Overactivated Neddylation Pathway as a Therapeutic Target in Lung Cancer

Lihui Li, Mingsong Wang, Guangyang Yu, Ping Chen, Hui Li, Dongping Wei, Ji Zhu, Li Xie, Huixun Jia, Jieyi Shi, Chunjie Li, Wantong Yao, Yanchun Wang, Qiang Gao, Lak Shin Jeong, Hyuk Woo Lee, Ju Mei, Ping Wang, Yiwei Chu, Hui Qi, Meng Yang, Ziming Dong, Yi Sun, Robert M. Hoffman, Lijun Jia

Manuscript received December 8, 2013; accepted March 4, 2014.

Correspondence to: Lijun Jia, PhD, Cancer Institute, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, 200032, China (e-mail: ljia@fudan.edu.cn).

Background A number of oncoproteins and tumor suppressors are known to be neddylated, but whether the neddylation pathway is entirely activated in human cancer remains unexplored.

Methods NEDD8-activating enzyme (NAE) (E1) and NEDD8-conjugating enzyme (E2) expression and global-protein neddylation were examined by immunohistochemistry, immunoblotting, and real-time polymerase chain reaction analysis. Cell proliferation, clonogenic survival, migration, and motility in vitro, as well as tumor formation and metastasis in vivo, were determined upon neddylation inhibition by MLN4924, an investigational NEDD8-activating enzyme inhibitor. Survival was analyzed with Kaplan–Meier methods and compared by the log-rank test. All statistical tests were two-sided.

Results The entire neddylation pathway, including NEDD8-activating enzyme E1, NEDD8-conjugating enzyme E2, and global-protein neddylation, is overactivated in both lung adenocarcinoma and squamous-cell carcinoma. Compared with lung adenocarcinoma patients with low expression, those with high expression had worse overall survival (NEDD8-activating enzyme E1 subunit 1 [NAE1]: hazard ratio [HR] = 2.07, 95% confidence interval [CI] = 0.95 to 4.52, P = .07; ubiquitin-conjugating enzyme E2M (UBC12): HR = 13.26, 95% CI = 1.77 to 99.35, P = .01; global protein neddylation: HR = 3.74, 95% CI = 1.65 to 8.47, P = .002). Moreover, inhibition of neddylation by the NAE inhibitor MLN4924 statistically significantly suppressed proliferation, survival, migration, and motility of lung cancer cells in vitro and tumor formation and metastasis in vivo. At the molecular level, MLN4924 inactivated Cullin-RING E3 ligases, led to accumulation of tumor-suppressive Cullin-RING E3 ligase substrates and induced phorbol-12-myristate-13-acetate-induced protein 1 (NOXA)-dependent apoptosis or cellular senescence.

Conclusions Our study highlights the overactivated neddylation pathway in lung cancer development and as a promising therapeutic target.


Protein neddylation is a newly-characterized posttranslational modification that adds the ubiquitin-like molecule neural precursor cell expressed, developmentally downregulated 8 (NEDD8) to substrate proteins (1–4). NEDD8 conjugation to its substrates is catalyzed in a three-step reaction by an NEDD8-activating enzyme (NAE, a heterodimer comprising NEDD8-activating enzyme E1 subunit 1 [NAE1] and ubiquitin-like modifier activating enzyme 3 [UBA3]), NEDD8-conjugating enzyme UBC12 (ubiquitin-conjugating enzyme E2M), and substrate-specific NEDD8-E3 ligases (1–4). The best known substrates of neddylation are members of the cullin family, which serve as the essential subunits of multiunit Cullin-RING E3 ligases (CRL) (5–7). CRL regulates diverse biological processes by targeting numerous proteins for proteasome-mediated degradation, whereas its dysfunction leads to carcinogenesis (8,9). Recently, several other proteins, including oncoproteins mouse double minute 2 homolog (Mdm2) (2) and Hu antigen R (HuR) (10,11), tumor suppressor p53 (2), and ribosomal proteins (12), have also been shown to be neddylated, further highlighting a pivotal role for neddylation in carcinogenesis and progression.

MLN4924 is an investigational NAE inhibitor that blocks protein neddylation, especially cullin neddylation, leading to the inactivation of CRL and accumulation of a mass of tumor-suppressive CRL substrates (13,14). The anticancer effects of neddylation inhibition by MLN4924 are attributable to the induction of the DNA damage response, cell cycle arrest, autophagy, apoptosis, or senescence due to the accumulation of CRL substrates in cancer cells (13,15–21). Preclinical studies have demonstrated the therapeutic efficacy of MLN4924 as a single anticancer agent (13,15–21) or in combination with chemoradiotherapy (22–24). Because of its promising anticancer efficacy in preclinical studies, MLN4924 is undergoing several Phase I clinical trials for several tumor types and hematological malignancies (14,25).
Lung cancer is one of the most common human malignancies and remains the leading cause of cancer-related deaths worldwide (26,27). Understanding the pivotal molecular events that drive lung carcinogenesis is thus crucial for the identification of novel therapeutic targets and the discovery of molecular-targeted agents (26,28). Here we hypothesized that the neddylation pathway in lung cancer is overactivated to facilitate lung carcinogenesis, whereas inhibition of this pathway suppresses the malignant phenotypes of lung cancer.

