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SIRT1 Inactivation Evokes Antitumor Activities in NSCLC through the Tumor Suppressor p27

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Abstract

P27^{Kip1} (CDKN1B) regulates cellular proliferation and senescence, and p27^{Kip1} deficiency in cancer is strongly correlated with poor prognosis of multiple cancer types. Understanding the mechanism of p27^{Kip1} loss in cancer and the consequences of restoring p27^{Kip1} levels is therefore critical for effective management during therapy. Here, SIRT1, a class III histone deacetylase (HDAC), is identified as an important regulator of p27^{Kip1} expression. Mechanistically, SIRT1 reduces p27^{Kip1} expression by decreasing p27^{Kip1} protein stability through the ubiquitin-proteasome pathway. In addition, SIRT1 silencing suppresses NSCLC proliferation and induces senescence in a p27^{Kip1}-dependent manner. Furthermore, SIRT1 silencing dramatically suppresses tumor formation and proliferation in two distinct NSCLC xenograft mouse models. Collectively, these data not only demonstrate that SIRT1 is an important regulator of p27^{Kip1} but that SIRT inhibition induces senescence and anti-growth potential in lung cancer *in vivo*.

Implications—SIRT1 is a key regulator of p27 protein levels and SIRT1 inhibition is a viable strategy for NSCLC therapy by means of p27 reactivation.

Keywords

SIRT1; p27^{kip1}; protein stability; non-small cell lung cancer; cell senescence

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The authors declare no conflict of interest.

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Introduction

The $p27^{kip1}$ protein is a member of a family of CDK inhibitors (CDKIs) and plays an important role in multiple fundamental cellular processes and in the pathogenesis of human malignancies, with roles in cell proliferation, cell senescence, cell differentiation, migration, and invasion [1]. $P27^{kip1}$ is frequently inactivated in human cancers through accelerated proteolysis, and reduced $p27^{kip1}$ expression has been shown to be associated with poor prognosis in a variety of human malignancies [2], whereas restoration of $p27^{kip1}$ levels can inhibit tumor growth and progression [3, 4]. Although it has been shown that $p27^{kip1}$ expression is regulated primarily at the posttranscriptional level [5–7], the mechanism of $p27^{kip1}$ reduction in cancer cells is still unclear. Identifying new pathways and molecules which regulate p27 expression is therefore critical for developing cancer treatments through restoration of p27 levels.

SIRT1 is a nicotinamide adenine dinucleotide (NAD)-dependent non-histone and histone deacetylase that has been reported to play an important role in a variety of physiological processes including aging, DNA repair, apoptosis and cell senescence [8–10]. SIRT1 overexpression has been shown to be correlated with advanced stages or poor prognosis in many cancer types [11–13], and recently there is growing evidence strongly implicating an important role for SIRT1 in cancer growth and progression [14–19]. In addition, several cellular proteins, such as p53, Foxo3, and E2F, have been identified to play an important role in SIRT1-mediated cancer cell survival [10]. P53 has been shown as an important target of SIRT1 to suppress apoptosis [20]; however, many studies show that SIRT1 regulates cancer cell growth regardless of p53 status [21–23]. The specific mechanism of SIRT1 regulating cancer cell growth and survival is largely unknown.

Recently, several studies have indicated that $p27^{kip1}$ may be a downstream target of SIRT1 [24, 25]. In particular, it has been established that $p27^{kip1}$ downregulation is consistently associated with poor prognosis in non-small cell lung cancer (NSCLC) [26–28] and similarly, SIRT1 overexpression is significantly associated with unfavorable clinicopathological factors, including high pathological T-stage and lymph node metastasis in NSCLC [29, 30]. Studies have also shown that SIRT1 inhibition suppresses NSCLC growth [25, 31–33], and SIRT1 knock-in in transgenic mice facilitates endothelial cell branching to increase vessel density and lung tumor growth [31]. In addition, SIRT1 activation sensitizes lung cancer cells to the antitumor activity of cisplatin [34]; in contrast, several other studies have shown that SIRT1 inhibition sensitizes lung and several other cancer types to the antitumor activity of cisplatin [19, 23, 30, 35–37]. Despite these findings, it is largely unknown for the role of SIRT1 in NSCLC tumor growth and progression, and the relationship between SIRT1 and $p27^{kip1}$ in the control of NSCLC growth and progression has not been established.

This study demonstrates for the first time that SIRT1 is a critical regulator of $p27^{kip1}$ reduction in NSCLC; it is shown that SIRT1 promotes $p27^{kip1}$ degradation through the ubiquitin-proteolysis pathway. We find that SIRT1 silencing or inhibition suppresses NSCLC proliferation and induces senescence in NSCLC cells, and we demonstrate that $p27^{kip1}$ is required for SIRT1-silencing-mediated cell senescence. Furthermore, we

demonstrate that SIRT1 silencing dramatically suppresses NSCLC tumor formation in xenograft mouse models. Thus, we discover SIRT1 as a new regulator of $p27^{kip1}$ degradation and identify a novel route of senescence induction in NSCLC cancer cells via the SIRT1-$p27^{kip1}$ pathway. Our results suggest the SIRT1-$p27^{kip1}$ axis plays an important role in controlling NSCLC growth and is a potential target for NSCLC treatment.

Materials and methods

Cell lines, plasmids and antibodies

NSCLC cell lines, including H460, H1299, A549, H1703, H1975, HCC827, and H1993 cells, were obtained from the American Type Culture Collection (Manasses, Virginia). H1299 and H1703 cells were maintained in 1× RPMI 1640 plus 10% FBS, and H460, A549, H1975, HCC827, and H1993 cells were maintained in 1× DMEM plus 10% FBS. The human SIRT1 expression vector pcDNA3.1-SIRT1 and the pSUPER.retro.puro-SIRT1 were generated or acquired as previously described [38]. Antibody p27 (3686) and Ki67 (9027) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to SIRT1 (sc-74504 & 19857) and p27 (sc-1641) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to β–actin was purchased from Sigma-Aldrich (St. Louis, MO).

