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Neddylation promotes protein translocation between the cytoplasm and nucleus

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ABSTRACT

Neddylation is an ubiquitin-like modification of proteins that affects the activity, stability and protein-protein interaction of its substrates. Apart from its role as a promoter for Cullin ring E3 ligase to positively regulate the ubiquitylation process, other functional studies about neddylation are still lacking. In this study, we developed a system to explore the impact of neddylation on changes in the subcellular localization of proteins at the omics level. By applying a method combining subcellular protein extraction and immunoprecipitation-mass spectrometry (IP-MS), 81 proteins with a tendency to shuttle between the cytoplasm and nucleus due to different neddylation levels were obtained. Among the 81 candidates, transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) and growth arrest and DNA damage protein 45a (Gadd45a) were confirmed as novel substrates of Nedd8, and neddylation promotes TAK1 nuclear import as well as Gadd45a nuclear export.

1. Introduction

Ubiquitination is one of the most common posttranslational modifications that affect substrate stability, activity, and subcellular localization [1]. Ubiquitin-like proteins (UBLs) are a group of proteins sharing similar amino acid sequences to ubiquitin, and neural precursor cell expressed developmentally downregulated protein 8 (Nedd8) is the one with the highest similarity to ubiquitin among the UBL family. The process of Nedd8 covalently conjugating to its target proteins is called neddylation, which requires an E1 activating enzyme (NAE1), an E2 conjugating enzyme (APPBP1-UBE2F) and a unique neddylation E3 ligase. The neddylated state of proteins can be reversed by two specific deneddylation enzymes called constitutive photomorphogenesis 9 (COP9) signalosome complex subunit 5 (CSN5/JAB1) and sentrin-specific protease 8 (SENP8/NEDP1), which mediate the deneddyation of Cullins and non-Cullins, respectively [2]. The most well-known substrates for Nedd8 are Cullins, and Cullins serve as scaffolds for Skip–Cullin–F-box (SCF) ubiquitin ligase, promoting the process of ubiquitination [3].

Different proteins are located in their specific subcellular compartments and play various roles in cell biology. However, the localizations of proteins can be changed in different biological and pathological situations [4]. The best known example for translocating protein is nuclear factor-κB (NF-κB), since it translocates from the cytoplasm to the nucleus when the immune pathway is activated by certain stimuli [5]. In addition, a large number of proteins shuttle between the nucleus and the cytoplasm under oxidative stress or apoptosis [6,7]. The unusual subcellular location of a protein may contribute to the onset of diseases [8]. In most instances, cytoplasmic or nuclear localization is related to the nuclear localization sequence (NLS) and the nuclear export sequence (NES). After the NLS/NES is recognized by importin or transportin proteins, which transport cargo through the nuclear pore complex (NPC) [9,10]. Apart from localization sequence, it has been indicated

Abbreviations: Nedd8, neural precursor cell expressed developmentally downregulated protein 8; TAK1, transforming growth factor beta-activated kinase 1; Gadd45a, growth arrest and DNA damage protein 45a; LFQ, label-free quantification.

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that posttranslational modifications of proteins such as ubiquitylation and phosphorylation may also affect their subcellular location [6].

Protein trafficking mediated by ubiquitin has been shown to be quite common [11–13]; crosstalk between ubiquitylation and neddylation has also been noticed in many pathways, but current research on neddylation and protein subcellular localization is still limited. As shown by previous studies, neddylation inhibition may promote p53 and Forkhead box protein O3 (FOXO3a) nuclear export [14,15], and decreased 60S ribosomal protein L11 (RPL11) neddylation contributes to RPL11 nucleoli export [16]; however, whether neddylation affects the subcellular localization of proteins remains unknown.

In this research, we aimed to explore cytoplasm-nucleus translocated proteins in response to neddylation in two cell lines exerting different levels of neddylation, wild-type (WT) and sentrin-specific protease 8 (SENP8/NEDP1) knockout (KO) 293T cells. By applying a system combining subcellular protein extraction with label-free quantification (LFQ) by MS, 77 nuclear export candidates and 4 nuclear import candidates were finally identified. Further immunofluorescence and western blotting confirmed that neddylation of TGF β-activated kinase 1 (TAK1) promotes its translocation from the cytoplasm to the nucleus, while growth arrest and DNA damage protein 45a (Gadd45a) undergoes nuclear export due to neddylation.

