Biochemical and Biophysical Research Communications 461 (2015) 653–658

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Yiyan Sui [1], Yaobin Liu [1], Guoqiang Xu [*]

Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases and College of Pharmaceutical Sciences, Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Soochow University, Suzhou, Jiangsu 215123, China

ARTICLE INFO

Article history:
Received 9 April 2015
Available online 24 April 2015

Keywords:
NEDD8
Neddylation
Cullins
Cullin-RING E3 ligases
Ubiquitination

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

* Corresponding author. Fax: +86 512 65882370.
E-mail address: gux2002@suda.edu.cn (G. Xu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.04.085
0006-291X/

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.

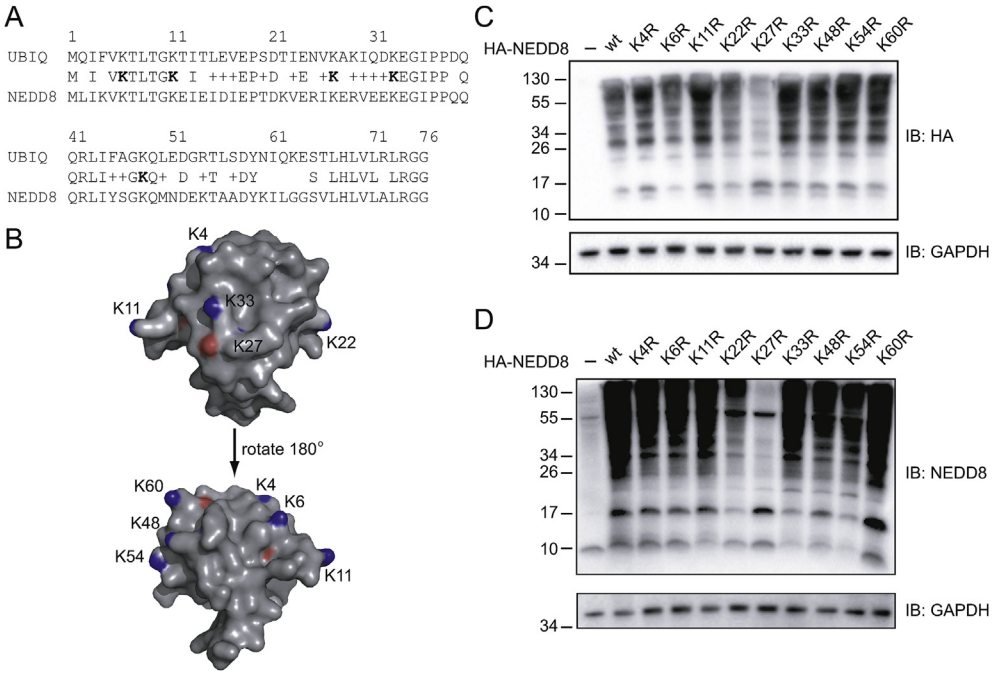

Fig. 1. The K27R NEDD8 mutant abolishes neddylation in cells. (A) Sequence alignment of human NEDD8 and ubiquitin (UBIQ). The conserved lysines are in the bold face. (B) The surface exposure of NEDD8. NEDD8 (PDB code: 1NDD) crystal structure was plotted with PyMol and the surface exposure of lysines 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.)

2.4. Affinity purification of proteins

Strep-FLAG-IκBα and Strep-FLAG-UBE2M were purified with Strep-Tactin Superflow agarose (IBA) according to a method described previously [21]. Briefly, cell lysate was incubated with beads for 4 h at 4 °C and washed with the modified RIPA buffer for four times and eluted with 5 mM desthiobiotin in RIPA buffer twice. FLAG-Cul1 and FLAG-Cul4A were purified with the pre-conjugated FLAG M2 antibody. Cell lysate was incubated with 20 μL of 50% antibody beads for 4 h and washed with the modified RIPA buffer for four times. Proteins were eluted with FLAG peptides (DYKDDDDK) twice. The eluate was combined for immunoblotting analysis.

