

ORIGINAL ARTICLE

Ubiquitin C-terminal hydrolase L1 promotes expression of programmed cell death-ligand 1 in non-small-cell lung cancer cells

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Abstract

Programmed cell death-ligand 1 (PD-L1) expressed on cancer cells can cause immune escape of non-small-cell lung cancer (NSCLC). Elucidation of the regulatory mechanisms of the PD-L1 expression is a prerequisite for establishing new tumor immunotherapy strategies. Ubiquitin C-terminal hydrolase L1 (UCHL1) is a regulator of cellular signaling transduction that is aberrantly expressed in NSCLC. However, it is not known whether UCHL1 regulates the expression of PD-L1 in NSCLC cells. In the present study, we found that UCHL1 promotes the expression of PD-L1 in NSCLC cell lines. In addition, UCHL1 expressed in NSCLC cells inhibited activation of Jurkat cells through upregulation of PD-L1 expression in *in vitro* experiments. Moreover, UCHL1 upregulates PD-L1 expression through facilitating activation of the AKT-P65 signaling pathway. In conclusion, these results indicated that UCHL1 promoted PD-L1 expression in NSCLC cells. This finding implied that inhibition of UCHL1 might suppress immune escape of NSCLC through downregulation of PD-L1 expression in NSCLC cells.

KEYWORDS

AKT, NSCLC, P65, PD-L1, UCHL1

1 | INTRODUCTION

The PD-L1 molecule is normally expressed on immune cells or in immune-privileged tissues. It can bind to its receptor PD-1 expressed in activated T cells, resulting in inhibition of T cell activation to prevent

immunopathological damage.¹ The PD-L1 molecule is also expressed in multiple cancer cells, including NSCLC cells, and constitutes an important immune escape mechanism in cancers. Programmed cell death-ligand 1 expressed by cancer cells can engage PD-1 expressed by cancer-specific CTLs, limiting the antitumor function of the CTLs.²

Abbreviations: CTL, cytotoxic T lymphocytes; FACS, fluorescence-activated cell sorting; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1; RT-PCR, reverse transcription PCR; siNC, siRNA of negative control; siRNA, small interfering RNA; UCH, ubiquitin C-terminal hydrolase.

Mao and Tan contributed equally to this work.

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Based on the immunosuppressive role of the PD-L1/PD-1 pathway, blocking Abs against PD-L1 or PD-1 have been used in clinical cancer immunotherapy with great success. Especially in patients with PD-L1-expressing NSCLC, therapy with Ab against PD-L1 improves overall survival by 5 months compared to standard chemotherapy.³ However, there are some disadvantages in the Ab therapy. One is insusceptibility or resistance to this treatment.^{4,5} The other is adverse effects, including organ-specific immune-related adverse events, eg colitis, hepatitis, pneumonitis, and hypothyroidism.⁶ Therefore, it is essential to research the regulatory mechanisms of the expression of PD-L1 in cancer cells. Clarification of these mechanisms is a prerequisite for development of new methods to suppress the PD-L1-mediated immune evasion of NSCLC.

Expression of PD-L1 in malignant cells is classified into inducible expression and constitutive expression according to the cause of PD-L1 expression. The inducible expression is caused by extrinsic stimuli, such as pro-inflammatory cytokines in the tumor microenvironment. The constitutive expression is caused by genetic alteration, such as oncogene mutation.⁷ Both forms of PD-L1 expression involve of cellular signaling cascades. For example, the oncogene RAS mutation is cancer-driving and increases cancer cell-intrinsic PD-L1 expression through MEK/ERK signaling.⁸ Tumor necrosis factor- α induces PD-L1 expression in cancer cells mainly through NF- κ B signaling.⁹⁻¹¹ Additionally, both forms of PD-L1 expression in cancer cells are regulated in multiple layers, such as chromatin modification, transcription, posttranscription, translation, and posttranslation.^{1,7}

Ubiquitination and deubiquitination are the most versatile post-translation modifications, participating in a plethora of biological processes, such as cell growth, differentiation, transcriptional regulation, and oncogenesis. Deubiquitination is mediated by deubiquitinases, which are emerging as important regulators of many pathways associated with cancer. They can regulate the stability of key oncogenic proteins or ubiquitin-dependent oncogenic signaling cascades.¹² Ubiquitin C-terminal hydrolase L1 is a member of the UCH subgroup of deubiquitinases and is expressed mainly in brain, testis, ovary, and placenta among normal tissues.^{13,14} It is also highly expressed in several forms of cancer, including NSCLC. Ubiquitin C-terminal hydrolase L1 is aberrantly expressed in NSCLC compared with normal lung tissues. Moreover, UCHL1 expression is strongly associated with the pathological stage of cancer.¹⁵ In addition, there is in vivo experimental evidence that UCHL1 transgenic mice have a striking tumor-prone phenotype with the development of lung tumors.¹⁶ These data therefore indicate that UCHL1 plays an oncogenic role in NSCLC. However, the oncogenic mechanisms are still elusive.

The expression of UCHL1 in the immune privileged organs, such as brain, testis, and placenta, implies its possible association with cancer immune evasion. Thus, we asked whether UCHL1 regulates expression of PD-L1 in NSCLC cells. In the present study, we found that UCHL1 promoted expression of PD-L1 in NSCLC cell lines through loss- and gain-of-function experiments. In addition, UCHL1 expression in NSCLC cells inhibited Jurkat cell activity through upregulation of PD-L1 in cancer cells. Furthermore, UCHL1 upregulated PD-L1 expression through the enhanced AKT-p65 signaling axis.

2 | MATERIALS AND METHODS

2.1 | Cancer cells and reagents

Human NSCLC cell lines (NCI-H460 and A549) and Jurkat cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Science. The cells were maintained at 37°C in DMEM. The media were supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells were incubated in a well-humidified incubator with 5% CO₂.

