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Original Article

EZH2 inhibitors transcriptionally upregulate cytotoxic autophagy and cytoprotective unfolded protein response in human colorectal cancer cells

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Abstract: Enhancer of zeste homolog 2 (EZH2) has been emerged as novel anticancer target. Various EZH2 small-molecule inhibitors have been developed in recent years. A major class of EZH2 inhibitors are S-adenosyl-*L*-methionine (SAM)-competitive inhibitors, such as EPZ005687, EI1, GSK126, UNC1999 and GSK343. Autophagy, a physiological process of self-digestion, is involved in the turnover of proteins or intracellular organelles. It can serve as cytoprotective or cytotoxic function in cancer. Our previous study has found that UNC1999 and GSK343 are potent autophagy inducers. In this study, the underlying molecular mechanisms were further investigated. Our results showed that UNC1999 and GSK343 transcriptionally upregulated autophagy of human colorectal cancer (CRC) cells through inducing LC3B gene expression. Besides, UNC1999/GSK343-induced autophagy was partially dependent on ATG7 but independent to EZH2 inhibition. Microarray and PCR array analyses identified that UNC1999 and GSK343 also induced endoplasmic reticulum (ER) stress and unfolded protein response (UPR). UNC1999/GSK343-induced ER stress/UPR contributed to the survival of cancer cells, which was opposite to UNC1999/GSK343-induced autophagy that promoted cell death.

Keywords: Autophagy, colorectal cancer, ER stress, EZH2, unfolded protein response

Introduction

Autophagy is a process for the degradation of proteins and organelles through the lysosomal pathway. It acts as a temporary survival mechanism in response to nutrient starvation through self-digestion to provide an alternative energy source [1]. Autophagy involves three stages including initiation (phagophore formation), elongation (autophagosome growth and closure) and maturation (autophagosome-lysosome fusion) [2, 3]. Various signaling pathways have been implicated in the regulation of autophagy. Autophagy is inhibited by mammalian target of rapamycin (mTOR)-dependent signaling and interruption of mTOR signaling by rapamycin is known to stimulate autophagy [4]. The biochemical mechanism by which mTOR inhibits autophagy is involved a protein complex associated with the kinase ATG1 (ULK1) [5]. The PI3K pathway is important for autophagy [6]. Class I and class III PI3K differently regulate autophagy. The class I PI3K is a negative regulator of autophagy. Activation of class I PI3K and AKT leads to the activation of mTOR and inhibits autophagy. By contrast, the class III PI3K promotes autophagy through interaction with Beclin 1 [7, 8]. The energy-sensing enzyme AMP-activated protein kinase (AMPK) plays a major role in the regulation of cellular lipid and protein metabolism [9]. Activation of AMPK is reported to induce autophagy through phosphorylation and activation of the tuberous sclerosis protein 1 (TSC1)/TSC2 complex that negatively regulates mTOR complex [10]. In addition, the autophagy-initiating kinase ULK1 is recently reported to be phosphorylated and activated by AMPK [11].

Overexpression of enhancer of zeste homolog 2 (EZH2), a histone H3 lysine 27 (H3K27)-specific methyltransferase, has been found in tumors to

inhibit the expression of tumor suppressor genes [12-14]. Inhibition of EZH2 to reactivate tumor suppressive genes is regarded as an attractive anticancer strategy [15, 16]. Several potent inhibitors of EZH2 have been developed in recent years [17]. S-adenosyl-*L*-methionine (SAM) is a universal methyl donor for catalytic reactions of histone methyltransferases. A major class of EZH2 inhibitors belong to SAM-competitive inhibitors, such as EPZ005687, EI1, GSK126, GSK343 and UNC1999 [18-22]. Our recent study has demonstrated for the first time that EZH2 inhibitors, GSK343 and UNC1999, induce autophagy in an EZH2-dependent manner, leading to cell death of cancer cells [23]. However, the underlying molecular mechanisms are still unclear. In this study, we demonstrated that EZH2 inhibitors transcriptionally induced autophagy in human colorectal cancer (CRC) cells through the upregulation of LC3B gene expression. In parallel, EZH2 inhibitor activated PERK/eIF2α arm of the UPR pathways that promoted cell survival. Therefore, inhibitor of ER stress enhanced EZH2 inhibitor-induced cytotoxicity.

Materials and methods

Materials

RPMI-1640 medium, L-glutamine, sodium pyruvate, and Antibiotic: Antimycotic Solution (penicillin G, streptomycin and amphotericin B) were purchased from Life Technologies (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY, USA). EZH2, LC3B, ATG5, ULK1, GRP78, TRB3, GAPDH, Tubulin, and β-Actin antibodies were purchased from GeneTex (Hsinchu, Taiwan). ATG7 and eIF2α antibodies was purchased from Santa Cruz (Island, CA, USA). Phospho-eIF2α (p-eIF2α) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). ATF4 antibody was purchased from ProteinTech Group (Chicago, IL, USA). Horseradish peroxidase-labeled goat anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). pCMV-EZH2 plasmid was purchased from Addgene (Cambridge, MA, USA). PolyJet™ In Vitro DNA Tranfection Reagent was purchased from SignaGen Laboratories (Ijamsville, MD, USA). siGENOME human EZH2 and ATG5 SMARTpool siRNAs, siGENOME Non-Targeting human siRNA Pool, ON-TARGETplus human LC3B SMARTpool siRNA, ON-TARGETplus human Non-Targeting siRNA Pool, and DharmaFECT 4 siRNA Transfection Reagent were purchased from Dharmacon (Lafayette, CO, USA). UNC1999 and 3-methyladenine (3-MA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). GSK343 was purchased from BioVision (Mountain View, CA, USA). Bafilomycin A1 was purchased from LC Laboratories (Woburn, MA, USA). GSK2606414 was purchased from ApexBio Technology (Houston, TX, USA). Dimethyl sulfoxide (DMSO) and 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Protease and phosphatase inhibitor cocktails were purchased from Roche (Indianapolis, IN, USA). Other chemicals or reagents not specified were purchased from OneStar Biotechnology (New Taipei City, Taiwan).

Cell culture

Human colorectal cancer cells (HCT116, LoVo, HCT-15, and DLD-1) were kindly provided by Prof. Ya-Wen Cheng (Taipei Medical University, Taipei, Taiwan). ATG7-wildtype (ATG7-WT), ATG7-knockout (ATG7-KO), ULK1-wildtype (ULK1-WT) and ULK1-dominant-negative mutant (ULK1-DN) DLD-1 cells were purchased from Horizon Discovery (Cambridge, UK). These cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 1% L-glutamine, 1% Antibiotic:Antimycotic Solution, and incubated at 37°C in a humidified incubator containing 5% CO_2.

Cell viability assay

Cell viability was measured with an MTT assay. Cells were plated in 96-well plates and treated with drugs. After 72 h of incubation, 0.5 mg/mL of MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was then dissolved in 200 μL of DMSO. The absorbance at 550 nm was measured on a multiwell plate reader.

