

Original Article

# Transcription factor EB is involved in autophagy-mediated chemoresistance to doxorubicin in human cancer cells

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## Abstract

Transcription factor EB (TFEB) is a master regulator of autophagy activity and lysosomal biogenesis, but its role in autophagy-mediated cell survival and chemotherapy resistance is not completely understood. In this study, we explored whether TFEB played an important role in autophagy-mediated chemotherapy resistance in human cancer LoVo and HeLa cells *in vitro*. Treatment of human colon cancer LoVo cells with doxorubicin (0.5  $\mu\text{mol/L}$ ) induced autophagy activation and nuclear translocation of TFEB, which resulted from inactivation of the mTOR pathway. In both LoVo and HeLa cells, overexpression of TFEB enhanced doxorubicin-induced autophagy activation and significantly decreased doxorubicin-induced cell death, whereas knockdown of TFEB with small interfering RNA blocked doxorubicin-induced autophagy and significantly enhanced the cytotoxicity of doxorubicin. In LoVo cells, autophagy inhibition by 3-methyladenine (3-MA) or knockdown of autophagy-related gene Atg5 increased cell death in response to doxorubicin, and abolished TFEB overexpression-induced chemotherapy resistance, suggesting that the inhibition of autophagy made cancer cells more sensitive to doxorubicin. The results demonstrate that TFEB-mediated autophagy activation decreases the sensitivity of cancer cells to doxorubicin.

**Keywords:** transcription factor EB (TFEB); LoVo cells; HeLa cells; doxorubicin; autophagy; apoptosis; chemotherapy resistance; 3-methyladenine

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## Introduction

Resistance of cancer cells to chemotherapeutic drugs remains the main obstacle to achieving effective treatment in human malignancies<sup>[1]</sup>. It has been found that various factors contribute to chemoresistance, including alterations in the drug target, ineffective induction of cell death pathways and activation of pro-survival pathways. Recent studies have revealed that autophagy activation is one of the key mechanisms by which cancer cells handle stress and promote survival during therapeutic treatment<sup>[1, 2]</sup>. Autophagy, a cellular adaptive response that helps organisms survive stress conditions, is emerging as an important mechanism in the development of chemoresis-

tance<sup>[3]</sup>. Autophagy is an intracellular degradation system that transports proteins and organelles to the lysosomes<sup>[4, 5]</sup>. Metabolic stress, such as starvation or hypoxia activates autophagy, providing necessary energy for tolerating adverse conditions. Although the functions of autophagy in regulating cancer initiation, development and chemotherapy remain controversial<sup>[6, 7]</sup>, many studies indicate that autophagy facilitates tumor development<sup>[5, 8]</sup>. In addition, increasing evidence indicates that autophagy acts as a pro-survival and resistance mechanism against chemotherapy<sup>[1, 9]</sup>. Our previous studies showed that epirubicin induces autophagy and that autophagy activation plays a vital role in drug resistance in tumor cells. Conversely, inhibition of autophagy augments epirubicin-induced apoptosis<sup>[10, 11]</sup>. Therefore, autophagy modulation, especially inhibition of autophagy, is emphasized as a novel strategy in cancer therapy<sup>[3]</sup>. However, the mechanisms by which autophagy participates in chemotherapy resistance have yet to be clarified.

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Transcription factor EB (TFEB), which is a member of the MiT/TFE family, is a major regulator of lysosomal biogenesis<sup>[12, 13]</sup>. Many target genes of TFEB participate in substrate degradation and lysosome-associated processes, such as autophagy and endocytosis<sup>[14]</sup>. mTORC1-mediated dephosphorylation of TFEB at the lysosomal membrane leads to TFEB nuclear translocation and regulates autophagosome formation and autophagosome-lysosome fusion under nutrient stress<sup>[15]</sup>. A recent study demonstrated that transcriptional activation of the lysosomal clearance pathway is a novel feature of aggressive malignancy<sup>[16]</sup>. Nuclear import of the MiT/TFE family, including transcription factors MITF, TFE3 and TFEB, is essential for pancreatic ductal adenocarcinoma growth by driving the expression of genes that promote high levels of lysosomal catabolic function<sup>[16]</sup>. Consistent with these findings, increased TFEB expression is associated with high levels of lysosomal markers and a poor prognosis in non-small cell lung cancer<sup>[17]</sup>. Furthermore, TFEB-mediated increases in lysosomal functions play a prominent role in multidrug resistance. Lysosomes sequester hydrophobic weak base chemotherapeutics away from their target sites. An increased number of lysosomes associated with nuclear translocation of TFEB is detected in cancer cells that are exposed to hydrophobic weak base drugs<sup>[18]</sup>. Considering that both autophagy and TFEB are potential targets of drug resistance, we predicted that autophagy activation mediated by TFEB nuclear translocation may be involved in the chemoresistance of tumor cells. In the present study, we found that TFEB nuclear translocation partially reduced the sensitivity of cancer cells to doxorubicin through activating autophagy.

## Materials and methods

### Antibodies, plasmids, and reagents

The following antibodies were used in this study: anti-TFEB and anti-GFP from Sigma (St Louis, MO, USA); anti-LC3 from MBL (Nagoya, Japan); anti- $\beta$ -actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-GAPDH, anti-histone H3, anti-p-mTOR, anti-mTOR, anti-p-p70S6K, anti-p70S6K and anti-Atg5 from Cell Signaling Technology (Danfoss, MA, USA); and anti-caspase-3 antibody from ENZO Life Sciences (Farmingdale, NY, USA). The EGFP-TFEB plasmid was kindly provided by Prof Guang-hui WANG (College of Pharmaceutical Sciences, Soochow University School of Pharmaceutical Science, Suzhou, China). Doxorubicin, 3-methyladenine (3-MA), and 4',6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich (St Louis, MO, USA). The Cell Counting Kit-8 was from Dojindo (CCK-8; Kumamoto, Japan). Crystal violet, Hoechst 33342 and Nuclear and Cytoplasmic Protein Extraction Kits were from Beyotime (Shanghai, China).

### Cell culture

LoVo and HeLa cells were from ATCC (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA) in a humidified incubator at 37°C with 5% CO<sub>2</sub> atmosphere. The cells

were passaged at pre-confluent densities every 2 to 3 d.

### Western blot analysis

Proteins were extracted from cells using cell lysis solution supplemented with protease inhibitors (Roche, 04693159001; Basle, Switzerland) and phosphorylase inhibitors (Roche, 04906845001). The protein concentration was determined with a BCA Protein Assay Kit (Pierce Biotechnology; Waltham, MA, USA). Equal concentrations of protein were separated on Tris-glycine SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% (*w/v*) nonfat milk and washed with TBST (TBS containing 0.1% Tween 20); then, they were incubated overnight at 4°C with the following antibodies: anti-TFEB, 1:1000; anti-histone H3, 1:10 000; anti-GFP, 1:2000; anti-LC3, 1:1000; anti-caspase-3, 1:1000; anti- $\beta$ -actin, 1:10 000; anti-p-mTOR, 1:1000; anti-mTOR, 1:1000; anti-p-p70S6K, 1:1000; anti-p70S6K, 1:1000; and anti-Atg5, 1:1000 in 5% (*w/v*) nonfat milk containing 0.02% NaN<sub>3</sub>. After being washed in TBST, the membranes were incubated with fluorescence secondary antibodies (1:10 000; Jackson ImmunoResearch, anti-rabbit, 711-035-152, anti-mouse, 715-035-150) at room temperature for 1 h. Immunoreactive protein bands were detected using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA). The signal intensity of the bands was quantitatively analyzed with ImageJ software (WS Rasband, ImageJ, NIH, Bethesda, MD, USA) and normalized to the loading control,  $\beta$ -actin.