To construct recombinant plasmid PRK5-UCHL1, the coding sequence of the human *UCHL1* gene from NCI-H460 cells was cloned into plasmid PRK5. The recombinant PRK5-UCHL1 codes for UCHL1 protein tagged with a Flag epitope at its C-terminal. The recombinant plasmid of pCMV3-PD-L1 was obtained from Sino Biological Company (Cat# HG10084-CH). All siRNAs were synthesized by Sigma-Aldrich. Two siRNAs targeting the *UCHL1* gene were designated as siUCHL1 #1 (5'-GGACAAGAAGUUAGUCCUATT-3') and siUCHL1 #2 (5'-GCACAAUCGGACUUAUUCATT-3'). The siRNA targeting the *P65* gene was designated as siP65 (5'-GGAGUACCCUGAGGCUAUATT-3'). The siRNA targeting PD-L1 was designated as siPD-L1 (5'-CACUAAUUGUCUAUUGGGATT-3'). The siNC represents the negative control siRNA (5'-UUCUCCGAACGUGUCACGUTT-3'). Transfection reagent RNAiMAX (Invitrogen) was used to transfect siRNA into NSCLC cells, and Lipofectamine 3000 (Invitrogen) was used for transfection of the plasmids. The UCHL1 inhibitor LDN-57444 was purchased from MCE company (Cat# HY-18637).

2.2 | Quantitative RT-PCR

Briefly, the total RNA of NSCLC cells was extracted with the RNA isolation kit RN07-EASYspin (Aidlab Biotechnologies) and transcribed into cDNAs using a Reverse Transcription System (Takara). Quantitative PCR was carried out using 2 \times SYBR Green qPCR Mix (Aidlab Biotechnologies) with primers specific for the *PD-L1* gene (forward, 5'-TGTGGCATCCAAGATACAAACTCAAAG-3'; reverse, 5'-TCCTCTCTGCTTTCGCCAGGTT-3'), the *UCHL1* gene (forward, 5'-AAGCCAATGTCGGGTAGAT-3'; reverse, 5'-ATGGTTCACCGGAAAAGGC-3'), and the β -actin gene (forward, 5'-GACTACCTCATGAAGATCCTCACC-3'; reverse, 5'-TCTCCTTAATGTCACGACGATT-3'). The β -actin gene served as an internal control. Relative expression of the target genes to the internal control gene was calculated using the formula: Relative expression = 2^{- Δ CT}, where Δ CT = CT_{target gene} - CT_{internal control}.^{17,18}

2.3 | Western blot assay

The total protein was extracted and subjected to electrophoresis. Cell lysates were prepared by using RIPA protein extraction reagent (Beyotime Biotechnology) and protein concentrations

were determined by Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Samples containing equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Amersham Hybond). Membranes were blocked by 5% BSA in TBS/0.1% Tween-20, then incubated overnight at 4°C. The expression level of each protein was detected by an enhanced chemiluminescence system (Vazyme Biotech). The primary Abs anti-PD-L1, anti-UCHL1, anti-STAT1, anti-phospho-STAT1, anti-ERK1/2, anti-phospho-ERK1/2, anti-P65, and anti-phospho-P65 were purchased from Cell Signaling Technology with catalog numbers E1L3N, D3T2E, 7649P, 9175S, 4695P, 4370S, 3033T, and 8242T, respectively. The primary Abs anti-AKT and anti-phospho-AKT were purchased from Epitomics with catalog numbers 1085-S and 2214-S, respectively. The primary Abs also included anti-Flag (Cat# F1804; Sigma-Aldrich) and anti- β -actin (Cat# PR-0255; ZSGB-BIO). The secondary Abs were goat anti-rabbit IgG Ab or goat anti-mouse IgG Ab (Cat# ZB-2301 or Cat# ZB-2305; ZSGB-Bio).

2.4 | Flow cytometry

Flow cytometry was used to quantify the expression of PD-L1 on NSCLC cells. The NSCLC cells were harvested and washed twice with ice-cold FACS buffer (0.5% of FBS in PBS). The cells were re-suspended in 100 μ L FACS buffer and stained with Abs conjugated with fluorescein for 40 minutes at 4°C. Anti-human-PD-L1 PE (329706; BioLegend) was used to detect the expression of PD-L1. Anti-human-PD-1 FITC (329903, BioLegend) was used to detect the PD-1 expression on Jurkat cells.

2.5 | Dual-luciferase reporter assay

The recombinant plasmid pGL3-PD-L1 containing the promoter region of the *PD-L1* gene was constructed based on promoterless firefly luciferase reporter vector pGL3-Basic (Promega) in our previous studies.¹⁹ Based on the pGL3-PD-L1, reporter plasmids pGL3-Mutp65 containing mutation of 2 binding sites of transcription factor P65 was constructed by site-directed mutagenesis.²⁰ *Renilla* luciferase plasmid pRL-TK served as the internal control. In order to determine whether UCHL1 regulates transcription of the *PD-L1* gene, a mixture of pGL3-PD-L1 and pRL-TK was cotransfected into NCI-H460 cells pretransfected with siNC or siUCHL1, or A549 cells pretransfected with PRK5 or PRK5-UCHL1. In order to determine whether UCHL1 inhibits transcription of the *PD-L1* gene through P65, A549 cells were transfected with PRK5 or PRK5-UCHL1, then cotransfected with a mixture of pGL3-MutP65 and pRL-TK, or a mixture of pGL3-PD-L1 and pRL-TK. Finally, all the cells were lysed and the luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.6 | Jurkat coculture and IL-2 production assay

Jurkat cells were pretreated with 10 μ g/mL PHA (Cat# P8090, Solarbio) for 48 hours so that the PD-1 expression was induced. The NSCLC cells (A549 or NCI-H460) were pretreated with plasmid (pRK5 or pRK5-UCHL1) or siRNA (siNC, siUCHL1, or siPD-L1) for 48 hours. The pretreated NSCLC cells (3×10^5) and pretreated Jurkat cells (3×10^5) were cocultured in U-bottomed 96-well plates in the presence of protein complex containing anti-CD3 and anti-CD28 Fab (Cat# 6-8901-000; IBA). After coculture for 48 hours, the supernatants were harvested for quantification of IL-2 by an ELISA kit (Cat# RK00002; ABclonal).

2.7 | Statistical analysis

GraphPad Prism software was used for statistical analysis of the experimental data. The data are presented as mean \pm SD. The difference between 2 experimental groups in the RT-PCR, flow cytometry, ELISA, or dual-luciferase reporter assay was analyzed by Student's unpaired *t* test. A probability level of 0.05 was considered to indicate a significant difference.

