

Temporal Evolution of Alveolar Epithelial Cell Dysfunction Induced by Cigarette Exposure plus Lipopolysaccharide in a Mouse Model of Chronic Obstructive Pulmonary Disease and the Protective Role of SIRT1

cheng liang

Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University

weijia zhou

Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University

wu li

Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University

qiang zeng

Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University

lei xue

Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University

xiaotian dai

Respiratory and Critical Care Medicine Department of First Affiliated Hospital of Army Medical University

wei xiong

xiongwei@tmmu.edu.cn

Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University

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**Temporal Evolution of Alveolar Epithelial Cell Dysfunction
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Model of Chronic Obstructive Pulmonary Disease and the Protective
Role of SIRT1**

Cheng Liang¹; Weijia Zhou¹; Wu Li¹; Qiang Zeng¹; Lei Xue¹; Xiaotian Dai^{2*}; Wei Xiong^{1*}.

Affiliations:

¹Department of Geriatrics, Chongqing Key Laboratory of Aging and Regeneration Medicine, The First Affiliated Hospital (Southwest Hospital) of Army Medical University, Chongqing 400038, China.

²Respiratory and Critical Care Medicine Department of First Affiliated Hospital of Army Medical University, Chongqing 400038, China.

***Correspondence:**

Xiaotian Dai daixiaot@tmmu.edu.cn

Wei Xiong xiongwei@tmmu.edu.cn

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is characterized by pathological alterations including alveolar structure destruction and small airway remodeling. However, the dynamic evolution of alveolar structural damage from the initiation of risk factor exposure to irreversible alveolar structural destruction of COPD remains to be fully elucidated. Concurrently, silent information regulator 1 (SIRT1), as a key regulator of metabolism and cellular stress, warrants further investigation regarding its potential role during the period from exposure to risk factors in COPD to the onset of irreversible alveolar structural destruction.

Methods: 120 male C57BL/6N mice were randomly divided into wild-type (WT) and cigarette smoke plus Lipopolysaccharide (CS-model) groups. CS-model was induced by 12-week cigarette smoke (CS) exposure combined with intratracheal lipopolysaccharide (LPS, 2mg/kg) twice on Day1 and Day14. Alveolar epithelial cell-specific SIRT1 gain- and loss-of-function models were constructed using adeno-associated virus (AAV6.2FF) with SIRT1 overexpression or inhibition vectors. Tissue samples were collected biweekly for 12 weeks. In vitro, A549 cells were treated with cigarette smoke extract (CSE, 50µg/mL) and LPS (10µg/mL). SIRT1 activation (SRT1720) and inhibition (EX527) as well as Nuclear factor erythroid 2-related factor 2(Nrf2) modulation (NK252, ML385) were applied to investigate mechanisms. Lung function, histology (HE, MASSON), protein expression (Western blot), and immunofluorescence were assessed at multiple time points(2,4,6,8,10 and 12 weeks).

Results: CS-model mice exhibited significant weight loss, alveolar destruction, impaired lung function, and elevated inflammatory markers (NLRP3, TNF-α). Type II alveolar epithelial cells(AT2) proliferation and differentiation declined as early as 2 weeks, while Type I alveolar epithelial cells(AT1) loss was evident at 6 weeks. The AT2/AT1 ratio gradually decreased from 4 weeks, reaching the lowest value at 12 weeks. SIRT1 and Nrf2 expression levels peaked at 2 weeks but significantly declined from 4 weeks, reaching nadir at 12 weeks. AAV-mediated SIRT1 overexpression improved lung histology and function, restored AT2/AT1 ratio, and reduced inflammation, apoptosis, and barrier dysfunction compared to the CS-model group. In vitro, SIRT1 activation with SRT1720 and Nrf2 activation with NK252 both improved cell viability and reduced apoptosis. In terms of cell proliferation, the SIRT1 agonist + Nrf2 inhibitor combination was superior to the SIRT1 inhibitor + Nrf2 agonist combination; in terms of cell apoptosis, the SIRT1 inhibitor + Nrf2 agonist combination was superior to the SIRT1 agonist + Nrf2 inhibitor combination.

Conclusions: During CS-model progression from onset to severe alveolar destruction, alveolar epithelial cells exhibited a time-dependent pattern of compensation at 2 weeks, decompensation at 4 weeks, and progressive injury thereafter. Abnormal AT2 proliferation and differentiation at 2 weeks suggests AT2 dysfunction maybe is an early driving event in CS-model. SIRT1 and Nrf2 expression dynamics closely mirrored AT2 cell changes, implying their regulatory role. SIRT1 overexpression conferred protective effects dependent on CS-model pathological background, with stronger regulation of AT2 than AT1 cells. SIRT1 promoted AT2-to-AT1 differentiation, restored proliferative capacity, suppressed TNF- α activation, maintained barrier integrity, and possibly inhibited apoptosis via Nrf2 enhancement. SIRT1 inhibition produced opposite effects. Notably, the differential effects of the SIRT1/Nrf2 agonist-inhibitor combinations on proliferation versus apoptosis were observed only in A549 cells in vitro, and thus the proposition that SIRT1 primarily regulates proliferation while Nrf2 governs apoptosis-functioning as a complementary regulatory network-should be regarded as a preliminary hypothesis that requires further in vivo validation.

Key Words: COPD SIRT1 Alveolar Epithelial Cells Cigarette Exposure
Alveolar Homeostasis

Background

COPD is a common, preventable chronic condition whose symptoms can be managed but not cured^{1,2}. Its pathological basis primarily includes the destruction of alveolar structures (emphysema) and small airway alterations^{2,3,4}. Globally, COPD stands as a predominant cause of morbidity and mortality, representing a major global health challenge which imposes a substantial health and socioeconomic burden^{5,6}. Historically, research has largely centered on the chronic inflammation within the small airways of established COPD^{7,8,9}. Although current treatments can alleviate symptoms in patients with COPD, they offer no cure. Given the substantial population at risk and those in early disease stages, how to delay or even reverse the progression of COPD has become a critical, unresolved challenge. Our research focuses on the longitudinal observation of structural changes in the lungs throughout the entire disease continuum—from the initiation of risk factor exposure to irreversible alveolar structural destruction—rather than solely examining the stable advanced phase. This time-course investigation aims to uncover the dynamics of disease evolution and identify potential therapeutic targets.

In recent years, with the advancement of single-cell sequencing technology, researchers have detected epithelial cell differentiation dysregulation as a potential key factor in the pathological progression of COPD through the analysis of lung tissues from both healthy individuals and COPD patients^{10,11}. This discovery provides a novel research perspective, namely focusing on the impact of dynamic cellular-level changes on COPD pathogenesis. In lung tissue architecture, although basal cells possess certain stem cell potential even during severe lung injury, alveolar epithelial cells both constitute the alveolar structure and maintain alveolar function, thereby playing a core and indispensable role. Among these cell types, AT2 warrant particular attention. In addition to secreting alveolar surfactant, AT2 serve as the primary source for alveolar repair and regeneration following injury, and can differentiate into AT1 to maintain alveolar structural stability^{12,13}. Although previous studies have identified key pathological alterations in AT2, including oxidative stress imbalance, mitochondrial dysfunction, and epithelial-mesenchymal transition (EMT), these investigations have been predominantly limited to static analyses at single time points in either acute lung injury models or chronic models^{14,15,16}. Systematic exploration of the dynamic evolutionary patterns of cells and key transition nodes during the critical window period from risk factor exposure to irreversible lung structural damage

remains lacking.

CS exposure stands as the predominant cause of COPD. This exposure triggers the irreversible breakdown of alveolar walls and remodels the airways via a web of linked pathological events. Key mechanisms involve persistent oxidative stress, ongoing inflammation, an imbalance between proteases and their inhibitors, and programmed cell death (apoptosis)^{17,18,19}. Within this destructive cascade, alveolar epithelial cells are central players. Special attention falls on AT2, which retain the crucial abilities to proliferate and differentiate²⁰. Beyond their essential job of producing pulmonary surfactant, AT2 also serve as the primary cellular source for repair and regeneration following alveolar injury. They uphold alveolar integrity by multiplying and maturing into thin, gas-exchanging type I alveolar epithelial cells (AT1 cells)^{21,22}. Substantial evidence indicates that during the pathogenesis of COPD/emphysema, CS exposure can directly induce cellular senescence, apoptosis, autophagy dysregulation, and aberrant epithelial-mesenchymal transition in AT2 cells²³. These alterations severely impair the regenerative repair capacity and barrier function of AT2 cells, which constitutes the core cellular biological basis for the persistent loss of alveolar structure^{24,25}. Despite these insights, a major gap remains in our understanding. The precise molecular circuitry that commands these fate choices in alveolar epithelial cells after CS—especially how this regulatory network changes over time—is still not clearly mapped.

SIRT1 (Silent information regulator 1), a core member of the highly conserved NAD⁺-dependent class III histone deacetylase family, serves as a central regulator in key biological pathways including cellular energy metabolism, stress response, senescence, apoptosis, and inflammation^{26,27}. In the field of pulmonary diseases, studies have suggested that SIRT1 may exert protective effects against inflammation, apoptosis, and oxidative stress in models of acute lung injury, asthma, and pulmonary fibrosis, likely through the deacetylation of downstream targets such as NF- κ B, p53, and FOXO^{28,29}. Particularly noteworthy is that recent research has unveiled the potential role of SIRT1 in maintaining airway epithelial barrier function in COPD. For instance, studies indicate that traditional Chinese herbal compounds can enhance autophagy by activating the SIRT1/AMPK/FOXO3 signaling axis, thereby protecting the integrity of airway epithelial tight junctions³⁰. Furthermore, in sepsis-induced acute lung injury, activation of SIRT1 has been demonstrated to ameliorate dysfunction of the alveolar epithelial barrier³¹. More direct evidence indicates that dysregulation of the SIRT1/p53 and SIRT1/FOXO3a signaling pathways is involved in the senescence process of AT2 in patients with COPD. However, most existing studies have focused on acute injury models or the airway level. There is still a lack of systematic in vivo experimental evidence regarding how SIRT1 dynamically regulates

the survival, senescence, barrier function, and differentiation potential toward AT1 of alveolar epithelial cells (particularly AT2) under the influence of chronic CS exposure, which is a core etiological factor of COPD. Over long-term CS exposure, how do alveolar epithelial cell phenotypes change dynamically?

This study aims to systematically detect the evolutionary patterns of alveolar epithelial cells during the critical period from COPD exposure initiation to the formation of irreversible alveolar structural destruction, while simultaneously exploring the regulatory role of SIRT1 on alveolar epithelial cells in COPD pathology. This research will provide experimental evidence for understanding the pathological and physiological processes of COPD, encompassing the transitional phase from reversible alveolar injury upon exposure to irritants to the eventual formation of irreversible alveolar structural destruction. Furthermore, our findings will offer a theoretical foundation for early intervention and treatment strategies targeting COPD, with the potential to slow down or even reverse the progression of lung structural damage.

Methods

Experimental Animal Models and Study Design

A total of 120 male C57BL/6N mice (8 weeks old, specific pathogen-free (SPF) grade) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were housed in the animal facility of the Science and Technology Center at the First Affiliated Hospital of Army Medical University and acclimatized for 7 days. The housing conditions were maintained at $25 \pm 2^{\circ}\text{C}$, 40–60% relative humidity, with a 12/12-hour light-dark cycle. Mice were fasted (food and water) for 12 hours prior to modeling. The animal experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University (Approval No. AMUWEC20252136). Mice were randomly divided into two groups: a wild-type control group (WT group, $n=50$) and a cigarette smoke-exposed group (CS group, $n=70$). Mice in the CS group received intratracheal instillation of lipopolysaccharide (LPS, 2 mg/kg) on day 1 and day 14^{32,33,34,35}, whereas the WT group received an equal volume of saline. LPS was purchased from Sigma-Aldrich (Catalog No. L2630). Prior to intratracheal instillation, mice were anesthetized via intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg). After anesthesia, mice were fixed on an operating platform. Following intratracheal administration, mice were held in an upright

position briefly to facilitate even distribution of LPS in both lungs. Cigarette smoke exposure was not performed on the days of LPS administration.

Cigarettes used for exposure were Hongmei brand (produced by Kunming Cigarette Factory, Yunnan Province), with specifications of 15 mg tar, 1.2 mg nicotine, and 13 mg carbon monoxide per cigarette. The CS exposure protocol was as follows: Four cigarettes were combusted per cycle for 12 minutes. Each exposure session lasted 1 hour (comprising 5 cycles), administered twice daily with an interval of at least 6 hours between sessions. This protocol was continued for 12 consecutive weeks²⁷. CS exposure commenced on the second day of the experiment. Exposure was conducted in a plexiglass chamber measuring 50 cm × 35 cm × 30 cm. Daily CS exposure was performed as scheduled, but was withheld on days when intratracheal instillation of LPS or adeno-associated virus (AAV) was administered. Mice in the WT group were never subjected to cigarette smoke exposure.

Body weight was measured for all mice every two weeks. Following measurement, 5 mice were randomly selected from each of the WT and CS-exposed groups for sample collection, according to the following procedure: Mice were anesthetized using 1% pentobarbital sodium. The anesthetized mouse was secured on a surgical platform. The trachea was exposed via dissection and cannulated. A syringe was used to draw 1 mL of ice-cold (4°C) PBS, which was then instilled into the lungs via the tracheal cannula. After three repeated lavages, the Bronchoalveolar Lavage Fluid (BALF) was recovered. The thoracic cavity and heart were exposed. Blood was collected from the right ventricle using an insulin syringe and transferred into an anticoagulant tube. An infusion needle was inserted into the right ventricle, and the right atrial appendage was cut. PBS was perfused until the effluent ran clear. The right lung was then excised, blotted dry, and immediately snap-frozen in liquid nitrogen for subsequent Western Blot (WB) analysis. The perfusate was switched to 4% Paraformaldehyde (PFA). Systemic perfusion fixation was continued until muscle twitching was observed. The left lung was harvested and immersed in 4% PFA for post-fixation, pending further processing. The collected BALF and blood samples were centrifuged in a refrigerated centrifuge (4°C, 3000 rpm, 10 minutes). The supernatant was collected and stored at -80°C for subsequent assays^{36,37,38,39}.

Due to the scheduled tissue collection protocol of 5 mice per group every 2 weeks for the WT and CS groups, the number of mice in both the WT and CS groups was reduced by 10 at the 4-week time point. As a result, the remaining animal numbers at Week 4 were 40 for the WT group and 60 for the CS group. At the 4th week of the experiment, mice were subjected to the following grouping and interventions: WT Groups: Based on body weight, mice were divided into five groups using stratified randomization: WT Control (n=20); WT + SIRT1 Overexpression (n=5); WT + SIRT1

Inhibition (n=5); WT + Empty Overexpression Control(n=5); WT + Empty Inhibition Control (n=5).

CS-Exposed Groups: Based on the rate of body weight loss, mice were divided into five groups using stratified randomization:CS Exposure Control (n=30); CS + SIRT1 Overexpression (n=10); CS + SIRT1 Inhibition (n=10); CS + Empty Overexpression Control (n=5); CS + Empty Inhibition Control (n=5).

All mice were anesthetized with 1% pentobarbital sodium and secured on an operating platform. Subsequently, the corresponding viral suspension (30 μ L per mouse) was administered via intratracheal instillation using a microsyringe: WT+SIRT1 Overexpression and CS+SIRT1 Overexpression groups: Received the SIRT1-overexpressing adeno-associated virus (serotype 6.2FF, titer $\geq 5.00 \times 10^{12}$ vg/mL, rAAV-SP-C-SIRT1-2A-EGFP-WPRE-hGH polyA).WT+SIRT1 Inhibition and CS+SIRT1 Inhibition groups: Received the SIRT1-inhibiting adeno-associated virus (rAAV-U6-shRNA(SIRT1)-CMV-mCherry-SV40 pA, serotype 6.2FF, titer $\geq 5.00 \times 10^{12}$ vg/mL).CS+Empty Overexpression Control group: Received the empty overexpression control virus (rAAV-SP-C-EGFP-WPRE-hGH polyA, serotype 6.2FF, titer 5.00×10^{12} vg/mL).CS+Empty Inhibition Control group: Received the empty inhibition control virus (rAAV-U6-shRNA(scramble)-CMV-mCherry-SV40 pA, serotype 6.2FF, titer 5.00×10^{12} vg/mL)^{40,41,42}. All remaining groups that did not receive viral intervention were administered an equal volume (30 μ L) of normal saline via intratracheal instillation as a control treatment.

During the subsequent experimental period, body weight was measured for all surviving mice every two weeks. Furthermore, tissue sampling was performed at specified time points (weeks 2, 4, 6, 8, 10, and 12), with 5 mice collected from each of the WT control group and the CS exposure control group at each time point. During the experimental period prior to the Week 12 sampling, the CS group experienced the loss of 2 mice, the CS+SIRT1 overexpression group lost 1 mouse, the CS+SIRT1 inhibition group lost 4 mice, and both the CS+empty overexpression and CS+empty inhibition groups each lost 1 mouse. The comprehensive experimental design including group allocation and flowchart is presented in Figure 1.

The total sample size for this experiment (N=120) was determined based on the following factors: (1) Longitudinal sampling: Six time points (Weeks 2, 4, 6, 8, 10, 12) were designated, with 5 mice sacrificed from both the WT control group and the CS exposure control group at each time point, requiring a minimum of 60 mice for the longitudinal observation of disease progression. (2) Subgroup comparisons after grouping at Week 4: The core experimental groups (CS + SIRT1 Overexpression group and CS + SIRT1 Inhibition group) were assigned a sample size of 10 mice per group to ensure adequate statistical power for detecting the intervention effect. Other

intervention subgroups (e.g., empty vector control groups) were set at 5 mice per group. (3) Mortality compensation: To account for anticipated mortality during CS exposure modeling and viral vector intervention (a total of 9 deaths were recorded in the CS groups and intervention subgroups in this experiment), the initial number of animals enrolled (50 WT + 70 CS) included a reasonable surplus to ensure that all groups at the study endpoint would retain the minimum valid sample size required for statistical analysis.

No inclusion or exclusion criteria were established a priori for this study.

Simple randomisation was used at the beginning of the study. Mice were allocated to the WT cohort and the CS cohort in a 5:7 ratio using an online randomisation tool. At Week 4, mice within the WT cohort and the CS cohort were separately ranked according to their rate of body weight change. Subsequently, stratified sampling was performed, again using an online randomisation tool, to allocate mice into the subsequent subgroups according to the corresponding ratios, in order to ensure similar sensitivity to cigarette smoke exposure across all groups. Randomisation was performed by Weijia Zhou .

