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Original Article

Prolactin-inducible EDD E3 ubiquitin ligase promotes TORC1 signalling, anti-apoptotic protein expression, and drug resistance in breast cancer cells

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Abstract: Previously, we identified a prolactin (PRL)-inducible gene encoding EDD E3 ubiquitin ligase in human breast cancer (BCa) cells. We reported that EDD binds the mTOR (TORC1)-associated α4 phosphoprotein-PP2Ac protein phosphatase complex that regulates initiation of translation and cell cycle progression, and that EDD targets PP2Ac for proteasomal degradation. The present study showed that EDD immunostaining was low in benign human breast tissues, but increased progressively in ductal carcinoma in-situ, low-grade, and high-grade BCa, and in triple-negative BCa (TNBC). EDD mRNA and protein levels varied in human BCa cell lines. In high-EDD expressing MCF-7 and T47D cells, siRNA knockdown of EDD arrested cells in the G2-phase of the cell cycle, decreased cell viability, and increased apoptosis. EDD siRNA-induced apoptosis in MCF-7 cells correlated with significantly increased levels of pro-apoptotic Bim and Bak mRNAs and proteins ($P < 0.05$, $n = 3$-6), and increased levels of pro-apoptotic Bax and MOAP-1 proteins ($P < 0.001$, $n = 3$-6), leading to increased cleavage of caspase-7 and caspase substrate poly-ADP-ribose polymerase-1 (PARP-1), as compared to control cells. Loss of EDD in MCF-7 cells decreased PRL-induced phosphorylation of eukaryotic initiation factor 4E-binding protein-1, a mediator of TORC1 signaling, resulting in decreased binding of 4E to γ-aminophenyl-m^{7}GTP agarose in Cap-binding assays. In low-EDD expressing MDA-MB-436 TNBC cell line, gain of EDD following pCMV-Tag2B.EDD transfection increased cell resistance to chemotherapeutic drugs cisplatin and doxorubicin, TORC1 inhibitor rapamycin, and TORC1/TORC2 inhibitor INK128, as compared to controls. In contrast, loss of EDD in MCF-7 cells increased cell sensitivity to cisplatin, doxorubicin, rapamycin, and selective estrogen receptor modulator tamoxifen. In summary, EDD levels increase with BCa progression *in vivo*. PRL-inducible EDD in BCa cells promotes TORC1 signaling, anti-apoptotic protein expression, and drug resistance *in vitro*. These findings implicate EDD as a potential therapeutic target and support PRL receptor blockade as an additional therapy for BCa.

Keywords: EDD E3 ubiquitin ligase, prolactin, TORC1, apoptosis, drug resistance, breast cancer

Introduction

EDD (E3 isolated by differential display) or UBR5, initially identified as a progestin-inducible gene in human T47D breast cancer (BCa) cells [1, 2], is the mammalian ortholog of the Drosophila hyperplastic discs gene that controls cell proliferation during development [3]. EDD is the only known E3 ligase with RING-like zinc-finger and HECT domains [2]. RING proteins display intrinsic E3 ligase activity or are part of E3 ligase complexes [4]. HECT proteins have E3 ligase activity [1] and also play critical roles in carcinogenesis [5]. Human EDD is ubiquitously expressed but frequent disruption of its gene locus at chromosome 8q22 in a variety of cancers [1] and EDD overexpression in many solid tumours have implied a role in tumourigenesis [6, 7]. EDD has emerged as a key regulator of various cellular processes in cancer, including gene expression, genome integrity, and chemoresistance [8]. High nuclear EDD expression in a cohort of 151 women with serous ovarian carcinoma was associated with an increased risk of disease recurrence following first-line chemotherapy, and siRNA-knock-

down of EDD gene expression partially restored cisplatin sensitivity in cisplatin-resistant ovarian cancer cells *in vitro* [9]. Loss of EDD induced cell-cycle arrest at G1 through upregulation of tumour suppressor p53 and p21 proteins in osteosarcoma cells *in vitro* [10]. Analysis of primary triple-negative BCa (TNBC) by whole-exon sequencing showed strong EDD gene amplification. EDD overexpression was confirmed in TNBC tissues and, using a murine TNBC model, CRISPR/cas9-mediated EDD deletion dramatically abrogated tumour growth and metastasis [11].

We identified EDD as a novel protein partner of a mTOR/TORC1-associated protein complex comprising α4-phosphoprotein and the catalytic subunit of protein phosphatase 2A (PP2Ac) [12]. The α4 protein physically interacted with PP2Ac and EDD at its N- and C-termini, respectively [12]. The α4-PP2Ac complex regulates TORC1 signaling through 4E-binding protein-1 (4EBP1), which binds eukaryotic initiation factor 4E (eIF4E), and ribosomal S6 kinase to initiate protein translation, cell-cycle progression, and cell proliferation [13-16]. Furthermore, we showed that EDD polyubiquitinated PP2Ac for proteasomal degradation [17]. Treatment of human MCF-7 and T47D BCa cell lines with progesterone and prolactin (PRL) upregulated EDD mRNA and protein levels with a concomitant decrease in PP2Ac levels [17], further supporting a role for EDD in PP2Ac turnover.

The present study investigated the role of EDD in breast cancer. EDD immunostaining was determined during tumour progression *in vivo*. The role of EDD in cell-cycle progression, the intrinsic apoptotic pathway, and PRL-stimulated TORC1 signaling was investigated in EDD-expressing MCF-7 and/or T47D breast cancer cell lines. The role of EDD on drug sensitivity was studied by ectopic expression of EDD in low EDD-expressing MDA-MB-436 BCa cells and by shRNA-knockdown in MCF-7 cells.

Materials and methods

Antibodies

Primary antibodies used were: rabbit polyclonal anti-EDD (1:500) (Abcam Inc., Toronto, ON, Canada); mouse monoclonal anti-EDD (1:200), anti-PARP-1 (1:1000; detected 116-kDa PARP-1, 24-kDa N-terminal cleavage product), anti-Mcl-1 (1:200; detected Mcl-1_L, Mcl-1_S), anti-4EBP1 (1:1000) and anti-Bax (1:200) from Santa Cruz Biotechnology (Dallas, TX); rabbit monoclonal anti-BAK (1:1000), anti-BIM (1:1000), anti-caspase-6 (1:1000) and anti-caspase-7 (1:1000), and rabbit polyclonal anti-4EBP1 (1:1000; detected α, β, γ-4EBP1) from Cell Signaling Technology (Danvars, MA); affinity-purified rabbit anti-Mcl-1 (1:1000) from Bethyl Labs (Montgomery, TX); mouse IgG from DakoCytomation, Agilent Technologies Canada Inc., (Mississauga, ON, Canada). Rabbit anti-MOAP-1 (1:1000) was from SinoBiologicals (Wayne, PA). Mouse anti-p53 (1 μg/ml), anti-β-tubulin (1:10,000), rabbit anti-β-actin (1:20,000) and secondary antibodies goat anti-rabbit IgG (1:5000) and goat anti-mouse IgG (1:3000) horse radish peroxidase (HRP) conjugates were from Sigma-Aldrich (Oakville, ON, Canada).

Tissue collection

Anatomical pathology electronic files (Cerner Millenium) for the Queen Elizabeth II Health Sciences Centre, Nova Scotia Health Authority were retrospectively searched for a cohort of invasive and in-situ breast carcinomas as previously described [18]. For this study, formalin-fixed paraffin-embedded (FFPE) breast tissues were used with approval from the QEII Research Ethics Board, and Materials Transfer and Collaboration Agreement between the Nova Scotia Health Authority, Central Zone, and Dalhousie University. A total of 56 cases were analyzed, comprising 10 ductal carcinoma in situ (DCIS, n = 10) and 46 invasive BCa of low-grade (Grades 1-2; n = 13), high-grade (Grades 3, n = 20) or the triple-negative subtype (n = 13), with matched benign breast tissues (n = 14).

Immunohistochemistry (IHC)

FFPE sections (5 μm) were deparaffinized, rehydrated and immunostained for EDD using UltraTek HRP Anti-Polyvalent Staining System (Cedarlane, Burlington, ON) following manufacturer's instructions. For IHC, mouse anti-EDD (sc-515494, Santa Cruz Biotech.) and mouse IgG (X0931; DakoCytomation) were used. Immunostaining was improved using antigen target retrieval for 30 min in 96°C citrate buffer (Dako Target Retrieval Solution). Computerized image analysis using ImageJ64 (NIH) was performed

as previously described [19]. All slides were evaluated by one person (LT) to ensure consistency.

Cell culture

Human breast cancer cell lines were maintained as follows: MCF-7 cells in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1X non-essential amino acids, 1 mM sodium pyruvate and 1% penicillin-streptomycin; T47D, SKBR3, and MDA-MB-231 cells in high-glucose DMEM containing 10% heat-inactivated FBS, 2 mM glutamine, 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfuric acid (HEPES) and 1% penicillin-streptomycin; MDA-MB-436 cells in Leibovitz L-15 medium containing 10% heat-inactivated FBS, 1X insulin-transferrin-selenium-ethanolamine, 16 µg/ml glutathione, and 1% penicillin-streptomycin. MDA-MB-436 cells were incubated in atmospheric air at 37°C. All other cell lines were in 5% CO_2 at 37°C. Before PRL treatment, cells were made quiescent for 24 h in phenol red-free DMEM containing 1% charcoal-stripped heat-inactivated FBS and all additives in the maintenance medium.

Preparation of total cell lysates

Cells were harvested in RIPA lysis buffer containing 100:1 (v/v) of protease inhibitor cocktail P8340 (Sigma-Aldrich), 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride, and homogenized through 21-gauge needles. The 13,000 rpm supernatant was collected as previously described [20] and used immediately or frozen at -30°C until further analysis.

Transfection of siRNA

Silencer® Select Negative Control #1 siRNA and pre-designed UBR5 Silencer® Select siRNA targeting hEDD (siEDD1, siEDD2) were from ThermoFisher Scientific (Burlington, ON, Canada). Sequences for siEDD1 and siEDD2 were 5'-AGA-CAA-AUC-UCG-GAC-UUG Att-3' and 5'-GCG-UGA-ACG-UGA-AUC-CGU-Utt-3', respectively, as previously described [17]. Cells were plated in complete media at 3-5 × 10^5 cells/well in 6-well plates for 24 h, then transfected with 40 pmol of siEDD1, siEDD2 or non-targeting siRNA (siNT) using RNAiMAX™ (Life Technologies) following the manufacturer's protocol. After 24-120 h (Days 1-5), cells were processed for immunoblotting, RT-PCR, or flow cytometry.

Trypan blue cell viability assay

Cells were detached using 0.25% trypsin-EDTA, then inactivated with an equal volume of growth medium before mixing with 0.4% trypan blue solution (1:1). Cells were counted using a TC20 cell counter (Bio-Rad Laboratories, Montreal, QUE, Canada).

MTS cell viability assay

Cell viability was assayed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI) according to the manufacturer's protocol. Cells were incubated for 20 min in 5% CO_2 at 37°C and absorbance was read at 490 nm using a Model 3550 Microplate Reader (Bio-Rad).