3 | RESULTS

3.1 | Ubiquitin C-terminal hydrolase L1 promotes PD-L1 expression in NSCLC cells

As deubiquitinase protein is druggable, we attempted to find deubiquitinases that can regulate the expression of PD-L1. First, we analyzed the expression profile of 93 deubiquitinases in NSCLC tissues on the GEPIA website (<http://gepia.cancer-pku.cn/>) (Figure S1). We selected 11 deubiquitinases that were highly expressed in these lung cancers to synthesize their siRNAs for screening the deubiquitinases that regulate expression of PD-L1 in NCI-H460 cells. The preliminary results showed that UCHL1 had potential to regulate PD-L1 expression (Figure S2). We used flow cytometry and western blot analysis to confirm that knockdown of UCHL1 inhibited PD-L1 expression in NCI-H460 cells. We also used an inhibitor of UCHL1 enzyme to inhibit the activity of UCHL1 (Figure 1A). We found that the UCHL1 inhibitor resulted in decreased expression of PD-L1 protein in NCI-H460 cells (Figure 1B). We evaluated UCHL1 expression in NCI-H460 and A549 cells and found that UCHL1 expression was lower in A549 cells than in NCI-H460 cells (Figure S3). Therefore, experiments for overexpression of UCHL1 were carried out in A549 cells. We found that overexpression of UCHL1 in A549 cells transfected with recombinant plasmid pRK5-UCHL1 gave rise to upregulated PD-L1 expression (Figure 1C). These results indicated that UCHL1 promoted expression of PD-L1 protein in NSCLC cells.

Next, we examined whether UCHL1 promoted PD-L1 expression at the mRNA level. First, quantitative RT-PCR was used to determine the expression of PD-L1. The results showed that knockdown

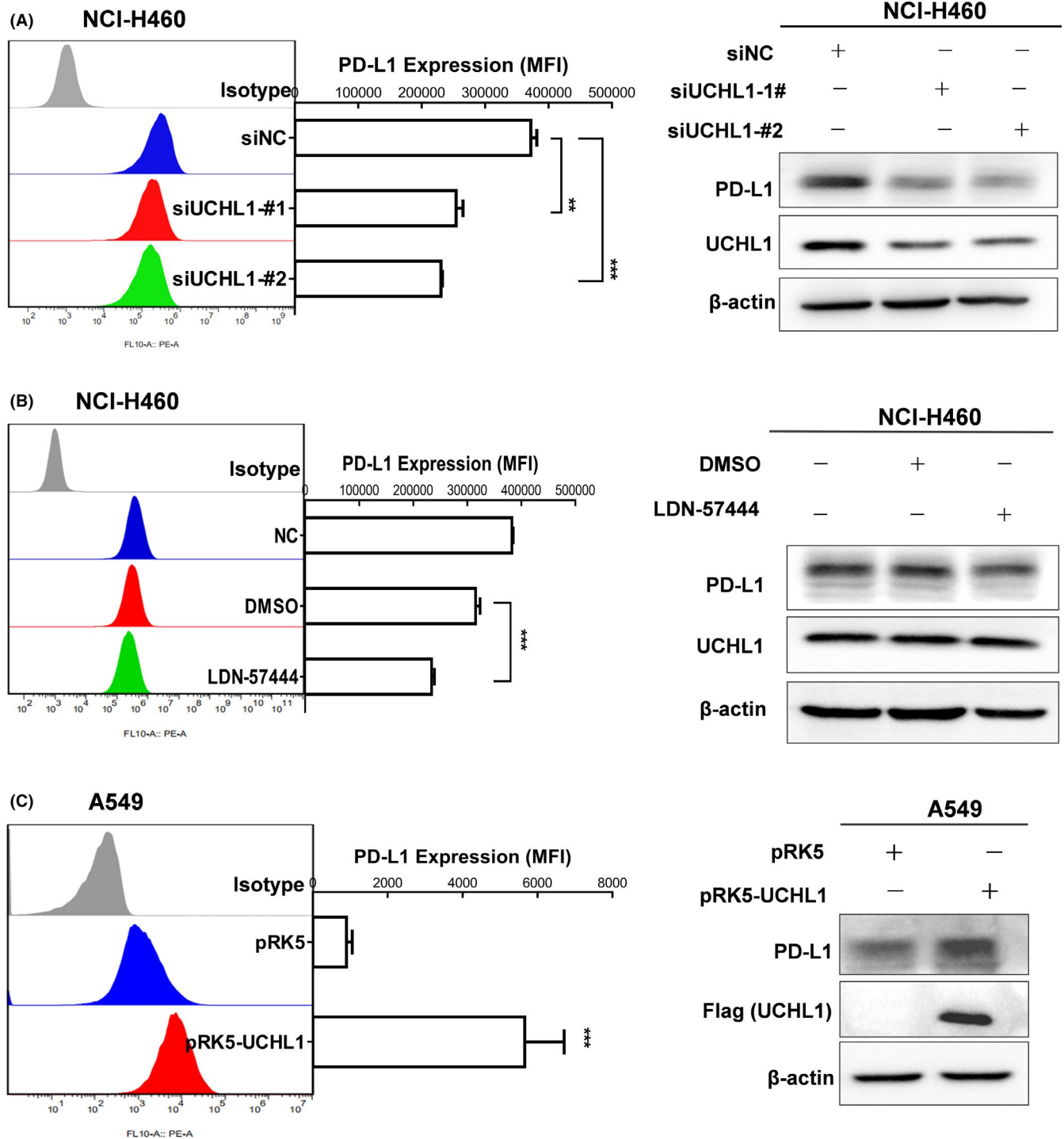


FIGURE 1 Ubiquitin C-terminal hydrolase L1 (UCHL1) promoted expression of programmed cell death-ligand 1 (PD-L1) at the protein level in non-small-cell lung cancer cells. A, NCI-H460 cells were transfected with siUCHL1 or negative control siRNA (siNC) for 48 h. Expression of PD-L1 was quantified by flow cytometry and western blot assay. B, NCI-H460 cells were treated with UCHL1 inhibitor LDN-57444 (10 μ M) for 48 h. Expression of PD-L1 was quantified by flow cytometry and western blot assay. C, A549 cells were transfected with pRK5 or pRK5-UCHL1 for 48 h. Expression of PD-L1 was quantified by flow cytometry and western blot assay. Similar results were obtained in 2 independent experiments carried out in triplicate. Data are shown as mean \pm SD. ** P < .01, *** P < .001. MFI, mean fluorescence intensity

of UCHL1 in NCI-H460 cells led to decreased mRNA expression of PD-L1 (Figure 2A,B). By contrast, the overexpression of UCHL1 in A549 cells promoted PD-L1 expression at the mRNA level (Figure 2C). Moreover, we undertook bioinformatic analyses of the

relationship between PD-L1 mRNA expression and UCHL1 mRNA expression in NSCLC using microarray data from R2: Genomics Analysis and Visualization Platform (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) and RNA sequencing data from the UCSC Xena

