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# New Insights into the Mechanisms Underlying NEDD8 Structural and Functional Specificities

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## Abstract

Ubiquitin (Ub) and ubiquitin-like (Ubl) proteins are small polypeptides that are conjugated to substrates affecting their activity and stability. Cells encode “receptors” containing Ub-/Ubl-binding domains that interpret and translate each modification into appropriate cellular responses. Among the different Ubls, NEDD8, which is the ubiquitin’s closest relative, retains many of the structural determinants that enable ubiquitin the ability to target proteins to degradation. Nevertheless, the direct involvement of NEDD8 conjugation to proteasome recruitment has been proved only in a few cases. To date, well-defined major NEDD8 substrates are primarily members of the cullin family, and cullin neddylation does not appear to mark these proteins for degradation. Various studies have demonstrated that selectivity between ubiquitin and NEDD8 is guaranteed by small but substantial differences. Nevertheless, several issues still need to be addressed, mainly concerning which interaction surfaces mediate NEDD8 function and what domains recognize them. Recently, two novel domains identified in KHNYN and N4BP1 proteins have shed new light on this research area. Here, I discuss some recent reports that contributed to shed light on the mechanisms underlining the discrimination between ubiquitin and NEDD8. Understanding the details of these molecular mechanisms represents a prominent facet for the identification of new therapeutic targets.

**Keywords:** NEDD8, ubiquitination, neddylation, ubiquitin-binding domains, KHNYN, N4BP1

## 1. Introduction

Protein diversity in living organisms is the result of several mechanisms acting at different steps of gene expression. Alternative splicing determines the production of a variety of proteins from a single pre-mRNA, and different promoters and termination sites increase the protein diversity during gene transcription. An additional level of complexity is achieved through posttranslational modifications (PTMs). More than 90,000 individual PTMs have been detected using biochemical and biophysical methods [1]. These modifications extend proteome diversity by inducing structural changes, such as the covalent binding of functional groups (phosphate, acetyl, methyl, lipids, and others) or the cleavage and the selective degradation of regulatory subunits. Such modifications in turn play a central role in regulating protein function, as they finely tune intermolecular interactions that modulate almost all biological processes. Thus, it is not surprising that almost 5% of the human genome

encodes enzymes in charge of catalyzing reactions leading to PTMs. Different from the majority of the biological processes that participate in increasing protein diversification, a key feature of PTMs is the reversibility. Indeed, with only a few exceptions such as proteolysis, deamidation [2], and the recently reported eliminylation catalyzed by phosphothreonine lyases [3], which are irreversible, PTMs are typically regulated by a set of enzymes that coordinate the temporary addition and removal of protein modifications, thus ensuring a finely tuned control of the process.

Typically, signal processing requires a third component that reads the PTM and transmits the signal to the downstream effectors of the signaling pathway. Since different signal transducers can recognize the same type of posttranslational modification, specificity is generally ensured by a context-specific recognition, meaning that the transducer interacts with the target molecule by recognizing a posttranslationally modified sequence motif that is unique for that target.

The specificity and cross talk underlining the signaling mechanisms have been illustrated by Wendell Lim and Tony Pawson, who coined the paradigm of the “three-part toolkit” applied to tyrosine phosphorylation [4]. Kinases “write” the modification mark, SH2 domain-containing proteins “read” it, and finally phosphatases “erase” it. Such a simple model, implying for each modification writer, eraser, and reader modules, can be easily extended to most modification systems. As might be expected, these three module toolkits are not isolated systems, and similar modifications have different degrees of cross talk. For instance, in the case of Ser/Thr or Tyr phosphorylation, writers and erasers display some promiscuity with a large number of kinases and phosphatases being able to phosphorylate or dephosphorylate both Ser/Thr and Tyr with low selectivity. The reader modules, on the other hand, are rather selective with SH2/PTB and 14-3-3/FHA being able to bind only pTyr- or pSer-/Thr-containing peptides, respectively.

The balance between specificity and promiscuity in modifications toolkits is particularly relevant in the ubiquitination process, where up to 16 different ubiquitin-like peptides can be covalently linked to a variety of proteins to support very diverse cellular events [5].

Ubiquitination is an ATP-dependent process that involves the action of at least three enzymes: an ubiquitin-activating enzyme, also known as E1 enzyme, which catalyzes the first step in the ubiquitination reaction; an ubiquitin-conjugating enzyme, dubbed E2, which performs the second step in the ubiquitination reaction; and the E3 ligase involved in the ligation step, consisting in the transfer of ubiquitin from the E2 to an internal lysine residue, thus generating an isopeptide bond. If the “three-part toolkit” paradigm is applied to the ubiquitination process, E3 ligases (writers), deubiquitinating enzymes (erasers), and ubiquitin-binding domains (readers), respectively, create, transmit, and cancel the ubiquitin signal. Moreover, the mechanisms underlying the conjugation, recognition, and removal of different Ubls have been modified by evolution into distinct systems with their associated, not interchangeable, modification toolkits, with the consequence that each Ubl-associated path has evolutionary gained a specific biological function.

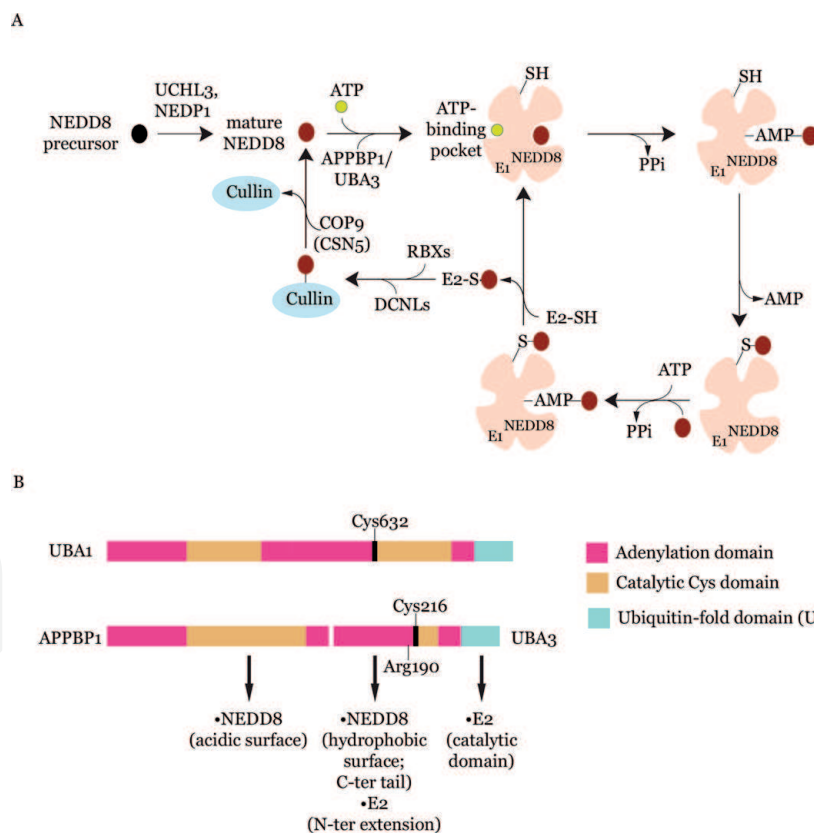
The case of ubiquitin and neural precursor cell expressed developmentally downregulated protein 8 (NEDD8) is particularly intriguing. NEDD8 is the ubiquitin's closest relative (58% sequence identity and 80% sequence similarity) and can be conjugated to target substrates in a process, called neddylation, which is similar to ubiquitination, but relies on its own enzymatic kit and targets a specific and limited set of substrates. The clear discrimination between conjugation and deconjugation of ubiquitin or NEDD8 is the result of a few subtle differences between these two molecules, primarily aimed at ensuring that the substrates targeted for neddylation are not modified by the addition of ubiquitin. In this way, the neddylation pathway remains insulated, only affecting defined biological processes.

Strikingly, alongside a clear function for neddylation, the ubiquitin readers are in general rather promiscuous and bind to either modifications, though not necessarily with similar affinity. Moreover, neddylation modulates ubiquitination efficiency. Indeed, NEDD8 conjugation to cullins is an essential step in the stimulation of the ubiquitin ligase activity of the Cullin RING-ligase (CRL) complexes, clearly indicating a cross talk between the two PTMs.

Here the current comprehension of the mechanisms underlining the discrimination between ubiquitin and NEDD8 is recapitulated, together with the emerging understanding of NEDD8 recognition domains.

