



## Validation of NEDD8-conjugating enzyme UBC12 as a new therapeutic target in lung cancer

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### ARTICLE INFO

#### Article history:

Received 11 May 2019

Received in revised form 31 May 2019

Accepted 5 June 2019

Available online 14 June 2019

#### Keywords:

Lung cancer

UBC12

Anticancer target

Cell-cycle arrest

Overcome drug resistance

### ABSTRACT

**Background:** The neddylation pathway is overactivated in human cancers. Inhibition of neddylation pathway has emerged as an attractive anticancer strategy. The mechanisms underlying neddylation overactivation in cancer remain elusive. MLN4924/Pevedonidistat, a first-in-class NEDD8-activating enzyme (NAE, E1) inhibitor, exerts significant anti-tumor effects, but its mutagenic resistance remains unresolved.

**Methods:** The expression of NEDD8-conjugating enzyme UBC12/UBE2M (E2) and NEDD8 were estimated by bioinformatics analysis and western blot in human lung cancer cell lines. The malignant phenotypes of lung cancer cells were evaluated both *in vitro* and *in vivo* upon UBC12 knockdown. Cell-cycle arrest was evaluated by quantitative proteomic analysis and propidium iodide stain and fluorescence-activated cell sorting (FACS). The growth of MLN4924-resistant H1299 cells was also evaluated upon UBC12 knockdown.

**Findings:** The mRNA level of UBC12 in lung cancer tissues was much higher than that in normal lung tissues, increased with disease deterioration, and positively correlated with NEDD8 expression. Moreover, the overexpression of UBC12 significantly enhanced protein neddylation modification whereas the downregulation of UBC12 reduced neddylation modification of target proteins. Functionally, neddylation inactivation by UBC12 knockdown suppressed the malignant phenotypes of lung cancer cells both *in vitro* and *in vivo*. The quantitative proteomic analysis and cell cycle profiling showed that UBC12 knockdown disturbed cell cycle progression by triggering G<sub>2</sub> phase cell-cycle arrest. Further mechanistical studies revealed that UBC12 knockdown inhibited Cullin neddylation, led to the inactivation of CRL E3 ligases and induced the accumulation of tumor-suppressive CRL substrates (p21, p27 and Wee1) to induce cell cycle arrest and suppress the malignant phenotypes of lung cancer cells. Finally, UBC12 knockdown effectively inhibited the growth of MLN4924-resistant lung cancer cells.

**Interpretation:** These findings highlight a crucial role of UBC12 in fine-tuned regulation of neddylation activation status and validate UBC12 as an attractive alternative anticancer target against neddylation pathway.

**Fund:** Chinese Minister of Science and Technology grant (2016YFA0501800), National Natural Science Foundation of China (Grant Nos. 81401893, 81625018, 81820108022, 81772470, 81572340 and 81602072), Innovation Program of Shanghai Municipal Education Commission (2019-01-07-00-10-E00056), Program of Shanghai Academic/Technology Research Leader (18XD1403800), National Thirteenth Five-Year Science and Technology Major Special Project for New Drug and Development (2017ZX09304001). The funders had no role in study design, data collection, data analysis, interpretation, writing of the report.

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**Abbreviations:** FACS, fluorescence-activated cell sorting; NAE, NEDD8-activating enzyme; CHX, cycloheximide; SPSS, Statistical Program for Social Sciences software; GFP, green fluorescent protein; CDKN1A, Cyclin Dependent Kinase Inhibitor 1A, p21; CDKN1B, Cyclin Dependent Kinase Inhibitor 1B, p27.

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### 1. Introduction

Post-translational protein neddylation is a process of the covalent attachment of NEDD8, an ubiquitin-like molecular, to substrate proteins, and thus regulates subcellular localization, stability, conformation and functions of targeted proteins. Neddylation is a three-step enzymatic cascade reaction, mediated by NEDD8-activating enzyme E1 (NAE, a

## Research in context

### Evidence before this study

The neddylation pathway is overactivated in human cancers. Inhibition of neddylation pathway has emerged as an attractive anti-cancer strategy. The mechanisms underlying neddylation overactivation in cancer remain elusive. MLN4924/Pevonedistat, a first-in-class NEDD8-activating enzyme (NAE, E1) inhibitor, exerts significant anti-tumor effects, but its mutagenic resistance remains unresolved. Based on this, we evaluated the role of UBC12 in the over-activated neddylation pathway of lung cancer, and identified whether UBC12 could be used as a potential molecular target in lung cancer.

### Added value of this study

In this study, we found that the mRNA level of UBC12 in lung cancer tissues was much higher than that in normal lung tissues, increased with disease deterioration, and positively correlated with NEDD8 expression. Functionally, neddylation inactivation by UBC12 knockdown suppressed the malignant phenotypes of lung cancer cells both *in vitro* and *in vivo*. Further mechanistical studies revealed that UBC12 knockdown inhibited Cullin neddylation, led to the inactivation of CRL E3 ligases and induced the accumulation of tumor-suppressive CRL substrates (p21, p27 and Wee1) to induce cell cycle arrest and suppress the malignant phenotypes of lung cancer cells. Finally, UBC12 knockdown effectively inhibited the growth of MLN4924-resistant lung cancer cells.

### Implications of all the available evidence

These findings highlight the crucial role of UBC12 in fine-tuned regulation of neddylation activation status and validate UBC12 as an attractive alternative anticancer target against neddylation pathway.

heterodimer consisting of NAE1 and UBA3), NEDD8-conjugating enzymes E2 (UBE2M/UBC12 or UBE2F), and NEDD8 ligases E3 (including RBX1/ROC1, RBX2/ROC2, MDM2, etc.) [1–3]. Among E2 enzymes, UBC12 pairs with RBX1/ROC1 to regulate the neddylation of Cullin 1, 2, 3, 4a, 4b and 7, while UBE2F pairs with RBX2/ROC2 to regulate the neddylation of Cullin 5 [4]. So far, the best identified neddylation substrates are the Cullin family members. NEDD8 conjugates to Cullin is necessary for the activation of CRLs, the largest multiunit ubiquitin ligase family in cells which is in charge of the ubiquitination and degradation of about 20% of ubiquitinated cellular proteins, including transcription factors, tumor suppressors, and onco-proteins [5]. It has been found that CRLs are dysregulated in many types of cancers, and thus leading to the accelerated degradation of tumor suppressors and promotion of tumorigenesis and tumor progression [6,7].

Recently, the neddylation pathway, including E1, E2, E3 and global neddylation of substrates, is reported to be hyperactivated in several types of cancers and associated with disease progression, such as worse patient overall survival [8,9]. Moreover, targeting overactivated protein neddylation pathway has been demonstrated as an effective anticancer strategy [10–16]. MLN4924, a small molecular inhibitor of NAE [17,18], has been developed as a first-in-class neddylation inhibitor. Intensive studies reported that MLN4924 induced the accumulation of tumor-suppressive CRLs substrates to suppress cancer cell growth and metastasis both *in vitro* and *in vivo*, owing to the inhibition of Cullin neddylation and CRLs activation [19–26]. Due to its high efficiency and well-tolerated toxicity in preclinical trials, MLN4924 has advanced

into clinical trials for human hematological malignancies and solid tumors [27–30].