2. Material and method

2.1. Antibodies and regents

All antibodies and reagents were purchased as follows: anti-FLAG (F7425, Sigma), anti-Myc (M192-3, MBL), anti-HA (M180-3, MBL), anti-NEDD8 (ab81264, Abcam), anti-NEDP1/SENP8 (SC271498, Santa Cruz), anti-β-tubulin (10094-1-AP, Proteintech), anti-TAK1 (ab10926, Abcam), anti-Lamin B1 (BA1228, Boster), anti-GAPDH (B1034, Biodragon), goat anti-rabbit IgG (111-005-003, Jackson), goat anti-mouse IgG (115-005-003, Jackson), Alexa Fluor 594 conjugated goat anti-rabbit IgG (ZF0516, Zsbio), Alexa Fluor 488 conjugated goat anti-mouse IgG (ZF0512, Zsbio), Protein A/G PLUS-Agarose (sc-2003, Santa), and DAPI (6057, Sigma).

2.2. Plasmid construction

Full-length TAK1 with a Myc tag was cloned from cDNA of 293T cells and inserted into pcDNA3.1(+) using a pair of primers, (forward: 5′-TTAAGCTTGGTACCGAGCTCGGATCCGCCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGATGTCTACAGCCTCTGCCG-3′, reverse:5′-CCACTGTGCTGGATATCTGCAGAATTCTCATGAAGTGCCTTGTCGTTTCT-3′). The FLAG-Nedd8, HA-Nedd8 and HA-Nedd8ΔGG plasmids were gifts from Dr. Ping Xie at Capital Medical University, China. FLAG-Gadd45a, FLAG-proliferating cell nuclear antigen (PCNA) and FLAG-methionine adenosyltransferase 2 subunit beta (MAT2A) were purchased from Sino Biological Company (Beijing).

2.3. Cell culture and transfection

HeLa cells and 293T cells were obtained from American Type Culture Collection (ATCC). The NEDP1−/− cells were gifted by Dr. Ping Xie from Capital Medical University, China. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 unit/ml), and the culture conditions were maintained at 37 °C with 5% CO_2. Cells were transfected with the indicated plasmids using PEI transfection reagent, according to the manufacturer's protocol.

2.4. Cell lysate, modification assay and western blotting

To prepare total cell lysates, modified lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1% SDS, pH 7.4) supplemented with protease inhibitor cocktail was used to solubilize cells. The cell lysate was incubated at 60 °C for 10 min and then diluted 10 times with modified lysis buffer without SDS, as described previously [17].

The subcellular fraction was analyzed using the *Cytoplasm and Nucleus Protein Extraction Kit* (P0028, Beyotime), according to the manufacturer's protocol. For further ubiquitylation or neddylation assays, cytoplasmic and nucleic protein extractions were incubated at 60 °C for 10 min before 0.1% SDS was added.

The lysate was incubated with the indicated antibody for 3 h at 4 °C before Protein A/G PLUS-Agarose was added, and the lysate was rotated overnight at 4 °C. The immunoprecipitants were washed at least three times in 2 × modified lysis buffer without SDS. The beads were boiled in 2 × SDS sample buffer before being resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

2.5. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 15 min and blocked with 0.8% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Slides were incubated with primary antibodies against TAK1, NEDP1 and HA overnight at 4 °C, followed by Alexa Fluor 488-conjugated goat anti-mouse secondary antibody, Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody and DAPI for 1 h. Slices were washed at least 3 times with ice-cold PBS after every step, and images were visualized with a Nikon A1R confocal microscope.

2.6. Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) analysis

The resulting peptides were analyzed on the LC-MS/MS platform of a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an EASY-nLC™ 1200 ultra-performance liquid chromatography (UPLC) system (Thermo Fisher Scientific, San Jose, CA, USA). Peptides were loaded on a 100 μm inner diameter (I.D.) × 2 cm precolumn (C18, 3 μm) and separated on a 75 μm I.D. × 15 cm capillary column (Beijing SpectraPeaks, Beijing, China) packed with 1.9 μm C18 reverse-phase fused-silica (Michrom Bioresources, Inc., Auburn, CA, USA). The LC nonlinear gradient with 78 min ramped from 7% to 95% of mobile phase B (phase B: 0.1% formic acid (FA) in acetonitrile (ACN), phase A: 0.1% FA + 2% ACN in water) at a nanoflow rate of 600 nL/min. The electrospray voltage was set to 2.2 kV and MS1 was detected with a mass range of 300–1400 at a resolution of 120,000 at m/z 400. The automatic gain control (AGC) was set as 3 × 106, and the maximum injection time (MIT) was 80 ms. The MS2 was detected in data-dependent mode for the 20 most intense ions subjected to high-energy collision dissociation (HCD) fragmentation cell with energy was 27% and detected in Orbitrap at a resolution of 15,000. For each scan, the AGC was set at $2 \times 10^{4,}$ and the MIT was set at 19 ms. The dynamic range was set at 15 s to suppress repeated detection of the same ion peaks.