Generation of SIRT1 or $p27^{kip1}$ knockdown in a NSCLC cell line

Establishment of Stable SIRT1-Knockdown Cell Lines—The Phoenix packaging cell line was transfected with shRNA expression plasmids pSUPER.retro.puro-SIRT1#1 (5′-GATGAAGTTGACCTCCTCA-3′) or pSUPER.retro.puro-SIRT1#2 (5-CTTGTACGACGAAGACGA-3), or pSUPER.retro.puro-non-targeting shRNA control separately, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 hours, the medium containing retrovirus was collected, filtered, treated with polybrene, and transferred to H1299, H460, and A549 cell cultures. Infected cells were selected with puromycin and the stably-infected colonies were pooled.

P27 knockdown—Two p27 siRNA (On-Target plus SMART pool, #L-003472-00-005, Thermo Scientific Dharmacon, and SignalSilence® p27 Kip1 siRNA I, #12324, Cell Signaling Technology) or non-targeting siRNA control (# D-001810-01-20, Thermo Scientific Dharmacon) were transfected at 25nM with DharmaFECT transfection reagent to NSCLC for 72 hours, and the p27 levels were determined by immunoblot using p27 antibody.

Qualitative reverse transcription real-time PCR (qRT-PCR) analysis

The qRT-PCR method has been previously described in detail [17]. The primers used were: p27 forward: CGCCATATTGGGCCACTAA; p27 reverse: CGCAGAGCCGTGAGC-AA; β-actin forward: AAGGCCAACCGC-GAGAAGAT; β-actin reverse: CACAGCC-TGGATAGCAACGTACA. PCR reactions were performed in triplicate. The mRNA levels were normalized to β-actin. The specificities of the RT-PCR products were monitored by melt curve analysis.

Immunoblotting

Cells were lysed with 1% Nonidet P-40 lysis buffer (1% NP40, 150 mm NaCl, 50 mM Tris-HCl pH8.0, EDTA 5mM, Na3VO4 1mM) with protease inhibitor cocktail tablets (Cat#14586500, Roche Diagnostics). Protein samples were subsequently separated on a 12% or 8% SDS PAGE gel and analyzed with anti-SIRT1 (sc-74504, 1000×), anti-p27 (sc-1641, 500×) and anti-β-actin (A1987, 5000×) antibodies.

In vivo experiments

The mouse work was performed at the Animal Core Facilities of the Boston University School of Medicine after approval by the Institutional Animal Care and Use Committee (IACUC). Briefly, SIRT1 (shRNA)-silenced or control (non-targeting shRNA) H1299 cells or H460 cells were made with specific SIRT1 shRNA expression vector and non-specific shRNA control vector. The SIRT1-silenced and shRNA control H1299 or H460 cells were diluted in 100 μl serum-free medium. The shRNA control cells were injected s.c. into the left flank of nude/nude male mice and the SIRT1-silenced cells were injected s.c. into the right flank of nude/nude male mice (Charles River Laboratories). The mice were observed every 3 days for the presence of tumors and the tumors were measured weekly with calipers. Each group contained four mice, and two groups of H1299 cells and two groups of H460 cells with independent SIRT1 silencing were studied. The tumor size was calculated based on the formula: 0.52×L×W×H.

SA-β-Gal analysis

The SIRT1-silenced and shRNA-control NSCLC cell lines were seeded in 6 well plates (5×10^5 cells per well). After 3 days of culture, the cells were washed three times with 1× PBS, and stained with X-gal solution (25 mg/ml X-gal in PBS buffer) overnight. The cells were observed under a microscope. Six or more separate fields were counted for each condition and the percentage of SA-β-Gal positive cells was calculated based on three independent analyses.

Ki67 Staining of Frozen Tissue Sections

At the endpoint of the *in vivo* mouse study, the tumors were removed and fixed in 4% paraformaldehyde overnight at 4°C, then changed to 15% sucrose for 4hr at RT. The tissue was embedded completely in OCT compound and kept at −80°C prior to cryostat sectioning. Cryostat sections were cut at 40 μm, mounted on gelatin-coated histological slides, and kept at −80°C. The tissue section was warmed at 37°C for 40 minutes upon removal of the tissues section from the freezer, and washed twice with PBS. The sections were further incubated for 3hr at RT with Ki67 antibodies (1:400, CST), then incubated with a biotin-conjugated secondary antibody followed by streptavidin-horseradish peroxidase with 3'-3'-diaminebenzine (DAB) as the substrate for immunodetection. Counter-staining was performed with hematoxylin. The detailed method followed the instructions of the Histostain Plus IHC Detection Kit (Invitrogen, #859673).

Results

SIRT1 negatively regulates p27^{Kip1} expression

It is well-established that lower levels of p27^{kip1} correlate with poor prognosis of NSCLC [26, 39–41] and overexpression of SIRT1 also correlates with unfavorable clinicopathological factors in NSCLC [29, 30]. To study whether SIRT1 plays a role to lower p27^{kip1} expression in NSCLC cells, we treated NSCLC cells with various SIRT1 inhibitors to determine whether SIRT1 inhibition upregulates p27^{Kip1} expression. SIRT1 inhibition by SIRT1 inhibitors, including Ex527, Sirtinol, and Nictotinamide, was found to greatly upregulate p27^{Kip1} level in NSCLC cells (Fig. 1A& 1B). To more specifically study the role of SIRT1 in controlling p27^{Kip1} levels, we knocked down SIRT1 in SIRT1-overexpressing NSCLC cells using SIRT1 shRNA to study the effect of SIRT1 silencing on p27^{Kip1} expression. Consistent with the data generated by SIRT1 inhibitor treatment, SIRT1 silencing by highly specific genetic methods greatly upregulates p27^{Kip1} expression (Fig. 1C & 1D). To further study the mechanism by which SIRT1 regulates p27^{Kip1} expression, we performed qRT-PCR analysis to study whether SIRT1 regulates p27^{Kip1} expression through regulating p27^{Kip1} transcription. The p27^{Kip1} mRNA level was unaffected by SIRT1 silencing (see Supporting data Fig. S1). This data suggests that SIRT1 plays an important role in p27^{kip1} downregulation in NSCLC cells, and that SIRT1-mediated regulation of p27^{Kip1} protein expression does not take place at the level of transcription or alter mRNA stability.

