



Senkyunolide A interrupts TRAF6-HDAC3 interaction to epigenetically suppress c-MYC and attenuate cholestatic liver injury

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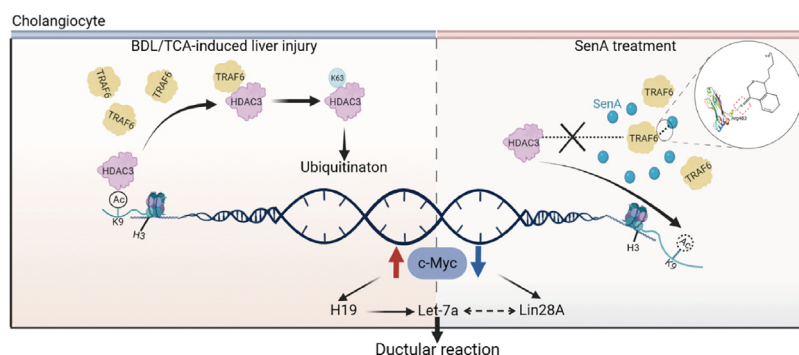
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HIGHLIGHTS

- SenA alleviated cholangiocyte proliferation by inhibiting c-MYC-related H19-Let-7a axis activation in cholestasis.
- SenA occupied binding site on TRAF6 that interacted with HDAC3 and rescuing HDAC3 from c-MYC promoters.
- SenA promoted H3K9 deacetylation and subsequently inhibited c-MYC transcription.
- This study identified SenA as a potential candidate drug for cholangiopathy-associated liver fibrosis.
- This study unveiled a novel approach for discovering epigenetic regulators by targeting protein-protein interactions.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 23 July 2024

Revised 12 March 2025

Accepted 1 April 2025

Available online 3 April 2025

Keywords:

Cholestatic liver diseases

c-Myc

H19

TRAF6

ABSTRACT

Introduction Cholestatic liver diseases are highly prevalent and lack effective treatment, ultimately progressing to end-stage liver diseases. Our recent study indicates that the interplay between c-MYC and lncRNA H19 exacerbates the ductular reaction during cholestasis. *Objective:* This study aims to unveil the underlying mechanisms of the protective effects of senkyunolide A (SenA) on cholangiocyte overproliferation in cholestatic liver diseases. *Methods:* Through comprehensive characterization using RNA sequencing, CHIP analysis, protein truncation, amino acid mutation or deletion, and the development of SenA derivatives, we explored the effects and mechanisms of SenA *in vivo* in bile duct ligation mice and *in vitro* in primary cholangiocytes. *Results:* We demonstrated that SenA effectively mitigates cholangiocyte hyperproliferation by epigenetically suppressing c-MYC expression and disrupting the downstream H19, Let-7a and Lin28a. Mechanically, we identified a potential interaction between the

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BDL, bile duct ligation; CETSA, Cellular thermal shift assay; c-Myc, cellular myelocytomatosis oncogene; DARTS, Drug affinity responsive target stability; DEGs, different expression genes; DR, ductular reaction; HAT, histone acetyltransferase; HDAC3, histone deacetylase 3; H3K9ac, acetylation of histone 3 lysine 9; ICR, imprinting control region; Pcn, proliferating cell nuclear antigen; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SenA, senkyunolide A; SPR, Surface plasmon resonance; TCA, taurocholate acid; TBA, total bile acid; TRAF6, TNF receptor associated factor 6.

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<https://doi.org/10.1016/j.jare.2025.04.002>

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HDAC3
Senkyunolide A

carbonyl group in SenA and Arg483 in TRAF6, disrupting the TRAF6-HDAC3 complex. This dissociation facilitates the binding of HDAC3 to the MYC promoter region, resulting in enhanced histone deacetylation and transcriptional suppression. **Conclusion:** We highlight the therapeutic potential of SenA in cholestatic liver diseases by elucidating its role in epigenetic regulation.

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Introduction

Cholestatic liver diseases, clinically manifested as primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC), intrahepatic cholestasis of pregnancy, progressive familial intrahepatic cholestasis and drug-induced cholestasis, were characterized by the gradual destruction of bile duct, impairment of bile flow and hepatic retention of bile acids (BAs), leading to progressive liver fibrosis, biliary cirrhosis and ultimately liver failure [1]. Under physiological conditions, BAs, as biological detergents synthesized in the liver, facilitate the digestion and absorption of lipids in the intestinal. While, because of the blockage of the biliary tract, BAs were accumulated in the liver and subsequently induced ductular reaction (DR), inflammatory response and even peroxide damage [2]. Meanwhile, a high concentration of BAs not only stressed cholangiocytes to undergo proliferation and differentiation but also could induce hepatocyte injury and immunity response [3]. In a common bile duct ligation (BDL)-stimulated cholestasis mouse model that mimics clinical symptoms of PSC and PBC, primary BAs especially for taurocholate acid (TCA) were significantly increased in serum and could be regarded as a biomarker of cholestatic liver injury [4]. Notably, it has been illustrated that multiple signals including cellular myelocytomatosis oncogene (c-MYC), long non-coding RNA (lncRNA) H19, sphingosine 1-phosphate receptor 2 (S1PR2) and sphingosine kinase 2 (SPHK2) are involved in BDL-stimulated ductular reaction (DR) [5], which were proven to be pharmacologically targetable.

c-MYC plays an essential role in balancing the fine line between quieting cells and proliferating cells, and the modification of c-MYC influences the multiple essential functions and biological processes, including cell proliferation, differentiation and DNA repair [6]. Although c-MYC was initially discovered as one of the oncogenes, the final effects exerted by c-MYC appear to be dependent on the diverse cellular context and target genes, like Lin28A/B and Cyclins [7]. It was noted that our previous study confirmed that c-MYC was observed to be abnormally hyperexpression in multidrug resistance 2 gene knockout (*Mdr2^{-/-}*) mouse livers or TCA-treated overproliferated cholangiocytes, which was positively correlated with cholestatic fibrosis marker H19 [8]. But until now, the exact regulation modes and underlying mechanisms of c-MYC in DR and cholestatic liver diseases are not well characterized and whether c-MYC-related signals could be prevented by any pharmacological therapies is unknown. Notably, c-MYC contains the basic helix-loop-helix leucine zipper (BHLH-LZ) spanning residues 357–439, providing a gathering place for transcriptional regulator and possibility for its epigenetic-related non-canonical regulation [9]. Epigenetics is functionally recognized as covalent changes to the genome with non-changes in the nucleotide sequence, thereby regulating transcriptional machinery [10]. Among different epigenetic modifications, histone acetylation, mediated by a dynamic balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC), seemed as an active mark of gene transcription [11]. Recently, numerous studies pinpointing the roles and mechanisms of epigenetics, especially histone acetylation, in cholestatic liver diseases with most relating to pharmacologic use of HDAC inhibitors [12]. Interesting, HDAC1/2/3 were observed to

bind to gathering place of transcriptional factors in MYC sequences, suggesting potential regulation involved in epigenetic modification of c-MYC [13]. However, how dose HDAC-related epigenetic modification affect c-MYC expression during BDL-stimulated DR remains elusive.

Multiple evidence identified that pharmacological approaches targeting c-MYC-mediated DR appear to block the cholestatic liver injury [14]. While, most of current therapies against cholestatic fibrosis are usually ignored epigenetic modification and their anti-fibrosis effects in clinical trials are limited. Notably, bioactive ingredients derived from natural medicines, are considered to weaken cholestatic liver injury. Recently, senkyunolide A (SenA, PubChem CID: 3085257) is a characteristic phthalide isolated from *Ligusticum wallichii* Franchat and exerts a broad spectrum of biological activities, including anti-migraine [15], anti-osteoarthritis [16] and anti-atherosclerosis [17], while other hepatoprotective effects of SenA still required more research to prove. Based on the above studies and existing questions, there is an unmet need for confirming the anti-cholestatic effects of SenA and investigating whether SenA is involved in epigenetic modification of c-MYC in cholestatic liver injury and underlying mechanisms.

In this work, we analyzed that SenA significantly alleviated c-MYC-associated DR and cholestatic liver injury both *in vivo* and *in vitro*. Mechanistically, SenA suppressed the combination of HDAC3 and TNF receptor associated factor 6 (TRAF6), helping the escaped HDAC3 return to the c-MYC promoter. Therefore, the acetylation of histone 3 lysine 9 (H3K9ac) was suppressed by HDAC3, which subsequently inhibited the transcription of c-MYC and suppressed c-MYC hyperexpression-induced liver fibrosis. Here, we highlighted the significance of HDAC3-mediated c-MYC hyper expression in cholangiocytes and further suggested the therapeutic potentials of SenA against cholestatic liver diseases in the clinic.

Materials and methods

Materials

SenA (PS2132) was obtained from Yuanye BioTechnology (Shanghai, China). HDAC3 inhibitor BG45 (926259–99–6) was purchased from Selleck (Shanghai, China). Recombinant protein of human TRAF6 was purchased from Origene (Shanghai, China). DiIc18(7) (DIR) was purchased from Aladdin (Shanghai, China). Other cell culture materials were obtained from Sigma (St. Louis, USA). Antibodies used in this study are shown in **Supplementary Table 1**.

Preparation of Lipo-SenA liposomes

The Lipo-Ct and Lipo-SenA cholangiocyte-targeting liposomes were prepared by thin lipid film technique and ammonium sulfate gradient loading method. Detailly, EPC, EDC, N-Hydroxy succinimide (NHS) and folic acid (FA) were dissolved in chloroform in a pear-shaped flask. The conjugation of targeting peptide and EDC-NHS-FA was achieved through a sulfhydryl-maleimide coupling method. Briefly, SenA (25 mM) was slowly added to EDC-NHS-FA

lipid film to prepare cholangiocyte-targeting liposomes containing SenA for the animal study in Fig. 8d–g.

Animal studies

Male C57BL/6J mice (7-week-old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). In Fig. 1, mice were randomly divided into 8 groups (n = 8): (1) Sham group; (2) SenA (low dose, 10 mg/kg); (3) SenA (medium dose, 20 mg/kg); (4) SenA (high dose, 40 mg/kg) (5) BDL group; (6) BDL + SenA (low dose, 10 mg/kg); (7) BDL + SenA (medium dose, 20 mg/kg); (8) BDL + SenA (high dose, 40 mg/kg). Mice in groups (1) and (5) underwent Sham or BDL surgery for 7 days, respectively. Mice in groups (2)–(4) and (6)–(8) were pre-treated with different doses of SenA by gavage for 2 days, underwent sham (2)–(4) or BDL (6)–(8) surgery and were treated with different doses of SenA for 4 days at 3 days post-operation. For experiments in **Supplementary Fig. 3**, mice were randomly divided into 5 groups (n = 8): (1) Sham group; (2) SenA (post-medium dose, 20 mg/kg); (3) BDL group; (4) BDL + SenA (pre-medium dose, 20 mg/kg); (5) BDL + SenA (post-medium dose, 20 mg/kg). Mice in groups (1) and (3) underwent Sham or BDL surgery for 7 days, respectively. Mice in group (4) were treated as same as mice in Fig. 1 group (7). Mice in groups (3) and (5) underwent BDL surgery and were treated with SenA for 4 days at 3 days post-operation. For experiments in Fig. 8a, mice were randomly divided into 5 groups (n = 8): (1) Sham group; (2) BDL group; (3) BDL + BG45 (20 mg/kg); (4) BDL + SenA (20 mg/kg); (5) BDL + SenA (20 mg/kg) + BG45 (20 mg/kg). Mice in groups (1) and (2) underwent sham or BDL surgery for 7 days, respectively. Mice in groups (4) and (5) were pre-treated with SenA by gavage for 2 days, underwent BDL surgery and were administrated with SenA by gavage for 4 days at 3 days post-operation. Mice in groups (3) and (5) underwent BDL surgery and were treated with BG45 (20 mg/kg) by gavage for 4 days at 3 days post-operation. For experiments in Fig. 8d, mice were randomly divided into 3 groups (n = 8): (1) BDL group; (2) BDL + Lipo-Ct group; (3) BDL + Lipo-SenA group. Mice in group (1) underwent BDL surgery for 7 days. Mice in groups (2) and (3) were tail vein injected with Lipo-Ct and Lipo-SenA for 2 days, underwent BDL surgery and were tail vein injected with Lipo-Ct and Lipo-SenA for 4 days at 3 days post-operation. At the end of different treatment, mice were sacrificed for collecting serum and liver tissues.