2.7. MS data analysis for protein identification and quantification

All raw files were searched by MaxQuant [18] (version 1.5.3.30) against the Swiss-Prot reviewed human database (version 2016.06). For proteome identification, searching parameters

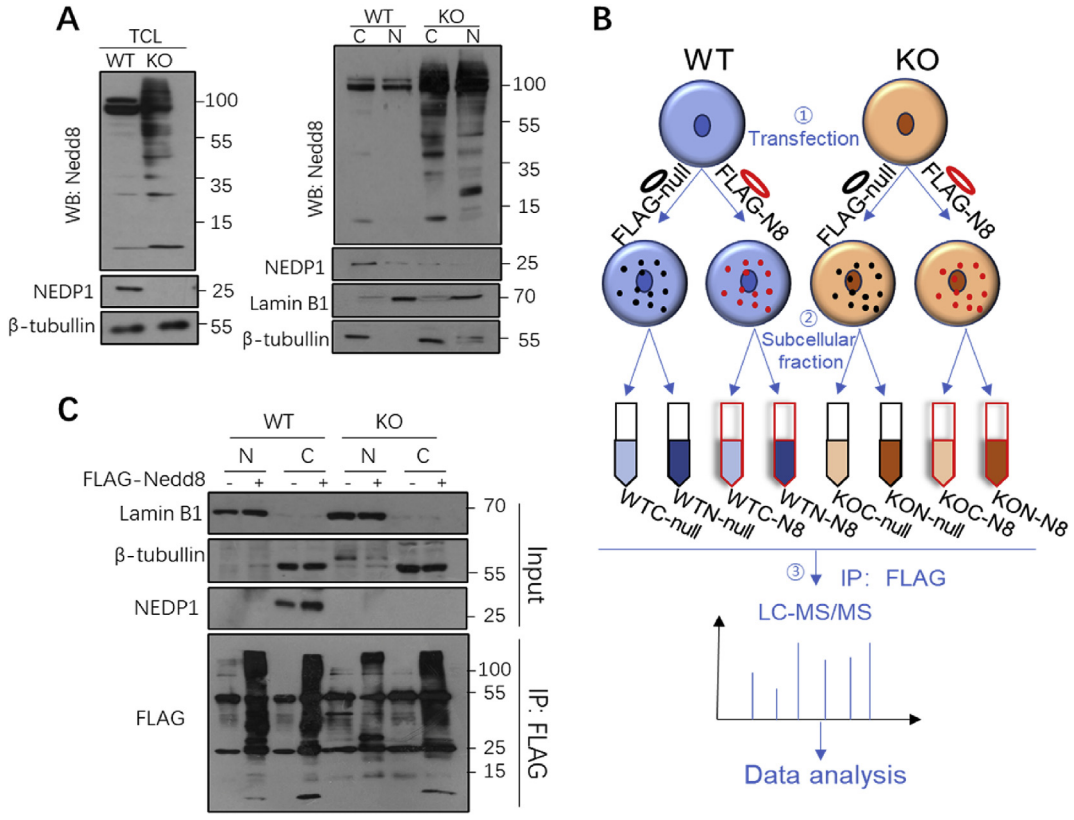

Fig. 1. Introduction and evaluation of the experimental system. (A) Western blot analysis for Nedd8 expression in total cell lysates (TCLs), the cytoplasm and the nucleus of wild-type and NEDP1 KO 293T cells. N, nucleus; C, cytoplasm. (B) Schematic representation of the work flow: ① Transfect FLAG-Nedd8 (treating group) or empty FLAG vector (negative control group) plasmids into WT or NEDP1 KO 293T cell lines; ② Extract the cytoplasm and the nucleus protein by subcellular lysis after 48 h of transfection; ③ The cytoplasm and the nucleus protein extracts were immunopurified with the anti-FLAG antibody and then eluted. The eluates were resolved by SDS-PAGE and Coomassie blue staining. Finally, the protein bands were retrieved and analyzed by LC-MS/MS. (C) The cytoplasmic and nuclear proteins in WT and NEDP1 KO cells before (input) or after immunoprecipitation and elution (IP: FLAG) were analyzed by western blotting with the indicated antibodies. Lamin B1 and β-tubulin were used as specific markers for the nucleus and cytoplasm, respectively.

