

# Knockdown of KAT5/KIF11 induces autophagy and promotes apoptosis in anaplastic thyroid cancer cells

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**Abstract.** K (lysine) acetyltransferase (KAT) 5, which is a member of the KAT family of enzymes, has been found to act as a regulatory factor in various types of cancer. However, the role of KAT5 in anaplastic thyroid carcinoma (ATC) and its underlying mechanism is still elusive. The expression levels of KAT5 and kinesin family member 11 (KIF11) in ATC cells were assessed utilizing reverse transcription-quantitative PCR and western blot analyses. The cell proliferative ability was assessed via Cell Counting Kit-8 assay and using 5-ethynyl-2'-deoxyuridine staining. Flow cytometry and western blot analyses were applied for the assessment of cell apoptosis. Cell autophagy was investigated by employing western blot analysis and immunofluorescence staining. In addition, the enrichment of histone H3 lysine 27 acetylation (H3K27ac) and RNA polymerase II (RNA pol II) was analyzed by chromatin immunoprecipitation assay. It was shown that KAT5 expression was markedly increased in ATC cells. KAT5 depletion suppressed the cell proliferative capability but promoted the induction of apoptosis and autophagy. In addition, the autophagy inhibitor 3-methyladenine reversed the effects of KAT5 deficiency on the proliferative and apoptotic activities of 8505C cells. With regard to the mechanism, it was found that KAT5 inhibited the expression of KIF11 by repressing the enrichment of H3K27ac and RNA pol II. Upregulation of KIF11 expression reversed the effects of KAT5 silencing on the proliferative activity, apoptosis and autophagy of 8505C cells. In conclusion, the results indicated that KAT5 induced autophagy and promoted apoptosis of ATC cells by targeting KIF11, which may provide a promising target for the treatment of ATC.

## Introduction

Thyroid cancer (TC) is the most frequent endocrine malignant tumor, accounting for >90% of all endocrine malignancies (1-3). TC can be divided into three main histological types: Differentiated (papillary and follicular TC), undifferentiated (poorly differentiated and anaplastic TC) and medullary TC, which originates from TC cells (4). Anaplastic thyroid carcinoma (ATC), which is also termed undifferentiated thyroid carcinoma, is a type of thyroid carcinoma featuring high-grade morphology and high aggressiveness (5). ATC has an incidence of 1.5% in all TCs and is the leading cause of all TC-related mortalities (6). Due to the high levels of extrathyroid invasion, distant metastasis and resistance to conventional treatment, the median survival time of ATC is 3-9 months (7). Despite the use of surgery, chemoradiotherapy, drug therapy and the development of other novel methods for ATC treatment, the survival rate of ATC patients is low because of high metastasis rate (8). Therefore, it is critical to explore the molecular mechanisms involved in ATC and identify improved therapeutic strategies for ATC.

K (lysine) acetyltransferase (KAT) is a class of enzymes that catalyze the transfer of acetyl groups from acetyl-CoA to the  $\epsilon$ -amino groups of lysine found on specific protein substrates (9). The acetylation of lysine residues can affect the functions of certain proteins, such as DNA-binding capacity, protein-protein interactions, enzyme activity, chromosomal structure and the regulation of nuclear transcription enzyme activity (10-12). KAT can be divided into several families of enzymes, among which Moz, YBF2, Sas2p and Tip (MYST), which is a pivotal one (13). Tip60, which is also termed KAT5, is a main member of the MYST family of enzymes and acts as a crucial participant in gene transcription, regulation of the cell cycle, autophagy, tumorigenesis and metastasis (14). A previous study has shown that KAT5 silencing can inhibit proliferation and induce apoptosis of liver cancer cells (15). KAT5 is also involved in the suppression of the cell cycle and in the induction of apoptosis of lung cancer cells (16). Overexpression of KAT5 increases the aggressiveness of papillary thyroid cancer cells (17). In addition, KAT5 has been shown to positively affect the invasive and migratory activities of ATC cells (18). However, whether KAT5 affects the induction of cell apoptosis in

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ATC is unknown. Therefore, the present study was designed to explore the effects of KAT5 in ATC and investigate its underlying mechanism of action.

## Materials and methods

**Cell culture and treatment.** The human ATC cell lines 8505C, FRO, KHM-5M and C643 and the normal thyroid cell line Nthy-ori 3-1 were provided by the American Type Culture Collection and were cultured in DMEM (Shanghai Zeye Biotechnology Co., Ltd.) in the presence of 10% FBS (Cytiva) and 1% antibiotics (MilliporeSigma) at 37°C with 5% CO<sub>2</sub>. To investigate the autophagic pathway, 10 mM 3-methyladenine (3-MA) was applied to treat ATC cells for 2 h at 37°C.

**Cell transfection.** The kinesin family member 11 (KIF11)-specific pcDNA overexpression vector (ov-KIF11) and corresponding negative control (ov-NC), the small interfering (si)-RNA targeting KAT5 (si-KAT5-1: GGACAGCUC UGAUGGAAUACC; si-KAT5-2: GCAAUAAAUUGUUUC UAUUAUG) and the corresponding negative control (si-NC: 5'-UUCUCCGAACGUGUCACGU-3') were provided by Genepharma, Inc. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for the transfection of the aforementioned recombinants into 8505C cells according to the manufacturer's protocols.

**Cell Counting Kit-8 (CCK-8) assay.** The evaluation of 8505C cell viability was assessed utilizing the CCK-8 assay. Following incubation in 96-well plates, the transfected cells were cultured in DMEM with 10% FBS. Subsequently, the cells were incubated with 10 µl CCK-8 solution (Abcam) for an additional 2 h. The absorbance was measured with a microplate reader at λ=450 nm.

