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Title Page

Betulinic acid decreases Sp1 level via increasing the sumoylation of Sp1 to inhibit lung cancer growth

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Running Title Page

Running title: Betulinic acid attenuates Sp1-regulated CCNA2 in lung cancer

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ABBREVIATIONS: BA, betulinic acid; Sp1, specificity protein 1; SUMO-1, small ubiquitin-like modifier-1; SENP1, SUMO1/sentrin specific peptidase 1; RNF4, RING finger protein 4; MMA, mithramycin A; CDK, cyclin-dependent kinase; pRb, phosphorylated retinoblastoma protein; SCLC, small cell lung cancer; NSCLC, non-

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small-cell lung cancer; FBS, fetal bovine serum; Scgb1a1, secretoglobin, family 1A, member 1; rtTA, reverse tetracycline transactivator; TetO, tetracycline operator element; DOX, doxycycline; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HE, hematoxylin and eosin; PI, propidium iodide; DMSO, dimethyl sulfoxide; CCNA2, gene of cyclin A2; GFP, green fluorescent protein; Rpt6, regulatory particle ATpase 6; PARP, poly ADP ribose polymerase; Aldh3a1, aldehyde dehydrogenase 3 family, member A1; Arg1, arginase 1; Ptx3, pentraxin-related protein, Tacstd2, tumor-associated calcium signal transducer 2; Tpx2, targeting protein for Xklp2; Ctsz, cathepsin Z; Il, interleukin; Lcn1, lipocalin-2 ; Ntrk3, neurotrophic tyrosine kinase, receptor, type 3; TRAIL, TNF-related apoptosis-inducing ligand; GADD45β, growth arrest DNA damage-inducible gene 45β; NAG-1, nonsteroidal anti-inflammatory drug -activated gene-1, NAG-1; CHX, cycloheximide.

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Abstract

Previous studies have shown that the inhibitory effect of betulinic acid (BA) on specificity protein (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 (SENP1) expression. The subsequent recruitment of E3 ubiquitin-protein ligase RNF4 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 downregulated 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 G2/M phase transition during cell cycle progression, was investigated in detail because its expression was regulated by Sp1. The downregulation of cyclin A2 by BA treatment resulted in decreased Rb phosphorylation and cell cycle G2/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.

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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 lung cancer patients die from either progressive local disease or metastatic disease. Clinical pathologists divide lung cancers into 2 broad categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLCs are further divided into 3 major types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. NSCLCs constitute 85% of all lung cancers, with adenocarcinoma accounting for 30–50% of all cases of lung cancer (Langer et al., 2010). Because the 5-year survival rate of lung cancer patients 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 (Alakurtti et al., 2006; Zuco et al., 2002). The administration of BA reduced the tumor burden of established melanoma and prostate cancer xenografts in nude mice (Chintharlapalli et al., 2007; Pisha et al., 1995). 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 (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-

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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; Tian et al., 2011; Yang et al., 2010). Because the downregulation 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 (CDK) 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 G1/S phase transition (Hilton et al., 2005; Kim et al., 2006). In addition to DNA synthesis in S phase, cyclin A2 also regulates the G2/M phase transition by promoting nuclear envelope breakdown, nuclear accumulation of cyclin B1, and activation of the M-phase promoting complex (Bendris et al., 2011; Gong et al., 2007). Despite the essential role of cyclin A2 in cell proliferation, few studies have addressed the importance of cyclin A2 in the therapeutic effect of anti-tumor 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 G2/M phase. At the molecular level, BA

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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 G2/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.

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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, USA). These cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS, 100 mg/ml streptomycin sulfate, and 100 U/ml penicillin G sodium at 37°C and 5% CO_2.

Chemicals

Both of betulinic acid ($C_{30}H_{48}O_3$) and mithramycin A (plicamycin; $C_{52}H_{76}O_{24}$) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 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 ml was measured by MTS490 assay kit (Promega, Madison, WI, USA). H1299 cells were infected with lentiviruses at 5 m.o.i. ,which is the ration of virus particles to infected cells, for 3 days to achieve effective Sp1 knockdown. Subsequently, H1299 cells, which infected with scramble- or Sp1 shRNA-expressed lentiviruses for 3 days, were treated with indicated dose of BA for additional 36 h.

Collection of clinically resected lung adenocarcinoma specimens

Clinical specimens were obtained from 36 of stage I lung adenocarcinoma patients, who underwent surgery at the Department of Surgery of National Cheng Kung

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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})

Both of Scgb1a1-rtTA and TetO-Kras^{G12D} transgenic mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and cared 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 per liter of doxycyline (Sigma) at the age of 2 months. The control group drank RO water. BA and MMA were administrated 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 concentration 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

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immunoreactivity was visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and photographed under Olympus BX-51 microscope (Olympus, Melville, NY, USA). 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.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using Trizol according to manufacturer's instruction. Three μg of RNA were used for RT with SuperScript III (Invitrogen). The primers for PCR are as follows. Human Sp1 (F), 5′-ACTAGTGTCCGGGTTCGCTTGCCT-3′, R, 5′-GGCGCCAAAGTTGTGTGGCTGTGA-3′; Human cyclin A2 (F), 5′-GGTACCCCATTTCAATAGTC-3′, R, 5′- GAGCTCAAATTGTTGGTTCA-3′; Mouse cyclin A2 (F), 5′-GAACGTTAATGAAGTACCTGAC-3′, R, 5′-CAGGGTCTCATTCTGTAGTTTA-3′; GAPDH (F), 5′-CCATCACCATCTTCCAGGAG-3′, 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 x 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

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The cells were trypsinized and washed with cold PBS. After fixation with cold 75% ethanol for 24 h, the cells were treated with 10 μg/ml RNase A (Qiagen, Cupertino, CA, USA) and stained with 50 μg/ml PI (Sigma) for 30 min at 37℃. To analyze cell distribution in cell cycle, PI-stained cells were analyzed by a flow cytometer (FACS-Calibur; BD Biosciences, Palo Alto, CA, USA). Data were analyzed by the WinMDI software.

Microarray analysis

Total RNA was extracted using Trizol (Invitrogen) from lungs of control, 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 the 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 control or different doses of BA for indicated time. To examine cell proliferation, cells were trypsinized and counted by hemacytometer after treatment with BA. Each experiment was duplicated and repeated 3 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′; R, 5′-AGATCTGGAGCGGCGGCTGTTCTT-3′. Deletion form (-1000/-151): F, 5′-GGTACCGGTTTTAAAATGGCATAC-3′; R, 5′-

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AGATCTGAGGTAGGATTTAGGGCC-3′. Deletion form containing putative Sp1-binding sites (-150/+50): F, 5′-GGTACCTCCCCGCCCCGCGCAGGC-3′; 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, USA)

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′; R, 5′-AGGAAACTGAGCAGGTTCGGGGCAGGAGGGAG-3′. For -144/-143C to -144/-143A: F, 5′-TCCTACCTCTCCCCGAACCGCGCAGGCGTTTT-3’; R, 5′-AAAACGCCTGCGCGGTTCGGGGAGAGGTAGGA-3′. Mutagenesis was performed by PCR using pfu DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA).

Transfection and luciferase assay

After cells were plated in an appropriate number in 6-well plates for 24 h, transfection was achieved by incubating cells with Opti-MEM (Invitrogen) containing 2 μg of the pGL2-vector, pGL2-SENP1 or pGL2-CCNA2 promoter plasmid, and 2 μl of lipofectamine (Invitrogen) at 37℃ and 5% CO_2 for 6 h. Subsequently, Opti-MEM was replaced with DMEM-10% FBS and incubated for 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

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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 the anti-Sp1 (Millipore, Bedford, MA, USA), 1 μg of anti-RNF4 (Sigma) or 2 μg of anti-ubiquitin antibodies (Santa Cruz Biotechnology, CA, USA) in modified RIPA buffer for 2 h at 4℃. Thirty microliter of protein A agarose beads (Millipore) were mixed with immunoprecipitated lysates and incubated for additional 1 h at 4℃. After washing by RIPA buffer for 4 times, beads were collected by centrifugation at 5000 rpm for 3 min at 4℃ and eluted by 1.5x 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, USA), RNF4, caspase 3 (Cell signaling Technology, Beverly, MA, USA), cyclin A2 (ProteinTech Group), SENP1 (Epitomics Inc., Burlingame, CA, USA), PARP (Epitomics), phospho-Rb (S807, Cell Signaling Technology), caspase 3 (Cell Signaling Technology), Rb (ProteinTech Group), Rpt6 (Biomol International L.P., Butler Pike, PA, USA), Flag (Sigma), tubulin (Sigma) and β-actin (Sigma).

MTT assay

After infection with lentiviruses expressing indicated shRNA for 3 days, cells were seeded in the 96-well plate (5000 cells/well) and treated with BA for additional 36 h,

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cell viability was determined by using MTT assay kit according to the manufacturer's instruction (Biovision Inc, Milpitas, CA, USA).

Chromatin immunoprecipitation (CHIP) assay

After cross-linking with 1% formaldehyde (Merck) for 10 min at room temperature, cells (1 x 10^8) were washed with ice-cold PBS 3 times and whole cell extracts were prepared with lysis buffer. The cellular DNA fragment (500 bp) 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℃ for 16 h with rotation. After incubation with 30 μl of protein A agarose beads at 4℃ for additional 2 h, beads were washed 3 times with high salt buffer (20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, 0.5% NP-40) and another 3 times with low salt buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.01% SDS). Subsequently, proteins binding to beads were eluted by 500 μl of TE buffer containing 1% SDS, and cross-links were reversed at 65℃ for 16 h. After protein digestion by 0.5 mg/ml proteinase K at 50℃ 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): Forward, 5′- TTAAAATAATCGGAAGCG- 3′; Reverse, 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 (ANOVA) was used to compare growth curve of tumors from DMSO- and BA-treated athymic nude mice.

Results

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BA decreases the Sp1 level in lung adenocarcinoma by modulating its posttranslational modification

A previous study showed that BA inhibits the growth of 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] and Supplementary Fig. S1) of SENP1 in lung adenocarcinoma cells. Finally, the interactions of Sp1 with the E3 ubiquitin-protein ligase RNF4 and the proteasome subunit regulatory particle

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ATPase 6 (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 to the Sp1 level in control (Fig. 2B). Taken together, a good correlation between cell growth inhibition and Sp1 downregulation with BA treatment suggests 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. 3). First, to clarify the clinical significance of Sp1 expression in tumorigenesis, Sp1 protein expression was detected in specimens from lung cancer patients by immunohistochemical staining (Fig. 3A and Table 1). Among 36 patients with stage I lung adenocarcinomas, 29 patients (81%) exhibited high Sp1 expression, whereas

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only 7 (19%) exhibited low Sp1 expression. This 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 severe combined immunodeficiency (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/3 days) in the fourth and fifth months of doxycycline treatment prevented tumor development. This 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 upregulation of Sp1, but not that of Kras, and upregulated caspase 3 (Fig. 3C[b]). This suggests 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 small hairpin RNA (shRNA) in lung cancer cells (Fig. 4). 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. 4A and 4B). 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-G1 fraction after treatment with 30 and 50 μM BA, respectively. In

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the Sp1 knockdown condition treated with 30 and 50 μM BA, 7.34% and 27.62% of cells were found in the sub-G1 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 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/week) during the last 2 months of a 5-month doxycycline treatment clearly prevented both tumor formation (Fig. 3C[a]) and Sp1 upregulation 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 downregulation 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 (Supplementary Fig. S2 and Supplementary Fig. S3). Functional grouping analyses 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, Supplementary Fig. S3,

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and Supplementary 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. 6 and Supplementary 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. 6A and 6B, 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 Sp1-binding sites in the *CCNA2* promoter by using the TFSEARCH bioinformatics software (Tsukuba, Japan). We found 2 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 (ChIP) 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*

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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 (Supplementary Fig. S5B, C, and D). We also showed that Sp1 overexpression significantly increased the protein level of cyclin A2 in H1299 cells (Supplementary 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 G1 to S and G2 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 pRb 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 G2/M phase in BA-treated cells

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was assessed by flow cytometry analysis. In A549 cells, the proportions of cells in the sub-G1 and G2/M fractions were 1.3% and 16.3%, respectively, whereas the proportions of cells in the sub-G1 and G2/M fractions increased to 17.03% and 22.91%, respectively, after treatment with 30 μM BA (Fig. 7C, upper panel). In H1299 cells, treatment with 30 μM BA increased the proportions of cells in the sub-G1 and G2/M populations from 2.5% to 10.75% and from 13.12% to 23.48%, respectively (Fig. 7C, lower panel). These results suggest that BA-mediated Sp1 downregulation blocks the cyclin A2/Rb signaling cascade, resulting in cell cycle arrest at the G2/M phase.

