

RETRACTED ARTICLE: SIRT6 overexpression induces apoptosis of nasopharyngeal carcinoma by inhibiting NF- κ B signaling

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Background: Previous reports show that SIRT6 serves as a critical modulator of the development of multiple malignancies as well as other disorders. However, its role in nasopharyngeal carcinoma (NPC) is unknown. Thus, we elucidated the effects of SIRT6 on the survival of NPC cells, and modulation of cell death.

Methods: We found that expression of SIRT6 is downregulated in ten human NPC specimens as well as in the human NPC cell lines, 5-8 F and CNE1, as compared with that in healthy tissues and normal nasopharyngeal NP69 cells. The MTT assay and colony formation assay revealed that upregulation of SIRT6 impaired the proliferation, as well as the survival of 5-8 F and CNE1 cells. The TUNEL assay, annexin V-FITC/propidium iodide, and flow cytometry were performed to detect apoptosis. The results revealed that the expression of SIRT6 resulted in increased apoptosis.

Results: Western blotting results showed that SIRT6 overexpression decreased anti-apoptotic Bcl-2 levels, whereas promoted an increase in pro-apoptotic Bax and cleaved caspase-3 levels. Moreover, NF- κ B levels were markedly reduced in cells expressing SIRT6, whereas they were increased in cells transfected with shRNA-SIRT6. Recovery of NF- κ B expression was found to counteract the suppressive influence of SIRT6 on NPC cell survival, whereas, NF- κ B knockdown increased apoptosis of NPC cells.

Conclusion: Thus, the findings of our study offer insight into the biological and molecular mechanisms underlying the development of NPC and may lead to the development of new and innovative strategies for the treatment of NPC.

Keywords: nasopharyngeal carcinoma, SIRT6, NF- κ B, apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) belongs to the family of squamous cell malignancies, with a high occurrence in Southeast Asia, especially in South China.¹ An increasing number of studies have revealed that NPC has a complicated pathogenesis owing to the interactions between various factors, such as gene susceptibility.² NPC is radiation-sensitive as compared with several other cancers, thus the major therapy used for treatment of this kind of cancer is radiotherapy. However, owing to the high rate of recurrence, and resistance to chemotherapy, patients with advanced stages usually have a poor prognosis.^{3,4} Therefore, the development of novel therapeutic targets, to improve the survival and quality of life of these patients is crucial.

Sirtuins belong to the family of NAD⁺-dependent deacetylases and have been considered to mediate multiple biological processes and the development of various diseases, including tumors and cancers.⁵ SIRT6 was first cloned from a human spleen

cDNA library.⁶ SIRT6 deacetylates histone H3 at specific sites to inhibit transcription of its target genes.^{7,8} In addition, SIRT6 controls or mediates various biological functions like maintenance of chromosome integrity, and inflammatory responses.^{7,8}

Accumulating reports have considered SIRT6 to be a potential target for the treatment of certain cancers.^{9,10} SIRT6 interacts with CDC25A, and it can suppress the proliferation of colorectal cancer stem cells via this interaction.¹¹ SIRT6 can activate the JAK2/STAT3 signaling pathway through interaction with miR-34c-5p, further promoting proliferation of colon cancer cells.¹² Some reports suggested that SIRT6 was able to suppress tumorigenesis in the liver, at the cellular level and in vivo.^{13–15} SIRT6 is downregulated in lung cancer, owing to various factors, including differentiation of cells and the tumor and TNM staging stage of the disease.¹⁶ SIRT6 has been found to function as an oncogene in melanoma and neuroblastoma.^{17–20} These findings indicate that SIRT6 may act in a tissue-dependent manner. However, the expression of SIRT6 in patients with NPC and its correlation with the clinical features of NPC are not completely elucidated. Moreover, the mechanisms underlying the function of SIRT6 in NPC and its dysregulation have not been shown.

Abrogation of SIRT has been reported to induce NF- κ B activity, causing significantly increased production of pro-inflammatory cytokines.²¹ Recently, several studies have demonstrated that SIRT6 plays a regulatory role by suppressing the c-JUN, Akt, and ROS-dependent pathways both in vivo and in vitro.^{22,23} However, no report has shown the effect of SIRT-NF- κ B interplay during the progression of NPC. Thus, expression of SIRT6 was compared between NPC cells and normal cells, and was altered in order to reveal its role in NPC cell proliferation, cell death, and in the NF- κ B P65 signaling pathway.

Material and methods

Cell culture

Human NPC cell lines 5-8 F and CNE1 were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, People's Republic of China). Cells were cultured in RPMI-1640 cell culture medium containing 10% FBS. RPMI-1640 cell culture medium and FBS were provided by Hyclone (Thermo Fisher Scientific, Waltham, MA, USA).

Tissue specimens

A total of ten NPC tissues (age range, 35–68 years; mean age, 45 years) and 110 normal nasopharyngeal epithelium specimens (age range, 35–67 years; mean age, 45 years) were

collected from patients receiving treatment in this hospital. All tissues were frozen in liquid nitrogen and stored at -70°C . Patients were screened according to the enrollment criteria (no history of blood transfusion, radio- or chemotherapy prior to this study). The TNM staging system was used to classify all patients with NPC.

Ethics statement

All patients agreed to participate in the study and gave written informed consent. Both this study and their consent were approved by the ethical board of the Second Xiangya Hospital and complied with the Declaration of Helsinki.

Western blotting (WB)

A protease inhibitor cocktail was added (Hoffman-La Roche Ltd., Basel, Switzerland) to the RIPA buffer (pH 8.0) and this was used for preparation of the whole cell lysate. The bicinchoninic acid protein quantitation kit was used for protein quantification, followed by the separation of proteins on a 10% polyacrylamide gel using SDS-PAGE (SDS-PAGE apparatus; Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins were transferred onto polyvinylidene fluoride membranes (PVDF; Millipore, Billerica, MA, USA). Immunoblots were blocked with 5% BSA at 25°C for 1 hour, followed by their incubation with primary antibodies (4°C overnight). Following incubation, the blots were treated with corresponding secondary antibodies at 25°C for 1 hour. Immunoreactivity was measured using the West Femto Maximum Sensitivity Substrate Kit (provided by Thermo Fisher Scientific). Images were taken using the C-DiGit Blot Scanner.

