

The CRTC1-NEDD9 Signaling Axis Mediates Lung Cancer Progression Caused by *LKB1* Loss

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Abstract

Somatic mutation of the tumor suppressor gene *LKB1* occurs frequently in lung cancer where it causes tumor progression and metastasis, but the underlying mechanisms remain mainly unknown. Here, we show that the oncogene *NEDD9* is an important downstream mediator of lung cancer progression evoked by *LKB1* loss. In *de novo* mouse models, RNAi-mediated silencing of *Nedd9* inhibited lung tumor progression, whereas ectopic *NEDD9* expression accelerated this process. Mechanistically, *LKB1* negatively regulated *NEDD9* transcription by promoting cytosolic translocation of *CRTC1* from the nucleus. Notably, ectopic expression of either *NEDD9* or *CRTC1* partially reversed the inhibitory function of *LKB1* on metastasis of lung cancer cells. In clinical specimens, elevated expression of *NEDD9* was associated with malignant progression and metastasis. Collectively, our results decipher the mechanism through which *LKB1* deficiency promotes lung cancer progression and metastasis, and provide a mechanistic rationale for therapeutic attack of these processes. *Cancer Res*; 72(24); 1–10. ©2012 AACR.

Introduction

Lung cancer is one of the most deadly diseases worldwide, mainly attributing to the high frequency of metastasis (1). Somatic inactivating mutation of *LKB1* is frequently observed in approximately 30% of non-small cell lung cancer (NSCLC; refs. 2–8) as well as several other epithelial carcinomas including prostate cancer, cervix cancer, and pancreas cancer (9). Our previous work in mouse models has uncovered an essential role of *LKB1* loss in promoting lung cancer progression and metastasis (4). However, the molecules mediating lung cancer progression and metastasis triggered by *LKB1* loss remain elusive. Identifying such mediators and deciphering the underlying mechanism are important for better understanding the

cancer progression and metastasis process, as well as finding potential therapeutic targets for effective lung cancer treatment in clinic.

As a master serine/threonine kinase and tumor suppressor, *LKB1* phosphorylates more than a dozen of downstream kinases including the well-known energy gauge kinase adenosine monophosphate kinase involved in mTOR signaling pathway (10). We have recently shown that the activation of mTOR signaling pathway by *LKB1* loss turns on lysyl oxidase (LOX) gene expression, which promotes lung cancer progression and metastasis through extracellular matrix remodeling (11). However, only partial inhibition of tumor progression was achieved by either LOX enzymatic inhibitor treatment or the combinational therapy using phosphatidylinositol 3-kinase-mTOR and mitogen-activated protein-extracellular signal-regulated kinase inhibitors (11, 12). Considering the limited efficacy of targeting either LOX or mTOR signaling pathway, we reason there may exist other pathways or molecules playing essential roles in lung cancer progression and metastasis induced by *LKB1* loss.

Through microarray data analysis, we have previously identified several potential candidates associated with metastasis in *Lkb1*-deficient murine lung cancer, including *Vegf* and *Nedd9* (neural precursor cell expressed, developmentally downregulated 9, also known as HEF1, Cas-L; ref. 4). While we have previously shown in mice that blocking *Vegfr* signaling is not effective in inhibition of *Lkb1*-deficient lung tumor metastasis (13), additional data support a potential role for *Nedd9*. *NEDD9* is a noncatalytic scaffold protein (14–16), and has been implicated in the metastatic behavior of several types of solid tumors (17, 18). In melanoma, *NEDD9* interacts with focal adhesion kinase (FAK) to promote cell invasion and metastasis (19). Similar observation has been reported in

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glioblastoma studies (20). These data support an important role of NEDD9 in cancer progression and metastasis of a number of malignancies. However, the interactive regulation between LKB1 and NEDD9, and the potential functional contribution of such a network to lung cancer progression and metastasis has not been explored yet.

Here, we provide strong evidences for NEDD9 as an important downstream mediator in *LKB1*-deficient lung cancer progression, and uncover a novel mechanism of LKB1 in contribution to lung cancer progression through CRTC1-NEDD9 axis. Our data provide novel functional evidences and mechanistic insights into lung cancer progression and metastasis evoked by *LKB1* loss and define a potential biomarker for lung cancer prognosis in clinic.

Materials and Methods

Mouse colony, mouse treatment, and mouse tumor analyses

Kras^{G12D} and *Lkb1*^{L/L} mice were generously provided by Drs. T. Jacks and R. Depinho, respectively (4). Nude mice (6 weeks old, male) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All mice were housed in a specific pathogen-free environment at Shanghai Institute of Biochemistry and Cell Biology and treated in strict accordance with protocols approved by the Institutional Animal Use Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

The *Kras*^{G12D} or *Kras*^{G12D}, *Lkb1*^{L/L} mice at 6 to 8 weeks old were virally infected with either 2×10^6 PFU Adeno-*Cre* or lentivirus (*Lenti-Cre*, *NEDD9-Cre*, *Ctrl-Cre*, or *shNedd9-Cre*) via nasal inhalation as previously described (21, 22). The lentiviruses were prepared as previously described (4). All mice were sacrificed for gross inspection and pathologic examination. Lung tumors were dissected for molecular analyses. The number of total tumor, well- or poorly differentiated tumors as well as the total tumor area were analyzed for each group from sectioned H&E slides as described before (23, 24) and the data were presented as mean \pm SEM. As for lung seeding assay, nude mice were injected with 1×10^6 cells via tail veins. After 10 weeks of inoculation, all mice were sacrificed and mouse lungs were weighted and lung metastasis was detected by H&E staining from series section.