To minimise potential confounders, the following measures were taken: (1) Order of CS exposure: The order in which cages were placed in the CS exposure chamber was rotated daily according to a pre-determined schedule. (2) Animal location: Cages were placed on the rack in an alternating pattern (CS vs WT cages separated by empty cages to prevent cross-contamination) and positions within the rack were rotated weekly to minimise positional effects.

Outcome assessment included the endpoint at the 12-week time point, evaluation of the magnitude of body weight loss, and pulmonary function test results.

In this study, because the mice were in the growth phase, a composite scoring system was adopted. Scores were assigned separately based on body weight changes, appearance, respiratory rate, abnormal behavior, and response to external stimuli. The endpoint was determined when the cumulative score reached 5 or when a single parameter reached the maximum score. The specific evaluation criteria are detailed in the attached document 'Humane endpoints standard'. Mice that have reached the endpoint will be euthanized.

All experimental mice were euthanized by intraperitoneal injection of 1% sodium pentobarbital at a dose of 200 mg/kg. The sodium pentobarbital was purchased from Sigma-Aldrich (USA). Following the injection, the mice rapidly lost consciousness due to profound central nervous system depression. Cessation of respiration and heartbeat, loss of the pupillary light reflex, and paleness of mucous membranes were assessed to confirm death. To further ensure that no animal revived, a secondary physical method (e.g., cervical dislocation) was performed after the animal was

unresponsive to deep pain stimuli. All efforts were made to minimize pain and distress during the procedure.

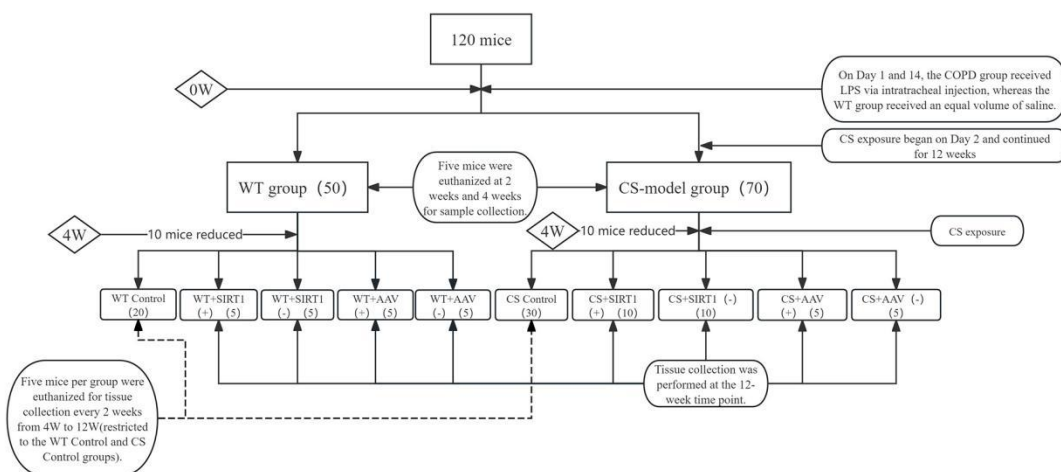


Figure 1. Flowchart of animal group allocation and experimental procedure.

HE and MASSON staining

Lung tissues were embedded in paraffin and sectioned at a thickness of 4 μ m. The sections were subsequently stained with Hematoxylin and Eosin (H&E) and Masson's trichrome. Stained sections were imaged under an optical microscope at 200 $\times$ magnification to observe morphological changes and perform quantitative analysis. The specific parameters analyzed were: H&E staining: Used to measure the mean linear intercept (MLI), the number of alveoli per field of view (MAN), and the destruction index (DI). Masson's trichrome staining: Used to assess collagen deposition. For quantification, five random fields of view were selected from each mouse lung section. Measurements were performed using Image-Pro Plus 6.0 software: the total number of alveoli, the number of alveolar walls intersecting a test line, and the area of the field of view were recorded. The following calculations were then applied: $MLI = \text{Total length of the test line} / \text{Number of alveolar walls intersecting the test line}$; $MAN = \text{Total number of alveoli} / \text{Area of the field of view}$; $DI = \text{Number of alveoli with structural destruction} / \text{Total number of alveoli}$ ^{43,44}. To minimize bias, counting and measurements were conducted in a blinded manner by personnel unaware of the group assignments. Subsequently, all data were compiled and subjected to statistical analysis.

Non-invasive pulmonary function testing

After the 12-week intervention period, pulmonary function in mice was assessed using a whole-body plethysmography (WBP) system, a non-invasive method. Pulmonary function was evaluated using a non-invasive whole-body plethysmography

system (Buxco, USA) according to the manufacturer's instructions. Prior to testing, the mice were weighed and recorded. The instrument software was opened, and the project name, mouse strain, test parameters, and test duration were configured as follows: adaptation period 10 min, detection period 20 min, and post-test adaptation time 1 min. Four plethysmograph chambers were prepared. The weighed mice were placed into the chambers, and the corresponding mouse IDs and body weights were entered into the software via the instrument interface. The parameters of chamber 1 were adjusted to normal baseline values, followed by sequential adjustments of chambers 2, 3, and 4. The non-invasive pulmonary function test was initiated. After each test cycle was completed, the mice were removed from the chambers and the next batch was prepared for testing. The measured parameters included minute ventilation (Mvb), Airway stenosis index (Penh), mid-expiratory flow (EF50), and Apnea index (PAU)^{45,46}. The data were exported for subsequent organization and analysis.

ELISA

ELISA was performed to measure SIRT1 levels in BALF and serum, using the Mouse NAD-dependent deacetylase sirtuin-1 (Sirt1) ELISA Kit (Cusabio, Cat. No. CSB-E16187m) according to the manufacturer's instructions.

Western Blotting

Mouse lung tissues were lysed using RIPA lysis buffer (Beyotime, Cat. #P0013B). The tissues were further disrupted by sonication on ice. Proteins were separated by SDS-PAGE gels of appropriate concentrations and then transferred onto PVDF membranes. After blocking with 5% skim milk for 2 h, the membranes were washed three times with TBST (≥ 5 min per wash) and incubated overnight at 4 °C with the following primary antibodies: HOPX (Proteintech, 11419-1-AP), CLDN4 (Abcam, ab210796), SIRT1 (SAB, 48501), SIRT1 (Prosci, 5765), HOPX (Santa Cruz, sc-398703), PCNA (Boster, A00125), NLRP3 (Boster, BA3677), E-cadherin (Proteintech, 20874-1-AP), TNF- α (Boster, BA0131), CLDN4 (Proteintech, 16195-1-AP), SP-C (Proteintech, 10774-1-AP), KRT8 (Boster, A01421). The next day, after three washes, the membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (Boster, BA1054) and HRP-conjugated goat anti-chicken secondary antibody (Abbkine, A21080). Following another three washes, protein bands were visualized and recorded using a chemiluminescence imaging system. Band intensities were analyzed with ImageJ software, normalized, and expressed as fold changes relative to the control group. The pre-stained protein marker used was the 26616 marker from Thermo Fisher Scientific, USA. The gradient

gel used for WB was either the 6% SDS-PAGE Gel One-Step Ultra-Fast Preparation Kit (Red Upper Gel) or the 12% SDS-PAGE Gel One-Step Ultra-Fast Preparation Kit (Red Upper Gel), with catalog numbers P0912S and P0919S, respectively. The former was used for molecules 50-150 kD, such as SIRT1 and E-cadherin, while the latter was used for molecules 12-60 kD, such as CLDN4, TNF- α , and KRT8.

Immunofluorescence assay and analysis

After embedding for 30 min, lung tissues were frozen and sectioned at 8-10 μ m thickness. The sections were mounted on slides and air-dried overnight at room temperature. The next day, antigen retrieval was performed using a frozen-section rapid antigen retrieval solution (Beyotime, P0090) for 10 min, followed by treatment with an autofluorescence quenching reagent (Applygen, C1212) for 90 min. Sections were then washed twice with pure water (3 min each), permeabilized with 0.5 Triton X-100 for 30 min, and washed three times (5 min each). After blocking with goat serum for 2 h and three washes (2 min each), the sections were incubated overnight at 4 °C with the following primary antibodies: HOPX (Oasis Biofarm, OB-PRT015-02), HOPX (Santa Cruz Biotechnology, sc-398703), SP-C (Proteintech, 10774-1-AP), SIRT1 (Prosci, 5765), Nrf2 (HUABIO, R1312-8-100ul), Cleaved-caspase-3 (Proteintech, 25128-1-AP), CLDN4 (Abcam, ab210796), E-cadherin (Proteintech, 20874-1-AP), TNF- α (Boster, BA0131), CLDN4 (Proteintech, 16195-1-AP), Ki-67 (Boster, PB9026). On the following day, after washing, sections were incubated in the dark for 1 h at room temperature with the corresponding secondary antibodies: Goat anti-chicken IgG (H+L) Alexa Fluor Plus 405 (Invitrogen, A48260), Goat anti-rabbit IgG H&L (Alexa Fluor 488) (Abcam, ab150077), Goat anti-rat IgG H&L (Alexa Fluor 555) (Abcam, ab150158), Alexa Fluor 647-conjugated goat anti-mouse antibody (Beyotime, A0473), Alexa Fluor 555-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, A-21428), DyLight 594-conjugated goat anti-mouse IgG (Abbkine, A23410), DyLight 680-conjugated AffiniPure goat anti-rabbit IgG (H+L) (Fdbio science, FD0133), AF647-conjugated goat anti-rabbit IgG (H+L) (Beyotime, A0468). After four washes, sections were air-dried in the dark, mounted with DAPI-containing mounting medium, and observed under an Olympus fluorescence microscope. Images were acquired and stored for subsequent analysis. Quantitative assessments, such as counting positive cells and measuring fluorescence intensity, were conducted in a batch-processing manner using ImageJ software. This was followed by data compilation and statistical evaluation.

Fluorescence quantification and comparability statement. To avoid spectral crosstalk caused by fluorescent proteins carried by the viral vectors (EGFP in the overexpression vector and mCherry in the knockdown vector) across different

experimental groups, different wavelengths of fluorescent secondary antibodies were used for the same target protein in different groups (e.g., green/red channels). It should be noted that different fluorophores exhibit inherent differences in quantum yield, and different secondary antibodies differ in their titers; thus, direct inter-group comparisons of fluorescence intensity or positive-cell counts without signal calibration may introduce bias. Therefore, in this study, the immunofluorescence images obtained with different fluorophores are used only for the qualitative visualization of target protein expression and localization, and are not used for inter-group quantitative comparisons; all quantitative conclusions related to immunofluorescence are based on Western blot results. In addition, in representative images, co-localization with an internal reference and negative controls (secondary antibody incubation alone) were included to verify staining specificity.

Cell culture and treatment

Human A549 cells (Procell; CL-0016) were cultured in DMEM medium supplemented with 10% fetal bovine serum (ZETA LIFE; Z7010FBS-500) and 1% penicillin-streptomycin solution (ZETA LIFE; BM0001-100) at 37 °C in a 5% CO₂ incubator. Based on preliminary experiments, the following working concentrations were used: Cigarette smoke extract (CSE) (PYTHONBIO; AAPR551-A10): 50 µg/mL; LPS (Sigma-Aldrich; L2630): 10 µg/mL; SIRT1 agonist SRT1720 (Beyotime; SC0267-5mg): 5 µM; SIRT1 inhibitor EX527 (Beyotime; SC0281-5mg): 10 µM; Nrf2 agonist NK252 (GLPBIO; GC13058): 10 µM; Nrf2 inhibitor ML385 (GLPBIO; GC19254): 5 µM; The CS-model group was treated with a combination of CSE and LPS, referred to hereafter as the CS group. Experimental Groups: The cells were divided into the following treatment groups: Control group; CS group (CSE + LPS); CS + SRT1720 group; CS + EX527 group; CS + NK252 group; CS + ML385 group; CS + SRT1720 + NK252 group; CS + SRT1720 + ML385 group; CS + EX527 + NK252 group; CS + EX527 + ML385 group; SRT1720 group (agonist alone); EX527 group (inhibitor alone); NK252 group (agonist alone); ML385 group (inhibitor alone); Drug vehicle control group (containing only the four drug vehicles, without cells); Assessment of Cell Proliferation and Apoptosis: Cell proliferation and apoptosis were evaluated using the following assays: Cell Counting Kit-8 (CCK-8) assay; ATPase activity assay; Apoptosis analysis via PI fluorescence staining. Cell experimental group allocation is illustrated in Figure 2.

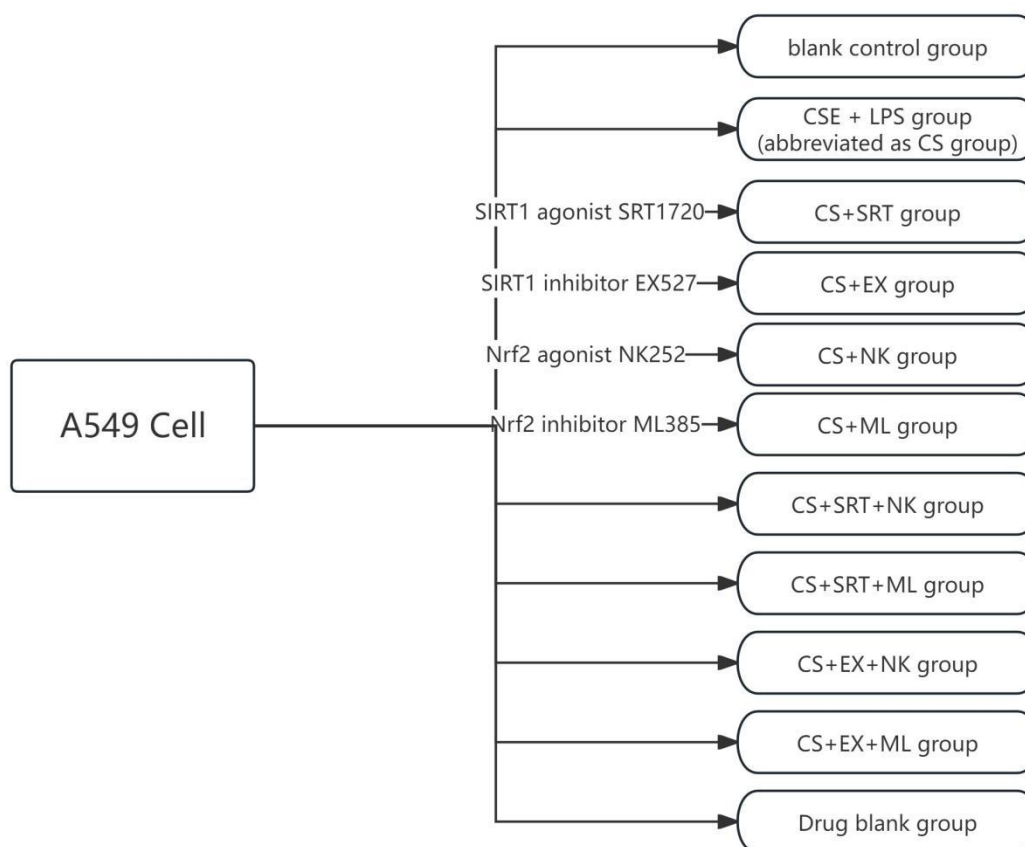


Figure 2. Cell experimental group allocation and experimental design.

Cell Counting Kit-8 (CCK-8) assay

A total of 100 μ L of cell suspension was seeded into each well of a 96-well plate. After 8 h of incubation, the medium was replaced, and the cells were treated with the pre-calculated drug concentrations for 48 h. 10 μ L of CCK-8 solution was added to each well. Care should be taken to avoid the formation of air bubbles. The 96-well plate was incubated in the cell culture incubator for 1 h. The plate was gently mixed on a shaker for 1 min, and the absorbance was measured at 450 nm using a microplate reader. Cell Counting Kit-8 (CCK-8) was purchased from Shanghai Hongye Biotechnology Co., Ltd., Shanghai, China (Cat. No.: GK10001). All cells used in this experiment were kept between passage 3 and passage 15.

CellTiter-Lumi™ II Luminescent Cell Viability Assay

White opaque 96-well plates were prepared, and cells were seeded at a density of 100 μ L per well. After 8 h of attachment, the culture medium was replaced with fresh medium containing drugs at pre-determined concentrations, and cells were incubated for an additional 48 h. Following treatment, the CellTiter-Lumi™ II assay buffer was thawed and kept on ice. The cell culture plates were allowed to equilibrate at room temperature for 10 min. Subsequently, 100 μ L of CellTiter-Lumi™ II detection

reagent was added to each well. The plates were gently mixed on a shaker for 2 min at room temperature to promote cell lysis. After incubation at room temperature (~25°C) for 10 min to stabilize the luminescent signal, chemiluminescence was measured using a multifunctional microplate reader. The CellTiter-Lumi Luminescent Cell Viability Assay Kit was purchased from Beyotime Biotechnology Co., Ltd., Shanghai, China (Cat. No.: C0065M). All cells used in this experiment were kept between passage 3 and passage 15.

PI Staining Cell Apoptosis Assay

Cell apoptosis was evaluated using Propidium Iodide (PI) staining according to the manufacturer's instructions. Briefly, cells were seeded in 12-well plates at a density of 1 mL per well. After 8 h of attachment, the culture medium was replaced with fresh medium containing drugs at pre-determined concentrations, and cells were incubated for an additional 48 h. Following treatment, 1 μ L of PI staining solution was added to each well, and the plates were incubated at 37°C for 30 min. After incubation, the plates were immediately transferred to a fluorescence microscope for observation. Images were captured and saved, and data were exported for subsequent statistical analysis. PI staining solution was purchased from Beyotime Biotechnology Co., Ltd., Shanghai, China (Cat. No.: C1008S). All cells used in this experiment were kept between passage 3 and passage 15.