While MLN4924 has been intensively investigated in preclinical and clinical trials, the induction of drug-resistant mutations in UBA3, the molecular target of MLN4924, raises the necessity to identify other therapeutic targets against neddylation pathway [31–33]. In this study, we found that UBC12 was overexpressed in human lung cancer, increased with disease deterioration, and positively correlated with NEDD8 expression. Moreover, targeting UBC12 effectively suppressed the malignant phenotypes of lung cancer both *in vitro* and *in vivo*. These findings validate UBC12 as a novel alternative therapeutic target against neddylation pathway.

## 2. Materials and methods

### 2.1. Cell culture and reagents

293T cells, human lung cancer cell lines A549 and H1299 were obtained from the American Type Culture Collection (Manassas, VA). Human lung cancer cell lines H1975, PC9, HCC1833, HCC1838, HCC827, HCC2030, HCC1438 and H596 were given by Dr. Yihua Sun (Department of Thoracic Surgery, Fudan University Shanghai Cancer Center). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, hyclone, Logan, UT), containing 10% fetal bovine serum (Biocrom AG, Berlin, Germany) and 1% penicillin-streptomycin solution at 37 °C with 5% carbon dioxide.

### 2.2. Analyzing of gene expression datasets from primary lung tumors

Two Affymetrix microarray datasets (Shedden and Hou) were obtained. The CEL files of microarray data were normalized using Robust Multi-Array Average (RMA) method and log 2 transformed data were used [34–36]. Hou's data (65 normal lung tissues, 45 lung adenocarcinomas, 19 large cell lung cancer, and 27 squamous cell lung cancer) was used for the comparison of tumor VS normal of UBC12. Shedden's data (442 lung adenocarcinomas) was used for the analysis of tumor differentiation and patient survival. We also obtained TCGA RNA-seq data from 500 lung adenocarcinomas. The clinical information from each patient was also obtained from the original publications.

### 2.3. Generation of stable cell lines by CRISPR/Cas9 system

For packaging lenti-virus used in UBC12 knockdown, three guide RNA sequences specifically against UBC12 were inserted into vector lenti-guide-puro, respectively. 293T cells were co-transfected with lenti-viral vectors lenti-guide-puro (4 µg) and packaging vectors AGP091 (3.0 µg) and AGP090 (1.2 µg). Forty-eight hours after transfection, the viral supernatants were collected, filtered, and infected A549 or H1299 cells. Polybrene (sigma-Aldrich, St. Louis, MO) was added into viral supernatant at the concentration of 10 µg/mL. Six hours after incubation, the viral supernatant was replaced with normal DMEM with 10% FBS.

### 2.4. Cell proliferation and clonogenic survival assays

Cell proliferation assay was determined with the ATPlite luminescence assay kit (PerkinElmer) according to the manufacturer's instruction. For clonogenic assay, cells were seeded into 6 cm dishes (300 cells per dish) in triplicate and cultured for 10 days. More information is provided in the Supplementary Methods. Representative results of three independent experiments with similar trends are presented.

### 2.5. Immunoblotting and cycloheximide (CHX) - chase analysis

For CHX-chase experiments, UBC12-knockdown cells and control cells were treated with 50 µg/mL CHX (sigma) for indicated time points.

Cell lysates were prepared for immunoblotting analysis using antibodies against UBC12, UBA3, Cullin1, Cullin2, Cullin5, p21 (abcam), NAE1, Cullin3, Cullin4a, p27, Wee1, p-H3, NEDD8 (Cell Signaling, Boston, MA), Cullin4b (protein Tech),  $\beta$ -actin (protein Tech) was used as the loading control.

### 2.6. Propidium iodide staining and fluorescence-activated cell-sorting analysis

For cell-cycle profile analysis, UBC12-knockdown cells and control cells were stained with propidium iodide (PI) and performed fluorescence-activated cell sorting (FACS) analysis as described previously [37]. More information is provided in the Supplementary Methods.

### 2.7. Transwell migration assay

The standard transwell migration assay, using a transwell polycarbonate filter (8- $\mu$ m pore size; Corning, Lowell, MA), was performed to analyze the cell migration abilities [10]. More information is provided in the Supplementary Methods.

### 2.8. Subcutaneous-transplantation tumor model and experimental lung metastasis in vivo

For tumor formation assay, five-week-old female athymic nude mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China).  $2 \times 10^6$  stable cells were subcutaneously injected into the right back or left back. Tumor size was measured by a vernier caliper and calculated as  $(\text{length} \times \text{width}^2)/2$ .

For experimental metastatic models, six-week-old female Balb/c-nude mice were intravenously injected with GFP conjugated A549 cells (control cells or UBC12-knockdown cells). Mice were sacrificed at the end of the study and detected as described in the Supplementary Methods (available online) [10]. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.9. Statistical analysis

Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test with Statistical Program for Social Sciences software (SPSS) Version 16.0. The overall survival time was defined as the duration from the date of diagnosis to the date of either death or censoring (which could occur either by loss to follow-up or by termination of the observation).

All data are presented as the mean  $\pm$  standard error from at least three independent experiments. The statistical significance of differences between groups was assessed using the Graph Pad 5 software (Graph Pad Software, San Diego, CA, USA). The unpaired 2-tailed *t*-test was used for the comparison of parameters between groups. For all the tests, three levels of significance (\**p* < .05, \*\**p* < .01, \*\*\**p* < .001) were used.

## 3. Results

### 3.1. Overexpressed UBC12 correlates with global neddylation and predicts poor survival in lung cancer

To evaluate the role of UBC12 in the occurrence and development of lung cancer, two published Affymetrix microarray datasets were used for the expression of UBC12 in tumor vs normal comparison analyses, tumor differentiation and patient survival [34,35]. Firstly, UBC12 mRNA expression in all three types of lung cancer was much higher than in normal lung tissues (lung adenocarcinoma vs normal, *p* = .001; large cell lung cancer vs normal, *p* < .001; squamous cell lung

cancer vs normal, *p* = .27) (Fig. 1a). Synchronously, UBC12 mRNA expression is much higher in poorly differentiated tumor in 442 lung adenocarcinomas (Fig. 1b). Moreover, Kaplan-Meier analysis revealed that the patients with high mRNA level of UBC12 conferred poorer overall survival than those with low expression in lung cancer patients (Fig. 1c). To further validate these findings, Kaplan-Meier analysis of the mRNA level of neddylation enzymes from TCGA datasets also showed that the overall survival rate was lower in lung cancer patients with the high mRNA level of UBC12 or NEDD8 than in the patients with low mRNA level of these two genes (Fig. 1d and e). In contrast, there was no significant correlation between the mRNA levels of NAE1 and UBA3, two E1 components, and the overall survival of lung cancer patients (Supplementary Fig. 1a and 1b). Finally, correlation analysis revealed that the mRNA level of NEDD8 and UBC12 had statistically significant correlation in lung cancer (Fig. 1f).

### 3.2. Direct regulation of protein neddylation modification by UBC12

To further evaluate the relevance between the expressions of neddylation enzymes NAE1, UBA3, UBC12 and the global protein neddylation (NEDD8-conjugated proteins) in lung cancer cells, we first determined the expression levels of NAE1, UBA3, UBC12 and global protein neddylation in 9 lung cancer cell lines. Interestingly, the expression level of UBC12, but not NAE1 and UBA3, displayed the consistent trends with the level of global protein neddylation (Fig. 2a and b). These findings suggested that the expression of UBC12 was positively correlated with protein neddylation modification levels.