2.5. Immunoblotting analysis

Samples were prepared according to the method described previously [21] except that in some cases the reducing agent, β-mercaptoethanol, was not added in the Laemmli sample buffer. Proteins were separated in 8% SDS-PAGE or in low-molecular-weight protein SDS-PAGE and transferred to PVDF membrane. The indicated primary and secondary antibodies were used for immunoblotting. The signal was visualized by chemiluminescent HRP substrate.

3. Results

3.1. K27R NEDD8 mutant almost completely abolishes protein neddylation

To explore the potential roles of lysines in NEDD8 on neddylation, we first aligned the sequence of mature human NEDD8 and ubiquitin using BLAST (Fig. 1A). These two proteins have about 44% sequence identity. Five lysines in NEDD8 are conserved in ubiquitin (indicated by the bold text in Fig. 1A). In a previous proteomic study, four lysines, K11, K22, K48, and K60, were detected to form potential NEDD8 chain linkages [10]. However, the conserved lysine K27 in NEDD8 has not been identified to form chain linkages. We further examined the location of these lysines in the NEDD8 crystal structure and depicted the solvent accessible surface of lysine residues (Fig. 1B). Eight lysines have different degree of solvent accessibility on the NEDD8 surface. However, the K27 residue is almost completely buried in the crystal structure. Five lysines, K11, K22, K48, K54, and K60, are in the loop region, and two lysines, K4 and K6, are in the β sheet (Fig. SI-1). The K33 residue is located at the end of an α helix and K27 is in the middle of the α helix. The fact that lysines in NEDD8 have very different solvent accessibility and are located at diverse secondary structures suggests that these lysines may have different roles in neddylation.

To examine this possibility, we mutated lysine to arginine individually on NEDD8 and obtained nine mutants (Fig. SI-2) which were cloned into a mammalian expression plasmid with an HA tag at the N-terminus. We expressed the wt and mutant NEDD8 in HEK293T cells and blotted whole cell lysates with anti-HA and anti-NEDD8 antibodies (Fig. 1C, D). The experiments showed that under a similar expression level, the wt and eight mutants produced approximately similar neddylation level. However, expression of the K27R NEDD8 mutant resulted in a near complete loss of neddylation although the unconjugated mutant has a slightly higher expression level than the wt and other mutants (Fig. 1C, D). This is consistent with its inability in conjugating on the substrates, leaving the expressed mutant in the unconjugated form. The K22R mutant also showed significant loss of neddylation although this reduction is much weaker than that for the K27R mutant. These experiments demonstrated that Lys27 on NEDD8 has distinct roles from other lysines in neddylation.

3.2. K27R NEDD8 mutant impairs NEDD8 conjugation

The next question we want to ask is which step the K27R NEDD8 mutant may affect in the neddylation cascades. To answer this, we first cotransfected two tagged subunits of the NEDD8 activating enzyme complex (Strep-FLAG-Uba3 and Myc-NAE1) with the wt, K27R, or K60R NEDD8 mutants to HEK293T cells. Cells were lysed after 48 h and whole cell lysates were blotted with anti-FLAG and anti-β-actin antibodies (Fig. 2A) in the absence or presence of the

