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Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells

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Abbreviations: AMPK, AMP-activated protein kinase; ATF4, activating transcription factor 4 (tax-responsive enhancer element B67); DDIT3/CHOP, DNA-damage-inducible transcript 3; CQ, chloroquine; EIF2A, eukaryotic translation initiation factor 2A, 65 kDa; EMT, epithelial-mesenchymal transition; ER, endoplasmic reticulum; ERN1, endoplasmic reticulum to nucleus signaling 1; XBP1, X-box-binding protein 1; MAP1LC3B, microtubule-associated proteins 1 light chain 3 beta; MTOR, mechanistic target of rapamycin (serine/threonine kinase); MTORC1, MTOR complex 1; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide-3 kinase; RPS6KB1, ribosomal protein S6 kinase, 70kDa, polypeptide 1; TRIB3, tribbles homolog 3 (Drosophila); 3-MA, 3-methyladenine; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; 4-PBA, 4-phenylbutyrate; AKT1, v-akt murine thymoma viral oncogene homolog 1; CDH1, cadherin 1, type 1, E-cadherin (epithelial); CTNNB1, catenin (cadherin-associated protein), beta 1, 88 kDa; CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Cip1); ATG5, autophagy related 5; ATG7, autophagy related 7; NUPR1, nuclear protein, transcriptional regulator, 1; CASP8, caspase 8, apoptosis-related cysteine peptidase; CASP3, caspase 3, apoptosis-related cysteine peptidase; PARP1, poly (ADP-ribose) polymerase 1

Salinomycin is perhaps the first promising compound that was discovered through high throughput screening in cancer stem cells. This novel agent can selectively eliminate breast and other cancer stem cells, though the mechanism of action remains unclear. In this study, we found that salinomycin induced autophagy in human non-small cell lung cancer (NSCLC) cells. Furthermore, we demonstrated that salinomycin stimulated endoplasmic reticulum stress and mediated autophagy via the ATF4-DDIT3/CHOP-TRIB3-AKT1-MTOR axis. Moreover, we found that the autophagy induced by salinomycin played a prosurvival role in human NSCLC cells and attenuated the apoptotic cascade. We also showed that salinomycin triggered more apoptosis and less autophagy in A549 cells in which *CDH1* expression was inhibited, suggesting that the inhibition of autophagy might represent a promising strategy to target cancer stem cells. In conclusion, these findings provide evidence that combination treatment with salinomycin and pharmacological autophagy inhibitors will be an effective therapeutic strategy for eliminating cancer cells as well as cancer stem cells.

Introduction

Salinomycin, a potassium ionophore,[1,2] which is extracted from *Streptomyces aibus* and previously has been used as a veterinary drug,[3,4] is potentially the first promising compound that has been screened through high throughput technology based on the cancer stem cell theory. Salinomycin can selectively target breast cancer stem cells in vitro and inhibit breast tumor seeding, growth and metastasis in vivo.[5] Moreover, salinomycin has been shown to kill a broad spectrum of transformed cells such as human colorectal cancer cells.

Many efforts have been made to decipher the molecular mechanism by which salinomycin induces cell death in cancer stem cells as well as cancer cells. Previous studies have shown that salinomycin acts as an effective inhibitor of ATP-binding cassette (ABC) transporter to overcome multidrug resistance and suppress the viability of cancer stem cells.[6,7] Recent studies indicate that salinomycin inhibits the WNT-CTNNB1 signaling pathway, which plays a crucial role in stem cell development and multiple malignancies.[8,9] Salinomycin is able to induce an increase in intracellular reactive oxygen species (ROS) levels, which contributes to BAX translocation to mitochondria and

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mitochondrial membrane depolarization. This results in cytochrome c release, activation of CASP3 and cleavage of its substrate PARP1, ultimately leading to apoptosis.[10] Salinomycin can elevate intracellular calcium levels via Na^+/Ca^{2+} exchangers, resulting in calpain activation and inducing caspase-dependent apoptosis in human neuronal cells.[11] In addition, salinomycin can increase DNA damage and decrease the expression of anti-apoptotic protein CDKN1A, which sensitizes cancer cells to the apoptotic effects of cytostatic drugs such as etoposide and doxorubicin.[12]

However, whether salinomycin induces autophagy, and the role it plays in cell death in human lung cancer cells, remain unclear. Our studies show that salinomycin induces apoptosis in a caspase-dependent manner while simultaneously inducing autophagy in human NSCLC cells. Macroautophagy (hereafter referred to as autophagy) is a highly conserved lysosomal degradation pathway in which unnecessary byproducts and damaged organelles are engulfed into double-membrane vesicles termed autophagosomes and transported to lysosomes. There, autophagosomes fuse with lysosomes and the inner cargoes are degraded and recycled. Therefore, autophagy is essential for maintaining homeostasis and it plays a prosurvival role. In other circumstances, it can stimulate a prodeath signal pathway.[13-16] Previous studies reported that autophagy was regulated by diverse signaling pathways, such as those controlled by class I PtdIns 3-kinase-AKT1 signaling, the mechanistic target of rapamycin (MTOR) kinase, the response to endoplasmic reticulum (ER) stress and the energy sensor AMP-activated protein kinase (AMPK).[17-20] In the present study, we demonstrated that salinomycin suppresses AKT1 activity through ATF4-DDIT3/CHOP-TRIB3-AKT1 axis in human cancer cells after activation of ER stress response, resulting in MTOR inhibition and autophagy consequently. Furthermore, autophagy induced by salinomycin plays a cytoprotective role for cell survival in human cancer cells. Based on our results, we postulate that combination therapy with salinomycin and pharmacological autophagy inhibitors will be a therapeutic strategy for killing cancer stem-like cells as well as cancer cells effectively.

Results

Salinomycin induces autophagy in human cancer cells. To examine the effects of salinomycin on cell survival in human cancer cell lines, we treated six human cancer cell lines including four human NSCLC cell lines A549, H460, Calu-1 and H157, one human esophageal carcinoma cell line TE3, and 1 human pancreatic carcinoma cell line PANC-1 with salinomycin at concentrations ranging from 1.25 to 5 μM. We found that salinomycin effectively decreased the survival of the indicated cells in a dose-dependent manner (**Fig. 1A**). To determine whether salinomycin induced autophagy, we treated three human NSCLC cell lines A549, Calu-1 and H157 with salinomycin. The conjugation of the soluble form of MAP1LC3 (MAP1LC3-I) with phosphatidylethanolamine and conversion to a nonsoluble form (MAP1LC3-II) is a hallmark of autophagy;[21] thus we examined the expression of MAP1LC3B-II formation. After treatment with salinomycin (2.5 μM) at the indicated times or with salinomycin at the indicated concentrations for 24 h, MAP1LC3B-II levels increased in all three cell lines in both time-dependent (**Fig. 1B**), and dose-dependent manner (**Fig. 1C**).

To monitor autophagosome formation, we constructed a Calu-1 cell line stably expressing the *EGFP-MAP1LC3B* fusion gene and designated it as Calu-1-EGFP-MAP1LC3B. We treated Calu-1-EGFP-MAP1LC3B with 2.5 μM salinomycin for the indicated times and quantified the EGFP-MAP1LC3B fluorescence. The results showed that the average number of EGFP-MAP1LC3B puncta per cell increased in a time-dependent manner (**Fig. 1D**). We also treated Calu-1-EGFP-MAP1LC3B cell line with salinomycin at the indicated concentrations for 24 h and found that the average number of EGFP-MAP1LC3B puncta per cell increased in a dose-dependent manner (**Fig. 1E**).

To monitor autophagic flux induced by salinomycin, we transfected both Calu-1 and Calu-1-EGFP-MAP1LC3B cells with *ATG5* or *ATG7* siRNAs to inhibit the expression of ATG5 or ATG7. Then, we measured the conversion of MAP1LC3B-II in Calu-1 cells and EGFP-MAP1LC3B puncta in Calu-1-EGFP-MAP1LC3B cells, respectively. After treatment with salinomycin (2.5 μM) for 12 h, the results showed that two different siRNAs against *ATG5* (#1 and #2) or *ATG7* (#1 and #2) decreased MAP1LC3B-II formation and *ATG5* siRNA (#1) or *ATG7* siRNA (#1) significantly and reduced EGFP-MAP1LC3B puncta (**Fig. 2A and B**). Furthermore, we cotreated both Calu-1 and Calu-1-EGFP-MAP1LC3B cells with salinomycin (2.5 μM) and autophagy inhibitors at different concentrations. The results demonstrated that coincubation with salinomycin and 3-MA (10 mM) or LY294002 (10 μM), both of which block the upstream steps of autophagy, reduced MAP1LC3B-II and EGFP-MAP1LC3B puncta accumulation (**Fig. 2C and D**). In contrast, coincubation with salinomycin (2.5 μM) and bafilomycin A_1 (20 nM) or chloroquine (CQ) (3 μM), both of which block the downstream steps of autophagy, increased conversion of MAP1LC3B-II and EGFP-MAP1LC3B puncta (**Fig. 2E and F**). Collectively, these data confirm that salinomycin induces autophagy in human NSCLC cells.

Salinomycin induces autophagy via ER stress-dependent upregulation of ATF4 and DDIT3. Since endoplasmic reticulum (ER) stress is one of the signaling pathways involved in regulation of autophagy,[22,23] we hypothesized that ER stress might play an important role in salinomycin-induced autophagy. To determine whether salinomycin induces ER stress, we measured the expressions of ERN1, phospho-EIF2A (p-EIF2A), ATF4 and DDIT3 proteins that are typically regarded as hallmarks of ER stress response. We treated indicated human NSCLC cell lines with (i) salinomycin at the indicated concentrations for 24 h or (ii) salinomycin (2.5 μM) at the indicated times. We measured the expression levels of ERN1, p-EIF2A, ATF4 and DDIT3 by western blot analysis. The levels of the four proteins increased in both dose-dependent and time-dependent manners (**Fig. 3A and B**), suggesting salinomycin activates ER stress in human NSCLC cells.

