Received: 19 July 2017 | Accepted: 1 October 2017

DOI: 10.1111/cpr.12402

ORIGINAL ARTICLE

WILEY Cell Proliferation

# Fluoxetine induces autophagic cell death via eEF2K-AMPK-mTOR-ULK complex axis in triple negative breast cancer

**Dejuan Sun**[1] | **Lingjuan Zhu**[1] | **Yuqian Zhao**[1] | **Yingnan Jiang**[1,2] | **Lixia Chen**[1] | **Yang Yu**[2] | **Liang Ouyang**[3]

[1]Wuya College of Innovation, School of Traditional Chinese Materia Medica, Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang, China

[2]Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou, China

[3]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University and Collaborative Innovation Center for Biotherapy, Chengdu, China

**Correspondence**
Lixia Chen, Wuya College of Innovation, School of Traditional Chinese Materia Medica, Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang, China.
Email: syzyclx@163.com
and
Yang Yu, Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou, China.
Email: 1018yuyang@163.com

**Funding information**
This work was supported by grants from National Key R&D Program of China (Grant No. 2017YFC0909301) and National Natural Science Foundation of China (Grant No. 81603275 and Grant No. 81602984).

## Abstract

**Objectives**: Triple negative breast cancer (TNBC) is a complex and intrinsically aggressive tumour with poor prognosis, and the discovery of targeted small-molecule drugs for TNBC treatment still remains in its infancy. In this study, we aimed to discover a small-molecule agent for TNBC treatment and illuminate its potential mechanisms.

**Materials and methods**: Cell viability was detected by using methylthiazoltetrazolium (MTT) assay. Electron microscopy, GFP-LC3 transfection, monodansylcadaverine staining and apoptosis assay were performed to determine Fluoxetine-induced autophagy and apoptosis. Western blotting and siRNA transfection were carried out to investigate the mechanisms of Fluoxetine-induced autophagy. iTRAQ-based proteomics analysis was used to explore the underlying mechanisms.

**Results**: We have demonstrated that Fluoxetine had remarkable anti-proliferative activities and induced autophagic cell death in MDA-MB-231 and MDA-MB-436 cells. The mechanism for Fluoxetine-induced autophagic cell death was associated with inhibition of eEF2K and activation of AMPK-mTOR-ULK complex axis. Further iTRAQ-based proteomics and network analyses revealed that Fluoxetine-induced mechanism was involved in BIRC6, BNIP1, SNAP29 and Bif-1.

**Conclusions**: These results demonstrate that Fluoxetine induces apoptosis and autophagic cell death in TNBC, which will hold a promise for the future TNBC therapy.

## 1 | INTRODUCTION

Triple negative breast cancer (TNBC) is a complex and intrinsically aggressive tumour characterized by lacking immunohistochemical expressions of oestrogen receptors (ER), progesterone receptors and human epidermal growth factor receptors.[1-3] It represents approximately 15%-20% of all diagnosed breast cancer patients and is highly prevalent in younger (under the age of 40) and African-American-originated women. Moreover, women diagnosed at the earliest stage of TNBC have an initial 5-year survival rate of 70%, which is lower than the 80% observed for other breast cancer subtypes.[4-6] Currently, the US FDA has not approved a standard therapy or proprietary medicine for TNBC. Chemotherapy including administering anthracyclines, taxanes or platinum to disrupt cancer cell functions, as well as radiation therapy, is the mainstay in the management of TNBC.[7] However, several difficulties such as multidrug resistance, strong systemic toxicity, rapid in vivo clearance and endosome degradation in cytosol have been encountered throughout the TNBC therapeutics.[6] Effective targeted treatment for TNBC is facing a significant clinical challenge, and the discovery of a novel targeted small-molecule drug has been emerging as the focus for the current TNBC therapeutics.

Autophagy, a term from Greek "auto" (self) and "phagy" (to eat), refers to an evolutionarily conserved cellular mechanism for the

Dejuan Sun and Lingjuan Zhu contributed equally to this work.

  

clearance of damaged or superfluous organelles, such as endoplasmic reticulum, golgi apparatus and mitochondria.[8-10] Recent work suggests that autophagy is important in cell death decisions and can protect cells from apoptosis and caspase-independent death.[11] However, unusual levels of autophagy can also do the opposite—it can also kill cells. Autophagic cell death is known as Type II programmed cell death to distinguish it from apoptosis or Type I programmed cell death. Although autophagic cell death and apoptosis bear distinct morphological characteristics and physiological process, there would be close connections between the regulatory machinery that control autophagy and apoptosis.[11-13] Sometimes apoptosis and autophagy could exert synergetic effect, while sometimes autophagy would be triggered only when apoptosis is suppressed. Moreover, recent studies have further pointed out that autophagy and apoptosis may be interconnected and even simultaneously regulated by the same trigger in tumour cells. Eukaryotic elongation factor-2 kinase (eEF2K), well-known to be a $Ca^{2+}$ calmodulin (CaM)-dependent kinase, is overexpressed in several types of malignancies, most notably TNBC.[14] Increasing evidence indicates that eEF2K may modulate the expression of some apoptotic proteins such as XIAP, c-FLIP$_L$, Bcl-X$_L$, PI3KCI and p70S6K to inhibit apoptotic process in cancer. On the other hand, it may regulate autophagy involved in AMPK, mTORC1 and ULK1, thereby promoting cancer cell survival.[15-17] Additionally, eEF2K may play a crucial role in the crosstalk between autophagy and apoptosis in TNBC. Collectively, these findings have led to the conclusions that eEF2K may be a novel anti-TNBC target, and inhibiting eEF2K would be a promising TNBC therapeutic strategy.

Previous reports showed that Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that has been widely used to modulate the concentration of serotonin in the central nervous system.[18] It is well-known as Prozac for the treatment of depression. Actually, there is encouraging evidence demonstrating that Fluoxetine interferes with mitochondrial function and may induce cell apoptosis.[19] The researches demonstrated that breast tumour cells possess the enzymatic machinery to synthesize serotonin, which acts by an autocrine or paracrine mechanism in conjunction with other serotonergic pathway components to maintain tumour growth, seed metastases and resist cytotoxic therapies.[20] A N-methyl-D-aspartic acid (NMDA) receptor blockade by ketamine led to an inhibition of eEF2K followed by enhanced protein synthesis and potentiation of hippocampal synapses that might mediate the antidepressant response of this compound.[21,22] In addition, the researchers stated that ketamine is metabolized in vivo to a ketamine derivative that shows antidepressant responses without blockage of NMDA receptors. The antidepressant effect of this derivative was still accompanied by a decrease in the phosphorylation of eEF2, an increase of synaptic transmission and neuronal network synchrony.[23,24] There is encouraging evidence demonstrating that Fluoxetine interferes with mitochondrial function and may changes autophagic flux and apoptosis. So, we choose Fluoxetine as candidate small molecule. In this study, we reported a small molecule, Fluoxetine that potently inhibits cell proliferation, associated with autophagy and apoptosis and inhibition of eEF2K. These findings highlight Fluoxetine that has the potential to impact the future TNBC therapy.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture and reagents

