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Original Articles

Ubiquitin-protein ligase E3C maintains non-small-cell lung cancer stemness by targeting AHNAK-p53 complex

Jie Gu[a,b,1], Wei Mao[a,b,1], Wenjia Ren[c,1], Fengkai Xu[a], Qiaoliang Zhu[a], Chunlai Lu[a], Zongwu Lin[a], Zhilong Zhang[d], Yiwei Chu[b], Ronghua Liu[b,**], Di Ge[a,*]

[a] Department of Thoracic Surgery, The Affiliated Zhongshan Hospital, Fudan University, Shanghai, 200032, PR China
[b] Key Laboratory of Medical Epigenetics and Metabolism, Institute of Biomedical Sciences, and Department of Immunology, School of Basic Medical Sciences, Fudan University, Shanghai, 200032, PR China
[c] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, 200032, PR China
[d] Department of Thoracic Surgery, Shanghai XuHui District Central Hospital, Shanghai, 200031, PR China

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ABSTRACT

Cancer stem-like cells (CSCs) are regarded as sources of tumorigenesis, metastasis, and drug resistance, which limits current cancer therapies. Elucidating the molecular modes governing CSC properties is necessary to optimize therapeutic approaches. In this study, we discovered that ubiquitin-protein ligase E3C (UBE3C)-mediated ubiquitination is a key posttranslational mechanism involved in maintaining CSC properties of non-small-cell lung cancer (NSCLC). UBE3C was overexpressed in stem-like NSCLC cells and acted as a stemness enhancer. Knockdown of UBE3C reduced NSCLC stemness and tumorigenesis both *in vivo* and *in vitro*. We further identified AHNAK as a novel UBE3C substrate, finding that UBE3C maintained stemness by ubiquitinating and promoting AHNAK degradation. AHNAK functioned as a cofactor assisting p53 binding to stemness-related gene promoters to inhibit transcription. Subsequent downregulation of AHNAK by UBE3C overexpression removed p53-mediated inhibition of gene expression, resulting in enhanced stemness. Clinical significance was investigated in 208 NSCLC patients and confirmed that attenuated UBE3C activity and elevated AHNAK protein levels correlated with extended survival time. Collectively, findings reveal the first global characterization of UBE3C-mediated ubiquitination as a key regulator of CSCs, with results suggesting involvement of the AHNAK-p53 complex.

1. Introduction

Cancer stem cells (CSCs) possess the capacity for self-renewal through both symmetric and asymmetric cellular division, with this activity recognized in various tumors, including lung cancer [1]. Unlike normal cancer cells, lung CSCs express different surface makers, including CD133, CD44, and CD166. Lung cancer cells positive for these markers exhibit significantly enhanced stem properties, including sphere formation and enhanced tumor-initiation potential [2-4]. Additionally, CSCs are often endowed with resistance to conventional chemotherapy and radiation along with tumor-initiating and metastasis properties [5-7]. According to these characteristics, identifying key molecular networks influencing CSC stemness renders such networks potential targets for cancer therapy.

Although transcriptional regulation of stem cell pluripotency and differentiation has been extensively studied [8-10], few studies focused on the roles of posttranslational modifications in these processes. Ubiquitination is a key posttranslational modification that play a central role in regulating various biological functions [11-14]. It is induced by a cascade of enzymatic reactions by the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase, with these processes reversed by deubiquitinating enzymes (DUBs) [15]. It has been reported that the CSC marker CD133 can be mono-ubiquitinated at lysine 848 [16], and ubiquitination of the marker CD166 has been found in head and neck cancer [17]. Additionally, tripartite-motif-containing 16, as a proteasome cofactor, directly regulated the

Abbreviations: NSCLC, non-small-cell lung cancer; CSC, cancer stem cell; AHNAK, Neuroblast differentiation associated protein AHNAK (Desmoyokin); UBE3C, ubiquitin-protein Ligase E3C; SOX2, sex-determining region Y-box 2; POU5F, octamer-binding transcription factor 4 (OCT4); NANOG, Homeobox protein NANOG; DUBs, deubiquitinating enzymes

* Corresponding author. 180 Fenglin Road, Shanghai, 200032, PR China.
** Corresponding author. 138, Yi Xue Yuan Rd, mail box 226, Shanghai, 200032, PR China.
E-mail addresses: ronghualiu@fudan.edu.cn (R. Liu), gedi6902@hotmail.com (D. Ge).
[1] These authors contributed equally to this paper.

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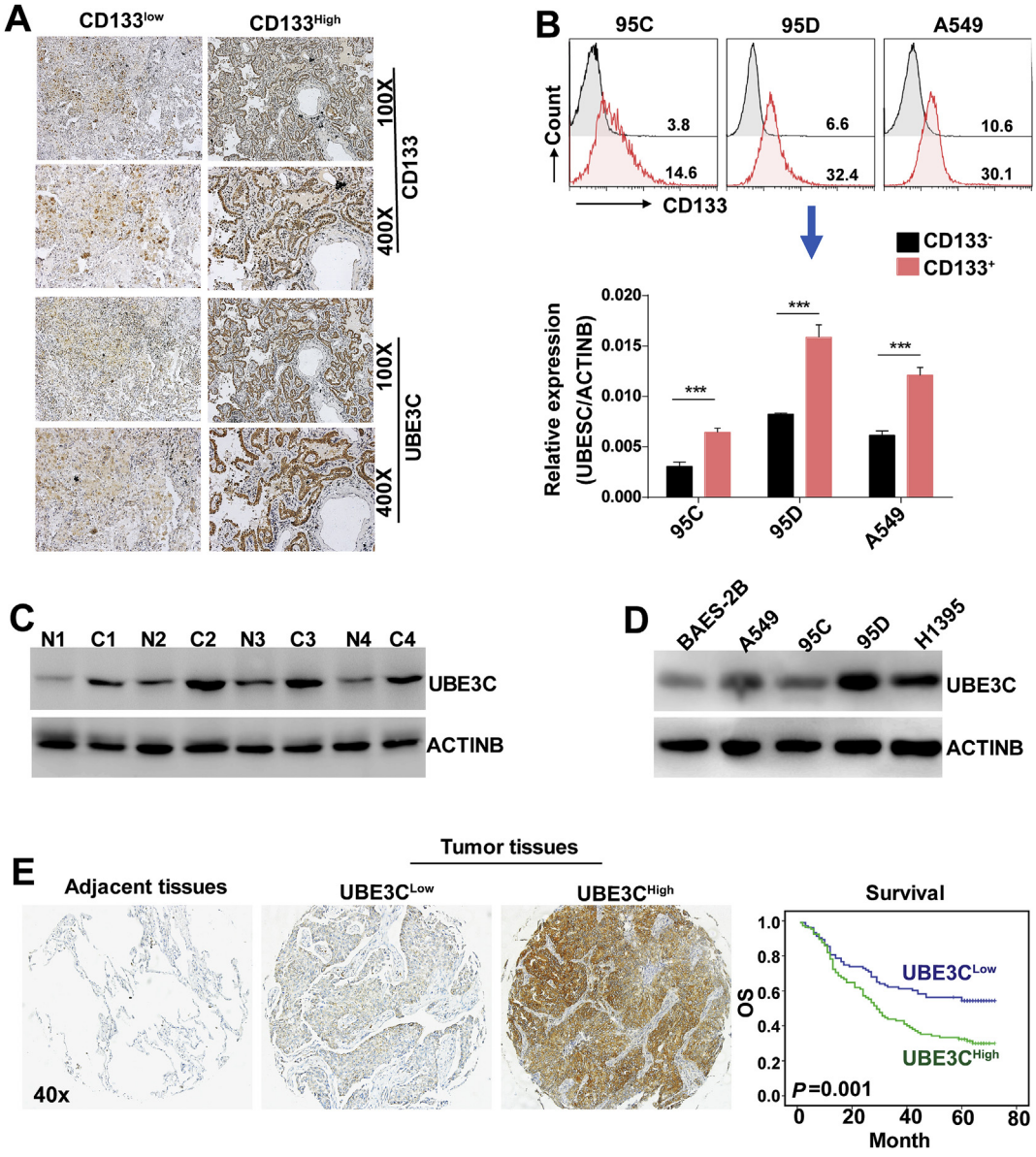