Western blot analysis

Cells were lysed in an ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1% NP-40, 10% glycerol, 1 mM DTT, 1 × protease inhibitor cocktail and 1 ×

phosphatase inhibitor cocktail at 4°C for 30 min. Cell lysates were separated on a sodium dodecylsulfate (SDS)-polyacrylamide gel, and then transferred electrophoretically onto the Hybond-C Extra nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was pre-hybridized in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween-20 (TBST buffer), and 5% skim milk for 1 h, and then transferred to a solution containing 1% bovine serum albumin (BSA)/TBST and a primary antibody and incubated overnight at 4°C. After washing with the TBST buffer, the membrane was submerged in 1% BSA/TBST containing a horseradish peroxidase-conjugated secondary antibody for 1 h. The membrane was washed with TBST buffer, and then developed with an enhanced chemiluminescence (ECL) system (Perkin-Elmer, Boston, MA, USA) and exposed to x-ray film (Roche, Indianapolis, IN, USA).

Fluorescence microscopic analysis of autophagic vacuoles

Formation of autophagic vacuoles was monitored using a Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's protocol. Briefly, cells were washed twice in phosphate-buffered saline (PBS) containing 5% FBS and then stained with Cyto-ID Detection Reagent and Hoechst 33342. After 30 min of incubation at 37°C, cells were washed and examined by fluorescence microscopy.

Transient transfection

For EZH2 overexpression, human EZH2-overexpressing (pCMV-EZH2) and its control (pCMV) plasmids were transiently transfected into cells with PolyJet™ In Vitro DNA Tranfection Reagent according to the manufacturer's instructions. For siRNA knockdown analysis, human EZH2, ATG5, LC3B and control siRNAs were transiently transfected into cells with DharmaFECT 4 siRNA Transfection Reagent according to the manufacturer's instructions. Twenty-four hours after transfection, the transfection mixture was replaced with fresh complete medium and cells were used for further experiments.

Microarray analysis and gene set enrichment analysis (GSEA)

Total RNA was extracted from HCT116 cells that were treated with 5 μM UNC1999 and 10 μM GSK343 for 4 h by the GENEzol TriRNA Pure Kit (Geneaid Biotech; New Taipei City, Taiwan). Microarray analysis using Agilent SurePrint G3 Human GE 8 × 60 K Microarray (Agilent Technologies) was performed by Welgene Biotech Company (Taipei, Taiwan). The raw data were deposited in NCBI GEO database (GSE83633). Gene set enrichment analysis (GSEA) was performed using GSEA v2.2.2 (http://www.broadinstitute.org/gsea/) provided by the Broad Institute of MIT and Harvard (Cambridge, MA, USA) [24, 25]. Enrichment analysis of the 50 cancer hallmarks from the Molecular Signatures Database (MSigDB) v5.1 was performed with default parameter setting [25, 26].

Real-time quantitative PCR (qPCR)

Total RNA (1 μg) was reverse-transcribed for 30 min at 42°C with the iScript cDNA Synthesis Kit according to the supplier's standard protocol (Bio-Rad Laboratories; Richmond, CA, USA). qPCR was performed using the following conditions: 10 min at 95°C and 45 cycles of 10 sec at 95°C and 30 sec at 60°C. The 2 × SYBR Green PCR Master Mix (Roche) and 200 nM of forward and reverse primers were used (human LC3B: forward 5'-AACGGGCTGTGTGAGAAA-AC-3' and reverse 5'-AGTGAGGACTTTGGGTG-TGG-3'; human β-Actin: forward 5'-GTTGCTAT-CCAGGCTGTGCT-3' and reverse 5'-AGGGCATA-CCCCTCGTAGAT-3'). Each assay was performed on a LightCycler Nano Real-Time PCR System (Roche) in triplicate, and the fold-changes in expression were derived using the comparative CT method calculated by LightCycler Nano Software v1.1 (Roche).

PCR array

Human Unfolded Protein Response Plus RT^2 Profiler PCR Array (Qiagen; Valencia, CA, USA) containing primers for 84 key genes of the UPR were used to examine the effects of EZH2 inhibitors the according to the manufacturer's protocol. Total RNA (0.5 μg) was reverse transcribed by a RT^2 First Strand Kit (Qiagen), prior to amplification using the RT^2 Profiler PCR Array. PCR amplifications were performed on a StepOne Plus Real-Time PCR System thermocycler (Life Technologies). The results were analyzed using a supplied online software. The comparative Ct method was used for relative transcript quantification against the average ΔCt derived from internal controls (β-Actin, β-2-microglobulin, GAPDH, HPRT1, and RPLP0).

