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Blockade of neddylation facilitates cancer cell migration by stabilizing c-Src

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이 건 우

A thesis of the Master's degree

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The Department of Biomedical Sciences,

Seoul National University

College of Medicine

Gunwoo Lee

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Blockade of neddylation facilitates cancer cell migration by stabilizing c-Src

by

Gunwoo Lee

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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Professor _____ **Chairman**

Professor _____ **Vice chairman**

Professor _____

Abstract

c-Src, a well-known proto-oncogene, mediates tumor progression properties including proliferation, angiogenesis, adhesion, and migration. It is well-characterized that the stability of active c-Src is regulated by ubiquitination while other post-translational modifications of c-Src have been unknown. Here, I found that the stability of c-Src is regulated by neddylation. Furthermore, c-Cbl turned out as an E3 ligase for conjugation of NEDD8 to c-Src. Moreover, the inhibition of c-Src neddylation markedly blocked its ubiquitination. To my surprise, the blockade of neddylation by NAE inhibitor MLN4924 or si-NEDD8 promoted cancer cell migration through the elevated stability of c-Src. Such consequences by inhibition of neddylation were abolished either by the treatment of Src-family kinase inhibitor PP2 or c-Src-targeting siRNA. Also the blockade of neddylation activated PI3K/Akt/mTOR pathway in a c-Src-dependent manner. Inhibitors of PI3K/Akt/mTOR pathway also could abolish cancer cell migration induced by MLN4924. Taken together, c-Src was identified as a novel substrate for neddylation that facilitates ubiquitination-dependent degradation, which blocks the migration of cancer cells via PI3K/Akt/mTOR pathway. My study provided a new insight about the effect of c-Src modification in tumor progression.

Key words: c-Src, c-Cbl, neddylation, ubiquitination, PI3K/Akt/mTOR, cell migration

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LIST OF ABBREVIATION

EGFR: Epidermal growth factor receptor

PI3K: Phosphoinositide 3-kinase

ERK: Extracellular signal-regulated kinase

MMP: Matrix metallopeptidase

FAK: Focal adhesion kinase

NEDD8: Neural precursor cell-expressed developmentally down-regulated 8

APPBP1: Amyloid beta precursor protein binding protein 1

SENP8: Sentrin-specific protease 8

Cbl : Casitas B-lineage lymphoma

mTOR : Mechanistic target of Rapamacin

INTRODUCTION

c-Src, a well known proto-oncogene, is activated or overexpressed in about 50% of tumors from colon, liver, lung, breast and the pancreas (Dehm and Bonham, 2004). As an upstream kinase of many signal pathways, c-Src mediates tumor progression properties including proliferation, angiogenesis, adhesion, and migration (Yeatman, 2004). Among these, c-Src can promote cell migration by activating diverse downstream molecules. EGFR, one of the major receptor tyrosine kinases (RTKs), is known for both enhancing the oncogenic properties with c-Src overexpression and causing aggressive migration in tumor cells (Maa et al., 1995; Bao et al., 2003). It was reported that the downstream kinase pathways of c-Src including PI3K/Akt and MEK/ERK participate in c-Src mediated cell migration (Guarino, 2010). It was presented that the RANKL-induced cell migration in breast cancer was mediated by c-Src through activating ERK and Akt (Zhang et al., 2012). IL-1β-induced MMP-9 expression and cell migration have been shown to be mediated via transactivation of EGFR/PDGFR/PI3K/Akt/NF-κB pathway by c-Src (Cheng et al., 2010). Also c-Src activates ERK/Sp1/MMP2 pathway, which elevates cell migration capacity (Kuo et al., 2006; Wu et al., 2016).

Although c-Src plays an important role in cancer cell migration and invasion, the regulations of its function and stability are not well-known.

Among the few, one of the known processes that regulates c-Src stability is ubiquitination. The active c-Src protein levels can be regulated by the ubiquitination-dependent degradation pathway. It was identified that the activation of c-Src increased its poly-ubiquitination (Harris et al., 1999; Hakak and Martin, 1999). In the process of ubiquitination for various tyrosine kinases, Cbl-family proteins are known to act as E3 ligases (Mohapatra et al., 2013). Cbl-family regulates the stability of c-Src as well. Cbl-c binds to phosphorylated c-Src at Y416 and induces ubiquitination of c-Src (Kim et al., 2004). Another protein of Cbl-family, c-Cbl, is phosphorylated by active c-Src, faciliating the ubiquitination of c-Src (Yokouchi et al., 2001). Consistent with these reports, c-Cbl also mediates cell migration through regulation of diverse molecules. For example, αPix-mediated cell migration and invasion were negatively regulated by c-Cbl (Seong et al., 2014). Furthermore, c-Cbl inhibited FAK/Src/GRB2 nexus, limiting cell migration in melanoma cells (Nihal and Wood, 2016). On the other hand, in glioma invasion, c-Cbl promoted upregulation of MMP2 (Lee and Tsygankov, 2010).

Apart from its ubiquitin-E3 ligase function, c-Cbl has another ligase function to conjugate small ubiquitin-like molecule, NEDD8. Protein neddylation is a process that NEDD8 covalently conjugates to other proteins (Kumar et al., 1993). Neddylation process is carried out through three steps similar to ubiquitination. First, E1 enzyme, namely NAE complex which is

composed of APPBP1 and UBA3, activates NEDD8. Followed by activation, conjugation of NEDD8 is conducted by unique neddylation E2 enzyme, UBC12. The last step determining substrate specificity is proceeded by various NEDD8-E3 ligases (Gong and Yeh, 1999). Neddylation has diverse effects on its substrate proteins. First, it can activate ubiquitin transfer function of E3 ligases. Cullin, a component of Cullin-RING ubiquitin ligases (CRLs), is conjugated with NEDD8 and activates the ubiquitin transfer activity (Bennett et al., 2010). Likewise, an ubiquitin-E3 ligase function of SMURF1, is facilitated by its neddylation (Xie et al., 2014). Secondly, neddylation regulates transcriptional activity of some transcription factors. p53 is identified to be neddylated by MDM2 NEDD8-E3 ligase, which inhibits the transcriptional activity (Xirodimas et al., 2004). Another transcription factor, E2F1, which controls cell proliferation and cell death, is also neddylated and results in interruption of the target specificity (Aoki et al., 2013). Neddylation is also well-known to alter the stability of its substrates. MDM2 conjugated NEDD8 to itself, thus bringing out its stabilization (Xirodimas et al., 2004; Watson et al., 2010). HIF-1α is also a target of neddylation. It promotes stabilization of this transcription factor (Ryu et al., 2011). When it comes to the function of c-Cbl as a NEDD8-E3 ligase, it causes the opposite effects to the known two substrates. c-Cbl conjugates NEDD8 to TGF-β type II receptor and improves protein stability by inhibiting

ubiquitination (Zuo et al., 2013). On the other hand, c-Cbl mediated neddylation of EGFR facilitates further ubiquitination and degradation (Oved et al., 2006).

Beyond the regulation of protein functions, neddylation also causes considerable changes to the cellular physiology. Given that a NEDD8 pathway affects many CRL substrates, it is not surprising that the neddylation process is involved in various types of cancers (Duncan et al., 2012). In fact, neddylation substrates found to be relevant in many kinds of cancers. For instance, MDM2 is included in typical cancers (Xirodimas et al., 2004). VHL, whose neddylation prevents interaction between CUL2 and fibronectin, is implicated in renal cancer (Ohh et al., 2003; Stickle et al., 2004). Gastrointestinal cancer involves disruption of IKKγ neddylation with increase of NF-κB signaling (Noguchi et al., 2011). Above these, many other neddylation substrates such as SCCRO, HuR, and CUL-1 showed relevancy with various cancers (Sakaria et al., 2006; Salon et al., 2007; Embade et al., 2012). Because of its interrelationship with tumor progression, neddylation has been receiving great attention as a therapeutic target for cancer treatments (Soucy et al., 2010). In particular, the inhibition of NEDD8-pathway was revealed to have a therapeutic potential (Abidi et al., 2015). One of the pharmacological methods to inhibit neddylation process is to treat MLN4924. MLN4924 is a selective inhibitor of neddylation-activating enzyme (NAE).

As MLN4924 has a similar structure with adenosine 5'-monophosphate (AMP), it bounds in the nucleotide-binding site of NAE (Bohnsack and Haas, 2003; Walden et al., 2003). Many studies reported about the fine effects of MLN4924 in cancers. It exhibited cytotoxic effects against variety of human-derived cancer cell lines and anti-tumor activity in xenografted mice (Soucy et al., 2009). Also MLN4924 was found to induce autophagy in liver cancer cells, promoting its apoptosis (Luo et al., 2012). In gastric cancer, this NAE inhibitor markedly suppresses cell migration via activating E-cadherin and reducing MMP-9 (Lan et al., 2016). Moreover, MLN4924 shows great effects for decreasing tumor progression in different types of cancers (Millhollen et al., 2010; Nawrocki et al., 2013; Wang et al., 2014). However, it was recently claimed that the treatment of MLN4924 facilitated formation of tumor-sphere via EGFR-dependent pathway *in vitro* and *in vivo* systems (Zhou et al., 2016).