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Institutional Animal Care and Use Committee of the Beijing University of Traditional Chinese Medicine (Approved project: BUCM-4-20200730023160).

Histopathologic analysis

After fixation by 4 % formaldehyde, tissues were embedded in paraffin and cut into sections of 4.5 µm thickness for hematoxylin and eosin (H&E) and Masson trichrome staining as previously described. The revised Knodell protocol (Histological Activity Index, HAI) was used to evaluate hepatic injury in H&E staining [18]. Briefly, 0, no inflammation or fibrosis; 1, confluent or intralobular inflammation; 2, focal necrosis or portal fibrosis; 3, serious confluent inflammation or bridging fibrosis; 4, cirrhosis [19]. Quantification of collagen deposition (the percentage of blue collagen area relative to the total tissue area) in Masson trichrome staining using Image J software. For immunohistochemical and immunofluorescent staining, sections were incubated with EpCAM antibody (21050–1-AP, proteintech), KRT19 antibody (10712–1-

AP, proteintech), Ki67 antibody (27309–1-AP, proteintech) at 4 °C overnight (detail antibodies information were shown in **Supplementary table 1**). The quantification of immunohistochemical staining of EpCAM or co-immunofluorescent staining of Ki67 and KRT19 were performed using Image J software.

Isolated mouse primary cholangiocytes

Mouse primary cholangiocytes were collected with a multi-step collagenase digestion method and cultured as previously described [20]. Mouse primary cholangiocytes were seeded into plate with DMEM/F12 medium containing 10 % fetal bovine serum (FBS), EGF (10 ng/ml) and HGF (10 ng/ml), dexamethasone and 1X insulin-transferrin-selenium and prepared for RNA isolation and RNA sequencing.

RNA sequencing of mouse primary cholangiocytes

Total RNA of mouse primary cholangiocytes was detected by NanoRhatometer® spectrophotometer (IMPLEN, USA). RNA integrity was evaluated using RNA Nano Assay kit. Subsequently, mRNA was purified from total RNA and cDNA was synthesis. The library fragments (250–300 bp) were purified with the AMPure XP system (Beckman Coulter, USA) and sequencing library was established by NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB) [21]. Finally, by applying edgeR R package, differentially gene expression was performed and Gene Ontology (GO) enrichment analysis were completed.

Cell culture and treatment

Human intrahepatic biliary epithelial cholangiocytes (HIBECs) were cultured in MEM containing 10 % fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin (100 µg/ml) at 37 °C and 5 % CO₂ in a humidified cell-culture incubator. HIBECs were first pre-treated with taurocholate acid (TCA) (100 µmol) then administrated with SenA (12.5, 25 and 50 µmol) for 24 h. For cell experiments in **Supplementary Fig. 4**, HIBECs were pretreated with 25 µmol BG45 for 1 h, then treated with 100 µmol TCA and SenA (25 µmol) for another 24 h.

Microscale thermophoresis (MST) assay

Firstly, 16 different concentrations of SenA (0.1526 µmol – 5 mmol) were prepared. 100 nmol TRAF6 protein were dissolved with PBS and labeled with RED dyes (MO-LO18, NanoTemper Technologies). Different concentrations of SenA were mixed with labeled TRAF6 protein (100 nmol), respectively. After 25 min, samples were detected by capillaries and MST evaluation was performed by Monolith NT.115 MST instruments (NanoTemper, Munich, Germany).

Molecular docking

The combination of SenA and TRAF6 (PDB: 6GCT) were detected by GLIDE software (Schrödinger, LLC, New York, NY, USA). Firstly, the structure of TRAF6 and SenA ligand were prepared by protein preparation wizard method and ChemDraw. TRAF6-SenA complex model was constructed by standard precision model method. Then, by applying PyMOL, the results with highest scoring orientation were showed in a stable conformation. Furthermore, Gromacs (v. 2019.1) software was used to provide a stable conformation for the MD simulation. Finally, the results of the combination of TRAF6 and SenA were analyzed by calculating the root-mean-square deviation (RMSF).

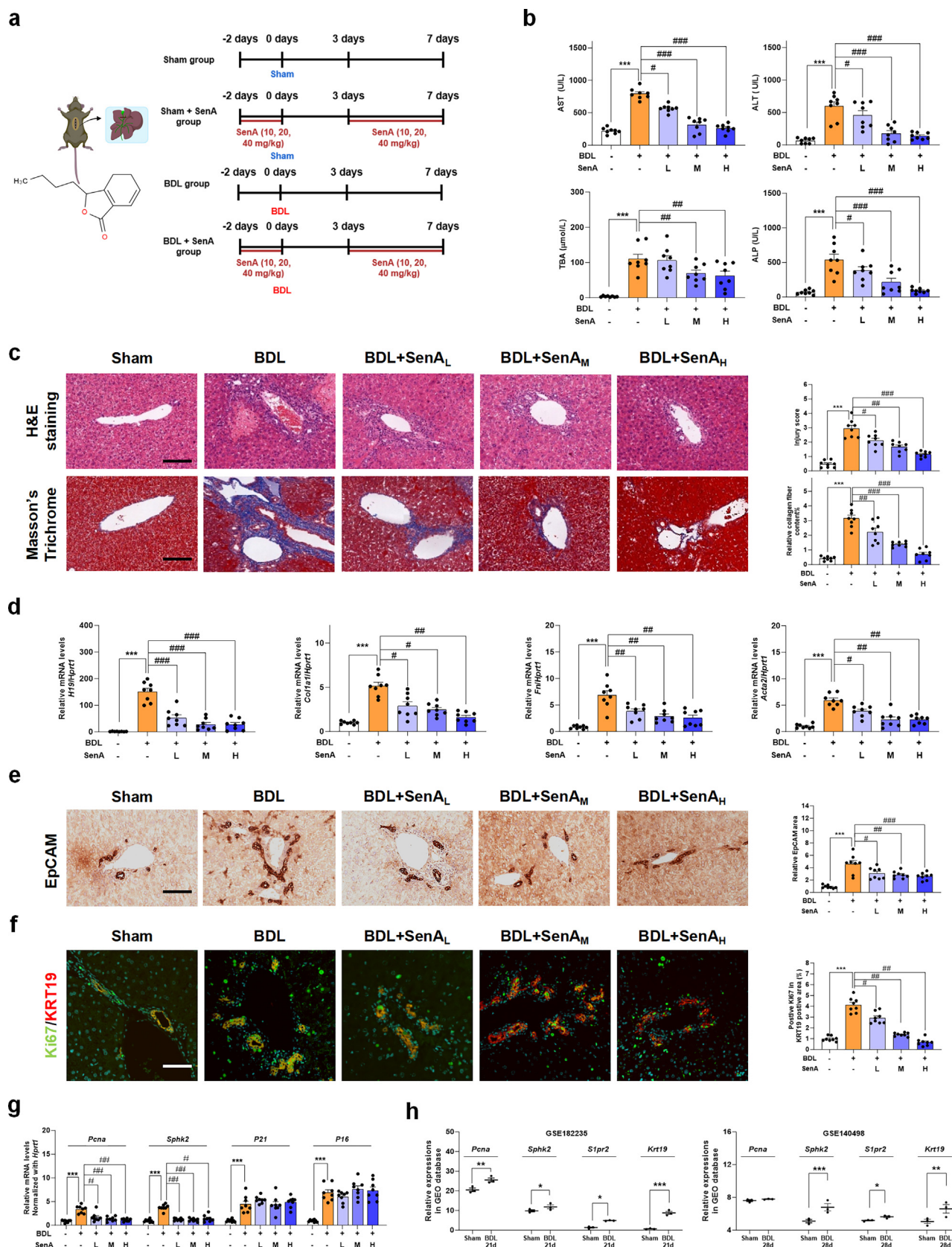


Fig. 1. SenA alleviates BDL-induced cholestatic liver injury. (a) Flowchart of animal experiments with associated photographic images of each group ($n = 8$). (b) Serum AST, ALT, TBA and ALP levels. (c) Representative images of H & E staining and Masson trichrome's staining was quantified using Image J software. Scale bar = 50 μm . (d) Relative mRNA levels of *H19*, *Col1a1*, *Fn1* and *Acta2*. *Hprt1* was utilized as an internal control. (e) Representative images of EPCAM immunohistochemistry staining. Scale bar = 50 μm . (f) co-immunohistochemistry staining of KRT19 with Ki67 were quantified using Image J software. Scale bar = 25 μm . (g) Relative mRNA levels of *Pcn*, *Sphk2*, *P21* and *P16* in BDL-induced mouse model. *Hprt1* was utilized as an internal control. (h) Analysis of genes downregulated by SenA in BDL mice from GEO database. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. Sham or Normal group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vs. Model group ($n = 8$).

Drug affinity responsive target stability (DARTS)

Firstly, the cell lysates were obtained from protein extraction buffer and the concentration was detected by BCA kit. Meanwhile, SenA (25 μ mol) was dissolved by DMSO. The cell lysates and SenA were mixed and the mixture were detected by western blot.

Cellular thermal shift assay (CETSA)

After treated with SenA (25 μ mol) for 24 h, the HIBECs were collected and divided into 8 parts. Meanwhile, as negative control, HIBECs treated without SenA were collected and detected by CETSA assay. Then, each part of samples was heated for 3 min under different temperature (37, 40, 45, 50, 55, 60, 65, 70°C). Cell lysates were obtained from HIBECs and proteins were collected by centrifuged at 20,000 g. The protein level of TRAF6 was detected by western blot.

Surface plasmon resonance (SPR) assay

Firstly, 20 μ g/ml TRAF6 protein was prepared in sodium acetate buffer and injected into the instrument. Meanwhile, a CM5 chip was first activated by a standard EDC/NHS method. Then the TRAF6 protein was injected into the channel of instrument and combined with chip. To evaluate the combination of SenA and TRAF6, SenA was dissolved into different concentrations. The different concentrations of SenA were injected into the channel of instrument. The data were obtained using SPR sensor (Biacore, GE Healthcare) at 25 °C and the results were analyzed by T200 software.

Chromatin immunoprecipitation (ChIP)-qPCR experiment

After treated with SenA, nuclear extract was collected using lysis buffer and were added protein occluded A/G microspheres with antibody against H3K9ac. The samples were rotated overnight at 4 °C and the eluted DNA were isolated from nuclear extract and further added with Proteinase K. Then, to detect the genes of interests, the primers were designed for the following detection. The experiments were performed using Thermo Pierce Agarose ChIP Kit (Thermo Scientific) and the fold enrichment of target genes were detected by q-PCR.

Co-immunoprecipitation (Co-IP)

After treated with SenA, HIBECs were collected using lysis buffer and added protein A/G microspheres with antibody HDAC3. The samples were rotated overnight at 4 °C. Then, the mix samples were washed by IP buffer for three times and the immune complexes were eluted with IP buffer. Finally, the protein samples were mixed with loading buffer and subsequently detected by western blot assay.

