survival and proliferation. A, LCC9 cells were pretreated with 50 μmol/L pan-caspase inhibitor (Z-VAD-FMK, FMK) for 30 minutes before transfection with control or GRP78 siRNA. These cells were then treated with ICI and/or Z-VAD-FMK for 6 days and cell density measured following crystal violet staining. B, protein homogenates from these treatment groups (100 nmol/L ICI for 6 days) were subjected to Western blotting hybridization to measure GRP78, cleaved caspase-7, LC3, and β-actin expression. C, LCC1 cells transfected with control or ATG5 siRNA and/or control or GRP78 cDNA and treated with 0.1% v/v ethanol vehicle or ICI. D, protein homogenates from these treatment groups (100 nmol/L ICI for 6 days) were subjected to Western blotting hybridization to measure GRP78, ATG5, cleaved caspase-7, p62, LC3, and β-actin. E, LCC9 cells constitutively expressing control shRNA or beclin-1 shRNA were transfected with control or GRP78 siRNA, treated with 0.1% v/v ethanol vehicle or ICI for 6 days, and cell density measured following crystal violet staining. F, protein homogenates from these treatment groups (100 nmol/L ICI for 6 days) were subjected to Western blotting hybridization to measure GRP78, beclin-1, cleaved caspase-7, p62, LC3, and β-actin. For all experiments, $n = 3$–4; one-way ANOVA with Bonferroni *post hoc* analysis; *, $P < 0.05$. Synergy is determined by RI [(survival A × survival B)/(survival A+B)] < 2.0.

microenvironment. A role for GRP78 in supporting neovascularization and angiogenesis has been reported (30) and may explain the role of GRP78 in the tumor microenvironment. Our data in human breast cancer cells growing *in vitro* show that its activities within cancer cells is sufficient to explain the role of GRP78 in acquired endocrine resistance.

Inhibition of GRP78 with RNAi restored a dose-dependent antiestrogen-mediated inhibition of proliferation in both LCC9 and MCF7-RR cells. Conversely, overexpression of GRP78 in LCC1 and MCF7 cells resulted in a loss of responsiveness when compared with their controls. GRP78 can protect some MCF7 cells against an estrogen deprivation–induced apoptosis (18, 19). However, this is a different phenotype from antiestrogen resistance, as evident in the responsiveness of many patient's tumors to an antiestrogen following failure on an aromatase inhibitor (31, 32), and the estrogen-independent (model of aromatase resistance) but tamoxifen- and ICI-sensitive LCC1 phenotype, which we show has lower expression of GRP78 (33). While overexpression of GRP78 results in a loss of antiestrogen sensitivity, there was a 20% to 25% decrease in relative cell density as measured by crystal violet assay. The overall decrease in cell density may result from an increase in apoptosis, cellular senescence, and/or a decrease in proliferation. Overexpression of GRP78 prevented endocrine

therapy–induced apoptosis (Fig. 3A–F) and stimulated autophagy (Fig. 4A–F). Autophagy was shown to decrease cell size and promote cellular senescence. Moreover, increased GRP78 expression inhibited the key proliferation regulator mTOR, which may explain the observed reduction in cell density with overexpression of GRP78. Further studies into the effect of GRP78 on cellular senescence and proliferation are being explored.