ERN1 activates the unconventional splicing of X-box binding protein 1 (*XBP1*) mRNA in response to ER stress.[24,25] The

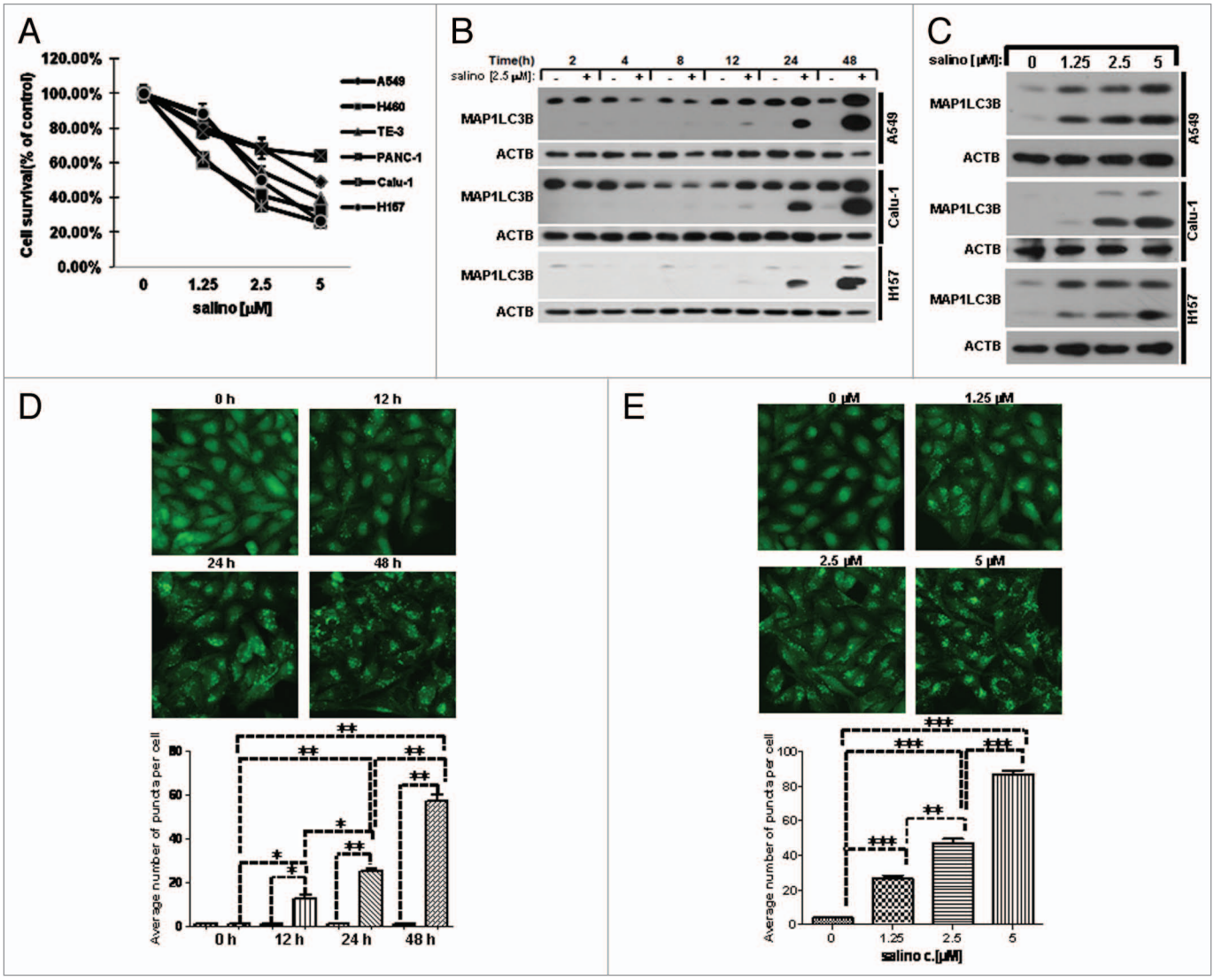

Figure 1. Salinomycin induces autophagy in human NSCLC cells. (**A**) The indicated cells were seeded in 96-well cell culture plates and treated with 1.25 μM, 2.5 μM and 5 μM of salinomycin on the second day. After treatment for another 48 h, the cells were fixed and subjected to estimate the cell number using the sulforhodamine B assay. (**B**) A549, Calu-1 and H157 cells were treated with 2.5 μM salinomycin for the indicated times. Levels of protein expression were analyzed by western blot using antibodies against MAP1LC3B and ACTB. (**C**) A549, Calu-1 and H157 cells were treated with the indicated concentrations of salinomycin and incubated for 24 h. Levels of protein expression were analyzed by western blot using antibodies against MAP1LC3B and ACTB. (**D**) Calu-1-EGFP-MAP1LC3B cells were treated with 2.5 μM salinomycin for the indicated period of time, and then EGFP-MAP1LC3B puncta were quantified. (**E**) Calu-1-EGFP-MAP1LC3B cells were treated with the indicated concentrations of salinomycin for 24 h, and then EGFP-MAP1LC3B puncta were quantified. Columns: mean of triplicate treatments; bars: ± SD. The statistical differences between the two treatments were analyzed by two-sided unpaired Student's t-tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

splicing results in the excision of a 26-nucleotide intron, leading to a spliced form that can be identified by the loss of a PstI restriction site.[24] To investigate *XBP1* splicing status in salinomycin-treated cells, we treated A549, Calu-1 and H157 with salinomycin at the indicated concentrations for 24 h. *XBP1* cDNA was amplified by PCR with primers encompassing the spliced sequences of *XBP1* and the PCR products were incubated with PstI. The spliced form of *XBP1* (*XBP1S*) was 447 bp and the unspliced form of *XBP1* (*XBP1U*) was digested into 290 bp and 183 bp by PstI. The data showed that the levels of *XBP1S* mRNAs were increased in a dose-dependent in all three cell lines (**Fig. 3C**), indicating salinomycin also activated the ERN1-XBP1 ER stress response branch in human cancer cells. Moreover, we noted that two fragments of 290 bp and 183 bp digested from *XBP1U* increased after salinomycin treatment. We speculate that salinomycin may enhance the transcription of *XBP1* by promoting the specific transcriptional factor to bind to the *XBP1* promoter.

To determine whether salinomycin induces autophagy via ER stress-mediated ATF4-DDIT3 axis, we transfected Calu-1 cells with *DDIT3* siRNA to inhibit DDIT3 expression. We measured DDIT3 and MAP1LC3B-II levels after treatment with salinomycin (2.5 μM) for 12 h by western blot. The results demonstrated that the level of MAP1LC3B-II significantly decreased after salinomycin exposure in DDIT3-knockdown cells compared with

