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Betulinic Acid Decreases Specificity Protein 1 (Sp1) Level via Increasing the Sumoylation of Sp1 to Inhibit Lung Cancer Growth[S]

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ABSTRACT

Previous studies have shown that the inhibitory effect of betulinic acid (BA) on specificity protein 1 (Sp1) expression is involved in the prevention of cancer progression, but the mechanism of this effect remains to be delineated. In this study, we determined that BA treatment in HeLa cells increased the sumoylation of Sp1 by inhibiting sentrin-specific protease 1 expression. The subsequent recruitment of E3 ubiquitin-protein ligase RING finger protein 4 resulted in ubiquitin-mediated degradation in a 26S-proteosome-dependent pathway. In addition, both BA treatment and mithramycin A (MMA) treatment inhibited lung tumor growth and down-regulated Sp1 protein expression in $Kras^{G12D}$-induced lung cancers of bitransgenic mice. In gene expression profiles of $Kras^{G12D}$-induced lung cancers in bitransgenic mice with and without Sp1 inhibition, 542 genes were affected by MMA treatment. One of the gene products, cyclin A2, which was involved in the S and G_2/M phase transition during cell cycle progression, was investigated in detail because its expression was regulated by Sp1. The down-regulation of cyclin A2 by BA treatment resulted in decreased retinoblastoma protein phosphorylation and cell cycle G_2/M arrest. The BA-mediated cellular Sp1 degradation and antitumor effect were also confirmed in a xenograft mouse model by using H1299 cells. The knockdown of Sp1 in lung cancer cells attenuated the tumor-suppressive effect of BA. Taken together, the results of this study clarify the mechanism of BA-mediated Sp1 degradation and identify a pivotal role for Sp1 in the BA-induced repression of lung cancer growth.

Introduction

Lung cancer is the most prevalent type of cancer and the leading cause of cancer-related death. Although chemotherapy can relieve symptoms and improve survival, most patients with lung cancer die of either progressive local disease or metastatic disease. Clinical pathologists divide lung cancers into two broad categories: small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLCs are further divided into three major types: squamous cell carcinoma,

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ABBREVIATIONS: NSCLC, non-small cell lung cancer; BA, betulinic acid; Sp1, specificity protein 1; MMA, mithramycin A; SUMO, small ubiquitin-like modifier; SENP1, SUMO1/sentrin specific peptidase 1; Rb, retinoblastoma protein; DMEM. Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; shRNA, short hairpin RNA; Scgb1a1, secretoglobin, family 1A, member 1; rtTA, reverse tetracycline transactivator; TetO, tetracycline operator element; PCR, polymerase chain reaction; HE, hematoxylin-eosin; PBS, phosphate-buffered saline; RT, reverse transcription; F, forward; R, reverse; PI, propidium iodide; GFP, green fluorescent protein; RIPA, radioimmunoprecipitation assay; RNF4, RING finger protein 4; PARP, poly(ADP-ribose) polymerase; Rpt6, regulatory particle ATPase 6; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

adenocarcinoma, and large cell carcinoma. NSCLCs constitute 85% of all lung cancers, with adenocarcinoma accounting for 30 to 50% of all cases of lung cancer (Langer et al., 2010). Because the 5-year survival rate of patients with lung cancer is low (Goldstraw et al., 2007), the development of more effective cancer therapies with fewer side effects is an important goal.

Betulinic acid (BA), a pentacyclic triterpenoid isolated from *Betula alba*, is a candidate for cancer therapy that has been shown to exhibit an antitumor effect without cytotoxicity (Zuco et al., 2002; Alakurtti et al., 2006). The administration of BA reduced the tumor burden of established melanoma and prostate cancer xenografts in nude mice (Pisha et al., 1995; Chintharlapalli et al., 2007). In particular, BA induces tumor cell death by inducing mitochondria-mediated apoptosis (Mullauer et al., 2010). In addition, BA inhibits prostate cancer tumor growth by degrading a transcription factor, specificity protein 1 (Sp1), which ultimately inhibits angiogenesis (Chintharlapalli et al., 2007). However, the effect of BA on cell cycle progression, the mechanism of BA-induced Sp1 degradation, and the relevance of Sp1 degradation to the antitumor effect of BA in other cancers, including lung cancer, have not been elucidated. Sp1 regulates several cellular functions and influences tumor growth by regulating the transcription of genes involved in proliferation, including cyclin D1, c-Jun, and c-Myc (Wierstra, 2008). The level of Sp1 increases in various types of cancers (Wang et al., 2008), and deletion of Sp1 reduces tumor growth in nude mice implanted with gastric cancer cells (Jiang et al., 2004). Sp1 is also involved in the nicotine-induced growth of lung cancer cells (Sun et al., 2009). Although the Sp1 inhibitor mithramycin A (MMA), which inhibits the binding of Sp1 to GC-rich sequences in gene promoters, including the Sp1 gene, has an antitumor effect, its cytotoxicity in normal tissues is very high (Guichard et al., 2006; Yang et al., 2010; Tian et al., 2011). Because the down-regulation of Sp1 is an effective therapeutic strategy in antitumorigenesis, it would be worthwhile to develop or discover new cancer therapy drugs that target Sp1 with less cytotoxicity.

Cell cycle progression is tightly controlled by the cyclin/cyclin-dependent kinase complex. Cell cycle deregulation caused by overexpression of cyclins, such as cyclin D1, occurs in various types of cancers (Musgrove et al., 2011). In particular, Sp1 has been shown to regulate the transcriptional expression of cyclins D1 and E, both of which are involved in the G_1/S phase transition (Hilton et al., 2005; Kim et al., 2006). In addition to DNA synthesis in the S phase, cyclin A2 also regulates the G_2/M phase transition by promoting nuclear envelope breakdown, nuclear accumulation of cyclin B1, and activation of the M phase promoting complex (Gong et al., 2007; Bendris et al., 2011). Despite the essential role of cyclin A2 in cell proliferation, few studies have addressed the importance of cyclin A2 in the therapeutic effect of antitumor drugs.

In this study, we show that BA inhibited lung tumor growth in a xenograft model and in bitransgenic mice with spontaneously developed lung tumors induced by Kras under the control of doxycycline. In lung adenocarcinoma cells, BA induced apoptosis and cell cycle arrest at the G_2/M phase. At the molecular level, BA decreased the level of Sp1 by repressing SENP1 and thereby inducing Sp1 sumoylation, which resulted in ubiquitin-mediated protein degradation of Sp1 in a proteasome-dependent manner. Moreover, the BA-mediated decrease in Sp1 blocked the cyclin A2/Rb signaling cascade, causing cell cycle arrest at G_2/M. Furthermore, Sp1 knockdown attenuated BA-induced cell cycle arrest and apoptosis. Taken together, our results support the hypothesis that attenuation of the cellular Sp1 level is a strategy to control lung tumorigenesis.

Materials and Methods

Cell Lines. The human lung cancer cell lines, A549 and H1299, and the human cervical cancer cell line, HeLa, were purchased from American Type Culture Collection (Manassas, VA). These cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS, 100 mg/ml streptomycin sulfate, and 100 U/ml penicillin G sodium at 37°C and 5% CO_2.

Chemicals. Both betulinic acid ($C_{30}H_{48}O_3$) and mithramycin A (plicamycin; $C_{52}H_{76}O_{24}$) were purchased from Sigma-Aldrich (St. Louis, MO). The stock solution was prepared in DMSO solvent.

Cellular Infection of Lentiviruses Expressing Scramble or Sp1 shRNA for Sp1 Knockdown. We purchased shRNA from National RNAi Core Facility in Academia Sinica of Taiwan (Taipei, Taiwan). The shRNA lentiviruses were obtained from the RNAi Core of Research Center of Clinical Medicine, National Cheng Kung University Hospital (Tainan, Taiwan). After collection of lentiviruses, the amount of virus particles per milliliter was measured with an MTS490 assay kit (Promega, Madison, WI). H1299 cells were infected with lentiviruses at 5 MOI multiplicity of infection, which is the ratio of virus particles to infected cells, for 3 days to achieve effective Sp1 knockdown. Subsequently, H1299 cells, which were infected with scramble- or Sp1 shRNA-expressed lentiviruses for 3 days, were treated with the indicated dose of BA for additional 36 h.

Collection of Clinically Resected Lung Adenocarcinoma Specimens. Clinical specimens were obtained from 36 patients with stage I lung adenocarcinoma, who underwent surgery at the Department of Surgery of National Cheng Kung University Hospital. The process of human specimen collection was approved by the Clinical Research Ethics Committee at National Cheng Kung University Medical Center.

Generation of Bitransgenic Mice (*Scgb1a1-rtTA/TetO-Kras^{G12D}*). *Scgb1a1-rtTA* and *TetO-Kras^{G12D}* transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and cared for at the National Laboratory Animal Center in Taiwan. The expression of rtTA protein was driven by the promoter of *Scgb1a1* in type II epithelial cells in lungs of *Scgb1a1-rtTA* transgenic mice. After mating, *Kras^{G12D}* expression was driven by the doxycycline-activated rtTA-bound tetracycline-responsive promoter element (TRE; TetO) in lungs from bitransgenic mice characterized by PCR genotyping. *Kras^{G12D}* expression was induced by drinking water containing 0.5 g/l doxycycline (Sigma-Aldrich) at the age of 2 months. The control group drank reverse osmosis water. BA and MMA were administered by intraperitoneal injection. After treatment for the indicated period, whole lung tissue containing lung tumors was homogenized and prepared as whole tissue protein for Western blotting. For histological analysis, whole lung tissue containing lung tumors was fixed in 10% formaldehyde.

