

# FAT10 is associated with the malignancy and drug resistance of non-small-cell lung cancer

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**Abstract:** Lung cancer has become one of the leading causes of cancer mortality worldwide, and non-small-cell lung cancer (NSCLC) accounts for ~85% of all lung cancer cases. Currently, platinum-based chemotherapy drugs, including cisplatin and carboplatin, are the most effective treatment for NSCLC. However, the clinical efficacy of chemotherapy is markedly reduced later in the treatment because drug resistance develops during the treatment. Recently, a series of studies has suggested the involvement of FAT10 in the development and malignancy of multiple cancer types. In this study, we focused our research on the function of FAT10 in NSCLC, which has not been previously reported in the literature. We found that the expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues, and we demonstrated that FAT10 promotes NSCLC cell proliferation, migration, and invasion. Furthermore, the protein levels of FAT10 were elevated in cisplatin- and carboplatin-resistant NSCLC cells, and knock-down of FAT10 reduced the drug resistance of NSCLC cells. In addition, we gained evidence that FAT10 regulates NSCLC malignancy and drug resistance by modulating the activity of the nuclear factor kappa B signaling pathway.

**Keywords:** FAT10, NSCLC, malignancy, drug resistance, NFκB

## Introduction

With >1.5 million new diagnoses per year, lung cancer has become one of the leading causes of cancer mortality worldwide.<sup>1</sup> Histologically, lung cancer can be categorized into two major types, small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC). NSCLC includes a variety of cancer subtypes with heterogeneous morphology and cell origin, such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. In total, these subtypes of NSCLC account for ~85% of all lung cancer cases.<sup>2</sup> The therapeutic outcome of NSCLC remains poor, despite advances in scientific and clinical research, primarily due to the fact that many cases of NSCLC are not diagnosed until they are already in advanced stages.<sup>3</sup> Cisplatin is one of the most effective chemotherapy drugs for NSCLC. Its mechanism of action involves forming DNA-platinum adducts, causing DNA lesions, and consequently inducing cell apoptosis.<sup>4</sup> Patients with NSCLC usually have a good initial response to cisplatin-based chemotherapy, but the clinical efficacy is significantly reduced later in the treatment because cisplatin resistance develops during the treatment. This resistance is mediated through multiple mechanisms, and extensive evidence has demonstrated that during this process, modulation in pathways supporting cell survival, including cell growth and proliferation, apoptosis, DNA damage repair, and endocytosis, is frequently observed.<sup>5</sup>

FAT10, originally identified through mapping the HLA-F genomic locus, is an 18 kDa protein composed of 165 amino acid residues.<sup>6</sup> It belongs to the ubiquitin-like (UBL)

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family of proteins and contains two UBL moieties fused in tandem.<sup>7</sup> Similar to other UBL proteins, FAT10 contains a C-terminal Gly-Gly motif that is essential for covalent conjugation to both substrates and itself.<sup>8,9</sup> The physiological function of FAT10 is largely unknown, although several interaction partners of FAT10 have been recently identified, including p53,<sup>10</sup> Mad2,<sup>11</sup> UBA6,<sup>12</sup> p62,<sup>8</sup> histone deacetylase 6,<sup>13</sup> and huntingtin.<sup>14</sup> Emerging evidence has suggested an association between increased FAT10 expression and progression of disease in a variety of cancer types. It has been shown that FAT10 is involved in the development of human hepatocellular carcinoma (HCC).<sup>15–18</sup> Recently, FAT10 expression was also found to be elevated in gastric cancer and became a potential biomarker for the survival of patients.<sup>19</sup> Furthermore, overexpression of FAT10 has been strongly linked to poor prognosis in glioma.<sup>20</sup> Interestingly, an indirect link of FAT10's involvement in cancer development was demonstrated by its ability to bind and interrupt the function of the spindle assembly checkpoint protein, Mad2, during mitosis,<sup>11</sup> thereby inducing an abbreviated mitotic phase and aneuploidy,<sup>21,22</sup> a signature of many cancers.<sup>23</sup> FAT10 was also shown to regulate other pathways that could affect cancer development and metastasis. For example, expression of FAT10 could be induced both by carcinogens in rodent models<sup>24</sup> and by proinflammatory cytokines in cultured cells,<sup>15,22</sup> supporting the hypothesis that FAT10 could play a role as a mediator of carcinogenesis associated with inflammation.

In this study, we focused our research on the roles FAT10 plays in NSCLC, which have not been previously reported by others. We demonstrated that the expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues and that knockdown of FAT10 inhibited NSCLC cell proliferation, migration, and invasion. In addition, the protein levels of FAT10 were elevated in cisplatin- and carboplatin-resistant NSCLC cells, and knockdown of FAT10 reduced the drug resistance of NSCLC cells. Finally, we provide preliminary evidence that FAT10 regulates NSCLC malignancy and drug resistance by modulating the nuclear factor kappa B (NFκB) signaling pathway.

## Materials and methods

### Reagents

Cisplatin and carboplatin were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), and primary antibodies (anti-FAT10, anti-p65, anti-GAPDH, and anti-histone H3) were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

## Cell culture

The human NSCLC cell lines, HCC827 and H1975, were maintained in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/mL penicillin, and 100 U/mL streptomycin (Thermo Fisher Scientific). Human lung fibroblast cell line MRC-5 was cultured in minimum essential medium (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were grown as adherent cells in a humidified atmosphere at 37°C in a 5% CO<sub>2</sub> incubator.

## Patient samples

From 2012 to 2014, 27 delayed chemoresistance samples and 18 quick chemoresistance samples were obtained from the Department of Medical Oncology, Harbin Medical University Cancer Hospital. In lung cancer patients, the recurrence of disease within 3 months after the initial treatment was defined as quick chemoresistance, and the recurrent disease appearing after 3 months was defined as delayed chemoresistance.<sup>25</sup> Tissue samples were acquired from routine therapeutic surgery of patients. Written informed consent was obtained from all patients, and the research was approved by the Ethics Committee of Harbin Medical University Cancer Hospital. Upon resection, surgical specimens were immediately frozen in liquid nitrogen and stored at –80°C in a freezer. The clinical characteristics of patients are presented in Table 1.

## Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene and rehydrated with gradient concentrations of ethanol. Endogenous peroxidase activity was blocked by H<sub>2</sub>O<sub>2</sub>, antigens were retrieved by microwaving, and nonspecific binding was blocked by 1% bovine serum albumin in phosphate-buffered saline. Sections were stained with FAT10 antibody (diluted 1:500) and visualized with DAB (Thermo Fisher Scientific). Representative fields were photographed using a scanner under microscope (Olympus Corporation, Tokyo, Japan).

## Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted with TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Two micrograms of the extracted total mRNA was used for cDNA synthesis, using the One Step RT-PCR Kit (TaKaRa, Tokyo, Japan). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with

**Table 1** Clinical characteristics of delayed chemoresistant and quick chemoresistant patients with NSCLC

Characteristics	Patient number	Delayed chemoresistant patients (n=27)	Quick chemoresistant patients (n=18)	P-value
Age, years				0.760
≥60	20	13	7	
<60	25	14	11	
Sex				0.721
Male	36	21	15	
Female	9	6	3	
Smoking status				0.111
Smokers	31	16	15	
Nonsmokers	14	11	3	
Stage				0.041
I-II	33	23	10	
III-IV	12	4	8	
Histology				0.539
Squamous cell carcinoma	19	10	9	
Adenocarcinomas	26	17	9	
Differentiation				0.013
Well/moderate	28	21	7	
Poor	17	6	11	
Tumor size				0.063
≤3 cm	15	12	3	
>3 cm	30	15	15	
FAT10 expression				0.001
High	23	8	15	
Low	22	19	3	

**Notes:** The clinical characteristics were analyzed by using chi-square test. A value of  $P < 0.05$  was considered statistically significant.

**Abbreviation:** NSCLC, non-small-cell lung cancer.

the  $\Delta\Delta C_t$  method, using a SYBR<sup>®</sup> Green PCR Master Mix kit (TaKaRa). As an internal control, human GAPDH was amplified to ensure cDNA quality and quantity for each RT-PCR reaction. The specific primer sequences of FAT10 primer sequences were 5'-TTGTTTCTTGTGGAGTCAGGTG-3' (sense) and 5'-AGTAAGTTGCCCTTTCTGATGC-3' (antisense); *TNF- $\alpha$* : 5'-GCCGCATCGCCGTCTCCTAC-3' (sense) and 5'-CCTCAGCCCCCTCTGGGGTC-3' (antisense); *IL-1b*: 5'-AATCTGTACCTGTCTGCGTGTT-3' (sense) and 5'-TGGGTAATTTTGGGATCTACACTCT-3' (antisense); *IL-6*: 5'-TTCTCCACAAGCGCCTTCGGTC-3' (sense) and 5'-TCTGTGTGGGGCGGCTACATCT-3' (antisense); *IL-8*: 5'-GTGCAGTTTTGCCAAGGAGT-3' (sense) and 5'-CTCTGCACCCAGTTTTCCTT-3' (antisense); *Cyclin D1*: 5'-CCGTCCATGCGGAAGATC-3' (sense) and 5'-GAAGACCTCCTCCTCGCACT-3' (antisense); *MYC*: 5'-GCCACGTCTCCACACATCAG-3' (sense) and 5'-TCTTGGCAGCAGGATAGTCCTT-3' (antisense); and *GAPDH*: 5'-GCTGAGAACGGGAAGCTTGT-3' (sense) and 5'-GCCAGGGGTGCTAAGCAGTT-3' (antisense).

qRT-PCR was run using an ABI PRISM 7500HT Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, USA).

## Drug-resistant cell establishment

Cisplatin- and carboplatin-resistant cells were established as previously described.<sup>26</sup>

Briefly, HCC827 and H1975 cells were treated with incrementally increasing concentrations of cisplatin or carboplatin. These cells were first maintained in a low dose of cisplatin or carboplatin (1  $\mu$ M, 2  $\mu$ M, 4  $\mu$ M, 6  $\mu$ M, 8  $\mu$ M, and 10  $\mu$ M). Cells were grown at each concentration for more than 1 month, and cell viability tests were then performed.

## Western blots

The cells were trypsinized and harvested by centrifugation, washed with phosphate-buffered saline, and resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 250  $\mu$ M phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 1% Nonidet P-40, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) on ice for 10 minutes. Cell debris was then removed by centrifugation (15 minutes, 12,000 $\times$  *g* at 4°C). The nuclear lysates were extracted with a Nuclear Protein Extraction Kit (Beyotime, Beijing, People's Republic of China) according to the manufacturer's protocol. The protein concentrations were determined using the enhanced BCA Protein Assay

Kit (Beyotime). Thirty micrograms of protein per well was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. Following electrophoresis, the separated proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membranes were immersed in blocking buffer (5% nonfat dry milk in Tris-buffered saline with Tween 20) for 1 hour at room temperature, then incubated with the primary antibodies against FAT10, p65, GAPDH, or histone H3 overnight at 4°C in Tris-buffered saline with Tween 20. Antibodies were recognized by species-specific secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories Inc.). Following final washing, the membranes were developed with the ECL kit (Beyotime) and exposed to X-ray film in a darkroom. Protein bands were processed using ImageJ software (NIH, Bethesda, MD, USA).

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays

NSCLC cells were seeded onto 96-well plates in complete medium ( $2 \times 10^3$  cells in 100  $\mu$ L of medium per well). For cell proliferation analysis, cells were incubated with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in a CO<sub>2</sub> incubator at 37°C for 4 hours. After removing all the media in the plates, 150  $\mu$ L of dimethyl sulfoxide was added to each well. Following incubation for a further hour, the absorbance at 490 nm in solution of each well was measured by a microplate reader.

### Migration and invasion assays

Cell migration and invasion abilities were evaluated with a transwell assay, using uncoated and matrigel-coated transwell systems, respectively (8  $\mu$ m pore size, polycarbonate membrane; BD Biosciences, San Jose, CA, USA). Briefly,  $5 \times 10^4$  cells were suspended in 200  $\mu$ L of Dulbecco's Modified Eagle's Medium/F12 medium, with 1% FBS added to the upper inserts and the lower chamber loaded with 500  $\mu$ L of medium supplemented with 10% FBS, serving as a chemoattractant. After 24 hours of incubation at 37°C, upper chamber (nonmigrated) cells were removed, and lower chamber (migrated) cells were fixed and stained with crystal violet and counted under an inverted microscope. Six random fields were chosen for cell quantification.

### Statistical analyses

Statistical analyses were performed using SPSS software Version 19.0 (IBM Corporation, Armonk, NY, USA). Values were expressed as mean  $\pm$  SD. One-way analysis of variance

and Student–Newman–Keuls tests were used to analyze statistics. Patient variables were analyzed using chi-square tests. A value of  $P < 0.05$  was considered statistically significant. All experiments were repeated in triplicate.