Human lung cancer specimen analyses

All 175 human lung cancer samples were collected with patient consents in Fudan University Shanghai Cancer Center (Shanghai, China) from October 2007 to February 2008 with approval from the Institute Research Ethics Committee.

Cell culture, plasmids, and antibodies

Plasmids construction and the detailed information for antibodies are described in Supplementary Materials and Methods. Human cell lines including A549, CRL-5907, CRL-5866, HEK-293, and HEK-293T were purchased from American Type Culture Collection (ATCC) and authenticated by ATCC by short tandem repeat (STR) profiling and used for functional studies within 6 months after thawed from liquid nitrogen tank. A549, CRL-5907, CRL-5866 and HEK-293, and

HEK-293T cells were, respectively, cultured in RPMI-1640 medium (Hyclone) or Dulbecco's Modified Eagle's Media (Hyclone) supplemented with 10% FBS (Biochrom AG) and antibiotics (100 U/mL streptomycin and 100 μ g/mL penicillin; Invitrogen).

Immunohistochemical analysis

Immunohistochemistry was conducted as previously described (24). The proliferation rate was assessed by counting Ki-67 positive nuclear staining at high-power field for more than 1,000 cells. The apoptosis was assessed by analysis of cleaved caspase 3 immunostaining. The NEDD9 immunostaining was reviewed and scored blindly into 2 criteria as low and high expression as previously described (25). The correlation between clinical features and NEDD9 expression in human lung cancer was analyzed by SPSS 13.0 statistical software, and *P* values were calculated by Pearson χ^2 test. A value of *P* < 0.05 was considered as significant (2 tailed).

RT-PCR, real-time RT-PCR, and Western blot analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and retrotranscribed into cDNA (Ferments). [Primers for reverse transcriptase (RT-PCR) and real-time RT-PCR are listed in Supplementary Table S1]. Western blot analysis was done as previously described (26). The samples of cytoplasm and nuclear protein were obtained using proteoextract subcellular extraction reagents (Calbiochem) according to the manufacturer's instruction.

Reporter gene assay

Luciferase activities were measured 48 hours after transfection using the Dual-Luciferase Assay kit (Promega) on a GloMax 20/20 luminometer (Promega). pRL-SV40 was cotransfected as internal control. Experiments were conducted in triplicates and repeated at least 3 times.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted as previously described (27). Briefly, 5 μ g antibody of rabbit anti-cAMP-responsive element binding protein (CREB) or rabbit anti-CRTC1 was added into each lytic sample at 4°C overnight. Rabbit IgG was used as negative control. Fifty microliter Protein A (Invitrogen) was added into each sample for immunoprecipitation, and then samples were rotated at 4°C for 2 hours. An aliquot of sonicated cleared extract (input) and the immunoprecipitated material were decross-linked in TE plus 1% SDS for at least 8 hours at 65°C. Primers for ChIP assay were listed in Supplementary Table S1. *NRAA2* and *GAPDH* served as positive and negative controls, respectively (28). Representative data were shown from 3 independent experiments.

Wound healing assay

Wound healing assay was conducted as described before (29). Cells at 100% confluence were starved in serum-free medium for more than 12 hours dependent on different cell lines. Cells were then lightly and quickly scratched with a

