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STAT3-induced SMYD3 transcription enhances chronic lymphocytic leukemia cell growth in vitro and in vivo

Fujia Lin[1] · Danjuan Wu[2] · Dan Fang[3] · Yao Chen[4] · Haitao Zhou[1] · Caiwen Ou[1]

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Abstract

Objective and design The purpose of this study was to investigate the roles of SMYD3 and STAT3 in chronic lymphocytic leukemia (CLL) and the possible underlying mechanisms.

Materials Blood samples were collected from 20 patients with CLL and 20 hematologically normal donors. Human cell lines K562, HL-60, MEG-1, and BALL-1 were performed in vitro and BALB/c nude mouse was used in subcutaneous tumor experiments.

Treatment WP1066 (30 mg/kg) was also injected intratumorally two days after the first lentivirus treatment and then every four days for a total of four injections and 3 μM WP1066 was carried out for 48 h to downregulate STAT3 phosphorylation.

Methods We performed studies using the human CLL cell line MEG-1 in vitro and nude mouse subcutaneous tumor experiments in vivo. Differential expression of RNAs was determined using qRT-PCR. The CCK-8 assay and colony formation assay were conducted to evaluate cell proliferation. Flow cytometry was performed to assess cell apoptosis. The relative protein levels were detected using western blotting. Chromatin immunoprecipitation (ChIP) assays, luciferase reporter assays and WP1066, a STAT3 inhibitor, were used to explore the regulatory mechanisms of proteases and transcription factors. A subcutaneous tumor model was constructed to verify the results in vivo.

Results SMYD3 and STAT3 expressions positively correlated with the progression of CLL. Upregulation of SMYD3 significantly promoted the proliferation and inhibited the expression of apoptosis-related genes. The results of the ChIP assays and luciferase reporter assays suggested that STAT3 targeted the promoter region of SMYD3 and, thus, promoted SMYD3 transcription. Downregulation of the phosphorylation of STAT3 by WP1066 notably inhibited the binding of STAT3 to the SMYD3 promoter, and subsequently downregulated SMYD3 transcription. The STAT3 inhibitor inhibited CLL cell growth in vivo, and overexpression of SMYD3 promoted CLL cell growth. Furthermore, overexpression of SMYD3 reversed the inhibitory effects of the STAT3 inhibitor on CLL cell growth.

Conclusions The STAT3-mediated transcription of SMYD3 plays a role in promoting the progression of chronic lymphocytic leukemia.

Keywords Chronic lymphocytic leukemia · STAT3 · SMYD3 · Transcription

Introduction

Chronic lymphocytic leukemia (CLL) is a common form of leukemia in Western countries that is characterized by an increasing accumulation of leukemic cells, limited apoptosis, and aberrant proliferation [1, 2]. Although current chemical immunotherapy has improved clinical outcomes, CLL still remains incurable. Therefore, studies aiming to understand the mechanisms that contribute significantly to the survival of CLL cells may lead to the development of additional and novel therapeutic strategies.

Signal transducer and activator of transcription (STAT) is a protein family consisting of transduction and transcription factors. The STAT family is implicated in many biological processes, including cell growth, differentiation, survival, and development [3]. STAT3 is abnormally activated in many cancer cells. This tumor-associated transcription factor

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Fujia Lin and Danjuan Wu contributed equally to this work.

✉ Caiwen Ou
ocw313@163.com

Extended author information available on the last page of the article

regulates gene expression, resulting in a significant upregulation of genes involved in tumor cell survival, proliferation, and immunosuppression [4, 5]. STAT3 is phosphorylated upon the activation of upstream receptor tyrosine kinases, and phosphorylated STAT3 (pSTAT3), which serves as a transcriptional factor, is transported into the nucleus. Then pSTAT3 binds to the promoter areas of genes that manage cell cycle progression and apoptosis [6]. STAT3 inhibition has been shown to reduce cell viability and promote apoptosis in various cancer models [7, 8]. STAT3 regulates the expressions of apoptosis-related genes, such as Bcl-2, Mcl-1, and Bax, to promote proliferation and inhibit the apoptosis of CLL cells [9].

The SET and MYND domain-containing 3 (SMYD3) protein is the most extensively characterized and well-researched SMYD family member. By activating the transcription of multiple target genes, SMYD3 facilitates cancer development [10]. Additionally, SMYD3 is overexpressed in many tumor types, such as mammary gland, gastric cancer, and liver carcinoma [11, 12]. Upregulated expression of the SMYD3 is required for proliferation, migration, and adhesion, while SMYD3 inhibition mediated by RNA interference or other reagents suppresses cell growth and migration [13]. However, the impacts of SMYD3 on CLL are largely unknown.

The function of SMYD3 depends upon the activation of signal transduction pathways during tumor progression to a large extent [13]. Although the mutual interaction between SMYD3 and STAT3 further increases cancer cell propagation and survival [14], researchers have not determined whether SMYD3 and STAT3 exert any impact on the development and progression of CLL.

Here, we investigated the functions of SMYD3 and STAT3 during CLL and their potential regulatory mechanisms. SMYD3 and STAT3 were expressed at high levels in CLL. Upregulated SMYD3 significantly promoted the growth of CLL cells, while the inhibition of STAT3 activation dramatically reversed this effect. Furthermore, STAT3 promoted *SMYD3* transcription and downregulated STAT3 phosphorylation to suppress SMYD3 expression. These results may provide the basis for the development of new therapeutic strategies to prevent CLL progression.

Materials and methods

Patients

The Ethics Committee of The Second Affiliated Hospital of Guangzhou University of Chinese Medicine approved in vitro studies on blood samples collected from 20 patients with CLL and 20 hematologically normal donors. All samples were obtained after the patient provided informed consent.