## Results

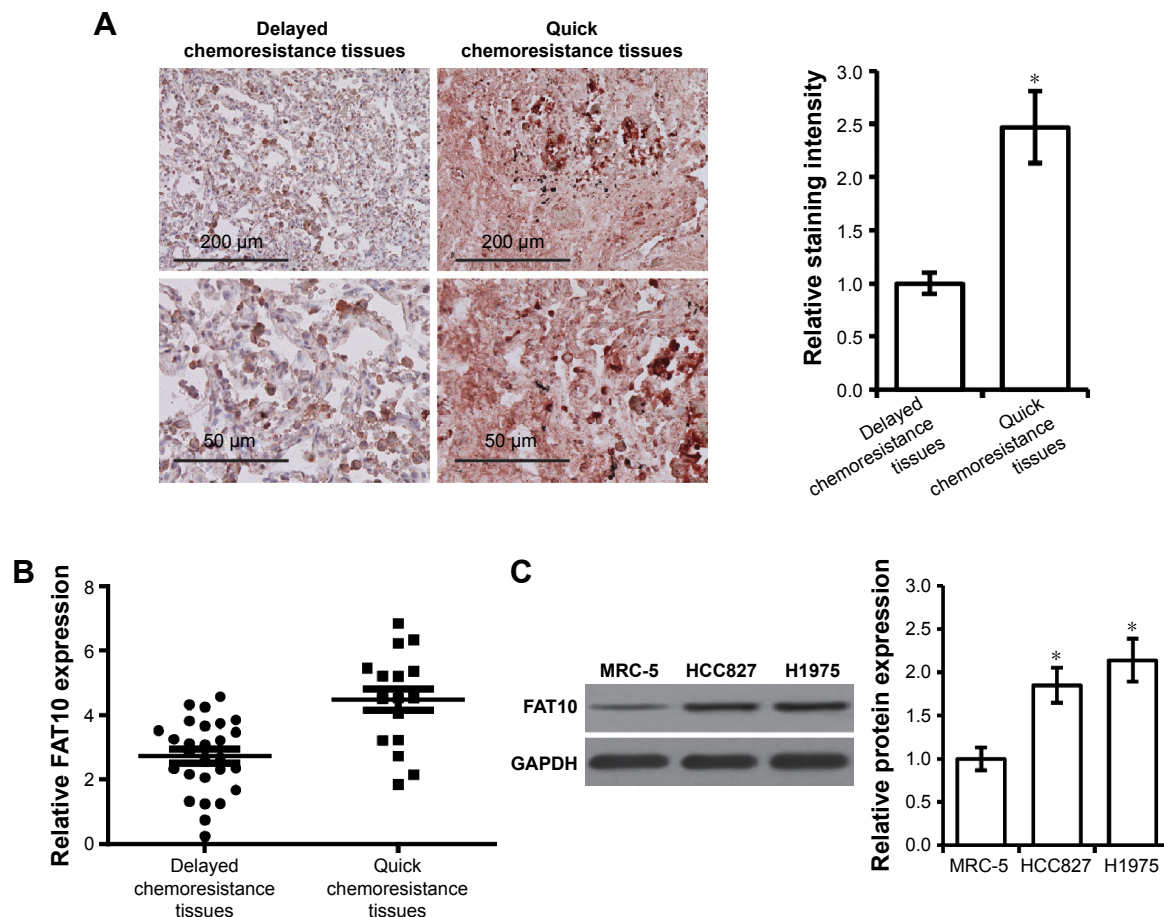
### The expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues

To examine the biological functions of FAT10 in development and drug resistance of NSCLC, we first sought to determine the expression levels of FAT10 in both delayed and quick chemoresistance NSCLC tissues. We found that the protein levels of FAT10 were significantly higher in quick chemoresistance tissues, as determined by immunohistochemistry staining (Figure 1A;  $P < 0.05$ ). We also examined the mRNA levels of FAT10 in 27 delayed chemoresistance NSCLC samples and 18 quick chemoresistance NSCLC samples by qPCR analysis and found that the mRNA levels of FAT10 were also significantly increased in quick chemoresistance NSCLC samples (Figure 1B;  $P < 0.001$ ). We next examined FAT10 expression in two NSCLC cell lines, HCC827 and H1975. Compared with MRC-5 cells, FAT10 was upregulated in both HCC827 and H1975 cells (Figure 1C;  $P < 0.05$ ). Taken together, these results indicated that the expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues.

### FAT10 regulated NSCLC cell proliferation, migration, and invasion

The fact that the expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues suggested that FAT10 could be involved in regulation of NSCLC development. Therefore, we sought to determine whether FAT10 plays roles in proliferation, migration, and invasion of NSCLC cells. To this end, we knocked down FAT10 expression in two independent NSCLC cell lines, HCC827, and H1975. The knockdown efficiency was confirmed by Western blot analysis (Figure 2A;  $P < 0.001$ ). For the FAT10 knockdown cells, we performed MTT cell proliferation assays, and found that knockdown of FAT10 significantly reduced cell proliferation in both HCC827 and H1975 cells (Figure 2B;  $P < 0.05$ ). Furthermore, overexpression of FAT10 significantly promoted NSCLC cell proliferation, as detected by MTT assays (Figure 2C and D;  $P < 0.05$ ). Additionally, we performed transwell-based cell migration and invasion assays on the FAT10 knockdown cells, and observed that knockdown of FAT10 led to significantly less cell migration





**Figure 1** Expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues.

**Notes:** (A) Immunohistochemistry staining of FAT10 in delayed and quick chemoresistance NSCLC tissues. Representative view fields and quantification of staining intensity are shown. (B) The relative mRNA levels of FAT10 in delayed and quick chemoresistance NSCLC tissues;  $P < 0.001$ . (C) Western blot analysis of FAT10 expression in HCC827 and H1975 cells compared with MRC-5 cells. GAPDH was used as a loading control, and quantification of Western blot intensity is shown in the right panel;  $*P < 0.05$ . Student's *t*-test and One-way ANOVA was performed to calculate *P*-values.

**Abbreviation:** NSCLC, non-small-cell lung cancer.

(Figure 3A;  $P < 0.05$ ) and cell invasion (Figure 3B;  $P < 0.05$ ) in both HCC827 and H1975 cells. Overexpression of FAT10 in HCC827 and H1975 cells consistently enhanced cell migration and invasion (Figure 3C and D). These results collectively indicate that FAT10 positively regulates proliferation, migration, and invasion of NSCLC cells and suggest that FAT10 might be required for NSCLC cells to maintain the capacity for robust proliferation, migration, and invasion.