Western analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10-20 µg protein/lane. After immunoblotting, immunoreactive signals were detected using Clarity Western ECL Substrate (Bio-Rad). Densitometry was performed using ImageJ64 (NIH, Bethesda, MD) with β-actin as a control.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA, extracted from cells using EZ-10 DNAaway RNA mini-prep kits (BioBasic, Markham, ON, Canada), was treated with DNase 1, reversed transcribed, and used (1 µg/reaction) for semi-quantitative PCR [21] or quantitative-PCR (qPCR) using the Comparative Cycle Threshold (Ct) method [20], with β-actin as a control. The following forward and reverse primer sets were used: Human EDD: 5'-GAC-GCG-AGA-ACT-CTT-GGA-AC-3' and 5'-TTC-AAA-TGG-ATT-TGG-GGG-TA-3' (196 bp amplicon; for qPCR) or 5'-AGA-TGC-TGA-CCC-TTC-TTC-TCT-CCT-GC-3' and 5'-GCA-CCC-AAT-TTC-CAG-TCT-TC-3' (209 bp; for semiquantitative PCR); Mcl-1: 5'-TAA-CTA-GCC-AGT-CCC-GTT-TTG-TCC-3' and 5'-CAT-GTT-TTC-AGC-GAC-GGC-GTA-A-3' (169 bp); Bim: 5'-CCG-AGA-AGG-TAG-ACA-ATT-GCA-G-3' and 5'-CCT-CTA-GGA-TGA-CTA-CCA-TTG-CAC-3' (191 bp); Bak: 5'-GAG-GTT-TTC-CGC-AGC-TAC-

GTT-T-3' and 5'-TAG-GTT-GCA-GAG-GTA-AGG-TGA-CCA-3' (106 bp); Bax: 5'-TTT-TCC-GAG-TGG-CAG-CTG-ACA-T-3' and 5'-GCA-CCA-GTT-TGC-TGG-CAA-AGT-A-3' (90 bp); Bcl-xL: 5'-TCC-CAG-AAA-GGA-TAC-AGC-TGG-AGT-3' and 5'-TCT-CCA-TCT-CCG-ATT-CAG-TCC-CTT-3' (88 bp); β-actin: 5'-AAA-CTG-GAA-CGG-TGA-AGG-TG-3' and 5'-AGA-GAA-GTG-GGG-TGG-CTT-TT-3' (172 bp). PCR cycle numbers were predetermined to ensure linear range of amplification. PCR cycles and annealing temperatures were: 30 cycles at 52°C for EDD primers (1 μM) and 25 cycles at 54°C for β-actin primers (0.5 μM). All other annealing temperatures were between 54-56°C. PCR products in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide were visualized using a Versadoc UV-transilluminator (Bio-Rad). Densitometry was performed using ImageLab™ (Bio-Rad) with β-actin as a control.

Propidium iodide staining for cell-cycle analysis

Cells, arrested for 24 h in medium containing 1% charcoal-stripped FBS to synchronize cell cycles, were seeded at 2 × 10^5 cells/well in 6-well plates. After 24 h, the cells were transfected with siEDD1, siEDD2, siNT, or left untransfected. After 48 h or 72 h, both floating and adherent cells were collected, washed once with ice-cold PBS, pelleted, and resuspended in 0.5 mL ice-cold PBS. The cell suspension was vortexed gently while slowly mixed with 4.5 mL of ice-cold 70% ethanol, incubated at -20°C for 3 h, and then washed in PBS. The cells were resuspended in 1 mL of cell cycle solution (0.1% Triton X-100, 0.2 mg/mL RNase A and 0.02 mg/mL propidium iodide in PBS). After 30 min at room temperature, the cells were analyzed using a FACS Canto 2 analyzer (BD Biosciences, San Jose, CA) with a minimum of 10,000 gated events/analysis. Cell-cycle analysis was performed using Modfit software package (Verity Software House, Topsham, ME).

Annexin-V staining for apoptosis

Annexin-V-FLUOS staining (Roche Diagnostics, Mannheim, Germany) was performed following the manufacturer's protocol. Briefly, cells were seeded at 2 × 10^5 cells/well in 6-well plates and transfected 24 h later with siEDD1, siEDD2, siNT or left untransfected. After 48 or 72 h, both floating and adherent cells were collected, washed once with PBS, pelleted, and resuspended in 100 μL of incubation buffer containing Annexin V-FLUOS labeling reagent and propidium iodide at the recommended concentrations. The samples were incubated in the dark at room temperature for 10-15 min and diluted with another 0.3 mL of incubation buffer before flow cytometry at λexcitation 488 nm and detection at λemission 515 nm (Annexin V) and 600 nm (propidium iodide). Data was analyzed using FCS Express-3 DeNovo Software (Glendale, CA). Percentage cell death was calculated from cells that stained positive for either Annexin V-FLUOS (early apoptosis) or Annexin V-FLUOS and PI (late apoptosis).

Generation of shEDD-MCF-7 clones

MCF-7 cells were stably transduced with shRNA targeting EDD through infection with lentiviral particles (sc-43744-V, Santa Cruz Biotechnology), following the manufacturer's protocol. Puromycin (4 μg/mL)-resistant clones were isolated with cloning rings. EDD mRNA/protein expressions were variable, ranging from 0 to 63% knockdown by RT-qPCR analysis, and from 0 to 100% knockdown by immunoblotting analysis. Specifically, EDD protein level was reduced by over 90% in clone 1.1C as compared to uninfected cells, but only by 22% in clone 1.1A. Clone 1.1A was used as a non-knockdown control cell line for comparison with clone 1.1C.

Transfection of EDD expression plasmids

MDA-MB-436 cells were transfected with pCMV-Tag2B.EDD (Addgene plasmid #37188) or pCMV-Tag2B control vector using GenJet Transfection Reagent (Version II; FroggaBio, Toronto, Canada) following the manufacturer's protocol. pCMV-Tag2B.EDD was a gift of Drs. Darren Saunders and Charles Watts (Garvan Institute of Medical Research, Australia).

Cap-binding assay

Immobilized γ-aminophenyl-m^7GTP (C_{10}-spacer) agarose beads (Jena Biosciences, Germany) were pre-washed thrice with freshly-prepared RIPA lysis buffer containing 100:1 (v/v) of protease inhibitor cocktail P8340 (Sigma-Aldrich), 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride. Cell lysates were prepared from cells treated ± PRL (10 ng/ml) and

transfected ± siNT or siEDD1. Each sample of cell lysate (100 µg protein) and 25 µl of pre-washed m^7GTP-agarose beads were incubated together in a microfuge tube, gently agitated on a nutator at 4°C for 3 h, then centrifuged at 500 × *g* for 1 min, and the supernatants were removed. The protein-bound m^7GTP-agarose beads in each tube were washed thrice with 1 ml RIPA buffer by inversion, re-centrifuged at 500 × *g*, and the supernatants were removed. The protein-bound m^7GTP-agarose beads, in 50 µl of 1X SDS-PAGE buffer, were boiled at 100°C for 15 min to elute m^7GTP cap-binding proteins. Eluted fractions and the respective whole cell lysates were resolved by SDS-PAGE and analyzed by western blotting for eIF4E, 4EBP1, and β-actin.

Statistics

Statistical analyses were performed using GraphPad Prism. Results were expressed as mean ± SEM. Student's unpaired, two-tailed t-tests and ANOVA were used to compare mean values. *P*-values ≤ 0.05 were considered significant.

Results

EDD immunostaining increases with BCa progression in vivo

EDD immunostaining was reported to be low or undetectable in normal breast tissues whereas low (37%) or high-intensity (63%) nuclear staining was detected in breast carcinomas [6]. In the present study, EDD immunostaining during BCa progression was determined using matched benign human breast tissues, ductal carcinoma in-situ (DCIS), low-grade, high-grade and triple-negative (TNBC) breast tumours (**Figure 1A**). EDD staining area, as a percent of total area, increased from 17.63 ± 6.25% in benign tissues to 25.65 ± 10.49, 45.13 ± 10.45, 71.29 ± 5.50 and 65.85 ± 7.71 in DCIS, low-grade, high-grade and TNBC breast tumours, respectively (**Figure 1B**). EDD staining was significantly higher in low-grade, high-grade and TNBC tumours, as compared to benign breast tissues. Although EDD staining increased in DCIS, it was not significantly higher than in benign tissues. EDD was detected mainly in the cytoplasm but also in the nucleus, especially in high-grade and TNBC tumours. Matched areas of low-grade and high-grade BCa were also immunostained with mouse anti-EDD or mouse IgG, and the absence of specific staining in the IgG controls indicated specificity of the EDD antibody (**Figure 1C**). Furthermore, Kaplan-Meier-Express analysis of the TCGA database (*http://ec2-52-201-246-161.compute-1.amazonaws.com/kmexpress/index.php*) showed that breast cancer patients (n = 943) with high EDD (UBR5) mRNA expression (Q3 vs Q1, which is top 25% vs bottom 25%, respectively) had a significantly lower (P = 0.0068) survival probability (**Figure 1D**).

EDD expression in BCa cell lines

BCa cell lines of different subtypes, MCF-7 and T47D (luminal A), SKBR3 (HER2-enriched), MDA-MB-231 (claudin-low TNBC) and MDA-MB-436 (basal-like TNBC) all expressed EDD but at varying levels (**Figure 2**). Relative EDD mRNA expression was high in T47D and MCF-7 cells and low in MDA-MB-436 cells (**Figure 2A**). A similar mRNA profile was previously reported [6]. At the protein level, EDD expression was high in T47D and MCF-7 cells, although MDA-MB-231 and MDA-MB-436 cells had the highest and lowest EDD levels, respectively (**Figure 2B**). Subsequently, MCF-7 and T47D cells were used in experiments using siRNA or shRNA to knockdown EDD gene expression. MDA-MB-436 cells, with the lowest EDD mRNA and protein levels, were used in experiments examining ectopic expression of EDD.

MCF-7 cells transfected with two sets of siRNAs targeting EDD showed a decrease in EDD mRNA levels from Day 1 (24 h) to Day 5 (**Figure 2C**), with siEDD1 consistently more effective than siEDD2. For example, on Day 3, EDD mRNA levels decreased by ~70% using siEDD1 and 50-60% using siEDD2 (**Figure 2C**), and each was accompanied by decreased EDD protein levels (**Figure 2D**).

Loss of EDD arrests MCF-7 and T47D cells in G2-phase

To investigate the effects of EDD on the cell cycle, MCF-7 and T47D cells were transfected with siEDD1 or siEDD2 for 48 and 72 h. Loss of EDD, confirmed using RT-PCR analysis (**Figure 3A**), caused an increase in cells arresting in G2 (**Figure 3B**, **3C**). For example, 34.31% of siEDD1-transfected MCF-7 cells were in G2 at 48 h, compared to 11.24% of siNT and 15.66%