**5-Ethynyl-2'-deoxyuridine (EdU) assay.** The 8505C cells were plated in six-well plates at a density of 4x10<sup>5</sup> cells/well and then incubated at 37°C overnight. 8505C cells were mixed with 4% polyformaldehyde for 15 min at room temperature, fixed and incubated further in the presence of 0.5% Triton X-100 for 15 min at room temperature. Subsequently, the cells were stained with Cell-Light™ EdU Cell Proliferation Detection Assay (Thermo Fisher Scientific, Inc.) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime Institute of Biotechnology) for 10 min at room temperature. Fluorescent microscopy was applied for counting the positive cells.

**Flow cytometry.** Cell apoptosis was detected by fluorescein isothiocyanate (FITC) Annexin V/propidium iodide (PI) Apoptosis Detection kit I (Guangzhou RiboBio Co., Ltd.). Briefly, the PBS-rinsed 8505C cells were re-suspended in binding buffer. Following 10 min of exposure to 5 µl of Annexin V-FITC according to the manufacturer's instructions, the cells were incubated with 10 µl PI at 4°C for 30 min in the dark. Samples were run on a FC500MPL flow cytometer (Beckman Coulter, Inc.) and FlowJo vX.0.7 software (FlowJo LLC) was employed for the analysis of cell apoptosis. The apoptotic rate was calculated using the formula: Apoptotic rate=Q2+Q3. The results of the cell apoptosis analysis were obtained from three different replications.

**Reverse transcription-quantitative PCR (RT-qPCR).** The extraction of total RNA from 1x10<sup>4</sup> treated cells was conducted employing TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The synthesis of RNA into cDNA was performed by using PrimeScript RT Master Mix (Takara Bio, Inc.) according to the manufacturer's instructions, which was provided by Takara Bio, Inc. Subsequently, the cDNA was amplified by RT-qPCR using the SYBR Premix Ex Taq II kit (Takara Bio, Inc.) according to the manufacturer's instructions. The thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min; followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The primer sequences for PCR are presented as below: KAT5: 5'-AGTGGAGGGAGGGAAGATGG-3' (forward) and 5'-TCT TCGTTGTCCTGGTTCCG-3' (reverse); KIF11: 5'-GCTTTC TCCTCGGCTCACTT-3' (forward) and 5'-GTGGGCACA GAGCCATTAGT-3' (reverse); GAPDH: 5'-GGGAAACTG TGGCGTGAT-3' (forward) and 5'-GAGTGGGTGTGCTG TTGA-3' (reverse). The comparative Cq method was employed for the estimation of the relative gene expression levels (19). The experiments were replicated for three times.

**Chromatin immunoprecipitation (CHIP) assay.** The EZ CHIP kit (Shanghai Huzheng Biotechnology Co., Ltd.) was employed for the CHIP assays. The cross-linking of the cells was performed with 1% formaldehyde, whereas their quenching with 2.5 M glycine. Then, 1x10<sup>6</sup> cells were collected via centrifugation at 300 x g for 3 min at 25°C and washed twice with pre-chilled PBS. DNA was immunoprecipitated from the sonicated cell lysates using 2 µg anti-KAT5 (1:30; cat. no. ab300521; Abcam) at 4°C overnight. The following day, the samples were conjugated with Protein A agarose (Invitrogen; Thermo Fisher Scientific, Inc.) for 6 h at 4°C. A ChIP DNA purification kit (Beyotime Institute of Biotechnology) was used to purify the immunoprecipitated DNA and it was amplified by qPCR. RT-qPCR was applied to analyze the enriched DNA.

**Immunofluorescence staining.** Initially, 8505C cells were subjected to 4% paraformaldehyde fixation as well as 0.25% Triton X-100 permeabilization (Triton X-100 was only used for α-smooth muscle actin, not cluster of differentiation 31). The overnight incubation of 8505C cells was performed at 4°C, in the presence of 2% BSA and the primary antibody microtubule-associated protein 1A/1B-light chain 3 (LC3) (cat. no. ab239416; Abcam). Subsequently, the samples were incubated with DAPI (BIOSS) and FITC-conjugated secondary antibodies IgG (H + L) (ProteinTech Group, Inc.) for LC3 in the dark. A fluorescence microscope was used for the visualization and the imaging of the cells.

**Western blot assay.** Total protein was extracted from 8505C cells using RIPA buffer (GCS Auragen). The determination of the concentration levels of the proteins was performed using the Detergent Compatible Bradford Protein Assay kit (Beyotime Institute of Biotechnology). Following their separation by PAGE in 10% SDS gels, the proteins (30 µg/lane) were transferred to polyvinylidene fluoride membranes. Subsequently, the membranes were blocked in 5% non-fat milk diluted in 0.1%

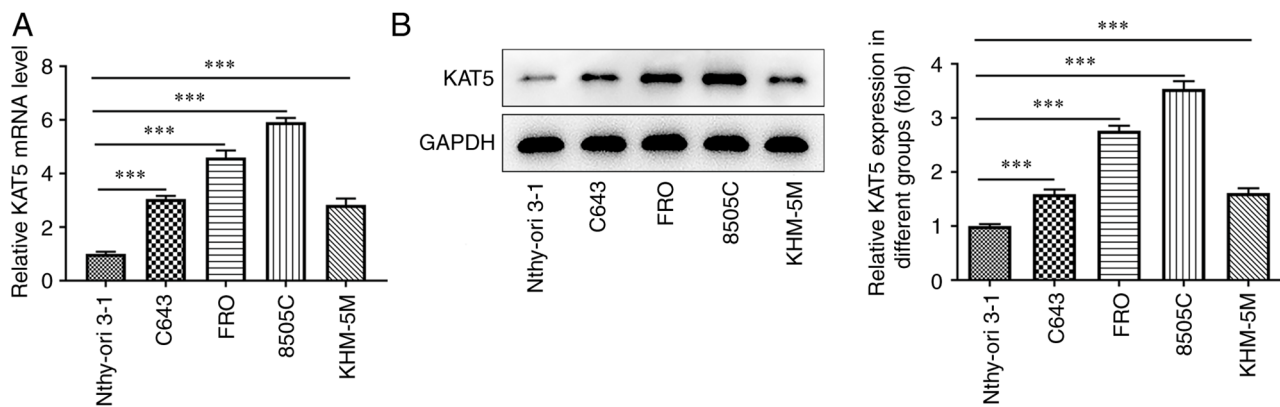


Figure 1. KAT5 is upregulated in ATC cells. The mRNA (A) and protein levels (B) of KAT5 in ATC cells and Nthy-ori 3-1 were detected by reverse transcription-quantitative PCR and western blotting. Data were expressed as mean  $\pm$  standard deviation. \*\*\* $P$ <0.001. KAT, K (lysine) acetyltransferase.

