The CRTC1-NEDD9 signaling axis mediates lung cancer progression caused by LKB1 loss

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Keywords: Lung cancer; progression; LKB1; NEDD9; CRTC1;
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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 demonstrate 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.
Introduction

Lung cancer is one of the most deadly diseases worldwide, mainly attributing to the high frequency of metastasis (1). Somatic inactivating mutation of \textit{LKB1} is frequently observed in ~30\% of non-small cell lung cancer (NSCLC) (2-8) as well as several other epithelial carcinomas including prostate cancer, cervix cancer, pancreas cancer (9). Our previous work in mouse models has uncovered an essential role of \textit{LKB1} loss in promoting lung cancer progression and metastasis (4). However, the molecules mediating lung cancer progression and metastasis triggered by \textit{LKB1} loss remains 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 AMPK involved in mTOR signaling pathway (10). We have recently shown that the activation of mTOR signaling pathway by \textit{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 PIK3-mTOR and MEK 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 \textit{LKB1} loss.

Through microarray data analysis, we have previously identified several potential candidates associated with metastasis in \textit{Lkb1}-deficient murine lung cancer, including \textit{Vegf} and \textit{Nedd9} (Neural precursor cell expressed, developmentally down regulated 9, also known as \textit{HEF1}, \textit{Cas-L}) (4). While we have previously shown in mice that blocking \textit{Vegfr} signaling is not effective in inhibition of \textit{Lkb1}-deficient lung tumor metastasis (13), additional data support a potential role for \textit{Nedd9}. \textit{NEDD9} is a non-catalytic scaffold protein (14-16), and has been implicated in the metastatic behavior of several types of solid tumors (17, 18). In melanoma, \textit{NEDD9} interacts with \textit{FAK} to promote cell invasion and metastasis (19). Similar observation has been reported in glioblastoma studies (20). These data support an important role of \textit{NEDD9} in cancer progression and metastasis of a number of malignancies. However, the interactive regulation between \textit{LKB1} and \textit{NEDD9}, and the potential functional contribution of such a network to lung cancer progression and metastasis have not been explored yet.

Here we provide strong evidences for \textit{NEDD9} as an important downstream mediator in \textit{LKB1}-deficient lung cancer progression, and uncover a novel mechanism of \textit{LKB1} in contribution to lung cancer progression through \textit{CRTC1-NEDD9} axis. Our data provide novel functional evidences and mechanistic insights into lung cancer progression and metastasis evoked by \textit{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\textsuperscript{G12D} and Lkb1\textsuperscript{L/L} mice were generously provided by Dr. T. Jacks and Dr. R. Depinho, respectively (4). Nude mice (6 weeks old, male) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). 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\textsuperscript{G12D} or Kras\textsuperscript{G12D}, Lkb1\textsuperscript{L/L} mice at 6–8 weeks old were virally infected with either 2x10\textsuperscript{6} PFU Adeno-Cre or lentivirus (Lenti-Cre, NEDD9-Cre, Ctrl-Cre, or shNedd9-Cre) via nasal inhalation as previously described (21, 22). The lentivirus were prepared as previously described (4). All mice were sacrificed for gross inspection and pathological 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 +/- s.e.m.. As for lung seeding assay, nude mice were injected with 1x10\textsuperscript{6} cells via tail veins. After ten 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 ATCC (American Type Culture Collection) and authenticated by ATCC by Short tandem repeat (STR) profiling and used for functional studies within six months after thawed from liquid nitrogen tank. A549, CRL-5907, CRL-5866 and HEK-293, HEK-293T cells were respectively cultured in RPMI-1640 medium (Hyclone) or DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany) and antibiotics (100 U/ml streptomycin and 100 μg/ml penicillin) (Invitrogen, USA).