Fig. 1. UBE3C is overexpressed in NSCLC stem like cells and predicts poor prognosis. (**A**) CD133$^+$ cell density in NSCLC tissues. The protein level of UBE3C were determined using IHC in NSCLC tissues with high density of CD133$^+$ cells (CD133 ^{high}) and low density of CD133$^-$cells (CD133^{low}). (**B**) The CD133$^+$ stem like cells and paired CD133$^-$ non-stem like cells were isolated from NSCLC 95C, 95D and A549 cells using flow cytometry. UBE3C expression was analyzed by quantitative PCR (qRT-PCR). (**C-D**) The protein level of UBE3C in NSCLC tissues and cells by western blot. (**E**) The immunohistochemistry staining of UBE3C in 208 human NSCLC specimens and its correlation with the clinical characteristics. The mean ± SD was determined from three replicates. ***$P < 0.001$.

degradation of Gli-1 protein via the ubiquitin-proteasome pathway, which suppressed the breast CSC properties [18]. Moreover, deubiquitylation acts to preserve the cancer stemness, with treatment with DUBs inhibitors resulting in reduction of CSCs and attenuated therapeutic resistance [19,20]. These findings suggest that ubiquitination represents a posttranslational modification capable of altering CSC properties.

The ubiquitin-protein ligase E3C (UBE3C) is an E3 ligase recently reported as being aberrantly expressed in breast cancer [21], hepatocellular carcinoma (HCC) [22], and renal cell carcinoma (RCC) [23], as well as in glioma tissues [24], thereby contributing to their development and progression. In these cases, UBE3C reportedly played different roles associated with regulation of microRNAs, downstream tumor-suppressor genes, or other signaling pathways. However, UBE3C participation in lung cancer growth, invasion and metastasis remains unknown, as does the possibility of altering lung cancer-cell stemness.

In this study, we assessed the role of UBE3C in promoting lung tumorigenesis and revealed its function in maintaining lung cancer-cell stemness. Moreover, we identified AHNAK is a new UBE3C substrate, with UBE3C maintaining lung cancer-cell stemness via AHNAK ubiquitination and degradation. Mechanistically, AHNAK was identified as a p53 cofactor required for p53-midiated inhibition of the transcription of stemness related genes through its binding to the respective promoter regions. Additionally, AHNAK downregulation by UBE3C overexpression eliminated p53-mediated inhibition of transcription, thereby resulting in enhanced cell stemness. These findings suggested that UBE3C functions as a tumor promotor in NSCLC by maintaining lung cancer-cell stemness through disruption of the AHNAK-p53 complex. Furthermore, this is the first study confirming that lower UBE3C and higher AHNAK protein correlate with longer survival time in NSCLC patients.

