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14. ABSTRACT

Previous genomic analysis has led us to identify the endoplasmic reticulum (ER) lipid raft-associated 2 (ERLIN2) gene as one of the candidate oncogenes within the 8p11-12 amplicon in a subset of aggressive breast cancer. We proposed that ERLIN2, an ER membrane protein, plays an unconventional oncogenic role through the endoplasmic reticulum (ER) stress pathway. In this study, we found: (1) ERLIN2 is required for cell proliferation and maintenance of transforming phenotypes in aggressive, ERLIN2-amplified breast cancer; (2) the UPR pathway, through the IRE1α/XBP1 axis, modulated the high-level expression of the ERLIN2 protein; (3) ERLIN2 also plays a key role in maintaining lipogenic phenotype of breast cancer cells by regulating activation of Sterol Regulatory Element-Binding Protein 1c (SREBP1c), the key lipogenic trans-activator; (4) ERLIN2 regulates activation of SREBP1c by interacting with Insulin-induced Gene 1 (INSIG1); (5) ERLIN2 had the ability to protect breast cancer cells from ER stress-induced cell death. The information provided here sheds new light on the mechanism of the novel ER factor ERLIN2 in promoting breast cancer progression.

15. SUBJECT TERMS
Gene amplification, Endoplasmic reticulum, ERLIN2

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Introduction

Breast cancer is the most common type of cancer for women in the United States and ranks second as a cause of cancer-related mortality. Increased *de novo* lipogenesis is one of the most important metabolic hallmarks of breast cancer [1-5]. Breast cancer cells contain a large number of genetic alterations that act in concert to create the malignant phenotype. For example, the up-regulation of oncogenes, such as *Her2, c-MYC and CCND1,* directly contributes to the uncontrolled proliferation of breast cancer cells. For cancer cells to survive, they must acquire the ability to tolerate a series of oncogenesis-associated cellular stress, such as proteotoxic-, mitotic-, metabolic-, and oxidative-stress [1, 2]. However, very little is known about the genomic basis and molecular mechanisms that allow breast cancer cells to tolerate and adapt to these stresses. Amplification of 8p11-12 occurs in approximately 15% of human breast cancer (HBC). This region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients [3-6]. Previous work in our laboratory, together with others, have identified the *endoplasmic reticulum (ER) lipid raft-associated 2* (*ERLIN2*, also known as *SPFH2*, *C8ORF2*) gene as one of several candidate oncogenes within the 8p11-12 amplicon, based on statistical analysis of copy number increase and over-expression [3, 4, 7]. Yet, the biological roles of ERLIN2 and molecular mechanisms by which ERLIN2 contributes to breast carcinogenesis remain unclear. In this research project, we hypothesized that *ERLIN2* plays an important role in the maintenance of malignancy and therapy-resistance through modulation of ER-associated signaling pathways in aggressive forms of human breast cancer. Accordingly, we propose that ERLIN2 represents a novel class of oncogenic factors and that targeting ERLIN2 may reduce the therapy resistance of aggressive breast cancers and thus improve the effectiveness of conventional anti-cancer drugs.

Body

1. Specific Aims

This project consists of 3 specific aims:
Aim 1: To investigate the role of ERLIN2 in the maintenance of stress- and apoptosis-resistant phenotypes of aggressive breast cancer cells.
Aim 2: To elucidate the molecular mechanism by which ERLIN2 increases ER protein folding capacity and suppresses ER stress-induced apoptosis in breast cancer cells.
Aim 3: To determine whether inhibition of ERLIN2 activity can enhance the effectiveness of the conventional anti-cancer drugs in aggressive breast cancers.

2. Studies and Results

Task 1 (Dr. Zeng-Quan Yang and Dr. Kezhong Zhang's labs). **To investigate the role of endogenous ERLIN2 up-regulation in the maintenance of stress- and apoptosis-resistant phenotypes of aggressive breast cancer cells. (**Completed)

In our previous annual reports, we have stated that we successfully knocked down ERLIN2 in *ERLIN2*-amplified SUM-44 and SUM-225 cells using the lentiviral-based shRNA system. Cell growth and proliferation analyses showed that knockdown of ERLIN2 slowed the proliferation rate of SUM-44 and SUM-225 cells, but not MCF10A control cells. We also revealed that knockdown of ERLIN2 in SUM-44 and SUM-225 cells also suppressed anchorage-independent growth in soft agar, one of the hallmark characteristics of aggressive cancer cells. Additionally, we found that amplification and over-expression of *ERLIN2* enhances the resistance to a variety of stressors, such as the ER stress-inducing reagents Tunicamycin or Thapsigargin, to promote breast cancer cell survival. Taken together, these results suggested ERLIN2 plays a role in cell proliferation and maintenance of transforming phenotypes in breast cancer cells with the *ERLIN2* amplification.

Previously, we evaluated the expression of ERLIN2 in normal and breast cancer tissues using immunohistochemistry (IHC) in tissue arrays. We found that the ERLIN2 protein is significantly upregulated in a subset of primary breast cancer cells compared with normal breast cells. Very recently, we searched the Cancer Genome Atlas database that contains 744 breast invasive carcinomas with survival data. We found a significantly worse overall survival for patients with ERLIN2 alteration, where the major samples are gene amplified and/or over-expressed ($p<0.05$). This new data further supports the findings that ERLIN2 plays an important role in promoting breast cancer progression.

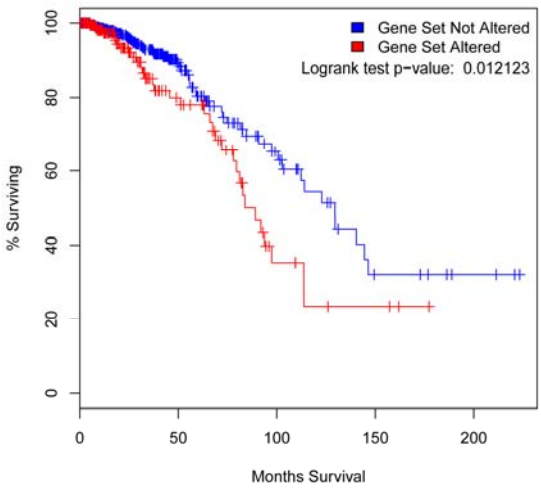

Fig.1. The overall survival of breast cancer patients with or without ERLIN2 gene alterations. The red curve in the Kaplan-Meier plot includes all tumors with an ERLIN2 copy number or expression alteration, and the blue curve includes all samples without the alteration.

Task 2 (Dr. Kezhong Zhang and Dr. Zeng-Quan Yang's labs). **To elucidate the molecular mechanism by which ERLIN2 regulates ER calcium levels, increases ER capacity, and suppresses ER stress-induced apoptosis in breast cancers. *(Completed).***

In the past progress reports, we have reported the following findings related to task 2: 1) the UPR signaling regulates ERLIN2 protein expression through IRE1α- XBP1 in human breast epithelial cells; 2) IRE1α-mediated regulation of ERLIN2 is through the IRE1α RNase activity, but not its kinase activity; 3) Over-expression of ERLIN2 leads to expansion of the ER compartment, a possible mechanism for stress- and apoptosis-resistance of human breast cancer cells; 4) Over-expression of ERLIN2 in human breast cancer cells did not affect ER calcium homeostasis, and thus, calcium signal alteration/ER calcium release is not likely the cause of resistance to apoptosis by ERLIN2 over-expression; 5) human breast cancer cells gain stress-adaption and apoptosis-resistance by up-regulating IRE1α/XBP1 UPR pathway but repressing ER stress-induced apoptotic pathway through CHOP in response to ER stress reagents or anti-cancer chemotherapeutic drugs; and 6) over-expression of ERLIN2 in human breast cancer cells promotes lipid droplet accumulation, a new mechanism to help cancer cells gain a growth advantage and protect from stress-induced apoptosis.

In the past year, we have accomplished all the remained tasks originally proposed for the Zhang lab. We not only accomplished all the experiments originally proposed in task 2, but also extended our study on delineating an unexpected molecular mechanism through which human breast cancer cells gain stress-resistance and apoptosis-evading capability. Specifically, we have made the following new processes:

Our studies suggested ERLIN2 does not function as a mediator of ER-associated protein degradation (ERAD) in human breast cancer cells. The levels of activated or polyubiquitinated IP3 receptor proteins, the key regulators of ER calcium homeostasis, were not changed in ERLIN2 over-expressing or knockdown breast cancer cells. As mentioned in the last report, ERLIN2 unlikely regulates ER calcium levels to prevent ER stress-induced apoptosis in breast cancers. This is opposite to our original hypothesis in potential roles of ERLIN2 in ER calcium homeostasis. However, we discovered that ERLIN2 over-expression promotes *de novo* lipogenesis and accumulation of cytosolic lipid droplets in breast cancer cells (reported in the last progress report), a new mechanism that help cancer cells survive from oncogenic stress and gain therapy resistance.

We investigate the molecular mechanism by which ERLIN2 regulates *de novo* lipogenesis and lipid droplet accumulation in human breast cancer cells. We examined activation of SREBP1c, an ER-transmembrane protein that plays the central roles in de novo lipogenesis[8], in MCF10A that over-expresses exogenous ERLIN2 or LacZ control. Levels of the activated form of SREBP1c were significantly higher in the MCF10A cells over-expressing ERLIN2,

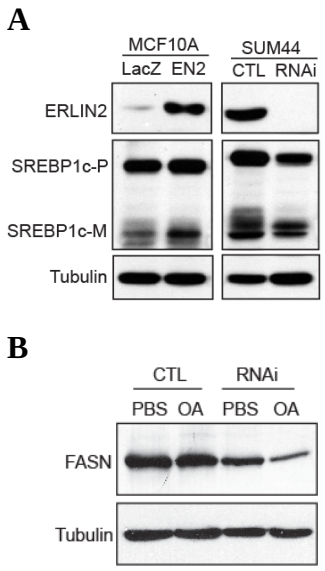

Fig 2. (**A**) Western blot analysis of ERLIN2 and SREBP1c levels in MCF10A cells expressing ERLIN2 or LacZ control and in ERLIN2-knockdown and control SUM44 cells. SREBP1c-P, SREBP1c precursor; SREBP1c-M, mature SREBP1c. (**B**) Western blot analysis of levels of fatty acid synthase (FASN) and tubulin (as a control) in ERLIN2 control (CTL) and knockdown (RNAi) Huh- 7cells. The cells were treated with vehicle PBS or oleic acid (OA) (0.5 mM) for 12 hr.

compared to that of the control cells (Figure 2A). Supporting a role of ERLIN2 in regulating SREBP1c activation in ERLIN2-amplified breast cancer cells, levels of cleaved SREBP1c protein were lower in the ERLIN2-knockdown SUM44 breast cancer cells than that in the control cells (Figure 2A). Supporting a regulatory role of ERLIN2 in SREBP1c activation, expression of fatty acid synthase (FASN), a key enzyme in de novo lipo genesis and a target of SREBP1c, was decreased in ERLIN2-knockdown hepatoma cell line Huh7 in the absence or presence of oleic acid (OA), a monounsaturated fatty acid that can stimulate cytosolic lipid accumulation (Figure 2B). Taken together, our gain- and loss-of-function analyses indicate that ERLIN2 regulates activation of SREBP1c, the key de novo lipogenic activator, in human breast cancer cells. This finding explains why the *ERLIN2*-amplified breast cancer cell lines, such as SUM225, possess abundant cytosolic lipid droplets, as we mentioned in the last progress report.

Next, we analyzed ERLIN2-binding partners in human breast cancer cells in order to understand the molecular mechanism underlying regulation of SREBP1c activation by ERLIN2. It is known that activation of SREBP is controlled by interactions involving ER-resident proteins that are regulated by metabolic signals [8, 9]]. In particular, the SREBP precursor proteins interact with ER membrane-SREBP cleavage-activating protein (SCAP), and SCAP binds to another ER membrane protein called insulin-induced gene 1 (INSIG1) to maintain SREBPs in an inactive state. In response to metabolic stimuli, INSIG1 dissociates with SCAP and is subsequently degraded through ERAD, thus allowing SREBP activation. We performed immunoprecipitation (IP)-western blot analysis to detect the interaction between ERLIN2 and INSIG1 in human breast cancer cells. In the absence of stress challenges, we detected only a nominal interaction between ERLIN2 and INSIG1 (Figure 3A). However, significant amounts of INSIG1 proteins associated with ERLIN2 were detected in the cells challenged by insulin or under the culture of lipoprotein-deficient serum (LPDS)-containing medium, a stress culture condition that stimulates de novo lipogenesis (Figure 3A) [8] . Additionally, we found that only a small portion of SCAP proteins associated with ERLIN2 in the presence of insulin or LPDS challenge. Moreover, we found that ERLIN2 does not function as a mediator of ERAD in facilitating INSIG1 degradation, as the levels of INSIG1 proteins were not significantly changed in ERLIN2-overexpressing cells compared to that in the control cells over-expressing LacZ (Figure 3B).

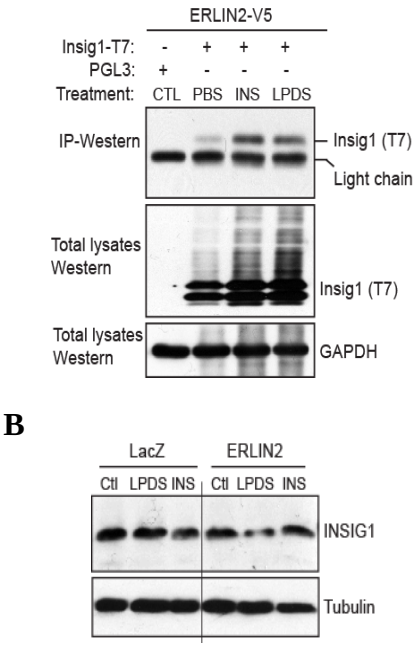

Fig 3. (**A**) IP-western blot analysis of the interaction between ERLIN2 and INSIG1. CHO cells stably expressing V5-tagged ERLIN2 were transfected with a plasmid vector expressing T7-tagged INSIG1. The transfected CHO cells were challenged with PBS, insulin (INS, 100 nM) for 6 hrs, or LPDS medium for 12 hrs. As a control, CHO cells were transfected with a plasmid vector PGL3. ER protein fractions were precipitated with an anti-V5 antibody and subjected to immunoblotting to detect the interaction between ERLIN2 and INSIG1. (**B**) Western blot analysis of INSIG1 protein levels in the Huh-7 cells overexpressing LacZ or ERLIN2. Cell lysates were prepared from the Huh-7 cells cultured in normal medium (Ctl), LPDS medium, or challenged with insulin (INS) for 6 hrs.

Task 3 (Dr. Zeng-Quan Yang). **To determine whether inhibition of ERLIN2 activity can enhance the effectiveness of conventional anti-cancer drugs in aggressive breast cancers *in vitro* and *in vivo,***

and to evaluate the potential of ERLIN2 as a therapeutic target in aggressive breast cancer. (No-cost extension)

In our previous annual reports, we have detailed that SUM-225 breast cancer cells with or without ERLIN2 knock-down were treated with conventional chemotherapeutic drugs, proteasome inhibitor Bortezonib or EGFR family inhibitors (lapatinib or erlotinib). We found that SUM-225 cells with ERLIN2 knockdown exhibited significantly lower Bortezonib IC50 values as compared with control SUM-225 cells without ERLIN2 knockdown. However, we did not detect significant changes of IC50 values for EGFR family inhibitors in SUM-225 cells with or without ERLIN2 knockdown. It is claimed that Bortezomib induces cell death by disrupting the ER stress responses in a wide variety of cancer cell lines. Our data suggests that the synergistic cooperation between knockdown of ERLIN2 and a proteasome inhibitor might lead to a significant decrease in proliferation in a subset of breast cancer cells *in vitro*.

In the past year, we performed *in vivo* studies with the two most effective ERLIN2 shRNAs determined in our previous *in vitro* experiments. Two breast cancer cell lines, SUM-225 and SUM-52, with the ERLIN2 gene amplification were used. The cells were orthotopically transplanted into the mammary fat pads of nude mice. Mice were examined for tumor growth by palpation two times every week. In order to avoid tumor necrosis and in compliance with regulations for use of vertebrate animals in research, the animals were euthanized when the largest tumors reached approximately 1.5 cm in diameter. We found that knockdown of ERLIN2 likely inhibits tumor growth of the SUM225 breast cancer line. Unfortunately, SUM-52 breast cancer cells didn't grow in mammary fat pads of nude mice. In our future experiments, we will mainly use the SUM-225 breast cancer line.

Remaining work for no-cost extension: *Task 3 (Dr. Zeng-Quan Yang)*: Since *in vivo* studies using human breast cancer xenografts are being conducted for more than 6 months, we are requesting a second no-cost extension in order to determine whether inhibition of ERLIN2 activity can enhance the effectiveness of the conventional anti-cancer drugs in breast cancer xenograft models. The results of *in vivo* experiments will provide more direct evidence that ERLIN2 represents a novel class of oncogenic factors and that targeting ERLIN2 can reduce the therapy resistance of aggressive breast cancers, and thus improve the effectiveness of conventional anti-cancer drugs. Breast cancer cell lines with or without ERLIN2 knockdown will be injected into the mammary fat pads of the left and right flanks of nude mice. Bi-weekly treatment of tumors with the drugs that have synergistic cooperation effects with ERLIN2 knockdown *in vitro*, or drug vehicles, will be initiated at 1 week after injection. Subcutaneous tumor volumes will be monitored by direct caliper measurement.

Key Research Accomplishments

The highlights of our accomplishments from the past years are: (1) we discovered that ERLIN2 is amplified and over-expressed in both aggressive luminal B and HER2 subtypes of breast cancer; (2) we stably knocked down or over-expressed ERLIN2 in various breast model cells utilizing lentiviral-based shRNA or over-expression systems, and demonstrated that ERLIN2 is required for cell proliferation and maintenance of transforming phenotypes in aggressive, ERLIN2-amplified breast cancer; (3) we determined that expression of ERLIN2 in human breast cancer cells is regulated by the unfolded protein response (UPR) pathway through the ER stress sensor IRE1α and its downstream trans-activator XBP1; (4) we revealed that ERLIN2 facilitates breast cancer cell adaptation to ER stress and resistance to ER stress-induced apoptosis; (5) we found that ERLIN2-amplified breast cancer cell lines, such as SUM225 and SUM44, possess abundant cytosolic lipid droplets; (6) we demonstrated that the levels of cytosolic lipid droplet and cellular triglyceride contents were significantly reduced in the ERLIN2-knockdown breast cancer cells; (7) we revealed that ERLIN2 modulates activation of ER-resident lipogenic regulators, including sterol regulatory element-binding protein 1c (SREBP1c) and fatty acid synthase (FASN), in breast cancer cells; (8) we discovered that ERLIN2 regulates activation of SREBP1c by interacting with the ER-resident, SREBP/SCAP retention protein INSIG1; (9)we found that knockdown of ERLIN2 enhances efficacy of chemo-therapeutic drugs, including proteasome inhibitor Bortezonib, in breast cancer *in vitro*.

Reportable Outcomes

Manuscript:

"ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways" BMC Cancer 2012,12:225.

"Endoplasmic Reticulum Factor ERLIN2 Regulates Cytosolic Lipid Contents in Cancer Cells" Biochem J. 2012, 446(3):415-425.

"Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation" Am J Transl Res. 2012, 4:102-113.

Conclusion

We have made significant progress in the past year in characterizing the endoplasmic reticulum factor, ERLIN2, in human breast cancer. We found that ERLIN2 confers a selective growth advantage on breast cancer cells by facilitating a cytoprotective response to various cellular stresses associated with oncogenesis. We demonstrated that ERLIN2 also plays a key role in maintaining lipogenic phenotype of breast cancer cells by regulating activation of SREBP, the key lipogenic trans-activator. Under oncogensis-associated metabolic stress, ERLIN2 interacts with INSIG1, thus leading to dissociation of SCAP from INSIG1 and subsequent activation of SREBP1c in human breast cancer cells. The activation of SREBP1c triggered by the interaction between ERLIN2 and INSIG1 represents an important

mechanism by which breast cancer cells increase *de novo* lipogenesis to gain growth advantage and stress-resistance capability. The information provided here sheds new light on the mechanism of breast cancer malignancy.

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RESEARCH ARTICLE Open Access

ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways

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Abstract

Background: Amplification of the 8p11-12 region has been found in approximately 15% of human breast cancer and is associated with poor prognosis. Previous genomic analysis has led us to identify the *endoplasmic reticulum (ER) lipid raft-associated 2* (*ERLIN2*) gene as one of the candidate oncogenes within the 8p11-12 amplicon in human breast cancer, particularly in the luminal subtype. ERLIN2, an ER membrane protein, has recently been identified as a novel mediator of ER-associated degradation. Yet, the biological roles of ERLIN2 and molecular mechanisms by which ERLIN2 coordinates ER pathways in breast carcinogenesis remain unclear.

Methods: We established the MCF10A-ERLIN2 cell line, which stably over expresses ERLIN2 in human nontransformed mammary epithelial cells (MCF10A) using the pLenti6/V5-ERLIN2 construct. ERLIN2 over expressing cells and their respective parental cell lines were assayed for *in vitro* transforming phenotypes. Next, we knocked down the ERLIN2 as well as the ER stress sensor IRE1α activity in the breast cancer cell lines to characterize the biological roles and molecular basis of the ERLIN2 in carcinogenesis. Finally, immunohistochemical staining was performed to detect ERLIN2 expression in normal and cancerous human breast tissues

Results: We found that amplification of the *ERLIN2* gene and over expression of the ERLIN2 protein occurs in both luminal and Her2 subtypes of breast cancer. Gain- and loss-of-function approaches demonstrated that ERLIN2 is a novel oncogenic factor associated with the ER stress response pathway. The IRE1α/XBP1 axis in the ER stress pathway modulated expression of ERLIN2 protein levels in breast cancer cells. We also showed that over expression of ERLIN2 facilitated the adaptation of breast epithelial cells to ER stress by supporting cell growth and protecting the cells from ER stress-induced cell death.

Conclusions: ERLIN2 may confer a selective growth advantage for breast cancer cells by facilitating a cytoprotective response to various cellular stresses associated with oncogenesis. The information provided here sheds new light on the mechanism of breast cancer malignancy

Keywords: Gene amplification, Breast cancer, Endoplasmic reticulum, ERLIN2

Background

Breast cancer cells contain a large number of genetic alterations that act in concert to create the malignant phenotype. For example, the up-regulation of oncogenes, such as *Her2, c-MYC and CCND1,* directly contributes to the uncontrolled proliferation of breast cancer cells. For cancer cells to survive, they must acquire the ability to tolerate a series of oncogenesis-associated cellular stressors, which include DNA damage, proteotoxic-, mitotic-, metabolic-, and oxidative-stress [1,2]. However, very little is currently known about the genomic basis and molecular mechanisms that allow breast cancer cells to tolerate and adapt to these stresses. Amplification of 8p11-12 occurs in approximately 15% of human breast cancer (HBC). This region of amplification is significantly associated with

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disease-specific survival and distant recurrence in breast cancer patients [3-6]. Previous work in our laboratory, together with others, have identified the *endoplasmic reticulum (ER) lipid raft-associated 2* (*ERLIN2*, also known as *SPFH2*, *C8ORF2*) gene as one of several candidate oncogenes within the 8p11-12 amplicon, based on statistical analysis of copy number increase and over expression [3,4,7]. Yet, the biological roles of ERLIN2 and molecular mechanisms by which ERLIN2 coordinates ER pathways in breast carcinogenesis remain unclear.