Statistical analysis

All data processing, statistical analysis, and visualization were performed using GraphPad Prism 9.5 software and IBM SPSS Statistics 27.0 software. First, normality testing was conducted for continuous variables using the Shapiro-Wilk test (for small sample sizes) or Kolmogorov-Smirnov test (for large sample sizes). Homogeneity of variance was assessed using Levene's test (when data distribution was non-normal) or Bartlett's test (when data distribution was normal). For normally distributed data, results were expressed as mean $\pm$ standard deviation (mean $\pm$ SD). Comparisons between two groups were performed using the independent samples t-test (when variances were homogeneous) or Welch's corrected t-test (when variances were heterogeneous). For comparisons among multiple groups, one-way analysis of variance (ANOVA) was used (when variances were homogeneous) or Welch's ANOVA was used (when variances were heterogeneous). For data that did not conform to normal distribution, results were expressed as median and interquartile range (IQR). Two-group comparisons were performed using the Wilcoxon rank-sum test, and multiple-group comparisons were performed using the Kruskal-Wallis test. Following significant results from ANOVA or Kruskal-Wallis tests, post-hoc analyses

were conducted using Tukey's HSD test (for all pairwise comparisons among groups) or Dunnett's test (for comparisons between experimental groups and the control group) to correct for Type I errors introduced by multiple comparisons. For repeated measurement data that changed over time, such as body weight, repeated measures ANOVA or linear mixed-effects models were used for analysis. In the models, "animal ID" was set as a random effect to control for the impact of individual differences on the results. Image analysis was performed using ImageJ and Image-Pro Plus 6.0 software. The reading and counting of all immunofluorescence images and histopathological sections were conducted using a blinded method, where two or more observers independently performed counting without knowledge of the experimental groupings, and the final mean values were taken to minimize subjective bias. All statistical tests were performed as two-tailed tests, and a P-value less than 0.05 ($P < 0.05$) was considered statistically significant. All cell experiments were performed with three independent biological replicates, where each independent experiment was conducted using cells from different passages. Technical replicates were performed within each experiment (three wells per treatment in parallel), and the mean values were used for statistical analysis.

Cheng Liang was aware of group allocation during the allocation, conduct of the experiment, outcome assessment, and data analysis stages. All other personnel were blinded to group allocation throughout all stages.

In this study, the experimental protocol was prepared prior to the research, including descriptions of the research questions, key time points, and specific experimental methods. The experimental protocol was not registered.

Results

1. Validation of the CS-model.

During the 0-4 week period, the body weight of CS-model mice under CS exposure continued to decline, whereas that of WT mice steadily increased (Fig. 3A-B). After 4 weeks, the body weight gain of CS-exposed mice was impeded, showing a persistent downward trend, in sharp contrast to the continued weight increase in WT mice (Fig. 3C-D). Histological examination of lung sections revealed progressively worsening alveolar damage and increased inflammatory cell infiltration in CS-model mice over the course of CS exposure, while the lung morphology of WT mice remained largely unchanged over time (Fig. 3E, H&E staining). Masson's trichrome staining showed that collagen deposition in the lungs of CS-model mice

intensified with prolonged CS exposure, with no significant changes observed in WT mice (Fig. 3F). Quantitative analysis demonstrated a significant increase in the MLI and the alveolar DI, along with a significant decrease in the MAN per field in the CS-model group compared to controls (Fig. 3G-I). We further assessed the dynamic changes in pulmonary inflammation during CS exposure. Immunofluorescence and Western blot analyses revealed that the expression of the pro-inflammatory cytokine TNF- α was significantly elevated in CS-model mice after CS exposure and remained high up to 12 weeks (Fig. 4A-D). Western blot results indicated that the expression levels of TNF- α and the key inflammasome protein NLRP3 were not significantly altered at the early stage (2 weeks) but began to increase markedly from the 4th week onward, sustaining high levels thereafter (Fig. 4C-F). Pulmonary function was evaluated in WT and CS-model mice after 12 weeks. Parameters including MVb, Penh, EF50, and the PAU were measured. The results showed that all pulmonary function indices were impaired in the CS-model group, with significant differences compared to the WT group (Fig. 12A-D). These findings collectively confirm the successful establishment of a valid mouse model induced by 12 weeks of CS exposure combined with early intratracheal LPS instillation.

2. CS exposure induces a time-dependent imbalance in the proportions of alveolar epithelial cell subpopulations

We first measured how the different types of alveolar epithelial cells changed over time. Using immunofluorescence and Western blot, we tracked these markers. The AT1 cell marker HOPX stayed fairly steady during the first 2 weeks of smoke exposure. However, it began to drop noticeably starting at week 4. On the other hand, the AT2 cell marker SP-C showed a small uptick at week 2. After that, it fell sharply. This decline in SP-C was much steeper than the drop in HOPX. As a result, the ratio of SP-C to HOPX quickly decreased (Fig. 5A-H). The specific changes in SP-C and HOPX in the CS model from 0 to 12 weeks are shown in Figure 6.

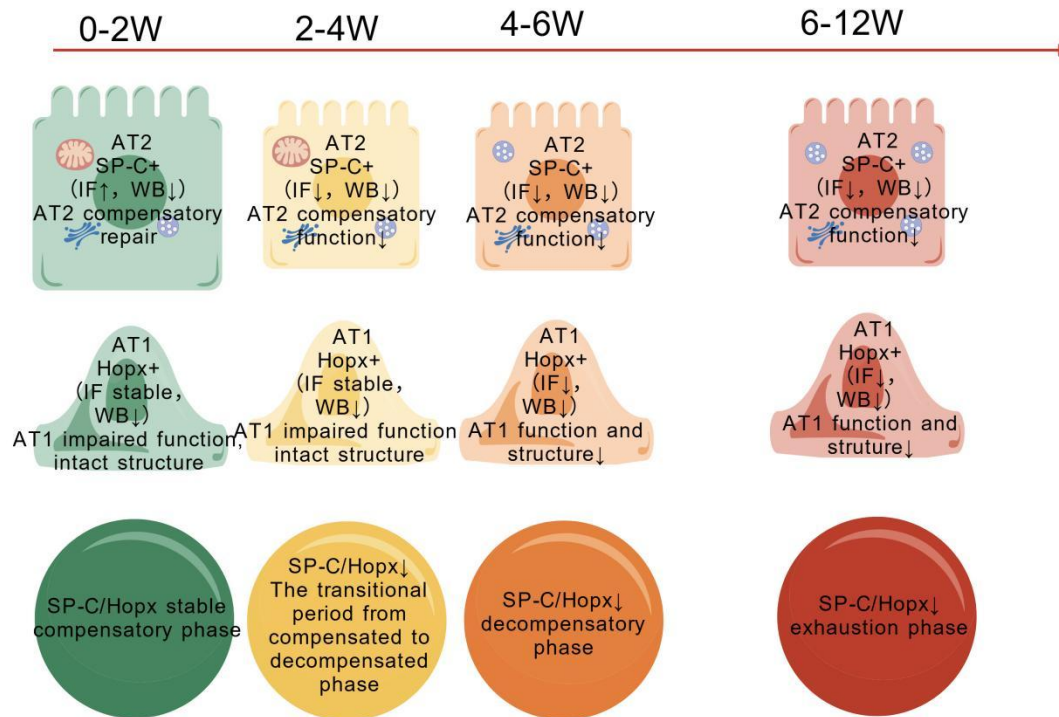


Figure 6. Schematic diagram of the alveolar epithelial injury time-course staging model.
(Created through BioGDP.com, with authorization obtained from the platform.)

Interestingly, Western blot analysis revealed that protein levels of KRT8, a marker associated with alveolar intermediate cells, did not change significantly at any time point compared to controls (Fig. 5I-J). However, both immunofluorescence and Western blot analyses showed that the expression of CLDN4, another marker associated with intermediate cell differentiation, began to gradually decrease after week 2. Prior research has delineated alveolar intermediate cells into five sequential stages: CTGF (connective tissue growth factor)-positive PATs (pre-alveolar type 1 transitional cells), LGALS3 (galectin-3)-positive PATs, KRT8+ ADI (alveolar differentiation intermediate cells), preAT1, and DATP (damage-associated transient progenitors), with the latter two stages expressing CLDN4. The transitional cells from AT2 to AT1 differentiation are shown in Figure 7.

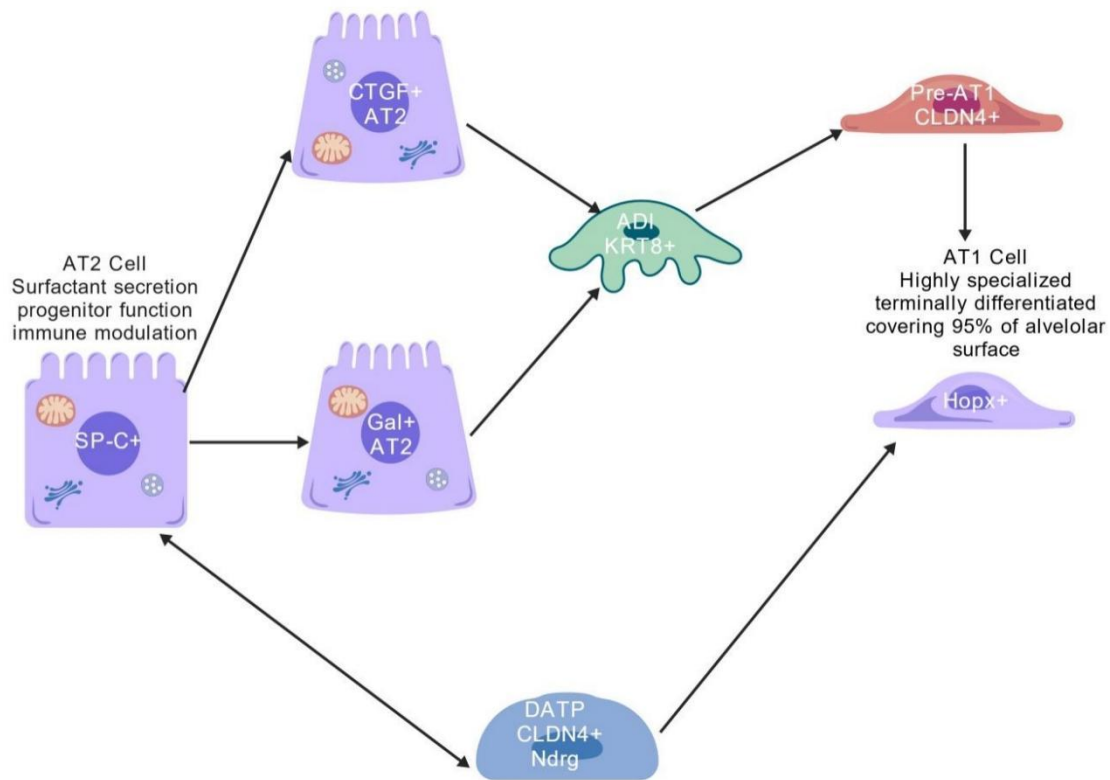


Figure 7. Transitional cells during AT2-to-AT1 differentiation.
(Created through BioGDP.com, with authorization obtained from the platform.)

3. Temporal Changes in Alveolar Epithelial Cell Barrier Function, Proliferation, and Apoptosis Status.

We next looked at proteins important for the barrier function of the alveolar epithelium. With longer CS, their levels progressively declined. Both immunofluorescence and Western blot showed that key tight junction proteins, as well as the adhesion protein E-cadherin, started to drop significantly in both positive cell counts and expression levels after two weeks (Fig. 8A-H). Assessing cellular proliferation revealed a complex pattern. The count of Ki-67-positive cells, a proliferation marker detected by immunofluorescence, was significantly reduced after two weeks of exposure (Fig. 9A, C). In contrast, protein levels of the proliferation marker PCNA, measured by Western blot, showed a significant initial increase at the two-week mark before declining rapidly in subsequent weeks (Fig. 9E-F). Apoptosis assessment indicated that the apoptosis marker cleaved-caspase-3 detected by immunofluorescence showed almost no increase at 2 weeks but began to rise significantly at 4 weeks of CS exposure (Fig. 9B,D).

The temporal changes in barrier function, proliferation, and apoptosis align with the overall alterations in alveolar epithelial cells. However, the peak of inflammatory indicators occurs later than these epithelial changes. Consequently, we propose that inflammation is likely not the primary cause of the alveolar epithelial cell alterations; instead, an earlier initiating stimulus probably triggers this process.

4. Temporal alterations in SIRT1/Nrf2 and validation of SIRT1 gain- and

loss-of-function models.

In our previous study, we observed that the expression of SIRT1 decreased early in a CS-model mice; its protein level increased at week 2 and then gradually declined (Fig. 10 C-D). This suggests that SIRT1 plays a role in the early stages of CS-model, and its temporal changes align with the alterations in alveolar epithelial cells. Furthermore, we examined the dynamics of Nrf2 expression and found that its level also rose at week 2 before gradually decreasing (Fig. 10 A-B), a pattern consistent with the changes in SIRT1 and alveolar epithelial cells.

Looking at the data over time, we saw that SIRT1 and Nrf2 levels change together, and this happens early in the disease. Interestingly, protein levels for both SIRT1 and Nrf2 went up after two weeks of exposure, but then fell later. This timing matches up closely with when the alveolar epithelial cells are lost and their function worsens. These results indicate that problems with the SIRT1/Nrf2 system are an early driver of the disease process, not just a late result. This gives us a reason, based on timing, to look at this system as a possible central control mechanism. There is also a proposed direct link between the SIRT1 and Nrf2 pathways. Earlier work suggests SIRT1 can activate Nrf2 by modifying both the Nrf2 protein itself and its inhibitor, Keap1. Since Nrf2 controls the cell's main defense against oxidative stress—a key starting point for smoke damage—this creates a logical chain. The pathway connects SIRT1's regulatory role to Nrf2 activation and finally to fighting oxidative damage. This makes it a strong candidate for directly linking smoke exposure (the cause) to oxidative injury in lung cells (the effect). We therefore suggest that in early exposure stage of COPD, the SIRT1/Nrf2 pathway is turned on. It likely helps AT2 cells differentiate and protects AT1 cells from dying. But with continued smoke exposure, this protective system gets worn down and depleted. Eventually, this leads to a failure in the cells' ability to proliferate and repair themselves.

Based on this hypothesis, at the fourth week of cigarette smoke exposure, we intratracheally administered two AAVs: one carrying a lung-specific promoter and green fluorescent protein for SIRT1 overexpression, and the other labeled with red fluorescent protein for SIRT1 inhibition. This gain- and loss-of-function experiment was conducted to test our proposed mechanism.

Body weight monitoring revealed that in both wild-type and CS-model groups, mice injected with the SIRT1-overexpressing virus exhibited higher absolute body weight and weight change rate compared to the saline control group. The control group, in turn, showed higher body weight metrics than mice injected with the SIRT1-inhibiting virus (Fig. 3C-D). Given the known role of SIRT1 in metabolism, this weight difference indirectly indicates successful expression and biological activity of the SIRT1 gain- and loss-of-function viruses.

Since the overexpression virus was tagged with EGFP and the inhibition virus with mCherry, both produce detectable fluorescence. Examination of lung tissue sections under a microscope revealed green fluorescence in the overexpression group and red fluorescence in the inhibition group (Fig. 11A). This visual evidence confirms successful viral transduction in the lung tissue.

We further assessed SIRT1 protein levels in mouse lung tissues using ELISA and Western blot. The measurements showed that SIRT1 expression was significantly elevated in the overexpression group compared to the control group. Conversely, SIRT1 levels in the control group were significantly higher than those in the inhibition group (Fig. 11B-J). Together, these results verify that the viral vectors not only reached the lung tissue but also effectively altered the expression of the target protein as intended.

Additionally, we included control groups receiving blank viruses (carrying fluorescent tags but without SIRT1 intervention). Measurements of body weight, fluorescence, and protein expression showed no significant differences between these blank virus groups and the saline control group, indicating that the observed effects were due to specific SIRT1 modulation rather than the viral vectors themselves.

In summary, we successfully established *in vivo* gain- and loss-of-function models for SIRT1.

5. Intervening in SIRT1 expression can alter the progression of CS-model through alveolar epithelial cells.

Respiratory function in mice was evaluated using non-invasive pulmonary function testing. Measured parameters included minute ventilation (reflecting overall ventilatory capacity), airway narrowing index (where a higher value indicates more severe obstruction), mid-expiratory flow (where a lower value suggests reduced lung elasticity), and the apnea-hypopnea index (where a higher value signifies increased airway resistance or worsened compliance). All pulmonary function parameters were significantly impaired in the CS-model group compared to wild-type mice. In CS-model mice, SIRT1 overexpression improved pulmonary function, whereas SIRT1 inhibition exacerbated its decline. Notably, modulating SIRT1 expression in wild-type mice did not produce a statistically significant effect on lung function under the experimental conditions of the present study. This suggests that the apparent role of SIRT1 observed here may be context-dependent. Furthermore, no significant difference in pulmonary function was observed between the blank virus control groups and the CS-model group, confirming that the viral vectors themselves did not influence respiratory function (Fig. 12A-D).

HE and MASSON staining of lung tissue sections showed that SIRT1 overexpression reduced cigarette smoke-induced lung damage, including emphysema,

inflammatory cell infiltration, and collagen buildup. In contrast, SIRT1 inhibition worsened these pathological changes (Fig. 12E). Further analysis of quantitative measures, such as the mean linear intercept and alveolar destruction index, confirmed these observations. These metrics were significantly better in the SIRT1-overexpression group compared to the cigarette smoke-exposed group, while SIRT1 inhibition led to further decline. No significant differences were found between the blank-virus control groups and the cigarette smoke-exposed group (Fig. 12F-H). These findings indicate that SIRT1 can rescue cigarette smoke-induced emphysematous structural alterations and improve lung function, suggesting a protective role for SIRT1 in CS-model.

Immunofluorescence and Western blot results showed that SIRT1 overexpression increased the numbers of both alveolar type I and type II epithelial cells compared to the CS-model group, while SIRT1 inhibition decreased them. No significant changes were seen between the blank-virus control groups and the CS-model group (Fig. 13). Further analysis indicated that the ratio of the AT2 cell marker SP-C to the AT1 cell marker HOPX was higher in the SIRT1-overexpression group than in the CS-model group, but lower in the inhibition group (Fig. 13D). This suggests that SIRT1 exerts a more pronounced effect on AT2 cells than on AT1 cells, indicating its role in modulating the compositional balance of alveolar epithelial cell subtypes.

