

Connexin32 promotes autophagy of cervical cancer to resistance to chemotherapy-induced apoptosis via AMPK pathway

lixia fan

foshan university

Liang Tao

sun yat-sen university

Yongchang Lai

The Eighth Affiliated Hospital of Sun Yat-Sen University

Shaoyi Cai

sun yat sen university

Ziyu Zhao

sun yat-sen university

Feng Yang

Sun Yat-Sen University

Riya Su

Sun Yat-Sen University

Qin Wang (✉ wangqin6@mail.sysu.edu.cn)

Sun Yat-Sen University

Research

Keywords: Connexin32, Cervical carcinoma, Autophagy, Apoptosis, AMP-activated protein kinase

Posted Date: March 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-337122/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

The roles of gap junction and its components, connexins in autophagy of cervical cancer cells are rarely investigated. Our previous study demonstrated that connexin32 (Cx32) exerted an anti-apoptotic effect on cervical cancer (CaCx). However, as an important regulator of apoptosis, whether the autophagy is involved in the function of Cx32 on cervical cancer cells are not well defined.

Methods

The expression of Connexin32 and the autophagy-associated protein LC3- β in paracancerous cervical tissues (n = 30) and cervical cancer (n = 50) tissues were determined by Western blotting. Forty-five cervical cancer specimens were used to evaluate the clinical relevance of Connexin32 and LC3- β . The effect of Connexin32 on autophagy was examined by detecting the change of LC3- β using Western blotting, transfection with enhanced green fluorescent protein-LC3 plasmid and transmission electron microscopy analysis. The mechanism of Connexin32-mediated autophagy was assessed by Western blotting and flow cytometry.

Results

Both Connexin32 and LC3- β are upregulated in cervical cancer tissues compared to those in paracancerous cervical tissues. Overexpression of Connexin32 significantly enhanced autophagy in HeLa-Connexin32 cells, whereas knockdown of Connexin32 suppressed autophagy in C-33A cells. Connexin32 inhibited apoptosis of cervical cancer cells by promoting autophagy. Moreover, Connexin32 triggered autophagy by activation of the AMP-activated protein kinase signalling, which was gap junction-independent.

Conclusion

Connexin32 exerts its anti-apoptotic effect by activating autophagy through the AMPK pathway in cervical cancer. This finding suggests that Connexin32 is a potential biomarker and a new therapeutic target for cervical cancer.

Trial registration

The study was approved by the Research Committee of Ethics of the Affiliated Cancer Hospital of Xinjiang Medical University (K-201354).

Background

Cervical cancer (CaCx) ranks third in morbidity and mortality among gynaecological malignancies worldwide (1, 2). Despite recent advances in diagnostic criteria and clinical therapeutic strategies, a great

number of women suffer from advanced disease (3). Surgery, radiotherapy, and chemotherapy are currently the three main treatments for CaCx, but the therapeutic efficiency is not so satisfactory (4). Connexin (Cx) is an important component of gap junction (GJ), which modulates various cellular processes, including electrical coupling, proliferation, differentiation, and apoptosis (5). Previous studies revealed that GJs and Cx are defective in tumour progression and that GJ recovery impedes cell proliferation and metabolism in tumours (6, 7). However, there are also emerging studies have demonstrated that Cx protein itself affects the development of carcinoma in a GJ-independent manner in recent years (8, 9). In fact, different connexin isoforms have various channel-dependent and-independent functions that are tissue and stage specific (10)

Among the 21 human connexin protein family, Cx32, Cx43 and Cx26 are widely studied. Our previous studies showed that unlike other Cxs, a specific type of Cx, connexin32 (Cx32) is aberrantly upregulated and mislocalized in human CaCx tissue, which mediates anti-apoptotic and pro-tumour effects via the EGFR and NF- κ B pathways in CaCx (11–14). However, besides apoptosis, the role of Cx32 on other types of cell death (e.g. autophagic cell death or necrosis) still needs to be further investigated in CaCx.

Autophagy is a conserved process for bulk degradation and recycling of cytoplasmic protein and organelles in lysosomes, providing free fatty acids and amino acids to maintain energy production and protein synthesis (15, 16). Autophagy contributes to the turnover of membrane proteins, including surface receptors and structural components (17, 18). As membrane protein-based structures, the stability and degradation of GJ plaques and Cx are regulated by macroautophagy via the ubiquitin–proteasome system and lysosomes (19–21). Endocytosed GJ is degraded by autophagy (22), while Cx43 degradation and gap junctional intercellular communication (GJIC) impairment induced by simulated ischaemia is prevented by chemical or genetic inhibitors of autophagy (23). On the other hand, Cx inversely acts as negative regulators of autophagic flux (24). In fact, Cx suppress autophagy by recruiting pre-autophagosomal Atg proteins and PI3K components (25). However, as a specific type of Cx, the role of Cx32 in the processes of autophagy formation is unclear, especially in CaCx.

In addition, to explore the correlation of Cx32 and autophagy in isolation cannot reflect the practical significance of Cx32 in CaCx progression. Autophagy and apoptosis are two distinct mechanisms that may be antagonistic in cancer cells (26). It has been reported that inhibition of autophagy enhances N, N-diethylnorspermine-induced apoptosis in colon cancer cells (27). Conversely, activation of autophagy by globular adiponectin attenuates ethanol-induced apoptosis in liver cancer cells (28). However, whether autophagy is involved in the anti-apoptotic effect of Cx32 in CaCx remains unclear. Therefore, we investigated the relationship between Cx32 and autophagy in the presence or absence of GJ formation, in order to elaborate new findings or novel mechanisms of Cx32 in CaCx cancerization.

Methods

Reagents and antibodies

Streptonigrin (SN), chloroquine (CQ), bafilomycin A1 (BA1), rapamycin (RAP), doxycycline (Dox), compound C, anti-GAPDH and anti-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). G418 and hygromycin B were purchased from Calbiochem (San Diego, CA, USA). Primary antibodies against GAPDH, β -tubulin, β -actin, LC3, Atg4, Atg5, Atg7, cleaved-caspase3, cleaved-PARP, AMPK/p-AMPK, and mTOR/p-mTOR were purchased from Cell Signalling Technology (Danvers, MA, USA). Anti-Cx32 antibody was obtained from Santa Cruz (Dallas, TX, USA). SiRNA targeting Cx32, Atg5 and AMPK were constructed by RiboBio (Guangzhou, China). EGFP-LC3 plasmids were constructed by Genecopoeia (Rockville, MD, USA). The BCA protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). An Annexin V-FITC apoptosis detection kit was purchased from Biotool (Houston, TX, USA). A Chemiluminescent HRP substrate kit was obtained from Millipore Corporation (Billerica, MA, USA). Secondary antibody, Lipofectamine™ 2000, DEME and Eagle's minimum essential medium (EMEM) media were purchased from Invitrogen (Carlsbad, CA, USA).

Human cervical specimens and clinical data

The study was approved by the Research Committee of Ethics of the Affiliated Cancer Hospital of Xinjiang Medical University (13, 14). Human cervical tissue samples were collected from patients from 2012 to 2014 at the Affiliated Cancer Hospital of Xinjiang Medical University, Xinjiang, China. None of the patients had received any chemoradiotherapeutic agents preoperatively. We collected 50 specimens of CaCx from patients who underwent total hysterectomy and 30 specimens of benign multiple uterine fibroids as normal cervical controls. All the CaCx tissue specimens, corresponding peritumoural tissues (< 3 cm distance from the tumour tissue), and remote normal liver tissues (5 cm away from the tumour tissue) were collected within 10 min. After hysterectomy, cervical specimens were stored in liquid nitrogen for protein extraction. The expression of Cx32 and LC3 was detected by Western blotting.

Cell lines and low-density cultures

The humanCaCx cell line (C-33A) was purchased from American Type Culture Collection (Manassas, VA, USA) and were cultured in EMEM; HeLa-Cx32 cells (a gift from Dr. Andrew L. Harris) are a stable transgenic cell line expressing Cx32 under the control of a bidirectional tetracycline-inducible promoter that was previously described and characterized (14). The cells were grown as monolayer cultures in DMEM supplemented with $100 \mu\text{g}\cdot\text{ml}^{-1}$ G418 sulfate and $200 \mu\text{g}\cdot\text{ml}^{-1}$ hygromycin B. Cx32 expression was induced with $1 \mu\text{g}\cdot\text{ml}^{-1}$ Dox for 48 h. All the cell lines were supplemented with 10% foetal bovine serum and were grown at 37°C in a 5% CO_2 atmosphere. To physically inhibit gap junction formation, we adopted the low-density culture method, and 1×10^5 cells were seeded in a 150 mm dish to ensure that the cells were not in direct contact with each other (11, 13).