The ER is a cellular organelle primarily responsible for protein folding, lipid and sterol biosynthesis, and calcium storage. Physiological processes that increase protein folding demand or stimuli that disrupt the ER protein folding process can create an imbalance between ER protein folding load and capacity. This imbalance leads to the accumulation of unfolded or misfolded proteins in the ER: a condition referred to as "ER stress" [8,9]. The ER has evolved highly specific signaling pathways, collectively termed the "unfolded protein response" (UPR), to ensure protein folding fidelity and to protect the cell from ER stress. Upon activation of UPR, inositol-requiring protein 1 (IRE1α), the conserved ER stress sensor from yeasts to mammals, mediates splicing of the mRNA encoding X-box binding protein 1 (XBP1). XBP1 serves as a potent UPR *trans*-activator that helps protein refolding, transportation, and degradation in order to bolster ER capacity and facilitate cell adaptation to stress [8]. However, if UPR fails to restore ER homeostasis, ER stress-associated apoptosis will occur [10]. As part of the UPR program, ER-associated degradation (ERAD) targets aberrantly folded proteins in the ER. In addition to this "quality control" function, ERAD also accounts for the degradation of several metabolically-regulated ER proteins [11].

Recent studies provide evidence that UPR and ERAD components are highly expressed in various tumors, including human breast cancer [12-21]. During tumor development and progression, increased amounts of misfolded proteins caused by gene mutations, hypoxia, nutrient starvation, and high-levels of reactive oxygen species lead to ER stress [22,23]. The activation of UPR and ERAD induces an adaptive response in which the tumor cell attempts to overcome ER stress to facilitate cytoprotection. In this study, we demonstrated that amplification and the resultant over expression of ERLIN2 occurred in both luminal and Her2 subtypes of breast cancer. We also found that the UPR pathway, through the IRE1α/XBP1 axis, modulated the high-level expression of ERLIN2 protein. Furthermore, ERLIN2 had the ability to protect breast cancer cells from ER stress-induced cell death. Thus, ERLIN2 is a novel mediator of ER stress response and thus amplification and over expression of ERLIN2 may facilitate the adaptation of breast cancer cells to the various cellular stresses associated with oncogenesis.

Materials and methods

Cell lines and cell culture conditions

The culture conditions of of SUM breast cancer cells and the immortalized non-tumorigenic MCF10A cells are described in the Additional file 1: Materials and Methods.

Genomic array CGH

Genomic array CGH experiments were performed using the Agilent 44 K human genome CGH microarray chip (Agilent Technologies, Palo Alto, CA). Agilent's CGH Analytics software was used to calculate various measurement parameters, including log2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe.

Semiquantitative RT-PCR reactions

Total RNA was prepared from human breast cancer cell lines and the MCF10A cell line by standard methods [3,24]. For RT-PCR reactions, RNA was converted into cDNA *via* a reverse transcription reaction using random hexamer primers. Primers were ordered from Invitrogen (Carlsbad, CA). A GAPDH primer set was used as a control. Semiquantitative RT-PCR was done using the iQSYBR Green Supermix (Bio-Rad, Hercules, CA).

Lentivirus construction and transduction of cells

The lentiviral expression construct containing the *ERLIN2* gene (pLenti-ERLIN2), was established as previously described [3]. The lentivirus for pLenti-ERLIN2 was generated and used to infect the immortalized, non-transformed mammary epithelial MCF10A cells. Control infections with pLenti-LacZ virus were performed in parallel with the pLenti-ERLIN2 infections. Selection began 48 h after infection in growth medium with 10 μg/mL blasticidin in the absence of either insulin or epidermal growth factor (EGF). Upon confluence, selected cells were passaged and serially cultured.

Three-dimensional morphogenesis assays in matrigel

For three-dimensional morphogenesis assays in Matrigel, cells grown in monolayer culture were detached by trypsin/EDTA treatment and seeded in Matrigel (BD Biosciences, San Jose, CA) precoated 8-well chamber slides. The appropriate volume of medium was added and cells were maintained in culture for 10–18 days. Phase-contrast images and immunostaining images were taken using bright-field and confocal microscopy.

Lentivirus-mediated shRNA knockdown of gene expression

We knocked down the expression of the human *ERLIN2* gene in breast cancer cell lines and in the MCF10A cell line using the Expression Arrest GIPZ lentiviral shRNA-mir system (OpenBiosystems, Huntsville, AL). Lentivirus was produced by transfecting 293FT cells with the combination of the lentiviral expression plasmid DNA and Trans-Lentiviral packaging mix (OpenBiosystems. Huntsville, AL). For cell infection, viral supernatants were supplemented with 6 μg/mL polybrene and incubated with cells for 24 hours. Cells expressing shRNA were selected with puromycin for 2–3 weeks for functional studies (cell proliferation and colony formation assays) and for 4 to 10 days after infection for RNA extraction.

Recombinant adenoviral or retrovirus infection

Adenoviruse vectors for expressing flag-tagged IRE1α isoforms, including wild type IRE1α (Ad-IRE1α WT), IRE1α kinase mutant (Ad-IRE1α K599A), and IRE1α RNase mutant (Ad-IRE1α K907A), were kindly provided by Dr. Yong Liu (Institute for Nutritional Sciences, Shanghai, China) and amplified using the AdEasy System (Stratagene) [25,26]. Retrovirus expressing spliced XBP1 was kindly provided by Dr. Lauri Glimcher (Harvard University) [27]. For infection of cells with adenovirus and retrovirus, cells were seeded in six-well plates. After 24 h, cells were infected with adenovirus expressing wild type IRE1α (Ad-IRE1α WT), IRE1α kinase mutant (Ad-IRE1α K599A), IRE1α RNase mutant (Ad-IRE1α K907A), and retrovirus expressing spliced XBP1 as described previously [28,29].

Tissue array and immunohistochemistry (IHC) staining

Human breast cancer tissue array was obtained from Nuclea Biotechnologies (Pittsfield, MA). Immunohistochemistry was performed on tumor tissue sections using the standard laboratory protocols [30]. Briefly, after deparaffinizing and hydrating with phosphate-buffered saline (PBS) buffer (pH 7.4), the sections were pretreated with hydrogen peroxide (3%) for 10 minutes to remove endogenous peroxidase, followed by antigen retrieval *via* steam bath for 20 minutes in EDTA. A primary antibody was applied, followed by washing and incubation with the biotinylated secondary antibody for 30 minutes at room temperature. Detection was performed with diaminobenzidine (DAB) and counterstaining with Mayer hematoxylin followed by dehydration and mounting. Immunostained slides were blindly evaluated under a transmission light microscope. Areas of highest staining density were identified for evaluating the expression in tumors.

Results

ERLIN2 is amplified and over expressed in human breast cancer cells

Recently, we used quantitative genomic PCR and array comparative genomic hybridization (CGH) to profile copy number alterations in 10 human breast cancer cell lines and 90 primary human breast cancers [3,6,31]. Analysis of our array CGH data showed that ERLIN2 gene was commonly amplified in 30% of the cell lines tested, as well as in 7.8% of breast cancer specimens tested (Figure 1a). Previously, we and several other laboratories have demonstrated that the 8p11-12 amplicon occurs mainly in the luminal subtype of breast cancer cells, such as the SUM-44 and SUM-52 cell lines. However, SUM-225 is a *Her2*-amplified HBC cell line [31,32]. We also found two primary tumors, 10173 and 9895, which have *Her2* gene amplifications in addition to the amplification of the ERLIN2 gene (Figure 1a). To obtain further support for a potential involvement of the ERLIN2 region in breast cancer, we searched the published database of the Affymetrix 250 K array CGH [33]. We found that 42 of the 243 HBC lines and primary samples in the array exhibited amplification of the ERLIN2 region. Interestingly, eight of the *ERLIN2*-amplified samples showed co-amplification of the *Her2* gene (Additional file 1: Figure S1). Next, we measured ERLIN2 protein levels in ten breast cancer cell lines by Western blot analysis. In correlation with *ERLIN2* gene amplification, ERLIN2 protein levels in SUM-44, SUM-52, and SUM-225 cells were dramatically greater than the levels in breast cancer cell lines without *ERLIN2* gene amplification (Figure 1b). The presence of the ERLIN2 amplification in both luminal and Her2 subtypes of breast cancer prompted us to further investigate the role of the *ERLIN2* gene in breast cancer progression.

ERLIN2 plays a functional role in breast cancer cells

Next, we addressed whether *ERLIN2* possess transforming properties. We transduced the immortalized, non-transformed mammary epithelial cell line, MCF10A, with lentivirus expressing ERLIN2 or control LacZ. Semi-quantitative RT-PCR (qRT-PCR), Western blot and immunofluorescence staining confirmed the over expression of ERLIN2 protein in MCF10A-ERLIN2 cells (Figure 2a and Additional file 1: Figure S2). The infected MCF10A cells were then subjected to analyses for growth rates, growth factor-independent proliferation, anchorage-independent growth, and three-dimensional morphogenesis assays. Growth curves and colony formation assays in MCF10A cells showed that forced expression of ERLIN2 resulted in growth factor-independent proliferation in insulin-like growth factor-deficient media. To further examine the effects of ERLIN2 in a context that more closely resembles *in vivo* mammary architecture, we assessed the consequences of ERLIN2

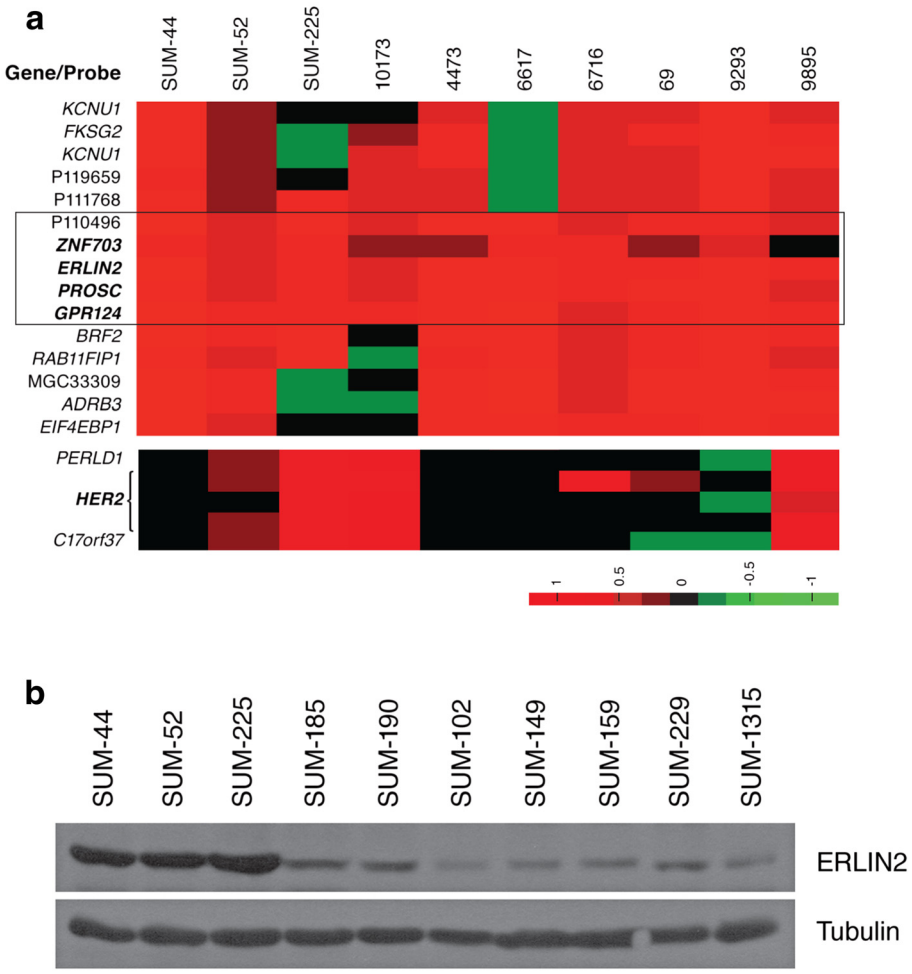

Figure 1 (a) Genomic copy number profiles of the ERLIN2 region analyzed on the Agilent oligonucleotide array CGH in 3 SUM breast cancer cell lines and 7 primary breast cancer specimens. Tumors are displayed vertically and array probes are displayed horizontally by genome position. Log2 ratio in a single sample is relative to normal female DNA and is depicted according to the color scale (bottom). Red indicates relative copy number gain, whereas green indicates relative copy number loss. (**b**) ERLIN2 protein levels were analyzed by Western blot in ten breast cancer cell lines with or without *ERLIN2* amplification.

over expression on three-dimensional morphogenesis in Matrigel. Although MCF10A cells formed polarized, growth-arrested acinar structures with hollow lumens similar to the glandular architecture *in vivo*, MCF10A-ERLIN2 cells formed abnormal acini at a high frequency that was grossly disorganized, and contained filled lumens (Figure 2b).

To further explore the pathophysiological function of ERLIN2 over expression, we stably silenced the *ERLIN2* gene in SUM-44 and SUM-225 breast cancer cells using the lentiviral-based shRNA system. To perform RNAi knockdown experiments, we utilized pGIPZ-ERLIN2 shRNA expression constructs in which TurboGFP and shRNA were part of a bicistronic transcript allowing for the visual marking of the shRNA-expressing stable cells. qRT-PCR and Western blot analysis indicated a marked reduction in expression levels of ERLIN2 mRNA and protein in the stable ERLIN2-shRNA-transduced SUM-44 and SUM-225 cell lines as compared with the control cell lines infected with a non-silencing shRNA lentiviral control (Figure 3a). Among the two targeted vectors used, ERLIN2-shRNA vector #1 produced a more striking knockdown effect: infected SUM-225 cells had a nearly complete loss of ERLIN2 protein expression (Figure 3a). We did not detect any change in ERLIN1 mRNA and protein levels in ERLIN2-shRNA knockdown cells, thus ruling out the possiblity of off-target effects by ERLIN2-shRNAs (Data not shown). Cell growth and proliferation analyses showed that knockdown of ERLIN2 slowed the proliferation rate of SUM-44 and SUM-225 cells, but had only a minor effect on SUM-102 and MCF10A cells, which lack ERLIN2 amplification (Figure 3b). Importantly, knockdown of ERLIN2 in SUM-44 and SUM-225 cells also suppressed anchorage-independent growth in soft agar, one of

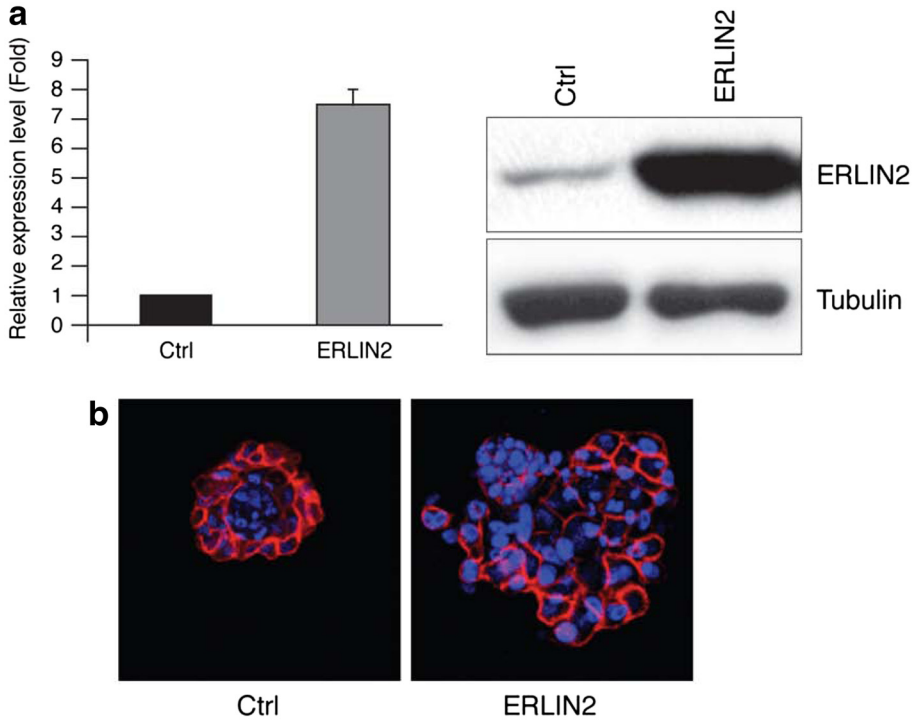

Figure 2 (a) Stable overexpressing ERLIN2 in MCF10A cells (MCF10A-ERLIN2) with the pLenti6/V5-ERLIN2 construct. Over expression of ERLIN2 mRNA and protein in this cell line was confirmed with semi-quantitative RT-PCR (right panel) and western blot assays (left panel). (**b**) Effects of ERLIN2 on mammary acinar morphogenesis. MCF10A-ERLIN2 and control cells were cultured on a bed of Matrigel. Representative images show the structures with staining for actin with phalloidin conjugated to Alexa Fluor-568 (red), and DAPI as a marker of nuclei (blue).

the hallmark characteristics of aggressive cancer cells. (Figure 3c). Taken together, results from over expression and knockdown experiments suggested *ERLIN2* plays a role in cell proliferation and maintenance of transforming phenotypes in breast cancer cells with the 8p11-12 amplification.

Expression of ERLIN2 is regulated by the ER pathway through IRE1α/XBP1

Recent studies have identified ERLIN2 both as a novel component of lipid raft domains in the ER membrane and as a substrate recognition factor during ERAD of activated inositol triphosphate receptors (IP3R) as well as other substrates [34-36]. IRE1α is the primary ER stress sensor implicated in the regulation of the ERAD pathway [37]. Under ER stress, IRE1α undergoes autophosphorylation to activate its RNase activity, which triggers one of the UPR cascades through splicing *Xbp1* mRNA [8]. Previous work has demonstrated that breast cancer cells over express XBP1 [38,39], while we observed that SUM-44, SUM-52 and SUM-225 cell lines over expressed total and activated XBP1 (Additional file 1: Figure S3 Additional file 2: Table S1). To evaluate the possibility of an association between ERLIN2 expression and the IRE1α-mediated UPR pathway in HBC, we inhibited IRE1α RNase or kinase activity in breast cancer cells. To accomplish this, we used adenoviral-based expression system to introduce the previously characterized IRE1 kinase dominant-negative mutant (IRE1 K599A) or the IRE1 RNase dominant-negative mutant (IRE1 K907A) into breast cancer cells [26,40,41]. We chose SUM-44 cells for this experiment because the SUM-44 cells are very amenable to adenovirus-mediated expression. The inhibition of the IRE1α RNase activity significantly reduced protein levels of ERLIN2 in SUM-44 cells (Figure 4a). In addition, forcible expression of wild-type IRE1α or spliced XBP1 in MCF10A cells resulted in increased expression levels of endogenous ERLIN2 protein (Figure 4b and c). However, quantitative real-time RT-PCR analysis showed that over expression of IRE1α or spliced XBP1 did not increase expression of the *ERLIN2* mRNA (Data not shown). Next, we asked whether ERLIN2 expression was induced by stress inducers in normal mammary epithelial cells. Our group routinely cultures MCF10A cells in serum-free, growth factor-supplemented media. Oncogenesis-associated conditions, such as nutrient or growth factor depletion, can cause pathophysiologic ER stress [22,23]. When MCF10A cells were cultured in media lacking insulin or EGF, expression levels of endogenous ERLIN2 protein in MCF10A were increased as compared with levels in cells cultured in normal media (Figure 4d). Our observations

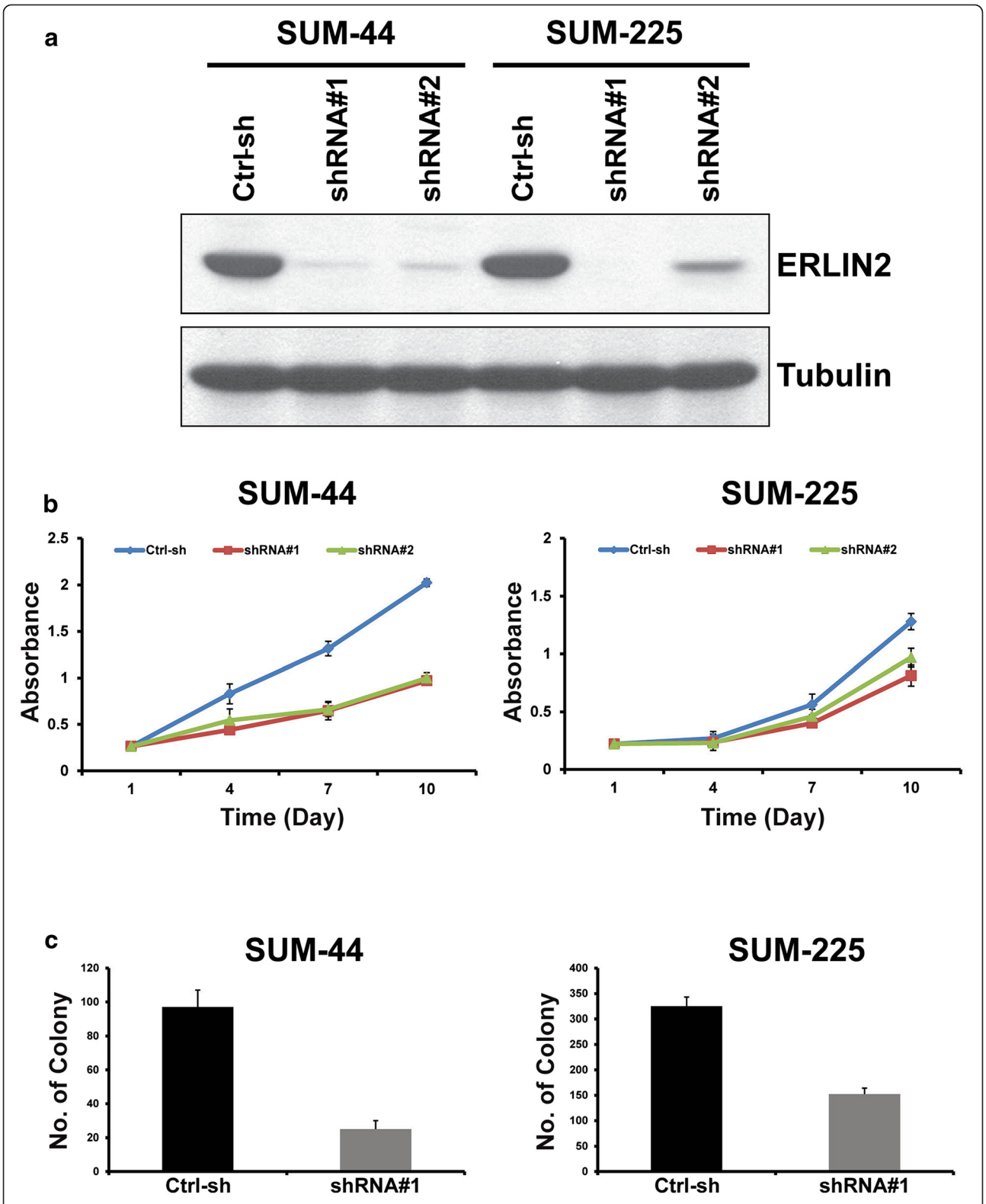

Figure 3 shRNA-mediated knockdown of ERLIN2 inhibits monolayer and anchorage-independent cell growth in breast cancer cells with ERLIN2 amplification. (**a**) Knockdown of ERLIN2 expression in SUM-44 and SUM-225 cells with two different shRNAs was confirmed by western blot. (**b**) *In vitro* growth rate of SUM-44 and SUM-225 cells with ERLIN2 shRNA treatment compared to cells with control shRNA treatment. (**c**) Knockdown of ERLIN2 reduces colony formation in soft agar. SUM-44 and SUM-225 cells were tranfected with ERLIN2 shRNA#1 or control shRNA. The colony numbers were counted 3 weeks later ($P < 0.05$).