Cell culture and reagents

The expression of SMYD3 and STAT3 was detected in the human CLL cell lines K562, HL-60, MEG-1, and BALL-1, which were obtained from the Bena culture collection (Suzhou, China). All cells were suspended in complete RPMI-1640 (w/o Hepes) + 15% FBS and cultured in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Venous blood was collected from healthy human subjects in heparinized tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden) and designated normal cells. WP1066 (Calbiochem, Merck, USA) was dissolved in dimethyl sulfoxide (DMSO; Solarbo, Beijing, China). WP1066 was added to cells at a final concentration of 3 μM for a 48-h treatment.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from blood samples and CLL cell lines using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's guidelines. For each swatch, the amount of total RNA was determined to be 200 ng using a NanoDrop 2000 instrument (Thermo Fisher Scientific Inc., USA). RNAs were reverse transcribed using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Then qRT-PCR was conducted using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Dusseldorf, Germany) and the following the reaction conditions and steps: 2 min at 94 °C; 30 cycles of 10 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C; followed by 10 min at 72 °C. Human β-actin was utilized as the internal control. All qRT-PCR experiments were repeated three times. The relative level of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

Plasmid construction and cell transfection

The SMYD3 overexpression vector (pCMV-SMYD3), knockdown vector (pCMV-sh-SMYD3), and empty pCMV vector were constructed and provided by GenePharma (Shanghai, China) and then packaged using the Lenti-Pac™ FIV Expression Packaging Kit (GeneCopoeia, Rockville, MD) according to the manufacturer's directions. Lentiviral particles were harvested from co-transfected HEK 293T cells. Forty-eight hours after transfection, cells were harvested for further analysis. The primers used to construct SMYD3 overexpression and knockdown vectors are listed in Table 1.

Table 1 Primers for qRT-PCR and the construction of plasmid vectors

Gene		Sequence (5′–3′)
SMYD3	Forward	TCGCAACCGCCAACAGGGGAAACGG
	Reverse	TCCCGAGAAGGCAGCGGTCGCAGAC
STAT3	Forward	AAACTGGAGGAGTTGCAGCAAAAG
	Reverse	CGGTCAGGATGCATGGGCATGCAGG
pCMV-SMYD3	Forward	CGGGAATTCATGGAGCCGCTGAAGGTGGAA
	Reverse	CCGCTCGAGTTAGGATGCTCTGATGTTGGCG
pCMV-sh-SMYD3	Forward	CACCGCACTACAGTATTTGGCGACGCCGAA GCGTCGCCAAATACTGTAGTG
	Reverse	AAAACACTACAGTATTTGGCGACGCTTCGG CGTCGCCAAATACTGTAGTGC
GAPDH	Forward	TGTGTCCGTCGTGGATCTGA
	Reverse	CCTGCTTCACCACCTTCTTGA

CCK-8 assay

Cell proliferation was examined using the Cell Counting Kit-8 (MedChemExpress, NJ, USA) according to the manufacturer's instructions. MEG-1 cells in exponential growth were prepared as single cell suspension in RPMI-1640 medium supplemented with 15% FBS. Briefly, cells transfected with different vectors (5000/well) were seeded into 96-well dishes and incubated in a 5% CO_2 atmosphere at 37 °C. On days 1, 2, 3, 4, and 5, cells were treated with 15 µl of CCK-8. Finally, the OD value of each sample was assessed at 450 nm using a microplate reader. Experiments were conducted in triplicate, and the average OD value was calculated.

Soft agar culture

Anchorage-independent growth was assessed by measuring colony formation in soft agar. Briefly, equal volumes of agar (1%, DNA grade) and 2 × RPMI-1640 (supplemented with 30% FBS) were mixed at 40 °C to prepare 0.5% agar in six-well tissue culture plates (Corning) as a base agar. Cells (0.1 mL of 2.0×10^5/mL) were suspended in 3 mL of 2 × RPMI-1640 (supplemented with 30% FBS) and 3 mL of 0.7% agar, and 1.5 mL of the cell suspension was then added to each well (as 0.35% top agar), with a final density of 5000 cells per well. The top agar was covered with culture medium. Plates were incubated at 37 °C in a humidified incubator with a 5% CO_2 atmosphere for 2–3 weeks and the medium was changed every 3–4 days. Colony formation was observed under a light phase-contrast microscope and images were captured.

Flow cytometry

Cells were cultured for 2 days after transfection, harvested, and washed five times with PBS to examine the impact of SMYD3 on the sensitivity of cells to apoptosis. Percentages of apoptotic cells were evaluated using Annexin V-FITC/PI double staining according to the manufacturer's instructions provided with the Annexin V-FITC Apoptosis Detection Kit (BD, San Jose, CA, USA). The final apoptosis rate was examined using a FACSCalibur flow cytometer with FACS Diva software. The experiment was repeated in triplicate.

Western blot

The cells and tumor tissues were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Keygen, Nanjing, China). A Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to determine the protein concentrations in the lysates and equal amounts of proteins were separated on SDS-PAGE gels and transferred to PVDF membranes using electrotransfer at a 200 mA constant current for 2 h. The membranes were immersed in TBST containing 5% skim milk powder, incubated with primary antibodies against SMYD3 (#4251S; dilution, 1:1000; Cell Signaling), STAT3 (sc-8019; dilution, 1:1000; Santa Cruz), p-STAT3 (sc-8001-R; dilution, 1:1500; Santa Cruz), Bcl-2 (sc-176463; dilution, 1:1000; Santa Cruz), Bax (sc-6236; dilution, 1:1000; Santa Cruz), and β-actin (as control; dilution,1:5000; Abcam, USA) at 4 °C for 12 h, followed by secondary antibodies conjugated to HRP (ab222759, 1:2000, Abcam, Cambridge, MA, USA) for 2 h at 23 °C. Finally, blots were visualized using the Life Technology Company ECL Plus reagent and the signals were detected using the Lab Works 4.5 imaging system.

Subcutaneous xenotransplanted tumor model

The study was approved by The Second Affiliated Hospital of Guangzhou University of Chinese Medicine and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals. Four-week-old

nude mice were purchased from Slac Laboratory Animal Company (Shanghai, China). The xenotransplanted tumor model was produced by subcutaneously injecting MEG-1 cells (1×10^6/200 µl PBS) into the left side of nude mice. When the average tumor size was approximately 100 mm^3 in tumor-bearing mice, mice were randomly divided into four groups (pCMV-SMYD3 group, pCMV-sh-SMYD3 group, empty vector group, and blank group) or five groups (pCMV-SMYD3 group, WP1066 group, pCMV-SMYD3 +WP1066 group, control (pCMV + DMSO), and blank group). Lentiviruses (1×10^8 pfu) in 100 µl of serum-free medium were injected into three sites of each tumor every 4 days for a total of four injections. DMSO or WP1066 (30 mg/kg) was also injected intratumorally 2 days after the first lentivirus treatment and then every 4 days for a total of four injections. Tumor diameters were measured using Vernier calipers. The maximum diameter (x) and its diameter (y) were measured, and the tumor volume was calculated as tumor volume (V) = 1/2 xy^2 (cm^3). Mice were evaluated every 7 days.