Immunohistochemistry. Lungs from bitransgenic mice and clinically resected specimens were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Histological sections with 5 μm were stained with hematoxylin-eosin (HE) after dewaxation in xylene and rehydration in decreasing concentrations of ethanol. For immunostaining, hydrogen peroxide (0.3% in PBS) was used to block endogenous peroxidase for 30 min at room temperature. Subsequently, sections were incubated in the appropriate primary antibody diluted in 2% BSA in PBS for 2 h at room temperature. Finally, the immunoreactivity was visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and photographed under an Olympus

BX-51 microscope (Olympus, Melville, NY). For the evaluation of Sp1 expression in clinically resected human lung adenocarcinomas, more than 30% of cells stained with Sp1 in the individual histological slide were considered as positive staining.

RT-PCR. Total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer's instructions. Three micrograms of RNA were used for RT with SuperScript III (Invitrogen). The primers for PCR were as follows: human *Sp1*, F, 5′-ACTAGTGTCCGGGTTCGCTTGCCT-3′ and R, 5′-GGCGCCAAAGTTGTGTGGCTGTGA-3′; human *cyclin A2*, F, 5′-GGTACCCCATTTCAATAGTC-3′ and R, 5′-GAGCTCAAATTGTTGGTTCA-3′; mouse *cyclin A2*, F, 5′-GAACGTTAATGAAGTACCTGAC-3′ and R, 5′-CAGGGTCTCATTCTGTAGTTTA-3′; and *glyceraldehyde-3-phosphate dehydrogenase*, F, 5′-CCATCACCATCTTCCAGGAG-3′ and R, 5′-CCTGCTTCACCACCTTCTTG-3′. PCR products were analyzed by ethidium bromide-containing agarose gel electrophoresis.

Xenograft Study. The animal experiment was approved by the Institutional Animal Care and Use Committee at National Cheng Kung University. Female athymic BALB/c nude mice (8 weeks old) were purchased from National Laboratory Animal Center in Taiwan. H1299 cells (1×10^6 cells) were suspended in 100 μl of PBS and implanted into the back of athymic nude mice. DMSO (for control) or BA (10 mg/kg) was injected intraperitoneally into mice every 3 days. The width (a) and length (b) of the tumor were measured by calipers for the evaluation of tumor volume according to the formula $V = ba^2/2$ (Conway et al., 2000).

Flow Cytometry for Cell Cycle Analysis. The cells were trypsinized and washed with 4°C PBS. After fixation with 4°C 75% ethanol for 24 h, the cells were treated with 10 μg/ml RNase A (QIAGEN, Valencia, CA) and stained with 50 μg/ml PI (Sigma-Aldrich) for 30 min at 37°C. To analyze cell distribution in the cell cycle, PI-stained cells were analyzed by a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA). Data were analyzed by WinMDI software.

Microarray Analysis. Total RNA was extracted using TRIzol from lungs of control mice, doxycycline-treated bitransgenic mice, and doxycycline-treated bitransgenic mice injected intraperitoneally with MMA. Microarray analysis was performed by Phalanx Biotech Group (Hsinchu, Taiwan). Microarray data were analyzed by using Ingenuity software.

Proliferation Assay. After plating cells (1×10^5) in the 6-cm dish for 24 h, the medium was changed to DMEM-10% FBS containing DMSO for the control or different doses of BA for the indicated time. To examine cell proliferation, cells were trypsinized and counted by hemacytometer after treatment with BA. Each experiment was duplicated and repeated three times.

Construction of *CCNA2* Promoters for Reporter Assay. For construction, genomic DNA of HeLa cells was prepared. *CCNA2* promoter (−1000/+50) was produced by PCR using primers: F, 5′-GGTACCGGTTTTAAAATGGCATAC-3′ and R, 5′-AGATCTGGAGCGGCGGCTGTTCTT-3′; deletion form (−1000/−151), F, 5′-GGTACCGGTTTTAAAATGGCATAC-3′ and R, 5′-AGATCTGAGGTAGGATTTAGGGCC-3′; and deletion form containing putative Sp1-binding sites (−150/+50), F, 5′-GGTACCTCCCCGCCCCGCGCAGGC-3′ and R, 5′-AGATCTGGAGCGGCGGCTGTTCTT-3′. After purification and amplification, plasmids were ligated to pGL2 vector using restriction enzymes, KpnI and BglII (New England Biolabs, Ipswich, MA).

Mutagenesis. The *CCNA2* promoter (−1000/+50) plasmid was used as the template for mutagenesis of Sp1-binding sites. Primers for mutations of −89/−88C to −89/−88A: F, 5′-CTCCCTCCTGCCCCGAACCTGCTCAGTTTCCT-3′ and R, 5′-AGGAAACTGAGCAGGTTCGGGGCAGGAGGGAG-3′; and for −144/−143C to −144/−143A: F, 5′-TCCTACCTCTCCCCGAACCGCGCAGGCGTTTT-3′ and R, 5′-AAAACGCCTGCGCGGTTCGGGGAGAGGTAGGA−3′. Mutagenesis was performed by PCR using plaque-forming unit DNA polymerase (Agilent Technologies, Santa Clara, CA).

Transfection and Luciferase Assay. After cells were plated in an appropriate number in six-well plates for 24 h, transfection was achieved by incubating cells with Opti-MEM (Invitrogen) containing 2 μg of the pGL2-vector or pGL2-SENP1 or pGL2-CCNA2 promoter plasmid and 2 μl of lipofectamine (Invitrogen) at 37°C and 5% CO_2 for 6 h. Subsequently, Opti-MEM was replaced with DMEM-10% FBS and incubated for an additional 36 h in the presence of DMSO or BA with GFP- or GFP-Sp1-expressed adenoviruses. Luciferase activity in cell lysates was determined by luminometer (LB9506; Berthold Technologies, Bad Wildbad, Germany).

Immunoprecipitation. Whole-cell lysates (500 μg), which were prepared by using radioimmune precipitation assay (RIPA) buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% Nonidet P-40, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), were immunoprecipitated with 1 μg of anti-Sp1 (Millipore Corporation, Billerica, MA), 1 μg of anti-RNF4 (Sigma-Aldrich), or 2 μg of anti-ubiquitin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in modified RIPA buffer for 2 h at 4°C. Protein A agarose beads (30 ml; Millipore Corporation) were mixed with immunoprecipitated lysates and incubated for additional 1 h at 4°C. After four washes with RIPA buffer, beads were collected by centrifugation at 5000 rpm for 3 min at 4°C and eluted by 1.5× sample buffer for electrophoresis and subsequent immunoblotting with the indicated antibody.

Western Blotting. Whole-cell lysates were collected for electrophoresis, transfer to polyvinylidene difluoride membrane (GE Healthcare, Baie d'Urfe, QC, Canada), and immunoblotting with antibodies against Sp1, Kras (ProteinTech Group, Chicago, IL), RNF4, caspase 3 (Cell Signaling Technology, Beverly, MA), cyclin A2 (ProteinTech Group), SENP1 (Epitomics Inc., Burlingame, CA), poly(ADP-ribose)polymerase (PARP) (Epitomics), phospho-Rb (Ser807; Cell Signaling Technology), caspase 3 (Cell Signaling Technology), Rb (ProteinTech Group), regulatory particle ATPase 6 (Rpt6) (Biomol International L.P., Butler Pike, PA), Flag (Sigma-Aldrich), tubulin (Sigma-Aldrich), and β-actin (Sigma).

MTT Assay. After infection with lentiviruses expressing the indicated shRNA for 3 days, cells were seeded in a 96-well plate (5000 cells/well) and treated with BA for an additional 36 h. Then cell viability was determined by using an MTT assay kit according to the manufacturer's instruction (Biovision Inc., Milpitas, CA).

Chromatin Immunoprecipitation Assay. After cross-linking with 1% formaldehyde (Merck, Darmstadt, Germany) for 10 min at room temperature, cells (1×10^8) were washed with ice-cold PBS three times, and whole-cell extracts were prepared with lysis buffer. The cellular DNA fragment (500 base pairs) was prepared by sonication. For immunoprecipitation, salmon sperm DNA-blocked cell lysate was incubated with 2 μg of anti-Sp1 antibody or IgG at 4°C for 16 h with rotation. After incubation with 30 μl of protein A agarose beads at 4°C for additional 2 h, beads were washed three times with high salt buffer (20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, and 0.5% Nonidet-40) and another three times with low salt buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.01% SDS). Subsequently, proteins binding to beads were eluted by 500 μl of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) containing 1% SDS, and cross-links were reversed at 65°C for 16 h. After protein digestion by 0.5 mg/ml proteinase K at 50°C for 2 h, DNA was extracted by phenol-chloroform and precipitated by ice-cold absolute alcohol. The purified DNA was analyzed by PCR using the primer for *CCNA2* promoter (−188/+1): F, 5′-TTAAAATAATCGGAAGCG-3′ and R, 5′-ACCAATGAAAGCGCTCGC-3′.

Statistical Analysis. Student's t test was used to compare the difference between DMSO- and BA-treated groups. Two-way analysis of variance was used to compare the growth curve of tumors from DMSO- and BA-treated athymic nude mice.