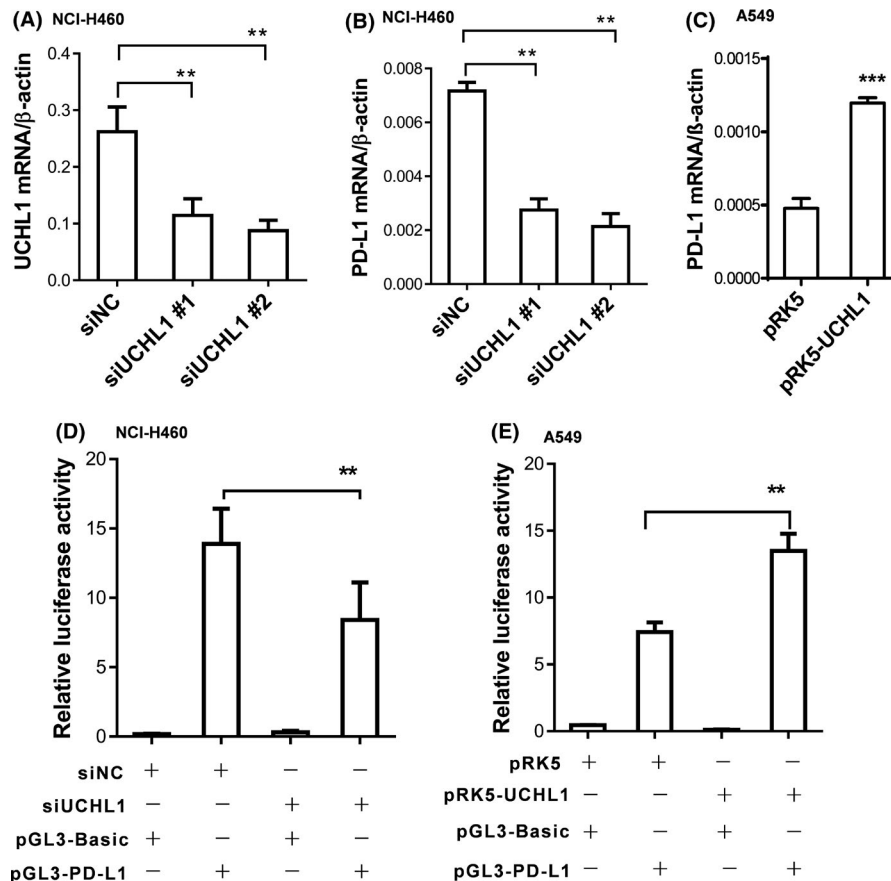


FIGURE 2 Ubiquitin C-terminal hydrolase L1 (UCHL1) promoted transcription of the *PD-L1* gene in non-small-cell lung cancer cells. NCI-H460 cells were transfected with siUCHL1 or negative control siRNA (siNC) for 24 h. The mRNA expression of *UCHL1* (A) or *PD-L1* (B) was detected by quantitative RT-PCR assay. C, A549 cells were transfected with pRK5 or pRK5-UCHL1 for 24 h and the mRNA expression of *PD-L1* was detected by quantitative RT-PCR. D, NCI-H460 cells were transfected with siNC or siUCHL1 for 24 h, then cotransfected with a mixture of pGL3-Basic + pRL-TK or pGL3-PD-L1 + pRL-TK for 24 h. Relative luciferase activities were measured by dual-luciferase reporter assay. E, A549 cells were transfected with pRK5 or pRK5-UCHL1 for 24 h, then cotransfected with a mixture of pGL3-Basic + pRL-TK or pGL3-PD-L1 + pRL-TK for 24 h. Relative luciferase activities were measured by dual-luciferase reporter assay. The experiment was carried out in triplicate and repeated at least twice. Data are shown as mean \pm SD. ** $P < .01$, *** $P < .001$

platform (<http://xena.ucsc.edu/>).²¹ We found that there was a correlation between them (Figure S4). To further elucidate the causes for the changed mRNA level of *PD-L1*, we undertook a dual-luciferase reporter assay to measure whether UCHL1 promoted the transcription of the *PD-L1* gene in NSCLC cells. The results showed that knockdown of UCHL1 expression decreased the *PD-L1* promoter activity in NCI-H460 cells (Figure 2D), whereas overexpression of UCHL1 increased that in A549 cells (Figure 2E). All these results suggest that UCHL1 promoted *PD-L1* expression in lung cancer cells at the transcriptional level.

3.2 | Ubiquitin C-terminal hydrolase L1 upregulated *PD-L1* expression in NSCLC cells, resulting in suppression of T cell activity

As UCHL1 upregulated the expression of *PD-L1* in lung cancer cells, we asked whether this event impaired the activity of cocultured T cells. We used PHA to simulate Jurkat cells to express *PD-1*, the

receptor of *PD-L1* (Figure 3A). Then we used a protein complex containing CD3 and CD28 Ab Fab to activate the Jurkat cells to produce IL-2 in the presence or absence of the lung cancer cells (Figure 3B). We found that the activated Jurkat cells cocultured with NCI-H460 cells transfected with siUCHL1 produced more IL-2 than those cocultured with NCI-H460 cells transfected with siNC. By contrast, overexpression of UCHL1 in A549 cells inhibited the production of IL-2 by the cocultured Jurkat cells (Figure 3C). These results indicated that UCHL1 expression in cancer cells led to suppression of T cell activity. To clarify that the suppression of T cell activity caused by UCHL1 expression in cancer cells was mediated by the upregulated *PD-L1*, we undertook rescue experiments. Overexpression of UCHL1 in A549 cells led to suppression of IL-2 secretion from the cocultured Jurkat cells. But simultaneous knockdown of *PD-L1* restored the IL-2 production by the cocultured Jurkat cells (Figure 3D). The siPD-L1 can efficiently silence the expression of *PD-L1* in NCI-H460 cells (Figure 3E,F). These results suggested that UCHL1 promoted *PD-L1* expression in NSCLC cells to suppress the activity of T cells.