SIRT1 regulates p27^{kip1} stability through the ubiquitin-proteolysis pathway

P27^{Kip1} proteolysis plays a major role in controlling p27^{kip1} levels [7]. We therefore sought to further determine whether increases in p27^{Kip1} protein levels by SIRT1 silencing is due to changes in p27^{Kip1} protein stability. The SIRT1 shRNA-silenced or non-targeting shRNA-control NSCLC cells were treated with cycloheximide (CHX) to inhibit protein synthesis, and the stability of p27^{Kip1} protein between SIRT1-silenced and shRNA-control cells was compared. The results show that the half-life of p27^{Kip1} protein was dramatically increased in SIRT1-silenced cells compared to that in shRNA-control cells (Fig. 2A& 2B). The p27 protein half-life was found to increase from 3.5hr to 17hr after SIRT1 silencing in H1299 cells (Fig. 2A), and to increase from 3.5hr to 15hr after SIRT1 knockdown in H460 cells (Fig. 2B). This data suggests that it is the alteration of p27^{Kip1} protein stability which is the major cause for increased levels of p27^{Kip1} protein expression in SIRT1-silenced cells.

Previous studies have shown that the ubiquitin-proteolysis pathway plays an important role in regulating p27^{Kip1} protein levels [1]. After finding that SIRT1 regulates p27^{kip1} stability, we next determined whether blocking proteasomal activity would reduce SIRT1-mediated p27^{Kip1} downregulation. We ectopically expressed SIRT1 in H1299 cells, which were then treated with MG132 to block proteasome activity, and determined the effects of SIRT1 overexpression on p27^{Kip1} expression. While SIRT1 overexpression reduced p27^{kip1} levels, blockade of proteasome activity by MG132 produced a dramatic increase in the p27^{Kip1} protein levels in SIRT1 ectopically-expressed H1299 cells (Fig. 2C). This result suggests that blocking proteasome activity by MG132 suppresses SIRT1-mediated p27^{kip1}

degradation, which is consistent with our other data that shows SIRT1 regulates $p27^{kip1}$ degradation through the ubiquitin-proteasome pathway.

SIRT1 inhibition/knockdown suppresses NSCLC proliferation

The role of $p27^{kip1}$ in suppressing cell proliferation and inducing cell senescence is well known [1]. After finding the importance of SIRT1 in controlling $p27^{kip1}$ expression and stability, we further studied the role of SIRT1 in controlling NSCLC proliferation. We treated a panel of NSCLC cell lines with SIRT1 inhibitors Sirtinol, and found that SIRT1 inhibition significantly decreases NSCLC proliferation in a dose-dependent manner (Fig. 3A). To validate the SIRT1 specificity of these results, we performed SIRT1 silencing by shRNA to study the effect of SIRT1 silencing on NSCLC proliferation. Responses similar to those of the SIRT1 inhibitor treatment experiment were seen, in that NSCLC proliferation was significantly suppressed by SIRT1 silencing (Fig. 3B).

SIRT1 silencing induces cell senescence through the $p27^{kip1}$ pathway

To further study the mechanism by which SIRT1 suppresses NSCLC proliferation, we first performed a SA-β-Gal analysis in SIRT1-silenced and shRNA-control NSCLC cells, including H460, H1299, and A549 cells. We detected much stronger SA-β-Gal positivity in SIRT1-silenced cells than in shRNA-control cells, suggesting that SIRT1 silencing induces cell senescence in NSCLC cells (Fig. 4A). We also assessed whether SIRT1 silencing induces apoptosis by analyzing PARP cleavage. We observed no obvious difference in PARP cleavage between SIRT1-silenced and shRNA-control NSCLC cells (see supporting data in Fig. S2). These results suggest that cell senescence is an important mechanism by which SIRT1 silencing suppresses NSCLC proliferation.

$P27^{kip1}$ has been shown to play an important role in the induction of cell senescence [42]. To clearly evaluate $p27^{Kip1}$ as a requirement in SIRT1 silencing-mediated cell senescence, we evaluated the effects of depleting $p27^{kip1}$ on the ability of SIRT1 inactivation to induce senescence. To this end, we reduced the levels of $p27^{Kip1}$ in SIRT1-silenced NSCLC cells using two independent $p27^{Kip1}$-specific siRNA to study whether $p27^{Kip1}$ depletion would rescue the cell senescent phenotype induced by SIRT1 silencing. $P27^{kip1}$ deletion in SIRT1-silenced cells greatly reduced the SA-beta-Gal staining compared to non-targeting siRNA-transfected control cells (Figs. 4B). This result demonstrates that $p27^{kip1}$ is required for SIRT1-silencing mediated cell senescence.

SIRT1 silencing exhibits strong anti-tumor activity *in vivo*

Since we have shown that SIRT1 inhibition or silencing induces cell senescence and inhibits NSCLC cell proliferation *in vitro*, we then examined the effect of SIRT1 silencing on suppression of tumor growth *in vivo*. We used H460 and H1299 NSCLC cell lines stably-expressing either SIRT1-specific shRNA or a non-targeting control shRNA to determine their respective tendencies for tumor formation and growth in xenograft mouse models. Both the SIRT1-silenced and the shRNA-control cells were injected into the same immunocompromised nu/nu mice to exclude individual variation (Fig. 5A & D). We performed two independent tumor injections with H1299 cells and two independent tumor injections with H460 cells, in which the SIRT1-silenced tumor cells were acquired using