Results

BA Decreases the Sp1 Level in Lung Adenocarcinoma by Modulating Its Post-Translational Modification. A previous study showed that BA inhibits the growth of

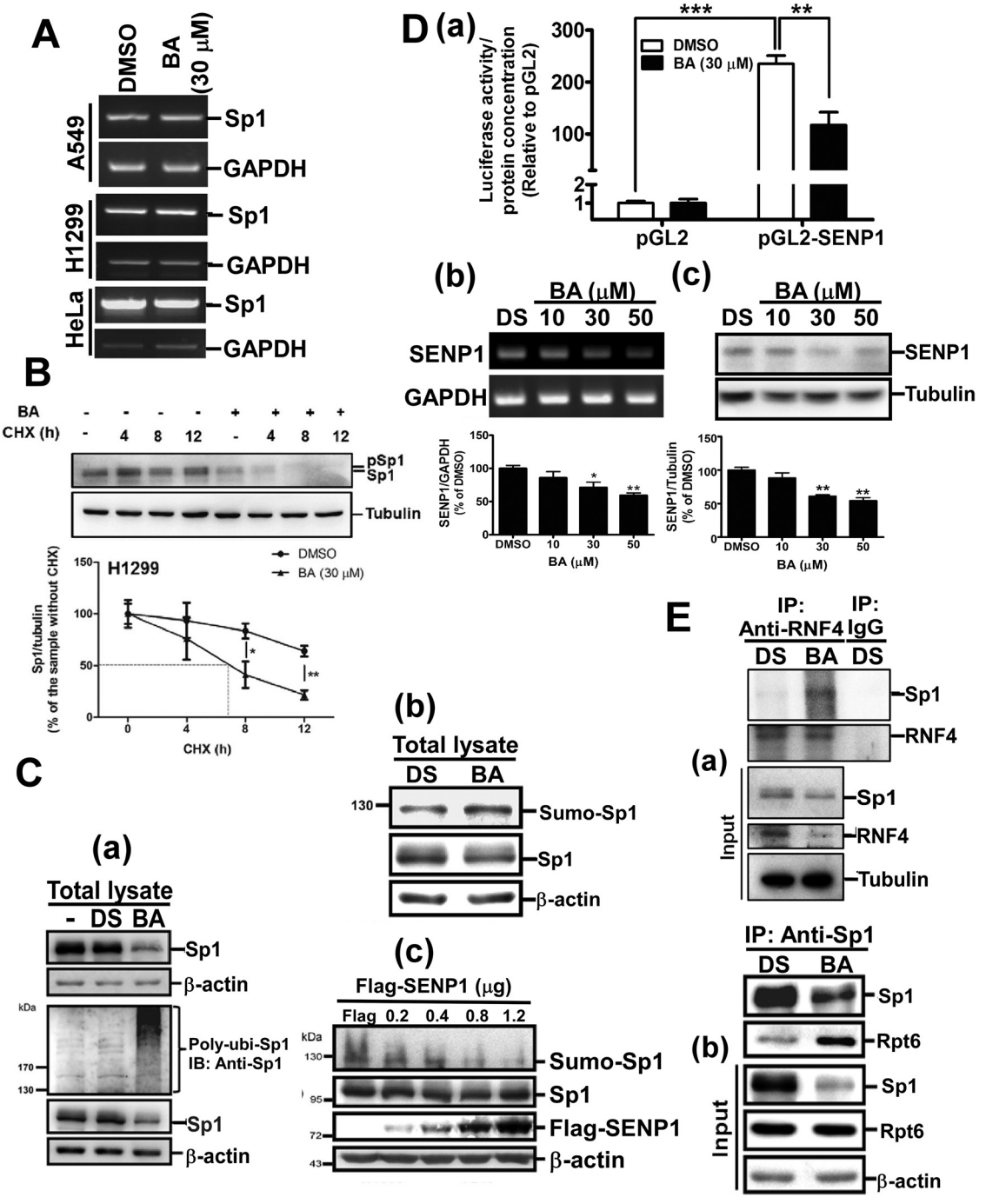

Fig. 1. Effects of BA on Sp1 degradation and SENP1 expression. A, after treatment with 30 μM BA for 36 h, cells were harvested, and RNA was extracted for RT-PCR. B, after treatment with DMSO (for control) or BA (30 μM) for 24 h, H1299 cells were treated with 100 μM cycloheximide (CHX) for indicated time periods. Subsequently, whole-cell lysate was prepared for Western blotting using the antibodies against Sp1 and tubulin (top panel). Quantitative results are shown in the bottom panel. Three experiments were conducted independently, and data are expressed as the mean ± S.E.M. (*, $P < 0.05$; **, $P < 0.01$, determined by Student's t test). C(a), HeLa cells treated with DMSO (DS) or BA for 36 h were harvested for preparation of whole-cell lysate for Western blotting with antibodies against Sp1 and β-actin. IB, immunoblotting; ubi, ubiquitin. C(b), cells transfected with SUMO1-GG and treated with BA for 36 h were harvested for Western blotting with antibodies against Sp1 and β-actin. C(c), 24 h after transfection with Flag-SENP1, HeLa cells were harvested for Western blotting with antibodies against Sp1, SENP1, and β-actin. D(a), after transfection with pGL2 or pGL2-SENP1 promoter plasmid, H1299 cells were treated with BA for 36 h and harvested for luciferase reporter assay. Experiments were performed independently three times, and data are expressed as the mean ± S.E.M. (**, $P < 0.01$; ***, $P < 0.001$,determined by Student's t test). D(b), H1299 cells were harvested for RT-PCR. Experiments were performed independently three times, and data are expressed as the mean ± S.E.M. D(c), H1299 cells were harvested for Western blotting using anti-SENP1 and anti-tubulin antibodies. (*, $P < 0.05$; **, $P < 0.01$, compared with the DMSO-treated control, determined by Student's t test). E(a), whole-cell lysate was immunoprecipitated (IP) by using antibody against RNF4 or IgG and then immunoblotted by using antibody against Sp1 or RNF4. E(b), whole-cell lysate was immunoprecipitated by using antibody against Sp1 and then immunoblotted by using antibody against Sp1 or Rpt6.

prostate cancer by degrading Sp1 protein, but it is still not clear how Sp1 is degraded (Chintharlapalli et al., 2007). First, we checked the transcriptional activity of *Sp1* by detecting Sp1 mRNA (Fig. 1A). No significant difference in the Sp1 mRNA levels was observed in cancer cells with and without BA treatment (30 μM) for 36 h. Therefore, Sp1 protein stability was studied under BA treatment (Fig. 1B). BA treatment significantly reduced the half-life of Sp1 in H1299 cells, suggesting that BA induces Sp1 protein instability. The ubiquitination of Sp1 was then measured in the absence or presence of BA. Ubiquitination of Sp1 was observed after BA treatment [Fig. 1C(a)]. Our previous studies revealed that sumoylation of Sp1 leads to Sp1 degradation, but phosphorylation of Sp1 increases its stability (Wang et al., 2008). The sumoylation and phosphorylation of Sp1 were then studied under BA treatment. No significant difference in the phosphorylation of Sp1 (Thr739) was observed with and without BA treatment (data not shown). On the other hand, sumoylation increased with BA treatment [Fig. 1C(b)]. To investigate whether the sumoylation of Sp1 was specific, Flag-SENP1 was overexpressed in cells, and Sp1 sumoylation was assessed. As indicated in [Fig. 1C(c)], Sp1 sumoylation decreased with Flag-SENP1 overexpression, indicating that the signal in [Fig. 1C(b)] is Sp1 sumoylation. To study whether endogenous SENP1 was affected by BA treatment, we examined the effect of BA on SENP1 expression. BA inhibited the transcriptional activity [Fig. 1D(a)], mRNA expression [Fig. 1D(b)], and protein expression [Fig. 1D(c); Supplemental Fig. S1] of SENP1 in lung adenocarcinoma cells. Finally, the interactions of Sp1 with the E3 ubiquitin-protein ligase RNF4 and the proteasome subunit Rpt6 were studied in cells with and without BA treatment. As shown in Fig. 1E, BA significantly enhanced the interaction of Sp1 with RNF4 [Fig. 1E(a)] and Rpt6 [Fig. 1E(b)]. These results suggest that BA increases the level of SUMO-Sp1 by repressing SENP1 expression, leading to degradation of Sp1 in a 26S-proteosome-dependent pathway in lung cancer cells.

A Decrease in Sp1 Level Is Important for the BA-Mediated Anti-Lung Cancer Effect. As indicated in Fig. 1, we determined the mechanism of Sp1 degradation by BA treatment in lung cancer cells. We then addressed the relationship between Sp1 degradation and BA-mediated inhibition of lung cancer growth. First, the cell morphology of lung cancer cells, H1299 and A549, was studied upon treatment with various doses of BA. The cell morphology and cell number of lung cancer cells were not affected by 10 and 20 μM BA, but an effect was observed with 30 and 40 μM BA (Fig. 2A). Next, the cell lysates were used to evaluate the Sp1 level (Fig. 2B). There was no difference in the Sp1 level after the 10 or 20 μM BA treatment, but there was an obvious decrease in the Sp1 level after 30 or 40 μM BA treatment, compared with the Sp1 level in controls (Fig. 2B). Taken together, these results