Mass spectrometry analysis

MS data was obtained using benchtop Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). For experiment, the heated capillary temperature was 320°C, the spray voltage was 3.5 kV, and auxiliary gas heater temperature was 400°C. The positive and negative ionization mode followed with the full scan analysis were performed in the mass spectrometry analysis with mass range of m/z 100–1500.

Nanoparticle tracking analysis (NTA)

Liposomes were performed on a LM10 nanoparticle analysis system. The NTA system were coupled with a syringe pump and

488 nm laser. The data were acquired and analyzed using the NTA 3.0 software (Malvern Panalytical).

Statistical analysis

Statistical analysis was performed using two-tailed Student *t* test between two groups or One-way analysis of variance with Tukey's post-hoc test among multiple groups using GraphPad Prism 8 software. All data were showed as mean $\pm$ SEM and difference were considered significant at a *P* value of ≤ 0.05 .

Please see the [Supporting Information](#) for other experimental materials and methods.

Results

SenA ameliorates experimental cholestatic liver injury induced by BDL surgery

Recently, extracts and active compounds of *Chuanxiong Rhizoma* have been considered to prevent diseases of hepatobiliary system. So far hundreds of chemicals have been identified in *Chuanxiong Rhizoma* including tetramethylpyrazine, ferulic acid and SenA. To screen out promising anti-fibrosis drugs in *Chuanxiong Rhizoma*, we preliminarily investigated the anti-proliferative activity of 11 compounds in *Chuanxiong Rhizoma* including SenA. As shown in [Supplementary Fig. 1a](#), we confirmed that TCA significantly stimulated cholangiocyte proliferation. TCA-induced upregulation of cholangiocyte proliferation-related genes, including EPCAM, KRT19, MYC and PCNA were all decreased by SenA. Meanwhile, tetramethylpyrazine, ligustilide, ferulic acid, chlorogenic acid, senkyunolide I, perillyrine, palmitic acid, levistilide A and coniferly had minimal effects on cholangiocyte proliferation. Thus, we focused on the effects of SenA on liver fibrosis.

To explore the effects of SenA on BDL-induced cholestatic liver injury, mice were pre-treated with SenA (10, 20, and 40 mg/kg) for 2 days, subjected to sham or BDL surgery, and then administrated with different doses of SenA for 4 days starting from 3 days post-operation ([Fig. 1a](#)). Firstly, we found that SenA had no effects on healthy livers ([Supplementary Fig. 1-2](#)). As shown in [Supplementary Fig. 1b](#), BDL surgery successfully induced cholestasis and SenA primarily remised pathology, as illustrated by the color and quantity of bile collected from different groups. BDL markedly stimulated cholestatic liver injury, as demonstrated by increased serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bile acid (TBA) and alkaline phosphatase (ALP), which was significantly and subsequently reduced by SenA treatment ([Fig. 1b](#) and [Supplementary Fig. 1c](#)). As shown in [Fig. 1c](#) and [Supplementary Fig. 1d](#), SenA significantly reversed BDL-caused lobular inflammation, collagen deposition and bile duct hyperplasia as illustrated by hematoxylin & eosin (H&E) and Masson's trichrome staining of liver tissues. The mRNA levels of hepatic markers including *H19*, *Col1a1* (encoding Collagen 1), *Fn1* (encoding Fibronectin) and *Acta2* (encoding α -SMA) were significantly elevated by BDL and dose-dependently reversed by SenA ([Fig. 1d](#) and [Supplementary Fig. 1e](#)). DR, characterized by the proliferation of reactive bile ducts induced by liver injuries, is the most common pathological changes in BDL mouse model. As shown in [Fig. 1e](#) and [Supplementary Fig. 2a](#), SenA significantly reversed BDL-stimulated DR, as illustrated by immunohistochemistry staining against EPCAM, a specific marker of DR. Meanwhile, Ki67 is a proliferation marker and KRT19 is a cholangiocyte marker. As shown in [Fig. 1f](#) and [Supplementary Fig. 2b](#), the co-immunohistochemistry staining of Ki67 with KRT19 indicated that BDL increased the expression of Ki67 in KRT19-positive area, indicating the enhanced proliferation of cholangiocytes, which was

dose-dependently reversed by SenA. Consistently, as shown in Fig. 1g and Supplementary Fig. 2c–d, SenA significantly downregulated the expression of proliferation-related genes in livers, including proliferating cell nuclear antigen (*Pcna*) and sphingosine kinase (*Sphk2*). Although proliferation and senescence usually coexist during DR [22], SenA had no effects on the expression of senescence-related genes, including *P21* and *P16*. Consistently, SenA dose-dependently decreased the protein expression of PCNA and SPHK2 stimulated by BDL but showed minimal effects on the protein levels of P21 and P16 (Supplementary Fig. 2e). In addition, SenA significantly reduced BDL-stimulated inflammation response, as illustrated by decreased mRNA expression of *Tgfb*, *Il1b* and *Il6* (Supplementary Fig. 2f). Meanwhile, the consistent efficacies were observed in mice treated with SenA only for 4 days after BDL surgery (Supplementary Fig. 3a–e). The genes downregulated by SenA, including *Pcna*, *S1pr2* and *Sphk2*, are potentially associated with the proliferation of cholangiocytes as suggested by previous studies. We found that the expressions of *Pcna*, *S1pr2* and *Sphk2* were elevated in PBC, PSC and biliary atresia patients, as illustrated by immunohistochemical and Western Blot assays [20,23–26]. Similarly, *Pcna* and *S1pr2* were both upregulated in BDL livers, accompanied by an increased expression of *Krt19*, as reported in datasets GSE182235, GSE140498, and GSE166867 (Fig. 1h). While increased *Sphk2* expression was not observed in GSE166867. These findings suggested that the protective effects of SenA were related to the inhibition of cholangiocyte dysplasia.

SenA restricts the DR in proliferating cholangiocytes

To investigate the underlying mechanisms and molecular targets of SenA, we determined the effects of SenA on BA accumulation and transformation. As shown in Supplementary Fig. 4a–b, BDL significantly induced TBA accumulation in liver and bile, which was gently decreased by SenA and the consistent results were observed in mice treated with SenA only for 4 days after BDL surgery (Supplementary Fig. 4c–d). Then, we confirmed that mRNA expression of *Ntcp* and *Cyp7a1* was not changed under the SenA administration (Supplementary Fig. 4e–f). Consistently, as shown in Supplementary Fig. 4g, the expression of *Ntcp*, *Cyp7a1* and *Bsep* was not affected by SenA in AML12 cell line, which encouraged us that the protective effects of SenA may be related to cholangiocytes other than hepatocytes. Subsequently, we isolated primary cholangiocytes from sham mice, mice-treated with BDL and BDL plus SenA and subsequently isolated RNA from cholangiocytes for RNA sequencing of single cell type (Fig. 2a). Furthermore, RNA-sequencing analysis was performed to determine the differential expressed genes (DEGs) between Sham group vs BDL group and BDL group vs BDL + SenA group. The DEGs implicated in cellular proliferation, cellular senescence and cellular migration between Sham group vs BDL group and BDL group vs BDL + SenA group were preliminary analyzed by GO analysis (Fig. 2b), suggesting the potential effects of SenA on bile duct hyperplasia. DEGs implicated in cellular proliferation and cellular senescence were plotted in Fig. 2c as a heatmap. The expression of several DEGs, including minichromosome maintenance (*Mcm*), *Mcm7*, cyclin dependent kinase 6 (*Cdk6*) and *Cdk4*, was remarkably downregulated in BDL + SenA group when compared to the BDL group. As shown in Fig. 2d, qPCR results demonstrated that SenA significantly decreased the mRNA expression of *Pcna*, *Epcam*, *Myc* and *Sphk2* stimulated by BDL in mouse primary cholangiocytes. Meanwhile, as shown in Fig. 2e and Supplementary Fig. 4h, TCA significantly increased the expression of Ki67 (red dots) in CK7-positive cells, which was markedly reversed by SenA. These results indicated that SenA significantly inhibited cholangiocyte proliferation-stimulated by TCA. As shown in Fig. 2f, TCA significantly promoted cell cycle, as illustrated by flow cytometry stud-

ies. While, SenA markedly upregulated the cell percentage in G0/G1 and S phase and decreased the cell population at G2 phase in HIBECs. Consistently, we confirmed that TCA significantly increased mRNA levels of proliferation-related genes like PCNA and SPHK2, which was consistent with our previous study, and was reversed by SenA (Fig. 2g). While, SenA rarely influenced senescence-related genes in TCA-treated HIBECs, including P21 and P16 (Fig. 2h). Consistently, SenA dose-dependently decreased the protein expression of PCNA and SPHK2 stimulated by TCA in cholangiocytes (Fig. 2i) but rarely influence the P21 and P16 protein expression (Fig. 2j). These results suggested that SenA prevented DR and cholestasis in a complex way associated with cholangiocyte hyperproliferation.

SenA suppresses cholangiocyte proliferation via targeting c-MYC/H19/Let-7a signaling

Notably, we have fully found that H19 is a key target in cholangiocyte proliferation and cholestatic liver injury induced by high dose of TCA [27]. Surprisingly, we found that SenA significantly decreased H19 expression *in vivo* based on Fig. 1. These events encouraged us to investigate the influence of SenA on the H19-associated signaling pathways. In human cholangiocyte cell line HIBEC cells, SenA significantly downregulated the mRNA level of H19 when compared to the TCA group (Fig. 3a). H19 has been illustrated to act as a molecular sponge to promote the transcription of LET7A, thus promoting DR [25,28] (Fig. 3b, left panel). Notably, TCA significantly increased the transcription of LET7A, Lin28A and CDK2, which was subsequently downregulated by SenA (Fig. 3b, right panel). Furthermore, to confirm the effects of SenA on H19, we established H19 overexpression (H19 OE) cholangiocytes. As shown in Fig. 3c, the abnormal hyperexpression of H19, EPCAM, KRT19, PCNA and CDK2 were stimulated by H19 OE while not significantly reversed by SenA treatment, suggesting the central role of H19 in anti-fibrosis effects of SenA [25]. As shown in Fig. 3d, Let-7a inhibitor (Let-7i) significantly downregulated the mRNA levels of EPCAM, KRT19, CDK2, CDK1 and PCNA induced by TCA, which were not affected by subsequent SenA administration. Consistently, the effects of H19 OE were intercepted by Let-7i and the effects of SenA against DR were overwhelmed by H19 OE and masked by Let-7i (Fig. 3e). We also detected the effects of SenA on H19 expression. As shown in Supplementary Fig. 5a, SenA failed to suppress H19 overexpression mediated by plasmid, which is independent of c-MYC regulation.