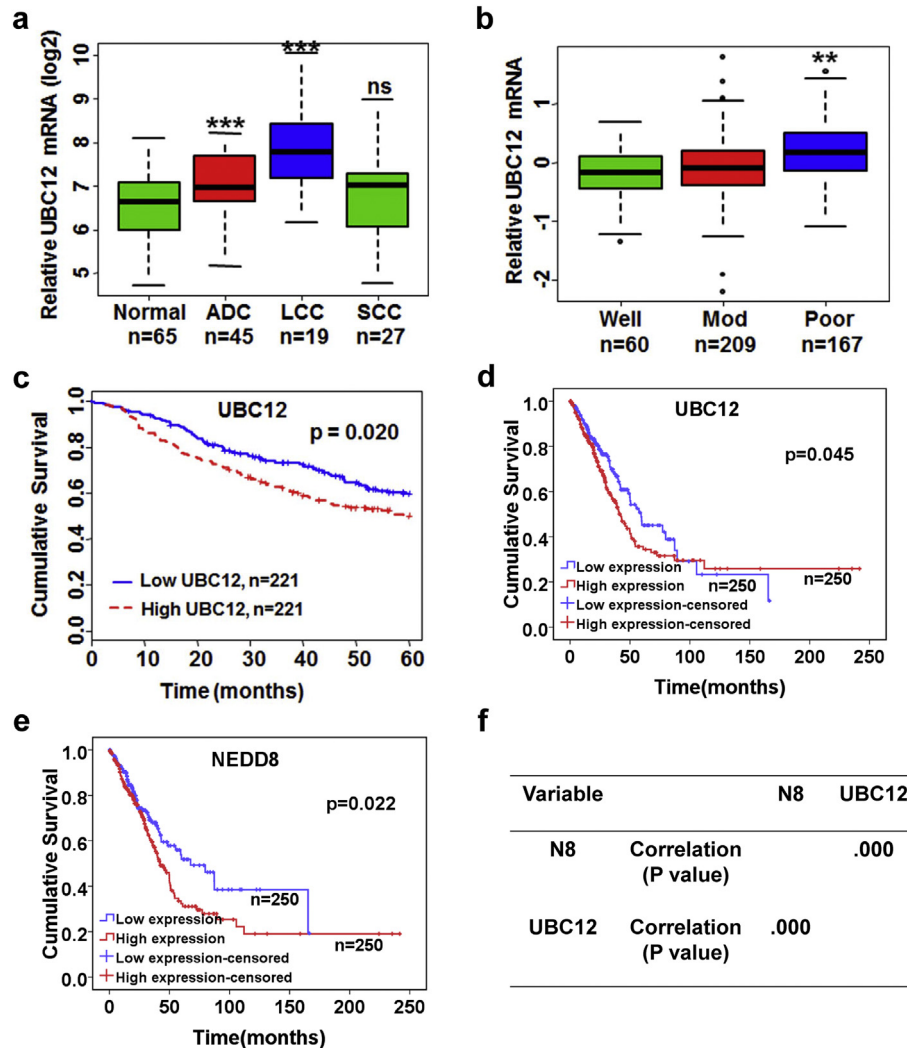
Next, we determine the effects of UBC12 expression on protein neddylation, two lung cancer cell lines (PC9 and HCC827) with low expression of UBC12 were genetically constructed to express UBC12 stably. As shown in Fig. 2c, UBC12 overexpression enhanced the neddylation levels of Cullin1, 2, 3, 4a and 4b as the classical UBC12 substrates, with no effect on Cullin 5. In contrast, downregulation of UBC12 expression in A549 and H1299 cells, in which displayed the high expression level of endogenous UBC12, significantly reduced the neddylation levels of Cullin1, 2, 3, 4a and 4b, but not Cullin 5 (Fig. 2d). These findings demonstrate that UBC12 expression tightly controls the protein neddylation.

### 3.3. Downregulation of UBC12 suppressed the malignant phenotypes of lung cancer cells in vitro

After demonstrating the regulatory effect of UBC12 on Cullin neddylation, we further evaluated the effects of UBC12 downregulation on the malignant phenotypes in A549 and H1299 cells by knocking down UBC12 using CRISPR/Cas9 system. We evaluated the effect of UBC12 knockdown on the cell proliferation of lung adenocarcinoma cells using ATP-lite luminescence assay. The results showed that UBC12 knockdown significantly inhibited cell proliferation (Fig. 3a). Similarly, UBC12 knockdown exerted remarkable suppressive effect on clonogenic survival of these two cell lines (Fig. 3b and c). Moreover, UBC12 downregulation significantly inhibited the transwell migration of A549 and H1299 cells (Fig. 3d and e). Altogether, these findings demonstrate that UBC12-mediated protein neddylation is required for the maintenance of malignant phenotypes of lung cancer cells.

### 3.4. UBC12 knockdown induced G<sub>2</sub> phase cell-cycle arrest in lung cancer cells

To investigate how UBC12 knockdown inhibits the growth of lung cancer cells, a quantitative proteomic strategy based on mass spectra was used to identify the up- and down-regulated proteins upon UBC12 knockdown in A549 cells. The results showed that there were 499 proteins were up-regulated >2 folds and 483 proteins were down-regulated >2 folds. KEGG enrichment analysis of up-regulated proteins revealed the cell cycle pathways were disturbed upon UBC12



**Fig. 1.** Overexpressed UBC12 correlated with global neddylation and predicted poor survival in lung cancer. a) UBC12 mRNA was lower in normal lung tissues as compared to tumors ( $p < .01$ , Hou's data). b) UBC12 mRNA was higher in poor differentiated tumor ( $p < .01$ , Shedden's data). (ADC, lung adenocarcinoma; LCC, large cell lung cancer; SCC, squamous cell lung cancer; Mod, Moderate). c) Kaplan–Meier curve was analyzed for overall survival rate of patients with lung adenocarcinoma according to the mRNA expression level of UBC12 (Shedden's data). d) and e) Kaplan–Meier curves were analyzed for overall survival rate of patients with lung adenocarcinomas (TCGA data) according to the mRNA expression level of NEDD8 (d) and UBC12 (e), respectively ( $p = .022$  for NEDD8;  $p = .045$  for UBC12, log-rank test). f) Correlation between neddylation enzymes and global protein neddylation was analyzed in lung cancer (Pearson chi-square test, TCGA data).