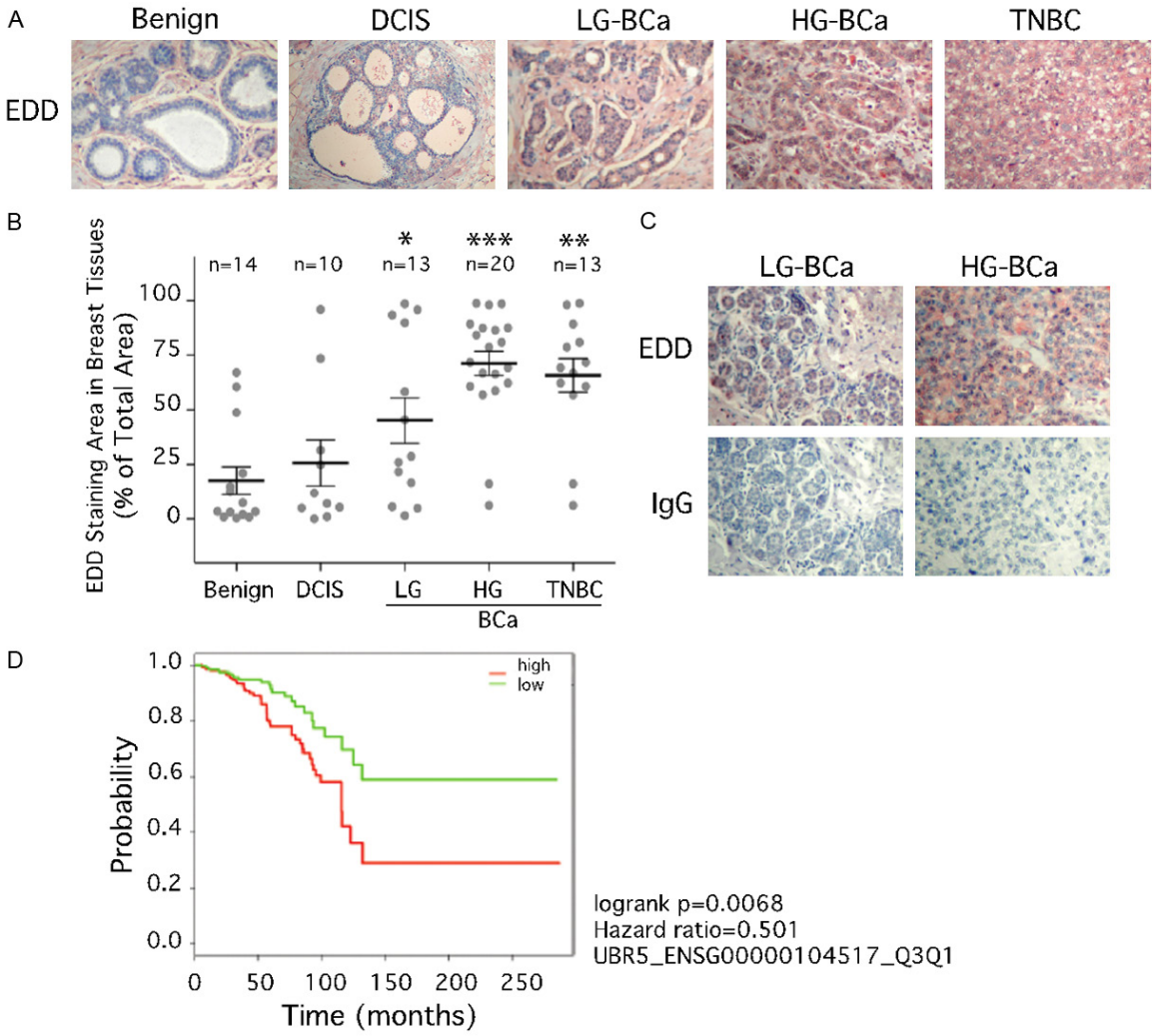

Figure 1. IHC: EDD increases with BCa progression *in vivo*. A. Immunostaining for EDD was performed using mouse anti-EDD Mab antibody and the percent of area stained was determined in benign tissues, DCIS, low-grade (LG) and high-grade (HG) BCa, and in TNBC, as described in *Materials and Methods*. B. EDD-stained area, as a percent of total area, of individual specimens was plotted. Mean ± SEM is indicated. *Significantly different from benign ($P < 0.05$, n = 13), **significantly different from benign and DCIS ($P < 0.05$, n = 13), ***significantly different from benign, DCIS and low-grade BCa ($P < 0.05$, n = 20). C. Immunostaining of matched areas of LG-BCa and HG-BCa with mouse anti-EDD Mab or normal mouse IgG. D. Online TCGA database analysis was used to determine the prognostic value of EDD (UBR5) mRNA expression in breast cancer patients (n = 943). Survival hazard ratio = 0.501 and log rank P = 0.0068 were displayed on the webpage. *P* value of < 0.05 was considered statistically significant.

of untransfected cells (**Figure 3B**). For T47D cells, 21.30% of siEDD1-transfectants were in G2 at 48 h, compared to 11.44% of siNT and 10.53% of untransfected cells (**Figure 3B**). SiEDD2 also increased G2 arrest in both cell lines but the effect was less pronounced (**Figure 3C**). The G2 arrest using siEDD1 was sustained through the 2 or 3 days of study (**Figure 3D**). The increased G2 arrest was accompanied by decreased cell viability and increased apoptosis (see below), indicating that these cells were not proliferating.

Loss of EDD decreases viability and increases apoptosis of MCF-7 and T47D cells

Using siEDD1, loss of EDD in MCF-7 and T47D cells was confirmed on Day 1 to Day 3 by RT-PCR (**Figure 4A**, **4B**; *upper panels*). MTS assays showed a significant decrease in the viability of cells transfected with siEDD1, as compared to siNT or untransfected controls (**Figure 4A**, **4B**; *lower panels*).

Cells with EDD loss by at least 70% at 48 h were used for flow cytometry (**Figure 5**). Loss of

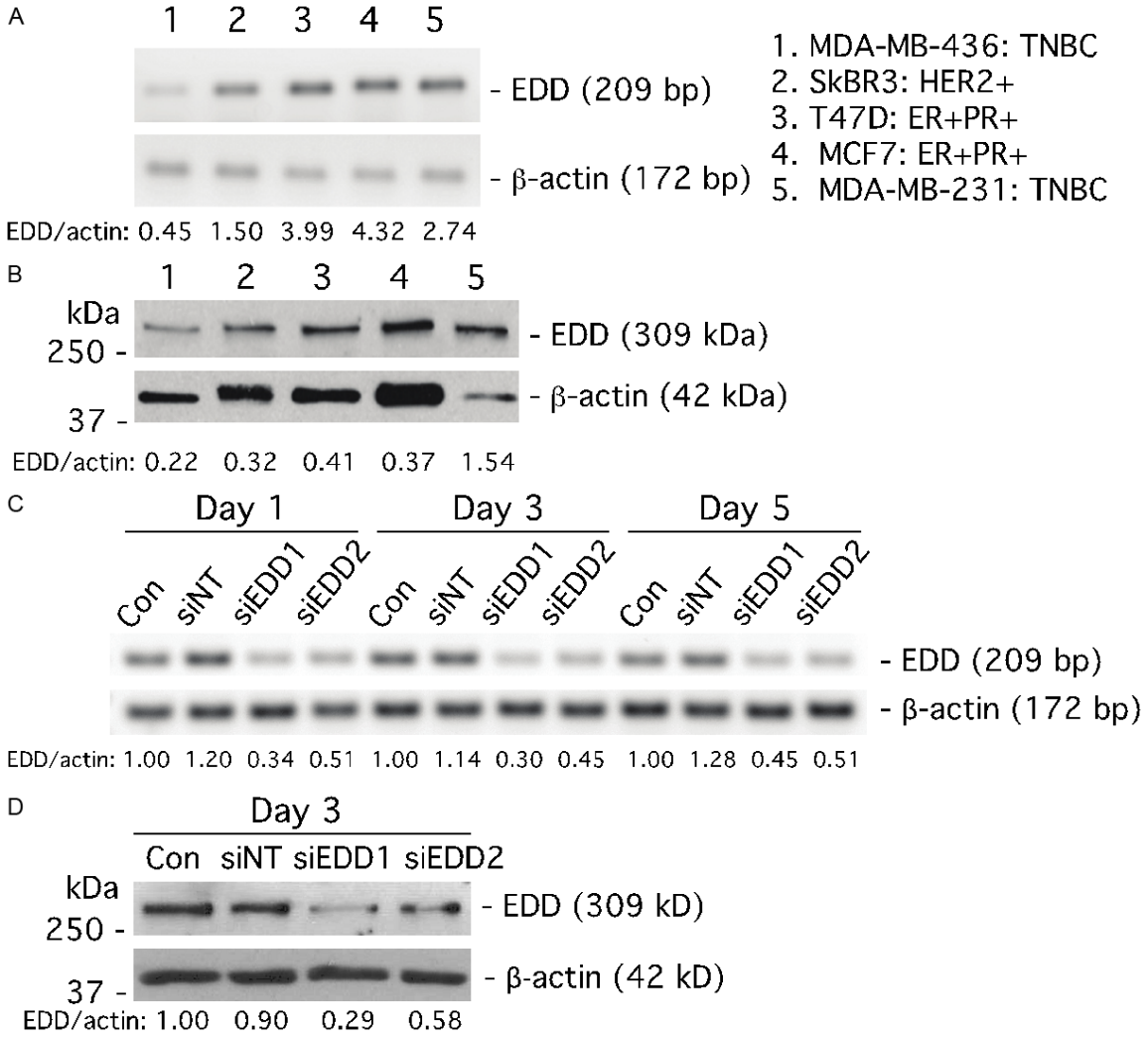

Figure 2. EDD expression in BCa cell lines. Actively growing BCa cell lines that were estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), epidermal growth factor receptor 2-positive (HER2+) or triple-negative (TNBC) were harvested for (A) total RNA extraction and semi-quantitative RT-PCR analysis or (B) total cell lysates and Western analysis. (C, D) MCF-7 cells were transfected with siEDD1, siEDD2, siNT or left untransfected (Con) for up to 5 days. Cells were harvested on Day 1 (24 h), Day 3, and Day 5 for RT-PCR analysis (C), or on Day 3 for Western analysis (D). Representative blots of at least 3 knockdown experiments.

EDD increased apoptosis, as compared to siNT and untransfected cells. Specifically, MCF-7 cells in late-stage apoptosis increased significantly from 2-8% in siNT and untransfected cells to 20-35% in siEDD1 cells (**Figure 5B**, **5D**). Although T47D cells were less affected by EDD loss, cells in early-stage apoptosis increased from 4-5% in siNT and untransfected cells to 13-20 % in siEDD1 cells (**Figure 5C**, **5E**).

Loss of EDD increases expression of pro-apoptotic Bim, Bak and Bax proteins

Semi-quantitative RT-PCR analysis of MCF-7 cells showed that EDD loss on Days 2 and 3 was accompanied by an increase in pro-survival Mcl-1 but not Bcl-X_L and increases in pro-apoptotic initiator Bim and pro-apoptotic effector Bak but not Bax (**Figure 6A**). Similarly, on Day 2, qPCR analysis showed significantly increased Mcl-1, Bim, and Bak mRNAs in siEDD-transfected cells, as compared to siNT cells (**Figure 6B**). Immunoblotting of the proteins of these three elevated genes showed increased Bim and Bak but not Mcl-1 (**Figure 6C**, **6D**). The Mcl-1 gene consists of three exons. Exclusion of exon 2 could produce pro-survival Mcl-1_L (40-kDa) and pro-apoptotic Mcl-1_S (32-kDa) isoforms [22]. Using another anti-