Given that MLN4924 induces phosphorylation of Y845 of EGFR, a well-known residue as the target of c-Src mediated phosphorylation (Sato, 2013; Zhou et al., 2016), I assumed that the phosphorylation of c-Src may also be influenced by the neddylation process. For now, there is no direct evidence that c-Src is post-translationally modified by neddylation. In this study, I identified that the stability of c-Src is regulated by neddylation. Furthermore, c-Cbl turned out as an E3 ligase for conjugation of NEDD8 to c-Src. The inhibition of c-Src neddylation markedly blocked its ubiquitination. Moreover,

blockades of neddylation by NAE inhibitor MLN4924 or si-NEDD8 facilitated cancer cell migration through the elevated stability of c-Src. Such consequences by the inhibition of neddylation were abolished either by the treatment of Src-family kinase inhibitor PP2 or c-Src-targeting siRNA. Furthermore, the activated form of c-Src downstream kinases; phospho-Akt/mTOR were increased by the blockade of neddylation and the inhibition of these kinases by inhibitors interrupted MLN4924-induced migratory effect of cancer cells. Taken together, c-Src was identified as a novel substrate for neddylation that facilitates ubiquitination-dependent stability, which mediates the migration of cancer cells through PI3K/Akt/mTOR pathway.

MATERIALS AND METHODS

Antibodies and Chemicals

Antibodies against APPBP1 (Novus Biologicals, Littleton, CO), FLAG-tag (Sigma-Aldrich, St.Louis, MO), HA-tag (Genetex, Irvine, CA), c-Src, phospho-Y416 Src, Myc-tag, NEDD8, Akt, phospho-S473 Akt, ERK, phospho-p44/42 (Erk1/2), phospho-S2448 mTOR (Cell Signaling Technology, Danvers, MA), β-tubulin, Cbl, and c-myc (Santa Cruz Biotechnology, Dallas, TX) were obtained from the indicated companies. Cycloheximide, MG132 (Sigma-Aldrich), PP2 (Calbiochem, La Jolla, CA), LY294002, MK-2206, and Rapamycin (Selleck Chemicals, Houston, TX) were purchased from the indicated companies. MLN4924 was synthesized as previously described (Lee et al., 2011).

Cell Culture

HEK293 (a human embryonic kidney cell), H1299 (a human non-small cell lung carcinoma cell), HCT116 (a human colon carcinoma cell), U87MG (a human glioblastoma), and PC3 (a human prostate cancer cell) cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK293, H1299 and HCT116 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). U87MG and PC3 cells were maintained in

MEM/EBSS and RPMI1640 medium, respectively. All medium were supplemented with 10% heat-inactivated fetal bovine serum, penicillin and streptomycin. All cells were incubated in 5% CO_2 at 37 ^{o}C.

Plasmids and Short interfering RNAs (siRNAs)

His_6-tagged NEDD8, mutant NEDD8 (NEDD8-ΔGG; which is unable to conjugate with target proteins), HA-Ubiquitin, and Myc-SENP8 were constructed as previously described (Park et al., 2016). PCR-amplified cDNAs of human c-Src were inserted into Myc-tagged pcDNA. FLAG-tagged constitutive active form (Y527F) and dominant negative form (K295R, Y527F) of Src were provided by Professor Yang Young (Park et al., 2015). FLAG-tagged c-cbl was given by Professor Chin-Ha Chung (Seong et al., 2014). The siRNAs used in experiments were synthesized by M.biotech (Hanam-si, Gyeongi-do). The targeting sequences of APPBP1 (NM_003905) were 5'-GGACAAUCCAGAUAAUGAAAUAGTG-3' (# 1) and 5'-GGAUC-UACGACUAGAUAAGCCAUTT-3' (# 2), corresponding to exon 17 and 8, respectively. The targeting sequence of c-Cbl (NM_005188) was 5'-GAAGUUACCCUAAUAAUCCAAAGATG-3', corresponding to exon 16. The sequences of siRNAs targeting NEDD8 (NM_006156) were 5'-AGCGGUAGGAGCAGCAAUUUAUCCG-3' (# 1) and 5'-GAAGAUGCU-AAUUAAAGUGAAGACG-3' (# 2), corresponding to exon 1 and 2,

respectively. The targeting sequence of c-Src (NM_198291) was 5'-AGAGAAACCUGGUGUGCAAAGUGGCC-3', corresponding to exon 10. The control siRNA sequence was 5'-UUGAGCAAUUCACGUUCAUTT-3'.

Western blotting and Immunoprecipitation

Total cells lysed with 2 X-SDS were separated on SDS/polyacrylamide gels and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk or 1% skim milk and BSA in TTBS (tris-buffered saline containing 0.1% Tween 20) for 1 h, and incubated overnight with primary antibody diluted 1 : 500 to 1 : 3000 in the blocking solution or TTBS with 1% BSA. The membranes were further incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h and visualized using the ECL Plus kit (Thermo Fisher Scientific, Waltham, MA).

To precipitate tagged proteins, transfected cells were lysed with buffer containing 5 mM EDTA, 50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 0.1% NP-40, and the protease inhibitor cocktail (Sigma-Aldrich). Cell lysates (1 mg proteins) were incubated with anti-HA or FLAG affinity beads (Sigma-Aldrich) at 4 °C for 4 h. Proteins bound to the beads were eluted with a 2 X-SDS or 3 X FLAG PEPTIDE (Sigma-Aldrich) and assessed to western blotting.

Identification of His_6-tagged NEDD8 Conjugates

Identification of NEDD8-conjugation was performed and modified based on the previous description (Jaffray and Hay., 2006). After transfection of plasmid DNA expressing His_6-tagged NEDD8 or NEDD8-ΔGG, cells were divided into two dishes. One was lysed with 2 X-SDS sample buffer and analyzed by Western blotting to check the expression level of proteins (input samples). The other was mixed with denaturing buffer (6 M guanidine hydrochloride, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-Cl (pH 8.0), 10 mM imidazole and 10 mM β-mercaptoethanol). The lysates were incubated with Ni-NTA-agarose beads (Qiagen, Valencia, CA), prewashed with lysis buffer, and rotated for 4 h at room temperature. The beads were washed for 2 minutes in each steps with the following solutions: lysis buffer (pH 8.0); washing buffer (pH 8.0) (Mixture of 8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 8.0, 20 mM imidazole and 10 mM β-mercaptoethanol); washing buffer (pH 6.3) plus 0.2% Triton X-100; and washing buffer (pH 6.3) plus 0.1% Triton X-100. Then, the beads were eluted with 2 X-SDS sample buffer and visualized by Western blotting.

Wound healing assay

H1299 cells transfected with appropriate vectors were cultured in 5% CO_2 incubator at 37 ^oC until they became confluent. They were scratched with an

autoclaved 200μl pipette tip and washed twice with serum-free DMEM to remove the debris. In order to prevent proliferation, the cells were incubated in serum-free DMEM with or without the indicated reagents for 24 h, and photographed using a camera-equipped microscope.

Transwell migration assay

Transwell migration assay was performed using polycarbonate inserts (Corning, Newyork, NY) which has 8.0 μm size pores in 24-well cell culture plates as described by the manufacturer. The lower part was filled with DMEM containing 10% FBS and the upper part was filled with serum-free media. Cells ($1.0 \sim 1.5 \times 10^4$) were added to upper chamber, and incubated for 18 h at 37 ^{o}C. Non-invaded cells were removed from the membrane surface of the upper part using cotton swab. The migrated cells were fixed by MeOH and stained by solution with 1% Crystal Violet and 2% MeOH. The stained cells were photographed by microscope and the migration was analyzed by counting the number of stained cells in four randomly chosen fields per sample.

RNA isolation and RT-qPCR

Total RNA was isolated using Trizol reagent (Invitrogen, Calsbad, CA) according to the manufacturer's protocol. cDNA synthesis and amplification

was performed with EasyScript cDNA Synthesis Kit (Applied Biological Materials Inc., Richmond, BC). cDNAs were amplified with EvaGreen qPCR master mix reagent (Applied Biological Materials) by StepOne Real-time PCR System (Applied Biosystems, Foster, CA). 18s ribosomal RNA was used as an internal control. The sequences of c-Src primer used in experiment were FOR: 5'-CAGAGAGGGAAAGCCACTTG-3' and REV: 5'-GGCTT-GCTCTTGTTGCTACC-3'.

RESULTS

Neddylation Inhibits Stabilization and Activation of c-Src.

Given that inhibition of neddylation by MLN4924 enhanced the phosphorylation of EGFR at Y845 mediated by c-Src kinase (Zhou et al., 2016), I examined whether c-Src is affected by the depletion of neddylation via the treatment of MLN4924. To remove the effects of various growth factors affecting c-Src activation, I incubated cells in serum-free media for 24 hours before MLN4924 treatment. Surprisingly, both the levels of c-Src and phospho-Y416 Src increased with the treatment of MLN4924 in H1299, HCT116, and PC3 cancer cell lines (Fig 1A). Consistently, the blockade of neddylation process through siRNAs targeting NEDD8 or APPBP1 enhanced the levels of both c-Src and phospho-Y416 Src in H1299 cell line (Fig 1B). To understand the mechanism underlying the alteration of the c-Src protein levels, I evaluated the synthesis and degradation of c-Src proteins. First, the stability of c-Src was investigated using Cycloheximide (CHX) with or without MLN4924 treatment in H1299 cells. The total c-Src protein levels slowly decreased with the treatment of MLN4924, compared to the control (Fig 2A). The half-life of c-Src protein was 7.9 ± 3.9 hours in the control, but markedly increased to 14.5 ± 5.0 hours with MLN4924 treatment. In order to rule out the possibility of newly synthesized c-Src proteins, mRNA level of c-Src was

checked with or without MLN4924 (Fig 2B). There were no changes in mRNA level of c-Src. These results suggest that neddylation negatively regulates the stability and activity of c-Src.