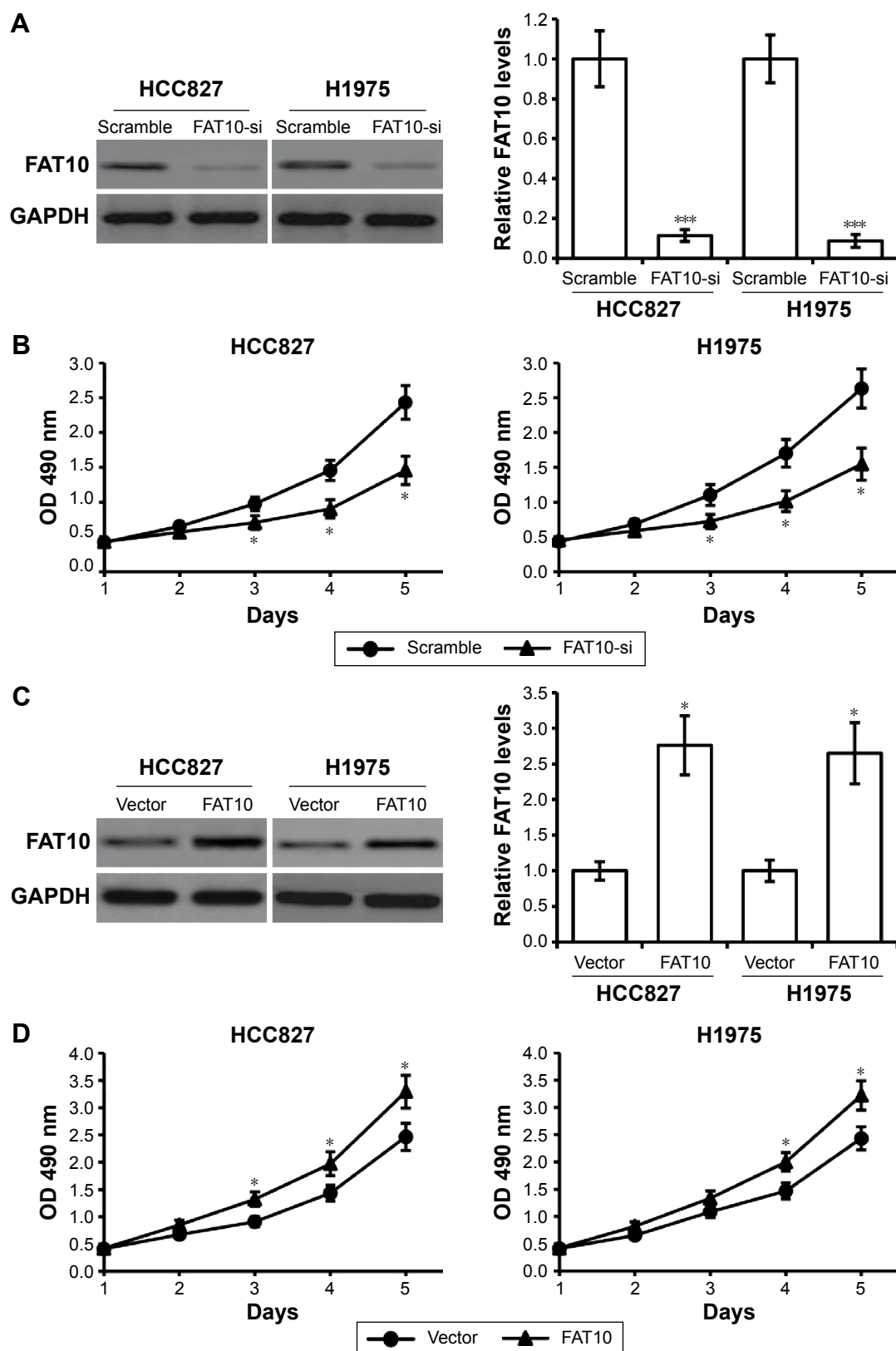
## FAT10 was associated with drug resistance in NSCLC cells

Since the expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues, it is possible that FAT10 could contribute to drug resistance in NSCLC cells. To test this possibility, we first induced cisplatin and carboplatin resistance in HCC827 and H1975 cells by treating them for extended periods. Drug resistance was confirmed by cell viability assays (Figure S1;  $P < 0.05$ ). Significantly, the

levels of FAT10 were increased in both cisplatin- (Figure 4A;  $P < 0.05$ ) and carboplatin-resistant cells (Figure 4B;  $P < 0.05$ ), as determined by Western blot analysis. Furthermore, we found that knockdown of FAT10 in both HCC827 and H1975 cells markedly reduced the percentages of viable cells when the cells were subjected to cisplatin (Figure 5A;  $P < 0.05$ ) or carboplatin (Figure 5B;  $P < 0.05$ ) treatment. As expected, overexpression of FAT10 significantly enhanced cisplatin and carboplatin resistance (Figure 5C and D;  $P < 0.05$ ). These data support the hypothesis that FAT10 levels are positively correlated with drug resistance of NSCLC cells.

## FAT10 regulated NSCLC malignancy and drug resistance via NF $\kappa$ B signaling

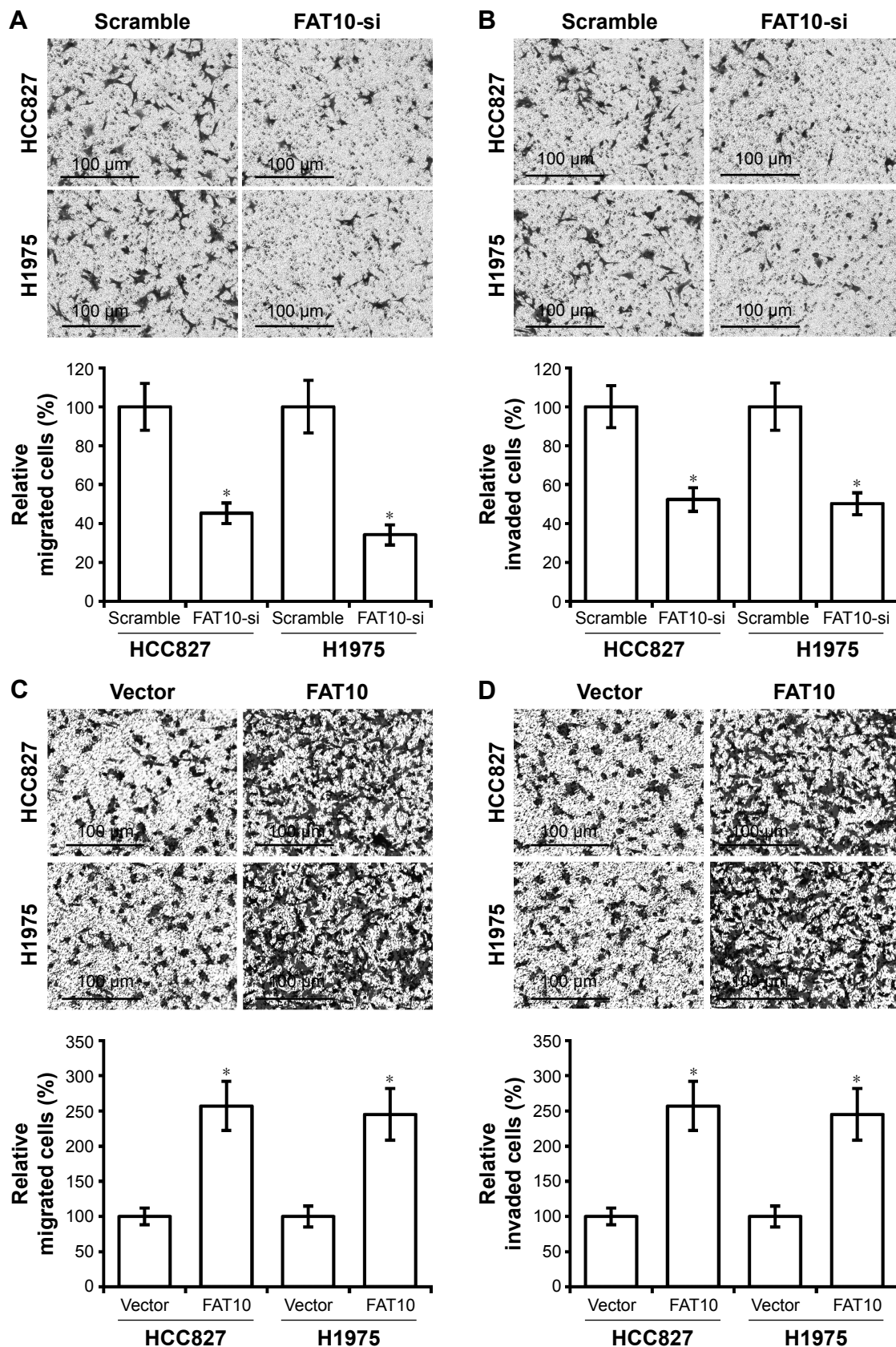
It has been reported in other cancer types that FAT10 regulates cancer development by modulating the NF $\kappa$ B signaling pathway.<sup>22,27</sup> To determine whether FAT10 could also regulate the malignancy and drug resistance of NSCLC cells via