Indeed, H19 transcription could be regulated by several transcription factors and organizer of genome spatial. Among these transcription factors, c-MYC promotes H19 transcription by binding to E-boxes close to imprinting control region (ICR) [29]. In order to further verify whether SenA affects H19 via targeting c-MYC, we observed the expression of c-MYC both in BDL-induced mouse livers and TCA-treated cholangiocytes. As shown in Fig. 3f, SenA markedly decreased the mRNA expression of c-MYC both *in vivo* and *in vitro*. Furthermore, SenA dose-dependently downregulated the protein expression of c-MYC stimulated by TCA or BDL surgery, as illustrated by immunofluorescence and western blot (Fig. 3g–h and Supplementary Fig. 5b). The correlation analysis in showed that mRNA expression of H19 is positively correlated with MYC (Fig. 3i), suggesting that SenA might be likely to affect the expression of H19 through c-MYC. Under physiological condition, SenA rarely affected the mRNA expression of *Myc* (Supplementary Fig. 5c). While, the mRNA expression of MYC, H19, CDK2, LET7A, LIN28A and SPHK2 were significantly inhibited by both c-Myc inhibitor 10058-F4 and SenA, suggesting that SenA inhibited proliferation via suppressing c-MYC (Supplementary Fig. 5d). Consistently, as shown in Fig. 3j and Supplementary Fig. 5e, SenA dose-dependently reversed the upregulation of c-

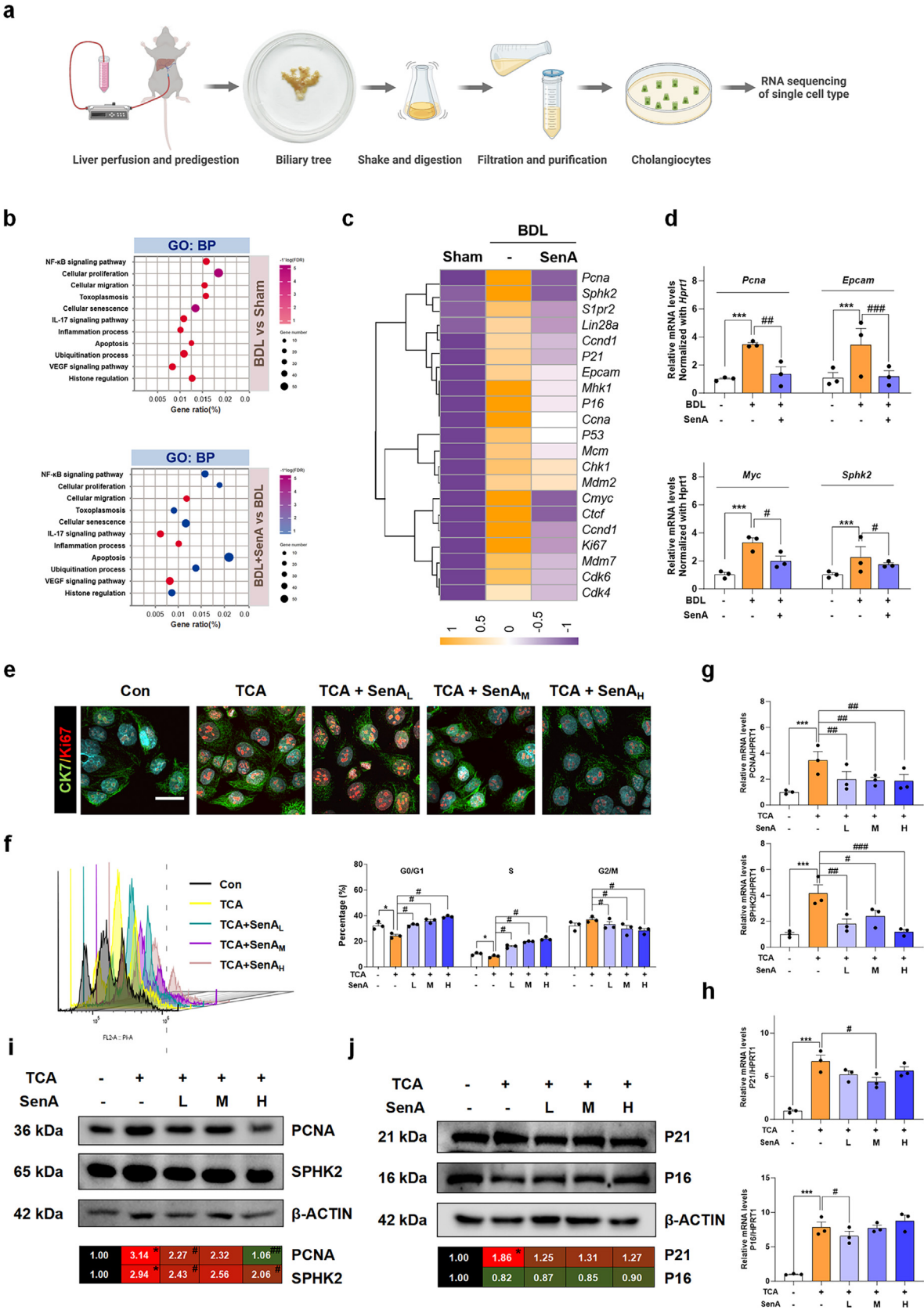


Fig. 2. RNA-sequencing analysis and experimental verification of the inhibitory effects of SenA on hyperproliferated cholangiocytes. (a) Flowchart of mouse primary cholangiocytes isolation with associated photographic images. (b) GO analysis of DEGs between BDL vs. Sham and DEGs between BDL + SenA vs. BDL by R software. The size of dots was used to describe the number of genes enriched in corresponding pathways and the color of dots indicates the significance. (c) The relative expression levels of DEGs implicated in cellular proliferation were shown as a heatmap. (d) Relative mRNA levels of *Pcn*, *Epcam*, *Myc* and *Sphk2* in mouse primary cholangiocytes. *Hprt1* was utilized as an internal control (n = 3). (e) Representative images of CK7 and Ki67 co-immunofluorescence staining. (f) The cell cycle was detected by PI staining. Relative mRNA levels of (g) PCNA and SPHK2 and (h) P21 and P16 in HIBECs. Representative immunoblots against (i) PCNA and SPHK2 and (j) P21 and P16 and β-ACTIN in HIBECs were shown.

*P < 0.05, ***P < 0.001, vs. Control group; #P < 0.05, ###P < 0.001, vs. Model group (n = 3).

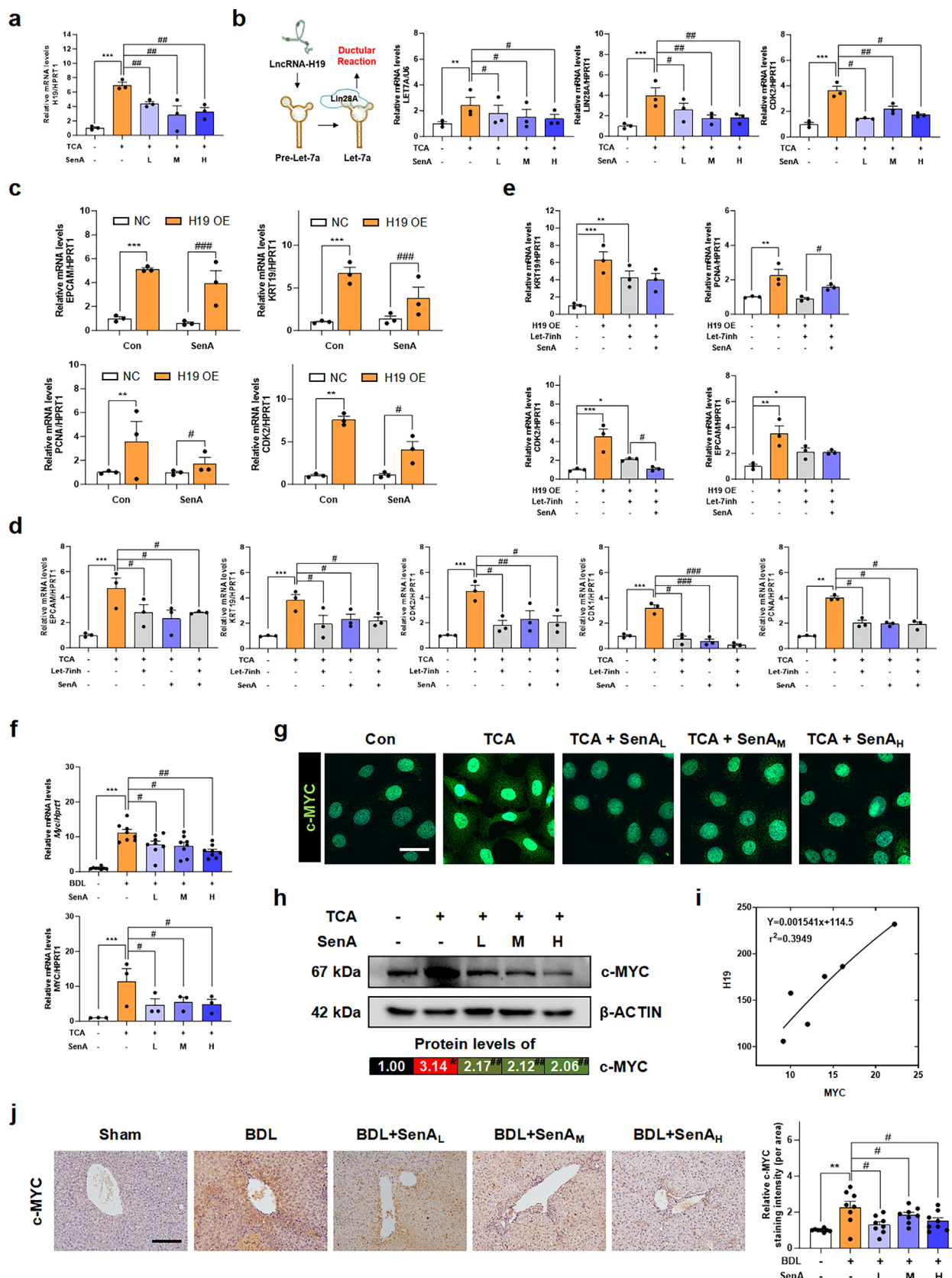


Fig. 3. SenA inhibits DR by regulating c-MYC-H19-Let-7a signaling in cholangiocytes. (a) Relative mRNA levels of H19, HPRT1 was utilized as an internal control. (b) The biological process of MYC-associated pathway was shown, LET7A, LIN28A and CDK2 in HIBECs treated with TCA and different dosages of SenA. HPRT1 was utilized as an internal control. (c) Relative mRNA levels of EPCAM, KRT19, PCNA and CDK2 in H19 overexpression-induced HIBECs. (d) Relative mRNA levels of EPCAM, KRT19, PCNA and CDK2 in H19 OE cells. (e) Relative mRNA levels of KRT19, PCNA, CDK1 and EPCAM in H19OE and Let-7a inhibitor-induced HIBECs. (f) Relative mRNA levels of MYC in livers and HIBECs. (g) Representative images of c-MYC immunofluorescence staining. (h) Representative immunoblots against c-MYC and β -ACTIN in HIBECs were shown. (i) The positive relationship between the mRNA expression of H19 and MYC. (j) Representative images of MYC immunohistochemistry staining were quantified using Image J software. Scale bar = 50 μ m. * P < 0.05, ** P < 0.01, *** P < 0.001, vs. Control group; # P < 0.05, ## P < 0.01, ### P < 0.001, vs. Model group (n = 3).

MYC in BDL mice. These results suggested that SenA prevented H19-related cholangiocyte proliferation through its modification on c-MYC.

