**NLK interacts with 14-3-3ζ to restore the expression of E-cadherin**

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**Abstract.** The Nemo-like kinase (NLK), a conserved serine/threonine kinase, plays a critical role in the regulation of a variety of transcription factors, with important roles in determining cell fate. Although recent studies have demonstrated decreased expression patterns of NLK in various types of human cancer, the functional mechanism of NLK in cancer development has not been elucidated. Here, in the present study overexpression of NLK was found to inhibit the growth and migration of the non-small cell lung cancer A549 cell line. NLK was subsequently found to interact with 14-3-3ζ (also known as YWHAZ), which is responsible for E-cadherin silencing during epithelial-mesenchymal transition (EMT). Furthermore, NLK overexpression was able to restore the expression of E-cadherin inhibited by 14-3-3ζ. Notably, NLK interacts with 14-3-3ζ and prevents its dimerization, which is essential for 14-3-3ζ stability and function. By fusing two copies of the 14-3-3ζ gene, via a Gly-rich linker, a non-dissociable dimer of 14-3-3ζ was formed. It was found that NLK was unable to restore the expression of E-cadherin inhibited by the overexpression of the fused dimer of 14-3-3ζ. In addition, the increased ability of migration induced by the overexpression of fused 14-3-3ζ dimer could not be altered by NLK overexpression. The results from the present study indicate that NLK is a negative regulator of 14-3-3ζ and plays a tumor suppressive role in the inhibition of cancer cell migration.

**Introduction**

Nemo-like kinase (NLK) is a conserved serine/threonine kinase and is involved in a variety of biological responses and is regulated by a variety of different transcription factors (1,2). Decreased expression of NLK was found in various types of human cancers. In human melanoma, decreased expression of NLK is associated with poor prognosis and increased vascularity, and metastasis (3). Decreased expression of NLK in human ovarian carcinomas is associated with poor patient survival (4,5). In addition, NLK is significantly downregulated in breast cancer tissues and non-small cell lung cancer (NSCLC) compared with that in corresponding normal tissues (6,7). NLK has also been identified as a tumor-suppressor gene in glioblastoma using an in vivo RNAi screen (8).

Epithelial-mesenchymal transition (EMT) is a biological process, involving the functional transition of polarized epithelial cells into mesenchymal cells, and is involved in cancer metastasis, migration, invasion, and progression (9-11). E-cadherin is typically repressed during EMT. Thus, E-cadherin is considered to be a suppressor of migration and invasion of malignant epithelial cancers (12,13). 14-3-3ζ (also known as YWHAZ) is a member of the evolutionally conserved regulatory family that mediates signal transduction by binding to phosphoserine-containing proteins (14). 14-3-3ζ was found to be responsible for silencing of E-cadherin during EMT (15).

In the present study, overexpression of NLK was found to inhibit the growth and migration of the NSCLC A549 cell line. Moreover, NLK interacts with 14-3-3ζ and prevents its dimerization. In addition, NLK overexpression was able to restore the expression of E-cadherin inhibited by 14-3-3ζ but not with the fused dimer of 14-3-3ζ. The results from the present study demonstrate that NLK is a negative regulator of 14-3-3ζ and plays a tumor-suppressive role by inhibiting the migration of cancer cells.

**Materials and methods**

**Cell culture.** The human A549, H358, H322, H23, H1437, H1650, Calu-3 and H441 lung cancer epithelial cell lines and human 293T cells were obtained from the Chinese Academy of Sciences cell bank, and were cultured in DMEM
Antibodies. Rabbit antibodies to 14-3-3ζ (cat. no. ab155037; dilution 1:1,000) and β-actin (cat. no. ab22783; dilution 1:2,000) were obtained from Abcam. Rabbit antibodies to NLK (cat. no. 94350; dilution 1:500), E-cadherin (cat. no. 3195; dilution 1:500) and HA tag (cat. no. 3724; dilution 1:2,000) were purchased from Cell Signaling Technology, Inc. Mouse antibodies to FLAG-M2 tag (cat. no. F1804; dilution 1:2,000), FLAG-M2 affinity gel (cat. no. A2220) and HA affinity gel (cat. no. A2095) were purchased from Sigma-Aldrich (Merck KGaA). Horseradish peroxidase (HRP)-linked anti-mouse IgG (cat. no. 7076; dilution 1:5,000) and anti-rabbit IgG (cat. no. 7074; dilution 1:5,000) were purchased from Cell Signaling Technology, Inc. HRP-linked light chain specific goat anti-mouse IgG (cat. no. 115-005-174) and mouse anti-rabbit IgG (cat. no. 211-002-171) for immunoprecipitation (IP) were purchased from Jackson ImmunoResearch Laboratories, Inc.