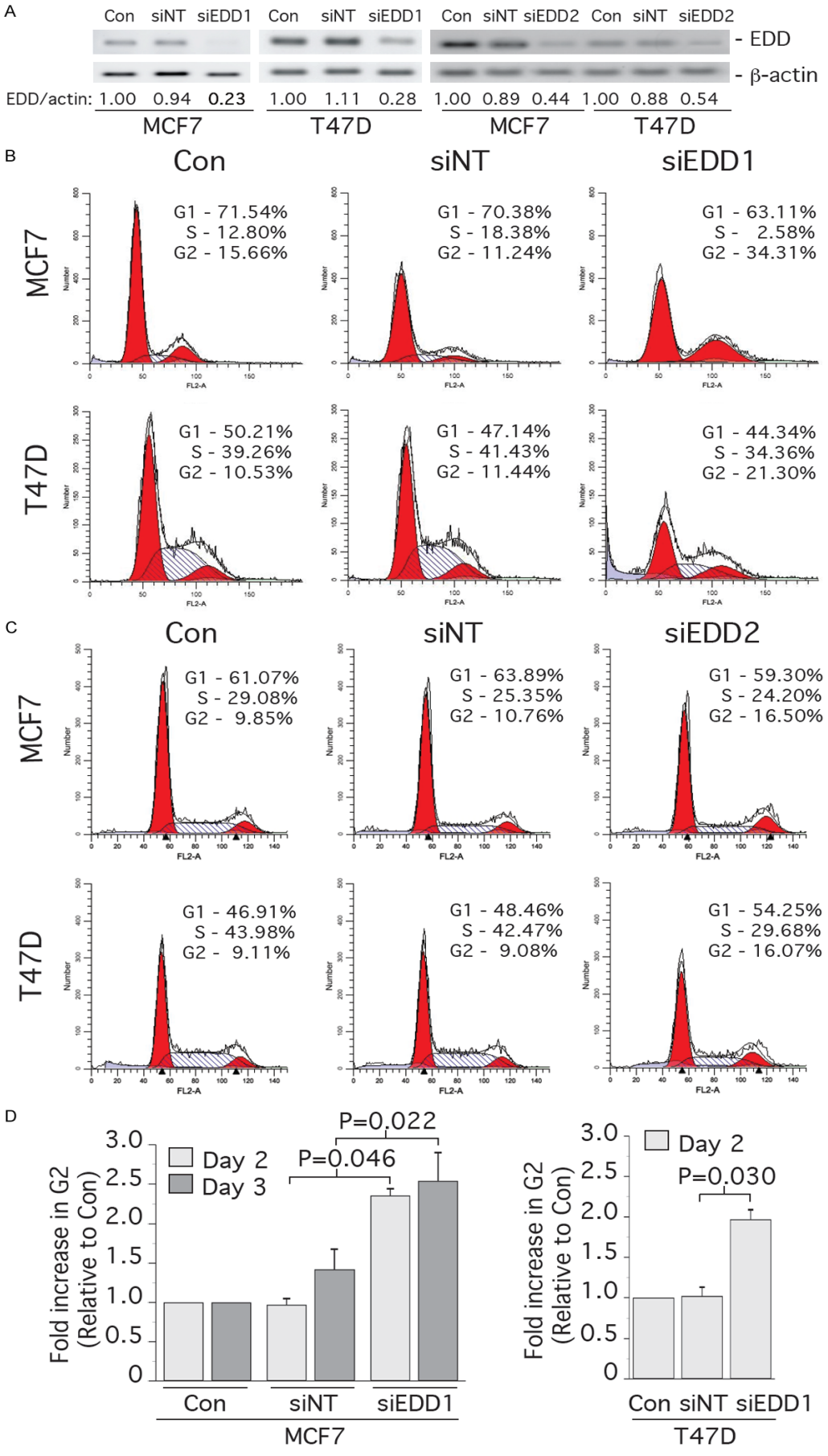
A
Con siNT siEDD1 Con siNT siEDD1 Con siNT siEDD2 Con siNT siEDD2
- EDD
- β-actin
EDD/actin: 1.00 0.94 0.23 1.00 1.11 0.28 1.00 0.89 0.44 1.00 0.88 0.54
MCF7 T47D MCF7 T47D
B
Con siNT siEDD1
MCF7
G1 - 71.54%
S - 12.80%
G2 - 15.66%
G1 - 70.38%
S - 18.38%
G2 - 11.24%
G1 - 63.11%
S - 2.58%
G2 - 34.31%
T47D
G1 - 50.21%
S - 39.26%
G2 - 10.53%
G1 - 47.14%
S - 41.43%
G2 - 11.44%
G1 - 44.34%
S - 34.36%
G2 - 21.30%
C
Con siNT siEDD2
MCF7
G1 - 61.07%
S - 29.08%
G2 - 9.85%
G1 - 63.89%
S - 25.35%
G2 - 10.76%
G1 - 59.30%
S - 24.20%
G2 - 16.50%
T47D
G1 - 46.91%
S - 43.98%
G2 - 9.11%
G1 - 48.46%
S - 42.47%
G2 - 9.08%
G1 - 54.25%
S - 29.68%
G2 - 16.07%
D
Fold increase in G2 (Relative to Con)
Day 2
Day 3
P=0.022
P=0.046
Con siNT siEDD1
MCF7
P=0.030
Con siNT siEDD1
T47D

Figure 3. Loss of EDD arrests BCa cells in G2-phase. MCF-7 and T47D cells were transfected with siEDD1, siEDD2, siNT or left untransfected (Con). On Day 2 (MCF-7 and T47D) or Day 3 (MCF-7) after transfection, cells were harvested for (A) total RNA and semi-quantitative RT-PCR analysis of EDD and β-actin or (B, C) cell cycle analysis (see *Materials and Methods*). Representatives of 2-4 experiments. (D) Results with siEDD1 were plotted as fold increase in G2. Mean ± SEM (n = 4), *p* values as shown (all < 0.05).

Mcl-1 antibody specific to both isoforms, immunoblotting showed Mcl-1_L was more abundant than Mcl-1_S in MCF-7 cells but their ratio (Mcl-1_S/Mcl-1_L) was not significantly changed in siEDD- and siNT-transfected cells. Tumour suppressor p53 protein was elevated with EDD loss in other studies [10], but we detected no change in p53 (**Figure 6C**, **6D**).

The pro-apoptotic effectors Bak and Bax perforate the outer mitochondria membrane, and the release of cytochrome C triggers downstream caspase cleavage and apoptosis [23]. Unlike increases in Bak mRNA and protein levels, EDD loss did not alter Bax mRNA levels (**Figure 6B**). However, Western analysis showed significant increases in the Bax protein and modulator of apoptosis protein 1 (MOAP-1) (**Figure 6E**, **6F**). Bak is a mitochondrial protein whereas Bax is cytosolic, and Bax binding to MOAP-1 translocates Bax to the mitochondria to initiate apoptosis [24]. Therefore, EDD loss led to increased pro-apoptotic Bim, Bak and Bax proteins, and Bax translocator MOAP-1.

Loss of EDD increases caspase-7 and PARP-1 cleavage in MCF-7 cells

The increase in pro-apoptotic proteins following EDD loss in MCF-7 cells (**Figure 6**) was accompanied by cleavage of caspase-7 at 6 h to 48 h, followed by cleavage of caspase substrate poly-(ADP-ribose)-polymerase-1 (PARP-1) at 24 h and 48 h (**Figure 7A**). Caspase-6 was not affected (**Figure 7B**).

Loss of EDD decreases PRL-induced phosphorylation of 4EBP1

The EDD-α4-PP2A complex is associated with mTOR/TORC1 signaling [12]. We reported that PRL rapidly activated TORC1 signaling by stimulating phosphorylation of 4EBP1 in rat Nb2 lymphoma cells [25] that are critically dependent on PRL for cell proliferation [26]. To assess the effect of EDD loss on PRL-induced phosphorylation of 4EBP1, quiescent MCF-7 cells were transfected with siEDD1 48 h prior to acute treatment with PRL for 1 h. SiEDD1 cells with 90% loss of EDD mRNA (**Figure 8A**) and protein (**Figure 8B**) were compared to siNT and untransfected cells. In control cells, immunoblots showed doublet α- and β-bands, with the lower molecular weight α-band representing hypophosphorylated 4EBP1 (**Figure 8B**; *lane 1*). Consistent with our findings [25], PRL-induced phosphorylation of 4EBP1 appeared as a higher molecular weight, hyper-phosphorylated γ-band after 1 h (**Figure 8B**; *lanes 2, 3*). PRL-induced phosphorylation of 4EBP1 decreased in siEDD1 cells, as shown by disappearance of the γ-band (**Figure 8B**; *lane 4*).

Densitometry showed that PRL significantly increased γ-4EBP1 in untransfected and siNT cells but this increase was significantly reduced with EDD loss. No difference was seen between α-4EBP1 and β-4EBP1 in the four treatment groups (**Figure 8C**).

Phosphorylation of 4EBP1 releases eIF4E and the freed eIF4E then binds to the 5'-cap of mRNAs to initiate translation [27]. Cap-binding assays showed that siEDD1-transfected cells with decreased PRL-induced phosphorylation of 4EBP1 also showed decreased eIF4E binding to γ-aminophenyl-m^7GTP agarose (**Figure 9**). As expected, no β-actin was recovered from the cap-binding assay.

Ectopic EDD promotes drug resistance in MDA-MB-436 cells

MDA-MB-436 cells, with low EDD levels (**Figure 2**), were transfected with pCMV-Tag2B.EDD or control pCMV-Tag2B at increasing plasmid concentrations to vary ectopic EDD levels, which were confirmed by RT-PCR (**Figure 10A**, **10B**; *upper panel*) and immunoblotting (**Figure 10B**, *lower panel*). Transfected cells were treated with IC_{50} doses of cisplatin (0.3125 μM; **Figure 10C**) or doxorubicin (0.0231 μM; **Figure 10F**) for 5 days. Cells receiving pCMV-Tag2B or pCMV-Tag2B.EDD above 250 ng/ml, grown in drug-free conditions, generally showed a progressive decrease in cell counts as compared to untransfected cells (**Figure 10D**, **10G**). Thus, in subsequent experiments, 500 ng/ml plasmid was the maximum concentration used.

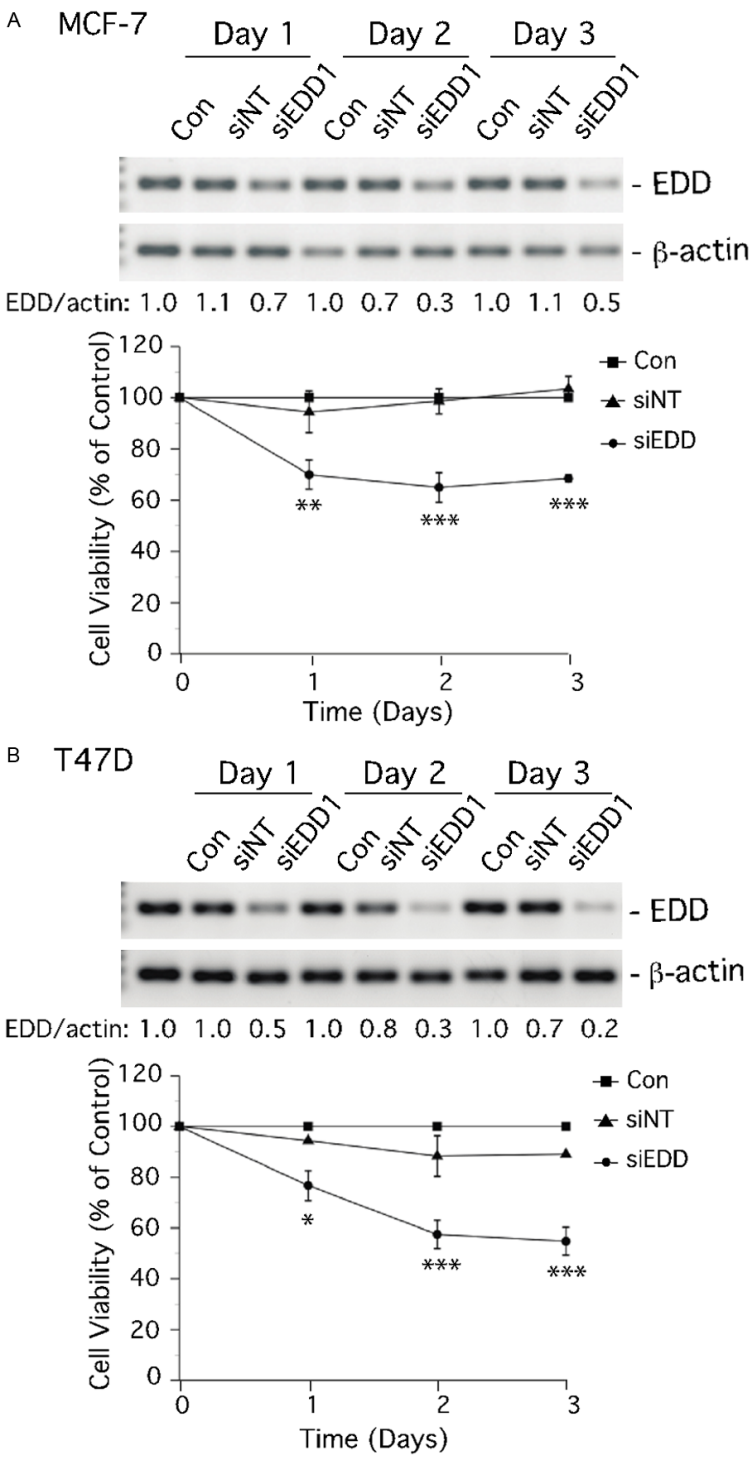

Figure 4. Loss of EDD decreases BCa cell viability. (A) MCF-7 and (B) T47D cells were transfected with siEDD1, siNT or left untransfected (Con) for up to 3 days. (A, B; *upper panels*) Semiquantitative RT-PCR analysis of EDD and β-actin mRNA levels. Representative blots of 3 experiments. (A, B; *lower panels*) MTS assays were performed on each day. Mean ± SEM (n = 3); *P < 0.05, **P < 0.01, ***P < 0.001 indicates significant difference between siEDD1- and siNT-transfected cells.

