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Original Article

The effect of cullin 4A on lung cancer cell chemosensitivity to paclitaxel through p33ING1b regulation

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Abstract: Cullin 4A (Cul4A) reportedly has oncogenic roles in several cancer types by regulating tumor suppressors through the ubiquitination and proteolysis of the tumor suppressor. In addition, Cul4A is associated with chemosensitivity to chemotherapy drugs. This study investigated the association between Cul4A and lung cancer cell chemosensitivity to paclitaxel, particularly with respect to the role of the p33 inhibitor of the growth 1 (p33ING1b) tumor suppressor. The results showed that the Cul4A knockdown upregulated the p33ING1b expression in lung cancer cells and increased the lung cancer cell and mice tumor xenograft chemosensitivity to paclitaxel. The Cul4A knockdown also inhibited the growth and increased the apoptosis in the tumor xenografts treated with paclitaxel. Notably, the p33ING1b overexpression increased the lung cancer cell chemosensitivity to paclitaxel, but the p33ING1b knockdown reduced the chemosensitivity. A further analysis demonstrated that Cul4A regulates the expression of p33ING1b through protein-protein interactions, ubiquitination, and protein degradation. In conclusion, the present findings suggest that Cul4A mediates the chemosensitivity of lung cancer cells to paclitaxel by regulating p33ING1b. These findings may offer novel insights into future therapeutic strategies for lung cancer that target Cul4A.

Keywords: Lung cancer, paclitaxel, cullin 4A

Introduction

The evolutionally conserved cullin protein cullin 4A (Cul4A) interacts with ring finger protein-1 as well as damaged DNA binding protein-1 to form a complex of multifunctional ubiquitin/protein ligase E3 [1, 2], and it drives the degradation of proteins. Through this ubiquitin-mediated proteolysis, Cul4A is crucial in numerous cellular functions, including genome stability, cell cycle regulation, histone modification, nuclear excision repair, and apoptosis [3-7].

Cul4A has also been reported to promote tumorigenesis in various types of cancer; its upregulated expression has been reported in breast cancer, mesothelioma, lung cancer, prostate cancer, osteosarcoma, and liver cancer [8-13]. Cul4A also drives the tumor suppressor ubiquitination and proteolysis for suppressors such as p27 [4], p53, p21, neurofibromin 2, transforming growth factor β-induced, and Ras association domain family 1 isoform A [14-18]. In one study, Cul4A overexpression promoted lung tumor development in transgenic mice [19]. An association exists between Cul4A knockdown and elevated sensitivity to chemotherapeutic drugs [17, 19]. These findings have highlighted the attractiveness of Cul4A as a target in treating cancers.

The inhibitor of growth (ING1) gene on chromosome 13q34 codes the ING1 protein. The type-II suppressor gene *ING1* encodes at least four protein isoforms: p24ING1c, p27ING1d, p33ING1b, and p47ING1a [20]. Among these isoforms, p33ING1b (ING1b) is extensively expressed in human tissues; consequently, it is currently the most investigated isoform. Significantly downregulated p33ING1b expression has been observed in breast, esophageal, gastric, and brain cancers in addition to leukemia [21-25]. Adenovirus (Ad)-mediated p33ING1b overexpression reportedly significantly suppresses proliferation and increases apoptosis in gastric adenocarcinoma and glioma [26, 27] cells. Notably, the overexpression of p33ING1b has been reported to enhance the chemosensitivity of osteosarcoma cells to paclitaxel [28].

Both Cul4A and p33ING1b play important roles in the carcinogenesis of lung cancer. In a previous study, the overexpression of Cul4A was observed in lung cancer tissues and was associated with the poor prognosis of lung cancer patients [10, 29]. Reduced expressions of the *p33ING1b* gene have been observed in lung cancer tissues and are associated with the *p21* and *bax* gene expressions. Thus, the *p33ING1b* gene might be a tumor suppressor gene in lung cancer [30]. To our knowledge, no association has been reported among Cul4A, chemosensitivity to paclitaxel, and the p33ING1b tumor suppressor. Therefore, we investigated this association in lung cancer. The underlying mechanisms of Cul4A regulating p33ING1b expression were also determined.

Material and methods

Cell lines and cultures

The American Type Culture Collection was the source of the non-small cell lung cancer cells (A549, NCI-H157, NCI-H322, and NCI-H460) and the 293T cells used in this study. Short tandem repeat profiling was performed to authenticate the cell lines. NCI-H460 and NCI-H322 cell culturing was performed using RPMI-1640 as the medium. Dulbecco's modified Eagle's medium with 10% fetal bovine serum plus penicillin and streptomycin (10 units/mL each) as supplements was used for culturing the A549, NCI-H157, and 293T cells; the cells were maintained in 5% CO_2 at 37°C.

Retrovirus production and transduction

The Cul4A gene was transduced into the pBABE-puro retroviral vector (Addgene, Inc.), and short hairpin RNA (shRNA/sh) targeting Cul4A (shCul4A) was cloned into the pSUPER. retro.puro vector (Oligoengine) as previously described [9]. After the retroviral transduction, the infected cells were cultured for 48 h in a puromycin (1 mg/mL)-containing medium (Sigma-Aldrich; Merck KGaA). The stably transduced cells were maintained in puromycin (0.5 mg/mL)-containing complete medium. Stable cells that had undergone empty vector (EV) transection were also produced using the aforementioned protocols.

Western blotting

M-PER Mammalian Protein Extraction reagent (Thermo Fisher Scientific, Inc.) supplemented with Complete Protease Inhibitor Cocktails (Roche Diagnostics) and a Phosphatase Inhibitor Cocktail Set II (Calbiochem; Merck KGaA) were used to extract the total protein from the cells. The protein separation and electroblotting onto Immobilon-P membranes (EMD Millipore) was achieved through 4%-15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Primary antibodies, including anti-Cul4A (Abcam), anti-p33ING1b (Santa Cruz Biotechnology, Inc.), anti-PARP (Cell Signaling Technology, Inc.), and anti-β-actin (Sigma, St. Louis, MO) were used. After incubation with specific primary antibodies, relevant secondary antibodies were used to incubate the membranes. The total protein was visualized using an enhanced chemiluminescent reagent and analysis system (Cytiva). The protein expression levels were measured using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining

Lung cancer cells were first cultured directly onto coverslips and subsequently fixed for 10 min in ice-cold methanol (-20°C) with blocking for 30 min using 2% bovine serum albumin (BSA). Subsequently, cell incubation was performed at room temperature (RT) for 1 h in 2% BSA with a primary anti-p33ING1b antibody (Santa Cruz Biotechnology, Inc.). The cells were washed with phosphate-buffered saline before undergoing 1 h of RT incubation in 2% BSA with

fluorescein-conjugated secondary antibodies and then counterstained with DAPI. They were placed onto slides using VECTASHIELD (Vector Laboratories, Inc.) after washing. A TCS SP5 confocal microscope (Leica Microsystems GmbH) was used to visualize the slides.