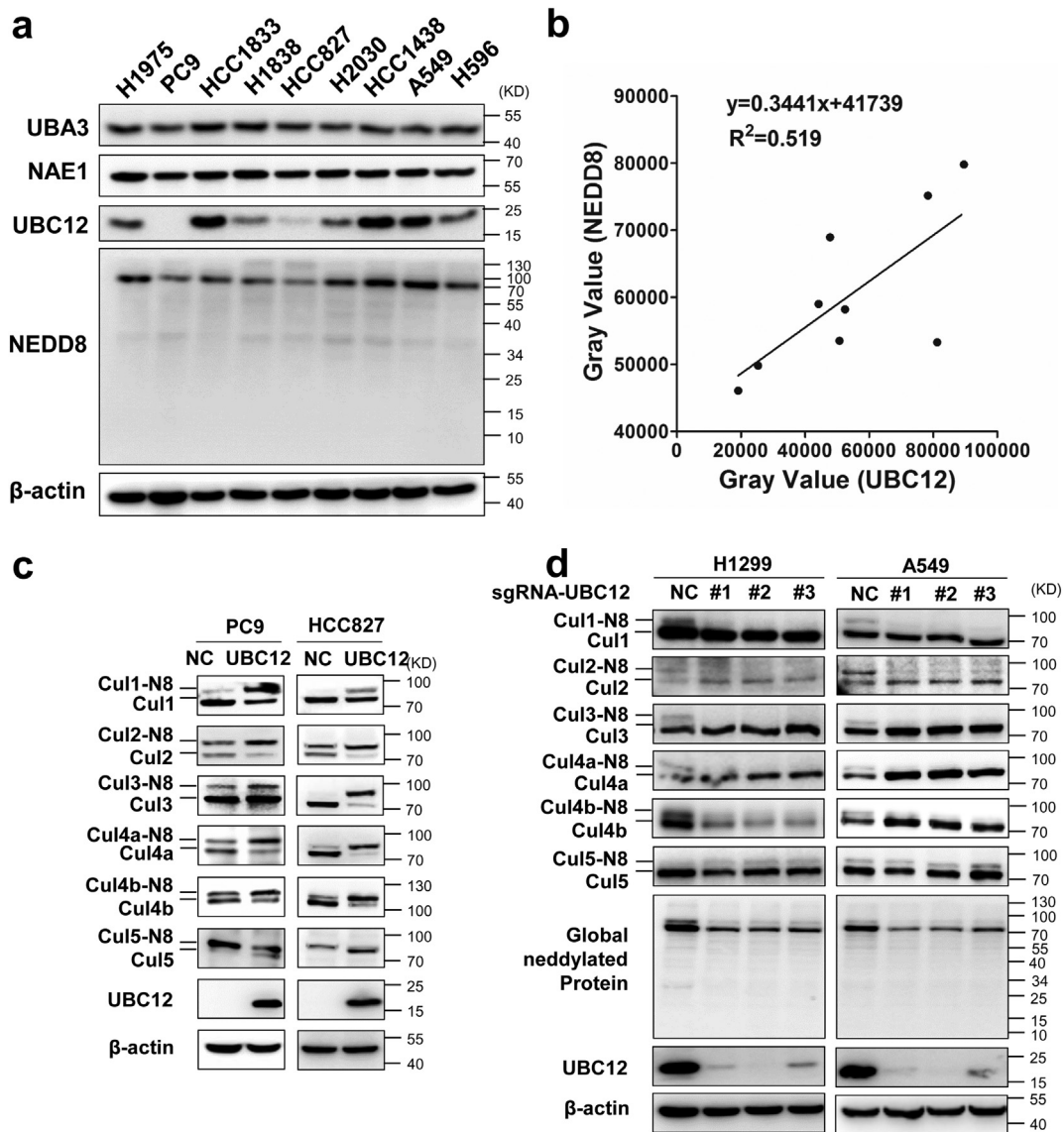
knockdown (Fig. 4a). Among these, 14 proteins were involved in cell cycle regulation as analyzed in sub protein–protein interaction network, including cyclin associated proteins (CCNB1, CCNA2, CCND3 and CDK4) and cyclin dependent kinase inhibitors (CDKN1A/p21 and CDKN1B/p27) (Fig. 4b). Consistently, we found that p21 and p27 were also significantly accumulated upon UBC12 knockdown (Fig. 4d). Based on these findings, cell-cycle profile of cells was further performed by PI staining and FACS analysis. As shown in Fig. 4c, downregulation of UBC12 triggered G<sub>2</sub>-M phase cell cycle arrest. To further determine at which phase of cell cycle was arrested upon UBC12 knockdown, we detected the expression status of Wee1, which is a well-defined CRLs substrate and an inhibitor of G<sub>2</sub>-M phase transition, as well as phosphorylated-Histone H3 (p-H3, ser10), a hallmark of M phase cells. As shown in Fig. 4d, Wee1 was significantly accumulated, whereas p-H3 sharply decreased upon downregulation of UBC12, indicating that UBC12-downregulated cells were arrested at G<sub>2</sub> phase and failed to pass to M phase.

Since p21, p27 and Wee1 serve as the substrates of CRL E3 ligases, we hypothesized that UBC12 knockdown blocks the turnover of these cell cycle inhibitors due to CRLs inactivation. To test this hypothesis, we used cycloheximide (CHX) to block protein translation and

determine the turnover rate of p21, p27 and Wee1 upon UBC12 knockdown. As shown in Fig. 4e and f, UBC12 knockdown significantly delayed the turnover of p21, p27 and Wee1, and extended the half-life of these cell cycle-inhibitory CRLs substrates. Taken together, these findings indicate that UBC12 knockdown induce cell cycle arrest by triggering the accumulation of p21, p27 and Wee1.

### 3.5. Targeting UBC12 suppressed cell growth and induced CRLs substrates accumulation in MLN4924-resistant cells

MLN4924, as a small molecular inhibitor of NAE, has been intensively investigated in preclinical and clinical trials. However, the induction of drug-resistant mutations in UBA3 impedes the subsequent development of MLN4924 and raises the necessity to identify the alternative therapeutic targets against neddylation pathway. To test the hypothesis that targeting UBC12 may be effective in MLN4924-resistant cells, we generated MLN4924-resistant H1299 cells (H1299-MR cells) by culturing cells in the media containing low dose of MLN4924 (330 nM) for 6 months. After that, a resistant population of H1299 cells that were capable of growth in the presence of 330 nM MLN4924 was selected. We found that the IC<sub>50</sub> of MLN4924 rose from 127.7 nM