6. SIRT1 regulates inflammation, barrier function, proliferation, and apoptosis in alveolar epithelial cells

Western blot analysis of TNF- α showed that SIRT1 overexpression led to higher levels of the TNF- α precursor but lower levels of the active soluble form, compared to the control group. Conversely, SIRT1 inhibition increased both the precursor and the active form. No significant changes were seen in the blank-virus control groups (Fig. 14A-F). NLRP3 expression was also affected: it decreased with SIRT1 overexpression and increased with SIRT1 inhibition, while blank-virus controls again showed no difference from the control group (Fig. 14D, H, I). Immunofluorescence staining for TNF- α supported these findings, with fewer TNF- α -positive cells after SIRT1 overexpression and more after its inhibition. These findings demonstrate that SIRT1 can ameliorate cigarette smoke-induced inflammatory responses in lung tissue. In a separate analysis of the tight junction protein CLDN4, Western blot showed that CLDN4 expression rose in the SIRT1-overexpression group—even exceeding levels in the wild-type group—but fell in the SIRT1-inhibition group. The blank-virus control groups did not differ from the control group (Fig. 15F-H). Immunofluorescence and Western blot analysis revealed higher E-cadherin expression in the SIRT1-overexpression group and lower expression in the SIRT1-inhibition group. The blank-virus control groups showed no significant change compared to the

control group (Fig. 15A-E). This points to a role for SIRT1 in enhancing barrier function. In addition, immunofluorescence staining revealed that the number of Ki-67-positive cells was increased in the SIRT1-overexpression group and decreased in the SIRT1-inhibition group, while no significant differences were observed between the blank-virus control groups and the control group (Fig. 16A-B). Western blot analysis indicated that PCNA expression was elevated in the SIRT1-overexpression group, exceeding that in WT group, whereas it was reduced in the SIRT1-inhibition group. The blank-virus control groups showed no notable change compared to the control group (Fig. 16C-E). These findings suggest that SIRT1 significantly influences the proliferative and differentiation functions of alveolar epithelial cells in CS-model. Finally, immunofluorescence for cleaved-caspase-3 demonstrated that SIRT1 overexpression reduced alveolar epithelial cell apoptosis, whereas inhibition worsened it. Blank-virus controls again showed no difference from the control group (Fig. 17A, C). This indicates that SIRT1 activation can alleviate apoptosis.

Immunofluorescence analysis indicated that SIRT1 overexpression up-regulated Nrf2 expression, whereas SIRT1 inhibition down-regulated it; no significant differences were observed between the blank-virus control groups and the normal control group (Fig. 17B, D). This suggests a functional link between SIRT1 and Nrf2 in the context of cigarette smoke-induced chronic obstructive pulmonary disease. We subsequently performed cell-based experiments to further investigate the interaction between SIRT1 and Nrf2.

7. Preliminary In Vitro Exploration of the SIRT1-Nrf2 Interaction in a Simulated Alveolar Epithelial Cell Injury Environment.

To precisely assess the direct reciprocal inhibition or synergistic activation relationship between SIRT1 and Nrf2 in a controlled in vitro environment, we established a simulated COPD alveolar epithelial cell injury model. This approach eliminated confounding factors from paracrine signaling and complex microenvironments present in in vivo lung tissue, including immune cells, stromal cells, and vascular endothelial cells. A549 human lung adenocarcinoma epithelial cells, which retain core biological characteristics of AT2, were subjected to inflammatory responses, oxidative stress, and apoptosis by CSE with LPS to mimic typical COPD-related pathological changes. Given that primary AT2 undergo spontaneous transdifferentiation within 72 hours under routine in vitro culture conditions, losing their characteristic AT2 phenotype, and exhibit significant batch-to-batch variability, we selected the A549 cell line for our in vitro experiments. Using SIRT1 and Nrf2 agonists and inhibitors, we further investigated the regulatory roles and interactions of these two proteins in alveolar epithelial cell proliferation and

apoptosis.

Western blot analysis showed that pharmacological treatment effectively activated or inhibited SIRT1 in cells (Fig. 18A-B).

CCK-8 and ATPase activity assays showed that activating either SIRT1 or Nrf2 alone partially restored the drop in cell viability and proliferation caused by CS. Inhibiting either pathway alone made the CS-induced damage worse. When one pathway was activated while the other was inhibited, the restorative effect was weaker, pointing to an interaction between SIRT1 and Nrf2 in fighting CS-induced proliferation problems. A closer look revealed that activating SIRT1 while blocking Nrf2 worked better than activating Nrf2 while blocking SIRT1. This suggests SIRT1 might have a stronger influence than Nrf2 on regulating cell proliferation in this context. Simultaneous inhibition of both SIRT1 and Nrf2 resulted in a substantial decrease in proliferative capacity, underscoring the critical protective roles both pathways play in normal cells responding to CS injury. Furthermore, treatment with the activators or inhibitors alone caused only a mild reduction in proliferation, indicating low inherent cytotoxicity of the compounds themselves. The results from the vehicle control groups confirmed that the color of the drugs did not significantly interfere with the assay readings (Fig. 18C-H).

Regarding apoptosis, CS treatment significantly increased cell apoptosis. Administration of SRT (a SIRT1 activator) or NK (an Nrf2 activator) alone effectively rescued the CS-induced apoptosis. Conversely, treatment with EX (a SIRT1 inhibitor) or ML (an Nrf2 inhibitor) alone exacerbated apoptosis. In the combination treatment experiments, the rescue effect achieved by co-administering SRT and ML (activating SIRT1 while inhibiting Nrf2) was inferior to that achieved by co-administering EX and NK (inhibiting SIRT1 while activating Nrf2). This result suggests that the Nrf2 pathway may play a more critical role than SIRT1 in counteracting CS-induced apoptosis. Control experiments validated the reliability of the assay system: the high apoptosis rate in the positive control group confirmed the effectiveness of the staining method. Meanwhile, the absence of apoptotic signals in the vehicle control groups indicated that the drugs themselves did not react with the apoptosis detection dye, thereby ruling out potential false-positive interference (Fig. 19).

Discussion

Traditionally, COPD has been associated with long-term airway inflammation caused by neutrophils. Our study, using data collected over time, reveals an earlier series of disease events in alveolar epithelial cells after smoke exposure. A key finding

is that an imbalance in the ratio of AT2 to AT1 cells, along with a significant drop in SIRT1 protein, was seen as early as 4 weeks into exposure—before any structural emphysema appeared. This suggests that problems in these lung lining cells are an early driver of CS-model, not just a late result. This idea fits with a recent single-cell study that found a unique, inflammatory type of AT2 in the lungs of human smokers^{10,11}, indicating these cells change their function right from the start. In more detail, after a brief increase, AT2 numbers dropped significantly and earlier than AT1. This caused a quick shift in the balance between their markers, SP-C for AT2 and HOPX for AT1. On the other hand, noticeable rises in the inflammatory signal TNF- α and the key inflammation protein NLRP3 only started after week 4. This clear order of events indicates that in CS-model, the alveolar epithelial cells are not just passive targets of inflammation. Instead, the breakdown in their own ability to proliferate and repair themselves likely acts as the initial problem that then triggers the later inflammation and tissue damage. In the CS exposure combined with LPS-induced model mice, we conducted longitudinal assessments from 2 to 12 weeks (at 2-week intervals) to evaluate the number and proportion of AT1 and AT2, along with their differentiation markers, barrier markers, proliferation markers, apoptosis markers, and the expression levels of SIRT1 and Nrf2. We observed that alveolar cells in CS model mice exhibited a temporal pathological pattern characterized by compensation at 2 weeks, decompensation at 4 weeks, and subsequent progressive injury. At 2 weeks, AT1 maintained intact structure, while AT2 showed a slight increase.

Markers of differentiation, barrier function, proliferation, and apoptosis did not demonstrate significant changes. At 4 weeks, AT2 proliferative capacity was exhausted, manifested by significantly decreased SP-C expression, reduced Ki-67-positive cell count, and decreased PCNA expression level. Differentiation arrest was indicated by stable KRT8 expression but significantly decreased CLDN4. Barrier function and HOPX protein in AT1 cells declined synchronously. Apoptotic programs were activated, indicating complete disruption of alveolar epithelial homeostasis. Consistent with the altered alveolar epithelial homeostasis described above, early focal inflammatory signals were already detectable by immunofluorescence as early as week 2. Specifically, immunofluorescence staining revealed a significantly higher number of TNF- α -positive cells in the CS2W group compared with WT controls, along with a significant increase in MAN at the same time point. It is important to note, however, that immunofluorescence is a semi-quantitative approach that reflects the local, spatially resolved abundance of the target within particular cell subpopulations, and is therefore particularly sensitive to early, focal elevations that may be confined to a minority of cells. By contrast, Western blotting measures the total protein content of the whole lung homogenate,

and thus represents an average signal across the entire tissue. When an inflammatory signal is still spatially restricted and has not yet undergone full tissue-wide amplification, the global protein level may fall below the threshold of statistical significance, even though locally abundant TNF- α -positive cells are already detectable. This explains why, at week 2, the CS2W group showed a significant increase in TNF- α -positive cells by immunofluorescence but no significant change in whole-lung TNF- α protein by Western blot. Notably, previous lung fibrosis-related studies reported significantly elevated KRT8, which differs from the stable KRT8 expression observed in this study. This discrepancy can be attributed to different pathological alterations between the two diseases: lung fibrosis is characterized by excessive and aberrant repair of alveolar epithelial cells, whereas COPD is characterized by exhaustion of the repair capacity of alveolar epithelial cells. Therefore, significant elevation of KRT8 does not occur in COPD. Taken together, these data support our staging model: at week 2 (compensatory phase), epithelial dysfunction manifests alongside only early, focal, low-grade inflammation that is captured by immunofluorescence; only by week 4 (decompensatory phase), do inflammatory mediators such as TNF- α rise to a significant and sustained tissue-wide level, coinciding with overt structural damage. The apparent discrepancy between the two techniques therefore does not contradict, but rather reinforces, the temporal ordering of epithelial dysfunction preceding overt inflammation and structural injury.

We discovered that in the CS exposure combined with LPS-induced model, the driving event of alveolar injury was unlikely to be direct damage to AT1 or stimulation by inflammatory factors, but rather abnormalities in the proliferative and differentiation capacity of AT2. Temporally, AT2 began to exhibit cell cycle arrest as early as 2 weeks, and the collapse of proliferative and differentiation function at 4 weeks preceded the elevation of inflammatory factors and extensive destruction of AT1 cell structure (which did not occur until 6 weeks). In terms of proportion changes, the continuous decrease in the SP-C/HOPX ratio indicated that AT2 loss exceeded AT1 loss, suggesting that the reduced self-renewal capacity of AT2 had a greater impact on alveolar homeostasis than AT1 apoptosis. Functionally, the arrest of AT2-to-AT1 differentiation directly resulted in the inability of AT1 to be replenished, leading to alveolar structural damage at its source. Therefore, our study suggests that the early driving event of alveolar injury is abnormalities in the proliferative and differentiation capacity of AT2, but this requires further validation through subsequent intervention experiments.

Regarding lung function assessment, the present study employed non-invasive pulmonary function testing. It should be noted that, although invasive measurements (with indices such as FEV0.1/FVC as the gold standard) more accurately reflect the

presence of airflow limitation, such procedures are highly invasive and lethal to experimental animals; mice generally expire shortly after the measurement, precluding repeated observations on the same animal. Because our study required dynamic, continuous tracking of lung function at multiple time points in the mice, in order to follow the evolution of lung function during disease progression, we selected the non-invasive approach, which allows repeated measurements on the same cohort across time points. While this choice sacrifices, to some extent, the precision of invasive indices in capturing the fine details of airflow limitation, it preserves the feasibility of longitudinal follow-up. As a complement, the establishment of the COPD model in this study is supported by relatively solid evidence (e.g., pathological and inflammatory parameters), and the non-invasive lung function data corroborate these histopathological/inflammatory findings, collectively supporting the presence of stable airflow limitation in the model. In addition, the modeling approach employed in this study has been previously validated and proven effective via invasive methodology in earlier investigations by our research group³⁰.

The temporal expression profiles of SIRT1 and Nrf2 were highly consistent with the progression of alveolar epithelial cell imbalance. At 2 weeks, both SIRT1 and Nrf2 were upregulated, supporting early proliferative repair of AT2 cells. At 4 weeks, both factors significantly decreased, resulting in reduced antioxidant capacity and impaired AT2 proliferative and differentiation capacity. After 4 weeks, both maintained persistently low expression levels, and the numbers and functions of both AT1 and AT2 cells continued to decline. Therefore, restoring the activity of SIRT1 or Nrf2 may be a key strategy to delay or reverse COPD progression. To investigate this, we performed intratracheal injection of SIRT1 overexpression and inhibition viruses in mice for *in vivo* validation.

The body weight change rate curves found that the SIRT1(+) group had the highest body weight change rate, the SIRT1(-) group had the lowest, and the control virus group showed no significant difference. This suggests that overexpression of SIRT1 can counteract the weight loss induced by CS exposure combined with LPS. SIRT1 may protect lung tissue structure by maintaining cellular metabolic capacity and preventing cellular functional failure. However, in the CS exposure combined with LPS-induced model, the effect of SIRT1 on body weight differed from previous studies. Previous studies found that increased SIRT1 led to weight loss, while decreased SIRT1 led to weight gain. In contrast, in our experiments, the SIRT1 overexpression group had higher body weight than the control group, while the SIRT1 inhibition group had lower body weight. This indicates that in the CS exposure combined with LPS-induced model, weight loss may be associated with reduced SIRT1 activity. SIRT1 overexpression rescued the weight loss caused by decreased

SIRT1 activity, while SIRT1 inhibition exacerbated weight loss. The result suggest that SIRT1 exerts a protective effect in the CS-induced mouse model.

SIRT1 overexpression significantly increased the number of SP-C and HOPX-positive cells and elevated the SP-C/HOPX ratio. Moreover, the protein expression level of SP-C was even slightly higher than that in the WT group. These findings suggest that SIRT1 not only restores AT2 numbers but also significantly enhances AT2 functional activity. This study demonstrates in an in vivo model that SIRT1 exerts protective effects in the CS exposure context primarily by targeting AT2 numbers and function. This finding provides a cellular-level mechanistic explanation for previously observed protective effects of SIRT1 against emphysema. Recent single-cell sequencing results revealed that the core mechanism of alveolar regeneration dysfunction in COPD patients is impaired self-renewal capacity of AT2 cells, and restoration of AT2 cell function can significantly ameliorate emphysema pathological changes. The regulatory effects of SIRT1 on AT2 cell numbers and function identified in our study provide new insights for regenerative therapy of COPD.

Our study demonstrates that SIRT1 can effectively block TNF- α activation. The observation that SIRT1 overexpression increased pro-TNF- α while decreasing soluble-TNF- α suggests that SIRT1 may inhibit the proteolytic cleavage of pro-TNF- α to its active form. Previous studies on SIRT1-mediated regulation of TNF- α have primarily focused on transcriptional control: SIRT1 is known to deacetylate the p65 subunit of NF- κ B, thereby inhibiting NF- κ B signaling activation and suppressing TNF- α mRNA expression at the transcriptional level. However, our findings raise the possibility of an additional, post-transcriptional/post-translational layer of regulation operating downstream of transcription. The conversion of pro-TNF- α to soluble-TNF- α is mediated by the proteolytic cleavage activity of A Disintegrin and Metalloproteinase Domain 17 (ADAM17), and previous research has shown that SIRT1 can inhibit ADAM17 activity and downregulate its expression. We therefore propose, as a hypothesis, that SIRT1 overexpression may inhibit ADAM17 activity, thereby limiting the proteolytic cleavage of pro-TNF- α to soluble-TNF- α ; this would explain the accumulation of pro-TNF- α and the marked reduction of the active form in the SIRT1 overexpression group. It should be emphasized that this proposed ADAM17-dependent, post-translational mechanism is a hypothesis inferred from our data and from previous reports, and it has not been directly tested in the present study. Direct measurements of ADAM17 activity/expression and functional dissection of this pathway will be required to validate this proposal. Future studies are therefore warranted to clarify the specific molecular mechanisms underlying this regulation.

Our findings align with the existing literature, further confirming the protective

role of SIRT1 in CS-model. Different from previous studies, our longitudinal intervention study provides direct *in vivo* causal evidence, moving beyond correlative observations. The data support that SIRT1 exerts its protective effects by inhibiting pro-apoptotic and pro-inflammatory pathways, consistent with its known functions. Notably, we found that in the context of CS-model, SIRT1 has a more pronounced impact on the proliferative and differentiation capacities of alveolar epithelial cells. SIRT1 not only promoted the proliferation of AT2 cells but, more importantly, enhanced their ability to differentiate into AT1 cells. This positive regulation of alveolar epithelial cell stemness and differentiation potential may be the core mechanism through which SIRT1 achieves long-term protection and promotes structural repair. Our data also suggest an interaction between SIRT1 and Nrf2 under CS-model conditions, but this relationship is not a simple hierarchical regulation. SIRT1 plays a more dominant role in rescuing cell viability and promoting proliferation, whereas Nrf2 has a greater influence in resisting apoptosis. This indicates that their downstream effectors differ: SIRT1 likely orchestrates a more global regulation of cellular metabolism, stress response, and epigenetic state, providing a foundation for cell survival and proliferation; whereas Nrf2 is more specialized in combating oxidative stress—the direct cause of apoptotic damage. This study used the A549 lung adenocarcinoma cell line for intervention experiments on the SIRT1-Nrf2 regulatory network at the cellular level. It must be acknowledged that A549 is of tumor origin, and its proliferation, apoptosis, and differentiation behaviors fundamentally differ from those of primary AT2 cells; consequently, the *in vitro* results carry intrinsic limitations regarding their physiological relevance to normal alveolar epithelium. The choice of A549 was based primarily on its extensive use in studies of alveolar epithelial oxidative stress and inflammatory mechanisms and on its high reproducibility for intervention, and it was positioned as a screening tool to drive mechanistic hypothesis generation.

The present study is not entirely without *in vivo* support: in the *in vivo* model, SIRT1 intervention was accompanied by concordant changes in Nrf2 expression, in line with the *in vitro* trend, providing whole-organism corroboration for the relationship between the two. However, we must candidly acknowledge that this *in vivo* evidence reflects correlation and cannot yet establish causality. We did not perform rescue experiments combining SIRT1 overexpression with specific Nrf2 inhibition *in vivo* or in primary AT2 cells; therefore, we cannot confirm whether SIRT1-mediated pulmonary protection is necessarily dependent on the Nrf2 pathway.

Given these limitations, we cautiously define the conclusion that “SIRT1 primarily regulates proliferation, Nrf2 primarily regulates apoptosis, and the two constitute a complementary regulatory network rather than a simple cascade” as a

preliminary mechanistic hypothesis supported by in vitro models, whose causal and physiological relevance still requires the following follow-up work: recapitulating the SIRT1-Nrf2 intervention findings in primary AT2 cells or organoids; performing in vivo Nrf2 blockade/rescue experiments to demonstrate pathway dependence. We believe these qualifications match the strength of the available evidence.