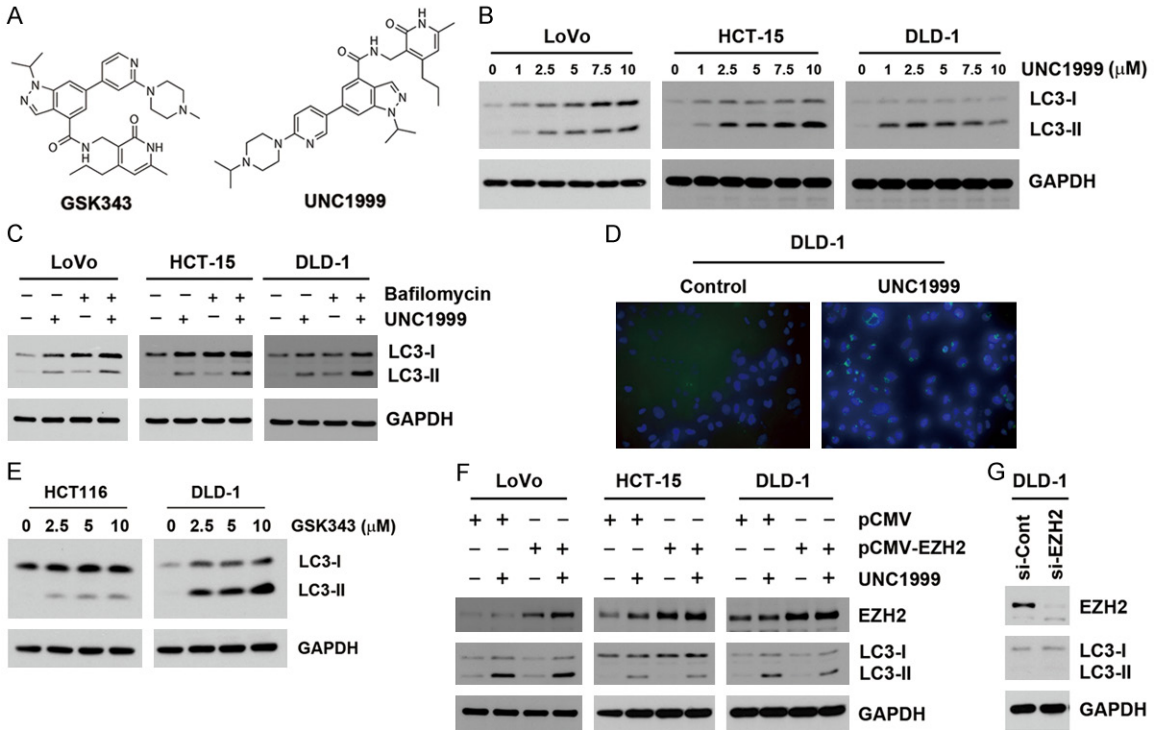

Figure 1. UNC1999 induced autophagy in human CRC cells in an EZH2-independent manner. A. The chemical structures of GSK343 and UNC1999. B. LoVo, HCT-15 and DLD-1 cells were treated with indicated doses of UNC1999 for 24 h. The protein expressions were analyzed by Western blots. C. LoVo, HCT-15 and DLD-1 cells were treated with 2.5 μM UNC1999 for 24 h in the absence or presence of 20 nM bafilomycin A1 (post-treatment for 4 h). The protein expressions were analyzed by Western blots. D. DLD-1 cells were treated with 2.5 μM UNC1999 for 24 h, and then stained with Cyto-ID Autophagy Detection Kit. The Cyto-ID fluorescence was observed by fluorescent microscopy. E. HCT116 and DLD-1 cells were treated with indicated doses of GSK343 for 24 h. The protein expressions were analyzed by Western blots. F. LoVo, HCT-15 and DLD-1 cells were transiently transfected with an EZH2-overexpressing (pCMV-EZH2) or a control (pCMV) plasmid for 48 h, and then treated with 2.5 μM UNC1999 for 24 h. The protein expressions were analyzed by Western blots. G. DLD-1 cells were transiently transfected with EZH2 siRNA for 72 h. The protein expressions were analyzed by Western blots.

Statistical analysis

Means and standard deviations of samples were calculated from the numerical data (at least 3 replica) generated in this study. Data were analyzed using Student's *t*-test, and *p* values of < 0.05 were considered significant (*).

Results

Characterization of autophagy induced by the EZH2 inhibitors in human colorectal cancer cells

Our previous study shows that SAM-competitive EZH2 inhibitors, GSK343 and UNC1999 (**Figure 1A**), induce autophagy in cancer cells [23]. The involved molecular mechanisms were further investigated in this study. First, we confirmed that GSK343 and UNC1999 induced autophagy in human colorectal cancer (CRC) cells. LoVo, HCT-15, and DLD-1 cells were treated with various doses of UNC1999 for 24 h, and autophagy was evaluated by the accumulation of LC3-II. As shown in **Figure 1B**, UNC1999 induced LC3-II accumulation in these cells. Because impaired autophagosome-lysosome fusion can result in LC3-II accumulation, autophagic flux was analyzed by treating bafilomycin A1, a vacuolar-type H^+-ATPase inhibitor that blocks autophagosome-lysosome fusion [27]. As shown in **Figure 1C**, UNC1999 induced more accumulation of LC3-II in the presence of bafilomycin A1, suggesting that the increase of LC3-II by UNC1999 was not due to the blockade of autophagic degradation. Furthermore, the formation of autophagic vacuoles was monitored using Cyto-ID Autophagic Detection Kit. UNC1999 increased the Cyto-ID fluorescence

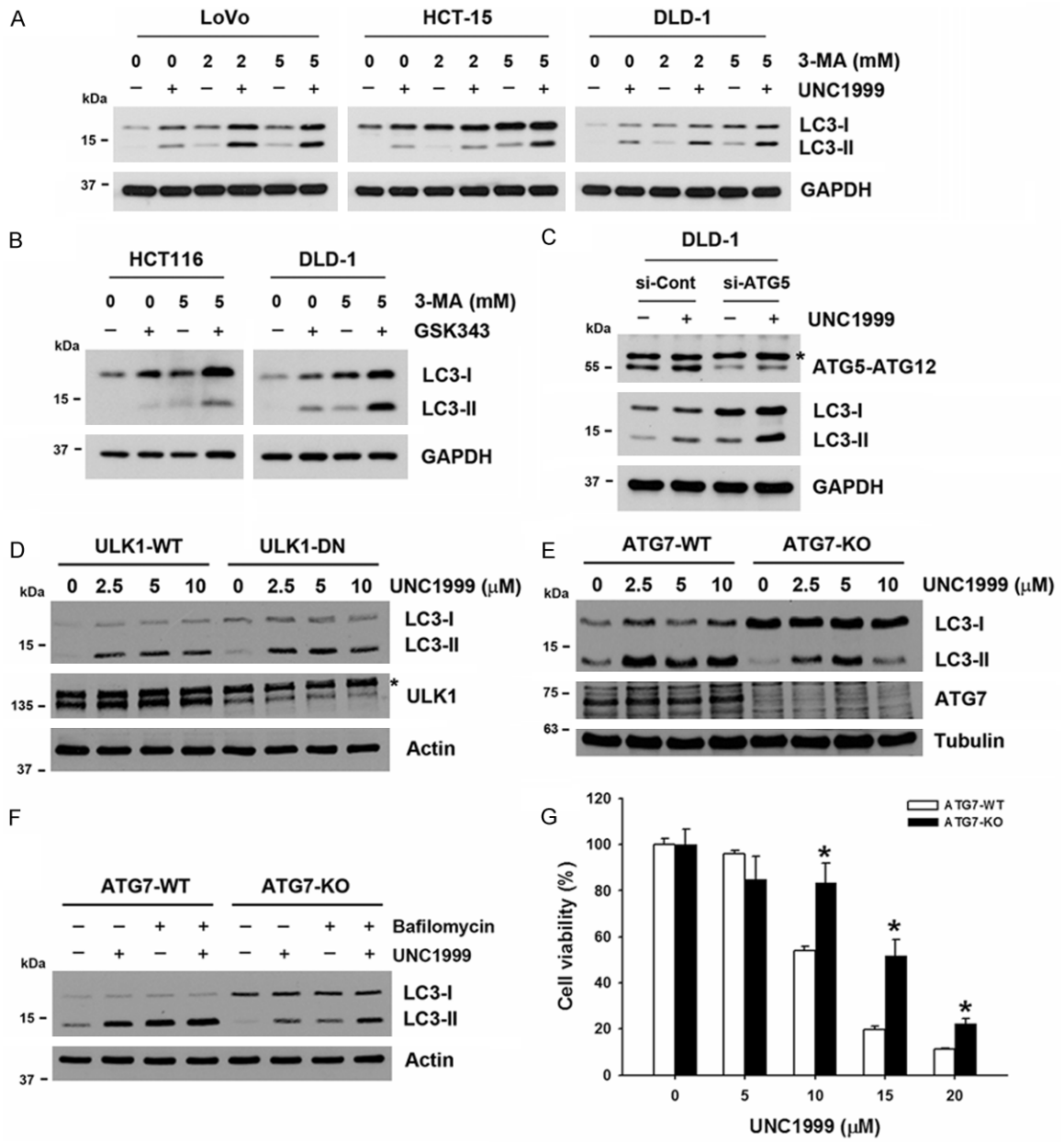

Figure 2. UNC1999 induced partially ATG7-dependent autophagy. A. LoVo, HCT-15 and DLD-1 cells were pretreated with 2 and 5 mM 3-MA for 1 h, and then exposed to 2.5 μM UNC1999 for 24 h. The protein expressions were analyzed by Western blots. B. HCT116 and DLD-1 cells were pretreated with 5 mM 3-MA for 1 h, and then exposed to 5 μM GSK343 for 24 h. The protein expressions were analyzed by Western blots. C. DLD-1 cells were transfected with ATG5 siRNA for 48 h, and then exposed to 2.5 μM UNC1999 for 24 h. The protein expressions were analyzed by Western blots. *nonspecific band. D and E. ATG7-KO and ULK1-DN DLD-1, as well as their control wildtype (WT) cells were treated with indicated doses of UNC1999 for 24 h. The protein expressions were analyzed by Western blots. F. ATG7-WT and ATG7-KO cells were treated with 5 μM UNC1999 for 24 h in the absence or presence of 20 nM bafilomycin A1 (post-treatment for 4 h). The protein expressions were analyzed by Western blots. G. ATG7-WT and ATG7-KO cells were treated with indicated doses of UNC1999 for 72 h. The cell viability was analyzed by an MTT assay. *$P < 0.05$ indicates significant differences between ATG7-WT and ATG7-KO cells.