Lentivirus packaging and transduction. The lentivirus pCDH vector containing NLK, C-terminal FLAG tagged NLK, 14-3-3ζ, C-terminal FLAG/HA tagged 14-3-3ζ, 14-3-3ζ-Fusion [by a 10-amino acid Glycine (Gly)-rich linker] and FLAG tagged 14-3-3ζ-Fusion were purchased from Genesent Biological Technology Co., Ltd. To prepare the lentivirus, 293T cells were transfected with the aforementioned lentiviral vectors and packaging plasmids from Genesent Biological Technology Co., Ltd. according to the manufacturer's instructions. Virus-containing medium was collected 48 h after transfection and filtered through 0.45 µM low protein-binding filters (Nalgene; Thermo Fisher Scientific, Inc.). For transduction, cells were infected with lentiviral vectors and selected with puromycin. Subsequently, the expression levels of the target genes were detected using western blot analysis.

Colony formation assay. For colony formation, A549 cells were seeded in 6-well plates at 500 cells per well, and cultured for 2 weeks. When clones were visible, the colonies were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) for 5 min. Subsequently images of the cells were obtained and the number of cells were counted under an Olympus BX51 fluorescence microscope (magnification x100; Olympus Corp.).

Wound healing assay. Total 5x10⁵ A549 cells infected with different lentivirus were cultured using 6-well plates. After cells had grown to produce a 90% confluent monolayer, a pipette tip (200 µl) was used to create a wound. The cells were washed twice with PBS to remove non-adherent cells and cultured in serum-free medium for 18 or 24 h. Subsequently, the images of wound healing were obtained an Olympus BX51 fluorescence microscope (magnification x100; Olympus Corp.) and the migration rate was calculated. The migration rate was calculated using the following formula: Migration rate=(Area of original wound - Area of actual wound)/Area of original wound x100%.

Sphere formation assay. Monolayer cultured A549 cells (1,000 cells per well) infected with different lentivirus were suspended in 24-well ultra-low attachment plates (Corning, Inc.) with serum-free DMEM/F12 containing 20 ng/ml of epidermal growth factor (Thermo Fisher Scientific, Inc.), 20 ng/ml of basic fibroblast growth factor (Sigma-Aldrich; Merck KGaA) and 2% B27 supplement (Thermo Fisher Scientific, Inc.). After 7 days of incubation, images of the spheres were obtained under a phase-contrast Olympus BX51 microscope (magnification x100; Olympus Corp.).

IP and liquid chromatography-mass spectrometry (LC-MS)/MS analysis. For the IP assay, cells were harvested and lysed with RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% NP-40, and 0.25% sodium deoxycholate] with protease inhibitor and phosphatase inhibitor cocktail (Roche Diagnostics, GmbH). Following brief sonication, the lysates were centrifuged at 15,000 x g for 15 min at 4°C and the supernatants were subsequently incubated with the indicated primary antibodies together with protein A/G-sepharose beads (Santa Cruz Biotechnology, Inc.) or direct with FLAG-M2 affinity gel at 4°C for 2 h. The beads were washed three times before heating or adding FLAG peptides (Sigma-Aldrich; Merck KGaA). Isolated protein, including NLK-FLAG and its associated proteins were forwarded to Taiyuan Rosetta Stone Biotech Co., Ltd. for LC-MS/MS analysis. The mass of the peptides was identified using LTQ-XL mass spectrometer (Thermo Fisher Scientific, Inc.).

SDS-PAGE and western blot analysis. Total proteins were lysed with RIPA lysis buffer (cat. no. 9806; Cell Signaling Technology, Inc.). For denaturing conditions, the cell lysates were mixed with 5X SDS denaturing sample buffer (cat. no. 39000; Thermo Fisher Scientific, Inc.), and incubated at 95°C for 5 min. For non-denaturing conditions, the cell lysates were mixed with 2X SDS non-denaturing sample buffer (cat. no. LC2673; Thermo Fisher Scientific, Inc.) without 2-mercaptoethanol and incubated at room temperature for 60 min. The protein was separated using 12% SDS-PAGE or NativePAGE™ (Thermo Fisher Scientific, Inc.), and subsequently transferred onto a PVDF membrane (EMD Millipore; Merck KGaA). The PVDF membranes were blocked in 5% skimmed milk for 1 h at room temperature, and incubated with aforementioned primary antibodies overnight at 4°C. Following which, the membranes were washed with TBS-Tween-20 and incubated with the aforementioned secondary antibodies for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence reagents (Roche Diagnostics GmbH). Densitometric analysis was performed using ImageJ software (version 1.48; National Institutes of Health).

