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A lysine-to-arginine mutation on NEDD8 markedly reduces the activity of cullin RING E3 ligase through the impairment of neddylation cascades

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ABSTRACT

Neural-precursor-cell-expressed developmentally down-regulated 8 (NEDD8) is a ubiquitin-like modifier, which forms covalent conjugates on lysines of its substrates. This post-translational modification, neddylation, plays important roles in tumor cell proliferation and viability. Ubiquitin can form diverse polyubiquitin chains, on its seven lysines, which play important functions in various biological processes. However, the roles of lysines in NEDD8 have not been explored. Here, we generated nine NEDD8 point mutants, each with one lysine replaced by an arginine, to study the putative function of lysines in NEDD8. Our experiments discover that Lys27 in NEDD8 is a critical residue for protein neddylation. Replacement of this residue with arginine almost completely eliminates the conjugation of NEDD8 to its substrates. Furthermore, we find that the K27R mutant impairs NEDD8 conjugation to the E2 enzyme, which normally forms thioester bonds for further transferring NEDD8 to its ligases and substrates. Therefore, this mutation completely inhibits global protein neddylation, including neddylation of cullin family proteins, resulting in decreased activity of cullin-RING E3 ligases. This work sheds new light on the roles of NEDD8 lysines on neddylation cascades and provides a dominant negative mutant for the study of neddylation and its biological functions.

1. Introduction

NEDD8 is one of the earliest identified ubiquitin-like modifiers, which plays important roles in regulating multiple biological processes, such as cell proliferation, cell viability, and apoptosis [1]. NEDD8 modification on lysines of its substrates (neddylation) occurs in a series of enzymatic reactions similar to protein ubiquitination, involving in one NEDD8-activating enzyme complex (NAE1-Uba3 heterodimer) [2], two E2 conjugating enzymes, UBE2F and UBE2M, and multiple E3 ligases [3–6]. The most extensively studied NEDD8 modified proteins are cullin family proteins [7] and their neddylation enhances the activity of cullin-RING E3 ligases (CRLs) [8,9], thus subsequently promoting the ubiquitination of their downstream targets. Other identified NEDD8 substrates or NEDD8-associated proteins have functions in transcription, DNA repair and replication, cell cycle, and chromatin structure regulation [10]. These results suggested that protein neddylation may have diverse biological functions.

Ubiquitin can form a variety of polyubiquitin chain linkages through its seven lysines on its surface [11]. These different chain linkages have distinct functions in regulating biological processes [12–15]. In general, the K48-linked polyubiquitin chain is a signal for proteasomal degradation of ubiquitinated substrates [12] whereas the K63-linked polyubiquitin chain has functions in kinase activation [14] and substrate internalization [16]. It has also been reported that K63-linked polyubiquitin chain is required for the degradation of EGF receptor [17] and plays key roles in regulating autophagy [18,19]. The unconventional K11 linkage-specific polyubiquitin chain is involved in endoplasmic reticulum-associated degradation of the modified substrates [15].

NEDD8 has high sequence similarity with ubiquitin and five of its nine lysines are conserved in ubiquitin. However, it has not been systematically studied whether these lysines have distinct roles in neddylation and whether they can form polyneddylation chains. A proteomic study of NEDD8-modified and associated proteins

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detected several peptides which contain the diglycine remnant putatively from the conjugated NEDD8 [10]. Four peptides are derived from NEDD8, suggesting the formation of potential chain linkages in NEDD8 [10]. An *in vitro* mechanistic study on the formation of polyneddylation chains on cullin-1 suggested that the formation of chain linkages may be a general phenomenon for ubiquitin-like modifiers [20].

Here, we examine whether lysine residues on NEDD8 play distinct roles in protein neddylation and the subsequent activation of CRL E3 ligases. We mutate the lysine residues in NEDD8 to arginine individually to generate nine NEDD8 single point mutants, which are used to study the effect of the lysine mutation on neddylation of whole cell lysate or specific NEDD8 substrates. Our experiments identify a critical lysine in NEDD8 and replacement of this lysine with arginine significantly impairs the neddylation enzymatic cascade. Detailed studies show that the conjugation is hindered due to its inability to form the thioester bond with the NEDD8 conjugating enzyme. Subsequently we found that this mutant can significantly affect the activation of CRL E3 ligases and thus the ubiquitination of their downstream targets.

2. Materials and methods

2.1. Materials

Human embryo kidney (HEK) 293T cells were obtained from American Type Culture Collection. High glucose Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Hyclone and Gibco, respectively. MG132 was from Santa Cruz Biotechnology and protease inhibitor cocktail tablet from Roche. Immobilon PVDF membrane and Western chemiluminescent HRP substrate were from Millipore. The antibodies used in this work were from the following companies: NEDD8 from Cell Signaling Technology, ubiquitin from Santa Cruz Biotechnology, FLAG M2 from Sigma, HA, Myc, GAPDH, and β-actin from HuaAn Biotechnology, and secondary antibodies from Beyotime Biotechnology.