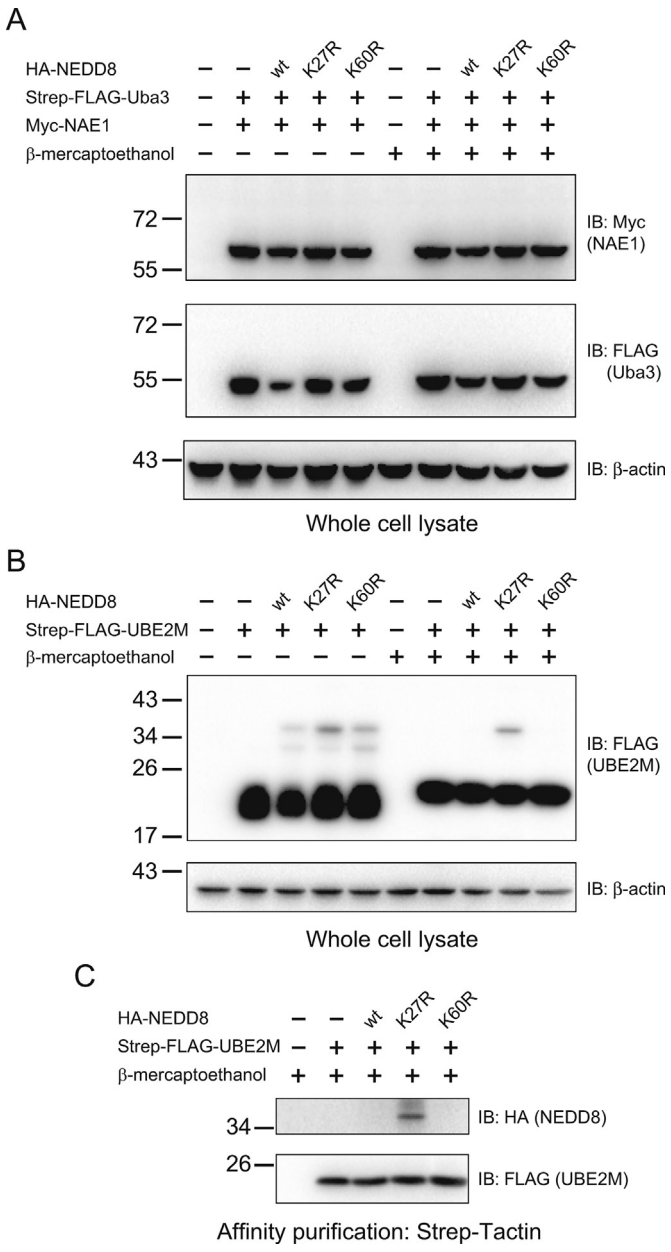

Fig. 2. The K27R NEDD8 mutant alters NEDD8 conjugation. (A) The K27R NEDD8 mutant does not affect NEDD8 activation. Strep-FLAG-Uba3 and Myc-NAE1 were cotransfected with wt and NEDD8 mutants to HEK293T cells. Cells were lysed after 48 h and cell lysates were blotted with the indicated antibodies in the absence and presence of β-mercaptoethanol. (B) The K27R NEDD8 mutant alters the formation of thioester bonds between the conjugating enzyme and the C-terminus of the NEDD8 mutant. UBE2M was cotransfected with the wt or NEDD8 mutants to HEK293T cells and whole cell lysates were blotted with anti-FLAG and anti-β-actin antibodies. (C) The K27R NEDD8 mutant forms a stable conjugate with UBE2M. The Strep-FLAG-UBE2M was purified with Strep-Tactin agarose and blotted with anti-HA and anti-FLAG antibodies in the presence of β-mercaptoethanol.

reducing agent, β-mercaptoethanol. The immunoblotting showed that there is essentially no change among the wt and two mutants. This result suggested that the NEDD8 activation is not significantly altered by the NEDD8 mutation.

Next, we asked whether the K27R mutant affects NEDD8 conjugation. UBE2M is the major E2 conjugating enzyme for neddylation and therefore we tested NEDD8 conjugation to UBE2M. We coexpressed Strep-FLAG-UBE2M with the wt, K27R, or K60R NEDD8 mutants to HEK293T cells. Whole cell lysates were blotted with an anti-HA antibody for NEDD8 (Fig. SI-3) and an anti-FLAG antibody for UBE2M (Fig. 2B and Fig. SI-4A for a long exposure) in the absence or presence of β-mercaptoethanol. On the immunoblotting analysis, we found that without NEDD8 expression, only a single band was detected for UBE2M, which represents the unmodified form. After coexpression with the wt or K60R mutant, weak bands with low mobility were detected for UBE2M in the absence of β-mercaptoethanol. These weak bands disappeared in the presence of β-mercaptoethanol in the sample buffer for SDS-PAGE. The increased molecular weight for the low mobility band is approximately equal to the addition of one NEDD8 molecule. Since this enzyme forms a thioester bond with NEDD8 during the activation and conjugation [2], the addition of reducing agent breaks this bond, resulting in the disappearance of the low mobility band at the high molecular weight region. However, when the K27R NEDD8 was expressed, the low mobility band for UBE2M was retained even in the presence of the reducing agent.