consisted of full tryptic restriction, and peptides were allowed up to 2 miss cleavages. The precursor mass tolerance was set at 20 ppm for the first search and 4.5 ppm for the main search. The tolerance of MS2 fragments was set at 20 ppm. Peptide matches were filtered by a minimum length of 7 amino acids. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was assigned as a variable modification. For ubiquitination identification, a research strategy was used in which di-Gly-lysine was added in the second search [19,20]. The peptides of the C-terminal lysine-modified residue with localization probability larger than 0.75 were removed from the dataset. The peptides, proteins and site identification were filtered with a false discovery rate (FDR) lower than 1% using a target-decoy search strategy [21,22]. For label-free quantification, intensity-based absolute quantification (iBAQ) in MaxQuant was performed on the identified peptides to quantify protein abundance. At least two unique or razor peptides were required for protein quantification.

2.8. Data analysis

To explore the substrates of Nedd8 that shuttle between the cytoplasm and the nucleus, the data screening strategy was set as follows: (1) fold change between every experimental group (transfection with FLAG-Nedd8) and corresponding control group (transfection with FLAG-null) >1.9; (2) The advantage intensity in the cytoplasm and the nucleus were different between WT and KO cells: (WTN−WTC) × (KON−KOC) < 0, the process of data screening was carried out by Excel. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed by Metascape [23].

3. Results

3.1. Experimental system for subcellular proteomics

NEDP1 acts as a deneddylation enzyme; once it was deleted, the neddylation level in whole cells was expected to increase [24]. It was confirmed by western blotting that more proteins remained modified by Nedd8 in NEDP1 KO cells, not only in total cell lysate but also in the cytoplasmic and nuclear protein extractions (Fig. 1A), indicating that it was reasonable to consider NEDP1 KO cells as a cell model with relatively high neddylation levels compared to WT cells. To explore the relationship between neddylation and protein subcellular location, we developed a system using WT and NEDP1 KO 293T cells. After transfecting these two cell lines with FLAG-tagged Nedd8 plasmid (experimental group) or vector (control group), the subcellular fraction was used to obtain cytoplasmic and nuclear proteins, and further IP-MS with the FLAG antibody was performed to screen the proteins with different distribution patterns in these two cell lines (Fig. 1B). The clearness of the subcellular lysis and the efficacy of the immunoprecipitation were further evaluated by Western blot analysis. As indicated by western