Paclitaxel chemosensitivity assay

The A549, NCI-H460, NCI-H157, and NCI-H322 lung cancer cells (1×10^4 cells/mL) stably transfected with shCul4A or EV underwent 48 h of culturing in six-well plates. After incubation, the cells received the mentioned concentrations of paclitaxel for the specified durations. The cells were trypsinized after the paclitaxel treatment. After the trypan blue dye exclusion, the viable cells were counted using a hemocytometer. The data are expressed as percentages relative to the control group of the EV-transfected cells. GraphPad Prism (version 6; GraphPad Software, Inc.) was employed to determine the half-maximal inhibitory concentrations (IC_{50}) using the log (inhibitor) versus response (variable slope) function.

Reverse transcription quantitative polymerase chain reaction

A RNeasy Mini kit (Qiagen GmbH) and TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) were employed for total RNA extraction from the lung cancer cells and tumor tissues, respectively. An iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc.) was used to reverse transcribe the total RNA into cDNA. Next, the reaction mixture (20 μL) was supplemented with 2 μL of cDNA. The quantitative polymerase chain reaction (qPCR) used the following primer pairs: p33ING1b, 5'-CCAAGGGCAAGTGGTACTGT-3' (forward) and 5'-CTGCCATCCCTATGAAAGGA-3' (reverse); actin, 5'-TCGTGCGTGACATTAAGGAG-3' (forward) and 5'-CCATCTCTTGCTCGAAGTCC-3' (reverse); and GAPDH, 5'-GCGGGGCTCTCCAGAACATCAT-3' (forward) and 5'-CCAGCCCCAGCGTCAAAGGTG-3' (reverse). The qPCR was conducted under the following thermocycling conditions: 5 min of initial denaturation at 95°C; 30 cycles of 20 s at 95°C, 30 s at 56°C, and 30 s at 72°C; and a 5-min final extension at 72°C prior to maintenance at 4°C by using a CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.).

Small interfering RNA and vector transfection

The cell culturing was performed using six-well plates with predesigned small interfering RNAs (siRNAs) targeting p33ING1b (Santa Cruz Biotechnology, Inc.) and Cul4A (GE Healthcare Dharmacon, Inc.) to an 80% confluence. Antibiotic-free media and Lipofectamine RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) were used to transfect the universal negative control siRNAs into the cells (final concentration: 50 nM).

OmniFect transfection reagent (Transomic Technologies, Inc.) was used to transfect the pCMV6-p33ING1b-GFP (OriGene Technologies, Inc.) and empty pcDNA3.1 (Invitrogen; Thermo Fisher Scientific, Inc.) vectors into the cells. In brief, the cells in the antibiotic-free media were plated into 96-well plates, and the transfection was executed when the cells reached 80% confluence with 0.1 μg of each vector per well.

A CellTiter-Glo luminescent cell viability assay (Promega Corporation) was employed for the evaluation of the cytotoxicity of the paclitaxel after the cells had been incubated for 72 h with the indicated doses of paclitaxel. In brief, the culture wells were directly supplemented with the CellTiter-Glo reagent (100 μL) in accordance with the manufacturer protocols. The reaction between luciferin and ATP catalyzed by luciferase produced luminescence, which was measured on a luminometer.

Protein degradation assay

The effects of Cul4A on the p33ING1b proteolysis in the lung cancer cells were evaluated using a protein degradation assay. The plating of the cells stably transfected with either the Cul4A overexpression plasmid or shCul4A onto 6-cm culture dishes was performed before the cells were treated with cycloheximide (100 mg/mL) after reaching 80% confluence. Cell harvesting was subsequently performed. Total protein was extracted, and the expression levels of p33ING1b were analyzed using western blotting. The loading control was β-actin.

Coimmunoprecipitation assay

Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was employed for co-transfecting pBABE-Cul4A-Myc-his [9] and pCMV6-p33ING1b-GFP (OriGene Technologies, Inc.) vectors into the 293T cells. After transfection for 24 h, MG132 (10 μg/mL; Sigma-Aldrich; Merck KGaA) was used for the 24-h cell treatment. The cells were sub-

sequently harvested and treated with an NP-40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP40] containing a cocktail of phosphatase and protease inhibitors (Roche Diagnostics). The Catch and Release v. 2.0 Reversible Immunoprecipitation system (EMD Millipore) and anti-Cul4A (Abcam) and anti-GFP (OriGene Technologies, Inc.) antibodies were used for the immunoprecipitation.

In vivo ubiquitination assay

pCMV6-p33ING1b-GFP and pBABE-Cul4A-Myc-his (OriGene Technologies, Inc.) co-transfection with or without pRK5-HA-Ubiquitin-wild-type (Addgene, Inc.) was performed on the 293T cells. All the cells were subjected to 24 h of treatment with 10 μg/mL MG132 before lysis using an NP-40 lysis buffer. The immunoprecipitation of the cell lysates was performed using an anti-GFP antibody. Western blotting was subsequently performed using an anti-HA tag antibody (Cell Signaling Technology, Inc.).

Cul4A RNA interference (RNAi) expression

Ads expressing Cul4A RNAi (AdsiCul4A) or EV (AdEV) were produced using the BLOCK-iT Adenoviral RNAi Expression system (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described [29]. An Adeno-X Rapid Titer kit (Clontech Laboratories, Inc.) was used to amplify and titer the Ads, which were used for the cytotoxicity and xenograft studies.

Mouse xenograft model

The Chang Gung Memorial Hospital Institutional Animal Care and Use Committee (Chiayi, Taiwan; approval no. 2013121003) approved the present study. Female 5-6-week-old BALB/c athymic nude mice were housed in conditions free of specific pathogens and given water and food. The mice were administered subcutaneous flank injections of 1×10^6 H460 lung cancer cells in a total of 20% Matrigel (BD Biosciences) mixed with 100 μL of serum-free RPMI-1640 medium. When the tumors reached 40-50 mm^3 in volume, the mice were separated to form four groups at random (five mice per group). They were administered intratumoral injections of AdEV or AdsiCul4A with or without 20 mg/kg paclitaxel intraperitoneally twice per week for 3 weeks. The AdEV and AdsiCul4A viruses (1×10^9 plaque-forming units) were injected intratumorally on days 0, 7, 14, and 21 after the day the mice were grouped. The tumor volumes were measured three times per week with a caliper and calculated using the formula $L/2\times W^2$, where L denotes the maximum tumor diameter and W denotes the width perpendicular to L. The relative tumor volume was calculated as TV_n/TV_0, in which TV_0 and TV_n are the volumes of the tumors at days 0 and n after the grouping, respectively [30]. On day 31, after the Ads injection, all the mice were sacrificed using CO_2 inhalation with a displacement rate of 30% of the chamber volume per minute. The tumors were excised, fixed, and embedded in paraffin or stored at -80°C for further study.