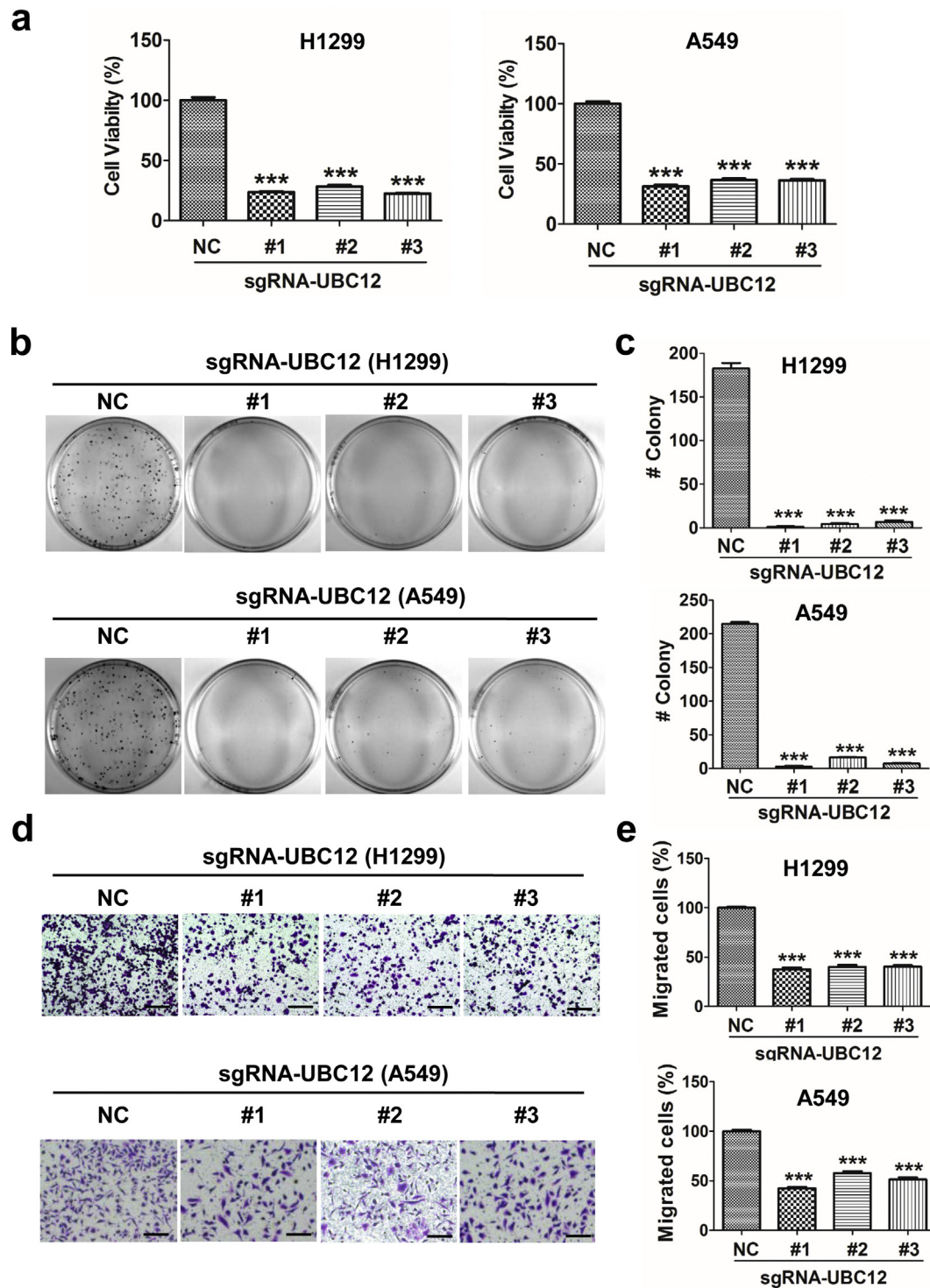
**Fig. 2.** Direct regulation of protein neddylation modification by UBC12. a) Immunoblotting was used to determine the expression levels of neddylation activating enzyme (NAE1 and UBA3), neddylation conjugating enzyme (UBC12/UBE2M) and the global protein neddylation. b) The expression levels of neddylation enzymes were further quantified by densitometric analysis using Image J software. The correlation was analyzed by GraphPad Prism5.0 software. c) Immunoblotting was used to analyze the neddylation levels of Cullin 1, 2, 3, 4a, 4b, 5 and global protein neddylation upon UBC12 overexpression in lung cancer cells PC9 and HCC827. d) Immunoblotting was used to analyze the neddylation levels of Cullin 1, 2, 3, 4a, 4b, 5 and global protein neddylation upon knocking down UBC12 in lung cancer cells A549 and H1299.

in H1299-wt cells to 2500 nM in H1299-MR cells (Fig. 5a). Mechanism studies revealed that, MLN4924 (1000 nM) almost completely inhibited Cullins neddylation in H1299-wt cells, while it only had minor to modest inhibitory effect on Cullins neddylation in H1299-MR (Fig. 5b, left panel). As a result, the classical substrates of CRLs, including Wee1, p27, p21, p-IkBa and ORC1, were significantly accumulated in H1299-wt cells, but not in H1299-MR cells upon MLN4924 treatment, indicating that MLN4924 failed to inactivate CRL E3 ligases due to the constant Cullins neddylation of H1299-MR cells (Fig. 5b, right panel).

Next, we test whether targeting UBC12 suppressed the growth of H1299-MR cells. As shown in Fig. 5c–e, the proliferation and colony formation of H1299-MR cells were significantly suppressed upon UBC12 knockdown. Mechanistic study revealed that UBC12 knockdown significantly inhibited neddylation modification of UBC12 classical substrates Cullin 1, 2, 3, 4a and 4b, but not non-UBC12 substrate Cullin5 in H1299-MR cells (Fig. 5f). As a result, CRLs substrates, including Wee1, p27, p21, CDT1 and ORC1, were significantly accumulated by UBC12 knockdown in MLN4924-resistant H1299-MR cells (Fig. 5f).

### 3.6. UBC12 knockdown inhibited the growth and metastasis of lung cancer *in vivo*

After demonstrating the anticancer effects of UBC12 knockdown *in vitro*, we finally investigated the anticancer effects of targeting UBC12 in two xenograft models *in vivo*. In the subcutaneous tumor model, we found that downregulation of UBC12 significantly suppressed tumor formation and growth when compared with the control groups. Strikingly, only 30% (3/10) mice transplanted with UBC12-knockdown A549 cells developed tiny tumors while 100% (10/10) mice transplanted with wild type cells developed large tumors (Fig. 6a–d). Furthermore, we evaluated the efficacy of UBC12 knockdown on the tumor growth and metastasis in an experimental metastatic model. For this purpose, we first established an experimental lung metastatic model by i.v. injection of A549-GFP (green fluorescent protein) cells, and then to measure the metastatic tumor nodules in lung with a fluorescence-based imaging system. Though fluorescence imaging analysis, micrometastases and metastases were easily visualized