Methods

Cell Culture and Reagents

Human lung cancer cell lines A549, H460, H1299 and murine Lewis lung carcinoma (LLC) cells were obtained from the American Type Culture Collection (Manassas, VA) and passaged five to six times before use. Cells were cultured in Dulbecco’s modified Eagle’s medium (Hyclone, Logan, UT), containing 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 1% penicillin-streptomycin solution at 37°C with 5% carbon dioxide. MLN4924, synthesized as previously described (29), was dissolved in dimethyl sulfoxide (DMSO) and kept at −20°C for in vitro studies. For in vivo studies, MLN4924 was dissolved in 10% 2-hydroxypropyl-β-cyclodextrin (Sangon Biotech, Shanghai, China). A solution of MLN4924 was freshly made every week and stored in the dark at room temperature before use.

Immunohistochemical Staining of a Human Tumor Tissue Array

Human lung cancer tissue arrays were immunohistochemically (IHC) stained with NAE1, UBC12, and NEDD8 antibodies from Shanghai Biochip (Shanghai, China). The tissue array sections (5 microns) were dehydrated and subjected to peroxidase blocking. Primary antibodies were added and incubated at room temperature for 30 minutes on the DAKO AutoStainer using the DakoCytomation EnVision-System-HRP detection kit (Dakocytomation, Carpenteria, CA). The slides were counterstained with hematoxylin. The stained slides were observed under microscope, and images were acquired. Based on staining intensity, we classified the samples into five groups with increasing staining intensity from the weakest (group 1) to the strongest (group 5) for staining-intensity analysis (30).

Collection of Lung Cancer Tissues and Clinicopathological Characteristics of Patients

All patients underwent surgery, followed by treatment in accordance with the National Comprehensive Cancer Network clinical practice guidelines. Fresh primary lung cancer tissues and adjacent lung tissues were collected from 75 lung adenocarcinoma and 75 lung squamous cell carcinoma patients undergoing resection from July 2004 to September 2007 at the Taizhou Hospital (Taizhou, China). Histological diagnosis and tumor–node–metastasis staging of cancer were determined in accordance with the American Joint Committee on Cancer manual criteria for lung cancer. The detailed clinicopathological characteristics of lung adenocarcinoma patients are described in Supplementary Table 1 (available online). Written informed consent regarding tissue and data use for scientific purposes was obtained from all participating patients. The study was approved by the Research Ethics Committee of Taizhou Hospital.

Cell Proliferation and Cell Clonogenic Assays

Cell proliferation assays and cell clonogenic assays were performed as previously described (15,30). For cell proliferation assays, cells seeded in 96-well plates with 1500 cells per well, in triplicate, and cultured overnight were treated with MLN4924 for 72 hours, followed by an ATPluminescence assay and Cell Counting Kit-8 assay, according to the manufacturer’s specifications. For clonogenic assays, cells were seeded in six-well plates (150 cells per well) in triplicate and cultured for 10 days. Representative results of three independent experiments with similar trends are presented. More information is provided in the Supplementary Methods (available online).

Real-Time Polymerase Chain Reaction Analyses

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and treated with RNase-free DNase. The reverse transcription reaction was performed on 2.5 μg of total RNA per sample using the PrimerScript reverse transcription reagent kit (TaKaRa, Shiga, Japan) according to the manufacturer’s protocol. After reverse transcription, the real-time polymerase chain reaction (PCR) was performed using the Power SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA) on the ABI 7500 thermocycler (Applied Biosystems) following the instrument manual. Primers used were designed by Primer 5.0 and Oligo 6.0. The sequences of the primers are as follows:

- Human β-actin: forward 5′-TGACGTGGACATCCGCAAAG-3′, reverse 5′-CTGGGAAAGGTGGCAGCGAGG-3′; Human NAE1: forward 5′-GCTGTGTGTCACTTCTCTC-3′, reverse 5′-TCCTGACATATCATCTC-3′; Human UBC12: forward 5′-TG TGTGCTGGCTGTCTTGAA-3′, reverse 5′-GTTGTGATCTTT CCTCCTCTAA-3′; Human UBA3: forward 5′-CAGAAGAAGG AGGAGGAGTC-3′, reverse 5′-GAAGTTGAGGAGGAGGCTC TG-3′; Human NEDD8: forward 5′-CAAACTCTGGAAGAGAGTGC TAAAT-3′, reverse 5′-TGCTGCGAGGTCATGCATAA-3′.