**Immunohistochemical analysis.** Immunohistochemistry was performed as previously described (24). The proliferation rate were assessed by counting Ki-67 positive nuclear staining at high-power field (HPF) for more than 1000 cells. The apoptosis was assessed by analysis of cleaved caspase 3 immunostaining. The NEDD9 immunostaining was reviewed and scored blindly into two 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 Chi-square test. A value of $P < 0.05$ was considered as significant (two 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 retro-transcribed into cDNA (Ferments). (Primers for RT-PCR and real-time RT-PCR were 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 regents (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 co-transfected as internal control. Experiments were performed in triplicates and repeated at least three times.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was performed as previously described (27). Briefly, 5μg antibodies of rabbit anti-CREB or rabbit anti-CRTC1 was added into each lytic sample at 4°C overnight. Rabbit IgG was used as negative control. 50 μl Protein A (Invitrogen) was added into each sample for immunoprecipitation, and then samples were rotated at 4°C for 2 hrs. An aliquot of sonicated cleared extract (input) and the immunoprecipitated material were de-crosslinked in TE plus 1% SDS for at least 8 hr at 65°C. Primers for ChIP assay were listed in Supplementary Table S1. NR4A2 and GAPDH served as positive and negative controls, respectively (28). Representative data were shown from three independent experiments.
**Wound healing assay.** Wound healing assay was performed as described before (29). Cells at 100% confluence were starved in serum-free medium for over 12 hrs dependent on different cell lines. Cells were then lightly and quickly scratched with a 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) 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 1~2 weeks. Photos were taken using a light microscope (Leica).

**Statistical analysis.** Data were analyzed by Student’s t test. $P<0.05$ was considered to be significant (two tailed). Error bars represent standard error of mean (s.e.m.).
Results

Transcriptional regulation of NEDD9 by LKB1 in mouse and human lung tumors. Our previous gene expression profiling analyses of mouse Kras\textsuperscript{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 correlation 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 \textit{in vivo} (Fig. 1H and I). These data demonstrate 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 over-expression 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 inhibition of tumor formation by LKB1 over-expression 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 demonstrated 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 pathological analyses at 21 weeks post viral administration. Knockdown of *Nedd9*, confirmed by real-time RT-PCR and immunostaining analyses (Fig. S2A and B), resulted in a slight decrease of the number of tumors on lung surface but not total tumor number and area (Supplementary Fig. S2C-E). 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 B), didn't affect the total number and area of lung tumors, while more nodules were observed on lung surface from NEDD9-Cre group than control (Supplementary Fig. S3C-E). Detailed pathological 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 over-expressed lung tumors (Supplementary Fig. S3F). Taken together, these data demonstrate 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, SRE, NF-κB, GRE, HSE, 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 co-activator CRTC1 (30, 31). We found that ectopic expression of CREB
and/or CRTC1 significantly up-regulated 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 (-146~166) with a non-classical CRE site ‘TGAGCTCA’(32), which could not be predicted by TFSCAN (http://www-bimas.cit.nih.gov/molbio/proscan/) (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 demonstrate 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 non-classical 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 translocation from nucleus to cytoplasm (Fig. 5H). This was further confirmed by western blot using cytoplasmic and nuclear fractions (Fig. 5I). Previous studies have shown that SIK (salt inducible
kinase), a downstream substrate of LKB1, is important in linking LKB1 and CRTC1/CREB transcription activity (31). Our data showed that ectopic LKB1 expression induced nuclear translocation of SIK2, which results in CRTC1 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 CRTC1 nuclear retention which eventually up-regulates NEDD9 transcript (Supplementary Fig. S10). These data convincingly showed that LKB1 regulates NEDD9 gene expression through CRTC1 cellular translocation via SIK2 in lung cancer.

We further found that ectopic CRTC1 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 demonstrate that CRTC1 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.
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 CRTC1, which in turn up-regulates 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 demonstrated that NEDD9 was up-regulated 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 HIF1α, downstream of mTOR pathway, regulates NEDD9 expression in colon and renal cancers (34, 35). We have previously shown that the mTOR-HIF1α axis actually upregulates lysyl oxidase (LOX), which in turn crosslinks collagen to remodel extracellular matrix and triggers β1-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, CRTC1/CREB transcription activation, is critical for mediating NEDD9 expression.
and potentially promotes lung tumor malignancy. Nuclear translocation of CRTC1 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 co-activator of CREB, CRTC1 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 CRTC/CREB transcription activity downstream of LKB1 (31). In LKB1-deficient cells, SIK is incapable of phosphorylating CRTC1, resulting in CRTC1 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 up-regulated by CRTC1/CREB pathway in LKB1 null lung cancer cells and promotes cell growth (42). Our data here indentify NEDD9 as another important downstream mediator of CRTC1/CREB pathway in lung cancer progression and metastasis caused by LKB1 loss. Further dissection of this 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, two 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's 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 a 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.
Authors' Contributions