Based on 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 G2/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 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

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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 (Ehrhardt et al., 2004; Fulda et al., 1997; Fulda et al., 1999; Pisha et al., 1995; Thurnher et al., 2003; Zuco et al., 2002). While 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 indicates 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 pro-apoptotic protein expression were significantly attenuated in Sp1 knockdown cells. As indicated in Fig. 4D, BA reduced Sp1 levels efficiently in cells containing a

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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 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 implies that sumoylation of Sp1 increases RNF4 recruitment, which leads to Sp1 degradation in a ubiquitination-dependent, 26S-proteasome–dependent pathway.

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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, 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 in order to elucidate the contributions of Sp1-regulated genes to tumor growth. Among the 68 Sp1-regulated genes related to proliferation, 7 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 (Diamandis et al., 2011; Kohmo et al., 2010; Lazer et al., 2009; Ma et al., 2006; Moreb et al., 2008; Rotondo et al., 2009; Shimada et al., 2005). In addition, *Ctsz*, *Il18*, *Il4ra*, *Lcn2*, *Ntrk3*, and *Ros1* have been shown to be involved in tumor growth and cancer pathogenesis (Berger et al., 2010; Rovina et al., 2011; Sevenich et al., 2010; Todaro et al., 2008; Wood et al., 2006). 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 *CD2*, and found that they were downregulated by BA treatment. Previous studies have shown that the strong expression of cyclin A2, which mediates cell cycle transitions from G1 to S and G2 to M phase, predicts poor prognosis for patients with several types of cancer (Chan et al., 2010; Yasmeen et al., 2003). Because of the critical role of cyclin A2 in cell cycle

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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 G2 by decreasing the level of cyclin A2. Previous studies have also reported that downregulation of cyclin A2 results in growth inhibition and G2 arrest in several cancer cell lines (Badie et al., 2000; Chan et al., 2010). Based on these findings, we speculate that cyclin A2 downregulation-mediated G2/M arrest is a prerequisite for subsequent apoptosis in BA-treated tumor cells. Previous studies showed that BA induces cell apoptosis through the CD95/TRAIL pathway (Fulda et al., 1997). In this study, we elucidated a new pathway by which BA can induce cell growth arrest. Therefore, these 2 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, Chang, Wang and Hung

Wrote or contributed to the writing of the manuscript: Hsu, Chang and Hung

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References

Alakurtti S, Makela T, Koskimies S, and Yli-Kauhaluoma J (2006) Pharmacological properties of the ubiquitous natural product betulin. Eur J Pharm Sci *29*, 1-13.

Badie C, Bourhis J, Sobczak-Thepot J, Haddada H, Chiron M, Janicot M, Janot F, Tursz T, and Vassal G (2000) p53-dependent G2 arrest associated with a decrease in cyclins A2 and B1 levels in a human carcinoma cell line. Br J Cancer *82*, 642-650.

Bendris N, Lemmers B, Blanchard JM, and Arsic N (2011) Cyclin A2 mutagenesis analysis: a new insight into CDK activation and cellular localization requirements. PLoS One *6*, e22879.

Berger T, Cheung CC, Elia AJ and Mak TW (2010) Disruption of the Lcn2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. Proc Natl Acad Sci U S A *107*, 2995-3000.

Chan QK, Lam HM, Ng CF, Lee AY, Chan ES, Ng HK, Ho SM and Lau KM (2010) Activation of GPR30 inhibits the growth of prostate cancer cells through sustained activation of Erk1/2, c-jun/c-fos-dependent upregulation of p21, and induction of G(2) cell-cycle arrest. Cell Death Differ *17*, 1511-1523.

Chintharlapalli S, Papineni S, Ramaiah SK and Safe S (2007) Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors. Cancer Res *67*, 2816-2823.

Chuang JY, Wang YT, Yeh SH, Liu YW, Chang WC and Hung JJ (2008) Phosphorylation by c-Jun NH2-terminal kinase 1 regulates the stability of transcription factor Sp1 during mitosis. Mol Biol Cell *19*, 1139-1151.

Conway TF Jr, Sabel MS, Sugano M, Frelinger JG, Egilmez NK, Chen F and Bankert RB (2000) Growth of human tumor xenografts in SCID mice quantified using an

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Molecular Pharmacology Fast Forward. Published on September 5, 2012 as DOI: 10.1124/mol.112.078485
This article has not been copyedited and formatted. The final version may differ from this version.

immunoassay for tumor marker protein in serum. J Immunol Methods *233*, 57-65.

Diamandis EP, Goodglick L, Planque C and Thornquist MD (2011) Pentraxin-3 is a novel biomarker of lung carcinoma. Clin Cancer Res *17*, 2395-2399.

Ehrhardt H, Fulda S, Fuhrer M, Debatin KM and Jeremias I (2004) Betulinic acid-induced apoptosis in leukemia cells. Leukemia *18*, 1406-1412.

Fisher GH, Wellen SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, Whitsett JA, Koretsky A and Varmus HE (2001) Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev *15*, 3249-3262.

Fulda S (2008) Betulinic Acid for cancer treatment and prevention. Int J Mol Sci *9*, 1096-1107.

Fulda S (2009) Betulinic acid: a natural product with anticancer activity. Mol Nutr Food Res *53*, 140-146.

Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer PH, Peter ME, and Debatin KM (1997) Betulinic acid triggers CD95 (APO-1/Fas)-and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res *57*, 4956-4964.

Fulda S, Jeremias I, Steiner HH, Pietsch T and Debatin KM (1999) Betulinic acid: a new cytotoxic agent against malignant brain-tumor cells. Int J Cancer *82*, 435-441.

Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, Postmus PE, Rusch V and Sobin L (2007) The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming

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Molecular Pharmacology Fast Forward. Published on September 5, 2012 as DOI: 10.1124/mol.112.078485
This article has not been copyedited and formatted. The final version may differ from this version.

(seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol *2*, 706-714.

Gong D, Pomerening JR, Myers JW, Gustavsson C, Jones JT, Hahn AT, Meyer T and Ferrell JE Jr (2007) Cyclin A2 regulates nuclear-envelope breakdown and the nuclear accumulation of cyclin B1. Curr Biol : CB *17*, 85-91.

Guichard SM, Else R, Reid E, Zeitlin B, Aird R, Muir M, Dodds M, Fiebig H, Sadler PJ and Jodrell DI (2006) Anti-tumour activity in non-small cell lung cancer models and toxicity profiles for novel ruthenium(II) based organo-metallic compounds. Biochem Pharmacol *71*, 408-415.

Hilton TL, Li Y, Dunphy EL, and Wang EH (2005) TAF1 histone acetyltransferase activity in Sp1 activation of the cyclin D1 promoter. Mol Cell Biol *25*, 4321-4332.

Jiang Y, Wang L, Gong W, Wei D, Le X, Yao J, Ajani J, Abbruzzese JL, Huang S and Xie K (2004) A high expression level of insulin-like growth factor I receptor is associated with increased expression of transcription factor Sp1 and regional lymph node metastasis of human gastric cancer. Clin Exp Metastasis *21*, 755-764.

Kim S, Kang J K, Kim YK, Seo DW, Ahn SH, Lee JC, Lee CH, You JS, Cho EJ, Lee HW and Han JW (2006) Histone deacetylase inhibitor apicidin induces cyclin E expression through Sp1 sites. Biochem Biophys Res Commun *342*, 1168-1173.

Kohmo S, Kijima T, Otani Y, Mori M, Minami T, Takahashi R, Nagatomo I, Takeda Y, Kida H, Goya S,Yoshida M, Kumagai T, Tachibana I, Yokota S and Kawasae I (2010) Cell surface tetraspanin CD9 mediates chemoresistance in small cell lung cancer. Cancer Res *70*, 8025-8035.

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Molecular Pharmacology Fast Forward. Published on September 5, 2012 as DOI: 10.1124/mol.112.078485
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Langer CJ, Besse B, Gualberto A, Brambilla E and Soria JC (2010) The evolving role of histology in the management of advanced non-small-cell lung cancer. J Clin Oncol *28*, 5311-5320.

Lazer G, Idelchuk Y, Schapira V, Pikarsky E and Katzav S (2009) The haematopoietic specific signal transducer Vav1 is aberrantly expressed in lung cancer and plays a role in tumourigenesis. J Pathol *219*, 25-34.

Ma Y, Lin D, Sun W, Xiao T, Yuan J, Han N, Guo S, Feng X, Su K, Mao Y, Cheng S and Gao Y (2006) Expression of targeting protein for xklp2 associated with both malignant transformation of respiratory epithelium and progression of squamous cell lung cancer. Clin Cancer Res *12*, 1121-1127.

Moreb JS, Baker HV, Chang LJ, Amaya M, Lopez MC, Ostmark B and Chou W (2008) ALDH isozymes downregulation affects cell growth, cell motility and gene expression in lung cancer cells. Mol Cancer *7*, 87.

Mullauer FB, Kessler JH and Medema JP (2010) Betulinic acid, a natural compound with potent anticancer effects. Anticancer Drugs *21*, 215-227.

Musgrove EA, Caldon CE, Barraclough J, Stone A and Sutherland RL (2011) Cyclin D as a therapeutic target in cancer. Nat Rev Cancer *11*, 558-572.

Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, Beecher C W, Fong HH, Kinghorn AD, Brown DM, Wani MC, Wall ME, Hieken TJ, Das Gupta TK and Pezzuto JM (1995) Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med *1*, 1046-1051.

Rotondo R, Barisione G, Mastracci L, Grossi F, Orengo AM, Costa R, Truini M, Fabbi M, Ferrini S and Barbieri O (2009) IL-8 induces exocytosis of arginase 1

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Molecular Pharmacology Fast Forward. Published on September 5, 2012 as DOI: 10.1124/mol.112.078485
This article has not been copyedited and formatted. The final version may differ from this version.

by neutrophil polymorphonuclears in nonsmall cell lung cancer. Int J Cancer *125*, 887-893.

Rovina N, Hillas G, Dima E, Vlastos F, Loukides S, Veldekis D, Roussos C, Alhanatis M and Bakakos P (2011) VEGF and IL-18 in induced sputum of lung cancer patients. Cytokine *54*, 277-281.

Sawada N, Kataoka K, Kondo K, Arimochi H, Fujino H, Takahashi Y, Miyoshi T, Kuwahara T, Monden Y and Ohnishi Y (2004) Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. Br J Cancer *90*, 1672-1678.

Sevenich L, Schurigt U, Sachse K, Gajda M, Werner F, Muller S, Vasiljeva O, Schwinde A, Klemm N, Deussing J, Peters C and Reinheckel T (2010) Synergistic antitumor effects of combined cathepsin B and cathepsin Z deficiencies on breast cancer progression and metastasis in mice. Proc Natl Acad Sci U S A *107*, 2497-2502.

Shimada A, Kano J, Ishiyama T, Okubo C, Iijima T, Morishita Y, Minami Y, Inadome Y, Shu Y, Sugita S, Takeuchi T and Noquchi M (2005) Establishment of an immortalized cell line from a precancerous lesion of lung adenocarcinoma, and genes highly expressed in the early stages of lung adenocarcinoma development. Cancer Sci *96*, 668-675.