NEDD8-conjugation of c-Src.

To examine whether c-Src is directly neddylated, His-N8 or His-N8ΔGG (conjugation-defective mutation of NEDD8) were co-transfected with Myc-WT-Src into HEK293 cells and the neddylated proteins were isolated by Ni^{2+} affinity beads. Ectopically expressed c-Src was firstly identified to be conjugated with NEDD8, while failed with NEDD8ΔGG (Fig 3A). Besides, the NEDD8-conjugation of c-Src was effectively removed by co-transfection of Myc-SENP8, a de-neddylating enzyme (Fig 3B). These results indicate that c-Src is directly conjugated with NEDD8.

The role of c-Cbl as a NEDD8-E3 ligase for c-Src.

c-Cbl, a known ubiquitin and NEDD8-E3 ligase, is involved in diverse cellular pathways through regulation of tyrosine kinases (Mohapatra et al., 2013). In particular, c-Cbl was identified to act as either ubiquitin or NEDD8-E3 ligase for EGFR and ubiquitin-E3 ligase for c-Src (Yokouchi et al., 2001; Oved et al., 2006). Accordingly, to examine whether c-Cbl plays a role as NEDD8-E3 ligase for c-Src, the interaction between c-Src and c-Cbl was first

evaluated by immunoprecipitation. Ectopically expressed c-Src binds to c-Cbl as previously reported (Fig 4A; Szymkiewicz et al., 2004). Next, the extent of neddylation of c-Src was measured when c-Cbl was depleted by siRNA in H1299 cells. As expected, knockdown of c-Cbl decreased the level of c-Src neddylation (Fig 4B). In contrast, when c-Cbl was overexpressed in H1299 cells, the neddylation of c-Src was increased (Fig 4C). Therefore, these results suggest that c-Cbl plays a role as a NEDD8-E3 ligase for c-Src.

c-Src activation facilitates its neddylation.

As the phosphorylation of c-Cbl at Y371 residue mediated by c-Src ubiquitinates itself as well as c-Src (Yokouchi et al., 2001), I examined whether the phosphorylation of c-Src could affect the neddylation of itself. The extent of neddylation of c-Src was measured using Ni^{2+} column in U87MG and H1299 cells expressing Myc-WT-Src and His-NEDD8 with or without PP2, a selective Src inhibitor. Neddylation of c-Src decreased in the presence of PP2 (Fig 5A). Consistent with this result, constitutive active form of c-Src (FLAG-CA-Src) was more neddylated than the dominant negative form (FLAG-DN-Src) (Fig 5B). These results indicate that the neddylation of c-Src is facilitated with its activation.

c-Src neddylation regulates its ubiquitination.

Given that EGFR conjugated with NEDD8 is facilitated to be ubiquitinated and degraded by the 26S proteasome system (Oved et al., 2006), I assumed that ubiquitination of activated-Src might also be regulated by its neddylation. To examine this, I assessed the level of ubiquitinated c-Src by immunoprecipitation with or without MLN4924 treatment in H1299 cells. As in Figure 6A, inhibition of neddylation by MLN4924 caused a decrease of c-Src ubiquitination. Also the de-neddylation through SENP8 effectively reduced the ubiquitination of c-Src (Fig 6B). These results suggest that the neddylation of c-Src facilitates its ubiquitination process.

Blockade of neddylation promotes cell motility in a c-Src-dependent pathway.

I have shown that c-Cbl facilitates conjugation of neddylation to c-Src, further inducing ubiquitination of the c-Src. c-Cbl is known to regulate the cell migration negatively, while c-Src promotes cell motility in cancers (Guarino, 2004; Seong et al., 2014; Nihal and Wood, 2016). Therefore, I examined whether neddylation can control cancer cell migration through c-Src-dependent pathway. First, metastasis in H1299 lung cancer cell line was evaluated. In a monolayer wound healing assay, the cells treated with MLN4924 has shown more migration than the untreated cells (Fig 7A).

Furthermore, the migration in the cells of which neddylation was blocked by the siRNA targeting NEDD8 increased than the control (Fig 7B). The migratory effect by inhibition of neddylation was further assessed by transwell migration assay. The MLN4924 treated H1299 cells showed greater migration capacity than the control (Fig 8A). Also the migration in the cells with knocked down expression of NEDD8 increased compared to the control (Fig 8B). Next, to examine whether the enhanced cell motility by blocking of neddylation is dependent on c-Src, the cell motility was evaluated in the cells expressing siRNA targeting c-Src with MLN4924 treatment. The rate of migration increased with MLN4924 in the cells expressing control siRNA, but decreased in cells expressing si-Src (Fig. 9A). However, the inhibition of c-Src could not block the cell motility completely. This could be reason that c-Src might not be sufficiently depleted with tested siRNA (Fig. 9A, lower panel) or other various pathways in cell motility might be influenced by neddylation inhibition. Furthermore, the elevated cell migration by MLN4924 was markedly reduced with the treatment of c-Src inhibitor, PP2 (Fig. 9B). Therefore, cell motility is increased with the blockade of neddylation via c-Src pathway.

Blockade of neddylation facilitates PI3K/Akt/mTOR signaling in a c-Src-dependent way.

Next, I investigated which downstream signaling is affected by the neddylation of c-Src. Previous reports have shown that c-Src mediates PI3K/Akt and MEK/ERK pathways, promoting cell migration (Kuo et al., 2006; Cheng et al., 2010; Zhang et al., 2012; Wu et al., 2016). Therefore, protein levels of Akt, ERK, and their kinase-active forms were evaluated with the treatment of MLN4924 and PP2 in H1299 cells. The level of kinase-active form of Akt (phospho-S473 Akt) dramatically increased with MLN4924 treatment while the levels of Akt, ERK, and phospho-ERK barely changed (Fig 10A). Furthermore, the downstream kinase of Akt, mTOR, was also activated in presence of MLN4924. Elevated levels of phospho-S473 Akt and phospho-S2448 mTOR by MLN4924 were reduced with the treatment of PP2. Next, to examine whether PI3K/Akt/mTOR pathway is involved in enhanced cell motility through the inhibition of neddylation, the cell migration was evaluated in H1299 treated with MLN4924 and various inhibitors. In line with PP2, treatment of inhibitors of PI3K (LY294002), Akt (MK-2206), and mTOR (Rapamycin) effectively interfered MLN4924-induced cell migration (Fig 10B). These results suggest that the blockade of neddylation promotes cancer cell migration through c-Src mediated PI3K/Akt/mTOR pathway.