## 2. The neddylation pathway

The *NEDD8* gene encodes a small protein of 81 amino acids, which is 58% identical and 80% homologous to ubiquitin. Like ubiquitin, the maturation of the precursor protein requires a proteolytic step that allows the exposure of the Gly<sub>75</sub>-Gly<sub>76</sub> residues, which are critical for the conjugation process (**Figure 1A**). This reaction is catalyzed by two enzymes: ubiquitin C-terminal hydrolase isozyme 3 (UCHL3), which can also process the ubiquitin precursor [6], and NEDD8-specific protease 1 (NEDP1), also called deneddylase 1 (DEN1) or SUMO-1-/sentrin-/SMT3-specific peptidase



**Figure 1.**

(A) Overview of NEDD8 activation, conjugation, and deneddylation. The NEDD8 precursor is cleaved at the carboxyl terminus to allow the exposure of the Gly<sub>75</sub>-Gly<sub>76</sub> residues. Free mature NEDD8 is adenylated by APPBP1/UBA3 in an ATP-dependent process; the APPBP1-UBA3-bound NEDD8 is transferred from AMP to the active-site Cys with the release of AMP; the ATP-dependent adenylation of a second free NEDD8 by the Cys-NEDD8-loaded APPBP1-UBA3 is followed by the transfer of the activated NEDD8 from APPBP1/UBA3 to the catalytic Cys of the E2 enzyme. The E2-bound NEDD8 is transferred to a Lys residue at the carboxyl-terminal ends of cullin proteins by RBXs RING-ligases in the context of the CRL complexes; this step requires the activity of the DCNL proteins. Deneddylation of cullins by CSN5 subunit of COP9 complex releases free NEDD8 for another cycle of conjugation/deconjugation. (B) Organization of the APPBP1/UBA3 heterodimer and comparison of the domain structures of NEDD8's E1 with the E1 for ubiquitin. Direct interactions between the heterodimer APPBP1-UBA3 and NEDD8, ATP, and the E2 enzymes are shown.



8 (SENp8). NEDP1 shows a remarkable specificity for the NEDD8 precursor as it cannot cleave ubiquitin nor the small ubiquitin-like modifier (SUMO) precursors [7]. Once converted into the mature form, NEDD8 is activated by the NEDD8-activating E1 enzyme, a heterodimer composed of amyloid- $\beta$  precursor protein-binding protein 1 (APPBP1, also called NAE1) and the ubiquitin-activating enzyme 3 (UBA3), the two subunits corresponding, respectively, to the N-terminal and C-terminal halves of a typical single-chain E1 [8–10]. Again, like all the E1 enzymes, three different domains can be identified in NAE1: an adenylation domain, a catalytic cysteine-containing domain harboring the Cys residue involved in the E1-NEDD8 thioester linkage, and a domain structurally resembling ubiquitin (called ubiquitin-fold domain, UFD) that binds the E2 [11]. The first step of the reaction requires the ATP-dependent adenylation of the NEDD8 C-terminus, mediated by the UBA3 adenylation domain. The Ubl is then transferred to the catalytic cysteine in UBA3 with the formation of a thioester intermediate and the release of AMP. At this step, the Cys~NEDD8-loaded E1 molecule goes through a second cycle of adenylation of a free NEDD8, which is followed by transfer of the first activated NEDD8 from the active cysteine of the E1 to the catalytic cysteine of the E2. Being NAE1 the only E1 enzyme for NEDD8, it has received attention as a promising target in new cancer therapeutic strategies, and several drugs have been developed in order to inhibit its activity. Among them, MLN4924, an analogue of adenosine 5'-monophosphate, binds to the ATP-binding site in UBA3 and forms an irreversible MLN4924-UBA3 adduct that inhibits NAE1, thus causing the disruption of protein turnover and cellular apoptosis by deregulation of DNA synthesis [12]. MLN4924 has shown significant anticancer efficacy in preclinical studies, and it has been advanced into several phase I clinical trials for certain solid tumors and hematological malignancies [12].

Structural studies of the heterodimer APPBP1-UBA3 in complex with NEDD8 showed that the interface between the two partners requires three contact sites: a hydrophobic surface in NEDD8 that contacts the adenylation domain portion of UBA3, an electrostatic interaction between NEDD8 and a charged surface on the E1-specific catalytic cysteine domain portion of APPBP1, and, finally, the partial stiffening of NEDD8's C-terminal tail that extends away from the globular domain and sits in a channel on the surface of UBA3 (**Figure 1B**). As shown below, each of these interactions participates in determining the specificity of NEDD8 for its E1 enzyme [9]. A second trans-thiolation reaction involves the transfer of NEDD8 from the active-site Cys of APPBP1-UBA3 onto the active-site Cys of the NEDD8 E2 enzyme, which in metazoans can be UBC12 (also known as UBE2M) or UBE2F. The E1-E2 interface involves two surfaces. The first one is shared with other E1-E2 pairs and mediates the interaction between the catalytic core domain of the E2 and the UBA3's UFD [13]. The second surface, which is unique for the NEDD8's E1-E2 pair, consists in an amino terminus extension in the NEDD8 E2s that interacts with a binding groove in the adenylation domain of UBA3. The combination of these two binding sites explains why the NEDD8 pathway remains substantially isolated from the ubiquitin enzymatic cascade despite sharing a common structural scaffold.

Once the E2 enzyme is charged, NEDD8 is conjugated to different substrates through the catalytic activity of several E3 ligases, resulting in the formation of an isopeptide bond linking the terminal carboxyl group of NEDD8 with the  $\epsilon$ -NH<sub>2</sub> group of a lysine residue of the substrate [14–16]. Interestingly, all the NEDD8 E3 enzymes that have been characterized so far also function as E3 ligases for ubiquitin. Among them, the best studied are the RING domain subunits RING-box proteins 1 and 2 (RBX1 and 2), which are components of the CRL complexes. RBX ligases transfer NEDD8 onto the cullins, a protein family acting as molecular scaffold of CRL complexes [17–19]. Cullin neddylation increases CRL ubiquitination activity via conformational changes that optimize ubiquitin transfer to the target proteins [20]. Considering that cullins

can potentially assemble hundreds of CRL complexes by utilizing different substrate receptors and switching from one receptor to another thanks to a finely tuned mechanism, CRLs are responsible for targeting a myriad of cellular substrates to degradation. Differently from RBX1, which recruits both NEDD8 E2s, RBX2 shows a clear preference for UBE2F [21]. Interestingly, biochemical and structural studies have demonstrated that binding of E1 and E3 to NEDD8 E2 UBC12 are mutually exclusive, so that the unidirectionality of the E1-E2-E3 conjugation cascade requires a switch based on the different binding affinities of NEDD8 toward charged and uncharged enzymes [13, 22–24].

RBX1 and 2 require auxiliary E3 factors to direct NEDD8 toward the correct lysine residue. These factors are encoded by proteins of the defective in cullin neddylation 1 (DCN1) family [25]. Human genome encodes for five DCN1-like proteins termed DCNL1–DCNL5. These DCNLs have distinct N-terminal domains but share a conserved C-terminal potentiating neddylation (PONY) domain that directly binds to cullins. Moreover, all DCNLs interact strongly with the CRL regulator CAND1, which promotes the release of old and the association of new substrate adaptors to cullin–RING core complexes [20, 26, 27]. These complexes including CAND1/deneddylated cullin/DCNL act as “reserve” CRLs that are ready to be rapidly activated [28]. Finally, it has been recently shown that additional factors can be required to ensure the efficient neddylation of specific cullins. TFB3, an essential subunit of the TFIIH complex, which mediates transcription initiation and is also involved in nucleotide excision repair (NER) [29], is needed for efficient neddylation of CUL3 and for neddylation and ubiquitylation of Rtt101, two yeast cullins regulating DNA-associated processes [30].

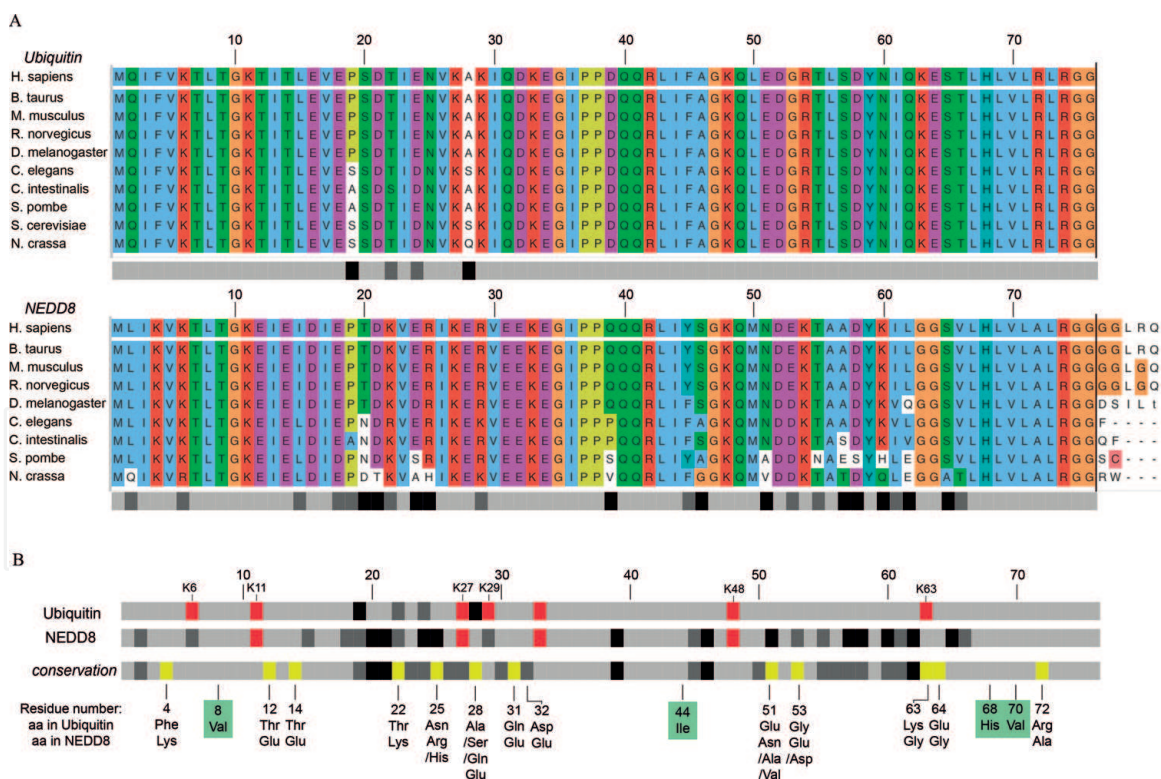
The COP9 signalosome (CSN) complex performs cullins deneddylation. Of the eight subunits that make up the complex, CSN5 is the one carrying the deneddylation activity. Furthermore, the CSN complex stably binds deneddylated CRLs and sterically inhibits RBX1-mediated E2 activation [31, 32]. The experimental evidences indicate that CSN5 is kept in an auto-inhibited state within the CSN complex and that its activation requires the interaction with neddylated CRLs [33]. It has been also observed that, despite being the major cullin deneddyase, CSN5 does not deconjugate hyper-neddylated cullins [33]. On the contrary, the deneddylation enzyme NEDP1 is responsible for the processing of non-cullin substrates [34], but it can also convert the hyper-neddylated cullin in the mononeddylated form [33]. Intriguingly, in *Arabidopsis*, NEDP1 has been shown to be involved in recovering NEDD8 moieties from autoneddylated NAE1 subunits in order to maintain the NEDD8 pathway activity, thus suggesting a more complex involvement of the deneddyase in NEDD8 homeostasis [34, 35]. Finally, NEDD8 peptides and fusion proteins can be also cleaved by USP21 [36], ataxin-3 [37], the parasite hydrolase PfUCH54 [38], UCH-L3 [6], and its orthologue in *S. cerevisiae* Yuh1 [39], all exhibiting a dual specificity for ubiquitin and NEDD8 precursors.