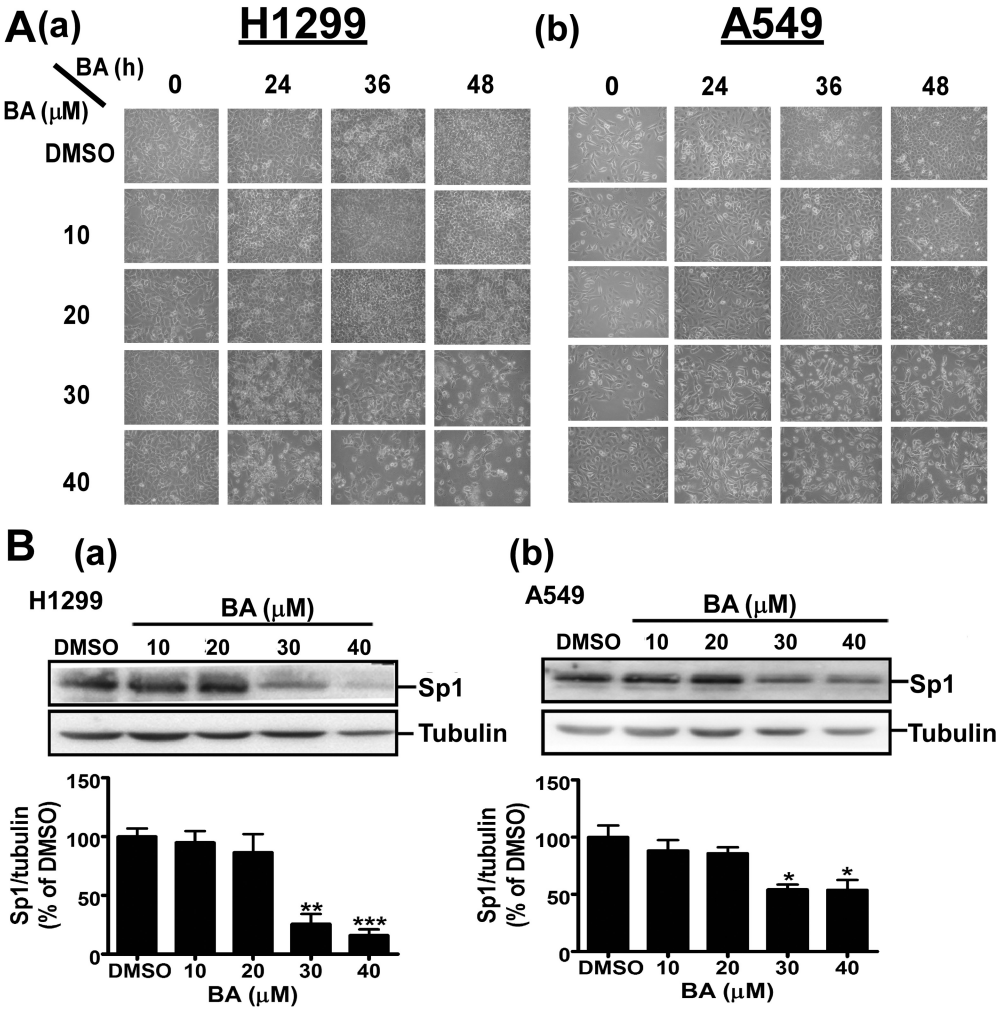

Fig. 2. Effect of BA on cellular proliferation and Sp1 expression in lung adenocarcinoma cells. A, cells were treated with different doses of BA for the indicated period, and photographed under a light microscope (×100). B(a) and B(b), DMSO- and BA-treated cells were harvested for Western blotting using antibodies against Sp1 and β-actin. Three experiments were conducted independently, and data are expressed as mean ± S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with the DMSO-treated control, determined by Student's t test).

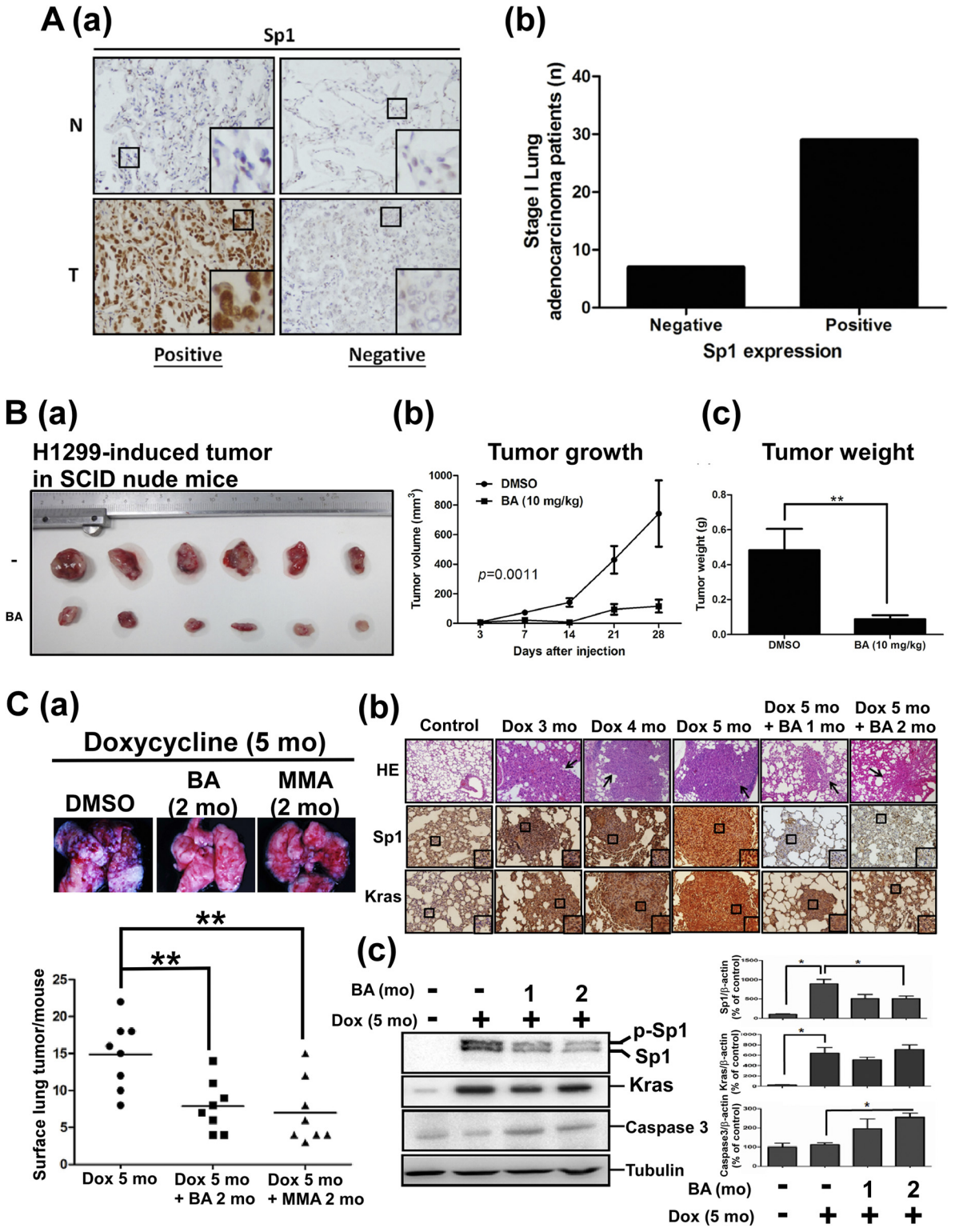

Fig. 3. Effect of BA and MMA on lung tumor growth in vivo. A(a), representative images of clinically resected lung adenocarcinomas (T) and normal lung tissues (N) with immunohistochemical staining using anti-Sp1 antibody. A(b), Sp1 expression in 36 patients with stage I lung adenocarcinoma. B, effect of BA on lung tumor growth in a xenograft model. B(a), images of tumors from athymic nude mice implanted with H1299 cells. After implantation with cells (1×10^6 cells/mouse), mice were treated with BA (10 mg/kg, once every 3 days) for 4 weeks, and resected tumors were then photographed. B(b), volumes of tumors formed after inoculation of cells were measured and calculated. Data are representative of six independent experiments and are presented as the mean ± S.E.M. $P = 0.0011$ was determined by two-way analysis of variance. B(c), tumors were resected 4 weeks after injection of cells and weighed. Data are representative of six independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$, determined by Student's t test). C, effect of Sp1 inhibitors on $Kras^{G12D}$-driven lung tumor formation in doxycycline-induced transgenic mouse model. C(a), effect of BA and MMA on lung tumor formation. Lung tumors in bitransgenic mice were induced by 5-month treatment with doxycycline (Dox). BA (20 mg/kg, once per day) and MMA (0.5 mg/kg, once per week) were intraperitoneally injected in the last 2 months (the 4th and 5th months). Representative images of lungs from the indicated mice are shown in the top panel, and numbers of tumors on the lung surfaces of bitransgenic mice are shown in the bottom panel. Each point represents one mouse (**, $P < 0.01$, determined by Student's t test). C(b), bitransgenic mice were treated

TABLE 1
Demographics of 36 patients with lung adenocarcinomas analyzed by immunohistochemical staining

	Patients with Sp1-Positive Staining (*n*)	Patients with Sp1-Negative Staining	*P*
No. male	12	3	1[a]
No. female	17	4	
Age, yr	69.81 ± 1.56	70.67 ± 4.82	0.82[b]

[a] Fisher's exact test.
[b] Student's *t* test.

indicating a good correlation between cell growth inhibition and Sp1 down-regulation with BA treatment suggest that Sp1 might be important for the anti-lung cancer effect of BA.