Transwell Migration and Cell Motility Assay

A transwell migration assay and cell motility assay were performed as described previously (31). The standard transwell migration assay, using a transwell polycarbonate filter (8-μm pore size; Corning, Lowell, MA), was performed to analyze the efficacy of MLN4924 on cell migration. Representative results of three independent experiments with similar trends are presented. More information is provided in the Supplementary Methods (available online).

Fluorescence-Activated Cell Sorting Analysis

Propidium iodide staining and fluorescence-activated cell sorting analysis of cells were performed as previously described (15,30). Cells treated with DMSO or MLN4924 were harvested and fixed in 70% ethanol at −20°C overnight, stained with a propidium iodide solution (36 μg/mL; Sigma, St. Louis, MO) containing RNase A (10 μg/mL; Sigma) at 37°C for 30 minutes, and analyzed for apoptosis and cell-cycle profile using CyAn ADP (Beckman...
Coulter, Fullerton, CA) (15,30,32). Apoptosis was measured by the percentage of cells in the sub-G1 population. Data were analyzed with ModFit LT software (Verity Software House, Topsham, ME). Representative results of three independent experiments with similar trends are presented.

**SA-β-Galactosidase Staining**

Cells were treated with MLN4924 for 72 hours. The expression of senescence-associated β-galactosidase was determined with the SA-β-galactosidase (SA-β-Gal) staining kit (Beyotime, Shanghai, China) according to the manufacturer’s specifications (16,30).

**Efficacy of MLN4924 on Experimental Lung Metastasis**

Experimental metastatic models of mouse and human lung cancer were established. Six-week-old female Balb/c-nude mice were intravenously injected with murine LLC cells or A549 human lung carcinoma cells. The next day the mice were randomly divided into 2 groups (n=10 per group) and treated with 10% 2-hydroxypropyl-β-cyclodextrin (vehicle control) or MLN4924. Mice were sacrificed at the end of the study by being placed in a carbon dioxide chamber. Lung tissues from the treated mice were collected for imaging with a fluorescence imaging system, as described in the Supplementary Methods (available online) (15).

**Statistical Analysis**

Data are presented as mean ± standard deviation. The Student’s t test was used for the comparison of parameters between two groups. The correlation of the expression of two given proteins was assessed by Pearson correlation analysis. Survival was analyzed using the Kaplan–Meier method and compared by the log-rank test. The Cox proportional hazards model was used to calculate hazard ratios (HRs) and their corresponding 95% confidence intervals (CIs) with adjustment for potential confounders. The proportional hazards assumption was examined by including time-dependent covariates in the model, and we found no violations of model assumptions except for UBC12. If one of the variables was not proportional, we included the time-dependent variable for the nonproportional variables in the model. SAS software version 9.2 (SAS Institute, Cary, NC) and Statistical Program for Social Sciences software 17.0 (SPSS, Chicago, IL) were used for statistical analyses. All statistical tests were two-sided.

**Results**

**Expression of NEDD8-Activating Enzyme E1 and NEDD8-Conjugating Enzyme E2 in Lung Cancer**

To evaluate the activation state of the neddylation pathway in lung cancer, we first determined the expression levels of NAE1 and UBC12 by IHC staining of tissue arrays derived from human lung adenocarcinoma and squamous-cell carcinoma, which contain 75 pairs of primary tumor vs normal tissues. NAE1 and UBC12 were overexpressed in the tumor tissues compared with adjacent normal tissues in both lung adenocarcinoma (Figure 1A) and squamous-cell carcinoma (Supplementary Figure 1A, available online). Based on staining intensity, we classified the samples into five groups with increasing staining intensity from the weakest (±, group 1) to the strongest (++++, group 5). Staining-intensity analysis demonstrated that the expression of NAE1 and UBC12 was low, falling into group 1 and group 2, in a majority of adjacent normal tissues, whereas it was high, falling into group 2 to group 5, in a majority of lung tumor tissues (Figure 1B; Supplementary Figure 1B, available online).

The expression levels of NAE1, UBC12, and UBA3, another subunit of the NAE heterodimer, in lung tumors vs normal tissues were further analyzed by immunoblotting. Overexpression was seen in the majority of tumor tissues (Figure 1, C and D; Supplementary Figure 1, C and D) compared with adjacent tissues (relative expression, mean ± SD, tumor tissue vs adjacent tissue: lung adenocarcinoma: NAE1: 0.46±0.25 vs 0.23±0.12, respectively, P=0.02; UBC12: 1.22±0.46 vs 0.82±0.47, respectively; P=0.02; squamous-cell carcinoma: NAE1: 0.29±0.17 vs 0.15±0.08, respectively, P=0.05; UBC12: 0.55±0.27 vs 0.27±0.13, respectively; P=0.02; UBC12: 0.88±0.49 vs 0.36±0.25, respectively, P=0.02) (see Supplementary Table 2, available online). In addition, Kaplan–Meier analysis showed that the overall survival rate was lower in lung adenocarcinoma patients with high expression of NAE1 or UBC12 than in patients with low expression of these proteins (NAE1: HR=2.07, 95% CI=0.95 to 4.59, P=0.04; UBC12: HR=0.23, 95% CI=0.12 to 0.42, P=0.007) survival analyses (Supplementary Table 2, available online).