Cell counts were further decreased with cisplatin (**Figure 10D**) or doxorubicin (**Figure 10G**) treatment but cells expressing ectopic EDD were more drug resistant than control cells. Specifically, the percent cell survival was significantly increased in cisplatin- (**Figure 10E**) and doxorubicin-treated (**Figure 10H**) cells that received 250 or 500 ng/ml pCMV-Tag2B.EDD, as compared to their respective pCMV-Tag2B control.

Since EDD modulates PRL stimulation of TORC1 signaling (**Figures 8**, **9**), MDA-MB-436 cell sensitivities to rapamycin (TORC1 inhibitor) and INK128 (TORC1/2 inhibitor) were investigated. IC_{50} doses of rapamycin and INK128 were 16.59 μM (**Figure 11A**) and 0.014 μM (**Figure 11D**), respectively. As before, cell counts decreased after pCMV-Tag2B or pCMV-Tag2B.EDD transfection for 5 days and further decreased with rapamycin (**Figure 11B**) or INK128 (**Figure 11E**) treatment. However, cells expressing ectopic EDD were more resistant to each inhibitor than controls, with the percent surviving rapamycin (**Figure 11C**) or INK128 (**Figure 11F**) treatment significantly increased. Therefore, ectopic EDD expression increased cell resistance to chemotherapy drugs, TORC1 and TORC2 inhibitors.

Loss of EDD decreases drug resistance in MCF-7 cells

Drug resistance in EDD-expressing MCF-7 cells was studied using lentiviral shRNA-EDD gene knockdown. West-

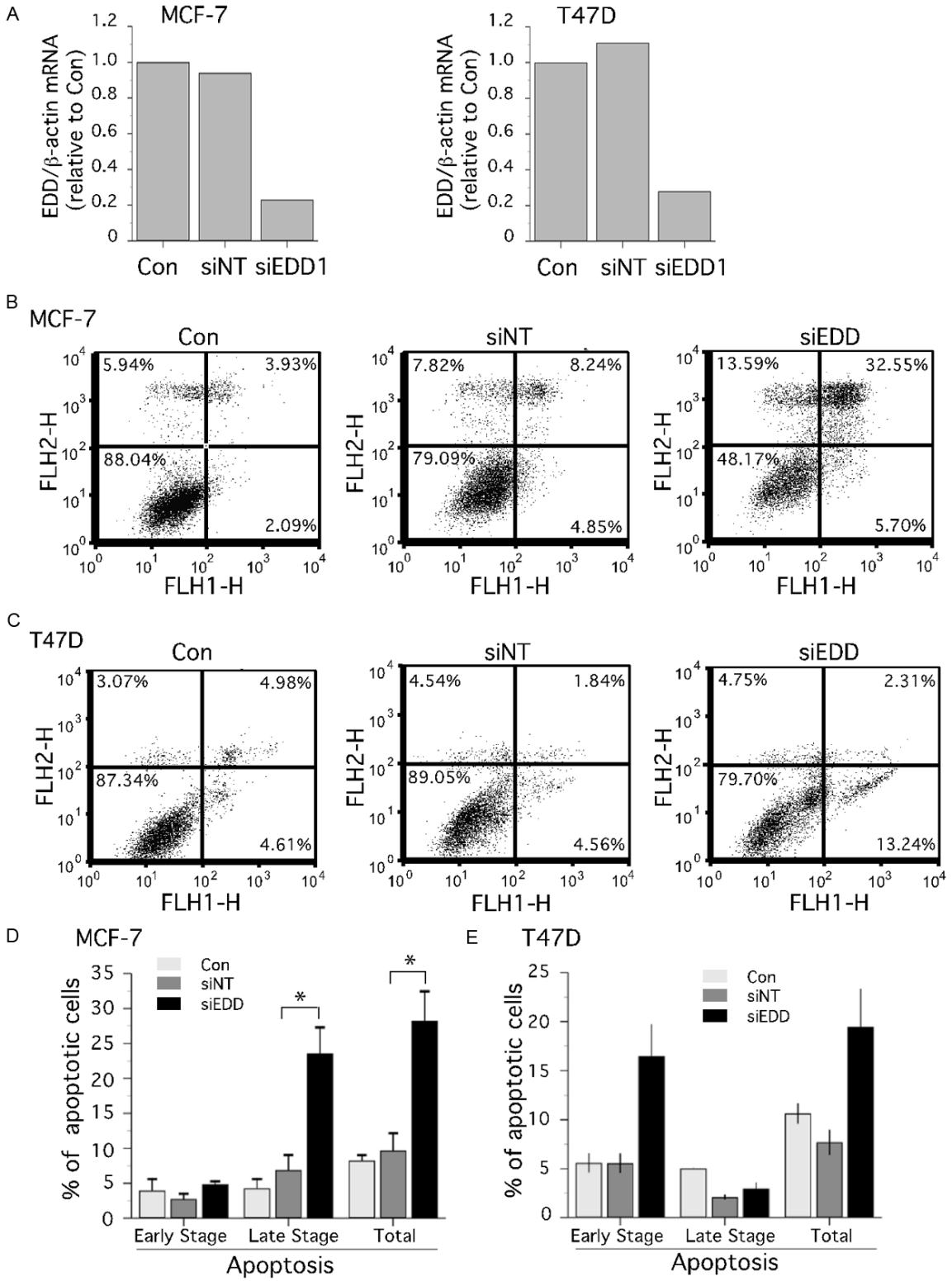

Figure 5. Loss of EDD increases cell apoptosis. MCF-7 and T47D cells were transfected with siEDD1, siNT or left untransfected (Con). On Day 2 (48 h), (A) EDD gene knockdown was confirmed by quantitative RT-PCR and (B, C) the cells were harvested, stained with Annexin-V and propidium iodide, and analyzed by flow cytometry (see *Materials and Methods*). Percentages of cells that were viable (lower left quadrant), in early-stage apoptosis (lower right quadrant), or in late-stage apoptosis (upper right quadrant) were determined. (D, E) Statistical analysis for % of apoptotic cells for (D) MCF-7 cells, mean ± SEM (n = 5), *P < 0.05 for siEDD *versus* siNT cells, and (E) T47D cells, mean ± range (n = 2), each in duplicate.

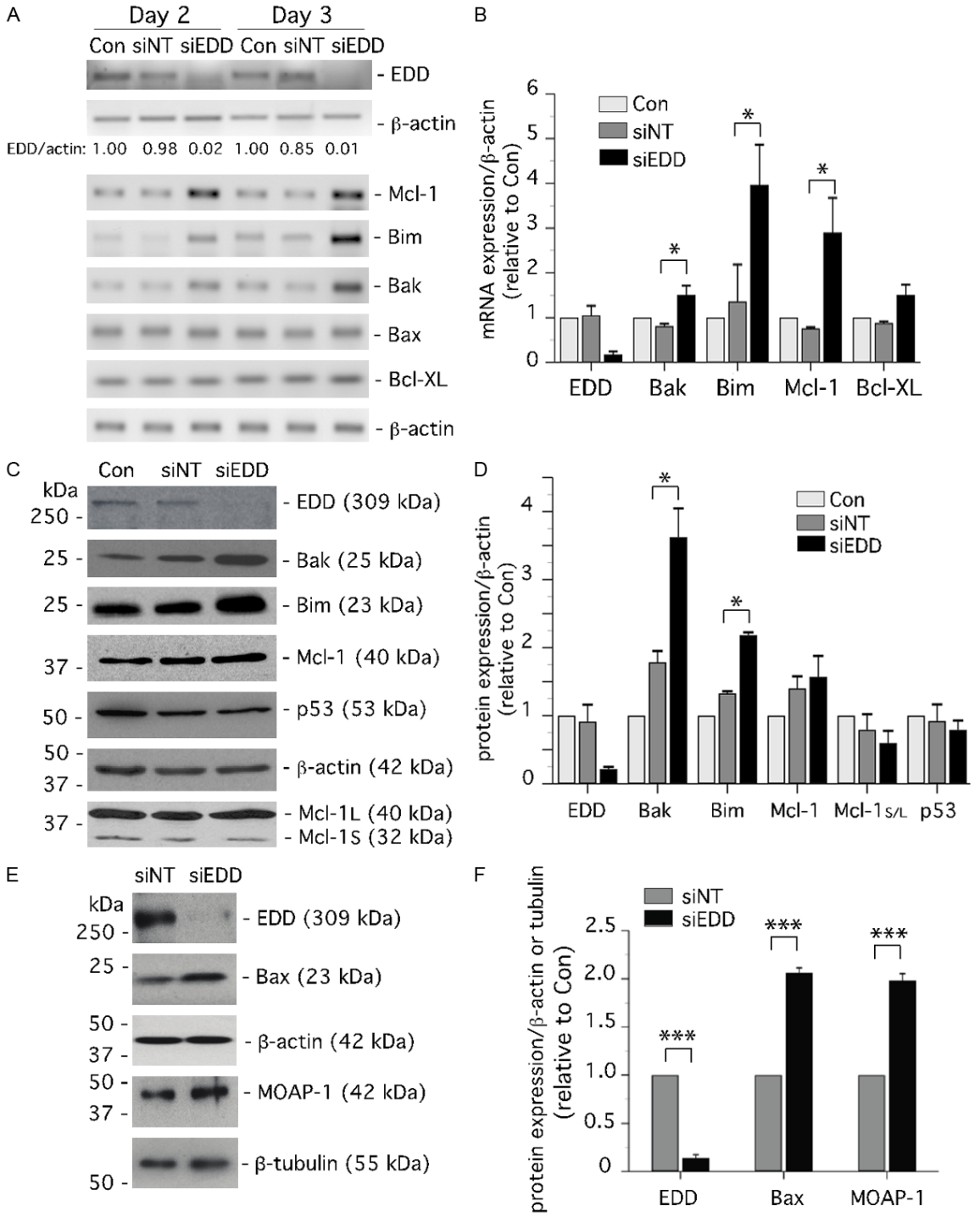

Figure 6. Loss of EDD increases pro-apoptotic proteins. MCF-7 cells were transfected with siEDD1, siNT or left un-transfected (Con) for 2-3 days. Cells were harvested for (A) total RNA and semi-quantitative RT-PCR analysis on Day 2 and Day 3 to analyze pro-survival and pro-apoptotic gene expression with β-actin as a control, representative of 2 independent experiments or (B) quantitative RT-PCR analysis on Day 2, mean ± SEM (n = 3-6), * ($P < 0.05$). (C-F) Cell lysates on Day 3 after transfection were used for SDS-PAGE (10 μg/protein per lane), Western blotting, and densitometric analysis. In (C), different anti-Mcl-1 and anti-Mcl-1_L/Mcl-1_S antibodies were used (see *Materials and Methods*); representative blots from 3 experiments, followed by (D) densitometry. Mean ± SEM (n = 3), *$P < 0.05$. In (E), representative Western blot for EDD, Bax, and MOAP-1, followed by (F) densitometry. Mean ± SEM (n = 3-6), ***$P < 0.001$.