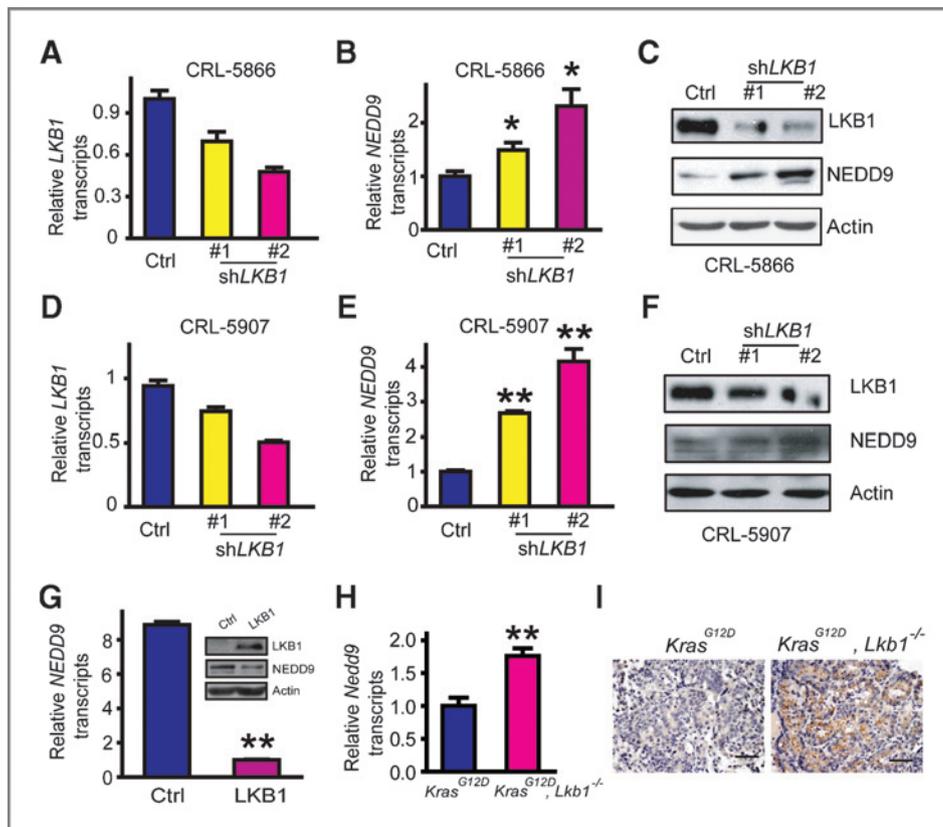


Figure 1. NEDD9 is transcriptionally regulated by LKB1 in human and mouse lung cancer. A, real-time RT-PCR analysis of *LKB1* knockdown efficiency in CRL-5866 cells. B and C, knockdown of *LKB1* upregulated NEDD9 expression at mRNA level (B) and protein level (C) in CRL-5866 cells assessed by real-time RT-PCR and Western blot analysis, respectively. Data are shown as mean \pm SEM. *, $P < 0.05$, compared with the control (Ctrl) group (B). D, real-time RT-PCR analysis of *LKB1* knockdown efficiency in CRL-5907 cells. E and F, knockdown of *LKB1* upregulated NEDD9 expression at mRNA level (E) and protein level (F) in CRL-5907 cells assessed by real-time RT-PCR and Western blot analysis, respectively. Data are shown as mean \pm SEM. **, $P < 0.01$, compared with the control (Ctrl) group (E). G, *NEDD9* mRNA and protein levels were downregulated in A549 cells by ectopic *LKB1* expression assessed by real-time RT-PCR and Western blot analysis, respectively. Data are shown as mean \pm SEM. **, $P < 0.01$. H and I, real-time RT-PCR (H) and immunostaining analyses (I) of Nedd9 expression level in *Kras*^{G12D} mouse tumors with or without *Lkb1* deficiency. Data are shown as mean \pm SEM. **, $P < 0.01$ (3 tumors in each group). Scale bar, 50 μ m (I).

pipette tip. The cell migration kinetics were closely monitored, photographed, and calculated by IncuCyte 2008B.

Three-dimensional cell culture

Cells were seeded in medium contain 2% Matrigel (BD Biosciences) on the top of another layer of solidified Matrigel. Cells were then cultured at 37°C incubators and monitored for the appearing morphologic changes in 1 to 2 weeks. Photos were taken using a light microscope (Leica).

Statistical analysis

Data were analyzed by Student *t* test. $P < 0.05$ was considered to be significant (2 tailed). Error bars represent SEM.

Results

Transcriptional regulation of NEDD9 by LKB1 in mouse and human lung tumors

Our previous gene expression profiling analyses of mouse *Kras*^{G12D} lung tumors with and without *Lkb1* deficiency identified *Nedd9* as a potential target gene downstream of *Lkb1* (4). To extend this finding, we investigated the functional corre-

lation of NEDD9 and LKB1 in human lung cancer. We found that knockdown of *LKB1* in human NSCLC cell lines CRL-5866 and CRL-5907, which express wild-type *LKB1*, resulted in increased NEDD9 mRNA and protein levels (Fig. 1A–F). Reciprocally, ectopic *LKB1* expression in the *LKB1*-mutant A549 human NSCLC cells inhibited NEDD9 expression (Fig. 1G). The regulation was associated with marked induction of Nedd9 expression in *Lkb1* deficient mouse tumors *in vivo* (Fig. 1H and I). These data show that *LKB1* is a negative regulator of *NEDD9* transcription in mouse and human lung tumors.

NEDD9 is an important downstream mediator of lung cancer progression and metastasis evoked by *Lkb1* deficiency

We next investigated the functional interaction between NEDD9 and LKB1 in lung cancer progression and metastasis. We found that *LKB1* overexpression in A549 cells blocked colony formation in soft agar, migration in wound healing assays, invasion in Matrigel as well as in boyden chamber assay whereas these effects were reversed by ectopic NEDD9 expression (Fig. 2A–E and Supplementary Fig. S1). Similarly, the