Sun X, Ritzenthaler JD, Zhong X, Zheng Y, Roman J and Han S (2009) Nicotine stimulates PPARbeta/delta expression in human lung carcinoma cells through activation of PI3K/mTOR and suppression of AP-2alpha. Cancer Res *69*, 6445-6453.

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Molecular Pharmacology Fast Forward. Published on September 5, 2012 as DOI: 10.1124/mol.112.078485
This article has not been copyedited and formatted. The final version may differ from this version.

Thurnher D, Turhani D, Pelzmann M, Wannemacher B, Knerer B, Formanek M, Wacheck V, and Selzer E (2003) Betulinic acid: a new cytotoxic compound against malignant head and neck cancer cells. Head Neck *25*, 732-740.

Tian H, Qian GW, Li W, Chen FF, Di JH, Zhang BF, Pei DS, Ma P and Zheng JN (2011) A critical role of Sp1 transcription factor in regulating the human Ki-67 gene expression. Tumour Biol *32*, 273-283.

Todaro M, Lombardo Y, Francipane MG, Alea MP, Cammareri P, Iovino F, Di Stefano AB, Di Bernardo C, Agrusa A, Condorelli, G., Walczak H, Stassi G (2008) Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. Cell Death Differ *15*, 762-772.

Wang YT, Chuang JY, Shen MR, Yang WB, Chang WC and Hung JJ (2008) Sumoylation of specificity protein 1 augments its degradation by changing the localization and increasing the specificity protein 1 proteolytic process. J Mol Biol *380*, 869-885.

Wang YT, Yang WB, Chang WC and Hung JJ (2011) Interplay of posttranslational modifications in Sp1 mediates Sp1 stability during cell cycle progression. J Mol Biol *414*, 1-14.

Wierstra I (2008) Sp1: emerging roles--beyond constitutive activation of TATA-less housekeeping genes. Biochem Biophys Res Commun *372*, 1-13.

Wood LD, Calhoun ES, Silliman N, Ptak J, Szabo S, Powell SM, Riggins GJ, Wang TL, Yan H, Gazdar A, Kern SE, Pennacchio L, Kinzler KW and Vogelstein B (2006) Somatic mutations of GUCY2F, EPHA3, and NTRK3 in human cancers. Hum Mutat *27*, 1060-1061.

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Molecular Pharmacology Fast Forward. Published on September 5, 2012 as DOI: 10.1124/mol.112.078485
This article has not been copyedited and formatted. The final version may differ from this version.

Yang TP, Chiou HL, Maa MC and Wang CJ (2010) Mithramycin inhibits human epithelial carcinoma cell proliferation and migration involving downregulation of Eps8 expression. Chem Biol Interact *183*, 181-186.

Yasmeen A, Berdel WE, Serve H and Muller-Tidow C (2003) E- and A-type cyclins as markers for cancer diagnosis and prognosis. Expert Rev Mol Diagn *3*, 617-633.

Zuco V, Supino R, Righetti SC, Cleris L, Marchesi E, Gambacorti-Passerini C and Formelli F (2002) Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. Cancer Lett *175*, 17-25.

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Footnotes

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Figure legends

Fig. 1. Effects of BA on Sp1 protein 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 (Upper panel). Quantitative results are shown in the lower panel. Three experiments were conducted independently, and data are expressed as mean ± SEM. (**P*<0.05 and ***P*<0.01 were determined by Student's *t* test) C(a). HeLa cells treated with DMSO (DS) or BA for 36 h were harvested for preparing whole cell lysate for Western blotting with antibodies against Sp1 and β-actin. C(b). Cells transfected with SUMO-1-GG and treated with BA for 36 h were harvested for Western blotting with antibodies against Sp1 and β-actin. C(c). Twenty-four hours 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 3 times and data are expressed as mean ± SEM. (***P*<0.01 and ****P*<0.001 were determined by Student's *t* test) D(b). H1299 cells were harvested for RT-PCR. Experiments were performed independently 3 times and data are expressed as mean ± SEM. D(c). H1299 cells were harvested for Western blotting using anti-SENP1 and anti-tubulin antibodies. (**P*<0.05 and ***P*<0.01, compared to DMSO-treated control, were determined by Student's *t* test) E(a). Whole cell lysate

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was immunoprecipitated 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.

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 indicated period, and photographed under light microscope (x 100). 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 **±** SEM. (**P*<0.05 and ***P*<0.01, compared to DMSO-treated control, were 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 and normal lung tissues with immunohistochemical staining using anti-Sp1 antibody. A(b). Sp1 expression in 36 stage I lung adenocarcinoma patients. 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 per mouse), mice were treated with BA (10 mg/kg, once/3 days) for 4 weeks, and resected tumors were then photographed. B(b). Volumes of tumors formed after innoculation of cells were measured and calculated. Data are representative of 6 independent experiments and are presented as the mean ± SEM. $P = 0.0011$ was determined by 2-way ANOVA. B(c). Tumors were resected 4 weeks after injection of cells and weighed. Data are representative of 6 independent experiments and are presented as the mean ± SEM.

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(*P < 0.05 was determined by Student's t test). C. Effect of Sp1 inhibitors on $Kras^{G12D}$-driven lung tumor formation in doxycycline-induced transgenic mouse model. (a) Effect of BA and mithramycin A (MMA) on lung tumor formation. Lung tumors in bitransgenic mice were induced by 5 months treatment with doxycycline (Dox). BA (20 mg/kg, once/days) and MMA (0.5 mg/kg, once/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 upper panel, and numbers of tumors on the lung surfaces of bitransgenic mice are shown in the lower panel. Each point represents one mouse (*P < 0.01 was determined by Student's t test). (b). Bitransgenic mice were treated with BA in the last one month and two months during doxycycline treatment period. Lungs were exercised, and immunohistochemical analysese were performed by using antibodies against Sp1 and Kras. Representative images of HE and immunohistochemical. (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. (upper panel). Data are representative of 8 independent experiments and are presented as the mean ± SEM. (lower panel, *P < 0.05 was 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 m.o.i.) 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 3 independent experiments and are presented as the mean ± SEM. (*P < 0.05 was determined by Student's t test) B. Cell viability was measured by MTT assay. Data are representative of 3 independent experiments and are presented as the mean ±

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SEM. (*P < 0.05, **P<0.01, ***P<0.001 and $^{\#}P$<0.05, all of which were compared to the scramble-treated group, were 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 sub-G1 population. Data are representative of 3 independent experiments and are presented as the mean ± SEM. (*P < 0.05 and **P<0.01 were determined by Student's *t* test) C(c). Statistical results for G2/M population. Data are representative of 3 independent experiments and are presented as the mean ± SEM. (*P < 0.05 was 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 3 independent experiments and are presented as the mean ± SEM. (*P < 0.05 and **P<0.01, compared to the DMSO-treated group, were 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 with MMA (0.5 mg/kg, one/week) in the last 2 months during 5 months of treatment period of doxycycline. A. Lungs were harvested and prepared for histological analysis. Representative images of lung sections with HE staining and with immunohistochemical were stained using antibodies against Sp1 and Kras. B. Heat map of 68 genes related to proliferation was created using the Genepattern website.

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 8 independent experiments and are presented as the mean ± SEM. (*P < 0.05 was

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determined by Student's *t* test) B. Protein was analyzed by Western blotting with anti-cyclin A2 antibody. Data are representative of 8 independent experiments and are presented as the mean ± SEM. (**P* < 0.05 and ***P* < 0.01 were determined by Student's *t* test) C. Lysates prepared from H1299 cells-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 6 independent experiments and are presented as the mean ± SEM. (**P* < 0.05 and ***P* < 0.01 were determined by Student's *t* test) D(a). Binding of Sp1 to the CCNA2 promoter (-188/+1) was analyzed by CHIP assay. (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 preparing whole cell lysates for luciferase activity assays. Data are representative of 3 independent experiments, each of which was performed in triplicate. Data are presented as the mean ± SEM. (***P* < 0.01 was determined by Student's *t* test) (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 preparing whole cell lysates for luciferase activity assays. *P*-value determined by Student's t test was indicated. (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 preparing whole cell lysates for luciferase activity assays. *P*-value determined by Student's t test was indicated. E. RNA from cells treated with DMSO (control) or BA for 36 h was extracted for RT-PCR. Data are representative of 3 independent

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experiments and are presented as the mean ± SEM. (*P < 0.05 and **P < 0.01 were 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 preparing whole cell lysates for Western blotting with antibodies against cyclin A2, pRb, Rb and β-actin. Left panel is representative images. Right panel is quantitative results. (*P < 0.05 and **P < 0.01 were determined by Student's t test) B. Lungs from control mice, 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 (S807) and Rb. Quantitative results are shown in lower panel. (*P < 0.05 and **P < 0.01 were 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 which results in the attenuation of CCNA2/Rb signaling and subsequent cell cycle arrest at the G2/M phase.

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

	Patients with Sp1 positive staining (n)	Patients with Sp1 negative staining (n)	*P*-value
Male	12	3	1†
Female	17	4	
Age	69.81±1.56	70.67±4.82	0.82*