Perturbation of GRP78 resulted in the altered regulation of its downstream UPR signaling components in the LCC1 (sensitive; GRP78 cDNA overexpressed) and LCC9 (resistant; GRP78 inhibited by RNAi) phenotypes, with the exception of ATF6. For example, LCC1-GRP78(+) showed an increase in the expression of antiapoptotic BCL2 family members including BCL2, BCL-X_L, and BCL-W, implicating their activities in preventing an apoptosis-mediated cell death. GRP78 overexpression also decreased expression of the endogenous phospho-mTOR/mTOR and TORC1 proteins, implying a role in regulating mTOR signaling. Because we also detected a concurrent increase in ATG9, a role for GRP78 in affecting the induction of autophagy was strongly implicated. Interestingly, GRP78 knockdown in LCC9 cells led to a potent stimulation of the endoplasmic reticulum chaperone GRP94, perhaps as a compensatory mechanism in the absence of GRP78. However, this induction of GRP94 is not sufficient to reverse the phenotype (34). We also detected a decrease in ER-α, perhaps reflecting GRP78 and estrogen interactions as reported in the endometrium (35). GRP78 knockdown in LCC9 cells reduced BCL-X_L and BCL-W protein expression with no effect on BCL2, suggesting an overall reduction in antiapoptotic BCL2 family members that could enable an apoptotic cell death. The effect of GRP78 on response to estrogen withdrawal is blocked when BIK is concurrently inhibited (18). While the effect of GRP78 on BCL2 family members may play a role in mediating antiestrogen resistance through apoptosis, these proteins are multifunctional and may regulate other cell fate pathways including autophagy.

Inhibition of GRP78 and treatment with antiestrogens in LCC9 and MCF7-RR cells produces a potent induction of apoptosis, as observed by increased cleaved caspase-7, cleaved PARP, and Annexin V staining. GRP78 knockdown further reduces ER expression in the LCC9, and RNAi knockdown of ER in these cells is growth-inhibitory (36). Endogenous ER levels are higher in MCF7-RR cells, perhaps explaining why tamoxifen treatment is necessary to stimulate cell death in the presence of GRP78 knockdown (Fig. 2C). Conversely, overexpression of GRP78 in LCC1 and MCF7 cells inhibited antiestrogen-stimulated apoptosis. GRP78 can bind procaspase-7 and inhibit its cleavage and subsequent activation of apoptosis (37), likely contributing to GRP78-mediated inhibition of an antiestrogen-induced increase in apoptosis. Pretreatment of LCC9 breast cancer cells with a pan-caspase inhibitor (Z-VAD-FMK) blocked the dose-dependent reduction of proliferation observed with GRP78 knockdown alone, suggesting a caspase-dependent mechanism for GRP78-mediated restoration of antiestrogen sensitivity. A 20% to 25% decrease of proliferation was also observed when GRP78-knockdown cells were pretreated with the pan-caspase inhibitor, suggesting the existence of another cellular mechanism of GRP78-mediated antiestrogen resistance.

Breast cancer cells have elevated basal autophagy when compared with immortalized breast epithelial cells, and antiestrogen-resistant cells have increased basal autophagy when compared with endocrine therapy–sensitive breast cancer cells (Supplementary Fig. S3; ref. 38). Knockdown of GRP78 in embryonic kidney cells inhibited autophagy through disruption of endoplasmic reticulum integrity, perhaps by preventing the translocation of ATG9 (34, 39). However, knockdown of GRP78 in LNCaP cells had no effect on basal autophagy (40), consistent with our results indicating that GRP78 knockdown has no effect on basal autophagy in either LCC9 or MCF7-RR cells, correlating with the higher level of autophagy observed in cancer cells than in normal cells.