SenA epigenetically inhibits MYC transcription via H3K9 deacetylation

Primary cholangiocytes from sham mice, BDL or BDL plus SenA-treated mice were isolated for RNA-sequencing and detailed analysis of the DEGs and key targets encouraged us to find out the potential mechanisms underlying the effects of SenA on MYC transcription. As shown in Fig. 4a, among the 2180 DEGs, transcripts of 454 genes were up-regulated, whereas transcripts of 321 genes were down-regulated in BDL-stimulated cholangiocytes compared to the those from Sham mice ($|\log_2$ fold change $|\geq 2$; P-value < 0.05). We also found 46 down-regulated and 24 up-regulated genes in cholangiocytes from SenA-treated BDL mice vs. BDL mice. Among the DEGs, *Myo* was conspicuously downregulated in the BDL + SenA group when compared with the BDL group. We further established 'Cluster analysis diagram' to explore the underlying mechanism of SenA in alleviating liver fibrosis-stimulated by BDL via using DEGs between BDL group and BDL + SenA group. Notably, we observed that several pathways, especially histone acetylation were enriched in SenA-treated mice (Fig. 4b). Consistently, GO enrichment analysis revealed that DEGs in BDL + SenA were significantly enriched in several GO: BP terms related to cellular proliferation, histone regulation, apoptosis, cellular senescence, inflammation process, NF- κ B and ubiquitination process in Fig. 4c. Histones modifications, especially histone acetylation, influence chromatin compaction and accessibility and thus regulating DNA availability to transcription factors and transcriptional activity to influence a variety of cellular processes, which attracted our attention and encouraged us to further investigate. It has been reported that H3K9ac was enriched in the promoter of *Fn1*, promoting DR and liver fibrosis in both knockout and diet-fed biliary fibrosis mice [30]. Therefore, we subsequently investigated the effects of SenA on H3K9 acetylation by immunofluorescence and western blot both *in vivo* and *in vitro*. As shown in Fig. 4d, BDL significantly stimulated H3K9ac accumulation in the liver, which was subsequently downregulated by SenA administration. Consistently, SenA significantly downregulated BDL-induced H3K9ac in cholangiocyte of portal area, which was illustrated by co-immunofluorescence of H3K9ac and glutamine synthetase (Gl Syn) in Fig. 4e and Supplementary Fig. 5f. Furthermore, SenA dose-dependently downregulated H3K9ac in TCA-stimulated cholangiocytes (Fig. 4f). Subsequently, we designed the specific primers at the promoter and exon regions of MYC to observe the effects of SenA on H3K9ac combination (Fig. 4g). As shown in Fig. 4h, H3K9ac markers were enriched at promoter 2 and Exon 1 of MYC in TCA-treated cholangiocytes, but significantly decreased by SenA. These results indicated that SenA suppressed MYC transcription by inhibiting H3K9ac and its enrichment in MYC promoter.

SenA enhances H3K9 deacetylation by interrupting the interaction between TRAF6 and HDAC3

Acetylation of histones was mediated by a dynamic balance between HATs and HDACs [31]: HATs were responded to transfer an acetyl from CoA to lysine residue, while hydrolyzing and removing acetyl groups was relied on HDACs [32]. Given the inhibitory effects of SenA on H3K9ac in cholangiocytes, we hypothesized that SenA might influence HATs and HDACs involved in H3K9 acetylation. The DEGs related to HATs and HDACs were shown in Fig. 5a as a heatmap. As shown in Fig. 5b and Supplementary Fig. 6a, among different classic HDACs, TCA and BDL mainly and significantly decreased the transcription of HDAC3 in cholangiocytes.

While, TCA rarely affected the mRNA expression of HATs, including KAT8, P300, CBP, PCAF and GCNA (Fig. 5c). Furthermore, we detected the effects of SenA on HATs activity both *in vivo* and *in vitro* and the results in Fig. 5d indicated that SenA had minimal effects on HATs activity. Interestingly, the activity of HDACs were concurrently decreased in BDL- or TCA-stimulated groups, which was subsequently reversed by SenA administration (Fig. 5e-f). We also found that SenA treatment resulted in HDAC3 enrichment at MYC promoter 2, which was associated with inhibited MYC transcription (Supplementary Fig. 6b). Thus, we further clarified the effects of SenA on cholangiocytes proliferation in the absence of HDAC3 by using HDAC3 inhibitor BG45 administration. BG45 significantly abolished the effects of SenA on reversing TCA-mediated upregulation of MYC, EPCAM, KRT19 (Fig. 5g) and H19, PCNA, S1PR2 and SPHK2 (Supplementary Fig. 6c). These findings highlighted that SenA-regulated H3K9ac accumulation is depending on regulating HDAC3 activation.

Considering that after activating HDAC3, SenA impotently inhibited the expression of cholangiocyte proliferation-related genes, we subsequently explored the internal mechanism by which SenA regulated HDAC3 expression. Based on DEGs between BDL group and BDL + SenA group, SenA was found to obviously alter ubiquitination-related genes. It has been reported that TRAF6 could serve as an E3 ubiquitin ligase and regulate proteasomal degradation of various proteins, including HDAC3 [33], NF- κ B [34] and YAP [35], which are essential for signal transduction or protein trafficking [36]. Recently, a recent study demonstrated that TRAF6 could interact with HDAC3 to promote its ubiquitination [37], which is believed to be involved in the regulation of HDAC3-mediated histone acetylation [38]. As shown in Fig. 5h and Supplementary Fig. 6d, SenA significantly decreased the ubiquitination of HDAC3 and this effect was abolished by TRAF6-Ubc13 inhibitor C25-140. These results strongly suggested that SenA restored the expression and activity of HDAC3 through a TRAF6-ubiquitination-related mechanism yet to be further elucidated. Since TRAF6 also plays critical role in the signaling transduction of inflammatory responses, DEGs implicated in TRAF6-related inflammation process between Sham group vs BDL group and BDL group vs BDL + SenA group were also plotted as heatmap (Fig. 5i). As shown in Fig. 5j, in BDL-stimulated mouse primary cholangiocytes, SenA markedly decreased the mRNA expression of TRAF6 signaling-related genes, such as nuclear factor kappa-B (*Nfkb*), *Traf3*, TGF-beta activated kinase 1 (*Tab1*) and inhibitor kappa B alpha (*Ikb α*). These results further confirmed that TRAF6 was a potential target of SenA.

Furthermore, co-immunofluorescence staining of TRAF6 and HDAC3 was performed to illustrate the effects of SenA on the interaction between TRAF6 and HDAC3. As shown in Fig. 5k, SenA significantly inhibited the co-localization of TRAF6 and HDAC3 in cytoplasm. Notably, TRAF6 (green dots) independent of the complex of TRAF6 and HDAC3 (red dots) were mostly seen in the cytoplasm rather than in the nucleus, suggesting that SenA may bind to TRAF6 and interrupted HDAC3-TRAF6 complex formation in the cytoplasm, which then licensed the nuclear translocation of HDAC3. Consistently, the disruption of TRAF6-HDAC3 complex by SenA was also observed in mouse primary cholangiocytes (Supplementary Fig. 6e) and livers (Supplementary Fig. 6f). Collectively these findings suggested that the effects of SenA on epigenetic regulation of MYC might attribute to the interruption of HDAC3 and TRAF6 interaction.

SenA disturbs the interaction between TRAF6 and HDAC3 in cholangiocytes

Based on the effects of SenA on decreasing the co-localization of TRAF6 and HDAC3 in Fig. 5, we further explored the underlying

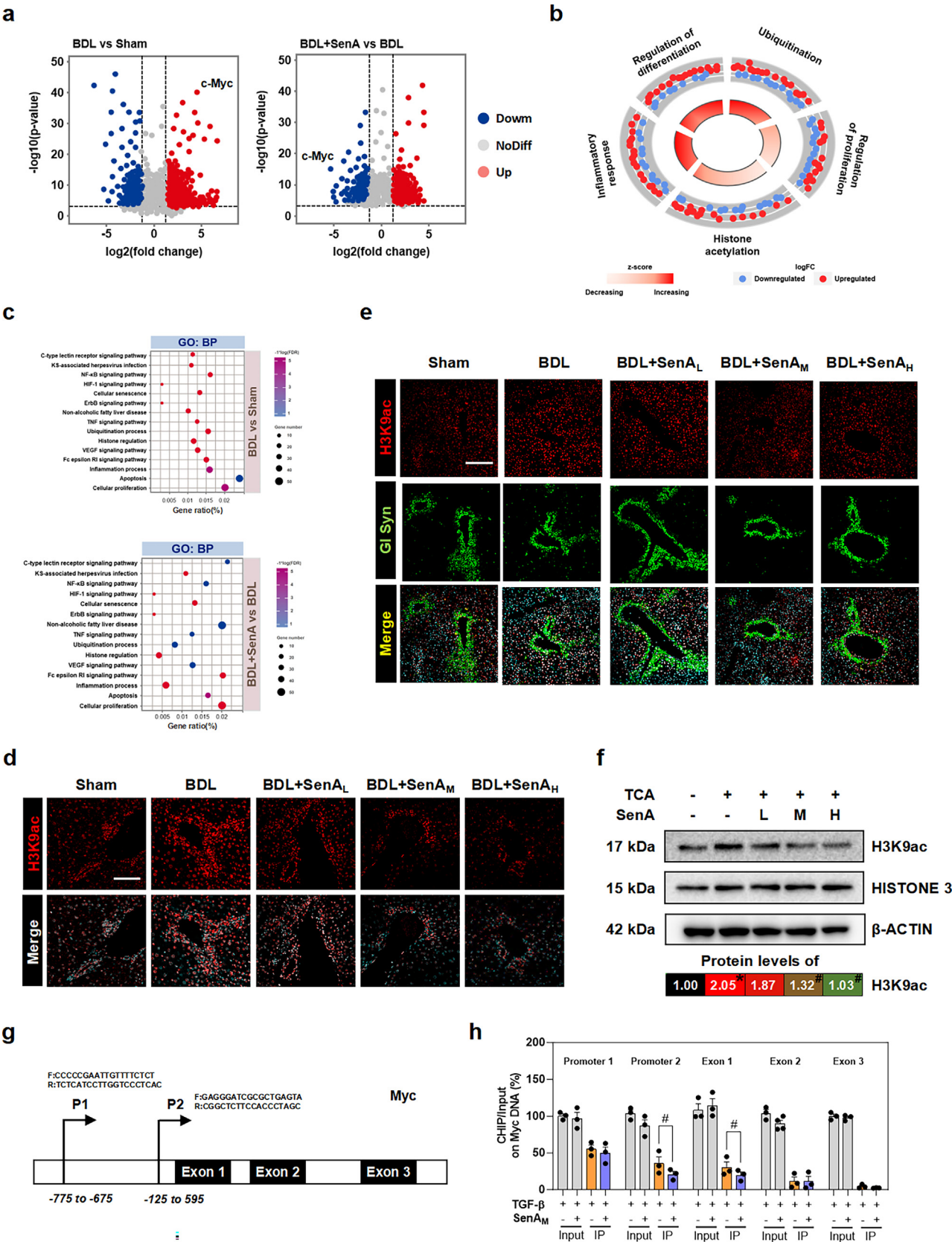


Fig. 4. SenA epigenetically inhibits the enrichment of H3K9ac at MYC promoter and exons. (a) The volcano map of DEGs between BDL vs. Sham and BDL + SenA vs. BDL. (b) “Targets-Pathways” network diagram. (c) GO analysis of DEGs between BDL vs. Sham and DEGs between BDL + SenA vs. BDL by R software. The size of dots was used to describe the number of genes enriched in corresponding pathways and the color of dots indicate the significance. (d) Representative images of H3K9ac immunohistochemistry staining. Scale bar = 50 μ m. (e) Representative images of H3K9ac (red) and Gl Syn (green) immunohistochemistry staining. Scale bar = 50 μ m. (f) Representative immunoblots against H3K9ac, HISTONE 3 and β -ACTIN in HIBECs were shown. (g) Location of primer sets relative to MYC transcription start site (TSS) for ChIP-qPCR analysis. (h) Input normalized H3K9ac enrichment levels were determined by ChIP-qPCR in the regions of promoter1, promoter2, exon1, exon2 and exon3 of c-MYC. * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model group (n = 3).