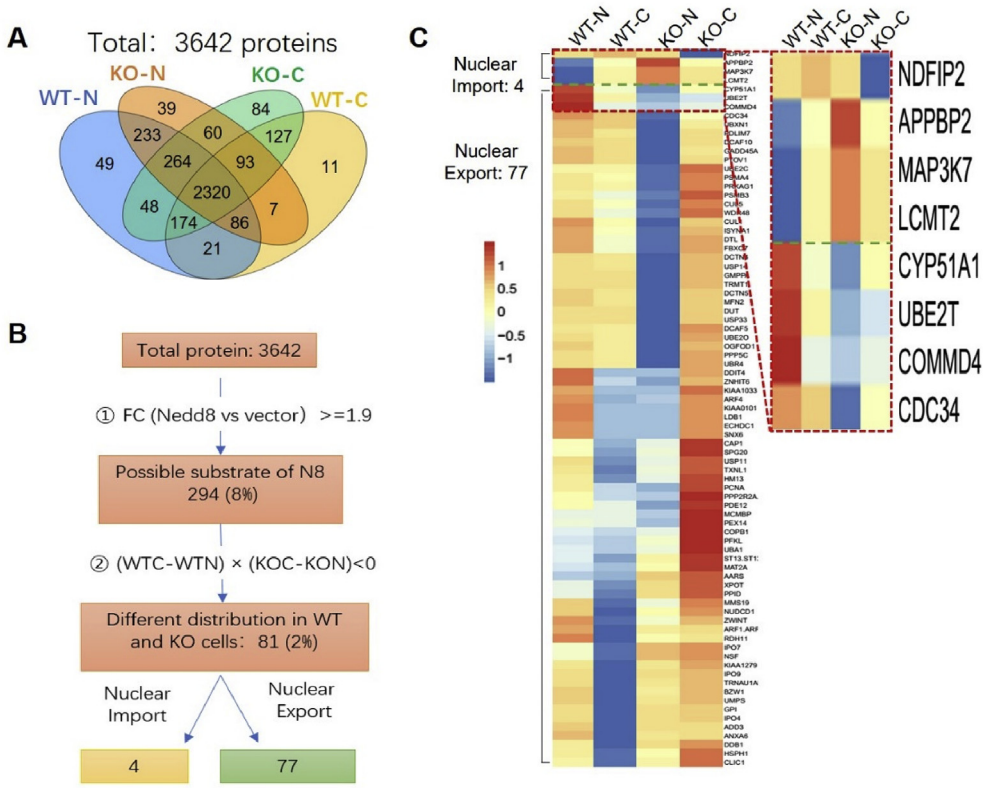

Fig. 2. Screening for proteins that undergo nuclear-cytoplasmic translocation. (A) Venn diagram showing the total 3642 protein distributions in the 8 groups identified by LC-MS/MS. (B) Schematic representation of the data screening strategy to select translocated proteins in response to neddylation from the total 3642 items: ① Set the fold change between FLAG-Nedd8 transfected groups with empty vector groups at 1.9 to select Nedd8 substrates. ② Set (WTN−WTC) × (KON−KOC) < 0 to screen the proteins with different dominant distributions between the cytoplasm and the nucleus between WT and KO cell lines. (C) Heat map clustered the 81 translocated candidates with their LFQ in the cytoplasm and the nucleus of WT and KO cells.

blotting, the components of cytoplasmic and nuclear proteins were highly specific to their subcellular compartments, and Nedd8-related proteins were highly enriched in the experimental groups (Fig. 1C). The immunoprecipitants were further analyzed by LC-MS/MS, and label-free quantification (LFQ) by MaxQuant was further performed. The credibility of subcellular proteomics was confirmed by subcellular LFQ data analysis (Supplementary Figs. 1 and 2).

3.2. Nedd8 overexpression causes protein translocation between the cytoplasm and the nucleus

A total of 3642 proteins were obtained from all samples by LC-MS (Fig. 2A). To further explore the nuclear-cytoplasmic shuttling proteins due to neddylation, we developed a data screening strategy to select proteins with both potentials to be Nedd8 substrates and opposite subcellular locations between WT and NEDP1 KO 293T cells (Fig. 2B). By setting the threshold of LFQ fold change between the relative experimental group and control groups at 1.9, 294 entries were obtained as potential Nedd8 substrates. GO and KEGG analyses of these proteins showed that these proteins were most related to the ubiquitin-proteasome system (Supplementary Fig. 3), which was consistent with a previous study about Nedd8. Finally, 81 proteins were identified as translocated proteins since they showed different locations in the cytoplasm or the nucleus between WT and NEDP1 KO 293T cells. Among them, 4 presented nuclear import potential, and another 77 tended to export from the nucleus due to NEDP1 KO (Fig. 2C).

3.3. Neddylation of TAK1 promotes its translocation from the cytoplasm to the nucleus

Mitogen-activated protein kinase kinase 7 (MAP3K7) was one of the nuclear import candidates, and it showed a dominant enrichment in the cytoplasm in WT cells, but it was mainly distributed in the nucleus in KO cells (Supplementary Fig. 3). MAP3K7 is also known as TAK1, and it plays an important role in both the MAPK pathway and NF-κB pathway [25–27]. Numerous studies have reported the necessity of the ubiquitin chain to TAK1 activity and stability [28,29]; although the cross talk between ubiquitylation and neddylation is quite a common phenomenon in many pathways [17,30–34], the relationship between Nedd8 and TAK1 has not been studied before, and TAK1 was previously accepted as a protein localized mainly in the cytoplasm [35]. The results of the modification assay showed that Nedd8 as well as ubiquitin could modify TAK1, but the Nedd8 mutant with the last two Glycines deleted (Myc-Nedd8ΔGG) lost the ability to modify TAK1 (Fig. 3A), supporting our hypothesis that TAK1 is a substrate of Nedd8.