With respect to the pharmacological manipulation of SIRT1, the activators and inhibitors used here primarily act by altering enzyme activity, whereas our WB measurement reflects protein abundance. It should nevertheless be recognized that SIRT1 protein abundance can partially mirror its state of activation: many SIRT1 activators enhance activity through allosteric regulation while also increasing protein stability, so treatment-associated changes in abundance are expected to accompany—rather than contradict—the changes in activity. Moreover, the specificity of the pharmacological interventions is supported by the existing functional validation in this study: the phenotypes induced by the activator/inhibitor (proliferation, apoptosis, and barrier function) are directionally consistent with those obtained through SIRT1 overexpression or knockdown. Together, these data support the effectiveness of SRT1720 and EX527 as a reasonable manipulation check for SIRT1 in the present study.

The clinical significance of this study lies in pointing out a clear therapeutic window for early intervention targeting the proliferative repair capacity of alveolar epithelial cells in at-risk individuals, before the formation of structural emphysema (approximately 4 weeks in this study). For smokers or early-stage COPD patients with preserved lung function, monitoring alveolar epithelial cell function or biomarkers related to SIRT1 activity may aid in the very early identification of disease risk. Secondly, targeting SIRT1 through early intervention, such as with SIRT1 activators (e.g., SRT1720, resveratrol), may delay or even halt the onset and progression of COPD. This treatment strategy, which addresses the early driver of the disease (impaired epithelial repair), may be more fundamental compared to traditional approaches that primarily target late-stage symptoms (e.g., bronchodilators or anti-inflammatory agents).

The investigation of COPD pathogenic mechanisms has historically been skewed toward end-stage pathological manifestations following disease establishment. The critical transitional period spanning from the initial exposure to risk factors through the onset of irreversible alveolar structural destruction remains substantially unexplored, representing a significant gap in current COPD research. In this study, we systematically investigated this pivotal window period and constructed a comprehensive temporal pathological change atlas of alveolar epithelial cells during early COPD progression. Our analysis preliminarily delineated the core window

during which alveolar injury transitions from a reversible to an irreversible state. Importantly, our findings suggest that dysregulation of AT2 cell proliferation and differentiation likely serves as a driving factor in the progressive destruction of alveolar structure, providing a potential mechanistic target for therapeutic intervention. Furthermore, our study identified SIRT1 as a critical protective target for pre-COPD intervention. We demonstrate that SIRT1 exerts lung-protective effects through the regulation of AT2 proliferation and differentiation. We also preliminarily identified a novel cooperative regulatory network between SIRT1 and Nrf2 under COPD pathological conditions, operating through a non-canonical mechanism that diverges from traditional upstream-downstream relationships. This research fills a significant knowledge gap regarding the functional roles and molecular mechanisms of SIRT1 during the pre-COPD disease course, before irreversible damage occurs. Our findings provide a solid experimental foundation for developing targeted interventional therapies aimed at early-stage COPD etiology, with potential implications for early screening protocols and high-risk population risk stratification strategies.

However, this study has several limitations. First, we only established the temporal relationship that alveolar structural damage precedes the elevation of inflammatory factors, without validating the causal relationship between the two through in vivo intervention experiments. Second, we only detected these changes at the whole tissue level using immunofluorescence and Western blot, without resolving the heterogeneity changes of alveolar epithelial cell subpopulations at single-cell resolution through techniques such as single-cell sequencing and lineage tracing. Third, our study only focused on the exposure-induced process of COPD development, without conducting a staged investigation of the disease course after successful COPD modeling. Our time line only covered the transition from health to pathology, without precisely distinguishing the various clinical stages after COPD formation. Fourth, we only detected the effects of SIRT1 overexpression and inhibition at a single time point, without performing temporal dynamic detection at various key nodes from 0 to 12 weeks. Fifth, we did not deeply investigate the specific mechanisms by which SIRT1 affects TNF- α at the post-translational level, only proposing hypotheses for future studies to elaborate. Sixth, the validation of the CSE and LPS co-induced model simulating COPD alveolar epithelial cell injury was insufficient. Additionally, the lack of single-cell sequencing and lineage tracing technologies prevented us from resolving the heterogeneity of cell subpopulations. Furthermore, the lack of clinical sample validation means that the generalizability of our animal study results to human patients still requires further verification. Moreover, although the in vivo measurements showed concordant changes in Nrf2 expression following SIRT1 intervention, these data provide correlative-rather than causal-evidence, because no in

vivo rescue experiment (e.g., SIRT1 overexpression combined with specific Nrf2 inhibition) was performed in the present study. We therefore interpret the “complementary regulatory network” between SIRT1 (predominantly regulating proliferation) and Nrf2 (predominantly regulating apoptosis) as a suggestive inference rather than a definitive conclusion. This pathway dependence requires further validation, ideally through Nrf2-blockade rescue experiments in primary AT2 cells and dedicated in vivo rescue studies, which we have identified as an important direction for future work.

We acknowledge a limitation in the temporal design of the intervention experiments. Post-intervention evaluation was confined to the week-12 endpoint, without intermediate time points (e.g., weeks 6, 8, and 10). The present results therefore reflect between-group differences at week 12 only and cannot distinguish whether SIRT1 intervention delayed disease progression or reversed established alveolar injury, nor can they directly validate the hypothesis that targeting the week-4 decompensation node halts disease progression. Accordingly, we describe the intervention effect as a difference observed at the week-12 endpoint, and consider its specific influence on the temporal dynamics of the disease, as well as the value of intervening at the week-4 node, as questions to be addressed by future multi-time-point studies.

To address the limitations identified in our current study, we propose a multifaceted research program designed to advance our understanding of COPD pathogenesis and therapeutic intervention. First, we will conduct comprehensive in vivo intervention studies to establish definitive causal relationships between alveolar structural destruction and inflammatory cytokine elevation. Concurrently, we will employ molecular biology approaches to further elucidate the underlying molecular mechanisms driving these pathological changes. Second, leveraging single-cell transcriptomics and lineage tracing methodologies, we will achieve high-resolution characterization of functional abnormalities within specific AT2 cell subpopulations. This will enable comprehensive mapping of the cellular evolution trajectory during the entire AT2-to-AT1 differentiation process. Third, we will establish a more physiologically relevant and refined COPD animal model by extending the disease induction duration. Integrating pulmonary function measurements with histopathological analysis, we will construct a precise staging framework (early: mild pulmonary function decline with mild alveolar destruction; middle: moderate impairment with significant damage; late: severe impairment with severe destruction). This staging system will facilitate investigation of optimal intervention windows and stage-specific therapeutic strategies. Fourth, we will implement multi-time-point SIRT1 interventions across the entire disease timeline. Through time-series data

analysis, we will identify the optimal timing for SIRT1-mediated intervention, precisely delineating the critical regulatory window from initial exposure to disease manifestation. Fifth, we will conduct mechanistic investigations into the hypothesis that SIRT1 overexpression inhibits ADAM17 activity, thereby preventing effective enzymatic cleavage of pro-TNF- α to soluble TNF- α , resulting in pro-TNF- α accumulation with concomitant reduction of the active form. Sixth, we will validate our in vitro COPD injury model through comprehensive inflammatory marker analysis and oxidative stress assessment to strengthen the scientific validity of our conclusions. Seventh, when resources permit, we will employ advanced single-cell and lineage tracing technologies to provide comprehensive insights into cell subpopulation dynamics in COPD. Finally, We will need to validate these findings in a simple chronic CS exposure model in the future.

List of abbreviations

AAV: adeno-associated virus

ADAM17: A Disintegrin and Metalloproteinase Domain 17

ADI: alveolar differentiation intermediate cells

AMPK: AMP-activated protein kinase

AT1: type I alveolar epithelial cells

AT2: type II alveolar epithelial cells

BALF: Bronchoalveolar Lavage Fluid

CCK-8: Cell Counting Kit-8

CLDN4: Claudin 4

COPD: Chronic obstructive pulmonary disease

CS: cigarette smoke

CSE: Cigarette smoke extract

CTGF: connective tissue growth factor

DATP: damage-associated transient progenitors

DI: destruction index

DMEM: Dulbecco's Modified Eagle Medium
EF50: mid-expiratory flow
EMT: Epithelial-Mesenchymal Transition
FOXO: Forkhead Box O
FOXO3: Forkhead box O3
HE: Hematoxylin and Eosin
HOPX: HOP homeobox
KRT8: Keratin 8
LGALS3: galectin-3
LPS: lipopolysaccharide
MAN: mean number of alveoli per field of view
MLI: mean linear intercept
Mvb: minute ventilation
NF- κ B: Nuclear Factor kappa-B
NLRP3: NOD-like receptor thermal protein domain associated protein 3
Nrf2: Nuclear factor erythroid 2-related factor 2
p53: Tumor Protein p53
PATs: pre-alveolar type 1 transitional cells
PAU: Apnea index
PCNA: Proliferating Cell Nuclear Antigen
Penh: Airway stenosis index
PFA: Paraformaldehyde
PI: Propidium Iodide
SIRT1: Silent information regulator 1
SP-C: surfactant protein C
SPF: specific pathogen-free
TNF- α : Tumor Necrosis Factor-alpha
WB: Western Blot
WBP: whole-body plethysmography
WT: wild-type

Declarations

Author's contributions

Wei Xiong and Xiaotian Dai conceived this project. Cheng Liang performed the major animal experiments, cell experiments and molecular experiments. Weijia Zhou and Cheng Liang contributed to the design of animal experiments and animal operations. Wu Li, Qiang Zeng, Lei Xue and Weijia Zhou contributed to the western blot, HE staining and Immunofluorescence analysis of data. Wei Xiong and Xiaotian

Dai provided the fund for this study. Cheng Liang, Wei Xiong and Xiaotian Dai wrote and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

Data and materials can be made available upon written request to the corresponding author.

Ethics approval and consent to participate

All animal studies were performed following guidelines from the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All protocols were approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University (Approval No. AMUWEC20252136).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure legend

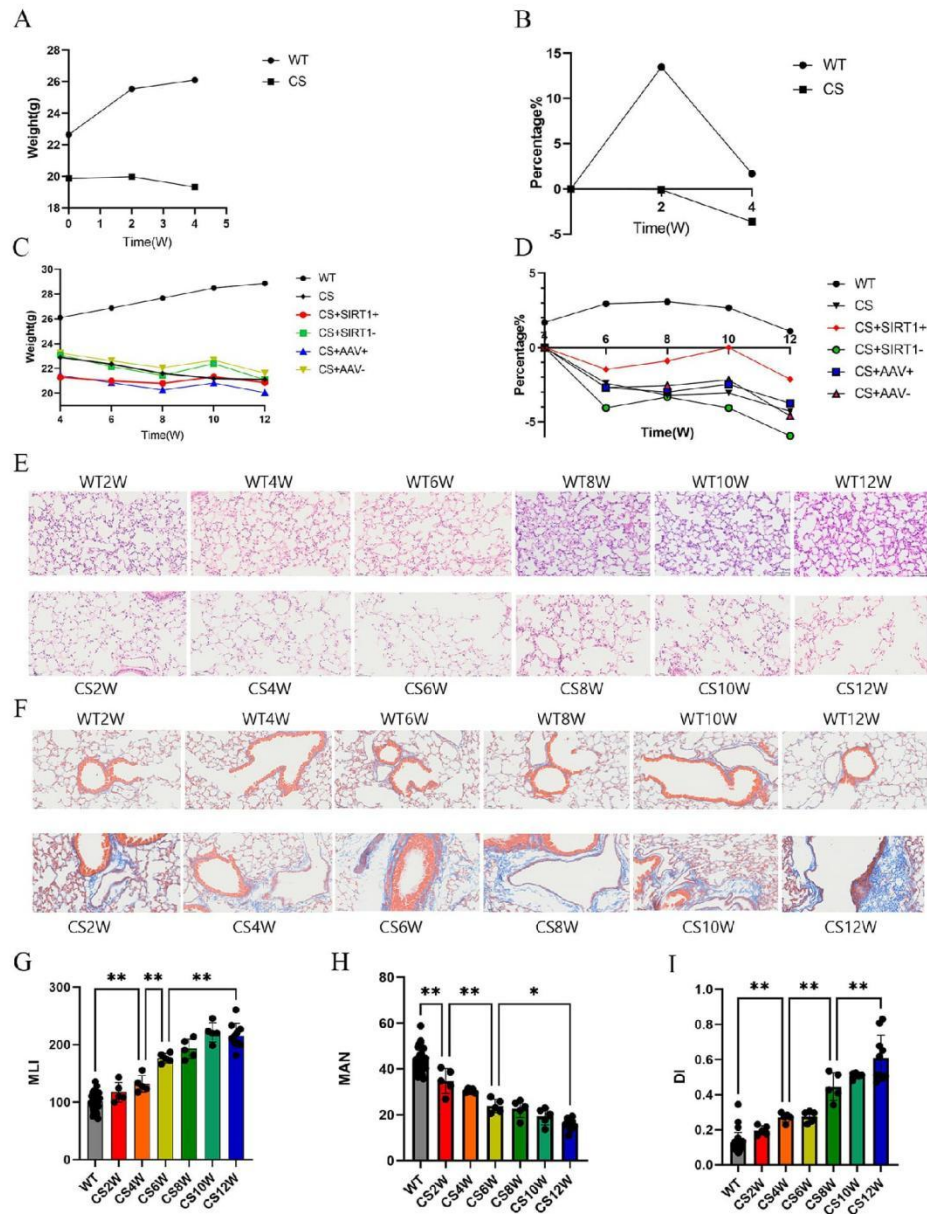


Figure 3. Effects of Cigarette Smoke Exposure on Mouse Body Weight and Lung Histopathological Changes (A) Absolute change curve of mouse body weight before grouping. (B) Rate of change curve of mouse body

weight before grouping. (C) Absolute change curve of mouse body weight after grouping. (D) Rate of change curve of mouse body weight after grouping. (E) H&E staining of mouse lung tissue (magnification $\times 200$; scale bar = 50 μm). (F) Masson staining of mouse lung tissue (magnification $\times 200$; scale bar = 50 μm). (G) Mean Linear Intercept (MLI) index for each group. (H) Mean Alveolar Number (MAN) per field for each group. (I) Destruction Index (DI) for each group.

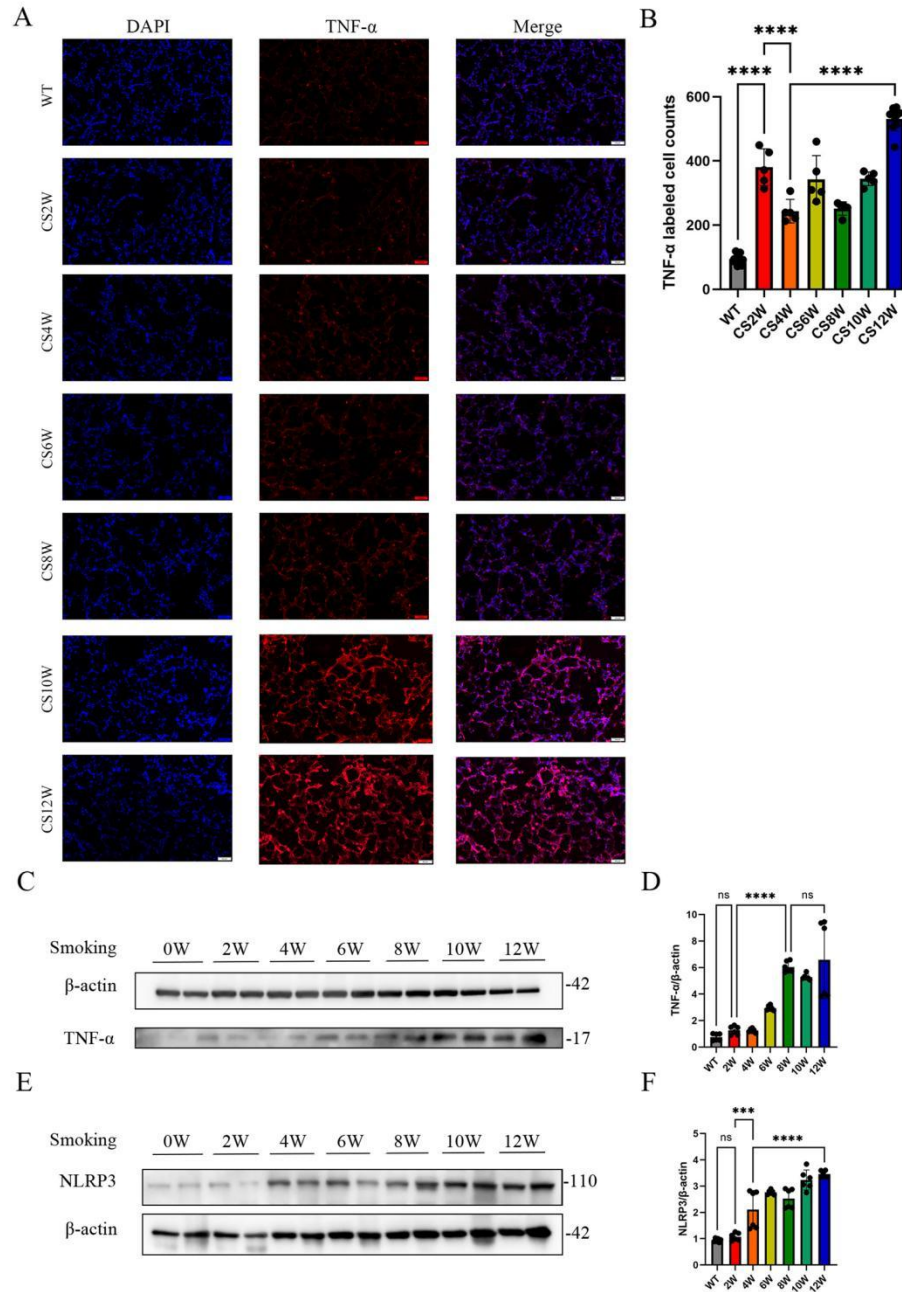


Figure 4. Effects of Cigarette Smoke Exposure on Inflammation in Mice (A) Immunofluorescence images of TNF- α for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of TNF- α fluorescent positive cells for each group. (C) Expression of TNF- α in lung tissues detected by Western blot for each group. (D) Relative protein expression level of TNF- α / β -actin for each group. (E) Expression of NLRP3

in lung tissues detected by Western blot for each group. (F) Relative protein expression level of NLRP3/ β -actin for each group.