**Activation Status of Global Protein Neddylation in Lung Cancer**

Because of overexpression of neddylation E1 (NAE1 and UBA3) and E2 (UBC12) enzymes in tumors, we hypothesized that global substrate neddylation may be overactivated in lung cancer. Indeed, IHC analysis of lung cancer tissue arrays, using a specific NEDD8 antibody recognizing NEDD8-conjugated proteins, revealed that global protein neddylation was substantially elevated in both lung adenocarcinoma (Figure 2A) and squamous-cell carcinoma (Figure 2B). Moreover, Kaplan–Meier analysis in patients with lung adenocarcinoma revealed that the overall survival rate in patients with elevated global neddylation was statistically significantly lower than that in patients with low expression of global neddylation (P=0.02, log-rank test) (Figure 2C). Also, elevated global neddylation was associated with poor overall survival of patients in both univariable (HR=2.27; 95% CI=1.11 to 4.66; P=0.03) and multivariable (HR=3.74; 95% CI=1.65 to 8.47; P=0.002) survival analyses (Supplementary Table 2, available online). Finally, we determined the neddylation status of a well-known neddylation substrate, cul1n1 (6,7,15) in lung adenocarcinoma and found that the ratio of NEDD8-conjugated to -unconjugated cul1n1 in tumor tissues was substantially higher than that in adjacent normal tissues (relative expression, mean ± SD, tumor tissue vs adjacent tissue: 3.17±0.32 vs 1.18±0.71; P=0.04, two-sided t test) (Figure 2D). Together with previous reports that Defective in Cullin Neddylation (DCN-1/Dcn1p) (33,34) and Regulator of Cullins 1 (ROC1) (35), two previously-identified NEDD8-E3 ligases responsible for cullin neddylation, are also overexpressed (32,36), we conclude that the entire
The neddylation pathway is overactivated in human lung cancer and associated with reduced overall survival of patients.

Correlation Analysis of Expression and Transcriptional Activation Status of NAE1, UBC12, and Global Neddylation

We next determined the correlation of expression levels of NAE1, UBC12, and global NEDD8 conjugation by IHC staining using successively sectioned tumor tissues (Figure 3A). The expression of NAE1 and UBC12 in tumors was statistically significantly correlated in both lung adenocarcinoma (P < .001, Pearson correlation) (Figure 3B) and squamous-cell carcinoma (P = .004, Pearson correlation) (Figure 3C). Positive correlations between global protein neddylation and NAE1 or UBC12 expression were also observed in adenocarcinoma (P = .02 for NEDD8 vs NAE1; P = .002 for NEDD8 vs UBC12, Pearson correlation) (Figure 3B) and squamous-cell carcinoma (P < .001 for NEDD8 vs NAE1 or UBC12, Pearson correlation) (Figure 3C) of the lung. These findings suggest that the entire neddylation pathway may be regulated in a coordinated manner.

After establishing the overexpression of NAE1, UBA3, UBC12, and global NEDD8 conjugation at the protein level, we measured...
the expression of their corresponding genes using real-time PCR for mRNA quantification. The expression of NAE1 mRNA was substantially elevated in adenocarcinoma (Figure 3D) and squamous-cell carcinoma (Figure 3E) of the lung, as compared with adjacent normal tissues. Moreover, the entire neddylation pathway was also overactivated in human lung cancer cells (H1299, A549, and H460) as compared with normal lung fibroblasts (WI38 and MRC-5) (Figure 3F). Similarly, the transcriptional activation of NAE1 was substantially elevated in lung cancer cells (Figure 3G).

In addition, we observed that the UBC12 and NEDD8 mRNAs were modestly upregulated in tumor tissues and lung cancer cells (Figure 3, D, E and G). These findings indicate that transcriptional activation may represent a molecular mechanism responsible for the overactivation of the neddylation pathway.