Immunofluorescence assays (IFA)

Cells were grown in 24-well plates with cover slides. Paraformaldehyde (4%) prepared in PBS was used to fix the cells for 1 hour at room temperature. Cells were permeabilized for 10 minutes using PBS with Tween 20 (PBST) at 25°C , followed by a 1-hour incubation with PBST (containing 0.4% BSA) at 37°C and a subsequent 1-hour incubation with the polyclonal anti-SIRT6 antibody, which was diluted 1:200 in PBST (containing 0.2% BSA) at 37°C . Cells were washed for 1 hour with PBST, then the cells were incubated for 1 hour with the tetramethylrhodamine-labeled goat anti-rabbit antibody diluted in 0.2% BSA and PBST at 37°C . The cells were then washed for 1 hour with PBST. Nuclei were stained with DAPI. A confocal laser scanning fluorescence microscope (Olympus LSCMFV500) was used to analyze SIRT6 staining in cells.

RNA isolation and qRT-PCR

After sample preparation, Trizol (Invitrogen, Thermo Fisher Scientific) was used for the extraction of total RNA. MiR-26a expression was quantified using the Roche Light-Cycler 480 Real-Time PCR system (provided by Hoffman-La Roche Ltd) and SYBR Green. Glyceraldehyde-3-phosphate was used as an internal control. qPCR was implemented as follows: initial denaturation for 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and extension for 30 seconds at 72°C using the Light-Cycler 480. Based on the $2^{-\Delta\Delta CT}$ method, the target values were calculated via normalization to the internal reference (mean of the control samples).

Transfection of cells

We used adenoviral vectors (generated by RAPAd® CMV Adenoviral Bicistronic Expression System; Cell Biolabs, San Diego, CA, USA) containing inserts of human *SIRT6* and *NF-κB* cDNA, for transfection of 293 cells using the Lipo 2000 reagent (Invitrogen, Thermo Fisher Scientific). Seven days later, we collected the supernatant medium to harvest the adenovirus, where cells harboring the adenovirus were frozen and thawed twice for the collection of virions, followed by preservation at -80°C. An amount of 20 µL of the suspension containing the adenovirus was added to 2 mL medium, and after 6 hours, cells were incubated further for 8 hours, with adenovirus expressing GFP (AD-negative control), as control or AD-SIRT6/NF-κB.

RNA interference

shRNA against *SIRT6* and *NF-κB* was used to silence the *SIRT6* and *NF-κB* gene sequences. shRNA targeting *SIRT6* and *NF-κB* P65 was designed as follows: *SIRT6*, 5'-CCG GTG GAA GAA TTT GCC AAG TGT ACT CGA GTA CAC TTG GCA TTT TCT TTT ATT TTT G-3'; P65, 5'-TGT GTC CAA TGT TTT TCT CCT CGA GGA GTG AGA CCA TGG TCA CA-3' and manufactured by Genomics Co., Beijing, People's Republic of China. The shRNA was added in RNase free water (5 µL) at a concentration of 100 pmol/µL, which was then mixed with the transfection reagent (10 µL), and followed by transfection of cells.

MTT assay

The MTT assay was conducted to evaluate cell survival. Cells were treated with MTT (20 µL, 0.5 mg/mL), with the supernatant being replaced by DMSO (150 µL) in each well, followed by rotation for 10 minutes to allow the incorporation of the formazan dye. The Infinite M200 microplate reader

(Tecan, Männedorf, Switzerland) was then used to measure the absorbance at 490 nm.

TUNEL

To assess the apoptosis-related changes in cells, we used the TUNEL assay with a TUNEL fluorescence kit (Hoffman-La Roche Ltd.) to stain cells, and DAPI (1:5,000; Beyotime, Beijing, People's Republic of China) to stain the nuclei. Using the Laser Scanning Confocal Microscope (SP8; Leica Microsystems, Wetzlar, Germany), we evaluated apoptosis of cells by counting the number of TUNEL-positive cells.

Colony generation assay

Cells were transfected using various treatments. Cells were resuspended 2 days following transfection in DMEM supplemented with 10% FBS and 5 mm 0.4% top agar prior to being transferred to 24-well plates containing 0.5 mL 0.5% bottom agar. After 7 days, three replicates were chosen randomly from each plate and colonies were quantified.

Evaluation of cell apoptosis

Cell apoptosis was evaluated using the annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA). After transfection, cells were resuspended in 100 µL of binding buffer and then incubated for 20 minutes with 5 µL of PI and 10 µL of annexin V-FITC in the dark.

Statistical analysis

The data obtained in this study are expressed as mean ± SD. The differences between two test groups were studied using ANOVA or two-tailed Student's *t*-test. A value of *P* < 0.05 indicates significant difference.

Results

SIRT6 levels are downregulated in NPC cells and tissues

Expression profiles of SIRT6 have been associated with the prognosis and diagnosis of different cancers. We tested the mRNA and protein expression of SIRT6 in the human NPC cell lines, 5-8 F and CNE1, and in the nasopharyngeal epithelial cell line NP69, using WB and qRT-PCR, respectively. Protein expression of SIRT6 was lower in NPC cells as compared to that in the normal nasopharyngeal epithelial cells (Figure 1A and B). IFA and qRT-PCR were also performed to confirm the protein and mRNA expression of SIRT6 in NPC cell lines. Diffuse SIRT6 staining was observed in 5-8 F and CNE1 cells, as compared with that observed in NP69 cells (Figure 1C and D). qRT-PCR data