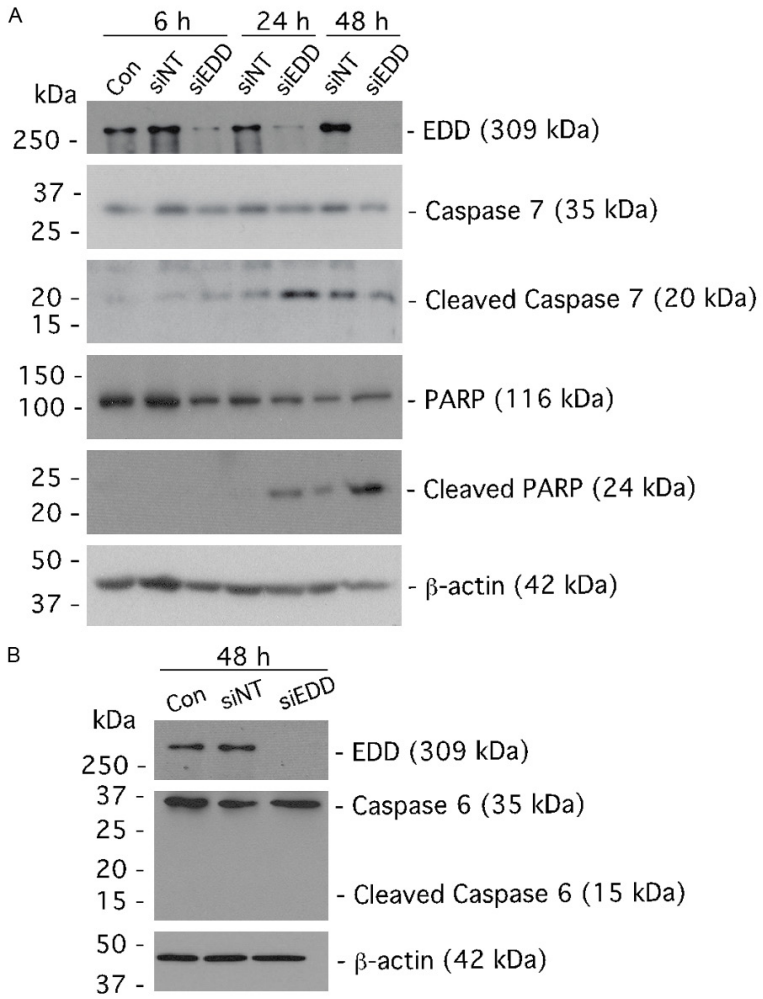

Figure 7. Loss of EDD increases caspase-7 and PARP-1 cleavage in MCF-7 cells. MCF-7 cells were transfected with siEDD1, siNT or left untransfected (Con) for up to 48 h. Cell lysates were then prepared for SDS-PAGE (10 μg protein/lane) and Western analysis. Representative immunoblots from 2-3 independent experiments.

ern analysis of several stable puromycin-resistant shRNA clones showed EDD-knockdown clones 1.1C and 3.1A, as well as clones 1.1A, 1.1B and 3.1C (**Figure 12A**). Clones 1.1C and 1.1A were selected as EDD knockdown and control clones, respectively. They were treated with IC_{50} doses of cisplatin (0.3125 mM), doxorubicin (0.0231 μM), rapamycin (16.59 μM) or tamoxifen (20 μM), and counted over 5 days. In the absence of drugs, EDD-knockdown clone 1.1C generally grew at a slower rate than clone 1.1A (**Figure 12B-D**; *left panels*). Cisplatin, doxorubicin or rapamycin treatment decreased growth of both clones (**Figure 12B-D**; *left panels*) but, in each case, clone 1.1C was less drug resistant than clone 1.1A on Day 4 and/or Day 5 (*middle panels*). The percent of clone 1.1C cells surviving cisplatin, doxorubicin or rapamycin treatment decreased significantly on Day 5 as compared to clone 1.1A (*right panels*). Resistance to estrogen receptor antagonist tamoxifen also decreased significantly in EDD-knockdown clone 1.1C as compared to clone 1.1A (**Figure 12E**). The percent of cells surviving tamoxifen treatment on Day 5 was significantly less in clone 1.1C. Therefore, EDD loss decreased cell resistance to chemotherapy drugs cisplatin and doxorubin, as well as to rapamycin and tamoxifen.

Discussion

Our present study showed that EDD immunostaining increased from low levels in benign breast tissues and ductal carcinoma in situ, to progressively higher levels in low-grade and high-grade tumours and TNBC, implicating a role for EDD in the progression of BCa. Indeed, Kaplan-Meier Plot analysis showed that high EDD (UBR5) mRNA expression correlated with a significantly lower probability of survival in breast cancer patients. EDD levels varied in BCa cell lines of different subtypes, and siRNA-knockdown of EDD decreased MCF-7 and T47D cell viability, increased apoptosis, and arrested cells in the G2/M phase. These events, measured at 24-72 h, correlated with increased expression of pro-apoptotic proteins Bim, Bak and Bax as well as MOAP-1, the activator and mitochondrial translocator of Bax, culminating in caspase-7 cleavage as early as 6 h, and PARP-1 cleavage at 24-48 h. Loss of EDD also inhibited TORC1 signaling, as indicated by decreased PRL-induced phosphorylation of 4EBP1 and decreased eIF4E binding to γ-aminophenyl-m^7GTP agarose, which represented the 5'-Cap of mRNAs. Furthermore, gain of EDD in low-EDD express-

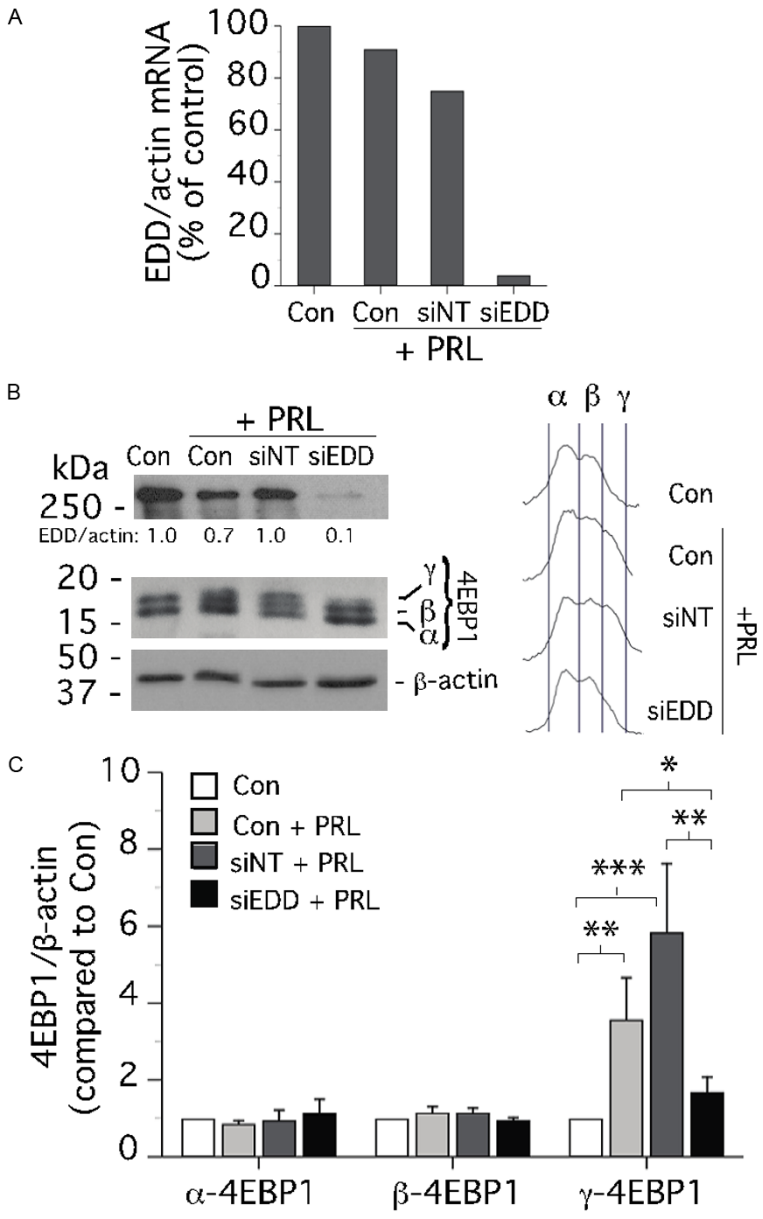

Figure 8. Loss of EDD decreases PRL-stimulated phosphorylation of 4EBP1. Quiescent MCF-7 cells (see *Materials and Methods*) were transfected with siEDD1, siNT or left untransfected (Con) for 48 h. Cells were then treated with PRL (10 ng/ml) for 1 h. In (A), total RNA was extracted for qPCR analysis of EDD and β-actin. In (B), cell lysates were prepared for SDS-PAGE (10 μg protein/lane), using 8% acrylamide gels for Western analysis of EDD, and 13% gels for analysis of 4EBP1 or β-actin, followed by densitometric analysis of the α, β, and γ bands of 4EBP1. Representative immunoblot of four independent experiments. In (C), densitometric analysis of α, β, and γ-4EBP1/β-actin. Mean ± SEM, n = 4; *P < 0.05; **P < 0.01; ***P < 0.001, indicates significant difference between the groups compared.

ing MDA-MB-436 cells promoted cell resistance to chemotherapeutic agents and TORC1/TORC2 inhibitors, whereas loss of EDD in EDD-expressing MCF-7 cells decreased cell resistance to these drugs and anti-estrogen tamoxifen.

By engaging the BCL-2 protein family, the intrinsic mitochondrial pathway commits cells to either survive or undergo apoptosis in response to cytotoxic stresses [23]. Apoptotic thresholds are set by interactions on the mitochondrial outer membrane between the BCL-2 homology 3 (BH3)-only proteins (e.g., Bim, Puma, Bad), pro-survival cell guardians (e.g., BCL-2, Bcl-XL, Mcl-1), and pro-apoptotic effector proteins (Bax, Bak, Bok). Successful initiation of apoptosis allows pro-apoptotic Bax and Bak to oligomerize and permeabilize the outer mitochondrial membrane to release cytochrome-C into the cytosol, leading to serial activation of caspases-3, -6, and -7, and consequently apoptosis.