Western blotting

Tissue or cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich). The proteins were quantified by using a Bio-Rad protein assay kit (Hercules, CA, USA), and equivalent amounts of protein

(20 µg) were resolved on 12% or 9% SDS-PAGE gels and transferred onto 0.2 µm or 0.45 µm Immobilon-P transfer membranes (Millipore, Billerica, MA, USA). The membranes were subsequently incubated in blocking buffer (5% nonfat milk) for 1 h at room temperature and then incubated with appropriate primary antibodies in blocking buffer at 4°C overnight. The dilutions of the antibodies were as follows: anti-LC3 and anti-Cx32 were 1:1000; anti-cleaved-caspase3, anti-cleaved-PARP, anti-Atg4, anti-Atg5, anti-Atg7, anti-AMPK/p-AMPK and anti-mTOR/p-mTOR were 1:1500; and anti-β-tubulin, anti-β-actin and anti-GAPDH were 1:10000. The proteins were probed with the relevant secondary antibody, detected with an ECL reagent (Millipore) and quantified by ImageQuant LAS 4000TM and ImageJ software. Anti-β-tubulin, anti-β-actin and anti-GAPDH were used as loading controls.

Hoechst 33258 staining

Briefly, HeLa cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with a solution containing 1% BSA and 0.5% Triton X-100 for 15 min at 37 °C. Then, the apoptotic cells were detected by staining with 0.1 µg·ml⁻¹ Hoechst 33258 for 15 min in the dark and washed with PBS. Finally, morphological changes in apoptotic nuclei were observed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) with an ultraviolet filter.

Apoptosis analysis

Apoptosis was induced in cells by incubation with 1 µM SN for 7 h (11, 13). Apoptosis was assessed via flow cytometry using an annexin V-FITC apoptosis detection kit according to the manufacturer's protocol. After exposure to SN, the cells were trypsinized, washed and collected. Then, the cells were resuspended in annexin-V binding buffer and incubated with 5µl fluorescein isothiocyanate (FITC)-Annexin V and 2 µl PI for 15 min in the dark at 37°C. The apoptotic cells were immediately analysed by flow cytometry using the Expo32 Software (Beckman XL, USA). The ratio of early apoptotic cells was compared to that of the controls for each experiment. Cell apoptosis was analysed with FlowJo 7.6 software.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 15 min, washed and blocked in 10% normal goat serum for 1 h. After blocking, the cells were incubated with a primary antibody against Cx32 (1:200) at 4°C overnight, washed and incubated with FITC-conjugated goat anti-mouse secondary antibody (1:400) in the dark at 37°C for 1 h. For identification of nuclear and cytoplasmic architecture, the cells were stained with 0.1 µg·ml⁻¹ Hoechst 33342 for 10 min and Alexa Fluor 488 Phalloidin (1:20) for 20 min in the dark. The cells were observed under a confocal microscope.

Transmission electron microscopy

Cells were harvested by trypsinization, washed and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. After washing in phosphate buffer, the samples were post-fixed in 1% osmium tetroxide, washed again, and dehydrated in a graded series of ethanol. Then, the cells were embedded in spur resin for

cutting into ultrathin sections. Transmission electron microscopy was used to observe all autophagosomal structures after staining the sections (a thickness of 50 nm) with uranyl acetate and lead citrate (29).

EGFP-LC3 plasmid transfection and siRNA interference

Cells were seeded in 6-well plates and grown to 80% confluence and then transfected with EGFP-LC3 plasmid using Lipofectamine 2000. For siRNA interference, the cells were grown to 30%-50% confluence, and then the cells were transfected for 6 h with the corresponding non-specific siRNA, targeted siRNA (50 nM) or scrambled control siRNA with Lipofectamine™ 2000. The cells were cultured in complete medium for 48h, and EGFP-LC3 plasmid transfection and siRNA interference were detected by immunofluorescence and Western blotting. The sequences for the synthetic DNA targeting EGFP-LC3 were as follows: forward primer: 5'ATGCCGTCGGAGAAGA

CCTTCAAG3' and reverse primer: 5'TTACAC TGACAATTTTCATCCCGAACGT3'. The sequences of the synthetic siRNAs targeting Cx32 (siCx32) were as follows: siCx32_1: 5'-CACCAACAACACATAGAAA-3', siCx32_2: 5-GCATCTGCATTAT

CCTCAA-3', and siCx32_3: 5'-GCCTCTCACCTGAATACAA-3'. The sequences of the synthetic siRNAs targeting Atg5 (siAtg5) were as follows: siAtg5_1: 5'-GGAATATCCTGCAGAAGAA-3', siAtg5_2:5'-GGAACATCACAGTACATT

T-3', and siAtg5_3:5'-GTGAGATATGGTTTGAATA-3'. The sequences of the synthetic siRNAs targeting AMPK (siAMPK) were as follows: siAMPK_1: 5'GAGGAGAGCTATTTGATTA_3', siAMPK_2:5'-GCAGAAGTATGTAGAGCAA

-3', and siAMPK_3: 5'-GATTGATGATGAAGCCTTA-3'.

Statistical analysis

All data are representative of at least three independent experiments and are presented as the mean ± standard error. Normal distribution test and homogeneity of variance test have been performed prior to statistical analysis. Analysis of variance (ANOVA), a Student's t-test, and a Wilcoxon test were used to evaluate statistical significance. Pearson's correlation analysis was used to analyse the correlation between Cx32 and LC3-II expression, and GraphPad Prism 6.0 software was used to create the histograms and scatter plots. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Cx32 positively correlates with autophagy in human CaCx

LC3-II is a well-known indicator of autophagosome formation (30). Thus, the expression of Cx32 and LC3-II was detected in normal cervix controls (n=30) and CaCx tissue (n=50) to evaluate the relationship between Cx32 and autophagy. Both Cx32 and LC3 were significantly increased in CaCx tissues compared with those in normal cervical tissues (Fig. 1a and 1b). Moreover, LC3-II was increased in cervical tissues with upregulated Cx32 expression, and there was a significant association between Cx32 and LC3-II (n=45, $r=0.625$, $P=0.00055$) (Fig. 1c).

Overexpression of Cx32 promotes autophagy in HeLa cells

Next, we examined the effect of Cx32 on autophagy in HeLa-Cx32 cell, a stable transgenic cell line expressing Cx32 by a bidirectional tetracycline-inducible promoter, since Cx32 is not naturally expressed in HeLa cells. Western blotting and immunofluorescence demonstrated that Cx32 expression was induced by doxycycline (Dox, $1\mu\text{g}\cdot\text{ml}^{-1}$, 48 h) (Fig. 2a and 2b). However, LC3-II expression was unchanged in the presence of Dox in control HeLa cells (Fig. 2c). Induction of Cx32 expression with Dox was accompanied by upregulation of LC3-II, autophagy-related gene 4 (Atg4), Atg5 and Atg7 (Fig. 2d). These data suggest that Cx32 promotes autophagy in HeLa-Cx32 cells.

To figure out whether the upregulation of LC3-II was due to autophagy induction or blockage of late steps of autophagy, such as autophagosome fusion with lysosomes and lysosomal degradation, HeLa-Cx32 cells were treated simultaneously with Dox and the lysosomal inhibitor CQ ($25\mu\text{M}$, 4 h), which blocks autophagic flux by impairing autophagosome-lysosome fusion (31). The expression of LC3-II in the Dox+CQ group was significantly increased compared to that of the Dox group (Fig. 2e), indicating an effect of Cx32 on autophagic flux. Dox-induced expression of Cx32 significantly increased LC3 puncta aggregation compared to that of control group and treatment with CQ further increased LC3 puncta accumulation induced by Cx32 (Fig. 2f). Transmission electron microscopy observation showed that Cx32 induction increased the number and size of autophagic vacuoles, whereas few vacuoles were observed in control cells. Additionally, Lysosomal inhibition by CQ further increased the number and size of autophagic vacuoles mediated by Cx32 (Fig. 2g). Together, these results suggest that Cx32 promotes the autophagosome formation and autophagic flux.

Knockdown of Cx32 negatively modulates autophagy in C-33A cells

Three different sequences of siCx32 was tested in C-33A cells that endogenously express Cx32 in cell membrane and cytoplasm. The results demonstrated that both siCx32-2 and siCx32-3 efficiently reduced Cx32 expression (Fig. 3a and 3b). Knockdown of Cx32 decreased the protein levels of LC3-II, Atg4, Atg5 and Atg7 (Fig. 3c), indicating that silencing of Cx32 inhibited autophagy in C-33A cells. Interestingly, the downregulated expression of LC3-II by Cx32 knockdown was restored by CQ ($25\mu\text{M}$, 4 h) (Fig. 3d). Next, C-33A was transfected with siCx32-3 for 24 h, followed by transfection with the EGFP-LC3 plasmid for 36 h and then treatment with or without CQ. The result showed that silencing of Cx32 decreased the aggregation of LC3 puncta and this effect was reversed by CQ (Fig. 3e). Similar result was observed in

the transmission electron microscopy experiment (Fig. 3f). These results indicate that knockdown of Cx32 blocks autophagy in C-33A cells.