### Plasmid and siRNA transfection

For plasmid transfection, cells were plated in 9-cm dishes at 30%–40% confluence, and the plasmid EGFP or EGFP-TFEB was transfected into the cells using Lipofectamine LTX and PLUS (Invitrogen, Burlingame, CA, USA) according to the manufacturer's instructions. For siRNA knockdown, the siRNA primers were synthesized by GenePharma (Shanghai, China) with the following target sense sequences: human *TFEB*, 5'-CCACGATGTATACAAGATA-3'; human *Atg5*, 5'-GCAACTCTGGATGGGATTG-3' and the negative control (NC), 5'-TAAGGCTATGAAGAGATAC-3'. Cells were transfected with siRNA primers using Lipofectamine RNAiMAX (Invitrogen, Burlingame, CA, USA) according to the manufacturer's instructions. The silencing efficiency of target proteins was examined with Western blot analysis 72 h after transfection.

### Immunofluorescence confocal microscopy

For monitoring the nuclear localization of TFEB, cells were plated on poly-L-lysine-coated glass coverslips in 24-well plates and cultured for 24 h; then, cells were treated with or without doxorubicin (0.5  $\mu$ mol/L) for 12 h. Then, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The slides were rinsed twice with PBS and were then permeabilized with 0.2% Triton X100 in PBS for 10 min at room temperature. After being rinsed twice with PBS, cells were incubated with the indicated primary antibodies in PBS containing 5% fetal bovine serum

and 0.1% (*w/v*) saponin for 1 h at room temperature. Then, cells were rinsed four times with PBS and incubated with the corresponding secondary antibodies for 45 min at room temperature in the dark. Afterward, cells were washed four times with PBS and counterstained with a DNA dye, DAPI, for the cell nucleus. After staining, coverslips were mounted onto glass slides with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA). Images were acquired with a laser scanning confocal microscope (Zeiss, Jena, German).

#### Nuclear and cytoplasmic fractionation

The isolation of the nucleus and cytoplasm was performed with a nuclear and cytoplasmic fractionation kit according to the manufacturer's procedures, as previously described by others in our laboratory<sup>[11]</sup>. The cytoplasmic and nuclear fractions were subjected to SDS-PAGE and immunoblotted for TFEB, cytoplasmic marker GAPDH and nuclear marker histone H3.

#### Cell viability assay

The short-term effect of doxorubicin on the viability of cells was evaluated using a Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. In brief, cells were cultured in 96-well plates ( $5 \times 10^3$  cells per well) in a final volume of 100  $\mu$ L/well medium and cultured in a humidified atmosphere (37°C, 5% CO<sub>2</sub>). After 24 h of culture, the cells were exposed to doxorubicin for 12 h and were then processed for a CCK-8 assay. CCK-8 reagent (10  $\mu$ L) was added to each well and then incubated for 1 h in a humidified atmosphere (37°C, 5% CO<sub>2</sub>). Quantification of the formazan dye produced by metabolically active cells was performed by measuring the absorbance at 450 nm using a microplate ELISA reader (ELx800; Bio-TEK Instruments, Winooski, VT, USA). In each experiment, quintuplicate wells were used for each drug concentration, and assays were repeated in three independent experiments.

#### Colony-formation assay

The long-term effect of TFEB knockdown on tumor cell proliferation was analyzed with the colony-formation assay. Cells transfected with NC or TFEB siRNA for 72 h were plated in 6-well plates at a density of 200 cells per well, and doxorubicin was added 24 h later. The medium was changed every 3 d. After 14 d, the colonies were stained with crystal violet for 15 min and rinsed with deionized distilled water. Then, the colonies were photographed using a Nikon D800 digital camera and scored using ImageJ software (WS Rasband, ImageJ, NIH, Bethesda, MD, USA).

#### Hoechst staining

The effects of doxorubicin and TFEB knockdown on apoptosis were evaluated using Hoechst 33342 staining. Cells were transfected with NC or TFEB siRNA for 72 h and then treated with doxorubicin (0.5  $\mu$ mol/L) for 12 h. Afterward, cells were fixed in 4% paraformaldehyde and stained with Hoechst 33342 (10  $\mu$ g/mL). Apoptotic nuclei were analyzed with laser scanning confocal microscopy (Nikon, C1S1, Tokyo, Japan), and

the apoptotic ratio was assessed in each group.

#### Flow cytometry

The apoptosis of LoVo cells was quantified with double staining of fluorescein isothiocyanate (FITC) conjugated Annexin-V and propidium iodide (PI; Biouniquer, BU-AP0103). Cells were transfected with NC or TFEB siRNA for 72 h and then treated with doxorubicin (0.5  $\mu$ mol/L) for 12 h. Ten thousand cells per sample were acquired with a FACScan flow cytometer (FACScan). Freshly trypsinized cells were pooled, washed twice with binding buffer, and processed according to the manufacturer's instructions<sup>[10]</sup>. Cells were analyzed with flow cytometry using Cell Quest Pro software (Beckman Coulter).

#### Statistical analysis

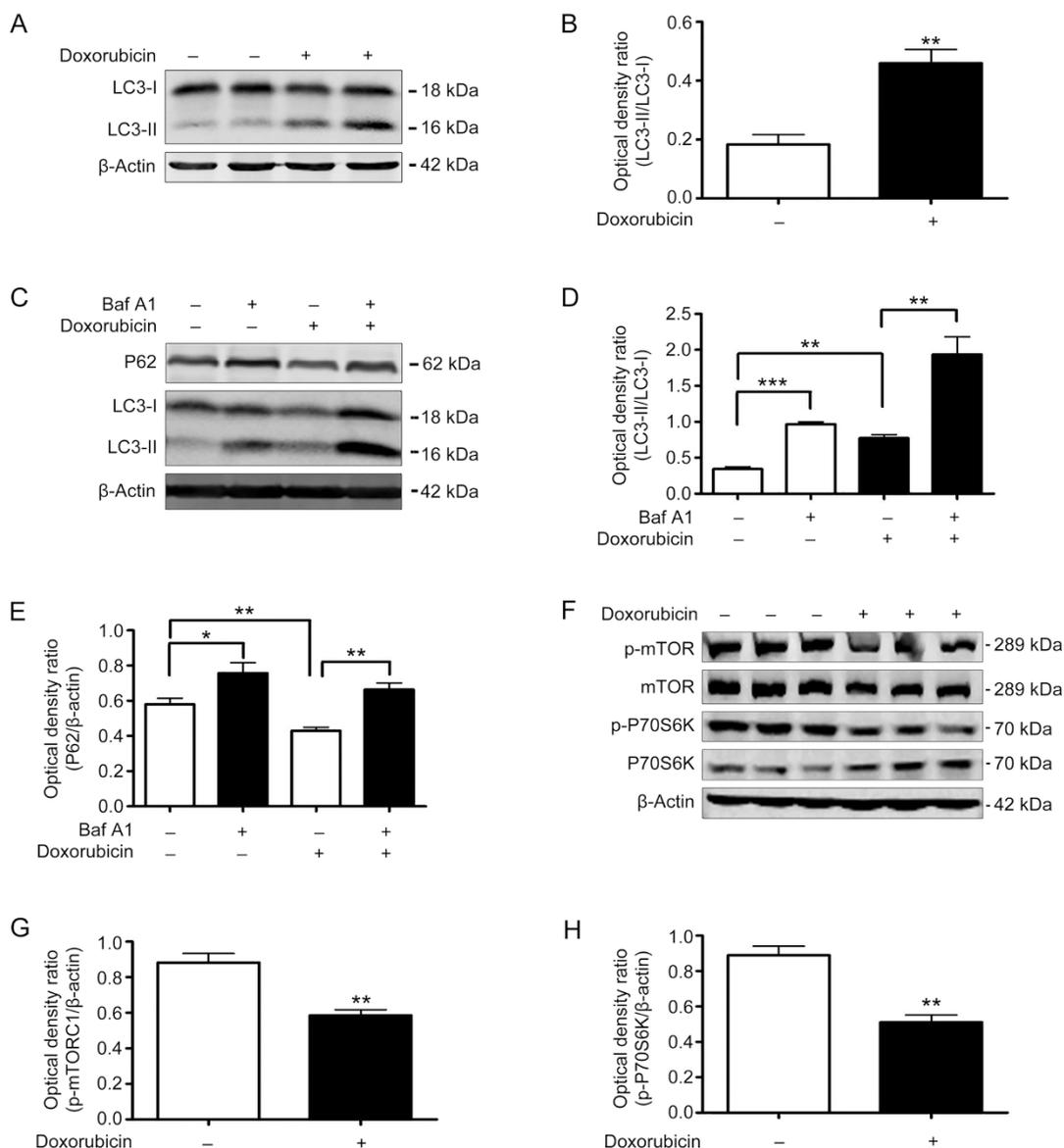
All data are presented as the mean $\pm$ SEM. Data were subjected to one-way ANOVA using the GraphPad Prism software statistical package (GraphPad Software, San Diego, CA, USA). When a significant group effect was found, *post hoc* comparisons were performed using the Newman-Keuls *t* test to examine special group differences. Independent group *t* tests were used for comparing two groups. Significant differences at  $P < 0.05$ ,  $< 0.01$ , and  $< 0.001$  are indicated with \*, \*\*, and \*\*\*, respectively.