†Fisher's extract test

*Student's t test

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Figure 1

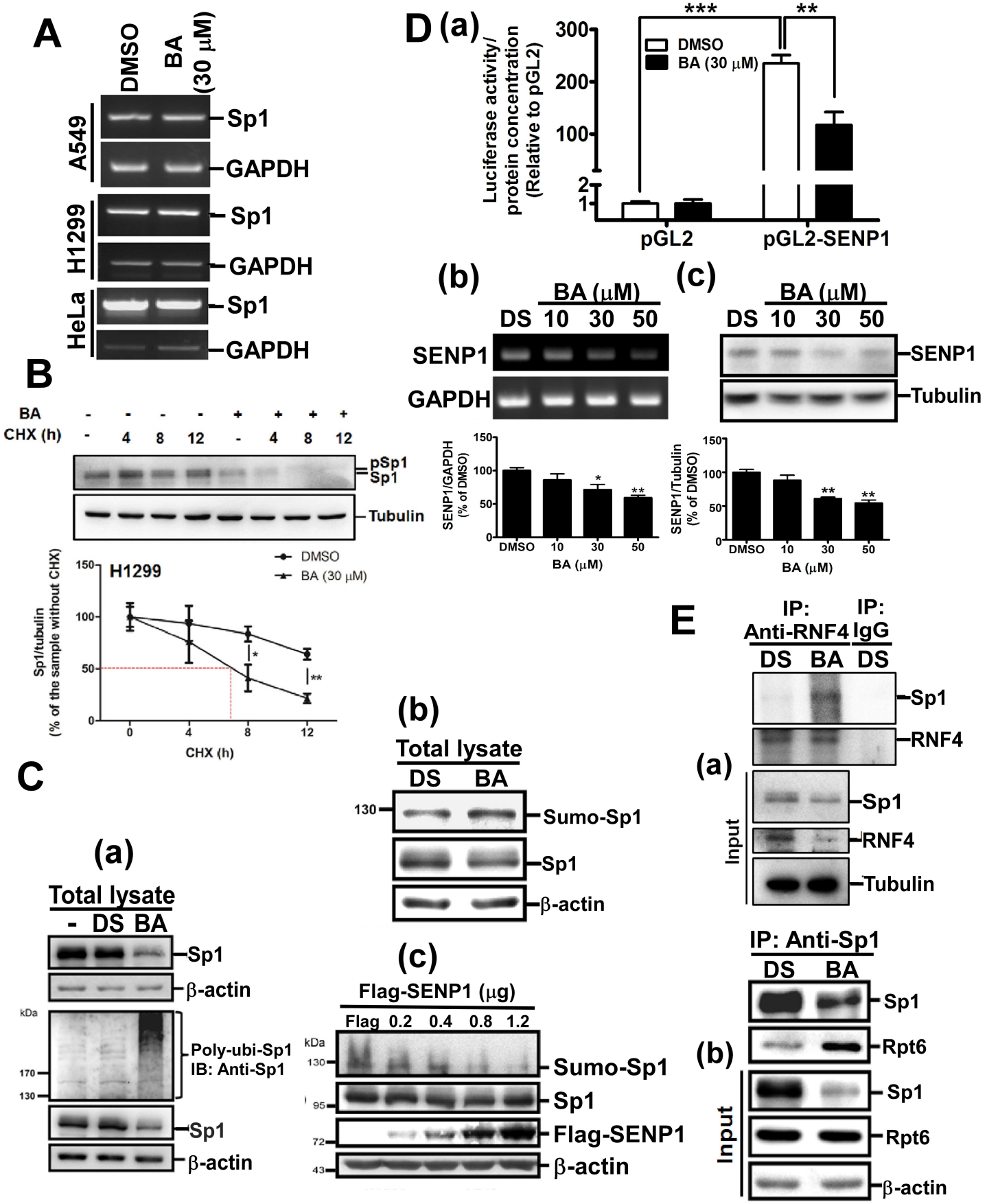

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Figure 2

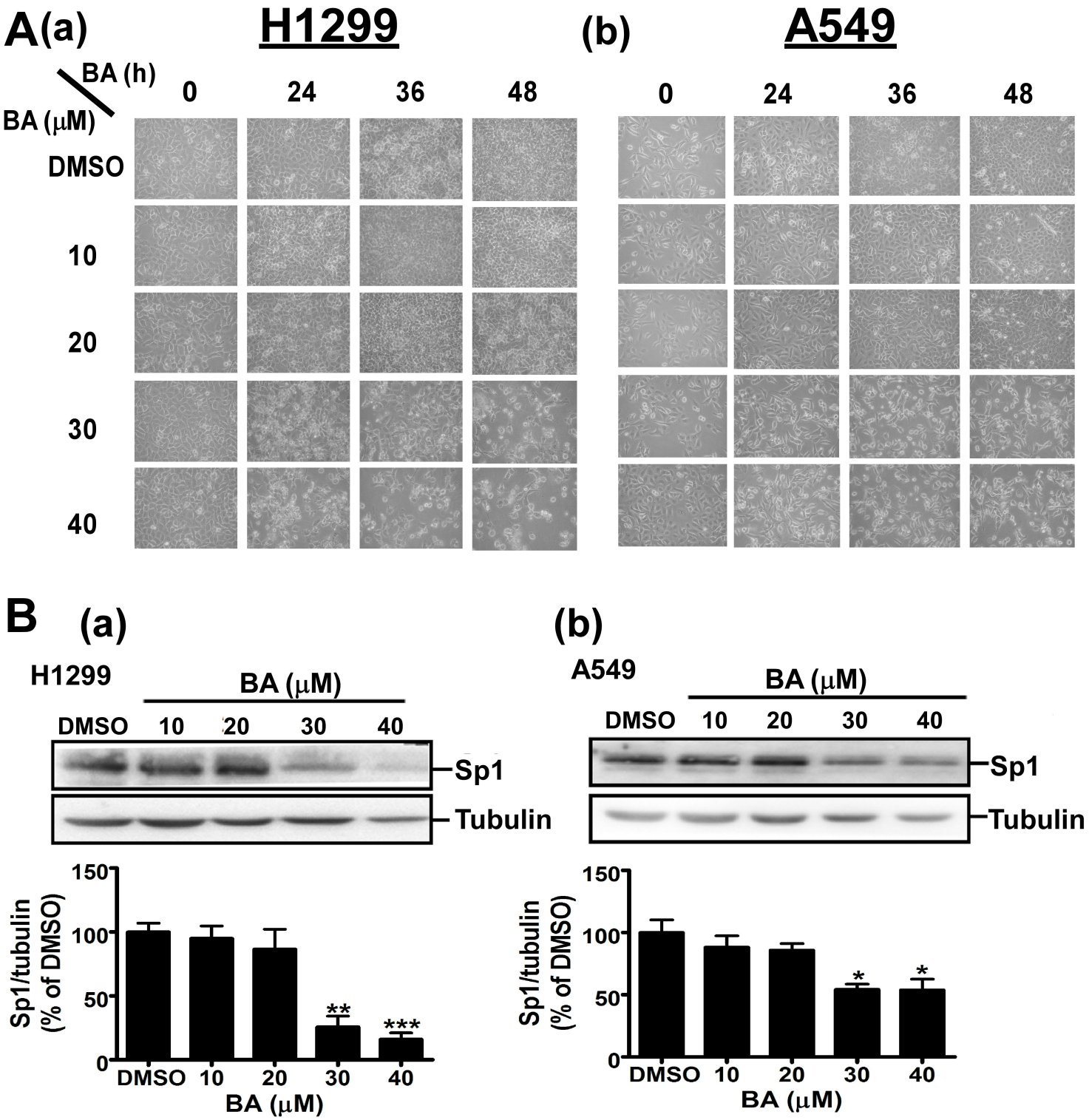

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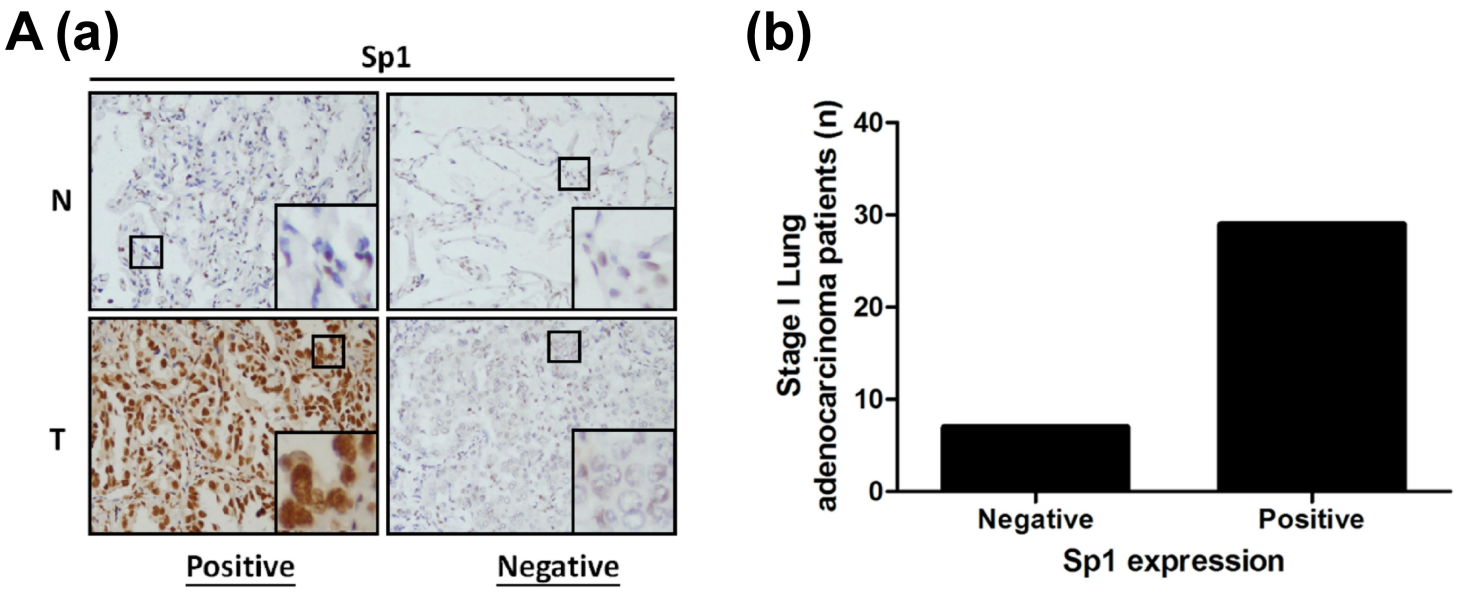
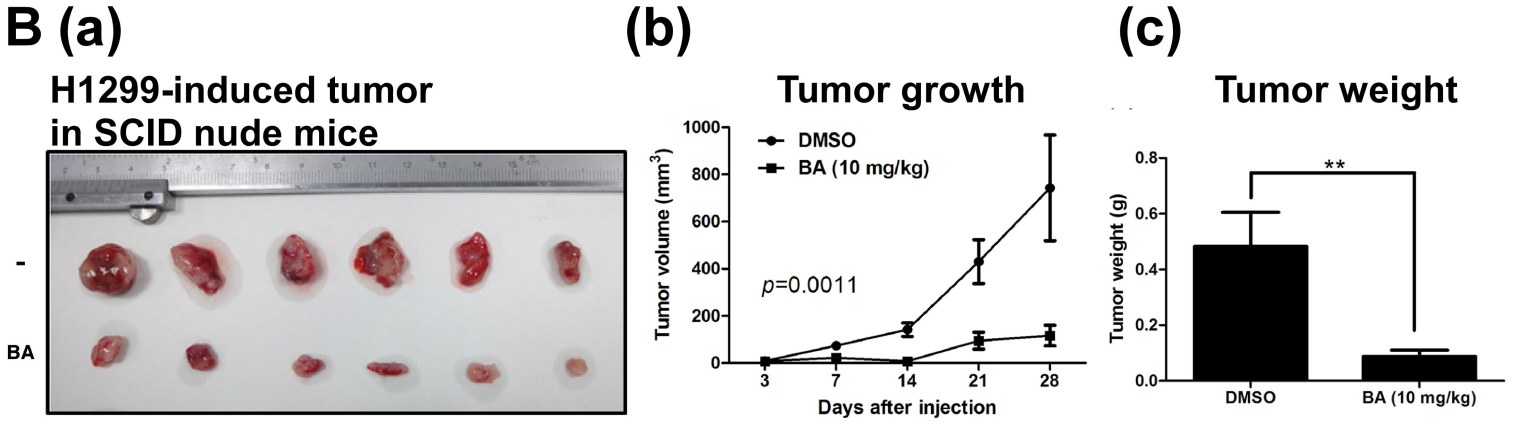
Figure 3