in DLD-1 cells (**Figure 1D**). Similarly, GSK343 also induced LC3-II accumulation in HCT116 and DLD-1 cells (**Figure 1E**). Therefore, EZH2 inhibitors can induce autophagy in human CRC cells. To investigate whether EZH2 inhibition was responsible for the effect of UNC1999 on

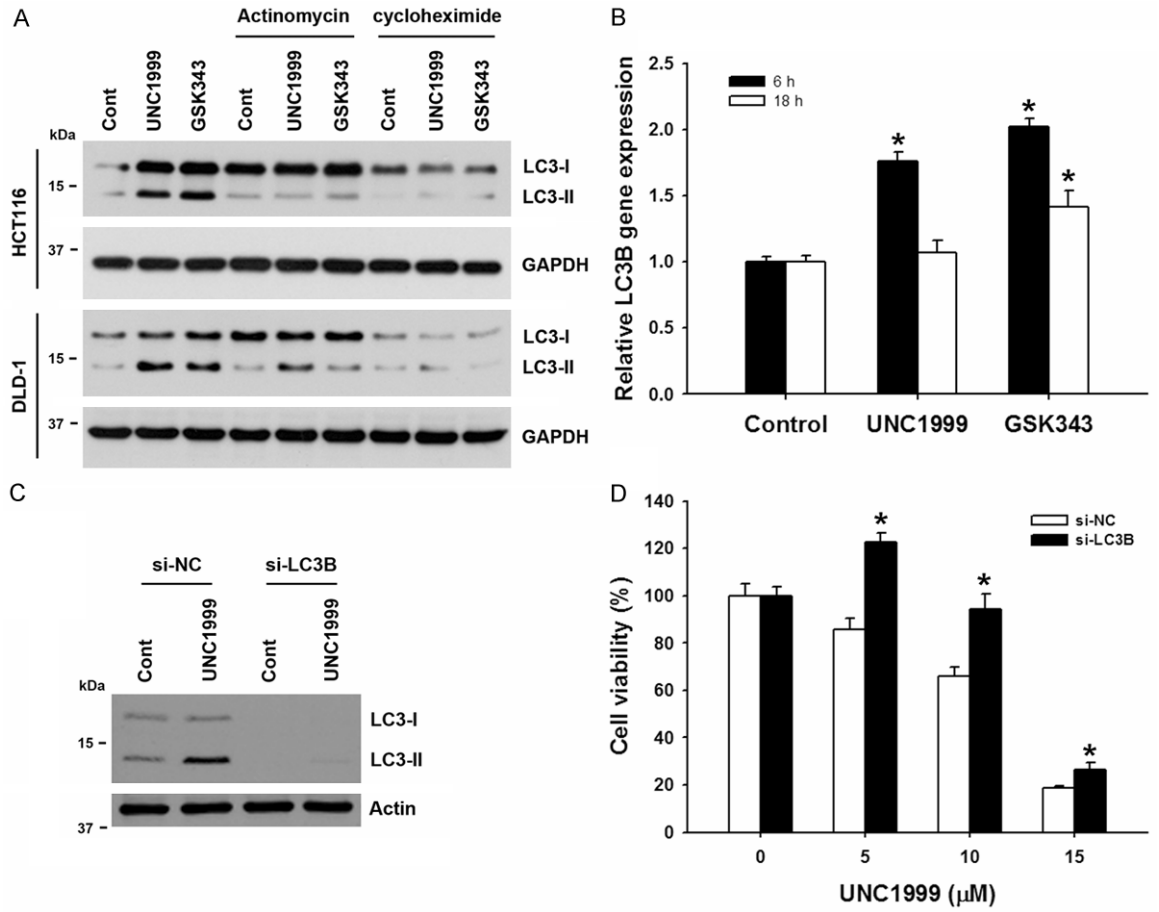

Figure 3. EZH2 inhibitors transcriptionally induced autophagy. A. HCT116 and DLD-1 cells were treated with 2 μg/mL antinomycin or 5 μg/mL cycloheximide for 1 h, and then exposed to 2.5 μM UNC1999 or 5 μM GSK343 for additional 24 h. The protein expressions were analyzed by Western blots. B. HCT116 cells were treated with 5 μM UNC1999 or 10 μM GSK343 for 6 or 18 h. The LC3B mRNA expression was analyzed by qPCR. **P*<0.05 indicates significant differences between drug-treated and control cells. C. HCT116 cells were transiently transfected with EZH2 siRNA for 48 h, and then treated with 5 μM UNC1999 for 24 h. The protein expressions were analyzed by Western blots. C. HCT116 cells were transiently transfected with EZH2 siRNA for 24 h, and then treated with indicated doses of UNC1999 for 72 h. The cell viability was analyzed by an MTT assay. **P*<0.05 indicates significant differences between si-NC- and si-LC3B-transfected cells.

inducing autophagy, LoVo, HCT-15 and DLD-1 cells were transfected with EZH2-overexpressing plasmids, and then treated with UNC1999 for 24 h. As shown in **Figure 1F**, UNC1999-induced LC3-II accumulation was not rescued by EZH2 overexpression. In addition, knockdown of EZH2 by siRNA was not sufficient to induce LC3-II accumulation in DLD-1 cells (**Figure 1G**). Therefore, UNC1999 induces autophagy of human CRC cells in an EZH2-independent manner.

EZH2 inhibitor-induced autophagy is partially dependent on ATG7

To investigate how EZH2 inhibitors induced autophagy in human CRC cells, a class III PI3K inhibitor, 3-methyladenine (3-MA), was used. Surprisingly, 3-MA enhanced UNC1999- and GSK343-induced LC3-II accumulation (**Figure 2A** and **2B**). To confirm the effect of 3-MA, an siRNA against ATG5 was transfected into DLD-1 cells. Consistently, inhibition of ATG5 expression potentiated UNC1999-induced LC3-II accumulation (**Figure 2C**). Furthermore, ULK1-dominant-negative mutant (ULK1-DN) and ATG7-knockout (ATG7-KO) DLD-1 cells were used. As shown in **Figure 2D** and **2E**, UNC1999 could still induce LC3-II accumulation in ULK1-DN and ATG7-KO DLD-1 cells. However, we noticed that UNC1999-induced LC3-II accumulation was partially inhibited in ATG7-KO cells (**Figure 2E**). To confirm this results, cells were treated

Table 1. The Gene Set Enrichment Analysis (GSEA) for pathways enriched in both UNC1999- and GSK343-treated HCT116 cells

Pathways	Number of Genes in Pathway	Number of Pathway Genes Differentially Expressed (% of Total)	*p* value
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	81	18 (22%)	0.000
HALLMARK_CHOLESTEROL_HOMEOSTASIS	61	13 (21%)	0.000
HALLMARK_MTORC1_SIGNALING	148	27 (18%)	0.500
HALLMARK_PI3K_AKT_MTOR_SIGNALING	72	7 (10%)	0.125

with UNC1999 with or without bafilomycin A. As shown in **Figure 2F**, the level of LC3-II was attenuated in ATG7-KO cells compared to ATG7-WT cells. Therefore, these results suggest that EZH2 inhibitors induce autophagy in human CRC cells, which was partially dependent on ATG7.