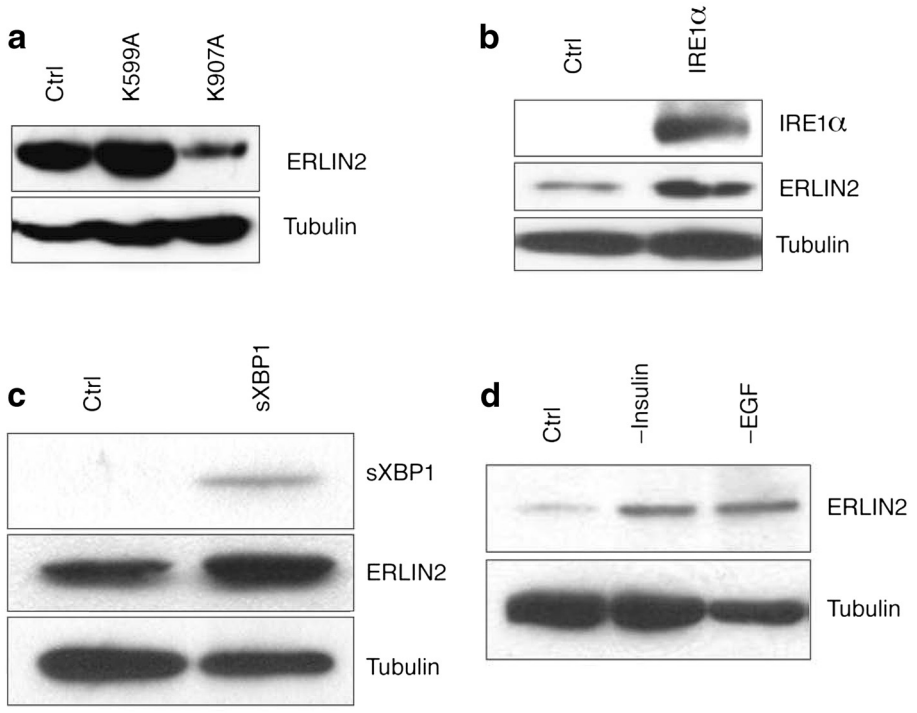

Figure 4 (a) The knockdown of the IRE1α RNase activity (K907A) reduced levels of ERLIN2 protein in SUM-44 cells. Forced expression of wild-type IRE1α (**b**) and its substrate, spliced XBP1 (**c**), leads to increased expression of ERLIN2 at protein level in MCF10A cells. (**d**) ERLIN2 expression in MCF10A cells was analyzed by western blot after culture 48 hours in insulin- or EGF-depleted media, compared to that in normal culture media.

suggest that the ER stress pathway likely regulates ERLIN2 protein expression through IRE1α-actived XBP1 in human breast epithelial cells.

ERLIN2 promotes breast cancer cell survival

Next, we tested if amplification and over expression of ERLIN2 enhances the resistance to a variety of stressors to promote cancer cell survival. Figure 5a shows the IC_{50} values for the ER stress-inducing reagent Tunicamycin (Tm), in ten breast cancer cell lines as well as in the nontransformed human mammary epithelial cell line MCF10A. SUM-44 and SUM-225 cells, which have ERLIN2 amplification, had significantly higher TM IC_{50} values than cell lines without ERLIN2 amplification ($P < 0.05$). We obtained similar results with Thapsigargin (Tg) treatment of SUM-225 cells (data not shown). Expression of the CCAAT/enhancer-binding protein (C/EBP) homology protein (CHOP) is characteristic of the ER stress–mediated apoptotic pathway. In response to treatment with Tm or Tg, expression of CHOP dramatically increased in control MCF10A cells (Figure 5b). However, induction of CHOP by Tm and Tg treatment was weaker or barely detectable in SUM-44 and SUM-225 cells (Figure 5b). Next, to determine whether suppressing ERLIN2 in breast cancer cells re-sensitize them to ER-stress, we challenged stable ERLIN2-knockdown SUM-44 and SUM-225 cells with Tm and Tg for 72 hours and evaluated their viability using the MTT assay. Knockdown of ERLIN2 resulted in increased sensitivity to Tm or Tg -induced cell death (Figure 5c). Our data suggested that over expression of ERLIN2 may facilitate the adaptation of breast epithelial cells to ER stress by supporting cell growth. Future investigations are required to more precisely address the mechanism by which ERLIN2 promotes breast cancer cell survival.

Expression of ERLIN2 in breast tissues: Carcinomas and normal

We evaluated the expression of ERLIN2 in normal and cancerous human breast tissues using immunohisyochemistry (IHC) in breast cancer tissue arrays. We first confirmed the specificity and sensitivity of the ERLIN2 antibody for visualizing ERLIN2 expression in formalin-fixed, paraffin-embedded breast cancer cell lines. Consistent with the immunoblotting data, SUM-225 cells displayed significantly higher levels of positive staining as compared with the MCF10A control cells (Additional file 1: Figure S4). The tissue array included 34 breast

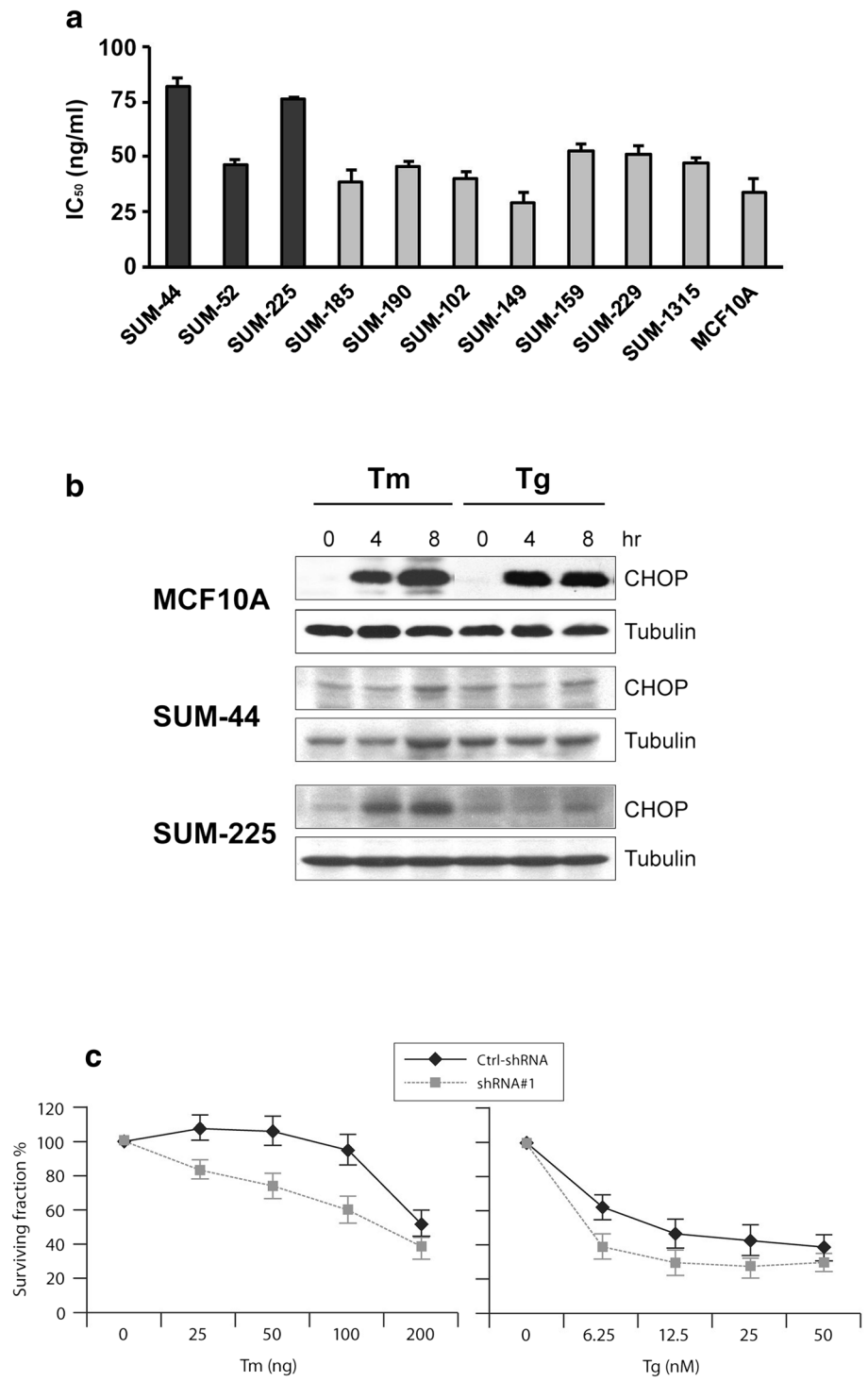

Figure 5 (a) IC_{50} values for the ER stress-inducing reagent Tm, in ten breast cancer cell lines as well as in the MCF10A cells (b) The expression level of CHOP in SUM-225, SUM-44 breast cancer cells and MCF10A control cells was analyzed with Western blot after Tm (500 ng) or Tg (400 nM) treatment. (**c**) Cell viability of ERLIN2 knockdown and control SUM-225 cells was measured with MTT assays after exposure to different concentrations of the Tm or Tg for 72 hours.

carcinomas and 17 normal breast tissue, which included 14 cases of adjacent normal counterparts. ERLIN2 expression was scored based on the staining intensity: 0 (negative), 1 + (weak), 2 + (low); 3+ (moderate) or 4 + (strong). In breast carcinomas samples, 11 (32.4%) stained ERLIN2 strongly and 13 (38.2%) moderately

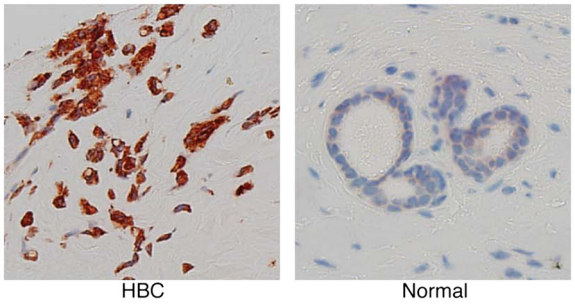

Figure 6 Immunohistotochemical staining of ERLIN2 protein on a representative HBC sample and a normal control.

(Figure 5, Additional file 1: Figure S5 Additional file 2: Table S2). In contrast, no strong or moderate staining was observed in the 17 normal breast tissues. The staining intensities of ERLIN2 were significantly higher in tumor cells than in normal tissue cells (P = 0.001).

Discussion

The 8p11-12 amplicon in HBC has been the subject of a number of studies using high-resolution genomic analysis of copy number and gene expression [3-6,42,43]. We previously found that the 8p11-12 amplicon has a highly complex genomic structure and that the size of the amplicon in three HBC lines, SUM-44, SUM-52 and SUM-225, is highly variable [6,31]. Moreover, the sub-amplicon of 8p11-12 that carries the *ERLIN2* gene amplification was more frequently identified in HBCs [4,7]. Previous studies have demonstrated that the 8p11-12 amplicon occurs mainly in the luminal subset of breast cancer cells, such as SUM-44 cells, a subset that also expresses the estrogen receptor [3,4,7,44-46]. Here we report that the co-amplification of the *ERLIN2* region occurred in a subset of *HER2*-amplified breast cancer cells, including SUM-225 cells. Our recent studies with Her2 model cells demonstrated that over expression of Her2 alone is not sufficient to induce full transformation *in vitro* and is not tumorigenic *in vivo* [47]. In contrast, *Her2*-amplified SUM225 breast cancer cells are fully transformed *in vitro* and tumorgic *in vivo* [48]. In this study, *in vitro* transforming and shRNA assays provided evidence that ERLIN2 is the most likely non- classical oncogene within this 8p11-12 minimal common amplified region. Our results suggest that the *ERLIN2* plays a role in cell proliferation and maintenance of transforming phenotypes in breast cancer cells with the 8p11-12 amplification.

ERLIN2 belongs to a larger family of proteins that share an evolutionarily conserved stomatin/prohibitin/flotillin/HflK/C (SPFH) domain. SPFH-containing proteins localize to different membranes, but have common characteristics. For example, N-terminal sequences are required for subcellular localization and membrane attachment, while the coiled-coil motifs located at the C-terminal side of SPFH domain mediate the assembly of high-molecular-weight complexes [49]. ERLIN2 and its homologue ERLIN1 were originally identified as components of lipid rafts that localize to the ER [36]. More recently, ERLIN2 has been recognized as a novel mediator of ERAD [34-36,50]. ERLIN2 binds to activated IP3Rs and other ERAD substrates, leading to polyubiquitination and subsequent degradation of these substrates [34,35].

Of particular interest in this study, we found that the UPR pathway modulated ERLIN2 protein expression in breast cancer cells through the IRE1α/XBP1 axis. Forced expression of IRE1α, or spliced XBP1, the target of IRE1α under ER stress, up-regulated expression of the ERLIN2 protein, while knockdown of IRE1α RNase activity decreased ERLIN2 expression in the *ERLIN2*-amplified breast cancer cells. These gain- and loss-of-function studies provided support that the IRE1α/XBP1-mediated UPR pathway in HBC regulated production of ERLIN2. Importantly, our study also showed that the depletion of nutrient and growth signals, a condition that is associated with oncogenesis and ER stress, can increase ERLIN2 production in breast epithelial cells. However, over expression of IRE1α or spliced XBP1 did not increase expression of the *ERLIN2* mRNA level, suggesting regulation at the post-transcription level. In the present study, we also showed that expression of primary breast cancer cells significantly up regulated ERLIN2 protein expression as compared with normal breast cells. As we had described earlier, amplification of the *ERLIN2* gene, as part of the 8p11-12 amplicon, occurs in approximately 15% of human breast cancer. It is reported that XBP1 is over expressed in aggressive breast cancer and associated with cancer cell survival and therapy resistance [51]. In the ten SUM breast cancer cell lines we investigated, three lines have both ERLIN2 gene amplification and up-regulation of activated XBP1, resulting in dramatically high-level expression of ERLIN2 protein. In contrast, two lines with up-regulation of the XBP1, but no ERLIN2 gene amplification, had moderately high-expression of the ERLIN2 protein. Taken together, our results raise an intriguing notion that the breast cancer cells may utilize gene amplification and the UPR pathway to regulate ERLIN2 protein over-production under oncogenic stress conditions.

In response to ER stress, cells activate UPR to reprogram gene transcription and translation. Depending on the type and/or degree of the stress, cells can differentially activate the UPR pathways in order to make survival or death decisions [52]. The literature indicates that the UPR branch, through IRE1α/XBP1, plays a critical role in cell adaptation to ER stress by increasing protein refolding and degradation of misfolded proteins, and by bolstering the protein-folding capacity and

secretion potential of the ER [20,52,53]. Cancer cells may adapt to the cellular stress and evade stress-induced apoptotic pathways by differentially activating the UPR branches. Indeed, tumor microenvironment has been characterized by a 'baseline' level of ER stress response that promotes tumor development and metastasis [20].

Conclusions

In the present study, we show that over expression of ERLIN2 may facilitate the adaptation of breast epithelial cells to ER stress by supporting cell growth and protecting the cells from ER stress-induced apoptosis. These results suggest that ERLIN2 confers a selective growth advantage for breast cancer cells by facilitating a cytoprotective response to various cellular stresses associated with oncogenesis. The information provided here sheds new light the mechanism of breast cancer malignancy.

Additional files

Additional file 1: Materials and Methods [54-58].

Additional file 2: Table S1. Expression Levels of XBP1, ERLIN1 and ERLIN2 in Ten SUM BreastCancer Cell Lines Using Our Affymetrix Array Database. Table S2: Expression of ERLIN2 in breast tissues:carcinomas and normal. Figure S1. Figure S2. Figure S3. Figure S4. Figure S5.

Abbreviations

Her2: v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian); c-MYC: v-myc myelocytomatosis viral oncogene homolog (avian); CCND1: cyclin D1; HBC: Human breast cancer; ER: Endoplasmic reticulum; ERLIN2: Endoplasmic reticulum lipid raft-associated 2; UPR: Unfolded protein response; IRE1: Inositol-requiring protein 1; XBP1: X-box binding protein 1; ERAD: ER-associated degradation; CGH: Comparative genomic hybridization; shRNA: Short hairpin RNA; IP3R: Inositol triphosphate receptors; Tm: Tunicamycin; Tg: Thapsigargin; CHOP: The CCAAT/enhancer-binding protein (C/EBP) homology protein; IHC: Immunohisyochemistry.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GHW, GL and XGW performed most of the experiments, participated in designing the study, analyzing the data. SS, RAF and ZZ were involved in IHC staining experiments. SE participated in design of the study. KZZ and ZQY conceived, coordinated, designed and procured funding for the study and wrote the manuscript. All authors gave final approval for the manuscript to be published.

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Endoplasmic reticulum factor ERLIN2 regulates cytosolic lipid content in cancer cells

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Increased *de novo* lipogenesis is a hallmark of aggressive cancers. Lipid droplets, the major form of cytosolic lipid storage, have been implicated in cancer cell proliferation and tumorigenesis. Recently, we identified the *ERLIN2* [ER (endoplasmic reticulum) lipid raft-associated 2) gene that is amplified and overexpressed in aggressive human breast cancer. Previous studies demonstrated that ERLIN2 plays a supporting oncogenic role by facilitating the transformation of human breast cancer cells. In the present study, we found that ERLIN2 supports cancer cell growth by regulating cytosolic lipid droplet production. ERLIN2 is preferably expressed in human breast cancer cells or hepatoma cells and is inducible by insulin signalling or when cells are cultured in lipoprotein-deficient medium. Increased expression of ERLIN2 promotes the accumulation of cytosolic lipid droplets in breast cancer cells or hepatoma cells in response to insulin or overload of unsaturated fatty acids. ERLIN2 regulates activation of SREBP (sterol regulatory element-binding protein) 1c, the key regulator of *de novo* lipogenesis, in cancer cells. ERLIN2 was found to bind to INSIG1 (insulin-induced gene 1), a key ER membrane protein that blocks SREBP activation. Consistent with the role of ERLIN2 in regulating cytosolic lipid content, down-regulation of ERLIN2 in breast cancer or hepatoma cells led to lower cell proliferation rates. The present study revealed a novel role for ERLIN2 in supporting cancer cell growth by promoting the activation of the key lipogenic regulator SREBP1c and the production of cytosolic lipid droplets. The identification of ERLIN2 as a regulator of cytosolic lipid content in cancer cells has important implications for understanding the molecular basis of tumorigenesis and the treatment of cancer.

Key words: cancer, endoplasmic reticulum (ER), ER membrane lipid raft-associated 2 (ERLIN2), lipogenesis, oncogenesis.

INTRODUCTION

Increased lipid and energy metabolism is a prominent feature of cancer [1]. It has been demonstrated that the lipogenic phenotype and activation of lipogenic enzymes correlate with a poorer prognosis and shorter disease-free survival for patients with different tumour types [2]. Production of lipid droplets, the main cytosolic lipid storage organelle in eukaryotic cells, is closely associated with *de novo* lipogenesis [3]. Indeed, elevated lipid droplet content is implicated in cancer cell proliferation and tumorigenesis and has been increasingly recognized as a hallmark of aggressive cancers [2,4].

ERLIN2 {ER (endoplasmic reticulum) membrane lipid raft-associated 2; also known as SPFH2 [SPFH (stomatin/prohibitin/flotillin/HflK/C) domain family, member 2] and C8ORF2 [chromosome 8 open reading frame 2]} and its homologue ERLIN1 belong to a larger family of proteins that share an evolutionarily conserved SPFH domain [5]. Recent high-resolution genomic analyses of copy number in human breast cancer specimens demonstrated that high-level amplification of the *ERLIN2* region occurs in 28 % of cases [6]. On the basis of statistical analysis of copy number increase and overexpression, we and others have identified the *ERLIN2* gene as one of several candidate oncogenes within the 8p11-12 amplicon [7–11]. Together with other identified oncogenes in the 8p11-12 amplicon, *ERLIN2* promotes transformation of human breast cancer cells, although it does not behave as a classical transforming oncogene, such as receptor tyrosine kinases and the small GTPase Ras [7,10–12]. Previous studies suggested that ERLIN2 might mediate ER-associated protein degradation [5,13,14]. ERLIN1 and ERLIN2 interact with each other to form a functional complex. ERLIN2 can bind to the activated inositol trisphosphate receptors and other ERAD (ER-associated degradation) substrates, leading to polyubiquitination and subsequent degradation of these substrates [13,14]. ERLIN2 can also interact with ER-resident proteins GP78 [AMFR (autocrine motility factor receptor)] and TMUB1 (transmembrane and ubiquitin-like domain containing 1) to mediate degradation of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase [15]. However, previous studies were focused on the biochemical characterization of ERLIN2 as a mediator of the ERAD pathway. The precise role and mechanism of ERLIN2 in aggressive cancer cells, where the *ERLIN2* gene is amplified and overexpressed, remain poorly understood.

In the present study, we found that ERLIN2 plays an important role in regulating cytosolic lipid content and activation of SREBP (sterol regulatory element-binding protein) 1c, a key lipogenic

Abbreviations used: AHF, atherogenic high-fat; BODIPY, boron dipyrromethene; CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylindole; DGAT, diacylglycerol *O*-acyltransferase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERLIN2, ER membrane lipid raft-associated 2; FAS, fatty acid synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; INSIG1, insulin-induced gene 1; IP, immunoprecipitation; LPDS, lipoprotein-deficient serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NP-40, Nonidet P40; PGC, peroxisome-proliferator-activated receptor γ coactivator; RT, reverse transcription; shRNA, small hairpin RNA; SPFH, stomatin/prohibitin/flotillin/HflK/C; SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; TG, triglyceride.

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regulator in human breast cancer cells and hepatoma cells. Furthermore, knockdown of endogenous ERLIN2 led to reduced cancer cell proliferation rates. Our finding that ERLIN2 regulates lipogenesis in cancer cells contributes to our understanding of the molecular basis governing lipid metabolism in tumorigenesis and could have important applications in cancer therapy.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma unless indicated otherwise. Synthetic oligonucleotides were purchased from Integrated DNA Technologies. Human insulin was purchased from Eli Lilly. Antibodies against FLAG, ERLIN1 and α-tubulin were purchased from Sigma. An antibody against ERLIN2 was purchased from Cell Signaling Technology. An antibody against SREBP1c was purchased from Thermo Scientific. ERLIN1 and ERLIN2 ON-TARGETplus siRNA SMARTpool® reagents were purchased from Dharmacon. Antibodies against T7, V5, INSIG1 (insulin-induced gene 1), SREBP1a and FAS (fatty acid synthase) were purchased from Santa Cruz Biotechnology. An antibody against SCAP (SREBP cleavage-activating protein) was purchased from Abcam. The monoclonal antibody against C-terminal SREBP2 was purchased from BD Pharmingen, and the polyclonal antibody against N-terminal SREBP2 was from Cayman Chemicals. The photo-reactive amino acids kit was from Thermo Scientific Pierce. The kit for measuring TGs (triglycerides) was from BioAssay System. The plasmid expressing T7-tagged human INSIG1 used in the present study was provided by Dr Jin Ye (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) [16].