## Results

### Autophagy activity is upregulated in doxorubicin-treated LoVo cells

Previous studies reported that doxorubicin could induce autophagy<sup>[19]</sup>. To investigate whether doxorubicin induces autophagy in LoVo cells, we treated the cells with doxorubicin (0.5  $\mu$ mol/L) for 12 h and determined the ratio of LC3-II/LC3-I, which is considered a measurement of autophagy activity and can be assessed with Western blot analysis. We found that doxorubicin treatment significantly upregulated the ratio of LC3-II/LC3-I (Figure 1A, 1B), indicating that autophagy was activated by doxorubicin treatment. To determine if doxorubicin enhanced the autophagy flux, we used bafilomycin A1 to inhibit autophagosome-lysosome fusion in LoVo cells. Both doxorubicin and bafilomycin A1 increased the ratio of LC3-II/LC3-I (Figure 1C, 1D). However, when the cells were treated with doxorubicin and bafilomycin A1 together, the ratio of LC3-II/LC3-I was higher than that in cells treated with doxorubicin and bafilomycin A1 alone (Figure 1C, 1D). p62, which binds ubiquitin and LC3 and is a selective substrate for autophagy, was increased when LoVo cells were treated with bafilomycin A1 alone (Figure 1C, 1E), indicating that bafilomycin A1 decreased the degradation of p62. In contrast, p62 was downregulated when LoVo cells were treated with doxorubicin (Figure 1C, 1E), which suggested activation of autophagy. However, when the cells were treated with doxorubicin and bafilomycin A1 together, the decrease in the p62 level was compromised (Figure 1C, 1E). These results indicate that doxorubicin increases the autophagy flux in LoVo cells.

mTOR is a major regulator of autophagy and its activity inhibition has been shown to induce activation of autophagy



**Figure 1.** Autophagy activity is upregulated in doxorubicin-treated LoVo cells. (A, B) LoVo cells were treated with 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h. Cells were then harvested and protein levels of LC3 and the loading control,  $\beta$ -actin, were analyzed using Western blotting. (C–E) LoVo cells were treated with 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h and then treated with bafilomycin A1 (baf A1, 100 nmol/L for 3 h) before cells were harvested. Protein levels of P62, LC3 and  $\beta$ -actin were analyzed using Western blotting. (F–H) LoVo cells were treated with 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h. Protein levels of LC3, p-mTORC1, mTORC1, p-p70S6K, p70S6K and the loading control,  $\beta$ -actin, were analyzed using Western blotting. Densities of protein bands were analyzed with SigmaScan Pro 5 and normalized to  $\beta$ -actin. Bars represent the mean  $\pm$  SEM;  $n=3$ . Statistical comparisons were performed with one-way ANOVA followed by the Bonferroni's *post hoc* test. **\*\*\*** $P<0.001$  vs control group and **\*\*** $P<0.01$  vs control group.

in response to nutrient starvation<sup>[20]</sup>. Therefore, we detected the phosphorylation levels of mTOR as well as its downstream protein, p70S6K, in response to doxorubicin. Doxorubicin treatment caused a robust decrease in the levels of phosphorylated mTOR and phosphorylated p70S6K in LoVo cells (Figure 1F–1H), suggesting that autophagy activation induced by doxorubicin was involved in mTOR pathway inactivation.

#### Doxorubicin induces TFEB nuclear localization in LoVo cells

A previous study showed that doxorubicin induced TFEB

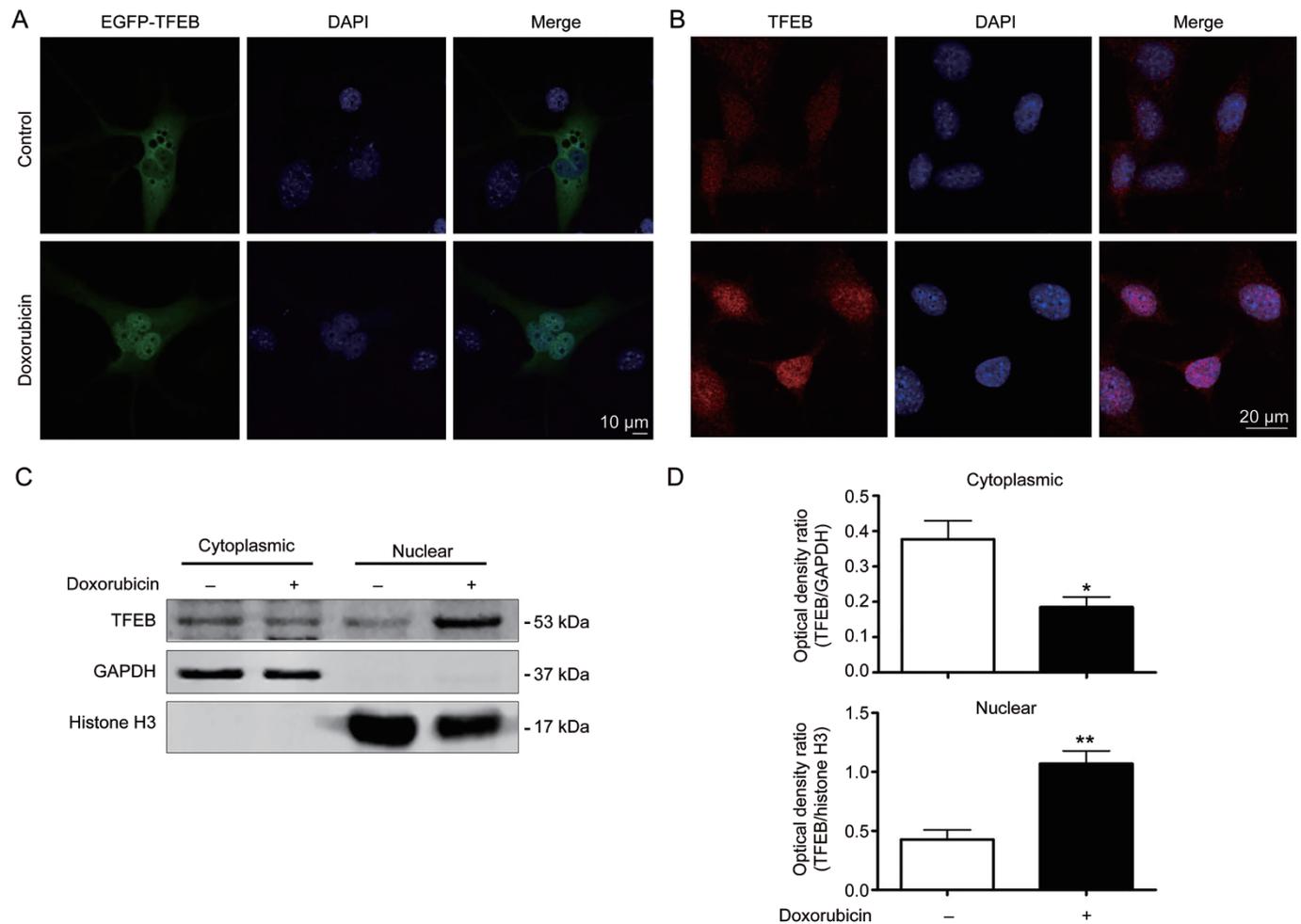
nuclear translocation in MCF-7, HeLa and HEK 293 cells<sup>[17]</sup>. mTOR-mediated dephosphorylation of TFEB at the lysosomal membrane, resulting in TFEB nuclear translocation, which then upregulates autophagic activity<sup>[12–15]</sup>. To determine whether the effect of doxorubicin on regulating autophagy activity is associated with TFEB nuclear translocation in LoVo cells, the cells were transiently transfected with EGFP-TFEB for 24 h and were then treated with doxorubicin for 12 h. Doxorubicin treatment induced dramatic nuclear translocation of EGFP-TFEB in LoVo cells (Figure 2A). To investigate the