In ovarian cancer cell lines, siRNA-depletion of EDD has also been reported to reduce cell viability, increase apoptosis, and enhance PARP cleavage [28]. However, in contrast to the current study, siEDD-induced apoptosis in ovarian cancer cells correlated with down-regulation of Mcl-1 [28]. Alternate splicing of the Mcl-1 gene could yield pro-survival Mcl-1_L (40-kDa) and pro-apoptotic Mcl-1_S (32-kDa) isoforms [29]. MCF-7 cells were reported to only produce high levels of Mcl-1_L [22]. Our study showed abundant Mcl-1_L and less, yet detectable, Mcl-1_S in MCF-7 cells, and the levels of both isoforms remained constant in controls, siNT- and siEDD-transfected cells. Therefore, EDD loss resulted in an overall increase in pro-apoptotic Bim, Bak and Bax over pro-survival Mcl-1. This is consistent with

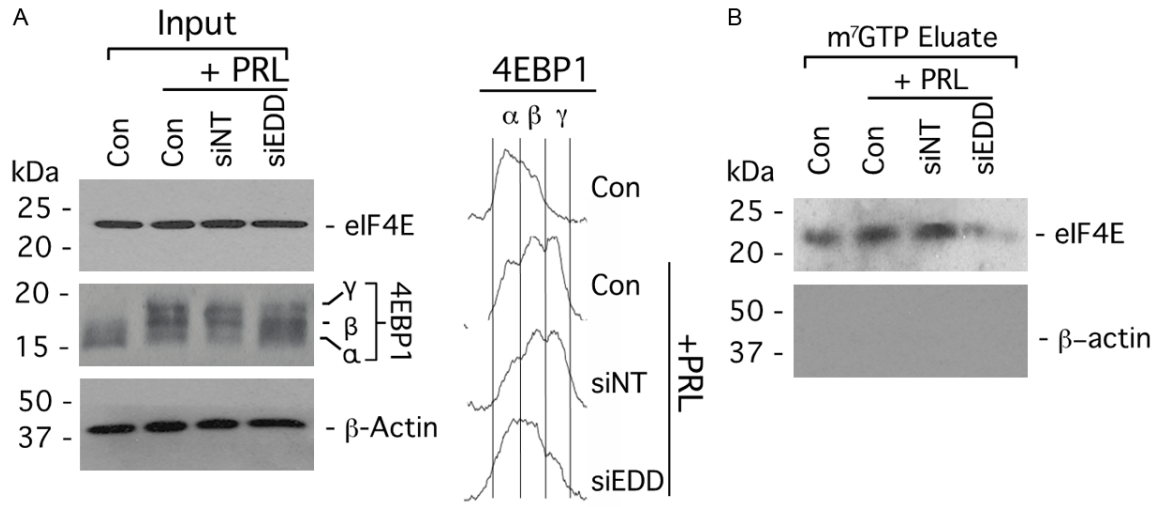

Figure 9. Loss of EDD decreases eIF4E binding to 5'-Cap of mRNAs. Quiescent MCF-7 cells were transfected with siEDD1 or siNT or left untransfected (Con) for 48 h, then treated ± PRL (10 ng/ml) for 1 h as in **Figure 8**. Each sample of cell lysate (100 µg protein) was incubated with 25 µl of pre-washed m^7GTP-agarose beads at 4°C for 3 h for the Cap-binding assay (see *Materials and Methods*). In (A), cell lysates were used for Western analysis (10 µg protein/lane) followed by densitometric analysis of α, β, and γ-4EBP1/β-actin. In (B), protein-bound m^7GTP-agarose beads were washed, the 500 x *g* supernatants collected, and resuspended in 50 µl of 1X SDS-PAGE buffer for Western analysis. Representative of 3 independent experiments.

elevation of EDD decreasing pro-apoptotic and/or increasing pro-survival gene expression to enable BCa cells to evade apoptosis.

Evasion of apoptosis also enables cancer cells to resist chemotherapy. The function of the pro-apoptotic protein Bax is enhanced by its binding to MOAP-1 [24]. In ovarian cancer cells, EDD (or UBR5) was shown to ubiquitinate MOAP-1 to destabilize it [30]. Importantly, MOAP-1 expression was low in cisplatin-resistant ovarian cancer cell lines but UBR5 knockdown increased MOAP-1 expression, enhanced Bax activation, and resensitized these cells to cisplatin-induced apoptosis. Furthermore, EDD/UBR5 expression was higher in ovarian cancers from cisplatin-resistant patients as compared to cisplatin-responsive patients [30], suggesting that EDD/UBR5 may confer cisplatin-resistance and, therefore, is an attractive therapeutic target for this cancer type [31]. Our studies using BCa cells also showed that EDD loss, through siRNA-knockdown in MCF-7 cells, elevated protein levels of MOAP-1 and Bax, as well as BIM, leading to cell apoptosis. EDD loss, through shRNA-knockdown in MCF-7 cells, also decreased drug resistance, leading to cell death. Together, our findings suggest that loss or inhibition of EDD decreases drug resistance, in part through upregulation of pro-apoptotic proteins.

We previously identified EDD as a binding partner of the α4 phosphoprotein-PP2Ac phosphatase complex that regulates TORC1 signaling, and we further determined that EDD targets PP2Ac for proteasomal degradation [17]. In the present study, EDD loss, that is, PP2Ac gain, decreased PRL-induced phosphorylation of 4EBP1, which decreased release of eIF4E, resulting in reduced binding of the freed eIF4E to the 5'-Cap of mRNAs. Therefore, EDD expression activates TORC1 signaling by promoting growth factor or hormone-induced phosphorylation of 4EBP1, culminating in the initiation of translation in BCa cells.

EDD is a regulator of cell cycle progression and the DNA damage signaling pathway [32, 33]. The DNA damage response is critical for the maintenance of genomic integrity and suppression of tumorigenesis. Following DNA damage, EDD accumulates in the nucleus, associates with the DNA damage checkpoint kinase CHK2, and mediates ATM (ataxia telangiectasia-mutated) phosphorylation of CHK2 to maintain cell-cycle checkpoints in MCF-7 and HeLa cells [32, 33]. EDD facilitates G1/S and intra S-phase DNA damage checkpoint activation and maintenance of G2/M arrest after double-strand DNA breaks in HeLa cells. However, EDD

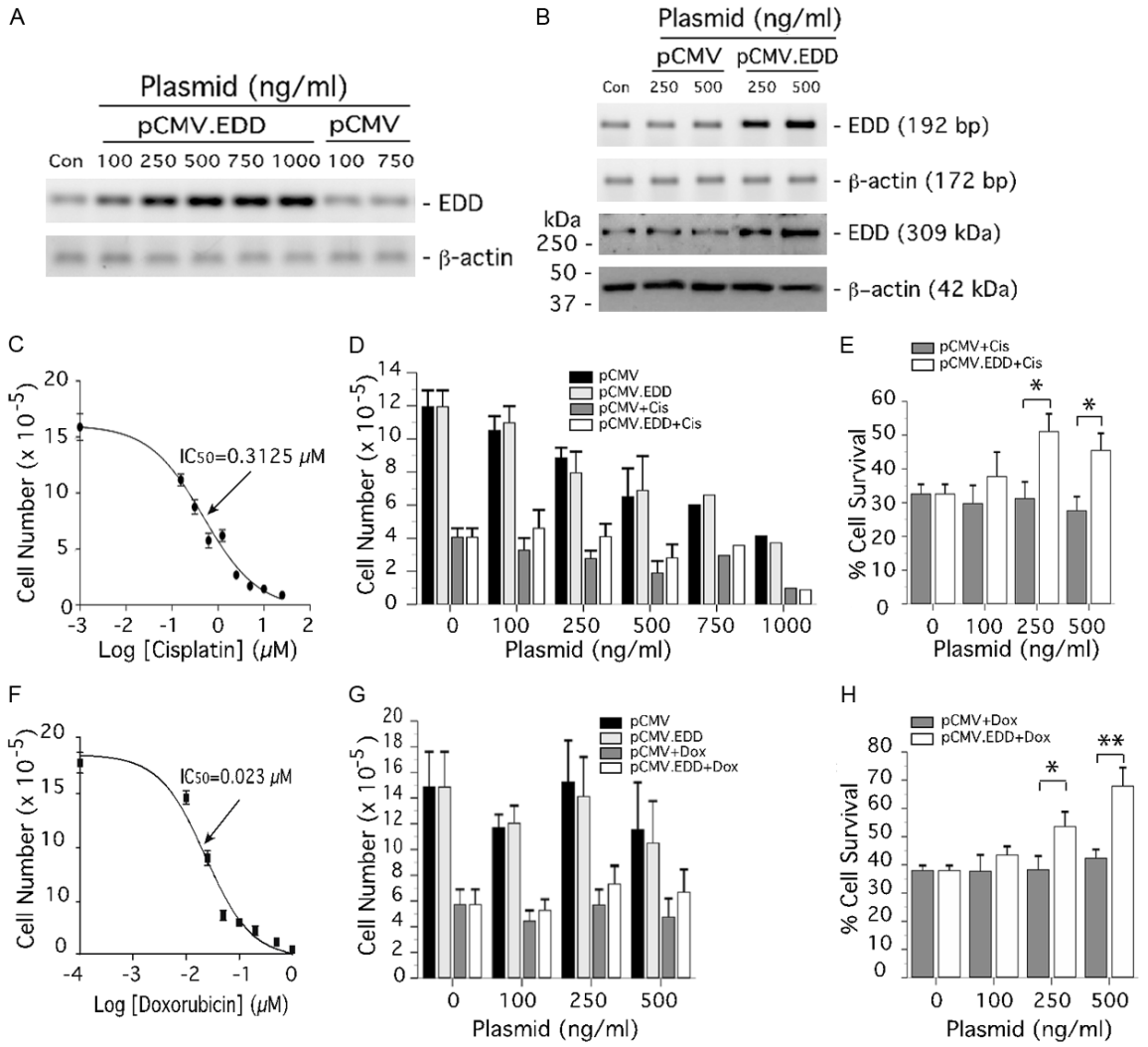

Figure 10. Ectopic EDD increases cisplatin/doxorubicin resistance in MDA-MB-436 cells. (A, B) MDA-MB-436 cells were transfected with increasing concentrations of plasmid pCMV-Tag2B or pCMV-Tag2B.EDD or left untransfected (Con). After 2 days, the cells were harvested for semi-quantitative RT-PCR (A, B; *upper panel*) or Western (B; *lower panel*) analyses to confirm elevated EDD expression. Each is a representative of 4 experiments. (C, F) MDA-MB-436 cells were treated with increasing doses of cisplatin (C) or doxorubicin (F) for 5 days and cells were counted to determine IC_{50} values. Mean ± SEM from 2 independent experiments, each in triplicate. (D, E, G, H) MDA-MB-436 cells were transfected with pCMV-Tag2B or pCMV-Tag2B.EDD. After 24 h (Day 1), the cells were treated ± IC_{50} doses of (D, E) cisplatin at 0.3125 μM or (G, H) doxorubicin at 0.023 μM. On Day 5, cells were counted using trypan-blue assay. The results were plotted as (D, G) cell numbers ± drug treatment or (E, H) % cell survival of drug-treated pCMV-Tag2B or pCMV-Tag2B.EDD transfectants, each compared to its untreated control. Mean ± SEM (n = 4-5), *P < 0.05, **P < 0.01.

depletion in DNA damaged HeLa cells impairs CHK2 activity, which can lead to radio-resistant DNA synthesis, premature entry into mitosis, accumulation of polyploid cells, and cell death [33]. This is in contrast to the role of EDD under normal conditions during which EDD depletion increases G2/M-arrest in HeLa cells [33] and, as observed in our present study, in MCF-7 cells, which then leads to decreased MCF-7 cell viability and apoptosis. EDD depletion induced G1-phase arrest by increasing p53 protein levels as early as 24 h, and further increased at 48 and 72 h, in two human osteosarcoma cell lines, [10]. EDD depletion, at 72 h, also increased p53 and p21 protein levels in MCF-7 and three human fibroblast cell lines [10], showing that this increase was not cell-specific. However, we showed that EDD loss at 48 h did not change p53 protein levels. This discrepancy could be due to the time points analyzed and/