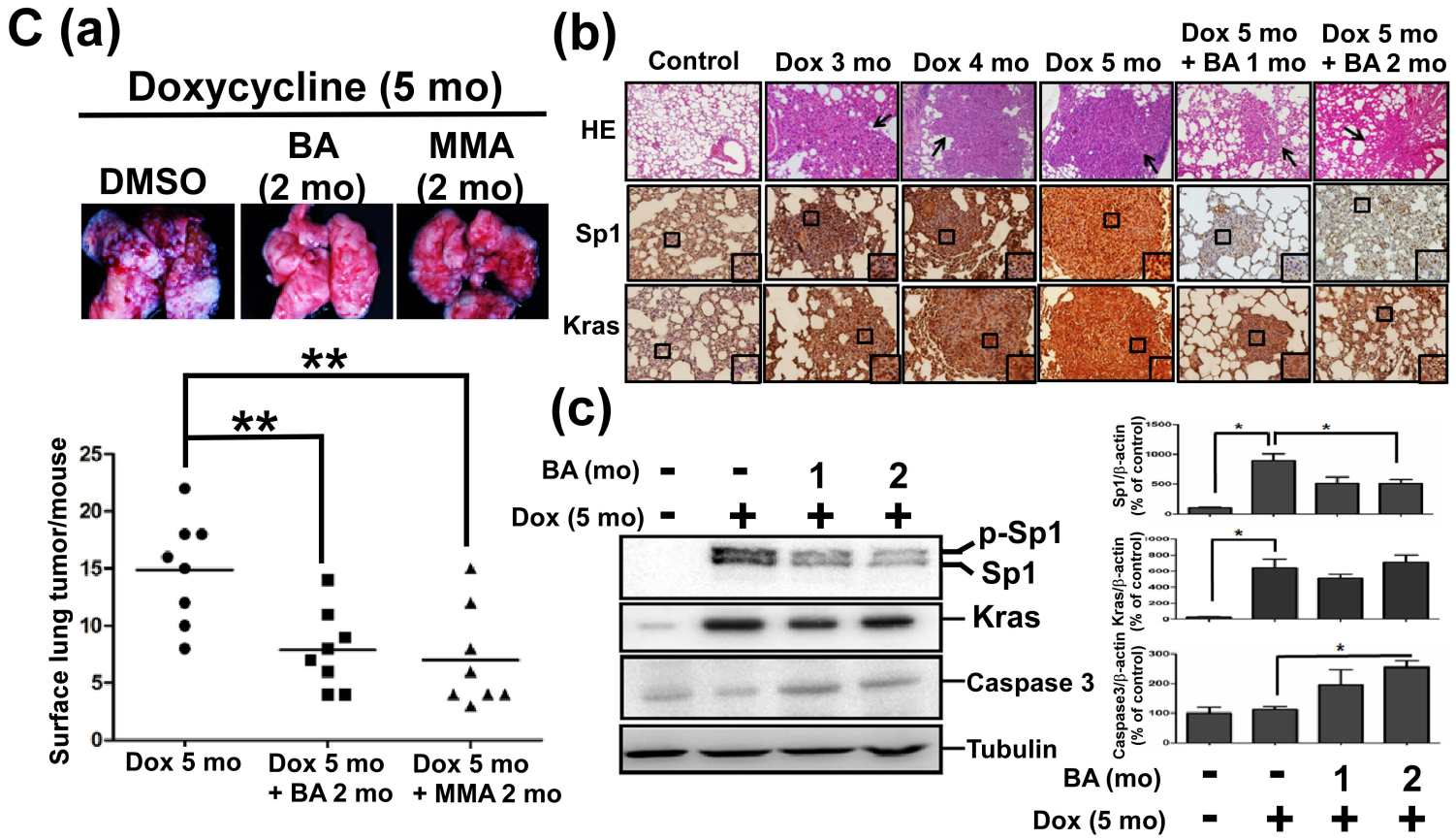


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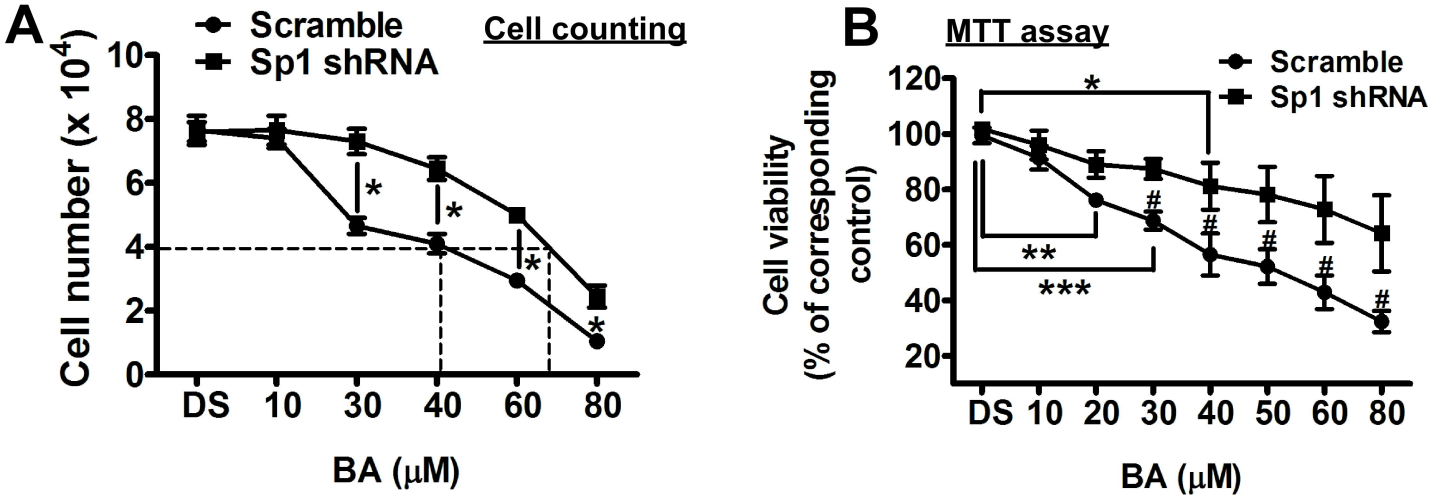
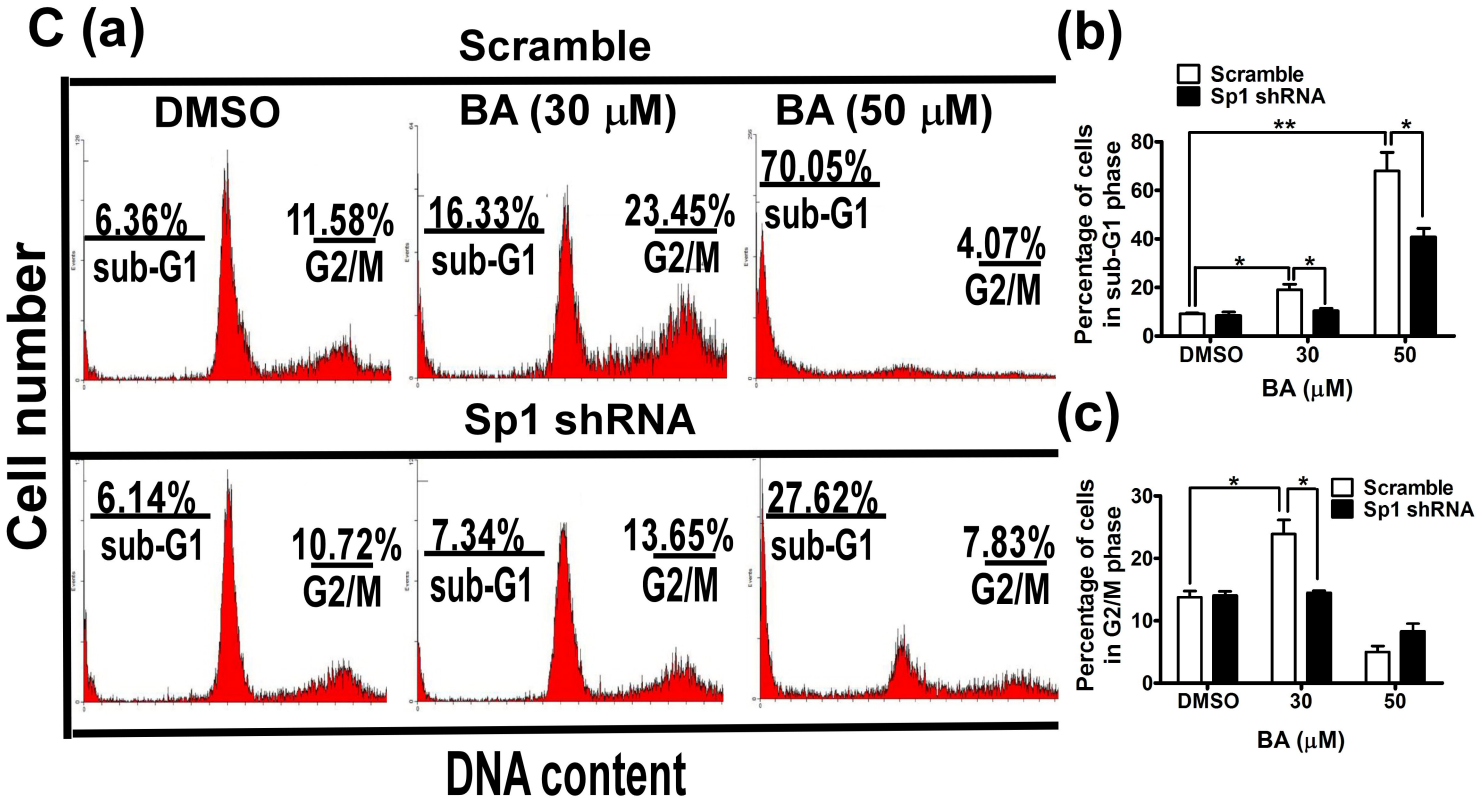
Figure 4