Immunohistochemistry

The xenograft tumor and lung cancer tissues (Institutional Review Board approval no. 103-6934B) were fixed with formalin, embedded in paraffin, cut into sections 4 μm in size, and placed onto slides. Xylene and a gradient ethanol series were used to deparaffinize and dehydrate the tissue sections, respectively. To retrieve the antigens, 97°C citric acid (pH 6.0) was applied for 30 min. The slides underwent incubation with 3% hydrogen peroxide and subsequently incubation overnight with anti-Cul4A (Abcam), anti-Ki-67 (Spring Bioscience; Abcam), anti-p33ING1b (Santa Cruz Biotechnology, Inc.), and anti-cleaved caspase 3 (Cell Signaling Technology, Inc.) antibodies at 4°C. For the lung cancer tissues, the Cul4A and p33ING1b expressions were calculated using their semi-quantitative immunoreactive scores (IRS) as described previously [31]. The percentage of the positively stained cells was defined by 0=0% of cells stained, 1= less than 10% of cells stained, 2=11-50% of cells stained, 3=5-80% of cells stained, and 4= more than 81% of cells stained. The staining intensity as defined by 0= no staining, 1= weak staining, 2= moderate staining, and 3= strong staining. The IRS scores were calculated by multiplying the percentage of the positively stained cells and the staining intensity. The integrated optical density (IOD) of the immunohistochemistry (IHC) staining was analyzed using Image-Pro 6.3 (Media Cybernetics, Inc.). GraphPad Prism (GraphPad Software, Inc.) was used to evaluate, calculate, and plot the IOD values.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.), with the data presented as means ± standard deviations. Unless otherwise specified, Student's *t* tests were conducted for the between-group comparisons including the densities of the western blot bands, the intensity of the immunofluorescence staining, the percentages of the surviving cells, and the density of the IHC staining. The correlations between the Cul4A and p33ING1b expressions in the lung cancer tissues were evaluated using the correlation coefficients calculated by MedCalc Version 19.6 (MedCalc Software Ltd). Two-way analyses of variance using Bonferroni's post hoc tests were conducted to analyze the mouse xenograft models. All the statistical testing was two-tailed, and $P<0.05$ indicated significance.

Results

The effect of the Cul4A knockdown on the p33ING1b expressions in the lung cancer cells

For the Cul4A expression knockdown, Cul4A siRNA was transfected into the NCI-H157, NCI-H322, NCI-H460, and A549 lung cancer cells (**Figure 1A** and **1B**). A subsequent analysis showed that the Cul4A knockdown upregulated the p33ING1b expression (**Figure 1A** and **1C**). The expressions of Cul4A and p33ING1b were also evaluated in the 28 lung cancer tissues using IHC staining. A negative correlation between Cul4A and p33ING1b was observed in the lung cancer tissues we studied (**Figure 1D**). To further confirm the findings, immunofluorescence staining for the p33ING1b expression was performed and visualized using a confocal microscope. The expression levels of p33ING1b were also upregulated in the shCul4A-transfected A549 and NCI-H460 cells (**Figure 2A** and **2B**). By contrast, the p33ING1b expression was downregulated in the A549 and NCI-H460 lung cancer cells overexpressing Cul4A (**Figure 2C** and **2D**).

The Cul4A knockdown increased the paclitaxel chemosensitivity in the lung cancer tumors and cells

The chemosensitivity of the shCul4A-transfected A549, NCI-H460, NCI-H157, and NCI-H322 lung cancer cells to paclitaxel was analyzed. The cells with knocked down Cul4A expressions exhibited increased chemosensitivity to paclitaxel and a significantly lower IC_{50} compared with the EV-transfected cells (**Figure 3A-D**).

The effects of the Cul4A siRNA transfection on the paclitaxel chemosensitivity were subsequently analyzed. NCI-H460 cell transduction was performed with AdsiCul4A or AdEV at a multiplicity of infection (MOI) of 200. Compared with the AdEV group, the AdsiCul4A group exhibited increased chemosensitivity to paclitaxel (**Figure 3E**).

To determine the therapeutic value of AdsiCul4A transfection *in vivo*, a mouse xenograft model of NCI-H460 lung cancer cells was established. The group injected with AdsiCul4A exhibited increased chemosensitivity to paclitaxel (**Figure 3F** and **3G**).

In the xenograft tumor tissues, the Cul4A expression was downregulated (**Figure 4A** and **4E**), but the p33ING1b expression was upregulated (**Figure 4B** and **4F**) in the AdsiCul4A siRNA group. The Ki-67 expression (**Figure 4C** and **4G**), which has an association with cancer cells' proliferative ability [32], was also downregulated in the Cul4A siRNA and paclitaxel groups. In addition, the cleaved caspase-3 expression (**Figure 4D** and **4H**), which is related to apoptosis [33], was upregulated in the Cul4A siRNA and paclitaxel groups. The mRNA expression levels of Cul4A were further evaluated using reverse transcription qPCR (RT-qPCR). The Cul4A mRNA expression was downregulated in the xenograft tumor tissues of the Cul4A siRNA group (**Figure 4I**).

The association between the p33ING1b expression and the chemosensitivity to paclitaxel

The association between the p33ING1b expression levels and the paclitaxel chemosensitivity was further evaluated in the A549 and NCI-H460 lung cancer cells with the silenced Cul4A expression. The chemosensitivity to paclitaxel decreased after the siRNA-induced knockdown of the p33ING1b expression (**Figure 5A** and **5B**).

The chemosensitivity to paclitaxel was also evaluated in the Cul4A-overexpressing A549 and NCI-H460 lung cancer cells. The cell chemosensitivity to paclitaxel increased after the p33ING1b overexpression (**Figure 5C** and **5D**).