To further investigate the effect of TAK1 neddylation on its localization within cells, HA-Nedd8 and Myc-TAK1 were coexpressed in WT and NEDP1 KO 293T cells, and Western blot analysis indicated that the overexpression of HA-Nedd8 rather than HA-Nedd8ΔGG obviously promoted nuclear enrichment of TAK1 in WT and KO 293T cells (Fig. 3B). Immunofluorescence assays validated this conclusion in both 293T cells and HeLa cells, showing that cytoplasmic TAK1 changed its location from the cytoplasm to the nucleus after Nedd8 transfection (Fig. 3C). Moreover, the enrichment of nuclear TAK1 had a relationship with the amount of Nedd8 expression. Coexpression of different amounts of Nedd8 with TAK1

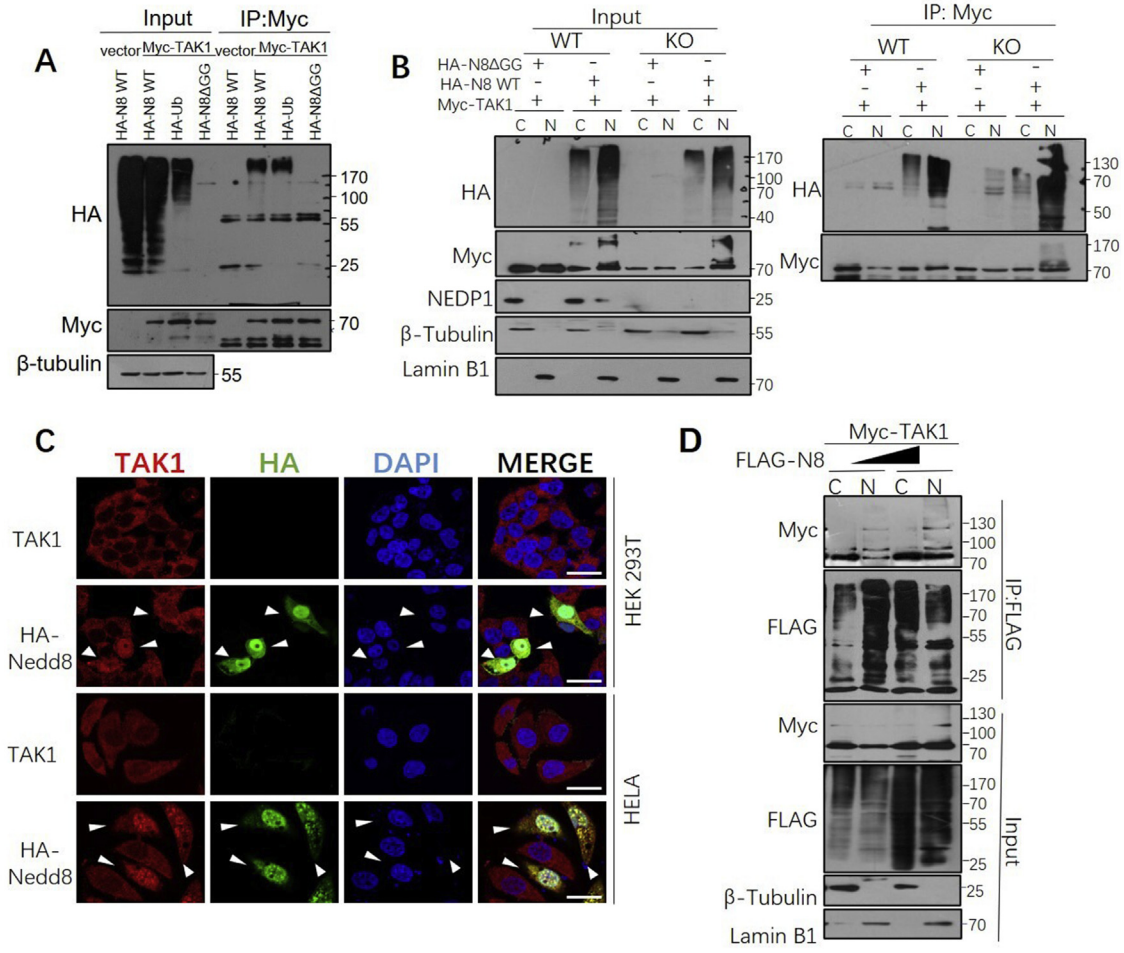

Fig. 3. Neddylation of TAK1 promotes its translocation from the cytoplasm to the nucleus. (A) Western blotting shows in vitro neddylation and ubiquitylation of TAK1 by coexpressing Myc-TAK1 and HA-Nedd8/HA-Ub/HA-Nedd8ΔGG in 293T cells. (B) Neddylation assay of cytoplasmic and nuclear TAK1 in WT and KO 293T cells followed by subcellular lysis. (C) Indirect immunofluorescence showing TAK1 location alone or after HA-Nedd8 transfection in 293T and HeLa cells. The white arrowheads indicate cells transfected with HA-Nedd8. Scale bar: 20 mm. (D) Western blotting shows TAK1 expression in the cytoplasm and nucleus after coexpression of different amounts of Nedd8 in wild-type 293T cells.