Chromatin immunoprecipitation (ChIP) Assay

Cells transfected with the appropriate plasmid were cross linked with 1% formaldehyde for 10 min at 37 °C. Cells were suspended in 300 µl of lysis buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS, and 1 mM PMSF) after washes with PBS. The DNA was sheared into small fragments by sonication. The supernatants were precleared using a herring sperm DNA/Protein G-Sepharose slurry (Sigma-Aldrich, USA). The retrieved supernatant was incubated with an anti-STAT3 antibody (ST3-5G7, Thermo Fisher Scientific, Waltham, MA, USA) in the presence of herring sperm DNA and Protein G-Sepharose beads for 2 h. Immunoprecipitated DNA was retrieved from the beads with 1% SDS and a 1.1 M $NaHCO_3$ solution at 65 °C for 6 h. The DNA was then purified using a PCR Purification Kit (Qiagen, USA). The primers are shown in Table 2.

Luciferase reporter assay

Luciferase activity was determined using the dual luciferase assay system (Promega, USA) according to the manufacturer's instructions. After 12–24 h of culture, MEG-1 cells were co-transfected with 0.6 µg of the expression vectors, 0.18 µg of the promoter–reporter plasmids, and 0.02 µg of the pRL-TK plasmids using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After 5 h of transfection, the supernatant was removed and cells were afforded to restore overnight in fresh medium complemented with 15% FBS for 2 days. Afterwards, the transfected cells in culture vessels were lysed with a lysis buffer, and the lysates were then centrifuged at maximum velocity in an Eppendorf microcentrifuge for 2 min. Relative luciferase activity was determined using a Modulus™ TD20/20 Luminometer (Turner Biosystems, USA), and the transfection efficiencies were normalized to the Renilla luciferase activity. Relevant primer sequences are provided in Table 2.

Statistical analysis methods

The experiments were implemented at least three times. The data are presented as the means ± standard deviations. The statistical significance of differences was analyzed using the

Table 2 Primers used for the SMYD3 promoter construct and ChIP

Primer name	Primer sequences (5′–3′)
Primers for the SMYD3 promoter construct:	
(− 1979/− 205) SMYD3 sense	TATAGAGCTCATGGGCCCACGGAGCAAGTG
(− 1608/− 205) SMYD3 sense	TATAGAGCTCGGAATGTTCTCAGCTCCCTC
(− 1246/− 205) SMYD3 sense	TATAGAGCTCATGACTTTAGATACACAGA
(− 884/− 205) SMYD3 sense	TATAGAGCTCTCGTACCACTGCATTCCAGC
(− 594/− 205) SMYD3 sense	TATAGAGCTCGATTGCACATGCCTGTAGT
Antisense	TATAACGCGTTGGCGACTGCGCAGAAGACA
Primers used for ChIP assays of the SMYD3 promoter:	
P1 sense	AAAATTAAGCTGGGTGTGGT
P1 antisense	CCAGAGACAGTCTACCTAT
P2 sense	ACTAAGAAATTTAGGCCGGG
P2 antisense	TCTCGATCTAACCTTGTGAT
P3 sense	GGCCCCACAACCCAAACAAG
P3 antisense	CTGTGAATATCTTTTAAGC
P4 sense	CCGTGGATGAGACACTTGCA
P4 antisense	TCCACAGTGTGATTAGCCTG

t test or one-way analysis of variance (ANOVA) when more than two groups were compared. Western blot band densities were assayed using the Kruskal–Wallis test followed by Dunn's post hoc test. Statistical analyses were performed using GraphPad Prism 6.0 software. The significance level was set to $P < 0.05$.

Results

SMYD3 and STAT3 were expressed at high levels in chronic lymphocytic leukemia (CLL) samples

Blood samples were isolated from 20 patients with CLL and 20 healthy donors (controls), and the expression of SMYD3 and STAT3 was increased in CLL samples, as detected using RT-qPCR (Fig. 1a, b). The expression of both the SMYD3 and STAT3 mRNAs in four CLL cell lines (K562, BALL-1, MEG01, and HL-60) and a normal cell line (control) was detected, and SMYD3 and STAT3 were upregulated in CLL cell lines (Fig. 1c). MEG-1 cells were chosen for further study.

Effects of SMYD3 on the proliferation of CLL cells in vitro

The expression of SMYD3 in MEG-1 cells transfected with pCMV-SMYD3 or pCMV-sh-SMYD3 was measured using RT-qPCR. Based on the RT-qPCR results, SMYD3 expression was significantly upregulated in the pCMV-SMYD3 group and downregulated in the pCMV-sh-SMYD3 group (Fig. 2a). CCK-8 and soft agar assays were performed to evaluate the viability of MEG-1 cells with altered expression of SMYD3. Compared with the control group, overexpression of SMYD3 increased cell proliferation (Fig. 2b). Downregulation of SMYD3 notably reduced the number and size of clones (Fig. 2c, d). Thus, SMYD3 promoted the proliferation of CLL cells in vitro.