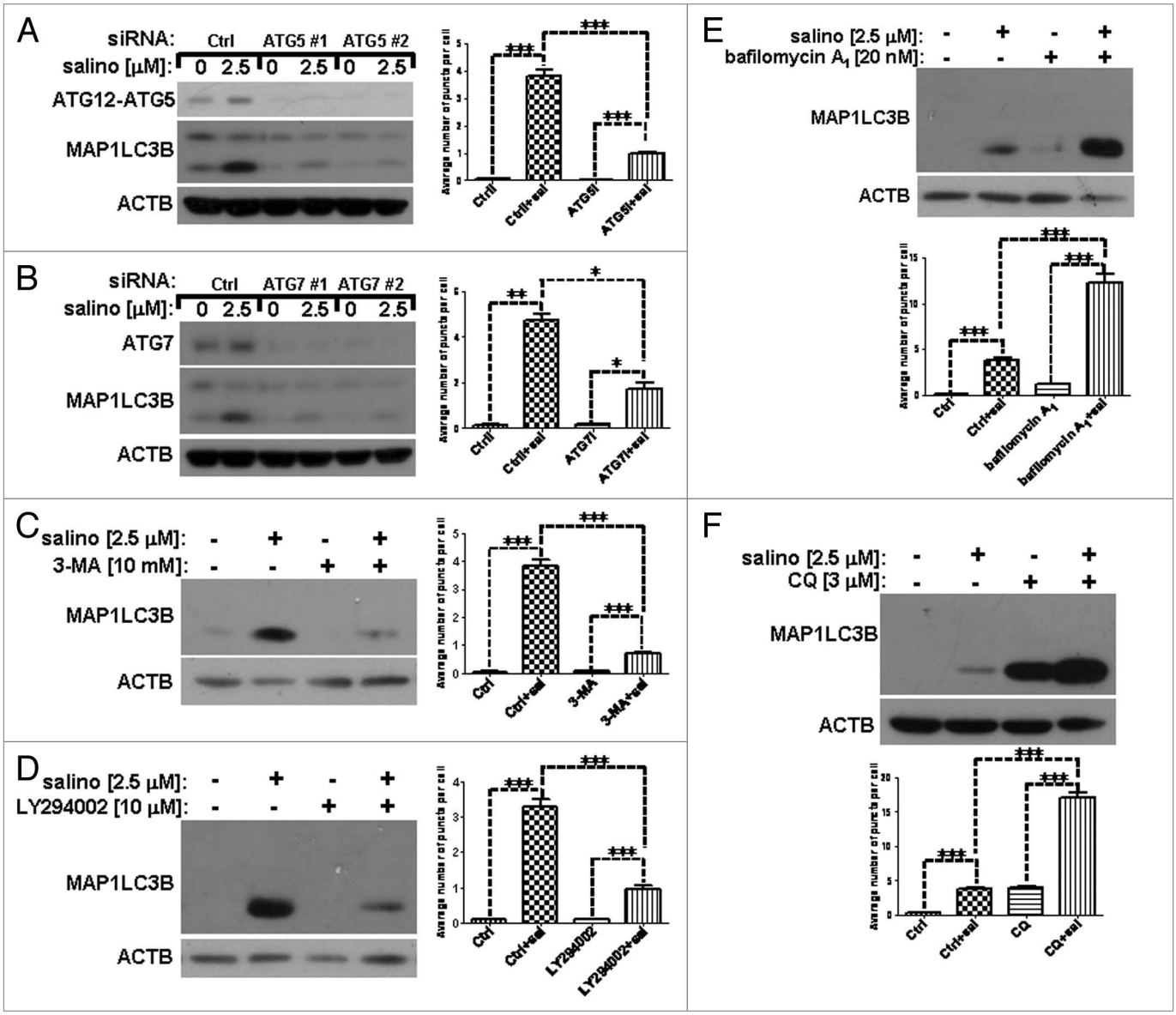

Figure 2. Salinomycin induces autophagic flux in human NSCLC cells. (**A and B**) Calu-1 cells were transfected with *ATG5* siRNAs (#1 and #2) or *ATG7* siRNAs (#1 and #2). 48 h later, cells were treated with 2.5 μM salinomycin or DMSO for 12 h, and then the samples were subjected to western blot analysis. Calu-1-EGFP-MAP1LC3B cells were transfected with *ATG5* siRNA (#1) or *ATG7* siRNA (#1). 48 h later, cells were treated with 2.5 μM salinomycin for 12 h, and then EGFP-MAP1LC3B puncta were quantified. (**C**) Calu-1 cells were pretreated with 3-MA (10 mM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h. Levels of protein expression were analyzed by western blot using antibodies against MAP1LC3B and ACTB. Calu-1-EGFP-MAP1LC3B cells were pretreated with 3-MA (10 mM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h, and then EGFP-MAP1LC3B puncta were quantified. (**D**) Calu-1 cells were pretreated with LY294002 (10 μM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h. Levels of protein expression were analyzed by western blot using antibodies against MAP1LC3B and ACTB. Calu-1-EGFP-MAP1LC3B cells were pretreated with LY294002 (10 μM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h, and then EGFP-MAP1LC3B puncta were quantified. (**E**) Calu-1 cells were pretreated with bafilomycin A_1 (20 nM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h. Levels of protein expression were analyzed by western blot using antibodies against MAP1LC3B and ACTB. Calu-1-EGFP-MAP1LC3B cells were pretreated with bafilomycin A_1 (20 nM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h, and then EGFP-MAP1LC3B puncta were quantified. (**F**) Calu-1 cells were pretreated with CQ (3 μM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h. Levels of protein expression were analyzed by western blot using antibodies against MAP1LC3B and ACTB. Calu-1-EGFP-MAP1LC3B cells were pretreated with CQ (3 μM) for 1 h, and then coincubation with salinomycin (2.5 μM) for another 12 h, and then EGFP-MAP1LC3B puncta were quantified. The average number of punctate structures per cell was quantified by confocal microscopy. Differences between groups were evaluated by Student's t-test. Columns: mean of triplicate treatments; bars: ± SD. The statistical differences between the two treatments were analyzed by two-sided unpaired Student's t-tests (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$).

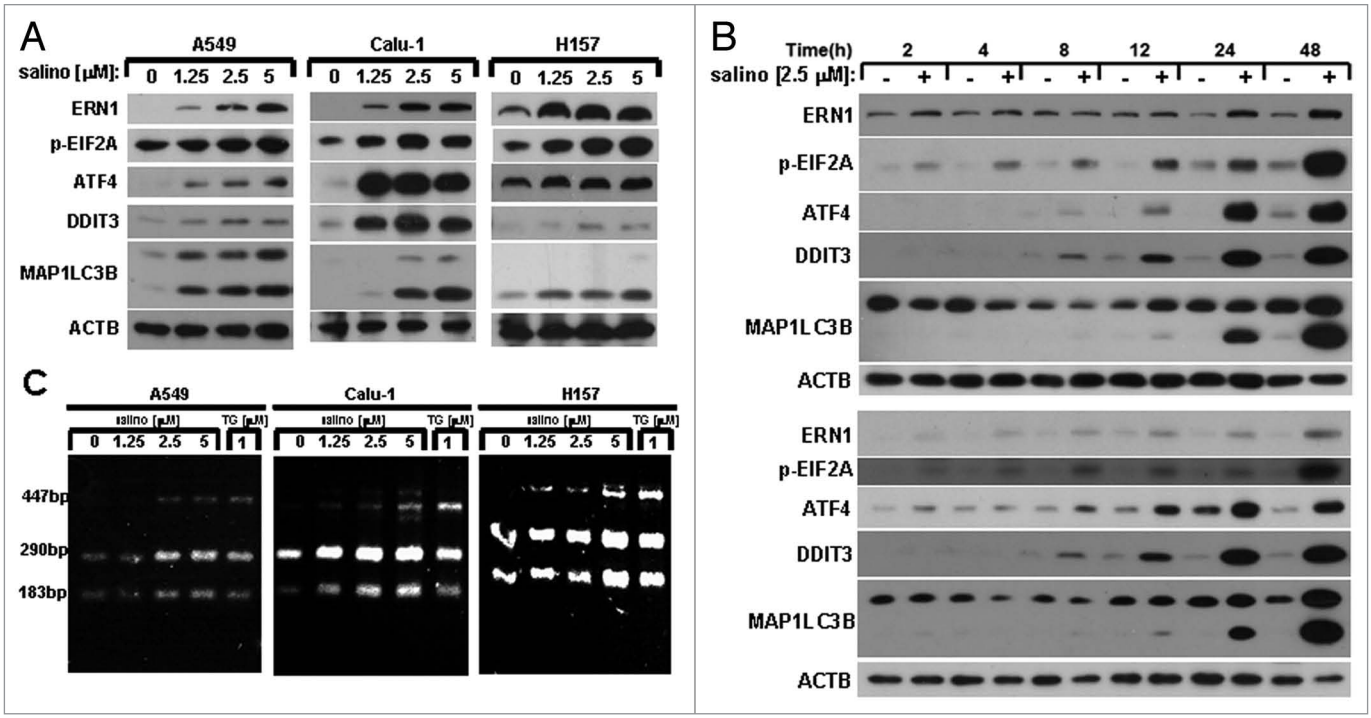

Figure 3. Salinomycin induces ER stress in human lung cancer cells. (**A**) A549, Calu-1 and H157 cells were treated with the given concentrations of salinomycin for 24 h. Levels of protein expression were analyzed by western blot using antibodies against ERN1, p-EIF2A, ATF4, DDIT3, MAP1LC3B and ACTB. (**B**) A549 and Calu-1 cells were treated with salinomycin (2.5 μM) for the indicated period of time. Levels of protein expression were analyzed by western blot using antibodies against ERN1, p-EIF2A, ATF4, DDIT3, MAP1LC3B and ACTB. (**C**) *XBP1* cDNA was amplified by PCR followed by incubation with PstI. The products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Treatment of cells with 1 μM thapsigargin for 24 h was used as a positive control.

control-knockdown cells. The identical results were obtained when using two different siRNAs against *DDIT3* (**Fig. 4A**). Then, we silenced ATF4 expression with *ATF4* siRNAs (#1 and #2) and evaluated the levels of DDIT3 and MAP1LC3B-II expression. After incubation with salinomycin (2.5 μM) for 12 h, the levels of DDIT3 and MAP1LC3B-II also dramatically decreased in ATF4-knockdown cells compared with control-knockdown cells (**Fig. 4B**). In addition, we pretreated Calu-1 and A549 cells with ER stress antagonist 4-phenylbutyrate (4-PBA) (1 mM) for 30 min followed by incubation with salinomycin (2.5 μM) for another 12 h and then measured the expressions of ATF4, DDIT3 and MAP1LC3B-II. This revealed that coincubation with salinomycin and 4-PBA decreased the expressions of ATF4, DDIT3 and MAP1LC3B-II (**Fig. 4C**). Thus, salinomycin induced autophagy via the ATF4-DDIT3 signaling axis. Taken together, these data demonstrated that salinomycin induces autophagy through activation of ER stress in human NSCLC cells.

Salinomycin induces autophagy via ATF4-DDIT3-TRIB3-AKT1-MTOR axis. MTOR acts as a central regulator in autophagy induction, and AKT1 modulates the activation of MTOR.[26,27] Thus, we investigated whether salinomycin-induced autophagy occurred via MTOR inhibition. We detected the levels of phospho-AKT1 (p-AKT1), phospho-RPS6KB1 (p-RPS6KB1) and phospho-EIF4EBP1 (p-EIF4EBP1) the latter two being MTORC1 substrates, in three human NSCLC cell lines A549, Calu-1 and H157. We treated all three cell lines with salinomycin at the indicated concentrations for 24 h, and then measured the expressions of p-AKT1, p-RPS6KB1 and p-EIF4EBP1 by western blot analysis. We found that the levels of p-AKT1, p-RPS6KB1 and p-EIF4EBP1 proteins decreased in a dose-dependent manner after salinomycin treatment (**Fig. 5A**), suggesting that autophagy is induced by salinomycin via AKT1-MTOR inhibition in human NSCLC cells.