### **3. Structure of the ubiquitin-like protein NEDD8**

Similar to ubiquitin and other UbLs, the structure of NEDD8 consists of a globular core called the  $\beta$ -grasp fold, comprising five-stranded mixed  $\beta$ -sheet and an  $\alpha$ -helix, and a flexible C-terminal tail that projects away from the body of the globular domain and terminates with the Gly-Gly motif (Gly<sub>75</sub>-Gly<sub>76</sub>) [40]. The surface distribution of charges is asymmetric, as in the ubiquitin structure, with a predominantly acidic face and a hydrophobic surface characterized by the Ile44-centered patch, including residues Leu8, Ile44, His68, and Val70. The Ile44 patch, which mediates the recognition of ubiquitin by the majority of the ubiquitin-binding domains, is perfectly conserved in NEDD8.



Only three amino acids differ between the ubiquitin orthologs in *H. sapiens* and *S. cerevisiae*. Similarly, a high conservation is also evident for NEDD8, with approximately 80% identity between the human and yeast orthologs. The sequence alignments of ubiquitin and NEDD8 from yeast to human, performed with protein visualization (ProViz) [41], are shown in **Figure 2A**. Below the alignments, colored boxes that mark identical residues, conservative substitutions, or non-conserved positions represent the sequence conservation degree of ubiquitin and NEDD8 in eukaryotes. In **Figure 2B**, residues that are divergent between the two molecules and not necessarily conserved during evolution within each sequence are highlighted [40]. As shown, positions 4, 12, 14, 31, 63, 64, and 72 share the common feature of being highly conserved in ubiquitin and NEDD8, while differing between the two molecules. Among them, Lys63 in ubiquitin is a substrate for the conjugation of polyubiquitin chains that are involved in several biological processes, primarily “proteasome-independent,” such as inflammatory signal transduction, DNA repair, and endocytosis [42, 43] (**Figure 2B**). Interestingly, unlike most of the lysine residues, which are conserved between ubiquitin and NEDD8, Lys63 is always a Gly in the Ubl, thus indicating that this position has acquired a completely different role in the Ubl. On the contrary, positions 22, 25, 28, 51, and 53, despite being divergent between the two molecules, show a certain degree of variability, to a different extent from one position to the other. For example, position 22 is almost always a Thr in ubiquitin, with the only exception of the Ser22 in *C. intestinalis*, representing a conservative substitution. In NEDD8, a Lys occupies position 22, with the single



**Figure 2.**

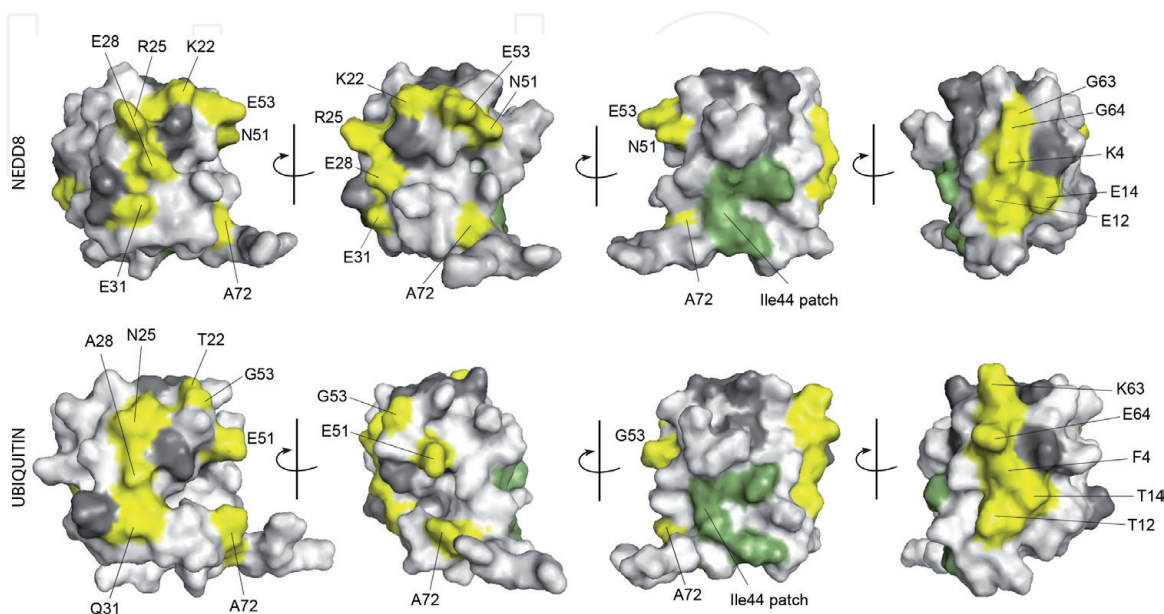
(A) Amino acid sequence alignment of ubiquitin and NEDD8 orthologs performed with the protein visualization (ProViz). The vertical black line indicates the C-termini of the mature proteins. Below the alignment conserved and non-conserved positions are shown as boxes marked with different colors. Invariant positions are shown in light gray; positions having similar chemical properties are marked in dark gray; divergent positions are shown in black. (B) Conservation between ubiquitin and NEDD8 sequences in human. The same colored boxes described in (A) are here represented with the addition of red boxes corresponding to the Lys residues. The conservation is shown below. Invariant positions are shown in light gray; positions having similar chemical properties in dark gray; conserved/divergent positions in yellow, while divergent positions are shown in black. The amino acids occupying the positions that are described in the text are indicated (aa in ubiquitin and aa in NEDD8). Residues included in the hydrophobic patch are in shown in green boxes.

exception of *S. cerevisiae* that has an Arg at that position. The negatively charged Glu in NEDD8 invariably occupies position 28, while in ubiquitin the small nonpolar Ala and Ser or Gln, which are both polar but uncharged, occupy this position. Position 51 is always a Glu in ubiquitin, a negatively charged residue; conversely, in NEDD8 there is almost always an Asn, which is a polar residue, or alternatively an Ala or a Val, which are both nonpolar aliphatic amino acids, suggesting that these positions allow a greater variability compared to the same position in ubiquitin. Finally, position 52 is always a Gly in ubiquitin, a residue that acts as a flexibility source, while a negatively charged residue (Glu or Asp) occupies this position in NEDD8.

The surface distribution of these residues on ubiquitin and NEDD8 is shown in **Figure 3**, together with the hydrophobic patch (shown in green). As previously observed, when mapped on the ubiquitin and NEDD8 surfaces, the conserved/divergent residues are organized in clusters along each side of the molecule [40]. In one cluster, positions 22, 25, 28, and 31 form a line that is characterized by positive (Arg, Lys) and negative (Glu, Glu) charges in NEDD8. In ubiquitin, this region is mainly uncharged, with three polar residues (Thr, Asn, Gln) and the nonpolar aliphatic Ala.

Positions 51 and 53 appear to be in continuity with the residue in position 22 and arranged like the short arm of an “L,” with the cluster constituting the longer arm. This “short arm” is charged/polar in NEDD8 (Glu, Asn) and nonpolar/charged in ubiquitin (Gly, Glu).