Human breast carcinoma MDA-MB-231, MDA-MB-436, MDA-MB-468, MDA-MB-453, MDA-MB-157 and MCF-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with 10% foetal bovine serum and incubated with 5% $CO_2$. Fluoxetine (#F132), 3-Methyladenine (#M9281), Z-VAD-FMK (#V116) and MTT (#M2128) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against eEF2K (#3692), eEF2 (#2332), p-eEF2$^{thr56}$ (#2331), AMPKα (#5831), p-AMPKα$^{thr172}$ (#5256), mTOR (#2983), p-mTOR$^{ser2448}$ (#2971), p70S6K (#2708), p-p70S6K$^{ser389}$ (#9234), ULK1 (#8054), p-ULK1$^{ser317}$ (#12753), p-ULK1$^{ser555}$ (#5869), mATG13 (#13273), Atg101 (#13492), FIP200 (#12436), Beclin-1 (#3495), p62 (#8025), LC3B (#3868), caspase-8 (#4790), caspase-3 (#9665), PARP (#9532), Bif-1 (#4467) and BIRC6 (#8756) were purchased from Cell Signaling Technologies (Boston, MA, USA). Bafilomycin A1 (#ab120497) and primary antibodies against BNIP1 (#ab151551), SNAP29 (#ab138500) were purchased from Abcam (Cambridge, UK). Primary antibodies against p-eEF2K$^{ser78}$ (#sc-33051), p-eEF2K$^{ser398}$ (#sc-33053) were purchased from Santa Cruz Biotechnology (CA, USA).

### 2.2 | Cell viability assay

Cells were dispensed in 96-well plates at a density of $5 \times 10^4$ cells/mL. After 24-hour incubation, cells were treated with different concentrations of Fluoxetine for the indicated time periods. Cell viability was measured by the MTT assay.

### 2.3 | Transmission electron microscopy

The ultra-structure of cytoplasmic vacuolization was observed using a Philips Tecnai-12 Biotwin transmission electron microscope similar to previous reports.[25] Briefly, collected cells were fixed with 2% glutaraldehyde for 2 hours, washed with PBS and then post-fixed with 1% OsO 4 for 1.5 hours at 4°C. The samples were then washed and dehydrated with graded alcohol. After dehydration, the samples were infiltrated and embedded in 618 epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and then examined under the transmission electron microscope (Hitachi 7000, Hitachi, Japan).

### 2.4 | GFP-LC3 transfection and monodansylcadaverine staining assays

For GFP-LC3 transfection and monodansylcadaverine (MDC) staining, cells were transfected with GFP-LC3 or stained with MDC (0.05 mmol/L) and observed under a fluorescence microscopy. MDC positive ratio was measured by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

### 2.5 | Apoptosis assay

For Annexin V-FITC/PI staining, the treated cells were collected, washed and then stained with Annexin V-FITC/PI at room temperature for 15 minutes. For PI staining, the collected cells were fixed with 500 μL 80% ethanol at 4°C overnight; then after washing twice with PBS, the cells were incubated with PI staining solution for 30 minutes at 4°C. The percentage of apoptotic cells and cells at different phases of the cell cycle or the sub-G1 DNA content was measured by flow cytometry (Becton Dickinson).

### 2.6 | RNA interference (RNAi)

MDA-MB-231 and MDA-MB-436 cells were transfected with eEF2K, negative control siRNAs at 100 nmol/L final concentration using Lipofectamine RNAiMAX reagent Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions. The transfected cells were used for subsequent experiments 36 hours later.

### 2.7 | Western blot analysis

Western blot analysis was carried out by the method as previous description.[26] Cells were treated with Fluoxetine for indicated times. Both adherent and floating cells were collected, washed twice with cold PBS and then lysed in a Ripa Buffer with Protease Inhibitor Cocktail (Sigma-Aldrich) after centrifugation (12 000 g at 4°C for 10 minutes); the protein concentration was determined by a BCA Protein Assay Kit (Beyotime, Jiangsu, China). The equal amount of protein was electrophoresed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with Tris Buffered Saline with Tween-20 (TBST) containing 5% skimmed milk at room temperature for 1 hour, incubated overnight with primary antibodies at 4°C and subsequently incubated with secondary horseradish peroxidase-conjugated, goat anti-rabbit or goat anti-mouse IgG (Abcam) at room temperature for 1 hour, then visualized by using ECL reagents.

### 2.8 | iTRAQ assay and network analysis

Two separate iTRAQ-based proteomics analysis were performed in MDA-MB-231 and MDA-MB-436 cells. Briefly, cells were dissolved in lysis buffer and labelled with iTRAQ labelling reagents. After 2D LC analysis and tandem mass spectrometry analysis, protein identification and relative iTRAQ quantification were performed with ProteinPilot™ Software 4.2 (AB SCIEX, Shanghai, China) using the Paragon™ algorithm for the peptide identification. Results with iTRAQ ratio cut-off values of 1.2 and 0.8 for fold-change and number cut-off values of 3 for quantifiable peptides were accepted in protein abundance. To build the global human protein-protein interaction (PPI) network, we collected protein interaction data from online database, PrePPI.[27] Pair-wise PPIs with high scores (>0.6) were selected to construct the global PPI network and eEF2K-related subnetwork. It only contained differentially expressed proteins in the results of iTRAQ-based proteomics analysis.

### 2.9 | Functional clustering analysis

Functional clustering was determined using the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/).[28] The functional categories used were GO term related to Biological Process and Molecular Function. The conforming differentially expressed proteins were used as the background set. And, the protein synthesis- and programmed cell death-related proteins were shown in further network analysis.

### 2.10 | Statistical analysis

All the presented data and results were confirmed in at least 3 independent experiments. The data are expressed as means ± SD and statistical comparisons were made by 1-way ANOVA and Student's *t* test of SPSS 17.0 (Chicago, IL, USA). $P < .05$ was considered statistically significant.

## 3 | RESULTS

### 3.1 | Fluoxetine induces autophagy in MDA-MB-231 and MDA-MB-436 cells

The effect of Fluoxetine on cell viability was determined on 6 breast cancer cell lines, including MDA-MB-231, MDA-MB-436, MDA-MB-468, MDA-MB-453, MDA-MB-157 and MCF-7 cells. We found that Fluoxetine showed more potent anti-proliferative activity on MDA-MB-231 and MDA-MB-436 cells (Figure 1A). Next, we examined Fluoxetine could induce autophagy in breast cancer cells. After treatment with Fluoxetine, we observed morphologic signs of autophagy under electron microscope, characterized by the extensive vacuoles in the cytoplasm (Figure 1B). The induction of autophagy was confirmed by GFP-LC3 transfection, which showed an increase in the GFP-LC3 puncta in the cells treated with Fluoxetine (Figure 1C). Monodansylcadaverine, a probe for the detection of autophagic vacuoles, was applied to Fluoxetine-treated cells, and the green fluorescent dots were observed and photographed under fluorescence microscopy (Figure 1D). To further confirm that Fluoxetine induces autophagy, we found that treatment with Fluoxetine resulted in a time-dependent elevation of LC3-II, which is a marker of autophagy, and increased expression of Beclin-1; both of these proteins are important markers of autophagy. Moreover, the level of the selective autophagy substrate, p62/SQSTM1, was reduced by treatment with Fluoxetine (Figure 1E). Next, we added the lysosomal inhibitor, bafilomycin A1 (BafA1), to validate the existence of autophagic flux. The results showed that LC3-II and p62/SQSTM1 significantly accumulated in the presence of BafA1, indicating that autophagic flux is enhanced by treatment with Fluoxetine (Figure 1F). These results collectively suggest that Fluoxetine can induce autophagy in breast cancer cells.