We then addressed the relationship between the Sp1 level and lung tumor formation and the influence of BA treatment on this relationship (Fig. 3A),). First, to clarify the clinical significance of Sp1 expression in tumorigenesis, Sp1 protein expression was detected in specimens from patients with lung cancer by immunohistochemical staining (Fig. 3A; Table 1). Among 36 patients with stage I lung adenocarcinomas, 29 patients (81%) exhibited high Sp1 expression, whereas only 7 (19%) exhibited low Sp1 expression. This result suggests that Sp1 accumulation is involved in human lung tumor growth. Although BA has been shown to be effective against prostate cancer (Chintharlapalli et al., 2007), its effect on other cancers, especially lung cancer, was not clear. Therefore, we evaluated the therapeutic effects of BA on lung cancer in a xenograft mouse model and in a bitransgenic mouse model. Treatment of animals with BA attenuated both tumor size and tumor weight in SCID mice implanted with H1299 cells (Fig. 3B). To confirm whether BA inhibits spontaneous lung tumor growth in vivo, we used a bitransgenic mouse model (*Scgb1a1-rtTA/TetO-Kras*^{G12D}). These mice spontaneously develop lung tumors through doxycycline-induced expression of mutant Kras (Fisher et al., 2001). As shown in Fig. 3C(a), the bitransgenic mice that received doxycycline alone for 5 months developed lung tumors, whereas BA treatment (20 mg/kg, once every 3 days) in the 4th and 5th months of doxycycline treatment prevented tumor development. This result indicates that BA inhibits the spontaneous lung tumor growth in vivo. Moreover, immunohistochemical staining [Fig. 3C(b)] and Western blotting [Fig. 3C(c)] showed that BA treatment blocked up-regulation of Sp1, but not that of Kras, and up-regulated caspase 3 [Fig. 3C(c)]. These results suggest that BA blocks lung tumor growth by inducing apoptosis.

To address more specifically the role of Sp1 in BA-mediated anti-lung tumorigenesis, Sp1 was knocked down by shRNA in lung cancer cells (Fig. 4A),). First, the cell number was counted, and viability was assayed after BA treatment with or without Sp1 knockdown. The data indicated that cells were more resistant to BA after Sp1 knockdown (Fig. 4, A and B). Cell death was then assayed using flow cytometry. As indicated in Fig. 4C, 16.33 and 70.05% of cells were found in the sub-G_1 fraction after treatment with 30 and 50 μM BA, respectively. In the Sp1 knockdown condition treated with 30 and 50 μM BA, 7.34 and 27.62% of cells were found in the sub-G_1 fraction, respectively. Finally, cleaved PARP and active caspase 3 were investigated in cells with or without Sp1 knockdown (Fig. 4D). Cleaved PARP and active caspase 3 were induced by various doses of BA in cells without Sp1 knockdown but not in the Sp1 knockdown condition. This result suggests that Sp1 is required for BA-mediated cell apoptosis.

Sp1-Mediated Genes Are Involved in the BA-Induced Repression of Lung Cancer in Kras^{G12D}-Driven Lung Tumors. In addition to BA, we also studied the effect of another Sp1 inhibitor, MMA, on Kras^{G12D}-driven lung tumors in the doxycycline-induced transgenic mouse model. In accordance with the results from BA treatment, treatment with MMA (0.5 mg/kg, once per week) during the last 2 months of a 5-month doxycycline treatment clearly prevented both tumor formation [Fig. 3C(a)] and Sp1 up-regulation in lung tumors, as analyzed by immunohistochemical analysis (Fig. 5A). The effects of treatment with BA and MMA, which induce degradation of Sp1 protein and inhibit binding of Sp1 to promoters, respectively, suggest that Sp1 down-regulation is involved in BA- and MMA-attenuated lung tumor formation in the bitransgenic mouse model.

To further address the functional role of Sp1 in lung cancer formation, total RNA was extracted from MMA-treated lung tissues containing tumors, and the gene expression profile was investigated by microarray analysis (Fig. 5). MMA treatment repressed 542 genes in Kras^{G12D}-driven lung cancer mice (Supplemental Figs. S2 and S3). Functional grouping analysis using Ingenuity software showed that 68 of these genes were related to cell proliferation. Of these 68, 40 genes have been reported to be involved in proliferation (Fig. 5B; Supplemental Fig. S3; Supplemental Table 1). We also used samples obtained from BA-treated lung cancer cells and BA- or MMA-treated mice bearing Kras-driven lung tumors to determine the expression level of important genes involved in cell proliferation, such as *CCNA2*, *EGR1*, *CTSZ*, *VAV1*, *TACSTD2*, *TPX2*, and *CD9*. The results from microarray analysis indicated that the mRNA expression of these important genes was inhibited by BA treatment in Kras-driven lung tumors and in H1299 cells (Fig. 6A),; Supplemental Fig. S4).

BA Decreases Cyclin A2 Expression by Suppressing Sp1 Expression in Lung Cancer Formation. Because cyclin A2 is essential for cell cycle progression and tumorigenesis (Yasmeen et al., 2003), we next examined cyclin A2 expression in Kras^{G12D}-driven lung tumors from doxycycline-induced transgenic mice to confirm the results from the microarray analysis. As shown in Fig. 6, A and B, treatment of Kras^{G12D}-induced lung cancer mice with either MMA or BA inhibited cyclin A2 expression at the mRNA and protein levels in the lung tissue containing tumors. In addition, lung tumor xenografts from BA-treated nude mice exhibited lower levels of cyclin A2 and Sp1 than those from DMSO-treated nude mice (Fig. 6C).

To determine whether the cyclin A2 gene (*CCNA2*) is regulated by Sp1 at the transcriptional level, we searched for

with BA in the last 1 month and 2 months during the doxycycline treatment period. Lungs were exercised, and immunohistochemical analyses were performed by using antibodies against Sp1 and Kras. Representative images of HE and immunohistochemical stains are shown. C(c), lungs from bitransgenic mice were used to prepare tissue lysates for Western blot analyses by using antibodies against Sp1, Kras, caspase 3, and β-actin (left panel). Data are representative of eight independent experiments and are presented as the mean ± S.E.M. (right panel). (*, $P < 0.05$, determined by Student's *t* test).

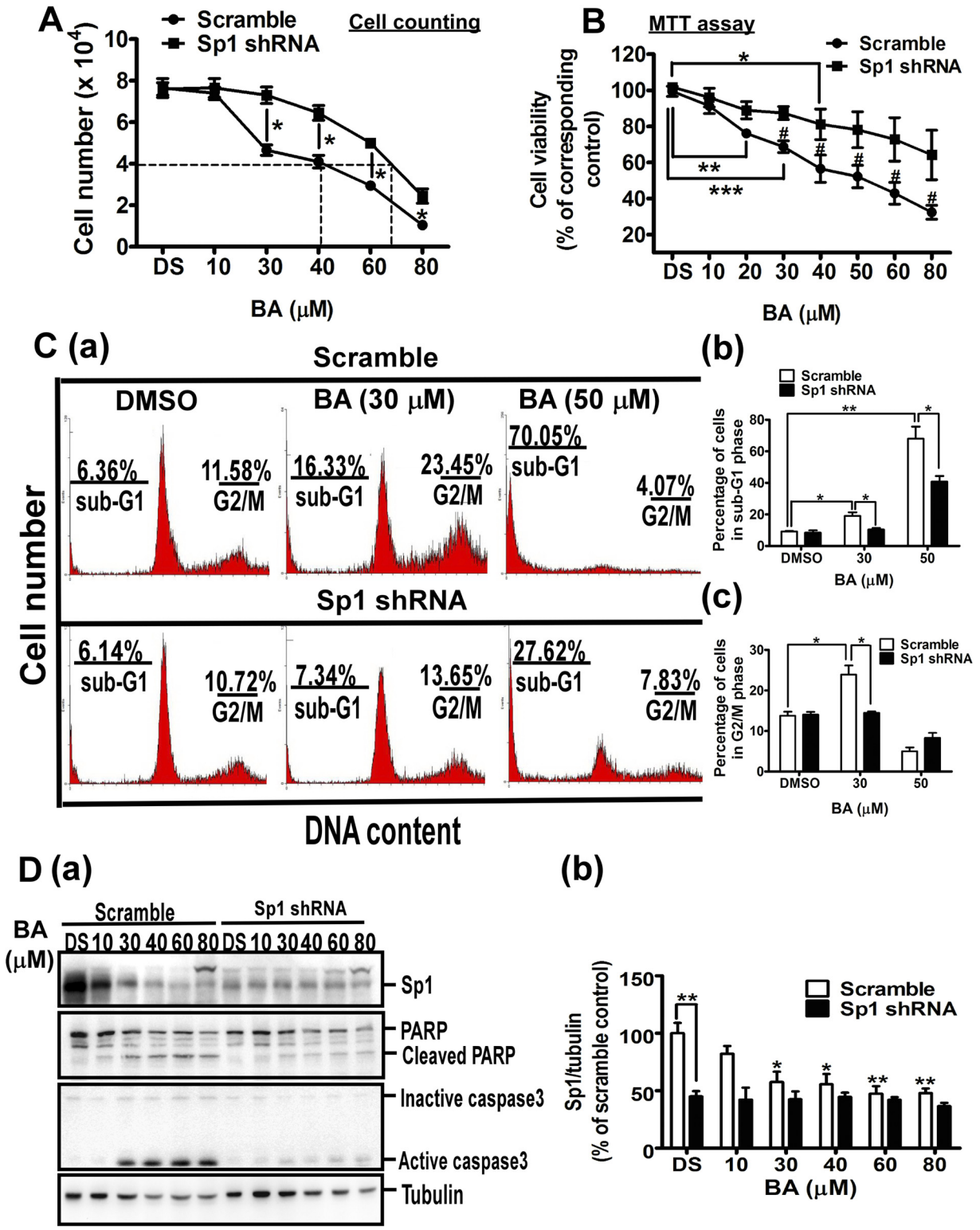

Fig. 4. Effect of Sp1 knockdown on BA-mediated antitumor effect in lung adenocarcinoma cells. A, after infection of H1299 cells with lentiviruses (5 multiplicity of infection) expressing scramble or Sp1 shRNA for 3 days, cells were treated with different doses of BA for 36 h. Cell number was determined by cell counting. Data are representative of three independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$, determined by Student's t test). B, cell viability was measured by MTT assay. Data are representative of three independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$, all of which were compared with the scramble-treated group, determined by Student's t test). C(a), cells were fixed with 75% ethanol, stained with PI, and subjected to flow cytometry. C(b), statistical results for the sub-G_1 population. Data are representative of three independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$; **, $P < 0.01$, determined by Student's t test). C(c), Statistical results for the G_2/M population. Data are representative of three independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$, determined by Student's t test). D(a), cells were harvested for Western blotting using antibodies against Sp1, PARP, caspase 3, and tubulin. D(b), data are representative of three independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$; **, $P < 0.01$, compared with the DMSO-treated group, determined by Student's t test).

