Novel substrates and functions for the ubiquitin-like molecule NEDD8

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Abstract
Genetic experiments have established an important role for the ubiquitin-like molecule NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) in the regulation of cell growth, viability and development. It is therefore essential to identify the molecular targets for the pathway. Until recently, the cullin family of proteins was characterized as the only substrates for NEDDylation. However, through either direct biological approaches or the use of proteomics, it is now evident that the NEDD8 proteome is more diverse than thought previously. The present review describes the biological significance of NEDDylation for the novel identified substrates and the emerging evidence for the co-operation between the ubiquitin and NEDD8 pathways to control protein function.

The NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) pathway
NEDD8 (or Rub1 in yeast) was originally discovered as one of a set of genes that are down-regulated in mouse brain during development and hence the name of these genes as multiple neural-precursor-cell-expressed developmentally down-regulated genes in mice [1]. Among the family of UBLs (ubiquitin-like proteins), NEDD8 has the highest identity with ubiquitin (59%). However, NEDDylation has its own unique set of enzymes to ensure a distinct conjugation pathway. NEDD8, as with the great majority of UBLs, is synthesized as a precursor with a C-terminal tail, the length of which varies in different species [2]. Processing of this tail by proteases will expose the diglycine motif, through which NEDD8 is covalently linked to substrates. NEDP1/DEN1/SENP8 was identified as a NEDD8 specific C-terminal hydrolase [3–5]. The ubiquitin hydrolase UCH-L3 (and its Saccharomyces cerevisiae homologue Yuh1) has a dual specificity and can also process NEDD8 [6,7]. Despite the high degree of identity to ubiquitin, NEDD8 activation and transfer to the Ubc12 E2 conjugation enzyme is highly specific. Structural studies on the E1–NEDD8 complex (APPBP1 [APP (amyloid precursor protein)-binding protein 1]–Uba3 dimer) showed that Ala72 in NEDD8 (Arg72 in ubiquitin) plays a crucial role in this specificity, ensuring that, under physiological conditions, NEDD8 and not ubiquitin or other UBLs are activated by the NEDD8 E1 enzyme [8]. Similarly, Ubc12 contains a unique N-terminus, which makes extra and specific interactions with the E1 heterodimer, again ensuring specificity in the pathway [9,10]. Upon charging of the correct E2, NEDD8 is covalently transferred to substrates. NEDDylation, similarly to ubiquitination and conjugation of other UBLs, is a dynamic process and proteases promote deconjugation of NEDD8 from its targets. The multi-component COP9 signalosome, the NEDD8-processing proteases NEDP1/DEN1/SENP8 and USP21 have been reported to deconjugate NEDD8 from substrates [11]. USP21 can also deconjugate ubiquitin from substrates. Similar dual specificity, at least at the recognition step, for ubiquitin and NEDD8 was demonstrated for UCH-L1, ataxin-3 and the parasite hydrolase PfUCH54 (but not its human homologue UCH34) [12,13].

Initial genetic experiments in S. cerevisiae where components of the NEDDylation machinery, such as rub1, ubc12, ula1 or uba3, were deleted, did not provide any encouraging data, as the mutants displayed no obvious defects. However, combination with temperature-sensitive mutants for ubc3/cdc34 ubiquitin-conjugating enzymes caused lethality [14–16]. Similar genetic experiments on components of the NEDD8 pathway in Schizosaccharomyces pombe, Caenorhabdities elegans, Drosophila and mice all provided a lethal phenotype, demonstrating an essential role for NEDD8 in cell viability [17–20]. In plants, recessive mutations in the axr1 gene which encodes for one of the components of the NEDD8 E1 enzyme, resulted in a decreased response to auxin, a phytohormone which controls gene expression involved in developmental processes [21]. Links between NEDD8 conjugation and cell proliferation were demonstrated in the TS-41 hamster cell line. These cells harbour a temperature-sensitive mutation in the Smc1 gene, which is almost identical with the human APPBP1, the regulatory component of the NEDD8 E1 enzyme. These cells at the restrictive temperature undergo repetitive cycles of G1/S-phases in the absence of any G2/M-phase. This dramatic

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Abbreviations used: APP, amyloid precursor protein; AICD, APP intracellular domain; APPBP1, APP-binding protein 1; BCA3, breast cancer-associated protein 3; EGF, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor 1α; IκB, inhibitor of nuclear factor κB; ISG15, interferon-stimulated gene 15; NEDD8, neural-precursor-cell-expressed developmentally down-regulated 8; Nfkb, nuclear factor κB; RING, really interesting new gene; SCF, Skp1/cullin/F-box; VEC, VHL/cullin-2/elongin B or C/Rbx1 complex; VHL, von Hippel-Lindau.