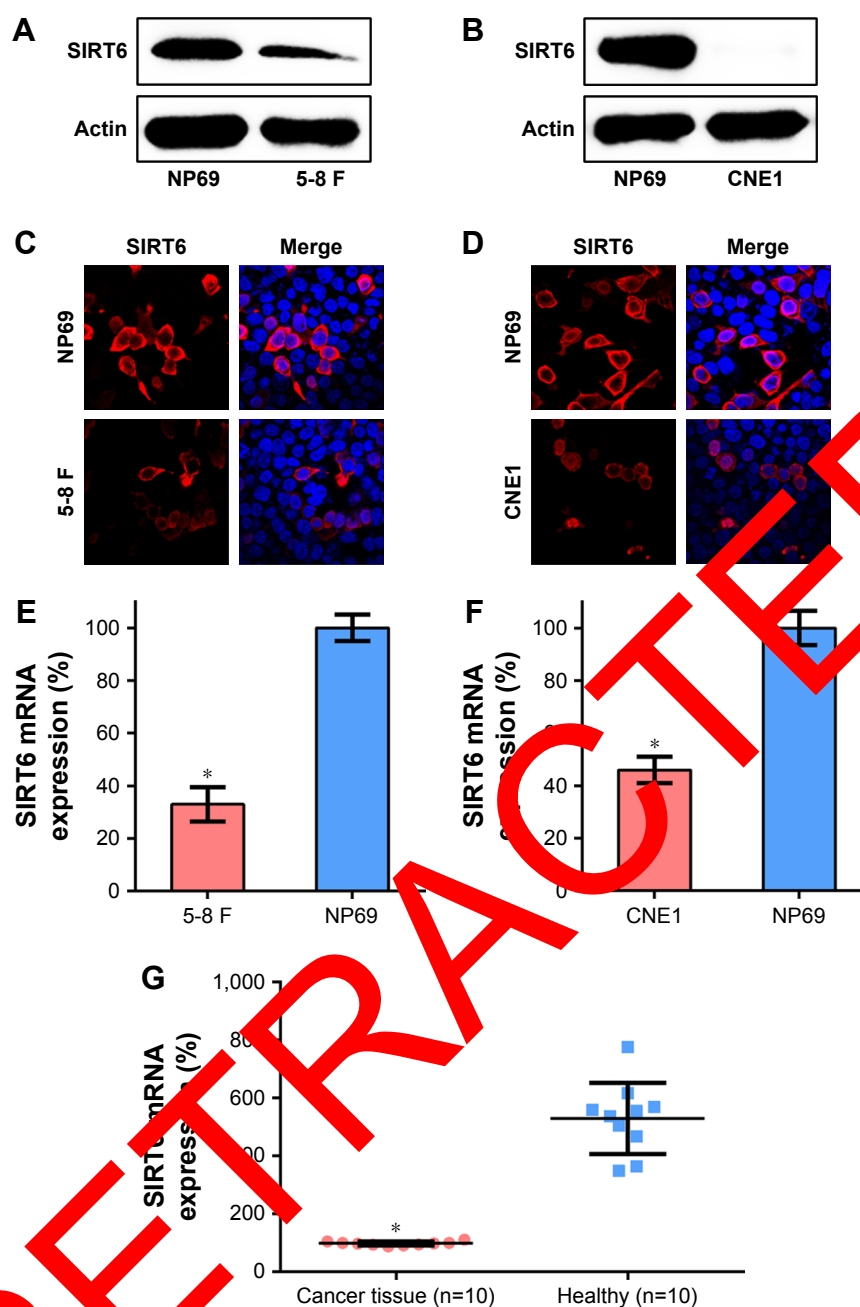


Figure 1 Expression of SIRT6 in NPC cells and tissue specimens.

Notes: (A, B) Expression of SIRT6 in NPC, 5-8 F, and CNE1 cells, as well as nasopharynx cells NP69, was detected using Western blotting. (C, D) Subcellular localization of SIRT6 in NPC cell lines 5-8 F and CNE1 was detected using immunofluorescence; [anti-SIRT6 antibody (red) and nuclear DNA (blue)]. (E, F) Expression of SIRT6 in 5-8 F and CNE1 cells determined using qRT-PCR. (G) Expression of SIRT6 in specimens obtained from NPC patients (n=10) vs normal healthy patients (n=10). Data are represented as mean \pm SD. * $P < 0.05$.

Abbreviation: NPC, nasopharyngeal carcinoma.

also showed that *SIRT6* mRNA levels were significantly reduced in 5-8 F and CNE1 cells, as compared to those in NP69 cells (Figure 1E and F). We then determined *SIRT6* expression levels in NPC tissue specimens and the normal nasopharyngeal epithelial specimens. *SIRT6* mRNA levels in NPC specimens were significantly downregulated, as compared with those in the normal nasopharyngeal epithelial specimens (Figure 1G).

Overexpression of SIRT6 reduced NPC cell viability

Next, we examined whether SIRT6 affects the viability of NPC cells, 5-8 F and CNE1. First, the overexpression of SIRT6 in 5-8 F and CNE1 cells was confirmed using WB and qRT-PCR (Figure 2A–D). Further, data from the MTT assay (Figure 2E and F) revealed that proliferation of 5-8 F and CNE1 cells transfected with SIRT6-expressing

adenovirus was significantly reduced 12–72 hours post-culture. SIRT6 overexpression also led to a notable decrease in colony numbers, evaluated using the colony generation assay, whereas continuous AD-NC transfection did not affect colony numbers of cells (Figure 2G–J).

SIRT6 triggers the death of NPC cells

The TUNEL assay and annexin V-FITC/PI FC staining were performed using the 5-8 F cells following transfection, to further evaluate the participation of SIRT6 in 5-8 F cell death. The number of TUNEL-positive AD-SIRT6 transfected 5-8 F cells was greater 36 hours post-transfection, as compared with that of the mock and AD-NC cells (Figure 3A). Furthermore, annexin V-FITC/PI FC staining demonstrated that high SIRT6 expression markedly increased the percentage of apoptosed 5-8 F cells (Figure 3B). It has been extensively reported that Bcl-2 promotes cell survival, while Bax promotes cell death. SIRT6 overexpression led to impaired BCL-2 expression and enhanced BAX expression at the protein level (Figure 3C), as compared with that in the mock and AD-NC cells. Additionally, cleaved caspase-3 levels, which are a typical biomarker of cell death, were significantly increased, and were quantified using WB following transfection (Figure 3D). Moreover, qRT-PCR results showed that expression of SIRT6 reduced BCL-2 transcription and elevated BAX transcription (Figure 3E and F). These data indicated that SIRT6 restoration-induced cellular apoptosis of 5-8 F cells.