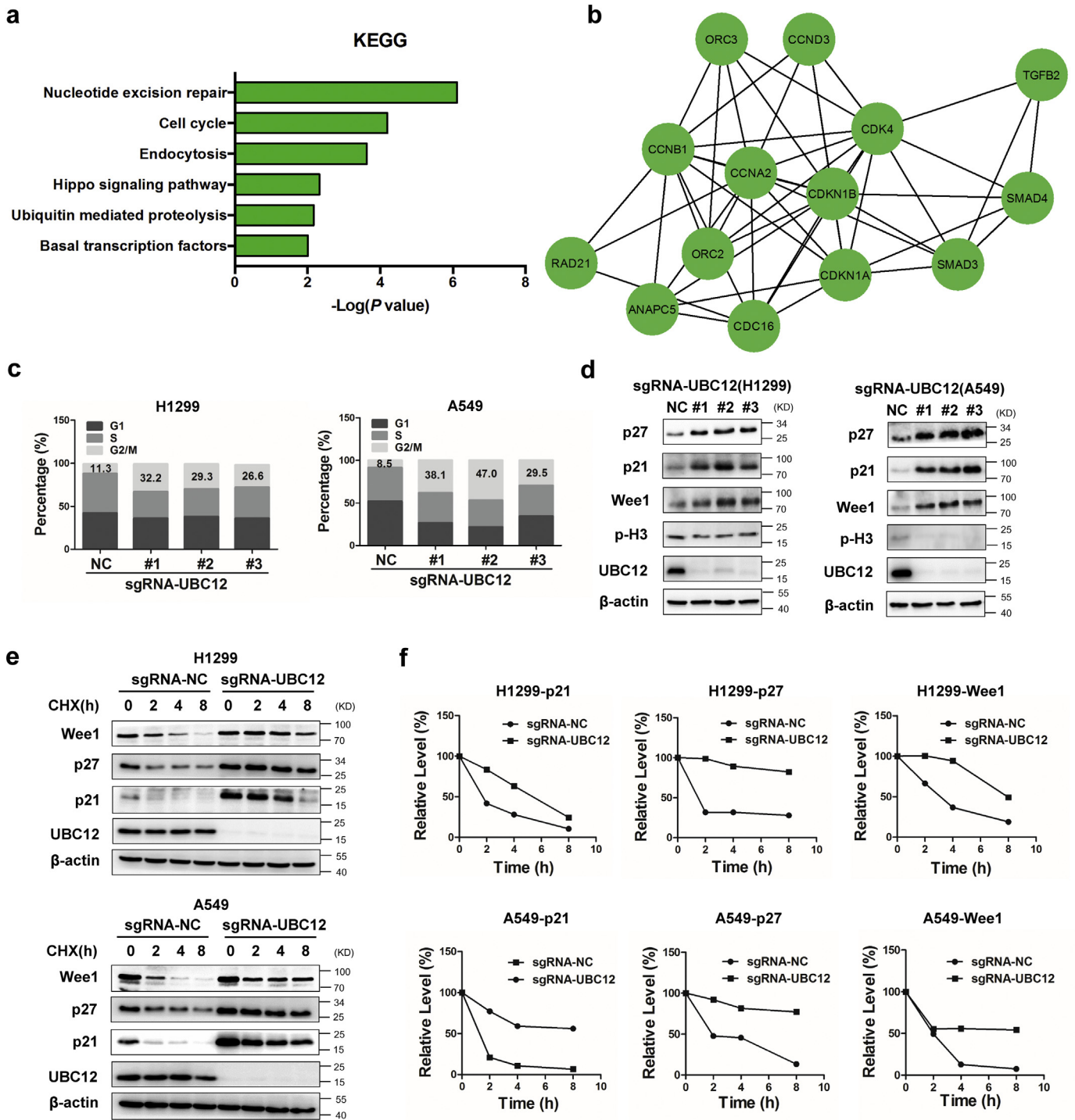
**Fig. 3.** Down regulation of UBC12 significantly suppressed the malignant phenotypes of lung cancer cells. a) Lung cancer cells were infected with lenti-guide-puro-control or lenti-guide-puro-UBC12, and subjected to cell proliferation analysis by ATPlite assay. The results were presented as mean value  $\pm$  S.E. from three independent experiments (two-sided *t*-test). b and c) UBC12 knockdown reduced cologenic survival in H1299 and A549 cells (two-sided *t*-test). d and e) UBC12 knockdown inhibited the transwell ability in H1299 and A549 cells. These data are representative results of three independent experiments with similar trends (scale bar = 200  $\mu$ m). Data represent means, and error bars are standard deviation (two-sided *t*-test).

and observed in the lungs of control mice, but not in the lungs of UBC12-knockdown mice (Fig. 6e and f). Collectively, UBC12 knockdown significantly inhibited the growth and metastasis of lung cancer *in vivo*.

#### 4. Discussion

The severe threat of lung cancer to human health raises an urgent necessity to further elucidate the mechanisms for lung carcinogenesis

and develop targeted drug. Our previous study reported that neddylation modification was over-activated in lung cancer and inversely correlated with the overall survival rate of lung cancer patients [10,38]. However, the mechanisms underlying neddylation over-activation have not been clearly elucidated. This study revealed that UBC12 played a crucial role in the fine-tuned regulation of the activity of neddylation pathway, with evidences: a) at mRNA level, the expression of UBC12 in lung cancer was much higher than that in normal