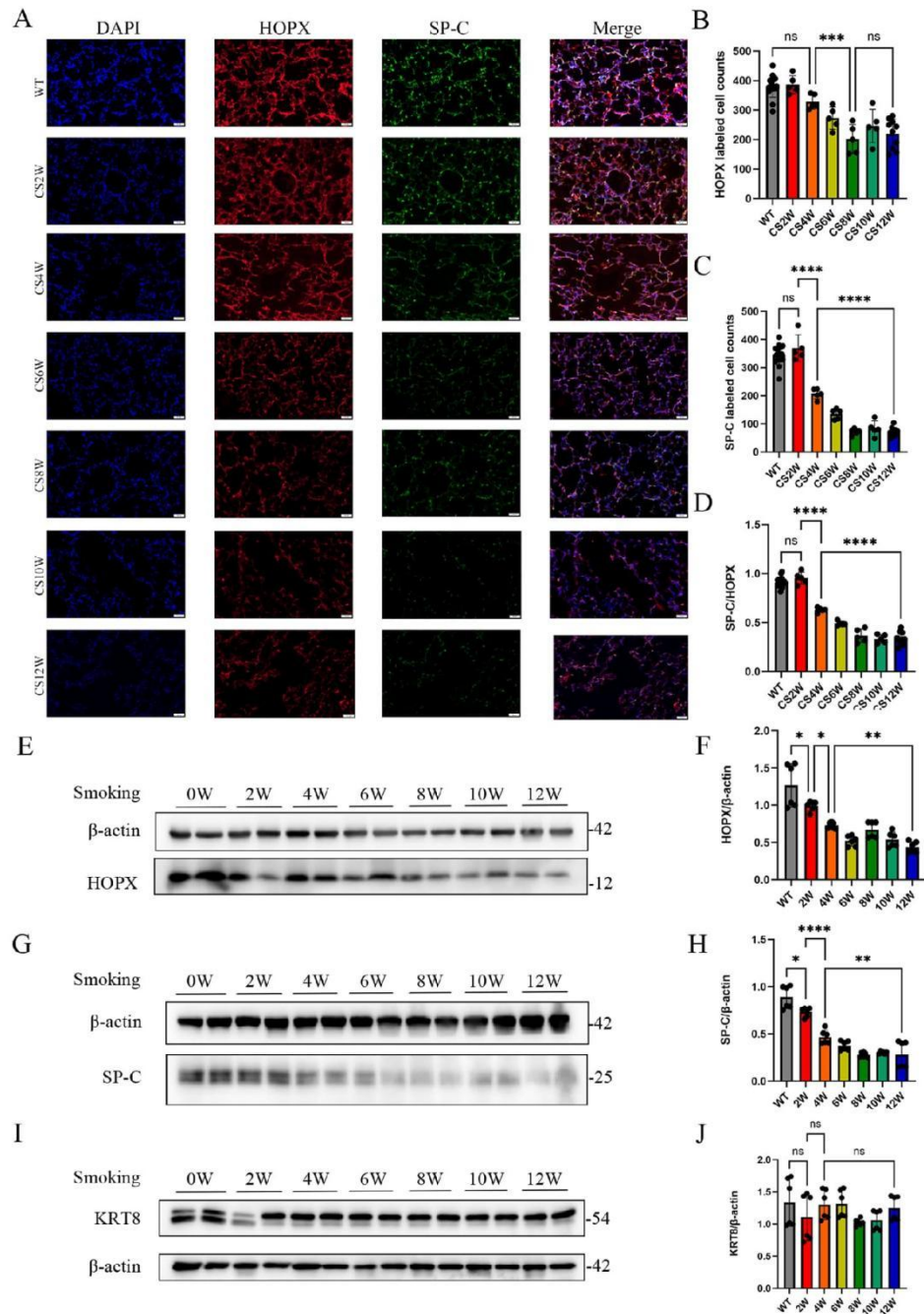


Figure 5. Temporal Changes in Alveolar Epithelial Cells Following CS Exposure (A) Immunofluorescence images of HOPX and SP-C for each group (magnification $\times 200$; scale bar = 50 μ m). (B) Count of HOPX fluorescent positive cells for each group. (C) Count of SP-C fluorescent positive cells for each group. (D) Ratio of SP-C/HOPX for each group. (E) Expression of HOPX in lung tissues detected by Western blot

for each group. (F) Relative protein expression level of HOPX/ β -actin for each group. (G) Expression of SP-C in lung tissues detected by Western blot for each group. (H) Relative protein expression level of SP-C/ β -actin for each group. (I) Expression of KRT8 in lung tissues detected by Western blot for each group. (J) Relative protein expression level of KRT8/ β -actin for each group.

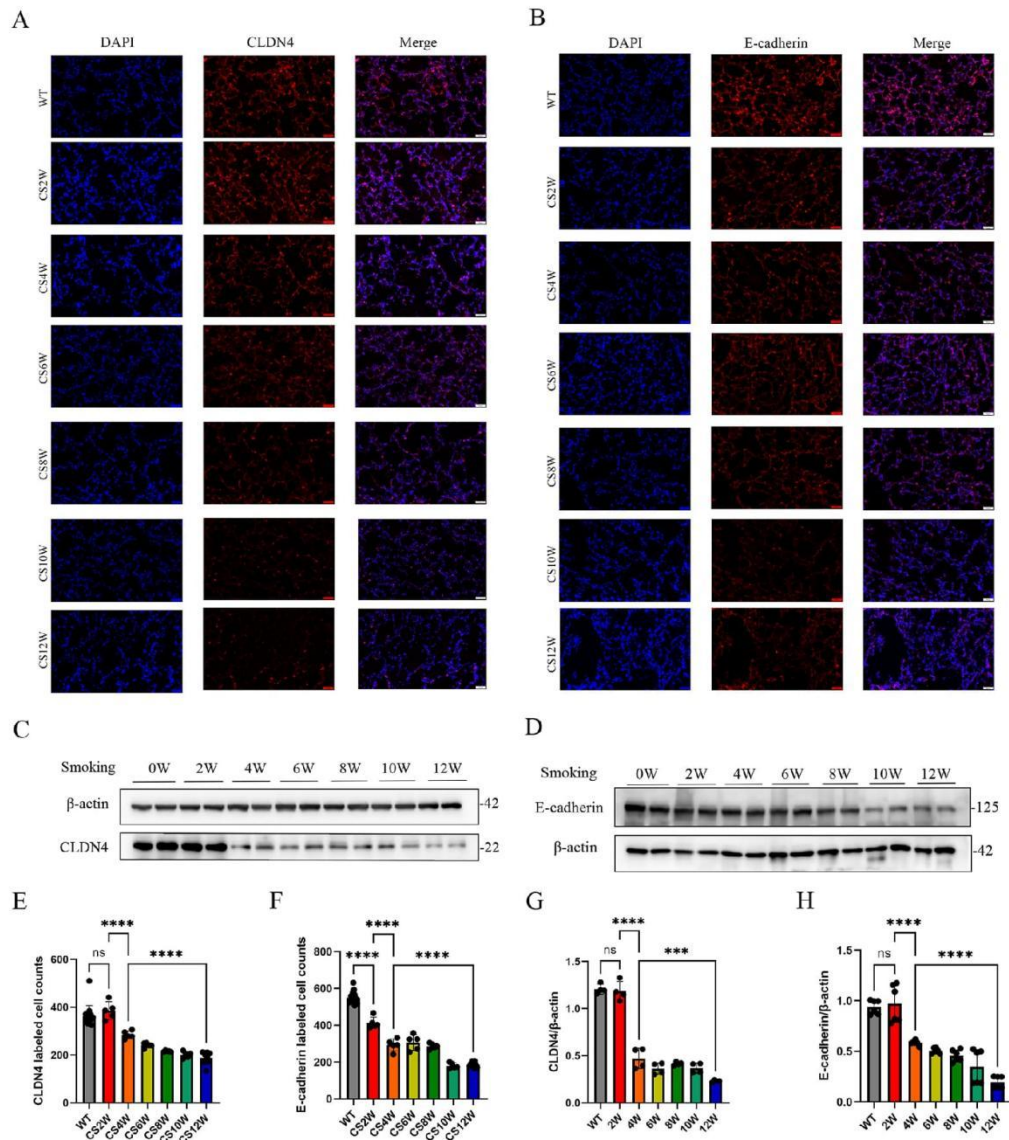


Figure 8. Temporal Changes in Pulmonary Barrier Function Following CS Exposure (A) Immunofluorescence images of CLDN4 for each group (magnification $\times 200$; scale bar = 50 μ m). (B) Immunofluorescence images of E-cadherin for each group (magnification $\times 200$; scale bar = 50 μ m). (C) Expression of CLDN4 in lung tissues detected by Western blot for each group. (D) Expression of E-cadherin in

lung tissues detected by Western blot for each group. (E) Count of CLDN4 fluorescent positive cells for each group. (F) Count of E-cadherin fluorescent positive cells for each group. (G) Relative protein expression level of CLDN4/ β -actin for each group. (H) Relative protein expression level of E-cadherin/ β -actin for each group.

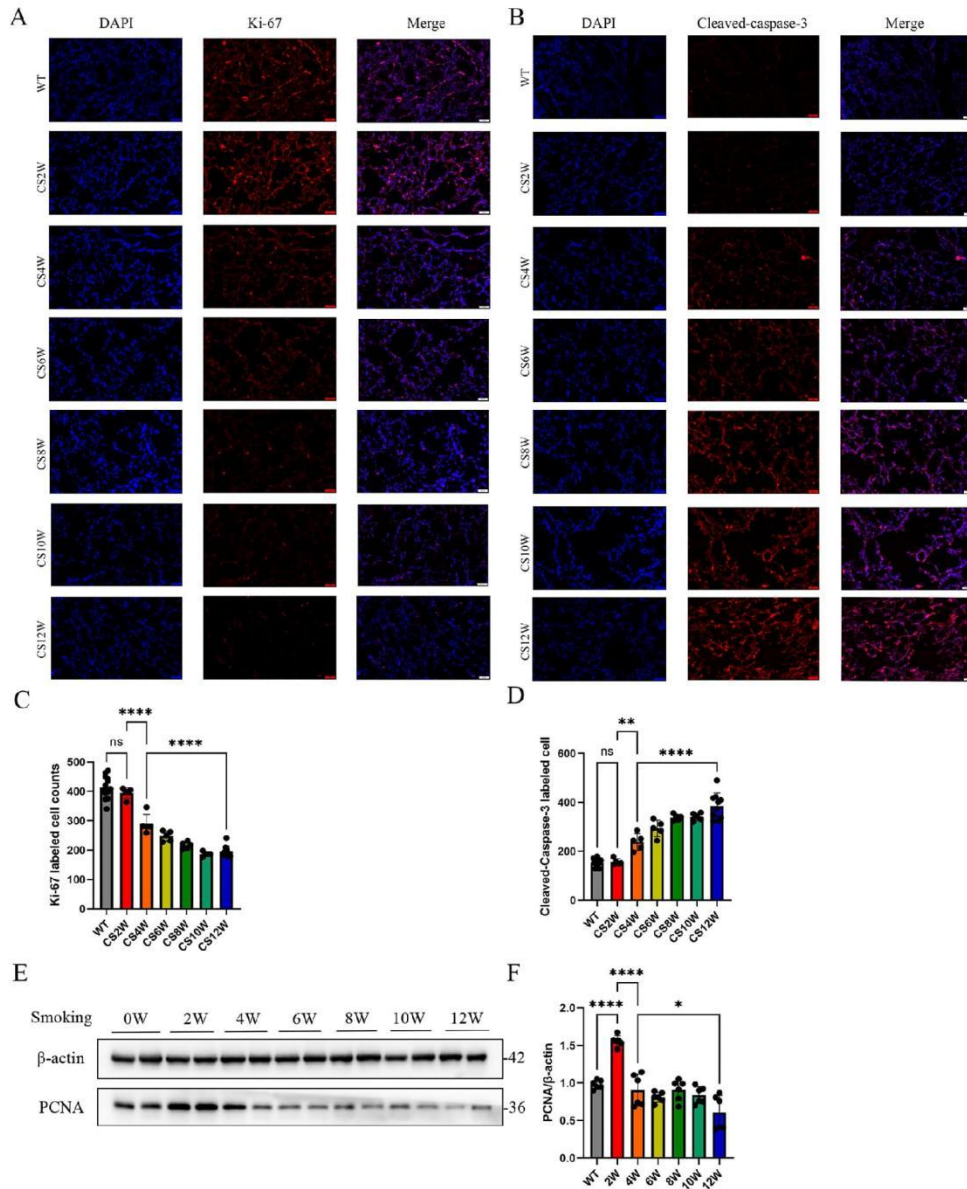


Figure 9. Temporal Changes in Lung Proliferation and Apoptosis Function Following CS Exposure (A) Immunofluorescence images of Ki-67 for each group (magnification $\times 200$; scale bar = 50 μ m). (B) Immunofluorescence images of cleaved-caspase-3 for each group (magnification $\times 200$; scale bar = 50 μ m). (C) Count of Ki-67 fluorescent positive cells for each group. (D) Count of cleaved-caspase-3 positive cells for each

group. (E) Expression of PCNA in lung tissues detected by Western Blot for each group. (F) Relative protein expression level of PCNA/ β -actin for each group.

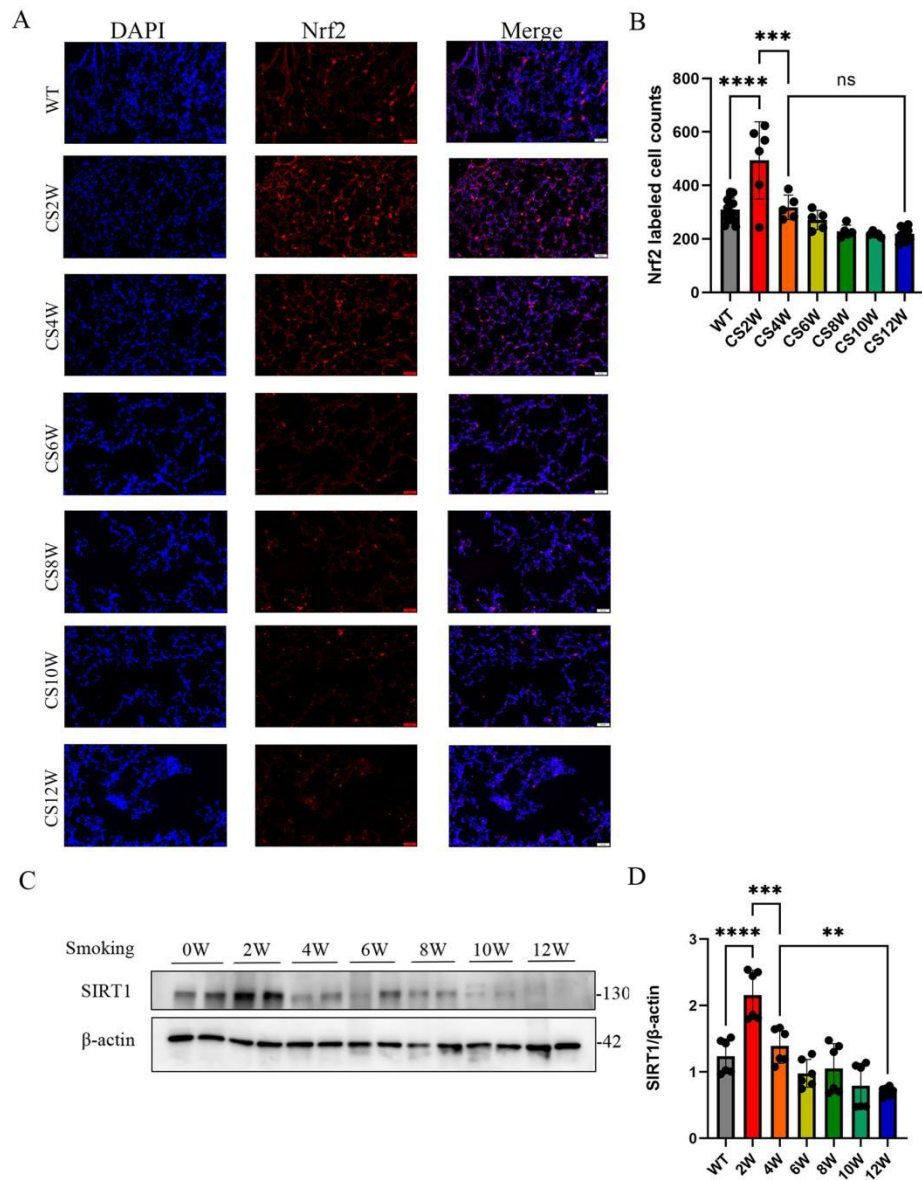


Figure 10. Temporal Changes in SIRT1 and Nrf2 Molecules Following CS Exposure
 (A) Immunofluorescence images of Nrf2 for each group (magnification $\times 200$; scale bar = 50 μ m). (B) Count of Nrf2 fluorescent positive cells for each group. (C) Expression of SIRT1 in lung tissues detected by Western Blot for each group. (D) Relative protein expression level of SIRT1/ β -actin for each group.