Cx32 promotes autophagy in CaCx cells in a GJ-independent manner

It is known that Cx exerts its biological function through GJ-dependent and GJ-independent mechanisms. To uncover whether Cx32-induced autophagy depends on GJ, low-density culture was used to physically inhibit GJIC. In low-density culture, Dox-induced Cx32 upregulated LC3-II expression in HeLa-Cx32 cells (Fig 4a). In contrast, knockdown of Cx32 decreased LC3-II expression in C-33A cells (Fig. 4b). These data suggest that the effect of Cx32 on autophagy is mediated by Cx32 protein rather than by GJ.

Cx32 promotes autophagy via the AMPK pathway in CaCx cells

Recent studies have shown that AMPK/mTOR signalling plays an important role in regulating autophagy (32). We then examined whether AMPK/mTOR signalling was involved in Cx32-induced autophagy. Induction of Cx32 increased the levels of LC3 and p-AMPK in HeLa-Cx32 cells (Fig. 5a). In contrast, knockdown of Cx32 decreased the levels of LC3 and p-AMPK in C-33A cells (Fig. 5b). To verify the involvement of AMPK in Cx32-induced autophagy, siAMPK and inhibitor (compound C) were used to inhibit AMPK expression (33). The upregulation of LC3-II and p-AMPK expression by Cx32 was reversed by siAMPK or compound C (10 μ M, 6 h) in HeLa-Cx32 cells (Fig.5c and 5d). Together, these data demonstrate that Cx32 mediates autophagy via the AMPK signalling pathway.

Cx32 inhibits apoptosis by promoting autophagy

Autophagy is supposed to be a crucial modulator of apoptosis (34) . We explored the relationship between autophagy and apoptosis in CaCx cells and found that pretreatment with an autophagy activator rapamycin (RAP, 2 μ M, 4 h) significantly inhibited SN (1 μ M, 6 h)-induced apoptosis in HeLa cells (Fig. 6a). In contrast, treatment with autophagy inhibitors CQ (25 μ M, 4 h) or Baf-A1 (100 nM, 4 h) enhanced apoptosis (Fig. 6a). The Atg5-Atg12 conjugation system is essential for autophagy, and inhibition of Atg5 specifically inhibits autophagy (35). Atg5 is key regulator controlling autophagy. Knockdown of Atg5 reduced LC3-II expression (Fig. 6b). Moreover, inhibition of autophagy by siAtg5-3 increased the level of apoptosis (Fig. 6c). Together, these data indicate that autophagy suppresses apoptosis in HeLa cells.

Next, we determined the effect of autophagy on Cx32-mediated apoptosis. Induction of Cx32 significantly inhibited SN-induced apoptosis in HeLa-Cx32 cells and this inhibition was blocked by pretreatment with autophagy inhibitor CQ (25 μ M, 4 h) or Baf-A1 (100 nM, 4 h) (Fig. 7a). In agreement with this result, overexpression of Cx32 reduced the levels of apoptosis-related protein, cleaved caspase3 and cleaved PARP, which was increased by SN. Moreover, pretreatment with CQ or Baf-A1 reversed the suppressive effect of Cx32 (Fig. 7b). Together, these results suggest that Cx32 inhibits apoptosis by activating autophagy in CaCx cells.

Discussion

Both autophagy and apoptosis are complex processes. Previously we had elucidated that Cx32 could inhibit apoptosis in CaCx by EGFR and NF- κ B pathways. However, besides apoptosis, various other functions of Cx32 in CaCx are still needed to be explored. In the present study, a positive correlation between Cx32 and LC3 was revealed in CaCx specimens. Importantly, by activation of AMPK signalling that promoted autophagy, Cx32 produces a resistance to chemotherapy-induced apoptosis. This work reveals a previously unknown mechanism between Cx32 and autophagy together with apoptosis in the process of CaCx progression.

Cx is an indispensable transmembrane protein with a short half-life and is suggested to be an autophagy substrate (22, 24). But increasing studies also have reported that Cxs themselves can suppress autophagy by recruiting pre-autophagosomal Atg proteins and PI3K components (25). With a series of experiments on CaCx cells, we explored the relationship between Cx32 and autophagy in CaCx and found that Cx32 was involved in the activation of autophagy.

Cx is an important component of GJs that maintains the normal activities of cells and its functions beyond GJs or hemichannels (36). Emerging studies have reported that in some tumour types, Cx facilitates specific stages of tumour progression through both junctional and non-junctional signalling pathways (37, 38). Cytoplasmic distribution of Cx32 exerts anti-apoptotic effects in hepatocellular carcinoma (14) and mediates cisplatin resistance in ovarian cancer cells (39, 40). Cytoplasmic expression of Cx26 in colorectal cancer was responsible for lung metastasis (41). However, the carboxy-tail of Cx43 localizes to the nucleus and inhibits cell growth (42). Therefore, the role of non-junctional Cx is still under controversial. In this study, the low-density cell culture data indicated that the induction of autophagy was mediated by the Cx32 protein rather than by its role in intercellular communication. This study further illuminated the role of the Cx32 protein itself in the progression of CaCx. In future studies, uncovering the turnover and trafficking of Cx would be indispensable in understanding the correlation between Cx and cancer progression and provide potential strategies for tumour treatment.

We also identified the molecular mechanism by which Cx32 promotes autophagy in CaCx. The AMPK/mTOR pathway is one of the classic autophagy regulatory pathways and plays an important role in regulating cell metabolism, growth, proliferation and apoptosis (43, 44). Therefore, we examined whether Cx32 promoted autophagy by this pathway. Our results demonstrated that Cx32 stimulated AMPK phosphorylation but had no effect on mTOR and p-mTOR protein levels. Accumulating evidence has reported that AMPK is an upstream molecule of mTOR and directly promotes autophagy by phosphorylating autophagy-related proteins in the mTORC1, ULK1, and PIK3C3/VPS34 complexes or indirectly by regulating the expression of autophagy-related genes downstream of transcription factors such as FOXO3, TFEB, and BRD4 (32). In addition, autophagy was reported to be induced by mTOR-independent Activation of the AMPK-ULK1 Pathway (45). Therefore, further investigations are still needed to explore the detailed mechanism of AMPK signalling involved in Cx32-mediated autophagy.

The role of autophagy in tumor growth remains controversial (46). On the one hand, autophagy inhibits tumour formation by reducing oxidative stress and DNA damage in normal tissues. On the other hand, autophagy provides cells with energy and vital compounds upon various stress stimuli in developed cancers, thus promoting tumour cell survival (47). Autophagy and apoptosis cross-regulate each other through an elaborate network (48). Autophagy reduces apoptosis by regulating the activity of caspase family proteins(49). In contrast, autophagy promotes apoptosis by autophagic degradation of certain types of IAPs (inhibitor of apoptosis proteins)(50, 51). In the present study, induction of autophagy reversed SN-induced apoptosis in HeLa cells, suggesting that autophagy antagonizes apoptosis in CaCx. To our knowledge, this is the first evidence showing that autophagy and apoptosis are antagonists in CaCx cells.

Our results further indicated that Cx32-induced autophagy antagonizes SN-induced apoptosis. These findings revealed a new mechanism by which Cx32 promotes autophagy and inhibits apoptosis, which accounts for the pro-tumour effect and drug resistance resulting from Cx32 in CaCx. This study may demonstrate a novel mechanism and provide a theoretical basis for Cx32 as a potential therapeutic target in CaCx

Conclusions

In conclusion, Cx32 promotes autophagy via the AMPK signalling pathway to protect CaCx cells from chemotherapy-induced apoptosis, suggesting that Cx32 may be a potential biomarker for chemotherapy resistance and may serve as a key link for autophagy and apoptosis in CaCx.

Abbreviations

CaCx, cervical cancer; Cx, Connexin; GJs, gap junctions; Cx32, Connexin32; GJIC, gap junctional intercellular communication; Atg, autophagy-related gene; PI3K, phosphoinositide 3-kinase; SN, Streptonigrin; CQ, chloroquine, BA1, bafilomycin A1; RAP, rapamycin; Dox, doxycycline; AMPK, AMP-activated protein kinase

Declarations

Ethics approval and consent to participate

Ethical approval (K-201354) of clinical tissues was obtained from the Research Committee of Ethics in the Affiliated Cancer Hospital of Xinjiang Medical University.

Consent for publication

Written informed consent form for the experimental studies was obtained from the patients or their guardians.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

All authors declare that they have no competing interests.

Funding

This work was supported by the Guangdong Basic and Applied Basic Research Foundation (Grant No. 2019A1515012215), the grant from Department of Science and Technology of Guangdong Province (Grant No.20160908), the National Natural Science Foundation of China (Grant No. 81473234) and the Joint Fund of the National Natural Science Foundation of China (Grant No. U1303221).

Authors' contributions

LT and QW designed research. LXF and LT performed the study. SYC and ZYZ participated in Western blotting of cervical tissues tissue. FY and RYS analyzed the data and prepared the figures and tables. LXF and YCL wrote the paper.

Acknowledgements

We thank the Affiliated Cancer Hospital of Xinjiang Medical University for providing parts of experimental assistance.