The second cluster is positioned at the opposite side of the Ubl like a stripe of aligned residues and includes positions 4, 12, 14, 63, and 64. The chemical nature of this pattern, looking from the top down in **Figure 3**, is nonpolar (Gly, Gly) and charged (Lys, Glu, Glu) in NEDD8 and charged (Lys, Glu), aromatic (Phe), and polar (Thr, Thr) in ubiquitin. Therefore, with the exception of Arg72 which is surrounded by residues that are identical between ubiquitin and NEDD8, the remaining divergent positions are structurally assembled in patterns characterizing different surface regions on NEDD8 and ubiquitin and having chemical features that are distinctive for each molecule. The features of these two groups of residues clearly suggest that they could mediate interactions with different NEDD8-specific partners. Accordingly, together with Ala72, most of these positions have been demonstrated to be involved in the discrimination between ubiquitin and NEDD8 by enzymes that are involved in the conjugation and deconjugation reactions, as described below.



**Figure 3.** Ribbon representation of NEDD8 (upper panels) and ubiquitin (lower panels). The conserved/divergent surfaces and the main amino acid differences are shown.



#### **4. Molecular mechanisms underlining the discrimination between ubiquitin and NEDD8 by neddylation and deneddylation enzymes**

The current knowledge regarding the mechanisms allowing the discrimination between ubiquitin and NEDD8 by neddylation and deneddylation enzymes is summarized in **Figure 2B**. The first key difference concerns position 72, which is the unique divergent residue in ubiquitin and NEDD8 C-terminal tails. As previously described, an Ala in NEDD8 and an Arg in ubiquitin occupy this position. Comparison of the crystal structures of NEDD8 alone [40] and within the complex with NEDP1 [44] or the E1-activating enzyme [45] reveals that, unlike both enzymes, NEDD8 does not undergo large conformational changes upon binding. The main exception is given by significant conformational changes observed in the NEDD8 C-terminal tail. Particularly, in the complex of NEDD8 with UBA3, the last three residues in the flexible tail, which are disordered in free NEDD8, adopt an extended more rigid conformation as it docks into the binding pocket in UBA3. This rearrangement allows the direct interaction of Leu71 and Ala72 with UBA3's residues Leu206 and Tyr207 in the so-called crossover loop, which is essential for the binding preference shown by UBA3 toward NEDD8 [40, 45]. Since all the remaining residues in NEDD8 that contact UBA3 are conserved in ubiquitin, the discrimination between NEDD8 and ubiquitin, at the level of the first step in the neddylation process, is entirely dependent on a single amino acid difference that is sufficient to ensure that ubiquitin is not mistaken for NEDD8. Accordingly, the substitution Ala72Arg can disrupt the specificity of the NEDD8 E1-activating enzyme [45]. On the other hand, it has been ascertained that NEDD8 is qualitatively competent in the interaction with the E1 ubiquitin enzyme. Indeed, the presence of an alanine in position 72 does not cause any repulsion with the binding groove on the E1 enzyme, but the kinetic of the reaction is significantly slower, thus making the NEDD8 thioesterification reaction by the ubiquitin E1 enzyme a kinetically disadvantaged process [40, 46, 47]. This intrinsic difference between process that is clearly prevented and another that is only disadvantaged indicates that NEDD8 performs functions that are strictly specific and not interchangeable. On the other hand, there are biological conditions in which, to a certain extent, NEDD8 can perform the functions typically absolved with ubiquitin. For example, under diverse stress conditions, the ubiquitin E1 enzyme UBE1 can activate NEDD8 [48]. This would suggest that the differences between the two molecules must be sufficient to guarantee their engagement in diverse functional contexts. At the same time, a high degree of similarity ensures a very tight cross talk when specific biological conditions take over.

Once transferred to the E2 enzymes UBC12 or UBE2F, the E3-ligase activity of RBX1/2 promotes cullin neddylation that switches the target preference and activates substrate polyubiquitination. A recent report described the crystal structure representing the RBX1-UBC12~NEDD8-CUL1-DCN1 intermediate and showed the mechanism of NEDD8 ligation to CUL1 by Rbx1 [49]. This study demonstrated that NEDD8 directs the juxtaposition of the UBC12~NEDD8 active site and the CUL1 acceptor site. This specificity is due to the side chain differences in positions 31 and 32, respectively, Glu-Glu in NEDD8, and Gln-Asp in ubiquitin [49]. As expected, at least one of these residues—specifically Glu31 in NEDD8 and Gln31 in ubiquitin—is divergent between ubiquitin and the Ubl. Both residues contact RBX1's Trp35, which is a key position in the NEDD8-binding site. Accordingly, mutation of Trp35 to Ala or to the aromatic residues Tyr and Phe only slightly decreases NEDD8 ligation to a cullin, whereas the Trp35Asp substitution abrogates the interaction with NEDD8 due to the repulsion with NEDD8's Glu31 and 32 [49]. On the other hand, the subtle differences in the side chain length between Glu and Asp in position 32 may be a determinant in the recognition by RBX1. Indeed, while the aliphatic portion of

NEDD8's Glu32 makes hydrophobic contacts with RBX1's Trp35, the latter would repel the ubiquitin's shorter Asp32. Notably, the swapping of residues 31 and 32 in ubiquitin with the corresponding amino acids in NEDD8 is sufficient to promote the ubiquitination of the C-terminal end of CUL1 by the E2 enzyme UBCH5, thus highlighting the crucial role of this pair in the discrimination between NEDD8 and ubiquitin by the CRL component RBX1.

CRL complexes recruit the E2 enzymes UBCH5 and CDC34, together with specific adaptor components, to the ubiquitin-conjugation machinery, and they carry out the substrate ubiquitination [50]. Given its dual capability to conjugate both NEDD8 and ubiquitin, depending on the specific E2 enzyme that is recruited (UBC12, UBE2F, UBCH5, and CDC34), Rbx1/2 constitutes a clear example of a multifunctional RING-ligase. This dual property is shared with other E3 ligases such as Mdm2, c-Cbl, IAPs, and RNF111, indicating that ubiquitination and neddylation of substrates are two closely connected processes.

As previously discussed, a dual specificity is also common among NEDD8 proteases, with few enzymes showing a clear selectivity for NEDD8 (CSN and NEDP1) and the majority catalyzing the C-terminal cleavage of both molecules (such as USP21, PfUCH54, Ataxin-3, UCH-L1, and UCH-L3 [51]). To date, no activity against neddylated cullins has yet been reported for the hydrolases of the second group [52]. Moreover, in addition to its role in cullin deneddylation, NEDP1 appears to control the deneddylation of many non-cullin proteins such as p53, Mdm2, Tap73, BCA3, and E2F1 [51, 53–56].

The NEDD8/NEDP1 structure has been solved by several groups [44, 55, 57]. In these complexes, Ala72 is oriented away from the NEDP1 surface [44, 55] with the majority of the contacts with NEDP1 involving the main chain of the C-terminal tail (Ala72-Gly76) and two amino acid stretches in the NEDP1 enzyme, including residues Trp26-Asp29 and Ala99-Thr101. Like in the case of UBA3 that has been previously described, NEDP1 undergoes a dramatic conformational change upon NEDD8 binding [55]. Conversely, the structure of NEDD8 is a little changed from the native protein [40]. Mutational studies have shown that two key residues, Ala72 and Asn51 (respectively, Arg and Glu in ubiquitin), determine the clear preference of this protease for NEDD8. Interestingly, while the A72R mutant is cleaved more slowly by NEDP1 than the wild-type NEDD8, cleavage is similarly abrogated in the presence of the single-mutation N51E or the double mutant N51E, A72R. Moreover, the mutation R72A in ubiquitin is not sufficient to redirect NEDP1 specificity, while the single-substitution E51N promotes recognition and cleavage of the mutant by NEDP1. Accordingly, the higher catalytic activity of NEDP1 is observed in the presence of the double mutant E51N, R72A. Therefore, at least in this case, the conserved/divergent position 51 seems to be the primary discrimination site for the recognition by NEDP1.

Additional information can be obtained from the work of Yung-Cheng Shin and collaborators. By using a biochemical approach, the authors demonstrated that the discrimination between ubiquitin and NEDD8 by the Ub-specific peptidase USP2 depends on the recognition of the conserved/divergent pattern including residues Phe4, Thr12, and Thr14 together with the C-terminal Arg72. Based on their model, USP2 firstly binds the N-terminus of ubiquitin allowing a stable interaction, which is followed by the recognition of the C-terminus of ubiquitin that ensures the substrate specificity. The relevance of both binding sites in the discrimination process is demonstrated by the observation that the NEDD8 Thr12/Thr14/Arg72 and the NEDD8 Phe4/Thr12/Thr14/Arg72 mutants are both accessible for hydrolysis by USP2, while the ubiquitin Lys4/Glu12/Glu14/Ala72 mutant completely prevents it [58].

Concluding, available data move toward demonstrating that divergences in the NEDD8 sequence, which have been fixed during the evolution of the Ubl, primarily concern the acquisition of binding sites that mediate the recognition by enzymes that

selectively recognize NEDD8. These binding properties are not at all associated with loss of ubiquitin recognition but rather with the acquisition of a potential dual-recognition mode, which could be possibly modulated by the specific asset of binding partners.