Since DDIT3 may inhibit AKT1 activation by upregulation of TRIB3 expression,[28,29] we treated A549, Calu-1 and H157 cells with salinomycin at the indicated concentrations for 24 h or treated A549 and Calu-1 cell with salinomycin (2.5 μM) at the indicated times, then measured the expression of TRIB3. The upregulation of the TRIB3 occurred in both dose-dependent and time-dependent manners, which happened after the expression of DDIT3 and ATF4 (**Fig. 5B and C**). To investigate whether the upregulation of TRIB3 is induced via the ATF4-DDIT3 pathway, we silenced *DDIT3* with siRNAs and measured the TRIB3 level after treatment with salinomycin (2.5 μM) for 12 h by western blot. The results demonstrated that the level of TRIB3 significantly decreased after salinomycin exposure in DDIT3-knockdown cells compared with control-knockdown cells (**Fig. 5D**).

AKT1 is a positive regulator of the MTOR pathway.[30] Therefore, we investigated whether AKT1-MTORC1 inhibition by salinomycin occurred through upregulation of TRIB3. We transfected Calu-1 cells with *TRIB3* siRNAs to knock

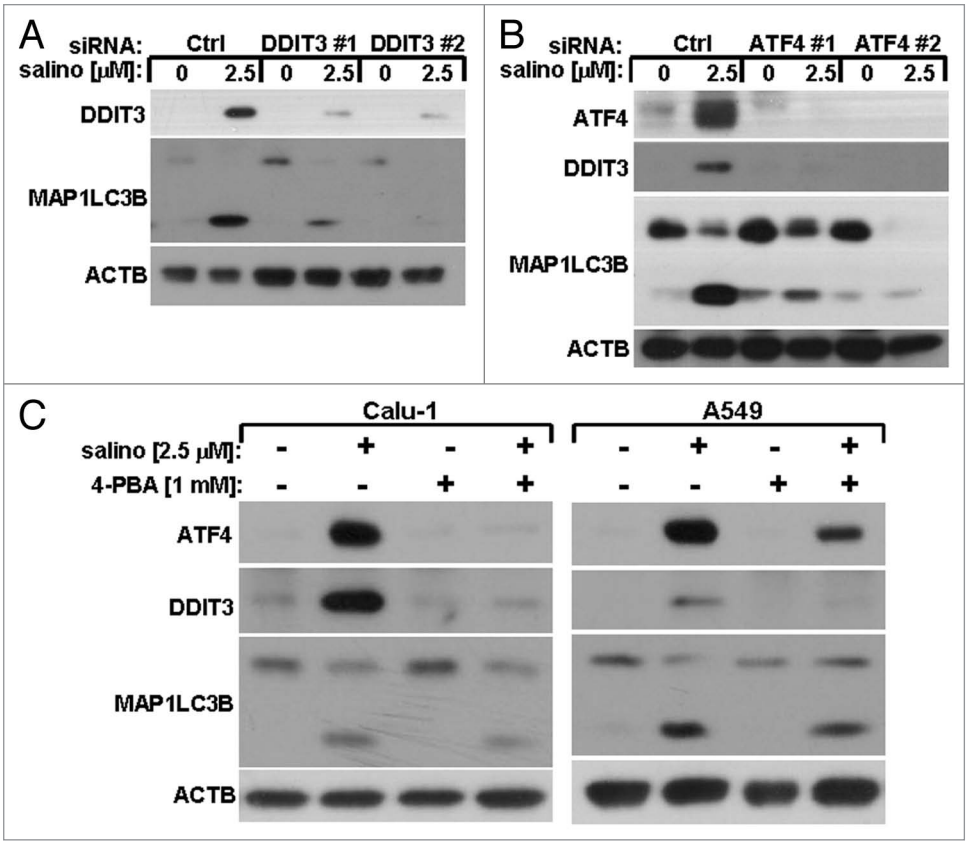

Figure 4. Salinomycin induces autophagy via ER stress-evoked ATF4-DDIT3 upregulation. (**A**) Calu-1 cells were transfected with control siRNA or *DDIT3* siRNAs (#1 and #2) for 48 h and then treated with salinomycin (2.5 μM) for 12 h. Levels of protein expression were analyzed by western blot using antibodies against DDIT3, MAP1LC3B and ACTB. (**B**) Calu-1 cells were transfected with control siRNA or *ATF4* siRNAs (#1 and #2) for 48 h and then treated with salinomycin (2.5 μM) for 12 h. Levels of protein expression were analyzed by western blot using antibodies against ATF4, DDIT3, MAP1LC3B and ACTB. (**C**) A549 and Calu-1 cells were pretreated with 4-PBA (1 mM) for 30 min and then cotreated with salinomycin (2.5 μM) for another 12 h. Levels of protein expression were analyzed by western blot using antibodies against ATF4, DDIT3, MAP1LC3B and ACTB.

down TRIB3 expression, and then we assessed the expression levels of TRIB3, p-AKT1, p-RPS6KB1, p-EIF4EBP1 and MAP1LC3B-II after 12 h treatment with 2.5 μM salinomycin. In TRIB3-knockdown cells, the expression of p-AKT1, p-RPS6KB1, and p-EIF4EBP1 recovered after salinomycin treatment. We observed similar results with two different siRNAs against *TRIB3* (**Fig. 5E**). We also transfected Calu-1 cells with *ATF4* siRNAs to knock down ATF4 expression and then assessed the levels of DDIT3, TRIB3, p-AKT1, p-RPS6KB1, p-EIF4EBP1 and MAP1LC3B-II after treatment with 2.5 μM salinomycin for 12 h. Consistent with our previous results, ATF4 knockdown reduced the levels of TRIB3, DDIT3 and MAP1LC3B-II. However, the levels of p-AKT1, p-RPS6KB1, p-EIF4EBP1 were partially recovered in ATF4-knockdown cells compared with control-knockdown cells following salinomycin treatment. We discovered similar results with two different siRNAs against *ATF4* (**Fig. 5F**), indicating the ATF4-DDIT3-TRIB3-AKT1-MTOR axis played a critical role in salinomycin-induced autophagy in lung cancer cells.

Autophagy plays a protective role in salinomycin-treated NSCLC cells. Since autophagy has dual roles in cell survival and cell death,[31,32] we next investigated the role of autophagy in salinomycin-induced cell death in human NSCLC cells. To determine whether salinomycin-induced autophagy promotes or attenuates apoptosis in lung cancer cells, we transfected A549 cells with *ATG5* or *ATG7* siRNAs to block the expression of ATG5 and ATG7. We then measured the cleavage of CASP3 and PARP1 after treatment with 5 μM salinomycin for 48 h. There was a pronounced increase in the cleavage of CASP3 and PARP1 in ATG5- or ATG7-depleted cells after treatment with salinomycin, compared with control cells (**Fig. 6A**). We also used *ATG5* siRNA (#1) and *ATG7* siRNA (#1) to knock down the expressions of ATG5 and ATG7 in A549, Calu-1 and H157 cells and then treated the cells with salinomycin (5 μM) for 72 h and measured DNA fragmentation. After treatment with salinomycin, the amount of DNA fragmentation increased in ATG5- or ATG7-knockdown cells compared with that in the control cells (**Fig. 6B**). Moreover, salinomycin treatment induced 31.1% apoptosis in ATG5-knockdown cells and 39.5% apoptosis in ATG7-knockdown cells, whereas it caused only 23.6% apoptosis in control-knockdown cells when evaluated by ANXA5-PE/7-AAD staining and flow cytometry analysis (**Fig. 6C**). Next, we examined the effects of combination treatment with autophagy inhibitors and salinomycin on cellular apoptosis in A549 cells. The combination of chloroquine and salinomycin synergized when compared with each single agent in inducing apoptosis. For example, chloroquine (3 μM) and salinomycin (5 μM) alone induced 4.1% and 31.3% of cells undergoing apoptosis, respectively, whereas their combination induced 42% of cells dying of apoptosis (**Fig. 6D**). Because salinomycin induces autophagy via an ER stress-related ATF4-DDIT3 axis, we also detected cell apoptosis in ATF4-knockdown cells treated with salinomycin. We knocked down *ATF4* expression by siRNA and then measured the cleavage of CASP3 and PARP1 after treatment with 5 μM salinomycin in A549, Calu-1 and H157 cells for 48 h. The cleavage of CASP3 and PARP1 in ATF4 depleted cells were augmented after treatment with salinomycin compared with the control cells (**Fig. S1A**). Furthermore,