References

1. Tsikouras P, Zervoudis S Fau - Manav B, Manav B Fau - Tomara E, Tomara E Fau - Iatrakis G, Iatrakis G Fau - Romanidis C, Romanidis C Fau - Bothou A, et al.: Cervical cancer: screening, diagnosis and staging. *J BUON* 2016; 21: 320-5.
2. Moshkovich OA-O, Lebrun-Harris LA-O, Makaroff L, Chidambaran PA-O, Chung M, Sripipatana AA-O, et al.: Challenges and Opportunities to Improve Cervical Cancer Screening Rates in US Health Centers through Patient-Centered Medical Home Transformation. *Adv Prev Med* 2015; 2015: 182073.
3. Boussios S, Seraj E, Zarkavelis G, Petrakis D, Kollas A, Kafantari A, et al.: Management of patients with recurrent/advanced cervical cancer beyond first line platinum regimens: Where do we stand? A literature review. *Crit Rev Oncol Hematol* 2016; 108: 164-74.
4. Marth C, Landoni F, Mahner S, McCormack M, Gonzalez-Martin A, Colombo N, et al.: Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2017; 28: iv72-iv83.
5. Sinyuk M, Mulkearns-Hubert EE, Reizes O and Lathia J: Cancer Connectors: Connexins, Gap Junctions, and Communication. *Front Oncol* 2018; 8: 646.

6. Wang Q, You T, Yuan D, Han X, Hong X, He B, et al.: Cisplatin and oxaliplatin inhibit gap junctional communication by direct action and by reduction of connexin expression, thereby counteracting cytotoxic efficacy. *J Pharmacol Exp Ther* 2010; 333: 903-11.
7. Zhang Y, Tao L, Fan L, Peng Y, Yang K, Zhao Y, et al.: Different gap junction-propagated effects on cisplatin transfer result in opposite responses to cisplatin in normal cells versus tumor cells. *Sci Rep* 2015; 5.
8. Li Q, Omori Y, Nishikawa Y, Yoshioka T, Yamamoto Y and Enomoto K: Cytoplasmic accumulation of connexin32 protein enhances motility and metastatic ability of human hepatoma cells in vitro and in vivo. *International Journal of Cancer* 2007; 121: 536-46.
9. Luo H, Wang X, Ge H, Zheng N, Peng F, Fu Y, et al.: Inhibition of ubiquitin-specific protease 14 promotes connexin 32 internalization and counteracts cisplatin cytotoxicity in human ovarian cancer cells. *Oncol Rep* 2019; 42: 1237-47.
10. Aasen T, Leithe E, Graham SV, Kameritsch P, Mayán MD, Mesnil M, et al.: Connexins in cancer: bridging the gap to the clinic. *Oncogene* 2019; 38: 4429-51.
11. Lai Y, Fan L, Zhao Y, Ge H, Feng X, Wang Q, et al.: Cx32 suppresses extrinsic apoptosis in human cervical cancer cells via the NF κ B signalling pathway. *Int J Oncol* 2017; 51: 1159-68.
12. Lai Y, Tao L, Zhao Y, Zhang X, Sun X, Wang Q, et al.: Cx32 inhibits TNF α -induced extrinsic apoptosis with and without EGFR suppression. *Oncol Rep* 2017; 38: 2885-92.
13. Zhao Y, Lai Y, Ge H, Guo Y, Feng X, Song J, et al.: Non-junctional Cx32 mediates anti-apoptotic and pro-tumor effects via epidermal growth factor receptor in human cervical cancer cells. *Cell Death and Disease* 2017; 8: e2773.
14. Xiang Y, Wang Q, Guo Y, Ge H, Fu Y, Wang X, et al.: Cx32 exerts anti-apoptotic and pro-tumor effects via the epidermal growth factor receptor pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res* 2019; 38: 145.
15. Ravanan P, Srikumar IF and Talwar P: Autophagy: The spotlight for cellular stress responses. *Life Sci* 2017; 1: 53-67.
16. Kardideh B, Samimi Z, Norooznejhad F, Kiani S and Mansouri K: Autophagy, cancer and angiogenesis: where is the link? *Cell Biosci* 2019; 9: 65.
17. Jin EJ, Kiral FR and Hiesinger PR: The where, what, and when of membrane protein degradation in neurons. *Dev Neurobiol* 2018; 78: 283-97.
18. Wang S, Zhu X, Xiong L, Zhang Y and Ren J: Toll-like receptor 4 knockout alleviates paraquat-induced cardiomyocyte contractile dysfunction through an autophagy-dependent mechanism. *Toxicol Lett* 2016; 22: 11-22.
19. Zhu X, Ruan Z, Fau - Yang X, Yang X, Fau - Chu K, Chu K, Fau - Wu H, Wu H, Fau - Li Y, Li Y, Fau - Huang Y, et al.: Connexin 31.1 degradation requires the Clathrin-mediated autophagy in NSCLC cell H1299. *J Cell Mol Med* 2015; 19: 257-64.
20. Shen ZA, Ohoo, Chen Q, Jin T, Wang M, Ying H, Lu J, et al.: Theaflavin 3,3'-digallate reverses the downregulation of connexin 43 and autophagy induced by high glucose via AMPK activation in

- cardiomyocytes. *J Cell Physiol* 2019; 234: 17999-8016.
21. Yang X, Xu S, Su Y, Chen B, Yuan H, Xu A, et al.: Autophagy-Src Regulates Connexin43-Mediated Gap Junction Intercellular Communication in Irradiated HepG2 Cells. *Radiat Res* 2018; 190: 494-503.
 22. Fong JT, Kells Rm Fau - Gumpert AM, Gumpert Am Fau - Marzillier JY, Marzillier Jy Fau - Davidson MW, Davidson Mw Fau - Falk MM and Falk MM: Internalized gap junctions are degraded by autophagy. *Autophagy* 2012; 8: 794-811.
 23. Martins-Marques T, Catarino S, Zuzarte M, Marques C, Matafome P, Pereira P, et al.: Ischaemia-induced autophagy leads to degradation of gap junction protein connexin43 in cardiomyocytes. *Biochem J* 2015; 467: 231-45.
 24. Iyyathurai J, Decuypere JP, Leybaert L, D'Hondt C and Bultynck G: Connexins: substrates and regulators of autophagy. *BMC Cell Biol* 2016; 17: 20.
 25. Bejarano E, Yuste A, Patel B, Stout RF, Jr., Spray DC and Cuervo AM: Connexins modulate autophagosome biogenesis. *Nat Cell Biol* 2014; 16: 401-14.
 26. Maiuri MC, Zalckvar E Fau - Kimchi A, Kimchi A Fau - Kroemer G and Kroemer G: Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2007; 8: 741-52.
 27. Gurkan AC, Arisan ED, Yerlikaya PO, Ilhan H and Unsal NP: Inhibition of autophagy enhances DENSpM-induced apoptosis in human colon cancer cells in a p53 independent manner. *Cell Oncol (Dordr)* 2018; 41: 297-317.
 28. Nepal S and Park PH: Activation of autophagy by globular adiponectin attenuates ethanol-induced apoptosis in HepG2 cells: involvement of AMPK/FoxO3A axis. *Biochim Biophys Acta* 2013; 1833: 2111–25.
 29. Martinet W, Timmermans JP and De Meyer GR: Methods to assess autophagy in situ—transmission electron microscopy versus immunohistochemistry. *Methods Enzymol* 2014; 543: 89-114.
 30. Mizushima N and Yoshimori T: How to interpret LC3 immunoblotting. *Autophagy* 2007; 3: 542-5.
 31. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema KJ, et al.: Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* 2018; 14: 1435-155.
 32. Li Y and Chen Y: AMPK and Autophagy. *Adv Exp Med Biol* 2019; 1206: 85-108.
 33. Hardie DG: AMPK and autophagy get connected. *EMBO J* 2011; 30: 634-5.
 34. Sun X, Momen A, Wu J, Noyan H, Li R, von Harsdorf R, et al.: p27 protein protects metabolically stressed cardiomyocytes from apoptosis by promoting autophagy. *J Biol Chem* 2014; 289: 16924-35.
 35. Mizushima N, Yoshimori T Fau - Ohsumi Y and Ohsumi Y: The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* 2011; 27: 107-32.
 36. Goodenough DA and Paul DL: Beyond the gap: functions of unpaired connexon channels. *Nat Rev Mol Cell Biol* 2003; 4: 285-94.
 37. Polusani SR, Kalmykov EA, Chandrasekhar A, Zucker SN and Nicholson BA-O: Cell coupling mediated by connexin 26 selectively contributes to reduced adhesivity and increased migration. *J Cell Sci* 2016; 129: 4399-410.