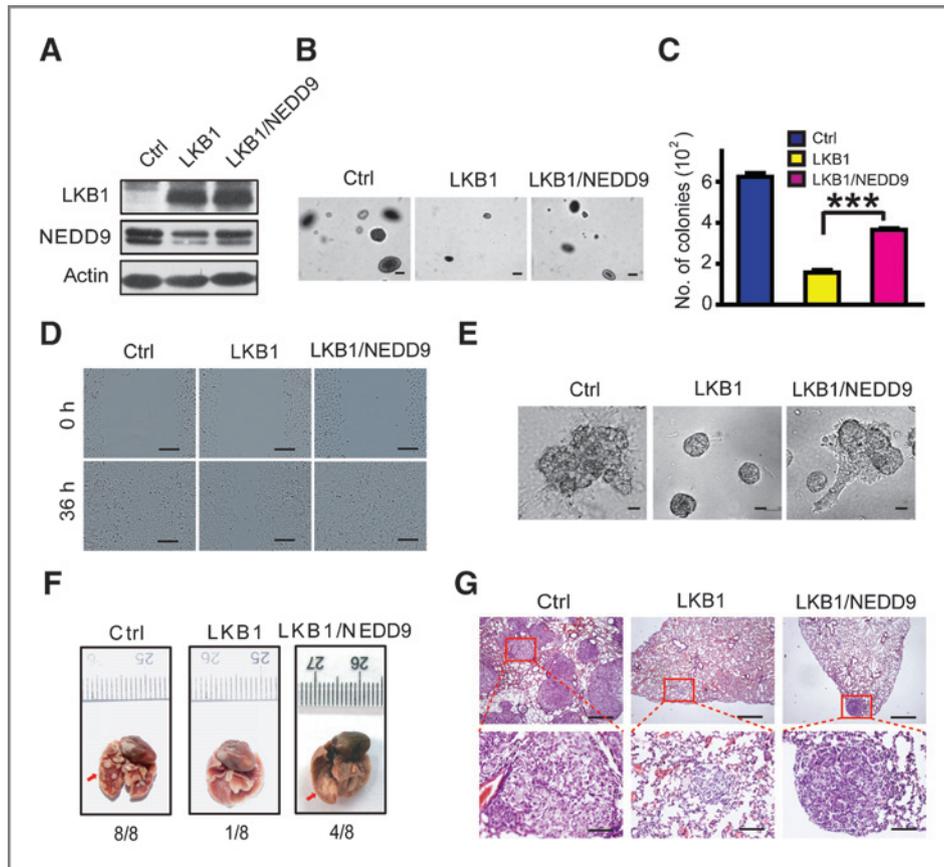


Figure 2. Ectopic NEDD9 expression partially reverses the inhibitory function of LKB1 upon colony formation, migration, and invasion of human lung cancer cells. **A**, Western blot detection of LKB1 and NEDD9 expression in A549 cells with ectopic LKB1 and/or NEDD9 expression. **B** and **C**, colony formation abilities of A549 cells with ectopic LKB1 and/or NEDD9 expression were assessed in soft agar. Representative photos (**B**) and the number of colonies (**C**) are shown. Scale bar, 100 μ m (**B**). Data are shown as mean \pm SEM. ***, $P < 0.001$. **D** and **E**, representative photos for migration in wounding assay (**D**) and invasiveness in Matrigel (**E**) of A549 cells with ectopic LKB1 and/or NEDD9 expression. Scale bar, 200 μ m (**D**) and 40 μ m (**E**). **F** and **G**, ectopic NEDD9 expression partially reverses the inhibitory function of LKB1 upon metastasis of A549 cells. Representative photos show the gross metastatic nodules on lung surface (**F**, indicated by the red arrows) as well as the histology of lung metastasis (**G**) from nude mice received with A549 cells with ectopic LKB1 and/or NEDD9 expression via tail vein injection. Metastatic incidence is shown on the bottom. Scale bars, 500 μ m (top) and 100 μ m (bottom).

inhibition of tumor formation by LKB1 overexpression in A549 lung seeding assay was partially overcome by ectopic NEDD9 expression; large nodules were detectable in all mice from the control group, in 1/8 mice from the LKB1 group, and in 4/8 mice from the LKB1/NEDD9 group (Fig. 2F and G). These data showed that NEDD9 can partially rescue the inhibitory function of LKB1 upon lung cancer invasion and metastasis.

Nedd9 knockdown inhibits *de novo* lung cancer progression in *Kras*^{G12D}, *Lkb1*^{L/L} mice model

We next asked if *Nedd9* knockdown affects *de novo* lung cancer progression (4). We infected *Kras*^{G12D}, *Lkb1*^{L/L} mice with lentivirus carrying Cre expression (Ctrl-Cre) or Cre expression together with *Nedd9* knockdown (sh*Nedd9*-Cre) as previously described (21). Mice were sacrificed for gross inspection and pathologic analyses at 21 weeks post viral administration. Knockdown of *Nedd9*, confirmed by real-time RT-PCR and immunostaining analyses (Supplementary Fig. S2A and S2B), resulted in a slight decrease of the number of tumors on lung surface but not total tumor number and area (Supplementary

Fig. S2C–S2E). More importantly, we found that the percentage of poorly differentiated tumors significantly decreased in *Nedd9*-knockdown group in comparison with control group (Fig. 3A and B). Consistently, *Nedd9* knockdown also resulted in a decrease of cell proliferation rate (Fig. 3C and D) while had no effect on cell apoptosis (Supplementary Fig. S2F). These data further support the essential role of *Nedd9* in regulating *de novo* lung cancer progression in context with *Lkb1* deficiency.

Ectopic NEDD9 expression promotes *de novo* lung cancer progression in *Kras*^{G12D} mouse model

We further investigated whether ectopic *Nedd9* expression is sufficient to promote lung cancer progression in *de novo* *Kras*^{G12D} mouse model. We treated *Kras*^{G12D} mice with lentivirus carrying either Cre (Lenti-Cre) or Cre with NEDD9 expression (*NEDD9*-Cre) via nasal inhalation and all mice were sacrificed for analyses at 24 weeks post viral administration. We found that ectopic NEDD9 expression, confirmed by real-time RT-PCR and immunostaining analyses (Supplementary Fig. S3A and S3B), did not affect the total number and area of