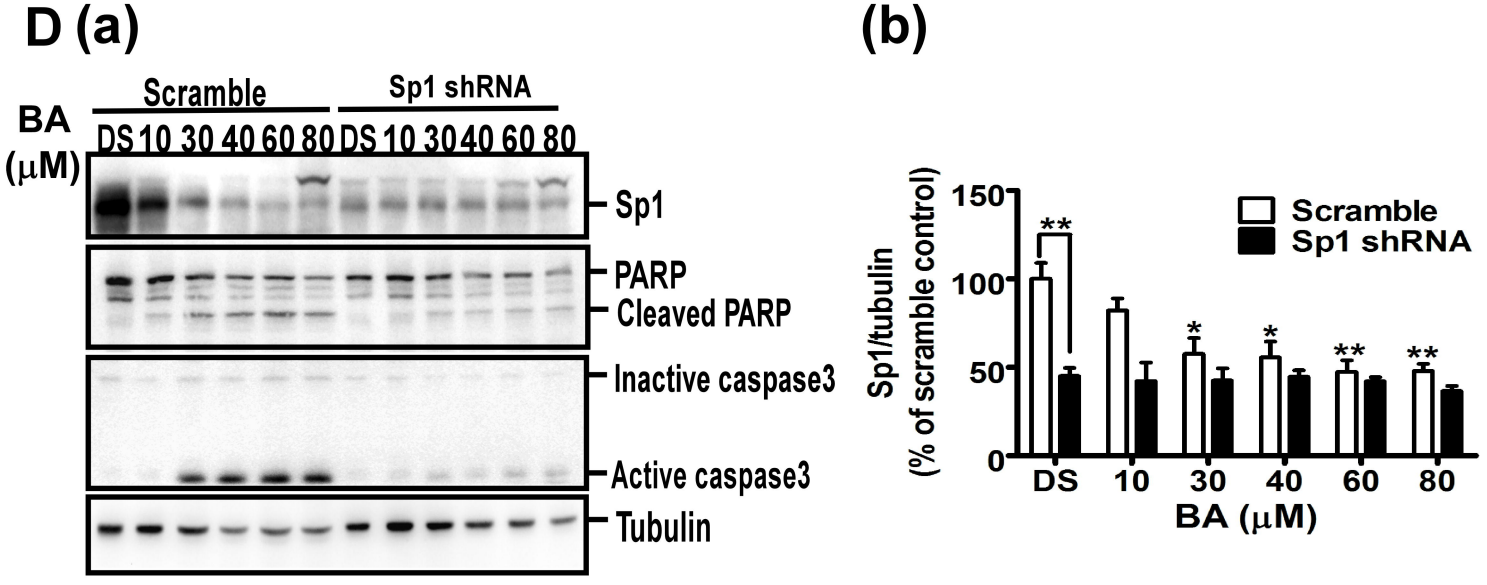


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Figure 5

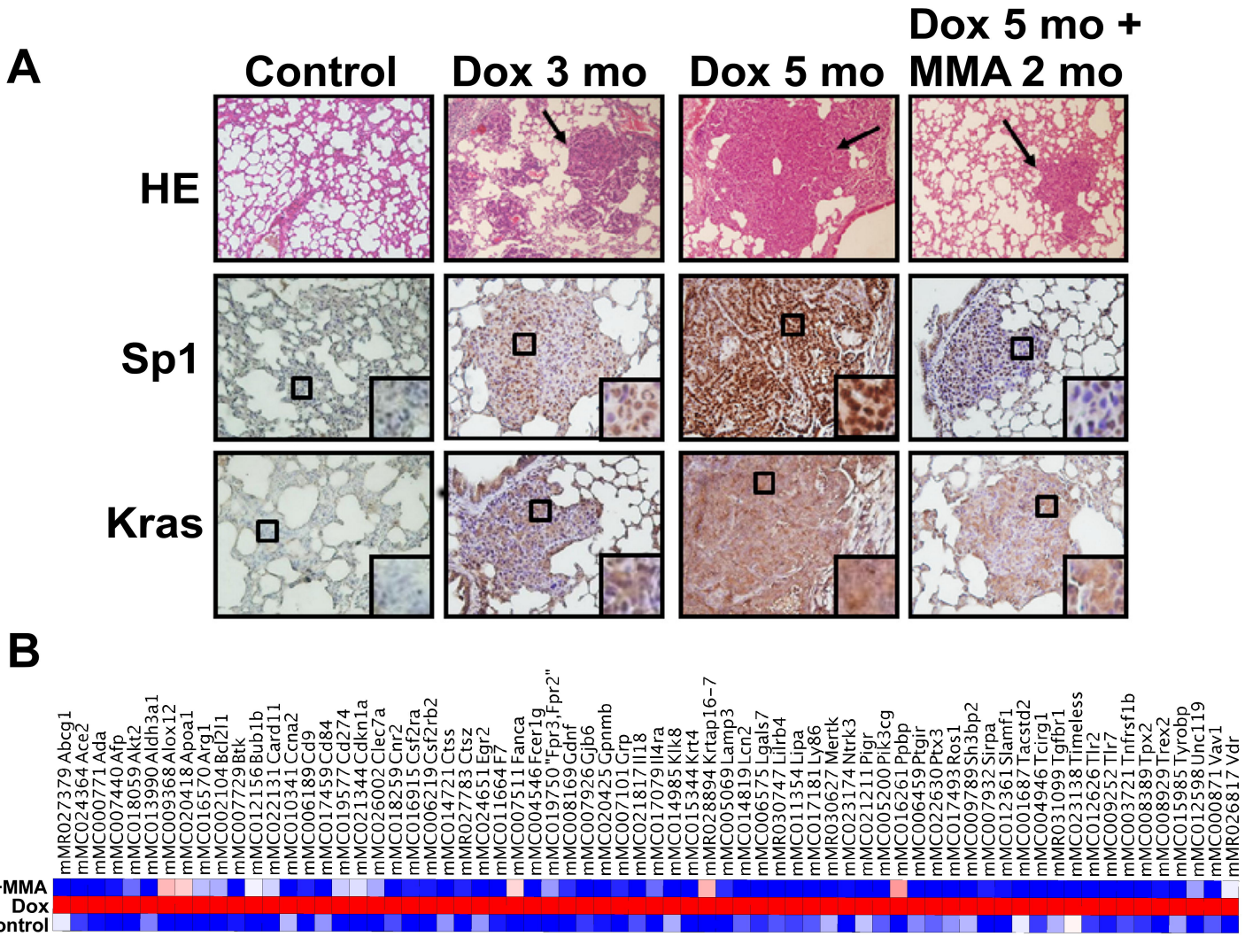

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Figure 6

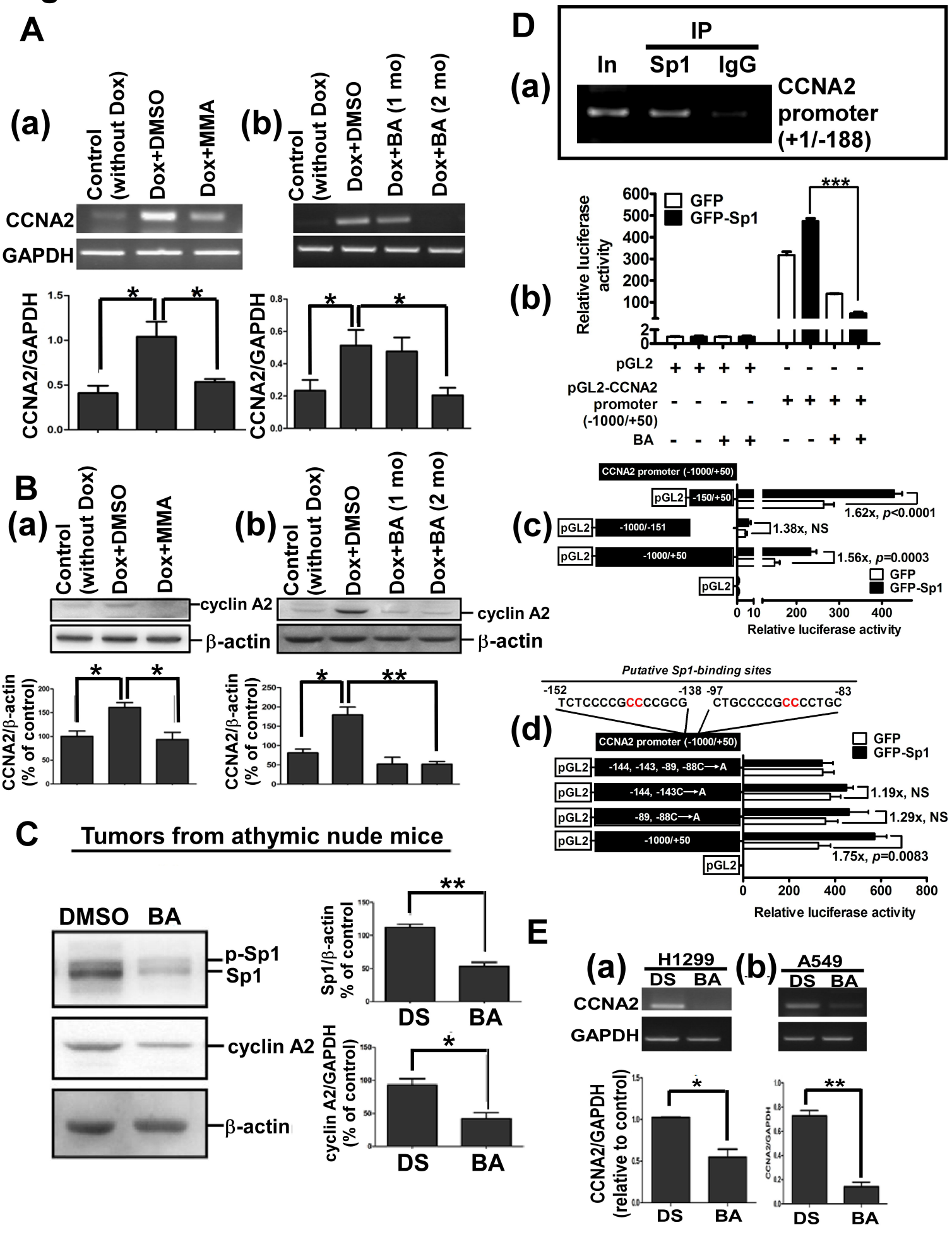

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Figure 7

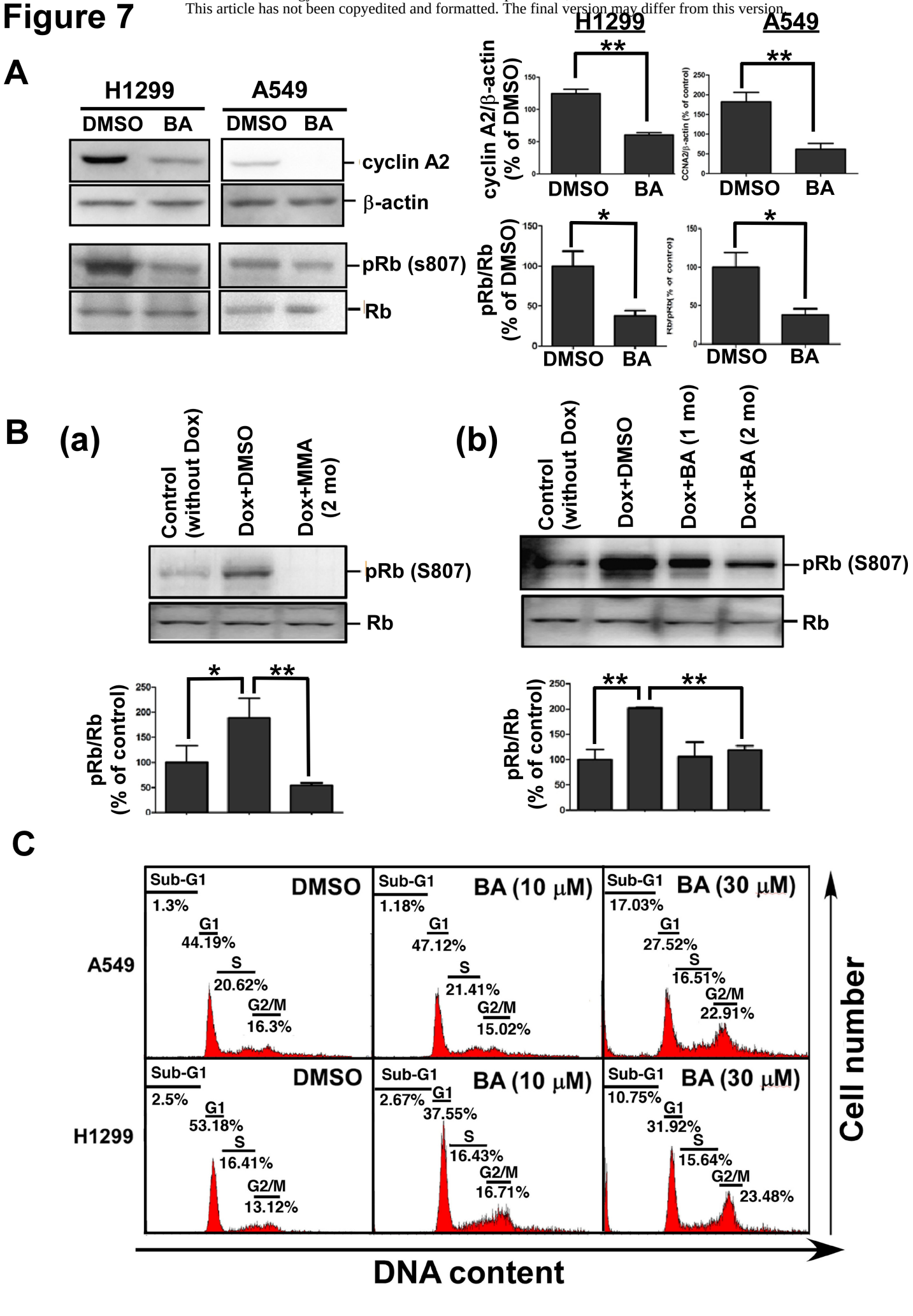

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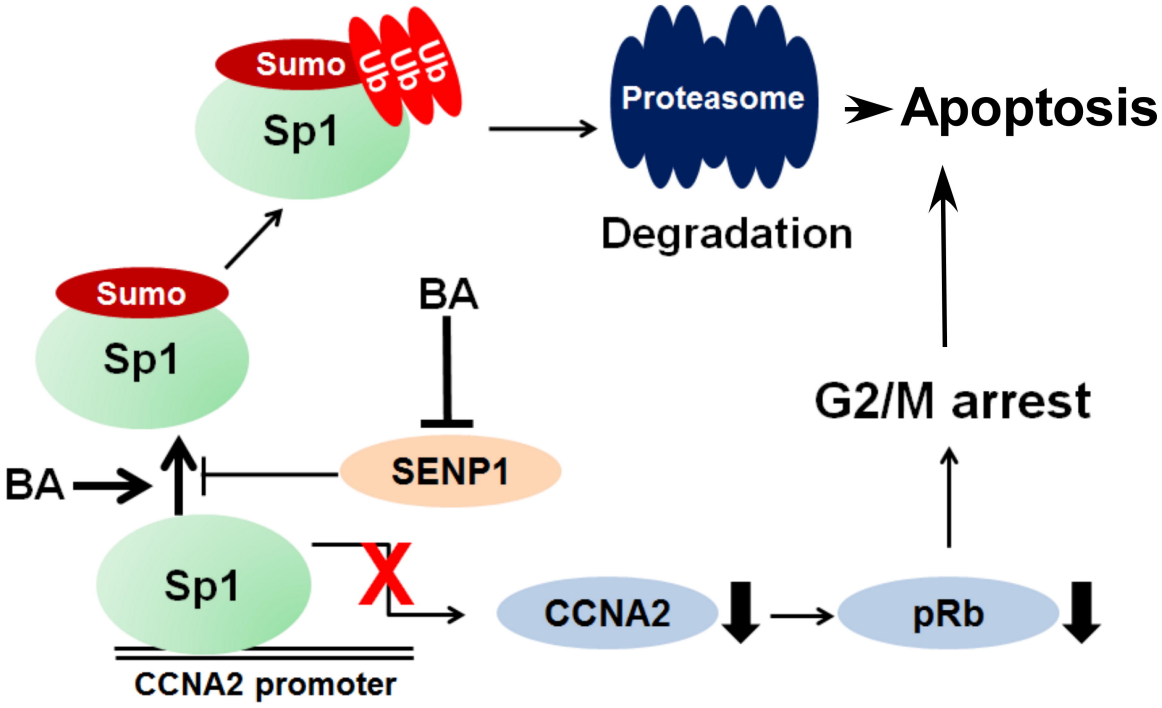
Figure 8