## **5. Old and new NEDD8 substrates**

In recent years, it has become increasingly evident that CRLs are the main targets of neddylation, but not the only ones (**Tables 1 and 2**). Indeed, nowadays a broad range of proteins besides cullins are known to be modified by NEDD8, and several non-RBX-family NEDD8 E3 ligases have been characterized, including Mdm2/HDM2, HUWE1, RNF111, c-Cbl, IAP1, and Parkin [59–68]. How neddylation controls protein function is still not fully understood, as well as how these dual specificity ligases distinguish signals promoting neddylation or ubiquitination of the same substrate. Nevertheless, the evidence that many of the reported non-cullin targets include key cell cycle regulators, tumor suppressors, signaling receptors, components of the apoptotic machinery, ribosomal proteins, and histones highlights the potential role for NEDD8 in controlling diverse cancer-related processes and the urgency of reaching a deeper understanding of these regulative mechanisms. Moreover, the auto-neddylation of several ubiquitin E3 ligases have revealed a more complex level of regulation of these enzymes, demonstrating that our knowledge about the spectrum of processes that are cooperatively regulated by ubiquitin and Ubls is still largely incomplete. A detailed description of the novel NEDD8 targets has been already carried out by others [23, 30, 69, 70]. Here, we recapitulate the current knowledge regarding the effects of E3-ligase neddylation and the involvement of NEDD8 in the cellular stress response.

### **5.1 General overview**

Similar to other posttranslational modifications, neddylation causes the structural modification of target proteins, thus affecting the enzymatic activity, the interaction with binding partners, and/or the subcellular localization. In addition to the well-known role in the activity of CRL complexes, NEDD8 has been shown to be also involved in the regulation of several transcription factors, by modulating their intracellular distribution and/or transcriptional activity. By comparing the effects that are associated to the conjugation of NEDD8 to non-cullin targets, the most frequent consequence of neddylation is a switch in protein stability, with neddylated targets usually being stabilized by the conjugation of the Ubl. For example, neddylation of p53 prevents the nuclear translocation and inhibits p53 transcriptional activity [59]. This effect is also common to TAp73 $\beta$ , BCA3, the ribosomal proteins L11 and L14, HBx, HuR, TGF $\beta$ RII, and Pink1 55 kDa fragment. Conversely, ITCH neddylates its substrate JunB and promotes its ubiquitin-dependent degradation, thus attenuating its transcriptional activity [71]. Differently from cullins that are neddylated on a single conserved lysine residue, all these novel substrates have been shown to be ubiquitinated as well as neddylated at multiple residues, which are in most cases overlapping. The only known exception is given by BRAP2 in which the neddylation site Lys432 is within an amino acid sequence that resembles the consensus neddylation sequence conserved in all cullin family proteins [72]. Consequently, while ubiquitination of these residues promotes the proteasomal degradation of target proteins, the addition of NEDD8 moieties to the same residues prevents substrate degradation. In line with this effect, the insulation of the substrate from the degradative pathway is frequently associated with the relocalization in a compartment in which the



<b>E3-ligase</b>	<b>E3-ligase function</b>	<b>Target</b>	<b>Substrate function</b>	<b>Effect of neddylation</b>	<b>Ref.</b>	
MDM2	E3 ubiquitin ligase localized in the nucleus. Targets tumor suppressor proteins for proteasomal degradation	MDM2			Neddylation promotes MDM2 protein stabilization and is reverted by NEDP1 activity. Cysteine C462, which is required for the E3 ubiquitin ligase activity of Mdm2, is also required for Mdm2-dependent neddylation.	[51, 59]
		p53	Short-lived tumor suppressor protein having transcriptional activity that responds to diverse cellular stresses	Neddylation of p53 inhibits its transcriptional activity and causes nuclear localization	[59, 153, 154]	
		TAp73 $\beta$	Member of the p53 family involved in cellular responses to stress and development	Neddylation of TAp73 promotes cytoplasmic localization and inhibits its transcriptional activity	[53]	
		VHL	Component of the E3-ligase complex including elongin B, elongin C, and cullin-2. It is involved in the ubiquitination and degradation of HIF1 $\alpha$	Neddylation of VHL promotes binding to fibronectin and prevents the incorporation of VHL within a CRL2 complex	[74, 75]	
		L11	Ribosomal protein component of the 60S subunit. It is located in the cytoplasm	Neddylation protects L11 from degradation by ensuring its nucleolar localization. Neddylation is reverted by NEDP1 activity	[115, 155, 156]	
		L14	Ribosomal protein component of the 60S subunit. It is located in the cytoplasm	Neddylated RPL14 localizes in the nucleolus. Neddylation is reverted by NEDP1	[115, 155]	
		MDMX	Structural homolog of MDM2 lacking ligase activity. In complex with MDM2 binds the transcriptional activation domain of p53 and inhibits its activity. It inhibits MDM2 degradation		[84]	
HDM2	Human homolog of MDM2	HBx	Transcriptional activator that modulates the expression of HBV and inflammatory genes	Neddylation enhances HBx stability by inhibiting its ubiquitination and promotes chromatin localization. Neddylation of HBx is reverted by NEDP1 activity	[60]	
		HuR	RNA-binding protein that selectively binds AU-rich elements (ARE) and stabilizes ARE-containing mRNAs.	Neddylation promotes HuR nuclear localization and protection from degradation. Deneddylation by NEDP1 reduces the nuclear localization of HuR	[61]	

E3-ligase	E3-ligase function	Target	Substrate function	Effect of neddylation	Ref.
FBXO11	Subunit of the ubiquitin-protein ligase SKP1-cullin-F-box (SCF)	p53		Neddylation of p53 inhibits its transcriptional activity	[86]
c-Cbl	RING finger E3 ligase acting as negative regulator of signal transduction. It interacts with Y-phosphorylated substrates and targets them for proteasome degradation	EGFR	Transmembrane glycoprotein with kinase activity that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation	NEDD8 conjugation enhances ligand-induced ubiquitination of the EGF receptor and clathrin-mediated endocytosis for lysosomal degradation.	[64]
		TGFβRII	Kinase that forms a heterodimeric complex with TGFβRI. Once activated by TGF-β, the complex phosphorylates target proteins, which enter the nucleus and regulate the transcription of several genes	Neddylation protects the receptor from ubiquitination by promoting the clathrin-mediated endocytosis of TβRII into EEA1-positive early endosomes. Neddylation is reverted by NEDP1	[118]
RBX1/2 (alias RNF7)	RING finger protein playing a key role in CRL complexes	CRL1, CRL2, CRL3, CRL4, CRL5	Cullins are scaffold proteins of CRL complexes that control the stability of proteins with diverse functions	Neddylation enhances the activity of CRLs and subsequent ubiquitination and degradation of the regulated substrates	[11, 21]
XIAP (alias IAP3)	RING-ligase is a negative regulator of apoptosis pathway	XIAP			[65, 157]
		Caspase-7	Cysteine-aspartic acid protease acting in the execution phase of cell apoptosis. Casp7 is activated upon cell death stimuli	Neddylation suppresses caspase activity, while deneddylation is required to execute apoptosis	[65, 157]
DIAP	DIAP is the <i>Drosophila</i> homolog of hIAP1	DIAP		Auto-neddylation of DIAP does not seem to affect its E3-ligase activity	[65]
		Drice	It is involved in the activation cascade of caspases in <i>Drosophila melanogaster</i>	Neddylation inhibits its proteolytic activity. The inhibitory effect is reverted by NEDP1	[65]
CRL2	E3 ubiquitin-protein ligase consisting of elongin C, elongin B, RBX1, cullin 2, and an E2 ubiquitin-conjugating enzyme	HIF1a	HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes, including those involved in energy metabolism, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia	The effect of neddylation on HIF-1 stability is contradictory. It has been suggested that the neddylation process is required for the ubiquitination and subsequent degradation of HIF-1	[79]

<b>E3-ligase</b>	<b>E3-ligase function</b>	<b>Target</b>	<b>Substrate function</b>	<b>Effect of neddylation</b>	<b>Ref.</b>
SMURF1	E3 ligase that is specific for receptor-regulated SMAD proteins in the bone morphogenetic protein (BMP) pathway	SMURF1		The auto-neddylation of SMURF1 stimulates the activation of its ubiquitin E3 ligase and the degradation of substrates. Neddylation of SMURF1 is reverted by NEDP1 activity	[66]
RSP5	Homolog of SMURF1 in <i>S. cerevisiae</i>	RSP5		Neddylation of Rsp5 is important for the Rsp5-mediated ubiquitylation of Vps9	[66]
RNF111	Nuclear RING E3 ligase promoting the ubiquitination and proteosomal degradation of inhibitor SMADs in the TGFβ/ NODAL signaling pathway	Histone H4	Basic nuclear protein that is a component of the nucleosome structure	DNA damage causes an increase in RNF111-mediated H4 neddylation and the recognition of the NEDD8 chains by RNF168, which is required for the subsequent recruitment of the latter to the sites of DNA damage	[63]
RNF168	RING finger E3 ligase involved in DNA double-strand break (DSB) repair	Histone H2A	Basic nuclear protein that is a component of the nucleosome structure	DNA damage causes a decrease in H2A-neddylation that allows its ubiquitination and the redistribution of RNF168 from H2A to H4	[63, 145]
TRIM40	Member of the tripartite motif (TRIM) protein family that plays a role as a negative regulator against inflammation and carcinogenesis	IKKγ	IKKγ (alias NEMO) is the regulatory subunit of the inhibitor of kappaB kinase (IKK) complex, which activates NFκB resulting in activation of genes involved in inflammation, immunity, cell survival, and other pathways	TRIM40-mediated neddylation of IKKγ inhibits the transcriptional activity of NFκB. Neddylation has been proposed to oppose the activation of IKKγ mediated by conjugation of K63-linked and linear polyubiquitin chains	[143]
FANCA	Member of the FANC complementation group, a nuclear protein complex required for the monoubiquitination and relocalization of FANCD2 to nuclear foci in response to DNA damage	CXCR5	Cytokine receptor that is expressed in mature B cells and Burkitt's lymphoma. Binds to B-lymphocyte chemoattractant and is involved in B-cell migration	Neddylation of CXCR5 receptor promotes membrane localization and is required for cell motility and migration of B lymphocytes to the germinal centers in response to its ligand, CXCL13	[158]