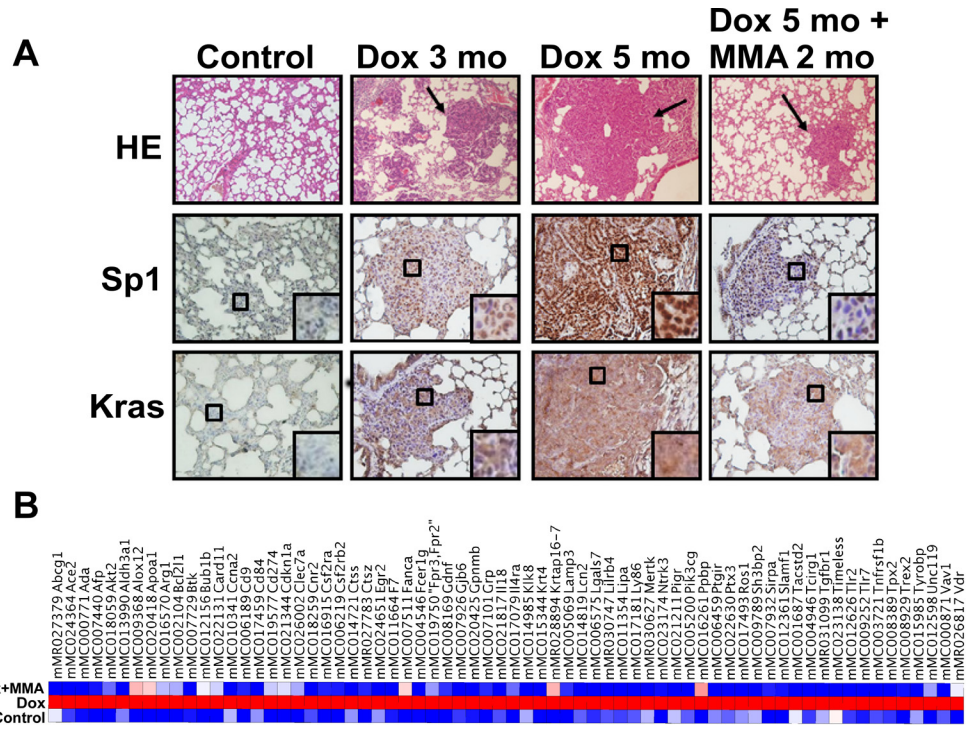

Fig. 5. Identification of Sp1-regulated gene expression related to proliferation in Kras^{G12D}-driven lung tumorigenesis. Bitransgenic mice were treated with doxycycline (Dox) with MMA (0.5 mg/kg, once per week) in the last 2 months during 5 months of treatment with doxycycline. A, lungs were harvested and prepared for histological analysis. Representative images of lung sections for HE staining and for immunohistochemical analysis were stained using antibodies against Sp1 and Kras. B, a heat map of 68 genes related to proliferation was created using the GenePattern Web site.

Sp1-binding sites in the *CCNA2* promoter by using bioinformatics software (TFSEARCH; Tsukuba, Japan). We found two Sp1-binding sites located at −149/−141 (score 95.9) and −94/−86 (score 94.5) ahead of the transcription start site (+1). Chromatin immunoprecipitation assays revealed that Sp1 bound to the *CCNA2* promoter (−188/+1) [Fig. 6D(a)]. In addition, the reporter assay indicated that GFP-Sp1 overexpression significantly enhanced *CCNA2* transcriptional activity in H1299 cells, whereas BA treatment significantly attenuated the effect of Sp1 [Fig. 6D(b)]. Subsequently, to establish whether Sp1-binding sites are important for *CCNA2* transcription, the transcriptional activity driven by *CCNA2* promoter (−150/+50) with Sp1-binding sites or by *CCNA2* promoter (−1000/−151) without Sp1-binding sites was analyzed. As shown in Fig. 6D(c), GFP-Sp1 overexpression significantly enhanced the activity of the promoter −150/+50 and not that of the promoter −1000/−151. Moreover, the transcriptional activity driven by promoter −1000/−151 was clearly lower than that driven by promoter −150/+50. Furthermore, mutations of Sp1-binding sites significantly blocked GFP-Sp1-enhanced *CCNA2* transcription [Fig. 6D(d)]. The expression of GFP or GFP-Sp1 in H1299 cells analyzed by a reporter assay was confirmed by Western blot analysis (Supplemental Fig. S5, B, C, and D). We also showed that Sp1 overexpression significantly increased the protein level of cyclin A2 in H1299 cells (Supplemental Fig. S5A). These results indicate that Sp1 directly regulates *CCNA2* promoter activity. In addition to the inhibition of *CCNA2* transcription by BA, the CCNA2 mRNA level was also decreased in BA-treated H1299 and A549 lung cancer cell lines (Fig. 6E).

A previous study showed that cyclin A2 is important for both G_1 to S and G_2 to M transitions of the cell cycle (Yasmeen et al., 2003). Because cyclin A2 is essential for Rb phosphorylation, the phosphorylation of Rb in BA-treated lung adenocarcinoma cells was studied. As shown in Fig. 7A, BA treatment simultaneously attenuated *CCNA2* expression and the phospho-Rb signal. Furthermore, Rb phosphorylation was also studied in MMA- and BA-treated Kras^{G12D}-driven lung tumor samples (Fig. 7B). The phosphorylated Rb signal was induced in Kras^{G12D}-driven lung tumors, but the signal was abolished after MMA or BA treatment. Finally, apoptosis or cell cycle arrest at the G_2/M phase in BA-treated cells was assessed by flow cytometry analysis. In A549 cells, the proportions of cells in the sub-G_1 and G_2/M fractions were 1.3 and 16.3%, respectively, whereas the proportions of cells in the sub-G_1 and G_2/M fractions increased to 17.03 and 22.91%, respectively, after treatment with 30 μM BA (Fig. 7C, top panel). In H1299 cells, treatment with 30 μM BA increased the proportions of cells in the sub-G_1 and G_2/M populations from 2.5 to 10.75% and from 13.12 to 23.48%, respectively (Fig. 7C, bottom panel). These results suggest that BA-mediated Sp1 down-regulation blocks the cyclin A2/Rb signaling cascade, resulting in cell cycle arrest at the G_2/M phase.

On the basis of these findings, we propose a model to explain the mechanism underlying BA-induced Sp1 degradation to inhibit lung cancer growth (Fig. 8). In lung tumor cells, BA induces sumoylation by decreasing SENP1 expression. The subsequent ubiquitination of Sp1 protein results in Sp1 degradation in a proteasome-dependent manner. The decrease in Sp1 reduces the level of cyclin A2 by attenuating *CCNA2* transcription. Decreased Rb phosphorylation then causes cell cycle arrest at the G_2/M phase and, finally, apoptosis.

Discussion

In this study, we found that BA decreased SENP1 levels, which increased Sp1 sumoylation and the recruitment of the Sp1 E3 ligase, RNF4, to degrade Sp1. We also observed that