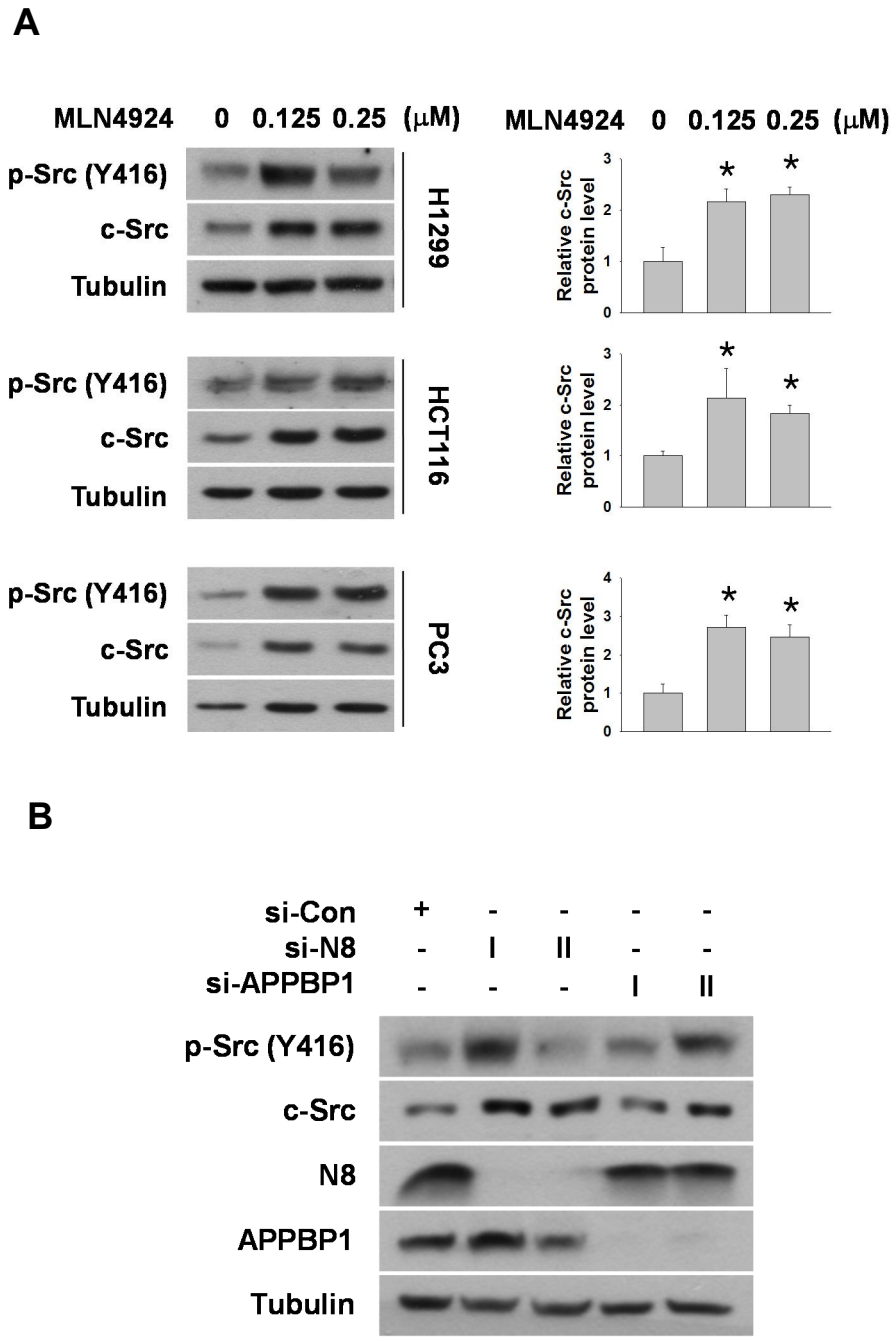
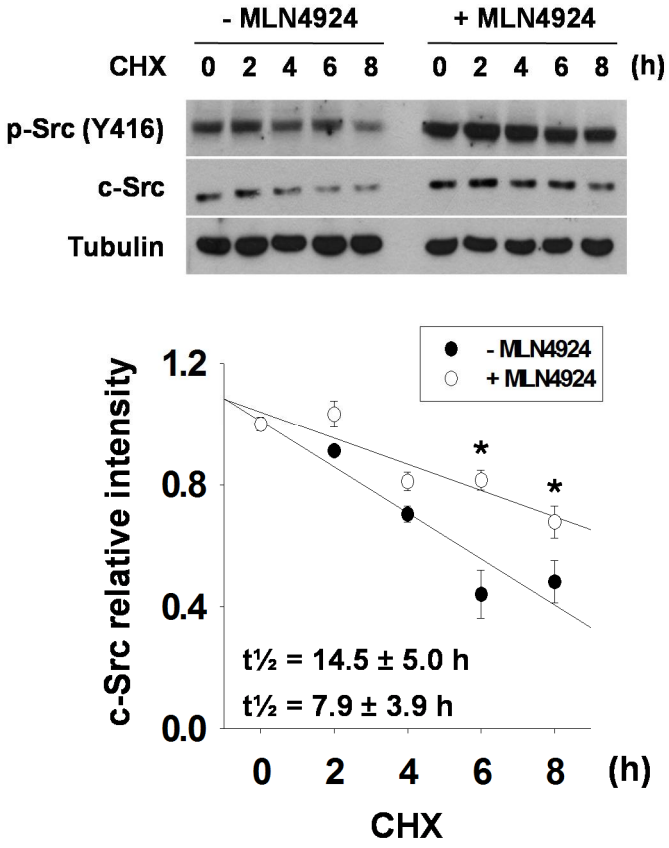

A
MLN4924 0 0.125 0.25 (μM)
p-Src (Y416)
c-Src
Tubulin
H1299
HCT116
PC3
Relative c-Src protein level
MLN4924 0 0.125 0.25 (μM)
B
si-Con + - - - -
si-N8 - I II - -
si-APPBP1 - - - I II
p-Src (Y416)
c-Src
N8
APPBP1
Tubulin

Figure 1. The protein levels of c-Src and phospho-Y416 Src are elevated with the inhibition of neddylation.

(A) H1299, HCT116, PC3 cell lines were incubated in serum-free media for 24 hours, following up with a treatment of MLN4924 for 24 hours in a dose dependent manner. The levels of c-Src and phospho-Y416 Src were analyzed by Western blotting. The band intensities of c-Src proteins (mean ± s.d, n=3) were calculated using ImageJ and plotted by the graph. (B) H1299 cells were transfected with siRNAs targeting NEDD8 or APPBP1. The proteins in total cell lysates were analyzed by immunoblotting using specific antibodies.

A


- MLN4924
+ MLN4924
CHX 0 2 4 6 8 0 2 4 6 8 (h)
p-Src (Y416)
c-Src
Tubulin
c-Src relative intensity
1.2
0.8
0.4
0.0
- MLN4924
+ MLN4924
*
*
t½ = 14.5 ± 5.0 h
t½ = 7.9 ± 3.9 h
0 2 4 6 8 (h)
CHX

B

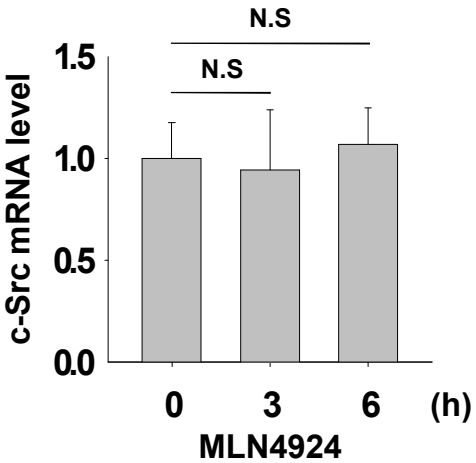
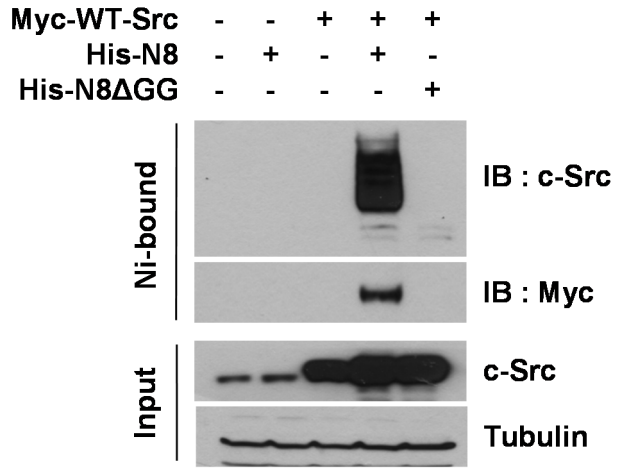

N.S
N.S
c-Src mRNA level
1.5
1.0
0.5
0.0
0 3 6 (h)
MLN4924

Figure 2. Half-life of c-Src is increased with the blockade of neddylation.

(A) H1299 cells were pre-incubated with MLN4924 for 24 hours in serum-free media and then incubated with cycloheximide for the indicated times. Cell lysates were subjected to Western blotting using anti-c-Src or anti-phospho-Y416 Src antibody (upper panel). Band intensities (mean ± s.d , n=3) on the blots were analyzed using ImageJ and plotted (lower panel). (B) H1299 cells were incubated with MLN4924 for the indicated times. Total RNAs were purified and the level of c-Src mRNA was analyzed by RT-qPCR.

A


Myc-WT-Src - - + + +
His-N8 - + - + -
His-N8ΔGG - - - - +
Ni-bound
IB : c-Src
IB : Myc
Input
c-Src
Tubulin

B

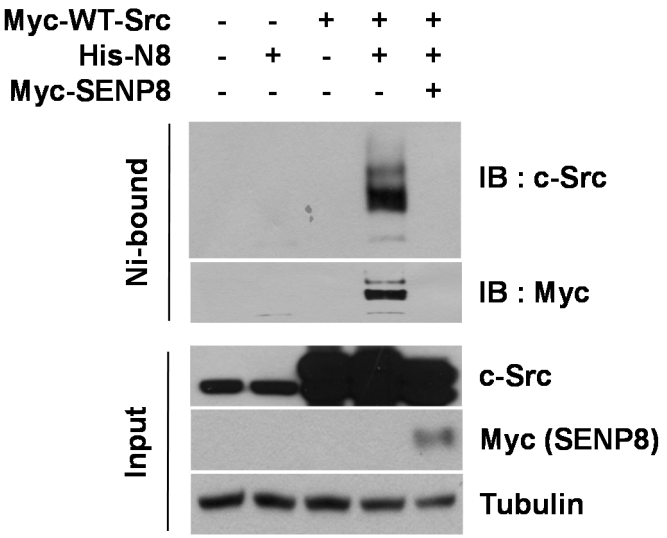
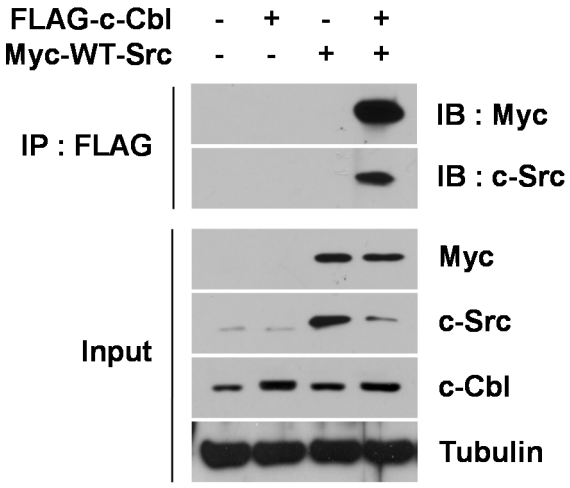