Tris-buffered saline with Tween 20 at room temperature for 2 h and incubated overnight with primary antibodies specific to KAT5 (1:1,000; cat. no. ab300521; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam), Bax (1:1,000; cat. no. ab32503; Abcam), cleaved caspase-3 (1:500; cat. no. ab32042; Abcam), Beclin1 (1:2,000; cat. no. ab207612; Abcam), p62 (1:10,000; cat. no. ab109012; Abcam), LC3II/I (1:2,000; cat. no. ab192890; Abcam), KIF11 (1:1,000; cat. no. ab254298; Abcam) and GAPDH (1:2,500; cat. no. ab9485; Abcam) at 4°C. The following morning, the membranes were incubated for 1 h with secondary antibodies (1:2,000; cat. no. ab6721; Abcam) at room temperature. Finally, the visualization of the protein bands was conducted by employing an ECL detection system (Shanghai Yeasen Biotechnology Co., Ltd.). QuantityOne 4.5.0 software (Bio-Rad Laboratories, Inc.) was used for densitometry.

**Statistical analysis.** The data are displayed in the format of mean  $\pm$  standard deviation. The analysis was performed with SPSS 22.0 software (IBM Corp.) and GraphPad Prism 6 software (GraphPad Software, Inc.). Significant differences were analyzed by one-way ANOVA followed by the Bonferroni post-hoc test for multiple comparisons.  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**KAT5 expression is upregulated in ATC cells.** The expression levels of KAT5 in ATC cell lines were initially appraised. The mRNA expression levels of KAT5 in the ATC cell lines including 8505C, FRO and KHM-5M and in the C643 cell line were increased relative to those noted in Nthy-ori 3-1 cells (Fig. 1A). The results obtained from the western blot assay indicated that KAT5 protein levels were also upregulated in ATC cell lines compared with those of the Nthy-ori 3-1 cell line (Fig. 1B). Among these ATC cell lines, 8505C cells exhibited the highest expression of KAT5. Therefore, 8505C cells were used for follow-up studies.

**Knockdown of KAT5 expression inhibits the proliferation and induces the apoptosis and autophagy of 8505C cells.** To explore the biological role of KAT5 in ATC, si-KAT5-1/2

was transfected into 8505C cells to knockdown KAT5 expression. RT-qPCR and western blot analyses indicated that si-KAT5-1 exhibited improved transfection efficiency. Therefore, si-KAT5-1 was selected for subsequent analysis (Fig. 2A and B). The results obtained from the CCK-8 assay indicated that the capacity of cell proliferation was remarkably inhibited following silencing of KAT5 compared with that of the negative control samples (Fig. 2C). EdU staining further revealed that the number of positive cells in the si-KAT5 group was significantly decreased (Fig. 2D). In addition, the induction of cell apoptosis was notably promoted by knockdown of KAT5 expression in 8505C cells (Fig. 2E). The data from the western blot analysis indicated that KAT5 silencing decreased Bcl-2 levels, whereas it also enhanced the contents of Bax and cleaved caspase-3 (Fig. 2F). In addition, silencing of KAT5 expression reduced p62 levels and promoted the levels of Beclin1 and LC3II/I compared with those of the negative control samples (Fig. 3A), which was consistent with the results of the immunofluorescence assay (Fig. 3B).

**Silencing of KAT5 expression reduces the proliferation and induces the apoptosis of 8505C cells by promoting autophagy.** To investigate the role of autophagy in KAT5-mediated proliferation and apoptosis of 8505C cells, 10 mM of the autophagy inhibitor 3-MA was administered to the cells. Treatment of the cells with 3-MA enhanced their decreased proliferation compared with the si-KAT5 group (Fig. 4A). Similarly, the EdU assay results revealed that 3-MA treatment significantly increased the number of positive cells (Fig. 4B). In addition, 3-MA repressed the induction of apoptosis in KAT5-silenced cells (Fig. 4C). Subsequently, Bcl-2 levels were elevated, while Bax and cleaved caspase-3 levels were decreased following the inhibition of autophagy (Fig. 4D).

**KAT5 depletion represses KIF11 expression in 8505C cells.** Subsequently, the potential mechanism underlying KAT5-mediated ATC cell apoptosis and autophagy was explored. The mRNA and protein expression levels of KIF11 were elevated in the ATC cell lines compared with those of the Nthy-ori 3-1 cell line (Fig. 5A and B). The levels of KIF11 were suppressed by KAT5 depletion (Fig. 5C). In addition,