EZH2 inhibitor-induced autophagy is associated with the upregulation of LC3B gene transcription

In addition to the cytosolic events that can regulate autophagy, accumulating evidences suggest the existence of transcriptional control of autophagy [28]. To investigate whether transcription and translation were involved in UNC1999-induced autophagy, a transcription inhibitor, antinomycin D, and a protein synthesis inhibitor, cycloheximide, were used. As shown in **Figure 3A**, UNC1999- and GSK343-induced LC3-II accumulation was inhibited by these two drugs, indicating that induction of gene expression and *de novo* protein synthesis are required for UNC1999- and GSK343-induced autophagy. Because treatment of 3-MA and ATG5 siRNA, as well as ATG7- and ULK1-deficiency did not inhibit EZH2 inhibitor-induced autophagy, we proposed that EZH2 inhibitors may directly induce the transcription of LC3B to trigger autophagy. Indeed, UNC1999 and GSK343 transiently increased the mRNA level of LC3B gene in HCT116 cells (**Figure 3B**). To investigate the role of autophagy in the anticancer activity of EZH2 inhibitors, endogenous LC3B expression was knocked down by transfecting siRNA (**Figure 3C**), and then cell viability was examined by MTT assay. As shown in **Figure 3D**, knockdown of LC3B gene rescued cell growth inhibition by UNC1999. Therefore, UNC1999 induced autophagy through transcriptional upregulation of LC3B gene. In addition, the partial ATG7-dependency of UNC1999-induced autophagy (**Figure 2E** and **2F**) may be due to that ATG7 is responsible for the LC3 lipidation [29].

Microarray analysis reveals that EZH2 inhibitors upregulate genes associated with an unfolded protein response

To further identify the biological pathways affected by UNC1999 and GSK343, microarray analyses were performed and analyzed by the Gene Set Enrichment Analysis (GSEA) software using hallmark gene sets [24-26]. The results indicated 4 out of 50 gene sets are upregulated in response to UNC1999 and GSK343 (**Table 1**). The top-ranking pathways with statistical significance (p value < 0.05%) included unfolded protein responses (UPR) and cholesterol biosynthesis (**Figure 4A** and **Table 1**). Activation of UPR drew our attention because UPR is known to connect ER stress to autophagy [30]. ER stress/UPR consists of three major arms (PERK/eIF2α, IRE1/XBP1, and ATF6). Each UPR pathway induces different target genes. ATF6 binds to ER-stress response elements (ERSEs) and induces transcription of several genes, including GRP78, CHOP and XBP1. XBP1 binds to UPR elements (UPREs) and activates many genes that are crucial for secretory function. One of these gene products, p58IPK, inhibits PERK activity. PERK-mediated phosphorylation of eIF2α suppresses global translation except the ATF4 mRNA. ATF4 can upregulate the third set of UPR target genes, one of which is CHOP that can induce apoptosis in cells with irrecoverable levels of ER stress [31]. The alterations of ER stress/UPR-related genes by UNC1999 and GSK343 were visualized by the Ingenuity Pathway Analysis (IPA). The results indicated that UNC1999 predominantly upregulated genes associated with PERK/eIF2α pathways (**Figure 4B**). However, GSK343 induced the expression of genes related to the three arms