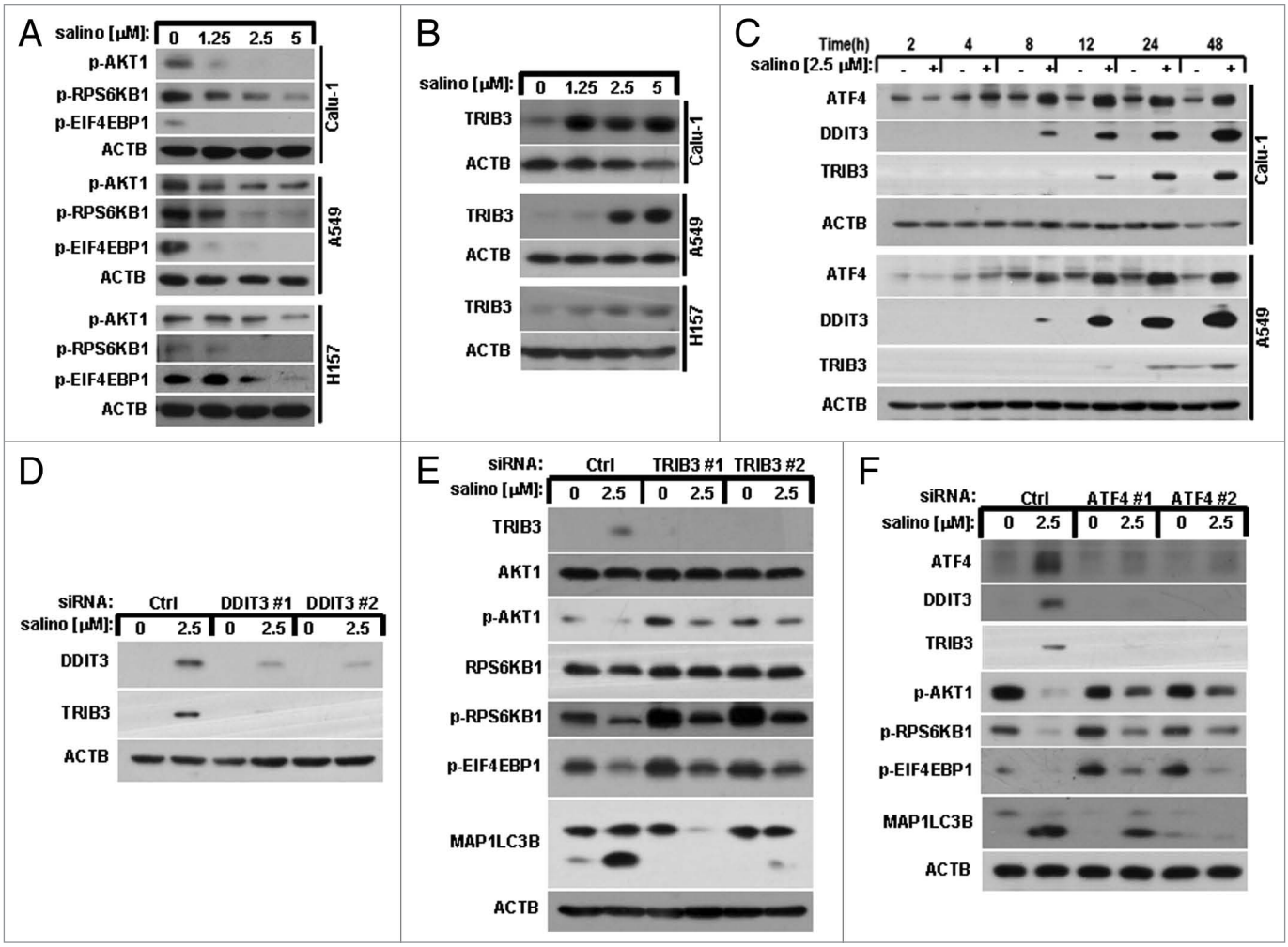

Figure 5. Salinomycin induces autophagy via the ATF4-DDIT3-TRIB3-AKT1-MTOR axis. (**A**) A549, Calu-1 and H157 cells were treated with the indicated concentration of salinomycin for 24 h. Levels of protein expression of p-AKT1, p-RPS6KB1, p-EIF4EBP1 and ACTB were measured by western blot analysis. (**B**) A549, Calu-1 and H157 cells were incubated with the given concentration of salinomycin for 24 h. Level of protein expression of TRIB3 was measured by western blot analysis. (**C**) A549 and Calu-1 cells were treated with salinomycin (2.5 μM) for the indicated period of time. Levels of protein expression were analyzed by western blot using antibodies against ATF4, DDIT3, TRIB3 and ACTB. (**D**) Calu-1 cells were transfected with control siRNA or *DDIT3* siRNAs (#1 and #2) for 48 h and then treated with salinomycin (2.5 μM) for 12 h. Levels of protein expression were analyzed by western blot using antibodies against DDIT3, TRIB3 and ACTB. (**E**) Calu-1 cells were transfected with control siRNA or *TRIB3* siRNAs (#1 and #2) for 48 h and then treated with salinomycin (2.5 μM) for 12 h. Levels of protein expression were measured by western blot analysis using antibodies against TRIB3, p-AKT1, AKT1, p-RPS6KB1, RPS6KB1, p-EIF4EBP1, MAP1LC3B and ACTB. (**F**) Calu-1 cells were transfected with control siRNA or *ATF4* siRNAs (#1 and #2) for 48 h and then treated with salinomycin (2.5 μM) for 12 h. Levels of protein expression were measured by western blot analysis using antibodies against ATF4, DDIT3, TRIB3, MAP1LC3B, p-AKT1, p-RPS6KB1, p-EIF4EBP1 and ACTB.

salinomycin treatment induced 29.17%, 29.6% and 40.68% in all three ATF4-knockdown cells compared with 18.22%, 20.24% and 25.6% in all three control cells, respectively (**Fig. S1B**). Collectively, these data confirmed that autophagy contributes to protecting cells from salinomycin-induced apoptosis in human NSCLC cells.

Salinomycin increases sensitivity to apoptosis in human NSCLC cells in which CDH1 expression is inhibited. Previous studies have shown that reduction of CDH1 level can induce the epithelial-mesenchymal transition (EMT) and lead to enrichment of cancer stem cells.[5,33,34] In addition, salinomycin can kill cancer stem cells. Therefore, we hypothesized that salinomycin might induce less autophagy in which CDH1 expression was inhibited and confer sensitivity to cell death. Accordingly, we infected A549 cells with the sh*CDH1* lentivirus to inhibit CDH1 expression. Cells infected with shCtrl lentivirus served as control. We treated cells with salinomycin at the indicated concentrations for 48 h and then assessed the expressions of MAP1LC3B and the apoptotic proteins CASP8, CASP3 and PARP1. The results showed that salinomycin increased the cleaved CASP8, CASP3 and PARP1, but decreased the MAP1LC3B-II level in A549/sh*CDH1* cells compared with A549/shCtrl cells (**Fig. 7A**). In a similar manner, salinomycin treatment increased the amount of DNA fragments (**Fig. 7B**) and induced 30.5% A549/sh*CDH1* cells compared with 19.5% A549/shCtrl cells to undergo apoptosis when measured using ANXA5-PE/7-AAD staining and flow cytometry analysis (**Fig. 7C**).