**Effect of Neddylation Inactivation on the Malignant Phenotype of Lung Cancer Cells**

Overactivation of the neddylation pathway in lung cancer implies that it may serve as an effective anticancer target. To validate this hypothesis, we systematically evaluated the efficacy of neddylation inactivation by MLN4924 in human (A549, H460, and H1299) and murine LLC lung cancer cells. First, we determined the specificity of MLN4924 for inhibition of the neddylation pathway when compared with MG132 and Bortezomib (originally code-named PS-341), two classical proteasome inhibitors (9,13). MLN4924, but neither MG132 nor Bortezomib, specifically suppressed global protein neddylation (Figure 4A; Supplementary Figure 2A, available online) or cullin neddylation (Figure 4B; Supplementary Figure 2B, available online). As a result, cyclin-dependent kinase inhibitor 1A (p21) and cyclin-dependent kinase inhibitor 1B (p27), two well-known substrates of CRL E3 ligases (8,15), accumulated in all treatments, whereas cyclin B1, a non-CRL substrate (13) only accumulated upon treatment with MG132 and Bortezomib, but not MLN4924 (Figure 4B; Supplementary Figure 2B, available online). These findings demonstrate that MLN4924 specifically inhibits protein neddylation, inactivates CRL, and leads to accumulation of CRL substrates.
Next we evaluated the effect of MLN4924 on the malignant phenotype of lung cancer cells in vitro. MLN4924 statistically significantly inhibited cell proliferation (Figure 4C; Supplementary Figure 3, available online) in a dose-dependent manner. Clonogenic survival (A549, control vs MLN 1.0 μM: 108.30 ± 5.56 vs 0.00 ± 0.00, respectively, \( P < .001 \); LLC, control vs MLN 1.0 μM: 66.00 ± 4.69 vs 0.00 ± 0.00, respectively, \( P < .001 \)) (Figure 4D); transwell cell migration (A549, control vs MLN 1.0 μM: 100.00 ± 9.11 vs 3.57 ± 1.01, respectively, \( P < .001 \); LLC, control vs MLN 1.0 μM: 100.00 ± 7.75 vs 8.56 ± 1.30, respectively, \( P < .001 \)) (Figure 4E), and motility (A549,
Figure 4. Effect of neddylation inactivation on the malignant phenotype of lung cancer cells. A and B) Specificity of the NAE inhibitor MLN4924 to inhibit the neddylation pathway and suppress CRL activity when compared with proteasome inhibitors MG132 and bortezomib. A549 cells were treated with DMSO, MLN4924 (1 μM), MG132 (20 μM), and Bortezomib (1 μM) for 15 minutes and 1 hour, followed by immunoblotting analysis to determine the change of global neddylation (A), cullin1 neddylation, and the levels of p21 and p27 as well as cyclin B1 with β-actin as a loading control (B).

C–F) A549 and Lewis lung carcinoma (LLC) cells were treated with MLN4924 at indicated doses to determine its therapeutic efficacy on cell proliferation (C; *P = .004; ***P < .001; CPS, counts per second), clonogenic survival (D; ***P < .001), transwell cell migration (E; ***P < .001), and motility (F; **P = .003; ***P < .001), as described in Supplementary Methods. These data were representative results of three independent experiments with similar trends. Data represent means, and error bars are standard deviation. Two-sided t test.

G) Chemosensitizing effect of MLN4924 to conventional cytotoxic agents. Cells were treated with MLN4924 (0.1 μM) alone or in combination with carboplatinum (10 μg/mL) or cisplatinum (1 μM) for 48 hours, followed by a cell viability assay (ATP[li]te luminescence assay) (***P < .001 for carboplatinum vs carboplatinum-MLN or **P = .003 for cisplatinum vs cisplatinum-MLN in A549 cells; ** P = .007 for carboplatinum vs carboplatinum-MLN or *P = .003 for cisplatinum vs cisplatinum-MLN in H460 cells). These data are representative results of three independent experiments with similar trends. Data represent means, and error bars are standard deviation. Two-sided t test.
control vs MLN 1.0 μM: 355.70±9.10 vs 117.10±8.83, respectively, P < .001; LLC, control vs MLN 1.0 μM: 468.30±14.49 vs 162.50±9.61, respectively, P < .001) (Figure 4F) were also inhibited in a dose-dependent manner.

Moreover, MLN4924 exerted chemosensitizing effects on carboplatinum or cisplatinum, two widely-used conventional cytotoxic agents for clinical treatment of lung cancer (37,38), in A549 and H460 cells in vitro (cell viability [%], mean ± SD; A549, carboplatinum vs carboplatinum-MLN: 44.01±3.47 vs 23.20±1.30, respectively, P = .001; cisplatinum vs cisplatinum-MLN, 44.44±3.79 vs 27.33±2.53, respectively, P = .003; H460, carboplatinum vs carboplatinum-MLN, 58.69±5.72 vs 36.45±5.07, respectively, P = .007; cisplatinum vs cisplatinum-MLN, 55.37±3.06 vs 41.06±2.16, respectively, P = .003, two-sided t test) (Figure 4G). These findings demonstrate that the neddylation pathway serves as a promising anticancer and chemosensitizing target.