Cancer cell culture, lentivirus construction and transduction of cells

The human breast cancer cell lines SUM44 and SUM225, and the human mammary epithelial cell line MCF10A were cultured as described previously [12,17,18]. The human breast cancer cell line ZR-75-1 was purchased from the A.T.C.C. The human hepatocellular carcinoma cell line Huh-7 was cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % FBS (fetal bovine serum), L-glutamine and antibiotics at 37 °C in a 5 % CO_2 environment [19]. The details regarding the origins and culture conditions of these cancer cell lines are described in the Supplementary Online data (at http://www.BiochemJ.org/bj/446/bj4460415add.htm). The lentiviral expression construct containing human *ERLIN2* (pLenti-ERLIN2) was established as described previously [7]. The lentivirus for pLenti-ERLIN2 was used to infect MCF10A or Huh-7 cells. Control infections with pLenti-LacZ virus were performed in parallel with the pLenti-ERLIN2 infections. Selection began 48 h after infection in growth medium with 10 μg/ml blasticidin. Upon confluence, selected cells were passaged and serially cultured.

Lentivirus-mediated shRNA (small hairpin RNA) knockdown of gene expression

We knocked down the expression of the human *ERLIN2* gene in human breast cancer cell lines SUM225 and SUM44 or human hepatoma cell line Huh-7 by using the Expression Arrest GIPZ lentiviral shRNAmir system (OpenBiosystems). Lentivirus was produced by transfecting HEK (human embryonic kidney)-293FT cells with a combination of the lentiviral expression plasmid DNA and the Trans-Lentiviral packaging mix (OpenBiosystems). For cell infection, viral supernatants were supplemented with 6 μg/ml polybrene and incubated with the cells for 24 h. The cells expressing shRNA were selected with puromycin for 2–3 weeks for functional studies (cell proliferation assays) and for 4–10 days after infection for RNA extraction.

BODIPY (boron dipyrromethene) staining of lipid droplets

The cells were washed with PBS, fixed with 3 % formaldehyde for 15 min and stained with BODIPY 493/503 (stock 1 mg/ml, working solution 1:1000 dilution; Invitrogen) for 15 min at room temperature (25°C). Cells were then mounted with Prolong gold antifade reagent containing DAPI (4′,6-diamidino-2-phenylindole; Invitrogen).

Oil Red O staining of lipid droplets

Frozen liver tissue sections were stained with Oil Red O according to the standard protocol to visualize lipid droplet content. Briefly, frozen liver tissue sections of 8 μm were air-dried and then fixed in formalin. The fixed sections were rinsed with 60 % propan-2-ol before they were stained with freshly prepared Oil Red O solution for 15 min. After Oil Red O staining, the liver sections were rinsed again with 60 % propan-2-ol followed by washing with water.

Incorporation of photo-reactive amino acids and UV cross-linking to analyse the protein binding complex

Cells at 60–70 % confluence were washed twice with PBS and cultivated with DMEM limiting medium (without L-leucine and L-methionine) containing 2 mM photo-reactive leucine and 4 mM photo-reactive methionine analogues supplemented with 10 % dialysed FBS for 24 h. After washing twice with PBS, cells were UV-irradiated using a Stratalinker 1800 (UVA output at 1 cm = 3000 μW/cm^2) for 12 min. The cell lysates were then collected in NP-40 (Nonidet P40) lysis buffer for IP (immunoprecipitation) Western blot analysis. The photo-reactive amino acids kit, DMEM limiting medium and dialysed FBS were purchased from Thermo Scientific Pierce.

Cell proliferation assay

Cell proliferation rates were determined using CellTiter 96 non-radioactive cell proliferation assay {MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide]} kits from Promega. Briefly, approximately 5000 cells per well were seeded in triplicate in 96-well culture plates and allowed to attach for 24 h. After cell culture for 3, 6, 9 and 12 days, 20 μl of MTT (5 mg/ml) solution was added to 200 μl of medium in each well. Cells were cultured for an additional 4 h to allow MTT to be well metabolized. After that, the medium was aspirated, and 200 μl of DMSO was added into the well to dissolve the purple formazan crystals. The absorbance of the plate was measured at 570 nm using a plate reader.

Western blot and IP Western blot analyses

To determine expression levels of ERLIN1, ERLIN2, SREBPs, INSIG1, α-tubulin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), total cell lysates were prepared from cultured cells or liver tissue by using NP-40 lysis as described previously [20]. Denatured proteins were separated by SDS/PAGE (10 %

Tris-Glycine polyacrylamide gels) and transferred on to a 0.45-mm PVDF membrane (GE Healthcare). Membrane-bound antibodies were detected by an enhanced chemiluminescence detection reagent (GE Healthcare). For IP Western blot analysis, total protein lysates from *in vitro* cultured cells were immunoprecipitated with the anti-ERLIN1, anti-ERLIN2 or anti-V5 antibody, followed by Western blot analysis with the anti-ERLIN1, anti-SCAP or anti-T7 antibody.

Quantitative real-time RT (reverse transcription)–PCR analysis

For real-time RT–PCR analysis, total cellular RNA was prepared using TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA using a random primer. The real-time PCR reaction mixture containing cDNA template, primers and SYBR Green PCR Master Mix (Invitrogen) was run in a 7500 Fast Real-time PCR System (Applied Biosystems). The sequences of the PCR primers used in the present study are provided in the Supplementary Table S1 (at http://www.BiochemJ.org/bj/446/bj4460415add.htm). Fold changes of mRNA levels were determined after normalization to internal control β-actin RNA levels.

Statistics

Experimental results (quantitative real-time RT–PCR analysis, MTT assay and quantitative Western blot analysis) were calculated as means $\pm$ S.E.M. (for variation between animals or experiments). The differences among means between multiple (more than two) groups were analysed by one-way ANOVA followed by post-hoc comparisons of group means with the Tukey–Kramer test. The mean values for biochemical data from two experimental groups were compared by a paired or unpaired two-tailed Student's *t* test. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

ERLIN2 is overexpressed in human cancer cells and inducible by metabolic stress signals

Previously we and others demonstrated that the *ERLIN2* gene was highly amplified in human breast cancer cell lines and breast tumours of aggressive forms [7–9]. Western blot analysis indicated that ERLIN2 was overexpressed in the aggressive human breast cancer cell lines SUM225, ZR-75-1, SUM44 and SUM52, and it was modestly expressed in the non-transformed human mammary epithelial cell line MCF10A (Figure 1A, [7]). Moreover, ERLIN2 was expressed in human hepatoma cell lines, including HepG2 and Huh-7, but only slightly expressed in murine primary hepatocytes (Figure 1A). Evidence suggests that elevated lipogenesis is essential for tumour cell survival and malignancy maintenance [2,4]. To elucidate the involvement of ERLIN2 in the lipogenic phenotype of human cancer cells, we exposed murine primary hepatocytes to metabolic stress conditions that are associated with lipogenesis and/or cancer cell growth. First, we found that expression of endogenous ERLIN2 in murine primary hepatocytes was inducible by insulin, a metabolic signal that induces activation of SREBP1c and *de novo* lipogenesis in the liver (Figure 1B) [21]. Secondly, expression of endogenous ERLIN2 was higher in primary hepatocytes cultured in medium containing LPDS (lipoprotein-deficient serum), a metabolic stress condition that triggers SREBP activation and *de novo* lipogenesis, in a time-dependent manner (Figure 1C). To further elucidate the effect of the insulin signal or LPDS on the induction of ERLIN2, we examined the induction of *ERLIN2* mRNA in murine primary hepatocytes in response to insulin or LPDS challenge. Quantitative real-time RT–PCR analysis indicated that expression of *ERLIN2* mRNA in primary hepatocytes is inducible by insulin or LPDS (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). However, there is a discrepancy in expression patterns between the ERLIN2 protein and mRNA levels. The discrepancy between protein and mRNA levels, which may be due to post-transcriptional regulation, has been observed with many genes involved in lipid metabolism [21–24].

We also evaluated the expression of ERLIN2 in the liver of mice receiving normal chow or an AHF (atherogenic high-fat) diet known to induce atherosclerosis and fatty liver disease in murine models [25,26]. Expression levels of the ERLIN2 protein in the steatoic livers of the mice fed the AHF diet were much higher than those in mice fed the normal diet (Figure 1D). Quantitative real-time RT–PCR analysis confirmed that expression of *ERLIN2* mRNA was significantly higher in the livers of the mice fed the AHF diet compared with the mice fed normal chow (Figure 1E). Together, the results suggest that expression of ERLIN2 is up-regulated in cancer cells and inducible by insulin or LPDS challenge. These findings motivated us to investigate the involvement of ERLIN2 in lipid metabolism associated with tumorigenesis.

ERLIN2 is required for cytosolic lipid droplet accumulation in cancer cells after insulin stimulation or overload of oleic acid

To assess the potential involvement of ERLIN2 in lipid metabolism in cancer cells, we analysed cytosolic lipid content in human mammary epithelial cells, breast cancer cells or hepatoma cells in which exogenous ERLIN2 was overexpressed or endogenous ERLIN2 was knocked down. Using a lentiviral expression system, we established a human mammary epithelial cell line (MCF10A) that stably expresses ERLIN2 or control LacZ (Figure 2A). Cytosolic lipid droplets, as indicated by BODIPY staining, accumulated in the MCF10A cells overexpressing exogenous ERLIN2, but not the LacZ control (Figure 2B). The human breast cancer cell line SUM225, in which the *ERLIN2* gene was amplified and overexpressed, possesses abundant cytosolic lipid droplet contents (Figures 2C and 2D). However, when the endogenous *ERLIN2* gene was knocked down, the levels of lipid droplet contents in the ERLIN2-knockdown SUM225 cells were significantly reduced. The reduction of lipid droplet contents in the absence of ERLIN2 was consistent with cellular TG levels in the ERLIN2-knockdown breast cancer cells (Supplementary Figure S2 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). Together, these results suggest a potential role for ERLIN2 in promoting lipid droplet production in human breast cancer cells.

To verify the role of ERLIN2 in cytosolic lipid accumulation, we used the human hepatocellular carcinoma cell line Huh-7 [19], which has been used to study tumorigenesis or hepatic lipid metabolism. The *ERLIN2* gene was stably silenced in Huh-7 cells by using a lentivirus-based shRNA system. The result of Western blot analysis indicated that expression levels of ERLIN2 protein were markedly reduced in the ERLIN2-knockdown Huh-7 cells, compared with the control cells transduced by a non-silencing shRNA control (Figure 3A). The ERLIN2-knockdown Huh-7 cells had much lower levels of lipid droplet content, indicated by Oil Red O staining, than the control cells (Figure 3B). Overexpression of ERLIN2 significantly increased lipid droplet

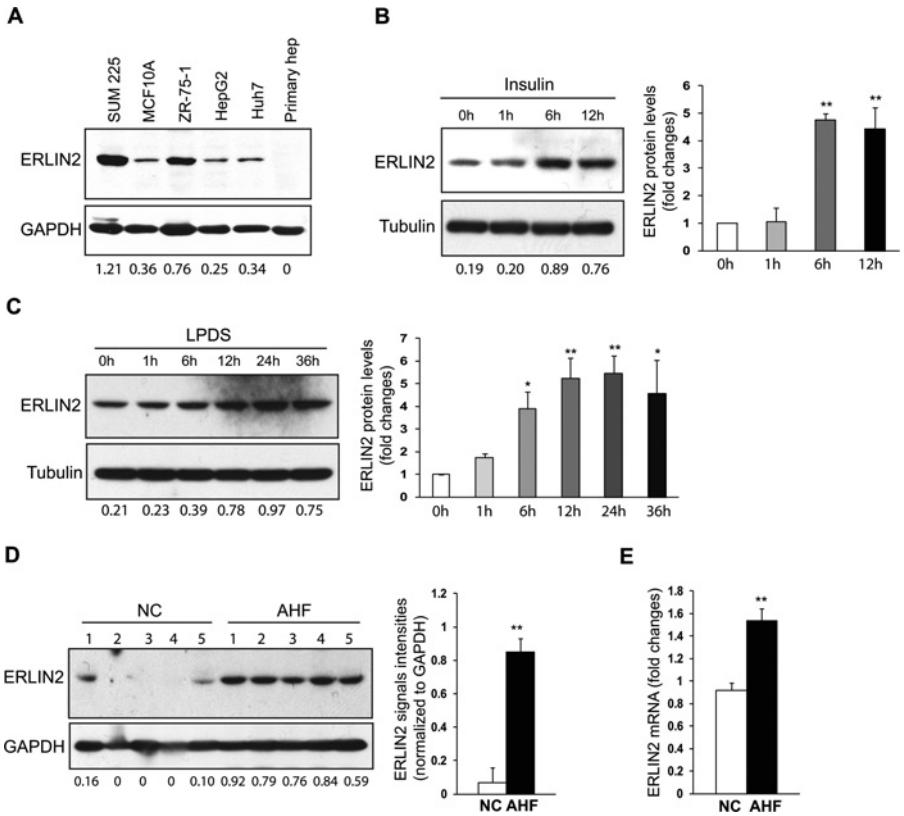

Figure 1 ERLIN2 is preferentially expressed in cancer cells and is inducible by metabolic signals

(**A**) Western blot analysis of ERLIN2 protein levels in the human breast cancer cell lines SUM225 and ZR-75-1, non-tumorigenic mammary epithelial cell line MCF10A, human hepatoma cell lines HepG2 and Huh-7, and murine primary hepatocytes (hep). Levels of GAPDH were included as loading controls. The values below the gels indicate ERLIN2 protein signal intensities [quantified using ImageJ (http://rsbweb.nih.gov/ij/)] after normalization to GAPDH signal intensities. (**B**) Western blot analysis of ERLIN2 protein levels in murine primary hepatocytes challenged with insulin (100 nM) for 1, 6 or 12 h. Murine primary hepatocytes were cultured in normal medium with vehicle buffer PBS added as a control (0 h under insulin). Tubulin was included as a loading control. The values below the gels indicate the ERLIN2 protein signal intensities after normalization to tubulin signal intensities. The graph beside the images shows fold changes of normalized ERLIN2 protein signal intensities (compared with 0 h control). Results are means ± S.E.M. ($n = 3$ experimental repeats); $^{**}P < 0.01$. (**C**) Western blot analysis of ERLIN2 protein levels in murine primary hepatocytes cultured in medium containing LPDS for 1, 6, 12, 24 or 36 h. As a control, murine primary hepatocytes were cultured in normal medium containing 10 % FBS (0 h under LPDS). Tubulin was included as a loading control. The values below the gels indicate the ERLIN2 protein signal intensities after normalization to tubulin signal intensities. The graph beside the images shows fold changes of normalized ERLIN2 protein signal intensities (compared with 0 h control). Results are means ± S.E.M. ($n = 3$ experimental repeats); $^{*}P < 0.05$; $^{**}P < 0.01$. (**D**) Western blot analysis of ERLIN2 protein levels in liver tissues of mice given normal chow (NC) or an AHF diet for 6 months. GAPDH was included as a loading control. The values below the gels indicate the ERLIN2 protein signal intensities after normalization to GAPDH signal. The graph beside the images shows ERLIN2 protein signal intensities in the livers of mice fed with normal chow or AHF diet after normalization to GAPDH. Results are means ± S.E.M. ($n = 5$); $^{**}P < 0.01$. For (**A**–**D**), the experiments were repeated three times and representative data are shown. (**E**) Quantitative real-time RT–PCR analysis of expression of *ERLIN2* mRNA in the liver of age-matched male mice given normal chow (NC) or the AHF diet for 6 months. Expression values were normalized to β-actin mRNA levels. The baseline of the *ERLIN2* mRNA level in one of the NC-fed mice was set to 1. Fold changes of the *ERLIN2* mRNA levels in other mice were calculated by comparing with the baseline mRNA level. Results are means ± S.E.M. ($n = 6$ mice per group). $^{**}P < 0.01$.

accumulation in Huh-7 cells, consistent with the observation that ERLIN2 overexpression increased lipid droplet production in MCF10A cells (Figure 2B). It is known that insulin can trigger *de novo* lipogenesis in hepatocytes, whereas overload of oleic acid, a monounsaturated fatty acid, can lead to cytosolic lipid droplet accumulation and steatosis in cultured hepatoma cells [27,28]. To further delineate the effect of ERLIN2 on cytosolic lipid droplet production, we challenged non-silencing control, ERLIN2-knockdown and ERLIN2-overexpressing Huh-7 cells with insulin or oleic acid. The ERLIN2-knockdown Huh-7 cells exhibited significantly less lipid droplet accumulation, whereas the ERLIN2-overexpressing Huh-7 cells displayed markedly greater hepatic steatosis compared with control Huh-7 cells, in response to insulin stimulation or oleic acid feeding (Figure 3). The lipid droplet staining results were consistent with the biochemical quantification of cellular TG levels in the related Huh-7 cells (Supplementary Figure S2). Together, these findings support the role of ERLIN2 in *de novo* lipogenesis and lipid droplet production.

ERLIN2 is associated with the activation of SREBPs in cancer cells

The ER is the organelle responsible for lipid and sterol biosynthesis. SREBP1 and SREBP2 are ER transmembrane proteins that play central roles in controlling expression of genes encoding key regulators and enzymes in *de novo* lipogenesis [21]. Among others, SREBP1c is inducible in liver and adipose tissue by insulin change or fasting/refeeding conditions, and it plays a critical role in nutritional regulation of lipogenic gene expression [21]. Induction of SREBP1c, but not SREBP1a or SREBP2, is evidenced in numerous primary human breast tumours and breast cancer cell lines [29]. Previous studies indicated that fatty acid synthesis and expression of lipogenic genes in breast

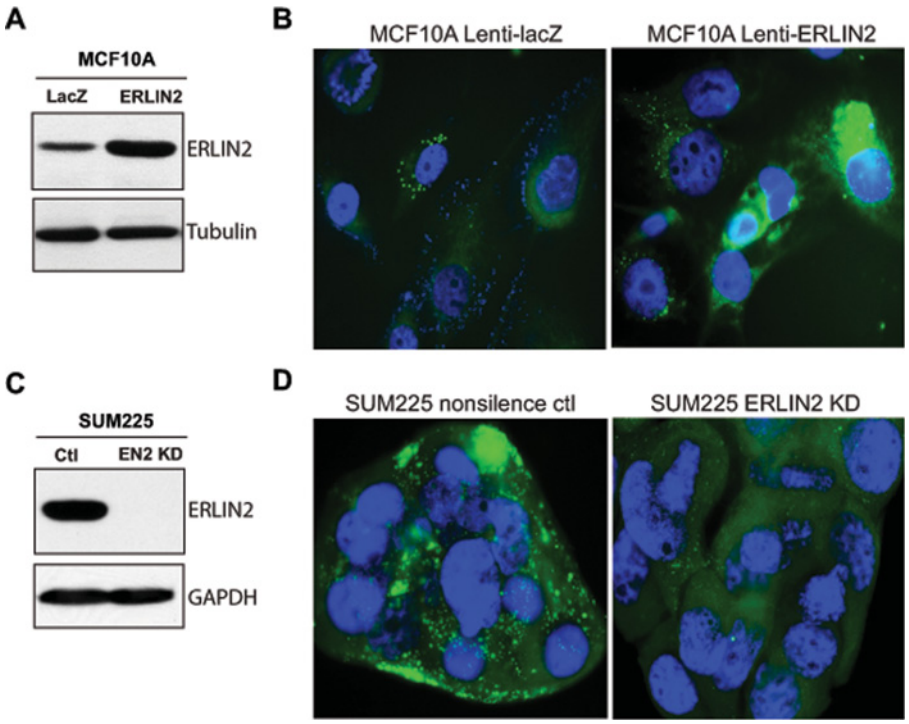

Figure 2 ERLIN2 regulates levels of cytosolic lipid content in human breast cancer cells

(**A**) Western blot analysis of ERLIN2 levels in MCF10A cells stably overexpressing LacZ or ERLIN2 through a lentivirus-based expression system. Tubulin was used as a loading control. (**B**) BODIPY staining of lipid droplets in MCF10A cells stably overexpressing LacZ or ERLIN2. The cells were counterstained with DAPI. Magnification is ×630. (**C**) Western blot analysis of ERLIN2 levels in human breast cancer cell line SUM225, in which ERLIN2 is stably knocked down (EN2 KD) and its control cell line (Ctl). GAPDH was used as a loading control. (**D**) BODIPY staining of lipid droplets in the ERLIN2 knockdown (KD) and control (ctl) SUM225 cells. Magnification is ×630. For (**A**–**D**) the experiments were repeated three times and representative data are shown.

cancer is achieved through modulation of SREBP1c, similar to its regulation in liver and adipose tissue, although the upstream regulation of lipogenesis differs in these tissues [21,29].

It has been shown that activation of SREBP is controlled by interactions involving ER-resident proteins that are regulated by metabolic signals [21]. In particular, the SREBP precursor proteins interact with ER membrane SCAP, and SCAP binds to another ER membrane protein called INSIG1 to maintain SREBPs in an inactive state [30]. In response to low sterol or insulin stimuli, INSIG1 dissociates from SCAP and is subsequently degraded through ERAD, thus allowing SREBP activation [30–32]. Because ERLIN2 is an ER lipid raft protein and has been characterized as a mediator of ERAD [13,14], we suspected that ERLIN2 might regulate lipogenesis by modulating the activation of SREBPs and/or ER-associated degradation of INSIG1. To explore this possibility, we first examined activation of SREBP1c in a mammary epithelial cell line (MCF10A) that overexpresses exogenous ERLIN2 or LacZ control. Levels of the cleaved/activated form of SREBP1c were significantly higher in the MCF10A cells overexpressing ERLIN2, compared with that of the cells overexpressing LacZ (Figure 4A). We further evaluated activation of SREBP1c in SUM44, an aggressive human breast cancer cell line in which the endogenous *ERLIN2* gene is amplified and overexpressed [7] (Figure 2C). We generated SUM44 stable cell lines in which ERLIN2 was knocked down through a lentivirus-based shRNA expression system. Supporting a role for ERLIN2 in regulating SREBP activation, the levels of total cleaved SREBP1c protein were lower in the ERLIN2 knockdown SUM44 cells, compared with that in the control cells (Figure 4B). Interestingly, SREBP cleavage products in SUM44 cells appeared as multiple isoforms that might represent phosphorylated or SUMOylated forms of mature SREBP under different metabolic conditions [33,34]. Moreover, the levels of the SREBP1c precursor were also modestly lower in the ERLIN2-knockdown SUM44 cells (Figure 4B). Additionally, we examined levels of SREBP1a in the ERLIN2-knockdown or control breast cancer cells. Consistent with the previous observation that SREBP1c, but not SREBP1a or SREBP2, is induced in human breast tumours and breast cancer cell lines [29], the ERLIN2 knockdown or overexpressing breast cancer cell lines express only trace levels of SREBP1a (Supplementary Figure S3 at http://www.BiochemJ.org/bj/446/bj4460415add.htm).