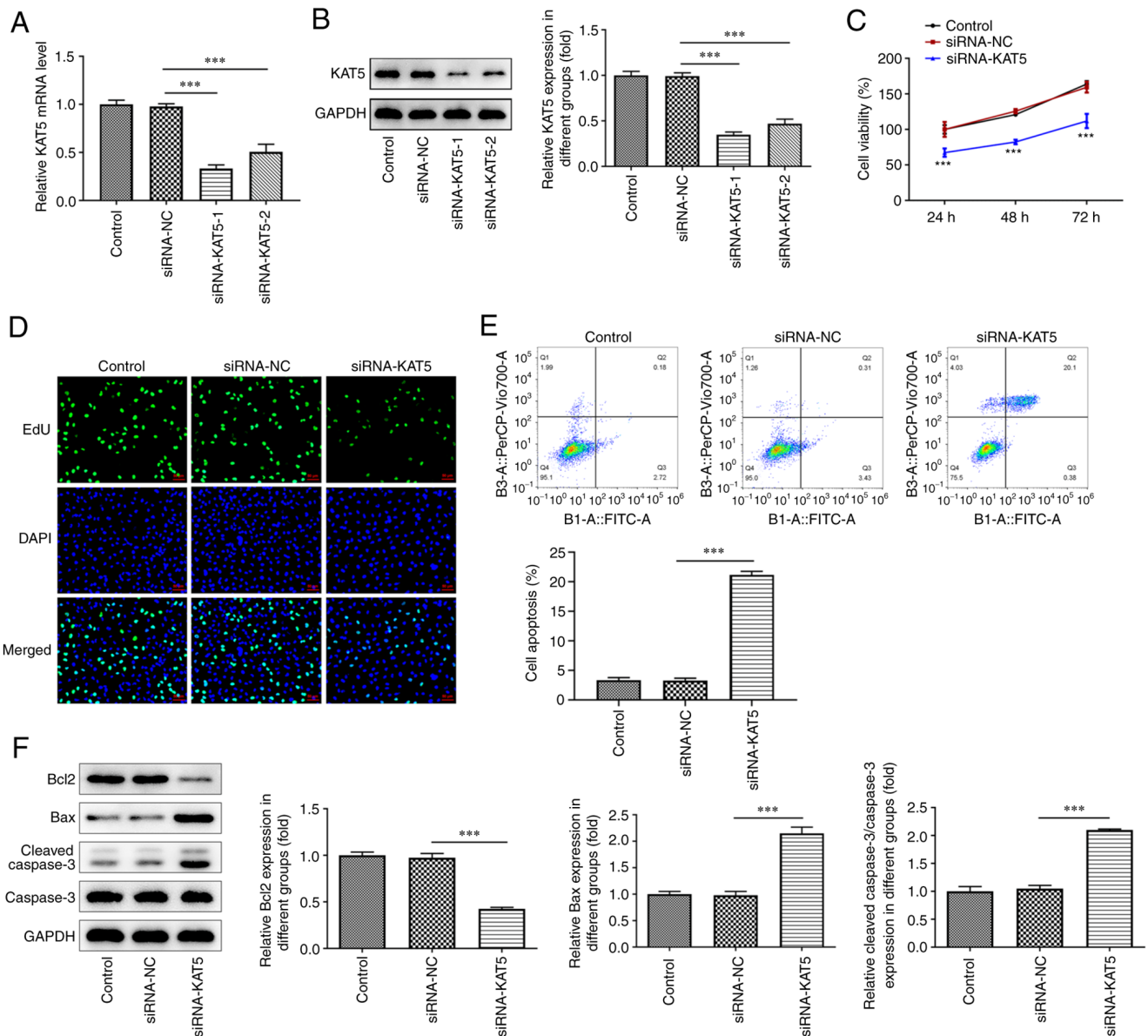


Figure 2. Knockdown of KAT5 inhibits the proliferation and induces the apoptosis of 8505C cells. The (A) mRNA and (B) protein levels of KAT5 in 8505C cells were detected by reverse transcription-quantitative PCR and western blotting. Cell proliferation was evaluated by (C) CCK-8 assay and (D) EdU staining (magnification,  $\times 200$ ). (E) Cell apoptosis was detected by flow cytometry analysis. (F) Western blot assay was used to assess apoptotic-related protein levels. Data were expressed as mean  $\pm$  standard deviation. \*\*\* $P < 0.001$ . KAT, K (lysine) acetyltransferase; EdU, 5-Ethynyl-2'-deoxyuridine; si, small interfering; NC, negative control.

the results obtained from the CHIP assay indicated that the enrichment of H3K27ac and RNA pol II on the promoter region of KIF11 was repressed following knockdown of KAT5 expression (Fig. 5D and E).

**Knockdown of KAT5 expression regulates proliferation, apoptosis and autophagy by inhibiting KIF11 expression.** Following the initial findings, the ability of KAT5 to affect ATC cell functions by targeting KIF11 expression was investigated. KIF11-overexpressing plasmids were constructed and transfected into 8505C cells. RT-qPCR and western blot analyses revealed that KIF11 overexpression promoted KIF11 expression (Fig. 6A and B). KIF11 overexpression significantly accelerated the production of p62 but reduced the levels of Beclin1 and LC3II/I in 8505C

cells co-transfected with siRNA-KAT5 and ov-KIF11 (Fig. 6C). The results of the immunofluorescence assay revealed that KIF11 overexpression evidently reduced the levels of LC3 compared with those of the cells transfected with siRNA-KAT5 (Fig. 6D). Furthermore, the results of the CCK-8 assay indicated that KIF11 overexpression reversed the effects caused on cell proliferation compared with the negative control cells (Fig. 7A); these effects were accompanied with an increased number of positive 8505C cells (Fig. 7B). In addition, the cell apoptotic rate in KAT5-silenced cells was reversed following transfection with ov-KIF11 plasmids (Fig. 7C). Finally, an increase in Bcl-2 levels and a reduction in the levels of Bax and cleaved caspase-3 were also noted in cells transfected with ov-KIF11 (Fig. 7D).