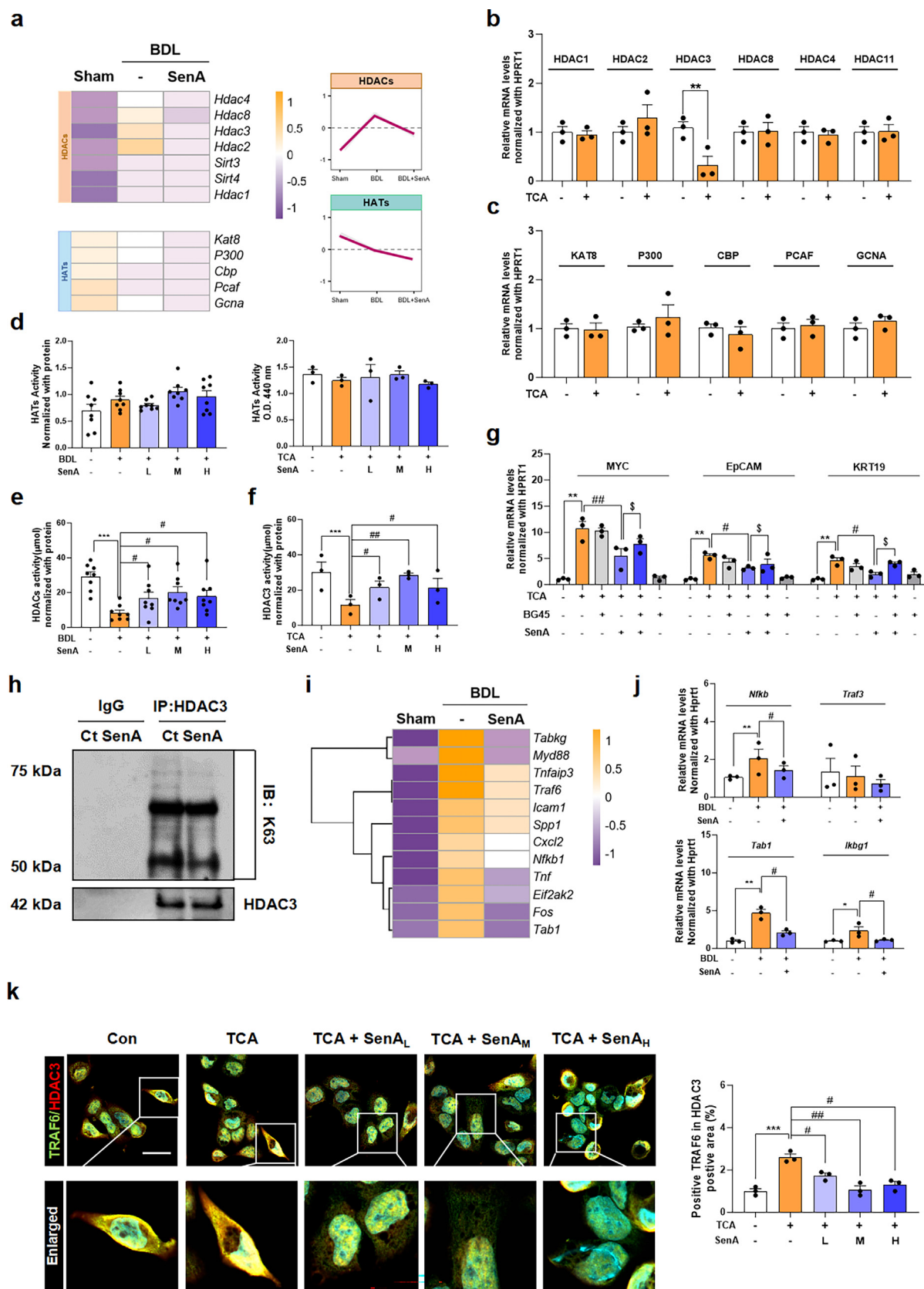


Fig. 5. SenA enhances HDAC3 activity via inhibiting the interaction between TRAF6 and HDAC3. (a) The relative expression levels of DEGs implicated in histone acetylation were shown as a heatmap. (b) Relative mRNA levels of HDAC1, HDAC2, HDAC3, HDAC8, HDAC4 and HDAC11 in HIBECs treated with TCA. (c) Relative mRNA levels of KAT8, P300, CBP, PCAF and GCNA in HIBECs treated with TCA. (d) HATs activities in HIBECs and livers. (e) HDACs activities in livers. (f) HDACs activities in HIBECs. (g) Relative mRNA levels of MYC, EpCAM and KRT19 in BG45-stimulated HIBECs. HPRT1 was utilized as an internal control. (h) Ubiquitination analysis of HDAC3. (i) The relative expression levels of DEGs implicated in inflammatory response were shown as a heatmap. (j) Relative mRNA levels of *Nfkb*, *Traf3*, *Tab1* and *Ikbk1* in primary cholangiocytes. *Hpvt1* was utilized as an internal control. (k) Representative images of TRAF6 (green) and HDAC3 (red) co-immunofluorescent staining were quantified using ImageJ software. ** $P < 0.01$, *** $P < 0.001$, vs. Control group; # $P < 0.05$, ## $P < 0.01$, vs. Model group; $^5P < 0.05$, vs. Model + SenA group ($n = 3$).

mechanism by molecular docking and Co-IP. As shown in Fig. 6a, a virtual TRAF6-HDAC3 complex was prepared and the binding mode was predicted by the Surflex-Dock program. Furthermore, the predicted binding interface, including ASP451, LYS469, TYR473, VAL474 and PHE410 of TRAF6 and ASN411, HIS418, PHE413 and TYR414 of HDAC3, was illustrated in Fig. 6b. Subsequently, Co-IP analysis results in primary cholangiocytes (Supplementary Fig. 7a) and HIBEC Fig. 6c suggested that the presence of TRAF6-HDAC3 complex was significantly decreased by SenA. Meanwhile, Co-IP analysis performed using cytoplasm and nucleus fractions further confirmed that the formation of tight complex between TRAF6 and HDAC3 was primarily in the cytoplasm and was significantly decreased by SenA, especially in cytoplasm (Supplementary Fig. 7b), which was consistent with our findings in IF staining (Fig. 5k). Next, we designed and expressed HA-tagged truncated forms of TRAF6 and HDAC3 proteins for Co-IP experiments to further confirm the protein–protein interaction sites proposed by molecular docking. Specifically, the FL TRAF6 coding sequence was split into two DNA fragments with HA tags: F1 (1–1050 bp) and F2 (1051–1566 bp, containing key residues for complex formation) (Fig. 6d, plasmid sequences provided in Supplementary table 2). Tight complex formation was observed only between TRAF6-F2 and HDAC3, as shown in Fig. 6e and Fig. 6f. Similarly, we truncated the coding sequence of HDAC3 (FL) into F1 (1–1119 bp) and F2 (1120–1287 bp, encoding critical interaction-related residues) (Fig. 6g). Fig. 6h and Fig. 6i demonstrated that deletion of 1120–1187 bp of HDAC3 abolished the formation of the TRAF6-HDAC3 complex. These results strongly support the predicted TRAF6-HDAC3 binding mode as suggested by molecular docking.

Notably, according to the molecular docking results in Fig. 6a, a stable hydrogen bond was formed between TRAF6 Arg483 and the carbonyl group of SenA. Furthermore, Arg483 presents on the interface of predicted protein–protein interaction between TRAF6 and HDAC3 (TRAF6: 351:522 aa), which has also been clearly reported [38]. Therefore, we deleted Arg483 in TRAF6 DNA sequence and established HA-TRAF6-d-intervened cholangiocytes (Fig. 6j). As shown in Fig. 6k, the formation of tight complex between TRAF6 and HDAC3 was disturbed by Arg483 deletion. Furthermore, the deletion of Arg483 completely blocked the binding of SenA to TRAF6, as illustrated by CETSA and DARTS experiments (Supplementary Fig. 7c–d). Collectively, these findings strongly suggested that Arg483 is critical for both the interaction between TRAF6 and HDAC3 and the regulative effects of SenA on HDAC activity.

SenA regulates HDAC3 function via directly binding with TRAF6

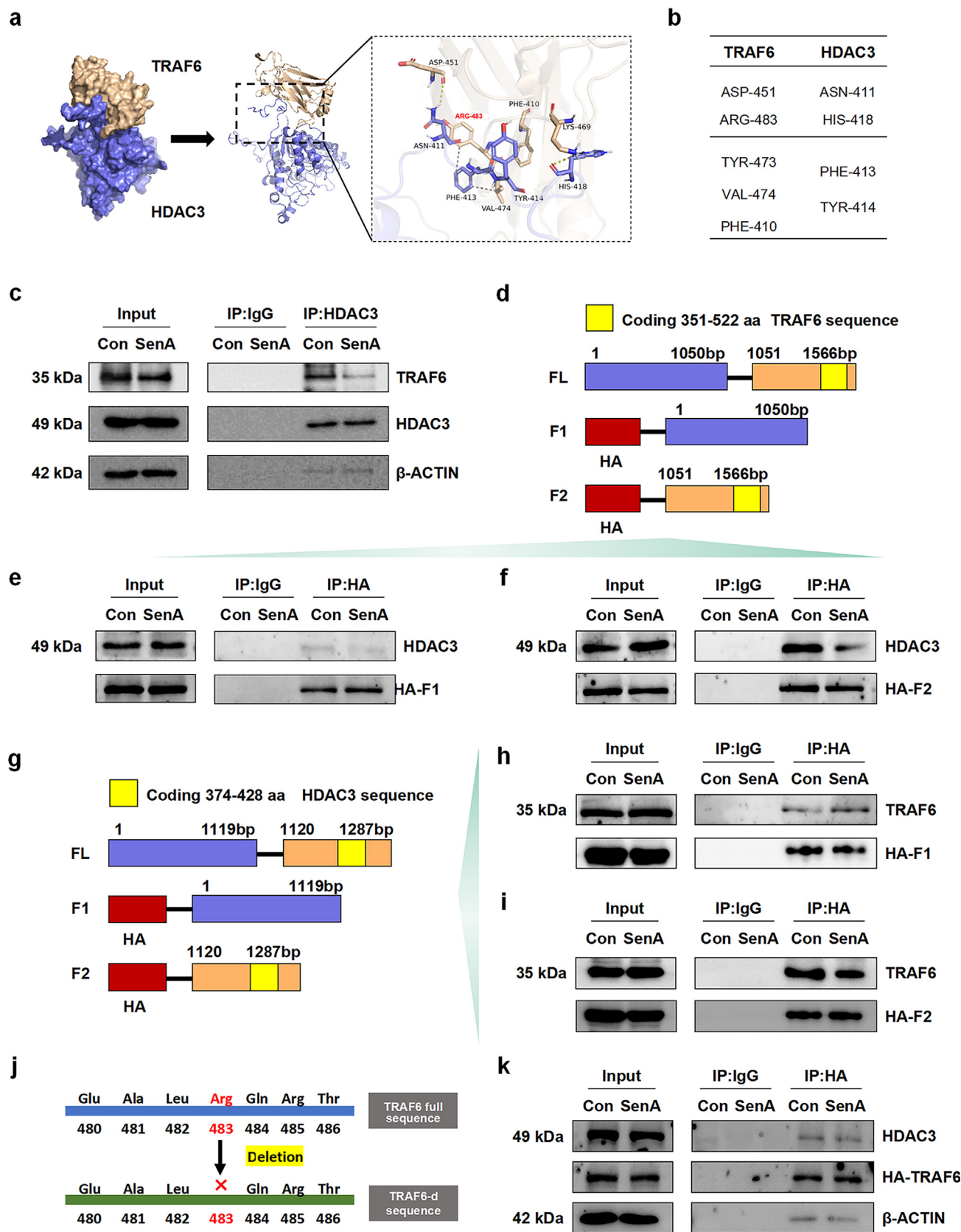
We hypothesized that SenA might disturb the combination of TRAF6 and HDAC3 by occupying the sites on TRAF6 used to bind with HDAC3, since SenA was highly unlikely to interact with HDAC3, as illustrated by CETSA and DARTS experiments (Supplementary Fig. 7e–f). To verify the hypothesis, we first examined whether SenA directly bound to TRAF6 using molecular docking, SPR, and MST assays. Molecular docking was used to simulate the binding of SenA to TRAF6. As shown in Fig. 7a, the docking score of SenA towards TRAF6 was -7.7 , suggesting that SenA might have a moderate binding affinity with TRAF6. Correspondingly, SenA interacted with TRAF6 by forming hydrogen bonds with catalytically active sites, Arg483, Leu364, Ile488, Leu482 and Leu479. Subsequently, Arg483 were identified as the most critical residue for the combination of TRAF6 and SenA. Additionally, SPR showed that the binding of SenA to TRAF6 was dose-dependent, exhibiting a classic association-dissociation process (Fig. 7b and Supplementary Fig. 8a). As shown in Fig. 7c and Fig. 7d, SenA improved the stability of TRAF6 by directly binding with TRAF6, as illustrated

by CETSA and DARTS assays. Furthermore, as shown in Fig. 7e, MST assay indicated that SenA directly bound to TRAF6 with a dissociation constant (KD) value of $10.25 \mu\text{mol}$.