Next, we confirmed the involvement of ERLIN2 in SREBP activation by using the hepatoma cell line Huh-7. In liver hepatocytes, activation of SREBP1c is tightly controlled by feedback regulation [21]. To circumvent the potential adaptation of SREBP activation in stable ERLIN2 knockdown Huh-7 cells, we transiently knocked down ERLIN2 and/or its functional binding partner, ERLIN1, in Huh-7 cells by using ON-TARGETplus siRNA SMARTpool® reagents [35]. Transient knockdown of ERLIN2 and/or ERLIN1 significantly reduced the levels of mature SREBP1c proteins in the Huh-7 cells in the absence or presence of insulin (Figure 4C). Moreover, levels of cleaved SREBP2 proteins were also reduced in the ERLIN2 and/or ERLIN1 knockdown Huh-7 cells, compared with those in control Huh-7 cells (Figure 4C and Supplementary Figure S4 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). These results suggest that ERLIN2 plays a role in regulating SREBP activation. Note that the results obtained with ERLIN1-knockdown cells suggested that ERLIN1 may also be involved in regulation of SREBP activation. Because ERLIN2 is known to

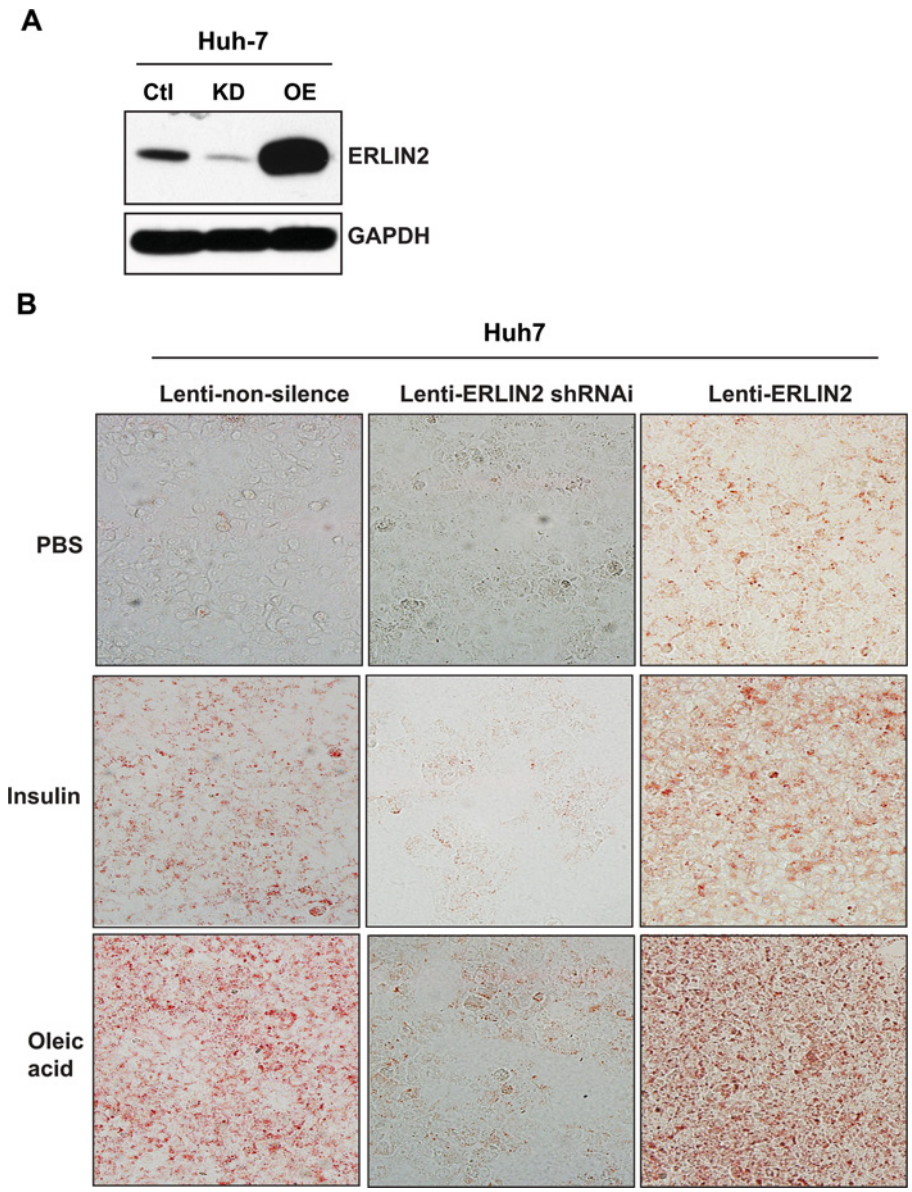

Figure 3 ERLIN2 regulates levels of cytosolic lipid content in human hepatoma cells in the absence or presence of metabolic signals

(**A**) Western blot analysis of ERLIN2 levels in the Huh-7 stable cell line, which was transduced with non-silencing control shRNA (Ctl), knocked down by ERLIN2 shRNAi (KD) or overexpressed ERLIN2 (OE) via lentivirus. GAPDH was included as a loading control. (**B**) Oil Red O staining of lipid droplets in the Huh-7 stable cell line, which was transduced with non-silencing control (lenti-non-silence), ERLIN2 shRNAi (lenti-ERLIN2 shRNAi) or ERLIN2 overexpression (lenti-ERLIN2) lentivirus and treated with vehicle PBS, insulin (100 nM) or oleic acid (0.5 mM) for 12 h. Magnification is ×200. The experiments were repeated three times and representative data are shown.

dimerize with ERLIN1 to form a functional complex [5,13,14], it is possible that knockdown of ERLIN1 may destabilize ERLIN2 and thus reduce SREBP activation in cancer cells. The involvement of ERLIN1 in regulating SREBP activation needs to be further elucidated in the future. Since ERLIN2 has been proposed as a mediator of ERAD [13,14], we wondered whether ERLIN2 regulates SREBP activation by facilitating INSIG1 degradation through the ERAD mechanism. The levels of INSIG1 proteins were not significantly changed in the ERLIN1- and/or ERLIN2-knockdown cells compared with the control cells (Figure 4C). Together, our data suggest that, although expression of ERLIN2 has a marginal effect on INSIG1 degradation, ERLIN2 regulates SREBP activation in cancer cells.

To further elucidate the role of ERLIN2 in *de novo* lipogenesis, we used ERLIN2 knockdown and control SUM44 or Huh-7 cells to examine the expression of genes that encode key lipogenic enzymes or regulators. Quantitative real-time RT–PCR analysis indicated that expression levels of SREBP1-regulated lipogenic genes, including *ACC1* (acetyl-CoA carboxylase 1) and *SCD1* (stearoyl-CoA desaturase 1) and other key lipogenic genes, including *DGAT* (diacylglycerol *O*-acyltransferase) 1, *DGAT2*, *ADRP* (adipose differentiation-related protein), *FIT1* (fat-inducing transcript 1), *FATP2* (fatty acid transport protein 2) and *FSP27* (fat-specific protein 27), were lower in ERLIN2 knock-down SUM44 and Huh-7 cells (Supplementary Figures S5A–S5C at http://www.BiochemJ.org/bj/446/bj4460415add.htm). We also

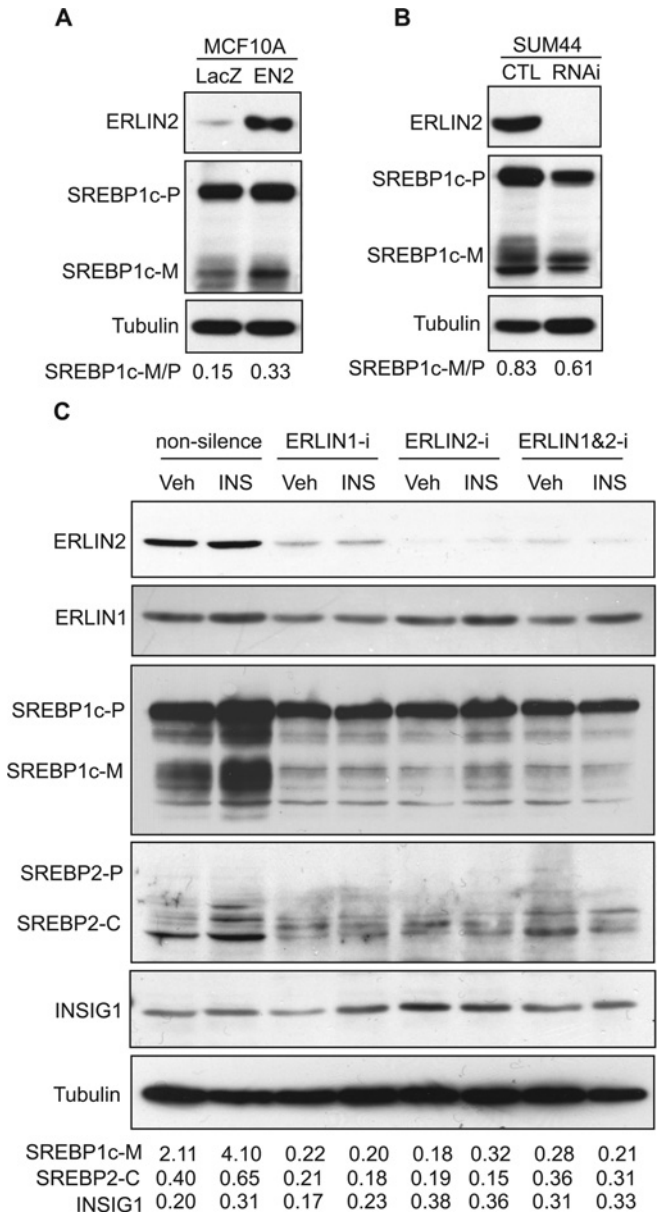

Figure 4 ERLIN2 regulates activation of SREBP1c

(**A**) Western blot analysis of ERLIN2 and SREBP1c levels in MCF10A cells that stably express exogenous ERLIN2 or LacZ control. Tubulin was included as a loading control. The values below the gels represent the ratios of signal intensities of mature SREBP1c to SREBP1c precursor. (**B**) Western blot analysis of ERLIN2, SREBP1c and tubulin levels in stable ERLIN2 knockdown SUM44 cells and control (CTL) SUM44 cells that were transduced by non-silencing shRNA. The values below the gels represent the ratios of signal intensities of mature SREBP1c to SREBP1c precursor. (**C**) Western blot analysis of ERLIN1, ERLIN2, SREBP1c, SREBP2 and INSIG1 protein levels in Huh-7 cells in which ERLIN1 and/or ERLIN2 were transiently knocked down. The *ERLIN1* and/or *ERLIN2* genes were transiently knocked down in Huh-7 cells by using ON-TARGETplus siRNA SMARTpool® reagents (Dharmacon). The Huh-7 cells transduced with non-silencing siRNA were included as the control. After 36 h, the control and knockdown cell lines were treated with PBS vehicle or insulin (100 nM) for 6 h followed by a collection of total cell lysates for Western blot analysis. The SREBP2 signal was detected by using a monoclonal antibody against a C-terminal SREBP protein fragment (BD Pharmingen). The values below the gels represent the ratios of mature SREBP1c to SREBP1c precursor, cleaved SREBP2 to tubulin, and INSIG1 to tubulin signal intensities. INS, insulin; RNAi, RNA interference; SREBP2-C, cleaved SREBP2 (C-terminal); SREBP1c-M, mature SREBP1c; SREBP1c-P, SREBP1c precursor; Veh, vehicle.

examined expression of lipogenic *trans*-activators in ERLIN2 knockdown and control Huh-7 cells. Expression levels of the genes encoding the lipogenic *trans*-activators *PGC* [PPARγ (peroxisome-proliferator-activated receptor γ) coactivator]-1α and *PGC-1*β were significantly lower in ERLIN2 knockdown Huh-7 cells (Supplementary Figure 5D). Additionally, we examined expression of FAS, a key SREBP1-regulated enzyme in *de novo* lipogenesis. Expression levels of *FAS* mRNA were only marginally altered by the absence of ERLIN2 (results not shown), whereas FAS protein levels were reduced in ERLIN2 knockdown Huh-7 cells (Supplementary Figure S6 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). The discrepancy in the expression patterns between the *FAS* mRNA and protein levels might be due to feedback regulation of the genes involved in lipid homoeostasis [21,23,24].

ERLIN2 interacts with SCAP and INSIG1

To gain further insight into the molecular basis by which ERLIN2 regulates SREBP activation, we tested whether ERLIN2 can interact with ER-resident protein factors that control SREBP activation. Through IP Western blot analysis, we first confirmed the strong interaction between ERLIN1 and ERLIN2 in Huh-7 cells (Figure 5A). We then examined potential interactions between ERLIN2 and the protein factors that regulate SREBP activation in the ER, particularly SCAP and INSIG1. Because ERLIN2, SCAP and INSIG1 are ER-resident proteins, we performed IP Western blot analyses with ER protein fractions to detect the interaction between ERLIN2, SCAP and INSIG1. IP Western blot analysis indicated that only a small portion of endogenous SCAP proteins associated with ERLIN2 in CHO (Chinese hamster ovary) cells exogenously expressing ERLIN2 and INSIG1 in the presence of insulin or LPDS challenge (Figure 5B). Moreover, we failed to detect ERLIN2 protein associated with SCAP in protein lysates pulled down by an anti-SCAP antibody (results not shown). Therefore the present study excludes the possibility of any strong or direct interaction between ERLIN2 and SCAP. Next, we evaluated the interaction between ERLIN2 and INSIG1. Because of a limitation of the anti-INSIG1 antibody in IP analysis, we expressed T7-tagged INSIG1 and V5-tagged ERLIN2 in CHO cells for IP Western blot analysis. In the absence of challenges, we detected only a nominal interaction between ERLIN2 and INSIG1 (Figure 5C). However, significant amounts of INSIG1 proteins associated with ERLIN2 were detected in the cells challenged by insulin or LPDS (Figure 5C). These results suggest a strong interaction between ERLIN2 and INSIG1 after insulin or LPDS challenge, the metabolic condition that triggers SREBP activation and *de novo* lipogenesis [21]. To further delineate the interaction between ERLIN2 and INSIG1, we endogenously incorporated photo-reactive amino acid analogues into the primary sequence of proteins during synthesis and then UV activated them to covalently cross-link proteins within protein–protein interaction domains in their native environment [36–38]. This powerful method enabled us to detect the intact protein interaction complex within live cells without the use of completely foreign chemicals or molecular modifiers that might adversely affect the interaction being studied [37]. Utilizing a photo-reactive amino acids kit, we incorporated photo-reactive leucine and methionine analogues into the CHO cells expressing both ERLIN2 and INSIG1. IP Western blot analysis with photo-reactive amino acid-incorporated UV cross-linked protein lysates revealed a significant amount of ERLIN2–INSIG1 binding complex formed in the CHO cells after insulin or LPDS challenge (Figure 5D).

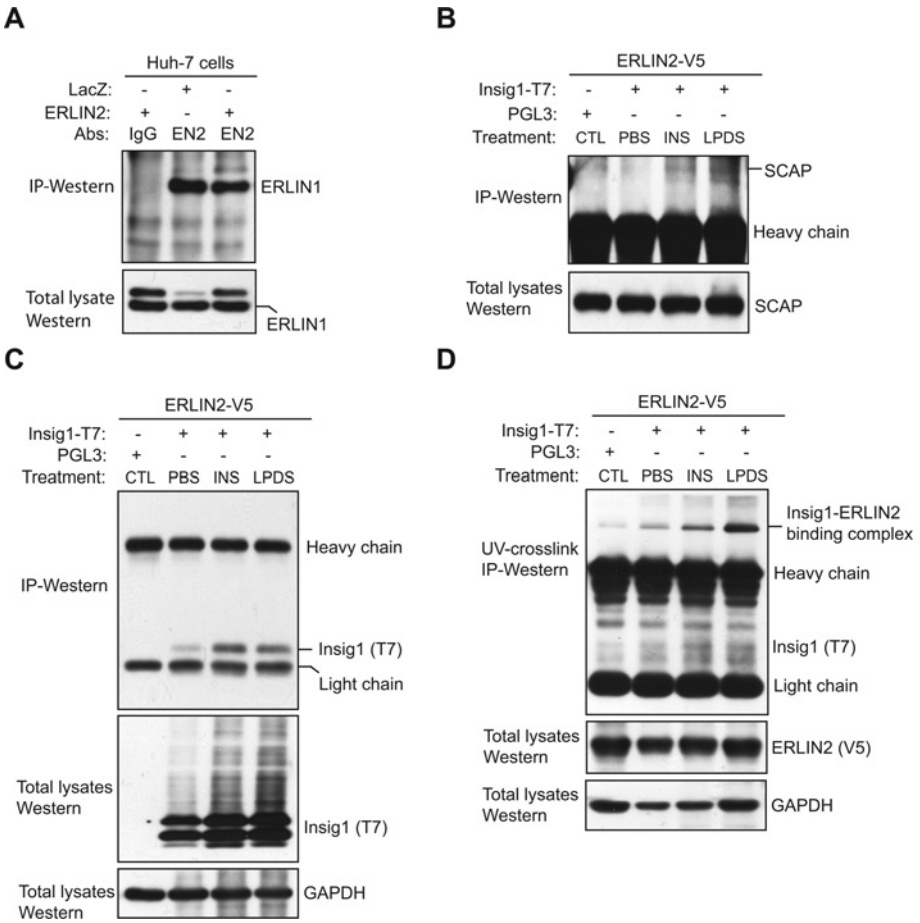

Figure 5 ERLIN2 interacts with SCAP and INSIG1

(**A**) IP Western blot analysis of the interaction between ERLIN1 and ERLIN2 in Huh-7 cells. T cell lysates from Huh-7 cells expressing exogenous ERLIN2 or LacZ were immunoprecipitated with an anti-ERLIN2 antibody (EN2) or rabbit IgG (negative control). The pulled-down proteins were subjected to immunoblotting analysis using an anti-ERLIN1 antibody. The levels of ERLIN1 in total cell lysates were included as input controls. (**B**) IP Western blot analysis of the interaction between ERLIN2 and SCAP. CHO cells stably expressing exogenous ERLIN2 protein with a V5 tag were transfected with a plasmid vector expressing INSIG1 protein with a T7 tag. The transfected CHO cells were treated with PBS vehicle, insulin (INS; 100 nM) for 6 h or were cultured in LPDS medium for 12 h. As a control (CTL), CHO cells stably expressing exogenous ERLIN2 protein were transfected with a plasmid vector control PGL3 and cultured in normal medium without insulin or LPDS challenge. ER protein fractions isolated from the CHO cells were immunoprecipitated with an anti-V5 antibody and then subjected to immunoblotting analysis using an anti-SCAP antibody to detect the interaction between ERLIN2 and SCAP. The levels of SCAP in total cell lysates were determined as input controls. (**C**) IP Western blot analysis of the interaction between ERLIN2 and INSIG1. The CHO cells, the plasmid transfection procedure, and the treatments are the same as described for (**B**). The ER protein fractions isolated from the CHO cells were immunoprecipitated with an anti-V5 antibody and then subjected to immunoblotting analysis using an anti-T7 antibody to detect the interaction between ERLIN2 and INSIG1. The levels of T7-tagged INSIG1 in total cell lysates were determined as input controls. (**D**) IP Western blot analysis of ERLIN2–INSIG1 binding complex by using a photo-reactive amino acid incorporation approach. The CHO cells, the plasmid transfection procedure, and the treatments are the same as described in (**B**), except that the cells were cultivated with DMEM limiting medium containing photo-reactive leucine and methionine analogues for 24 h before UV cross-linking and cell lysate collection. The photo-reactive amino acids incorporated UV cross-linked protein lysates were immunoprecipitated with an anti-V5 antibody and then subjected to immunoblotting analysis using an anti-T7 antibody to detect the ERLIN2–INSIG1 binding complex. The levels of ERLIN2 in total cell lysates were determined as input controls.

To verify whether ERLIN2 is involved in ER-associated degradation of INSIG1, we determined the levels of INSIG1 in Huh-7 cells overexpressing ERLIN1 or ERLIN2 after insulin or LPDS challenge. Consistent with our previous observation using transient ERLIN2-knockdown cells (Figure 4C), the levels of INSIG1 were marginally lower in the Huh-7 cells overexpressing ERLIN2, compared with those in cells overexpressing LacZ or ERLIN1 (Supplementary Figure S7 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). Therefore ERLIN2 is not likely to play a significant role in mediating INSIG1 degradation. The interaction between ERLIN2 and INSIG1, and loosely with SCAP, might be required for efficient SREBP activation by facilitating the dissociation of the SREBP–SCAP complex from INSIG1 in cancer cells (Supplementary Figure S8 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). This hypothesis needs to be further investigated in future studies.

Knockdown of ERLIN2 leads to reduced proliferation rates in cancer cells

Cancer cells, especially aggressive forms, have a high demand for lipid supplies for unlimited cell proliferation. The SREBP activities and *de novo* lipogenesis are functionally relevant to the cell proliferation rate [39]. Having established the role of ERLIN2 in regulating SREBP activation and lipid droplet production, we determined whether down-regulation of endogenous ERLIN2 in cancer cells affects cancer cell growth. To address this question, we examined cell proliferation rates in the human hepatoma cell line Huh-7 or the human breast cancer cell line SUM225 in which ERLIN2 had been knocked down. We observed that in the absence of ERLIN2 the Huh-7 cells or SUM225 cells displayed a significant reduction in both size and number of cell aggregates (Figures 6A and 6B).