two specific SIRT1 shRNA expression vectors. We injected 4 mice each for the shRNA control group and for the SIRT1 silenced group. The results show that while 4 out of 4 H460 shRNA control cells formed tumors, only 1 out of 4 H460 SIRT1-silenced H460 mouse cells formed tumors; additionally, the tumorigenic growth of SIRT1-silenced cells was significantly retarded when compared with that of shRNA-control cancer cells (Fig. 5B). Similar results were obtained from another independent H460 group: while 4 out of 4 control injected mice formed tumors, only 2 out of 4 SIRT1-silenced mouse cells formed tumors, and the tumor growth in the SIRT1-silenced cells was greatly reduced (Fig. 5C). In parallel with these H460 studies, we also performed two independent experiments with SIRT1-silenced and shRNA-control H1299 cells. The tumor formation rate of control cells was 8 out of 8 (100%), while the rate for SIRT1-silenced cells was 2 out of 8, and the tumor volume of SIRT1 knockdown tumors was greatly reduced compared to that of shRNA control H1299 cells (Figs. 5D, E & F). This data demonstrates that SIRT1 silencing has a dramatic effect on inhibiting lung tumor formation and growth *in vivo* in xenograft mouse models. At the endpoint of the mouse model experiment, we isolated the tumors and analyzed SIRT1 and $p27^{kip1}$ levels by immunoblot analysis. The data shows that small tumor size correlated with SIRT1 silencing and $p27^{kip1}$ upregulation (Fig. 5G & H). We also show that SIRT1-silenced tumors had a lower proliferating cell fraction, as determined by IHC detection of Ki67 (Fig. 5I). Collectively, these results demonstrate that SIRT1 knockdown greatly reduces tumor formation and growth in NSCLC cells, and that $p27^{kip1}$ is an important downstream target of SIRT1.

Discussion

This study presents three new findings with important therapeutic implications. First, SIRT1 is a regulator of $p27^{kip1}$ expression by controlling $p27^{kip1}$ stability through the ubiquitin-proteolysis pathway, and SIRT1 inhibition can upregulate $p27^{kip1}$ expression. Targeting SIRT1 could therefore be a novel strategy for moderating $p27^{kip1}$ levels in cancer cells. Second, we show that SIRT1 silencing can induce cell senescence in NSCLC, and that this response is dependent on $p27^{kip1}$. Thus, we demonstrate a new route for senescence induction in established tumors. Third, we show that SIRT1 silencing profoundly suppresses NSCLC tumor formation and growth *in vivo*, and SIRT1 silencing-mediated growth suppression is associated with upregulated $p27^{kip1}$ levels. Thus, our results suggest that SIRT1 inhibition could be a potential strategy for NSCLC treatment *via* its upregulation of $p27^{kip1}$ expression.

SIRT1 induces p27-dependent, but p53- or p16-independent, cell senescence

Our data shows that SIRT1 regulates $p27^{kip1}$ expression by controlling $p27^{kip1}$stability through the ubiquitin-proteolysis pathway, and SIRT1 silencing-mediated cell senescence depends on $p27^{kip1}$. It has been established that cyclin inhibitors, including $p27^{kip1}$, and the p16 and p53-p21 pathways, play an important role in cell senescence regulation [42, 43]. Several studies have shown that SIRT1 inhibition increases p53 acetylation or upregulates p16 expression to induce cell senescence in endothelial cells or fibroblasts [44, 45]. In NSCLC, a large percentage of cells contain either deleted or mutated p16 or p53, while $p27^{kip1}$ remains intact [46]. In the NSCLC cells we studied, 5 out of 7 cell lines had p53

deletion or mutation (Supplementary data Table S1) and only 2 out of 7 (H460, A549) had wild-type p53. The p16 pathway was largely disrupted either through deletion (H460, A549 and H1993), methylation (H1299 and HCC827), or mutation (H1975) (Supplementary data Table S1). Our result shows that SIRT1 silencing can induce cell senescence in p53-deficient or p16-deficient cells lines, suggesting that the p53-p21 axis or p16 alone cannot be the only mediator of cell senescence after SIRT1 silencing. To test whether the p53-p21 pathway may be involved in SIRT1 silencing-mediated cell senescence in p53 wild-type cells, we checked the acetylated p53 and p21 levels in H460 cells. The results show that although SIRT1 silencing can increase acetylated p53 levels, the level of the p53-responsive gene p21 is downregulated instead of upregulated (supporting data Fig. S3A). This is consistent with the report that adriamycin-induced p21 protein levels were not higher in $SIRT1^{\Delta/\Delta}$ and $SIRT1^{\Delta ex4/\Delta ex4}$ MEFs than in the corresponding WT and heterozygous MEFs [47], although there are other reports which show that p53 hyperacetylation in response to overexpression of a dominant-negative SIRT1 protein was accompanied by increased induction of the p53-responsive gene p21 [20]. In addition to p53, our data also shows that SIRT1 silencing can induce cell senescence in p16-deficient cell lines (H460 and A549) (supporting data Fig. S3B), indicating that p16 also does not play an important role in SIRT1 silencing-mediated cell senescence. Collectively, these findings suggest that $p27^{kip1}$ rather than the p53-p21 pathway or p16 plays an important role in SIRT1 inhibition-mediated cell senescence. Thus, we have identified a new route for cell senescence induction through the SIRT1-p27 pathway in NSCLC cells.

SIRT1 inhibition is a potential consideration for NSCLC therapy

Cell senescence plays an important role in cancer development and progression [48], and therapy-induced senescence (TIS) has emerged as a novel therapeutic approach for cancer eradication [42, 49, 50]. Several TIS pathways have been identified that induce cell senescence and the eradication of established tumors, such as inactivation of MYC, SKP2, or CK2 [51, 52]. Our data shows that SIRT1 is a critical regulator of $p27^{kip1}$ reduction in NSCLC cells, SIRT1 silencing induces cell senescence in a $p27^{kip1}$ dependent manner, and also that SIRT1 silencing suppresses NSCLC tumor formation and growth in xenograft mouse models. Although NSCLC is a leading cause of cancer death, current treatment for advanced NSCLC is only moderately effective. Consistent with our findings, recent publications have shown that SIRT1 promotes NSCLC growth and progression, and SIRT1 inhibition induces anti-tumor activity in NSCLC [30, 31, 53]. Moreover, SIRT1 overexpression has been shown to be significantly associated with poor prognosis of NSCLC [29, 30, 53, 54]. Together with these results, our findings suggest that targeting the SIRT1-$p27^{kip1}$ axis represents an important potential targeted strategy for treating NSCLC.