### 3.2 | Fluoxetine induces autophagy through eEF2K inhibition and activation of AMPK-mTOR-ULK complex pathway

To illuminate the mechanism for Fluoxetine-induced autophagy, we examined the expression of autophagy-associated proteins. Firstly,

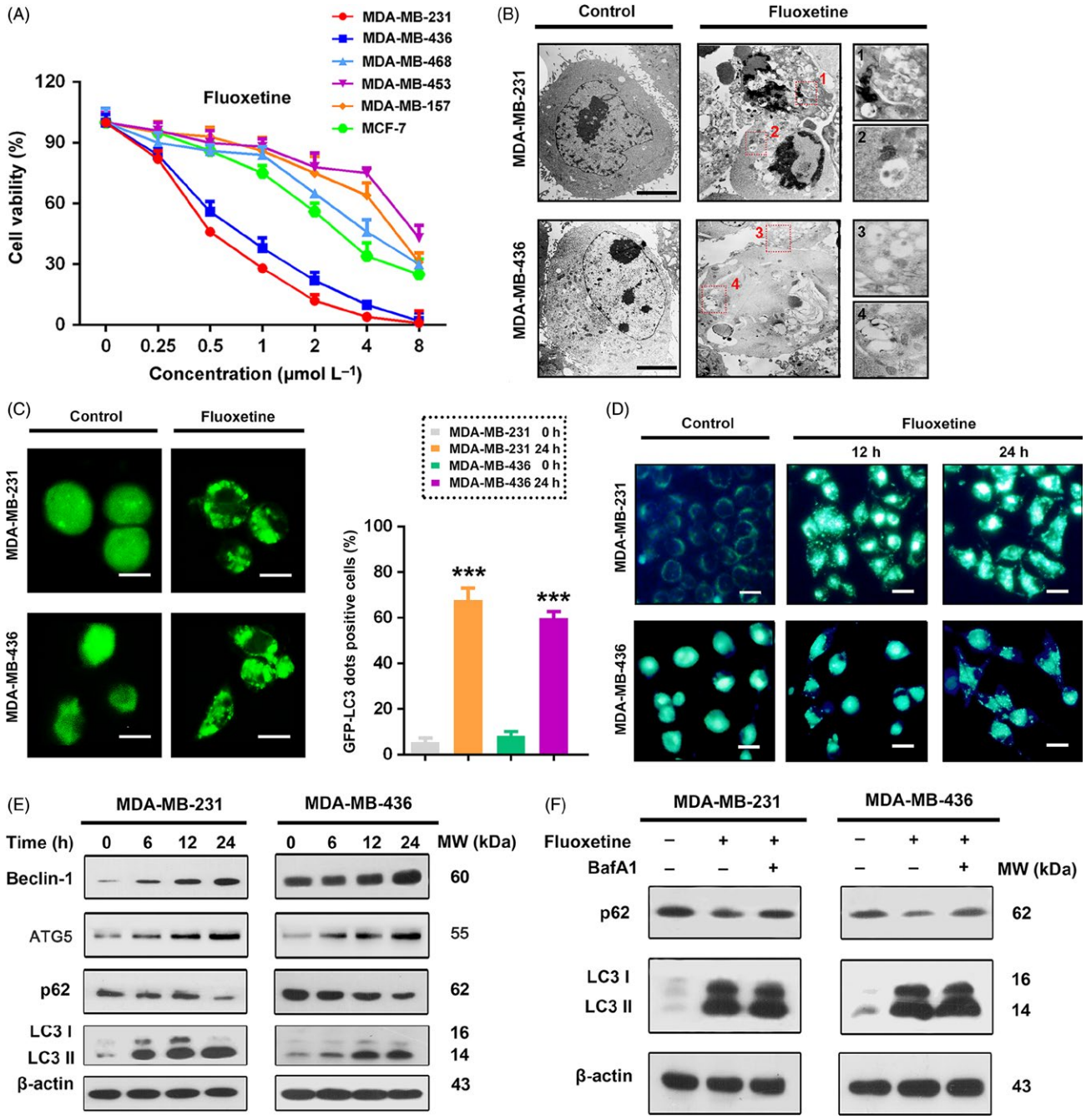


**FIGURE 1** Fluoxetine induces autophagy in MDA-MB-231 and MDA-MB-436 cells. A, Cell viabilities were measured by the MTT assay in Fluoxetine-treated breast cancer cell lines, including MDA-MB-231, MDA-MB-436, MDA-MB-468, MDA-MB-453, MDA-MB-157 and MCF-7 cells. B, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 μmol/L Fluoxetine for the indicated times. Then cells were processed for electron microscopy. Scale bar = 5 μm. C, MDA-MB-231 and MDA-MB-436 cells were transfected with a GFP-LC3 plasmid, followed by treatment with 0.5 μmol/L Fluoxetine. Then, the cells were inspected under a fluorescence microscope. The numbers of GFP-LC3 dots positive cells were analysed from at least 20 randomly chosen fields. ***$P < .001$ compared with control group. Scale bar = 20 μm. D, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 μmol/L Fluoxetine for the indicated times. Then, the cells were detected by MDC fluorescence staining under a fluorescence microscope. Scale bar = 20 μm. E, Fluoxetine or MDA-MB-436 cells were treated with Fluoxetine for the indicated times. The expression levels of Beclin-1, p62 and LC3 were examined by Western blot analysis. F, MDA-MB-231 or MDA-MB-436 cells were treated with Fluoxetine for 24 hours in the presence or absence of bafilomycin A1 (BafA1). The autophagy flux was determined by Western blot analysis of LC3 and p62. β-actin was used as a loading control

we found that Fluoxetine significantly decreased the phosphorylation of eEF2K at ser$^{78}$ and ser$^{398}$, indicating the inhibition of eEF2K activity. In addition, we also found that Fluoxetine inhibited the phosphorylation of eEF2 at thr$^{56}$ and had little effect on eEF2 expression, suggesting the activation of eEF2 caused by eEF2K inhibition (Figure 2A). To further demonstrate whether Fluoxetine has

a coordinative inhibition efficacy with eEF2K siRNA, we detected the expressions of eEF2K, p-eEF2K (ser$^{398}$), eEF2, p-eEF2 (thr$^{56}$) and LC3 after eEF2K knockdown, in the absence or presence of Fluoxetine. As expected, eEF2K knockdown could markedly reduce the expression of eEF2K, p-eEF2K (ser$^{398}$) and p-eEF2 (thr$^{56}$), while the expression of eEF2 was barely changed. Compared with Fluoxetine alone treatment, using Fluoxetine in combination with eEF2K siRNA resulted in more potent effect on these protein expressions (Figure 2B). As AMPK plays a key role in the regulation of energy homeostasis and eEF2K is regulated by the mTOR-p70S6K pathway, we subsequently examined the expression of AMPK, mTOR and p70S6K. These results showed that after treatment with Fluoxetine, the phosphorylation of mTOR and p70S6K decreased, while AMPK was activated, suggesting that Fluoxetine may regulate autophagy through the AMPK and mTOR pathways (Figure 2C). Because the ULK1-mAtg13-FIP200 complex is closely related with autophagosome formation, we next examined the expression levels of ULK1, mAtg13, Atg101 and FIP200. As a result, the expression levels of p-ULK1 (phosphorylation at ser$^{317}$ and ser$^{555}$), mAtg13, Atg101 and FIP200 increased in a time-dependent manner after treatment with Fluoxetine (Figure 2D). Taken together, these results indicate that Fluoxetine induces autophagy through inhibition of eEF2K and modulation of AMPK-mTOR-ULK complex pathway.