**Efficacy of Neddylation Inhibition on Experimental Lung Metastasis**

Next we investigated the efficacy of MLN4924 on tumor growth and metastasis in two experimental metastatic models. We first established an experimental lung metastatic model using aggressive LLC-green fluorescent protein (GFP)-red fluorescent protein (RFP) cells (LLC-Dual, which are LLC cells expressing GFP in the nucleus and RFP in the cytoplasm) which enabled us to measure tumor growth with a fluorescence-based imaging system. With fluorescence imaging numerous micrometastases and metastases were visualized in the lung in the control group; however, only a few micrometastases and metastases were visualized in the lung in the MLN4924-treated groups (Figure 5A). MLN4924 statistically significantly suppressed in vivo tumor formation and growth (mean fluorescence intensity, mean ± SD; control vs MLN4924: 26.40±15.47 vs. 9.70±5.58, respectively, P = .005, two-sided t test) (Figure 5B), leading to smaller tumor size (Figure 5C) and weight (grams) (mean ± SD; control vs MLN4924: 0.19±0.13 vs 0.05±0.05, respectively, P = .009, two-sided t test) (Figure 5D) compared with the control groups. During the treatment, no obvious adverse effects, such as body weight loss, were observed (Figure 5E). Similarly, MLN4924 remarkably suppressed the formation and growth of the A549-GFP tumor (No. of tumor nodules per lung, mean ± SD; control vs MLN4924, 63.50±29.84 vs 34.00±20.31, respectively, P = .02, two-sided t test) (Figure 5, F–H). Similarly, no observable adverse effects were observed during the treatment (Figure 5I). Moreover, we found that the expression of p21 and p27, two well-established substrates of CRL (8,15), was notably increased in tumor tissues upon MLN4924 treatment (Figure 5J), indicating that MLN4924 reached the tumor site to cause the inactivation of CRL and accumulation of its substrates. These findings demonstrate the in vivo anticancer efficacy of MLN4924.

**Molecular and Cellular Responses to Neddylation Inhibition**

Mechanistic analysis revealed that MLN4924 completely inhibited cullin neddylation, inactivated CRLs, and led to accumulation of CRL substrates, including cell cycle inhibitors p21, p27, and Wee1; NF-κB inhibitor IκB-α, as well as DNA replication licensing proteins CDT1 (chromatin licensing and DNA replication factor 1) and ORC1 (origin recognition complex, subunit 1) (Figure 6A; Supplementary Figure 4A, available online). As a result, MLN4924 triggered a DNA damage response, as reflected by increased levels of phosphorylated H2AX and CHK1 (Figure 6A; Supplementary Figure 4A, available online). MLN4924 also induced G1 phase cell cycle arrest, as demonstrated by the accumulation of G1-M phase transition inhibitor WEE1, the downregulation of M phase marker p-histone H3 (Figure 6A; Supplementary Figure 4A, available online) and the increased frequency of cells in G1 phase (Figure 6B; Supplementary Figure 4B, available online). Lung cancer cells arrested in G1 phase eventually died by either apoptosis or senescence in a cell-line-dependent manner. In H460 and LLC cells, MLN4924 induced apoptosis, as reflected by cleaved-caspase 3 and cleaved-PARP (poly ADP-ribose polymerase) (Figure 6C) and the appearance of a sub-G1 peak observed by cell sorting (Supplementary Figure 4B, available online), whereas in A549 and H1299 cells MLN4924 triggered senescence, as demonstrated by an enlarged and flattened cellular shape and the expression of senescence-associated β-galactosidase (Figure 6D and data not shown).

**Role of NOXA in Cell-Fate Determination (Apoptosis vs Senescence) Upon Neddylation Inhibition**

Previous studies have demonstrated a causal role of p21 and p27 upregulation in cellular senescence induced by neddylation inhibition/CRL inactivation (16,30,39,40). To further investigate the potential mechanisms underlying apoptotic induction, we measured the expression of classical pro-apoptotic proteins (Figure 7A) and anti-apoptotic proteins (Figure 7B) in apoptotic H460 and senescent A549 cells. Among these proteins, pro-apoptotic NOXA, a potential CRL substrate (41), was overexpressed in apoptotic H460, but not senescent A549 cells, suggesting a causal role of NOXA in the induction of apoptosis upon neddylation inhibition (Figure 7A). To test this hypothesis, the expression of NOXA was downregulated by small interfering RNA in H460 cells, and its effect on the induction of apoptosis upon neddylation inhibition was determined. As shown in Figure 7C, NOXA knockdown substantially attenuated the levels of cleaved PARP and caspase 3, demonstrating a causal role of NOXA in apoptosis induction upon neddylation abrogation. We further found that NOXA-silenced H460 cells displayed classical senescence morphology with an enlarged and flattened cellular shape when MLN4924-induced apoptosis was blocked; this indicated a conversion of the cell death phenotype from apoptosis to senescence in these cells (Figure 7D). These findings indicate that cell-line dependent induction of NOXA plays a critical role in cell-fate determination upon neddylation inhibition.