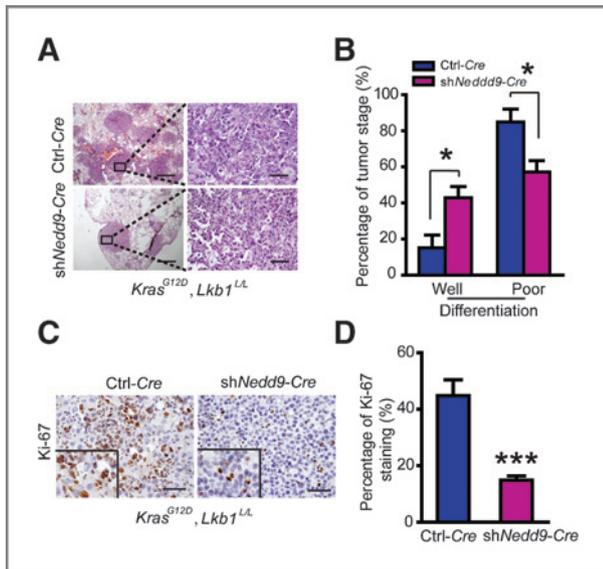


Figure 3. *shNedd9* alleviates *de novo* lung cancer progression evoked by *Lkb1* deficiency. **A**, pathologic photos of lung tumors from *Kras*^{G12D}, *Lkb1*^{LL} mice at 21 weeks post viral infection of Ctrl-Cre or *shNedd9-Cre* (7 mice per group). Scale bars, 500 μ m (top) and 50 μ m (bottom). **B**, statistic analyses of well- or poorly differentiated lung tumors from *Kras*^{G12D}, *Lkb1*^{LL} mice receiving Ctrl-Cre or *shNedd9-Cre* infection. Data are shown as mean \pm SEM. *, $P < 0.05$ ($n = 7$). **C**, Ki-67 immunostaining of lung sections from *Kras*^{G12D}, *Lkb1*^{LL} mice virally infected with Ctrl-Cre or *shNedd9-Cre*. Scale bars, 50 μ m. **D**, bar diagrams illustrate the percentage of Ki-67-positive staining in lung tumors from *Kras*^{G12D}, *Lkb1*^{LL} mice virally infected with either Ctrl-Cre or *shNedd9-Cre*. The percentage of Ki-67 staining was quantified by counting more than 1,000 nuclei staining tumor cells. Data are shown as mean \pm SEM. ***, $P < 0.001$.

lung tumors, whereas more nodules were observed on lung surface from *NEDD9-Cre* group than control (Supplementary Fig. S3C–S3E). Detailed pathologic analyses further revealed that the percentage of poorly differentiated tumors was significantly higher in *NEDD9-Cre* group (Fig. 4A and B). Consistently, a higher proliferation rate was observed in lung tumors with ectopic *NEDD9* expression (Fig. 4C and D). However, no significant change of apoptosis was found in *NEDD9* overexpressed lung tumors (Supplementary Fig. S3F). Taken together, these data show that ectopic *NEDD9* expression significantly promotes lung cancer progression in *de novo* mouse model.

CRTC1 regulates *NEDD9* transcription and contributes to *LKB1*-deficient lung tumor progression

To determine how *LKB1* negatively controls *NEDD9* expression, we initially screened seven reporters including CRE, serum response element (SRE), NF- κ B, glucocorticoid response element (GRE), heat-shock element (HSE), TATA-like promoter (TAL), and AP1 for their response to *LKB1* knockdown in CRL-5866 cells. Interestingly, CRE reporter was the only one regulated by *LKB1* (Supplementary Fig. S4), consistent with the inhibitory role of *LKB1* upon the transcription activity of CREB and its coactivator CRTC1 (30, 31). We found that ectopic expression of CREB and/or

CRTC1 significantly upregulated *NEDD9* mRNA and protein levels (Fig. 5A and B). Conversely, knockdown of *CRTC1* dramatically decreased the *NEDD9* mRNA level (Supplementary Fig. S5). To further explore the regulation of *NEDD9* by CRTC1/CREB, we cloned the 3kb genomic DNA fragment containing *NEDD9* promoter for reporter gene assay analyses. Through a series of deletion/mutation analyses (Supplementary Fig. S6), we had narrowed down the CRTC1/CREB-responsive element to a 20bp fragment (–146 to –166) with a nonclassical CRE site "TGAGCTCA" (32), which could not be predicted by TFSCAN (<http://www-bimas.cit.nih.gov/molbio/proscan/>; ref. 33). Mutation of this non-classical CRE site abolished the response of *NEDD9* promoter to either CREB or CRTC1 expression (Fig. 5C and D). Results from ChIP assay confirmed the *de novo* binding of either CREB or CRTC1 to *NEDD9* promoter region (Fig. 5E). These data convincingly show that CRTC1/CREB participates in regulating *NEDD9* gene transcription.

Moreover, we found that *LKB1* knockdown or ectopic *LKB1* expression regulated the transcriptional activity of *NEDD9* promoter with wild-type nonclassical CRE site but not with mutated site (Supplementary Fig. S7). Ectopic CRTC1 expression partially reversed the decrease of *NEDD9* mRNA and protein levels caused by ectopic *LKB1* expression in A549 cells (Fig. 5F and G). Immunofluorescence analysis showed that ectopic *LKB1* expression resulted in CRTC1 subcellular

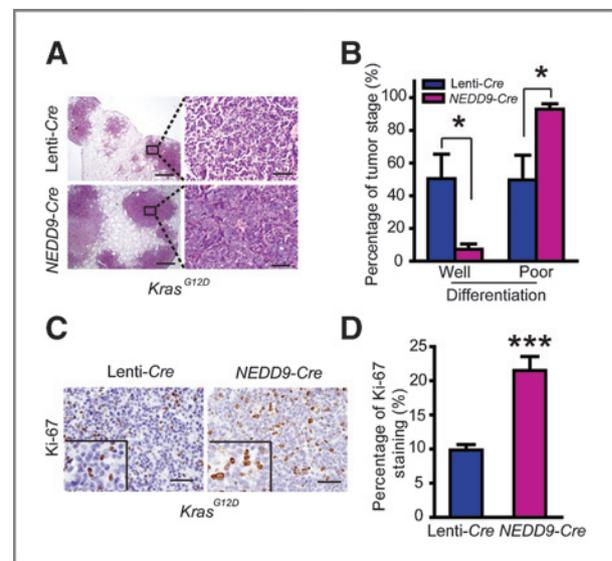


Figure 4. *NEDD9* promotes *de novo* lung cancer progression of *Kras*^{G12D} mice. **A**, pathologic photos of lung tumors from *Kras*^{G12D} mice at 24 weeks post Lenti-Cre or *NEDD9-Cre* infection (5 mice per group). Scale bars, 500 μ m (top) and 50 μ m (bottom). **B**, statistic analyses of well- or poorly differentiated lung tumors from *Kras*^{G12D} mice receiving Lenti-Cre or *NEDD9-Cre*. Data are shown as mean \pm SEM. *, $P < 0.05$ ($n = 5$). **C**, Ki-67 immunostaining of lung sections from *Kras*^{G12D} mice virally infected with either Lenti-Cre or *NEDD9-Cre*. Scale bars, 50 μ m. **D**, bar diagrams illustrate the percentage of Ki-67-positive staining in lung tumors from *Kras*^{G12D} mice treated with Lenti-Cre or *NEDD9-Cre* infection. The percentage of Ki-67 staining was quantified by counting more than 1,000 nuclei staining tumor cells. Data are shown as mean \pm SEM. ***, $P < 0.001$.