SIRT6 reduced apoptosis-associated NF- κ B level in NPC cells

As a pivotal immune sensor, NF- κ B P65 plays a critical role in the regulation of cellular apoptosis. Therefore, we used WB analysis to assess whether the expression of NF- κ B corresponded with that of SIRT6 in 5-8 F and CNE1 cells. At first, we tested the protein expression of P65 in the 5-8 F, CNE1, and NP69 cells. Protein expression of NF- κ B P65 was higher in NPC cells as compared to that in the NP69 cells (Figure 4A and B). Cells were transfected with the SIRT6-expressing adenovirus, and then we examined NF- κ B expression. As shown in Figure 4C and D, NF- κ B expression was increased in NPC cell lines, however, overexpression of SIRT6 impaired its expression. To further confirm the inhibitory effect of SIRT6 on NF- κ B expression, SIRT6 was knocked down by transfection with shRNA-SIRT6 and shRNA-NC. Consequently, NF- κ B levels were markedly elevated due to reduced SIRT6 expression in 5-8 F and CNE1 cells (Figure 4E and F). IFA was also performed to detect whether the cellular location of P65 was altered by SIRT6

expression. Nuclear P65 location was observed in untreated 5-8 F and CNE1 cells, as compared with cytoplasmic location of NF- κ B P65 observed in the cells transfected with shRNA-SIRT6 (Figure 4G and H).

Ectopic expression of NF- κ B mediates apoptosis of NPC cells

To determine whether the restoration of NF- κ B can suppress the influence of SIRT6 on apoptosis of NPC cells, NF- κ B was overexpressed in 5-8 F cells expressing SIRT6, presumably expressing lower levels of NF- κ B. WB was performed in order to verify the change of NF- κ B levels (Figure 5A). We showed that enhanced expression of NF- κ B restored Bcl-2 and Bax mRNA and protein levels mediated by SIRT6 overexpression (Figure 5A–C). IFC was performed to evaluate cell death of 5-8 F cells. Ectopic overexpression of 5-8 F resulted in a notable elevation in apoptotic cells that were transfected with the SIRT6-expressing adenovirus (Figure 5D).

To further confirm the role of NF- κ B in SIRT6-regulated apoptosis of NPC cells, 5-8 F cells were transfected with shRNA-NF- κ B to impair the expression of NF- κ B in cells treated with shRNA-SIRT6, in which NF- κ B expression was presumably upregulated. First, we verified that NF- κ B expression levels were reduced (Figure 6A). We found that Bcl-2 and Bax levels were not altered by SIRT6 downregulation. However, NF- κ B knockdown remarkably decreased Bcl-2 mRNA and protein levels and increased Bax mRNA and protein levels (Figure 6A–C). FC was also used to evaluate the correlation between apoptosis of 5-8 F cells and reduced NF- κ B levels. The number of dead cells was notably increased in shRNA-SIRT6 and shRNA-NF- κ B transfected 5-8 F cells (Figure 6D). Thus, these data show that NF- κ B expression was related to cell apoptosis induced by SIRT6 expression.

Discussion

This study revealed that the expression of SIRT6 was reduced in NPC cell lines (5-8 F and CNE1), and overexpression of SIRT6 in the 5-8 F and CNE1 cells restrained cell viability and proliferation and promoted apoptosis. Further investigation revealed that SIRT6 overexpression abrogates NF- κ B levels; in contrast, silencing of SIRT6 expression resulted in the stimulation of NF- κ B expression. Moreover, NF- κ B overexpression restored cell viability affected by SIRT6, whereas knockdown of NF- κ B-induced apoptosis of 5-8 F cells as revealed by flow cytometry and Bcl-2/Bax modulation. Our findings suggest that SIRT6 acts as suppressor of

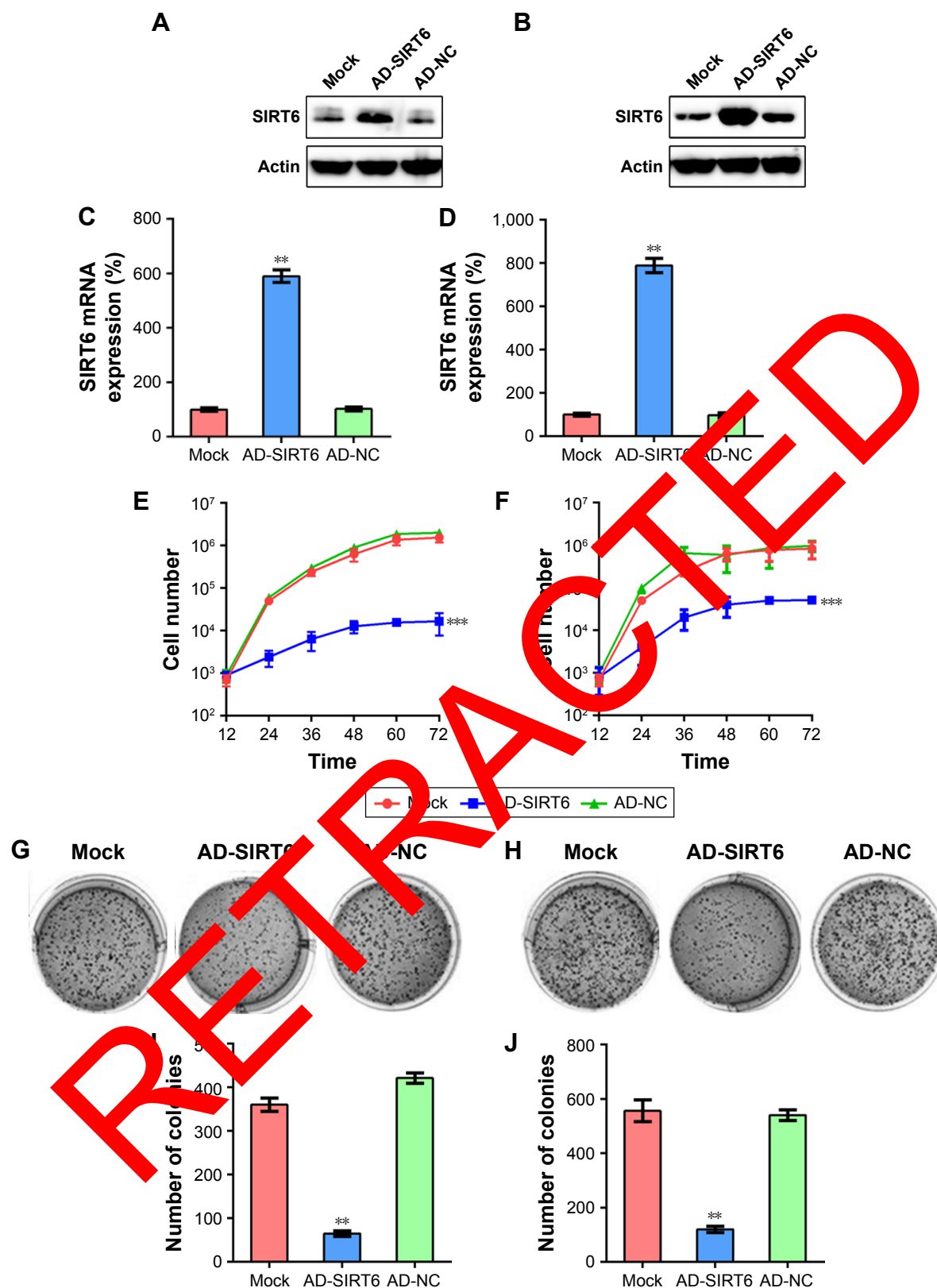


Figure 2 SIRT6 overexpression affected viability of NPC cells.