Myc-WT-Src - - + + +
His-N8 - + - + +
Myc-SENP8 - - - - +
Ni-bound
IB : c-Src
IB : Myc
Input
c-Src
Myc (SENP8)
Tubulin

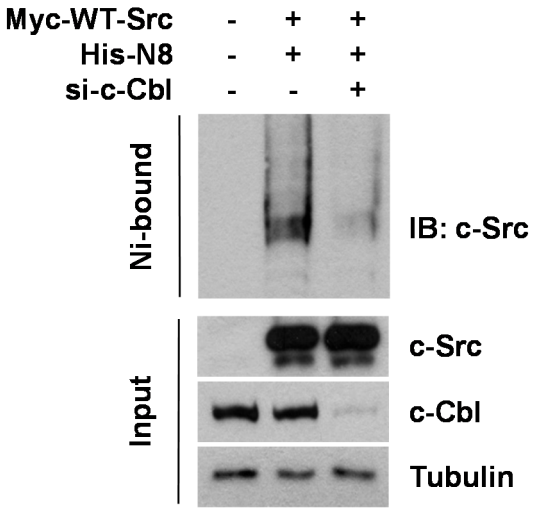
Figure 3. NEDD8-conjugation of c-Src.

(A) Myc-WT-Src plasmid was co-transfected with His-N8 or His-N8ΔGG into HEK293T cells. The protein lysates were purified with Ni^{2+} column and assessed by immunoblotting using specific antibodies. (B) HEK293T cells were transiently transfected with vectors encoding Myc-WT-Src, His-N8 and Myc-SENP8 (a de-neddylating enzyme). Proteins isolated using Ni^{2+} column were analyzed by Western blotting.

A


FLAG-c-Cbl - + - +
Myc-WT-Src - - + +
IP : FLAG
IB : Myc
IB : c-Src
Input
Myc
c-Src
c-Cbl
Tubulin

B


Myc-WT-Src - + +
His-N8 - + +
si-c-Cbl - - +
Ni-bound
IB: c-Src
Input
c-Src
c-Cbl
Tubulin

C

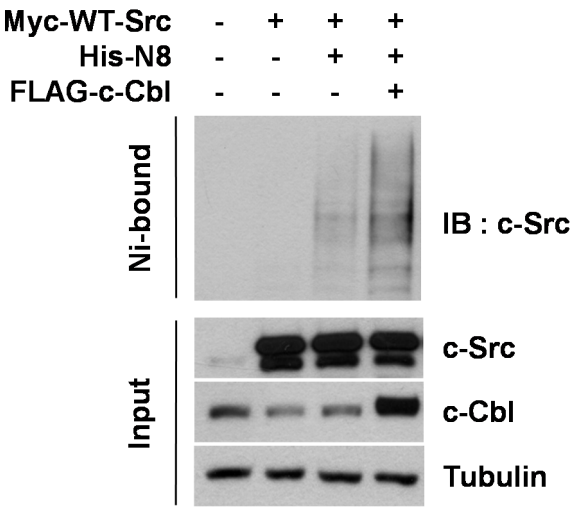
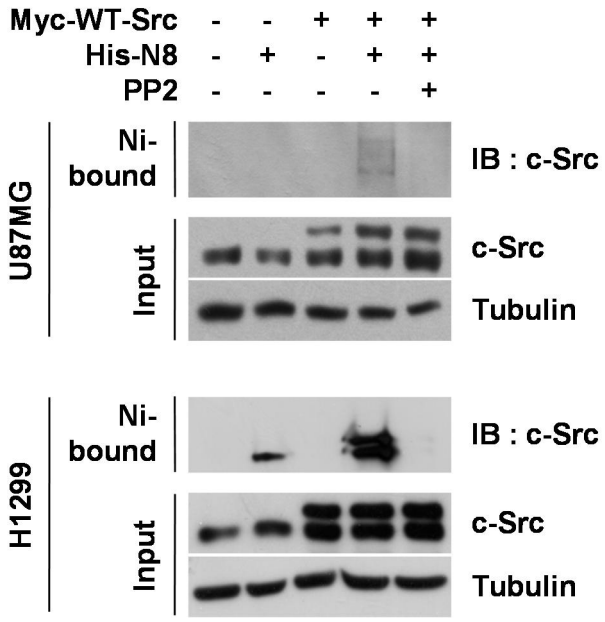


Figure 4. Neddylation of c-Src is regulated by c-Cbl.

(A) H1299 cells expressing FLAG-c-Cbl and Myc-WT-Src were subjected to immunoprecipitation using Anti-FLAG affinity beads. Purified proteins were analyzed by Western blotting using indicated antibodies. (B) H1299 cell was co-transfected with indicated vectors with or without the siRNA targeting c-Cbl. Proteins were filtered by Ni^{2+} column and visualized by immunoblotting. (C) FLAG-c-Cbl was co-transfected with Myc-WT-Src and His-N8 into H1299 cells. Purified lysates by Ni^{2+} column were analyzed by Western blotting.

A


Myc-WT-Src - - + + +
His-N8 - + - + +
PP2 - - - - +
U87MG
Ni-bound
IB : c-Src
Input
c-Src
Tubulin
H1299
Ni-bound
IB : c-Src
Input
c-Src
Tubulin

B

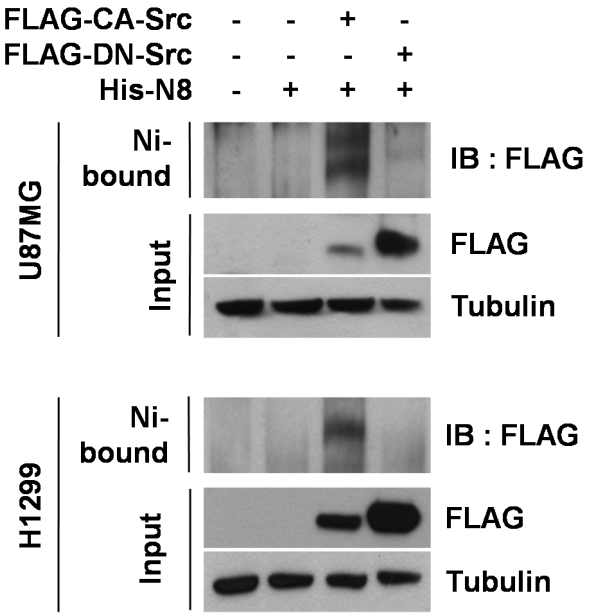
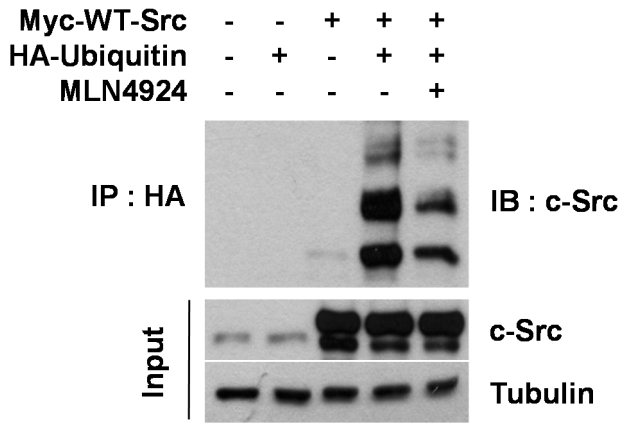

FLAG-CA-Src - - + -
FLAG-DN-Src - - - +
His-N8 - + + +
U87MG
Ni-bound
IB : FLAG
Input
FLAG
Tubulin
H1299
Ni-bound
IB : FLAG
Input
FLAG
Tubulin

Figure 5. Activation of c-Src facilitates its neddylation.

(A) U87MG and H1299 cells were transfected with Myc-WT-Src and His-N8. 48 hours after transfection, 10 μM of PP2 was added to the cells. Cell lysates were purified with Ni^{2+} column. (B) FLAG-tagged constitutive active form of Src or FLAG-tagged dominant negative form of Src was co-transfected with His-N8 into U87MG and H1299 cells. After purified with Ni^{2+} column, lysates were assessed by immunoblotting with anti-FLAG antibody.