Effects of SMYD3 on the apoptosis of CLL cells

Cells were incubated for 48 h after transfection with pCMV-SMYD3 and pCMV-sh-SMYD3 to upregulate or silence SMYD3 expression, respectively. The percentages of apoptotic cells in different groups were determined using flow cytometry following AnnexinV FITC/PI dual staining of MEG-1 cells. SMYD3 overexpression inhibited apoptosis, while SMYD3 knockdown promoted apoptosis (Fig. 3a, b). The levels of proteins encoded by apoptosis-related genes in MEG-1 cells transfected with pCMV-SMYD3 and

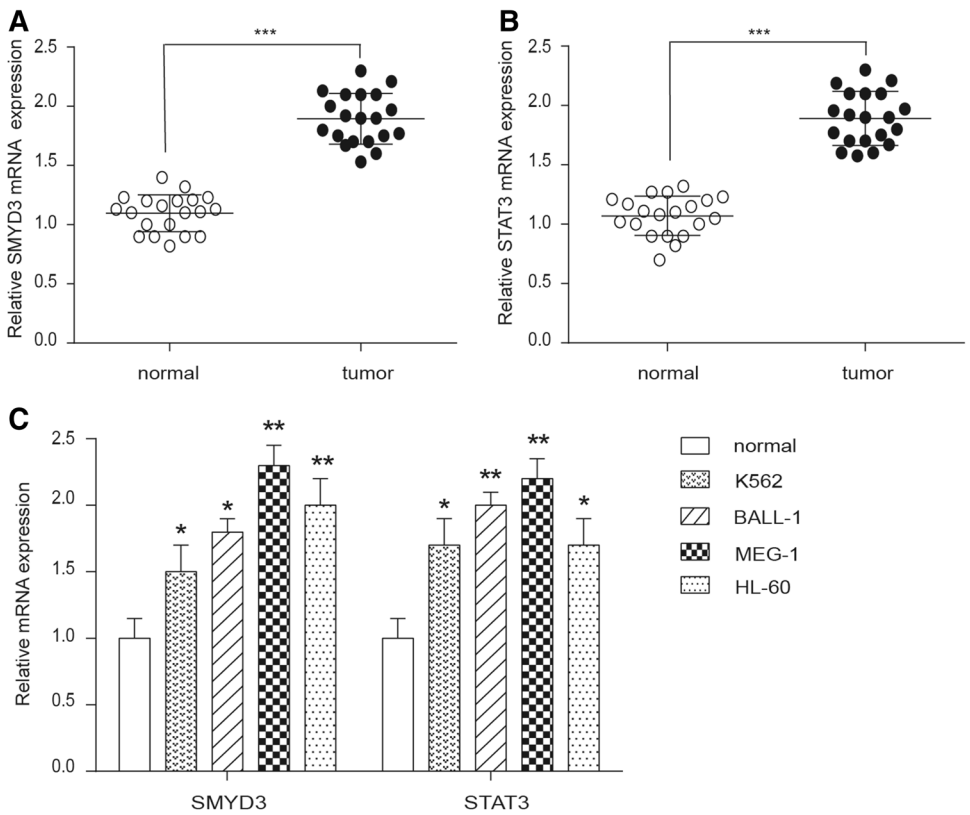

Fig. 1 SMYD3 and STAT3 are expressed at high levels in chronic lymphocytic leukemia (CLL) samples. **a, b** The expression of the SMYD3 and STAT3 mRNAs in samples from 20 patients with CLL and 20 normal controls were examined using RT-qPCR. **c** The expression of the SMYD3 and STAT3 mRNAs in four CLL cell lines and normal cells was detected using RT-qPCR. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the normal group

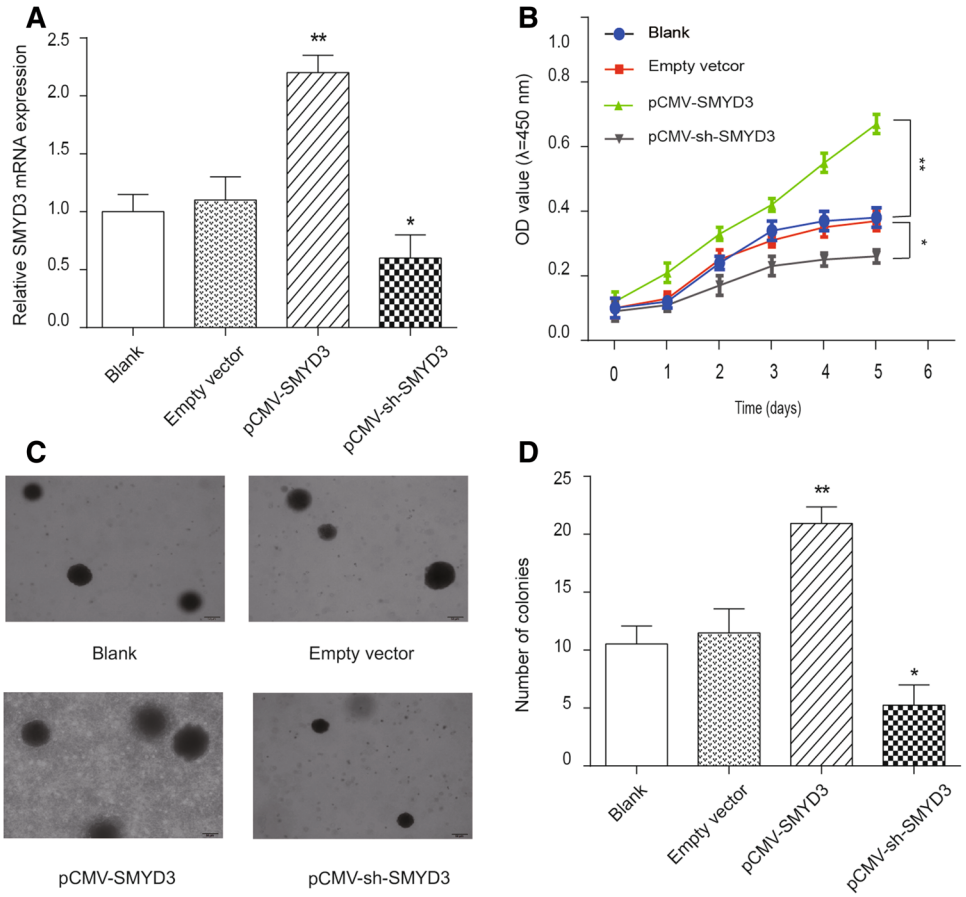

Fig. 2 Effects of SMYD3 on the proliferation of CLL cells. **a** The expression of SMYD3 in MEG-1 cells transfected with pCMV-SMYD3 and pCMV-sh-SMYD3 was measured using RT-qPCR. **b** The effects of different levels of SMYD3 expression on the proliferation of MEG-1 cells were detected using the CCK-8 assay. **c** Photomicrograph of a typical colony growing in soft agar. **d** Colony numbers of cells growing in soft agar. $^{*}P<0.05$ and $^{**}P<0.01$ compared with empty vector group

pCMV-sh-SMYD3 were also assayed using western blot analysis. Overexpression of SMYD3 dramatically reduced the levels of apoptosis-promoting proteins, such as Bax, and upregulated anti-apoptosis proteins such as Bcl-2. In contrast, SMYD3 knockdown exerted the opposite effect (Fig. 3c–e).