**Figure 2** FAT10 regulated NSCLC cell proliferation.

**Notes:** (A) The protein levels of FAT10 in HCC827 and H1975 cells transfected with scrambled siRNA or FAT10-si as determined by Western blot analysis. GAPDH was used as a loading control. (B) MTT cell proliferation assays in HCC827 and H1975 cells transfected with scrambled siRNA or FAT10-si; \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with scrambled siRNA control cells. (C) The protein levels of FAT10 in HCC827 and H1975 cells transfected with vector or FAT10-pcDNA3.1 plasmid (FAT10) as determined by Western blot analysis. GAPDH was used as a loading control. (D) MTT cell proliferation assays in HCC827 and H1975 cells transfected with vector or FAT10; \* $P < 0.05$  compared with vector control cells. Student's t-test was performed to calculate  $P$ -values.

**Abbreviations:** NSCLC, non-small-cell lung cancer; FAT10-si, siRNA targeting FAT10; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density.

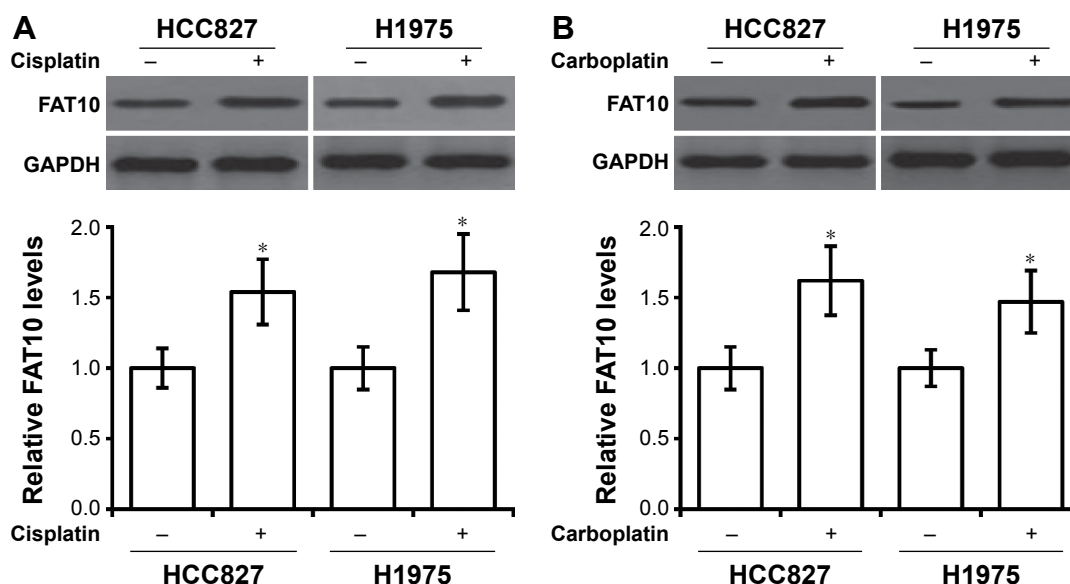


**Figure 3** FAT10 regulated NSCLC cell migration and invasion.

**Notes:** (A) Cell migration and (B) cell invasion as determined by transwell analysis in HCC827 and H1975 cells transfected with scrambled siRNA or FAT10-si; \* $P < 0.05$  compared with scrambled siRNA control cells. (C) Cell migration and (D) cell invasion as determined by transwell analysis in HCC827 and H1975 cells transfected with vector or FAT10; \* $P < 0.05$  compared with vector control cells. Student's *t*-test was performed to calculate *P*-values.

**Abbreviations:** NSCLC, non-small-cell lung cancer; FAT10-si, siRNA targeting FAT10.





**Figure 4** The protein levels of FAT10 were elevated in cisplatin- and carboplatin-resistant NSCLC cells.

**Notes:** The protein levels of FAT10 in (A) cisplatin- and (B) carboplatin-resistant HCC827 and H1975 cells, as determined by Western blot analysis. GAPDH was used as a loading control; \* $P < 0.05$  compared with cells without treatment. Student's *t*-test was performed to calculate *P*-values. – represents without treatment; + represents with treatment.