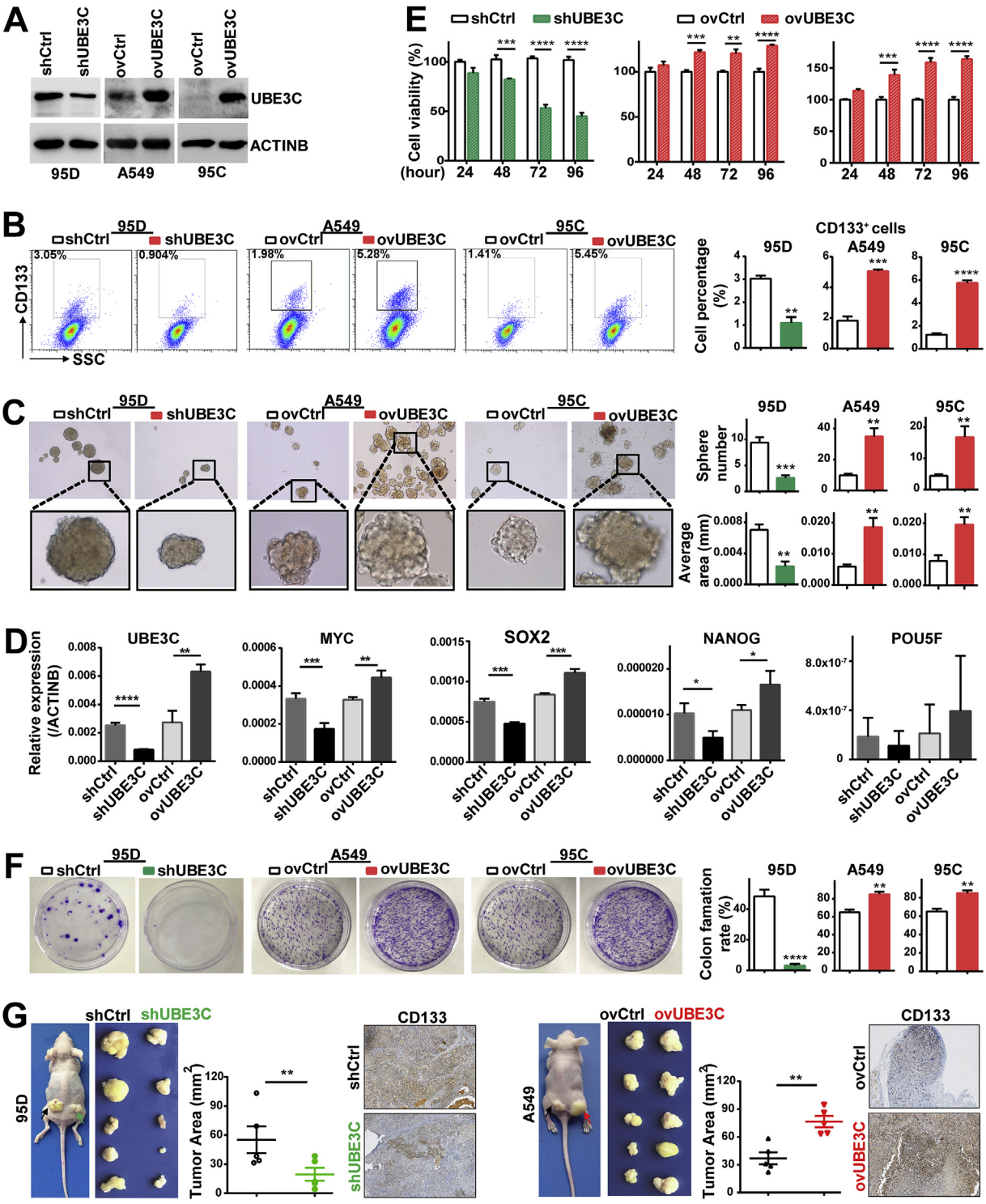

Fig. 2. UBE3C contributes to NSCLC growth by maintaining cancer cell stemness. (**A**) Overexpressing UBE3C in both 95C cells and A549 cells using a lentivirus harboring *UBE3C* (ovUBE3C), and attenuating UBE3C expression in 95D cells using a lentivirus harboring shRNAs against *UBE3C* (shUBE3C). UBE3C protein levels were determined by western blot. siCtrl and ovCtrl are the negative control. (**B**) The percentage of $CD133^+$ cells were analyzed after downregulation or overexpression of UBE3C. (**C**) Tumorspheres were established in ultra-low-adherence culture conditions with 2% B27, 20 ng/mL human recombinant epidermal growth factor, and 20 ng/mL basic fibroblast growth factor. The number of tumorspheres were counted (diameter > 50 μm) and their average volume were analyzed. (**D**) Levels of stem-related genes, including *sex-determining region Y-box 2* (*SOX2*), *MYC*, *NANOG*, and *octamer-binding transcription factor 4 (OCT4)/POU5F*, as well as UBE3C were determined by qRT-PCR. (**E-F**) Cell viability and colony formation were analyzed in NSCLC cells differentially expressing UBE3C. The mean ± SD was determined from three replicates. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (**G**) UBE3C overexpressing (ovUBE3C) A549 cells, UBE3C deficient 95D cells and their paired control (ovCtrl or shCtrl) cells were implanted subcutaneously into the bilateral posterior flank of nude mice. After nude mice were sacrificed, tumor size was measured and $CD133^+$ cell density was determined by IHC straining. The data shown of each group are pooled with 5 mice and are expressed as mean ± SD. $^{**}P < 0.01$.

2. Materials and methods

2.1. Samples and cells

NSCLC specimens embedded in paraffin were obtained from 208 patients who underwent complete surgical resection at Zhongshan Hospital (Shanghai, People's Republic of China) in 2005 following their providing written informed consent. The informed consent, and clinicopathological characteristics of patients were reported in the previous studies [25], and relisted here (Supplementary Table S1). All prognostic studies followed the REMARK reporting guidelines (Supplementary Table S2), and the description of the specimens was carried out per the BRISQ reporting guidelines (Supplementary Table S3). Study approval for the study was obtained from the Research Ethics Committee of Zhongshan Hospital.

Cell line BEAS-2B were originally obtained from ATCC (Manassas, VA, USA). Cell lines A549, H1395 and 95D were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China), and 95C cells were from BeNa Culture Collection (Kunshan, China). These cells were authenticated by short-tandem-repeat DNA profiling. All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS, 2 μM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin sulfate.

2.2. Statistical analysis

Data were analyzed using GraphPad Prism software package (version 5; GraphPad Software Inc., La Jolla, CA, USA) and are presented as the mean ± standard deviations (SD). Differences between two groups were analyzed using the Student's unpaired *t*-test. Pearson's correlation coefficient was used to analyze correlations between groups. The cumulative survival time was calculated using the Kaplan–Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. UBE3C is overexpressed in stem-like NSCLC cells and predicts poor prognosis

Previous reports identified CD133 as a surface marker of lung cancer stem cells. In the present study, we found that UBE3C expression in NSCLC tissues harboring a high density of $CD133^+$ cells ($CD133^{high}$) was higher than that in tissues exhibiting a low density of $CD133^-$ cells ($CD133^{low}$) (Fig. 1A). To confirm a positively correlation between UBE3C expression and NSCLC stemness, we isolated the $CD133^+$ stem-like NSCLC cells and compared them with $CD133^-$ non-stem like cells from NSCLC 95C, 95D and A549 cells, respectively. Subsequent analysis of UBE3C expression showed that $CD133^+$ cells have exhibited higher UBE3C expression than paired $CD133^-$ cells (Fig. 1B). Moreover, UBE3C protein levels in NSCLC tissue and cells and cells confirmed that UBE3C was overexpressed in cancer tissues as compared with levels in paired adjacent normal tissues (Fig. 1C). Consistently, UBE3C expression was higher in NSCLC cell lines, including A549, 95C, 95D and H1395, relative to levels in normal bronchial epithelial cells (BEAS-2B) (Fig. 1D).

To determine the clinical significance of UBE3C overexpression in stem-like NSCLC cells, we analyzed UBE3C expression by IHC staining in 208 human NSCLC specimens and evaluated its correlation with the clinical characteristics. The results showed upregulated UBE3C levels in NSCLC tissues, and the higher UBE3C levels correlated with shorter patient survival time and advanced tumor stage (Fig. 1E, and Supplementary Table S1). These results indicate UBE3C is overexpressed in stem-like NSCLC cells, and that elevated UBE3C expression is correlated with the shorter survival time in NSCLC patients.

3.2. UBE3C contributes to NSCLC growth by maintaining cancer-cell stemness

To investigate the role of UBE3C in NSCLC cell self-renewal and tumorigenesis, we overexpressed UBE3C in both 95C cells and A549 cells using a lentivirus harboring *UBE3C* (ovUBE3C). Additionally, we attenuated UBE3C expression in 95D cells and A549 cells using a lentivirus harboring shRNAs against *UBE3C* (shUBE3C) (Fig. 2A). Our results showed that the percentage of $CD133^+$ cells increased in ovUBE3C-treated NSCLC cells, but decreased in shUBE3C-treated cells relative to paired control cells (Fig. 2B and Supplementary Fig. S1). Subsequent detection of cell stemness revealed a higher number of tumor spheres, as well as increases in average volume, in ovUBE3C-treated NSCLC cells relative to that in paired control cells. However, both number and volume were lower following UBE3C silencing (Fig. 2C). Consistently, the secondary spheres of ovUBE3C-treated NSCLC cells were also increased in number and size compared to control cells (Supplementary Fig. S2). Furthermore, we consistently found that levels of stem-related genes, especially *sex-determining region Y-box 2* (*SOX2*) and *MYC* were higher in the UBE3C-overexpressing cells, but lower after UBE3C knockdown (Fig. 2D). These data demonstrate that UBE3C is functionally required to maintain NSCLC stemness.