To further demonstrate the high molecular weight band in Fig. 2B is indeed the UBE2M conjugated with the K27R mutant, we used Strep-Tactin agarose to purify Strep-FLAG-UBE2M and blotted with anti-HA and anti-FLAG antibodies (Fig. 2C and Fig. SI-4B). Our result showed that the anti-HA blot has a high molecular weight band when UBE2M is coexpressed with K27R NEDD8 while this band is not present when UBE2M is coexpressed with wt or K60R NEDD8 in the presence of β-mercaptoethanol. This experiment demonstrated that the K27R NEDD8 mutant is indeed conjugated onto UBE2M.

Together, these experiments indicate that the conjugation between K27R NEDD8 and UBE2M is resistant to the reducing environment and thus is not formed through a labile thioester bond. This bond must be another type of covalent conjugation, which cannot be destroyed by the reducing environment. Therefore, the conjugated NEDD8 mutant could not be further transferred to E3 ligases and substrates for subsequent neddylation. These experiments suggested that the K27R NEDD8 mutant impairs the NEDD8 conjugation by forming stable covalent bond(s) with the E2 enzyme.

3.3. K27R NEDD8 mutant reduces cullin neddylation and ubiquitination

The next question we sought to ask is whether the K27R NEDD8 acts as a dominant negative mutant for neddylation in cells. Previous studies have demonstrated that cullins are post-translationally modified by NEDD8 [7]. In order to explore the effect of the K27R mutant on cullin neddylation, we co-expressed Cul1 and Cul4A with the wt and mutant NEDD8 in HEK293T cells. Whole cell lysate was blotted with FLAG, NEDD8, and GAPDH to show similar protein expression (Fig. SI-5). The two cullins were purified through the FLAG affinity tag at their N-terminus and blotted with an anti-NEDD8 antibody. The result showed that neddylation of Cul1 and Cul4A was clearly detected when the wt NEDD8 was expressed, which is consistent with the previous discovery of cullin neddylation [22,23]. However, when the K27R mutant was co-expressed with cullins, cullin neddylation was almost completely lost (Fig. 3A). This result suggested that the K27R mutant prevents cullins from neddylation.

Cullins are scaffold proteins in CRL E3 ligases, such as SCF (Skp1-cullin 1-F box) E3 ligases [24]. It has been reported that cullin