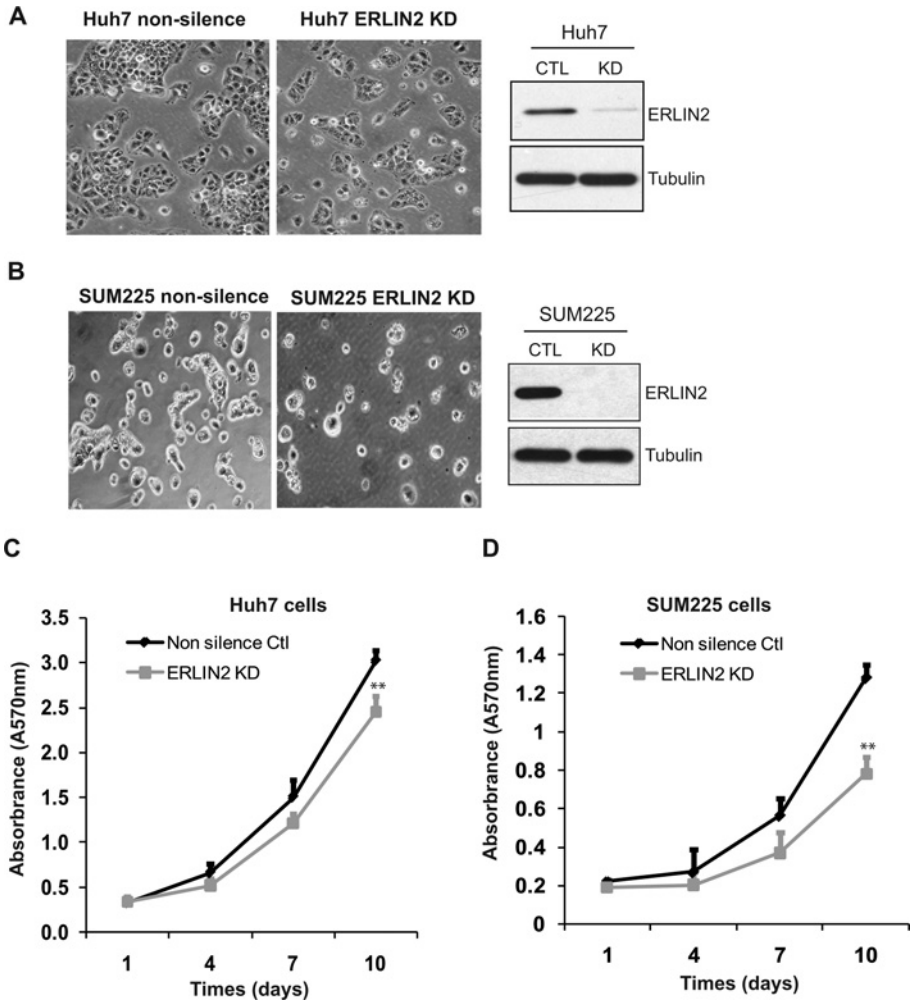

Figure 6 Down-regulation of ERLIN2 reduces cancer cell proliferation rates

(**A**) Left-hand panel, morphology of ERLIN2 knockdown and control Huh-7 cells. Magnification is ×400. Right-hand panel, levels of ERLIN2 protein in knockdown (KD) and control (CTL) Huh-7 cells were determined by Western blot analysis. (**B**) Morphology of ERLIN2 knockdown and control SUM225 cells. Magnification is ×400. Levels of ERLIN2 protein in knockdown and control SUM225 cells were determined by Western blot analysis. (**C** and **D**) Cell proliferation assays with the stable ERLIN2 knockdown Huh-7 (**C**) or SUM225 (**D**) cells and control cells. On day 1, the same number of ERLIN2 knockdown or control cells was seeded (5000 cells per well). The cell proliferation rate at each time point was represented by a 570-nm absorbance reading determined by MTT assay. Results are means ± S.E.M. ($n = 3$ biological samples). $^{**}P < 0.01$.

Cell growth and proliferation analyses indicated that knockdown of ERLIN2 reduced proliferation rates of Huh-7 cells and SUM225 cells (Figures 6C and 6D). After 10 days in cell culture, the proliferation rates of ERLIN2 knockdown Huh-7 cells or SUM225 cells were significantly lower than that of control cells. The reduced cancer cell proliferation rates, caused by down-regulation of ERLIN2, are consistent with the role of ERLIN2 in regulating *de novo* lipogenesis. This result implies that targeting ERLIN2 could be an effective therapeutic approach for aggressive cancers by down-regulating *de novo* lipogenesis in cancer cells.

DISCUSSION

The present study provides important new information about the role and mechanism of the ER lipid raft protein factor ERLIN2 in lipid metabolism associated with tumour cell growth and malignancy maintenance. The *ERLIN2* gene is amplified and overexpressed in the luminal subtype of human breast cancer that is associated with reduced metastasis-free survival rate [7–9,12]. Our work demonstrated that ERLIN2 is preferably expressed in aggressive breast cancer cell lines and in mouse fatty liver tissue, and it is inducible by insulin or LPDS-containing culture medium (Figure 1). We found that ERLIN2 modulates the activation of SREBP1c, the key regulator of lipid and cholesterol metabolism, in cancer cells (Figure 4). Consistently, cytosolic lipid droplet production, a reflection of *de novo* lipid/cholesterol metabolism, could be modulated by up- or down-regulation of ERLIN2 in human breast cancer cells or hepatoma cells (Figures 2 and 3). As a result of decreased lipogenesis, and other possible effects, cancer cell proliferation rates were reduced when ERLIN2 was down-regulated (Figure 6). These findings not only contribute to our understanding of the regulatory mechanism of activation of SREBPs in cancer cells, but could also inform novel therapy and pharmaceutical interventions to control cancers, especially aggressive forms.

Previously, ERLIN2 was characterized as a mediator of ERAD of activated inositol trisphosphate receptors, the key component of the ER Ca^{2+}-release channel, and of the cholesterol biosynthetic enzyme HMG-CoA reductase [13–15]. The ERLIN1–ERLIN2 complex interacts with the membrane-bound ubiquitin ligase GP78 and the substrate inositol trisphosphate receptors or HMG-CoA reductase, leading to polyubiquitination and subsequent degradation of these substrates. Activation of SREBPs, the key regulators of lipid and sterol biosynthesis, is regulated by the binding activities of ER-resident proteins, including INSIG1 and SCAP [21]. Because SCAP escorts SREBP from the ER to the Golgi for proteolytic processing into an active transcription factor, the binding of SCAP by INSIG1 effectively prevents SREBP activation [30]. Degradation of the INSIG1 protein through ERAD is an important process that is associated with the activation of SREBPs [31,32]. The present study showed that ERLIN2 interacts with INSIG1 and is weakly associated with SCAP after insulin signalling or LPDS culture (Figure 5). Because INSIG1 interacts with SCAP, it is possible that ERLIN2 indirectly associates with SCAP by interacting with INSIG1. We showed that ERLIN2 regulates cleavage of SREBP1c in human breast cancer cells or hepatoma cells (Figure 4). However, ERLIN2 does not likely play a significant role in degrading INSIG1, although it interacts directly with INSIG1 in response to metabolic signals (Figure 4C and Supplementary Figure S7). On the basis of these results, we propose that ERLIN2 might interact with the INSIG1–SCAP binding complex by directly binding to INSIG1 after insulin or LPDS challenge. Consequently, ERLIN2 and INSIG1 interaction facilitates the dissociation of SCAP from INSIG1, thus promoting SREBP activation and *de novo* lipogenesis in cancer cells (Supplementary Figure S8). The ERLIN2-mediated regulation of SREBP and thus of *de novo* lipogenesis might represent an important enhancing mechanism in lipid and energy metabolism that helps cancer cells gain their growth advantage.

During tumorigenesis, uncontrolled proliferation of cancer cells requires elevated *de novo* lipogenesis to meet the high demand for lipids and energy [2]. Shifting lipid acquisition toward *de novo* lipogenesis dramatically changes membrane properties and protects cells from both endogenous and exogenous insults. Our work suggests that ERLIN2, which is highly expressed in aggressive human breast cancer cells, supports malignancy by promoting *de novo* lipogenesis. Down-regulation of ERLIN2 can reduce cytosolic lipid droplet content and slow the proliferation rate of cancer cells. Therefore targeting ERLIN2 might reduce resistance of aggressive cancers to therapy and thus improve the effectiveness of conventional anti-cancer drugs. The present study has also raised many immediate and important questions. For example, what is the precise mechanism by which ERLIN2 regulates activation of SREBPs? Does ERLIN2 interact with other ER-resident lipogenic regulators, such as the hepatocyte-specific CREBH (cAMP responsive element binding protein) [40,41], to regulate lipid metabolism in cancer cells? Our data showed that ERLIN2 is involved in lipid droplet accumulation in the cells incubated with oleic acid (Figure 3B). Given that the oleic acid-induced response is not dependent upon *de novo* lipogenesis, ERLIN2 may also act on other pathways to facilitate cytosolic lipid accumulation. In the future, it will be interesting to investigate additional roles of ERLIN2 in promoting lipid accumulation. Additionally, the present study only demonstrated the regulation of SREBP activation and lipogenesis by ERLIN2 in a panel of cancer cells. Interestingly, expression of ERLIN2 was elevated in fatty liver tissues (Figure 1). Therefore, it is plausible to speculate that ERLIN2 may also regulate lipid metabolism in fatty liver disease. All of these questions merit future research in ERLIN2.

AUTHOR CONTRIBUTION

Kezhong Zhang, Zeng-Quan Yang and Guohui Wang designed the study; Guohui Wang, Xuebao Zhang, Jin-Sook Lee, Xiaogang Wang and Kezhong Zhang performed the experiments; Guohui Wang, Xuebao Zhang, Kezhong Zhang and Zeng-Quan Yang analysed the data; and Kezhong Zhang and Zeng-Quan Yang wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Endoplasmic reticulum factor ERLIN2 regulates cytosolic lipid content in cancer cells

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Origins and culture conditions for the cancer cell lines used in the present study

The SUM44 cell line was established from pleural effusion-derived breast cancer cells [1]. SUM44 cells were cultured in Ham's F12 medium supplemented with 0.1 % BSA, fungizone (0.5 μg/ml), gentamicin (5 μg/ml), ethanolamine (5 mmol/l), Hepes (10 mmol/l), transferrin (5 μg/ml), T3 (3,3′,5-triiodo-L-thyronine; 10 μmol/l), selenium (50 μmol/l), hydrocortisone (1 μg/ml) and insulin. The SUM225 cell line was established from a chest wall recurrence of ductal carcinoma *in situ* of breast [2]. SUM225 cells were cultured with 5 % FBS, fungizone (0.5 μg/ml), gentamicin (5 μg/ml), hydrocortisone (1 μg/ml) and insulin (5 μg/ml). Each of these cell lines, from a single human patient, represents a different subtype of breast cancer [1, 2]. All of the currently known oncogenes with altered expression patterns in breast cancer are well represented and have been characterized in the SUM lines. These cell lines have been described in over 50 peer-reviewed publications in cancer research.

MCF10A is a spontaneously immortalized, but non-transformed, human mammary epithelial cell line derived from the breast tissue of a 36-year-old patient with fibrocystic changes [3]. MCF10A cells were cultured in Ham's F12 medium supplemented with 0.1 % BSA, fungizone (0.5 μg/ml), gentamicin (5 μg/ml), ethanolamine (5 mmol/l), Hepes (10 mmol/l), transferrin (5 μg/ml), T3 (10 μmol/l), selenium (50 μmol/l), hydrocortisone (1 μg/ml), insulin (5 μg/ml) and 10 ng/ml epidermal growth factor.

Huh-7 is a hepatocellular carcinoma cell line that was originally derived from a liver tumour in a Japanese male [4]. This cell line was cultured in DMEM containing 10 % FBS, L-glutamine and antibiotics at 37 °C in a 5 % CO_2 environment.

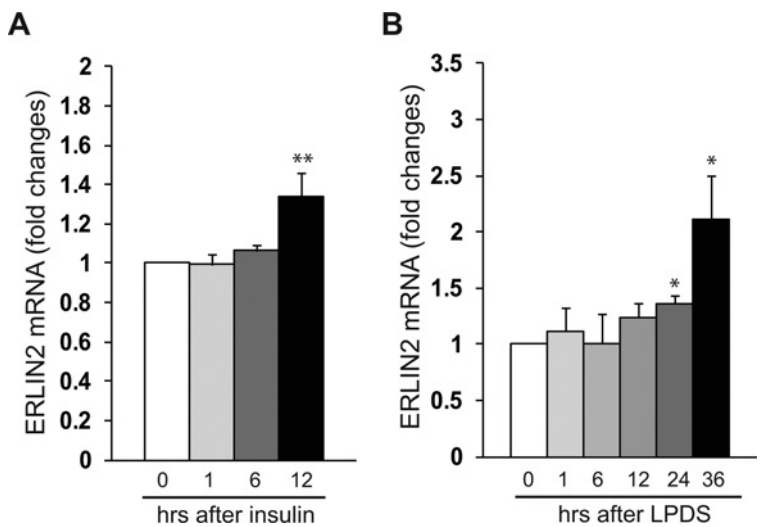

Figure S1 Quantitative real-time RT–PCR analysis of *ERLIN2* mRNA expression in murine primary hepatocytes in response to insulin or LPDS challenge

(**A**) Murine primary hepatocytes were challenged with insulin (100 nM) for 1, 6 or 12 h. Murine primary hepatocytes were cultured in normal medium with vehicle buffer PBS added as a control (0 h under insulin). (**B**) Murine primary hepatocytes were cultured in medium containing LPDS for 1, 6, 12, 24 or 36 h. As a control, murine primary hepatocytes were cultured in normal medium containing 10 % FBS (0 h under LPDS). For (**A** and **B**), total RNAs were isolated from the primary hepatocytes after the treatment, and quantitative real-time RT–PCR was performed to determine *ERLIN2* mRNA expression levels in the primary hepatocytes after insulin or LPDS challenge. The mRNA expression values were determined after normalization to internal control *GAPDH* mRNA levels. The baseline of the *ERLIN2* mRNA level in the hepatocytes challenged with insulin or LPDS at 0 h was set to 1. After the treatments, fold changes of *ERLIN2* mRNA levels in the hepatocytes were calculated by comparison to the baseline mRNA level. Results are means ± S.E.M. ($n = 3$ experiments). *$P < 0.05$; **$P < 0.01$.

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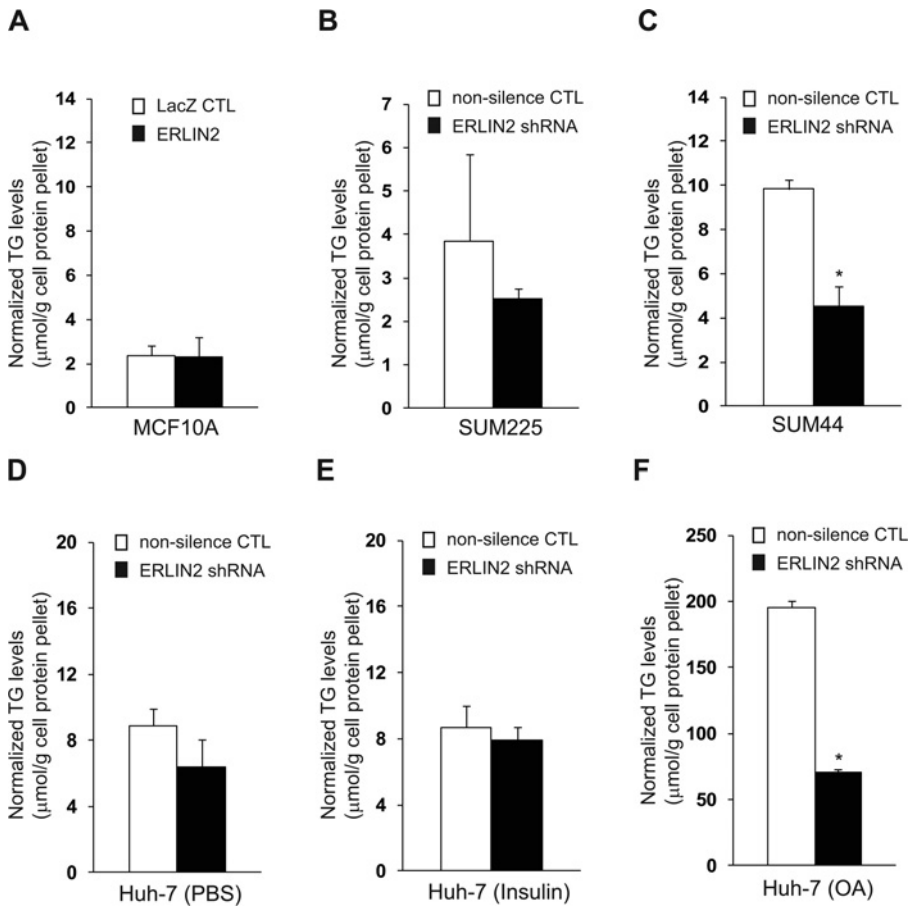

Figure S2 Biochemical quantification of cellular TG levels in MCF10A, SUM225, SUM44 and Huh-7 cells

Levels of total cellular TG were determined using a TG measurement kit from BioAssay System. TG levels were presented after normalization to net weights of cellular pellets. For Huh-7 cells, ERLIN2 knockdown or control (CTL) stable cell lines were incubated with vehicle PBS, insulin (100 nM), or oleic acid (OA) (0.5 mM) for 12 h. (**A**) Cellular TG levels in MCF10A cells expressing exogenous LacZ or ERLIN2. (**B**) Cellular TG levels in ERLIN2 knockdown or control SUM225 cells. (**C**) Cellular TG levels in ERLIN2 knockdown or control SUM44 cells. (**D**) Cellular TG levels in ERLIN2 knockdown or control Huh-7 cells treated with PBS vehicle. (**E**) Cellular TG levels in ERLIN2 knockdown or control Huh 7 cells treated with insulin. (**F**) Cellular TG levels in ERLIN2-knockdown or control Huh-7 cells incubated with oleic acid. Results are means $\pm$ S.E.M. ($n = 3$). *$P < 0.05$.

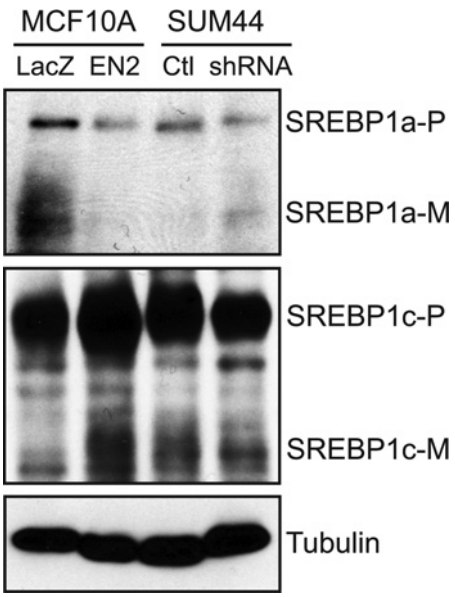

Figure S3 Western blot analysis of SREBP1c and SREBP1a protein levels in MCF10A expressing LacZ control or ERLIN2 and in ERLIN2 knockdown or control SUM44 cells

Tubulin was included as a loading control. LacZ, LacZ overexpression; EN2, ERLIN2 overexpression; Ctl, non-silence control; shRNA, ERLIN2 shRNA knockdown; SREBP1a-P, SREBP1a precursor; SREBP1a-M, mature SREBP1a; SREBP1c-P, SREBP1c precursor; and SREBP1c-M, mature SREBP1c. Note that our data showed that SUM44 and MCF10A cells only express trace levels of SREBP1a, which is consistent with the published conclusion that SREBP1c, but not SREBP1a or SREBP2, is induced in human breast cancer cell lines [5]. Interestingly, our data shows that expression levels of SREBP1a were decreased in MCF10A cells overexpressing ERLIN2, whereas SREBP1a levels were increased in ERLIN2 knockdown SUM44 cells. The correlation of SREBP1a levels with ERLIN2 induction and malignancy states is an interesting question to be elucidated in the future.

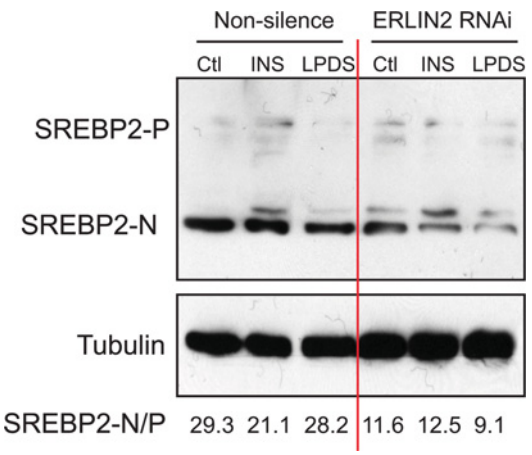

Figure S4 Western blot analysis of cleaved SREBP2 protein levels in Huh-7 cells that were transduced by non-silencing or ERLIN2 shRNAi lentivirus

The Huh-7 cells were treated with vehicle PBS or insulin (100 nM) for 6 h or cultured in LPDS-containing medium for 12 h. Tubulin was included as a loading control. SREBP2 protein signals were detected by using an antibody against the N-terminal SREBP protein fragment (Cayman Chemicals). The values below the gels represent the ratios of mature cleaved SREBP2 to SREBP2 precursor signal intensities. Ctl, control cells treated with vehicle PBS; INS, insulin; SREBP2-N, cleaved SREBP2 (N-terminal); SREBP2-P, SREBP2 precursor.

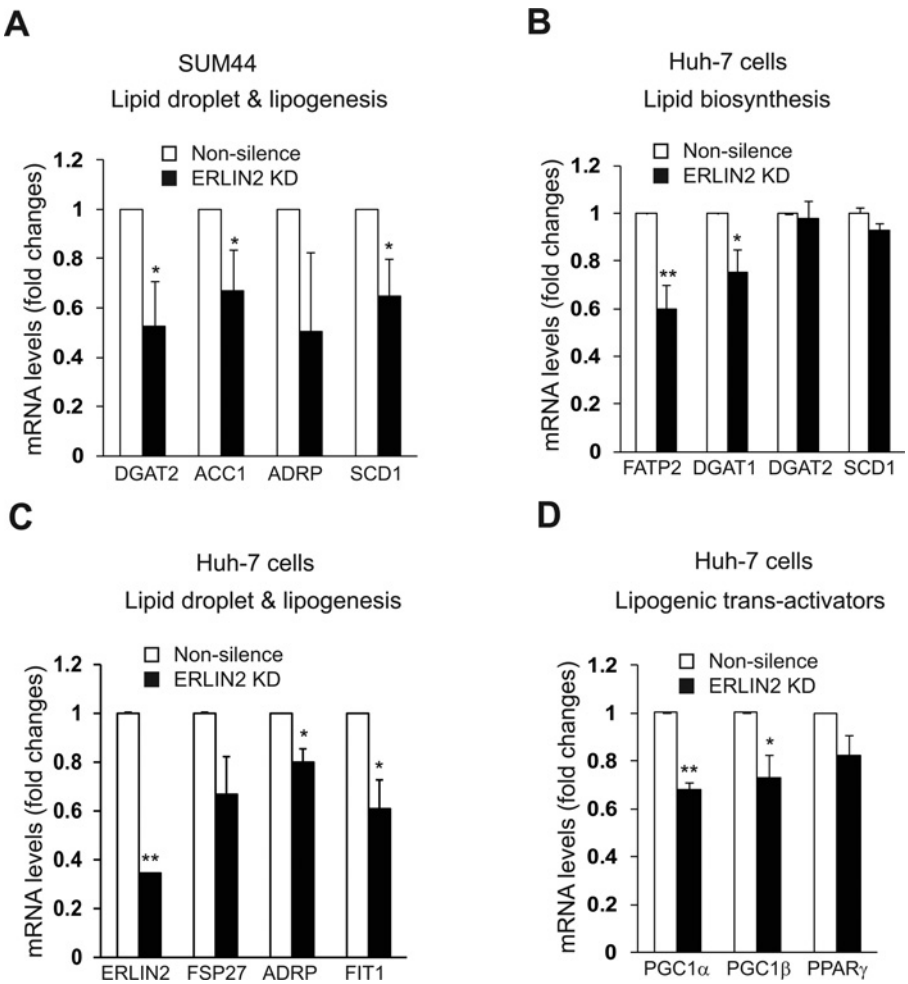

Figure S5 Quantitative real-time RT–PCR analysis of expression of lipogenic genes in ERLIN2 knockdown and non-silenced control Huh-7 or SUM44 cells

(**A**) Expression of the genes involved in lipid droplet formation and lipogenesis, including *DAGT2*, *ACC1*, *ADRP*, and *SCD1*, in ERLIN2-knockdown and control SUM44 cells. (**B**) Expression of the genes involved in lipid biosynthesis, including *FATP2*, *DGAT1*, *DAGT2* and *SCD1*, in ERLIN2 knockdown and control Huh-7 cells. (**C**) Expression of the *ERLIN2* gene and the genes involved in lipid droplet formation and lipogenesis, including *FSP27*, *ADRP* and *FIT1*, in ERLIN2 knockdown and control Huh-7 cells. (**D**) Expression of the genes encoding the lipogenic *trans*-activators PGC1α, PGC1β and PPARγ2 in ERLIN2 knockdown and control Huh-7 cells. For (**A**–**D**), total RNAs were isolated from the cells, and quantitative real-time RT–PCR was performed to determine mRNA expression levels. The mRNA expression values were determined after normalization to internal control *GAPDH* mRNA levels. To determine the expression profile for a particular gene, the baseline mRNA level in control cells was set to 1. Fold changes in the mRNA levels of the ERLIN2-knockdown cells were calculated by comparison with the baseline mRNA level. Results are means $\pm$ S.E.M. ($n = 3$ experimental repeats). *$P < 0.05$; **$P < 0.01$. ACC1, acetyl-CoA carboxylase 1; ADRP, adipose differentiation-related protein; FATP2, fatty acid transport protein 2; FIT1, fat-inducing transcript 1; FSP27, fat-specific protein 27; PPAR, peroxisome-proliferator-activated receptor.