According to the previous findings, CSCs share properties, such as self-renewal, tumorigenic and metastatic abilities. To test the ability of UBE3C to initiate and promote NSCLC growth, we analyzed the cell proliferation and metastasis, as well as tumorigenesis, in cell differentially expressing UBE3C. The results showed that UBE3C overexpression enhanced cell viability (Fig. 2E) and promoted colony formation of NSCLC cells (Fig. 2F). Whereas, both cell viability and colony formation rates were reduced following UBE3C silencing (Fig. 2E and F). Furthermore, $CD133^+$ cells exhibit stronger proliferative capacity than $CD133^-$ cells especially in serum-free stem cell growth conditions. Loss of UBE3C expression impaired both $CD133^+$ and $CD133^-$ cell proliferation (Supplementary Fig. S3). Additionally, UBE3C levels affected cell apoptosis, but not the cell cycle (Supplementary Fig. S4). To confirm the growth-enhancing role of UBE3C in NSCLC, we investigated the effect of UBE3C on NSCLC tumor growth *in vivo*. UBE3C overexpressing (ovUBE3C) A549 cells, UBE3C deficient (shUBE3C) 95D cells and their paired control (ovCtrl, shCtrl) cells were implanted subcutaneously into the bilateral posterior flank of nude mice. We observed that the UBE3C- overexpressing cells yielded tumors with a larger size than the same dose of control cells. While, the tumor growth rate of UBE3C-knockdown group was significantly slower (Fig. 2G). Moreover, results from transwell assays and showed that UBE3C promoted NSCLC cell migration (Supplementary Fig. S5). Considering the involvement of cancer stem cells in drug resistance, we further validated that knockdown of UBE3C enhanced paclitaxel-induced suppression of 95D cell viability, and increased cell apoptosis (Supplementary Fig. S6). These results indicate UBE3C that promoted cell-stemness function as a tumor-promoting factor in NSCLC.

3.3. AHNAK is a direct target of UBE3C

To determine the mechanisms underlying UBE3C induction of NSCLC stemness, we screened UBE3C-interacting proteins in A549, 95D cells and UBE3C-overexpressing 95C cells by co-IP-MS analysis. We found a total of 20 proteins significantly interacting with UBE3C, with overlaps in the three cells. Of them, AHNAK, an unusual and somewhat mysterious scaffolding protein, were highlighted, because AHNAK peptides were found most frequently in the UBE3C-precipitation complex (Fig. 3A). Moreover, recent evidence suggests that AHNAK might be an accomplice in the development of tumor metastasis. In the present study, we found that fewer AHNKA peptides were detected in UBE3C-overexpressing 95C cells or 95D cells relative to those found in A549 cells exhibiting lower UBE3C expression, suggesting a negative

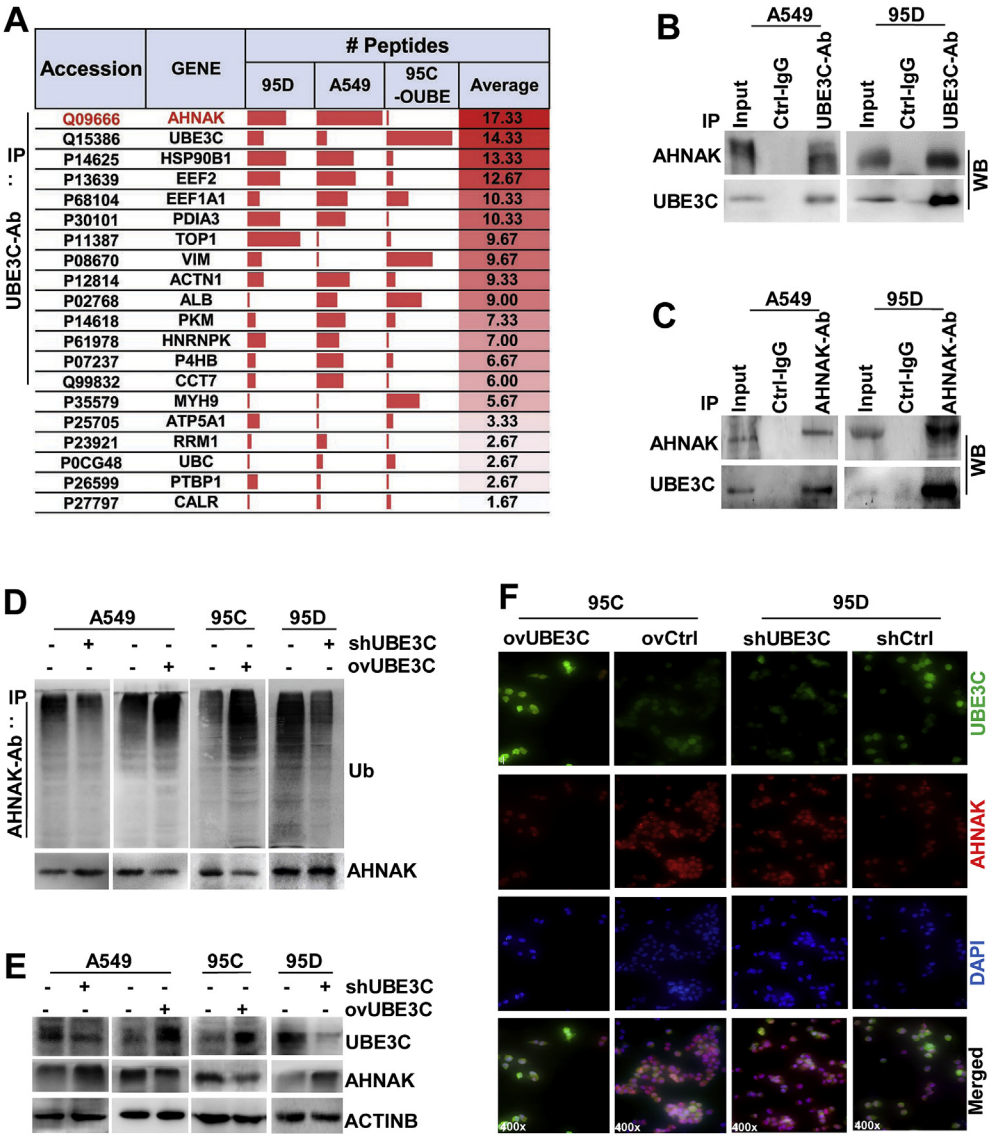

Fig. 3. AHNAK is the direct target of UBE3C. (**A**) UBE3C-interacting proteins in A549, 95D cells and UBE3C overexpressing 95C cells (95C-OUBE) were screened by co-immunoprecipitation (co-IP)-mass spectrometry (MS) analysis. (**B-C**) Co-IP and western blot (WB) analysis. UBE3C antibodies (UBE3C-Ab) pulled down AHNAK, and UBE3C was pulled down using AHNAK antibodies (AHNAK-Ab), respectively in A549 and 95D cells. (**D**) To determine the ubiquitination, the endogenous AHNAK was precipitated by anti-AHNAK antibody and the ubiquitin (Ub) chain conjugates on AHNAK were determined by western blot with anti-Ub antibody. (**E**) Protein levels of AHNAK were determined in UBE3C deficient (with shUBE3C) or overexpressing (with ovUBE3C) cells. (**F**) Immunofluorescence straining was performed using anti-UBE3C antibody and anti-AHNAK antibody. DAPI were used to indicate the nucleus. The data shown are repeated three times and show the consistent results.

correlation between UBE3C and AHNAK. Furthermore, in reciprocal co-IP assays, UBE3C Abs pulled down AHNAK, and UBE3C was pulled down using AHNAK Abs (Fig. 3B and C). These results strongly suggest that endogenous UBE3C forms a physical complex with AHNAK.