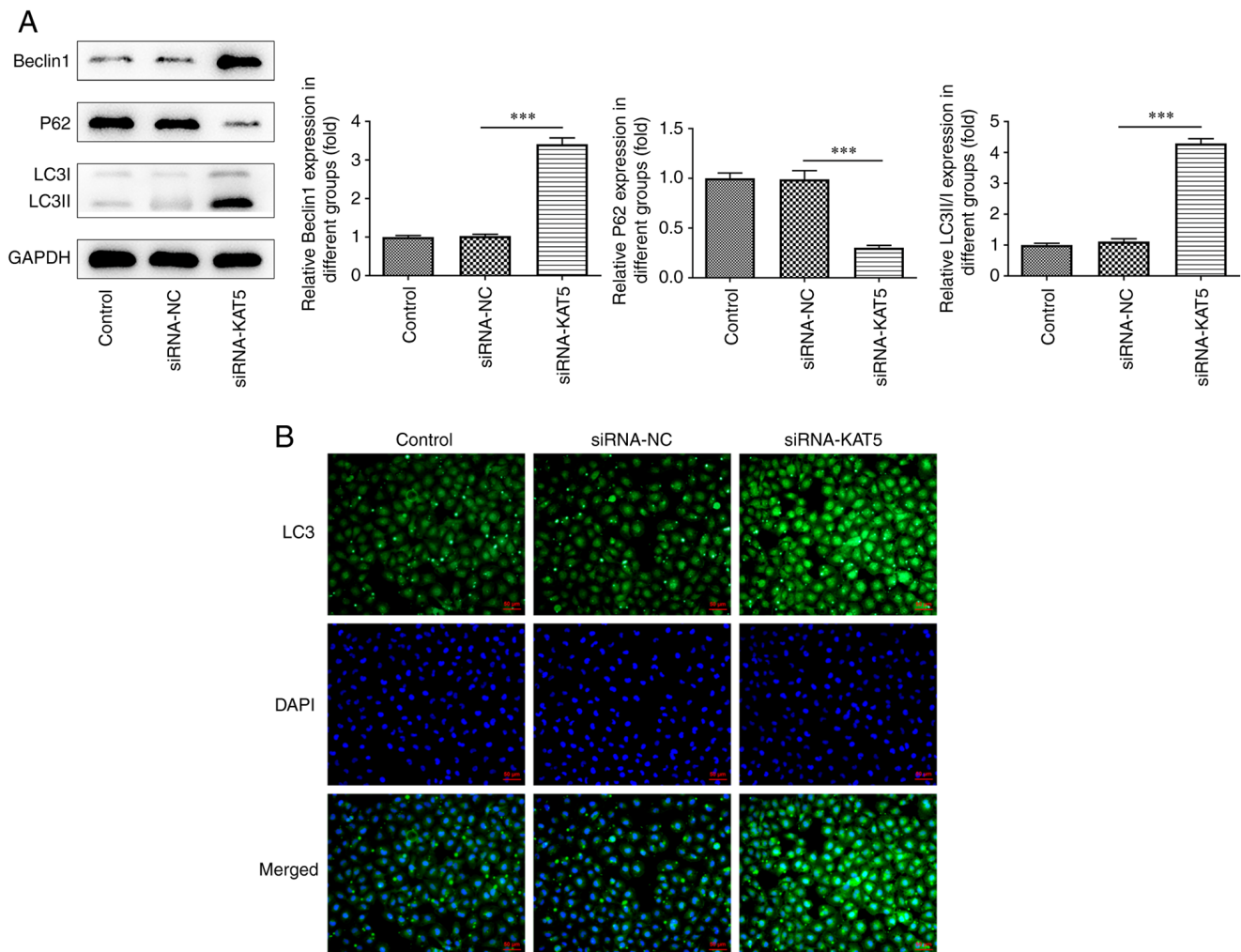


Figure 3. Knockdown of KAT5 promoted the cell autophagy of 8505C cells. (A) Western blot assay was used to evaluate the levels of p62, Beclin1 and LC3II/I in 8505C cells transfected with siRNA-KAT5. (B) Immunofluorescence assay was used to assess the level of LC3 in 8505C cells transfected with siRNA-KAT5 (magnification, x200). Data were expressed as mean  $\pm$  standard deviation. \*\*\* $P < 0.001$ . KAT, K (lysine) acetyltransferase; si, small interfering; NC, negative control.

## Discussion

As one of the deadliest human cancers, ATC accounts for <2% of TCs (20). The treatment for ATC includes surgery, chemotherapy, such as cisplatin or doxorubicin, as well as accelerated hyperfractionated external beam radiation therapy (21). Due to the fact that ATC is a rare and aggressive tumor, it is still challenging to predict the clinical responsiveness of patients to treatment (22). Recently, several genetic mutations and different molecular pathways, which are involved in tumor progression, have been explored in ATC; moreover, novel therapies acting on these molecular pathways have been developed to improve the quality of life of patients with ATC (23-25). The present study aimed to explore the mechanism of KAT5/KIF11 in ATC. The data demonstrated that KAT5 deficiency reduced KIF11 expression to promote the induction of apoptosis and autophagy in ATC cells.

KATs are emerging as candidate therapeutic targets for several cancer types; several inhibitors have been discovered to target these proteins (26). The MYST family, including KAT5-KAT8, has diverse functions that affect a variety of cellular processes, including gene regulation,

cell cycle, stem cell homeostasis and development as well as the DNA damage repair process (27). KAT5, which is a critical element of the MYST family, is the catalytic subunit of the NuA4 histone acetyltransferase complex. This protein participates in the transcriptional activation of specific genes (28) and the complex is essential for triggering transcriptional programs which have close relation with oncogene and proto-oncogene-modulated growth induction, tumor suppressor-regulated growth arrest and induction of apoptosis (29,30). Feng *et al* (31) demonstrated that KAT5 was involved in the progression of gallbladder carcinoma tumorigenesis and was closely related to disease prognosis. It was shown that KAT5 silencing induced apoptosis of cells via the p38MAPK-mediated upregulation of cleaved caspase-9. Kim and Lee (32) demonstrated that KAT5 regulated the proliferation of prostate cancer cells by a caspase-3-dependent apoptotic pathway, suggesting that it was probably to be involved in the induction of apoptosis of ATC cells. Therefore, the present study explored the mechanism by which KAT5 participates in the induction of ATC apoptosis. The data indicated that KAT5 expression was upregulated in ATC cells compared with the