According to the results of molecular docking in Fig. 7a, the carbonyl group is supposed to be critical for the pharmacological effects of SenA. Thus, we subsequently produced several derivatives of SenA, namely S1, 2, and 3, through Grignard reaction-mediated removal of 1'-carbonyl group, hydrogenation, and alkaline hydrolysis, respectively. The structures of these derivatives were determined by MS spectrum (Fig. 7f and Supplementary Fig. 8b). Subsequently, we investigated the effects of S1, S2 and S3 on cholangiocytes proliferation and the qPCR results demonstrated that S1 had no effect, yet S2 and S3 had partial effects, on TCA-induced upregulation of KRT19, EPCAM and CDK2 (Fig. 7g). While, S1, S2 and S3 failed to influence the upregulation of TCA-stimulated MYC (Fig. 7g) and PCNA expression (Supplementary Fig. 8c). MST assay further confirmed that S1, S2 and S3 failed to bind with TRAF6 (Fig. 7h and Supplementary Fig. 8d). Therefore, the partial effects of S2 and S3 on cholangiocytes proliferation may not be entirely dependent on TRAF6. Meanwhile, we observed that Ligustilide, owing similar structure (1'-carbonyl group and lactone ring) with SenA, could not bind with TRAF6, as demonstrated by CETSA and DARTS experiments (Supplementary Fig. 8e–f). Compared with a rotatable 3,8-single bond in SenA, the 3,8-double bond contributed to a rigid planar conformation of Ligustilide and thus limited its hydrogen bond formation between 1'-carbonyl group and Arg483 of TRAF6. Overall, these results confirmed that SenA reverse HDAC3 function via directly binding with TRAF6.

The anti-fibrotic mechanisms of SenA are confirmed by HDAC3 inhibition and cholangiocyte-targeted SenA delivery

According to the above findings, SenA alleviated liver fibrosis by binding with TRAF6 and facilitating HDAC3 activation, suggesting that inhibiting HDAC3 enzymatic activity might, at least partially, diminish the therapeutic effects of SenA. To confirm whether HDAC3 activity is critical for the pharmacological effects of SenA, mice were pre-treated with BG45 (HDAC3 inhibitor, 20 mg/kg) or BG45 plus SenA (20 mg/kg) for 2 days, underwent BDL surgery and were administrated with BG45 or BG45 plus SenA for another 4 days from the 3rd day post-surgery (Fig. 8a). BG45 aggravated BDL-induced serum levels of AST, ALT, TBA and ALP at least partly blunted the therapeutic effects of SenA (Supplementary Fig. 9a). As shown in Fig. 8b, BG45 aggravated BDL-induced lobular inflammation, collagen deposition and bile duct hyperplasia as illustrated by H&E staining, Masson's trichrome staining and EpCAM staining of liver tissues and the antifibrotic activity of SenA was significantly reduced when HDAC3 was inhibited by BG45. Consistently, in the presence of BG45, the inhibitory effects of SenA on BDL-associated upregulation of *H19*, *Myc*, *Epcam*, *Cdk2* (Fig. 8c), *Krt19*, *Pcna*, *Lin28a*, *S1pr2*, *Sphk2* and *Cdk2* (Supplementary Fig. 9b) were partially reversed. To further validate whether the therapeutic effect of SenA in liver fibrosis relies on its anti-cholangiocyte proliferation activity, we employed a FA-conjugated liposomal delivery system (Lipo-SenA) to achieve targeted delivery of SenA to cholangiocytes. The physical and chemical properties, including particle size, encapsulation efficiency, and Zeta potential, were determined (Supplementary Fig. 9c). As shown in Supplementary Fig. 9d, mice were pre-treated with Lipo-SenA for 2 days and *in vivo* fluorescence imaging was applied to identify the efficacy of Lipo-SenA-mediated targeted delivery. Subsequently, mice underwent BDL surgery and were administrated with Lipo-SenA for 4 days from the 3rd day post-operation (Fig. 8d). As shown in Fig. 8e, BDL-induced lobular inflammation and collagen accumulation, was reversed by Lipo-SenA. Lipo-SenA decreased the mRNA



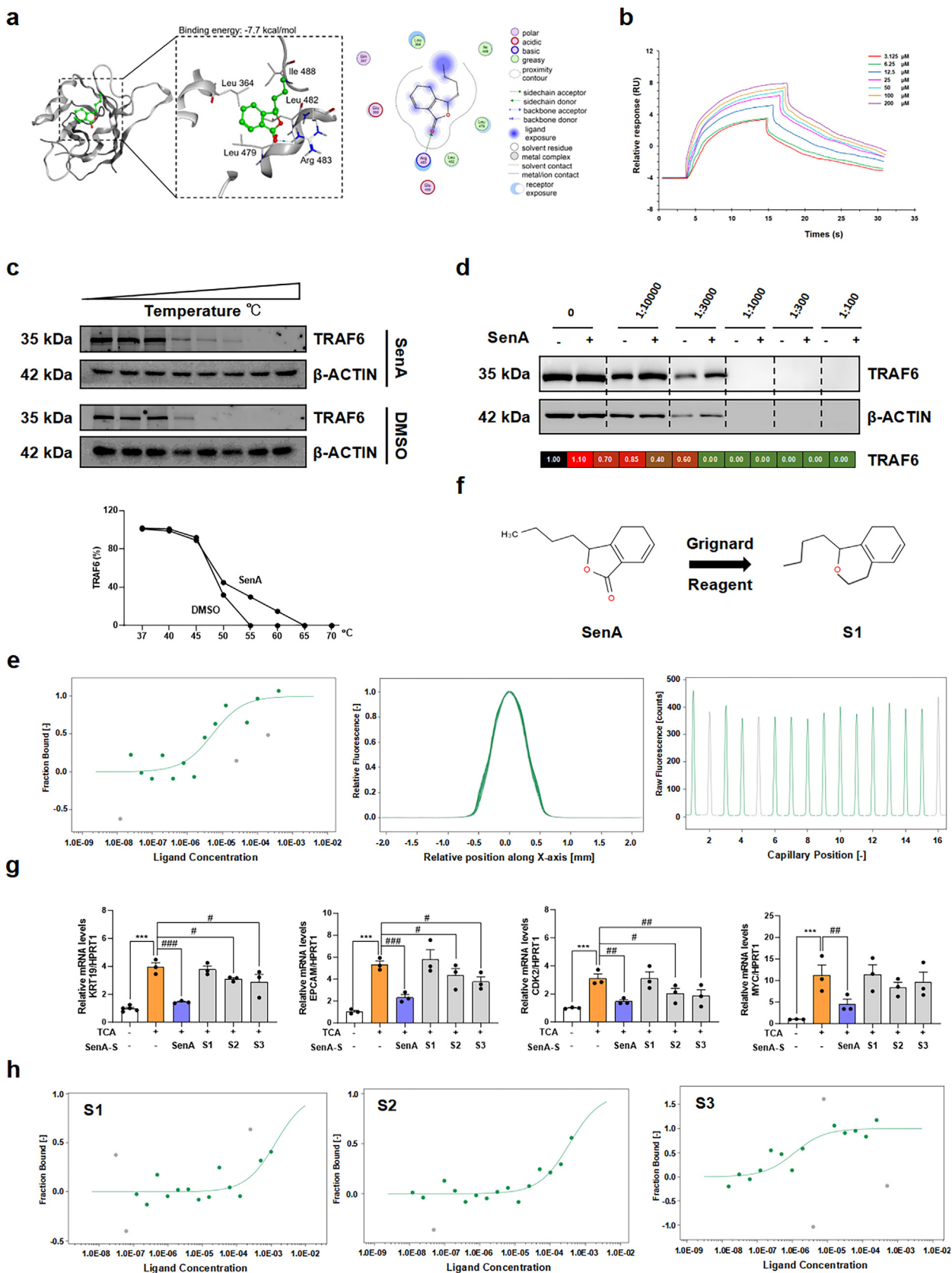


Fig. 7. The carbonyl group of SenA is critical for its binding to TRAF6. (a) Molecular docking of SenA and TRAF6. (b) SPR analysis of SenA and TRAF6. (c) CETSA analysis of SenA and TRAF6. (d) DARTS analysis of SenA and TRAF6. (e) MST analysis of SenA and TRAF6. (f) Structural modification (S1) of SenA via Grignard reagent reaction. (g) Relative mRNA levels of KRT19, EPCAM, CDK2 and MYC in TCA plus S1, S2 and S3-treated HIBECs. HPRT1 was utilized as an internal control. (h) MST analysis for S1, S2 and S3. *** $P < 0.001$, vs. Control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. Model group ($n = 3$).