Conception and design: H. Ji, Y. Feng

Development of methodology: H. Ji, Y. Feng

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

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

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

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Wong, N. Bardeesy, H. Chen, Y. Sun, Z. Xiong, Y. Zhou, X. Liu, F. Li, Z. Wang, Y. Gao

Study supervision: H. Ji
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References

34. Kim SH, Xia D, Kim SW, Holla V, Menter DG, Dubois RN. Human enhancer of filamentation 1 Is a mediator of hypoxia-inducible factor-1alpha-mediated migration in colorectal carcinoma cells. Cancer Res 2010;70:4054-63.


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

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<td></td>
<td></td>
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<td>High 58.4%</td>
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<tr>
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<td>98</td>
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<td>39(32.2%)</td>
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<tr>
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<tr>
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Abbreviation: ADC, adenocarcinoma; SCC, squamous cell carcinoma; LN metastasis, lymph node metastasis. n, number. *P < 0.05; **P < 0.01.
Figure legends

Figure 1. NEDD9 is transcriptionally regulated by LKB1 in human and mouse lung cancer. A, real-time RT-PCR analysis of \textit{LKB1} knockdown efficiency in CRL-5866 cells. B and C, knockdown of \textit{LKB1} up-regulated NEDD9 expression at mRNA level (B) and protein level (C) in CRL-5866 cells assessed by real-time RT-PCR and western blot, respectively. Data were shown as mean ± s.e.m.. * \( P < 0.05 \), compared with the control (Ctrl) group (B). D, real-time RT-PCR analysis of \textit{LKB1} knockdown efficiency in CRL-5907 cells. E and F, knockdown of \textit{LKB1} up-regulated NEDD9 expression at mRNA level (E) and protein level (F) in CRL-5907 cells assessed by real-time RT-PCR and western blot, respectively. Data were shown as mean ± s.e.m.. ** \( P < 0.01 \), compared with the control (Ctrl) group (E). G, NEDD9 mRNA and protein levels were down-regulated in A549 cells by ectopic LKB1 expression assessed by real-time RT-PCR and western blot, respectively. Data were shown as mean ± s.e.m.. ** \( P < 0.01 \). H and I, real-time RT-PCR (H) and immunostaining analyses (I) of Nedd9 expression level in \textit{Kras}^{G12D} mouse tumors with or without \textit{Lkb1} deficiency. Data were shown as mean ± s.e.m.. ** \( P < 0.01 \) (3 tumors in each group). Scale bar: 50\( \mu \text{m} \) (I).
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) were shown. Scale bar: 100μm (B). Data were shown as mean ± s.e.m.. *** 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: 200μm (D) and 40μm (E). F and G, ectopic NEDD9 expression partially reverses the inhibitory function of LKB1 upon metastasis of A549 cells. Representative photos showed 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 was shown on the bottom. Scale bars: 500μm (top panels) and 100μm (bottom panels).
**Figure 3.** shNedd9 alleviates *de novo* lung cancer progression evoked by Lkb1 deficiency. A, pathological photos for lung tumors from *Kras*\(^{G12D}\), *Lkb1*\(^{L/L}\) mice at 21 weeks post viral infection of Ctrl-Cre or shNedd9-Cre (7 mice per group). Scale bars: 500\(\mu\)m (*top panels*) and 50\(\mu\)m (*bottom panels*). B, statistic analyses of well- or poorly-differentiated lung tumors from *Kras*\(^{G12D}\), *Lkb1*\(^{L/L}\) mice receiving Ctrl-Cre or shNedd9-Cre infection. Data were shown as mean±s.e.m.. *P* < 0.05 (n=7). C, Ki-67 immunostaining of lung sections from *Kras*\(^{G12D}\), *Lkb1*\(^{L/L}\) mice virally infected with Ctrl-Cre or shNedd9-Cre. Scale bars: 50\(\mu\)m. D, bar diagrams illustrated the percentage of Ki-67 positive staining in lung tumors from *Kras*\(^{G12D}\), *Lkb1*\(^{L/L}\) 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 were shown as mean±s.e.m.. ***P* < 0.001.
Figure 4. NEDD9 promotes de novo lung cancer progression of Kras$^{G12D}$ mice. A, pathological photos for lung tumors from Kras$^{G12D}$ mice at 24 weeks post Lenti-Cre or NEDD9-Cre infection (5 mice per group). Scale bars: 500μm (top panels) and 50μm (bottom panels). B, statistic analyses of well- or poorly-differentiated lung tumors from Kras$^{G12D}$ mice receiving Lenti-Cre or NEDD9-Cre. Data were shown as mean ± s.e.m.. * 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: 50μm. D, bar diagrams illustrated 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 were shown as mean ± s.e.m.. *** P < 0.001.
Figure 5. LKB1 regulates NEDD9 expression by modulating CRTC1 nuclear translocation. A and B, both NEDD9 mRNA (A) and protein level (B) were up-regulated by expression of CRTC1 and/or CREB in CRL-5907 cells assessed by real-time RT-PCR and western blot. Data were shown as mean ± s.e.m.. *** \( P < 0.001. \) C and D, ectopic expression of either CREB (C) or CRTC1 (D) significantly increased the transcriptional activity of \( NEDD9 \) promoter with wild-type but not mutated non-classical CRE site in CRL-5866 cells. E, ChIP assay confirmed the binding of CREB or CRTC1 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 CRTC1 reversed the down-regulation of \( NEDD9 \) mRNA (F) and protein levels (G) by LKB1 in A549 cells. Data were shown as mean ± s.e.m.. * \( P < 0.05. \) H, immunofluorescence assay showed that ectopic Flag-LKB1 expression promoted CRTC1 translocation into cytoplasm. Flag-LKB1 (green), CRTC1 (red), DAPI (blue). Scale bar: 10µm. I, western blot analyses showed that ectopic LKB1 expression decreased the nuclear CRTC1 but increased the amount of CRTC1 in cytoplasm. HSP90 and Fibrillarin served as loading controls for cytoplasm and nuclear fractions, respectively.
**Figure 6.** Ectopic expression of CRTC1 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: 100μm (A). Data were shown as mean ± s.e.m.. **P < 0.01. C and D, ectopic expression of CRTC1 partially reserved the inhibitory effect of LKB1 upon A549 cell migration ability (C) and cell invasiveness in matrigel (D). Scale bar: 200μm (C) and 40μm (D). E and F, ectopic expression of CRTC1 partially reverses the inhibitory function of LKB1 upon metastasis of A549 cells. Representative photos showed 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 CRTC1 expression via tail vein injection (5 mice per group). Metastatic incidence was also shown on the bottom. Scale bars: 500μm (top panels) and 100μm (bottom panels).
Figure 1