We further determined whether UBE3C ubiquitylates AHNAK, thereby marking it for ubiquitin-mediated proteolysis. IP using an Ab against endogenous AHNAK, followed by detection of ubiquitin conjugates, revealed that AHNAK was multi-ubiquitinated in NSCLC cells. The level of ubiquitin conjugates on the AHNAK protein was lower in UBE3C-deficient cells, but higher in UBE3C overexpressing cells (Fig. 3D). Consistently, AHNAK protein levels were dramatically decreased in UBE3C overexpressing cells, but upregulated in those with UBE3C deficiency (Fig. 3E). Moreover, immunofluorescence identified co-localization of UBE3C and AHNAK along with a negative correlation in their relative expression levels (Fig. 3F). These results indicate that AHNAK is a direct substrate of UBE3C, and that elevated UBE3C levels controlled the basal turnover of AHNAK by ubiquitin-mediated proteolysis.

3.4. AHNAK reverses UBE3C mediated-regulation of NSCLC stemness

To determine whether AHNAK as a substrate mediates the effect of UBE3C on NSCLC stemness, we downregulated AHNAK expression by RNA inference in UBE3C-deficient NSCLC cells (Supplementary Fig. S7A). The results showed a decreased percentage of $CD133^+$ cells in the presence of UBE3C deficiency; however, this was reversed following downregulation of AHNAK expression (Fig. 4A). Consistently, decreases in both the number and volume of tumorspheres caused by downregulation of UBE3C was also reversed by AHNAK downregulation (Fig. 4B).

Furthermore, investigation of stemness-related gene expression revealed that AHNAK downregulation in NSCLC cells increased the expression of stemness related genes, especially *MYC* and *SOX2*. This was similar to results observed following *UBE3C* overexpression. Moreover, both mRNA and protein levels of *MYC* and *SOX2* were upregulated following AHNAK downregulation in UBE3C-deficient cells (Fig. 4C and D; Supplementary Fig. S7B). Inhibition of cell proliferation and cell migration caused by UBE3C deficiency were also reversed by AHNAK downregulation (Fig. 4E and Supplementary Fig. S8). These results indicate that AHNAK is a factor that weakens stem cell characteristics, and that its downregulation contributes to UBE3C-mediated enhancement of stemness. Additionally, we found that UBE3C levels were upregulated following AHNAK downregulation (Fig. 4F), suggesting a possible feedback loop that synergistically regulates NSCLC cell stemness.

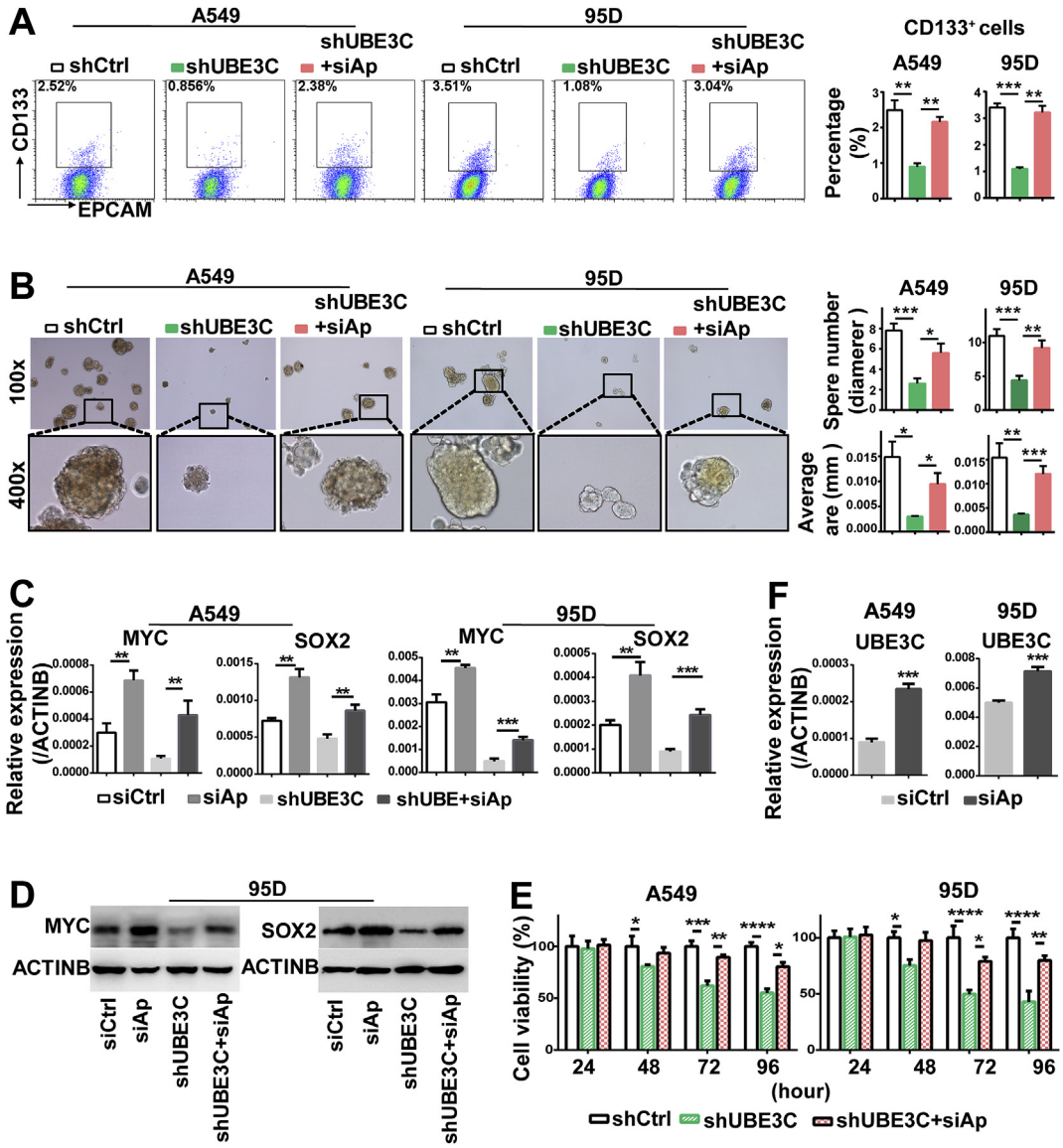

Fig. 4. AHNAK reverses UBE3C mediated-regulation of cancer cell stemness. Downregulating AHNAK expression by transfecting a small RNA mix (siAp, containing three small RNAs targeting different regions of AHNAK) in UBE3C deficient A549 and 95D cells respectively. (**A**) The percentage of CD133$^+$ stem like cells, (**B**) the number and volume of tumorspheres, (**C**) and the RNA levels of the stemness related genes, *MYC* and *SOX2* were determined. Following AHNAK downregulation, (**D**) protein levels of MYC and SOX2 were confirmed in UBE3C deficient 95D cells, (**E**) cell viability were analyzed by performing CCK-8 assay, (**F**) and UBE3C expression was analyzed by qRT-PCR. The mean ± SD was determined from three replicates. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.5. UBE3C targets AHNAK to remove p53 inhibition of stemness-related gene transcription

Given that downregulation of AHNAK is crucial for UBE3C-mediated NSCLC cell stemness, we investigate the underlying mechanisms associated with this role. The role of p53 as a key tumor suppressor reportedly involves suppression of stemness in multiple cancers. Consistent with these reports, we found that inhibition of p53 dramatically increased stemness-related gene expression, especially that of *SOX2* and *MYC*, with higher basal levels in NSCLC cells (Fig. 5A). However, p53 expression was not changed following AHNAK downregulation (Fig. 5B). Because AHNAK is a scaffolding protein frequently found in the nucleus, we hypothesized that it might play a role in p53 binding to target-gene promoter regions. In reciprocal co-IP assays using Abs against AHNAK, p53 was also pulled down (Fig. 5C), suggesting that endogenous AHNAK forms a physical complex with p53.