3.3 | Ubiquitin C-terminal hydrolase L1 promoted PD-L1 expression through Akt-P65 signaling axis

Previous studies reported that activation of the NF- κ B signaling pathway led to increased expression of PD-L1 in lung cancer cells.²² In line with the research, our results showed that knockdown of P65 decreased PD-L1 expression at both protein and mRNA levels in NCI-H460 cells (Figure 4A,B). Next, we asked whether UCHL1 promoted PD-L1 expression through the P65 signaling pathway. Knockdown of UCHL1 in NCI-H460 cells decreased, whereas overexpression of UCHL1 in A549 cells increased, phosphorylation of P65 (Figure 4C,D). Furthermore, a rescue experiment was undertaken by simultaneous overexpression of UCHL1 and knockdown of P65 in A549 cells. Overexpression of UCHL1 alone induced elevated expression of PD-L1. Simultaneous knockdown of P65 attenuated the upregulation of PD-L1 (Figure 4E). These results indicated that UCHL1 promoted PD-L1 expression through the P65 signaling pathway. Previous studies showed transcription factor P65 bound to the promoter region of the *PD-L1* gene to initiate the mRNA expression

of PD-L1. Therefore, we used the dual-luciferase reporter assay to undertake rescue experiments to clarify that UCHL1 promoted PD-L1 expression through P65 signaling. Overexpression of UCHL1 increased the activity of the promoter region of PD-L1 but did not increase the activity of the PD-L1 promoter region with mutation of p65 binding sites (Figure 4F). This result indicated that UCHL1 promoted the transcription of the *PD-L1* gene, depending on P65 signaling.

Previous studies also showed that activation of the PI3K/Akt signaling pathway gave rise to expression of PD-L1 in lung cancer cells.^{23,24} In agreement with these reports, we found that knockdown of Akt in NCI-H460 cells led to decreased expression of PD-L1, not only at the protein level but also at the mRNA level (Figure 5A,B). Subsequently, we asked whether UCHL1 promoted PD-L1 expression through Akt signaling. Knockdown of UCHL1 in NCI-H460 cells contributed to reduced phosphorylation of Akt, whereas overexpression of UCHL1 in A549 cells led to increased phosphorylation of Akt (Figure 5C,D). Rescue experiments were undertaken with simultaneous overexpression of UCHL1 and knockdown of Akt in A59