distribution of endogenous TFEB in response to doxorubicin treatment, LoVo cells were exposed to doxorubicin for 12 h. Then, immunofluorescence as well as a nuclear and cytoplasmic fractionation assay were performed to detect the nuclear levels of TFEB. Immunofluorescence staining showed that TFEB was diffusely distributed in both the cytoplasm and nucleus in untreated cells, and doxorubicin treatment induced distinct nuclear localization of endogenous TFEB in LoVo cells (Figure 2B). Consistent with the results of immunofluorescence, the nuclear and cytoplasmic fractionation assay also showed that doxorubicin treatment decreased the levels of TFEB in the cytoplasm and dramatically increased the levels of TFEB in the nucleus (Figure 2C, 2D).

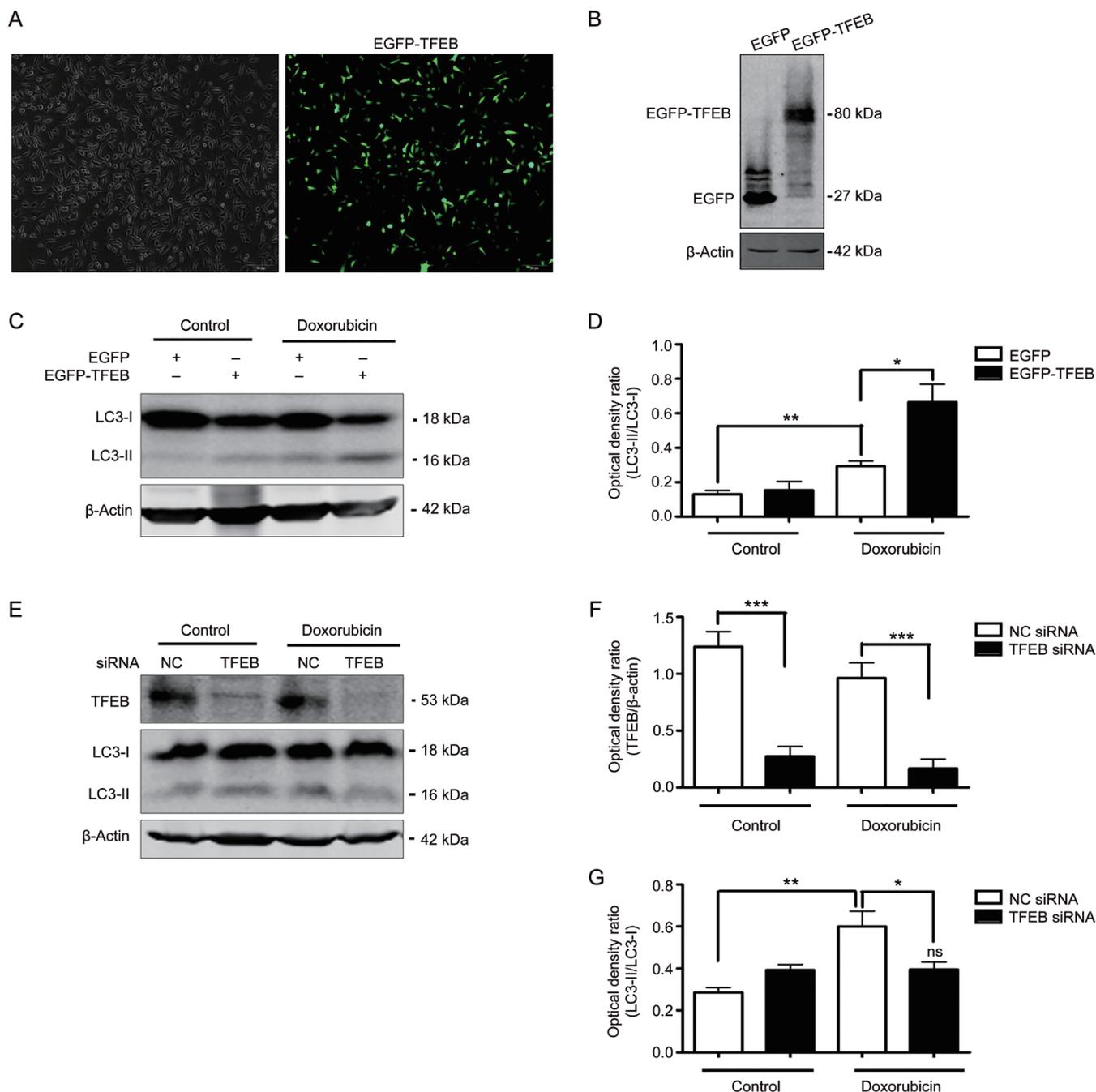
#### Doxorubicin-induced autophagy activation is TFEB-dependent in LoVo cells

Next, we assessed the role of TFEB in doxorubicin-induced

autophagy. LoVo cells were subjected to EGFP-TFEB overexpression and TFEB knockdown manipulations. The plasmid EGFP or EGFP-TFEB was transiently transfected into LoVo cells (Figure 3A), and the EGFP-TFEB protein was successfully overexpressed in the cells (Figure 3B). Doxorubicin increased the ratio of LC3-II/LC3-I in EGFP overexpressing LoVo cells (Figure 3C, 3D). Doxorubicin induced much higher levels of autophagy activity in EGFP-TFEB overexpressing LoVo cells compared with EGFP overexpressing LoVo cells (Figure 3C, 3D). As for TFEB knockdown, approximately 78% and 83% of the TFEB protein was silenced by TFEB siRNA in control and doxorubicin treated LoVo cells, respectively (Figure 3E, 3F). Doxorubicin treatment upregulated the ratio of LC3-II/LC3-I in NC siRNA LoVo cells (Figure 3E, 3G). However, doxorubicin treatment could not upregulate the ratio of LC3-II/LC3-I in TFEB knockdown LoVo cells (Figure 3E, 3G). Overall, these results suggest that doxorubicin-induced



**Figure 2.** Doxorubicin induces TFEB nuclear localization in LoVo cells. (A) LoVo cells were transiently transfected with EGFP-TFEB plasmid for 24 h and were then treated with 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h. Then, cells were visualized with a confocal microscope. EGFP-TFEB was green and the nucleus was stained blue from DAPI. Bar=10  $\mu\text{m}$ . (B) LoVo cells were treated with 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h and immunofluorescence was performed. Endogenous TFEB was stained red and the nucleus was stained blue from DAPI. Bar=10  $\mu\text{m}$ . (C, D) LoVo cells were treated with 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h. Cells were subjected to nuclear and cytoplasmic fractionation. Protein levels of TFEB were analyzed using Western blotting. H3 and GAPDH were used as the nuclear and cytoplasmic markers, respectively. \*\* $P < 0.01$  vs control group and \* $P < 0.05$  vs control group.