**Discussion**

Lung cancer remains the most common and deadly human malignancies worldwide, and there are many critical molecular events involved in lung cancer development remaining to be discovered (26–28). In this study, we found that the entire neddylation pathway is overactivated in both adenocarcinoma and squamous-cell carcinoma of the lung. Moreover, the neddylation pathway is functionally required for the maintenance of the malignant phenotype of lung cancer because abrogation of neddylation statistically significantly inhibits the proliferation, survival, migration, and motility

jnci.oxfordjournals.org
Figure 5. Efficacy of neddylation inhibition on experimental lung metastasis. A–D) MLN4924 inhibited tumor formation and growth in a Lewis lung carcinoma (LLC)–green fluorescent protein (GFP)-red fluorescent protein (RFP) (LLC-Dual) experimental lung metastasis model. LLC-Dual experimental metastatic models were established and treated with MLN4924 (n = 10 per group; as described in Supplementary Methods, available online). A) Lung metastasis of LLC-Dual visualized by GFP and RFP expression in either control or MLN4924-treated groups visualized by fluorescence microscopy. Lung tissues of 5 of 10 mice per group were shown. Scale bar = 2 mm. B) The fluorescence intensity of the lung tumors was calculated by ModFit LT software (n = 10;10 entire lungs from 10 mice per group). Sum of GFP and RFP is a relative value of mean fluorescence intensity (MFI). Data are means, and error bars represent standard deviation. **P = .005 by two-sided t test. C) Hematoxylin and eosin (HE) staining of lung sections. Tumor nodules are indicated with arrows. Scale bar = 500 μm; D) Weight of control and MLN4924-treated tumors. Horizontal lines indicate means ± standard deviation. **P = .009 by the two-sided Student t test. E) Body weight change during treatment (n = 10 per group). NS = not statistically significant. F–H) Efficacy of MLN4924 on A549-GFP experimental lung metastasis. A549-GFP experimental lung metastatic models were established and treated with MLN4924 (n = 10 per group), as described in Methods. F) 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 is shown. Scale bar = 2 mm. Tumor nodules are indicated with arrows. G) The number of tumor nodules on lung surfaces emitting green fluorescence was calculated (n = 10; 10 entire lungs from 10 mice per group). Data are mean, and error bars represent standard deviation. *P = .02 by the two-sided t test. H) H & E staining of lung sections. Scale bar = 500 μm. I) Body weight change during treatment (n = 10 per group). NS = not statistically significant. J) Accumulation of p21 and p27 in MLN4924-treated tumors, determined by immunohistochemical staining. Scale bar = 100 μm.
of lung cancer cells. Thus, our studies identify the activation of the neddylation pathway as a critical molecular event associated with lung carcinogenesis and validate this overactivated pathway as a promising therapeutic target against lung cancer.

Based on our findings reported in this study, we propose a working model regarding the potential role of neddylation in lung carcinogenesis (Figure 7E). During lung cancer development, neddylation enzymes are overexpressed, leading to the elevated global neddylation of substrates, such as cullins, to promote the degradation of tumor suppressors (such as p21 and p27) and facilitate carcinogenesis and progression. In contrast, inhibition of neddylation by the NAE inhibitor MLN4924 blocks protein neddylation, especially cullin neddylation, inactivates CRL, results in the accumulation of tumor-suppressive CRL substrates, and induces apoptosis or senescence to inhibit tumor growth and metastasis.

Furthermore, our study indicates that transcriptional activation may represent a molecular mechanism responsible for the overactivation of the neddylation pathway in lung cancer. Meanwhile, there may be different regulatory mechanisms responsible for the overexpression of different neddylation components. This notion is further supported by the fact that NEDD8 conjugation to targets is a rather complicated process involving multiple steps that are tightly regulated, positively or negatively (42). For example, the COP9 signalosome, an enzyme for deneddylation, removes NEDD8 from substrates (such as cullins) (42). Moreover, NUB1 and NUBIL induce proteasome-mediated degradation of NEDD8 and NEDD8 conjugates (43–45). Thus, the altered regulation of neddylation and deneddylation may contribute to the overactivation of the neddylation pathway during carcinogenesis and progression, solely or jointly.

We also demonstrated statistically-significant anticancer and chemosensitizing effects of neddylation inhibition by MLN4924, a first-in-class anticancer agent, both in vitro and in vivo, with low toxicity. Moreover, we characterized NOXA as a critical determinant for apoptotic induction by MLN4924 treatment, whereas p21 and p27 expression, upon neddylation inhibition/CRL inactivation, contributes to senescence induction (16,30,39,40). The findings extend our understanding of the intrinsic cellular responses to neddylation inactivation. Our studies provide a sound rationale and molecular basis for future clinical trials of MLN4924 in the treatment of lung cancer as a single agent or in combination with conventional anticancer drugs.