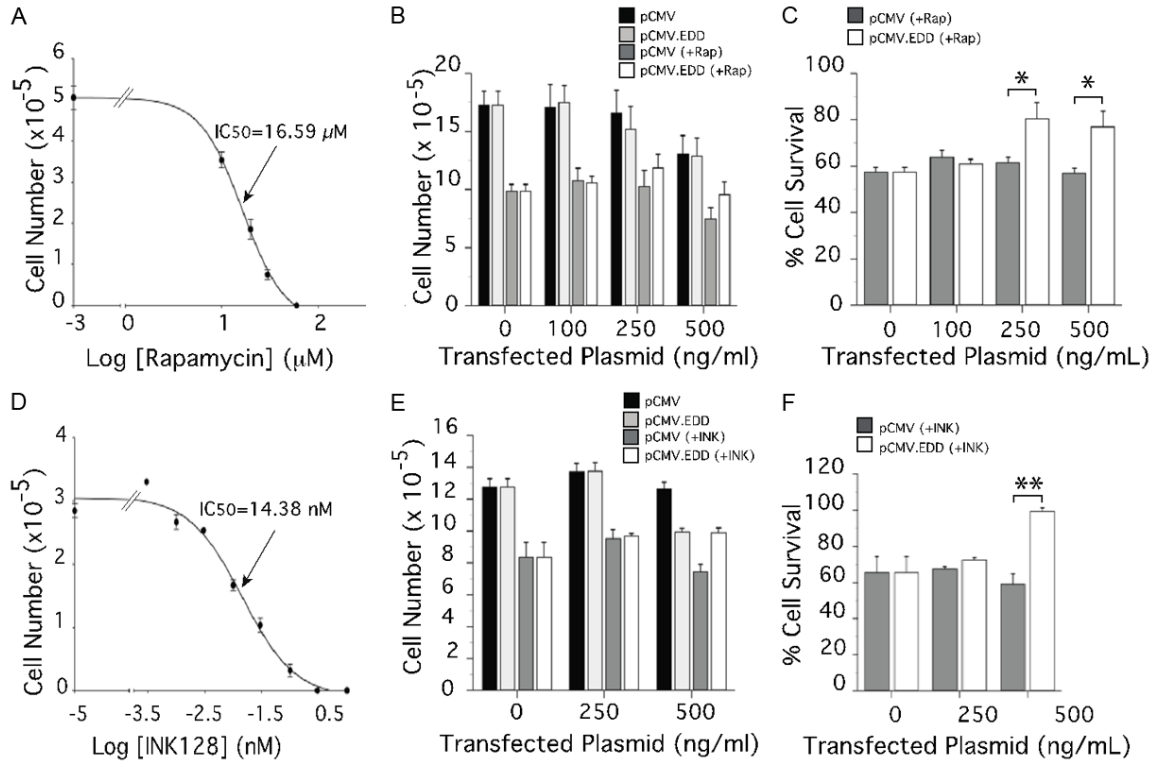

Figure 11. Ectopic EDD increases resistance to mTOR inhibitors in MDA-MB-436 cells. As in **Figure 10**, MDA-MB-436 cells were treated with increasing doses of rapamycin (A) or INK128 (D) for 5 days to determine drug IC_{50}'s. Mean ± SEM from 2 independent experiments, each in triplicate. (B, C, E, F) MDA-MB-436 cells were transfected with pCMV-Tag2B or pCMV-Tag2B.EDD for 24 h and then treated ± IC_{50} doses of (B, C) rapamycin at 16.59 μM or (E, F) INK128 at 14.38 nM. On Day 5, viable cell numbers were counted and the results were plotted as in **Figure 10**. (B, E) cell numbers ± drug treatment or (C, F) % cell survival of drug-treated pCMV-Tag2B or pCMV-Tag2B.EDD transfectants, each compared to its untreated control. Mean ± SEM (n = 3-5). *P < 0.05, **P < 0.01.

or clonal differences in the MCF-7 cells lines, since p53 is a caspase-3 substrate, and MCF-7 cells generally lack caspase-3 [34].

Regulation of cell cycle progression, DNA damage signaling, and the evasion of apoptosis are some of the processes that enable cancer cells to be resistant to chemotherapy. Our study showed that gain of EDD in low-EDD expressing MDA-MB-436 cells promoted cell resistance to chemotherapeutic agents and TORC1/TORC2 inhibitors, whereas loss of EDD in EDD-expressing MCF-7 cells decreased cell resistance to these drugs and anti-estrogen tamoxifen. In serous ovarian carcinoma, EDD gene amplification and overexpression have been linked to platinum resistance [9]. Although EDD expression was not directly correlated with relative cisplatin sensitivity in ovarian cancer cells, sensitivity to cisplatin was partially restored in platinum-resistant cells following EDD knockdown [9]. EDD overexpression in recurrent, platinum-resistant ovarian cancers also suggest a role in tumour survival and/or platinum-resistance [28]. EDD loss enhanced cisplatin sensitivity in ovarian cancer cells, and the dual activity of EDD as a regulator of cell survival and drug resistance supports EDD as a therapeutic target such as with siRNA, in combination with chemotherapy, for this disease [28]. Our current findings suggest that EDD is also a promising therapeutic target for BCa.

Since EDD is implicated in various aspects of cancer biology, its potential as a target in cancer therapy has been recognized [8]. It has been proposed that as an E3 ubiquitin ligase, a systematic approach to define EDD substrates in various contexts, particularly cancer, is a priority in future research [8]. Therapies targeting EDD expression, such as EDD siRNA delivered in nanoparticles, also appears to be most beneficial, since small molecule inhibitors of ubiquitin ligases have not been adequately effective [28].

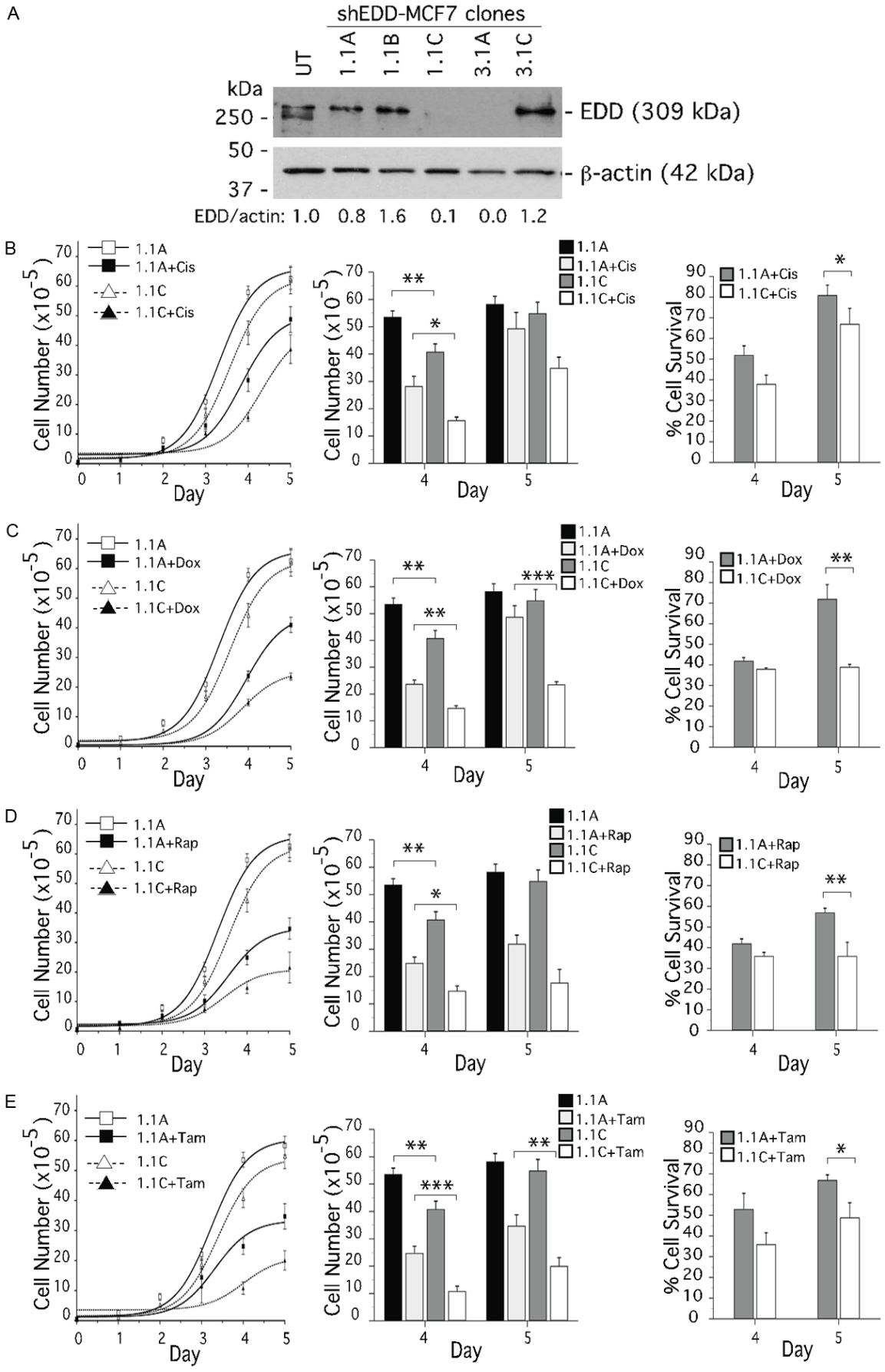
A
shEDD-MCF7 clones
UT
1.1A
1.1B
1.1C
3.1A
3.1C
kDa
250 -
50 -
37 -
- EDD (309 kDa)
- β-actin (42 kDa)
EDD/actin: 1.0 0.8 1.6 0.1 0.0 1.2
B
C
D
E
Cell Number (x10^-5)
Day
1.1A
1.1A+Cis
1.1C
1.1C+Cis
1.1A+Dox
1.1C+Dox
1.1A+Rap
1.1C+Rap
1.1A+Tam
1.1C+Tam
% Cell Survival

Figure 12. Loss of EDD decreases drug resistance in MCF-7 cells. MCF-7 cells were infected with shRNA lentiviral particles to knockdown EDD and puromycin-resistant clones were selected. In (A), Western analysis showed EDD gene knockdown in clone 1.1C but not in clone 1.1A. Each clone was treated with (B) 0.3125 μM cisplatin, (C) 0.023 μM doxorubicin, (D) 16.59 μM rapamycin or (E) 20 μM tamoxifen for up to 5 days. On Day 1-5, viable cell counts ± drug treatment were measured (*left panels*). Day 4 and 5 cell counts were replotted to clearly show statistical significance (*middle panels*) or expressed as % cell survival of drug-treated clone 1.1A or 1.1C, each compared to its untreated control (*right panels*). Mean ± SEM (n = 3-4). *P < 0.05, **P < 0.01, ***P < 0.001.

In summary, EDD levels increase with BCa progression *in vivo*. EDD expression in BCa cells promotes PRL-induced TORC1 signaling for the initiation of translation, anti-apoptotic protein expression, and drug resistance *in vitro*. Our study supports EDD as a therapeutic target for BCa and suggests that EDD expression may predict BCa responsiveness to various drug treatments. Furthermore, since EDD is a PRL-inducible gene [17], PRL receptor blockade holds promise as an additional therapy for BCa.

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Disclosure of conflict of interest

None.

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