Effects of different levels of SMYD3 expression on CLL cell growth in vivo

For the in vivo assay, MEG-1 cell lines were transplanted into nude mice and then injected with lentiviruses expressing the pCMV-SMYD3, pCMV-sh-SMYD3 or empty vector. Macroscopic images of tumor xenografts and the growth curve of the subcutaneous tumor volume in different groups revealed that SMYD3 overexpression significantly increased tumor growth, whereas SMYD3 knockdown negatively affected the progression (Fig. 4a, b). The expression of the SMYD3 mRNA was significantly increased in subcutaneously implanted tumors in nude mice (Fig. 4c). Levels of the SMYD3 and apoptosis-related proteins in the subcutaneous tumors expressing pCMV-SMYD3 and pCMV-sh-SMYD3 were examined using western blot analysis. SMYD3 overexpression decreased Bax levels and increased Bcl-2 levels, while SMYD3 knockdown exerted the opposite effect (Fig. 4d–f).

STAT3 targeted the SMYD3 promoter and activated SMYD3 transcription in CLL cells

WP1066 (an inhibitor of STAT3 activation) was employed in subsequent studies to investigate the interaction between SMYD3 and STAT3 in CLL cells. Levels of the SMYD3, STAT3, and p-STAT3 proteins in MEG-1 cells were examined using western blot analysis. WP1066 reduced SMYD3 and p-STAT3 levels, but not the levels of the total STAT3 protein. More importantly, SMYD3 overexpression reversed the inhibitory effects of WP1066 on levels of the SMYD3 protein (Fig. 5a, b). A sequence analysis identified four possible STAT3 binding sites in the SMYD3 promoter (Fig. 5c). Continuous deletion mutagenesis suggested that the P3 and P4 STAT3 binding sites were required for STAT3-induced SMYD3 transactivation (Fig. 5c). ChIP assays further confirmed that STAT3 directly bound to the SMYD3 promoter in chronic lymphocytic leukemia cells (Fig. 5d). Based on these findings, SMYD3 was a direct transcriptional target of STAT3. As shown in Fig. 5e and f, WP1066 markedly decreased the binding of STAT3 to the SMYD3 promoter,

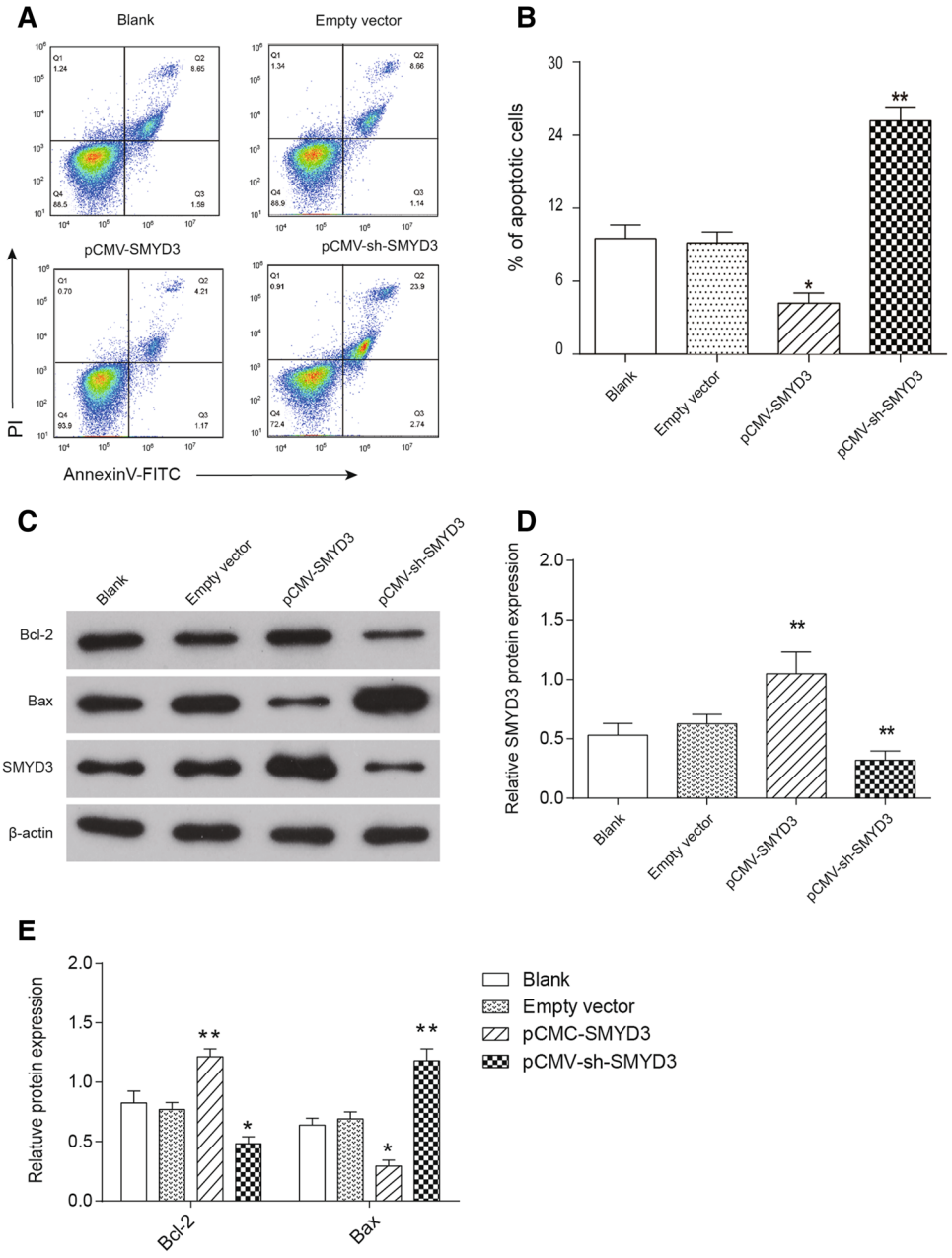

Fig. 3 Effects of SMYD3 on the apoptosis of CLL cells. **a** Apoptotic MEG-1 cells in different group were examined using flow cytometry. **b** Percentages of apoptotic cells in different groups. **c**–**e** Levels of the SMYD3, Bcl-2, and Bax proteins in MEG-1 cells were determined using western blotting. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the empty vector group

indicating that STAT3 phosphorylation was required for its transcriptional activity.