A


Myc-WT-Src - - + + +
HA-Ubiquitin - + - + +
MLN4924 - - - - +
IP : HA
IB : c-Src
Input
c-Src
Tubulin

B

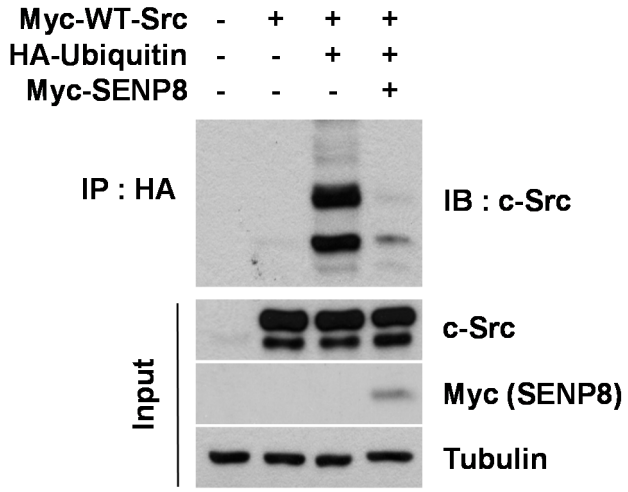
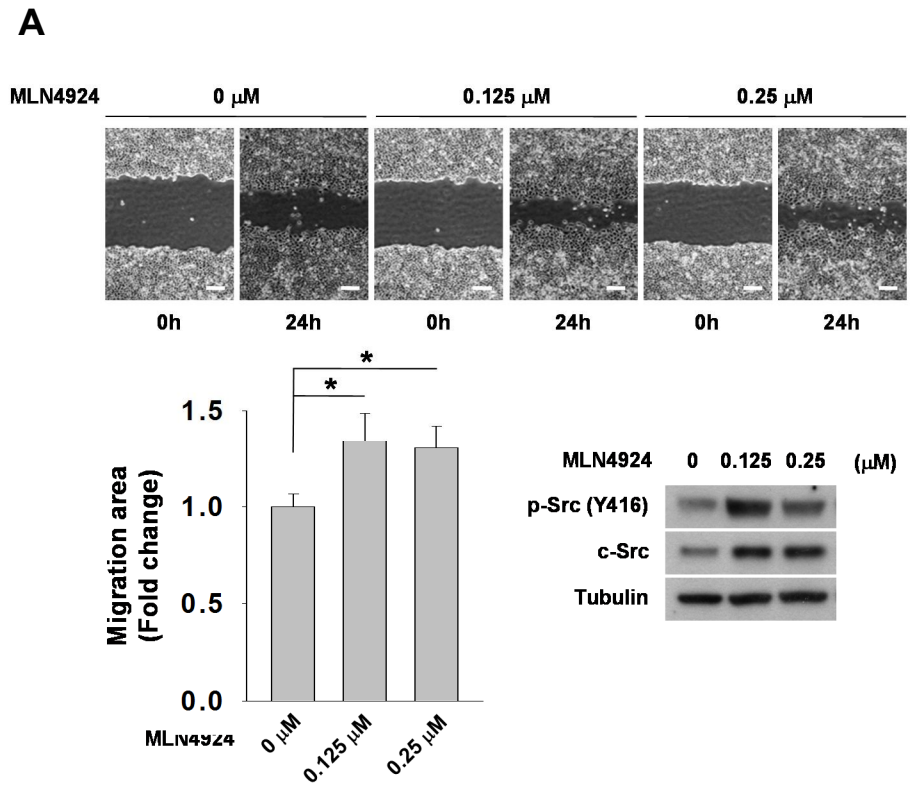

Myc-WT-Src - + + +
HA-Ubiquitin - - + +
Myc-SENP8 - - - +
IP : HA
IB : c-Src
Input
c-Src
Myc (SENP8)
Tubulin

Figure 6. Neddylated c-Src is further ubiquitinated and degraded.

(A) H1299 cells transfected with indicated plasmids were incubated in serum-free media and exposed to MLN4924 for 24 hours. Cell lysates were subjected to immunoprecipitation with HA-affinity beads and assessed to Western blotting. (B) Myc-SENP8 plasmid was co-transfected with Myc-WT-Src and HA-Ubiquitin into H1299 cells. Cell lysates were analyzed by immunoprecipitation and immunoblotting.


A
MLN4924
0 μM
0.125 μM
0.25 μM
0h
24h
0h
24h
0h
24h
*
*
1.5
1.0
0.5
0.0
Migration area
(Fold change)
MLN4924
0 μM
0.125 μM
0.25 μM
MLN4924 0 0.125 0.25 (μM)
p-Src (Y416)
c-Src
Tubulin

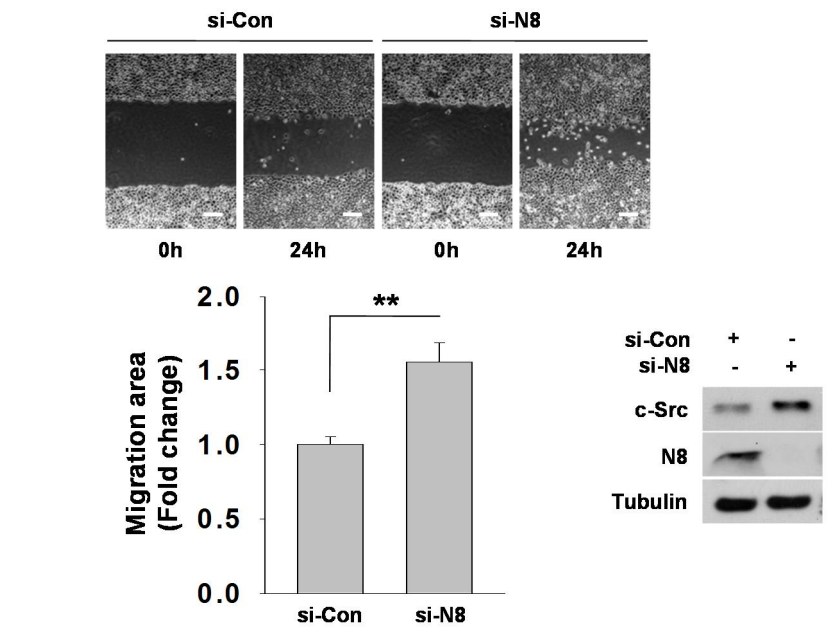
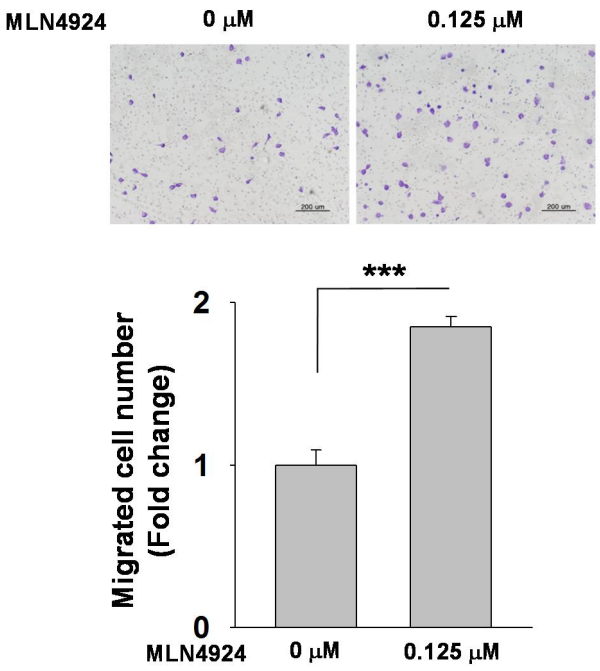

B
si-Con
si-N8
0h
24h
0h
24h
**
2.0
1.5
1.0
0.5
0.0
Migration area
(Fold change)
si-Con
si-N8
si-Con + -
si-N8 - +
c-Src
N8
Tubulin

Figure 7. Blockade of neddylation promotes cancer cell migration.

(A) H1299 cells were pre-incubated with serum-free media for 24 hours and subjected to wound healing assay with or without MLN4924 treatment. (B) H1299 cells expressing si-Control or si-N8 were subjected to wound healing assay. Empty areas in A~B were quantified by using Image J software and plotted. Cells were subjected to immunoblot analysis to verify the expression of c-Src and phospho-Y416 Src proteins.

A


MLN4924
0 μM
0.125 μM
***
Migrated cell number
(Fold change)
2
1
0
MLN4924
0 μM
0.125 μM

B

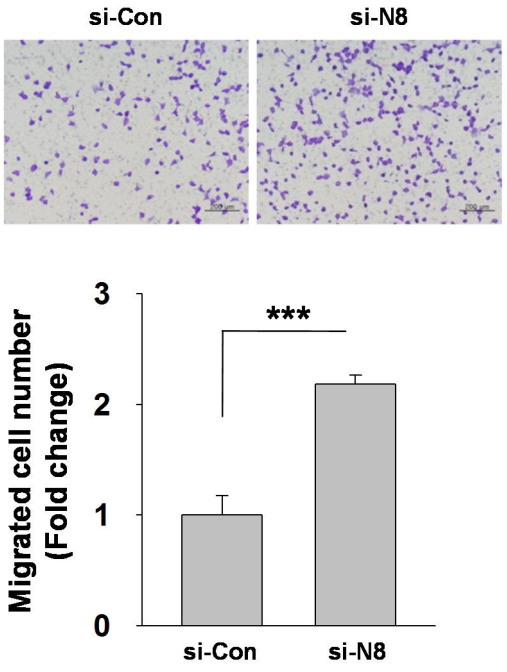
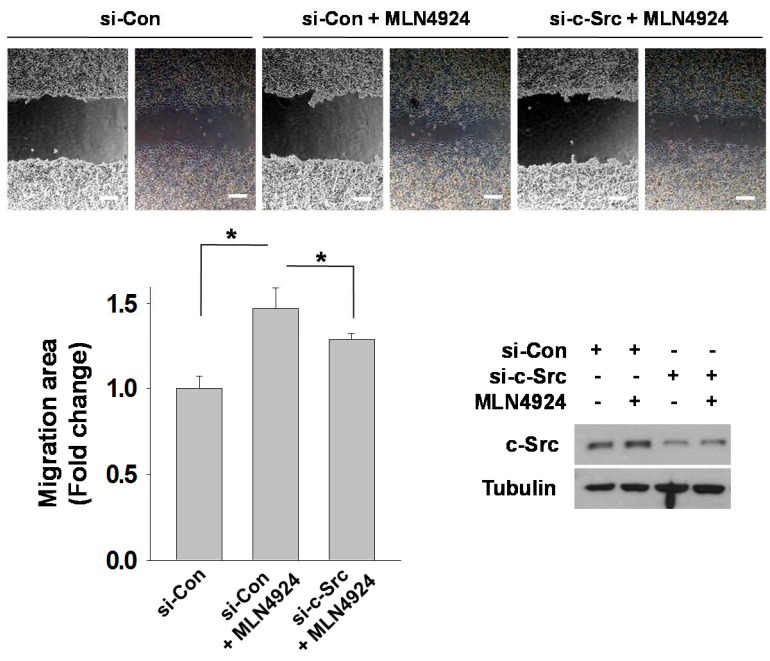