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cell phenotype suggests a role for NEDD8 in regulating the coupling of DNA replication and cell division [22].

**Targets for NEDDylation**

At the molecular level, the most well-characterized substrate for the NEDD8 pathway is the cullin family of proteins [23]. Cullins are scaffold components of the SCF (Skp1/cullin/F-box) ubiquitin ligase complex, which controls the ubiquitination and proteasomal degradation of proteins involved in cell-cycle regulation (p27 and cyclin E), transcriptional regulation and signal transduction (IKB, inhibitor of NF-kB [nuclear factor kB] α, β-catenin), O2 regulation (HIF-1α [hypoxia-inducible factor 1α], -catenin) and in centrosome and cytoskeletal regulation [24–29]. The Rbx1 RING (really interesting new gene) finger E3–ubiquitin ligase, which mediates substrate ubiquitination in the SCF complex, is also reported to promote cullin NEDDylation [30]. Recently, the Dcn1 protein was demonstrated as a NEDD8 E3 ligase for cullins. Dcn1 lacks a typical RING or HECT (homologous with E6-associated protein C-terminus) domain found in the majority of E3 ligases, but fulfills criteria for an E3 ligase [31]. However, the fact that Dcn1 can also interact with Rbx1 raises the possibility of a heterodimeric ligase complex [32]. NEDDylation of cullins modulates the ubiquitin–ligase activity of the complex, resulting in increased ubiquitination and proteasomal degradation of the substrates [23]. Mechanistically, NEDDylation of cullins reverses the effect of CAND1, which is reported to be a natural inhibitor of cullin-based SCF ligases [33]. Other studies showed that, upon cullin NEDDylation, the recruitment of the E2 ubiquitin-conjugating enzyme is now more efficient [34]. Finally, another role for NEDD8 in this process is to promote cullin dimerization [35]. However, in recent studies, either through direct biological approaches or use of proteomics, novel substrates for the pathway have been identified. This suggests that the NEDD8 proteome may be more diverse than thought previously.

The stability and function of the p53 tumour suppressor is very tightly controlled through its modification with ubiquitin and UBLs. In this process, the Mdm2 oncogene plays a vital role, as, by acting as an E3 ligase, it promotes p53 proteasomal degradation and inhibition of p53 transcriptional activity. The specificity of Mdm2 as an E3 ligase was extended to NEDDylation, as it was shown that Mdm2 can dramatically stimulate p53 modification with NEDD8, but not with SUMO-1 (small ubiquitin-related modifier 1) or ISG15 (interferon-stimulated gene 15). As it is common with RING-type E3 ligases, Mdm2 was also shown that it is modified by NEDD8 itself [11]. This dual activity of Mdm2 for ubiquitin and NEDD8 also suggests that E3 ligases have more diverse roles in regulating protein function and can provide an integration/cross-talk point for different conjugation pathways. Mutations which abolish Mdm2 ubiquitin ligase activity also inhibit its stimulatory effect on p53 NEDDylation, suggesting the existence of common components for these modifications [36]. However, there is also evidence to suggest that Mdm2-mediated ubiquitination and NEDDylation of p53 are differentially regulated. Although six lysine residues in the C-terminus of p53 (Lys370, Lys372, Lys375, Lys381, Lys382 and Lys386) are required for efficient ubiquitination, mutation of three lysine residues within this group (Lys370, Lys372 and Lys375) is sufficient to reduce p53 NEDDylation [11]. Recently, the Tip60 acetyltransferase, which is a known regulator of the p53–Mdm2 pathway was shown to preferentially inhibit Mdm2-mediated p53 NEDDylation, but not ubiquitination [37]. Furthermore, experiments where ubiquitin or NEDD8 were fused at the C-terminus of p53 showed that ubiquitin could promote the cytoplasmic localization of p53, whereas the p53–NEDD8 fusion was found almost exclusively in the nucleus [38,39]. Functionally, NEDDylation of p53 inhibits its transcriptional activity. This was shown with the use of p53 mutants deficient in NEDDylation and the TS-41 cell line [11].