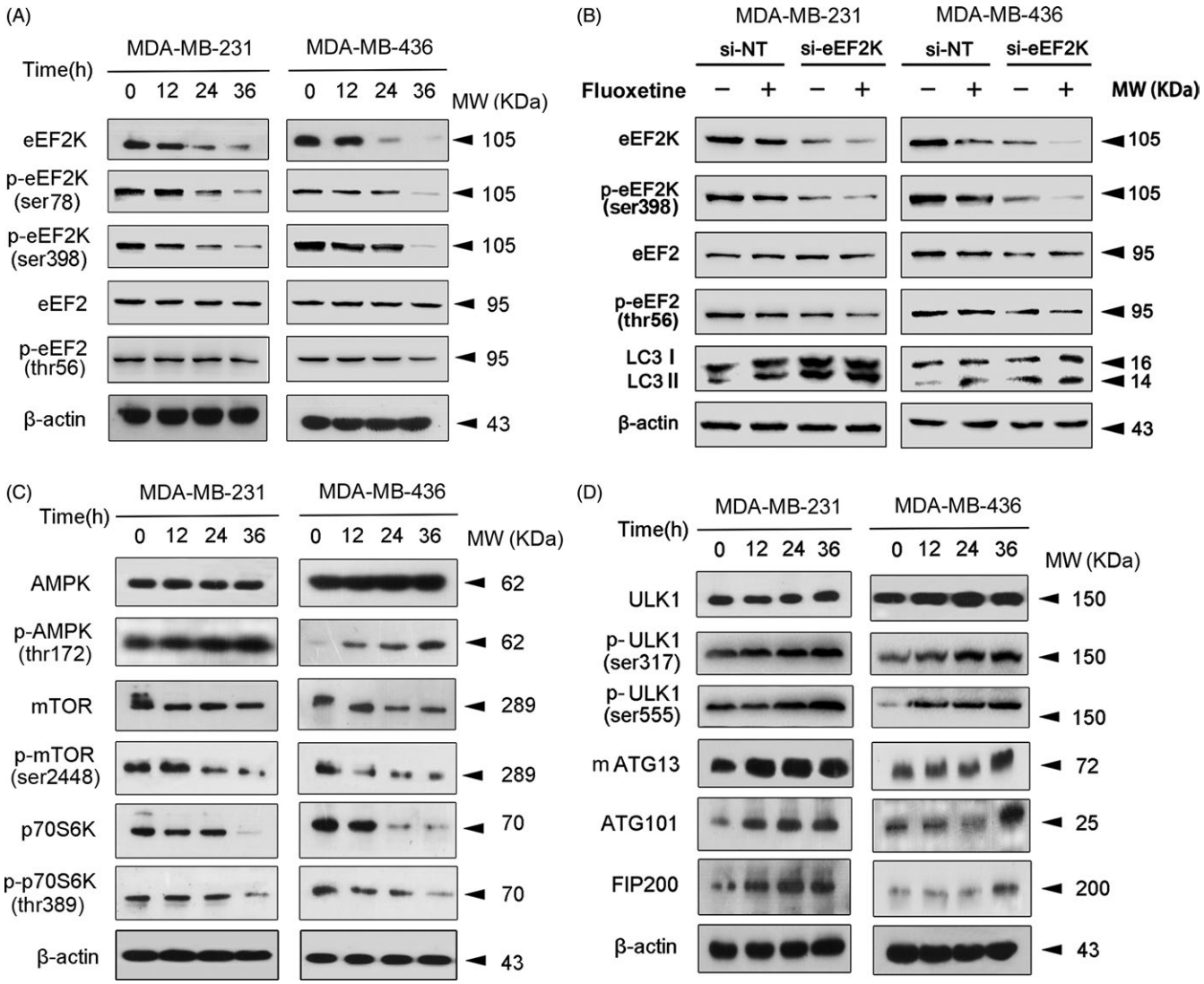


**FIGURE 2** Fluoxetine induces autophagy-associated with inhibition of eEF2K and modulation of AMPK-mTOR-ULK complex pathways. A, Fluoxetine-induced eEF2K suppression. MDA-MB-231 or MDA-MB-436 cells were treated with Fluoxetine. Then, the expression levels of eEF2K, p-eEF2K (ser$^{78}$), p-eEF2K (ser$^{398}$), eEF2 and p-eEF2 (thr$^{56}$) were examined by Western blot analysis. B, Fluoxetine displayed almost coordinative effects with eEF2K siRNA on EEF2K inhibition and autophagy induction. MDA-MB-231 or MDA-MB-436 cells were treated with Fluoxetine in the presence or absence of eEF2K siRNA. Then the expression levels of eEF2K, p-eEF2K (ser$^{398}$), eEF2, p-eEF2 (thr$^{56}$) and LC3 were examined by Western blot analysis. C, Fluoxetine affected the AMPK-mTOR-p70S6K pathway. After treatment, the expression levels of AMPK, p-AMPK, mTOR, p-mTOR, p70S6K and p-p70S6K were examined by Western blot analysis. D, Fluoxetine activated the ULK complex. After treatment, the expression of the ULK complex was examined by Western blot analysis. β-actin was used as a loading control

### 3.3 | Fluoxetine induces autophagic cell death in MDA-MB-231 and MDA-MB-436 cells

To determine the role of autophagy induced by Fluoxetine, the autophagy inhibitor 3-methyladenine (3-MA), which inhibits autophagy by blocking autophagosome formation via inhibiting type III phosphatidylinositol 3-kinases (PI3KIII), was employed to block the induction of autophagy. The MTT assay was carried out to assess the cell viability. We found that the cell viabilities were significantly increased following treatment with 2 mmol/L 3-MA ($P < .01$) (Figure 3A). Subsequently, flow cytometry analysis with Annexin V/PI demonstrated that 3-MA suppressed the level of apoptotic cells upon Fluoxetine stimulation. The apoptotic cell ratio decreased in cells treated with 3-MA ($P < .05$) (Figure 3B). Flow cytometry PI staining showed that 3-MA suppressed the accumulation of sub-G1 (Figure 3C,D). Western blot analysis results revealed that 3-MA could obviously inhibit caspase activities (Figure 3E). These results suggest that Fluoxetine induces autophagic death in MDA-MB-231 and MDA-MB-436 cells.