si-Con
si-N8
***
Migrated cell number
(Fold change)
3
2
1
0
si-Con
si-N8

Figure 8. Cell migration is promoted with blockade of NEDD8 process.

(A) H1299 cells were pretreated with DMSO or MLN4924 for 24 hours and subjected to transwell migration assay with or without MLN4924. (B) H1299 cells expressing si-Control or si-N8 were subjected to transwell migration assay. The number of cells in four randomly chosen fields were counted and averaged. Data are the mean ± s.d (n = 3).

A


si-Con
si-Con + MLN4924
si-c-Src + MLN4924
Migration area (Fold change)
1.5
1.0
0.5
0.0
*
*
si-Con
si-Con + MLN4924
si-c-Src + MLN4924
si-Con + - - +
si-c-Src - - + +
MLN4924 - + - +
c-Src
Tubulin

B

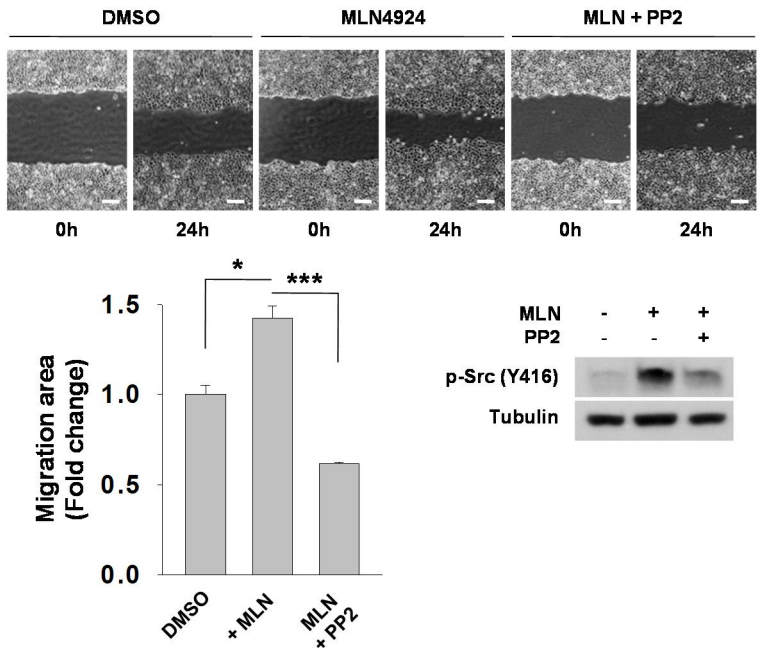
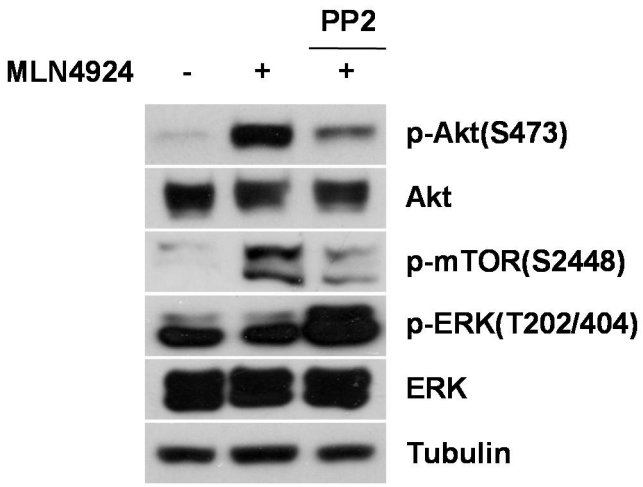

DMSO
MLN4924
MLN + PP2
0h
24h
0h
24h
0h
24h
Migration area (Fold change)
1.5
1.0
0.5
0.0
*
***
DMSO
+ MLN
MLN + PP2
MLN - + +
PP2 - - +
p-Src (Y416)
Tubulin

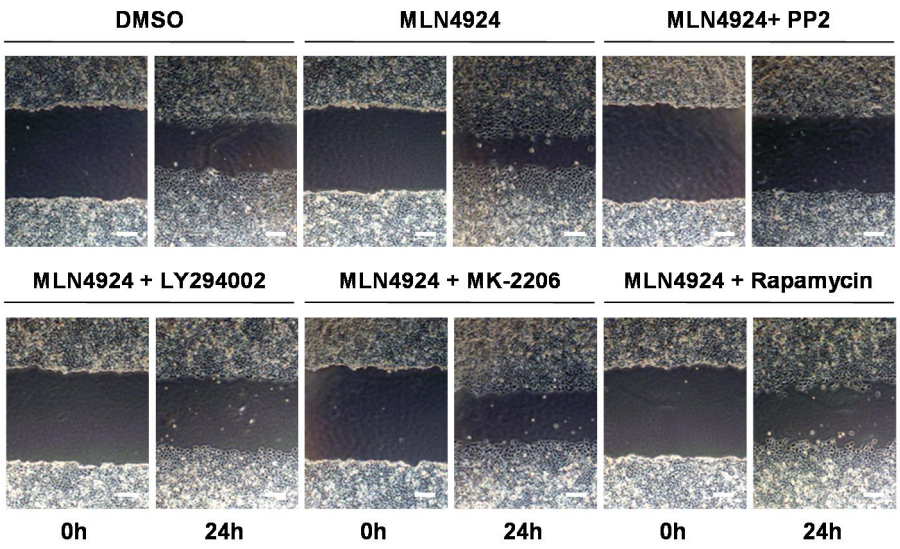
Figure 9. Neddylation-mediated cancer cell migration is regulated via c-Src-dependent way.

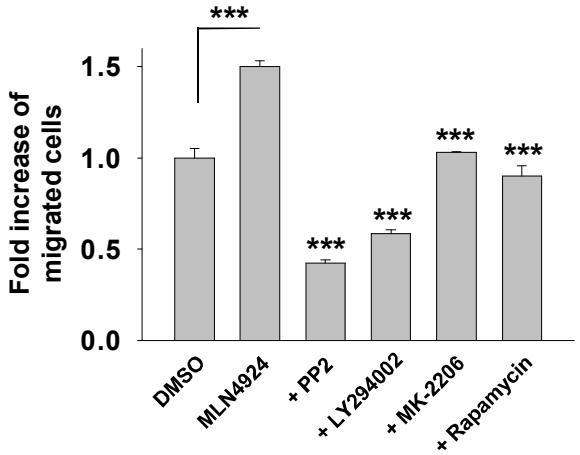
(A) H1299 cells transfected with si-Control or si-c-Src were treated with DMSO or 0.125 μM of MLN4924 and analyzed by wound healing assay. (B) Cells pre-incubated with serum-free media for 24h were subjected to wound healing assay with 0.125 μM of MLN and 10 μM PP2 for indicated times. Empty areas in A~B were quantified by using Image J software and plotted. Cells were subjected to immunoblot analysis to verify the expression of c-Src and phospho-Y416 Src proteins.

A


PP2
MLN4924 - + +
p-Akt(S473)
Akt
p-mTOR(S2448)
p-ERK(T202/404)
ERK
Tubulin

B


DMSO
MLN4924
MLN4924+ PP2
MLN4924 + LY294002
MLN4924 + MK-2206
MLN4924 + Rapamycin
0h
24h
0h
24h
0h
24h


Fold increase of migrated cells
1.5
1.0
0.5
0.0
***
***
***
***
***
DMSO
MLN4924
+ PP2
+ LY294002
+ MK-2206
+ Rapamycin

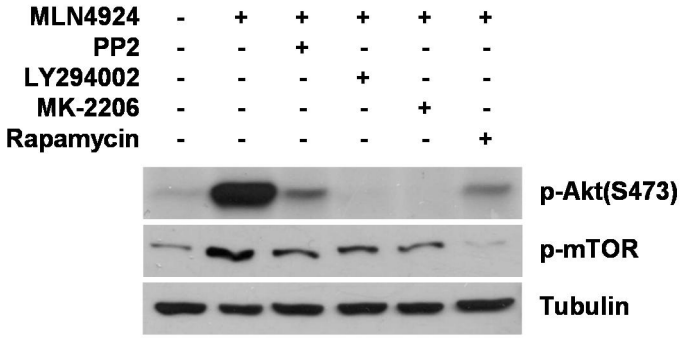

MLN4924 - + + + + +
PP2 - - + - - -
LY294002 - - - + - -
MK-2206 - - - - + -
Rapamycin - - - - - +
p-Akt(S473)
p-mTOR
Tubulin

Figure 10. Inhibition of neddylation promotes cell migration through the c-Src/PI3K/Akt/mTOR pathway.