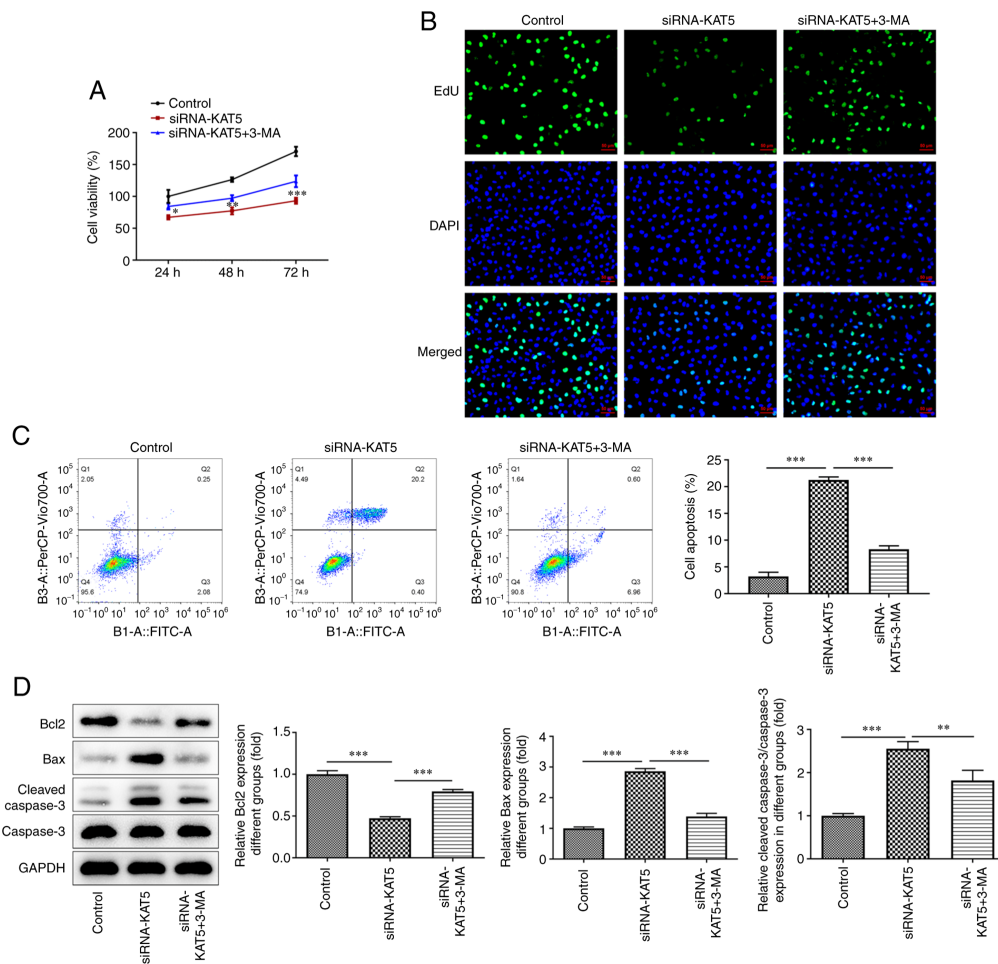


Figure 4. KAT5 silencing reduces the proliferation and induces the apoptosis of 8505C cells by promoting autophagy. Cell proliferation was evaluated by (A) CCK-8 assay and (B) EdU staining (magnification, x200). (C) Cell apoptosis was detected by flow cytometry analysis. (D) Western blot assay was used to assess apoptotic-related protein levels. Data were expressed as mean  $\pm$  standard deviation.  $P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ . KAT, K (lysine) acetyltransferase; si, small interfering; NC, negative control.

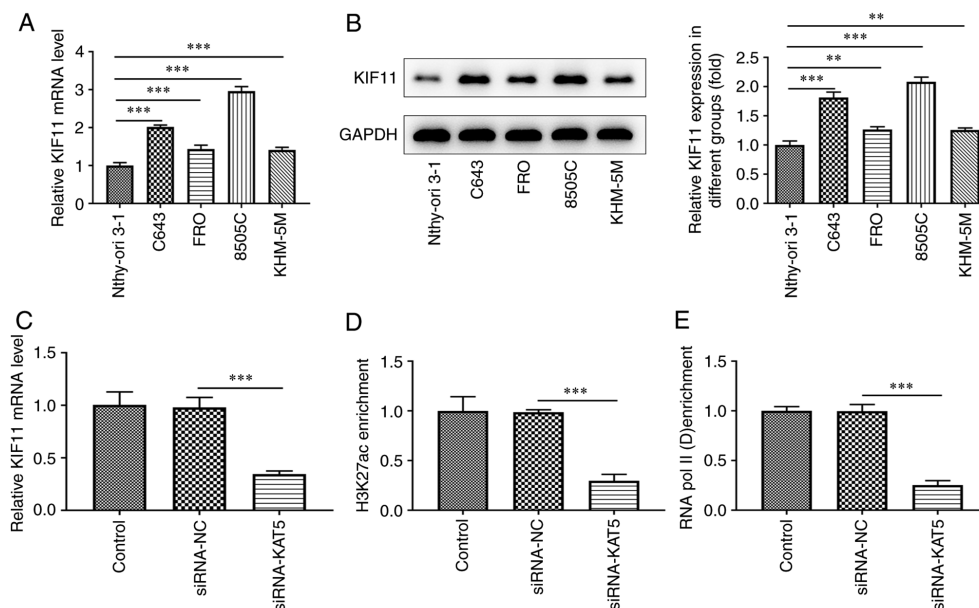


Figure 5. KAT5 depletion represses KIF11 expression in 8505C cells. The (A) mRNA and (B) protein levels of KIF11 in ATC cells and Nthy-ori 3-1 were detected by RT-qPCR and western blotting. (C) The mRNA of KIF11 in 8505C cells was detected by RT-qPCR. CHIP assay was performed to assess the enrichment of (D) H3K27ac and (E) RNA pol II on the promoter region of KIF11 after KAT5 knockdown. Data were expressed as mean  $\pm$  standard deviation.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ . KAT, K (lysine) acetyltransferase; KIF11, kinesin family member 11; RT-qPCR, reverse transcription-quantitative PCR; CHIP, chromatin immunoprecipitation.