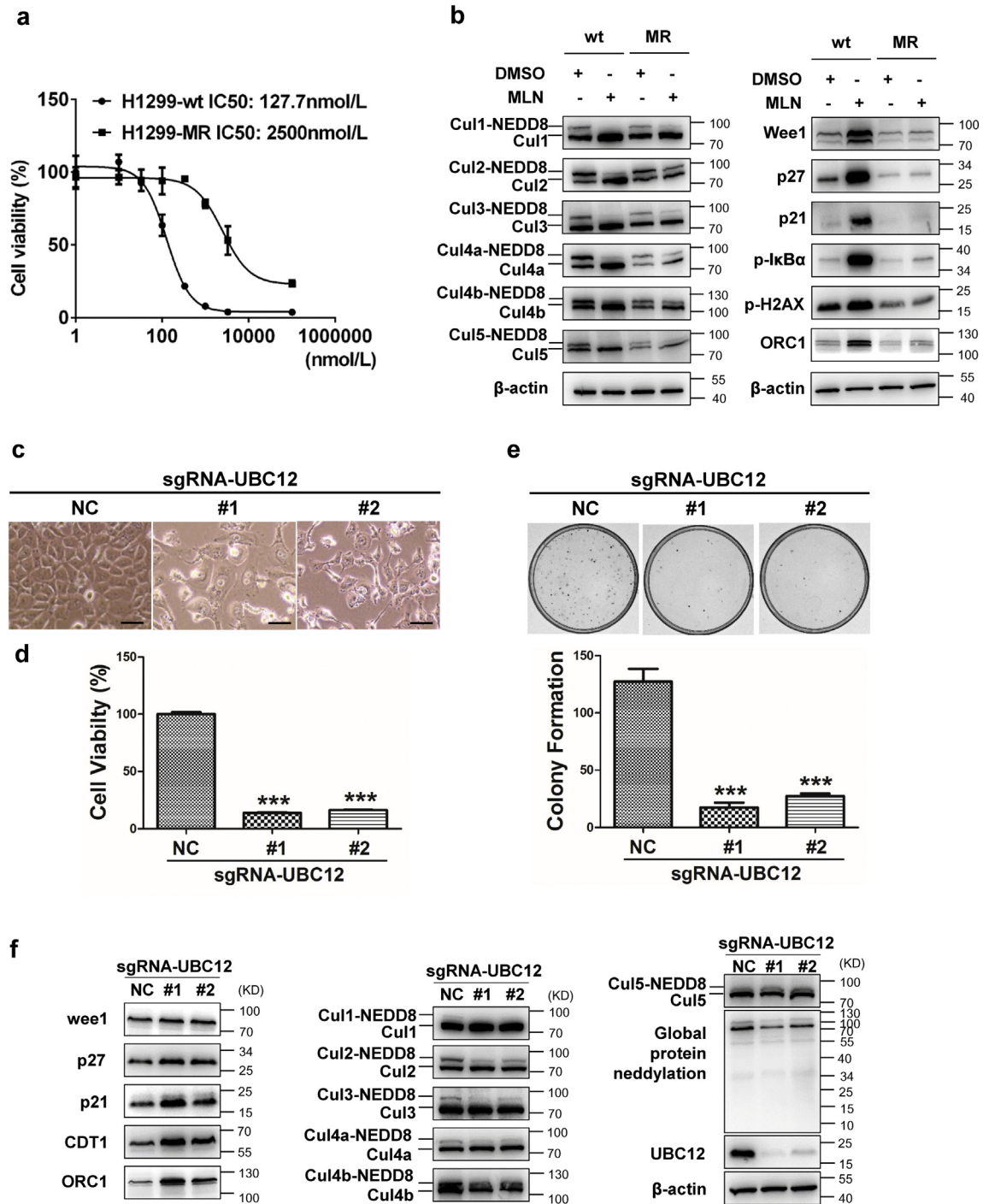


**Fig. 4.** UBC12 knockdown induced G<sub>2</sub> cell-cycle arrest in lung cancer cells. a) A quantitative proteomic strategy based on mass spectra was used to identify the up- and down-regulated proteins upon UBC12 knockdown in A549 cells. KEGG enrichment analysis was used to reveal the changed biological pathways. b) 14 proteins involving in cell cycle were mapped the sub protein-protein interaction network. c) Lung cancer cells were infected with lenti-guide-puro-control or lenti-guide-puro-UBC12, and subjected to PI staining and FACS analysis. The percentage of cells at the G<sub>2</sub>-M phase and sub-G<sub>1</sub> phase was indicated. d) UBC12 knocking down decreased the expression levels of p-H3, but induced the accumulation of p21, p27 and Wee1. A549 and H1299 cells were infected with lenti-guide-puro-control or lenti-guide-puro-UBC12 and subjected to immunoblotting analysis for p21, p27, Wee1 and p-H3 with  $\beta$ -actin as a loading control. e) and f) Half-life of p21, p27 and Wee1 was extended upon down regulation of UBC12. CHX was used to block protein synthesis, and protein lysates were extracted and subjected to immunoblotting against p21, p27 and Wee1 in A549 and H1299 cells (e). The protein levels were quantified by densitometric analysis with Image J software (f).

tissues, and increased with the aggravation of tumor deterioration; b) overexpressed UBC12 mRNA in lung cancer predicted poor survival, and was positively correlated with the mRNA expression of NEDD8 in lung cancer tissues; c) at protein level, the expression of UBC12, but not E1 subunits (NAE1 and UBA3) was positively associated with the level of global protein neddylation in diverse lung cancer cell lines;

and d) overexpression of UBC12 significantly strengthened the protein neddylation whereas downregulation of UBC12 reduced protein neddylation modification.

Targeting neddylation pathway has been demonstrated as a promising anticancer strategy, as supported by the development of MLN4924 (Pevonedistat/TAK924), an investigational small molecular inhibitor of

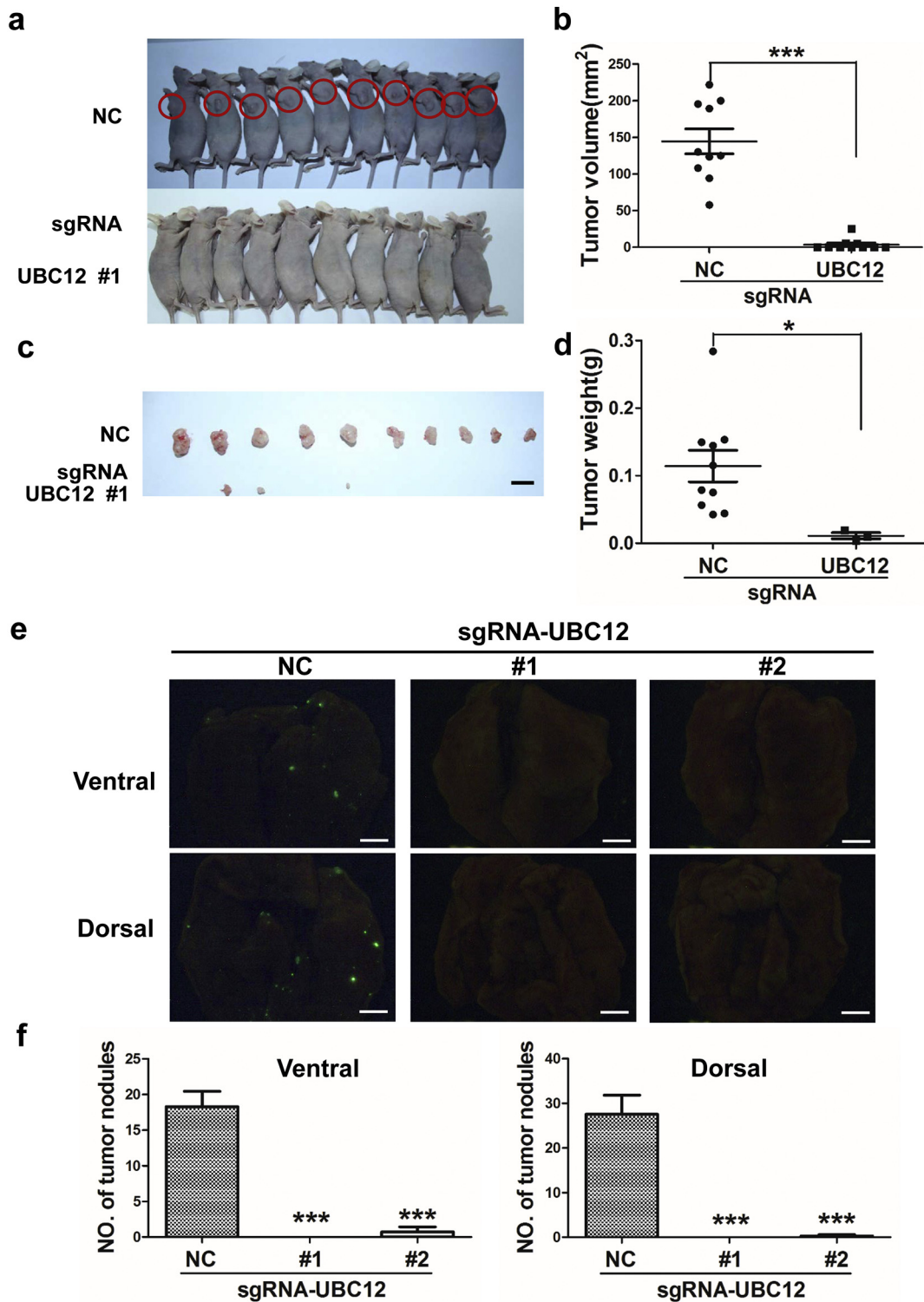


**Fig. 5.** Targeting UBC12 suppressed cell growth and induced CRL substrates accumulation in MLN4924-resistant cells. **a)** IC50 of MLN4924-resistant H1299 cells (H1299-MR) increased to 2500 nM. Wild type H1299 (H1299-wt) cells were cultured in the media containing low dose of MLN4924 (330 nM) for 6 months. 1500 H1299-MR and H1299-wt cells were seeded in 96-well plates in triplicates and then treated with indicated dose of MLN4924 for 72 h and then lysed for ATPlite assay. **b)** H1299-wt and H1299-MR cells were treated with MLN4924 at the concentration of 1000 nM. Protein was extracted and subjected to immunoblotting against Cullin1–5 and CRLs substrates. **c–e)** UBC12 knocking down suppressed the viability of H1299-MR cells. H1299-MR cells were infected with lenti-guide-puro-control or lenti-guide-puro-UBC12. 1500 cells were seeded in 96-well plates in triplicates and cultured for 72 h and then lysed for ATPlite assay (**c** and **d**) (scale bar = 50 μm, two-sided *t*-test). 400 cells were seeded in 6 cm plates in triplicates and cultured for 10 days and then stained with crystal violet (**e**). **f)** UBC12 knocking down suppressed neddylation of Cullin1, Cullin 2, Cullin3, Cullin4a, Cullin4b, induced accumulation of CRLs substrates in H1299-MR cells. H1299-MR cells were infected with lenti-guide-puro-control or lenti-guide-puro-UBC12. Protein was extracted and subjected to immunoblotting against Cul1–5 and CRLs substrates. (MR, MLN4924 resistant).