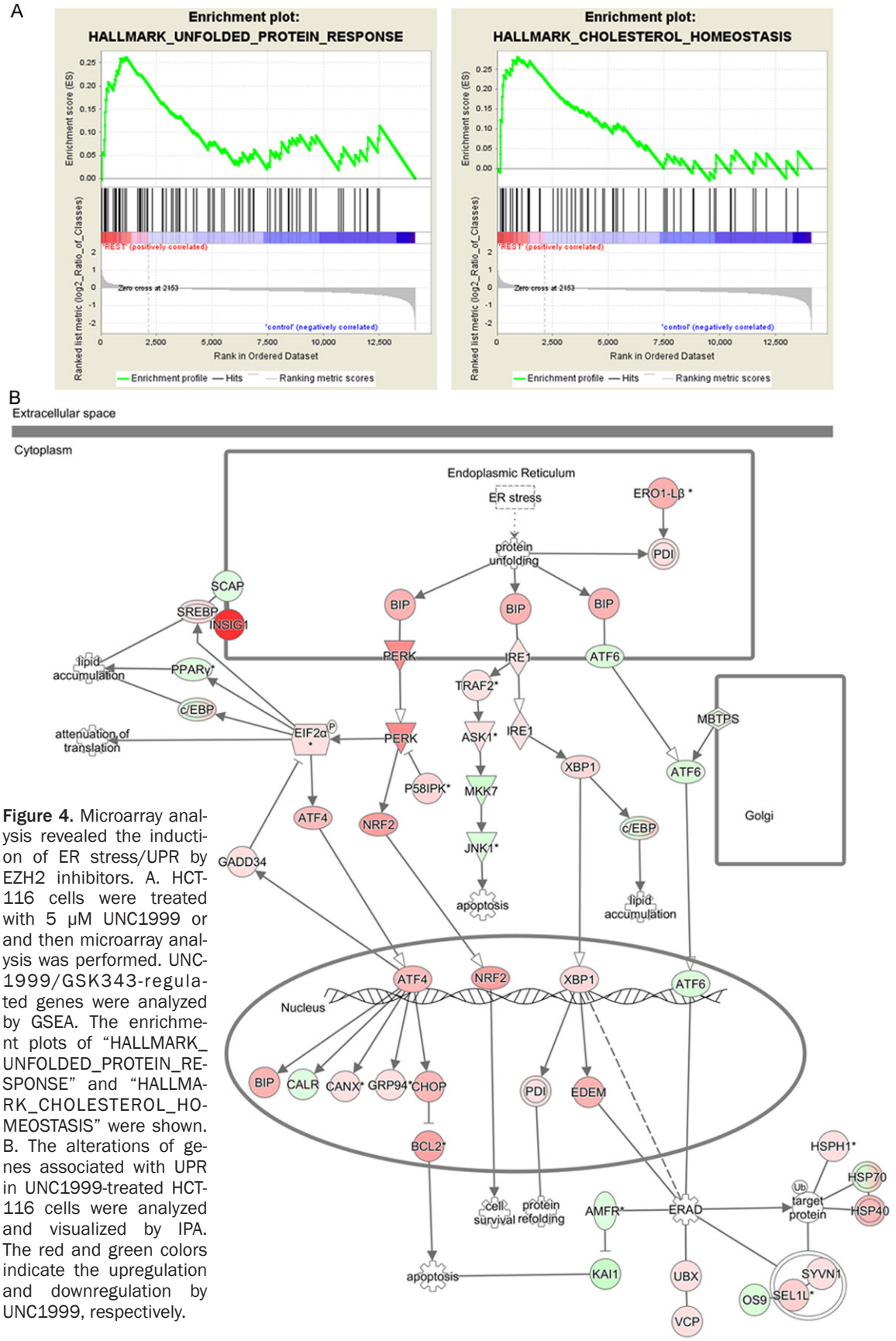

Figure 4. Microarray analysis revealed the induction of ER stress/UPR by EZH2 inhibitors. A. HCT-116 cells were treated with 5 μM UNC1999 or and then microarray analysis was performed. UNC-1999/GSK343-regulated genes were analyzed by GSEA. The enrichment plots of "HALLMARK_UNFOLDED_PROTEIN_RESPONSE" and "HALLMARK_CHOLESTEROL_HOMEOSTASIS" were shown. B. The alterations of genes associated with UPR in UNC1999-treated HCT-116 cells were analyzed and visualized by IPA. The red and green colors indicate the upregulation and downregulation by UNC1999, respectively.

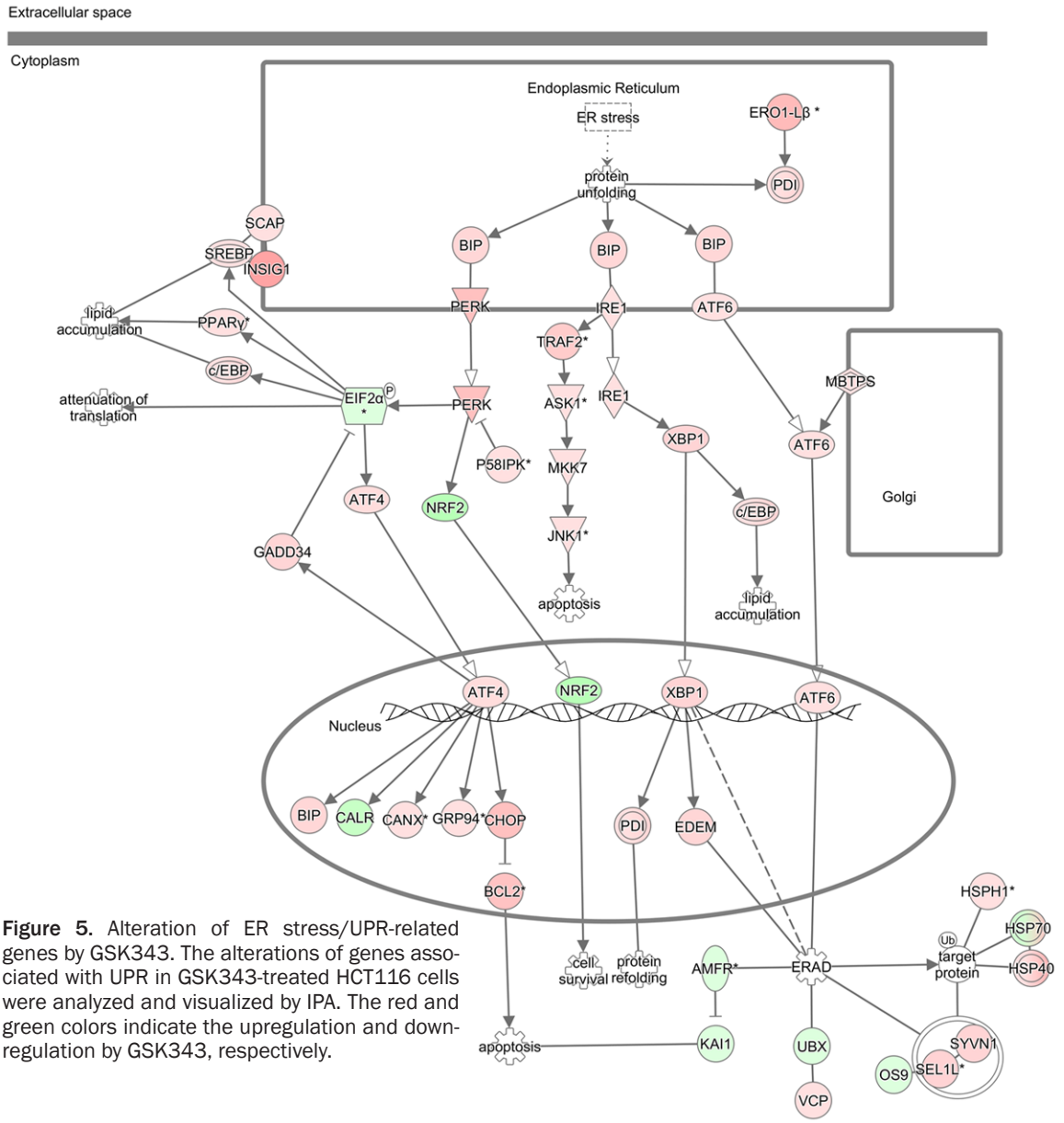

Figure 5. Alteration of ER stress/UPR-related genes by GSK343. The alterations of genes associated with UPR in GSK343-treated HCT116 cells were analyzed and visualized by IPA. The red and green colors indicate the upregulation and downregulation by GSK343, respectively.

of UPR (**Figure 5**). Therefore, EZH2 inhibitors may induce autophagy through the activation of UPR pathways.

EZH2 inhibitors activate the PERK/eIF2α arm of the unfolded protein response pathways

To confirm the result of microarray analysis, the Human Unfolded Protein Response Plus PCR Array analysis was performed. As shown in **Figure 6A**, the common genes induced by UNC1999 and GSK343 were ATF4 and DDIT3 (CHOP/GADD153) genes at 6 h, and TRB3 gene at 6 and 18 h. Induction of ATF4 and CHOP represents the downstream of PERK/eIF2α arm of UPR [32]. TRB3, a mammalian homolog of Drosophila tribbles, functions as a negative modulator of AKT/mTOR [33]. TRB3 is induced by ATF4/CHOP pathway and is involved in CHOP-dependent cell death during ER stress [34]. It has been reported that ATF4 and CHOP directly bind and regulate LC3B gene promoter to promote autophagy [35]. Our result indicated that the transient induction of ATF4 and DDIT3/CHOP gene expression was correlated with the upregulation of LC3B genes (**Figure 3B**). Therefore, we proposed that EZH2 inhibitors can activate autophagy through PERK/eIF2α signaling