Statistical analysis. GraphPad Prism 5 software (GraphPad Software, Inc.) was used to analyze the data. Data are presented as the mean ± standard deviation. All experiments were repeated 3 times. Two-tailed Student's t-test was used when two independent groups were compared, while one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison was used for multiple comparison tests. A P<0.05 was considered to indicate a statistically significant difference.
Results

**NLK inhibits cell growth and migration.** To examine whether overexpression of NLK affects cell growth, lentivirus transfection was used to upregulate the gene expression of NLK in A549 cells. From the western blot analysis, A549 cells showed stable overexpression of NLK compared with that in the control cells transfected with the vector only (Fig. 1A). Colony formation assay (CFA) demonstrated that overexpression of NLK significantly represses colony formation ability, and overexpression of NLK significantly repressed sphere formation capabilities (SFA) in the A549 cells (Fig. 1B). Wound healing assay was used to determine whether overexpression of NLK affected the migration ability of A549 cells, and it was found that overexpression of NLK resulted in significantly decreased migration of A549 cells compared with that in cells transfected with the vector only (Fig. 1C). The results suggest that NLK significantly inhibited cell growth and migration capabilities.

**NLK interacts with 14-3-3ζ.** To further understand the mechanism of NLK in the regulation of cell growth and migration, IP and MS analyses were used to identify NLK binding proteins in A549 cells stably expressing C-terminally FLAG-tagged NLK (NLK-FLAG) created using lentivirus transfection. Cell extracts were subjected to IP with FLAG agarose beads. The specific bands of NLK-FLAG in isolated proteins were identified using western blot analysis (Fig. 2A). Subsequently, the isolated proteins, including NLK-FLAG and its associated proteins were analyzed using LC-MS/MS to identify NLK-interacting proteins. The tryptic peptides
corresponding to 14-3-3ζ protein with a high sequence coverage were identified (Fig. 2B). The interaction between NLK-FLAG and 14-3-3ζ was further verified using western blot analysis with the precipitated proteins (Fig. 2C). Moreover, endogenous NLK and 14-3-3ζ also showed an interaction in A549 cells (Fig. 2D). These data indicate that 14-3-3ζ is a novel NLK-interacting protein.

**NLK inhibits the dimerization of 14-3-3ζ and restores 14-3-3ζ-repressed E-cadherin expression.** A previous study has shown that 14-3-3ζ is responsible for silencing of E-cadherin during EMT (15). Lentivirus transfection was used to upregulate the gene expression of 14-3-3ζ in A549 cells (Fig. 3A). It was subsequently confirmed that overexpression of 14-3-3ζ induced a more spindle-like cell shape and a scattered distribution, suggesting decreased epithelial cell-to-cell contacts in the A549 cells (Fig. 3A). In addition, A549 cells stably overexpressing 14-3-3ζ were transfected with the NLK lentivirus to create NLK/14-3-3ζ double-overexpressing cells. EMT morphological changes induced by 14-3-3ζ overexpression were inhibited by NLK overexpression (Fig. 3A). Subsequently it was confirmed that overexpression of 14-3-3ζ inhibited the expression of E-cadherin (Fig. 3A). Furthermore, NLK/14-3-3ζ double-overexpressing cells revealed restored expression of E-cadherin compared with that in cells overexpressing 14-3-3ζ (Fig. 3B). It has been reported that dimerization is essential for 14-3-3ζ stability and the ability of 14-3-3ζ to bind to its target proteins (16,17). Using non-denaturing conditions, which would preserve any disulfide linkages during western blot analysis, an endogenous 14-3-3ζ dimer band was found in A549 cells (Fig. 3C). Moreover, the 14-3-3ζ monomer band and 14-3-3ζ dimer band were found to be increased in A549 cells stably overexpressing 14-3-3ζ. On the other hand, the 14-3-3ζ dimer band was inhibited in NLK/14-3-3ζ double-overexpressing cells as compared with that in 14-3-3ζ overexpressing cells (Fig. 3C).
At the protein level, expression of E-cadherin was evaluated in 8 human lung cancer cell lines using western blot analysis. There were no differences in the expression levels of 14-3-3ζ in these cells, and the expression levels between NLK and E-cadherin were highly correlated (Fig. 3D). These results suggest that NLK may be involved in 14-3-3ζ-regulated E-cadherin expression by the interaction with 14-3-3ζ. Using lentivirus transfection, A549 cells stably sole-expressing or co-expressing FLAG-14-3-3ζ and HA-14-3-3ζ were generated. As shown in Fig. 4A, FLAG-14-3-3ζ was co-immunoprecipitated with HA-14-3-3ζ in FLAG-14-3-3ζ and HA-14-3-3ζ co-expressing A549 cells. Notably, overexpression of untagged NLK in FLAG-14-3-3ζ and HA-14-3-3ζ co-expressing cells inhibited the interaction between FLAG-14-3-3ζ and HA-14-3-3ζ (Fig. 4B and C). These results suggest that NLK disturbs 14-3-3ζ-14-3-3ζ dimerization.