38. Chen Q, Boire A, Jin X, Valiente M, Er EE, Lopez-Soto A, et al.: Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. *Nature* 2016; 533: 493-8.
39. Wu W, Fan L, Bao Z, Zhang Y, Peng Y, Shao M, et al.: The cytoplasmic translocation of Cx32 mediates cisplatin resistance in ovarian cancer cells. *Biochem Biophys Res Commun* 2017; 487: 292-9.
40. Zhang Y, Tao L, Fan LX, Huang K, Luo HM, Ge H, et al.: Cx32 mediates cisplatin resistance in human ovarian cancer cells by affecting drug efflux transporter expression and activating the EGFR/Akt pathway. *Mol Med Rep* 2019; 19: 2287-96.
41. Ezumi K, Yamamoto H, Murata K, Murata K, Higashiyama M, Higashiyama M, Damdinsuren B, Damdinsuren B, Nakamura Y, Nakamura Y, Kyo N, et al.: Aberrant expression of connexin 26 is associated with lung metastasis of colorectal cancer. *Clin Cancer Res* 2008; 14: 677-84.
42. Dang X, Doble B, Kardami E and Kardami E: The carboxy-tail of connexin-43 localizes to the nucleus and inhibits cell growth. *Mol Cell Biochem* 2003; 242: 35-8.
43. Tamargo-Gomez I and Marino G: AMPK: Regulation of Metabolic Dynamics in the Context of Autophagy. *Int J Mol Sci* 2018; 19: pii: E3812.
44. Visnjic D, Dembitz V and Lalic H: The Role of AMPK/mTOR Modulators in the Therapy of Acute Myeloid Leukemia. *Curr Med Chem* 2019; 26: 2208-29.
45. Li RL, Wu SS, Wu Y, Wang XX, Chen HY, Xin JJ, et al.: Irisin alleviates pressure overload-induced cardiac hypertrophy by inducing protective autophagy via mTOR-independent activation of the AMPK-ULK1 pathway. *J Mol Cell Cardiol* 2018; 121: 242-55.
46. Cristofani RA-OX, Montagnani Marelli M, Cicardi ME, Fontana F, Marzagalli M, Limonta P, et al.: Dual role of autophagy on docetaxel-sensitivity in prostate cancer cells. *Cell Death Dis* 2018; 9: 889.
47. Cotzomi-Ortega I, Aguilar-Alonso P, Reyes-Leyva J and Maycotte P: Autophagy and Its Role in Protein Secretion: Implications for Cancer Therapy. *Mediators Inflamm* 2018; 2018: 4231591.
48. Chung Y, Lee J, Jung S, Lee Y, Cho JW and Oh YJ: Dysregulated autophagy contributes to caspase-dependent neuronal apoptosis. *Cell Death Dis* 2018; 9: 1189.
49. Tsapras P and Nezis IP: Caspase involvement in autophagy. *Cell Death Differ* 2017; 24: 1369-79.
50. He W, Wang Q, Srinivasan B, Xu J, Padilla MT, Li Z, et al.: A JNK-mediated autophagy pathway that triggers c-IAP degradation and necroptosis for anticancer chemotherapy. *Oncogene* 2014; 33: 3004-13.
51. Xu J, Xu X, Shi S, Wang Q, Saxton B, He W, et al.: Autophagy-Mediated Degradation of IAPs and c-FLIP(L) Potentiates Apoptosis Induced by Combination of TRAIL and Chal-24. *J Cell Biochem* 2016; 117: 1136-44.

Figures

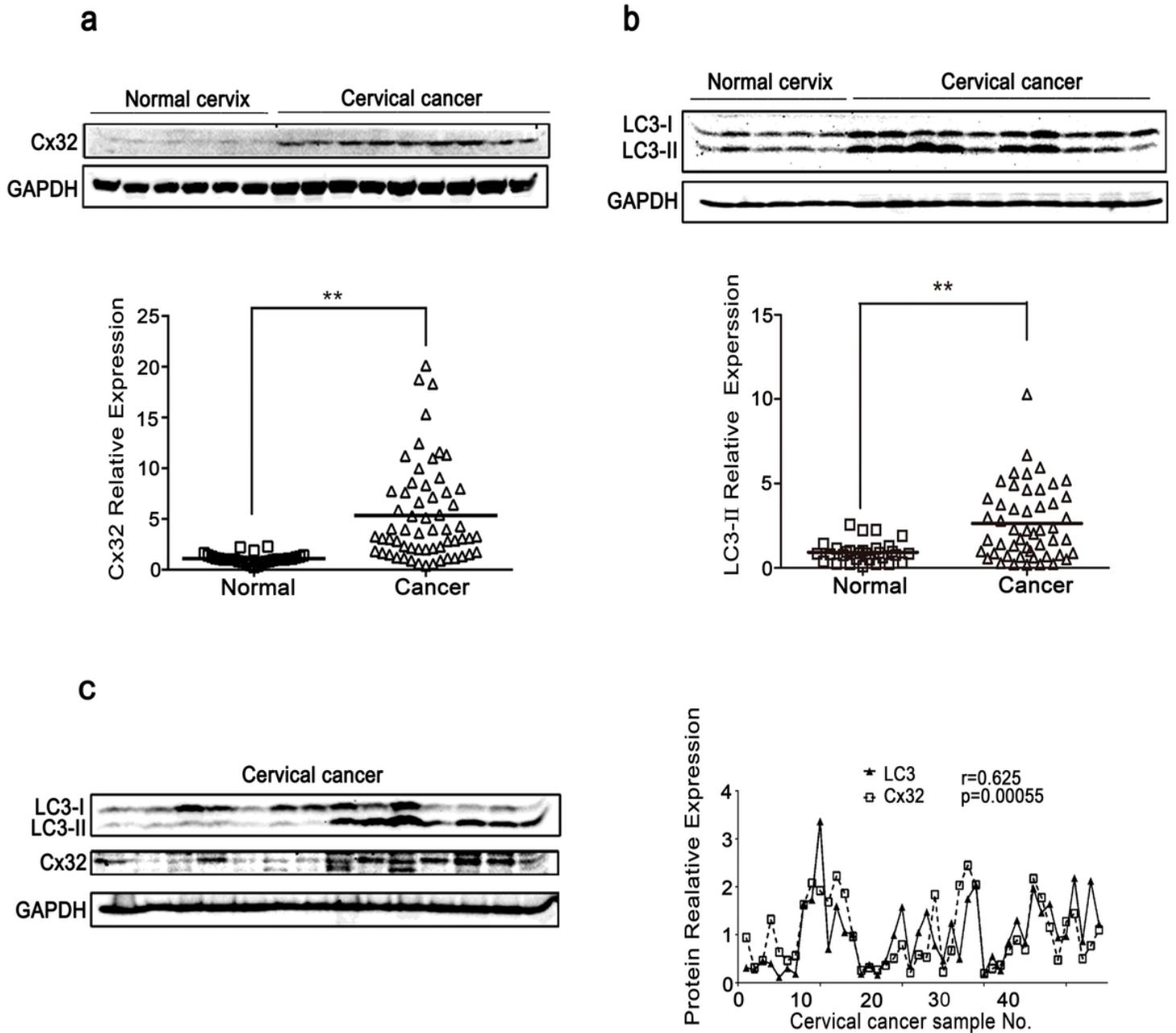


Figure 1

The relationship between Cx32 and autophagy in human CaCx tissue. a-b. The protein expression of Cx32 and LC3 was determined by Western blot analysis in CaCx tissue (n=50) and normal cervical tissue (n=30). c. The protein expression of Cx32 and LC3-II was determined by Western blotting analysis in CaCx tissue (n=45). The correlation analysis of Cx32 and LC3-II expression was performed using GraphPad Prism 6.0 (P=0.00055, r=0.625). The data are presented as the mean \pm SEM. ** P<0.01, vs. normal.