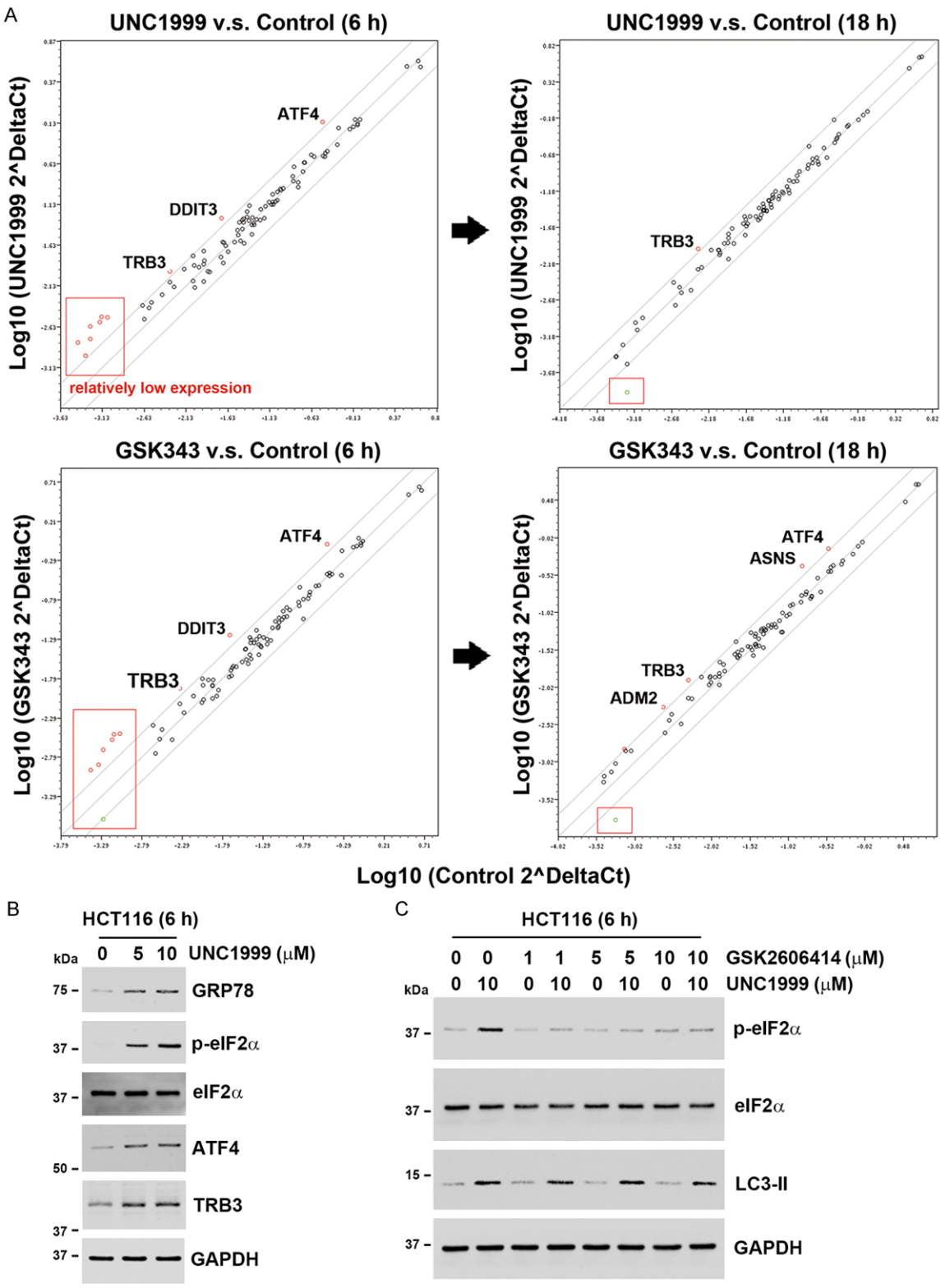

Figure 6. PCR array analysis identified the activation of the PERK/eIF2α arm by EZH2 inhibitors. A. HCT116 cells were treated with 5 μM UNC1999 or 10 μM GSK343 for 6 and 18 h, and the ER stress/UPR-associated genes were analyzed by the Human Unfolded Protein Response Plus PCR Array. The scatter plot compared normalized expression of every gene on the array between control and test samples. The central line indicates unchanged gene expression. The fold regulation cut-off (boundary) was set to "2-fold". The red circles indicated that genes were up-regulated and the green circles indicated that genes were down-regulated. The genes (red square) with relatively higher average threshold cycle (>30) were not considered significant. The names of significant genes were

shown in diagrams. B. HCT116 cells were treated with indicated doses of UNC1999 for 6 h. The protein expressions were analyzed by Western blots. C. HCT116 cells were treated with indicated doses of UNC1999 with or without GSK2606414 for 6 h. The protein expressions were analyzed by Western blots.

axis. Indeed, UNC1999 induced the phosphorylation of eIF2α, as well as the expression of ATF4 (**Figure 6B**). In addition, the expression of GRP78, a central regulator for ER stress [36], was also induced by UNC1999 (**Figure 6B**). Therefore, UNC1999 indeed induces ER stress and UPR in human CRC cells. To investigate the role of ER stress in UNC1999-induced autophagy, a PERK inhibitor, GSK2606414, was used. As shown in **Figure 6C**, phosphorylation of eIF2α by UNC1999 was attenuated by GSK-2606414. However, GSK2606414 did not alter UNC1999-induced LC3-II accumulation. Therefore, UNC1999 induced autophagy in an ER stress-independent manner.

Inhibition of ER stress enhances UNC1999-induced cell death

To investigate the role of ER stress/UPR in the anticancer activity of EZH2 inhibitors in CRC cells, HCT116 cells were treated with GSK-2606414, and then exposed to UNC1999 for 48 h. The cell viability was examined by a MTT assay. As shown in **Figure 7A**, synergistic inhibition of cell viability by GSK2606414 and UNC1999 was found. The inhibition of ER stress by GSK2606414 was ascertained by reduction of the eIF2α phosphorylation (**Figure 7B**). To confirm the enhancement of UNC1999-inducedcell death by GSK2606414, cell apoptosis was examined by the cleavage of poly (ADP-ribose) polymerase (PARP). Indeed, UNC-1999-induced PARP cleavage was augmented in the presence of GSK2606414. In addition, UNC1999-induced autophagy (LC3-II accumulation) was not altered by GSK2606414 (**Figure 7B**). Therefore, ER stress/UPR plays a cytoprotective role in the anticancer activity of UNC1999.

Discussion

Recently, non-canonical autophagy has been characterized [37-41]. Unlike canonical autophagy, the formation of the double-membraned autophagosome does not require the hierarchical intervention of all of the ATG proteins [39]. For example, non-canonical autophagy can occur in the Beclin 1/VPS34-, ATG5/ATG7- and ULK1/ULK2-independent manners [37, 40, 41]. Even so, non-canonical autophagy pathways and structures have the same function as canonical autophagy in sequestering some of the cytoplasm and compartmentalizing pathogens. In addition, material sequestered by non-canonical autophagy is ultimately degraded in the lysosomal compartment [42]. However, no specific markers are able to distinguish between the non-canonical and canonical autophagic pathways. In our study, EZH2 inhibitor induced 3-MA-insenstitive, as well as ATG5- and ULK1-indpendent autophagy despite the partial dependency of ATG7, suggesting that EZH2 inhibitors may also induce non-canonical autophagy. Further investigation is required to clarify the underlying molecular pathways.