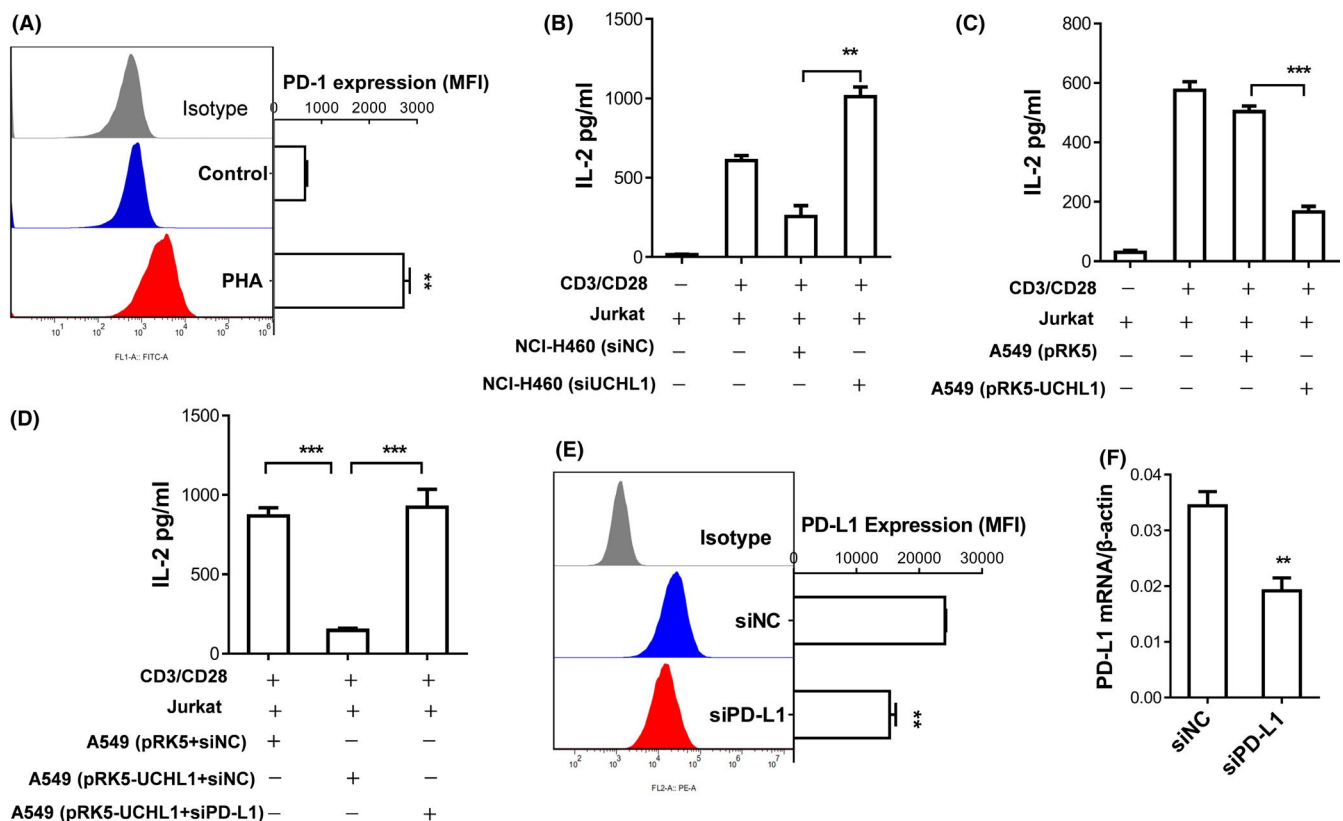


FIGURE 3 Non-small-cell lung cancer cells with high expression of ubiquitin C-terminal hydrolase L1 (UCHL1) suppressed activity of T cells depending on programmed cell death-ligand 1 (PD-L1). A, Jurkat cells were activated by phytohemagglutinin (PHA; 10 μ g/mL) for 48 h. Expression of programmed cell death protein 1 (PD-1) was measured by flow cytometry. B, NCI-H460 cells were transfected with negative control siRNA (siNC) or siUCHL1, then cocultured with activated Jurkat cells for 48 h. The concentration of interleukin-2 (IL-2) in the supernatant of the coculture was detected by ELISA. C, A549 cells were transfected with pRK5 or pRK5-UCHL1, then cocultured with activated Jurkat cells for 48 h. The concentration of IL-2 in the supernatant from the coculture was detected by ELISA. D, A549 cells were transfected with pRK5, pRK5-UCHL1, siNC, or siPD-L1, then cocultured with activated Jurkat cells for 48 h. The concentration of IL-2 in the supernatant from the coculture was detected by ELISA. E, F, The efficacy of siPD-L1 was assessed by flow cytometry and RT-PCR. Similar results were obtained in 2 independent experiments carried out in triplicate. Data are shown as mean \pm SD. ** P < .01, *** P < .001. MFI, mean fluorescence intensity

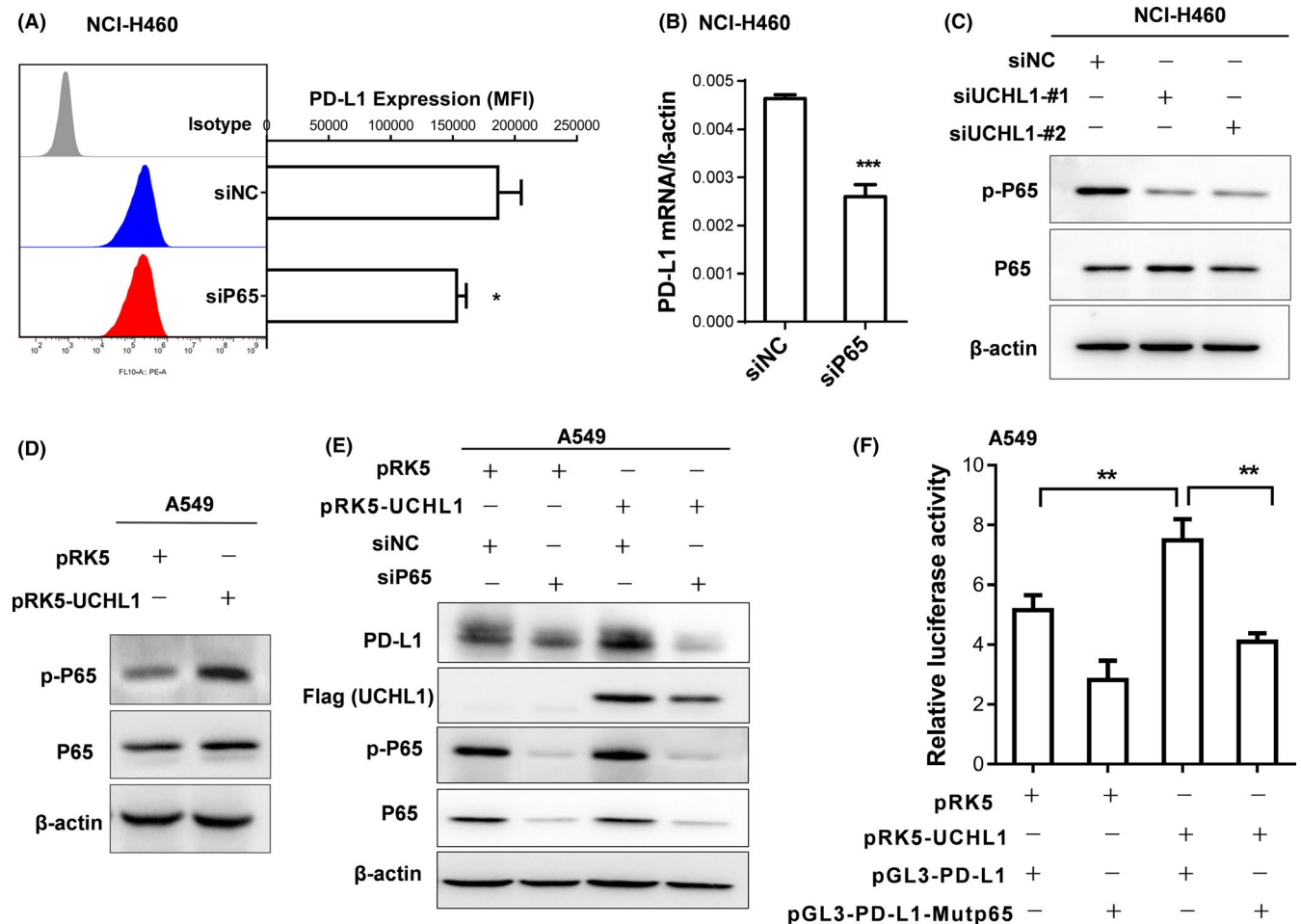


FIGURE 4 Ubiquitin C-terminal hydrolase L1 (UCL1) promoted programmed cell death-ligand 1 (PD-L1) expression through the P65 signaling pathway. A, B, NCI-H460 cells were transfected with negative control siRNA (siNC) or siP65 for 48 h. Expression of PD-L1 was quantified by flow cytometry and RT-PCR. C, NCI-H460 cells were transfected with siNC or siUCL1 for 48 h. The activation of P65 was measured by western blot. D, A549 cells were transfected with pRK5 or pRK5-UCL1 for 48 h. Activation of P65 was measured by western blot. E, A549 cells were transfected with pRK5, pRK5-UCL1, siNC, or siP65 for 48 h. Expression of PD-L1 and the activation of P65 were determined by western blot. F, A549 cells were transfected with pRK5 or pRK5-UCL1 for 24 h, then cotransfected with pRL-TK + pGL3-PD-L1 or pRL-TK + pGL3-Mutp65 for 24 h. Relative luciferase activity was measured by dual-luciferase reporter assay. Western blot assays were carried out at least twice. Flow cytometry, quantitative RT-PCR, and dual-luciferase reporter assay were carried out in triplicate and repeated at least twice. Data are shown as mean \pm SD. * $P < .05$, ** $P < .01$, *** $P < .001$. MFI, mean fluorescence intensity

cells. Overexpression of UCL1 alone promoted PD-L1 expression, but simultaneous knockdown of Akt attenuated the upregulation of PD-L1 (Figure 5E,F). These results indicated that UCL1 promoted PD-L1 expression depending on Akt signaling.