**Figure 3.** Doxorubicin-induced autophagy activation is TFEB-dependent in LoVo cells. (A, B) LoVo cells were transiently transfected with EGFP or EGFP-TFEB for 24 h. GFP-green fluorescence was detected with a fluorescence microscopy. Protein levels of EGFP, EGFP-TFEB and  $\beta$ -actin were analyzed using Western blotting. (C, D) LoVo cells were transiently transfected with EGFP or EGFP-TFEB for 24 h and were then treated with 0.5  $\mu$ mol/L doxorubicin for 12 h. LC3 and  $\beta$ -actin were analyzed using Western blotting. (E-G) LoVo cells were transiently transfected with NC or TFEB siRNA for 72 h and were then treated with 0.5  $\mu$ mol/L doxorubicin for 12 h. Protein levels of TFEB, LC3 and  $\beta$ -actin were analyzed using Western blotting. \*\*\* $P$ <0.001 vs control group and \*\* $P$ <0.01 vs control group. NS, not statistically significant.

autophagy depends on increasing the nuclear translocation of TFEB in LoVo cells.

#### TFEB promotes survival of LoVo and HeLa cells in response to doxorubicin

To confirm whether TFEB plays a role in doxorubicin-induced

drug resistance, we assessed the effects of TFEB on cell survival in response to doxorubicin treatment in LoVo and HeLa cells. The EGFP-TFEB protein was successfully overexpressed in HeLa cells (Figure 4A), and approximately 82% of the TFEB protein was silenced by *TFEB* siRNA (Figure 4B, 4C). LoVo and HeLa cells were transfected with EGFP or EGFP-TFEB for

24 h; then, they were treated with different concentrations of doxorubicin (0.25–4.0  $\mu\text{mol/L}$ ) for 12 h, and the cell viability was measured with the CCK-8 assay. Doxorubicin decreased the cell viability in a concentration-dependent manner in both EGFP and EGFP-TFEB overexpressed cells (Figure 4D, 4E). Although overexpression of EGFP-TFEB alone did not affect the viability of untreated tumor cells, EGFP-TFEB overexpression increased the cell viability in the presence of doxorubicin compared with EGFP overexpression for a certain range of concentrations in LoVo and HeLa cells (Figure 4D, 4E). Consistent with EGFP-TFEB overexpression, although knockdown of TFEB alone did not impair cell viability, knockdown of TFEB combined with doxorubicin treatment significantly reduced the cell viability in LoVo and HeLa cells compared with doxorubicin treatment alone (Figure 4F, 4G). Moreover, the decrease in the cell viability by TFEB deficiency with doxorubicin treatment was confirmed in a long-term survival assay. Knockdown of TFEB alone did not reduce the cell viability in CCK-8 assay, but knockdown of TFEB alone decreased the cell clonogenic potential, which indicates that TFEB inhibition might repress long-term survival of the cells. TFEB deficiency combined with doxorubicin treatment caused a clear decrease in the clonogenic potential of LoVo cells (Figure 4H, 4I). These findings suggest a key role of TFEB in doxorubicin-induced drug resistance.

#### Knockdown of TFEB sensitizes tumor cells to doxorubicin-induced apoptosis

In addition, we assessed whether knockdown of TFEB aggravated apoptosis induced by doxorubicin treatment. Indeed, Hoechst staining showed that doxorubicin induced more cell death in the TFEB knockdown group than in the control group (Figure 5A, 5B). In agreement with Hoechst staining, flow cytometry assays indicated that TFEB knockdown significantly promoted doxorubicin-induced apoptosis (Figure 5C, 5D). Additionally, when approximately 77% and 87% of TFEB protein was silenced by TFEB siRNA in control and doxorubicin treated LoVo cells, respectively (Figure 5E, 5F), doxorubicin induced more cleaved caspase-3 in TFEB-deficient cells compared with control cells (Figure 5G, 5H). Taken together, these data suggest that TFEB overexpression partially counteracts the anti-tumor effect of doxorubicin; however, TFEB knockdown augments doxorubicin-induced apoptosis.

#### Inhibition of autophagy attenuates TFEB-induced cell survival in response to doxorubicin

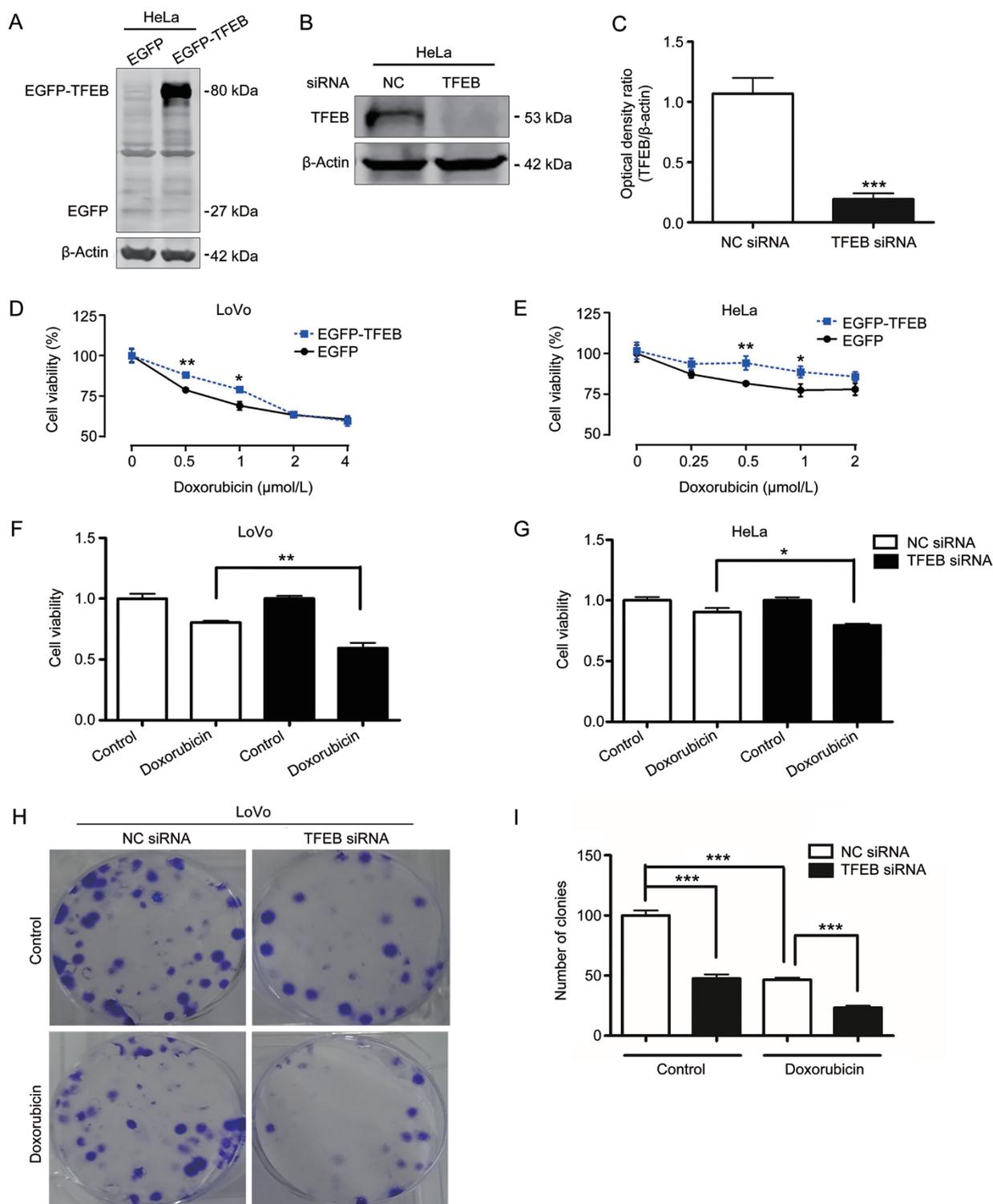
As TFEB nuclear translocation and autophagy were induced in doxorubicin-treated cells, we next investigated the role of autophagy activation in the TFEB-mediated cell protection in response to doxorubicin. 3-MA, an autophagy inhibitor, has been reported to inhibit the activity of PI3 kinase and block the formation of autophagosomes<sup>[21]</sup>. LoVo cells were transfected with EGFP or EGFP-TFEB for 24 h and were then exposed to doxorubicin with or without 3-MA for 12 h. The cell viability was assessed with the CCK-8 assay. Overexpressing EGFP-TFEB increased the cell viability with doxorubicin-induced