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Betulinic acid decreases Sp1 level via increasing the sumoylation of Sp1 to inhibit lung cancer growth

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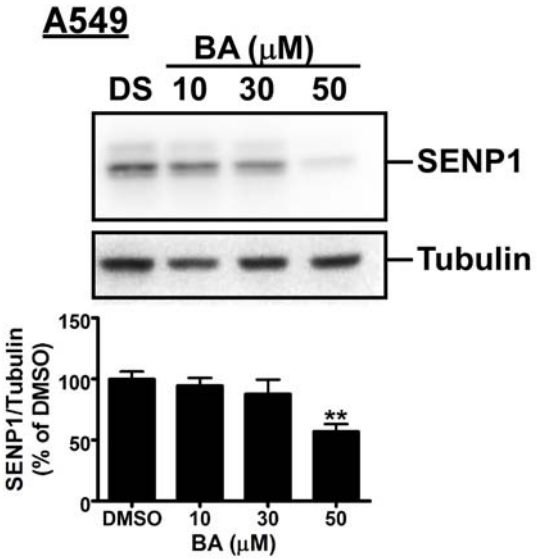

Supplementary Figure S1. Effect of BA on the protein level of SENP1 in A549 cells. Whole cell lysate was prepared for Western blotting using the antibodies against SENP1 and tubulin (Upper panel). Quantitative results are shown in the lower panel. Three experiments were conducted independently, and data are expressed as mean ± SEM. (**P<0.01)

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Supplementary Figure S2. Heatmap of microarray data indicating that 542 genes are possibly regulated by Sp1 in the growth of lung tumor. RNA of lungs from control, Dox-treated bitransgenic in the presence or absence of MMA was prepared for microarray analysis. The heatmap was made by the Genepattern website. A. 1-180. B. 181-360. C. 361-542

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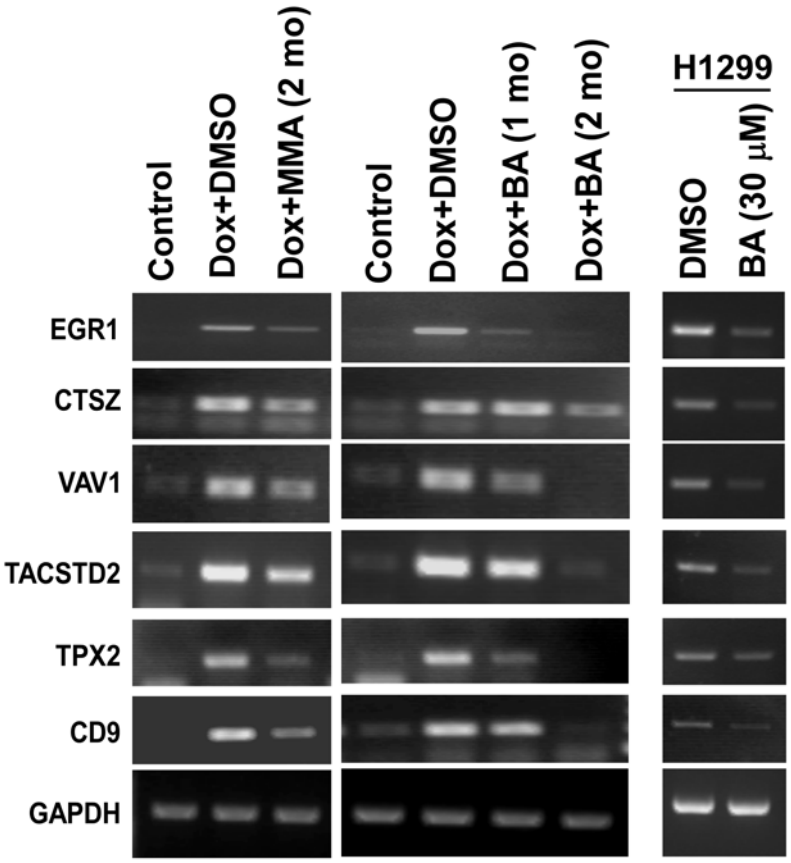

Supplementary Figure S3. Several important genes involved in cell proliferation are inhibited by BA or MMA treatment in Kras-driven lung tumors mouse model and in H1299 cells. Treatment of cells with BA was performed after treatment for 24 h.

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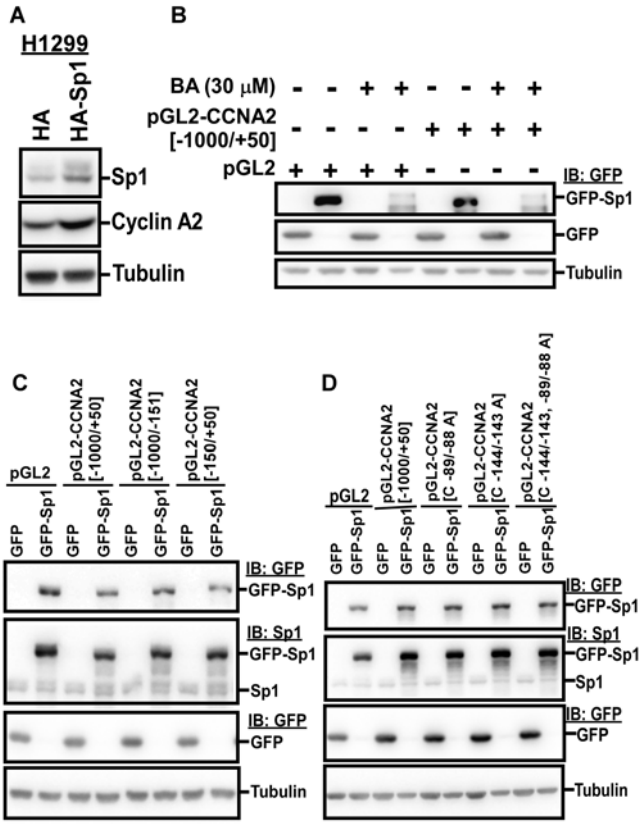

Supplementary Figure S4. Sp1 overexpression in H1299 cells for reporter assay. A. After transfection with HA or HA-Sp1 (2 μg) for 36 h, cell lysates were prepared for Western blotting using antibodies targeting Sp1, cyclin A2 and tubulin. B, C, D. The confirmation of GFP-Sp1 expression in cells used for luciferase activity assay. H1299 cells, which were transfected with 2 μg of the indicated 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 preparing whole cell lysates for Western blotting and luciferase activity assays (Fig. 6).

Supplementary Table S1. Microarray analyses indicating that 40 of well-known genes related to proliferation are regulated by Sp1 in lung tumor growth.

ID	Gene symbol	Gene name	References
mMR027379	Abcg1	ATP-binding cassette sub-family G member 1	Immunol. 2010 184:173-83 (Armstrong et al., 2010)
mMC024364	Ace2	angiotensin-converting enzyme 2	Am J Physiol Lung Cell Mol Physiol. 2009 297:L631-40 (Zhang et al., 2009)
mMC007440	Afp	alpha fetoprotein	Gene Ther. 2010 17:905-12 (Ma et al., 2010)
mMC018059	Akt2	beta serine/threonine-protein kinase	Genes Dev. 2003, 17:1352-65 (Peng et al., 2003)
mMC013990	Aldh3a1	aldehyde dehydrogenase 3 family, member A1	Chem Biol Interact. 2009,178:48-55 (Ucar et al., 2009)
mMC009368	Alox12	arachidonate 12-lipoxygenase	Exp Dermatol. 2009, 18:939-46 (Agarwal et al., 2009)
mMC000771	Ada	adenosine deaminase	J Immunol. 1995 155:2813-21 (Antonysamy et al., 1995)
mMC020418	Apoa1	apolipoprotein A1	Eur J Cancer Care. 2009, 18:625-35 (Wang et al., 2009)
mMC016570	Arg1	arginase 1	Am J Pathol. 2003 162:203-11 (Bruch-Gerharz et al., 2003)
mMC007729	Btk	bruton agammaglobulinemia tyrosine kinase	J Leukoc Biol. 2006, 79:852-9 (Schmidt et al., 2006)
mMC010341	Ccna2	cyclin A2	Curr Biol. 2003, 13:1775-85 (Berthet et al., 2003)

mMC017459	Cd84	cd84 antigen	Eur J Immunol. 2002, 32:1640-9 (Tangye et al., 2002)
mMC016915	Csf2ra	colony stimulating factor 2 receptor, alpha	Am J Hum Genet. 1993, 53:1146-53 (Milatovich et al., 1993)
mMC014721	Ctss	cathepsin S	Mol Cell Endocrinol. 2010, 323:277-81 (Ushizawa et al., 2010)
mMC024651	Egr2	early growth response 2	Cell Death Differ. 2009 16:782-9 (Boyle et al., 2009)
mMR027783	Ctsz	cathepsin Z	Proc Natl Acad Sci U S A. 2010, 107:2497-502 (Sevenich et al., 2010)
mMC006001	Erbb4	v-erb-a erythroblastic leukemia viral	Cancer Res. 1999, 59:5315-22 (Tang et al., 1999)
mMC011664	F7	coagulation factor VII	Oncol Rep. 2008, 19:1445-52 (Li et al., 2008)
mMC007511	Fanca	fanconi anemia, complementation group A	Biochem J. 2009, 422:161-70 (Ibanez et al., 2009)
mMC019750	Fpr2	formyl peptide receptor 2	FASEB J. 2011, 25:483-96 (Khau et al., 2011)
mMC008169	Gdnf	glial cell derived neurotrophic factor	Mol Cell Biochem. 2011, 347:95-101 (Liu et al., 2011)
mMC007926	Gjb6	gap junction protein, beta 6, 30kDa	Br J Dermatol. 2006, 155:844-6 (Lemaitre et al., 2006)
mMC020425	Gpnmb	glycoprotein (transmembrane) nmb	Cancer Res. 2010, 70:5778-87 (Tomihari et al., 2010)
mMC007101	Grp	gastrin releasing peptide	Am J Physiol Lung Cell Mol Physiol. 2004,

			286:L165-73 (Shan et al., 2004)
mMC021817	Il18	interleukin 18	Cancer Res. 2008 68:7237-45 (Zhong et al., 2008)
mMC017079	Il4r	interleukin 4 receptor	Carcinogenesis. 2010, 31:1010-7 (Koller et al., 2010)
mMC012846	Krt4	keratin 4	J Biol Chem. 1998, 273:23904-11 (Ness et al., 1998)
mMR027795	Lamp3	lysosomal-associated membrane protein 3	Am J Pathol. 2004, 164:861-71 (Salaun et al., 2004)
mMC014819	Lcn2	lipocalin 2	Mol Cell Biochem. 2011, 351:165-72 (Jin et al., 2011)
mMC011354	Lipa	lysosomal acid lipase A	Am J Pathol. 2005, 167:813-21 (Qu et al., 2009)
mMC030627	Mertk	c-mer proto-oncogene tyrosine kinase	Mol Cancer Ther. 2010, 9:1298-307 (Keating et al., 2010)
mMC023174	Ntrk3	neurotrophic tyrosine kinase, receptor, type 3	Proc Natl Acad Sci U S A. 2007, 104:7957-62 (Laneve et al., 2007)
mMC005200	Pik3cg	phosphoinositide-3-kinase, catalytic	EMBO J. 2004, 23:2586-96 (Baki et al., 2004)
mMC016261	Ppbp	pro-platelet basic protein	J Biol Chem. 2008 283:24155-66 (Nesmelova et al., 2008)
mMC012361	Slamf1	signaling lymphocytic activation molecule	Blood. 2002, 100:2899-907 (Howie et al., 2002)
mMC008389	Tpx2	microtubule-associated protein	Mol Cell. 1998, 2:417-25 (Wood et al., 2006)
mMC031099	Tgfbp1	transforming growth factor, beta 1	Biol Reprod. 2011,

			84:553-9 (Lafontaine et al., 2011)
mMR027071	Tnfrsf13b	tumor necrosis factor receptor superfamily	Dev Comp Immunol. 2010, 34:1199-208 (Zhang et al., 2010)
mMC012598	Unc119	unc-119 homolog	J Exp Med. 2004, 199:369-79 (Gorska et al., 2004)
mMC000871	Vav1	vav 1 oncogene	Hematol Oncol. 2010, 28:142-50 (Hollmann et al., 2010)

Supplementary references

Agarwal, S., Achari, C., Praveen, D., Roy, K. R., Reddy, G. V., and Reddanna, P. (2009). Inhibition of 12-LOX and COX-2 reduces the proliferation of human epidermoid carcinoma cells (A431) by modulating the ERK and PI3K-Akt signalling pathways. Exp Dermatol *18*, 939-946.