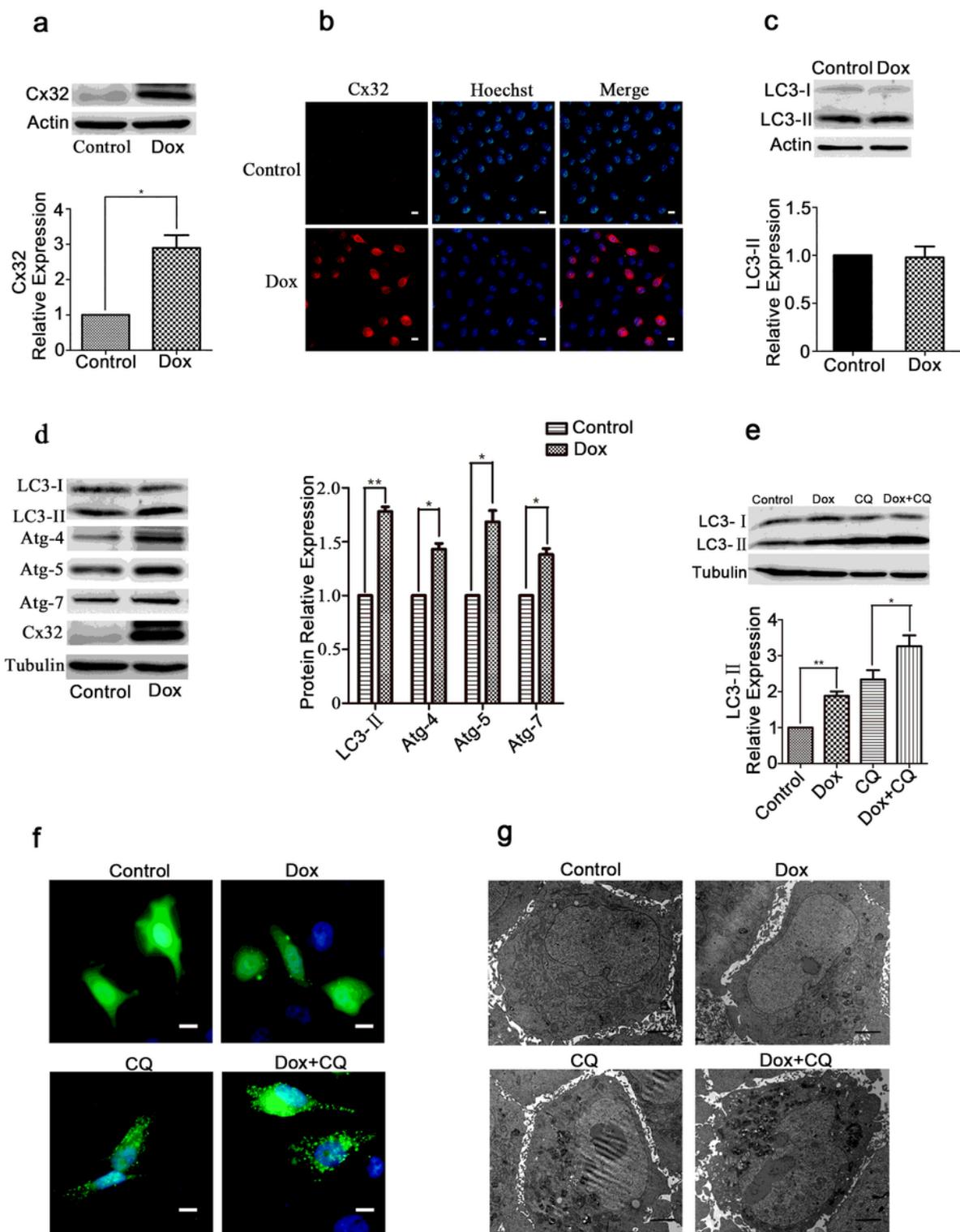


Figure 2

Cx32 overexpression promotes autophagy in HeLa-Cx32 cells. a-b. HeLa-Cx32 cells were treated with Dox ($1\mu\text{g}\cdot\text{ml}^{-1}$, 48 h), and the expression of Cx32 was detected by Western blotting (n=4) and immunofluorescence (n=3). The scale bars represent $20\mu\text{m}$. c. LC3 remained unchanged in HeLa cells with Dox treatment, as determined by Western blotting (n=5). d. Western blotting analysis showed that the autophagy-associated proteins LC3, Atg4, Atg5 and Atg7 were increased with Dox treatment ($1\mu\text{g}\cdot\text{ml}^{-1}$

-1, 48 h) in HeLa-Cx32 cells (n=5). e. LC3-II protein levels in the presence of CQ (25 μ M, 4 h) were determined by Western blotting (n=4). f. EGFP-LC3 puncta were examined by fluorescence microscopy and the number of puncta per cell was quantified. The scale bars represent 20 μ m (n=3). g. Autophagic vesicles containing cell organelles were observed by electron microscopy analysis. The scale bars represent 2 μ m (n=3). The data are presented as the mean \pm SEM. * P<0.05; ** P<0.01 vs control.

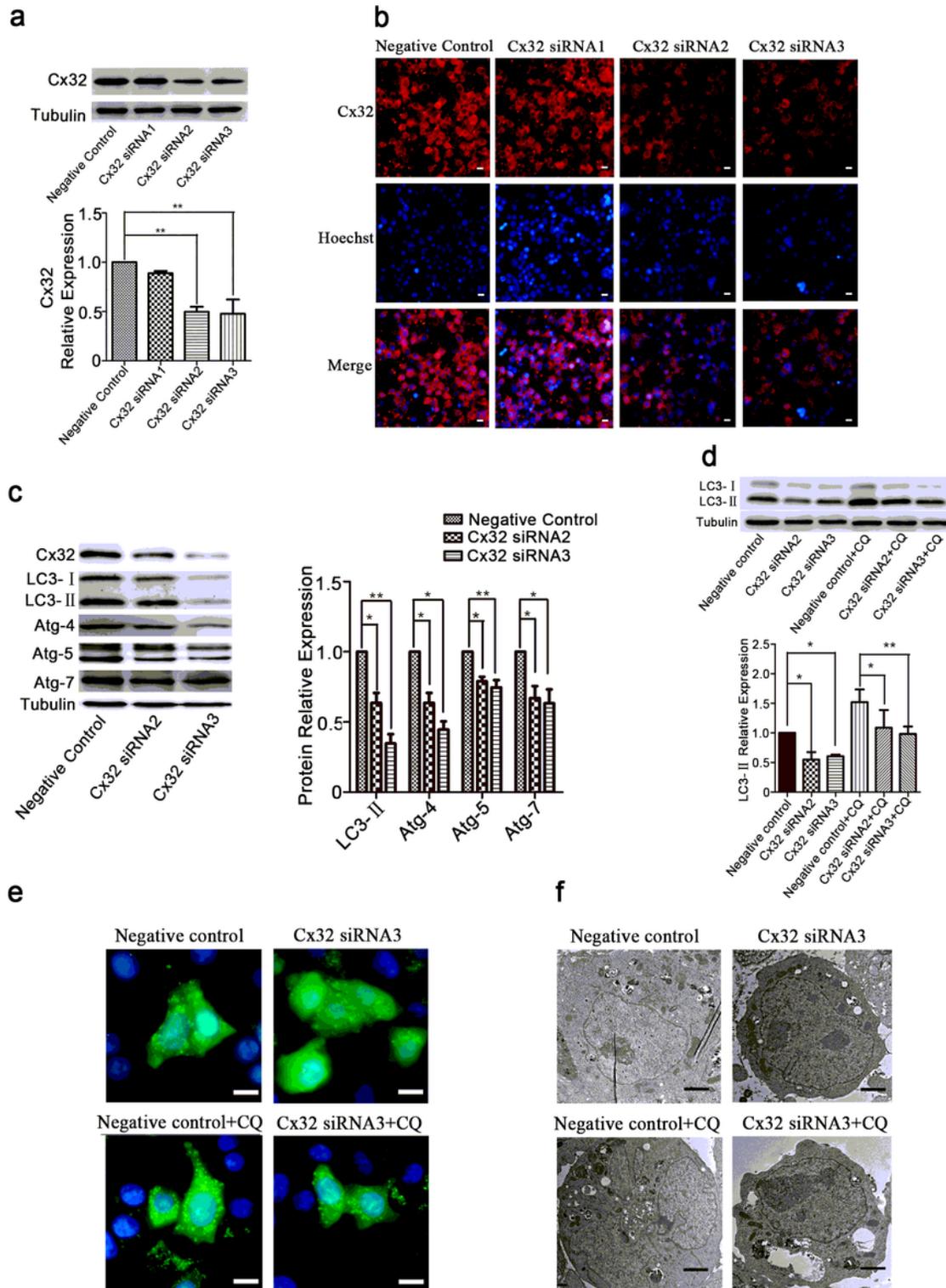
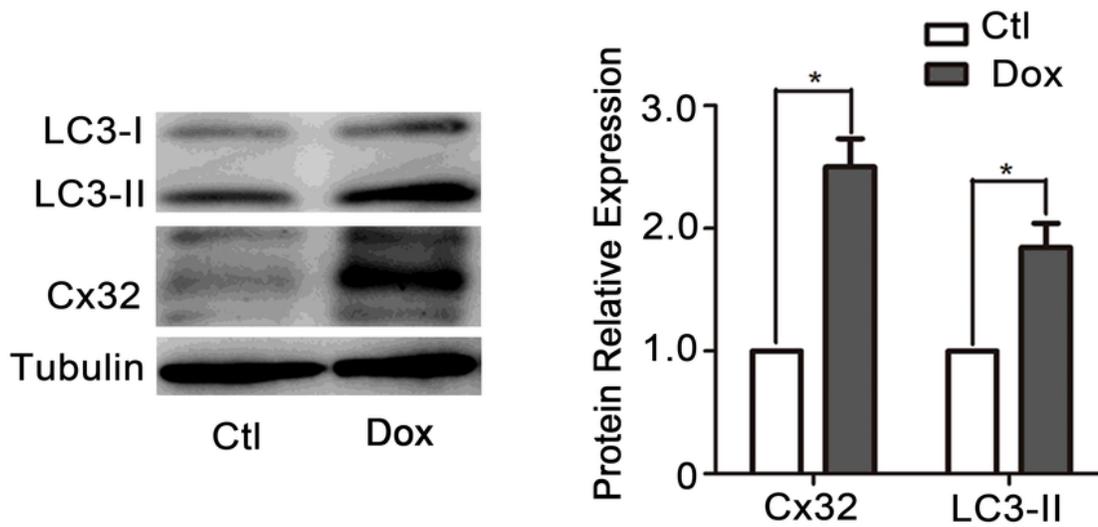