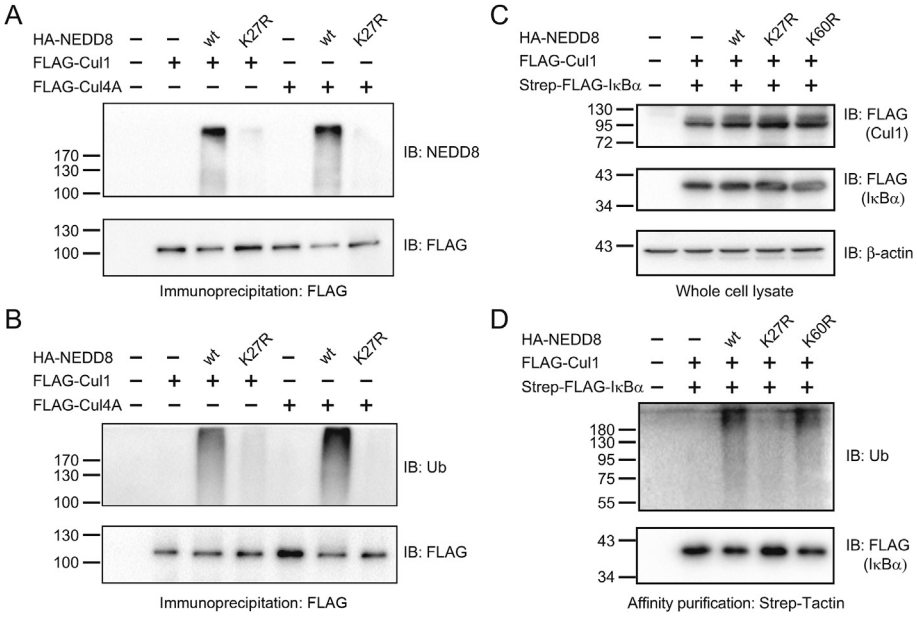

Fig. 3. The K27R NEDD8 mutant prevents cullins from becoming neddylated and ubiquitinated and affects ubiquitination of the downstream target of a CRL E3 ligase, IκBα. (A) Cullin neddylation is diminished by the K27R NEDD8 mutant. GFP (−), wt and NEDD8 mutants were cotransfected with FLAG-Cul1 or FLAG-Cul4A in HEK293T cells. Cul1 and Cul4A were purified by an anti-FLAG M2 antibody and blotted with anti-NEDD8 and anti-FLAG antibodies. (B) The K27R NEDD8 mutant significantly reduces cullin ubiquitination. The above affinity purified samples were blotted with anti-ubiquitin and anti-FLAG antibodies. (C) Expression of FLAG-Cul1 and Strep-FLAG-IκBα in HEK293T cells. Strep-FLAG-IκBα was cotransfected with FLAG-Cul1 and the wt or mutant NEDD8 in HEK293T cells. Cells were treated with MG132 (10 μM) for 10 h. Cell lysates were blotted with anti-FLAG and anti-β-actin antibodies. (D) The K27R NEDD8 mutant remarkably reduces IκBα ubiquitination. Strep-FLAG-IκBα in cell lysates from (C) was affinity purified and blotted with anti-ubiquitin and anti-FLAG antibodies.

neddylation enhances the activity of their associated E3 ligases [25,26]. E3 ligases can not only ubiquitinate their substrates but also undergo autoubiquitination [27–29]. Here we further examined the effect of K27R NEDD8 mutant on cullin ubiquitination and found that K27R NEDD8 completely blocked the ubiquitination of Cul1 and Cul4A while wt NEDD8 leads to their ubiquitination (Fig. 3B). These results demonstrated that Lys27 on NEDD8 can alter both neddylation and ubiquitination of cullin family proteins.

3.4. K27R NEDD8 mutant reduces ubiquitination of the CRL E3 ligase substrate

Next we asked whether the alteration of cullin neddylation and ubiquitination by the K27R NEDD8 mutant affects their associated E3 ligase function. To do so, we chose a known substrate of an SCF E3 ligase, IκBα [25]. Immunoblotting of cell lysates showed that Cul1 and IκBα were expressed in a similar level in different samples (Fig. 3C). Then we purified Strep-FLAG-IκBα with Strep-Tactin agarose and blotted with anti-ubiquitin and anti-FLAG antibodies. When the wt or K60R mutant is cotransfected with Cul1 and IκBα, ubiquitination of purified IκBα is readily detected, which is consistent with a previous finding [25]. However, upon cotransfection of K27R NEDD8 with Cul1 and IκBα, ubiquitination of IκBα is almost completely reduced (Fig. 3D). These results suggested that the activity of the cullin-associated E3 ligase is reduced upon the expression of the K27R NEDD8 mutant, presumably through the reduction of neddylation.

4. Discussion

Neddylation involves in a series of enzymatic reactions similar to ubiquitination. Five lysines in NEDD8 are conserved in ubiquitin. This led us to the conjecture that lysines in NEDD8 may also have distinct biological functions. Here, we discovered a critical lysine residue K27 on NEDD8 which significantly alters the neddylation reactions, cullin neddylation and ubiquitination, and thus affects the activity of CRL E3 ligases. Substitution of this lysine with arginine significantly reduces the global protein neddylation. Expression of the K27R NEDD8 mutant almost completely hampers cullin neddylation and ubiquitination. The reduction of cullin neddylation by this mutant significantly diminishes the activity of cullin-associated E3 ligases.