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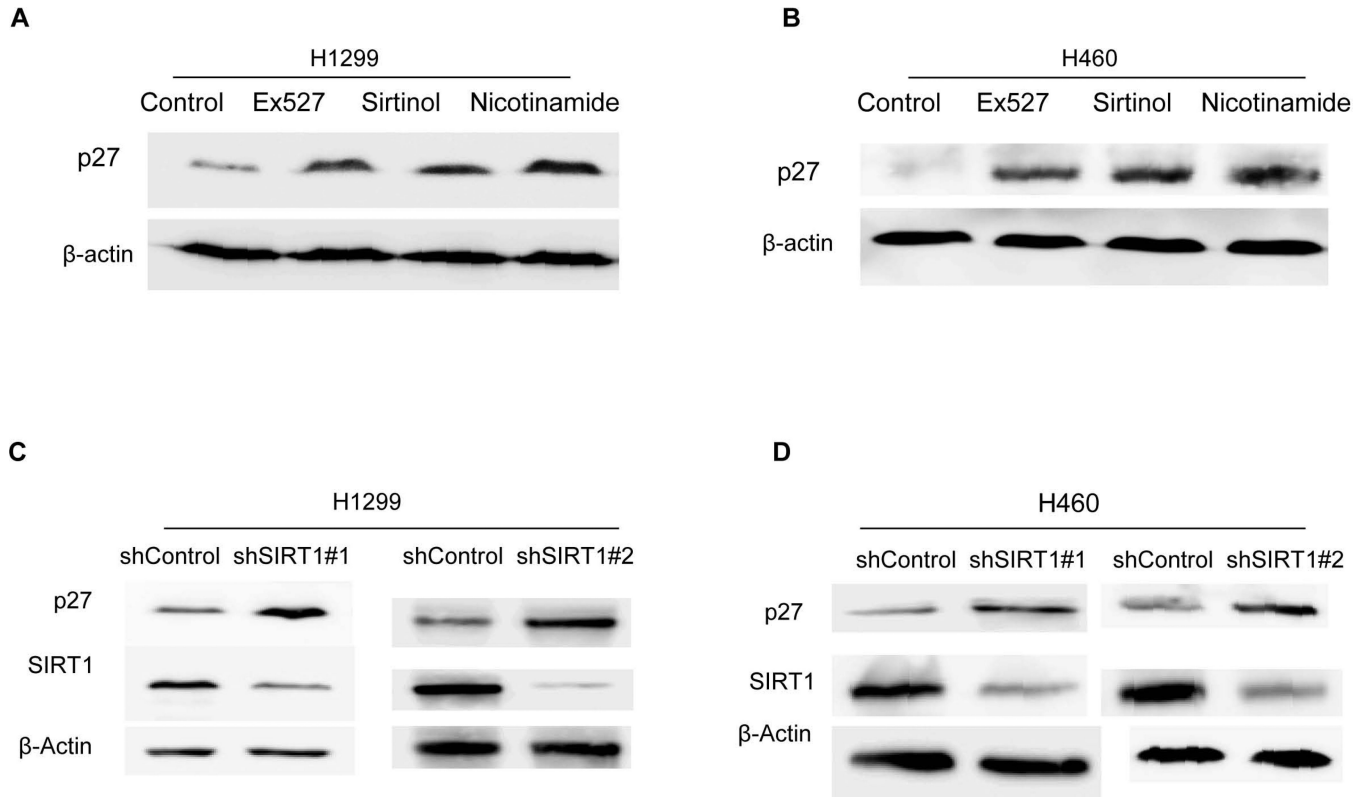

Figure 1.

SIRT1 regulates p27 protein expression. **A & B.** SIRT1 inhibition with SIRT1 inhibitors upregulates p27 expression. H1299 (A) and H460 (B) cells were treated with Ex527 1 uM, Sirtinol 100 uM or Nicotinamide 10 mM for 12 hrs. Immunoblot analysis was performed with *p27*^{kip1} and β-actin antibodies. **C & D.** SIRT1 knockdown results in p27^{kip1} upregulation. H1299 (C) and H460 (D) cells were infected with SIRT1 shRNA #1 or SIRT1 shRNA #2 or non-specific shRNA control retrovirus, and SIRT1 stable knockdown colonies were collected. Cell extracts were made from SIRT1-silenced and shRNA control H1299 and H460 cells, and immunoblot analysis was performed with p27, SIRT1 and β-actin antibodies.