As UCL1 promoted PD-L1 expression relying on both P65 and Akt signaling in NSCLC cells, the relationship between Akt signaling and P65 signaling should be explored. We found that knockdown of Akt reduced phosphorylation of P65 in NCI-H460 cells (Figure 5G). This implied that Akt signaling might be upstream of P65 signaling. We undertook rescue experiments with the combination of overexpression of Akt and knockdown of P65 in A549 cells. The overexpression of Akt induced elevated expression of PD-L1, but the simultaneous knockdown of P65 attenuated the elevation of PD-L1 expression (Figure 5H,I). The results from the rescue experiments indicated that PD-L1 expression was regulated by the Akt-p65

signaling axis in NSCLC cells. Taken together, these results suggested that UCL1 promoted PD-L1 expression through the Akt-p65 axis in NSCLC cells.

4 | DISCUSSION

Programmed cell death-ligand 1 is a critical target molecule for cancer immunotherapy. Clarification of the regulatory mechanism of PD-L1 expression in cancer cells can contribute to discovery of new strategies for blockade of the PD-L1 checkpoint. Ubiquitin C-terminal hydrolase L1 plays an oncogenic role in NSCLC development, but the specific oncogenic mechanism is still elusive. In the present study, we found that UCL1 promoted PD-L1 expression in NSCLC cells. Moreover, UCL1 mediated the impairment of T cell