Phosphorylated STAT3 physically bound to the SMYD3 promoter and promoted CLL growth by inducing SMYD3 transcription in vivo

MEG-1 cells were transplanted into the nude mice and then treated with empty vector, pCMV-SMYD3, WP1066 or pCMV-SMYD3 plus WP1066 to further elucidate the interaction between SMYD3 and STAT3 during chronic lymphocytic leukemia in vivo. The growth curve showed that WP1066 significantly inhibited tumor growth, and the inhibitory effects were reversed by SMYD3 overexpression (Fig. 6a). Levels of SMYD3, STAT3, p-STAT3, and apoptosis-related proteins in the subcutaneously implanted tumors were assayed using western blotting. WP1066 reduced the levels of p-STAT3 and SMYD3. More importantly, similar to the effects on cells, WP1066 inhibited the effects of pCMV-SMYD3 on the levels of these proteins (Fig. 6b, c). Overexpression of SMYD3 reduced Bax levels and increased Bcl-2 levels, while SMYD3 knockdown exerted the opposite effect (Fig. 6b–d).

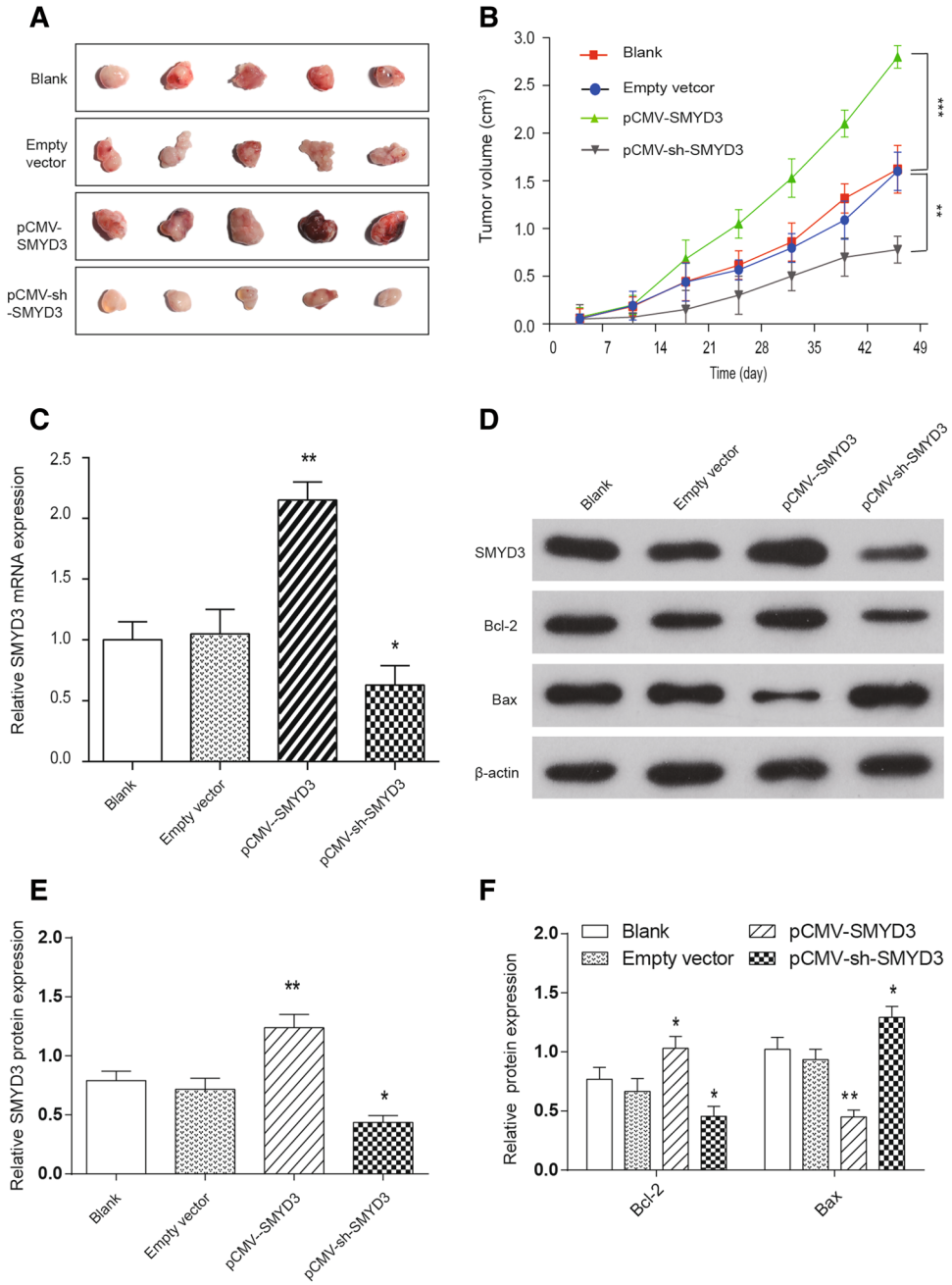

Fig. 4 Effects of different levels of SMYD3 expression on chronic lymphocytic leukemia in vivo. **a** Macroscopic images of xenografts from control, SMYD3 overexpression, and SMYD3 knockdown tumors at the end of the experiment. **b** Growth curves of subcutaneously implanted tumor volumes in different groups. **c** The expression of the SMYD3 mRNA in subcutaneously implanted tumors in nude mice. **d–f** Levels of the Bcl-2, Bax, and SMYD3 proteins in tumor tissues were detected using western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with empty vector group

Discussion

CLL is characterized by an imbalance between the proliferation and apoptosis of tumor B cells [15, 16]. The phenotypic pattern of B cells in leukemia differs from B cells in healthy patients. Although the pathophysiological mechanisms that lead to CLL are still poorly understood, cell accumulation in CLL undisputedly results from the anergic malignant lymphocytes in peripheral blood that undergo cell cycle arrest at G0/G1 phase and display resistance to apoptosis. Here, similar to the tyrosine phosphorylation of STAT3 in other tumors, serine phosphorylation of STAT3 stimulates the transcription of survival and proliferation genes in CLL [17].