2.2. Plasmids

The cDNA library was obtained from HEK293T cells according to a method described previously [21]. The wild-type (wt) NEDD8, IκBα, Uba3, NAE1, UBE2M, and cullin-1 (Cul1) were amplified by polymerase chain reaction. A hemagglutinin (HA) tag and Myc tag were added to the N-terminus of NEDD8 and NAE1, respectively. Strep-FLAG tag was introduced to the N-terminus of IκBα, Uba3, and UBE2M. They were then cloned to the pcDNA3.1 vector. The NEDD8 point mutation was carried out using the Easy Mutagenesis System kit (Transgen). The original cullin-4A (Cul4A) plasmid was kindly provided by Dr. Pengbo Zhou at Weill Cornell Medical College and subcloned to a pcDNA3.1 vector with the addition of FLAG-tag at their N-termini.

2.3. Transfection and cell lysate preparation

HEK293T cells were transfected with the appropriate amount of plasmids using polyethylenimine (Sigma) when cells were at ~70% confluent on the day after seeding. Forty-eight hours after transfection, cells were treated with DMSO or MG132 for 12 h and washed with ice-cold PBS, lysed on ice for 15 min in the modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 10% glycerol) with freshly added protease inhibitor cocktail. Cell lysates were centrifuged at 4 °C for 10 min and the supernatant was used for the subsequent experiments.

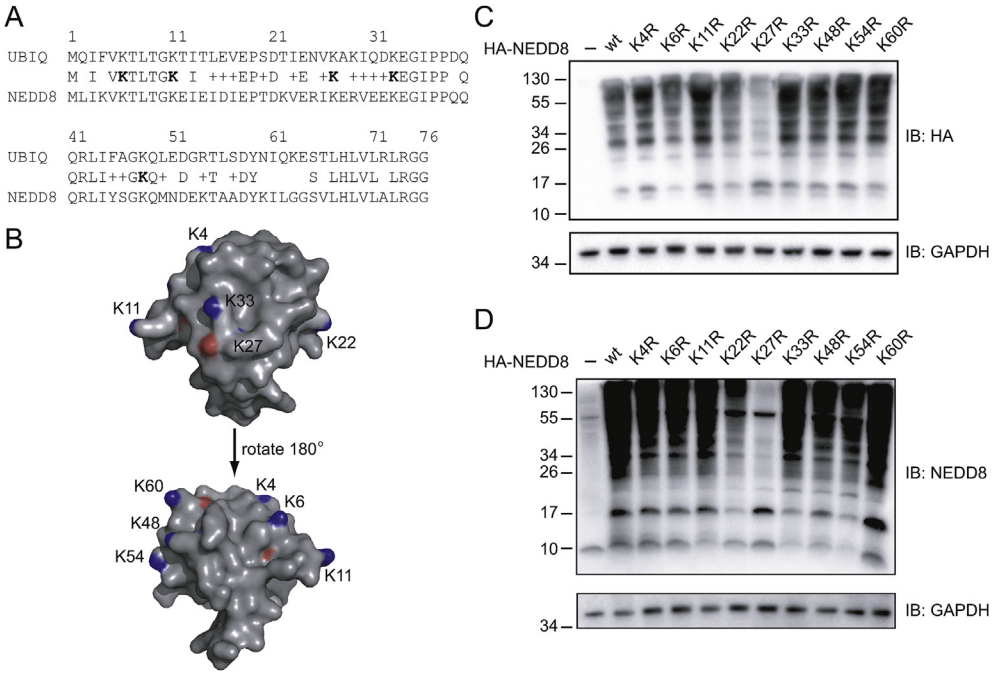

Fig. 1. The K27R NEDD8 mutant abolishes neddylation in cells. (A) Sequence alignment of human NEDD8 and ubiquitin (UBIQ). The conserved lysines are in the bold face. (B) The surface exposure of NEDD8. NEDD8 (PDB code: 1NDD) crystal structure was plotted with PyMol and the surface exposure of lysines was indicated by blue (nitrogen) or red (oxygen) color. The residue number for lysines was labeled. (C) The K27R mutant almost completely abolishes NEDD8 conjugation in cells. Wild-type (wt) and NEDD8 mutants were transiently transfected to HEK293T cells and lysates were blotted with an anti-HA antibody. GAPDH was used as a loading control. (D) The same set of samples in (C) were blotted with an anti-NEDD8 antibody on a different PVDF membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)