The role of NEDD8 in transcriptional regulation was extended to other members of the p53 family. The TAp73 splice variant is NEDDylated in an Mdm2-dependent manner. This modification is specific for TAp73, as the ΔNp73 variant, which lacks the N-terminal Mdm2-binding domain, is not modified by NEDD8. This also shows the important role for Mdm2 in this post-translational modification. As for p53, NEDDylation of TAp73 also inhibits its transcriptional activity, at least partly due to preferential localization of NEDDylated TAp73 in the cytoplasm [40]. Additional regulators of p53 NEDDylation have been identified as the F-box protein FBX011 binds directly to p53, but, in contrast with Mdm2, it promotes only p53 NEDDylation, but not ubiquitination. Consistent with an inhibitory role for NEDD8 for p53 function, FBX011 suppresses p53 transcriptional activity [41].

The role of NEDD8 in controlling the activity of NF-kB is well established. NEDDylation of cullin 1 is regarded as an important regulatory step in the degradation of IκBα, which is the natural inhibitor of NF-kB [26]. However, recent studies suggested a nuclear role for NEDD8 in controlling NF-kB-dependent transcription. BCA3 (breast cancer-associated protein 3) was identified as a NEDD8 substrate, shown to interact directly with NF-kB (p65) and inhibit its transcriptional activity. This effect is mediated at least partly through the recruitment of the histone deacetylase SIRT1. NEDDylation of BCA3 is required for SIRT1 recruitment, suggesting that NEDD8 can negatively regulate NF-kB function [42].

Another example of transcriptional regulation by NEDD8 was demonstrated recently on AICD (APP intracellular domain). APP is a membrane protein with an extracellular and intracellular domain and is physiologically processed through the action of α-, β- and θ-secretases. Increased processing of APP is believed to play an important role in the development of Alzheimer’s disease. Initial cleavage creates the production of Aβ (amyloid β-peptide), which, upon further processing, creates the C-terminal APP fragment, the AICD fragment. Upon its production, AICD translocates to the nucleus and interacts with the transcriptional co-activators Fe65 and Tip60 acetyltransferase. This causes changes in
gene expression leading to the induction of apoptosis [43]. APP was shown to be NEDDylated and mutational analysis has identified that AICD contains acceptor lysine residues. Functionally, NEDDylation inhibits AICD-mediated transcriptional activation and this is due to inhibition of its interaction with the co-activators Fe65 and Tip60 [44].

The above data are consistent with a role for NEDD8 in transcriptional regulation. Additional novel NEDD8 targets include VHL (von Hippel–Lindau) tumour suppressor, EGFR (epidermal growth factor receptor) and ribosomal proteins.

VHL is a component of a cullin-2-based SCF ligase complex, which includes elongin B or C and Rbx1 (VEC). VHL provides the specificity of the ligase as it interacts directly with the targeted substrates. Two main functions have been identified for the VEC ligase: regulation of the stability of HIF-1α, which, upon its prolyl-hydroxylation, is recognized by VEC through VHL and targeted for degradation. The other role for VHL is for the formation of extracellular matrix though its interaction with fibronectin. Importantly, cancer-derived mutants for VHL are deficient for interaction with fibronectin [45]. VHL is covalently modified with NEDD8, and, although this modification does not affect the ubiquitin ligase activity of the VEC, it controls the interaction of VHL with fibronectin. VHL mutants deficient in NEDDylation were also shown to be deficient in promoting fibronectin assembly, suggesting a direct role for NEDD8 in this process. Mechanistically, NEDDylation of VHL prevents the association of cullin 2 and the engagement of VHL in an SCF complex and promotes VHL association with fibronectin [46].