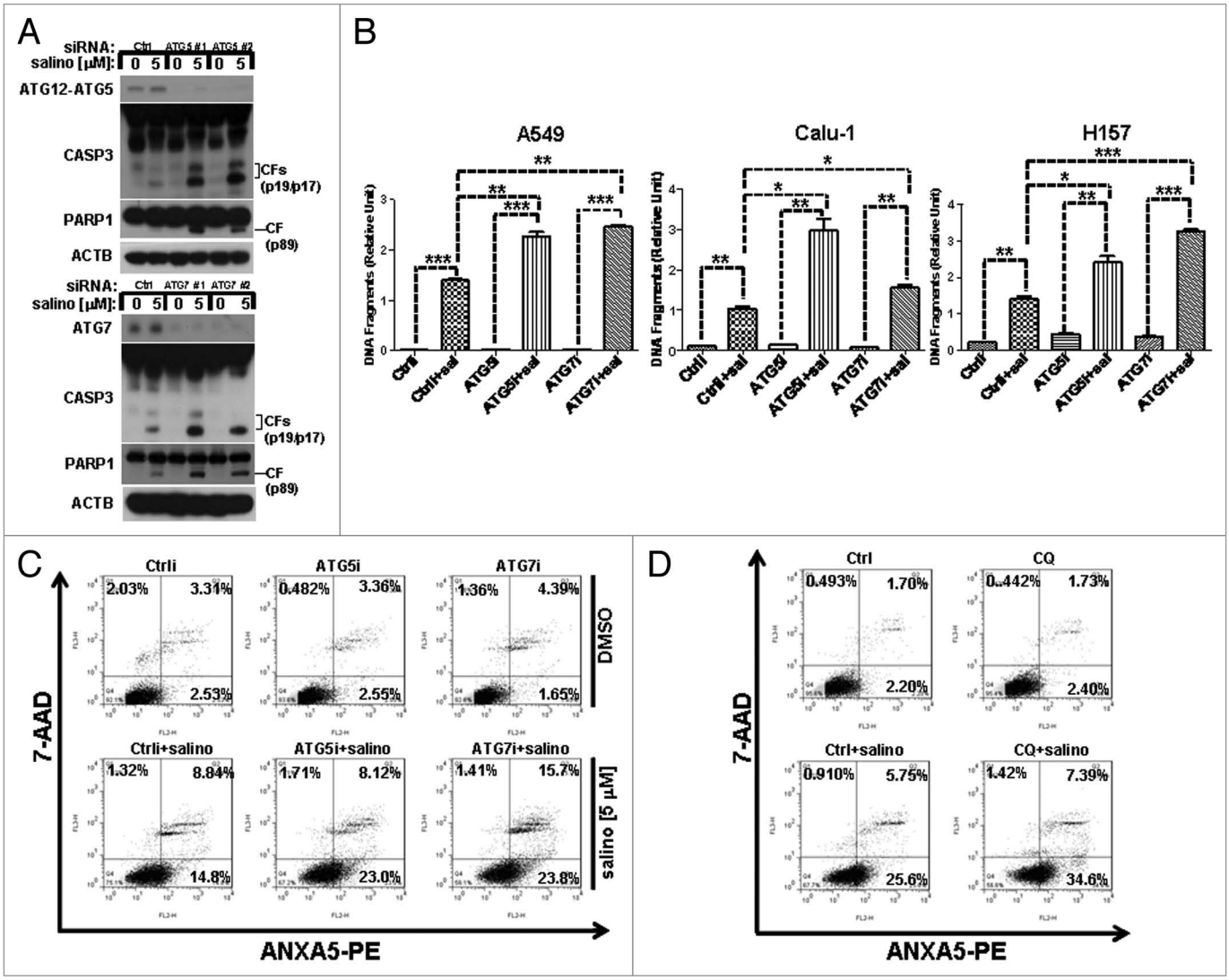

Figure 6. Autophagy plays a protective role in salinomycin-treated human NSCLC cells. (**A**) A549 cells were transfected with control siRNA, *ATG5* siRNAs (#1 and #2) or *ATG7* siRNAs (#1 and #2) for 48 h and then treated with salinomycin (5 μM) for another 48 h. Levels of protein expression were measured by western blot analysis using antibodies against ATG5, ATG7, CASP3, PARP1 and ACTB. (**B**) A549 cells were transfected with control siRNA, *ATG5* siRNA (#1) or *ATG7* siRNA (#1) for 48 h. After treatment with salinomycin (5 μM) for 72 h, cells were measured for DNA fragmentation by ELISA assay. (**C**) A549 cells were transfected with control siRNA, *ATG5* siRNA (#1) or *ATG7* siRNA (#1) for 48 h. After treatment with salinomycin (5 μM) for 72 h, cells were stained with ANXA5-PE/7-AAD and detected by flow cytometry analysis. (**D**) A549 cells were pretreated with CQ (3 μM) for 1 h, then cotreated with salinomycin (5 μM) for 72 h and were stained with ANXA5-PE/7-AAD and detected by flow cytometry analysis. Columns: mean of triplicate treatments; bars: ± SD. The statistical differences between the two treatments were analyzed by two-sided unpaired Student's t-tests (*p < 0.05; **p < 0.01; *** p < 0.001).

To monitor the changes of EGFP-MAP1LC3B puncta, we constructed A549/sh*CDH1* and A549/shCtrl cell lines stably expressing the *EGFP-MAP1LC3B* fusion gene and designated A549/sh*CDH1*-EGFP-MAP1LC3B and A549/shCtrl-EGFP-MAP1LC3B, respectively. We treated A549/sh*CDH1*-EGFP-MAP1LC3B and A549/shCtrl-EGFP-MAP1LC3B cells with 2.5 μM salinomycin for the indicated times and quantified the EGFP-MAP1LC3B-positive dots. The results showed that the average number of puncta per cell was reduced in the A549/sh*CDH1*-EGFP-MAP1LC3B cell line compared with the A549/shCtrl-EGFP-MAP1LC3B cell line (**Fig. 7D**).

Moreover, the levels of ATF4 and DDIT3 were lower in A549/sh*CDH1* cells than those in A549/shCtrl cells after salinomycin treatment. Accordingly, phosphorylation levels of RPS6KB1 and EIF4EBP1, which are essential substrates of MTOR, were partially recovered in A549/sh*CDH1* cells after salinomcyin treatment (**Fig. 7A**). Together, our data suggest that salinomycin induces more apoptosis and less autophagy in A549/sh*CDH1* cells compared with A549/shCtrl cells because MTOR inhibition is alleviated in CDH1-knockdown cells.

To determine whether CDH1 expression influences the sensitivity of cells to salinomycin, we examined the levels of CDH1 in six cell lines by western blot (**Fig. S2A**) and noted that three cell lines A549, TE3 and PANC-1 are CDH1 positive and exert more resistance when treated with 2.5 μM and 5 μM salinomycin than the other three, Calu-1, H157 and H460, in which CDH1 are

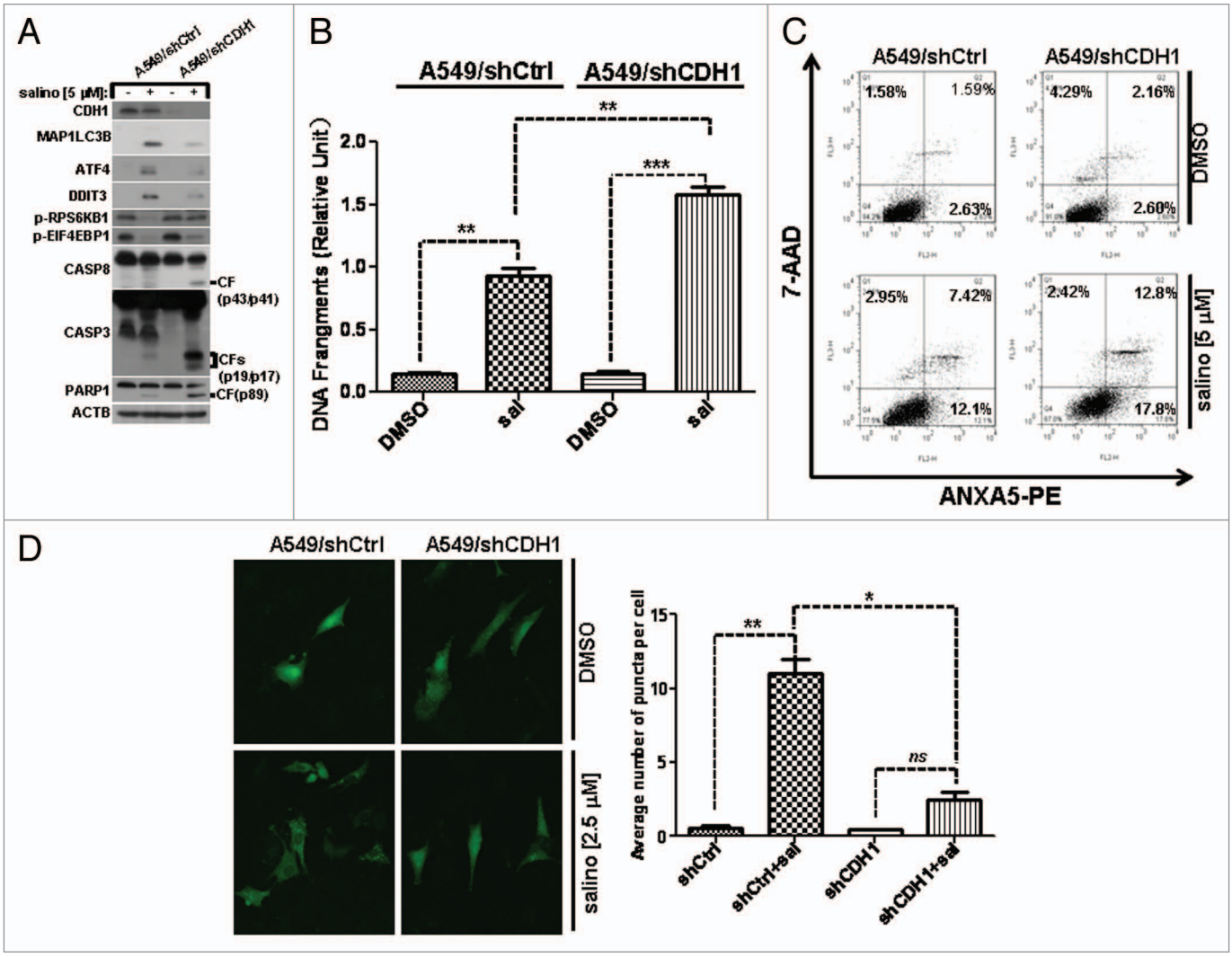

Figure 7. Salinomycin increases sensitivity to apoptosis in human NSCLC cells in which CDH1 expression is inhibited. (**A**) A549/shCtrl cells and A549/sh*CDH1* cells were treated with salinomycin (5 μM) for 48 h. Levels of protein expression were measured by western blot analysis using antibodies against CDH1, MAP1LC3B, ATF4, DDIT3, p-RPS6KB1, p-EIF4EBP1, CASP8, CASP3, PARP1 and ACTB. (**B**) A549/shCtrl cells and A549/sh*CDH1* cells were treated with salinomycin (5 μM) for 72 h, then cells were measured for DNA fragmentation by the ELISA assay. (**C**) A549/shCtrl cells and A549/sh*CDH1* cells were treated with salinomycin (5 μM) for 72 h, then cells were stained with ANXA5-PE/7-AAD and detected by flow cytometry analysis. (**D**) A549/shCtrl-EGFP-MAP1LC3B and A549/sh*CDH1*-EGFP-MAP1LC3B cell lines were treated with salinomycin (2.5 μM) for 12 h. The average number of punctate structures per cell was quantified by confocal microscopy. Columns: mean of triplicate treatments; bars: ± SD. The statistical differences between the two treatments were analyzed by two-sided unpaired Student's t-tests (*p < 0.05; **p < 0.01; *** p < 0.001; ns: no significant difference).

negative (**Fig. 1A**). In addition, we transfected TE3 and PANC-1 cells with *CDH1* siRNA to inhibit CDH1 expression and then measured CDH1, CASP3, PARP1 and MAP1LC3B-II levels after salinomycin (5 μM) for 48 h by western blot. The results demonstrated that the levels of cleaved CASP3 and PARP1 significantly increased after salinomycin exposure in CDH1-knockdown cells compared with control cells and the level of MAP1LC3B-II decreased inversely (**Fig. S2B**). In a similar manner, salinomycin treatment induced 28.9% TE3/siEcad cells or 35.3% PANC-1/siEcad cells compared with 16.3% TE3/siCtrl cells or 19.8% PANC-1/siCtrl cells to undergo apoptosis respectively (**Fig. S2C**). The results indicated that salinomycin increased sensitivity to apoptosis in human cancer cells in which CDH1 expression was inhibited.

Discussion

Salinomycin, a potassium ionophore, is the first drug reported to selectively eliminate breast cancer stem cells in vitro and inhibit breast tumor seeding, growth and metastasis in vivo effectively,[5] though the molecular mechanism remains unclear. Many efforts have been made to decipher the molecular mechanism by which salinomycin induces cell death in cancer stem cells as well as cancer cells. We, for the first time, demonstrated that salinomycin induces autophagy in human lung cancer cells.