**NLK regulation of E-cadherin expression depends on the homodimer of 14-3-3ζ.** Gly-rich linkers are flexible and naturally exist in a number of proteins of their Gly residues (18-20). Thus, Gly-rich linkers have been used to create fusion proteins with independent sequences, including the connection with the homodimer, ligand-receptor and co-activators (21-23). By fusing two copies of the 14-3-3ζ gene via a 10-amino acid Gly-rich linker, heterodimer formation between 14-3-3ζ and 14-3-3ζ was observed. This interaction was inhibited by overexpression of untagged NLK in A549 cells. These results suggest that NLK may regulate 14-3-3ζ homodimerization through the interaction with 14-3-3ζ.
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A linker, a non-dissociable dimer of 14-3-3ζ (14-3-3ζ-Fusion) with lentiviral vector (Fig. 5A) was generated. As shown in Fig. 5A, the expression of the fused dimer of 14-3-3ζ and endogenous 14-3-3ζ were examined using western blot analysis, under denaturing and non-denaturing conditions.

Moreover, spindle-like cell shape and a scattered distribution were also found in cells stably expressing 14-3-3ζ-Fusion. The EMT morphological changes were not inhibited by overexpression of NLK (Fig. 5A). Meanwhile, 14-3-3ζ-repressed E-cadherin expression could not be restored by NLK overexpression (Fig. 5B).

To further determine whether NLK binds to the monomer or dimer form of 14-3-3ζ, the endogenous interaction between NLK and 14-3-3ζ under non-denaturing conditions was investigated. As shown in Fig. 5C, NLK was found to be associated with the monomer form but not with the dimer form of 14-3-3ζ under non-denaturing conditions. Moreover, exogenous FLAG-tagged 14-3-3ζ dimer fusion protein (14-3-3ζ-Fusion-FLAG) could not bind to endogenous NLK in 14-3-3ζ-Fusion-FLAG expressing cells (Fig. 5C). In addition, wound healing assay revealed that 14-3-3ζ overexpression enhanced the migration of A549 cells. NLK was able to inhibit the increased migration from 14-3-3ζ overexpression. On the other hand, 14-3-3ζ-Fusion overexpression revealed a higher migration ability compared with that in 14-3-3ζ-overexpressing cells and this effect was not regulated by NLK (Fig. 5D). Together, these results suggest that NLK regulation of E-cadherin expression depends on the homodimer of 14-3-3ζ.