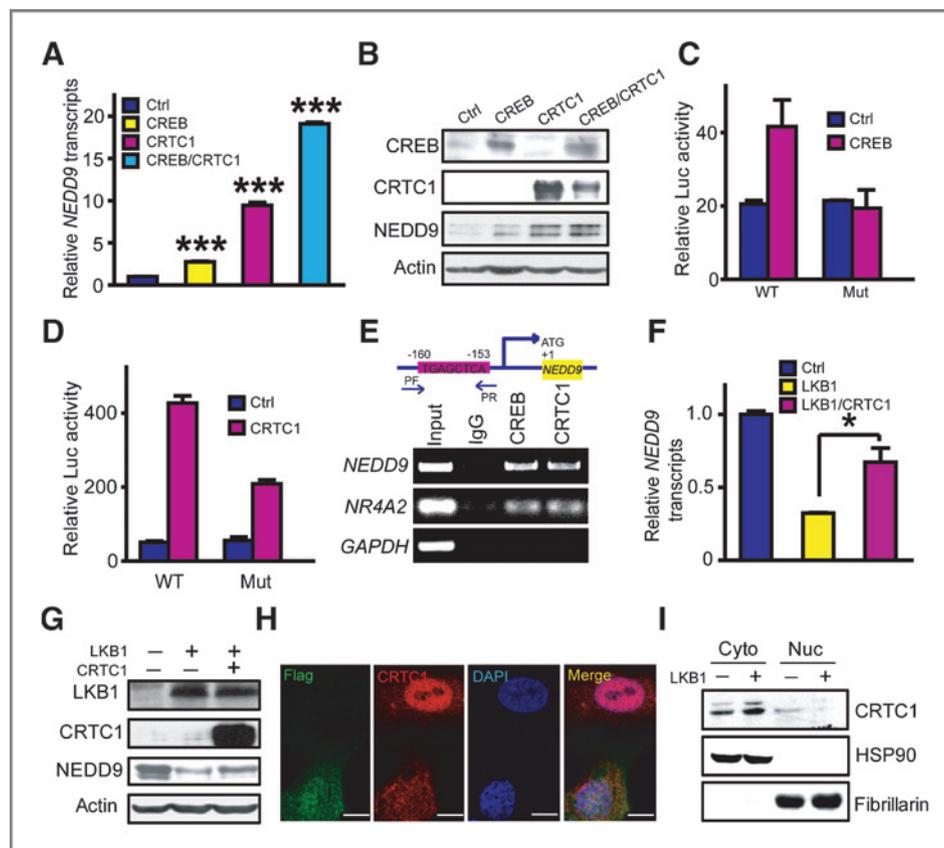


Figure 5. LKB1 regulates NEDD9 expression by modulating CRTCl nuclear translocation. A and B, both NEDD9 mRNA (A) and protein level (B) were upregulated by expression of CRTCl and/or CREB in CRL-5907 cells assessed by real-time RT-PCR and Western blot analysis. Data are shown as mean \pm SEM. ***, $P < 0.001$. C and D, ectopic expression of either CREB (C) or CRTCl (D) significantly increased the transcriptional activity of NEDD9 promoter with wild-type but not mutated nonclassical CRE site in CRL-5866 cells. E, ChIP assay confirmed the binding of CREB or CRTCl to NEDD9 promoter in A549 cells. PF, forward primer; PR, reverse primer. NR4A2 and GAPDH served as positive and negative controls respectively. F and G, ectopic expression of CRTCl reversed the downregulation of NEDD9 mRNA (F) and protein levels (G) by LKB1 in A549 cells. Data are shown as mean \pm SEM. *, $P < 0.05$. H, immunofluorescence assay showed that ectopic Flag-LKB1 expression promoted CRTCl translocation into cytoplasm. Flag-LKB1 (green), CRTCl (red), and DAPI (blue). Scale bar, 10 μ m. I, Western blot analyses showed that ectopic LKB1 expression decreased the nuclear CRTCl but increased the amount of CRTCl in cytoplasm. HSP90 and fibrillarlin served as loading controls for cytoplasm and nuclear fractions, respectively.

translocation from nucleus to cytoplasm (Fig. 5H). This was further confirmed by Western blot analysis using cytoplasmic and nuclear fractions (Fig. 5I). Previous studies have shown that salt inducible kinase (SIK), a downstream substrate of LKB1, is important in linking LKB1 and CRTCl/CREB transcription activity (31). Our data showed that ectopic LKB1 expression induced nuclear translocation of SIK2, which results in CRTCl nuclear exportation in human lung cancer cells (Supplementary Fig. S8). This regulation was further confirmed by LKB1 knockdown experiment (Supplementary Fig. S9). Moreover, SIK2 knockdown resulted in CRTCl nuclear retention which eventually upregulates NEDD9 transcript (Supplementary Fig. S10). These data convincingly showed that LKB1 regulates NEDD9 gene expression through CRTCl cellular translocation via SIK2 in lung cancer.