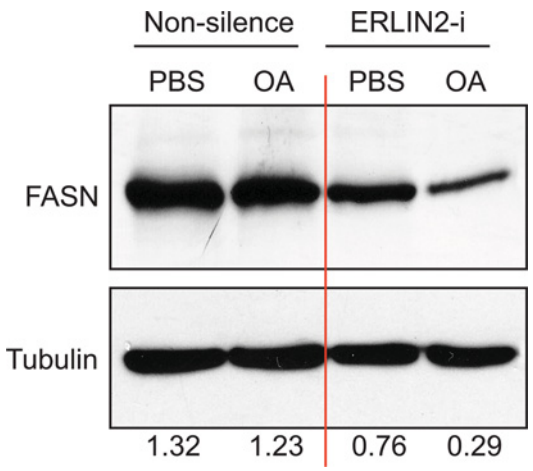

Figure S6 Western blot analysis of FAS protein levels in Huh-7 cells that were transduced by non-silencing or ERLIN2 shRNAi lentivirus in the presence or absence of oleic acid treatment

The non-silencing control or ERLIN2 knockdown Huh–7 cells were treated with vehicle PBS or oleic acid (OA) (0.5 mM) for 12 h. Tubulin was included as a loading control. The values below the gels represent the ratio of FAS to tubulin signal intensities.

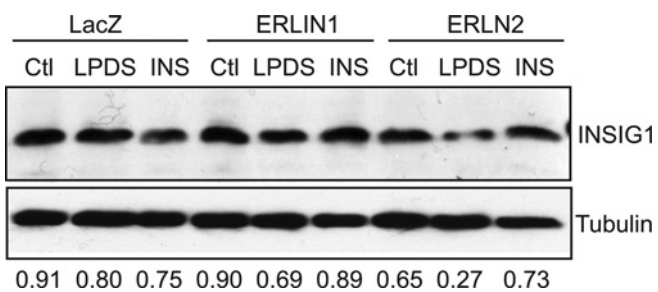

Figure S7 Western blot analysis of INSIG1 protein levels in the Huh-7 cell line that was transduced by lentivirus overexpressing LacZ, ERLIN1 or ERLIN2

Cell lysates were prepared from the Huh-7 cell lines cultured in normal medium (Ctl), LPDS medium for 12 h, or challenged with insulin (INS, 100 nM) for 6 h. Tubulin was included as a loading control. The values below the gels represent INSIG1 signal intensities after normalization to tubulin signal intensities.

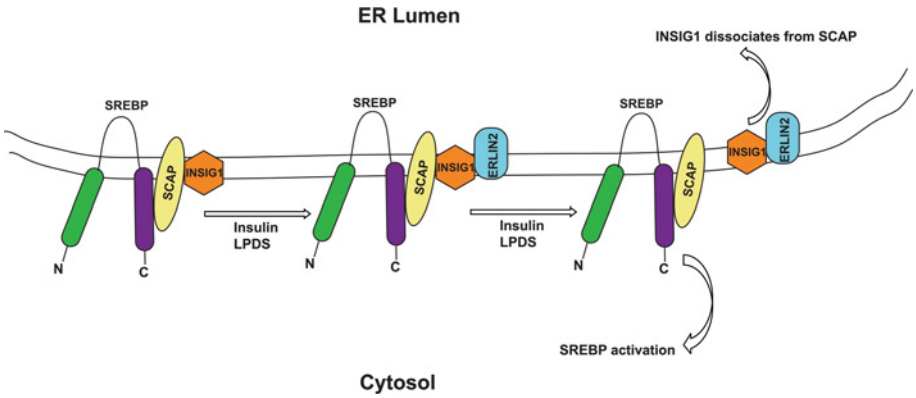

Figure S8 A working model for regulation of SREBP activation by ERLIN2

In response to insulin or LPDS challenge, ERLIN2 interacts with the INSIG1–SCAP binding complex by directly binding to INSIG1. The interaction between ERLIN2 and INSIG1 facilitates the dissociation of SCAP from INSIG1, thus promoting SREBP–SCAP complex release from the ER to Golgi for SREBP processing.

Table S1 Sequence information for the real-time PCR analysis

ACC1, acetyl-CoA carboxylase 1; ACTB, β-actin; ADRP, adipose differentiation-related protein; FATP2, fatty acid transport protein 2; FIT1, fat-inducing transcript 1; FSP27, fat-specific protein 27.

Gene symbol	Forward primer (5′→3′)	Reverse primer (5′→3′)
PGC1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
PGC1β	AGAAGCTCCTCCTGGCCACATCCT	GCCTTCTTGTCTTGGGTGCTGTCC
ERLIN2	GAACCAGTGGTGGTGTGATG	TATGAACGCTGCAGAACTGG
FSP27	GAGTCCAACGCAGTCCAGCTGAC	GCAGCTGCTGGGTCACCACAG
ACC1	AGGGCTAGGTCTTTCTGGAAGTGGA	TCAGCTCCAGAGGTTGGGCCA
DGAT1	CCGTGAGCTACCCGGACAAT	AGGATCCGTCGCAGCAGAA
DGAT2	TTTCGAGACTACTTTCCCATCCA	TGGCCTCTGTGCTGAAGTTG
ADRP	GATGGCAGAGAACGGTGTGAA	TCAATCCTGTCTAGCCCCTTACAG
SCD1	CTGCCCCTACGGCTCTTTCT	ACGTCGGGAATTATGAGGATCA
PPARγ2	CCTATTGACCCAGAAAGCGATT	CATTACGGAGAGATCCACGGA
FIT1	TTCGCCAGCCACGGCAACTT	GCGCCGTGTAGCCAGGAACA
FATP2	CCACAGGTCTTCCAAAAGCAGCCA	GTGCAGCACTGTGGTAAAAGGGCA
ACTB	AGCCTCGCCTTTGCCGATCCG	ACATGCCGGAGCCGTTGTCGA

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Original Article

Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation

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Abstract: Endoplasmic Reticulum (ER) stress refers to a condition of accumulation of unfolded or misfolded proteins in the ER lumen. A variety of biochemical stimuli or pathophysiologic conditions can directly or indirectly induce ER stress, leading to activation of an ER-originated adaptive signaling response called Unfolded Protein Response (UPR). Recent studies demonstrated that ER stress and UPR signaling are critically involved in the initiation and progression of many diseases, such as metabolic disease, cardiovascular disease, neurodegenerative disease, and cancer. In this study, we show that ER stress induced by pharmacologic reagents, including tunicamycin (TM) and thapsigargin (Tg), promotes hepatic lipogenesis and lipid droplet formation. Using quantitative gene expression analysis, we identified 3 groups of key lipogenic regulators or enzymes that are inducible by pharmacological ER stress in a human hepatoma cell line Huh-7. These ER stress-inducible lipogenic factors include: 1) lipogenic trans-activators including CCAAT/enhancer binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), PPARγ coactivator 1-alpha (PGC1α), and Liver X receptor alpha (LXRα); 2) components of lipid droplets including fat-specific protein 27 (FSP27), adipose differentiation related protein (ADRP), fat-inducing transcript 2 (FIT2), and adipocyte lipid-binding protein (AP2); 3) key enzymes involved in *de novo* lipogenesis including acetyl-CoA carboxylase 1 (ACC1) and stearoyl-CoA desaturase-1 (SCD1). Supporting the role of pharmacologic ER stress in up-regulating *de novo* lipogenesis, TM or Tg treatment significantly increased accumulation of cytosolic lipid droplet formation in the hepatocytes. Moreover, we showed that forced expression of an activated form of X-box binding protein 1 (XBP1), a potent UPR *trans*-activator, can dramatically increase expression of PPARγ and C/EBPα in Huh-7 cells. The identification of ER stress-inducible lipogenic regulators provides important insights into the molecular basis by which acute ER stress promotes *de novo* lipogenesis. In summary, the findings from this study have important implication in understanding the link between ER stress and metabolic disease.

Keywords: Endoplasmic reticulum (ER) stress, hepatic lipogenesis, lipid droplet formation

Introduction

In eukaryotic cells, the ER is the site of folding of membrane and secreted proteins, synthesis of lipids and sterols, and storage of free calcium [1, 2]. As a protein-folding compartment, the ER is exquisitely sensitive to alterations in homeostasis, and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi and unfolded or misfolded proteins are retained in the ER and ultimately degraded. A number of biochemical stimuli and physiological and pathological processes, such as perturbation in calcium homeostasis, elevated secretory protein synthesis, and expression of misfolded proteins, can disrupt ER homeostasis, impose stress to the ER, and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen. To cope with accumulation of unfolded or misfolded proteins in the ER, the cell has evolved highly specific signaling pathways called the unfolded protein response (UPR) to reduce the amount of new proteins translocated into the ER lumen, increase retrotranslocation and degradation of ER-localized proteins, and bolster the protein-folding capacity and secretion potential of the ER [2]. The UPR is orchestrated by transcriptional activation of multiple genes mediated by the protein kinase/endoribonuclease IRE1

(inositol-requiring 1) and the b-ZiP transcription factor ATF6 (activating transcription factor 6), and a general decrease in translation initiation and the selective translation of specific mRNAs mediated by the protein kinase PERK (double-strand RNA-activated kinase-like ER kinase) [1-4].

Liver is a major organ responsible for lipid and glucose metabolism. Dysregulation of hepatic lipid metabolism is closely associated with the initiation and progression of metabolic syndrome. Recent studies suggest that ER stress response plays important roles in maintaining lipid homeostasis [5-9]. The UPR branches through IRE1α and/or ATF6 is required to prevent hepatic steatosis upon acute ER stress [6, 7, 10]. It has also been shown that the IRE1α/XBP1 UPR branch is activated by the dietary high-carbohydrate and controls the expression of lipogenic enzymes, such as ACC2, DGAT2 and SCD1, that are essential for fatty acid and cholesterol biosynthesis [11]. Moreover, the UPR pathway through PERK/eIF2α was documented to be required for the expression of lipogenic genes and the development of hepatic steatosis [9]. Together, these observations suggest that ER stress and the UPR signaling are critically involved in regulating hepatic lipid metabolism.

In this study, we utilized two structurally-unrelated ER stress-inducing reagents, tunicamycin (TM) and Thapsigargin (Tg), to induce pharmacologic ER stress in Huh-7, a human hepatoma cell line that maintains key features of hepatic lipid metabolism [12]. Through this approach, we confirmed the effect of pharmacologic ER stress in up-regulating *de novo* lipogenesis and lipid droplet formation. Importantly, we have identified a subset of genes encoding key lipogenic trans-activators and enzymes, which are inducible by acute ER stress. The results from this study provide important insights into ER stress-induced hepatic lipogenesis

Materials and methods

Materials

Chemicals were purchased from Sigma unless indicated otherwise. Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Antibodies against XBP1, C/EBPα, and PPARγ were from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA). Antibodies against GAPDH and β-actin were purchased from Sigma (St. Louis, MO). Tunicamycin was from Sigma. BODIPY staining kit was purchased from Invitrogen. Human hepatoma cell line Huh-7 was kindly provided by Drs. Christopher M. Schonhoff (Tufts University Cummings School of Veterinary Medicine).

Huh-7 cell culture and TM and Tg treatment

HuH-7 cells were cultured at 37 °C and 5% CO_2 in DMEM containing high glucose (25 mM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Huh-7 cells at 70% confluency were treated with tunicamycin (TM; 5, 10, and 20 μg/ml) or thapsigargin (Tg; 0.5, 1, and 1.5 μM) or vehicle PBS for 6, 12, and 24 hrs.

BODIPY staining of lipid droplets

Cells were washed with PBS, fixed with 3% formaldehyde for 15 min, and stained with BODIPY 493/503 (Invitrogen, stock concentration 1mg/ml, working solution 1:1000 dilution) for 15min at room temperature. Cells were then mounted with Prolong gold anti-fade reagent (Invitrogen) followed by washing in PBS for 3 times.

Western Blot and IP-Western blot Analyses

To determine expression levels of XBP1, PPARγ, C/EBPα, and GAPDH, total cell lysates were prepared from cultured Huh-7 cells using NP-40 lysis as previously described [13]. Denatured proteins were separated by SDS-PAGE on 10% Tris-glycine polyacrylamide gels and transferred to a 0.45-mm PVDF membrane (GE Healthcare). Membrane-bound antibodies were detected by an enhanced chemiluminescence detection reagent (GE Healthcare).

Recombinant adenoviral infection

Huh-7 cells at 60% confluency were infected by recombinant adenovirus expressing GFP or an activated form of XBP1 protein at an MOI of 100 for 48 hours before cell lysates were collected for Western blot analysis. Adenovirus expressing spliced XBP1 was kindly provided by Dr. Umut Ozcan (Harvard University) [14]. Recombinant adenovirus expressing GFP was kindly provided by Dr. Jiande Lin (University of Michigan).

Quantitative real-time RT-PCR analysis

For real-time PCR analysis, the reaction mixture containing cDNA template, primers, and SYBR Green PCR Master Mix (Invitrogen) was run in a 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA). The real-time PCR primer sequences used in this study are described in supplemental information. Fold changes of mRNA levels were determined after normalization to internal control β-actin RNA levels.

Statistics analysis

Experimental results are shown as mean ± STDEV (for variation between experiments). The mean values for biochemical data from the experimental groups were compared by a paired or unpaired, 2-tailed Student's *t* test. Statistical tests with $P < 0.05$ were considered significant.

Results

Pharmacologic ER stress induced by TM or Tg promotes lipid droplet formation

To study the effect of pharmacologic ER stress on hepatic lipid metabolism, we challenged a human hepatoma cell line, Huh-7, with two structurally-unrelated ER stress-inducing drugs, tunicamycin (TM) and Thapsigargin (Tg). Huh-7 is a human hepatocellular carcinoma cell line that has been used for studying hepatic lipid metabolism [12]. TM is a bacterial nucleoside antibiotic that can block N-linked glycoproteins and cause accumulation of unfolded or misfolded proteins in the ER [15]. Tg is a specific inhibitor of intracellular SERCA-type Ca^{2+} pumps present in the sarcoplasmic/ER [16, 17]. Tg treatment can disrupt ER calcium homeostasis, leading to accumulation of unfolded or misfolded proteins in the ER lumen. Both TM and Tg have been routinely used as experimental tools to induce pharmacologic ER stress [18]. To delineate gene expression profiles in hepatic lipid metabolism upon pharmacologic ER stress challenge, Huh-7 cells were treated with TM at doses ranging from 5 to 20 μg/ml or Tg at doses ranging from 0.5 to 1.5 μM. The time intervals for each treatment were 6, 12, and 24 hours. Quantitative real-time RT-PCR analysis indicated that expression of the UPR target mRNA or genes, including spliced *Xbp1* mRNA, *Bip*, and *Chop*, was increased upon TM or Tg treatment in a dose-and time-dependent manner (**Figure 1**). This result suggests that TM and Tg can efficiently induce ER stress and activation of the UPR signaling in Huh-7 cells. Note that the levels of the spliced *XBP1*, *CHOP*, and *BiP* mRNAs in Huh-7 cells under different doses of TM treatment were comparable at 24 hours post TM treatment (**Figure 1A-C**), suggesting that Huh-7 cells can adapt to ER stress at the late stage of TM treatment.

Next, we evaluated the impact of pharmacologic ER stress in *de novo* hepatic lipogenesis, a key lipid synthesis progress that is tightly regulated by multiple layers of metabolic and stress signals [19, 20]. We examined the production of cytosolic lipid droplets, a major indicator of *de novo* lipogenesis, in the Huh-7 cells upon TM or Tg challenge. Production of cytosolic lipid droplets, as indicated by Bodipy staining, was significantly increased in the Huh-7 cells after 6 hours of TM or Tg treatment, compared to that after vehicle treatment (**Figure 2**). This result suggests that pharmacologic ER stress induced by TM or Tg can promote hepatic lipid droplet formation.

Challenge of TM or TG up-regulates expression of the genes encoding key lipogenic trans-activators

To understand the mechanism by which pharmacologic ER stress promotes lipid droplet formation, we first tested whether TM or Tg can upregulate *trans*-activators in *de novo* lipogenesis. Huh-7 cells were treated with different doses of TM or Tg for a time course from 6, 12, to 24 hours. Quantitative real-time RT-PCR analysis was performed with the TM or Tg-treated Huh-7 cells to determine ER stress-inducible target genes in *trans*-activation of *de novo* lipogenesis. Among known lipogenic *trans*-activators we examined, expression of the genes encoding lipogenic *trans*-activators including CCAAT/enhancer binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma 2 (PPARγ2), PPARγ coactivator 1-alpha (PGC1α), and Liver X receptor alpha (LXRα) was significantly increased in the Huh-7 cells under the treatment of TM or Tg (**Figure 3A-D**). Although all these genes are inducible by TM or Tg, the expression dynamics of these genes were different upon TM or Tg challenge. Expression of the *C/EBPα*, *LXRα*, and *PGC1α* genes was inducible by TM or Tg in the time windows from 6 to 24

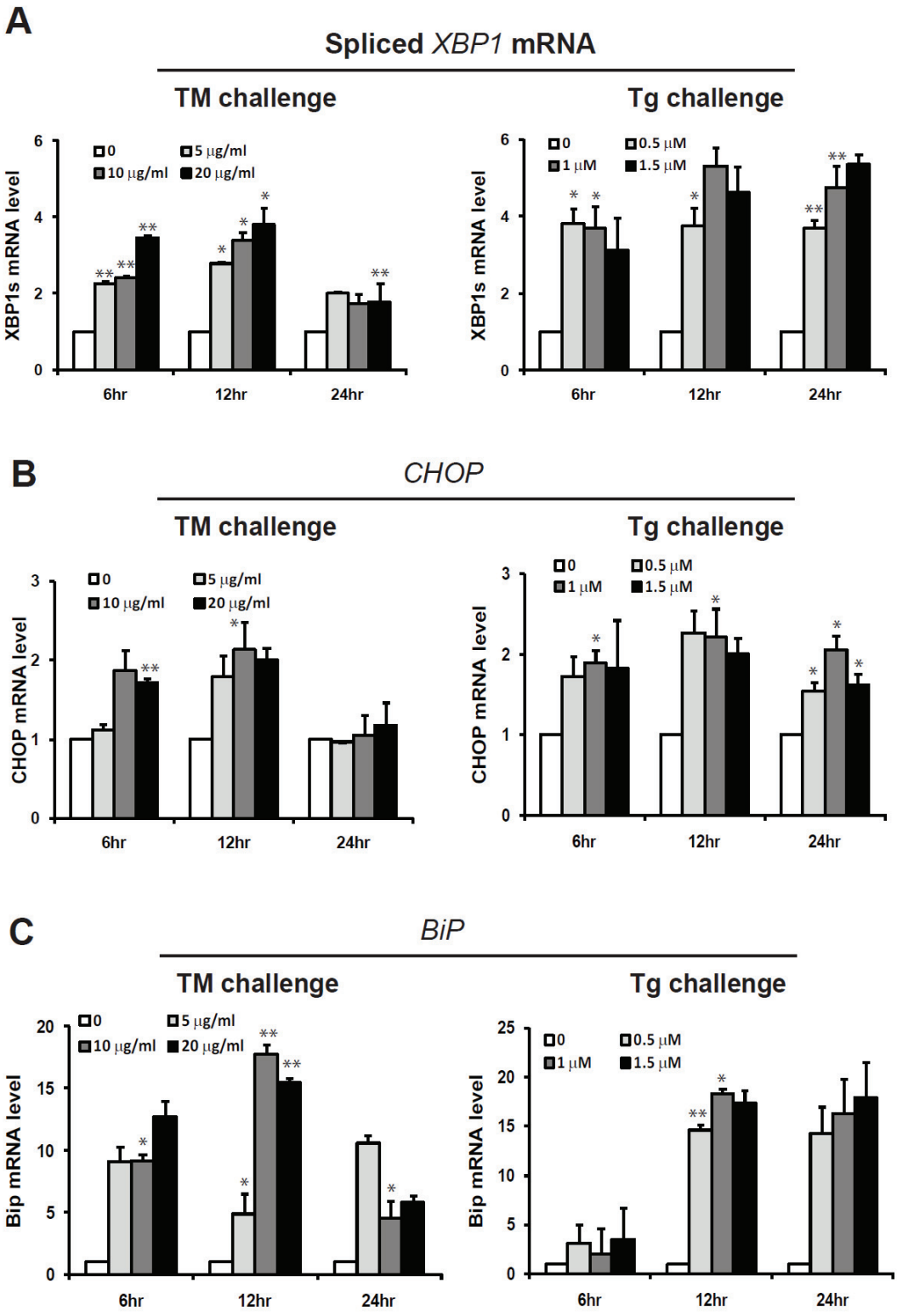


Figure 1. Quantitative real-time RT-PCR analysis of the mRNAs encoding spliced XBP1 (A), CHOP (B), and BiP (C) in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

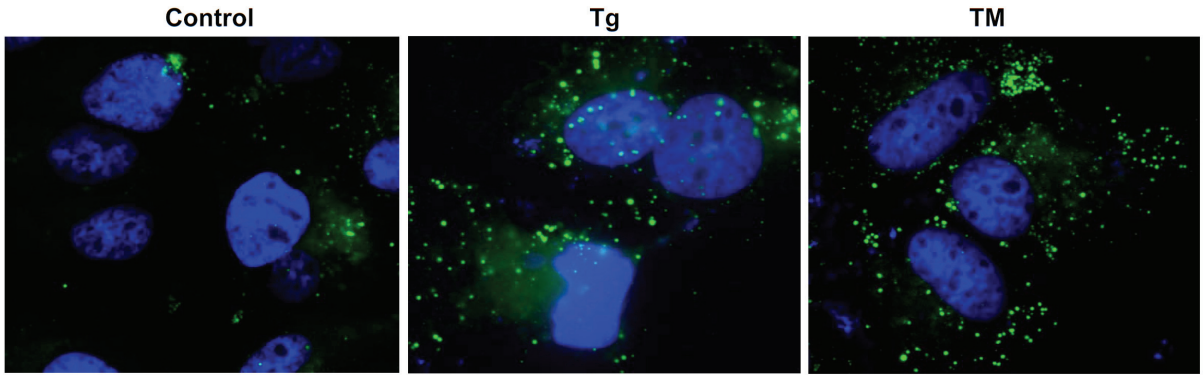


Figure 2. Bodipy staining of lipid droplets in Huh-7 cells. Huh-7 cells were treated with TM (10 μg/ml) or Tg (1 μM) for 6 hrs and then stained with Bodipy for lipid droplets. Magnification: 630 ×.

hours post treatment (**Figure 3A**, **C** and **D**). However, expression of the gene encoding PPARγ2 was only inducible at 12 hours after TM or Tg treatment (**Figure 3B**). Both TM and Tg challenge failed to increase PPARγ2 expression at either early 6 hours or late 24 hours post treatment. It has been documented that PPARγ2, which is usually expressed in adipose tissue, is inducible in steatotic livers, and contributes to increased *de novo* lipogenesis [19, 21-23]. Our data suggest that pharmacologic ER stress can induce expression of PPARγ2 in hepatocytes that may contribute to ER stress-induced lipogenesis and lipid droplet formation.