SET domain-containing proteins, a class of lysine histone methyltransferases, play important roles in carcinogenesis [18]. According to Pawel et al., SMYD3 interacts with MAP3K2 in Ras-driven carcinomatosis [19]. SMYD3 overexpression correlates with the development and progression of tumors. As shown in the study by Chen et al., SMYD3 knockdown in liver cancer induces apoptosis and prevents cell growth and migration [12]. In addition, SMYD3 overexpression impacts cell viability, adhesion, migration, and aggression [13]. Wang et al. reported that SMYD3

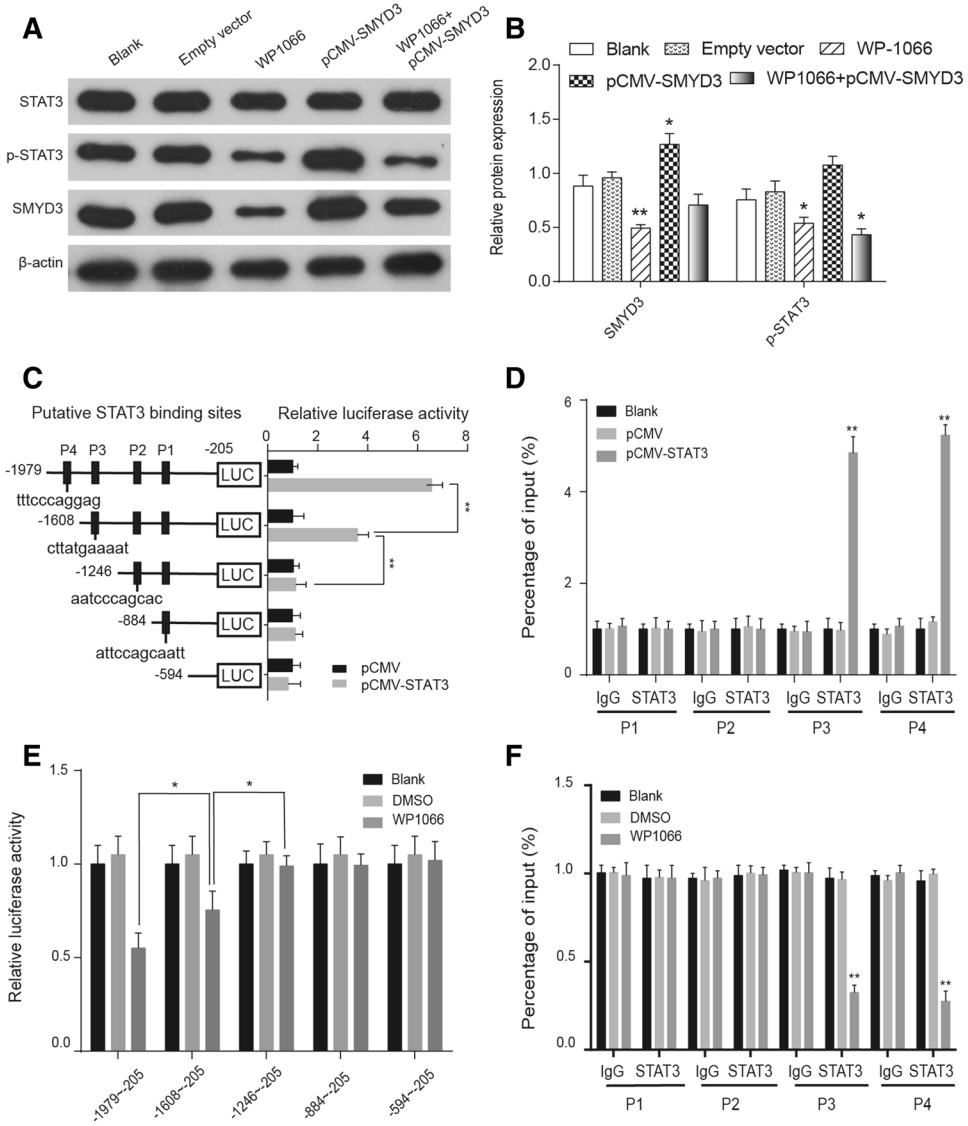

Fig. 5 STAT3 binds to the SMYD3 promoter and activates SMYD3 in CLL cells. **a, b** MEG-1 cells transfected with the empty vector, pCMV-SMYD3, WP1066, and pCMV-SMYD3 plus WP1066. Western blotting was used to analyze the levels of the SMYD3, STAT3, and p-STAT3 proteins in MEG-1 cells. **c** Deletion mutation analyses identified STAT3-responsive regions in the SMYD3 promoter using a luciferase reporter assay after STAT3 overexpression. The schematic constructs are shown (left panel), and the bar graphs present the relative levels of luciferase activity in each of the samples (right panel). **d** ChIP assays were performed to identify STAT3-responsive regions in the SMYD3 promoter after STAT3 overexpression. **e** WP1066 inhibited STAT3 binding to the SMYD3 promoter and subsequently decreased the luciferase activity. **f** The WP1066 treatment inhibited STAT3 binding to the SMYD3 promoter. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the empty vector group or DMSO groups

knockdown prevents cervical carcinoma cell proliferation and aggression in vitro [20]. According to other researchers, the SMYD3 mRNA is overexpressed in CLL cells. Wilson et al. reported the overexpression of SMYD2 and SMYD3 in patients with CLL who presented a high white blood cell count and complex karyotype [21]. However, the specific features of SMYD3 in CLL are rarely reported. In our study, SMYD3 functioned as an oncogene that promoted CLL cell growth and inhibited apoptosis in vitro and in vivo.