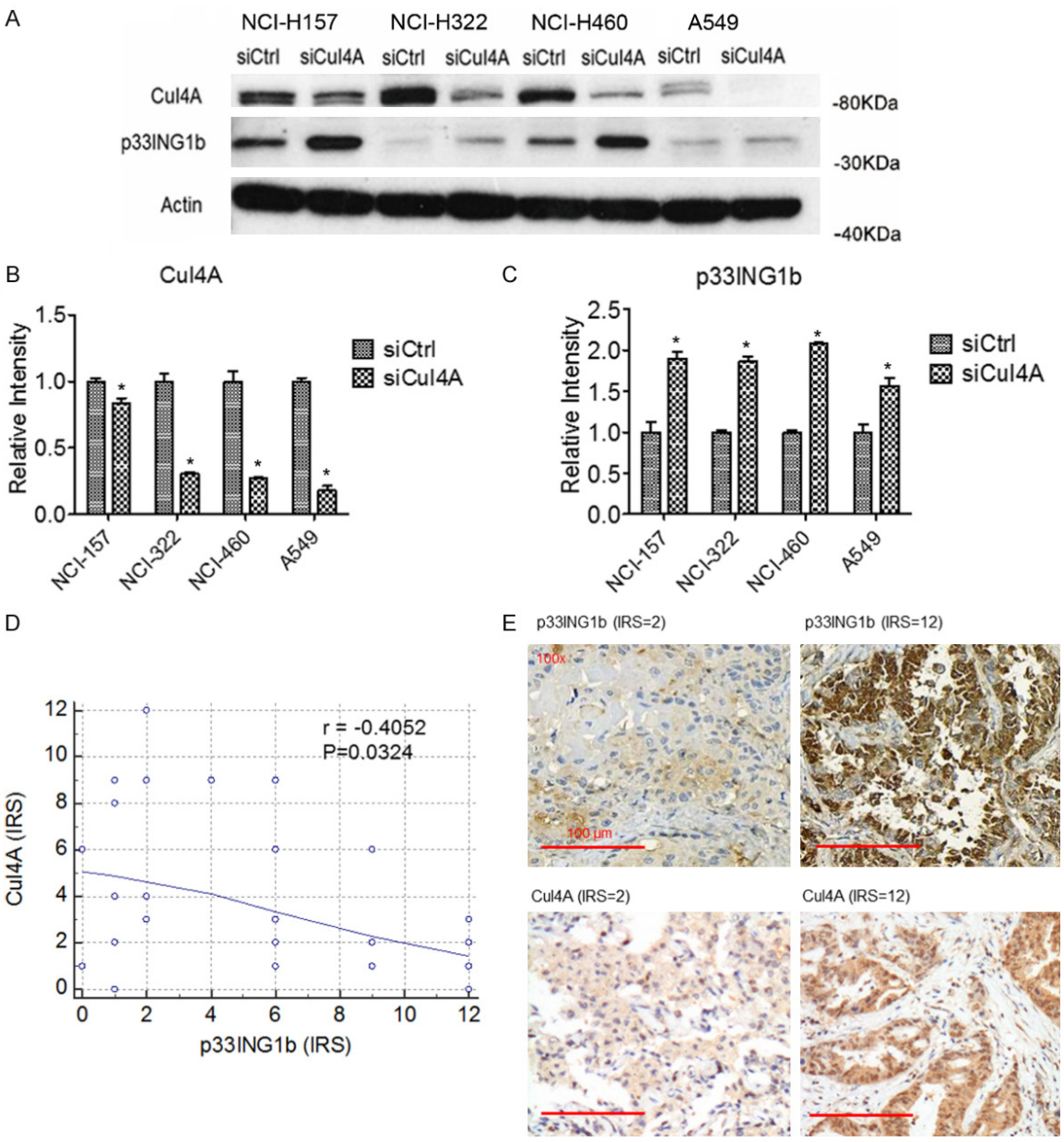

Figure 1. The knockdown of Cul4A increases the expression of p33ING1b in lung cancer cells, and Cul4A negatively correlates with p33ING1b in lung cancer tissues. (A) Western blot analysis of the p33ING1b and Cul4A expression levels in NCI-H157, NCI-322, A549, and NCI-H460 cells. The internal control was actin. The bar graphs show the relative (B) Cul4A and (C) p33ING1b expression levels in Western blot analysis. The Cul4A and p33ING1b expression levels were normalized to actin with the siCtrl group as the control. *P<0.05 (compared with the siCtrl group). Cul4A, cullin 4A; p33ING1b, p33 inhibitor of growth 1b; si, small interfering RNA; Ctrl, control. (D) Correlation of the expressions of Cul4A and p33ING1b in the lung cancer tissues. (E) The representative IHC stainings of Cul4A and p33ING1b are also shown. Original magnification: 100×; scale bar: 100 μm; IRS: immunoreactive score; IHC: immunohistochemistry.

The association between the p33ING1b expression and apoptosis

The association between the p33ING1b and apoptosis was evaluated through the siRNA knockdown of the paclitaxel treated H460 lung cancer cells. An increased expression of cleaved PARP, which is related to apoptosis, was observed in the lung cancer cells treated with Cul4A siRNA and paclitaxel. However, the

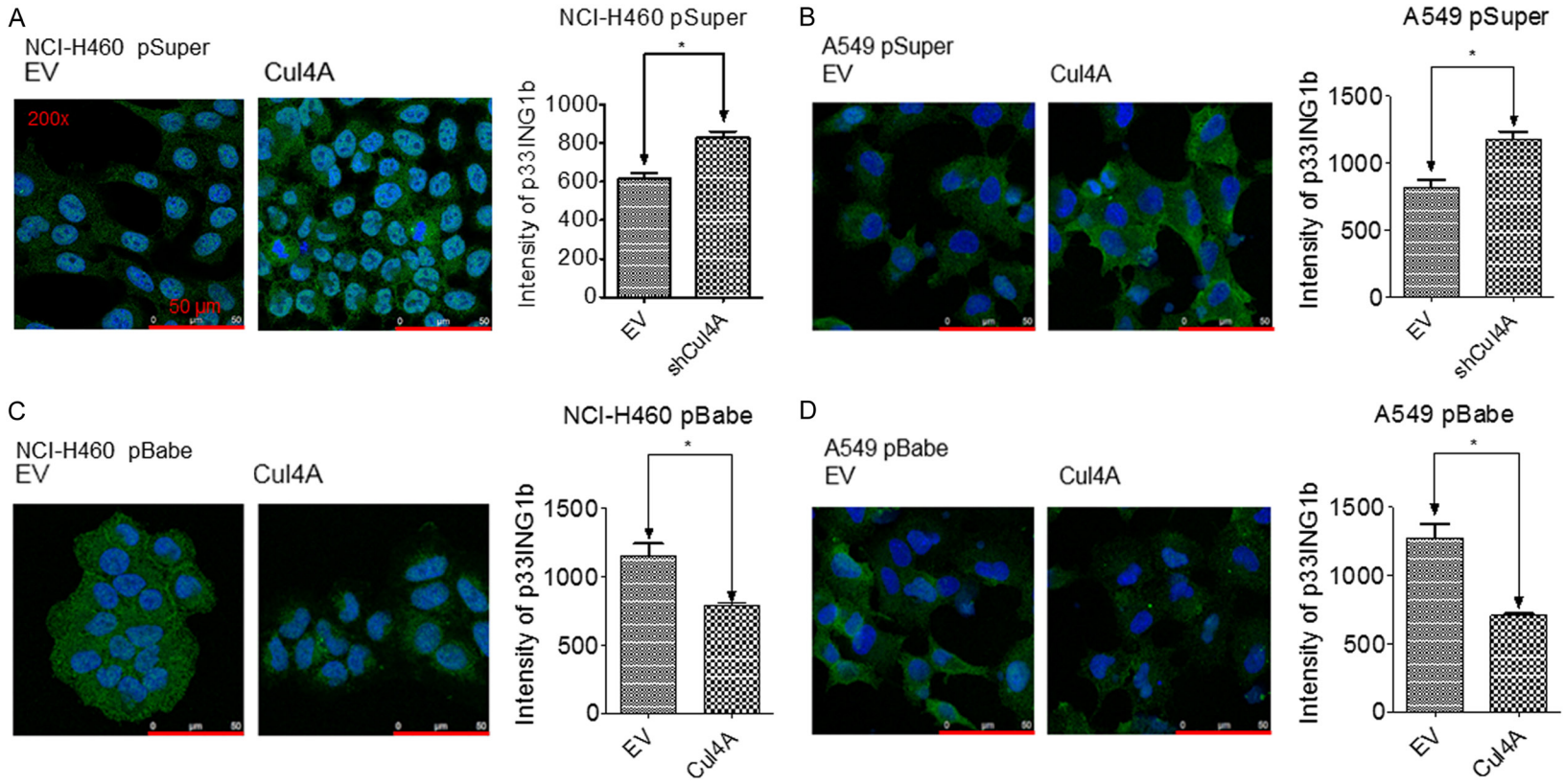

Figure 2. The knockdown of Cul4A increases and the overexpression of Cul4A decreases the p33ING1b expression in lung cancer cells. An immunofluorescence analysis of the shCul4A-containing pSUPER vector-transfected (A) NCI-H460 and (B) A549 lung cancer cells. An immunofluorescence analysis of the Cul4A-overexpressing pBABE vector-transfected (C) NCI-H460 and (D) A549 lung cancer cells. The control group consisted of EV-transfected cells. The p33ING1b positive stains are indicated in green; the nuclear staining is indicated in blue (original magnification, 200×; scale bar, 50 μm). Three independent experiments were conducted to measure the p33ING1b intensity, which is presented as the mean intensity ± standard deviation. $^{*}P<0.05$. sh, short hairpin RNA; Cul4A, cullin 4A; p33ING1b, p33 inhibitor of growth 1b; EV, empty vector.