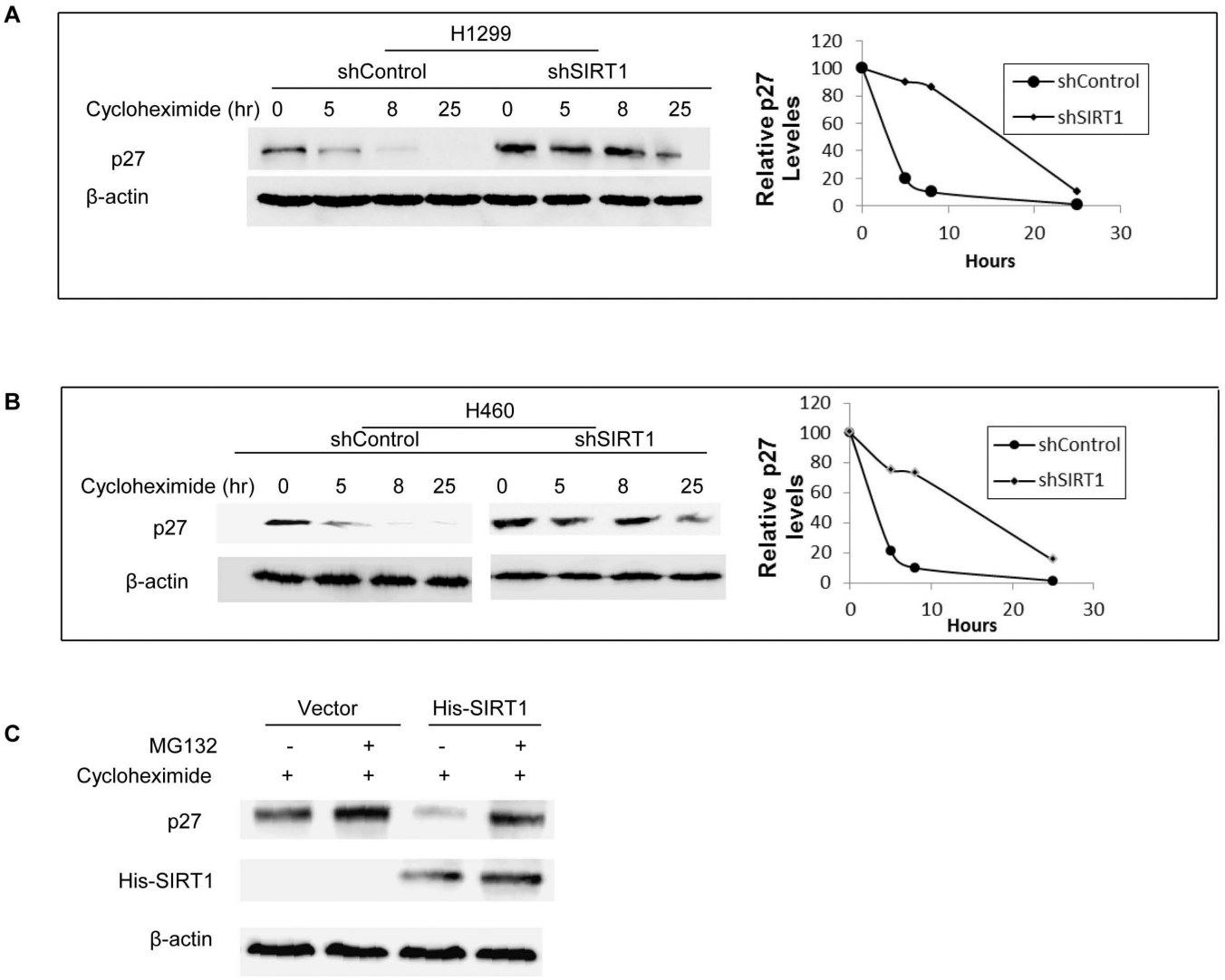

Figure 2.

SIRT1 regulates $p27^{kip1}$ stability through the ubuquitin-proteolysis pathway. **A & B.** SIRT1 silencing induces significant increase in $p27^{kip1}$ protein half-life. SIRT1-silenced and shRNA-control H1299 (A) and H460 (B) cells were treated with cycloheximide (10 μg/ml) for 0, 5, 8, 25 hr. Cell extracts were made from the treated cells and immunoblot analysis was performed with p27 and β-actin antibodies (**left panels**). The p27 intensity was analyzed using image J software and the p27 half-lives were calculated as the time required for each p27 protein decrease to 50% of its initial level (**right panels**). **C.** Proteasome activity inhibition with MG132 blocks SIRT1 overexpression-mediated $p27^{Kip1}$ downregulation. His tagged-SIRT1 expression plasmid and vector control plasmid were transfected into H1299 cells. After 48 hrs, the cells were further treated with cycloheximide (10μg/ml) together with MG132 (10uM) or vehicle for 4hrs. Immunoblot analysis was performed with p27, His-tag, and β-actin antibodies.

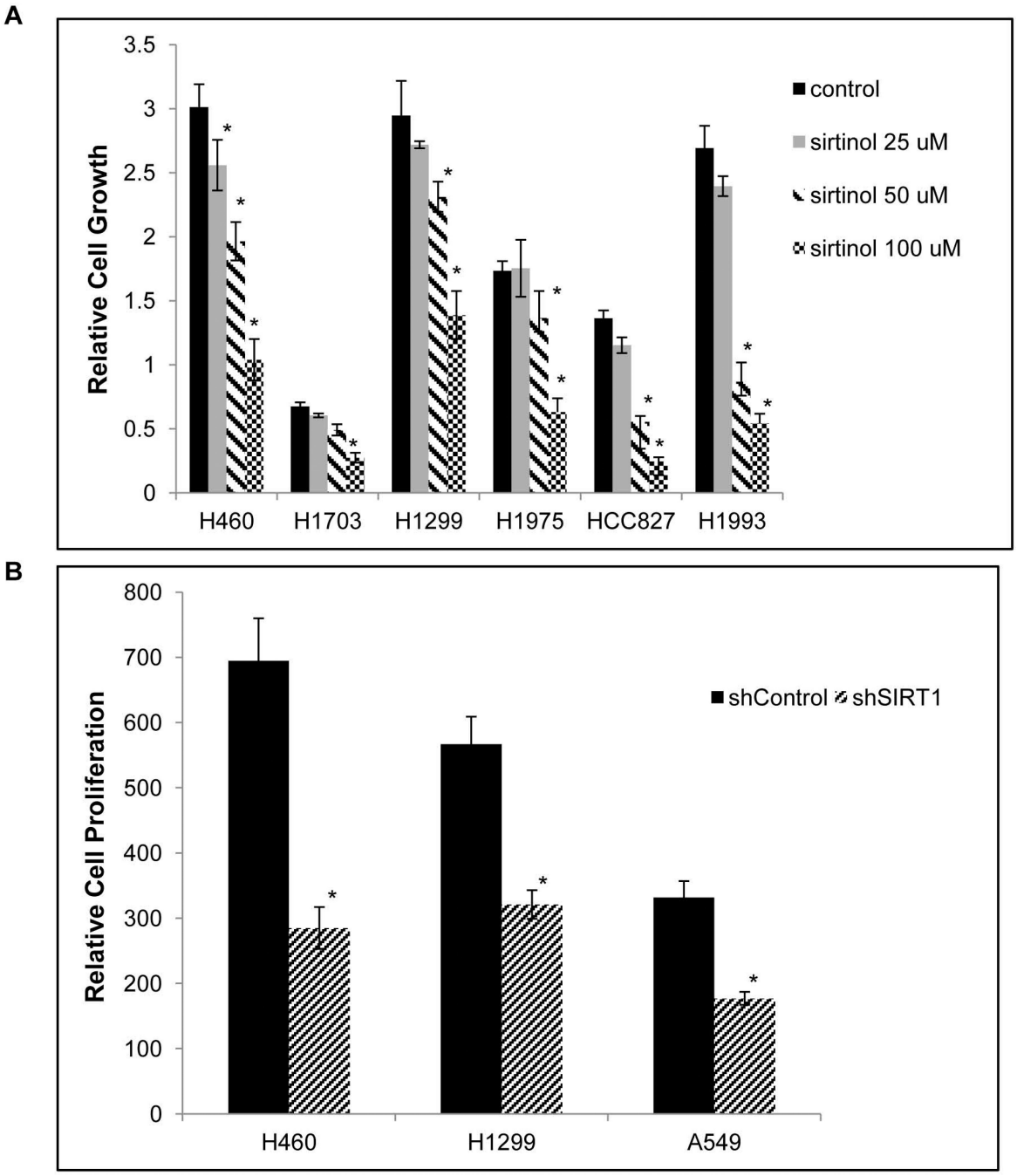

Figure 3.