Discussion

EMT is involved in cancer metastasis, migration, invasion, and progression. E-cadherin is essential for the maintenance of intercellular adhesion as a key component of the adherens junctions (24). Thus, the loss of E-cadherin is considered to be a major hallmark of EMT and has been reported in various types of cancers (25). 14-3-3ζ, a member of the evolutionally conserved family, mediates signal transduction by binding to phosphoserine-containing proteins. Dimerization is essential for 14-3-3ζ stability and the ability of 14-3-3ζ to bind its target proteins (16). 14-3-3ζ was found to be responsible for silencing of E-cadherin during EMT (15). In the present study, a novel NLK-interacting protein was identified. NLK was found to inhibit cell growth capabilities using colony formation assay. The number or size of spheres partly reveals the proliferation of cancer stem/progenitor cells (26). The sphere formation assay revealed that NLK markedly repressed the sphere formation capacities in A549 cells, in the present study. Furthermore, wound healing assay was performed to examine the effect of NLK on migration ability. Overexpression of NLK markedly inhibited cell migration. As an NLK-interacting protein, 14-3-3ζ overexpressing cells revealed enhanced migration capacity. NLK and 14-3-3ζ co-expressing cells revealed decreased migration compared with that in cells only expressing 14-3-3ζ. These findings suggest that NLK inhibition of migration may be dependent on the regulation of 14-3-3ζ function.
14-3-3ζ has been previously found to promote EMT and inhibit the expression of E-cadherin (15). In the present study, 14-3-3ζ was found to induce EMT and repress E-cadherin in A549 cells. Co-immunoprecipitation assay was performed with exogenous tagged protein or endogenous proteins to verify the interaction between NLK and 14-3-3ζ identified by LC-MS/MS analysis of NLK-interacting proteins. In light of the key role of NLK in the regulation of 14-3-3ζ-induced E-cadherin repression, A549 cells stably co-expressing FLAG-14-3-3ζ and HA-14-3-3ζ were constructed. FLAG-14-3-3ζ was found to be co-immunoprecipitated with HA-14-3-3ζ. Notably, overexpression of untagged NLK in FLAG-14-3-3ζ and HA-14-3-3ζ co-expressing cells inhibits the interaction between FLAG-14-3-3ζ and HA-14-3-3ζ. These results suggest that NLK disturbs the 14-3-3ζ-14-3-3ζ dimerization, and hence the function of NLK to regulate E-cadherin.

Gly-rich linkers naturally exist in a number of proteins and have been used to create fusion proteins with independent sequences, such as the connection with homodimer, ligand-receptor and co-activators (18). Overexpression of 14-3-3ζ-Fusion created by Gly-rich linker successfully repressed the expression of E-cadherin, promoted EMT morphological changes and cell migration. Notably, NLK lost its ability to regulate the expression of E-cadherin,

Figure 5. The function of non-dissociable homodimer of 14-3-3ζ. (A) Two copies of the 14-3-3ζ gene via a 10-amino acid Gly-rich linker to get a non-dissociable dimer of 14-3-3ζ (14-3-3ζ Fusion) with lentiviral vector. Expression of endogenous 14-3-3ζ and 14-3-3ζ Fusion was detected by western blot analysis in A549 cells transfected with the vector and 14-3-3ζ-Fusion lentivirus (left: Under denaturing condition, right: Under non-denaturing condition). Densitometric analysis of the relative expression level of dimer and monomer of 14-3-3ζ. *P<0.005 compared to the Vector group. Lower images: The cells exhibited different morphologies with the transfection of the vector, 14-3-3ζ-Fusion or 14-3-3ζ-Fusion plus untagged NLK lentivirus (scale bar, 100 µm). (B) Expression of E-cadherin and β-actin was detected by western blot analysis in A549 cells transfected with the vector lentivirus, 14-3-3ζ-Fusion or 14-3-3ζ-Fusion plus untagged NLK lentivirus. Densitometric analysis of the relative expression level of E-cadherin. *P<0.001 compared to the Vector group. (C) Left: A549 cells were lysed and subjected to IP with NLK antibodies, controlled by corresponding IgG. Immunoprecipitated protein were then analyzed by western blot analysis under non-denaturing condition. Right: Immunoprecipitated 14-3-3ζ-Fusion-FLAG-associated protein in A549 cells was subjected to western blot analysis. (D) Wound healing analysis of A549 cells transfected with the vector, 14-3-3ζ, 14-3-3ζ-Fusion, 14-3-3ζ plus NLK or 14-3-3ζ-Fusion plus NLK lentivirus. *P<0.005 compared to the Vector or 14-3-3ζ+NLK groups (scale bar, 200 µm). NLK, nemo-like kinase; IP, immunoprecipitation.
EMT morphological changes and cell migration induced by 14-3-3ζ-Fusion overexpression. In conclusion, the results from the present study reveals that NLK interacts with 14-3-3ζ to repress the expression of E-cadherin by interrupting 14-3-3ζ dimer formation.

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Availability of data and materials
The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions
JC, QL, TN, DS and WM conceived and designed the experiments. JC, QL and TN performed the majority of the experiments and assembled the data. JZ, FL, XL, YL, SR, TL, TZ and SH participated in the other experiments and article revision. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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