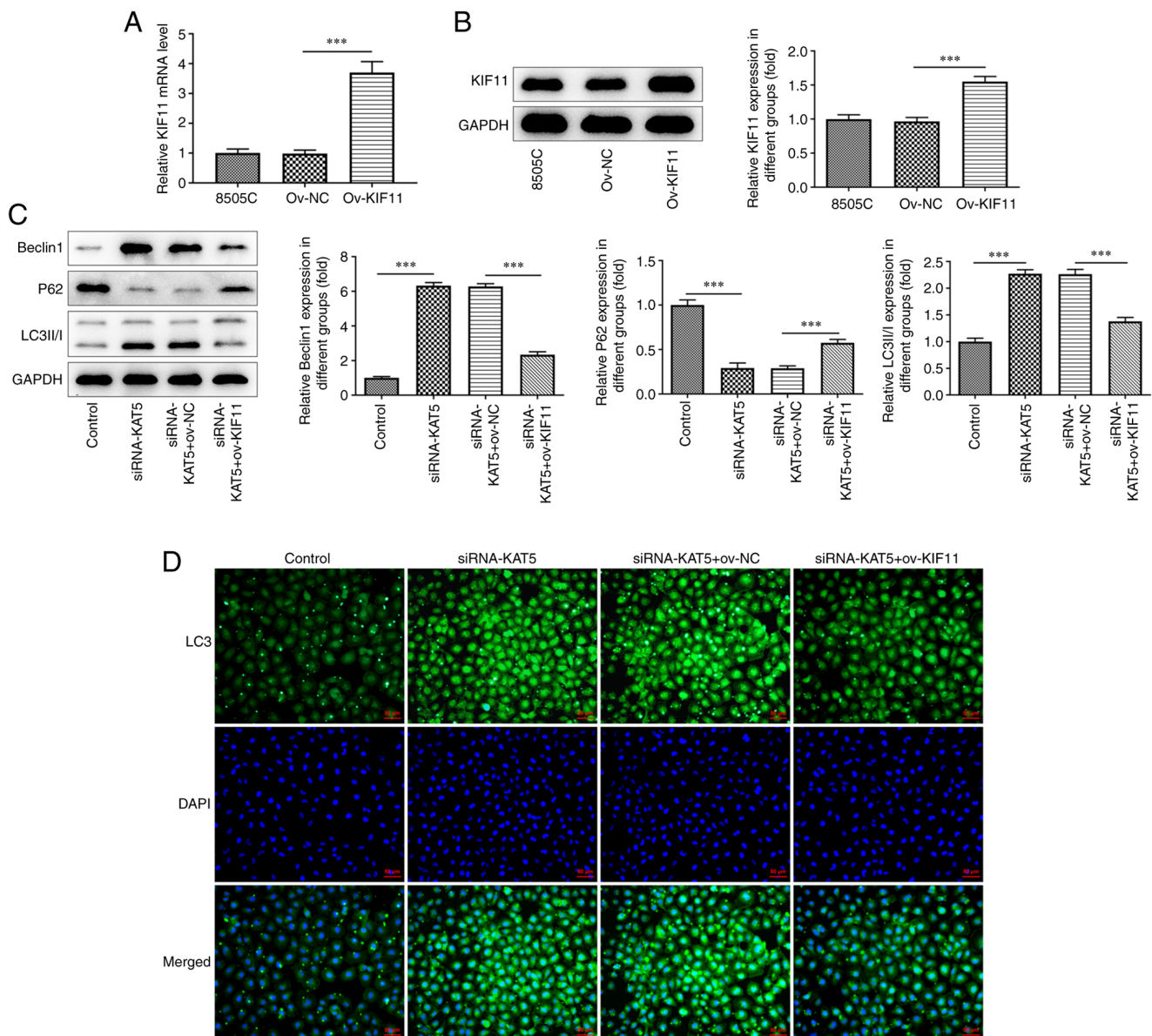


Figure 6. KAT5 knockdown regulates autophagy by inhibiting KIF11 expression. The (A) mRNA and (B) protein levels of KIF11 in 8505C cells were detected by reverse transcription-quantitative PCR and western blotting. (C) Western blot assay was used to evaluate the levels of p62, Beclin1 and LC3II/I in 8505C cells transfected with Ov-KIF11. (D) Immunofluorescence assay was used to assess the level of LC3 in 8505C cells transfected with Ov-KIF11 (magnification, x200). Data were expressed as mean  $\pm$  standard deviation. \*\*\*P<0.001. KAT, K (lysine) acetyltransferase; KIF11, kinesin family member 11; Ov, overexpression; si, small interfering; NC, negative control.

corresponding expression in Nthy-ori 3-1 cells. KAT5 interference repressed the proliferation of 8505C cells and induced their apoptosis. Furthermore, the tumor-suppressive role of KAT5 silencing was associated with the induction of autophagy.