Antonysamy, M. A., Moticka, E. J., and Ramkumar, V. (1995). Adenosine acts as an endogenous modulator of IL-2-dependent proliferation of cytotoxic T lymphocytes. J Immunol *155*, 2813-2821.

Armstrong, A. J., Gebre, A. K., Parks, J. S., and Hedrick, C. C. (2010). ATP-binding cassette transporter G1 negatively regulates thymocyte and peripheral lymphocyte proliferation. J Immunol *184*, 173-183.

Baki, L., Shioi, J., Wen, P., Shao, Z., Schwarzman, A., Gama-Sosa, M., Neve, R., and Robakis, N. K. (2004). PS1 activates PI3K thus inhibiting GSK-3 activity and tau overphosphorylation: effects of FAD mutations. EMBO J *23*, 2586-2596.

Berthet, C., Aleem, E., Coppola, V., Tessarollo, L., and Kaldis, P. (2003). Cdk2 knockout mice are viable. Curr Biol *13*, 1775-1785.

Boyle, K. B., Hadaschik, D., Virtue, S., Cawthorn, W. P., Ridley, S. H., O'Rahilly, S., and Siddle, K. (2009). The transcription factors Egr1 and Egr2 have opposing influences on adipocyte differentiation. Cell Death Differ *16*, 782-789.

Bruch-Gerharz, D., Schnorr, O., Suschek, C., Beck, K. F., Pfeilschifter, J., Ruzicka, T.,

and Kolb-Bachofen, V. (2003). Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation. Am J Pathol *162*, 203-211.

Gorska, M. M., Stafford, S. J., Cen, O., Sur, S., and Alam, R. (2004). Unc119, a novel activator of Lck/Fyn, is essential for T cell activation. J Exp Med *199*, 369-379.

Hollmann, A., Aloyz, R., Baker, K., Dirnhofer, S., Owens, T., Sladek, R., and Tzankov, A. (2010). Vav-1 expression correlates with NFkappaB activation and CD40-mediated cell death in diffuse large B-cell lymphoma cell lines. Hematol Oncol *28*, 142-150.

Howie, D., Okamoto, S., Rietdijk, S., Clarke, K., Wang, N., Gullo, C., Bruggeman, J. P., Manning, S., Coyle, A. J., Greenfield, E., Kuchroo, V., and Terhorst, C. (2002). The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. Blood *100*, 2899-2907.

Ibanez, A., Rio, P., Casado, J. A., Bueren, J. A., Fernandez-Luna, J. L., and Pipaon, C. (2009). Elevated levels of IL-1beta in Fanconi anaemia group A patients due to a constitutively active phosphoinositide 3-kinase-Akt pathway are capable of promoting tumour cell proliferation. Biochem J *422*, 161-170.

Jin, D., Zhang, Y., and Chen, X. (2011). Lipocalin 2 deficiency inhibits cell proliferation, autophagy, and mitochondrial biogenesis in mouse embryonic cells.

Mol Cell Biochem *351*, 165-172.

Keating, A. K., Kim, G. K., Jones, A. E., Donson, A. M., Ware, K., Mulcahy, J. M., Salzberg, D. B., Foreman, N. K., Liang, X., Thorburn, A., and Graham, D. K. (2010). Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. Mol Cancer Ther *9*, 1298-1307.

Khau, T., Langenbach, S. Y., Schuliga, M., Harris, T., Johnstone, C. N., Anderson, R. L., and Stewart, A. G. (2011). Annexin-1 signals mitogen-stimulated breast tumor cell proliferation by activation of the formyl peptide receptors (FPRs) 1 and 2. FASEB J *25*, 483-496.

Koller, F. L., Hwang, D. G., Dozier, E. A., and Fingleton, B. (2010). Epithelial interleukin-4 receptor expression promotes colon tumor growth. Carcinogenesis *31*, 1010-1017.

Lafontaine, L., Chaudhry, P., Lafleur, M. J., Van Themsche, C., Soares, M. J., and Asselin, E. (2011). Transforming growth factor Beta regulates proliferation and invasion of rat placental cell lines. Biol Reprod *84*, 553-559.

Laneve, P., Di Marcotullio, L., Gioia, U., Fiori, M. E., Ferretti, E., Gulino, A., Bozzoni, I., and Caffarelli, E. (2007). The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of

human neuroblastoma cells. Proc Natl Acad Sci U S A *104*, 7957-7962.

Lemaitre, G., Sivan, V., Lamartine, J., Cosset, J. M., Cavelier-Balloy, B., Salomon, D., Waksman, G., and Martin, M. T. (2006). Connexin 30, a new marker of hyperproliferative epidermis. Br J Dermatol *155*, 844-846.

Li, Y., Ishiyama, S., Matsueda, S., Tsuda, N., and Ioannides, C. G. (2008). HER-2 peptides p776 and F7, N-terminal-linked with Ii-Key tetramer (LRMK) help the proliferation of E75-TCR+ cells: The dependency of help on the side chains of LRMK-extended peptide pointed towards the T cell receptor. Oncol Rep *19*, 1445-1452.

Liu, H., Ma, Q., and Li, J. (2011). High glucose promotes cell proliferation and enhances GDNF and RET expression in pancreatic cancer cells. Mol Cell Biochem *347*, 95-101.

Ma, S. H., Chen, G. G., Yip, J., and Lai, P. B. (2010). Therapeutic effect of alpha-fetoprotein promoter-mediated tBid and chemotherapeutic agents on orthotopic liver tumor in mice. Gene Ther *17*, 905-912.

Milatovich, A., Kitamura, T., Miyajima, A., and Francke, U. (1993). Gene for the alpha-subunit of the human interleukin-3 receptor (IL3RA) localized to the X-Y pseudoautosomal region. Am J Hum Genet *53*, 1146-1153.

Nesmelova, I. V., Sham, Y., Gao, J., and Mayo, K. H. (2008). CXC and CC

chemokines form mixed heterodimers: association free energies from molecular dynamics simulations and experimental correlations. J Biol Chem *283*, 24155-24166.

Ness, S. L., Edelmann, W., Jenkins, T. D., Liedtke, W., Rustgi, A. K., and Kucherlapati, R. (1998). Mouse keratin 4 is necessary for internal epithelial integrity. J Biol Chem *273*, 23904-23911.

Peng, X. D., Xu, P. Z., Chen, M. L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararajan, D., Chen, W. S., Crawford, S. E., Coleman, K. G., and Hay, N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev *17*, 1352-1365.

Qu, P., Du, H., Wilkes, D. S., and Yan, C. (2009). Critical roles of lysosomal acid lipase in T cell development and function. Am J Pathol *174*, 944-956.

Salaun, B., de Saint-Vis, B., Pacheco, N., Pacheco, Y., Riesler, A., Isaac, S., Leroux, C., Clair-Moninot, V., Pin, J. J., Griffith, J., Treilleux, I., Goddard, S., Davoust, J., Kleijmeer, M., and Lebecque, S. (2004). CD208/dendritic cell-lysosomal associated membrane protein is a marker of normal and transformed type II pneumocytes. Am J Pathol *164*, 861-871.

Schmidt, N. W., Mayo, L. D., Donner, D. B., and Kaplan, M. H. (2006). p53 regulates

Btk-dependent B cell proliferation but not differentiation. J Leukoc Biol *79*, 852-859.

Sevenich, L., Schurigt, U., Sachse, K., Gajda, M., Werner, F., Muller, S., Vasiljeva, O., Schwinde, A., Klemm, N., Deussing, J., Peters, C., and Reinheckel, T. (2010). Synergistic antitumor effects of combined cathepsin B and cathepsin Z deficiencies on breast cancer progression and metastasis in mice. Proc Natl Acad Sci U S A *107*, 2497-2502.

Shan, L., Emanuel, R. L., Dewald, D., Torday, J. S., Asokanathan, N., Wada, K., Wada, E., and Sunday, M. E. (2004). Bombesin-like peptide receptor gene expression, regulation, and function in fetal murine lung. Am J Physiol Lung Cell Mol Physiol *286*, L165-173.

Tang, C. K., Concepcion, X. Z., Milan, M., Gong, X., Montgomery, E., and Lippman, M. E. (1999). Ribozyme-mediated down-regulation of ErbB-4 in estrogen receptor-positive breast cancer cells inhibits proliferation both in vitro and in vivo. Cancer Res *59*, 5315-5322.

Tangye, S. G., van de Weerdt, B. C., Avery, D. T., and Hodgkin, P. D. (2002). CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2. Eur J Immunol *32*, 1640-1649.

Tomihari, M., Chung, J. S., Akiyoshi, H., Cruz, P. D., Jr., and Ariizumi, K. (2010).

DC-HIL/glycoprotein Nmb promotes growth of melanoma in mice by inhibiting the activation of tumor-reactive T cells. Cancer Res *70*, 5778-5787.

Ucar, D., Cogle, C. R., Zucali, J. R., Ostmark, B., Scott, E. W., Zori, R., Gray, B. A., and Moreb, J. S. (2009). Aldehyde dehydrogenase activity as a functional marker for lung cancer. Chem Biol Interact *178*, 48-55.

Ushizawa, K., Takahashi, T., Hosoe, M., Kizaki, K., and Hashizume, K. (2010). Cleaved bovine prolactin-related protein-I stimulates vascular endothelial cell proliferation. Mol Cell Endocrinol *323*, 277-281.

Wang, X., Dai, S., Zhang, Z., Liu, L., Wang, J., Xiao, X., He, D., and Liu, B. (2009). Characterization of apolipoprotein A-I as a potential biomarker for cholangiocarcinoma. Eur J Cancer Care (Engl) *18*, 625-635.

Wood, L. D., Calhoun, E. S., Silliman, N., Ptak, J., Szabo, S., Powell, S. M., Riggins, G. J., Wang, T. L., Yan, H., Gazdar, A., Kern, S. E., Pennacchio, L., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. (2006). Somatic mutations of GUCY2F, EPHA3, and NTRK3 in human cancers. Hum Mutat *27*, 1060-1061.

Zhang, J. X., Ma, H. W., Sang, M., Hu, Y. S., Liang, Z. N., Ai, H. X., Zhang, J., Cui, X. W., and Zhang, S. Q. (2010). Molecular structure, expression, cell and tissue distribution, immune evolution and cell proliferation of the gene encoding bovine (Bos taurus) TNFSF13 (APRIL). Dev Comp Immunol *34*, 1199-1208.

Zhang, R., Wu, Y., Zhao, M., Liu, C., Zhou, L., Shen, S., Liao, S., Yang, K., Li, Q., and Wan, H. (2009). Role of HIF-1alpha in the regulation ACE and ACE2 expression in hypoxic human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol *297*, L631-640.

Zhong, L., Roybal, J., Chaerkady, R., Zhang, W., Choi, K., Alvarez, C. A., Tran, H., Creighton, C. J., Yan, S., Strieter, R. M.,Pandey, A., and Kurie, J. M. (2008). Identification of secreted proteins that mediate cell-cell interactions in an in vitro model of the lung cancer microenvironment. Cancer Res *68*, 7237-7245.