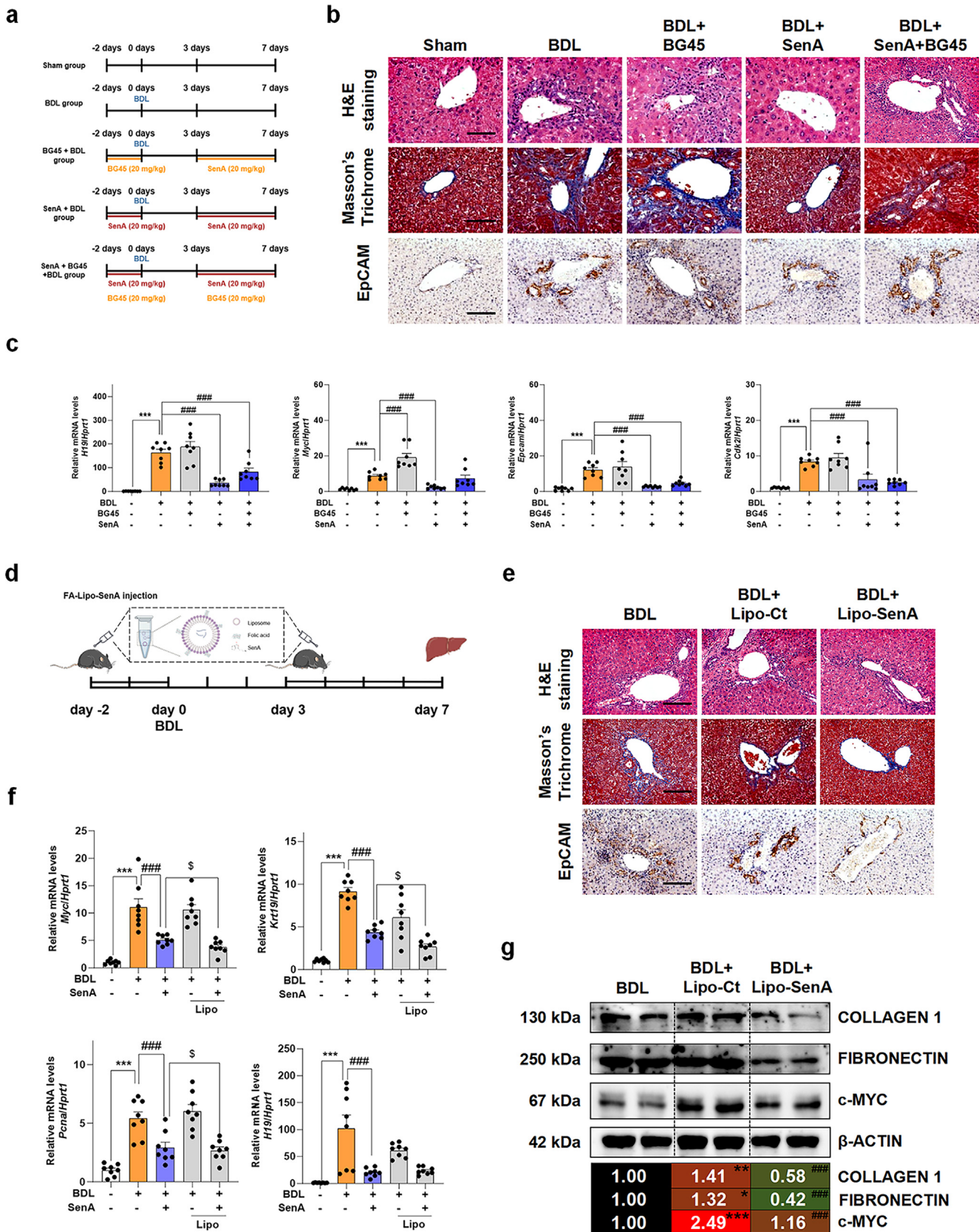


Fig. 8. The anti-fibrotic mechanisms of SenA are confirmed by HDAC3 inhibition and cholangiocyte-targeted delivery of SenA. (a) The experimental design for HDAC3 inhibition. (b) Representative images of H & E staining, Masson trichrome's staining and EPCAM immunohistochemistry staining were quantified using Image J software. Scale bar = 50 μ m. (c) Relative mRNA levels of *H19*, *Myc*, *Epcam* and *Cdk2* in livers treated with BDL, BDL + BG45, BDL + SenA and BDL + BG45 + SenA. *Hprt1* was utilized as an internal control. (d) The experimental design for cholangiocyte-targeted delivery of SenA. (e) Representative images of H & E staining, Masson trichrome's staining and EPCAM immunohistochemistry staining were quantified using Image J software. Scale bar = 50 μ m. (f) Relative mRNA levels of *Myc*, *Krt19*, *H19* and *Pcna* in the liver of mice treated with BDL plus Lipo-Ct, Lipo-SenA, and SenA. *Hprt1* was utilized as an internal control. (g) Representative immunoblots against COLLAGEN 1, FIBRONECTIN, c-MYC and β -ACTIN in the liver of mice treated with BDL plus Lipo-Ct or Lipo-SenA. *** P < 0.001, vs. Control group; ### P < 0.001, vs. Model group; * P < 0.05, vs. Model + SenA group (n = 8).

expression of *Myc*, *Krt19*, *H19*, *Pcna* and *H19* (Fig. 8f). As shown in Fig. 8g, the protein levels of COLLAGEN 1, FIBRONECTIN and c-MYC were increased both in the BDL group and BDL + Lipo-PBS (Lipo-Ct) group, which was markedly decreased by Lipo-SenA treatment. Collectively, these findings commonly illustrated that SenA reversed liver fibrosis by targeting HDAC3 in proliferating cholangiocytes.

Discussion

As a transcription factor, c-MYC regulated gene transcription *via* binding to DNA sequence elements E-boxes, which providing possibility for c-MYC to regulate various physiological process including proliferation, cell cycle progression and apoptosis [39]. Notably, the transcription of c-MYC were regulated by several mechanisms [40]. It has been widely accepted that abnormal expression of c-MYC is an indicator of tumorigenesis in tumor microenvironment. Hence, the identification of key mediators in c-MYC transcription regulation are critical for discovering novel targets and therapies for the cholestatic liver injury. In the current study, we highlighted the important roles of c-MYC in DR and further demonstrated that SenA inhibited cholestatic liver diseases by suppressing c-MYC transcription and its downstream targets in cholangiocytes. Furthermore, our findings in this manuscript suggested that TRAF6/HDAC3 may be the central event for SenA to inhibit transcription of c-MYC.

DR in cholangiocytes is the most immediate and sensitive event in BDL mouse model [41]. The abnormally high expression of c-MYC in cholangiocytes is achieved by gene amplification, insertion and ectopic. While, the findings in this manuscript suggested that TRAF6 may affect c-MYC transcription through an underlying mechanism that is not yet known. TRAF6, an important inflammatory signal, played a pivotal role in NF- κ B activation and macroautophagy activation induced by toll like receptor 4 signaling [42]. Cholestatic liver injury is accompanied by significant inflammatory response, characterized by a large number of inflammatory cell infiltration and activation of inflammatory pathways. Thus, it may be widely accepted that TRAF6 affecting cholestatic liver injury *via* regulating inflammation processes. Whereas, we innovatively identified that TRAF6 affected MYC transcription *via* binding with HDAC3. Indeed, the roles of TRAF6 in inflammatory signals were relied on its ubiquitin-ligase activities [43]. Consistently, TRAF6 induced HDAC3 ubiquitin through directly binding with HDAC3, and SenA disturbed the HDAC3 ubiquitin and leaved HDAC3 in the MYC promotor by occupying binding sites of HDAC3 in TRAF6. Thus, the roles of TRAF6 in c-MYC transcription are also relied on its ubiquitin-ligase activities. Notably, C-terminal domain (351–522 aa) is responsible for substrate binding. On the other hand, C-terminal domain (374–428 aa) was critical for HDAC3 to interact with other proteins. Hua W reported that HDAC3 carried the TRAF6-binding motif residue [38]. SenA directly combined with TRAF6 at Arg483, and thus impaired the interaction between HDAC3 and TRAF6. Cholangiocyte proliferation appeared to be indispensable during cholestasis. The effects of TRAF6 in c-MYC transcription suggested a potential to relieve early fibrosis.

Currently, the agonists and inhibitors of HDACs, including broad-spectrum HDACs and SIRT1 inhibitors, are continuous exploitation [44]. Indeed, several HDAC inhibitor, including vorinostat, VPA, entinostat, BRD4884 and NW21, are being clinically used in lymphatic cancer and hepatoma [45,46]. While, a great deal of HDACs is being addressed in clinical trials due to the low clinical compliance [47]. However, there is still a gap to be filled in the clinical application of histone deacetylases agonists and inhibitors. Meanwhile, HDAC3 belongs to the first class of histone deacetylases, which are the most widely accepted and potential class among the four HDAC families to develop inhibitors

for clinical applications [48]. Currently, antifibrotic effects of several inhibitors of HDACs have been illustrated [49,50], which only focused on fibroblasts and inflammatory pathways. Indeed, Hua W and his colleagues [38] leaded HDAC3 at c-MYC promoters *via* depleting TRAF6. Therefore, HDAC3 continuously moved acetylation away from H3K9 and thus inhibited c-MYC transcription. Here, we applied BDL-stimulated cholestatic liver injury and demonstrated that SenA bound with TRAF6 to leave HDAC3 in the promoter region of MYC and thus inhibited the transcription of c-MYC in cholangiocytes, highlighting the complexity of targeting HDAC3. Meanwhile, c-MYC, also acted as a transcription factor, could be bound to the DNA promoter region of multiple downstream targets, including H19 [51]. Our previous studies demonstrated that H19 activated S1PR2 and SPHK2 and thus promoted cholangiocyte proliferation and fibrotic responses. Meanwhile, H19 promoted biliary atresia *via* increasing LET7A transcription [25]. Here, we showed that as the downstream of c-MYC, H19 promoted LET7A, LIN28A and CDK2 in cholangiocytes, bridging the MYC-H19 and proliferation-related genes in cholangiocytes. Thus, our study providing a new perspective for the discovery of SenA-based therapeutic agents against cholestatic liver injury. Indeed, targeted therapy for specific cell population has attracted much attention.

We recently established FA-Lipo-SenA liposome targeted delivery to anchor tumor cells and thereby inhibited tumor growth, fibrosis and reeducated immunosuppressive tumor microenvironment [52]. Meanwhile, Fei W and his colleagues established liposomes containing ASCT2 shRNA/ASCT2 plasmid targeted hepatic stellate cells through vitamin A [53]. Cholangiocyte-targeted liposome is being used in cholangiocarcinoma, while it is still rarely used in cholestatic liver injury. Notably, after verifying the effects of SenA on c-MYC-associated cholangiocyte proliferation, we also constructed liposomes containing SenA by using FA-targeting cholangiocytes as previously described [52]. Until now, the cholangiocyte-targeted drug delivery system of SenA has not been reported. Comparing with BG45-stimulated HDAC3 inhibition, Lipo-SenA delivery system seemed to show better anti-cholestatic effects, including lower lobular inflammation, less collagen deposition and genital DR. While, the drug delivery system still need improvement. Detailly, Lipo-SenA targeting proliferating cells in the liver through FA, a cellular proliferation marker. As treatment of SenA continues to improve, cholangiocytes will gradually drop proliferation and the targeting of liposomes might be slowly weakened, leading to the efficacy reduction. Taken together, our findings reprovved the cholangiocytes as the centrality cell types in BDL-stimulated liver fibrosis [27] and provide a novel strategy for cholestatic hepatic fibrosis.

Herein, we confirmed that SenA alleviated cholangiocyte proliferation by inhibiting c-MYC-related H19-Let-7a axis activation in cholestatic liver injury. Mechanistically, SenA occupies the binding site on TRAF6 that interacts with HDAC3, rescuing HDAC3 from the inhibitory complex and enhancing its enrichment at the MYC promoter region, ultimately suppressing MYC transcription through H3K9 deacetylation. Our study not only identified SenA as a potential candidate drug for cholangiopathy-associated liver fibrosis but also unveiled a novel approach for discovering epigenetic regulators by targeting protein–protein interactions.

Compliance with Ethics Requirements

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Institutional Animal Care and Use Committee of the Beijing University of Traditional Chinese Medicine (Approved project: BUCM-4-20200730023160).

CRediT authorship contribution statement

Yajing Li: Investigation, Methodology, Formal analysis, Writing – original draft. **Runping Liu:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Jianan Li:** Methodology. **Feng Gao:** Methodology. **Zhi Ma:** Methodology. **Kaihong Xie:** Methodology. **Fanghong Li:** Methodology. **Bing Xu:** Methodology. **Qi Zheng:** Methodology. **Yajie Cai:** Methodology. **Jiaorong Qu:** Validation. **Xiaoyong Xue:** Methodology. **Kexin Jia:** Methodology. **Xiaojiayang Li:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (Grant NO. 82274186 to XL and 82322075 to RL); National High-Level Talents Special Support Program to XL; Scientific research innovation capability support project for young faculty in central universities to XL.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2025.04.002>.

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