SIRT1 inhibition or knockdown suppresses NSCLC growth and proliferation. **A.** Treatment with the SIRT1 inhibitor Sirtinol suppresses NSCLC cell growth. 5×10^3 NSCLC cells, including H460, H1299, H1975, H1993, H1703 and HCC827 cells, were seeded to 96 well plates and treated with vehicle or Sirtinol at 25 uM, 50 uM, and 100 uM for 72 hrs, and a MTS assay was performed. The error bars represent the SEM. **B.** SIRT1 knockdown suppresses NSCLC proliferation. 4×10^4 SIRT-silenced or shRNA-control H1299, H460, and A549 cells were seeded onto 24 well plates and counted after 72 hrs of culture. The error

bars represent the SEM. Asterisks indicate significant differences between experimental versus control group (* $p<0.05$).

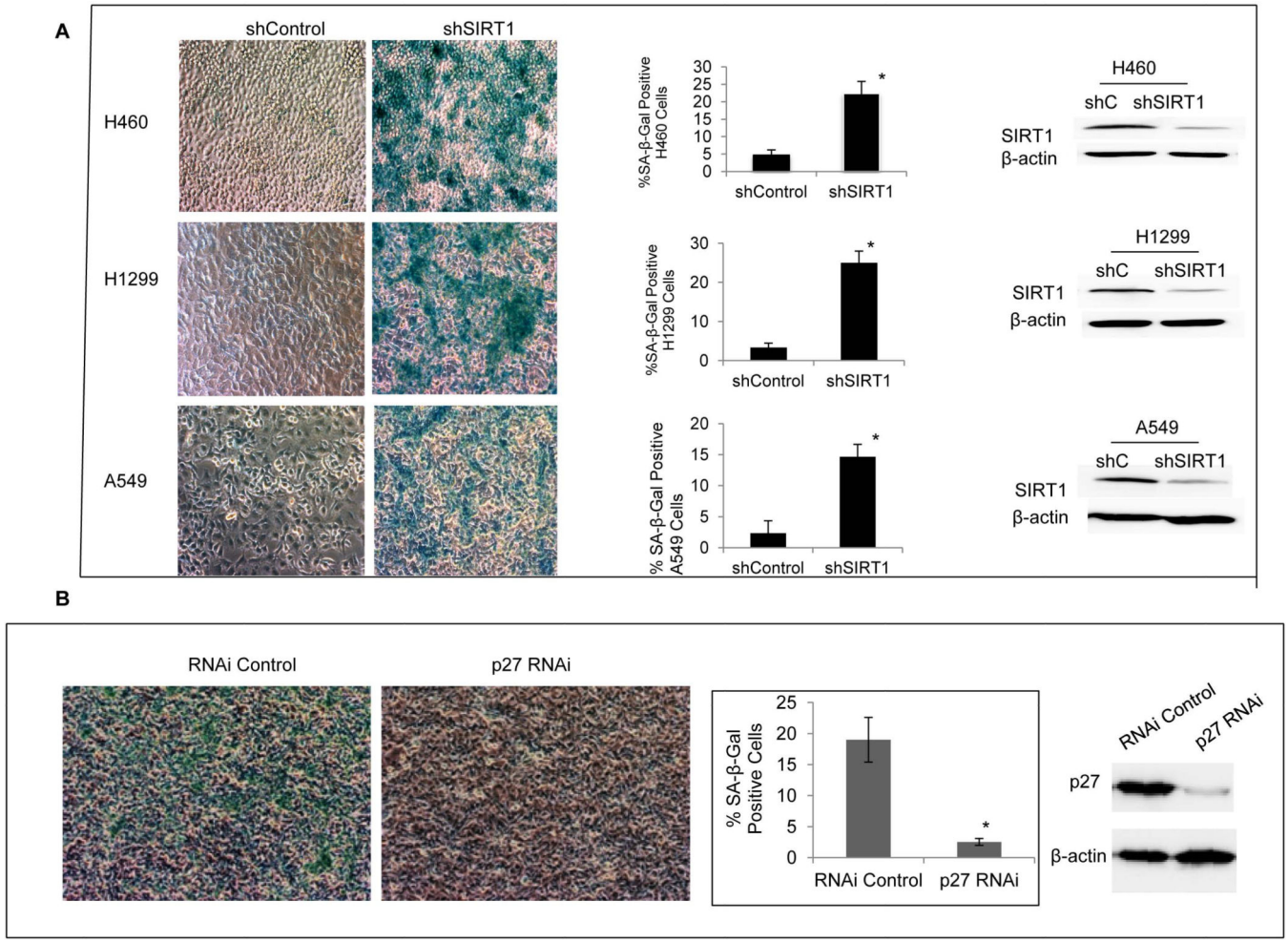

Figure 4.

SIRT1 silencing induces cell senescence through the $p27^{kip1}$ pathway. **A.** SIRT1 knockdown induces cell senescence in NSCLC cells. 5×10^5 SIRT-silenced or shRNA-control H1299, H460 and A549 cells were seeded onto 6 well plates and cultured for 72 hrs, then SA-β-Gal analysis was performed. A representative experiment from three independent experiments is shown (**left panels**). The SA-β-Gal positive cells were quantitated under the microscope and the percentage of SA-β-Gal positive cells in each condition was calculated. The histograms represent the mean ± SD of triplicates (**middle panels**). Asterisks indicate significant differences between two groups (* $p<0.01$). Immunoblot analysis shows the SIRT1 levels in the H1299, H260 and A549 knockdown cells that were used for the SA-β-Gal analysis (**right panels**). **B**. $p27^{kip1}$ knockdown rescues SIRT1-silencing-induced cell senescence. SIRT1-silenced H1299 cells were transfected with two independent p27 siRNA (#L-003472-00-005 & #12324) or non-targeting siRNA control (# D-001810-01-20) at 25nM for 72 hrs, then the SA-β-Gal analysis was performed. A representative experiment from three independent experiments is shown (the **left panels**). SA-β-Gal positive cells were quantitated under the microscope and the percentage of SA-β-Gal positive cells in each condition was calculated (the **middle panel**). Asterisks indicate significant differences

between two groups (* $p<0.01$). The p27 levels were determined by immunoblot analysis with p27 antibody (**the right panel**).