Autophagy is one of the key mechanisms to degrade and recycle damaged and harmful cellular components to mediate metabolic adaptation and maintain energy homeostasis under the integrated stress response.[20] Our data demonstrated that salinomycin is able

to stimulate the conversion of MAP1LC3B-I to MAP1LC3B-II and increase the number of EGFP-MAP1LC3B puncta in lung cancer cells. In addition, we examined whether salinomycin could induce the autophagic flux in cancer cells. Cotreatment with salinomycin and autophagy inhibitors, such as 3-MA and LY294002, or genetic inhibition of autophagy by knocking down either the *ATG5* or *ATG7* gene decreased MAP1LC3B-II formation and reduced EGFP-MAP1LC3B puncta. In contrast, coincubation with salinomycin and autophagy inhibitors, such as bafilomycin A_1 or chloroquine, increased MAP1LC3B-II formation and EGFP-MAP1LC3B puncta, indicating salinomycin induces autophagy in human lung cancer cells.

The endoplasmic reticulum is an organelle in which synthesis, folding and maturation of proteins occur. Growing evidence indicates that the ER may act as a source of the membrane of autophagosomes.[35,36] ER stress activates the unfolded protein response (UPR), which initiates the inhibition of protein translation through phosphorylation of EIF2A.[37] The phosphorylation of EIF2A stimulates the selective translation of transcription factors NUPR1, ATF4, and DDIT3 as well as the pseudokinase TRIB3.[38,39] The pseudokinase TRIB3 inhibits the AKT1-MTORC1 axis to stimulate autophagy. Actually, many anticancer compounds (e.g., some derivatives of indole-3-carbinol) have been reported to act primarily via ER stress induction.[40-42] In our study, we demonstrated that salinomycin could upregulate ER stress-related proteins such as phospho-EIF2A, ATF4, DDIT3 in both a time-dependent and dose-dependent manner in human NSCLC cells. Furthermore, using RNA interference against *ATF4* or *DDIT3* and inhibitors of ER stress in combination with salinomycin, we confirmed that the EIF2A-ATF4-DDIT3 axis was the crucial mediator of salinomycin-induced autophagy. Moreover, our data demonstrated that salinomycin reduces activation of AKT1 as well as its downstream substrate MTOR. Taken together, we speculate that salinomycin upregulates DDIT3 due to an ER stress response and, in turn, induces TRIB3 expression, which consequently inhibits AKT1 activation and MTORC1, leading to autophagy.

The role of autophagy in cancer cells highly depends on cell types and inducers.[24,39] In our study, we investigated the relationship between autophagy and apoptosis in salinomycin-treated NSCLC cells and we found that when combined with salinomycin, apoptosis was enhanced upon ATG5 and ATG7 depletion or in autophagy inhibitor-treated cells, which suggests that autophagy contributes to promoting cell survival in salinomycin-treated human NSCLC cells.

Emerging evidence has shown that the induction of the EMT can lead to acquisition of cancer stem cells, and CDH1 inhibition by sh*CDH1* vector induced the EMT and resulted in enrichment of cells with stem cell-like traits.[5] Interestingly, we confirmed here that salinomycin increased apoptosis but reduced autophagy in A549 cells in which *CDH1* expression was silenced. These data may provide an explanation why salinomycin is able to kill migratory cancer stem cells.

In summary, in the present study, we demonstrated that salinomycin induces autophagy via ER stress response, which plays a protective role for cell survival in human NSCLC cells. Prior studies have indicated that autophagy suppression may be a therapeutic strategy for cancer treatment.[24,43] We predict that combination treatment with salinomycin and pharmacological autophagy inhibitors will be an effective therapeutic strategy since it can induce cell death not only in migratory cancer stem cells but also in cancer bulk cells.

Materials and Methods

Cell lines and cell culture. All cell lines used in this study were obtained from the American Type Culture Collection. A549 cell line was recently authenticated in Microread Gene Technology by STR analysis. The cells were maintained in RPMI 1640 medium (Sigma-Aldrich, R6504) supplemented with 5% (v/v) fetal bovine serum (FBS) (SAFC® Global, 12003C) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were treated with salinomycin (Sigma-Aldrich, S4526), 3-methyladenine (3-MA) (Sigma-Aldrich, M9281), LY294002 (Sigma-Aldrich, L9908), bafilomycin A_1 (Sigma-Aldrich, B1793), chloroquine (CQ) (Sigma-Aldrich, C6628) and 4-phenylbutyrate (4-PBA) (Sigma-Aldrich, SML0309), among which 3-MA was dissolved in water; salinomycin, LY294002, bafilomycin A_1, CQ and 4-PBA were dissolved in DMSO. The control cells were treated with DMSO only. The incubation times and concentrations of agents are stated in the figure legends.

Establishment of stable cell lines overexpressing EGFP-MAP1LC3B. The coding region of the *EGFP-MAP1LC3B* fusion gene was amplified by PCR using EGFP-MAP1LC3B plasmid as the template, which was obtained from Addgene. Lentivirus production in 293FT cells was performed as previously described.[44] Calu-1 cells were infected with EGFP-MAP1LC3B lentivirus and the stable transfectant Calu-1-EGFP-MAP1LC3B was established as previously described.[44]

Establishment of stable cell lines knocking down *CDH1*. A549 cells expressing either control shRNA (shCtrl) or *CDH1* shRNA (sh*CDH1*) were generated by infection with lentivirus encoding the pLKO.1-shCtrl and pLKO.1-sh*CDH1* plasmid, respectively. Lentivirus production in 293FT cells and the stable transfectants A549/shCtrl and A549/sh*CDH1* were established as previously described.[44]

Western blot analysis. Preparation of whole-cell protein lysates and the procedures for the western blot were previously described.[45] Primary antibodies against MAP1LC3B (2775), ATG5 (2630), ATG7 (2631), CASP8 (9746), PARP1 (9542), ERN1 (3294), phospho-EIF2A (3398), phospho-RPS6KB1 (9205), phospho-EIF4EBP1 (9451, Cell Signaling Technology), ATF4 (Santa Cruz, sc-200), phospho-AKT1 (Abcam, ab66138) were used in our study.

RNA preparation and *XBP1* splicing assay. Total RNAs from cultured cells were prepared with TRIzol Reagent (Invitrogen, 15596-018). The cDNA was reverse transcribed from 2 μg of total RNA with oligo (dT) using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, 04896866001). *XBP1* cDNA was amplified by PCR with PrimeSTAR Max DNA Polymerase (Takara, DR045S). PCR was then performed

using the sense primer 5'-AAA CAG AGT AGC AGC TCA GAC TGC-3' and antisense primer 5'-TCC TTC TGG GTA GAC CTC TGG GAG-3' encompassing the spliced sequences of human *XBP1*. The fragments were further digested by PstI (Takara, D1073A) at 37°C for 1 h, then were separated by electrophoresis on a 2% agarose gel (Lonza, 50002) and visualized by ethidium bromide staining.

siRNA transfections. siRNAs were synthesized by GenePharma (Shanghai, China). The transfection of siRNA was conducted as previously described.[45] *ATF4* #1 and *ATF4* #2 siRNAs target the sequences 5'-TCC CTC AGT GCA TAA AGG A-3' and 5'-GCC TAG GTC TCT TAG ATG A-3',[46] respectively. *DDIT3* #1 and *DDIT3* #2 siRNAs target the sequences 5'-GCC TGG TAT GAG GAC CTG C-3' and 5'-AAG AAC CAG CAG AGG UCA CAA-3',[46] respectively. *ATG5* #1 and *ATG5* #2 siRNAs target the sequences 5'-CCT TTG GCC TAA GAA GAA A-3' and 5'-CAT CTG AGC TAC CCG GAT A-3'. *ATG7* #1 and *ATG7* #2 siRNAs target the sequences 5'-GGA GTC ACA GCT CTT CCT T-3' and 5'-CAG CTA TTG GAA CAC TGT A-3', respectively.

Cell survival assay. Cells were seeded in 96-well cell culture plates and treated with salinomycin at the indicated concentrations on the second day. After treatment for another 48 h, the cells were fixed and subjected to estimating the cell number using the sulforhodamine B assay as previously described.[47]

Apoptosis assays. Apoptosis was evaluated by the ANXA5-PE/7-AAD apoptosis detection kit purchased from BD Biosciences (11774425001) following the manufacturer's instructions. Caspase activation was detected by western blot. Cytoplasmic histone-associated DNA fragments of apoptotic cells were measured by enzyme-linked immunosorbent assay (Cell Death Detection ELISA Plus kit; Roche Molecular Biochemicals, 559763), according to the manufacturer's instructions.[47]

Confocal microscopy. Calu-1-EGFP-MAP1LC3B cells were seeded into 24-well cell culture plates with 4×10^4 cells in each well. After indicated treatments, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Fixed cells were washed with PBS for three times and then examined under laser scanning fluorescence confocal microscope (Leica TCS SP5). Eight images were randomly selected for the average number of EGFP-MAP1LC3B puncta per cell.