followed by subcellular lysis and Western blot analysis showed that more nuclear TAK1 accumulated when more Nedd8 was transfected, and migrated bands corresponding to TAK1 with greater molecular mass appeared in the nucleus as the Nedd8 expression increased (Fig. 3D). Taken together, TAK1 can be neddylated, and neddylation promotes TAK1 translocation from the cytoplasm to the nucleus in a dose-dependent manner.

3.4. Gadd45a neddylation promotes its export from the nucleus to the cytoplasm

To verify the MS results of nuclear export candidates, we first selected three of the 77 candidates for further exploration: proliferating cell nuclear antigen (PCNA), Gadd45a and MAT2A. As previous studies have shown, these three proteins are located in the nucleus, and in our MS data, they have a relatively high content in the cytoplasm of WT cells and nucleus of KO cells. The western blotting results showed that both PCNA and Gadd45a can be neddylated, while MAT2A cannot (Fig. 4A). PCNA has been well studied for its key role in the DNA damage response (DDR) [36], and ubiquitylation and neddylation of PCNA have also been reported previously [37]. We decided to focus on Gadd45a since no previous studies have shown that Gadd45a is a substrate of Nedd8. Further modification assays using ubiquitin and NEDD8ΔGG as controls proved that Gadd45a can be modified by Nedd8 rather than by ubiquitin (Fig. 4B). To observe the effect of neddylation on the subcellular location of Gadd45a, we transfected FLAG-Gadd45a alone or with Nedd8 into 293T cells. The immunofluorescence results showed that FLAG-Gadd45a was in the nucleus alone, while coexpression with HA-Nedd8 and Gadd45a showed obvious colocalization with HA-Nedd8 in the cytoplasm (Fig. 4C), indicating that nuclear Gadd45a changes its location from the nucleus to the cytoplasm due to neddylation. The results of the modification assay followed by subcellular lysis validated the immunofluorescence results, as more cytoplasmic Gadd45a appeared in the cotransfection group than in the control group, and the relative contents of cytoplasmic Nedd8 coprecipitated by Gadd45a in the IP samples were higher than that of the whole Nedd8 in the input samples (Fig. 4D). These results together confirmed that Gadd45a is a substrate of Nedd8, and the neddylated Gadd45a may undergo translocation from the nucleus to the cytoplasm.

4. Discussion and conclusion

Proteostasis is the balanced state of proteins, and once proteostasis is out of control, it may lead to aging and other pathologies [38]. To achieve proteostasis, cooperation among protein synthesis, degradation and transport is needed. Synthesis ensures the protein