impairment, whereas 3-MA blocked the cell protection by EGFP-TFEB overexpression in response to doxorubicin (Figure 6A). These results indicated inhibition of autophagy partially abolished the protective role of TFEB against doxorubicin. To further study the role of autophagy in the chemosensitivity of tumor cells to doxorubicin, we suppressed autophagy activity by silencing *Atg5*, an autophagy-related gene. LoVo cells were transfected with *Atg5* siRNA for 48 h, and approximately 82% of *Atg5* was silenced (Figure 6B, 6C). Knockdown of *Atg5* significantly downregulated the ratio of LC3-II/LC3-I (Figure 6B, 6D). LoVo cells transfected with NC or *Atg5* siRNA as well as EGFP or EGFP-TFEB were exposed to doxorubicin for 12 h; then, the cell viability was assessed with the CCK-8 assay. EGFP-TFEB overexpression partially protected cells from doxorubicin-induced cytotoxicity in NC siRNA transfected LoVo cells. By contrast, the protective effect of EGFP-TFEB overexpression was completely abolished by *Atg5* knockdown (Figure 6E), suggesting that the protective function of TFEB against doxorubicin occurred through increasing autophagy activity.

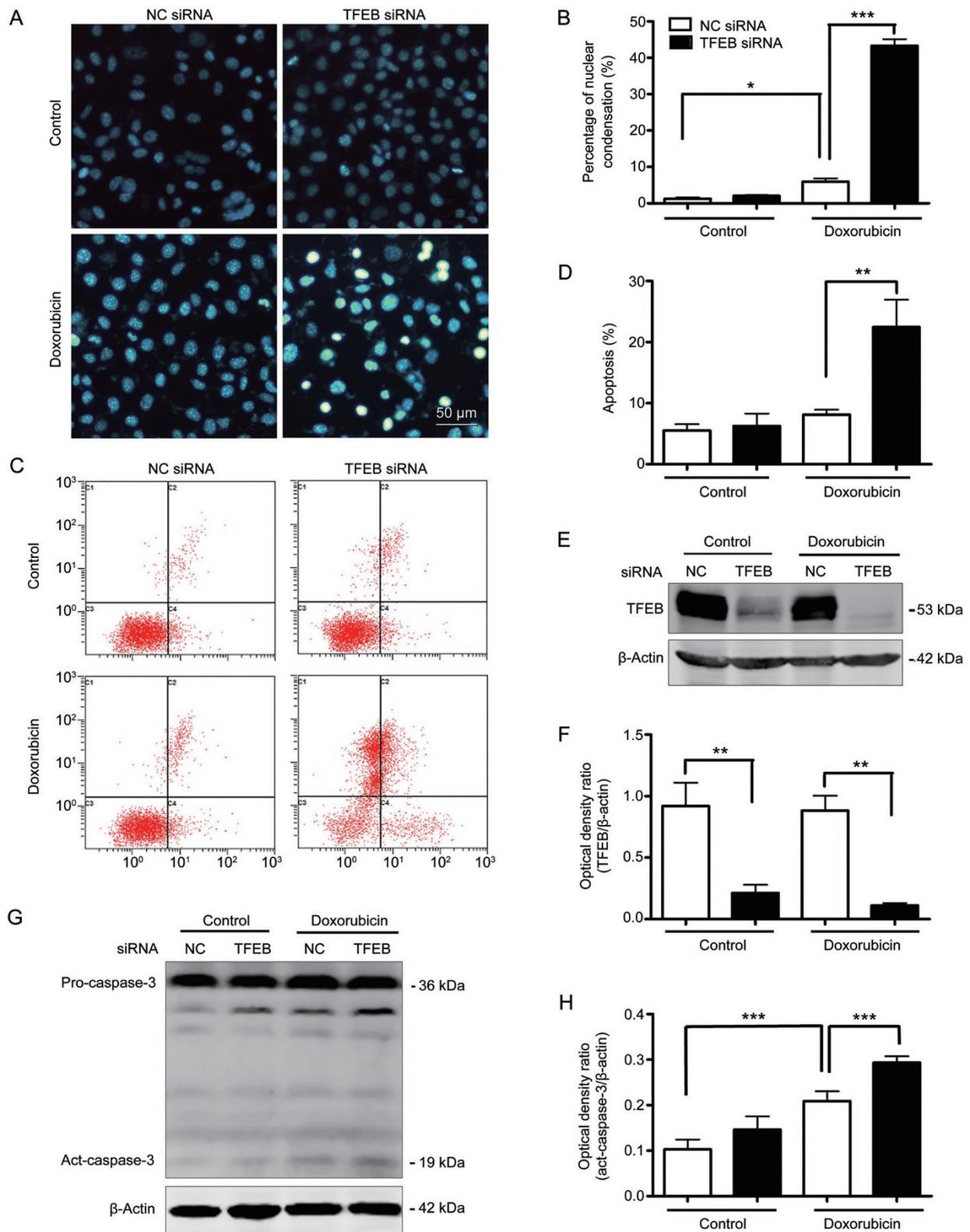
#### Discussion

Chemotherapy is one of the primary clinical treatment strategies for various human malignancies. However, chemotherapy resistance is considered a major hindrance to the efficacy of chemotherapeutic drugs<sup>[22]</sup>. Therefore, it is urgent to reveal the molecular mechanisms of chemotherapy resistance and find novel drug resistance-related targets that are crucial to the use of novel anticancer drugs or therapeutic strategies. The mechanisms of chemotherapy resistance involve a variety of signaling pathways, and autophagy is considered as a closely correlated factor<sup>[23]</sup>. Here, we showed that autophagy activation that is mediated by TFEB is probably responsible for cancer cell escape from apoptosis in colon LoVo cells in response to doxorubicin treatment. Inhibition of TFEB or autophagy increased the cytotoxicity of doxorubicin (Figures 4–6).