Based on previous findings on the neddylation and our results presented here, we propose the following mechanism for the alteration of neddylation by the K27R NEDD8 mutant (Fig. 4). The wt NEDD8 is first activated by the E1 complex consisting of NAE1 and Uba3, then conjugated to NEDD8 E2, and further transferred to cullins by NEDD8 E3 ligases for the activation of CRL ubiquitin E3 ligases for subsequent ubiquitination of their substrates [30]. However, the K27R NEDD8 mutant is unable to form a thioester bond with the active cysteine of NEDD8 E2 for subsequent neddylation of its cullin substrates. Instead, this mutant forms a stable covalent bond with NEDD8 E2. The model is supported by that fact that mutating the active cysteine in UBE2M (also called UBC12) to serine can form a stable covalent bond different from the thioester bond between UBE2M and the wt NEDD8 [31]. Therefore, the K27R NEDD8 mutant impairs NEDD8 conjugation, blocks cullin neddylation, and alters subsequent ubiquitination of CRL E3 ligase substrates. These results suggested the importance of this lysine in NEDD8 conjugation and of its biological functions in the downstream neddylation cascades.

Our experiments also provided a useful reagent for the study of neddylation and its functions. Because the K27R NEDD8 mutant almost completely blocks protein neddylation compared with the wt counterpart, K27R NEDD8 could serve as a useful dominant

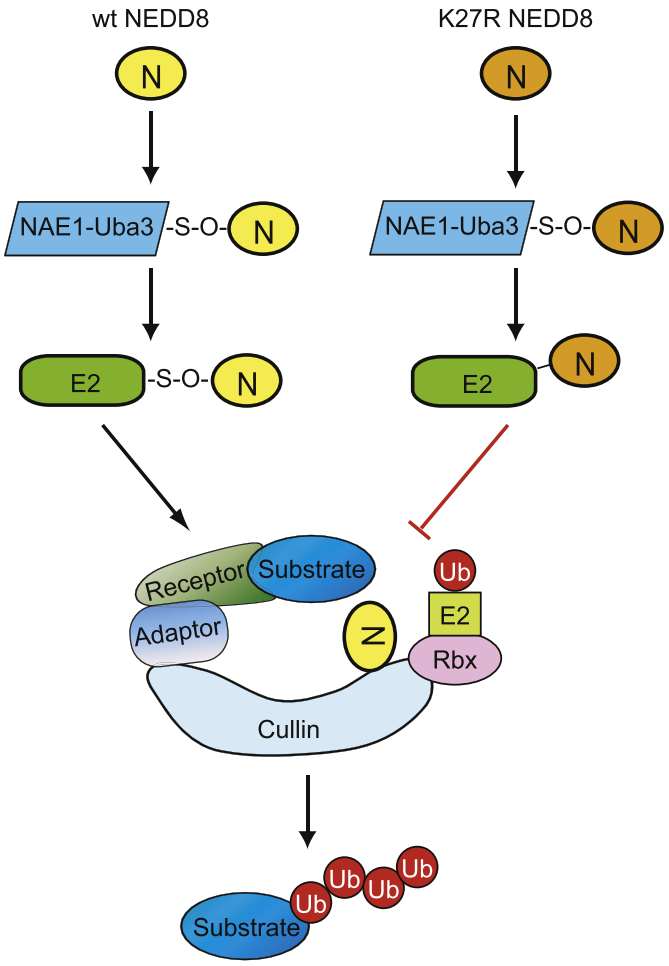

Fig. 4. A proposed model for the impairment of NEDD8 enzymatic cascades by the K27R NEDD8 mutation. Yellow ellipse: wt NEDD8; orange ellipse: K27R NEDD8; cyan quadrangle: E1 NAE1-Uba3 heterodimer; green round rectangle: NEDD8 E2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

negative mutant in investigating the biological functions of protein neddylation. The corresponding conserved lysine residue in ubiquitin, Lys27, which is located in the center of the α helical structure, similar as Lys27 in NEDD8, can also form polyubiquitin conjugates [11]. It may be possible for K27 in NEDD8 to form polyneddylation conjugates. However, the amino acid sequence in the proximity to K27 of the wt NEDD8 is RI^{27}KER, which generates a very short peptide, IKER, after trypsinolysis, preventing this chain linkage from being identified by proteomic studies. In addition, this conjugate might have much lower abundance than other types of conjugates, as evidenced in polyubiquitin chains.