**Abbreviation:** NSCLC, non-small-cell lung cancer.

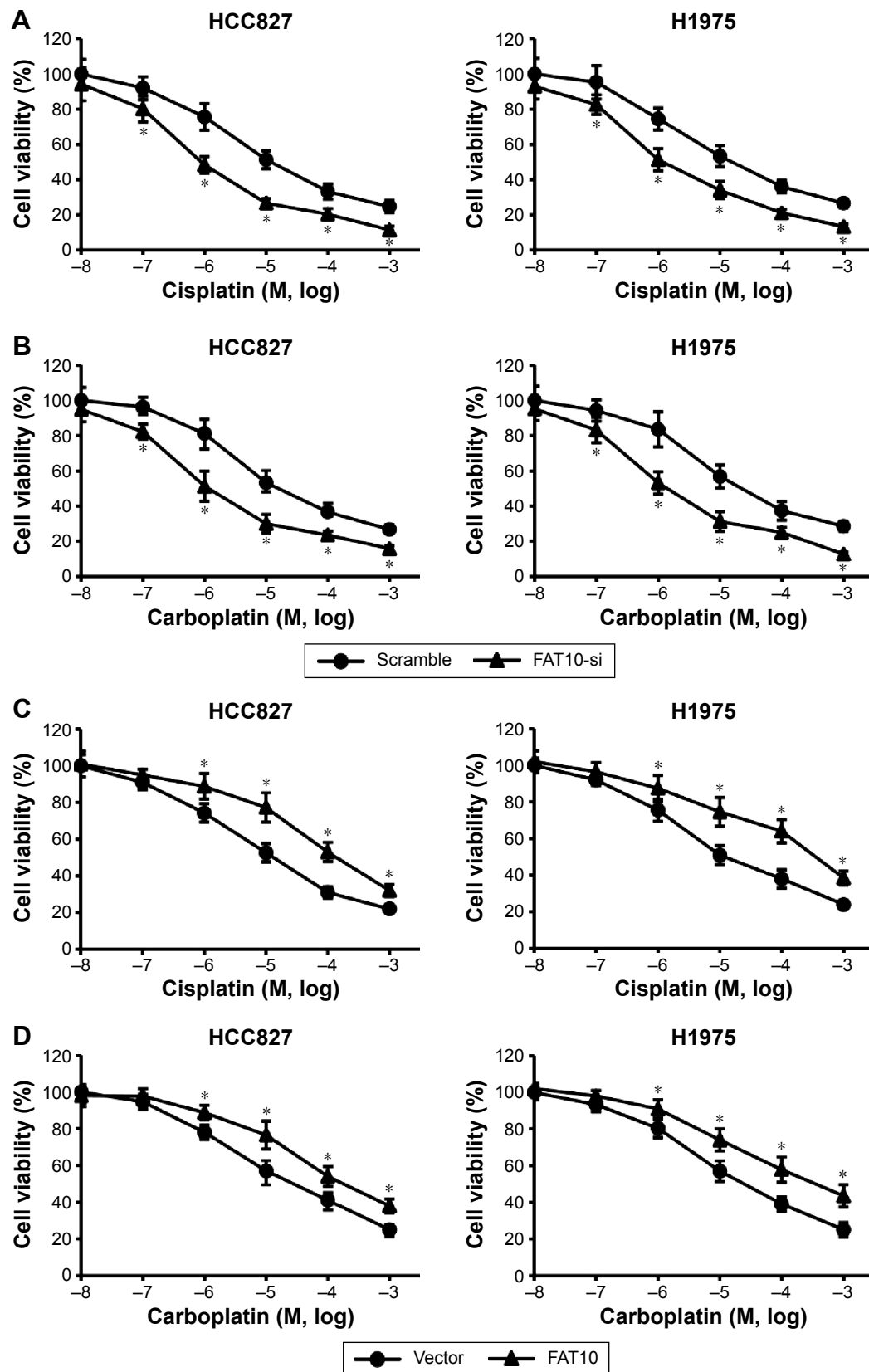
the NF $\kappa$ B signaling pathway, we first determined the levels of whole-cell and nuclear forms of p65 in HCC827 cells transfected with either scrambled siRNA or siRNA targeting FAT10. We found that the levels of nuclear p65 were significantly reduced in FAT10 knockdown NSCLC cells but did not affect p65 levels in whole cells (Figure 6A;  $P < 0.05$ ). To confirm that FAT10 regulates NF $\kappa$ B signaling, we performed NF $\kappa$ B luciferase assays and qRT-PCR for NF $\kappa$ B signaling downstream gene expression, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, cyclin D1, and MYC.<sup>28</sup> The results revealed that FAT10 deletion significantly decreased NF $\kappa$ B luciferase activity and NF $\kappa$ B signaling downstream gene expression (Figure 6B and C;  $P < 0.05$ ). We reasoned that if FAT10 regulates NSCLC development through the NF $\kappa$ B signaling pathway, loss of p65 would abolish the regulatory ability of FAT10. We knocked down p65 in HCC827 cells and observed that the baseline levels of cell proliferation, migration, and invasion significantly decreased. Importantly, in these p65 knockdown cells, knocking down FAT10 (Figure 7A;  $P < 0.001$ ) could no longer inhibit cell proliferation (Figure 7B;  $P < 0.05$ ), cell migration (Figure 7C;  $P < 0.05$ ), or cell invasion (Figure 7D;  $P < 0.05$ ). In addition, in p65 knockdown NSCLC cells, the ability of FAT10 to affect drug resistance against both cisplatin and carboplatin was also abolished (Figure 7E;  $P < 0.05$ ). Taken together, these results were consistent with the theory that FAT10 regulates NSCLC malignancy and drug resistance by modulating the NF $\kappa$ B signaling pathway.

## Discussion

Several recent studies have shown that there is an association between FAT10 levels and malignant progression of a variety of cancer types.<sup>15,17–20,29</sup> However, there have been no prior reports about the roles of FAT10 in NSCLC. In this study, we demonstrated that expression levels of FAT10 were elevated in quick chemoresistance NSCLC tissues. Development of drug resistance in cancer cells is a complex process and could involve numerous epigenetic and genetic changes, resulting in activation of many pathways at both the molecular and cellular levels that affect survival of cells. The observation that expression levels of FAT10 were elevated in NSCLC tissues that were resistant to chemotherapy drugs was consistent with previous findings that FAT10 had profound effects on promoting proliferation and survival of cells. For example, FAT10-deficient mouse lymphocytes are more susceptible to spontaneous cell death.<sup>30</sup> Increased FAT10 levels in HCT116 cells conferred a protective effect against toxin-induced apoptosis.<sup>22</sup> Overall, our observation supported a survival role for FAT10.

We demonstrated that knockdown of FAT10 inhibited NSCLC cell proliferation, migration, and invasion. In addition, protein levels of FAT10 were elevated in cisplatin- and carboplatin-resistant NSCLC cells, and knockdown of FAT10 reduced drug resistance of NSCLC cells. These data clearly indicate that elevation of FAT10 expression in cancerous tissues is not a coincidental effect of malignant