(A) H1299 cells pre-incubated in serum-free media for 24h were treated with 0.125 μM of MLN4924 and 10 μM of PP2. The cell lysates were subjected to immunoblotting. (B) Cells pre-incubated with serum-free media for 24h were subjected to wound healing assay with the treatment of various inhibitors (MLN4924: 0.125 μM; PP2: 10 μM; LY294002: 50 μM; MK-2206: 1 μM; Rapamycin: 0.1 μM). Empty areas were quantified by using Image J software and plotted. Cells were subjected to immunoblot analysis to verify the expression of the proteins.

DISCUSSION

Although Src is the first confirmed oncogene in 1970s (Martin, 2001), its post-translational modifications have been scarcely identified so far. It is well defined that the activity of c-Src is controlled by its phosphorylation states. While phosphorylation of Y529 makes c-Src 'inactive form', phosphorylation of Y416 makes it 'active form'. Also a phospho-Y416 Src is ubiquitinated and further degraded by 26S proteasome system (Guarino, 2004). Here, for the first time, I discovered that c-Src is regulated by another protein modification, neddylation. I ascertained that c-Src is directly neddylated through an E3 ligase, c-Cbl (Fig. 3, 4). c-Cbl is well-known for its ubiquitin-E3 ligase activity targeting protein tyrosine kinases (PTKs) (Mohapatra et al., 2013). Moreover, it was identified that c-Cbl can also act as a NEDD8-E3 ligase. Zuo et al reported that c-Cbl carries out neddylation of TGF-βIIR and it antagonizes conjugation of ubiquitins (Zuo et al., 2013). On the other hand, the other group found that c-Cbl mediated neddylation of EGFR promotes its ubiquitination (Oved et al., 2006). Interestingly, c-Cbl showed dual roles as neddylation and ubiquitin-E3 ligases of EGFR. Similar with this report, my results suggested that c-Cbl shows a NEDD8-E3 ligase activity for c-Src (Fig 4). Furthermore, Blockade of neddylation efficiently prevented ubiquitination of c-Src. Given that c-Cbl conjugates ubiquitin to c-Src (Yokouchi et al.,

2001), it seems that c-Cbl can play roles as both NEDD8 and ubiquitin-E3 ligases of c-Src.

The activity of c-Cbl is regulated by c-Src and EGFR. c-Src and EGFR phosphorylate Y371 residue of c-Cbl, facilitating its ubiquitin-E3 ligase function (Levkowitz et al., 1999; Yokouchi et al., 2003). EGFR-dependent phosphorylation of c-Cbl positively mediates its NEDD8-E3 ligase activity as well (Oved et al., 2006). According to my results, the activity of c-Src also seems to be important for its neddylation (Fig 5). It is likely that c-Src mediated phosphorylation of c-Cbl facilitates its NEDD-E3 function, which promotes c-Src neddylation. Thus, based on my results, I could formulate a stepwise model for c-Src modification. First, c-Src is activated through phosphorylation of Y416. Next, c-Src phosphorylates c-Cbl, promoting its NEDD8-E3 ligase activity. In turn, the activated c-Cbl conjugates NEDD8 to the c-Src. Then, ubiquitination of c-Src is further facilitated by c-Cbl. Finally, c-Src is destructed by 26S proteasome system. Consistent with my model, inhibition of neddylation effectively increased the stability and activity of c-Src (Fig 1, 2). However, although neddylation of c-Src was elevated with phosphorylation of Y416 residue, it is uncertain that activation of c-Cbl was requisitely involved in this process. The precise mechanism underlying interaction between c-Src and c-Cbl remains to be elucidated.

Neddylation controls stability and activity of c-Src, most likely affecting

the downstream molecules. Under the inhibition of neddylation by MLN4924, not only Y416 of c-Src but also Y845 of EGFR, which is c-Src-dependent phosphorylated residue, was phosphorylated. Also the target kinases of c-Src and EGFR such as ERK and Akt, proteins that regulate proliferation and migration, were activated through the blockade of neddylation (Zhou et al., 2016). My results proposed that the inhibition of c-Src neddylation induces activation of PI3K/Akt/mTOR pathway, which results in promotion of cell migration in lung cancer cell line, H1299 (Fig 10). A selective Src inhibitor, PP2, interrupts activation of Akt and mTOR, suggesting c-Src is an upstream kinase of these molecules. Furthermore, inhibitors of this pathway effectively blocked cell migration induced by MLN4924. It was reported that c-Src mediates cell motility through diverse pathways including PI3K/Akt, Ras/ERK/MAPK, and plenty of other adhesion proteins (Kumar et al., 2003; Guarino, 2004; Zhang et al., 2012; Wu et al., 2016). Among these, Akt mediates cell migration through activation of various transcription factors such as HIF-1α, SNAI1, TWIST (Xue et al., 2012; Filippi et al, 2014; Yang et al., 2016). The accurate mechanisms underlying regulation of cell migration mediated by c-Src neddylation ought to be discovered.

To inhibit conjugation of NEDD8, I used MLN4924, a promising anti-cancer agent. Many studies reported that MLN4924 prevented tumor cell proliferation and migration in various cancers (Wang et al., 2014; Kuo et al.,

2015; Lan et al., 2016, 44, 58). However, latest research detected that the physiological outcomes of MLN4924 were reversed in case of low concentrations. Zhou et al reported that treatment of MLN4924 caused striking effects on tumor sphere formation and wound healing of epithelial cells (Zhou et al., 2016). Also our laboratory presented that blocking of neddylation through MLN4924 elevated cell motility via caveolin-1 dependent way (unpublished data). In line with these results, my data showed that the inhibition of c-Src neddylation by MLN4924 improved c-Src activity, which promotes cell motility (Fig 7). Considering c-Src is one of the main upstream kinases in the cell, it is reasonable to suggest that the blockade of c-Src neddylation can be a crucial mechanism in MLN4924-induced cell migration. More research on this novel medicine is crucial and necessary.

In conclusion, Neddylation of c-Src mediated by c-Cbl promotes further ubiquitination of itself. Also the blockade of neddylation elevates tumor migration through PI3K/Akt/mTOR pathway in a c-Src-dependent manner. These consequences can provide better understanding for the role of c-Src modification and NEDD8 process in cancer physiology.

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국 문 초 록

c-Src은 최초로 발견된 종양 유전자로서 세포의 증식, 부착, 이동성, 혈관 신생 등의 종양 진행을 조절하는 인자이다. Tyrosine 416이 인산화된 c-Src의 안정성은 유비퀴틴화에 의해 조절된다는 것이 잘 알려져 있으나, 다른 번역 이후의 구조변환에 의한 조절은 밝혀지지 않았다.

본 연구에서는 c-Src의 안정성이 네딜화에 의해 조절된다는 것을 처음으로 밝혔다. 더욱이, c-Cbl에 의해 c-Src의 네딜화가 매개됨을 밝힘으로써 c-Cbl이 E3 ligase로서 작용하는 것을 밝혔다. 네딜화 억제제인 MLN4924 또는 si-NEDD8 또는 네딜화 E1효소인 APPBP1의 siRNA로 발현을 억제시킴으로써 네딜화를 차단시켰을 때, c-Src의 안정성이 증가하였으며 그 결과 암 세포의 이동이 증가하였다. 네딜화 억제로 유도된 위 현상들은 PP2로 c-Src의 활성을 억제하거나, c-Src의 siRNA를 처리하여 c-Src의 발현을 억제하였을 때, 네딜화 억제에 의해 증가되었던 세포이동이 다시 감소하였다. 또한 네딜화 억제는 c-Src 의존적으로 Akt/mTOR 신호전달 과정을 활성화시켰으며, 이 신호전달 경로 억제제들과 네딜화 억제제인 MLN4924의 동시 투약은 MLN4924에 의해 증가되었던 세포 이동을 다시 억제하였다.

연구 결과를 종합해 볼 때, c-Src이 c-Cbl에 의해 네딜화되는 새로운 표적 기질이며, c-Src의 네딜화은 단백질의 유비퀴틴화를 유도하여 c-

Src을 단백질 수준에서 파괴를 촉진하는 현상임을 밝혔다. 또한 c-Src의 네딜화에 의해 유도된 단백질의 불안정성은 결과적으로 Akt/mTOR 신호전달 경로를 억제하게 되어 암 세포의 이동을 감소시킨다는 것을 발견하였다. 본 연구결과를 통해 종양의 진행과정에서 c-Src의 네딜화는 암세포의 이동을 억제하여 전이를 막는 새로운 조절기전이 될 가능성을 제시하였다.

주요어: c-Src, c-Cbl, neddylation, ubiquitination, PI3K/Akt/mTOR, 세포 이동

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