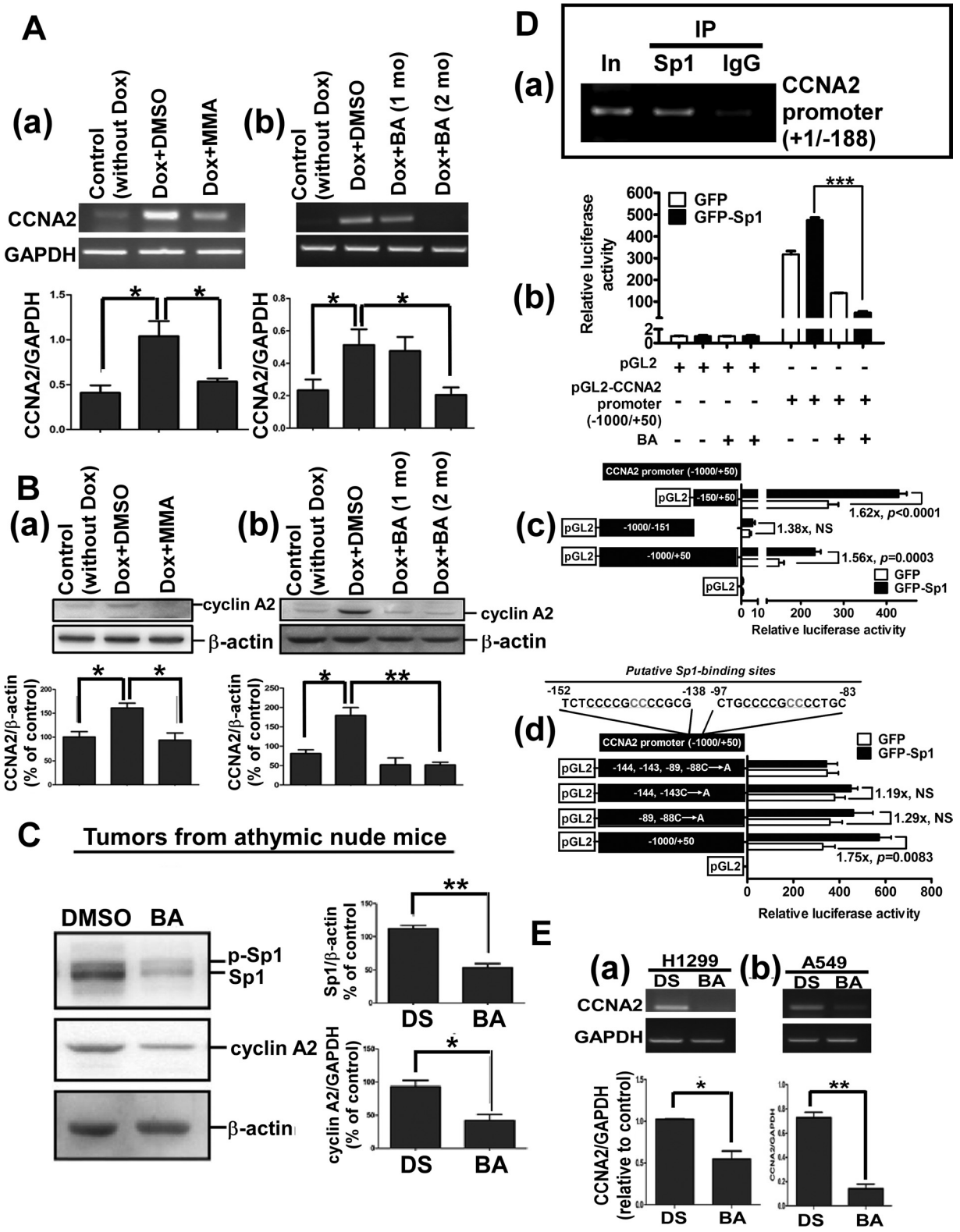

Fig. 6. Effect of BA on Sp1-regulated cyclin A2 expression in lung adenocarcinomas. A, RNA from lungs of bitransgenic mice treated with MMA (a) or BA (b) was analyzed by RT-PCR using primers for CCNA2. Data are representative of eight independent experiments and are presented as the mean ± S.E.M. (∗, $P < 0.05$, determined by Student's t test). Dox, doxycycline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B(a) and B(b), protein was analyzed by Western blotting with anti-cyclin A2 antibody. Data are representative of eight independent experiments and are presented as the mean ± S.E.M. (∗, $P < 0.05$; ∗∗, $P < 0.01$, determined by Student's t test). C, lysates prepared from H1299 cell-xenografted tumors in athymic nude mice treated as indicated were prepared as lysates for Western blotting with antibodies against Sp1, cyclin A2, and β-actin. Data are representative of six independent experiments and are presented as the mean ± S.E.M. (∗, $P < 0.05$; ∗∗, $P < 0.01$, determined by Student's t test). DS, DMSO. D(a), binding of Sp1 to the CCNA2 promoter (−188/+1) was analyzed by a chromatin immunoprecipitation (IP) assay. D(b), H1299 cells, which were transfected with 2 μg of pGL2 or pGL2-CCNA2 promoter plasmid and cultured in the presence or absence of BA (30 μM) for 36 h with GFP- or GFP-Sp1-expressed adenoviruses, were harvested for preparation of whole-cell lysates for luciferase activity assays. Data are representative of three

the Sp1 level is important for the BA-mediated anti-lung cancer effect.

BA is a naturally occurring pentacyclic triterpene that exhibits a variety of biological activities, including potent antiviral and anticancer effects (Alakurtti et al., 2006). A previous study indicated that the anticancer activity is contributed by its ability to trigger mitochondrial membrane permeabilization (Fulda, 2009). The anticancer activity of BA has been studied in a wide variety of cancer cell lines, primarily in tumor samples and xenograft mouse models (Fulda, 2008) of melanoma, neuroectodermal tumors, leukemia, and head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian, and cervical cancers (Pisha et al., 1995; Fulda et al., 1997, 1999; Zuco et al., 2002; Thurnher et al., 2003; Ehrhardt et al., 2004). Whereas most studies addressed the antitumor activity of BA in cell lines, some were performed in nude mice. In an in vivo model of metastatic melanoma, the addition of BA augmented vincristine suppression of experimental lung metastasis of melanoma cells (Sawada et al., 2004). Past studies on the effect of BA on lung cancer focused on cancer cell lines such as H460 cells (Zuco et al., 2002) without in vivo studies to address the role of BA in lung tumorigenesis. In this study, we not only used xenograft experiments to examine the effect of BA on lung cancer formation but also used a bitransgenic lung tumor mouse model, in which conditional expression of $Kras^{G12D}$ promotes spontaneous tumor development, to study the effect of BA on lung cancer formation. The data shown in Fig. 3C indicate that BA significantly represses lung tumor growth. In the bitransgenic lung tumor mouse model, the Sp1 level decreased, and caspase 3 was activated by BA treatment (Fig. 3C). Taken together, these in vivo results support the hypothesis that BA inhibits lung cancer growth by influencing Sp1 level, thereby triggering apoptosis.

We also investigated whether the antitumor effect of BA was dependent on Sp1 expression. As shown in Fig. 4, we demonstrated that Sp1 knockdown decreased the sensitivity of lung cancer cells to BA treatment. The effects of BA on the growth parameters obtained from a proliferation assay, cell cycle analysis, apoptosis assay, and proapoptotic protein expression were significantly attenuated in Sp1 knockdown cells. As indicated in Fig. 4D, BA reduced Sp1 levels efficiently in cells containing a high level of Sp1, but it did not further decrease Sp1 when Sp1 was knocked down. The underlying mechanisms by which BA only affects Sp1 in cells with a high level of Sp1 requires further study. We also found that a decrease in the Sp1 level was not enough to induce cancer cell death because cell apoptosis was not induced when we used shRNA to knockdown Sp1. Therefore, BA not only induces Sp1 degradation but also modulates other factor(s) in cancer cells to induce cell apoptosis.

Sp1 regulates the expression of genes related to several cellular functions, and its expression is essential for tumor growth (Wierstra, 2008). Previous studies showed that Sp1 accumulates in most cancer cell types (Wang et al., 2008). Both transcriptional activity and protein stability contribute to the increased Sp1 level observed during tumorigenesis (Wang et al., 2008). Recent studies found that post-translational modification of Sp1 modulates Sp1 stability. Sp1 is stabilized by phosphorylation at Thr739 but is destabilized by sumoylation (Chuang et al., 2008; Wang et al., 2008). Our recent finding indicates that the interplay between phosphorylation and sumoylation modulates the ubiquitination of Sp1 by recruiting the E3 ubiquitin ligase RNF4 (Wang et al., 2011). Although a previous study indicated that BA inhibits Sp1 in LNCaP cells, the mechanism of this inhibition remained unclear (Chintharlapalli et al., 2007). Herein, we clarified the mechanism by which BA induces Sp1 degradation: BA increased the sumoylation of Sp1 by decreasing the level of SENP1 (Fig. 1). We also determined the effect of BA on the phosphorylation of Sp1 at Thr739 and found no significant effect (data not shown), but we did observe that the interaction between Sp1 and RNF4 increased with BA treatment [Fig. 1E(a)]. This finding implies that sumoylation of Sp1 increases RNF4 recruitment, which leads to Sp1 degradation in a ubiquitination-dependent, 26S-proteasome-dependent pathway.

In addition to revealing the mechanism by which BA treatment leads to Sp1 degradation, we also elucidated, in detail, the molecular mechanism by which BA-mediated Sp1 degradation inhibits lung tumor growth. In particular, we analyzed the change in global gene expression induced by the Sp1 inhibitor MMA in lung tissue containing few lung tumors (Fig. 5). A number of genes contain one or more Sp1-binding elements within their promoter regions, implying that Sp1 could regulate these genes. This observation, in turn, implies that the enhancement of Sp1-mediated gene expression facilitates lung tumor formation. Because of the difficulty of confirming the roles of all Sp1-regulated genes identified by the microarray analysis, we examined proliferation-related genes that were potentially regulated by Sp1 to elucidate the contributions of Sp1-regulated genes to tumor growth. Among the 68 Sp1-regulated genes related to proliferation, seven genes, namely, *Aldh3a1*, *Arg1*, *Cd9*, *Ptx3*, *Tacstd2*, *Tpx2*, and *Vav1*, have been reported to be overexpressed in lung tumors or to be biomarkers for the diagnosis of lung cancer (Shimada et al., 2005; Ma et al., 2006; Moreb et al., 2008; Lazer et al., 2009; Rotondo et al., 2009; Kohmo et al., 2010; Diamandis et al., 2011). In addition, *Ctsz*, *Il18*, *Il4ra*, *Lcn2*, *Ntrk3*, and *Ros1* have been shown to be involved in tumor growth and cancer pathogenesis (Wood et al., 2006; Todaro et al., 2008; Berger et al., 2010; Sevenich et al., 2010; Rovina et al., 2011). The microarray data suggest that several genes involved in tumorigenesis are regulated by Sp1. We examined the gene expression of several important genes, such as *CCNA2*, *EGR1*, *CTSZ*, *VAV1*, *TACSTD2*, *TPX2*, and

independent experiments, each of which was performed in triplicate. Data are presented as the mean ± S.E.M. (***, $P < 0.001$, determined by Student's t test). D(c), H1299 cells, which were transfected with 2 μg of pGL2, pGL2-CCNA2 (−1000/+50), pGL2-CCNA2 (−1000/−151), or pGL2-CCNA2 (−150/+50) promoter plasmid and cultured for 36 h with GFP- or GFP-Sp1-expressed adenoviruses, were harvested for preparation of whole-cell lysates for luciferase activity assays. P values determined by Student's t test are indicated. NS, not significant. D(d), H1299 cells, which were transfected with 2 μg of pGL2, pGL2-CCNA2 (−1000/+50), mutant pGL2-CCNA2 (C −144, −143 A) or mutant pGL2-CCNA2 (C −89, −88 A) promoter plasmid and cultured for 36 h with GFP- or GFP-Sp1-expressed adenoviruses, were harvested for preparation of whole-cell lysates for luciferase activity assays. P values determined by Student's t test are indicated. E(a) and E(b), RNA from cells treated with DMSO (control) or BA for 36 h was extracted for RT-PCR. Data are representative of three independent experiments and are presented as the mean ± S.E.M. (*, $P < 0.05$; **, $P < 0.01$, determined by Student's t test).