**Table 1.**  
*Neddylated substrates and E3 ligases responsible for the modification.*

proteasomal degradation is precluded. NEDD8-conjugated proteins predominately reside in the nucleus, thus supporting the idea that nuclear compartmentalization is a feature frequently associated with the neddylation process. Examples are the Mdm2-mediated neddylation of p53, which causes its nuclear localization [59], while p53 degradation is mainly cytoplasmic. Neddylation of the ribosomal proteins L11



E2F-1	Transcription factors playing a crucial role in the control of cell cycle and tumor suppressor proteins. E2F binds to retinoblastoma protein pRB in a cell cycle-dependent manner. It can mediate both cell proliferation and p53-dependent/p53-independent apoptosis	Neddylated negatively regulates E2F-1 activity. The effect is reverted by the action of the NEDP1 enzyme or upon DNA damage. Neddylated sites are also targets of methylation	[54, 78]
BRAP2	Cytoplasmic RING E3 ligase which may regulate nuclear targeting by retaining proteins with a nuclear localization signal in the cytoplasm. It binds to the nuclear localization signal of BRCA1 and other proteins	Unknown	[72]
AICD	Fragment produced by $\gamma$ -secretases; it binds to the transcriptional coactivator Fe65 and forms a complex in conjunction with Tip60 to activate the transcription of a target genes	Neddylated of AICD inhibits its interaction with FE65 and Tip60 transcriptional co-regulator, thus resulting in the impairment of AICD-Fe65-Tip60 complex formation	[159]
Parkin	Component of a multiprotein E3 ubiquitin ligase complex that mediates the targeting of substrate proteins for proteasomal degradation	Neddylated promotes ubiquitin E3-ligase activity toward synphilin-1 and increases Parkin auto-ubiquitination	[67, 68]
Pink1	Serine/threonine protein kinase that localizes to mitochondria. It is thought to protect cells from stress-induced mitochondrial dysfunction	PINK1 neddylated increases the stability of the PINK1 55 kDa fragment, a processed form of PINK1 that is found in the cytoplasm and forms a complex with Parkin	[67]
BCA3 (alias AKIP1)	Nuclear protein that interacts with protein kinase A catalytic subunit and regulates the effect of the cAMP-dependent protein kinase signaling pathway on the NF-kappa-B activation cascade	BCA3 neddylated suppresses NFkB transcription via its ability to associate with nuclear p65. Moreover, neddylated BCA3 associates with cyclin D1 promoter	[56]

**Table 2.**  
Neddylated substrates for which the E3 ligase is unknown.

and L14 ensures their localization in the nucleolar compartment, protecting L11 from degradation occurring in the nucleus. Relocalization following neddylated is also observed for TAp73 $\beta$  that, unlike p53, accumulates in the cytoplasm [53]. Moreover, the RNA-binding protein HuR relocalizes in the nucleus following Mdm2-mediated neddylated, and it is protected from degradation. Pink1 55 kDa fragment is stabilized following neddylated and relocalizes from mitochondria to the cytoplasm, where it forms a complex with Parkin [67]. If NEDD8 conjugation promotes the relocalization of the target protein, thus it is plausible to assume that the differential intracellular distribution would be dependent on the recognition of the NEDD8 modification by shuttling proteins (spatial sequestration). Interestingly, the majority of the transcription factors that are neddylated by the same E3 ligases responsible for their ubiquitination show reduced transcriptional activity and intracellular relocalization. A molecular switch affecting the enzymatic activity (conformational inactivation) could explain this effect. However, neddylated may also control the differential incorporation in complexes that, similarly to the case of shuttling proteins, drive the transcriptional activity mediated by the neddylated targets toward different promoters (selective recruitment), thus inhibiting the main downstream effects and

promoting alternative pathways that must be activated in specific cellular conditions. The observation that transcription factors such as p53 are also involved in cytoplasmic processes like endocytic degradative pathways of membrane receptors and actin remodeling [73] supports the notion that differential posttranslational modifications could be key events underlying triggering of alternative pathways. As an example, the Hdm2-mediated neddylation of hepatitis B virus X protein (HBx), a transcriptional activator that modulates the expression of hepatitis B virus (HBV) genes and inflammatory genes, enhances HBx stability and promotes chromatin localization, which in turn favors HBx-dependent transcriptional regulation, cell proliferation, and HBV-driven tumor growth [60].

Another example of switch mediated by target neddylation is given by the Von Hippel-Lindau (VHL) tumor suppressor [59, 74]. VHL is a component of the VHL tumor suppressor-containing E3 ubiquitin ligase complex (ECV), a class of CRL2 complexes that controls the stability of the transcription factor hypoxia-inducible factor 1- $\alpha$  (HIF1 $\alpha$ ) upon hypoxic conditions [75]. The function of VHL is to recruit the substrate HIF1 $\alpha$  by directly interacting with the  $\alpha$ -subunits of the transcription factor, thus promoting its degradation by the CRL2 complex. Interestingly, VHL can also interact with fibronectin and promote extracellular matrix assembly [76]. The switch between these two functions (negative regulator of HIF1 $\alpha$  and positive regulator of fibronectin) is regulated by the neddylation of VHL. Indeed, NEDD8 conjugation precludes ECV complex formation by steric hindrance, causing the exclusion of CUL2 from the complex and the stabilization of HIF1 $\alpha$ . At the same time, neddylation of VHL confers the ability to interact with fibronectin and to promote extracellular matrix assembly [75].

Neddylation of several membrane receptors has been recently described. The EGFR and TGF $\beta$ RII have been shown to be neddylated by c-Cbl, a RING E3 ligase containing an N-terminal phosphotyrosine-binding domain, which allows it to interact with numerous tyrosine-phosphorylated substrates, targeting them for proteasomal degradation. Following NEDD8 modification, two opposite effects have been described: while EGFR degradation is promoted following ligand stimulation, neddylation of TGF $\beta$ RII reduces the receptor degradation rate by shifting the receptor internalization from caveolae to clathrin-coated pits. In both cases, neddylation of these receptors results in the inhibition of proliferation and cell cycle arrest. Therefore, despite promoting different outcomes, the resulting effects of neddylation converge toward the same signaling output. Shc, an adaptor that transduces signals from several receptor tyrosine kinases and cytokine receptors, has been recently identified as a NEDD8 target. Neddylation of Shc promotes downstream signaling by favoring the formation of a ZAP70-Shc-Grb2 complex that is required for downstream Erk activation [77].

It must be pointed out that several reports analyzed the neddylation of substrates using an experimental approach that is based on the transient overexpression of tagged NEDD8, which is known to cause nonphysiological effects due to “atypical” neddylation of substrates [47]. This is particularly relevant in those papers in which the E3 ligase responsible for target neddylation has not been identified [78–80]. Nevertheless, the parallel use of complementary approaches, such as knockdown or inhibition of APP-BP1 activity, the use of a dominant-negative mutant of Ubc12, and NEDP1-mediated deneddylation, supports the main statement and encourages in the direction of deepening the unexpected functions of neddylation and how ubiquitin and Ubl conjugations are combined in modulating biological processes.

## 5.2 The Mdm2/p53 pathway

In unstressed conditions, Mdm2/Hdm2, which heterodimerize with MdmX, mainly controls the levels of the transcription factor p53. Mono-ubiquitination of

p53, promoted by Mdm2 low levels, is sufficient for nuclear export, whereas high levels of Mdm2 activity induce p53's polyubiquitination and proteasome-dependent degradation [81]. Several evidences suggest that neddylation, promoted by Mdm2, regulates p53 signaling at different levels. Under DNA damage conditions, the phosphorylation of Mdm2 and MdmX by c-Abl promotes the association of the heterodimer, with Mdm2 acting as a ubiquitinating enzyme that destabilizes MdmX resulting in p53 stabilization [82–84]. On the contrary, in growing conditions under c-Src activity, Mdm2 acts as neddylation enzyme that neddylates p53, Mdm2 itself, and MdmX [84, 85]. Neddylation of p53 prevents the nuclear translocation and inhibits p53 transcriptional activity; in contrast the neddylation-resistant mutants retain the transcriptional activity [59].