Figure 3

Knockdown of Cx32 negatively modulates autophagy in C-33A cells. a-b. Western blotting and immunofluorescence results demonstrated that both siCx32-2 and siCx32-3 effectively silenced the expression of Cx32 (n=3). The scale bars represent 20 μ m. c. The protein levels of Cx32, LC3, Atg4, Atg5 and Atg7 were reduced when Cx32 was knocked down, as detected by Western blotting (n=5). d. LC3-II was decreased by siCx32-2 and siCx32-3 but was restored by incubation with CQ (25 μ M, 4 h) (n=4). e. EGFP-LC3 puncta were examined by fluorescence microscopy. The scale bars represent 20 μ m. f. Autophagosomes were detected by transmission electron microscopy in C-33A cells. Bar represents 2 μ M. The data are presented as the mean \pm SEM.*P<0.05; **P<0.01 vs control.

a Low-density culture (HeLa-Cx32 cells)



b Low-density culture (C-33A cells)

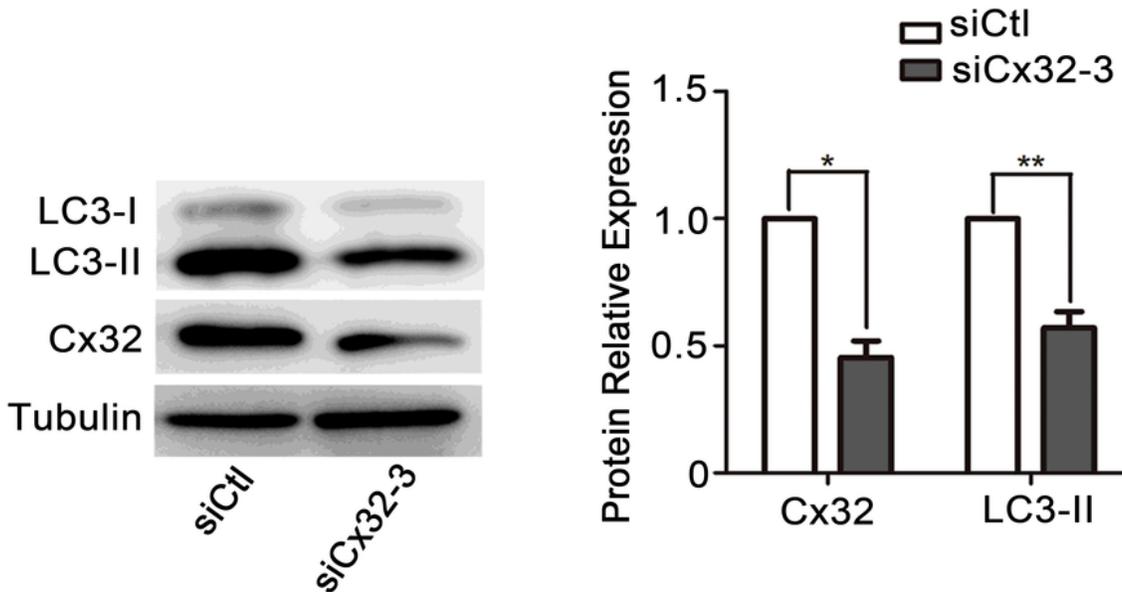


Figure 4

Cx32 promotes autophagy in CaCx cells in a GJ-independent manner. Under the circumstances of low-density culture, a. Cx32 induced by Dox increased the protein level of LC3 in HeLa-Cx32 cells (n=4). b. Cx32 knockdown by siCx32-3 decreased the protein level of LC3 in C-33A cells (n=4). The data are presented as the mean \pm SEM. *P<0.05; **P<0.01 vs control.

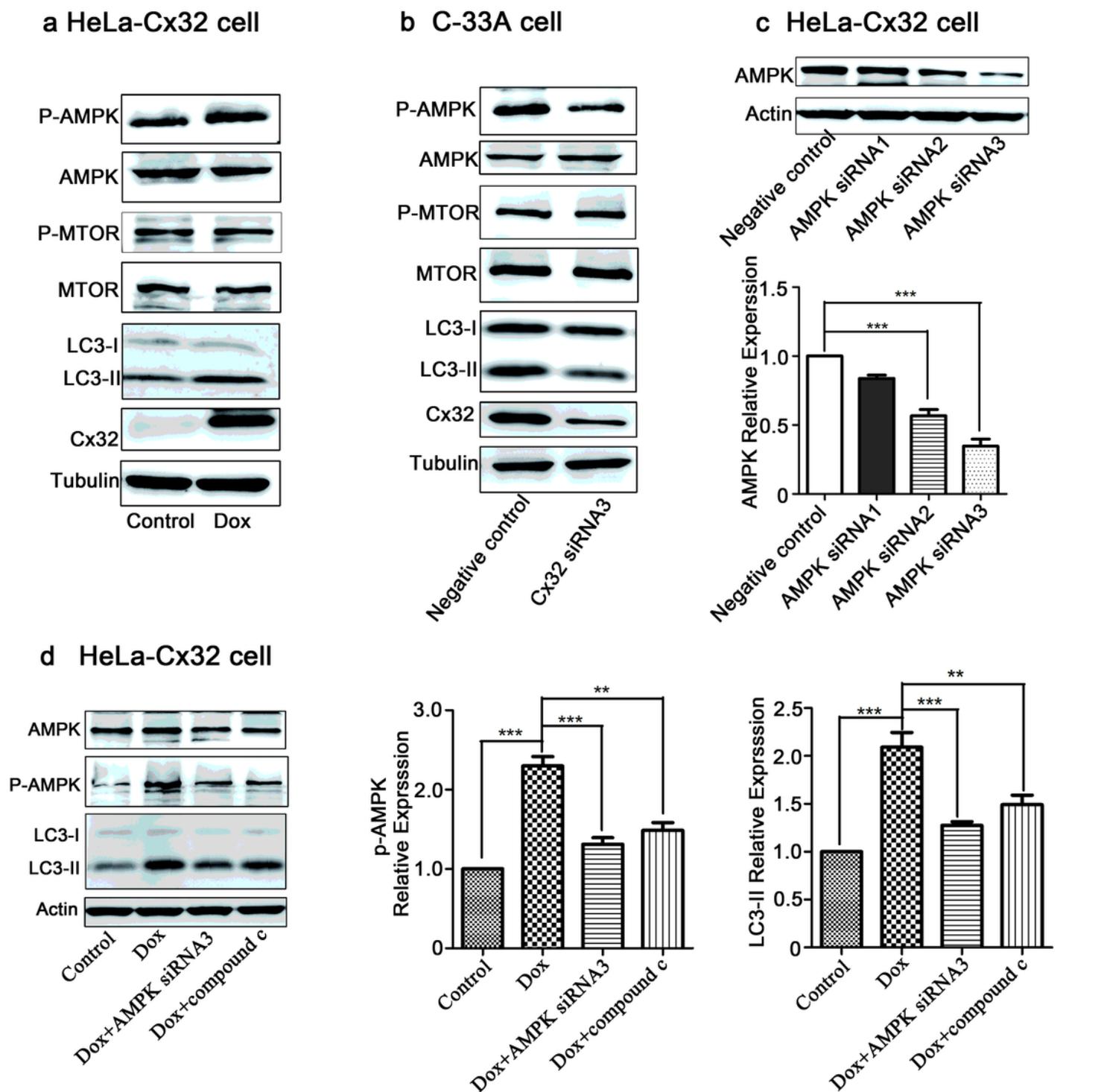


Figure 5

Cx32 promotes autophagy via the AMPK pathway in CaCx cells. a. Cx32 overexpression in HeLa-32 cells increased the protein levels of LC3 and p-AMPK (n=4). b. Cx32 knockdown in C-33A cells decreased the protein levels of LC3 and p-AMPK (n=4). c. Western blotting showed that siAMPK-3 had the greatest efficacy in reducing the expression of AMPK in HeLa-Cx32 cells (n=4). d. The upregulation of LC3-II and p-

AMPK expression by Cx32 in HeLa-Cx32 cells was reversed by siAMPK-3 and compound C (10 μ M, 6 h) (n=4). The data are presented as the mean \pm SEM. **P<0.01; ***P<0.001, vs control.