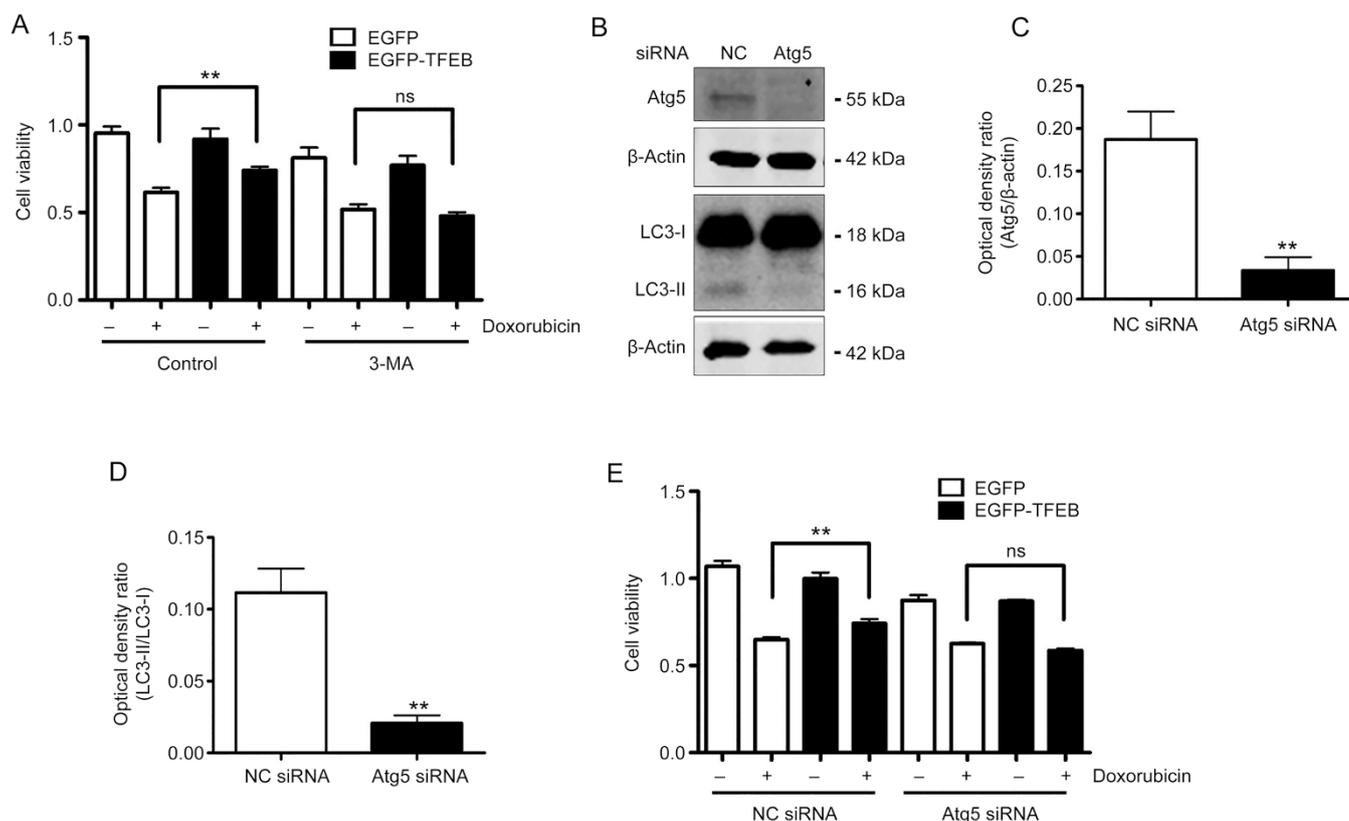
Autophagy is an evolutionarily conserved mechanism for degrading intracellular components, and autophagy is responsible for recycling metabolic substances and maintaining intracellular homeostasis. Autophagy is closely related to tumor development and drug resistance of tumors<sup>[24, 25]</sup>. Although some studies suggest that autophagy acts as a tumorigenesis-suppression factor, many studies support that autophagy facilitates tumorigenesis<sup>[5, 8]</sup>. The concept that autophagy mediates tumor suppression is based on earlier reports that an important autophagy-related gene, *beclin1*, is monoallelically lost in 40%–75% of human breast and prostate cancers<sup>[26, 27]</sup>. Additionally, *in vivo* studies found that single allelic loss of *beclin1* renders mice prone to spontaneous malignancies<sup>[28, 29]</sup>. However, conditional knockout of autophagy-related gene *Atg5* or *Atg7* in mice only results in benign tumors<sup>[30]</sup>. These findings indicated that tumor suppression by autophagy may be tumor type- or stage-dependent and should be further studied. Indeed, autophagy maintains the stability of the genome, decreases oxidative stress and alleviates the DNA damage response, which contribute to tumorigenesis<sup>[31, 32]</sup>. In addition,



**Figure 4.** TFEB promotes survival in response to doxorubicin in LoVo and HeLa cells. (A) HeLa cells were transiently transfected with EGFP or EGFP-TFEB for 24 h. Protein levels of EGFP, EGFP-TFEB and  $\beta$ -actin were analyzed. (B, C) HeLa cells were transiently transfected with TFEB siRNA for 72 h. Protein levels of TFEB and  $\beta$ -actin were analyzed. (D, E) LoVo and HeLa cells were transiently transfected with EGFP or EGFP-TFEB for 24 h and were then treated with 0.5, 1, 2, or 4  $\mu$ mol/L doxorubicin for 12 h, respectively. Cell viability was tested using CCK-8. \* $P$ <0.05 EGFP-TFEB vs EGFP with 1  $\mu$ mol/L doxorubicin; \*\* $P$ <0.01 EGFP-TFEB vs EGFP with 0.5  $\mu$ mol/L doxorubicin. (F, G) LoVo and HeLa cells were transiently transfected with NC or TFEB siRNA for 72 h and were then treated with 0.5  $\mu$ mol/L doxorubicin for 12 h. Cell viability was tested using CCK-8. (H) LoVo cells were transiently transfected with NC or TFEB siRNA for 72 h and were then treated with 0.5  $\mu$ mol/L doxorubicin for 1 to 2 weeks. Cell growth was assessed with the clone formation assay. (I) Quantitative analysis of clone numbers in (H) was counted. The results were repeated in three independent experiments. \*\*\* $P$ <0.001 vs control group, \*\* $P$ <0.01 vs control group, and \* $P$ <0.05 vs control group.



**Figure 5.** Knockdown of TFEB sensitizes tumor cells to doxorubicin-induced apoptosis. LoVo cells were transiently transfected with NC or TFEB siRNA for 72 h and were then treated with 0.5 μmol/L doxorubicin for 12 h. (A) The nuclear condensation was measured using Hoechst 33342 staining. The brighter nuclei were indicated as Hoechst 33342-positive apoptotic cells. Scar bar=50 μm. (B) Quantitative analysis of the percentage of the nuclear condensation in (A) was evaluated. (C) Flow cytometry assays were preformed using FITC conjugated Annexin-V and propidium iodide (PI). (D) Quantitative analysis of the percentage of apoptosis in (C) was counted. (E, F) TFEB and β-actin were analyzed using Western blotting. (G, H) Caspase-3 and β-actin were analyzed using Western blotting. \*\*\**P*<0.001 vs control group, \*\**P*<0.01 vs control group, and \**P*<0.05 vs control group.



**Figure 6.** Inhibition of autophagy attenuates TFEB-induced cell survival in response to doxorubicin. (A) LoVo cells were transiently transfected with EGFP or EGFP-TFEB for 24 h and were then treated with or without 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h in the presence or absence of 3-MA (10 mmol/L), as indicated. Cell viability was assessed using a CCK-8 kit. (B–D) LoVo cells were transiently transfected with NC or *Atg5* siRNA for 48 h. *Atg5*, LC3 and  $\beta$ -actin were analyzed using Western blotting. (E) LoVo cells were transiently transfected with NC or *Atg5* siRNA for 48 h and were then transfected with EGFP or EGFP-TFEB for another 24 h. The cells were treated with or without 0.5  $\mu\text{mol/L}$  doxorubicin for 12 h. Cell viability was tested using a CCK-8 kit. \*\* $P < 0.01$  vs control group. ns, not statistically significant.

a recent, large-scale genomic analysis of human cancers failed to identify any mutations in essential autophagy genes<sup>[33]</sup>. These studies raised questions on the role of autophagy in tumor suppression.

Tumor cells frequently experience nutrient and oxygen deprivation due to the rapid growth of tumor cells and inadequate blood supply. Autophagy is activated in tumor cells under stress conditions<sup>[34, 35]</sup>. There is evidence that autophagy occurs in hypoxic regions of tumors to mitigate metabolic stress and support tumor cell survival<sup>[36]</sup>. Indeed, autophagy activation with stress plays a particularly important role in maintaining the long-term survival of tumor cells<sup>[36]</sup>. The activation of autophagy followed by chemotherapy tends to have a protective effect against therapeutics rather than a damaging effect on tumor cells. Therefore, it induces chemotherapy tolerance, which was not conducive to cancer chemotherapy<sup>[37]</sup>. Autophagy inhibitors targeting the later stage of autophagy, lysosomes, in combination with chemotherapy drugs are frequently evaluated in preclinical models<sup>[38]</sup>.