Interestingly, also the F-box protein FBX011, a component of the CRL1 complex, promotes p53 neddylation by inhibiting p53 transactivation function. Nevertheless, differently from Mdm2, it does not ubiquitinate p53 nor affect its stability [86]. Deneddylation of p53 is promoted by two different mechanisms: the activity of NEDP1 [51] and the interaction of p53 with NUB1, a NEDD8-interacting protein that acts as negative regulator of NEDD8 and neddylated proteins [87]. NUB1 associates with neddylated p53, decreases p53 neddylation, and stimulates p53 mono-ubiquitination, resulting in p53 nuclear export. Recently, it has been found that NUB1 is a target of Mdm2-mediated ubiquitination. Instead of representing a degradative signal, Mdm2 conjugates a di-ubiquitin signal on NUB1 that is thought to be necessary for exposing the NEDD8-binding site following the intramolecular recognition between the UBA domain of NUB1 and the di-ubiquitin signal [87].

### **5.3 Dual E3 enzymes: insights into the regulation of HECT and RBR ligases**

Based on their structural organization and conjugation mechanism, E3 enzymes are commonly grouped into three classes. Really interesting new gene (RING) E3s act as allosteric activators of E2 that promotes the transfer of ubiquitin from the E2 to the target protein. Homologous to E6AP C-terminus (HECT)- and RING-between-RING (RBR)-type ligases, although being structurally distinct, require an intermediate step where the ubiquitin is first transferred from the E2 to an active-site cysteine residue on the E3 ligase to be then conjugated to the target protein [88, 89]. The NEDD4 subfamily is the best-characterized family of the HECT E3s. It contains an N-terminal C2 domain that binds Ca<sup>2+</sup> and phospholipids followed by two up to four WW domains, responsible for the substrate recognition [90, 91]. Auxiliary or inhibitory factors, respectively, facilitate or interfere with the substrate recruitment [91–93]. A distinct level of regulation instead affects the HECT domain and depends on intramolecular interactions between the HECT and the C2 domains that keep the E3 ligase in an auto-inhibited conformation. Typically, signals that trigger the addition of phosphorylations along the amino acid chains promote relief of the inhibited state and substrate recruitment [94]. In the only case of SMURF1, homo-dimerization inhibits the catalytic domain by promoting its association with the C2 domain of the partner in the dimer; the relief is achieved by the interaction with adaptor proteins including CKIP, CDH1, or CCM2 [95–97]. Among the PTMs that are known to regulate HECT E3 ligases, auto-ubiquitination, together with the reverse reaction promoted by deubiquitinating enzymes, defines the rate of HECT downregulation mediated by the proteasomal degradation [98]. Interestingly, a non-covalent interaction of the HECT domain with ubiquitin has also been reported, at least for Rsp5 and NEDD4 [99, 100]. The ubiquitin-binding site, called “exosite,” is located in the N-terminal lobe of the HECT domain of these E3 ligases and contacts the hydrophobic patch residues of ubiquitin. Disruption of the interaction, obtained by mutagenesis of the HECT-/ubiquitin-binding interface, resulted in defects in the



substrate polyubiquitination, leading to the interpretation that the N-lobe ubiquitin-binding site promotes the elongation of the ubiquitin chain by orienting the distal end of the ubiquitin chain in the optimal position for the next conjugation event.

In addition to ubiquitination, also neddylation regulates E3-ligase activity. Indeed, it has been shown that several E3 ligases have neddylation activity and are regulated by auto-neddylation. Typically, neddylation promotes both the activity toward substrates and the increase in auto-ubiquitination, leading to ubiquitination and subsequent degradation of the ligase. The first ubiquitin E3 ligases identified as subjected to such regulative mechanism were Mdm2, Parkin, and XIAP, all belonging to the RING-ligase family. Auto-neddylation has been then demonstrated to regulate also SMURF2, SMURF1, and its homolog in *S. cerevisiae* RSP5 [66, 101]. Even though the details of the activation mechanism have not been yet clarified, it is interesting to observe the active site responsible for SMURF1 auto-neddylation maps in the HECT N-lobe (Cys426), thus being different from the ubiquitination catalytic cysteine typically located in the C-lobe (Cys716). This spatial separation underlies the potential autonomy of the NEDD8-regulated mechanism. Conversely, the homolog RSP5 uses the same catalytic site Cys777 both for neddylation and ubiquitination [66]. A similar mechanism could also involve other members of the HECT family, such as ITCH, NEDL1, and NEDL2, but not in NEDD4.1 and NEDD4.2 [66]. Moreover, in addition to promoting its auto-neddylation, ITCH neddylates its substrate JunB and attenuates its transcriptional activity by promoting JunB ubiquitination-dependent degradation. This catalytic mechanism requires the same Cys residue (Cys830) that is involved in the ubiquitination reaction [71]. Whether the remaining HECT-type ubiquitin ligases can also catalyze the neddylation of protein substrate remains to be experimentally verified.

Interestingly, together with the capability to conjugate NEDD8, a Ubl non-covalent-binding site has been identified in SMURF2, and it has been shown to be conserved also in SMURF1 [102] (see below). The evidence that, following mutations that disrupt the non-covalent interaction with NEDD8, SMURF neddylation is reduced and the E3-ligase stabilized due to reduced self-ubiquitylation highlights the importance of this interaction in regulating SMURF enzymatic activity. Accordingly, NEDD8 binding to SMURF plays important roles in the regulation of cell migration and in the BMP and TGF $\beta$  signaling pathways, both biological processes in which SMURFs are known to play a key role [102].

#### **5.4 Neddylation in the nucleolar stress response**

In eukaryotes, ribosomes are preassembled in pre-ribosomal particles in a subnuclear compartment called nucleolus and then transferred to the cytoplasm where the assembly of the two subunits takes place. The limiting factor of ribosome assembly is the rate of rRNA transcription in the nucleus; therefore the formation and accumulation of unusable components are minimized through the rapid ubiquitination and proteasome-dependent degradation of free ribosomal proteins [103]. Since ribosome biogenesis is a complex and highly resource-consuming process, several control mechanisms are in charge of monitoring any insult that causes activation of stress signaling, thus leading to rapid repression of protein synthesis and ribosome biogenesis in response to stress, as well as rapid derepression in response to improved conditions. The nucleolus, long known primarily for its role in ribosome biosynthesis, has assumed a function in recent years as a sensor for those cellular stresses that lead to the impairment of ribosome biogenesis homeostasis. This function has led to the coining of the term “nucleolar stress” or “ribosomal stress,” highlighting that perturbations in ribosome biogenesis are accompanied by morphological changes, functional defects, and eventually disruption of nucleolus. The nucleolus is in continuity with the surrounding nucleoplasm, so that

any soluble molecule is potentially free to traffic between the nucleolus and the nucleoplasm. Accordingly, several proteins enriched in the nucleolus are frequently shuttled between the nucleolus and the nucleoplasm. Among them, nucleophosmin (NPM1), an abundant nucleolar protein, is massively moved to the nucleoplasm and cytoplasm following various cellular insults, so that its redistribution upon stress has been set as indicator of nucleolar stress [104].

Nucleolar stress is induced by various stressors such as heat shock, chemotherapeutic agents, UV, starvation, etc., resulting in the activation of both p53-dependent and p53-independent signaling pathways and cell cycle arrest. The p53-dependent response relies on the disruption of the interaction between p53 and Mdm2/Hdm2, which results in p53 stabilization and activation. For example, NPM1 undergoes S-glutathionylation upon nucleolar oxidative stress, translocates to the nucleoplasm, and disrupts the p53-Hdm2 interaction [105]. Interestingly, over the last years, several studies have shown that most of the p53-dependent but also p53-independent stress responses are dependent on the activity of free ribosomal proteins (RPs) that have been shown to perform multiple extra-ribosomal functions, including regulation of apoptosis, cell cycle arrest, cell proliferation, and DNA damage repair [106, 107]. In response to nucleolar stress, the RPs L5, L11, and L23 bind to Mdm2 and block Mdm2-mediated p53 ubiquitination and degradation, leading to p53 stabilization and activation [108–111]. Similarly, L5, L11, and L23 have been shown to bind and suppress c-Myc and E2F-1, two transcription factors that, respectively, control ribosome biogenesis and promote cell proliferation, thus leading to p53-independent inhibition of cell proliferation [112–114]. Interestingly, also the switch between the ribosome biogenesis pathway and the incorporation of free ribosomal proteins within transcription factor complexes is regulated by neddylation. Indeed, it has been shown that Mdm2 promotes the neddylation of L11, which accumulates in the nucleolus, thus protecting it by the ubiquitin-dependent degradation in the nucleoplasm. Under nucleolar stress, a decrease in L11 neddylation promotes its nucleoplasmic relocalization, where it binds and inhibits Mdm2. This not only provides a trigger for p53 activation but also makes L11 susceptible to ubiquitin-mediated degradation. Analogously to L11, the ribosomal protein L14 binds to Hdm2 and inhibits Hdm2-mediated p53 polyubiquitination and degradation, thus increasing p53 stability and activity. Upon neddylation, L14 associates with hCINAP, which in turn recruits NEDP1 on L14. Deneddylation by NEDP1 promotes the release of L14 from the complex and the relocalization of L14 to the nucleoplasm [115].