This study has a few limitations. The mechanisms underlying neddylation overactivation in lung cancer have not been fully defined. For example, it is unclear how NAE1 is transcriptionally activated and how other neddylation enzymes are upregulated in lung cancer. Additionally, the statistical analysis of the clinicopathological characteristics of the patients with lung squamous-cell

Figure 6. Cellular responses to neddylation inhibition. A) MLN4924 inhibited cullin neddylation and induced the accumulation of CullinRING E3 ligases (CRL) substrates. A549 and H460 cells were treated with MLN4924 for 72 hours and subjected to immunoblotting analysis for specific proteins with β-actin as a loading control. B) A549 (top panel) and H460 (bottom panel) cells were treated with MLN4924 for 72 hours and subjected to propidium iodide (PI) staining and fluorescence-activated cell sorting analysis to determine cell cycle profiles. These data are representative results of three independent experiments with similar trends. C and D) MLN4924 induced apoptosis or senescence in a cell line–dependent manner. Cells were treated with MLN4924 for 72 hours and subjected to immunoblotting to determine the induction of cleaved-caspase 3 (C-casp3) and cleaved-PARP (C-PARP) (C) or subjected to morphological observation (D, top panels) and expression analysis of senescence-associated β-galactosidase by β-galactosidase staining (D, bottom panels). Scale bar = 100 μm.
carcinoma was not performed in this study. Therefore, further investigations are required to address these fundamental issues.

In conclusion, our study reveals that the neddylation pathway is overactivated in lung cancer and suggests this pathway as a promising therapeutic target. This provides impetus for clinical trials of neddylation inhibitors. Moreover, the activation status of the neddylation pathway could be predictive for enrollment of an appropriate patient population for effective clinical trials of neddylation inhibitors. Finally, the successful development of new neddylation-pathway inhibitors, such as inhibitors of neddylation E2 or E3, may provide additional choices for targeting the overactivated neddylation pathway and could potentially overcome emerging resistance to E1 inhibitors (eg, MLN4924) as a result of treatment-related mutations (46,47).

Figure 7. Role of NOXA in cell-fate determination (apoptosis vs senescence) upon neddylation inhibition. A and B) Expression of pro-apoptotic proteins (A) and anti-apoptotic proteins (B) in MLN4924-treated A549 and H460 cells. Cells were treated with MLN4924 for 72 hours and subjected to immunoblotting for proteins involved in apoptotic induction with β-actin as a loading control. C and D) NOXA knockdown blocked apoptotic induction (C) and induced cell senescence (D) in MLN4924-treated cells. H460 cells transfected with either siControl or siNOXA were treated with MLN4924 (1 μM) for 72 hours and subjected to immunoblotting for cleaved PARP and caspase 3 with β-actin as a loading control (C) and senescence morphology observation (D). Scale bar = 100 μm. E) A working model of the role of neddylation during lung cancer development. NAE = NEDD8-activating enzyme; NOXA = phorbol-12-myristate-13-acetate-induced protein 1; RING = Small RING Finger Proteins RBX1 or RBX2; SRS = substrate recognition subunit; Ub = ubiquitin, UBC12 = ubiquitin-conjugating enzyme E2M.
Thomas MP, Narayanan U, et al. Treatment-emergent...

Yamaguchi Y, Miyauchi Y, et al. Direct interactions between...

Saville MK, Bourdon JC, et al. Mdm2-mediated...

O-charoenrat P, Talbot SG, et al. Squamous cell carcinoma...

jnci.oxfordjournals.org


This work was supported by the National Basic Research Program of China (973 program, 2012CB910302), the National Natural Science Foundation Grant

Funding
of China (grant numbers 81172092, 81372196, 31071204), the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning, Shanghai Pujiang Talent Program (12PJ1400600), and a grant from Fudan University Shanghai Cancer Center in China to LJ. This work was also partially supported by the grants from Mid-career Research Program (2010-0026203) through the National Foundation, Korea to LSJ.

Notes
L Li, M. Wang, and G. Ya contributed equally to this work. The authors are solely responsible for the study design, data collection, analysis and interpretation of the data, writing the manuscript, and decision to submit the manuscript for publication.

Affiliations of authors: Cancer Institute, Fudan University Shanghai Cancer Center (LL, GY, PC, DW, CL, WY, YW, LJ), Department of Oncology, Shanghai Medical College (LL, GY, PC, DW, CL, WY, YW, LJ), Department of Immunology, School of Basic Medical Sciences (LL, GY, CL, YW, YC, LJ), Clinical Statistics Center, Department of Radiation Oncology, Fudan University Shanghai Cancer Center (JZ, LX, HJ), and Liver Cancer Institute, Zhongshan Hospital (LS, QG), Fudan University, Shanghai, 200032, China; Department of Thoracic Cardiovascular Surgery, Xinhua Hospital of Shanghai Jiaotong University School of Medicine, Shanghai, China (MW, FH, JVM); College of Basic Medical Sciences, Zhengzhou University, Zhengzhou, China (PC, ZD); Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai, China (HL, PW); College of Pharmacy, Seoul National University, Seoul, Korea (LSJ); College of Pharmacy, Ewha Womans University, Seoul, Korea (LSJ, HJ, Y); AntiCancer Biotech Beijing Co. Ltd., Beijing, China (HQ, MY); Division of Radiation and Cancer Biology, Department of Radiation Oncology, University of Michigan, Ann Arbor, MI (YS); Department of Surgery, University of California, San Diego, CA (RMH); AntiCancer, Inc., San Diego, CA (RMH, MY).