TM or TG treatment promotes expression of genes encoding key enzymes in lipid droplet formation and triglyceride synthesis

We extended our effort in understanding pharmacologic ER stress-induced hepatic lipogenesis by identifying ER stress-inducible target genes in lipid droplet formation and triglyceride synthesis [19]. Through quantitative real-time RT-PCR analysis, we found that expression of the genes encoding key factors in lipid droplet formation, including fat-specific protein 27 (FSP27), adipose differentiation related protein (ADRP), fat-inducing transcript 2 (FIT2), and adipocyte lipid-binding protein (AP2), was increased in Huh-7 cells challenged with TM or Tg (**Figure 4**). All these genes were inducible by TM or Tg at 6 hours post treatment. However, expression of ADRP and FSP27 was reduced in Huh-7 cells at 24 hours post TM treatment (**Figure 4A-B**). The expression patterns of *ADRP* and *FSP27* were similar to those of the classic ER stress targets including *XBP1*, *CHOP*, and *BiP* (**Figure 1A-C**), implying that an ER stress-associated negative feedback regulation may exist for expression of the *ADRP* and *FSP27* genes (**Figure 4**). In contrast, expression of AP2, a protein factor involved in lipid transport and storage in lipogenesis and lipolysis [24], was increased in response to TM or Tg treatment from 6 to 24 hours (**Figure 4D**), suggesting a prominent regulation of AP2 gene expression by ER stress. Moreover, we identified two key enzymes required for triglyceride synthesis, acetyl-CoA carboxylase 1 (ACC1), and stearoyl-CoA desaturase-1 (SCD1), were inducible by TM or Tg in Huh-7 cells in a dose- and time-dependent manner (**Figure 5**). Because triglyceride is the core component of lipid droplet, increased expression of key enzymes or protein regulators in triglyceride synthesis and lipid droplet formation may account for ER stress-induced lipid droplet formation.

Activated XBP1 increases expression of PPARγ and C/EBPα in hepatoma cells

To verify the role of the UPR signaling in regulating expression of lipogenic genes, we forcibly expressed an activated form of human XBP1, an ER stress-inducible transcription factor, in Huh-7 cells by utilizing an adenoviral-based over-expression system. As a control, Huh-7 cells were infected by an adenovirus over-expressing GFP. Under ER stress, the UPR transducer IREα is activated to function as an RNase that splices the mRNA encoding X-box binding protein 1 (XBP1) [25-27]. The spliced *XBP1* mRNA, but not the unspliced *XBP1* mRNA, encodes an activated transcription factor that potently activates the UPR target genes. Through Western blot

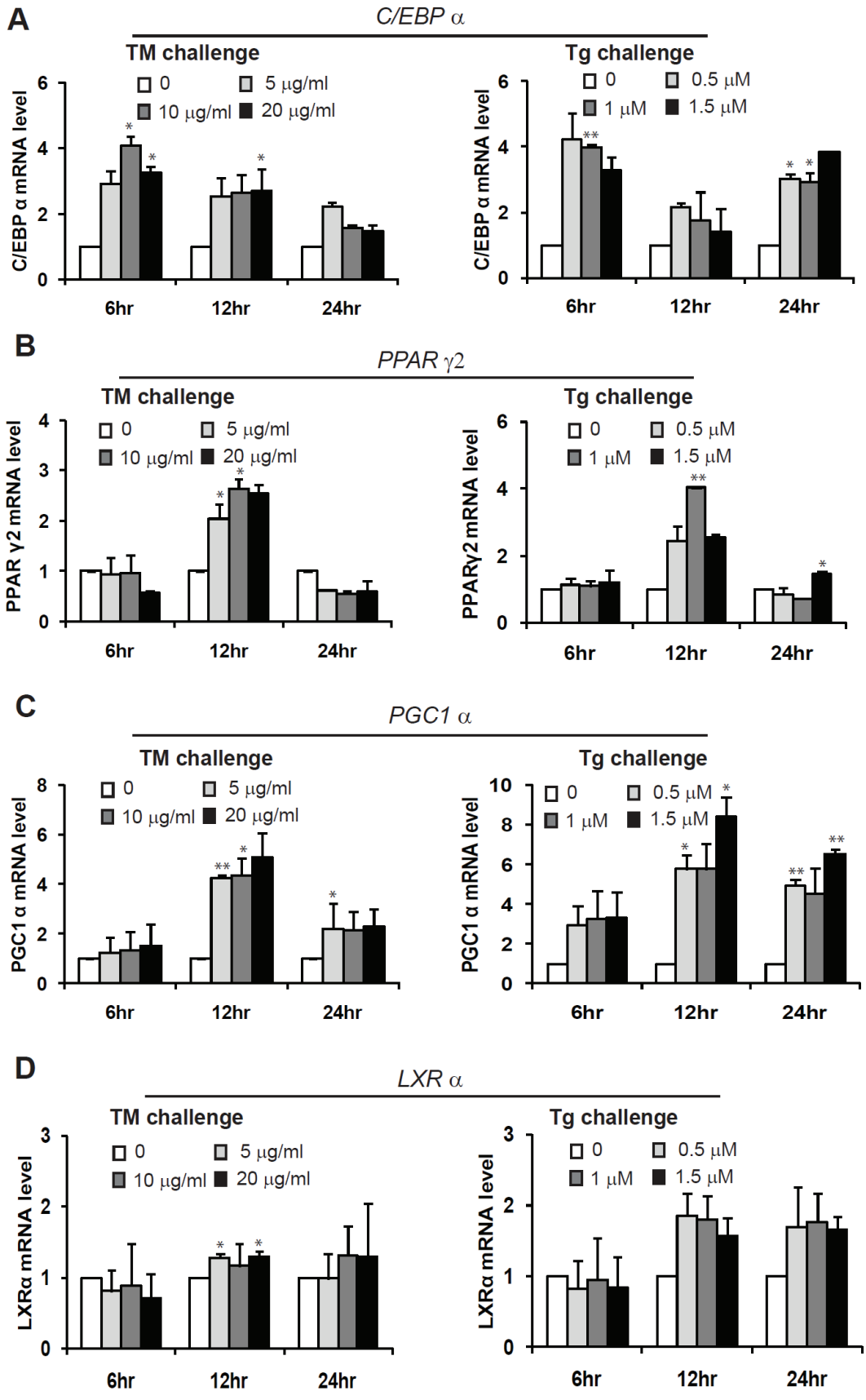

A
C/EBP α
TM challenge
0
5 μg/ml
10 μg/ml
20 μg/ml
C/EBP α mRNA level
6hr
12hr
24hr
Tg challenge
0
0.5 μM
1 μM
1.5 μM
B
PPAR γ2
PPAR γ2 mRNA level
PPARγ2 mRNA level
C
PGC1 α
PGC1 α mRNA level
D
LXR α
LXRα mRNA level

Figure 3. Quantitative real-time RT-PCR analysis of the mRNAs encoding key lipogenic trans-activators, including C/EBPα (A), PPARγ2 (B), PGC1α (C), and LXRα (D), in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

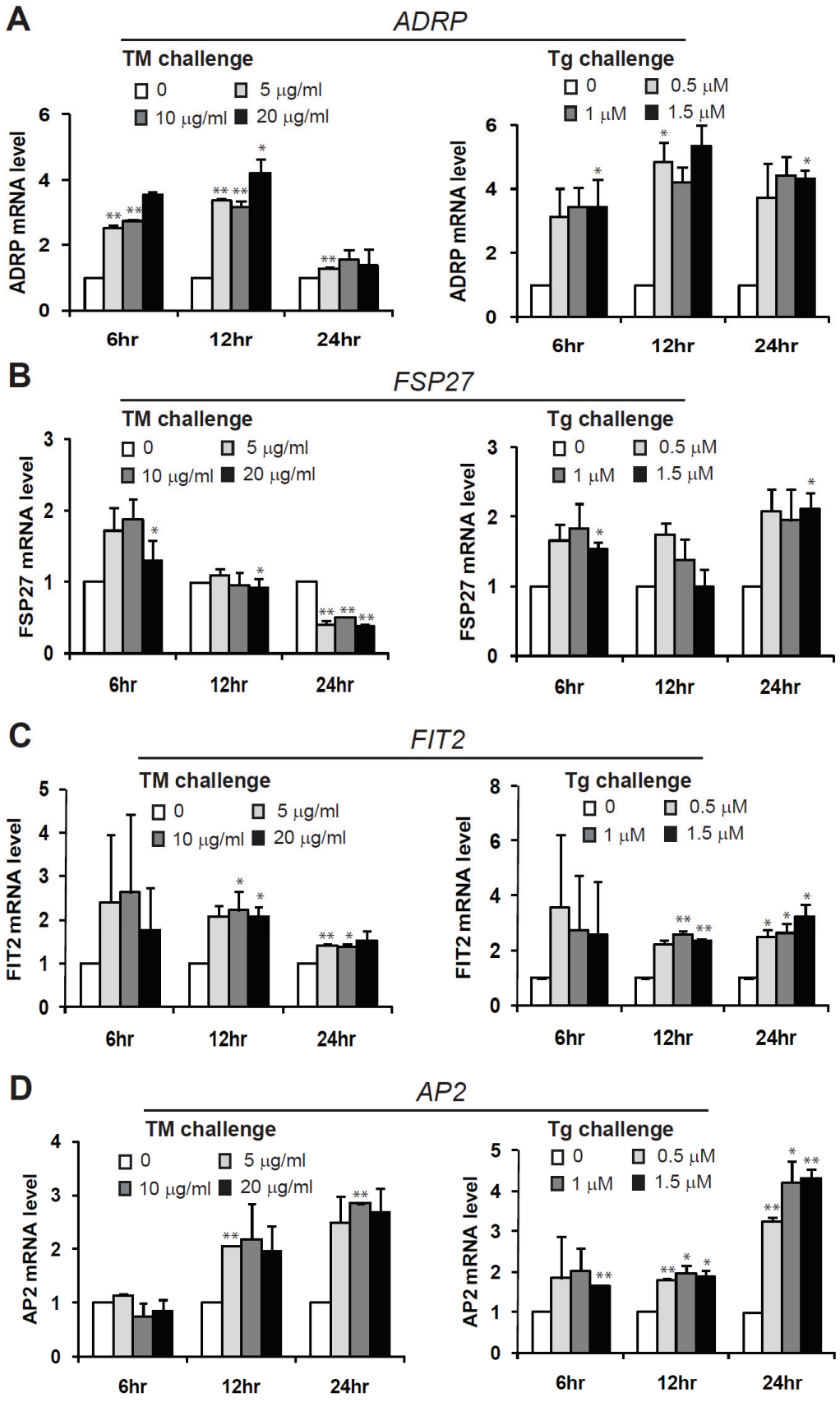
analysis, we confirmed that expression levels of the activated form of XBP1 protein (encoded by the spliced *XBP1* mRNA), but not the inactivated XBP1 protein, were significantly increased in Huh-7 cells infected by the adenovirus expressing the activated form of human XBP1 (**Figure 6A-B**). Consistent with the gene expression analysis, Huh-7 cells expressing the activated XBP1 produced much higher levels of PPARγ and C/EBPα, two key lipogenic *trans*-activators, compared to those expressing GFP (**Figure 6A-B**). These results confirm the role of the UPR in activating hepatic lipogenesis. It should be noted that over-expression of the activated XBP1 has mild or no effects on activating expression of other lipogenic regulators or enzymes that are inducible by ER stress challenge (data not shown), suggesting that ER stress may regulate the ER stress-inducible lipogenic factors through the other UPR branches. Nevertheless, our study indentified two ER stress-inducible lipogenic *trans*-activators, C/EBPα and PPARγ, that are under the regulation of the IRE1α/XBP1-mediated UPR pathway.

Discussion

In this study, we demonstrated that pharmacologic ER stress, induced by two structurally-unrelated ER stress-inducing reagents TM and Tg, can promote *de novo* lipogenesis in the hepatoma cell line Huh-7. Both lipid droplet phenotype and gene expression profile further validated the effect of pharmacologic ER stress in promoting lipogenesis and lipid droplet formation (**Figures 2-5**). Importantly, we identified three groups of ER stress-inducible regulators and enzymes in *de novo* lipogenesis (**Figures 3-5**). In particular, we demonstrated that the ER stress-inducible lipogenic *trans*-activators, C/EBPα and PPARγ are regulated by the UPR *trans*-activator XBP1 (**Figure 6**). These results have important implications in the understanding of the upstream signals that facilitate *de novo* lipogenesis.

The UPR signaling is an adaptive response that protects cells from ER stress [28]. The UPR signaling mediated through IRE1α/XBP1, ATF6, and PERK/eIF2α reprograms transcription and translation of stressed cells, leading to alterations in cell physiology that helps the stressed cells adapt to ER stress. However, when ER stress gets more severe or prolonged, the same UPR signaling can activate cell death programs to remove the stressed cells. Lipid droplet is a dynamic organelle composed of a monolayer phospholipid embedded with numerous proteins without trans-membrane spanning domains, and a hydrophobic core that contains triglycerides and sterol esters [29]. Under normal physiological conditions, hepatic lipid droplets are important to maintain lipid and energy homeostasis at the cellular and organismal levels. As a defense response to acute liver injuries, accumulation of lipid droplets is increased in the liver of animal models [6, 7, 30]. Our study suggests that the UPR-regulated *de novo* lipogenesis and accumulation of cytosolic lipid droplets may be parts of the protective response of liver hepatocytes to pharmacologic ER stress. On the other hand, excessive accumulation of lipid droplets is closely associated with the development of metabolic disease [31]. If ER stress-induced lipid droplet accumulation cannot be resolved, prolonged hepatic lipid droplet accumulation may result in metabolic deterioration. This is consistent with the dual roles of the UPR in mediating survival and death signals in the context of cell pathophysiology.

Our work demonstrated that pharmacologic ER stress represents a strong stimulus that triggers *de novo* lipogenesis and lipid storage. In addition to TM or Tg, many pharmaceutical drugs, for example, clinically-used anti-cancer drug Bortezomib, are strong inducers of pharmacologic ER stress [7, 32, 33]. Although the mechanisms involved in its anticancer activity are still being elucidated, Bortezomib has been shown to cause the accumulation of misfolded proteins in the ER by inhibiting the 26S proteasome activity and subsequent ER-associated protein degradation machinery [34-36]. Previously we demonstrated that Bortezomib induces pharmacologic ER stress, causes hepatic steatosis, and increases hepatotoxicity in an animal model [7]. Our work here confirmed that phar-


A
ADRP
TM challenge
0
5 μg/ml
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FSP27
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FSP27 mRNA level
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TM challenge
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AP2 mRNA level
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Tg challenge
0
0.5 μM
1 μM
1.5 μM
AP2 mRNA level
6hr
12hr
24hr

Figure 4. Quantitative real-time RT-PCR analysis of the mRNAs encoding protein factors in lipid droplet formation, including ADRP (A), FSP27 (B), FIT2 (C), and AP2 (D), in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

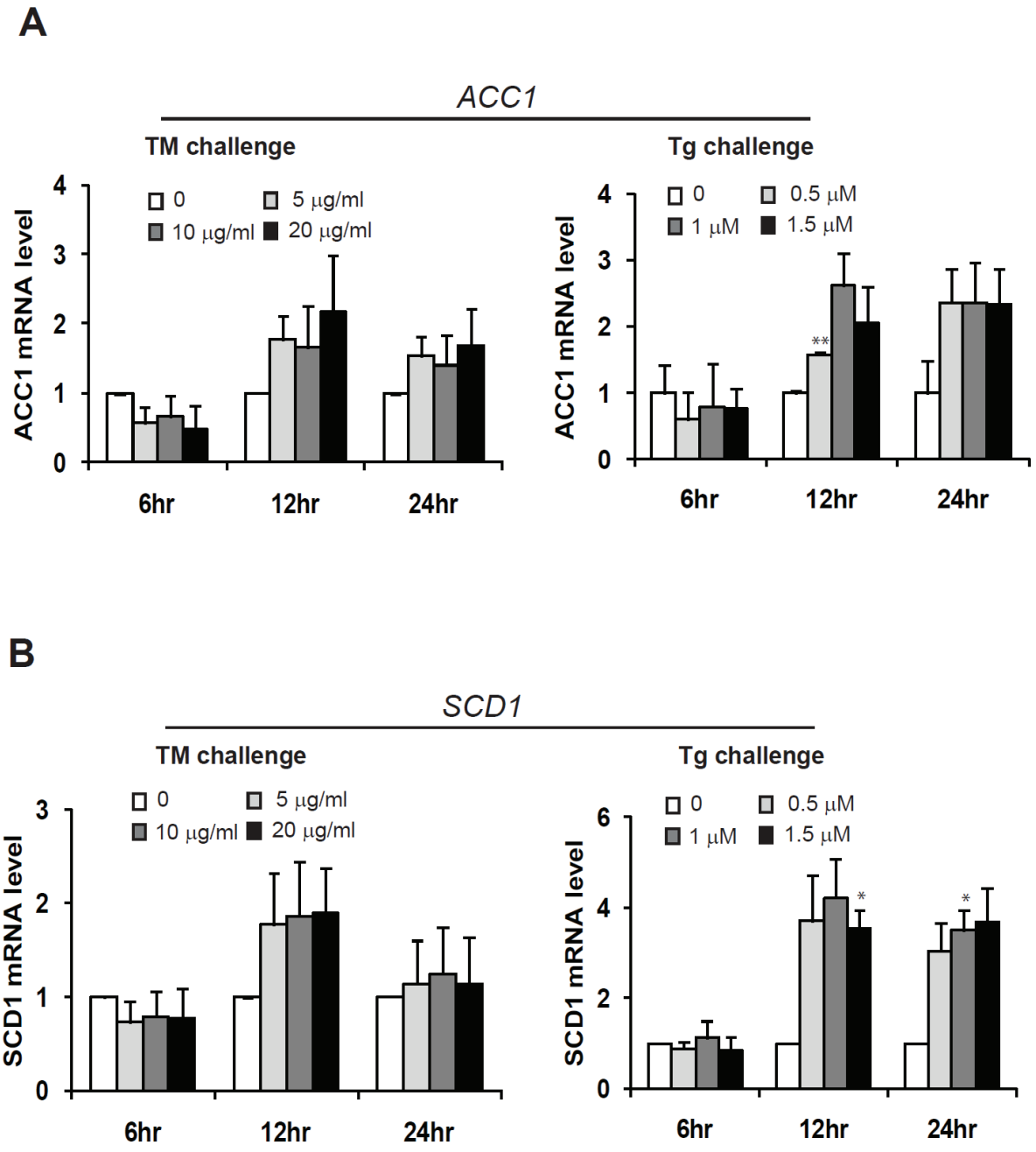

Figure 5. Quantitative real-time RT-PCR analysis of the mRNAs encoding key enzymes in triglyceride synthesis, including ACC1 (A) and SCD1 (B), in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

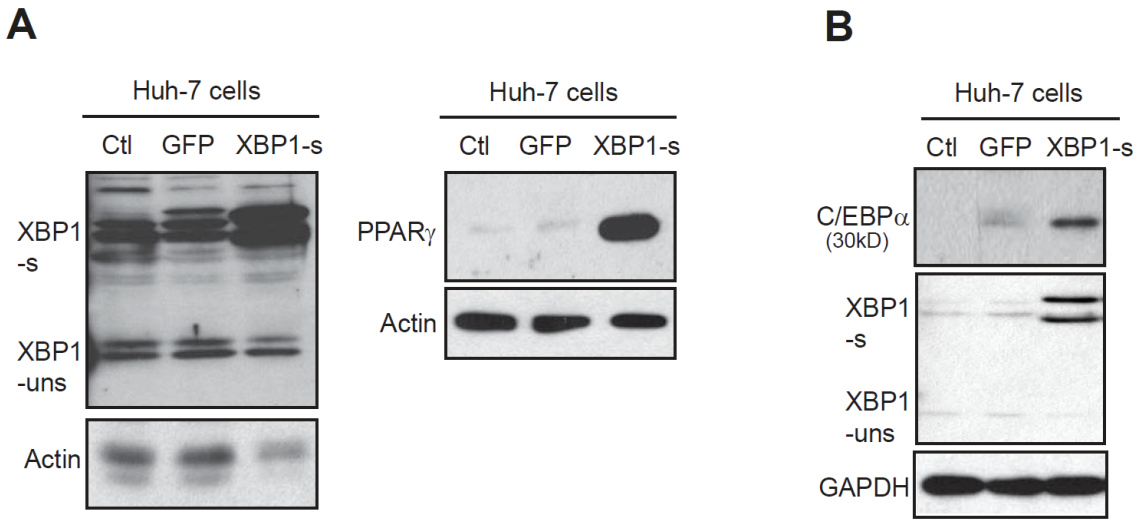

Figure 6. Western blot analysis of expression levels of PPARγ2 (A) and C/EBPα (B) proteins in Huh-7 cells. Huh-7 cells were infected with an adenovirus expressing an activated form of human XBP1 protein or GFP control. Levels of β-actin or GAPDH were determined as a loading control. XBP1-s, the activated form of XBP1 protein encoded by the spliced *XBP1* mRNA; XBP1-uns, the inactivated XBP1 protein encoded by the un-spliced *XBP1* mRNA.

maceuticals that directly or indirectly induce ER stress *in vivo* may have side-effects on induction of hepatic steatosis by promoting lipogenesis and lipid deposition.

In summary, our study provides mechanistic evidence that pharmacologic ER stress and its associated UPR signaling can directly regulate hepatic lipid metabolism by stimulating lipogenesis and lipid droplet accumulation. The identification of the ER stress-inducible lipogenic regulators and enzymes provides important insights into the molecular link between ER stress and lipid metabolism. Additional investigations need to be done in the future in order to delineate the regulation of these individual ER stress-inducible targets by the UPR branches. Nevertheless, the findings from this study significantly contribute to our understanding of pathophysiological roles of ER stress and the UPR as well as potential side effects of ER stress-inducing clinically-used drugs.

Acknowledgement

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Conflict of Interest

None.

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; TM, tunicamycin; Tg, thapsigargin. PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha; XBP1, X-box binding protein 1.

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