A. CRL-5866

B. CRL-5866

C. CRL-5866

D. CRL-5907

E. CRL-5907

F. CRL-5907

G. Relative LKB1 transcripts

H. Relative NEDD9 transcripts

I. Relative Nedd9 transcripts

Notes:
- **Ctrl** and **#1** and **#2** represent different conditions.
- **shLKB1** indicates knockdown of LKB1.
- **LKB1**, **NEDD9**, and **Actin** are shown in the corresponding panels.
- **Kras** and **Lkb1** knockout conditions are indicated.
- Significance levels are denoted as * (p < 0.05) and ** (p < 0.01).
Figure 2

A

B

C

D

E

F

G
Figure 3

A

Kras<sup>G12D</sup>, Lkb1<sup>L1</sup>

shNedd9-Cre Ctrl-Cre

B

Percentage of tumor stage (%)

Well Differentiation

C

D

Percentage of Ki-67 staining (%)

Ctrl-Cre shNedd9-Cre

Kras<sup>G12D</sup>, Lkb1<sup>L1</sup>
Figure 5

A

B

C

D

E

F

G

H

I

Relative NEDD9 transcripts

Ctrl CREB CRTC1 CREB+CRTC1

***

CTRL

CRTC1

NEDD9 Actin

Relative Luc activity

WT Mut

Relative Luc activity

WT Mut

Relative NEDD9 transcripts

Ctrl LKB1 LKB1+CRTC1

NEDD9 NR4A2 GAPDH

Cyto Nuc

LKB1 LKB1+CRTC1

CRTC1 HSP90 Fibrillin
Figure 6

A

B

C

D

E

F
The CRTC1-NEDD9 signaling axis mediates lung cancer progression caused by LKB1 loss

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