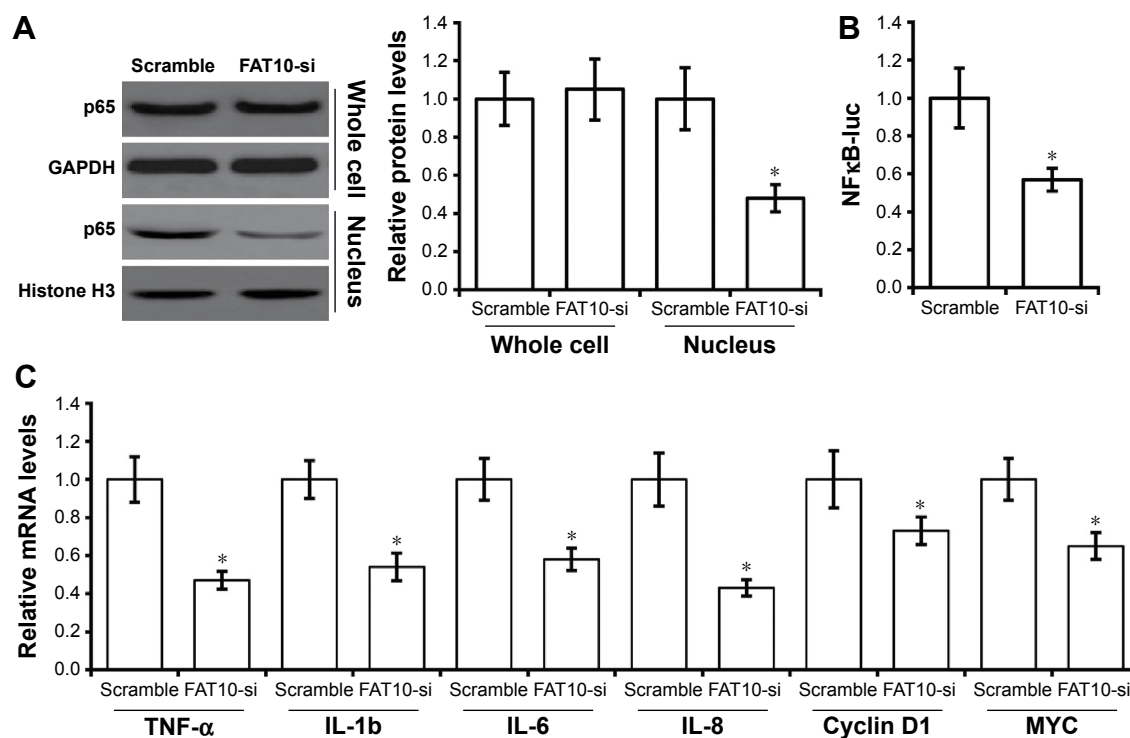




**Figure 5** FAT10 regulated the drug resistance of NSCLC cells.

**Notes:** MTT cell viability assays in HCC827 and H1975 cells transfected with scrambled siRNA or FAT10-si, treated with different concentrations of (A) cisplatin or (B) carboplatin (as indicated); \* $P < 0.05$  compared with scrambled control cells. MTT cell viability assays in HCC827 and H1975 cells transfected with vector or FAT10 and treated with different concentrations of (C) cisplatin or (D) carboplatin; \* $P < 0.05$  compared with vector control cells. Student's *t*-test was performed to calculate *P*-values.

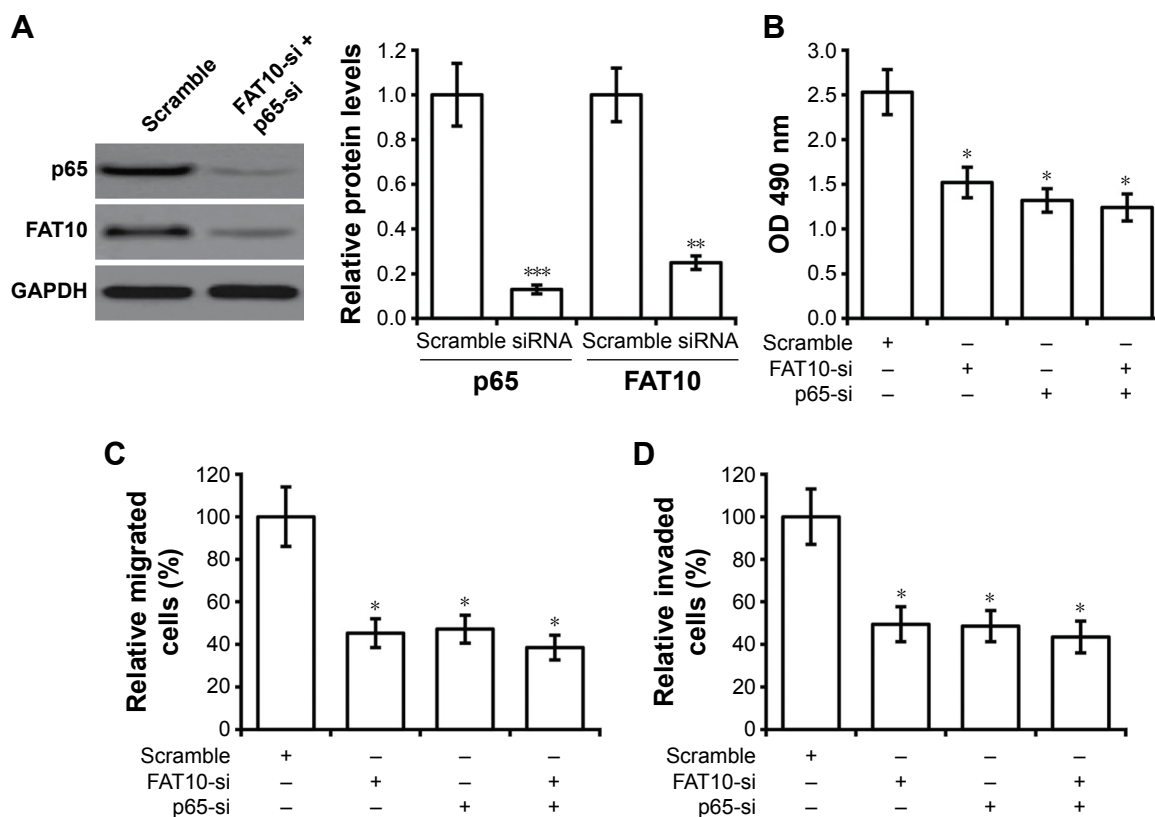
**Abbreviations:** NSCLC, non-small-cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FAT10-si, siRNA targeting FAT10; M, mol/l.



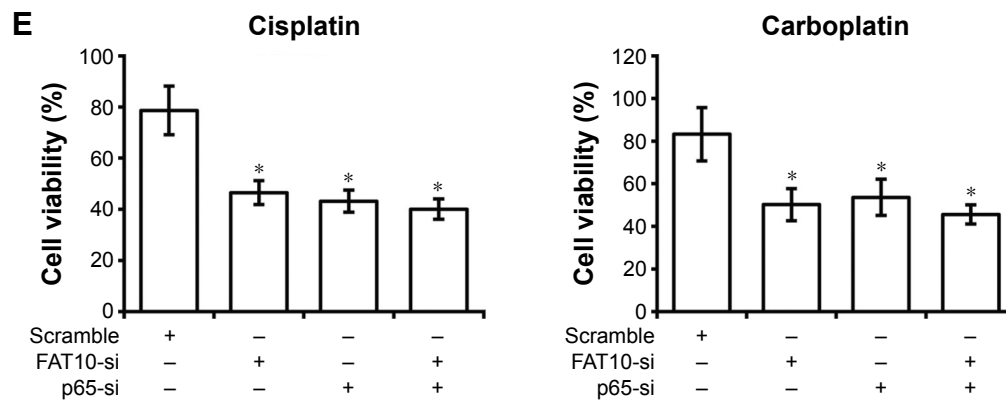
**Figure 6** FAT10 affected NFκB signaling.

**Notes:** (A) Whole-cell and nuclear p65 levels in HCC827 cells transfected with scrambled siRNA or FAT10-si, as determined by Western blot analysis. GAPDH and histone H3 were used as loading controls, respectively. Quantification of p65 is shown in the right panel. (B) NFκB luciferase activity was performed in HCC827 cells transfected with scrambled siRNA or FAT10-si. (C) Expression of NFκB signaling targeted genes (TNF-α, IL-1b, IL-6, IL-8, cyclin D1, and MYC) were determined by qRT-PCR analysis. \* $P < 0.05$ , compared with Scramble in each group. Student's *t*-test was performed to calculate *P*-values.