NAE (E1) [39,40]. Currently, MLN4924 is evaluated in Phase II/III clinical trials for multiple hematologic malignancies and solid tumors [27,29,41]. However, the emergence of drug-induced mutations in UBA3, the molecular target of MLN4924 [31,32,42], makes it necessary to identify other potential targets against neddylation pathway. In this study, UBC12 was validated as an alternatively attractive therapeutic

target in lung cancer. We found that the expression of UBC12 was not only positively correlated with the status of neddylation activity, but also required for the maintenance of the malignant phenotypes of lung cancer cells. Consistently, genetically inhibition of UBC12 potently inhibited the growth of lung cancer cells both *in vitro* and *in vivo*. Moreover, UBC12 knockdown significantly suppressed the growth of





**Fig. 6.** Downregulation of UBC12 suppressed tumor growth and metastasis *in vivo* in nude mice. a–d) UBC12 knockdown inhibited tumor formation and growth in an A549 experimental subcutaneous xenograft tumor model in nude mice.  $2 \times 10^6$  A549 cells infected with lenti-guide-puro-control or lenti-guide-puro-UBC12 were injected subcutaneously and subjected to tumor growth analysis. Mice were sacrificed and photographed at 35th day after treatment (the end of study,  $n = 10$ ) (a). Tumor tissues of mice were collected, photographed (b,  $<0.01$  and c scale bar = 1 cm), and weighed (d;  $p < .05$ ). e–f) Efficacy of silencing UBC12 on A549-GFP experimental lung metastasis. UBC12 knockdown inhibited tumor formation and growth in an experimental metastatic model in nude mice.  $2 \times 10^6$  A549 cells infected with lenti-guide-puro-control or lenti-guide-puro-UBC12 were injected through caudal vein and subjected to tumor growth analysis. Mice were sacrificed at the end of study, and lung tissues were collected and imaged with a fluorescence imaging system. A representative lung per group was shown. Tumor nodules were indicated with arrows (scale bar = 2 mm). f) The number of tumor nodules on lung surfaces emitting green fluorescence was calculated ( $n = 10$ ; 10 entire lungs from 10 mice per group) (two-sided *t*-test).

MLN4924-resistant cells by inhibiting Cullins neddylation and inducing the accumulation of CRLs substrates effectively.

In mammalian cells, there are one NEDD8-activating enzyme (E1) and two NEDD8-conjugating enzymes (E2, UBC12/UBE2M and UBE2F) in neddylation pathway. For the two E2 enzymes, UBC12, pairing with RBX1/ROC1 as the NEDD8 E3 ligase, promotes the neddylation of most of Cullin proteins (Cul1, Cul2, Cul3, Cul4a, Cul4b and Cul7). However, UBE2F, pairing with RBX2/SAG as the NEDD8 E3 ligase, regulates the neddylation of Cul5 [4,7]. While NAE E1 enzyme has been well-defined as a promising anticancer target, the feasibility of screening E2 enzymes as therapeutic targets remains elusive. In this study, UBC12 was validated as an alternative anticancer target against neddylation pathway. Compared to targeting E1, which inactivates the whole neddylation pathway, inhibition of E2 may offer better cytotoxic selectivity by inhibiting the NEDD8 conjugation of a subset, but not all of neddylation substrates.

Unlimited proliferation and rapid growth are the characteristics of cancer cells, which can be attributed to the sustaining proliferative signaling and the accelerating cell cycle process. In cancer cells, the oncogenic proteins promoting cell cycle progression are usually overexpressed while the cyclic inhibitor proteins (such as p21 and p27) are generally downregulated. Therefore, the blockage of cell cycle procession by restoring the expression of tumor-suppressive cell cycle inhibitors has been a long-term goal and a major strategy to treat cancer [43–45]. In this study, we revealed that targeting UBC12 halted the cell cycle progression at G<sub>2</sub> phase by inducing the accumulation of p21, p27 and Wee1 as CRLs substrates due to the inactivation of CRL ubiquitin ligases. Consistently, inhibition of neddylation with MLN4924/Pevonedistat, the first-in-class NAE inhibitor, also induced G<sub>2</sub> phase cell cycle arrest in diverse types of cancer cells by upregulating the expression of tumor-suppressive CRLs substrates [11,44–47]. These findings further highlight UBC12 as an alternative therapeutic target against neddylation pathway.

Based on our findings reported in this study, we propose a working model regarding the crucial role of UBC12 in regulation of protein neddylation and the malignant phenotypes of cancer cells. During lung cancer development, overexpression of UBC12 and global protein neddylation enhance the activation of CRLs ligases to promote the degradation of tumor suppressors (such as p21 and p27) and facilitate carcinogenesis and cancer progression. In contrast, neddylation inhibition by targeting UBC12 blocks protein neddylation and inactivates CRLs, thus induces the accumulation of tumor-suppressive CRLs substrates to induce cell-cycle arrest and inhibit tumor growth and metastasis. Therefore, the further development of UBC12 inhibitors may provide another choice for targeting the overactivated neddylation pathway and potentially overcome emerging resistance to NAE (E1) inhibitors (e.g. MLN4924).

## Acknowledgements

This work was supported by the Chinese Minister of Science and Technology grant (2016YFA0501800), National Natural Science Foundation of China (Grant Nos. 81401893, 81625018, 81820108022, 81772470, 81572340 and 81602072), Innovation Program of Shanghai Municipal Education Commission (2019-01-07-00-10-E00056), Program of Shanghai Academic/Technology Research Leader (18XD1403800), National Thirteenth Five-Year Science and Technology Major Special Project for New Drug and Development (2017ZX09304001).

## Author contributions

Lijun Jia designed and supervised the project. Lihui Li carried out the experiments and drafted the manuscript, Lijun Jia finalized the manuscript. Jihui Kang and Wenjuan Zhang performed the TCGA analysis. Lili Cai performed KEGG enrichment analysis. Guo'an Chen performed

analysis of primary tumor-derived gene expression datasets. Shiwen Wang, Yupei Liang, Yanyu Jiang, Xiaojun Liu, Yunjing Zhang, Hongfeng Ruan and Mingsong Wang performed statistical analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All experimental protocols were approved by the Animal Experimental Ethics Committee of Longhua Hospital of Shanghai University of Traditional Chinese Medicine.

## Conflicts of interest statement

The authors agree with the content of this manuscript and declare there are no competing interests about this study.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.06.005>.

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