### 3.4 | Fluoxetine induces apoptosis in MDA-MB-231 and MDA-MB-436 cells

To determine the mechanism of Fluoxetine-induced cell death, we employed flow cytometry analysis with PI staining to detect the occurrence of apoptosis. The results suggested that Fluoxetine-induced apoptosis in MDA-MB-231 and MDA-MB-468 cells, which was determined by the increasing percentage of cells in sub-G1 phase (Figure 4A,B). Moreover, Western blot analysis revealed that the expression levels of caspase-3/8 and PARP were remarkably up-regulated after Fluoxetine treatment (Figure 4C). Taken together, these results indicate that Fluoxetine could induce apoptosis in MDA-MB-231 and MDA-MB-468 cells.

### 3.5 | The intricate relationship between Fluoxetine-induced autophagic cell death and apoptosis

To explore the intricate relationship between Fluoxetine-induced autophagic cell death and apoptosis, we next added pan-caspase inhibitor

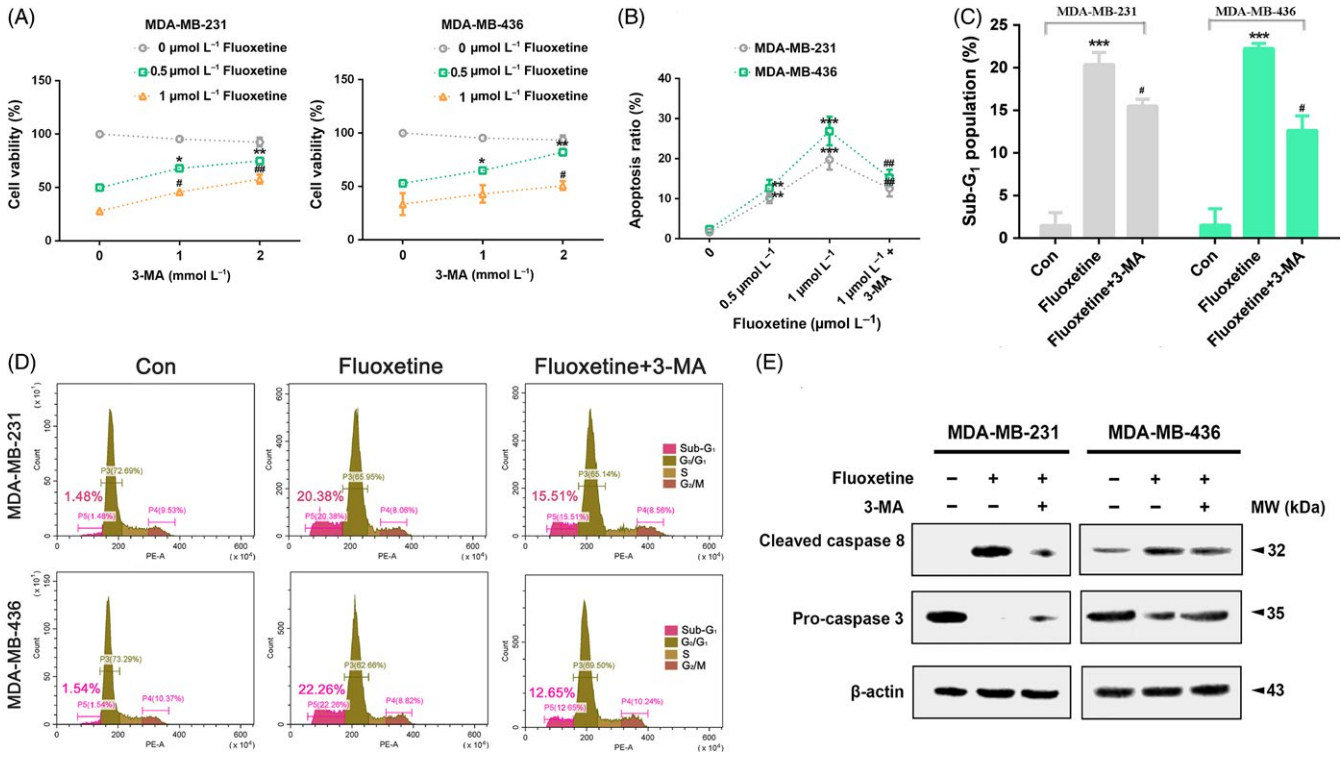


**FIGURE 3** Fluoxetine induces autophagic cell death in MDA-MB-231 and MDA-MB-436 cells. A, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 and 1 μmol/L of Fluoxetine, 3-MA (1 and 2 mmol/L) was added 1 hour before treated with Fluoxetine respectively. After treatment, cell viabilities were detected by MTT assay. $^{*}P < .05$; $^{**}P < .01$ compared with Fluoxetine-treated group (0.5 μmol/L), $^{\#}P < .05$; $^{\#\#}P < .01$ compared with Fluoxetine-treated group (1 μmol/L). B, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 and 1 μmol/L Fluoxetine for indicated times, apoptosis ratios were determined by flow cytometry analysis of Annexin V/PI staining. 3-MA (2 mmol/L) was added 1 hour before treated with Fluoxetine. $^{**}P < .01$; $^{***}P < .001$ compared with control group, $^{\#\#}P < .01$ compared with Fluoxetine-treated group. C, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 μmol/L Fluoxetine for indicated times. Sub-G1 phase population was determined by flow cytometry analysis of PI staining. 3-MA (2 mmol/L) was added 1 hour before treated with Fluoxetine. D, Bar graph depicting sub-G1 population. $^{***}P < .001$ compared with control group, $^{\#}P < .05$ compared with Fluoxetine-treated group. E, MDA-MB-231 or MDA-MB-436 cells were treated with Fluoxetine in the presence or absence of 3-MA. Then the expression levels of caspase 8 and caspase 3 were examined by Western blot analysis