In our experiments, we found that reduction of neddylation on cullins significantly reduces its own ubiquitination. This result provides the evidence that these two modifications may crosstalk with each other on cullins. The interplay between neddylation and ubiquitination may alter the biological functions of the modified proteins, such as the subcellular localization and stability. Indeed, under stresses, such as proteasomal inhibition, heat shock, and oxidative stress, the neddylated proteins can also be ubiquitinated and it is possible that NEDD8 utilizes the ubiquitin-like modifier-activating enzyme 1 (Uba1/Ube1) for the activation of NEDD8 for subsequent modification [32]. Our experiments cannot rule out the possibility that the K27R NEDD8 mutant uses Ube1 for its

activation. Additional experiments are required in order to answer this question.

In summary, using mutagenesis and biochemical approaches, we have identified a key lysine residue, K27, in NEDD8. Replacement of this residue with arginine significantly alters NEDD8 conjugation and subsequent NEDD8 modification on cullins, thus affecting the biological function of cullin-associated E3 ligases.

Conflict of interest

None.

Acknowledgments

We are grateful to Dr. Pengbo Zhou at Weill Cornell Medical College for his kindly providing the Cul4A plasmid. The work was supported by the National Basic Research Program of China (973 Program: 2012CB947602), the National Natural Science Foundation of China (31270874), Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases (BM2013003), a project funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.085.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.085.