Because both AHNAK downregulation and treatment with a p53 inhibitor increased the mRNA levels of *SOX2* and *MYC*, we evaluated whether p53 binds directly to the *SOX2* and *MYC* promoter regions to regulate transcription and whether this process is controlled by the UBE3C-AHNAK axis. ChIP and qRT-PCR assays confirmed recruitment of p53 to the putative *MYC* and *SOX2* promoter regions in NSCLC cells; however, attenuated AHNAK levels dramatically reduced p53 binding (Fig. 5D). Consistently, in UBE3C-overexpressing NSCLC cells harboring low levels of AHNAK, p53 binding to the *MYC* and *SOX2* promoter regions was also suppressed (Fig. 5E). By contrast, UBE3C knockdown improved p53 binding to these regions, whereas AHNAK silencing by RNAi decreased p53 binding (Fig. 5F). These results indicate that AHNAK is required for p53 binding to the promoter regions of stemness-related genes, thereby controlling cell stemness. While, AHNAK downregulation derived by UBE3C *o*verexpression reduce the gene transcription and the subsequent cell stemness by inhibiting p53 binding to their respective promoter regions.

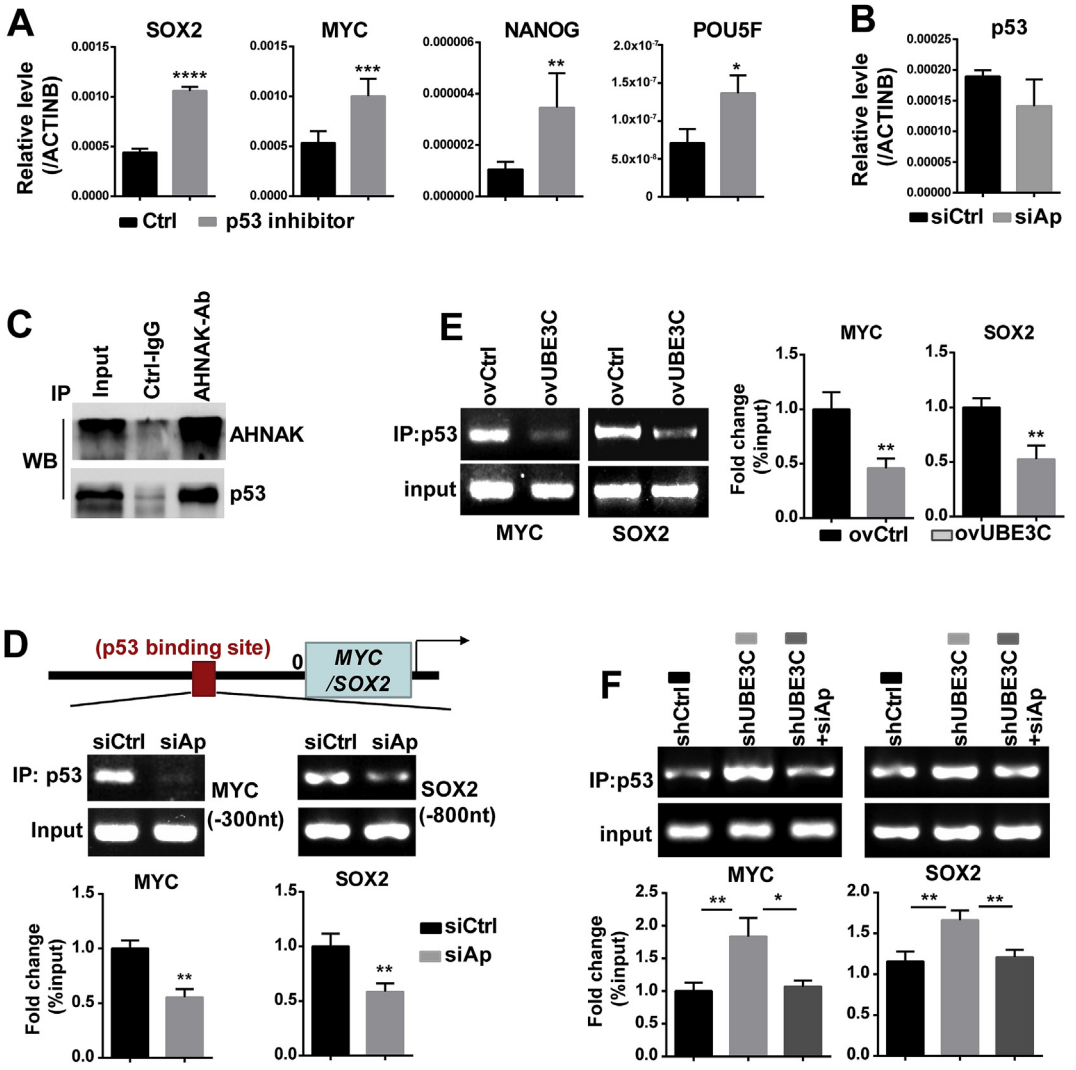

Fig. 5. UBE3C targets AHNAK to remove p53 inhibition on stemness related gene transcription. (**A**) A549 cells were treated with p53 inhibitor (Pifithrin-α-HBr, 10 μM), and then RNA levels of *SOX2, MYC*, *NANOG* and *POU5F* were determined by qRT-PCR. (**B**) Relative expression of p53 in mRNA level. (**C**) Co-IP assays were performed using antibodies against AHNAK (AHNAK-Ab) and its control IgG (Ctrl-IgG). p53 was detected in the co-immunoprecipitation complex using western blot (WB). (**D**) The binding site of p53 on the promoter of *MYC* and *SOX2*. After silencing AHNAK by transfection of siAp, chromatin immunoprecipitation and PCR (ChIP-PCR) assays were performed to confirm recruitment of p53 to the putative *MYC* and *SOX2* promoter regions in NSCLC cells. (**E-F**) The binding of p53 to the *MYC* and *SOX2* was also determined by ChIP-PCR assay in UBE3C-overexpressing or -deficient NSCLC cells, as well as in cells with double deficiency of AHNAK and UBE3C (shUBE3C + siAHNAK). The mean ± SD was determined from three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.6. AHNAK negatively correlates with UBE3C levels in NSCLC and predicts longer survival of patients

To assess the clinical significance of the UBE3C-AHNAK regulatory axis in patients, we confirmed a negative relationship between AHNAK and UBE3C levels, as well as their correlation with the prognosis of NSCLC patients. Our results showed that AHNAK protein levels in NSCLC cancer tissues were lower those in adjacent normal tissues (Fig. 6A). Moreover, these levels negatively correlated with UBE3C levels in NSCLC tissues (Fig. 6B). Additionally, compared with normal bronchial epithelial BAES-2B cells, AHNAK levels were reduced in most NSCLC cell lines, but especially in 95D, A549, and H1395 cells exhibiting high levels of UBE3C (Fig. 6C).