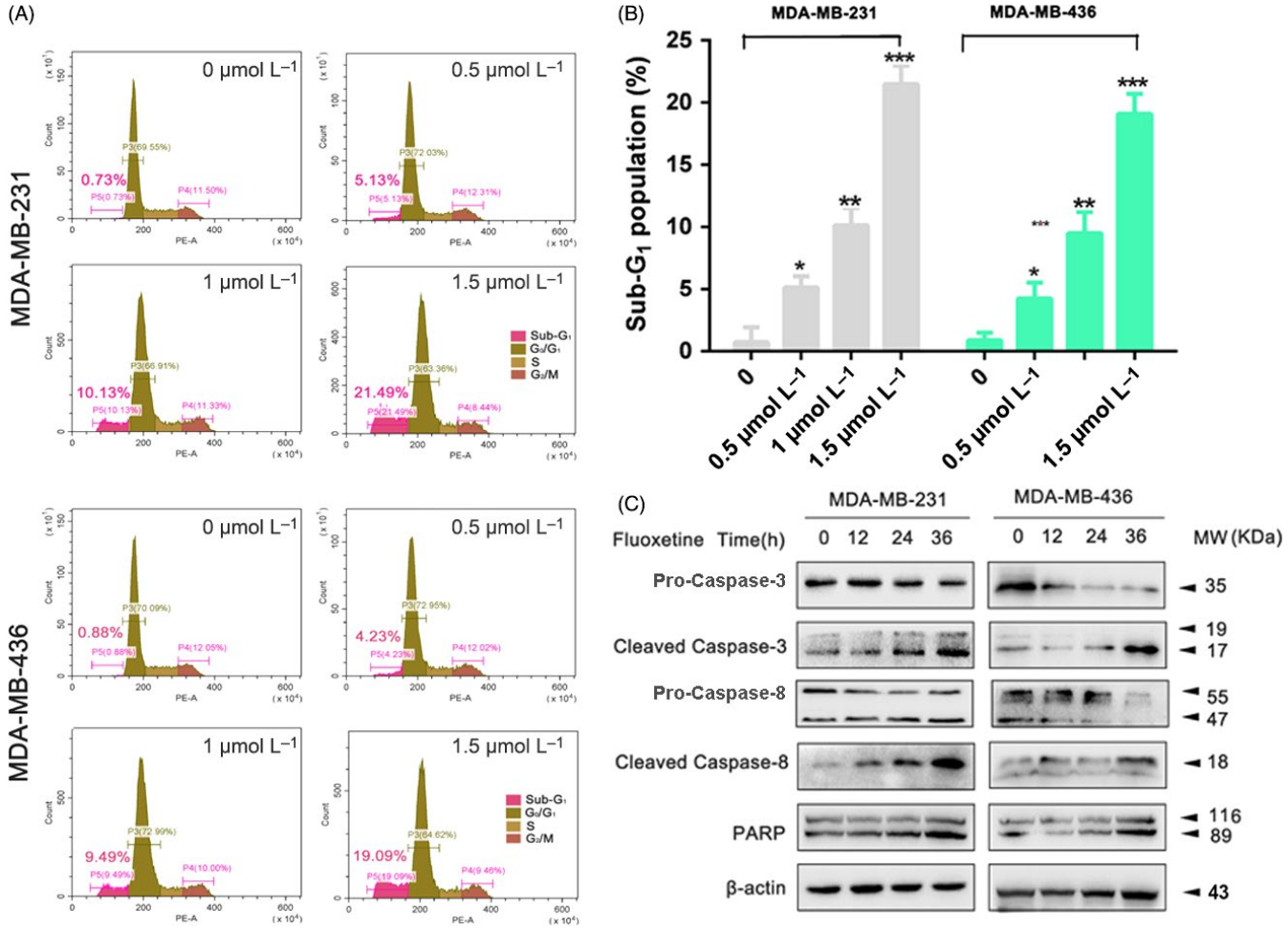


**FIGURE 4** Fluoxetine induces apoptosis in MDA-MB-231 and MDA-MB-436 cells. A, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 μmol/L Fluoxetine for indicated times, and the number of Fluoxetine-treated cells in the sub-G1 phase was analysed by flow cytometry of PI staining. The increased percentage of cells in the sub-G1 phase showed that Fluoxetine-induced apoptosis. B, Bar graph depicting sub-G1 population. *$P$ < .05; **$P$ < .01; ***$P$ < .001 compared with control group. C, The expression levels of apoptosis-related proteins, including Caspase 3, Caspase 8 and PARP were examined by Western blot analysis. β-actin was used as a loading control

Z-VAD to our experiments. Interestingly, we found that the autophagy ratio was not changed after treatment with Z-VAD, suggesting that caspase inhibition cannot block Fluoxetine-induced autophagy (Figure 5A). Moreover, the MTT assay further demonstrated that Z-VAD did not increase cell viabilities markedly (Figure 5B), whereas Z-VAD could not inhibit autophagy induction as determined by p62 degradation and LC3-II accumulation (Figure 5C). Together, above-mentioned results indicate that Fluoxetine induces autophagic cell death, then leading to apoptosis in TNBC cells.

### 3.6 | iTRAQ-based proteomics analyses of Fluoxetine-induced autophagic mechanisms

To reveal the molecular mechanisms underlying Fluoxetine-induced autophagic cell death with eEF2K, iTRAQ-based proteomics analysis was employed to profile differentially expressed proteins in MDA-MB-231 and MDA-MB-436 cells treated with Fluoxetine respectively. Hundreds of proteins were retrieved from the proteomics results, among which only differentially expressed proteins (>1.2, <0.8) were selected for further analysis. Given the pivotal role that eEF2K plays in protein translation elongation and programmed cell death, proteins that were differentially expressed in iTRAQ analysis and that were associated with both protein synthesis and programmed cell death were considered highly relevant to the mechanisms with Fluoxetine. Moreover, functional annotation and clustering were processed in both cell lines (Figure 6A).

We also built the global human PPI network and modified this network into the eEF2K-related PPI network. Then, we identified differentially expressed proteins that interacted with eEF2K in this network (Tables S1 and S2). Four differential expression proteins, BIRC6, SNAP29, Bif-1 and BNIP1, were predicted to interact with eEF2K or to be affected by eEF2K (Figure 6B). Next, we found that BIRC6 was down-regulated, while SNAP29, BNIP1 and Bif-1 were up-regulated after Fluoxetine treatment in MDA-MB-231 cells (Figure 6C). To further validate the possible mechanisms under eEF2K inhibition, we used eEF2K siRNA to compare with Fluoxetine. Interestingly, we found that Fluoxetine displayed almost coordinative effects with eEF2K siRNA on the expressions of novel eEF2K

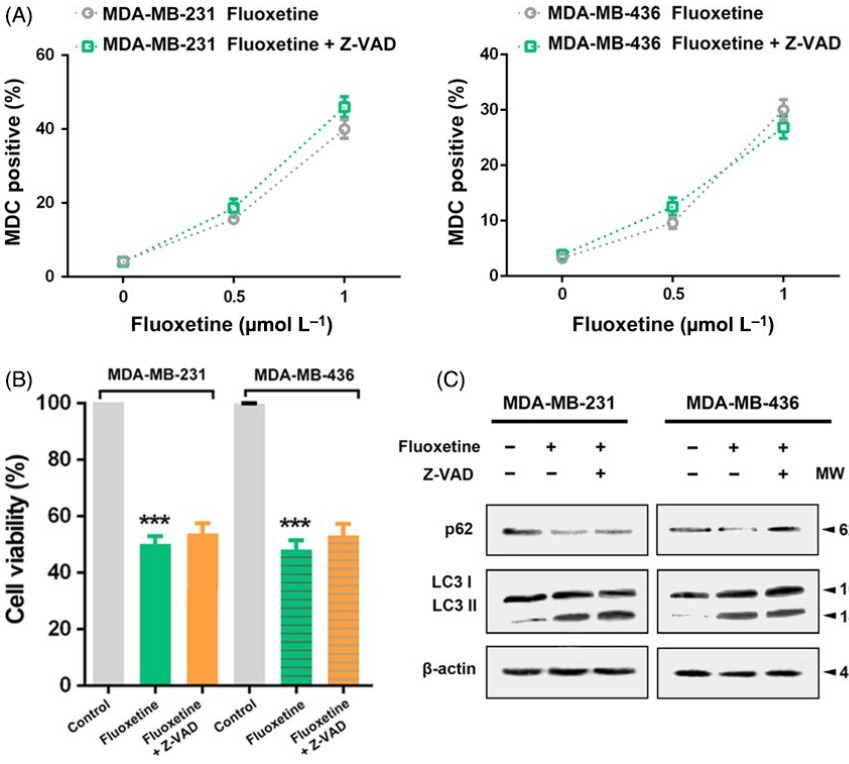


**FIGURE 5** The intricate relationship between Fluoxetine-induced autophagic cell death and apoptosis. A, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 and 1 μmol/L of Fluoxetine, Z-VAD (5 μmol/L) was added 1 hour before treated with Fluoxetine. After treatment, cell viabilities were detected by MTT assay. B, MDA-MB-231 or MDA-MB-436 cells were treated with 0.5 and 1 μmol/L Fluoxetine for indicated times, autophagy ratios were determined by flow cytometry analysis of MDC staining. Z-VAD (5 μmol/L) was added 1 hour before treated with Fluoxetine ***$P < .001$ compared with control group. C, MDA-MB-231 or MDA-MB-436 cells were treated with Fluoxetine in the presence or absence of Z-VAD. Then the expression levels of p62 and LC3 were examined by Western blot analysis