References

[1] D.P. Xirodimas, Novel substrates and functions for the ubiquitin-like molecule NEDD8, Biochem. Soc. Trans. 36 (2008) 802–806.
[2] L. Gong, E.T. Yeh, Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway, J. Biol. Chem. 274 (1999) 12036–12042.
[3] M. Morimoto, T. Nishida, Y. Nagayama, H. Yasuda, Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability, Biochem. Biophys. Res. Commun. 301 (2003) 392–398.
[4] T. Kurz, Y.C. Chou, A.R. Willems, N. Meyer-Schaller, M.L. Hecht, M. Tyers, M. Peter, F. Sicheri, Dcn1 functions as a scaffold-type E3 ligase for cullin neddylation, Mol. Cell 29 (2008) 23–35.
[5] M. Broemer, T. Tenev, K.T. Rigbolt, S. Hempel, B. Blagoev, J. Silke, M. Ditzel, P. Meier, Systematic in vivo RNAi analysis identifies IAPs as NEDD8-E3 ligases, Mol. Cell 40 (2010) 810–822.
[6] S. Kamada, Inhibitor of apoptosis proteins as E3 ligases for ubiquitin and NEDD8, Biomol. Concepts 4 (2013) 161–171.
[7] T. Hori, F. Osaka, T. Chiba, C. Miyamoto, K. Okabayashi, N. Shimbara, S. Kato, K. Tanaka, Covalent modification of all members of human cullin family proteins by NEDD8, Oncogene 18 (1999) 6829–6834.
[8] D.M. Duda, L.A. Borg, D.C. Scott, H.W. Hunt, M. Hammel, B.A. Schulman, Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation, Cell 134 (2008) 995–1006.
[9] M. Morimoto, T. Nishida, R. Honda, H. Yasuda, Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1), Biochem. Biophys. Res. Commun. 270 (2000) 1093–1096.
[10] J. Jones, K. Wu, Y. Yang, C. Guerrero, N. Nillegoda, Z.Q. Pan, L. Huang, A targeted proteomic analysis of the ubiquitin-like modifier nedd8 and associated proteins, J. Proteome Res. 7 (2008) 1274–1287.
[11] J. Peng, D. Schwartz, J.E. Elias, C.C. Thoreen, D. Cheng, G. Marsischky, J. Roelofs, D. Finley, S.P. Gygi, A proteomics approach to understanding protein ubiquitination, Nat. Biotechnol. 21 (2003) 921–926.
[12] V. Chau, J.W. Tobias, A. Bachmair, D. Marriott, D.J. Ecker, D.K. Gonda, A. Varshavsky, A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein, Science 243 (1989) 1576–1583.
[13] E.S. Johnson, P.C. Ma, I.M. Ota, A. Varshavsky, A proteolytic pathway that recognizes ubiquitin as a degradation signal, J. Biol. Chem. 270 (1995) 17442–17456.
[14] L. Deng, C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, Z.J. Chen, Activation of the IkB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain, Cell 103 (2000) 351–361.
[15] P. Xu, D.M. Duong, N.T. Seyfried, D. Cheng, Y. Xie, J. Robert, J. Rush, M. Hochstrasser, D. Finley, J. Peng, Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation, Cell 137 (2009) 133–145.
[16] B. Varghese, H. Barriere, C.J. Carbone, A. Banerjee, G. Swaminathan, A. Plotnikov, P. Xu, J. Peng, V. Goffin, G.L. Lukacs, S.Y. Fuchs, Polyubiquitination of prolactin receptor stimulates its internalization, postinternalization sorting, and degradation via the lysosomal pathway, Mol. Cell. Biol. 28 (2008) 5275–5287.
[17] F. Huang, X. Zeng, W. Kim, M. Balasubramani, A. Fortian, S.P. Gygi, N.A. Yates, A. Sorkin, Lysine 63-linked polyubiquitination is required for EGF receptor degradation, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 15722–15727.
[18] J.A. Olzmann, L.S. Chin, Parkin-mediated K63-linked polyubiquitination: a signal for targeting misfolded proteins to the aggresome-autophagy pathway, Autophagy 4 (2008) 85–87.
[19] C.S. Shi, J.H. Kehrl, TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced autophagy, Sci. Signal 3 (2010) ra42.
[20] Y. Ohki, N. Funatsu, N. Konishi, T. Chiba, The mechanism of poly-NEDD8 chain formation in vitro, Biochem. Biophys. Res. Commun. 381 (2009) 443–447.
[21] G. Xu, X. Jiang, S.R. Jaffrey, A mental retardation-linked nonsense mutation in cereblon is rescued by proteasome inhibition, J. Biol. Chem. 288 (2013) 29573–29585.
[22] F. Osaka, H. Kawasaki, N. Aida, M. Saeki, T. Chiba, S. Kawashima, K. Tanaka, S. Kato, A new NEDD8-ligating system for cullin-4A, Genes Dev. 12 (1998) 2263–2268.
[23] G. Bornstein, D. Ganoth, A. Hershko, Regulation of neddylation and deneddylation of cullin1 in SCF^{Skp2} ubiquitin ligase by F-box protein and substrate, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11515–11520.
[24] N. Zheng, B.A. Schulman, L. Song, J.J. Miller, P.D. Jeffrey, P. Wang, C. Chu, D.M. Koepp, S.J. Elledge, M. Pagano, R.C. Conaway, J.W. Conaway, J.W. Harper, N.P. Pavletich, Structure of the Cul1-Rbx1-Skp1-F box^{Skp2} SCF ubiquitin ligase complex, Nature 416 (2002) 703–709.
[25] M.A. Read, J.E. Brownell, T.B. Gladysheva, M. Hottelet, L.A. Parent, M.B. Coggins, J.W. Pierce, V.N. Podust, R.S. Luo, V. Chau, V.J. Palombella, Nedd8 modification of cul-1 activates $SCF^{\beta TrCP}$-dependent ubiquitination of IκBα, Mol. Cell. Biol. 20 (2000) 2326–2333.
[26] M. Ohh, W.Y. Kim, J.J. Moslehi, Y. Chen, V. Chau, M.A. Read, W.G. Kaelin Jr., An intact NEDD8 pathway is required for cullin-dependent ubiquitylation in mammalian cells, EMBO Rep. 3 (2002) 177–182.
[27] P. Zhou, P.M. Howley, Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases, Mol. Cell 2 (1998) 571–580.
[28] U. Nuber, S.E. Schwarz, M. Scheffner, The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate, Eur. J. Biochem. 254 (1998) 643–649.
[29] A. Chen, F.E. Kleiman, J.L. Manley, T. Ouchi, Z.Q. Pan, Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase, J. Biol. Chem. 277 (2002) 22085–22092.
[30] H. Walden, M.S. Podgorski, B.A. Schulman, Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8, Nature 422 (2003) 330–334.
[31] H. Wada, E.T. Yeh, T. Kamitani, A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo, J. Biol. Chem. 275 (2000) 17008–17015.
[32] O. Leidecker, I. Matic, B. Mahata, E. Pion, D.P. Xirodimas, The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions, Cell Cycle 11 (2012) 1142–1150.