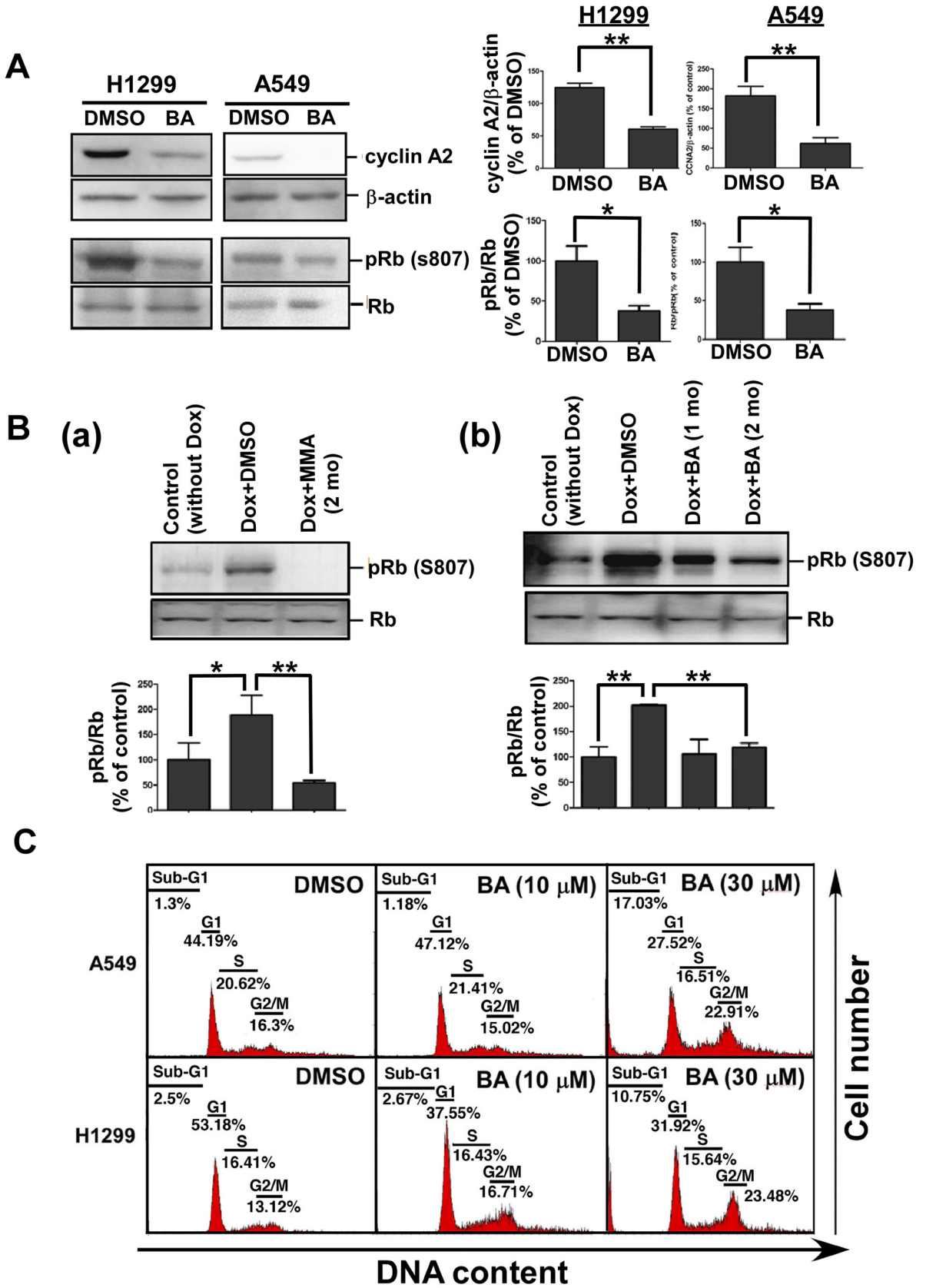

Fig. 7. Effects of BA on RB phosphorylation and cell cycle progression in lung adenocarcinomas. A, cells treated for 36 h with DMSO (control) or BA were harvested for preparation of whole-cell lysates for Western blotting with antibodies against cyclin A2, phosphorylated Rb (pRb), Rb, and β-actin. Left panel represents representative images. Right panel represents quantitative results (*, $P < 0.05$; **, $P < 0.01$, determined by Student's t test). B(a) and B(b), Lungs from control mice, doxycycline (Dox)-treated bitransgenic mice, and Dox-treated bitransgenic mice treated with MMA or BA for the last 2 months were prepared as lysates for Western blotting with antibodies against pRb (Ser807) and Rb. Quantitative results are shown in the bottom lower panel (*, $P < 0.05$; **, $P < 0.01$, determined by Student's t test). C, cells were harvested, fixed with 75% ethanol, washed with PBS, stained with PI, and analyzed by flow cytometry.

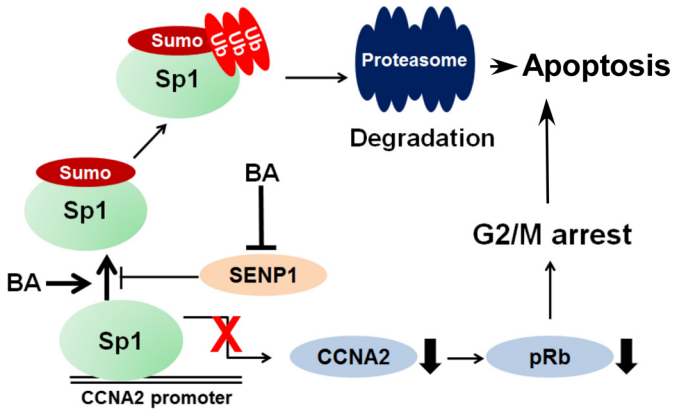

Fig. 8. The proposed model illustrates that BA induces proteasome-dependent degradation of Sp1 through increasing sumoylation (Sumo), which results in the attenuation of CCNA2/Rb signaling and subsequent cell cycle arrest at the G_2/M phase. Ub, ubiquitin.

CD2, and found that they were down-regulated by BA treatment. Previous studies have shown that the strong expression of cyclin A2, which mediates cell cycle transitions from the G_1 to S and G_2 to M phase, predicts poor prognosis for patients with several types of cancer (Yasmeen et al., 2003; Chan et al., 2010). Because of the critical role of cyclin A2 in cell cycle progression, it should be targeted by tumor therapy. Herein, we showed that the levels of Sp1 and cyclin A2 significantly increased in lung tumors, whereas BA blocked both of these increases and cyclin A2-mediated Rb phosphorylation. Furthermore, cyclin A2 expression was regulated transcriptionally by Sp1 through the 2 Sp1-binding sites (−149/−141 and −94/−86). Deletion of the Sp1-binding sites dramatically attenuated *CCNA2* transcriptional activity, indicating that Sp1 was absolutely required for *CCNA2* gene expression. These results also suggest that BA inhibits lung tumor growth by blocking the Sp1-mediated cyclin A2/Rb signaling cascade, which is required for cell cycle progression. It has been reported that BA induces tumor cell death by inducing apoptosis via a mitochondria-dependent pathway (Mullauer et al., 2010). We showed that in addition to apoptosis, BA induces cell cycle arrest at G_2 by decreasing the level of cyclin A2. Previous studies have also reported that down-regulation of cyclin A2 results in growth inhibition and G_2 arrest in several cancer cell lines (Badie et al., 2000; Chan et al., 2010). On the basis of these findings, we speculate that cyclin A2 down-regulation-mediated G_2/M arrest is a prerequisite for subsequent apoptosis in BA-treated tumor cells. Previous studies showed that BA induces cell apoptosis through the CD95/TNF-related apoptosis-inducing ligand pathway (Fulda et al., 1997). In this study, we elucidated a new pathway by which BA can induce cell growth arrest. Therefore, these two pathways might be involved in BA-induced apoptosis in parallel. In addition, although regulation of *CCNA2* by Sp1 in BA-induced tumor suppression is important, other Sp1-regulated genes might also be involved in the inhibitory effect of BA on lung cancer formation.

In this study, we determined the mechanism of BA-mediated Sp1 degradation and reported a novel BA-induced apoptotic pathway. These findings provide supportive evidence for treating lung tumors by inhibiting Sp1.

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Authorship Contributions

Participated in research design: Hsu and Hung.
Conducted experiments: Hsu, Wang, Chen, and Huang.
Contributed new reagents or analytic tools: Hsu, Yeh, Su, and Hung.
Performed data analysis: Hsu, Wang, Chang, and Hung.
Wrote or contributed to the writing of the manuscript: Hsu, Chang, and Hung.

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