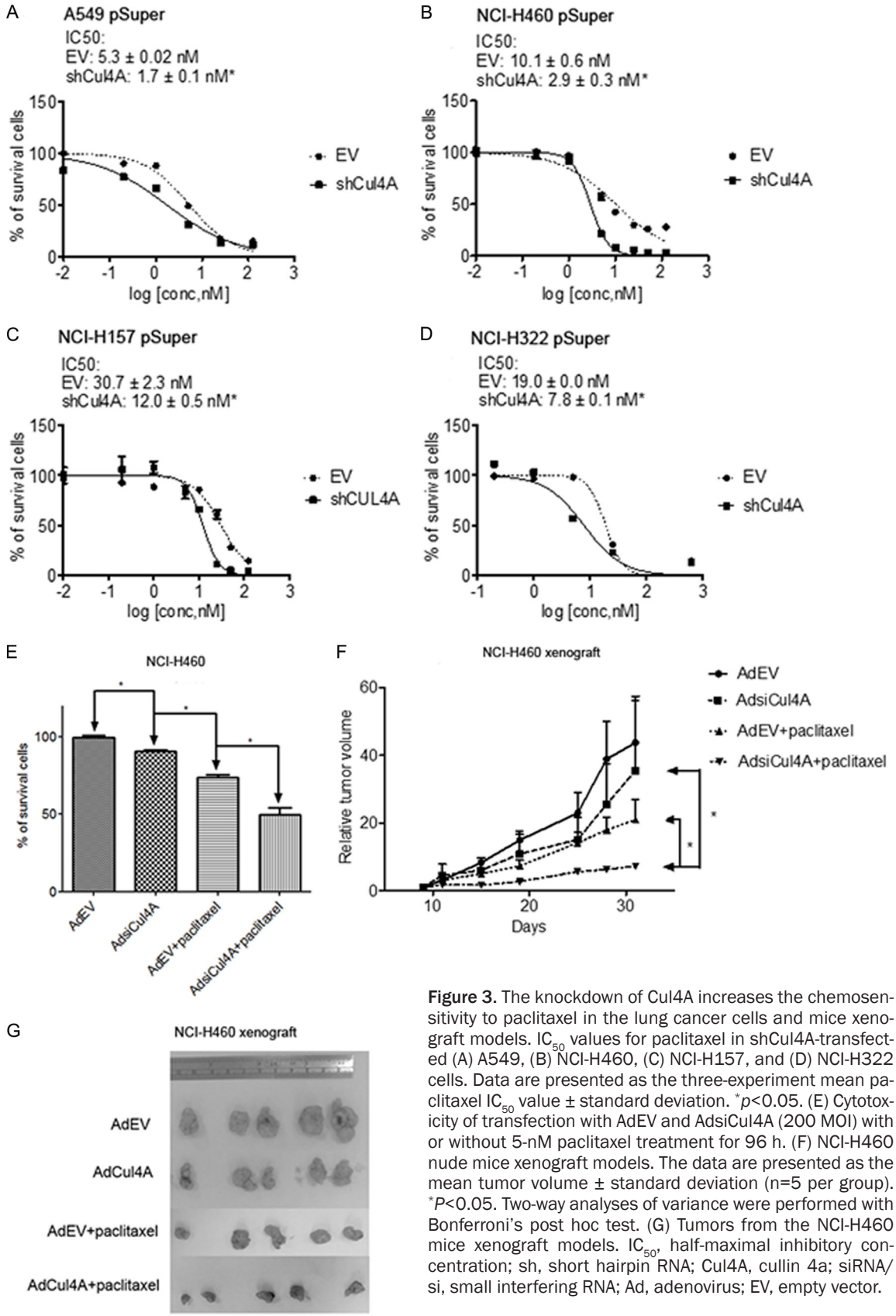

Figure 3. The knockdown of Cul4A increases the chemosensitivity to paclitaxel in the lung cancer cells and mice xenograft models. IC_{50} values for paclitaxel in shCul4A-transfected (A) A549, (B) NCI-H460, (C) NCI-H157, and (D) NCI-H322 cells. Data are presented as the three-experiment mean paclitaxel IC_{50} value ± standard deviation. $^{*}p<0.05$. (E) Cytotoxicity of transfection with AdEV and AdsiCul4A (200 MOI) with or without 5-nM paclitaxel treatment for 96 h. (F) NCI-H460 nude mice xenograft models. The data are presented as the mean tumor volume ± standard deviation (n=5 per group). $^{*}P<0.05$. Two-way analyses of variance were performed with Bonferroni's post hoc test. (G) Tumors from the NCI-H460 mice xenograft models. IC_{50}, half-maximal inhibitory concentration; sh, short hairpin RNA; Cul4A, cullin 4a; siRNA/si, small interfering RNA; Ad, adenovirus; EV, empty vector.