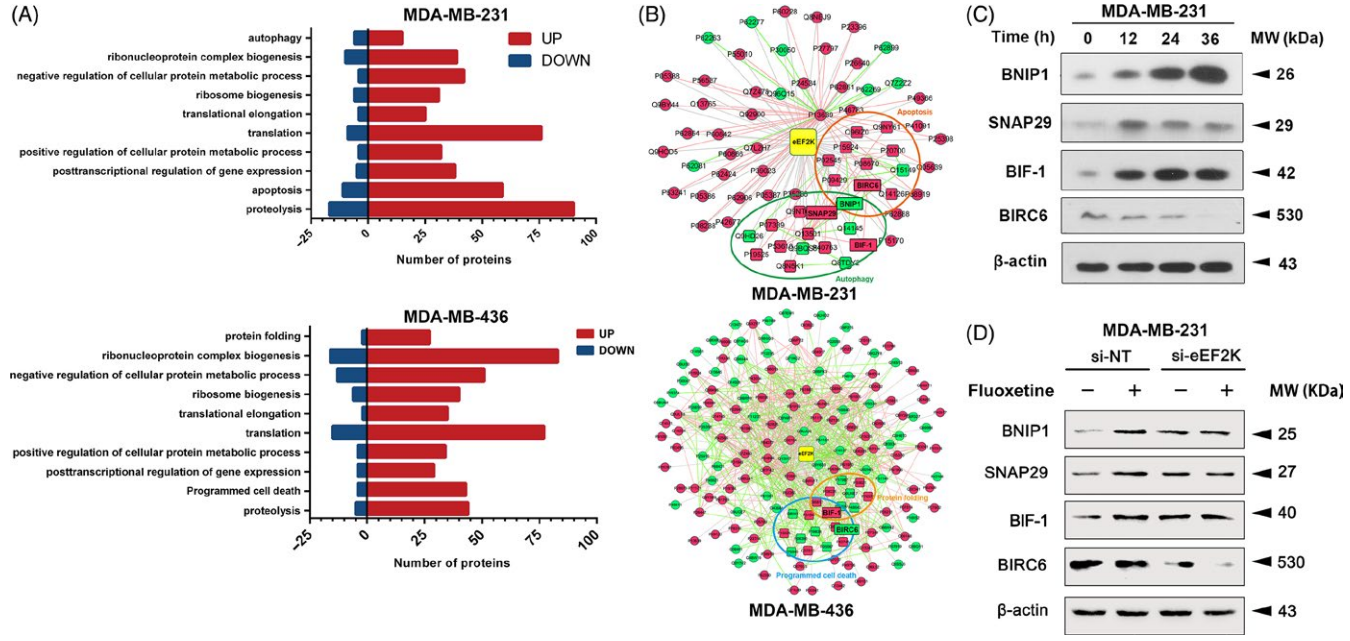


**FIGURE 6** Proteomics-based identification of novel Fluoxetine-regulated pathways. A, Enrichment analyses in Fluoxetine-treated MDA-MB-231 and MDA-MB-436 cells. B, Proteomics-based identification of novel pathways in Fluoxetine-treated MDA-MB-231 and MDA-MB-436 cells. C, MDA-MB-231 cells were treated with Fluoxetine. Then, the expression levels of BNIP1, SNAP29, Bif-1 and BIRC6 were examined by Western blot analysis. D, MDA-MB-231 cells were treated with Fluoxetine in the presence or absence of eEF2K siRNA. Then the expression levels of BNIP1, SNAP29, Bif-1 and BIRC6 were examined by Western blot analysis. β-actin was used as a loading control

interactors. When Fluoxetine was used in combination with eEF2K siRNA, we found more markedly decreased expression of BIRC6, as well as up-regulation of SNAP29, BNIP1 and Bif-1 (Figure 6D). These results indicate that the Fluoxetine-induced mechanism is involved BIRC6, BNIP1, SNAP29 and Bif-1, which may be associated with inhibition of eEF2K.

## 4 | DISCUSSIONS

Triple negative breast cancer constitutes a heterogeneous subtype of breast cancers characterized by its biological aggressiveness, worse prognosis and lacking of therapeutic targets. A majority of TNBC patients still exhibit poor outcomes, with only 30%-45% of patients

achieving a pathological complete response (pCR) and survival rates similar to other breast cancer subtypes. Consequently, TNBC remains a significant challenge for the current TNBC therapeutics. Previous studies have demonstrated that Fluoxetine treatment changes autophagic flux and apoptosis, and the PI3K-AKT-mTOR regulatory pathway is involved in the mechanism of Fluoxetine.[29] In our study, we demonstrated that Fluoxetine could induce autophagic cell death in TNBC cells. Interestingly, we found that the autophagy-modulating mechanism of Fluoxetine was dependent on the AMPK-mTOR-ULK complex pathways. A recent study has revealed that silencing eEF2K induced AMPK-ULK1-dependent autophagy in colon cancer cells, suggesting eEF2K can regulate ULK1 indirectly.[30] And it is also well-known that ULK1 and ULK complex, as the initiator of autophagy, are regulated by AMPK and mTOR.[31] Therefore, Fluoxetine-induced eEF2K inhibition may lead to a feedback regulation on AMPK-mTOR-p70S6K pathways, thereby activating ULK complex to induce autophagy. Distinct from these above-mentioned mechanisms, our study also identified some novel key proteins such as BINP1, SNAP29, Bif-1 and BIRC6, which could be associated with eEF2K in Fluoxetine-induced autophagic cell death. BNIP1 is capable of recruiting autophagy receptor p62, which simultaneously binds both ubiquitin and LC3 to link ubiquitination and autophagy.[32] And recent evidence suggests that SNAP29 is essential for the fusion between autophagosomes and lysosomes controlled by Atg14/Atg14L.[33] Bif-1, a membrane curvature-inducing protein, interacts with Beclin-1 through UVRAG and regulates the post-Golgi trafficking of membrane-integrated Atg9a for autophagy.[34] BIRC6, as an inhibitor of apoptosis, has recently shown to be overexpressed in different types of cancer such as breast cancer.[35] Among these proteins, Bif-1 and BNIP1 exert significant roles in both autophagy and apoptosis, whereas SNAP29 is necessary to autophagosome maturation and BIRC6 is very important to inhibit apoptosis. Our findings would uncover more potential mechanisms for Fluoxetine-induced autophagic cell death, which remain to be further investigated.