Notes: (A, B) Western blotting and (C, D) qRT-PCR were performed to evaluate SIRT6 expression in 5-8 F and CNE1 cells following infection with adenovirus-SIRT6 (AD-SIRT6) or AD-NC. (E, F) MTT assay was used to examine the proliferation of 5-8 F and CNE1 cells 12–72 hours post-culture. (G, H) Soft agar colony formation assay for the 5-8 F and CNE1 cells infected with AD-SIRT6, AD-NC, and that for uninfected cells. (I, J) The number of colonies formed in each group. Data are shown as mean \pm SD, ** $P < 0.01$, *** $P < 0.001$.

Abbreviations: AD, adenovirus; NC, negative control; NPC, nasopharyngeal carcinoma.

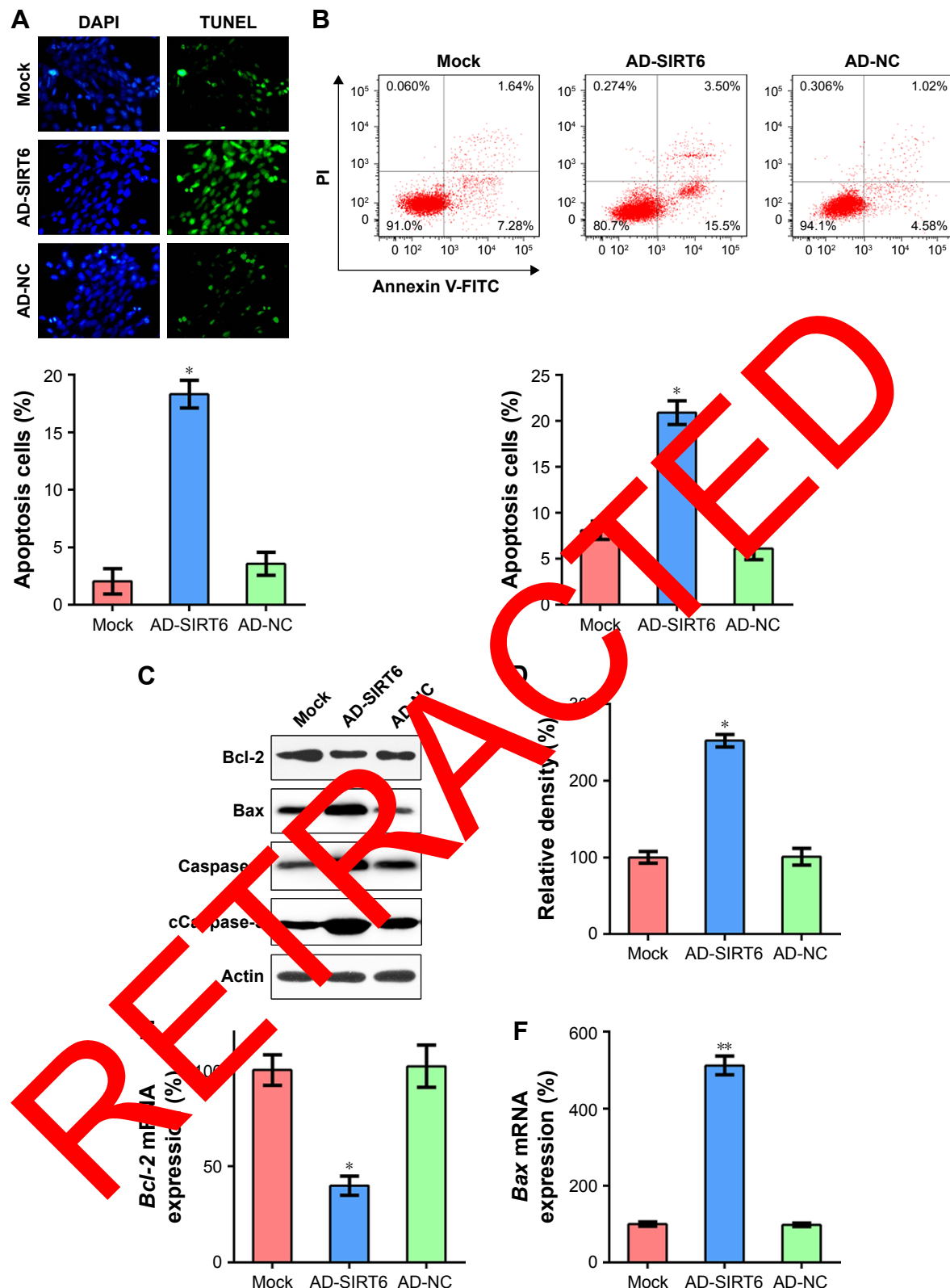


Figure 3 Overexpression of SIRT6 enhanced apoptosis of NPC cells.

Notes: (A) TUNEL assay was performed in each group of 5-8 F cells expressing SIRT6 or NCs. Magnification, 400 \times . The number of TUNEL-positive 5-8 F cells is displayed in the lower panel. (B) Annexin V-FITC/PI staining and flow cytometry were performed to evaluate the number of apoptotic cells. Early apoptotic cells are shown in the upper right quadrant of each plot, and later apoptotic cells are displayed in the lower right quadrant of each plot. Analysis of the apoptotic rate of 5-8 F cells in all groups is shown in the lower panel. (C) Expression levels of SIRT6-regulated Bcl-2 and Bax, total caspase-3, and cleavage of caspase-3 in the Hep-2 cells. (D) Relative density of cleaved caspase-3 bands. (E, F) qRT-PCR was performed to assess the influence of SIRT6 on mRNA levels of Bcl-2 and Bax. Data are shown as mean \pm SD. * P <0.05, ** P <0.01.