TFEB is the one of the critical regulators of autophagy, and it has a potential role in chemotherapy resistance. Therefore, in the present study, we attempted to explore the effects of TFEB

in autophagy-mediated chemoresistance. We observed the distribution pattern of TFEB protein after doxorubicin treatment in cancer cells and found that treatment of cancer cells with doxorubicin resulted in a robust translocation of TFEB from the cytoplasm to the nucleus. The nuclear localization of TFEB was probably regulated by the mTORC1 pathway, as we found that mTOR was inactivated in response to doxorubicin treatment. Then, we demonstrated that doxorubicin-induced autophagy activation was involved in TFEB nuclear translocation into tumor cells. Next, we observed that overexpression of TFEB decreased the chemosensitivity of cancer cells to doxorubicin, while knockdown of TFEB increased the chemotherapeutic efficacy of doxorubicin (Figure 4D–4G). These results are correlated with autophagy upregulation by TFEB overexpression or autophagy suppression by TFEB knockdown in doxorubicin treated cells, respectively (Figure 3C–3G). Knockdown of TFEB did not reduce the cell viability in CCK-8 assays; however, it decreased cell clonogenic potential (Figure 4F–4I), which indicates that TFEB silencing might inhibit long-term cell survival via repressing the expression of autophagy and lysosomal genes. Furthermore, the pro-survival role of TFEB was completely abolished by autophagy

inhibition using the autophagy inhibitor 3-MA or *Atg5* siRNA (Figure 6A–6E). Doxorubicin-induced caspase-3 activation and apoptosis were increased by knockdown of TFEB, and these results suggested that the promotion of chemosensitivity by TFEB knockdown was also involved in the caspase-dependent apoptotic pathway (Figure 5A–5H). Therefore, our and previous studies demonstrated an essential role of the autophagy pathway in promoting tumor cell growth and drug resistance in a TFEB-dependent manner<sup>[18]</sup>.

In summary, an anti-cancer drug, doxorubicin, promoted TFEB nuclear translocation and autophagy activation in LoVo cancer cells. TFEB overexpression increased autophagy activation and rendered tumor cells insensitive to doxorubicin, while TFEB deficiency reduced autophagy activation and enhanced the cytotoxicity of doxorubicin. The doxorubicin chemoresistance caused by TFEB was inhibited by autophagy repression. Overall, our results demonstrated that cancer cells could activate the TFEB-mediated autophagy pathway to reduce the cytotoxicity of doxorubicin and that TFEB may be a novel target for cancer therapy.

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### Author contribution

All listed authors contributed to the idea generation, design, and completion of this study. Li-mei FANG and Bin LI contributed equally to the idea generation, experimental work and manuscript preparation; Hai-dong XU, Jun-jie GUAN, and Gen-hai SHEN contributed to the experimental work and manuscript preparation; Quan-gen GAO and Zheng-hong QIN guided the idea generation, experimental work and manuscript preparation. All authors reviewed the manuscript.

### References

- 1 Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis* 2013; 4: e838.
- 2 Jin S, White E. Tumor suppression by autophagy through the management of metabolic stress. *Autophagy* 2008; 4: 563–6.
- 3 Wang C, Hu Q, Shen HM. Pharmacological inhibitors of autophagy as novel cancer therapeutic agents. *Pharmacol Res* 2016; 105: 164–75.
- 4 Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008; 132: 27–42.
- 5 Vitale I, Manic G, Dandrea V, De Maria R. Role of autophagy in the maintenance and function of cancer stem cells. *Int J Dev Biol* 2015; 59: 95–108.
- 6 Klionsky DJ. Citing recent declines in the discovery of new ATG genes, some scientists now suggest that the end of autophagy research may be within sight. *Autophagy* 2014; 10: 715–6.
- 7 Wesselborg S, Stork B. Autophagy signal transduction by ATG proteins: from hierarchies to networks. *Cell Mol Life Sci* 2015; 72: 4721–57.
- 8 Qian HR, Yang Y. Functional role of autophagy in gastric cancer. *Oncotarget* 2016; 7: 17641–51.
- 9 Pan ST, Li ZL, He ZX, Qiu JX, Zhou SF. Molecular mechanisms for tumour resistance to chemotherapy. *Clin Exp Pharmacol Physiol* 2016; 43: 723–37.
- 10 Xie JM, Li B, Yu HP, Gao QG, Li W, Wu HR, et al. TIGAR has a dual role in cancer cell survival through regulating apoptosis and autophagy. *Cancer Res* 2014; 74: 5127–38.
- 11 Yu HP, Xie JM, Li B, Sun YH, Gao QG, Ding ZH, et al. TIGAR regulates DNA damage and repair through pentosephosphate pathway and Cdk5-ATM pathway. *Sci Rep* 2015; 5: 9853.
- 12 Sardiello M, Ballabio A. Lysosomal enhancement: a CLEAR answer to cellular degradative needs. *Cell Cycle* 2009; 8: 4021–2.
- 13 Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, et al. A gene network regulating lysosomal biogenesis and function. *Science* 2009; 325: 473–7.
- 14 Palmieri M, Impey S, Kang H, di Ronza A, Pelz C, Sardiello M, et al. Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. *Hum Mol Genet* 2011; 20: 3852–66.
- 15 Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Huynh T, et al. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J* 2012; 31: 1095–108.
- 16 Perera RM, Stoykova S, Nicolay BN, Ross KN, Fitamant J, Boukhali M, et al. Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. *Nature* 2015; 524: 361–5.
- 17 Giatromanolaki A, Kalamida D, Sivridis E, Karagounis IV, Gatter KC, Harris AL, et al. Increased expression of transcription factor EB (TFEB) is associated with autophagy, migratory phenotype and poor prognosis in non-small cell lung cancer. *Lung Cancer* 2015; 90: 98–105.
- 18 Zhitomirsky B, Assaraf YG. Lysosomal sequestration of hydrophobic weak base chemotherapeutics triggers lysosomal biogenesis and lysosome-dependent cancer multidrug resistance. *Oncotarget* 2015; 6: 1143–56.
- 19 Pan Y, Gao Y, Chen L, Gao G, Dong H, Yang Y, et al. Targeting autophagy augments *in vitro* and *in vivo* antimyeloma activity of DNA-damaging chemotherapy. *Clin Cancer Res* 2011; 17: 3248–58.
- 20 He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 2009; 43: 67–93.
- 21 Chen S, Rehman SK, Zhang W, Wen A, Yao L, Zhang J. Autophagy is a therapeutic target in anticancer drug resistance. *Biochim Biophys Acta* 2010; 1806: 220–9.
- 22 Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 2013; 13: 714–26.
- 23 Lozy F, Karantz V. Autophagy and cancer cell metabolism. *Semin Cell Dev Biol* 2012; 23: 395–401.
- 24 Dalby KN, Tekedereli I, Lopez-Berestein G, Ozpolat B. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. *Autophagy* 2010; 6: 322–9.
- 25 Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev* 2011; 25: 717–29.
- 26 Bestebroer J, V'kovski P, Mauthe M, Reggiori F. Hidden behind autophagy: the unconventional roles of ATG proteins. *Traffic* 2013; 14: 1029–41.
- 27 Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999; 402: 672–6.
- 28 Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003; 112: 1809–20.
- 29 Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene

- essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A* 2003; 100: 15077–82.
- 30 Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, *et al*. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* 2011; 25: 795–800.
- 31 Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, *et al*. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev* 2007; 21: 1621–35.
- 32 Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, *et al*. Autophagy suppresses tumorigenesis through elimination of p62. *Cell* 2009; 137: 1062–75.
- 33 Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, *et al*. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 2014; 505: 495–501.
- 34 Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer* 2007; 7: 961–7.
- 35 White E, Karp C, Strohecker AM, Guo Y, Mathew R. Role of autophagy in suppression of inflammation and cancer. *Curr Opin Cell Biol* 2010; 22: 212–7.
- 36 Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, *et al*. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 2006; 10: 51–64.
- 37 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.
- 38 Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, *et al*. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 2007; 117: 326–36.