To determine the clinical significance of these findings, AHNAK protein levels were detected by IHC in 208 NSCLC specimens, with results showing that NSCLC patients exhibiting high levels of AHNAK displayed longer survival times. The 5-year disease-specific survival rate in patients with high level of AHNAK was higher than that with low AHNKA level ($P = 0.043$) (Fig. 6D). Moreover, further analysis showed that patients with both high levels of UBE3C and low levels of AHNAK have the shortest survival time. (Fig. 6E). In multivariate analysis, lymph node metastasis, tumor size and combination of UBE3C/AHNAK were identified as independent prognostic factors in patients' disease-specific survival (Table 1). These results suggested that elevated AHNAK levels and lower UBE3C levels predict better patient survival and might represent a prognostic indicator of prognosis in NSCLC.

4. Discussion

According to CSC characteristics, identifying key regulatory pathways controlling stemness could suggest potential targets for cancer therapy. In this study and for the first time, we found that UBE3C-mediated ubiquitination regulated the maintenance of cell stemness in NSCLC. Our results demonstrated the AHNAK was a novel substrate of UBE3C and required for p53 binding to the promoter regions of stemness-related genes to inhibit their transcription. Moreover, ubiquitin-mediated degradation of AHNAK attenuated p53 binding to these promoter regions, thereby allowing stemness-related gene transcription

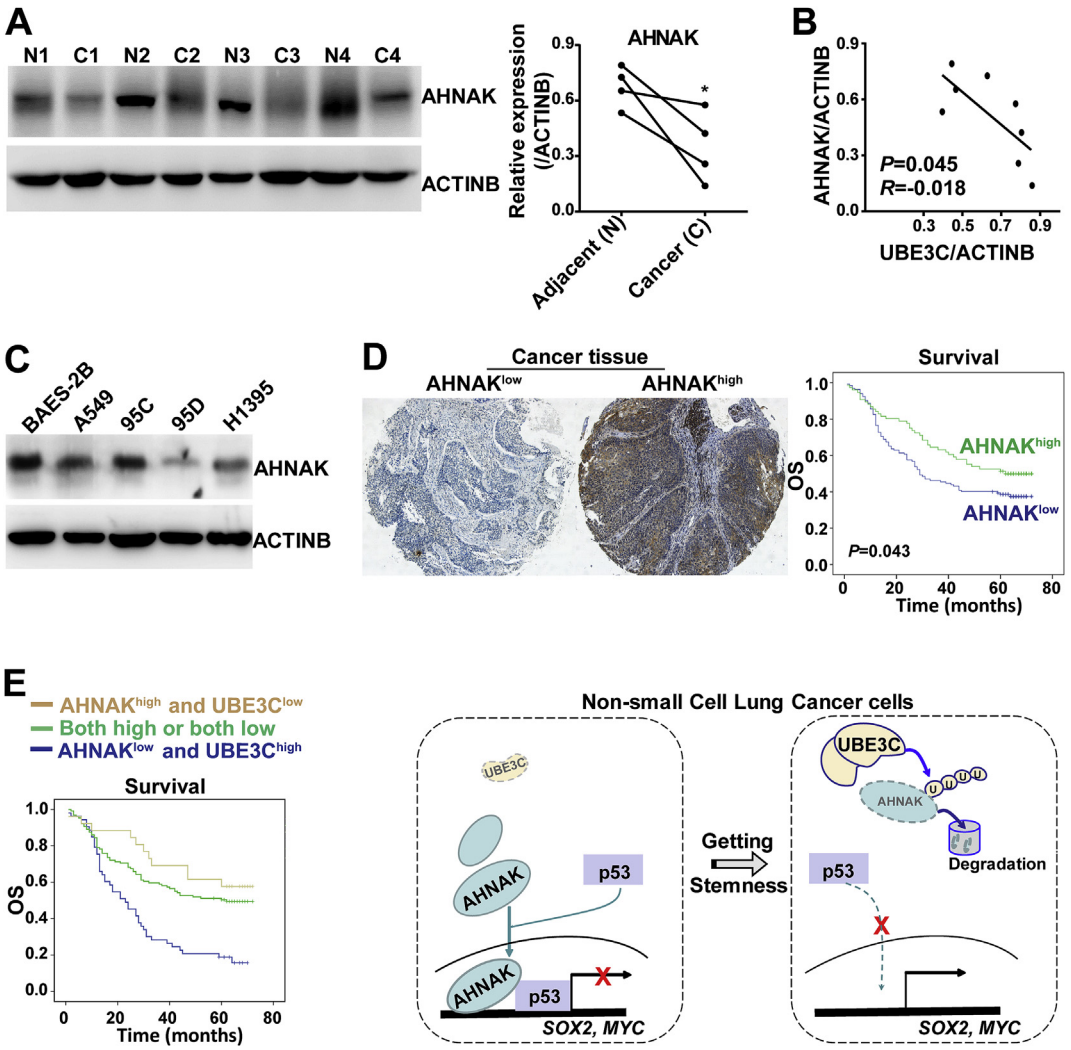

Fig. 6. AHNAK negatively correlates with UBE3C in NSCLC and predicts a longer survival of patients. (**A**) AHNAK protein levels was determined in human NSCLC cancer tissues and their adjacent normal tissues, (**B**) and then their correlation with UBE3C levels were analyzed. The data are expressed as mean ± SD. *$P < 0.05$. (**C**) AHNAK levels in NSCLC cell line 95D, A549 and H1395, as well as in the normal bronchial epithelial BAES-2B cells. (**D**) AHNAK protein levels were detected by IHC in 208 NSCLC specimens, and their correlation with the prognosis of NSCLC patients were analyzed. $AHNAK^{high}$ indicated the group with high level of AHNAK, while, $AHNAK^{low}$ indicated the group with low level of AHNAK. (**E**) Survival analysis. The "both high or both low" group were composed of patients with both high level of AHNAK and UBE3C, or patients with both low level of AHNAK and UBE3C. (**F**) A proposed model for all the results. AHNAK is a novel substrate of UBE3C, and required for the binding of p53 to the promoter regions of stemness-related genes (*e.g. SOX2* and *MYC*) to inhibit their transcription. When NSCLC cells turn into stem like cells or obtain stemness, UBE3C level is frequently upregulated, leading to ubiquitination of AHNAK. Subsequently, ubiquitin-mediated degradation of AHNAK attenuates p53 binding to these promoter regions, thereby allowing stemness-related gene transcription and promoting stem-like characteristics.