Abbreviations: AD, adenovirus; NC, negative control; NPC, nasopharyngeal carcinoma; PI, propidium iodide.

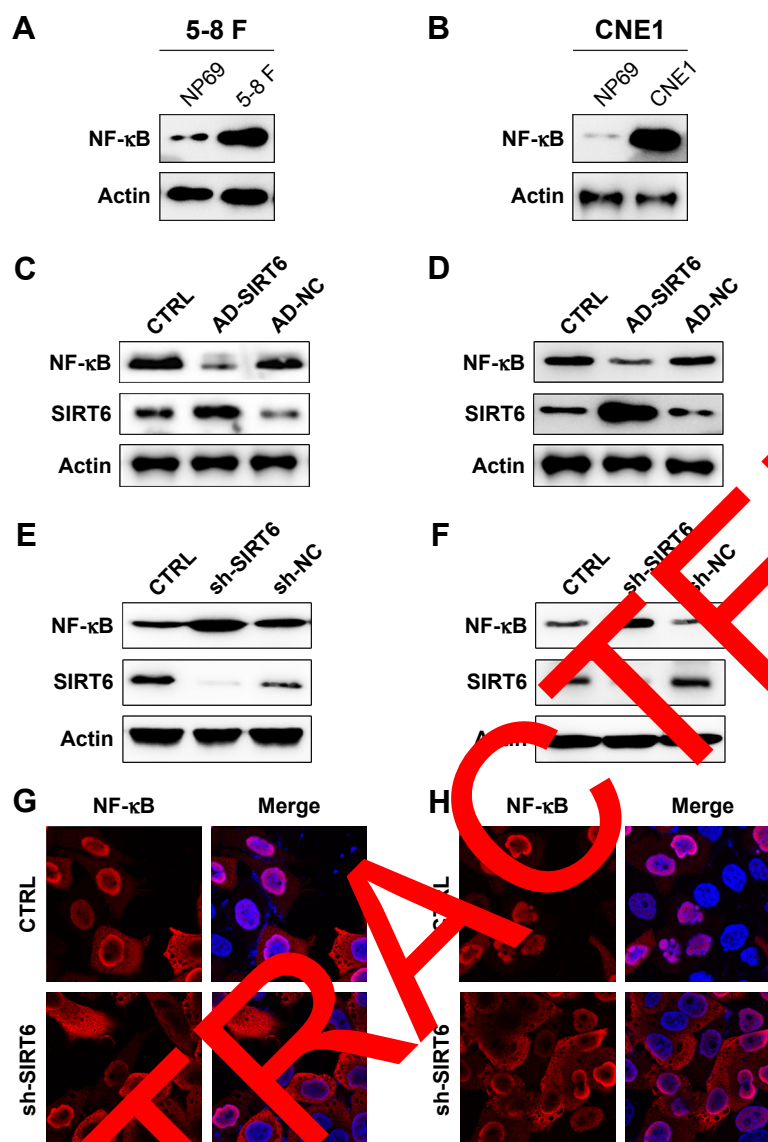


Figure 4 SIRT6 regulated NF- κ B expression in NPC cells.

Notes: (A, B) Expression of NF- κ B in NPC, 5-8 F, and CNE1 cells, as well as nasopharynx cells NP69, was detected using Western blotting. (C, D) 5-8 F and CNE1 cells were infected with AD-SIRT6 or AD-NC. Protein expression of SIRT6 and NF- κ B was detected using Western blotting. (E, F) 5-8 F and CNE1 cells were transfected with shRNA-SIRT6 and shRNA-NC to knock down *SIRT6* expression. Protein expression of SIRT6 and NF- κ B was then analyzed using Western blotting. (G, H) Subcellular localization of NF- κ B in NPC cells transfected with shRNA-SIRT6 or not, was detected using immunofluorescence; [anti-NF- κ B antibody (red) and nuclear DNA (blue)]. Data are shown as mean \pm SD.

Abbreviations: AD, adenovirus; CTRL, control; NC, negative control; NPC, nasopharyngeal carcinoma.

NPC tumors by regulating NF- κ B protein expression, and thus is a potential candidate for the treatment of NPC.

Previously, apoptosis was believed to be a crucial factor inducing cell death via SIRT1. Several previous reports show that SIRT1 is required for the rapid and efficient activation of apoptosis in multiple human cells (human tenocytes, chondrocytes, vascular endothelial cells, and osteoblast cells).^{24–27} This process is dependent on the modulation of mitochondria-related apoptotic signals by SIRT. However, the role of SIRT6 in apoptosis is still not well-documented. In this study, SIRT6-induced apoptosis in NPC cell lines (5-8 F and CNE1) as indicated by decreased Bcl-2 expression, as

well as by increased annexin V/PI staining, and TUNEL-positive cells. Cleaved caspase-3 corresponded with the activation of apoptosis upon SIRT6 overexpression in 5-8 F cells. In accordance with our study, SIRT6 overexpression-induced apoptosis, activated caspase-9 and caspase-3 in human gastric cancer cells, resulting in cell cycle arrest, induced cellular apoptosis through inactivation of JAK2/STAT3 signaling, and by downregulation of cyclin D1 and Bcl2.²⁸ Apoptosis was also observed in A549 non-small-cell lung cancer cells,²⁹ mouse embryonic fibroblasts,³⁰ and hepatocellular carcinoma,¹⁰ with downregulation of cell survival proteins (JAK2/STAT3 and Bcl-2), and upregulation of