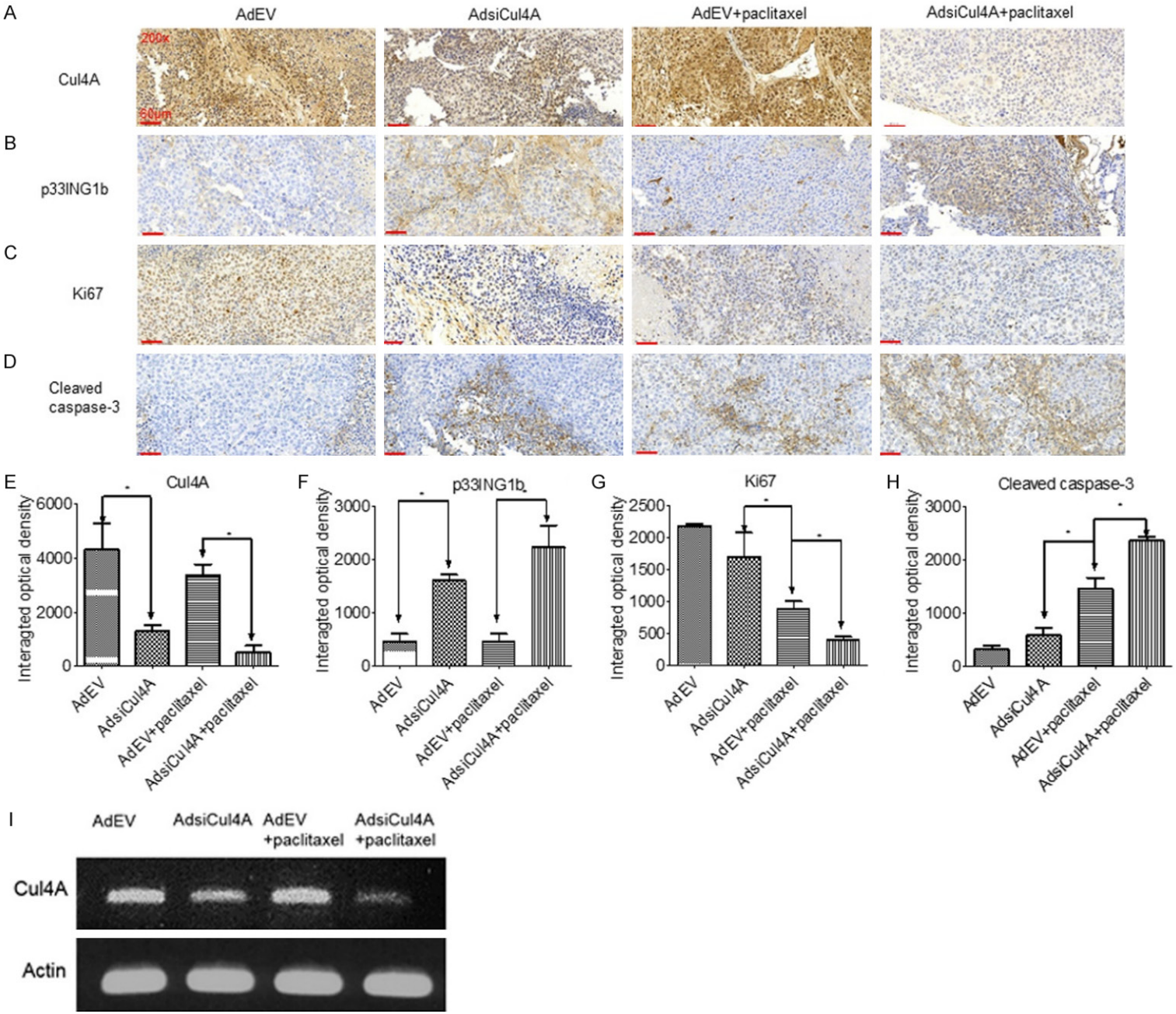
A
AdEV
AdsiCul4A
AdEV+paclitaxel
AdsiCul4A+paclitaxel
Cul4A
200x
50μm
B
p33ING1b
C
Ki67
D
Cleaved caspase-3
E
Cul4A
Interagted optical density
6000
4000
2000
0
AdEV
AdsiCul4A
AdEV+paclitaxel
AdsiCul4A+paclitaxel
F
p33ING1b
Interagted optical density
3000
2000
1000
0
G
Ki67
Interagted optical density
2500
2000
1500
1000
500
0
H
Cleaved caspase-3
Interagted optical density
3000
2000
1000
0
I
AdEV
AdsiCul4A
AdEV +paclitaxel
AdsiCul4A +paclitaxel
Cul4A
Actin

Figure 4. The knockdown of Cul4A with paclitaxel treatment increases the p33ING1b and cleaved caspase-3 and decreases the Ki-67 expressions in mice xenograft tumors. IHC analysis of (A) Cul4A, (B) p33ING1b, (C) Ki-67, and (D) cleaved caspase-3 expression in H460 xenograft tumors. Original magnification, ×200. The IOD of (E) Cul4A, (F) p33ING1b, (G) Ki-67, and (H) cleaved caspase-3 was quantified. The data points represent the mean IOD ± the standard deviations of three representative xenograft tumors. $^{*}P<0.05$. (I) Reverse transcription quantitative polymerase chain reaction analysis of the Cul4A and p33ING1b expression levels in the tumors of the NCI-H460 mice xenograft models transfected with AdEV and AdsiCul4A with or without paclitaxel treatment. The internal control was actin. IHC, immunohistochemistry; Cul4A, cullin 4A; p33ING1b, p33 inhibitor of growth 1b; IOD, integrated optical density; Ad, adenovirus; EV, empty vector; si, small interfering RNA.

expression of cleaved PARP decreased after the knockdown of p33ING1b (**Figure 5E-G**). Our study results showed that decreased p33ING1b levels may decrease the apoptosis related to the Cul4A knockdown and paclitaxel treatment.

Regulation of p33ING1b expression by Cul4A-mediated protein degradation

The lung cancer cell expression levels of p33ING1b mRNA after the Cul4A knockdown by siRNA were analyzed using RT-qPCR; no significant changes in the p33ING1b mRNA expressions were observed (**Figure 6A**). Therefore, Cul4A was hypothesized to regulate p33ING1b through ubiquitination and protein degradation. The protein degradation assays indicated that the p33ING1b degradation rate in the A549 and NCI-H460 cells with the Cul4A knockdown was less than it was in the cells transfected with EV (**Figure 6B** and **6C**). By contrast, a higher p33ING1b degradation rate was observed in the Cul4A-overexpressing A549 and NCI-H460 lung cancer cells (**Figure 6D** and **6E**).

Regulation of the p33ING1b expressions by the Cul4A-mediated ubiquitination

To investigate the interaction between p33ING1b and Cul4A, a reciprocal coimmunoprecipitation assay was performed. An interaction between the two proteins was observed (**Figure 7A**). In addition, the ubiquitination assay indicated that the p33ING1b ubiquitination was mediated by Cul4A (**Figure 7B**).

Discussion

The present results revealed that the Cul4A knockdown in the lung cancer cells upregulated the expressions of p33ING1b, a tumor suppressor. The Cul4A knockdown also increased the cell and tumor xenograft chemosensitivity to paclitaxel. The combination of the transfection with Cul4A siRNA with the paclitaxel treatment also reduced the proliferation and increased the apoptosis in the tumor xenografts. The overexpression and knockdown of p33ING1b upregulated and downregulated the lung cancer cell chemosensitivity to paclitaxel, respectively. The results also demonstrate that the Cul4A knockdown reduced the p33ING1b degradation, but the Cul4A overexpression increased its degradation. Finally, Cul4A was found to be involved in the p33ING1b ubiquitination. Our findings suggest that Cul4A regulates the lung cancer cell chemosensitivity to paclitaxel by regulating p33ING1b.

Upregulated Cul4A expression can increase the chemoresistance to various chemotherapeutic agents, including cisplatin, gemcitabine, docetaxel [10] and doxorubicin [34]. The present results suggest that Cul4A has an association with the chemosensitivity of lung cancer cells to paclitaxel; the results also provide additional evidence that Cul4A has a crucial role in regulating the chemosensitivity to chemotherapy drugs. The present findings concur with the results published in another study, in which MLN4924, a NEDD8-activating enzyme small molecule inhibitor, increased the chemosensitivity of cancer cells to paclitaxel [35]. Because Cul4A is an E3 ubiquitin ligase family member whose activity requires cullin neddylation, MLN4924 may block such neddylation, indirectly inhibiting Cul4A. Cul4A activity has been reported in various types of cancer; therefore, therapeutic strategies targeting Cul4A may be a promising adjuvant treatment method. The current study also showed that the Ad-mediated Cul4A siRNA transfection with the paclitaxel treatment reduced the growth of the tumors and increased the apoptosis in the lung cancer xenograft mice, indicating that the combined treatment strategy is clinically relevant for future lung cancer therapy. However, further research to validate these findings is warranted.