Table 1
Univariate and Multivariate analysis of factors associated with OS.

Variables	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Gender (female vs. male)	0.789	0.526–1.183	0.251			
Smoking status (non-smokers vs. smokers)	0.779	0.543–1.118	0.175			
Differentiation (Poor vs. Well/Moderate)	1.431	1.000–2.049	0.050			
Tumor stage (III-IV vs. I-II)	2.771	1.922–3.993	< 0.001	1.295	0.818–2.049	0.270
Lymph node metastasis (Yes vs. No)	3.042	2.103–4.399	< 0.001	2.240	1.440–3.486	< 0.001
Tumor size (≥ 3 cm vs. < 3 cm)	2.755	1.758–4.318	< 0.001	2.090	1.315–3.320	0.002
Combination: UBE3C/AHNAK expression			< 0.001			0.003
Group II vs. Group III	0.449	0.305–0.661	< 0.001	0.511	0.344–0.759	0.001
Group I vs. Group III	0.324	0.167–0.629	0.001	0.471	0.232–0.955	0.037

Abbreviations: OS, overall survival; 95%CI, 95% confidence interval. Group I, AHNAK high expression and UBE3C low expression; Group II, both AHNAK and UBE3C high or low expression; Group III, AHNAK low expression and UBE3C high expression.

and promoting stem-like characteristics. Furthermore, NSCLC patients exhibiting both high levels of AHNAK and low levels of UBE3C displayed longer survival times relative to other phenotypes, with these levels also indicating NSCLC prognosis (Fig. 6F).

UBE3C belongs to the HECT family of E3 ubiquitin ligases and is implicated in the ubiquitin-proteasome pathway [15,26]. Genetic aberrations and altered expression of HECT E3 ligases are often observed in tumors [22]. We confirmed that UBE3C expression was markedly increased in NSCLC tissues, played a tumorigenic role. This role has been reported in other tumors, including those associated with hepatocellular carcinoma [22], renal cell carcinoma [23], glioma [24], melanoma [27], and breast cancer [21]. An important finding from our study was that UBE3C was preferentially overexpressed in CSCs and essential for maintaining stemness.

CSCs harbor the potential of self-renewal, tumor-initiating, recurrence, and resistance to conventional chemotherapy and radiation. Tumor with high proportions of CSCs is an unfavorable prognostic indicator for patients. We identified UBE3C as a prognostic indicator for NSCLC. Patients with high level of UBE3C exhibited markedly shorter survival time. The 5-year recurrence rate for patients with high level of UBE3C was 68.6%, which was much higher than those with low level of UBE3C. Moreover, we validated the role of UBE3C in sensitizing NSCLC cells to paclitaxel, one of the first-line chemotherapy drugs. Considering the involvement of cancer stem cells in drug resistance, we speculated that UBE3C overexpression in CSCs might be a primary explanation for its tumor-promoting effect and chemotherapy-resistance. Additionally, we identified for the first time AHNAK as a binding target of UBE3C.

Although other tumor related molecules have been identified as UBE3C substrates, including Annexin A7 [24] and Wnt/β-catenin [23]. AHNAK was the first target associated with maintaining cell stemness. ANXA7 is a target of UBE3C, whereas, our results showed that loss of ANXA7 had no significant effect on the expression of stemness related genes, indicating it is not responsible for UB3C-maintained stemness (data not shown). Although the precise role of AHNAK remains unknown, previous studies report that AHNAK participates in multi-protein complexes, most likely acting as a structural scaffold. Furthermore, several intracellular locations have been reported for AHNAK, including the nucleus, cytoplasm, and plasma membrane, with both nuclear-localization and -export signals identified in AHNAK and indicating a possible role in intracellular trafficking [28,29]. Additionally, studies have implicated AHNAK in distinct cellular processes and/or pathways ranging from formation of the blood–brain barrier and cell architecture and migration to regulation of cardiac calcium channels and muscle-membrane repair [30-33].

The present study is the first to demonstrate AHNAK downregulation in NSCLC tissue, its status as a UBE3C substrate, and a role as a tumor suppressor in NSCLC. Studies specifically investigating the role of AHNAK in cancer remain especially limited, although AHNAK was recently reported as a tumor suppressor in breast cancer based on its ability to activate the TGFβ-signaling pathway [34-36]. Consistently, Park et al. showed that *AHNAK*-knockout mice displayed increased lung volume and thicker alveolar walls along with type II pneumocyte hyperplasia [37]. Distinct functions of AHNAK might due to specific targets and the surrounding conditions. Lee et al. described AHNAK as capable of binding to regulatory Smad proteins, resulting in attenuated cell proliferation, which depended TGFβ stimulation [35]. In the present study, we found that AHNAK played a suppressive role in NSCLC that was p53 dependent. Our results represent the first report of a relationship between AHNAK and p53.

The regulatory role of p53 in CSCs has been reported in various tumors [38-41]. P53 deficiency or inactivation can induce cancer stem cells. In our study, p53 inhibitor, PFTα [42] downregulated cell growth related endogenous cellular p53-responsive genes (e.g. *MDM2*, *p21* and *BAX*) (data not shown), but promoted expression of stemness related genes. Consistently, HepG2 cells directly treated with PFTα or transfected with the p53 siRNA showed decreased $CD133^+$ cells and sphere-forming ability [39]. Other results from Tschaharganeh [43], Aloni-Grinstein [41], and Sui [44] confirmed that p53 served as a barrier to CSC formation. Significantly, we confirmed p53 binding to the promoter regions of the stemness-related genes *SOX2 and MYC* to suppress their expression. In this process, AHNAK was required. The frequent downregulation of AHNAK levels in NSCLC tissues might represent a reason for the failure of p53 to inhibit cell stemness. Since direct or indirect proteins coupled with the target protein can be pulled down in co-IP assay, p53 was detected in the complex precipitated by UBE3C Abs because of their interaction with AHNKA. It implies that p53, ANNAK and UBE3C may exist in the same protein complex, although functional structure of the three proteins in this complex is still unclear. While, overexpression of UBE3C had no effect on p53 expression (data not shown), at least suggesting that p53 is not a direct target of UBE3C.

In conclusion, our findings suggest UBE3C-mediated AHNAK ubiquitination and degradation accompanied by subsequent blockage of p53 inhibition of stemness-related-gene transcription as a new post-translational regulatory model describing maintenance of NSCLC cell stemness. According to our result, UBE3C knockdown increased p53 binding to the promoter regions of stemness-related genes, thereby inhibiting their transcription and preventing NSCLC tumorigenesis. Therefore, repressing UBE3C-mediated ubiquitination in order to promote formation of the AHNAK-p53 complex might represent a promising therapeutic strategy for NSCLC.

Author contributions

J.G., W.M and W.R. performed the research, discussed and analyzed the data, and wrote the paper. R.L., and D.G. designed the research, discussed and analyzed the data, and wrote the paper. F.X., Q.Z., C.L., Z.L. and Z.Z. developed methods and provided material supports. Y.C. revised the paper and provided technical supports.

Conflicts of interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.029.

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