EGFR is ubiquitinated upon ligand binding and this induces the internalization and lysosomal degradation of the receptor. The c-Cbl E3-ligase mediates this ubiquitination step. As is the case for Mdm2, c-Cbl displays a dual specificity and can also mediate modification of EGFR with NEDD8. NEDDylation increases the efficiency of EGFR ubiquitination and facilitates its lysosomal degradation [47]. This is another example of the close co-operation between the ubiquitin and NEDD8 pathway to control protein function.

The recent advances in proteomics have made possible the identification and quantification of target substrates for post-translational modifications. Since there is a limited knowledge of molecular targets for the NEDD8 pathway, different groups have applied proteomics to identify novel substrates for NEDDylation [48–51]. The potential identified targets suggest that NEDD8 may be involved in mRNA splicing, DNA replication and repair, chromatin remodelling and proteasomal degradation. However, the technical aspects of these proteomics studies cannot distinguish between non-covalent interactors with NEDD8 and covalently modified substrates. Validation experiments in tissue culture systems have shown that proteins such as Rpt6 (a component of the 19S proteasome), SMC1 (a member of chromosome-associated ATPases) and DNA-PK (DNA-dependent protein kinase) interact non-covalently with NEDD8 [50]. Ribosomal proteins were also identified as major potential substrates for NEDDylation. Experiments performed in tissue culture systems validated the covalent modification of five out of six tested ribosomal proteins with NEDD8. Using L11 as a model ribosomal protein, it was shown that NEDDylation protects L11 from destabilization. Importantly, under conditions of reduced NEDDylation where the levels of L11 are decreased, the production of ribosomes is not affected [51]. This suggests that ribosomal proteins may not be limiting for ribosome biogenesis. Interestingly, the stability of the mature ribosome is regulated by ubiquitination [52]. Whether ubiquitin and NEDD8, in this case, control similar or separate steps in ribosome biogenesis and stability is not currently known. Another point that arose from the proteomic studies is the presence of ubiquitin in the isolated NEDD8 proteome [51]. This is consistent with the idea that a lot of proteins can be modified with ubiquitin and NEDD8 and that these pathways co-operate to control protein function. Indeed, a number of studies have implicated NEDD8 as a regulator of protein degradation. For cullins, disruption of continuous cycles of NEDDylation and de-NEDDylation leads to their destruction [53,54]. Furthermore, NUB-1, which interacts with NEDD8 and subunits of the 26S proteasome, was shown to target both free NEDD8 and NEDDylated proteins for proteasomal degradation [55,56].

NEDD8 was thought to modify substrates as a single moiety and not be able to form chains. The proteomic studies also showed that internal NEDD8 lysine residues can act as acceptor sites for the formation of chains. However, the data cannot indicate whether these lysine residues are NEDDylated or modified with ubiquitin or other UBLs such as ISG15 [50,51].

**Future directions**

The plethora of genetic evidence derived from yeast, plants, C. elegans, Drosophila and mice strongly suggest an important role for NEDD8 in developmental regulation, cell viability and growth. Clearly, the cullin family of proteins is the best-characterized substrate for NEDD8, and a lot of phenotypes observed in the genetic studies could be attributed through the regulation of the SCF ligases and the substrates that they control. However, it is now evident that additional molecular targets exist for NEDDylation with diverse functional outcomes such as transcriptional regulation, membrane receptor cycling, extracellular matrix formation and ribosomal protein stability. At least for some of the new substrates, such as p53, p73 and EGFR, their modification with NEDD8 is controlled by E3–ubiquitin ligases. Consistent with this, most of the novel identified NEDD8 substrates are also modified with ubiquitin, and this is confirmed by proteomic studies. Given the specificity of the pathways at the E1–E2 step to ensure the entry of the correct molecule, the cross-talk at the E3 level may highlight the diversity of ligases in controlling protein function (Figure 1). However, the precise mechanism(s) and the components involved in the modification of these substrates by NEDD8 are not currently known. It will be important in the future.
Figure 1 | Cross-talk between ubiquitin and NEDD8 pathways

E3 ligases act as integration points for regulation of protein function.

References


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