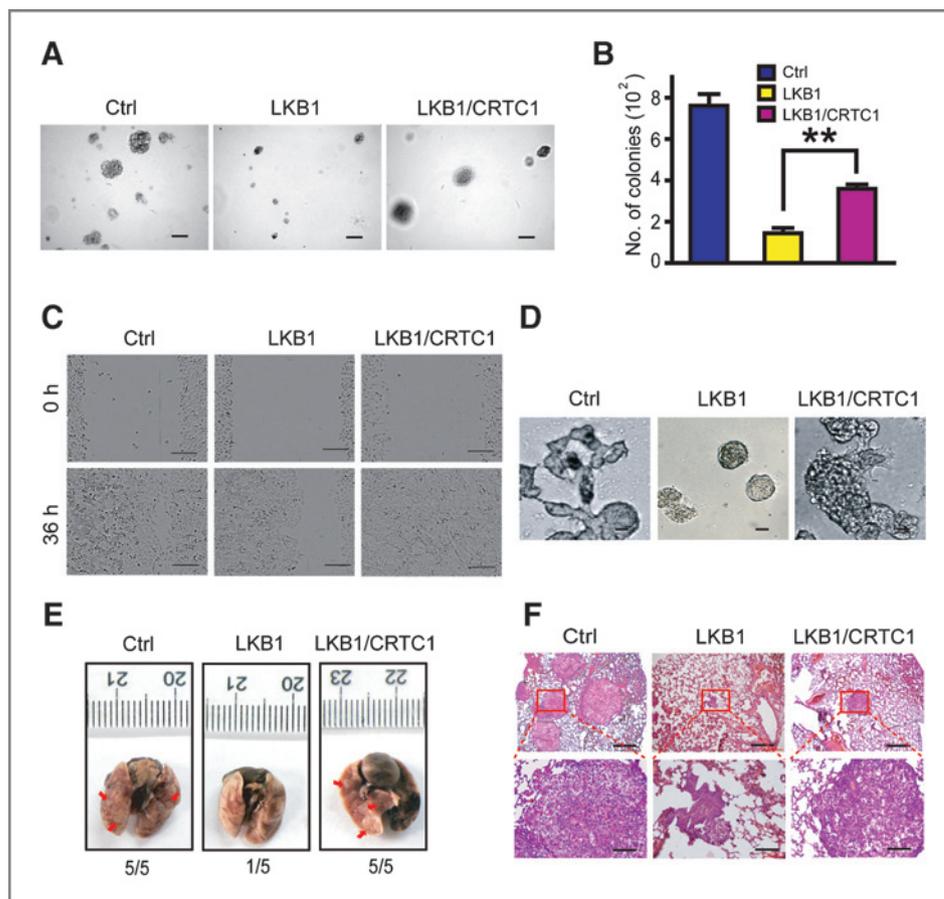
We further found that ectopic CRTCl expression partially rescued the inhibition of colony formation, cell migration, and invasion caused by LKB1 expression in A549 cells (Fig. 6A–D

and Supplementary Fig. S11), as well as the inhibitory effect of LKB1 upon lung cancer metastasis in lung seeding assay (Fig. 6E and F). Taken together, these data show that CRTCl regulates NEDD9 gene expression and contributes to LKB1-deficient lung tumor progression.

High NEDD9 expression correlates with human lung cancer malignancy progression and metastasis

Finally, we examined the clinical relevance of NEDD9 expression in human lung cancer. A cohort of 175 human NSCLC samples was used for NEDD9 immunostaining (Supplementary Fig. S12). Remarkably, high NEDD9 expression was strongly correlated with lymph node metastasis and advanced clinical stage, as well as with smoking and low differentiation (Table 1). No significant correlations were observed between NEDD9 expression and other clinical features, including NSCLC subtypes and tumor size (Table 1). Therefore, high NEDD9 level appears to be an indicator of poor prognosis for human lung cancer.

Figure 6. Ectopic expression of CRTCC1 partially reverses the inhibitory function of LKB1 upon colony formation, migration, invasion, and metastasis of lung cancer cells. A and B, representative photos (A) and statistic analyses (B) of colonies formed in soft agar of three different groups of A549 cells as indicated. Scale bar, 100 μ m (A). Data are shown as mean \pm SEM. **, $P < 0.01$. C and D, ectopic expression of CRTCC1 partially reversed the inhibitory effect of LKB1 upon A549 cell migration ability (C) and cell invasiveness in Matrigel (D). Scale bar, 200 μ m (C) and 40 μ m (D). E and F, ectopic expression of CRTCC1 partially reverses the inhibitory function of LKB1 upon metastasis of A549 cells. Representative photos show the gross metastatic nodules on lung surface (E, indicated by the red arrows) as well as the histology of lung metastasis (F) from nude mice received with A549 cells with ectopic LKB1 and/or CRTCC1 expression via tail vein injection (5 mice per group). Metastatic incidence is also shown on the bottom. Scale bars, 500 μ m (top) and 100 μ m (bottom).



Discussion

Metastasis is a major factor contributing to the high mortality of lung cancer. Here, we discover an important novel pathway involved in lung cancer progression and metastasis triggered by *LKB1* loss: *LKB1* loss triggers the nuclear translocation of CRTCC1, which in turn upregulates NEDD9 and promotes lung cancer progression and metastasis. Our study highlights NEDD9 as an important downstream mediator in lung cancer progression evoked by *LKB1* loss. High NEDD9 expression strongly associates with poor differentiation, advanced clinical stage, as well as lymph node metastasis. Collectively, these data provide a novel mechanism by which *LKB1* loss promotes cancer progression and metastasis as well as the potential biomarker for human NSCLC prognosis.