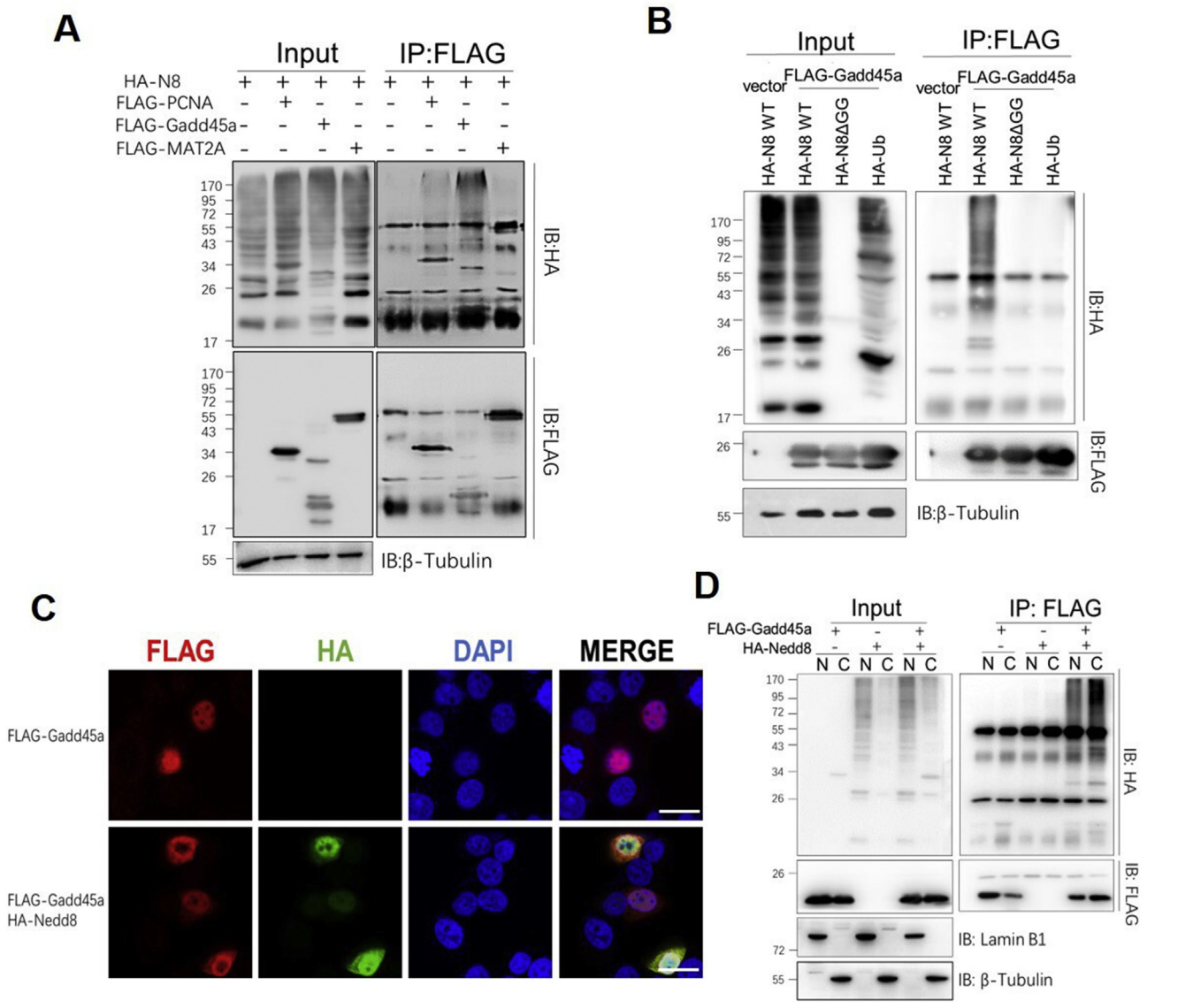

Fig. 4. Gadd45a neddylation promotes its export from the nucleus to the cytoplasm. (A) Neddylation assay of three nuclear export candidates, PCNA, Gadd45a and MAT2A, by coexpressing FLAG-PCNA/FLAG-Gadd45a/FLAG-MAT2A with HA-Nedd8 in 293T cells. (B) Western blotting shows the modification assay of Gadd45a by coexpressing FLAG-Gadd45a and HA-Nedd8/HA-Ub/HA-Nedd8ΔGG in 293T cells. (C) Indirect immunofluorescence showing the subcellular location of FLAG-Gadd45a alone or with HA-Nedd8 cotransfection in 293T cells. Scale bar: 20 mm. (D) Neddylation assay of cytoplasmic and nuclear Gadd45a in 293T cells followed by subcellular lysis.

supply; degradation controls the quality and quantity of the proteins; and transport enables the supplement and removal of proteins. As ubiquitylation is mainly involved in proteasome-related protein degradation [39], this study revealed a novel role of Nedd8 in proteostasis to regulate the protein subcellular location.

By applying a method of subcellular lysis combined with IP-MS technology, this study explored the role of a ubiquitin-like protein, Nedd8, in the regulation of its protein subcellular location at the proteomics level for the first time. Eighty-one proteins were identified as translocated candidates in response to Nedd8 overexpression, among which TAK1 and Gadd45a were proven to be novel substrates of Nedd8. Neddylation of TAK1 promotes its relocation from the cytoplasm to the nucleus, while Gadd45a shows an opposite translocation from the nucleus to the cytoplasm due to neddylation. However, the mechanism of neddylation-mediated protein translocation and the biological functions of these translocated proteins are worthy of further exploration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.07.012.

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