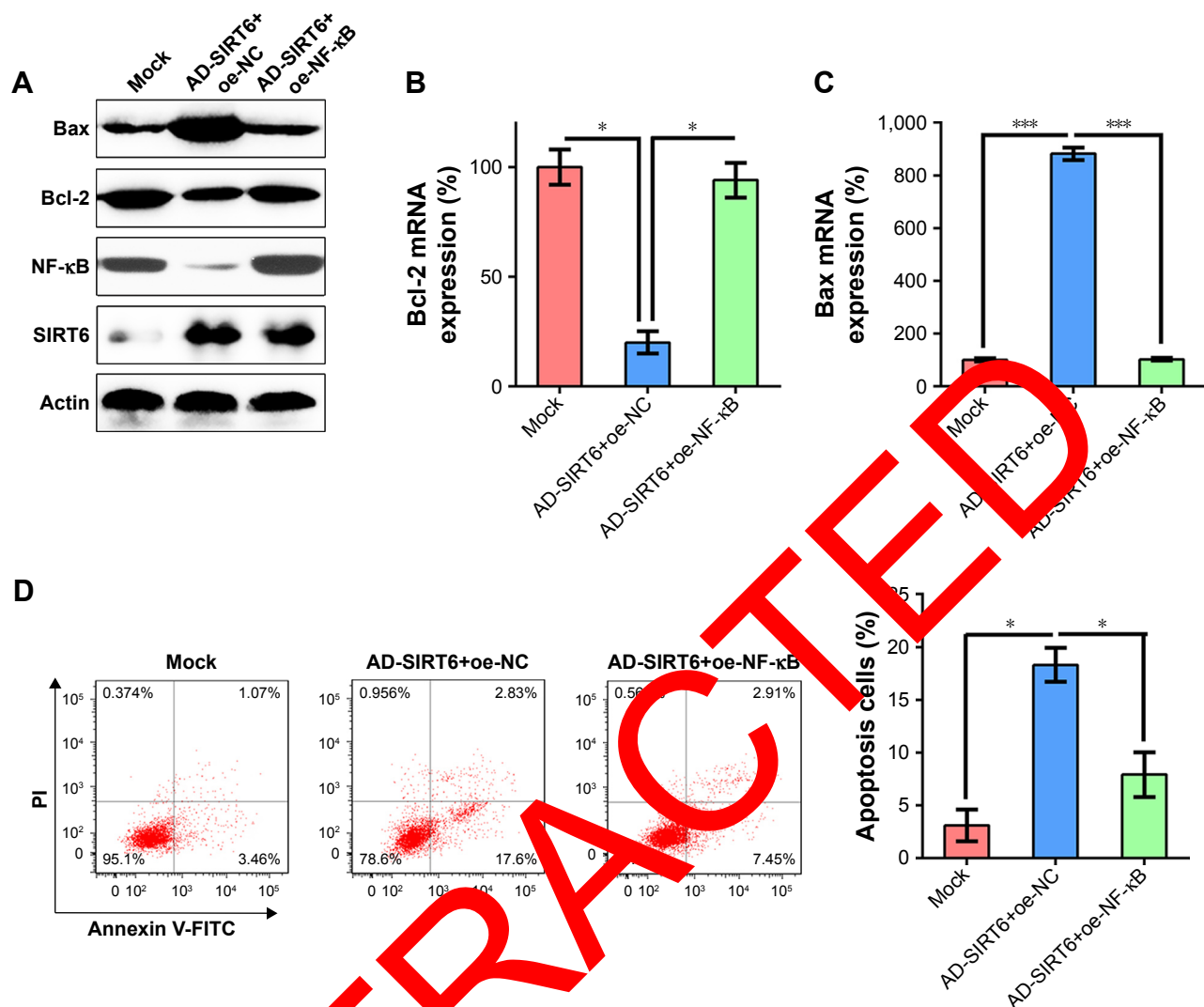


Figure 5 NF-κB overexpression restores cell viability impaired by SIRT6.

Notes: 5-8 F cells were first infected with AD-SIRT6, and then transfected with the NF-κB-expressing vector to elevate NF-κB levels. **(A)** Protein expression of SIRT6, NF-κB, Bcl-2 and Bax in each group was detected using Western blotting. **(B, C)** qRT-PCR was performed to assess the influence of NF-κB on mRNA expression of Bcl-2 and Bax. **(D)** Annexin V-FITC/PI staining and flow cytometry were performed to evaluate the number of apoptotic cells. Early apoptotic cells are shown in the upper right quadrant of each plot, and late apoptotic cells are displayed in the lower right quadrant of plot. Analysis of the apoptotic rate of 5-8 F cells in all groups is shown in the lower panel. Data are shown as mean \pm SD. * $P < 0.05$, *** $P < 0.001$.

Abbreviations: AD, adenovirus; NC, negative control; PI, propidium iodide.

pro-apoptotic proteins (p53 and E2F-1, Bid and bax). However, several reports implicate a suppressive role of SIRT6 in cellular apoptosis, which is inconsistent with the results of our study. Chen et al indicated a protective effect for Sirt6 overexpression against apoptosis and premature senescence in intervertebral disc degeneration.³¹ Zhang et al reported that SIRT6 provides protection against hepatic ischemia/reperfusion injury by inhibiting apoptosis and autophagy-related cell death.³² Based on the finding of Loris et al, SIRT6 suppressed cancer stem-like capacity in tumors with PI3K activation independently of its deacetylase activity. The effect of SIRT6 on apoptosis may also be independent of its deacetylase activity.³³ These paradoxical conclusions drawn

from the interplay between SIRT6 and apoptosis might suggest that SIRT6 acts in a tissue-dependent manner.

Increasing evidence has shown that activation of apoptosis-associated signaling pathways during tumorigenesis is one of the major factors responsible for the pathogenesis of various cancers.^{34,35} Apoptosis, known as programmatic cell death, maintains the balance between survival and death of cells. Dysfunction of apoptosis brings about severe outcomes like cancer, however, abnormally enhanced apoptosis contributes to the development of conditions associated with degeneration. NF-κB forms different complexes and can bind to consensus DNA sequences, to stimulate responsive genes for the regulation of biological processes.³⁶ The NF-κB signaling pathway