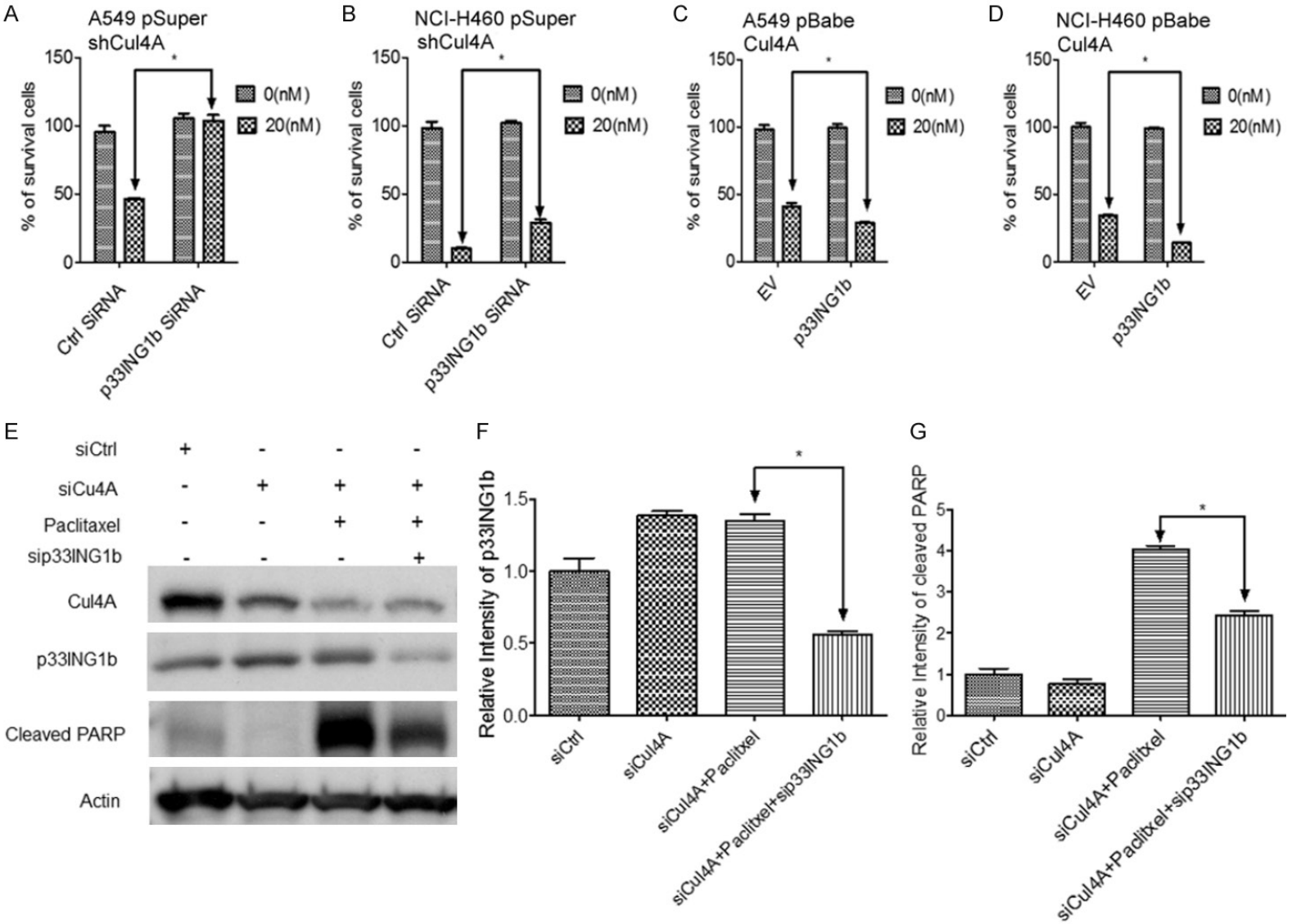

Figure 5. p33ING1b is associated with chemosensitivity to paclitaxel and apoptosis in lung cancer cells. Cytotoxicity of shCul4A-transfected (A) A549 and (B) NCI-H460 cells treated with paclitaxel. For both cell types, control siRNA or p33ING1b siRNA transfection for 96 h was performed and followed by 72 h of treatment with the indicated concentrations of paclitaxel. The groups not treated with paclitaxel were used to normalize the proportions of the surviving cells. Data are presented as the three-experiment mean ± standard deviation. $^{*}P<0.05$. Cytotoxicity of (C) A549 and (D) NCI-H460 lung cancer cells overexpressing Cul4A and treated with paclitaxel. Both of the Cul4A-overexpressing lung cancer cell types were transfected for 96 h with either the EV or the pCMV6-p33ING1b-GFP vector and subse-

quently subjected to 72 h of paclitaxel treatment. *The non-paclitaxel-treated group was used to normalize the proportion of the surviving cells. (E) Western blot analysis of Cul4A, p33ING1b, and cleaved PARP in H460 lung cancer cells treated with 100 nM of control siRNA (siCtrl), Cul4A siRNA (siCul4A), p33ING1b siRNA (sip33ING1b) and 100 nM of paclitaxel for 72 h. (F) The p33ING1b and (G) cleaved PARP expression levels in Western blot analysis. The p33ING1b and cleaved PARP expression levels were normalized to actin with the siCtrl group as the control. The data represent three-experiment means ± standard deviations. *P<0.05. sh, short hairpin RNA; Cul4A, cullin 4A; p33ING1b, p33 inhibitor of growth 1b; siRNA, small interfering RNA; EV, empty vector.