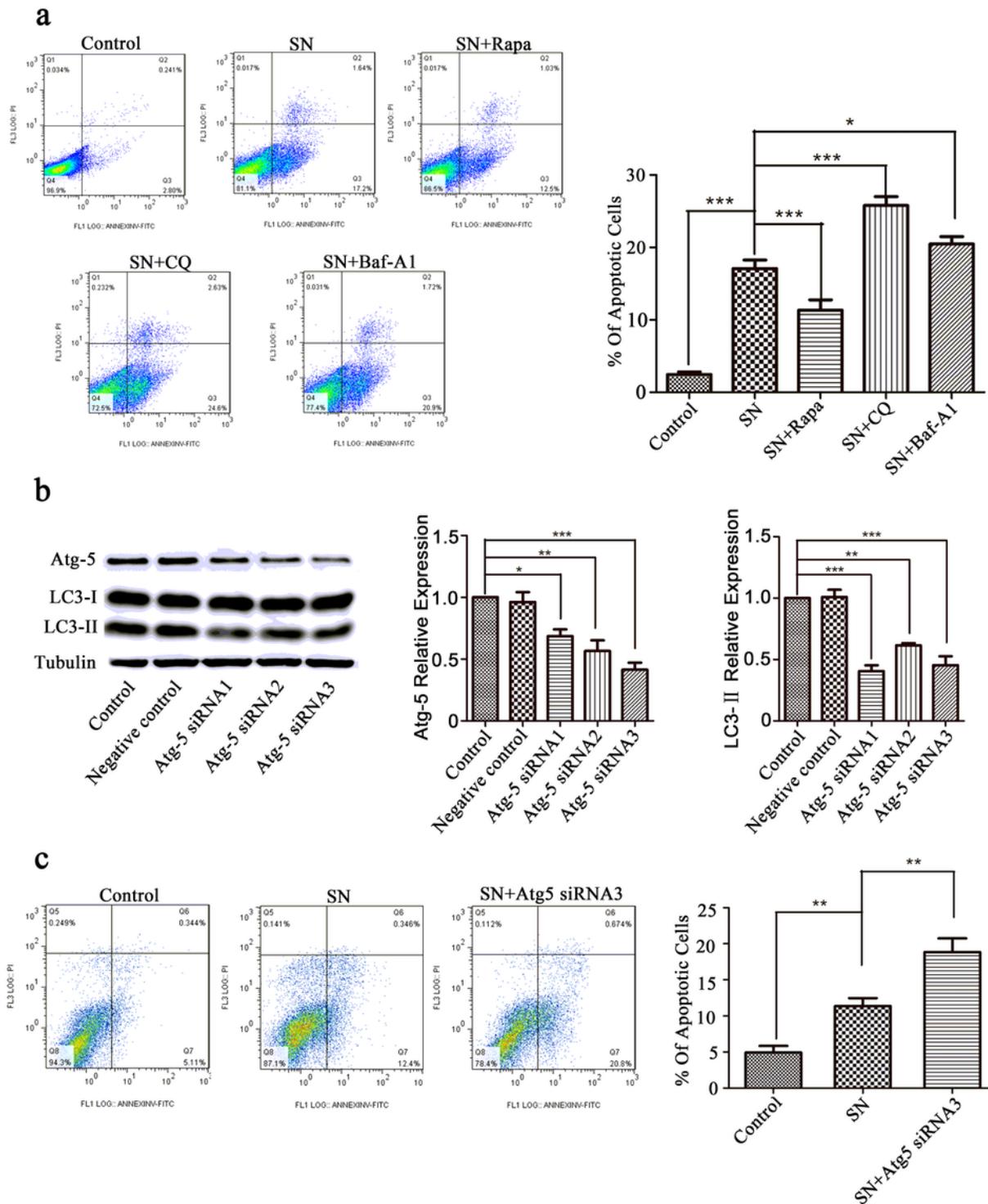
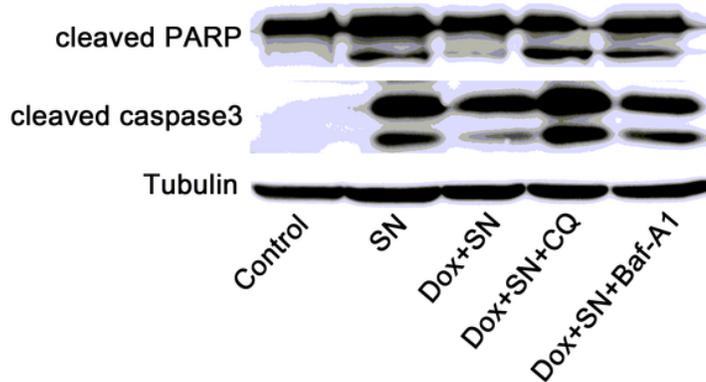


Figure 6

Effects of autophagy promoters or autophagy inhibitors on SN-induced apoptosis in HeLa cells. a. Autophagy activator (RAP) and autophagy inhibitors (CQ or Baf-A1) significantly influenced SN-induced apoptosis, and cell apoptosis (n=3). b. Western blot analysis of the effectiveness of three siAtg5 in

reducing the expression of Atg5 and autophagy levels (n=3). c. HeLa cells were transfected with siAtg5 for 48 h before exposure to 1 μ M SN for 6 h. Cell apoptosis was analysed using an annexin V/PI assay (n=3). The data are presented as the mean \pm SEM. *P<0.05; **P<0.01; ***P<0.001 vs control.

a Low density culture



b Low density culture

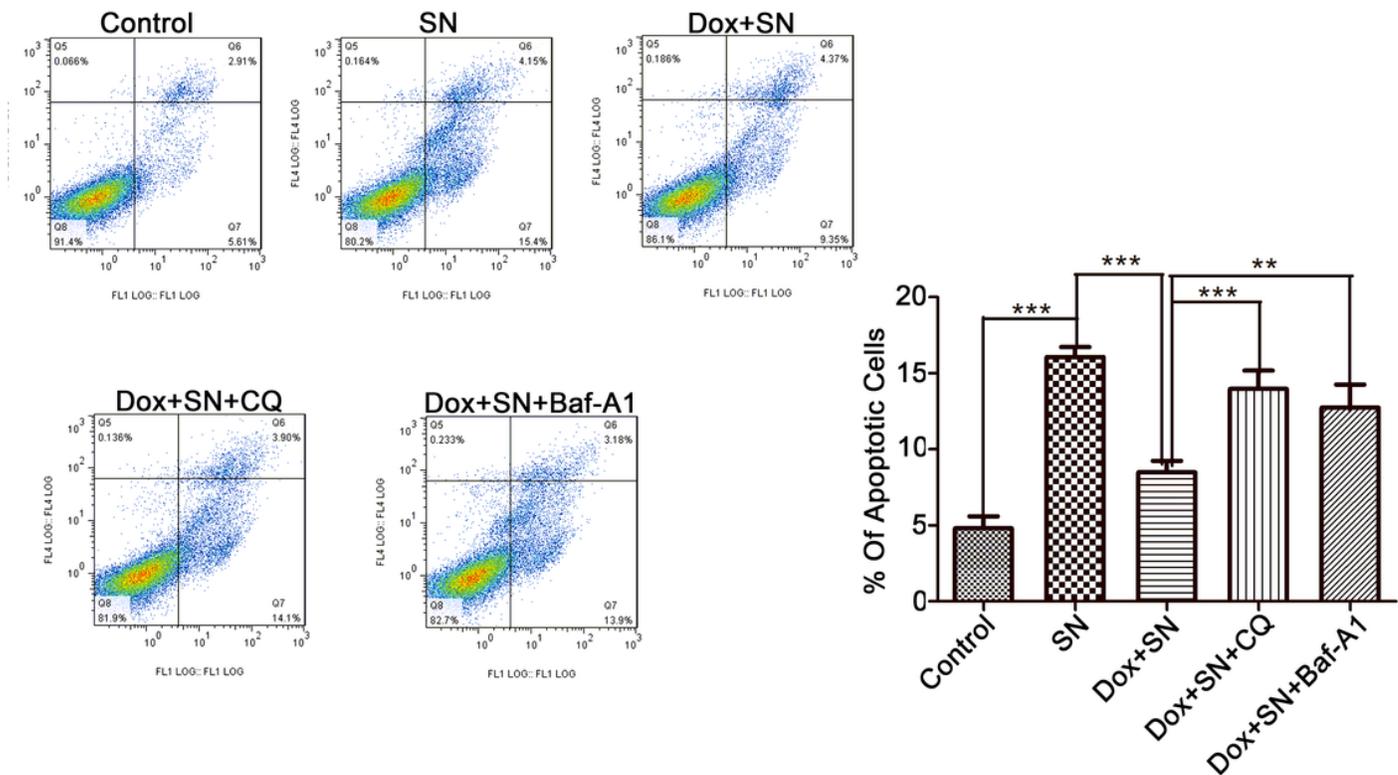


Figure 7

Cx32 inhibits apoptosis by promoting autophagy in HeLa-Cx32 cells. a. Western blotting analysis of cleaved caspase-3 and cleaved PARP expression (n=3). b. HeLa-Cx32 cell apoptosis was determined by flow cytometry (n=3). The data are presented as the mean \pm SEM. **P < 0.01, ***P < 0.001 vs control.