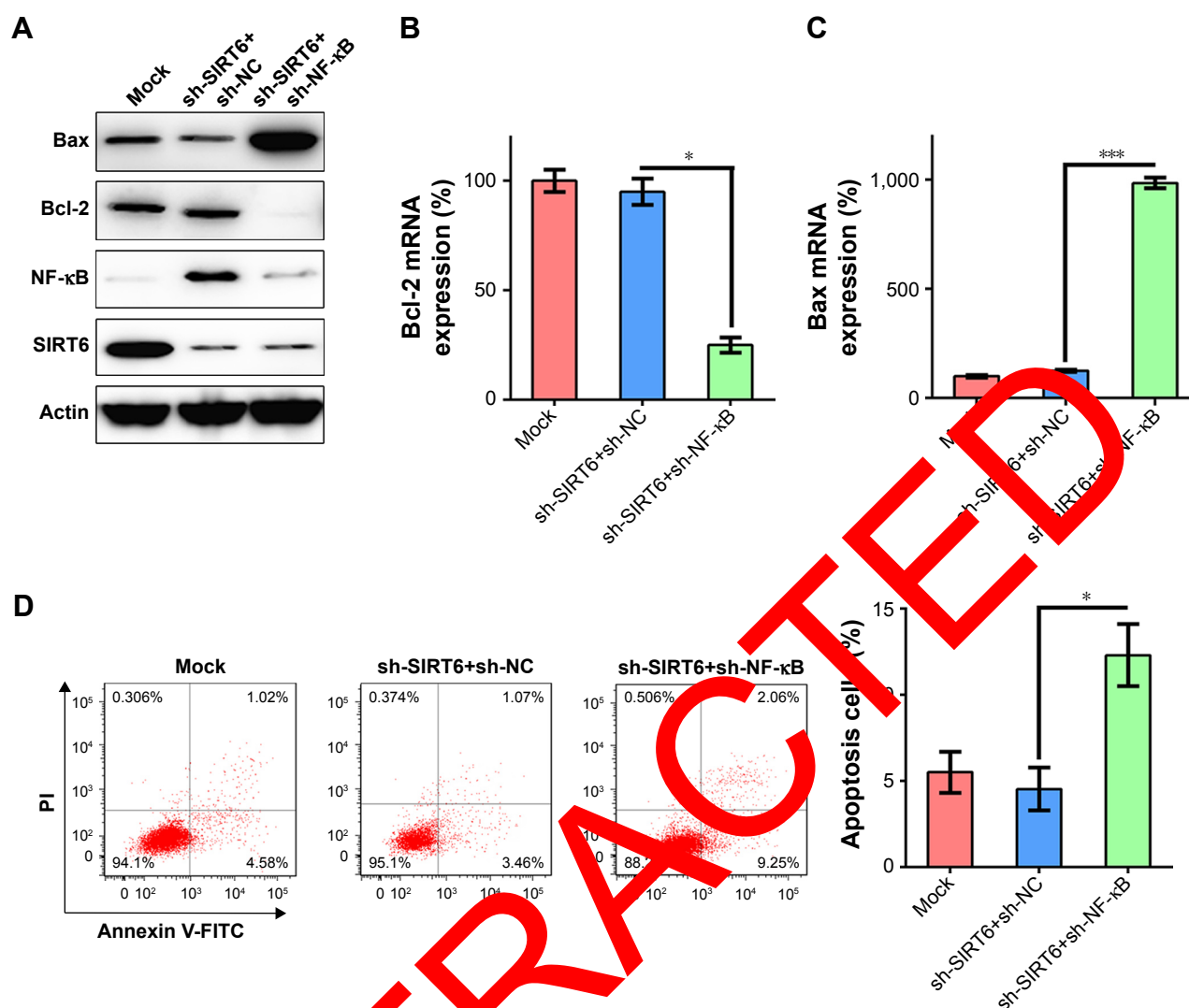


Figure 6 NF-κB knockdown had a similar effect on cell viability as that of SIRT6 overexpression.

Notes: 5-8 F cells were transfected with sh-SIRT6 or co-transfected with shRNA-SIRT6 and shRNA-NF-κB. (A) Protein expression of SIRT6, NF-κB, Bcl-2, and Bax in each group was assessed using Western blotting. (B, C) RT-PCR was performed to examine the mRNA expression of Bcl-2 and Bax. (D) Annexin V-FITC/PI staining and flow cytometry were performed to count the number of apoptotic cells. Early apoptotic cells are shown in the upper right quadrant of each plot, and later apoptotic cells are displayed in the lower right quadrant of plot. Analysis of the apoptotic rate of 5-8 F cells in all groups is shown in the lower panel. Data are shown as mean ± SD. * $P < 0.05$, *** $P < 0.001$.

Abbreviations: NC, negative control; PI, propidium iodide.

regulates many genes related to cell differentiation, proliferation, apoptosis, and innate immune response. NF-κB initiates the transcription of genes antagonizing apoptosis, such as FLIP (caspase-8 inhibitor), c-IAP1/2, and XIAP (inhibitor of apoptosis proteins).³⁷ A previous report has revealed the critical role of NF-κB in preventing apoptosis in some cancers (such as prostate cancer,³⁸ hematological malignancies,³⁹ and pancreatic cancer).⁴⁰ However, despite the availability of a large number of reports about NF-κB, none of them clearly show the mechanism of action of NF-κB in many cancers. In the present study, in NPC cells, the overexpression of SIRT6 suppressed NF-κB expression, which increases cellular apoptosis and abrogates viability of cells. Furthermore, NF-κB

overexpression in 5-8 F cells stably expressing SIRT6 inhibited apoptosis, whereas silencing of NF-κB stimulated the induction of apoptosis of 5-8 F cells. However, elucidation of the molecular mechanisms underlying regulation of NF-κB expression by SIRT6 during NPC development and progression requires further investigation. Previous studies suggest that NF-κB itself is subject to regulation by acetylation, and can be deacetylated by several deacetylases including SIRT1.^{41,42} Therefore, it is possible that SIRT6 might also directly modulate acetylation levels of NF-κB. Kawahara et al have reported that SIRT6 did not show significant deacetylation of RELA in vitro or in cells.⁴³ Therefore, NF-κB expression might be regulated by SIRT6 in ways other than modification by deacetylation.

Conclusion

Collectively, our data, for the first time, indicate the absence of SIRT6 in human NPC tissues or cells, and indicate that overexpression of SIRT6 inhibits cell proliferation and viability, and induces apoptosis in NPC cell lines (5-8 F and CNE1). Overexpression of SIRT6 may be a crucial link between inactivation or downregulation of NF- κ B, and SIRT6 is a potential target for the development of treatment strategies for NPC.

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Author contributions

In this work, LO, LY and XY conceived the study and designed the experiments. JL and SY contributed to the data collection, SL and PL performed the data analysis and interpreted the results. LO and LY wrote the manuscript; PL and XY contributed to the critical revision of article. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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