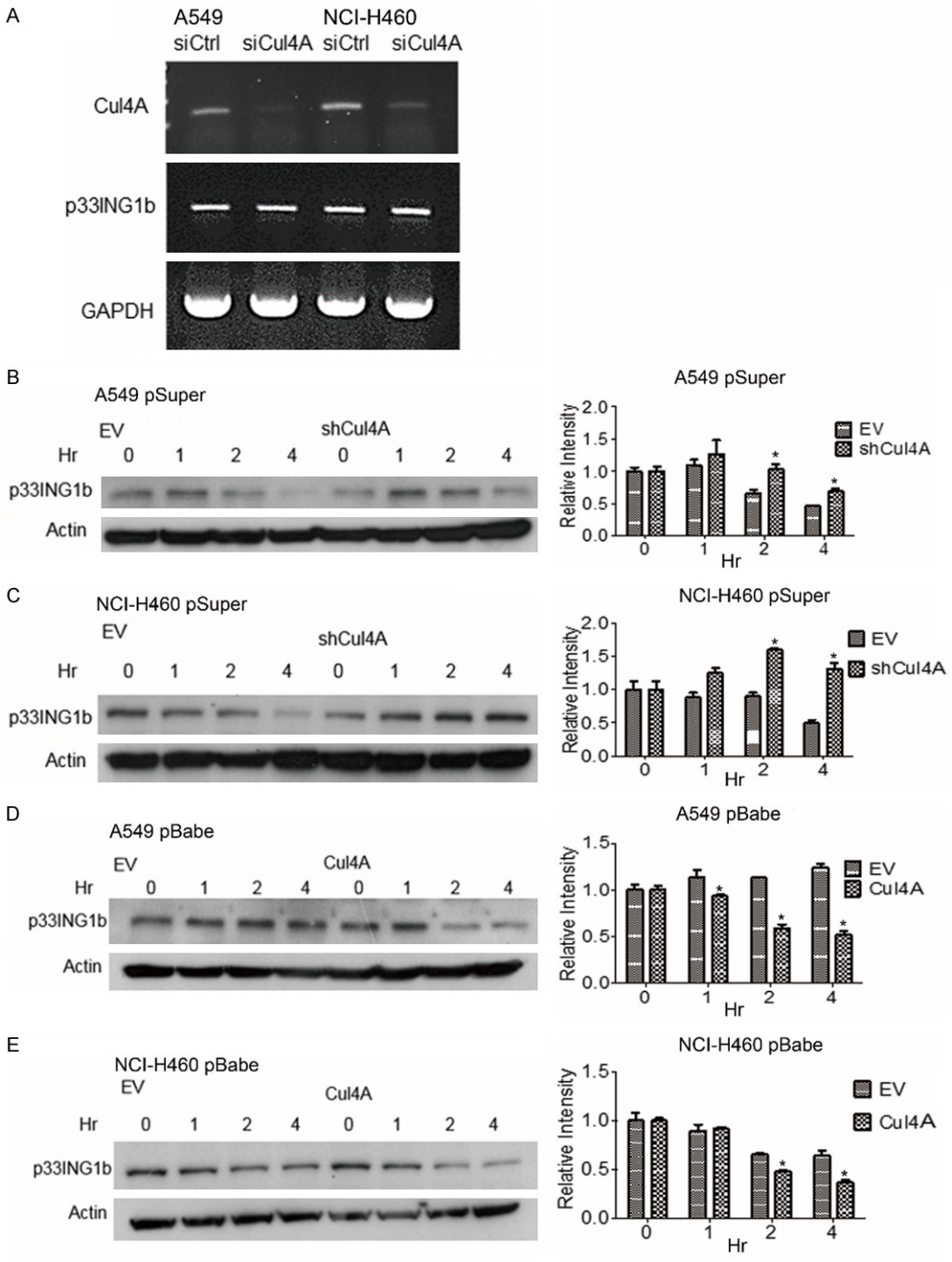

Figure 6. Cul4A mediates p33IN1b protein degradation in lung cancer cells. (A) Reverse transcription quantitative polymerase chain reaction analysis of Cul4A and p33ING1b expression in siCtrl- or siCul4A-transfected A549 and NCI-H460 cells. The internal control was GAPDH. To analyze the protein degradation, the p33ING1b expressions in the (B) A549 and (C) NCI-H460 cells stably transfected with shCul4A or EV were analyzed. Lung cancer cells were incubated for the indicated durations with 20 μg/mL cycloheximide. The p33ING1b expression was analyzed through western blotting of (D) A549 and (E) NCI-H460 cells stably transfected with EV or Cul4A overexpression plasmid. Lung cancer cells were incubated for the indicated durations with 20 μg/mL cycloheximide. The p33ING1b protein expression levels were normalized to actin with the 0-h group as the control. The bar graphs show the relative p33ING1b expression in Western blot analysis. $^{*}P<0.05$ (compared with the EV group). Cul4A, cullin 4A; p33ING1b, p33 inhibitor of growth 1b; Ctrl, control; si, small interfering RNA; sh, short hairpin RNA; EV, empty vector.

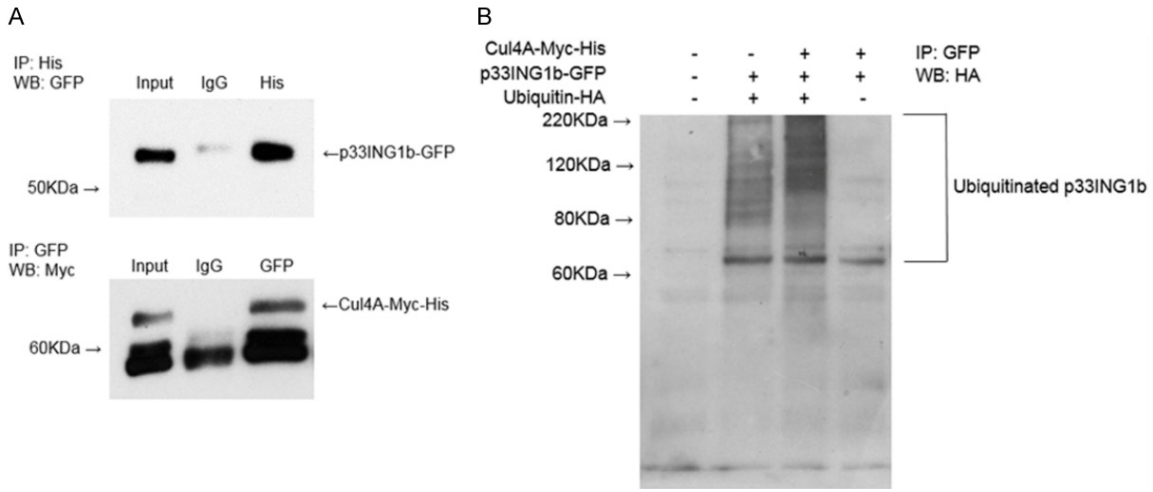

Figure 7. Cul4A mediates p33ING1b ubiquitination in lung cancer cells. A. Cul4A and p33ING1b immunoprecipitation. The 293T cells underwent co-transfection with pBABE-Cul4A-Myc-his and pCMV6-p33ING1b-GFP vectors. Anti-GFP for the pCMV6-p33ING1b-GFP vector and anti-His tag antibody for the pBABE-Cul4a-Myc-his vector were employed for the immunoprecipitation. B. *In vivo* effects of p33ING1b and Cul4A overexpression on ubiquitination. The transfection of the 293T cells was performed using pBABE-Cul4A-Myc-his, pCMV6-p33ING1b-GFP, and pRK5-HA-Ubiquitin-wild-type vectors. Prior to the lysis, all the cells were treated for 24 h with 10 μM MG132. Anti-GFP antibody was used to perform the immunoprecipitation, and anti-HA antibodies were employed to perform the Western blotting analysis. Cul4A, cullin 4A; p33ING1b, p33 inhibitor of growth 1b.

To our knowledge, research on the function of p33ING1b in lung cancer has been insufficient. The present findings reveal that p33ING1b regulates the lung cancer cell chemosensitivity to paclitaxel. In osteosarcoma, the underlying mechanism of the increase in chemosensitivity induced by the p33ING1b expression was found to be associated with the upregulated Bax, p21, and p53 protein expressions in addition to the caspase 3 activation [28]. Notably, it was hypothesized that p33ING1b physically binds to the tumor suppressor p53 and activates the transcription mediated by p53 while inhibiting the cell proliferation [36]. Moreover, p33ING1b has been reported to upregulate the expressions of the cell cycle regulatory and proapoptotic proteins, such as the p21 cyclin-dependent kinase inhibitor [37] and Bax [38], respectively. Another study reported an increased chemosensitivity to paclitaxel in both mutant (H157) and wild-type (A549 and H460) p53 lung cancer cells [39]. Because Cul4A knockdown also upregulates the p53 and p21 expression levels, the data in the current study strongly suggest that a complex set of pathways modulates increased chemosensitivity to paclitaxel after the Cul4A knockdown. However, p33ING1b still plays an essential role in mediating the chemosensitivity to paclitaxel in lung cancer.

In conclusion, our findings show that Cul4A may mediate lung cancer cell chemosensitivity to paclitaxel by regulating p33ING1b expression. p33ING1b is also associated with this chemosensitivity. These findings may be useful for the development of therapeutic strategies that target Cul4A in patients with lung cancer.

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Disclosure of conflict of interest

None.

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