In conclusion, our results demonstrate that a small molecule, Fluoxetine, potently inhibits tumour growth of TNBC, as well as inducing apoptosis and autophagic cell death associated with inhibition of eEF2K, modulation of multiple signalling pathways, including AMPK-mTOR-ULK, BIRC6, BNIP1, SNAP29 and Bif-1, which may provide a promising avenue for future TNBC therapy.

## CONFLICTS OF INTEREST

We declare that none of the authors have financial interest related to this work.

## ORCID

*Lixia Chen* http://orcid.org/0000-0003-2196-1428

## REFERENCES

1. Kalimutho M, Parsons K, Mittal D, López JA, Srihari S, Khanna KK. Targeted therapies for triple-negative breast cancer: combating a stubborn disease. *Trends Pharmacol Sci*. 2015;36:822-846.
2. Balanis N, Carlin CR. Stress-induced EGF receptor signaling through STAT3 and tumor progression in triple-negative breast cancer. *Mol Cell Endocrinol*. 2017;451:24-30.
3. Ouyang L, Zhang L, Fu L, Liu B. A small-molecule activator induces ULK1-modulating autophagy-associated cell death in triple negative breast cancer. *Autophagy*. 2017;13:1-2.
4. Bosch A, Eroles P, Zaragoza R, Viña JR, Lluch A. Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. *Cancer Treat Rev*. 2010;36:206-215.
5. Stagg J, Allard B. Immunotherapeutic approaches in triplenegative breast cancer: latest research and clinical prospects. *Ther Adv Med Oncol*. 2013;5:169-181.
6. Bakrania AK, Variya BC, Patel SS. Novel targets for paclitaxel nano formulations: hopes and hypes in triple negative breast cancer. *Pharmacol Res*. 2016;111:577-591.
7. Kumar P, Aggarwal R. An overview of triple-negative breast cancer. *Arch Gynecol Obstet*. 2016;293:247-269.
8. Zhang L, Guo M, Li J, et al. Systems biology-based discovery of a potential Atg4B agonist (Flubendazole) that induces autophagy in breast cancer. *Mol BioSyst*. 2015;11:2860-2866.
9. Zhang L, Fu LL, Zhang SY, et al. Discovery of a small molecule targeting ULK1-modulated cell death of triple negative breast cancer in vitro and in vivo. *Chem Sci*. 2017;8:2687-2701.
10. Shi Z, Li CY, Zhao S, et al. A systems biology analysis of autophagy in cancer therapy. *Cancer Lett*. 2013;337:149-160.
11. Thorburn A. Apoptosis and autophagy: regulatory connections between two supposedly different processes. *Apoptosis*. 2008;13:1-9.
12. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. *Biochim Biophys Acta*. 2013;1833:3448-3459.
13. Liu JJ, Lin M, Yu JY, Liu B, Bao JK. Targeting apoptotic and autophagic pathways for cancer therapeutics. *Cancer Lett*. 2011;300:105-114.
14. Fu LL, Xie T, Zhang SY, Liu B. Eukaryotic elongation factor-2 kinase (eEF2K): a potential therapeutic target in cancer. *Apoptosis*. 2014;19:1527-1531.
15. White-Gilbertson S, Kurtz DT, Voelkel-Johnson C. The role of protein synthesis in cell cycling and cancer. *Mol Oncol*. 2009;3:402-408.
16. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
17. Chen Z, Gopalakrishnan SM, Bui MH, et al. 1-Benzyl-3-cetyl-2-methylimidazolium iodide (NH125) induces phosphorylation of eukaryotic elongation factor-2 (eEF2): a cautionary note on the anticancer mechanism of an eEF2 kinase inhibitor. *J Biol Chem*. 2011;286:43951-43958.
18. Caiaffo V, Oliveira BDR, Sa FB, Neto JE. Anti-inflammatory, antiapoptotic, and antioxidant activity of fluoxetine. *Pharmacol Res Perspect*. 2016;4:e00231.
19. Oliveira MR. Fluoxetine and the mitochondria: a review of the toxicological aspects. *Toxicol Lett*. 2016;258:185-191.
20. Gwynne WD, Hallett RM, Girgis-Gabardo A, et al. Serotonergic system antagonists target breast tumor initiating cells and synergize with chemotherapy to shrink human breast tumor xenografts. *Oncotarget*. 2017;8:32101-32116.
21. Autry AE, Adachi M, Nosyreva E, et al. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature*. 2011;475:91-95.
22. Monteggia LM, Gideons E, Kavalali ET. The role of eukaryotic elongation factor 2 kinase in rapid antidepressant action of ketamine. *Biol Psychiatry*. 2013;73:1199-1203.
23. Malinow R. Depression: ketamine steps out of the darkness. *Nature*. 2016;533:477-478.
24. Zanos P, Moaddel R, Morris PJ, et al. NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature*. 2016;533:481-486.
25. Wang WB, Feng LX, Yue QX, et al. Paraptosis accompanied by autophagy and apoptosis was induced by celastrol, a natural compound

with influence on proteasome, ER stress and Hsp90. *J Cell Physiol*. 2012;227:2196-2206.
26. Fu LL, Wang SY, Wang X, Wang PQ, Zheng YX, Yao DH. Crystal structure-based discovery of a novel synthesized PARP1 inhibitor (OL-1) with apoptosis-inducing mechanisms in triple-negative breast cancer. *Sci Rep*. 2016;6:3.
27. Zhang QC, Petrey D, Deng L, et al. Structure-based prediction of protein-protein interactions on a genome-wide scale. *Nature*. 2012;490:556-560.
28. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4:44-57.
29. Yang Y, Hu Z, Du X, Davies H, Huo X, Fang M. miR-16 and fluoxetine both reverse autophagic and apoptotic change in chronic unpredictable mild stress model rats. *Front Neurosci*. 2017;11:428.
30. Xie CM, Liu XY, Sham KW, Lai JM, Cheng CH. Silencing of EEF2K (eukaryotic elongation factor-2 kinase) reveals AMPK-ULK1-dependent autophagy in colon cancer cells. *Autophagy*. 2014;10:1495-1508.
31. Alers S, Löffler AS, Wesselborg S, Stork B. Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Mol Cell Biol*. 2012;32:2-11.
32. Tang F, Wang B, Li N, et al. RNF185, a novel mitochondrial ubiquitin E3 ligase, regulates autophagy through interaction with BNIP1. *PLoS ONE*. 2011;6:e24367.
33. Liu R, Zhi X, Zhong Q. ATG14 controls SNARE-mediated autophagosome fusion with a lysosome. *Autophagy*. 2015;11:847-849.
34. Takahashi Y, Young MM, Serfass JM, Hori T, Wang HG. Sh3glb1/Bif-1 and mitophagy: acquisition of apoptosis resistance during Myc-driven lymphomagenesis. *Autophagy*. 2013;9:1107-1109.
35. Luk SU, Xue H, Cheng H, et al. The BIRC6 gene as a novel target for therapy of prostate cancer: dual targeting of inhibitors of apoptosis. *Oncotarget*. 2014;5:6896-6908.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Sun D, Zhu L, Zhao Y, et al. Fluoxetine induces autophagic cell death via eEF2K-AMPK-mTOR-ULK complex axis in triple negative breast cancer. *Cell Prolif*. 2018;51:e12402. https://doi.org/10.1111/cpr.12402