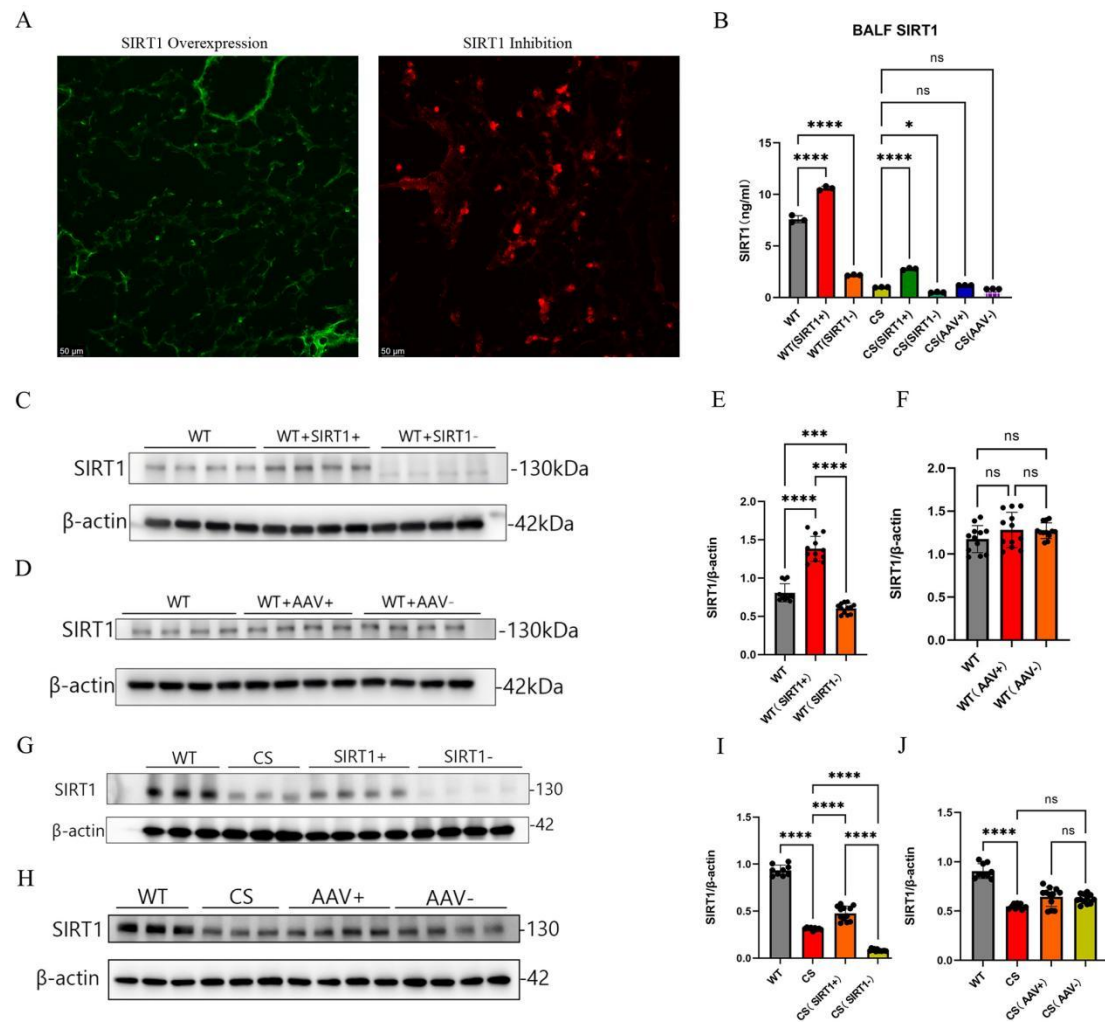


Figure 11. Validation of SIRT1 Overexpression and Inhibition Models
 (A) Fluorescent images of lung tissue adenovirus for SIRT1 overexpression and inhibition. (B) Changes in SIRT1 content in bronchoalveolar lavage fluid (BALF) of each group. (C) Expression of SIRT1 in lung tissues of WT, WT + SIRT1 overexpression, and WT + SIRT1 inhibition groups detected by Western Blot. (D) Expression of

SIRT1 in lung tissues of WT, WT + blank overexpression (AAV+), and WT + blank inhibition (AAV-) groups detected by Western Blot. (E) Relative protein expression level of SIRT1/ β -actin for each group. (F) Relative protein expression level of SIRT1/ β -actin for each group. (G) Expression of SIRT1 in lung tissues of WT, CS, CS + SIRT1 (+), and CS + SIRT1 (-) groups detected by Western Blot. (H) Expression of SIRT1 in lung tissues of WT, CS, CS + AAV (+), and CS + AAV (-) groups detected by Western Blot. (I) Relative protein expression level of SIRT1/ β -actin for each group. (J) Relative protein expression level of SIRT1/ β -actin for each group.

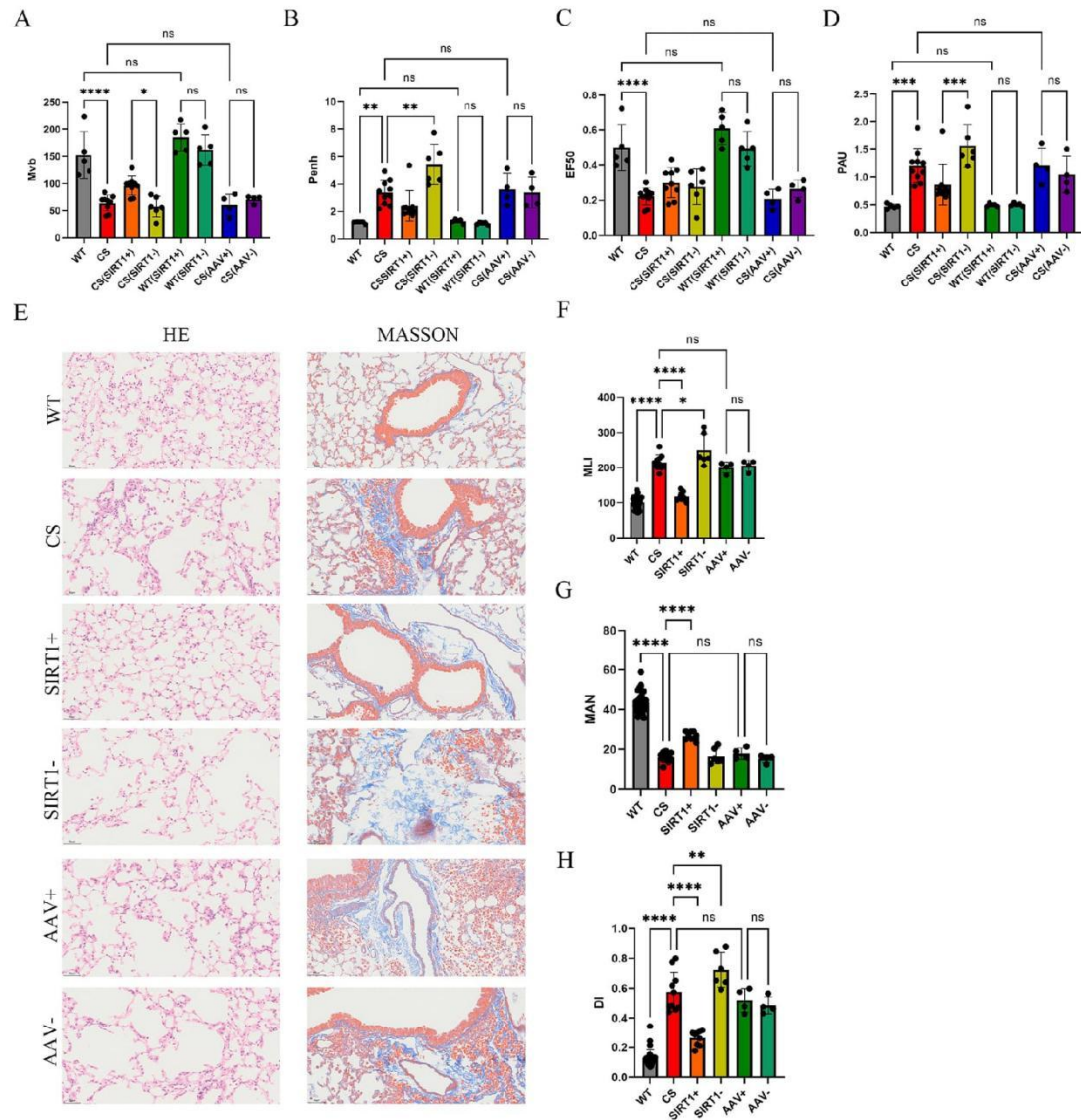


Figure 12. Effects of SIRT1 Overexpression and Inhibition on Lung Function and Lung Histopathology in the CS Model
 (A) Measurement of minute ventilation (Mvb) by non-invasive lung function test for each group. (B) Measurement of airway narrowing index (Penh) by non-invasive lung function test for each group. (C) Measurement of mid-expiratory flow (EF50) by non-invasive lung function test for each group. (D) Measurement of apnea index

(PAU) by non-invasive lung function test for each group. (E) HE and MASSON staining of mouse lung tissues for each group (magnification $\times 200$; scale bar = 50 μm). (F) Mean linear intercept (MLI) index for each group. (G) Number of alveoli per field of view (MAN) index for each group. (H) Alveolar destruction index (DI) for each group.

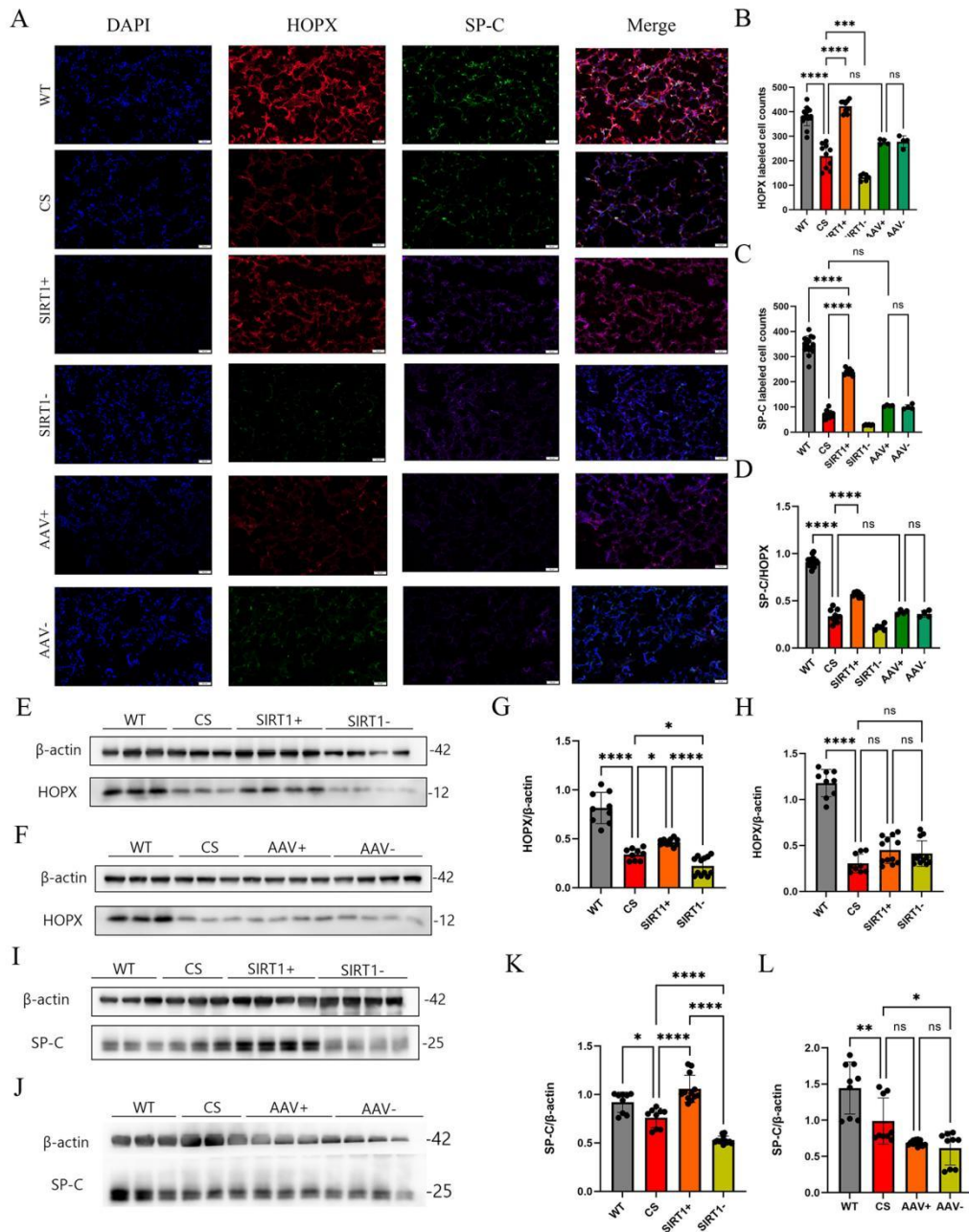


Figure 13. Effects of SIRT1 Overexpression and Inhibition on the Number of Alveolar Epithelial Cells (A) Immunofluorescence images of HOPX and SP-C for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of HOPX fluorescence-positive cells for each group. (C) Count of SP-C fluorescence-positive cells for each group. (D) SP-C/HOPX ratio for each group. (E) Expression of HOPX in lung tissues of SIRT1 overexpression and inhibition groups detected by Western blot. (F) Expression of HOPX in lung tissues of blank overexpression and inhibition groups detected by Western blot. (G) Quantification of HOPX protein levels (HOPX/ β -actin) for each group. (H) Quantification of HOPX protein levels (HOPX/ β -actin) for each group. (I) Expression of SP-C in lung tissues of SIRT1 overexpression and inhibition groups detected by Western blot. (J) Expression of SP-C in lung tissues of blank overexpression and inhibition groups detected by Western blot. (K) Quantification of SP-C protein levels (SP-C/ β -actin) for each group. (L) Quantification of SP-C protein levels (SP-C/ β -actin) for each group.

and inhibition groups detected by Western blot. (G-H) Relative protein expression level of HOPX/ β -actin for each group. (I) Expression of SP-C in lung tissues of SIRT1 overexpression and inhibition groups detected by Western blot. (J) Expression of SP-C in lung tissues of blank overexpression and inhibition groups detected by Western blot. (K-L) Relative protein expression level of SP-C/ β -actin for each group.

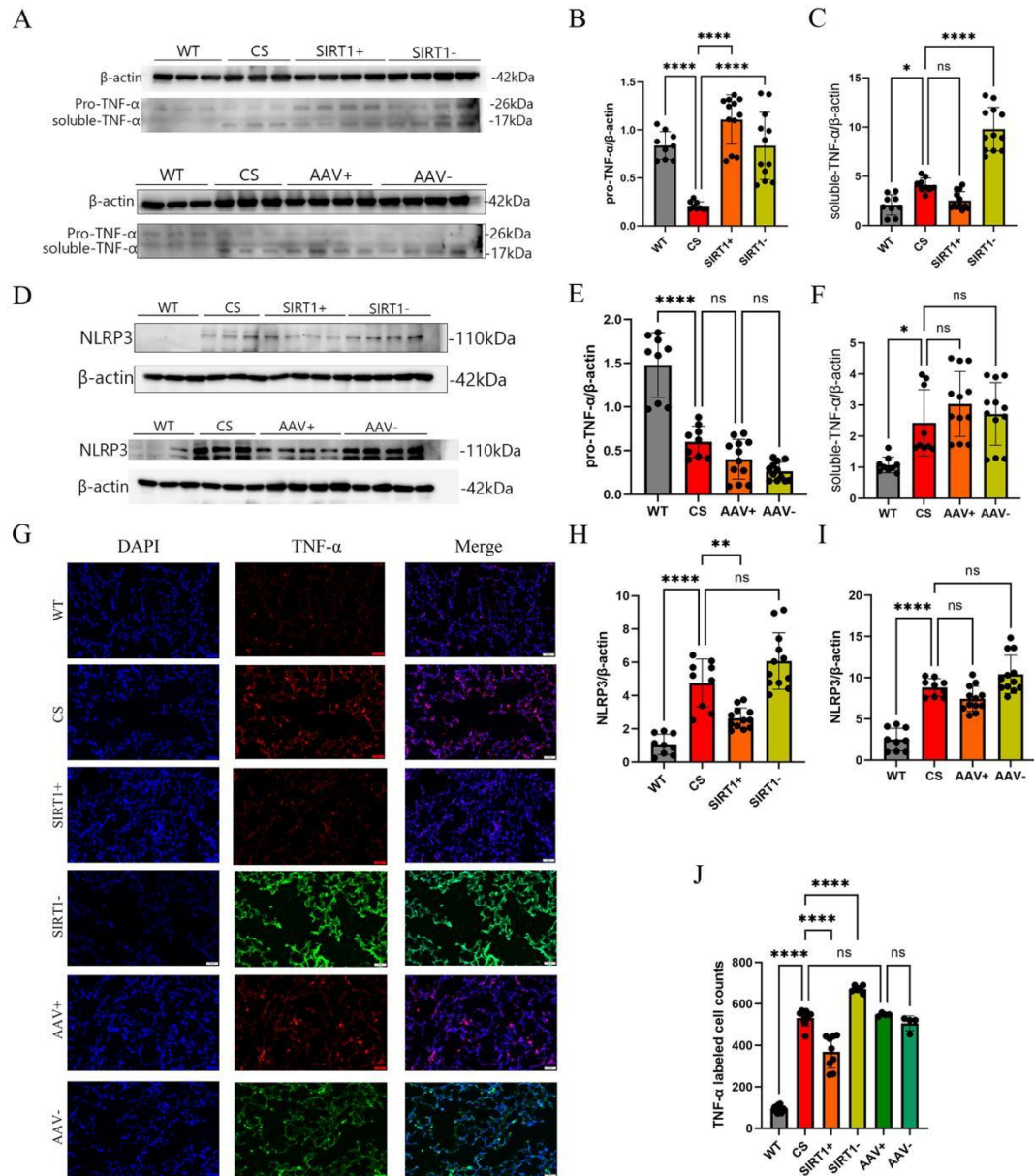


Figure 14. Effects of SIRT1 Overexpression and Inhibition on Inflammation in CS Model (A) Expression of TNF- α in lung tissues of each group detected by Western blot. (B) Relative protein expression level of pro-TNF- α / β -actin for each group. (C) Relative protein expression level of soluble-TNF- α / β -actin for each group. (D) Expression of NLRP3 in lung tissues of each group detected by Western blot. (E) Relative protein expression level of pro-TNF- α / β -actin for each group. (F) Relative protein expression level of

soluble-NLRP3/ β -actin for each group. (G) Immunofluorescence images of TNF- α for each group (magnification $\times 200$; scale bar = 50 μ m). (H-I) Relative protein expression level of NLRP3/ β -actin for each group. (J) Count of TNF- α fluorescence-positive cells for each group.