Our previous work showed that NEDD9 was upregulated by *LKB1* loss in lung cancer metastasis but did not resolve how NEDD9 potentially links *LKB1* loss to the malignant phenotype (4). Recent studies have shown that activation of hypoxia-inducible factor-1 α (HIF-1 α), downstream of mTOR pathway, regulates NEDD9 expression in colon, and renal cancers (34, 35). We have previously shown that the mTOR-HIF-1 α axis actually upregulates LOX, which in turn cross-links collagen to remodel extracellular matrix and triggers β 1-integrin signaling through FAK activation, and thus

promotes lung cancer metastasis (11). However, no evidence of the involvement of mTOR activation was found in NEDD9 regulation (4). Interestingly, CRTCC1/CREB transcription activation is critical for mediating NEDD9 expression and potentially promotes lung tumor malignancy. Nuclear translocation of CRTCC1 regulated by *LKB1* loss promotes *NEDD9* gene expression in lung cancer, which may indicate a cell type-specific pattern of *NEDD9* gene regulation. As a coactivator of CREB, CRTCC1 forms complex with CREB in nucleus and turns on the CREB transcriptional activity (30, 31, 36), which plays important roles in glucose and lipid metabolism (31, 37–39). However, apart from its role in metabolic regulation, less is known about its potential contribution in lung cancerigenesis. Previous studies have identified SIK as an important mediator of CRTCC1/CREB transcription activity downstream of LKB1 (31). In *LKB1*-deficient cells, SIK is incapable of phosphorylating CRTCC1, resulting in CRTCC1 nucleus retention where it interacts with CREB and promotes certain gene expression (30, 31, 36, 40, 41). Previous study has shown that *NR4A2* is transcriptionally upregulated by CRTCC1/CREB pathway in *LKB1* null lung cancer cells and promotes cell growth (42). Our data here identify NEDD9 as another important downstream mediator of CRTCC1/CREB pathway in lung cancer progression and metastasis caused by *LKB1* loss. Further dissection of this

Table 1. The clinical correlation of NEDD9 expression in human lung cancer

Characteristics	n	NEDD9		P
		Low	High	
Smoking				
No	77	32 (41.6%)	45 (58.4%)	0.025 ^a
Yes	98	25 (25.5%)	73 (74.5%)	
Histology				
ADC	121	39 (32.2%)	82 (67.8%)	0.886
SCC	54	18 (33.3%)	36 (66.7%)	
Differentiation degree				
High	19	12 (63.2%)	7 (36.8%)	0.001 ^b
Moderate	104	36 (34.6%)	68 (65.4%)	
Low	52	9 (17.3%)	43 (87.2%)	
T classification				
T1-2	146	49 (33.6%)	97 (66.4%)	0.531
T3-4	29	8 (27.6%)	21 (72.4%)	
LN metastasis				
Negative	90	38 (42.2%)	52 (57.8%)	0.005 ^b
Positive	85	19 (22.4%)	66 (77.6%)	
Clinical stage				
I/II	102	42 (41.2%)	60 (58.8%)	0.004 ^b
III/IV	73	15 (20.5%)	58 (79.5%)	

Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; LN metastasis, lymph node metastasis; n, number.

^aP < 0.05.

^bP < 0.01.

circuitry may provide important hints for targeted therapy in *LKB1*-deficient lung cancer.

With integrative studies using *de novo* animal model, lung seeding assay as well as *in vitro* migration and invasion systems, we have convincingly shown that NEDD9 plays a positive role in lung cancer progression and metastasis. Previous studies have supported that NEDD9 promotes solid tumor metastasis via FAK and Src activation (19, 20, 43) through direct interaction (18). Interestingly, 2 recent studies have implicated the potential involvement of NEDD9 in epithelial–mesenchymal transition (EMT) process in breast cancer (44, 45). EMT is thought to be an important event involved in early stages of cancer metastasis (46). It is attempting to ask if Nedd9 mediates the EMT process observed in *Lkb1*-deficient lung cancer progression and metastasis (12).

We further investigated the clinical correlation of NEDD9 expression level in 175 lung cancer specimens. Interestingly, we have found that high NEDD9 expression is positively correlated with lymph node metastasis and malignant progression, suggesting that NEDD9 is an indicator for poor prognosis of lung cancer patients. Future studies with an even larger sample number will be needed to establish a correlation between NEDD9 expression and survival.

In summary, our work has provided mechanistic insights into how *LKB1* loss-of-function mutations promote lung cancer progression. Identification of CRTC1/CREB as important regulators of NEDD9 gene expression may support mechanistic

insights into lung cancer progression and metastasis evoked by *LKB1* loss. NEDD9 also serves as a potential biomarker for lung cancer prognosis.

Disclosure of Potential Conflicts of Interest

K.-K. Wong has a commercial research grant from AstraZenca and Millenium, and is a consultant/advisory board member of Molecular MD. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: Y. Feng, H. Ji

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Feng, Y. Wang, Z. Wang, F. Li, Y. Gao, Y. Zhou, Q. Zhai, Y. Sun, N. Bardeesy, H. Chen, H. Ji

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Feng, Z. Fang, N. Bardeesy, K.-K. Wong, H. Ji

Writing, review, and/or revision of the manuscript: Y. Feng, Y. Gao, T. Xiao, F. Li, N. Bardeesy, K.-K. Wong, H. Ji

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Wang, Z. Fang, Y. Gao, H. Liu, F. Li, X. Liu, H. Chen, H. Ji

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The CRTC1-NEDD9 Signaling Axis Mediates Lung Cancer Progression Caused by *LKB1* Loss

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