Despite the fact that the execution of autophagy includes a unique set of cytoplasmic events, nuclear events, in particular transcriptional programs, have emerged as an important regulator of this process [28, 43]. Particularly, recent studies have suggested the existence of epigenetic mechanism for autophagy. For example, the methyltransferase G9a, that catalyzes the dimethylation of histone H3 lysine 9 (H3K9-me2), directly represses genes known to participate in the autophagic process [44]. In addition, induction of autophagy is coupled with the reduction of histone H4 lysine 16 acetylation (H4K16-ac) through downregulation of the histone acetyltransferase hMOF (also called KAT8 or MYST1). H4K16 deacetylation is associated predominantly with the downregulation of autophagy-related genes, and antagonizing H4K16-ac downregulation upon autophagy induction results in the promotion of cell death [45]. Most recently, EZH2 has been shown to inhibit autophagy through epigenetically repressing the negative regulators of mTOR [46]. However, this study is controversial to our results showing that knockdown of EZH2 by siRNA was not sufficient to induce autophagy and overexpression of EZH2 cannot block EZH2 inhibitor-induced autophagy. In addition, our previous study demonstrated that 3-deazaneplanocin A (DZNep), an indirect EZH2 inhibitor through depleting EZH2 [16], fails to induce autophagy [23]. It is likely that cell type or con-

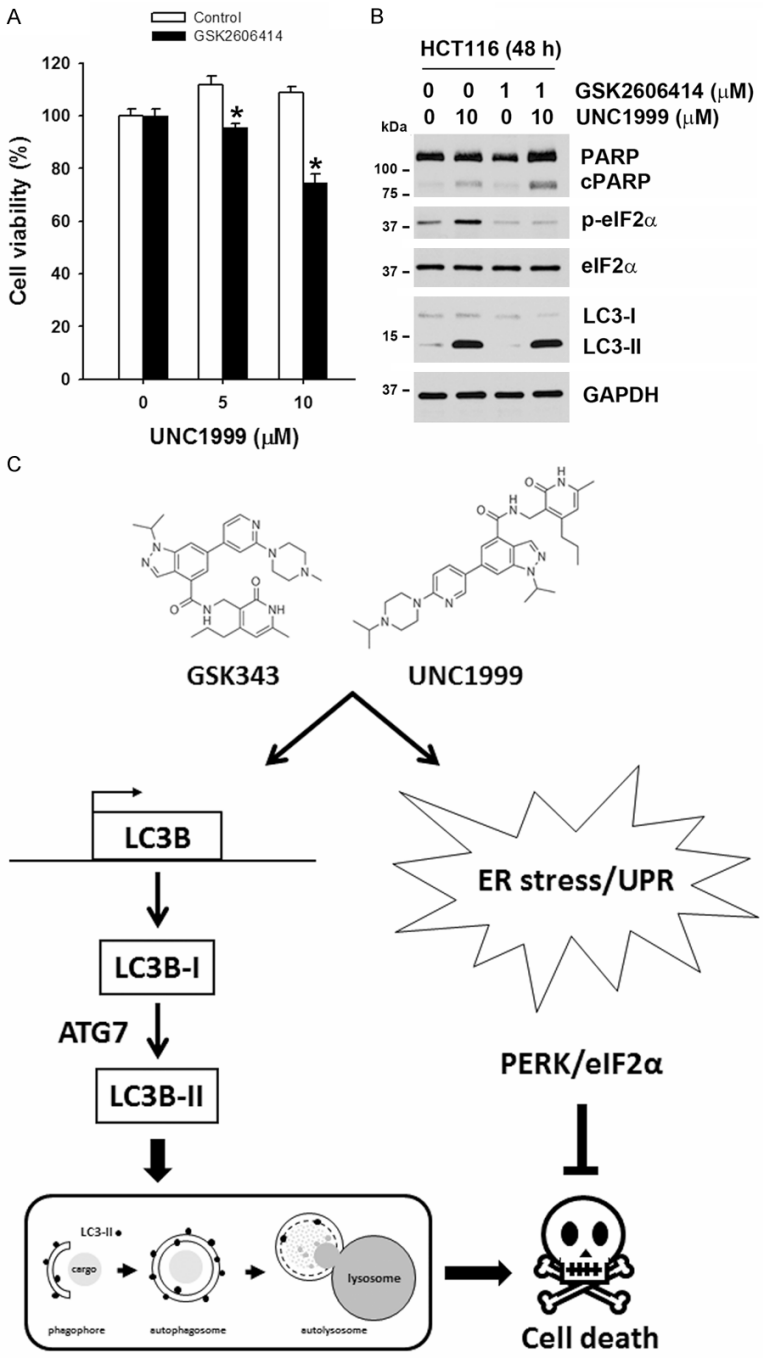

Figure 7. Inhibition of ER stress enhanced UNC1999-induced cell death. (A and B) HCT116 cells were treated with indicated doses of UNC1999 with or without 10 μM GSK2606414 for 48 h. The cell viability was analyzed by an MTT assay (A). The protein expressions were analyzed by Western blots (B). *$P < 0.05$ indicates significant differences between GSK2606414-treated and control cells. (C) A working model for the action mechanism of EZH2 inhibitors.

text specificity for EZH2-dependent regulation of autophagy may exist.

In addition to a basic role in the turnover of proteins and organelles, autophagy is observed under pathological conditions including myopathy, neuronal degeneration, infectious disease, and cancer [47, 48]. Previous studies have reported that either blockage of autophagy or induction of autophagy could lead to tumor growth. The impact of autophagy appears to vary with intrinsic properties of the tumor [49]. Accumulating evidences indicate that autophagy facilitates the resistance of cancer cells to chemotherapy and radiation [50]. On the other hand, total destruction of the cells by autophagy is served as type II programmed cell death [51]. Autophagic cell death has been reported to be activated in cancer cells in response to various anticancer therapies. Therefore, investigating the role of autophagy in cancer therapy and then modulating the activity of autophagy will improve the efficacy of cancer therapy, which relies on more understanding the mechanism of autophagy. In this notion, our previous and current studies showed that UNC1999 and GSK343 induced autophagic cell death [23]. Therefore, enhancement of autophagy by small molecules, such as rapamycin, might enhance the anticancer activity of UNC1999 and GSK343.

ER stress/UPR could result in both adaptive and apoptotic outputs and is associated with a wide range of diseases including cancers [52]. Generally, the ability of cells to respond to ER stress is critical for cell survival. However, chronic or unresolved ER stress can lead to apoptosis [53]. Our study showed that induction of ER stress/UPR belonged to an immediate response to EZH2 inhibitors, which play a protective role from cancer cell death. Besides, microarray and hallmark gene set analyses found that, in

addition to UPR, EZH2 inhibitors may also activate mTOR, PI3K/AKT survival signaling pathways despite of no statistical significance.

Taken together, we proposed a working model (**Figure 7C**) to describe the action mechanism of EZH2 inhibitors (UNC1999 and GSK343). EZH2 inhibitors exhibit anti-CRC activity through inducing autophagic cell death. EZH2 inhibitors induce autophagy via transcriptionally upregulated the expression of LC3 gene. In contrast, EZH2 inhibitors also trigger ER stress to activate PERK/eIF2α pathway, which plays a protective role in cancer cells. Thus, combination therapy of ER stress and EZH2 inhibitors may have synergistic anti-CRC activity.

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Disclosure of conflict of interest

None.

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