**Abbreviations:** NFκB, nuclear factor kappa B; FAT10-si, siRNA targeting FAT10; qRT-PCR, quantitative reverse transcription polymerase chain reaction.



**Figure 7** (Continued)



**Figure 7** FAT10 regulated NSCLC malignancy and drug resistance through NF $\kappa$ B signaling.

**Notes:** (A) p65 and FAT10 levels in HCC827 cells transfected with scrambled siRNA or siRNA targeting p65 (p65-si) and FAT10-si, as determined by Western blot analysis. Quantification of p65 and FAT10 is shown in the right panel. (B) MTT cell proliferation assay, (C) cell migration assay, and (D) cell invasion assay in HCC827 cells transfected with the indicated combinations of siRNA targeting FAT10 and p65 after 50  $\mu$ M cisplatin or carboplatin treatment for 48 hours; \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 compared with scrambled siRNA control cells. One-way ANOVA and Student's  $t$ -test was performed to calculate  $P$ -values. – represents without treatment; + represents with treatment.

**Abbreviations:** NSCLC, non-small-cell lung cancer; NF $\kappa$ B, nuclear factor kappa B; FAT10-si, siRNA targeting FAT10; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density.

progression; rather, FAT10 is likely one of the causal factors of a series of cellular events that lead to malignancy, including cell proliferation, cell migration, and cell invasion. FAT10 is one of the downstream molecules in the pathway of inflammation-induced tumorigenesis.<sup>15,27</sup> The expression of FAT10 can be induced by TNF- $\alpha$ .<sup>31</sup> The upregulation of FAT10 observed in chemoresistance tissues might be induced by the inflammatory tumor microenvironment during the development of chemoresistance.

We provide evidence that FAT10 regulates NSCLC malignancy and drug resistance by modulating the activity of the NF $\kappa$ B signaling pathway. We show that decreased FAT10 levels in NSCLC cells reduced the nuclear fraction of NF $\kappa$ B p65 protein and that knockdown of p65 abolished the ability of FAT10 to regulate cell invasion and drug resistance. These results were consistent with the requirement for FAT10 in TNF $\alpha$ -induced NF $\kappa$ B activation in renal cells<sup>32</sup> and were also in agreement with the fact that FAT10 expression was inducible by cytokines in immune cells and cancer cells.<sup>11,15,22</sup> It is interesting to note that there were putative NF $\kappa$ B-binding sites at the FAT10 promoter region,<sup>33</sup> and in some cell types, cytokine-dependent induction of FAT10 expression required activation of the NF $\kappa$ B signaling pathway.<sup>22</sup> These reports collectively suggest that a positive feedback loop might exist, by which cytokine-induced FAT10 stimulates NF $\kappa$ B signaling, which in turn induces FAT10 production. Whether this feedback loop is present in NSCLC cells and whether it is required for malignant development and drug resistance of NSCLC remain to be investigated.

Our study demonstrated novel promalignant functions of FAT10 in NSCLC, as evidenced by the requirement for

FAT10 to support malignant progression of NSCLC cells. Further elucidation of the mechanisms involved would contribute to new insights into the development of NSCLC, as well as contributing to strategies for new anticancer approaches.

## Acknowledgment

This work was supported by the National Natural Science Foundation of China (nos 81572276 and 81172214).

## Disclosure

The authors report no conflicts of interest in this work.

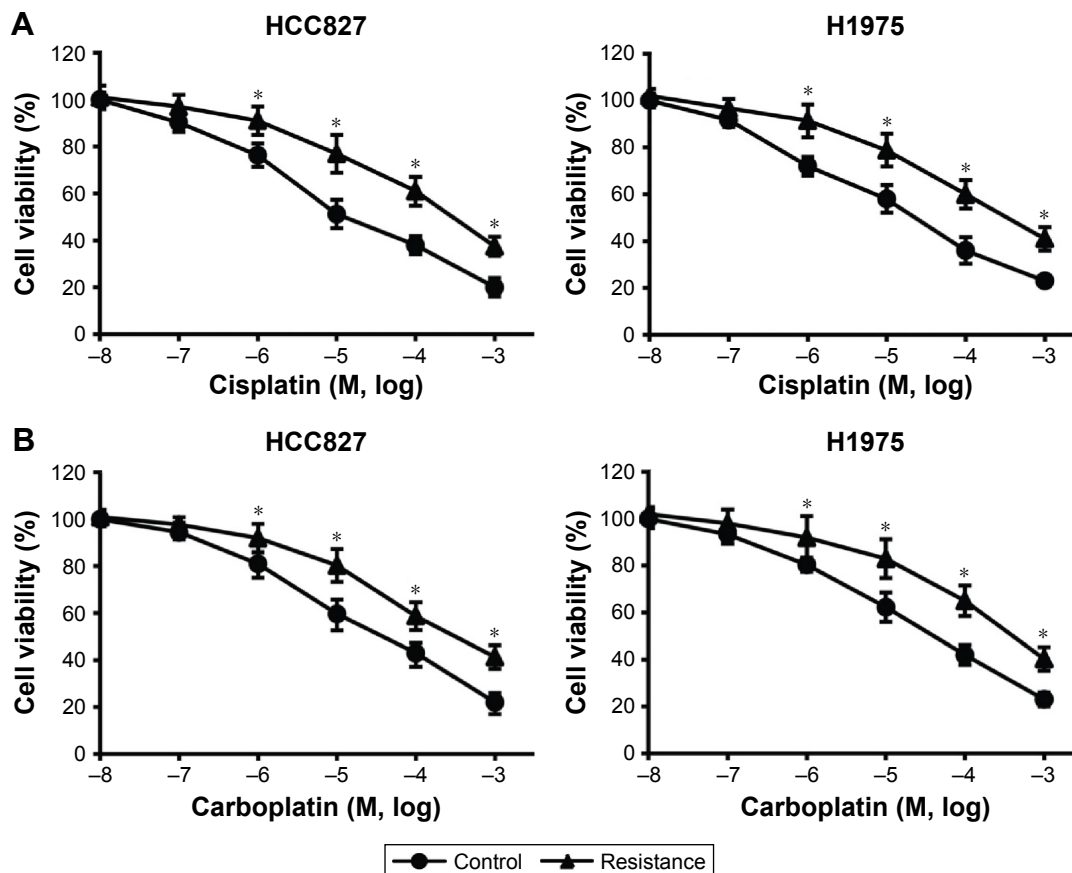
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## Supplementary material



**Figure S1** Drug-resistant NSCLC cell generation.

**Notes:** MTT cell viability assays in HCC827 and H1975 cisplatin- or carboplatin-resistant cells treated with different concentrations of (A) cisplatin or (B) carboplatin (as indicated). \* $P < 0.05$  compared with control cells. Student's *t*-test was performed to calculate *P*-values.

**Abbreviations:** NSCLC, non-small-cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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