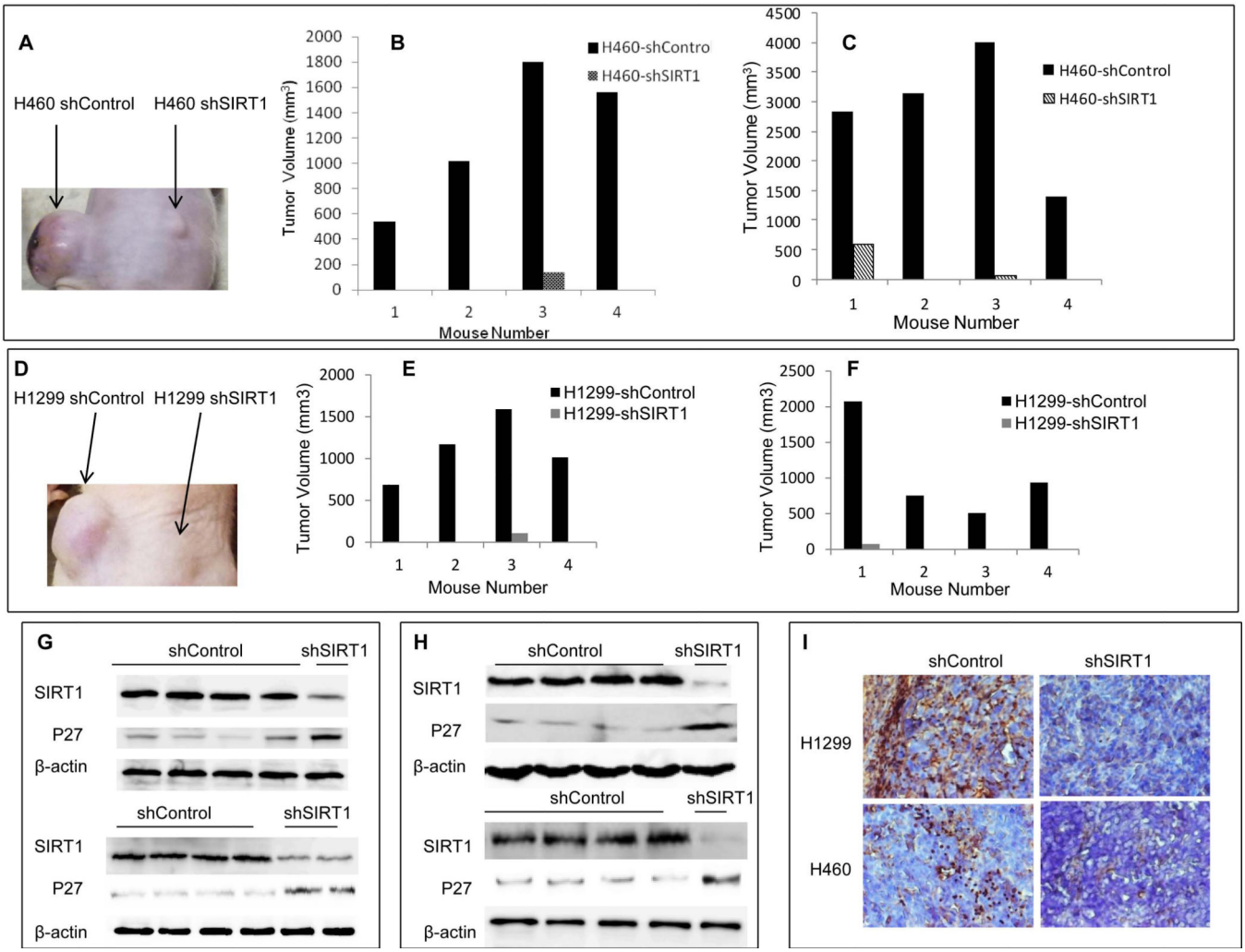

Figure 5.

SIRT1 knockdown dramatically suppresses NSCLC tumor formation and growth in vivo. The SIRT1-silenced and shRNA-control H460 or H1299 cells were injected subcutaneously in the dorsal flanks of same athymic nude mice (the shRNA control cells is on the left side and SIRT1-silenced cells is on the right side). **A**. Representative of H460 injected mouse (Left: shRNA Control H460; right: SIRT1-silencedd H460). **B.** 1×10^6 H460 SIRT1-silenced and shRNA-control cells were injected and tumor volumes were measured 28–34 days (n=4). **C.** 1.5×10^6 SIRT1-silenced and shRNA control H460 cells were injected and tumor volumes were measured at 21–27 days (n=4). The SIRT1-silenced or shRNA-control H460 cells used in B & C were generated from independent infections with two specific SIRT1 shRNA. **D.** Representative of H1299 injected mouse (Left: shRNA control H1299; right: SIRT1-silenced H1299). **E.** 2×10^6 SIRT1-silenced and shRNA control H1299 cells were injected and tumor volumes were measured at 27–33 dasy (n=4). **F.** 1×10^6 SIRT1-silenced and shRNA-control H1299 cells were injected and tumor volumes were measured at 40–46 days (n=4). The SIRT1-silenced or shRNA-control H1299 cells used in E & F were generated from independent infections with two specific SIRT1 shRNA. **G.** Cell extracts were made from collected tumors of two independent H460 injections in group B & C, and

immunoblot analysis was performed with p27, SIRT1, and β-actin antibodies. **H**. Cell extracts were made from collected tumors of two independent H1299 injections in group E & F, and immunoblot analysis was performed with $p27^{kip1}$, SIRT1 and β-actin antibodies. **I.** Frozen sections of the collected tumor tissues from SIRT1-silenced and shRNA-control H460 and H1299 xenografts were stained with the proliferation marker Ki-67.