Statistical analysis. The data of cell viability and EGFP-MAP1LC3B puncta were expressed as the mean ± SD and differences between groups were evaluated by Student's t-test. In all statistical analyses, results were considered to be statistically significant when the P-value was less than 0.05.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/24632

References

1. Ruben GC. The iso-competition point, a new concept for characterizing multivalent versus monovalent counterion competition, successfully describes cation binding to DNA. Biophys J 1999; 77:1-2; PMID:10388735; http://dx.doi.org/10.1016/S0006-3495(99)76867-0
2. Mitani M, Yamanishi T, Miyazaki Y, Otake N. Salinomycin effects on mitochondrial ion translocation and respiration. Antimicrob Agents Chemother 1976; 9:655-60; PMID:131509; http://dx.doi.org/10.1128/AAC.9.4.655
3. Daugschies A, Gässlein U, Rommel M. Comparative efficacy of anticoccidials under the conditions of commercial broiler production and in battery trials. Vet Parasitol 1998; 76:163-71; PMID:9615950; http://dx.doi.org/10.1016/S0304-4017(97)00203-3
4. Callaway TR, Edrington TS, Rychlik JL, Genovese KJ, Poole TL, Jung YS, et al. Ionophores: their use as ruminant growth promotants and impact on food safety. Curr Issues Intest Microbiol 2003; 4:43-51; PMID:14503688
5. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 2009; 138:645-59; PMID:19682730; http://dx.doi.org/10.1016/j.cell.2009.06.034
6. Fuchs D, Daniel V, Sadeghi M, Opelz G, Naujokat C. Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. Biochem Biophys Res Commun 2010; 394:1098-104; PMID:20350531; http://dx.doi.org/10.1016/j.bbrc.2010.03.138
7. Riccioni R, Dupuis ML, Bernabei M, Petrucci E, Pasquini L, Mariani G, et al. The cancer stem cell selective inhibitor salinomycin is a p-glycoprotein inhibitor. Blood Cells Mol Dis 2010; 45:86-92; PMID:20444629; http://dx.doi.org/10.1016/j.bcmd.2010.03.008
8. Lu D, Choi MY, Yu J, Castro JE, Kipps TJ, Carson DA. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. Proc Natl Acad Sci U S A 2011; 108:13253-7; PMID:21788521; http://dx.doi.org/10.1073/pnas.1110431108
9. Aranda V. Cancer: Salinomycin acts through Wnt in CSCs. Nat Med 2011; 17:1059; http://dx.doi.org/10.1038/nm.2476
10. Kim KY, Yu SN, Lee SY, Chun SS, Choi YL, Park YM, et al. Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization. Biochem Biophys Res Commun 2011; 413:80-6; PMID:21871443; http://dx.doi.org/10.1016/j.bbrc.2011.08.054
11. Boehmerle W, Endres M. Salinomycin induces calpain and cytochrome c-mediated neuronal cell death. Cell Death Dis 2011; 2:e168; PMID:21633391; http://dx.doi.org/10.1038/cddis.2011.46
12. Kim JH, Chae M, Kim WK, Kim YJ, Kang HS, Kim HS, et al. Salinomycin sensitizes cancer cells to the effects of doxorubicin and etoposide treatment by increasing DNA damage and reducing p21 protein. Br J Pharmacol 2011; 162:773-84; PMID:20973777; http://dx.doi.org/10.1111/j.1476-5381.2010.01089.x
13. Eskelinen EL. The dual role of autophagy in cancer. Curr Opin Pharmacol 2011; 11:294-300; PMID:21498118; http://dx.doi.org/10.1016/j.coph.2011.03.009
14. Chen HY, White E. Role of autophagy in cancer prevention. Cancer Prev Res (Phila) 2011; 4:973-83; PMID:21733821; http://dx.doi.org/10.1158/1940-6207.CAPR-10-0387
15. Yang SY, Winslet MC. Dual role of autophagy in colon cancer cell survival. Ann Surg Oncol 2011; 18(Suppl 3):S239; PMID:21603870; http://dx.doi.org/10.1245/s10434-011-1789-x
16. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther 2011; 10:1533-41; PMID:21878654; http://dx.doi.org/10.1158/1535-7163.MCT-11-0047
17. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol 2009; 335:1-32; PMID:19802558; http://dx.doi.org/10.1007/978-3-642-00302-8_1
18. Levine B. Cell biology: autophagy and cancer. Nature 2007; 446:745-7; PMID:17429391; http://dx.doi.org/10.1038/446745a
19. Mizushima N. Autophagy: process and function. Genes Dev 2007; 21:2861-73; PMID:18006683; http://dx.doi.org/10.1101/gad.1599207

20. Kroemer G, Mariño G, Levine B. Autophagy and the integrated stress response. Mol Cell 2010; 40:280-93; PMID:20965422; http://dx.doi.org/10.1016/j.molcel.2010.09.023
21. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010; 140:313-26; PMID:20144757; http://dx.doi.org/10.1016/j.cell.2010.01.028
22. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol 2006; 26:9220-31; PMID:17030611; http://dx.doi.org/10.1128/MCB.01453-06
23. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. J Biol Chem 2006; 281:30299-304; PMID:16901900; http://dx.doi.org/10.1074/jbc.M607007200
24. Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002; 415:92-6; PMID:11780124; http://dx.doi.org/10.1038/415092a
25. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001; 107:881-91; PMID:11779464; http://dx.doi.org/10.1016/S0092-8674(01)00611-0
26. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 2007; 8:741-52; PMID:17717517; http://dx.doi.org/10.1038/nrm2239
27. Mehrpour M, Esclatine A, Beau I, Codogno P. Overview of macroautophagy regulation in mammalian cells. Cell Res 2010; 20:748-62; PMID:20548331; http://dx.doi.org/10.1038/cr.2010.82
28. Du K, Herzig S, Kulkarni RN, Montminy M. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science 2003; 300:1574-7; PMID:12791994; http://dx.doi.org/10.1126/science.1079817
29. Matsushima R, Harada N, Webster NJ, Tsutsumi YM, Nakaya Y. Effect of TRB3 on insulin and nutrient-stimulated hepatic p70 S6 kinase activity. J Biol Chem 2006; 281:29719-29; PMID:16887816; http://dx.doi.org/10.1074/jbc.M511636200
30. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004; 18:1926-45; PMID:15314020; http://dx.doi.org/10.1101/gad.1212704
31. Thorburn A. Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis 2008; 13:1-9; PMID:17990121; http://dx.doi.org/10.1007/s10495-007-0154-9
32. Debnath J, Baehrecke EH, Kroemer G. Does autophagy contribute to cell death? Autophagy 2005; 1:66-74; PMID:16874022; http://dx.doi.org/10.4161/auto.1.2.1738
33. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133:704-15; PMID:18485877; http://dx.doi.org/10.1016/j.cell.2008.03.027
34. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 2008; 68:3645-54; PMID:18483246; http://dx.doi.org/10.1158/0008-5472.CAN-07-2938
35. Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol 2009; 11:1433-7; PMID:19898463; http://dx.doi.org/10.1038/ncb1991
36. Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 2009; 5:1180-5; PMID:19855179; http://dx.doi.org/10.4161/auto.5.8.10274
37. Milani M, Rzymski T, Mellor HR, Pike L, Bottini A, Generali D, et al. The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with Bortezomib. Cancer Res 2009; 69:4415-23; PMID:19417138; http://dx.doi.org/10.1158/0008-5472.CAN-08-2839
38. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010; 140:900-17; PMID:20303879; http://dx.doi.org/10.1016/j.cell.2010.02.034
39. Salazar M, Carracedo A, Salanueva IJ, Hernández-Tiedra S, Lorente M, Egia A, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest 2009; 119:1359-72; PMID:19425170; http://dx.doi.org/10.1172/JCI37948
40. Galluzzi L, De Santi M, Crinelli R, De Marco C, Zaffaroni N, Duranti A, et al. Induction of endoplasmic reticulum stress response by the indole-3-carbinol cyclic tetrameric derivative CTet in human breast cancer cell lines. PLoS One 2012; 7:e43249; PMID:22905241; http://dx.doi.org/10.1371/journal.pone.0043249
41. Savino JA 3rd, Evans JF, Rabinowitz D, Auborn KJ, Carter TH. Multiple, disparate roles for calcium signaling in apoptosis of human prostate and cervical cancer cells exposed to diindolylmethane. Mol Cancer Ther 2006; 5:556-63; PMID:16546969; http://dx.doi.org/10.1158/1535-7163.MCT-05-0355
42. Abdelrahim M, Newman K, Vanderlaag K, Samudio I, Safe S. 3,3'-diindolylmethane (DIM) and its derivatives induce apoptosis in pancreatic cancer cells through endoplasmic reticulum stress-dependent upregulation of DR5. Carcinogenesis 2006; 27:717-28; PMID:16332727; http://dx.doi.org/10.1093/carcin/bgi270
43. Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, et al. Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res 2011; 17:654-66; PMID:21325294; http://dx.doi.org/10.1158/1078-0432.CCR-10-2634
44. Liu X, Yue P, Schönthal AH, Khuri FR, Sun SY. Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. Cancer Res 2006; 66:11115-9; PMID:17145853; http://dx.doi.org/10.1158/0008-5472.CAN-06-2471
45. Liu X, Yue P, Zhou Z, Khuri FR, Sun SY. Death receptor regulation and celecoxib-induced apoptosis in human lung cancer cells. J Natl Cancer Inst 2004; 96:1769-80; PMID:15572759; http://dx.doi.org/10.1093/jnci/djh322
46. Liu G, Su L, Hao X, Zhong N, Zhong D, Singhal S, et al. Salermide up-regulates death receptor 5 expression through the ATF4-ATF3-CHOP axis and leads to apoptosis in human cancer cells. J Cell Mol Med 2012; 16:1618-28; PMID:21801305; http://dx.doi.org/10.1111/j.1582-4934.2011.01401.x
47. Sun SY, Yue P, Dawson MI, Shroot B, Michel S, Lamph WW, et al. Differential effects of synthetic nuclear retinoid receptor-selective retinoids on the growth of human non-small cell lung carcinoma cells. Cancer Res 1997; 57:4931-9; PMID:9354460