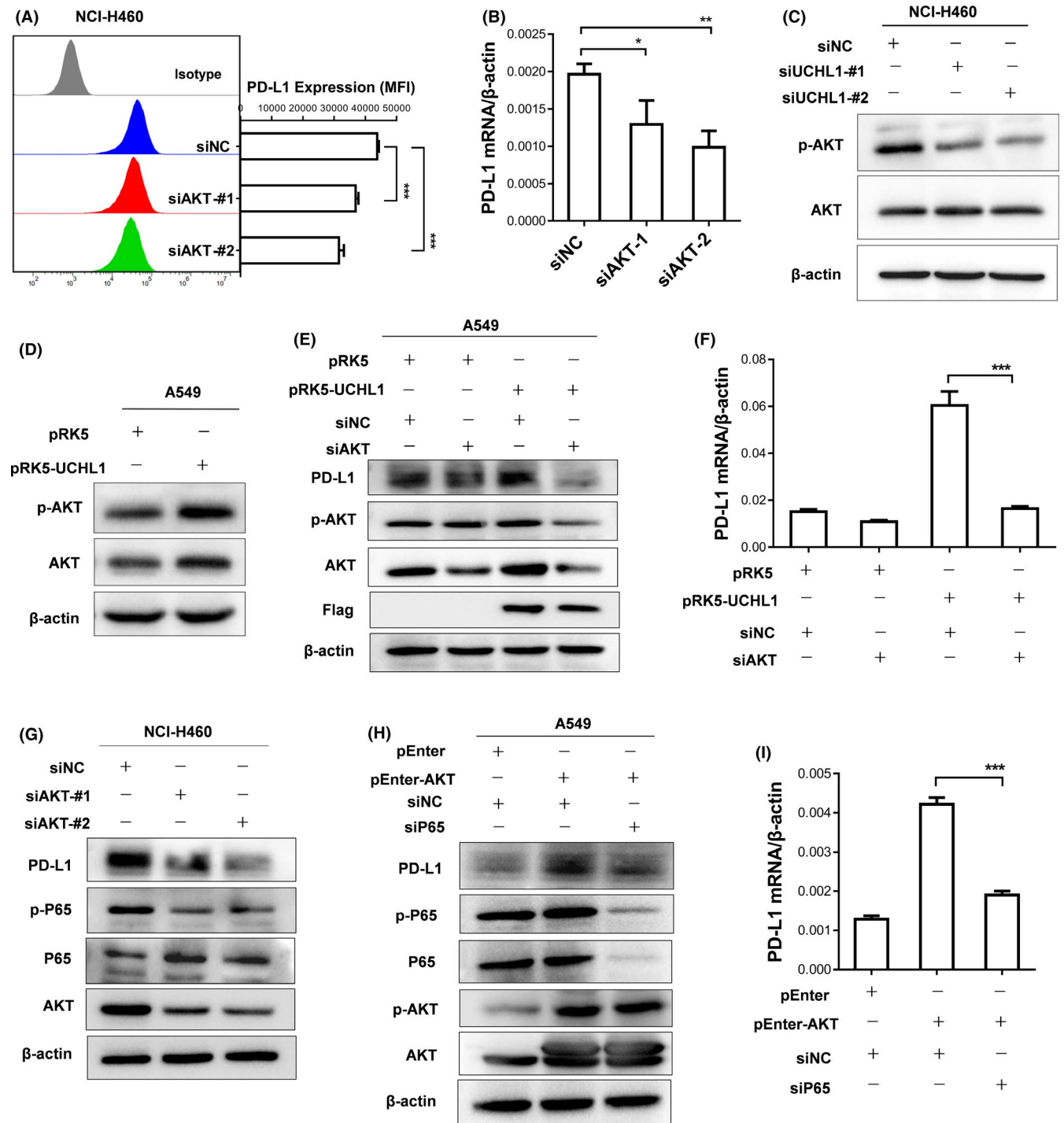


FIGURE 5 Ubiquitin C-terminal hydrolase L1 (Uchl1) promoted programmed cell death-ligand 1 (PD-L1) expression through the Akt-P65 signaling axis in non-small-cell lung cancer cells. A, B, NCI-H460 cells were transfected with negative control siRNA (siNC) or siAKT for 48 h. PD-L1 expression was quantified by flow cytometry and western blot assay. C, NCI-H460 cells were transfected with siNC or siUchl1 for 48 h. Phosphorylation of Akt was measured by western blot. D, A549 cells were transfected with pRK5 or pRK5-Uchl1 for 48 h. Phosphorylation of Akt was measured by western blot. E, A549 cells were transfected with pRK5, pRK5-Uchl1, siNC, or siAKT for 24 h. PD-L1 expression was measured by western blot. F, A549 cells were transfected with pRK5, pRK5-Uchl1, siNC, or siAKT for 24 h. PD-L1 expression was measured by quantitative RT-PCR. G, NCI-H460 cells were transfected with siNC or siAKT for 48 h. Phosphorylation of P65 was measured by western blot. H, A549 cells were transfected with pEnter, pEnter-AKT, siNC, or siP65 for 48 h. PD-L1 expression was measured by western blot. I, A549 cells were transfected with pEnter, pEnter-AKT, siNC, or siP65 for 24 h. PD-L1 expression was measured by quantitative RT-PCR. Similar results were obtained in 2 independent experiments carried out in triplicate by flow cytometry and quantitative RT-PCR assays. For western blot assay, similar results were obtained from at least 2 experiments. Data are shown as mean \pm SD. * $P < .05$, ** $P < .01$, *** $P < .001$. MFI, mean fluorescence intensity

activity through upregulation of PD-L1 in NSCLC cells. We further revealed the mechanisms whereby UCHL1 promoted PD-L1 expression. Ubiquitin C-terminal hydrolase L1 facilitated PD-L1 expression through the Akt-P65 signaling axis in NSCLC cells.

The combination of our results and data from previous studies can explain the mechanism by which UCHL1 promotes PD-L1 expression in NSCLC cells in detail. Our results showed that UCHL1 promoted PD-L1 expression through the AKT-P65 signaling axis in NSCLC cells. That is, both the P65 and Akt signaling pathways were implicated in promotion of PD-L1 expression by UCHL1. Moreover, the AKT signaling was upstream of the P65 pathway. This is consistent with previous reports that activated AKT can phosphorylate IKK α , resulting in activation of the IKK complex. Then the activated IKK complex phosphorylates I κ B and p65 proteins, leading to nuclear translocation of the activated P65 to initiate transcription of the target genes.^{25,26} In addition, the activated AKT can also directly phosphorylate P65 to enhance NF- κ B signaling.²⁷ As for regulation of UCHL1 on AKT activation, a published study showed that UCHL1 boosted the AKT pathway by downregulating the antagonistic phosphatase PHLPP1, an event that requires its deubiquitinase activity.¹⁶ However, the mechanism by which UCHL1 affects the levels of PHLPP1 is currently not clear. Another explanation for regulation of UCHL1 on AKT activation is that UCHL1 increases mTORC2 activity to enhance AKT activation. Ubiquitin C-terminal hydrolase L1 counteracts Raptor ubiquitination, leading to mTORC1 dissolution and a secondary increase in mTORC2.²⁸ The specific mechanisms whereby UCHL1 regulates AKT activation in NSCLC cells remains to be clarified in our future research.

Ubiquitin C-terminal hydrolase L1 could become a therapeutic target in NSCLC. It is a deubiquitinase and is druggable. Ubiquitin C-terminal hydrolase L1 is a cysteine protease, consisting of 223 amino acid residues. It possesses a defined active site, which is a catalytic triad of the aspartate¹⁷⁶, histidine¹⁶¹, and cysteine⁹⁰ residues. The cysteine residue in the active site is expected to be reactive to various electrophiles.²⁹ Therefore, some compounds can modulate the site to inactivate its catalytic activity. For example, small molecule LDN-57444 is an active site-directed inhibitor of UCHL1.²⁹ A metabolite of prostaglandin D₂, 15 Δ -PGJ₂, modifies a cysteine in UCHL1 protein, resulting in inhibition of enzyme activity.³⁰ Hence, UCHL1 is easily targeted. However, previous studies provide in vitro and in vivo evidence that UCHL1 is associated with the oncogenesis of NSCLC. Ubiquitin C-terminal hydrolase L1 is highly expressed in NSCLC cells¹⁵ and UCHL1 transgenic mice are prone to spontaneous lung cancer.¹⁶ Furthermore, previous studies show the oncogenic mechanism of UCHL1. It can regulate many cellular signaling cascades germane to cancer. For instances, UCHL1 boosts activation of the AKT signaling pathway to enhance the invasion and migration capability of NSCLC cells.^{16,31} Ubiquitin C-terminal hydrolase L1 also increases activity of oncogenic transcription factor hypoxia-inducible factor-1 α to facilitate tumor metastasis.³² Additionally, UCHL1 upregulates oncogenic β -catenin/T-cell factor signaling through a positive feedback in transformed cells.³³ We found that UCHL1 promoted PD-L1 expression

in NSCLC cells, resulting in reduced activity of the cocultured Jurkat cells. This finding suggested that UCHL1 might facilitate immune evasion by upregulating PD-L1 expression in NSCLC cells. Thus, our data support the oncogenic roles of UCHL1 in NSCLC. The therapeutic strategy targeting UCHL1 not only inhibits the malignant behavior of cancer cells per se but also could boost antitumor immunity. Taken together, the vulnerability of UCHL1 to its inhibitors and the oncogenic properties of UCHL1 make it a promising candidate for targeted cancer therapy.

In summary, we analyzed the relationship between deubiquitinase UCHL1 and immune checkpoint molecule PD-L1 in NSCLC cells. Our results showed that UCHL1 promoted expression of PD-L1 through the Akt-P65 signaling axis. Non-small-cell lung cancer cells with high UCHL1 expression inhibited the activity of T cells. These findings not only revealed the regulatory mechanisms for PD-L1 expression in NSCLC cells but also provided evidence for the oncogenic role of UCHL1 in NSCLC.

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DISCLOSURE

None of the authors has any conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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