How UPR and GRP78 regulate autophagy requires further study. Upregulation of GRP78 inhibits UPR signaling (Table 1), which is expected to inhibit UPR-initiated autophagy by preventing an ATF4-mediated induction of ATG12 (6). However, this outcome lead to an increase in autophagy as indicated by increased autophagic flux (decreased p62) and increased LC3-II formation and puncta (Fig. 4D–F). We propose that a GRP78-mediated upregulation of prosurvival autophagy can occur outside of the canonical GRP78 UPR response. The antiapoptotic BCL2 family members were shown to contribute to autophagy by binding to the BH3 domain of BECN1, thereby preventing BECN1 from initiating autophagy. We show in Table 1 that GRP78 overexpression in LCC1 breast cancer cells induces BCL2, BCL-X_L, and BCL-W expression, with no apparent change in BECN1 levels. In contrast to the elevated expression of BCL2 family members that might be expected to inhibit autophagy, we show elevated autophagy. Because GRP78 can bind to several BCL2 family members, overexpressing GRP78 may sequester the elevated BCL2 preventing BCL2 from inhibiting BECN1, thereby enabling autophagy (19). mTOR regulation of autophagy is also well-documented (39, 41) but much less is known of the effects of mTOR on UPR signaling. TSC1- and TSC2-null mouse embryonic fibroblasts exhibit increased UPR signaling, suggesting that TSC deficiency leads to increased TORC1 activity and dysregulated protein synthesis that could activate UPR (42), perhaps through an ATF6-dependent activation of mTOR (43). However, GRP78 modulation in LCC1 and LCC9 cells had no effect on ATF6 levels (Table 1), implicating another mechanism of UPR/GRP78 regulation of mTOR. Modulation of GRP78 resulted in perturbations in mTOR expression, with a corresponding change in phospho-TSC2 and phospho-AMPK. Using RNAi against TSC2 and AMPK, we showed that GRP78-mediated activation of AMPK and TSC2 results in TORC1 inactivation and autophagy stimulation. These data highlight a novel signaling mechanism of GRP78, in which GRP78-mediated autophagy is due to the modulation of mTOR signaling. Therefore, UPR-induced changes in GRP78 expression may affect subcellular localization to regulated changes to AMPK, TSC2, and TORC1.

To investigate further the role of autophagy in GRP78 mediated resistance, LCC1-GRP78(+) transfected with ATG5 siRNA exhibited a resensitization to antiestrogen treatment,

suggesting that a key component to GRP78-mediated endocrine resistance is the stimulation of autophagy. Because of lack of specificity of chemical inhibitors, we used ATG5 siRNA to inhibit autophagy. Increased cell death in response to ICI treatment was observed in LCC1-ATG5–knockdown cells when compared with control cells expressing intact autophagy, highlighting the prosurvival role of autophagy in antiestrogen-resistant breast cancer (Fig. 6C and D). Moreover, concurrent inhibition of BECN1 and GRP78 in LCC9 cells produced a synergistic inhibition of proliferation in response to ICI (Fig. 6E and F). Thus, how autophagy is inhibited may influence subsequent responses. For example, the observed synergy may reflect that knockdown of GRP78 would activate an mTOR-mediated inhibition of autophagy (Fig. 5A), whereas inhibition of BECN1 would block BECN1-dependent autophagy.

We show that resistance to endocrine therapies requires the concurrent inhibition of prodeath signaling (apoptosis) and an increased ability to respond to the stress of the therapy (prosurvival autophagy). These integrated actions are controlled, at least partly, by signaling initiated within the UPR in response to endoplasmic reticulum stress and the activation of GRP78. In antiestrogen-resistant breast cancer cells, elevated levels of GRP78 support cell survival by inhibiting apoptosis through inducing antiapoptotic BCL2 family members and inhibiting caspase-7 activation. To ensure that cells can respond to therapy-induced stress, which includes loss of growth factor signaling (44), GRP78 activates an mTOR-regulated prosurvival autophagy (GRP78-mediate prosurvival signaling summarized in Supplementary Fig. S4). Thus, the cell can recover energy and intermediate metabolites from the autophagic cannibalization of damaged subcellular organelles and misfolded/unfolded proteins (6, 7). The data presented here now show how cells integrate prodeath and prosurvival signaling, how this is altered in sensitive and acquired resistant cells, and implicates UPR and GRP78 as central components in the critical cross-talk between UPR signaling, apoptosis, and autophagy signaling that determines cell fate outcome in response to antiestrogens.

Disclosure of Potential Conflicts of Interest

L.A. Hilakivi-Clarke provided expert testimony in a court case involving DES exposures in women and breast cancer risk for the Aaron Levine law firm. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.L. Cook, A. Warri, L. Jin, R. Clarke
Writing, review, and/or revision of the manuscript: K.L. Cook, A.N. Shajahan, A. Warri, R. Clarke
Study supervision: A.N. Shajahan, R. Clarke

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