As an acetyltransferase, KAT5 is involved in the process of acetylation (33). It has been suggested that histone acetylation can activate gene transcription (34). For example, KAT5 binds to programmed death-ligand 1 (PD-L1) and glutathione peroxidase (GPX) 4 promoters through the H3K27ac acetylation site to promote the transcription of PD-L1 and GPX4, indicating that it may recruit transcription factors through the H3K27ac acetylation site (35,36). In addition, Li *et al* (37) revealed that KIF11 was increased in ATC tissues. KIF11 has been shown to possess the binding site of H3K27ac

acetylation (38). A previous study has also shown that KIF11 can activate the expression levels of AKT signaling members; the activation of these proteins can inhibit the occurrence of autophagy (39). A previous study suggested that the activation of autophagy can induce apoptosis of TC cells (40). In the present study, it was found that silencing of KAT5 expression inhibited the expression levels of KIF11 by repressing the enrichment of H3K27ac and RNA pol II. KIF11 overexpression counteracted the effects of KAT5 silencing on the proliferation, apoptosis and the induction of autophagy of 8505C cells.

The present study concluded that silencing of KAT5 expression suppressed the proliferation and induced the apoptosis and autophagy of ATC cells by targeting KIF11. The latter may serve as a potential therapeutic target for ATC.

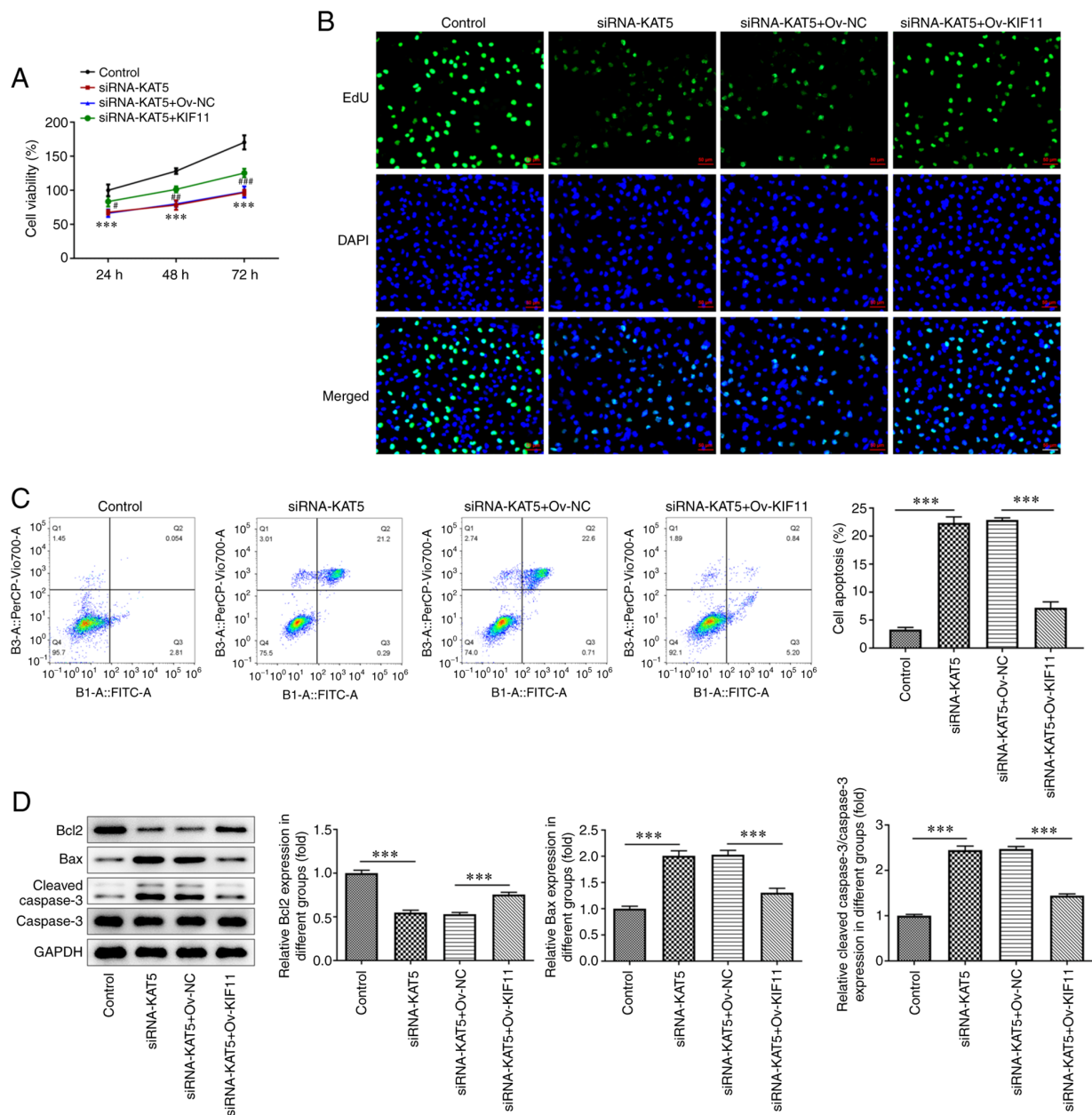


Figure 7. KAT5 knockdown regulates proliferation and apoptosis by inhibiting KIF11 expression. Cell proliferation was evaluated by (A) CCK-8 assay and (B) EdU staining (magnification, x200). (C) Cell apoptosis was detected by flow cytometry analysis. (D) Western blotting assay was used to assess apoptotic-related protein levels. Data were expressed as mean  $\pm$  standard deviation. \*\*\* $P$ <0.001; \*\* $P$ <0.05, # $P$ <0.01 and ### $P$ <0.001. KAT, K (lysine) acetyltransferase; KIF11, kinesin family member 11; Ov, overexpression; si, small interfering; NC, negative control.

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## Availability of data and materials

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

## Authors' contributions

LF and BZ designed the study, drafted and revised the manuscript. LinZ, CL, PW, QW, JX and LixZ analyzed the data and searched the literature. YG, SL, LS and XL confirm



the authenticity of all the raw data. All authors performed the experiments. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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