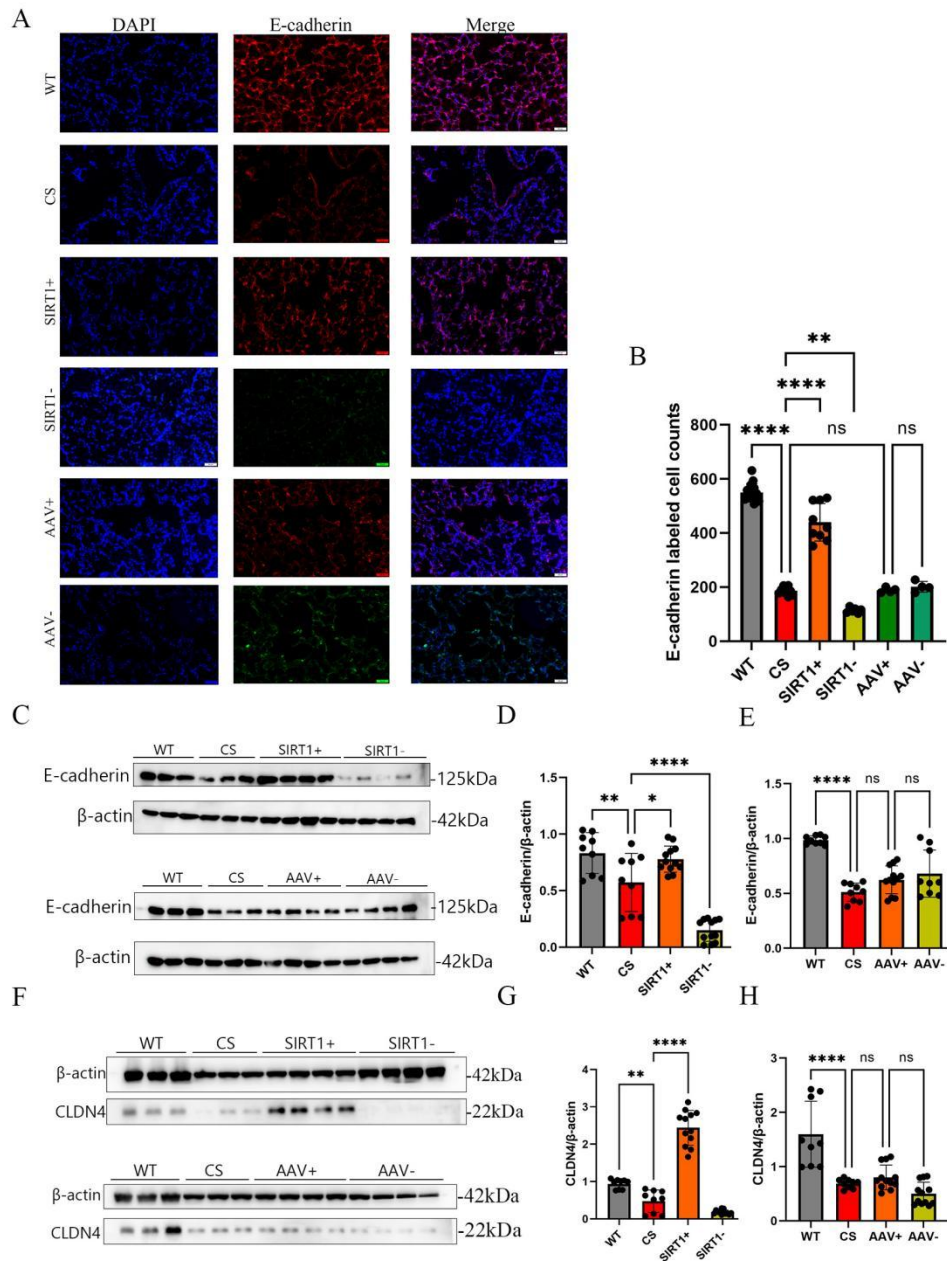


Figure 15. Effects of SIRT1 Overexpression and Inhibition on Barrier Function in CS Model (A) Immunofluorescence images of E-cadherin for each group (magnification $\times 200$; scale bar = 50 μ m). (B) Count of E-cadherin fluorescence-positive cells for each group. (C) Expression of E-cadherin in lung tissues of each group detected by Western blot. (D-E) Relative protein expression level of E-cadherin/ β -actin for each group. (F) Expression of CLDN4 in lung tissues of each group detected by Western blot. (G-H) Relative protein expression level of CLDN4/ β -actin for each group.

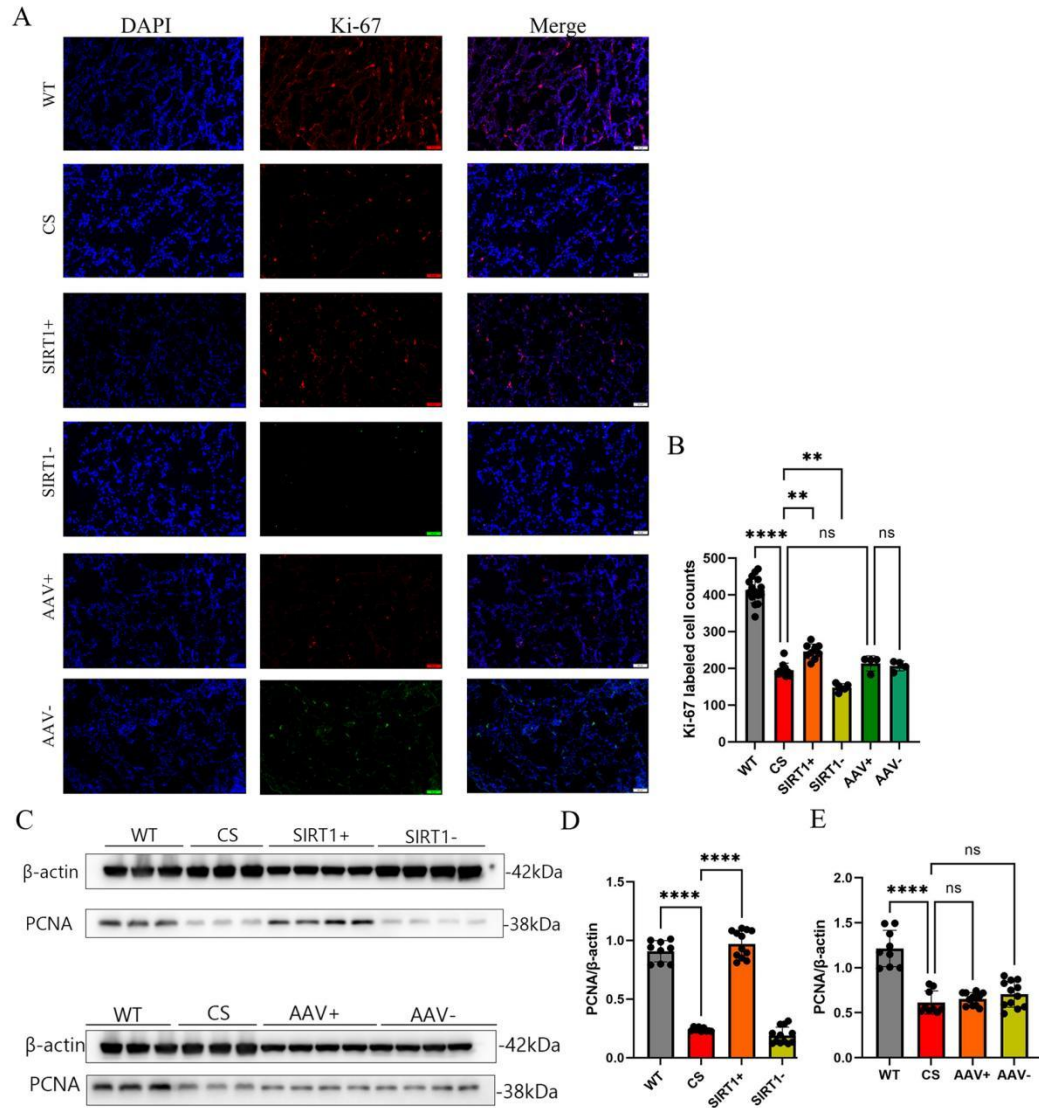


Figure 16. Effects of SIRT1 Overexpression and Inhibition on Proliferative Function in CS Model
 (A) Immunofluorescence images of Ki-67 for each group (magnification $\times 200$; scale bar = 50 μ m). (B) Count of Ki-67 fluorescence-positive cells for each group. (C) Expression of PCNA in lung tissues of each group detected by Western blot. (D-E) Relative protein expression level of PCNA/ β -actin for each group.

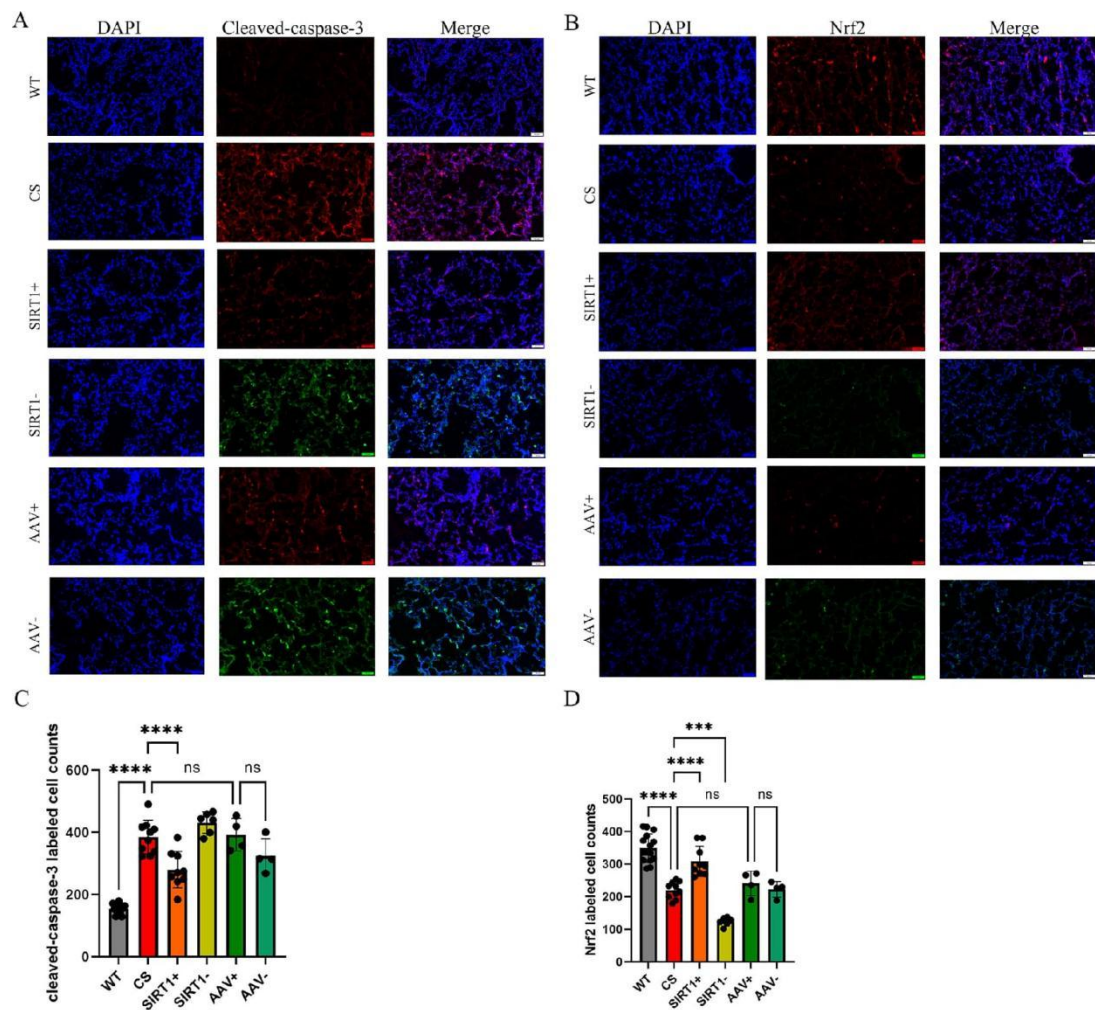


Figure 17. Effects of SIRT1 Overexpression and Inhibition on Apoptotic Function and Nrf2 in CS Model
 (A) Immunofluorescence images of Cleaved-caspase-3 for each group (magnification $\times 200$; scale bar = 50 μm). (B) Immunofluorescence images of Nrf2 for each group (magnification $\times 200$; scale bar = 50 μm). (C) Count of Cleaved-caspase-3 fluorescence-positive cells for each group. (D-E) Count of Nrf2 fluorescence-positive cells for each group.

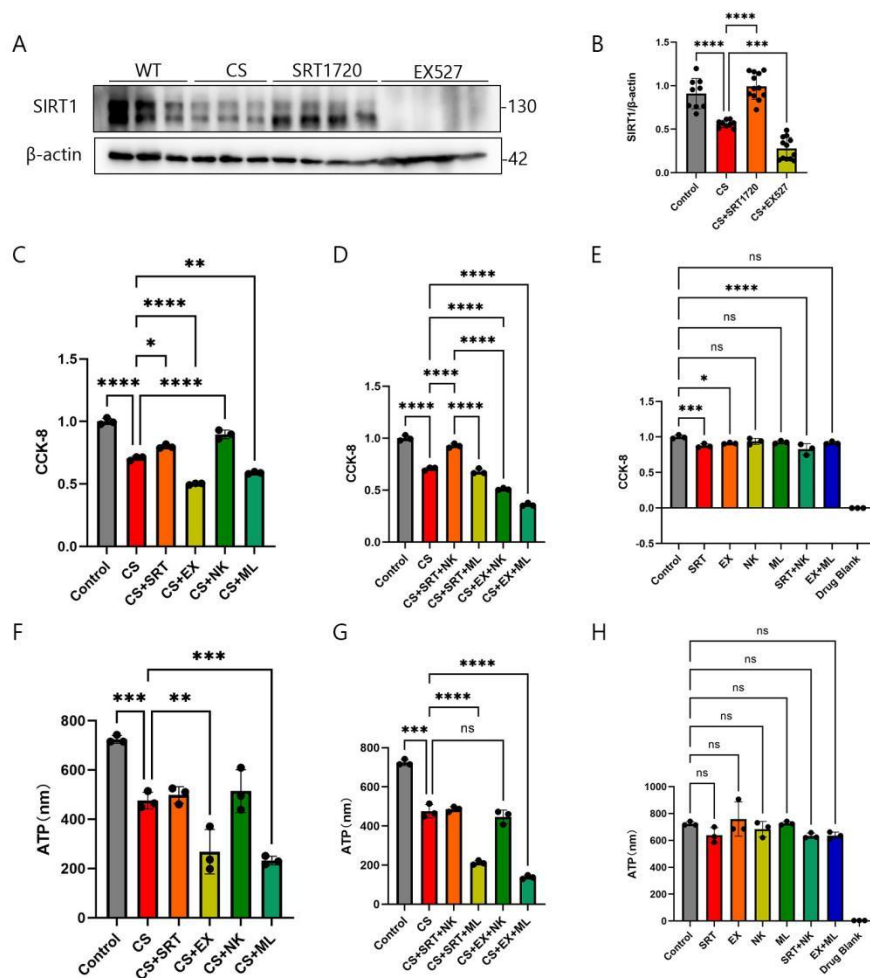


Figure 18. Validation of the SIRT1 Agonist and Inhibitor Models in A549 Cells and CCK-8 and Cell Viability Assays
 (A) Expression of SIRT1 in A549 cells after treatment with WT, CS, SIRT1 agonist (SRT1720), and SIRT1 inhibitor (EX527), detected by Western Blot. (B) Relative protein expression level of SIRT1/ β -actin for each group. (C-E) Cell survival rate measured by CCK-8 assay for each group. (F-H) ATP content measured by cell viability assay for each group.

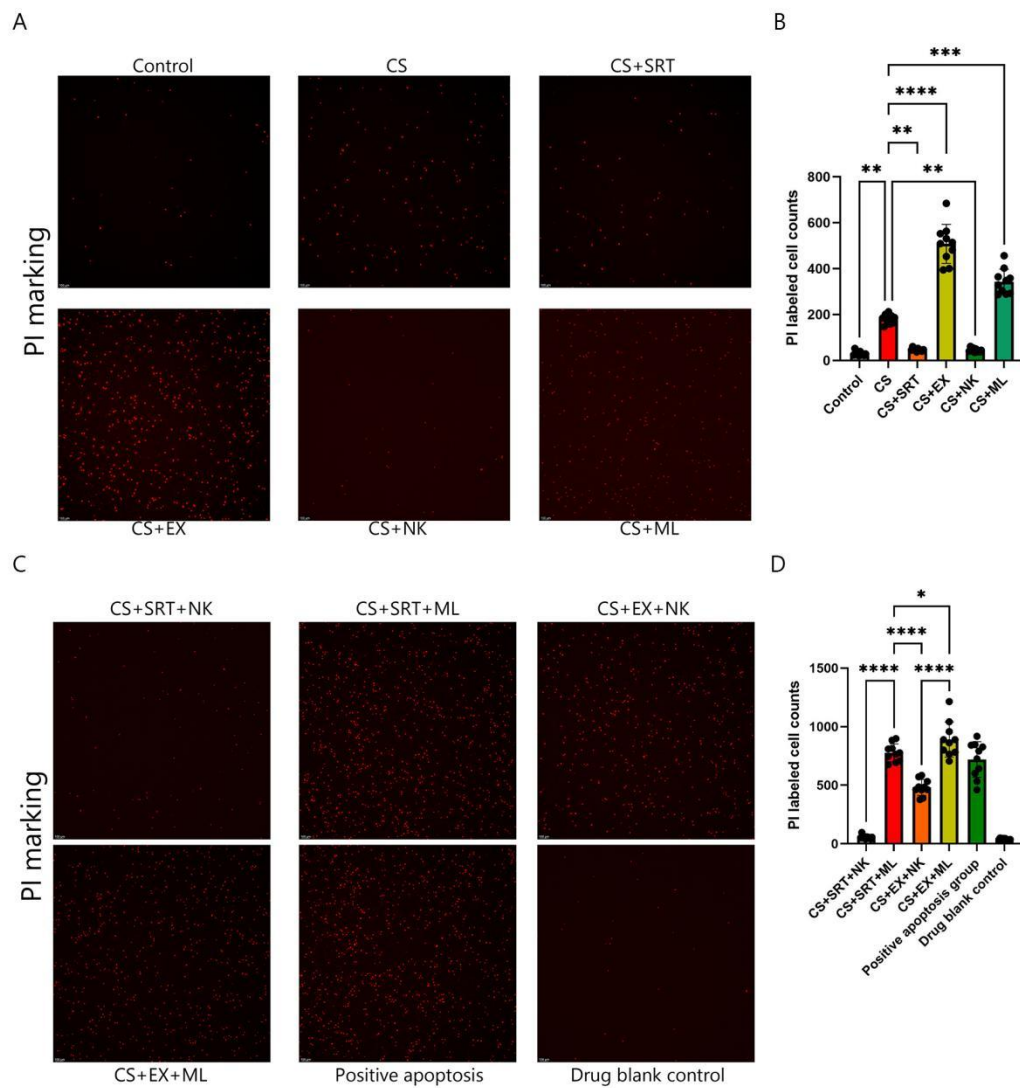


Figure 19. PI Fluorescence Cell Apoptosis Results in A549 Cells for Each Group
(A, C) PI fluorescence images of cell apoptosis for each group (magnification $\times 100$; scale bar = 100 μm). (B, D) Count of PI fluorescence-positive cells for each group.

Supplementary Files

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- [Rawimageofwb.pdf](#)