Constitutively active STAT3 has been observed in various cancer cells, animal models, and human cancer tissue specimens [22]. Improper STAT3 activation inhibits tumor cell apoptosis and increases cancer cell proliferation [23, 24]. Feng et al. reported that STAT3 and NF-kappaB synergistically control spontaneous apoptosis and poor chemo responsiveness in CLL [25]. Liu et al. reported a correlation between the overexpression of SMYD3 and increased activation of STAT3 in stomach cancer [14]. Nevertheless, the mutual effects of SMYD3 and STAT3 on CLL have not been thoroughly studied. In our study, STAT3 induced SMYD3 transcription and subsequently promoted CLL progression.

We performed a series of experiments with WP1066, an inhibitor of STAT3, to determine whether a similar correlation between SMYD3 and STAT3 activation existed in CLL. WP1066 inhibited the phosphorylation of STAT3 in MEG-1 cells. However, WP1066 is a promiscuous inhibitor that inhibits STAT1, STAT3, and STAT5 [26]. In addition, WP1066 also elicits significant off-target activities, particularly NF-κB, STAT5, and STAT1 reporter cells [26]. Iwamaru et al. also observed an effect of the WP1066 treatment on the phosphorylation of STAT5 in U87-MG cells [27]. Since this article focuses on the correlations between STAT3, SMYD3, and CLL progression, the underlying

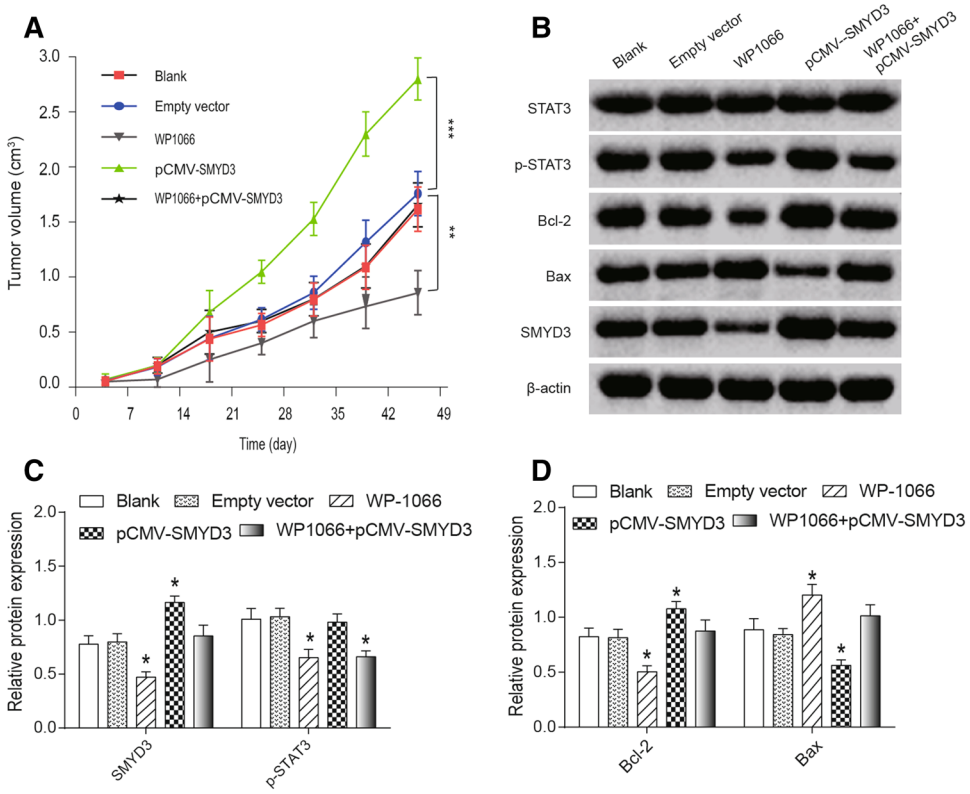

Fig. 6 WP1066 inhibits CLL cell growth by regulating SMYD3 transcription in vivo. **a** Growth curve of subcutaneously implanted tumor volumes in the five groups. **b**–**d** Levels of the SMYD3, STAT3, p-STAT3, Bcl-2, and Bax proteins in tumor tissues were examined using western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the empty vector group

mechanism of WP1066 treatment is not well explored and is a limitation of our study.

The STAT3 inhibitor, WP1066, significantly decreased the levels of the p-STAT3 and SMYD3 proteins in CLL. Based on these observations, phosphorylated STAT3 plays an important role in CLL carcinogenesis and tumor progression. Hanzan et al. reported that phosphorylated STAT3 was a trait of CLL and was regarded as a treatment target in CLL [17]. Upon phosphorylation, STAT3 molecules dimerize through a reciprocal interaction of their phosphorylated SH2 domains, which in turn promotes translocation to the nucleus and binding to specific DNA elements to regulate the transcription of target genes involved in proliferation, apoptosis, and differentiation [28].

Taken together, activated STAT3 physically binds to the SMYD3 promoter and induces the transcription of SMYD3 in CLL. These results might improve our understanding of the mechanism that governs chronic lymphocytic leukemia and serve as a stepping stone for achieving progress in new medical treatments.

In conclusion, phosphorylated STAT3 directly bound to the SMYD3 promoter, enhanced the SMYD3 transcription, and promoted CLL progression. Decreased phosphorylation of STAT3 or SMYD3 knockdown significantly inhibited CLL cell growth in vitro and *in vivo*, suggesting that STAT3 and SMYD3 are potential therapeutic targets in CLL.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and animals All procedures performed in studies involving human participants were in accordance with the ethical standards of The Second Affiliated Hospital of Guangzhou University of Chinese Medicine and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Affiliations

Fujia Lin[1] · Danjuan Wu[2] · Dan Fang[3] · Yao Chen[4] · Haitao Zhou[1] · Caiwen Ou[1]

[1] Department of Clinical Laboratory, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, No 111 Dade Road, Yuexiu District, Guangzhou 510120, Guangdong, China

[2] Department of Clinical Laboratory, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong, China

[3] Department of Hematology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong, China

[4] Department of Hematology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510120, Guangdong, China