Recently, a novel function of NEDD8 in proteotoxic stress has been identified [62]. Exposure of cells to heat shock, proteasome inhibitors, or oxidative stress increases neddylation through an enzymatic chain that requires the ubiquitin E1-activating enzyme UBA1 instead of the NEDD8-activating E1 enzyme. It also involves the formation of hybrid NEDD8/ubiquitin conjugates [47, 48]. The authors demonstrated that NEDD8 promotes the transient and reversible nuclear protein aggregation during proteotoxic stress and colocalizes with ubiquitin in the nuclear aggregates but not in the cytoplasmic aggresomes. These nuclear aggregates, in which RNA transport and ribosomal proteins account for almost half of the total components, require the activation of NEDD8 by UBA1, as demonstrated by the observation that a drastic decrease of protein aggregation follows treatment of cells with the specific UBA1 inhibitor MLN7243 but not the NAE1 inhibitor MLN4924 [62]. Interestingly, even though they are also enriched in RPs, the NEDD8/ubiquitin nuclear aggregates are distinct from the stress-induced nucleolar structures. A proteomic study identified the HECT E3-ligase HUWE1 as a component of nuclear aggregates, and immunofluorescence and biochemical assays strongly suggested that HUWE1 is the major E3 ligase that specifically promotes neddylation during proteotoxic stress [62]. Accordingly, depletion of HUWE1 does not affect the

global ubiquitination or the amount of ubiquitin staining in the nuclear aggregates while causing a dramatic decrease in neddylation, thus compromising the NEDD8-mediated cellular response to proteotoxic stress. Moreover, HUWE1 has been also reported to be a neddylation substrate in a proteomic analysis [116]. Finally, given that ubiquitin-NEDD8 mixed chains are resistant to proteasome degradation, the stress-induced aggregation of substrates modified with both molecules could be intended as a way to sequester a certain group of proteins from the ubiquitin-mediated degradative route, in order to promptly restore their functions once the external conditions return to be favorable to cellular growth and proliferation.

Concluding, neddylation is clearly emerging as an important regulator of several pathways, governing proliferation, differentiation, and survival. However, despite the importance of this posttranslational modification in cell biology, the mechanism through which neddylation controls protein properties is still a matter of debate. Neddylation influences the stability of the target protein, but it also determines its intracellular distribution by promoting the interaction with shuttling proteins. Most of all, neddylation seems to be involved in cellular stress signaling, by providing a regulatory mechanism that acts like a “switch,” allowing the activation of alternative pathways dictating the key steps of the cellular stress response. This switch is promoted by the disruption of protein complexes acting in unstressed conditions and the assembly of new complexes in which seemingly secondary functions of key components become decisive for the correct signaling output.

## **6. NEDD8 chains and mixed ubiquitin-NEDD8 chains**

Traditionally, neddylation consisted in the addition of a single NEDD8 molecule to a target protein. Nevertheless, a significant amount of experimental evidences gradually emphasized that the neddylation signal, as also happened for ubiquitin, was more detailed and complex than initially thought.

The first argument is the evidence that several lysine residues in NEDD8 can be used for chain extension [116] and that, at least in vitro, poly-NEDD8 chains can be synthesized [117]. Moreover, evidence that NEDD8 chains can be generated in vivo has been recently recovered, even though the biological significance of poly-neddylation remains unknown [116]. The second point that supports the existence of poly-neddylation is the evidence that high-molecular-weight NEDD8 conjugates have been observed through affinity purification and mass spectrometry [54, 67, 68, 118]. Again, whether these conjugates are the result of poly-neddylation or correspond to mono-neddylation events remains to be elucidated.

Finally, clear experimental evidence demonstrated that, upon proteotoxic stress, proteins are simultaneously modified by NEDD8 and ubiquitin mixed chains [48]. One possible explanation is that NEDD8 acts as a ubiquitin substitute, but, being less easily ubiquitinated, it caps the ubiquitin chain and prevents the excessive extension, a cellular response that could be a key event to avoid a further escalation of the proteotoxic stress, especially in those conditions in which the ubiquitin pool is depleted. Another possibility is based on the evidence that several ubiquitin-binding domains have been shown to interact poorly with NEDD8 [119] (see below). Based on this assumption, protein domains that recognize ubiquitin chains are disfavored when dealing with mixed NEDD8-ubiquitin chains. Consequently, the conjugation of these signals would trigger the exclusion of binding partners that transduce the ubiquitin signal under unstressed conditions, for example, favoring the interaction with protein domains that specifically recognize these mixed chains. This interpretation is consistent with the model that sees neddylation as a switch in the outcome of unstressed/stresses responses. Moreover, several proteomic studies have reported



phosphorylation, acetylation, and succinylation sites on NEDD8 [120–122]. One could speculate that mixed chains are assembled and further modified in order to “create” an entirely new signal, which goes well beyond the simple random mix between ubiquitin and NEDD8. In order for poly-neddylated and mixed NEDD8-ubiquitin signals to be effective, however, the existence of a system that unambiguously discriminates between different combinations of two strictly similar molecules should be assumed. The identification of protein domains capable of recognizing unusual combinations of these signals and the need to clarify to what extent known NEDD8-binding proteins are capable to do it are research topics required for a deeper comprehension of the cross talk between these two posttranslational modifications.

## **7. Ubiquitin-binding domains: a general overview**

The first ubiquitin receptors to be identified were intrinsic components of the proteasome that directly bind polyubiquitin chains driving the recruitment and the degradation of the substrates [123–125]. Subsequently, polyubiquitin-binding domains have been also found in mobile shuttling factors that direct polyubiquitinated proteins to the proteasome, and, in a relatively short space of time, it has been clear that ubiquitin functions spread in modern eukaryotes to play key roles in several cellular processes. Many of these functions are mediated by the association with ubiquitin-binding domains (UBDs), which usually bind to ubiquitin only weakly [126]. Nevertheless, several mechanisms work together in order to increase both affinity and avidity of the recognition. For example, specific binding can be greatly enhanced by polymerization of the ubiquitin signal, in the form of homologous, mixed, and branched ubiquitin chains. In this way, multiple surfaces can be generated on a target protein, thus increasing the number of different UBDs that can simultaneously recruit the substrate. Moreover, the arrangement in tandem of more UBDs along the same amino acid chain increases the contact sites between the substrate and the ubiquitin receptor.

To date, at least 20 structurally distinct UBD classes have been characterized that vary considerably in size (from ~30 to 150 residues) and tertiary structure [126]. They interact with ubiquitin monomers or chains, usually recognizing diverse ubiquitin signals. UBDs that bind to ubiquitin chains are often selective for a specific type of chain linkage. This selectivity may arise from the recognition of a unique orientation of the chain and distinct surfaces on the ubiquitin moieties or via direct interaction with the linker region connecting the two ubiquitins. Ubiquitin/UBD interactions have been thoroughly reviewed in recent years [126–128]. The canonical ubiquitin/UBD complex requires the recognition of the Ile44-centered hydrophobic patch in ubiquitin, including residues Ile44, Val8, His68, and Val70. Residues peripheral to the hydrophobic patch are also important for UBD binding. Indeed, distinct structural motifs have been shown to recognize the hydrophobic patch in a variety of orientations that can be attributed to specific contacts between each UBD and residues surrounding the hydrophobic patch on ubiquitin. Accordingly, mutations outside the hydrophobic patch that disrupt one ubiquitin/UBD pair do not necessarily affect the recognition by another domain [129]. In some cases, however, Ile44 does not constitute the “center” of the interaction site and contributes only marginally to the binding affinity of an interaction [130]. Moreover, although the hydrophobic patch plays a role in many interactions with UBDs, other regions can solve this function, among them the Ile36 patch (Leu8-Ile36-Leu71-Leu73), the C-terminal end (Gly75-Gly76), and the surface including residues of and around the flexible loop between  $\beta$ 1 and  $\beta$ 2 and Lys6 and Lys11. The Ile36-centered hydrophobic patch is the contact site for a relatively small number of binding partners.

Among them, contacts between the E3 enzyme and the Ile36 patch of the ubiquitin moiety conjugated to the E2 have been shown to favor the ubiquitin transfer [131]. Interestingly, due to the close proximity of different binding sites in ubiquitin, multiple contact sites can increase the interaction affinity for a given partner [132].

Like ubiquitin, the NEDD8 backbone is relatively rigid, although slight structural changes occur during binding. On the contrary, the C-terminus of both proteins is highly flexible in solution and adopts a more rigid conformation only following the interaction with the enzymes responsible for the conjugation process [44, 45]. Finally, while the main contact sites (Ile44-patch, Ile36 patch, and  $\beta$ 1- $\beta$ 2 loop) are well conserved in NEDD8, polar and charged surfaces show a greater differentiation, and they are expected to influence specific binding properties [40].

## 8. NEDD8 interacting partners

Small ubiquitin-like modifier (SUMO), which is the Ubl that is more similar to ubiquitin after NEDD8, shows only 17% of identity and, similarly to NEDD8, is involved in many cellular processes where it exerts non-proteolytic functions. The conjugation of SUMO leads to consequences that are similar to NEDD8 conjugation. For example, sumoylation of transcription factors and histones is generally associated with decreased gene expression and intracellular relocation. Moreover, SUMO and ubiquitin can compete for the same target lysines [133]. Both a sumoylation consensus motif and a non-covalent SUMO-interaction/SUMO-binding motif have been identified in selected SUMO enzymes, targets, and downstream effectors [134]. Despite a certain degree of cross talk between ubiquitin and these Ubls in several biological processes, it seems that NEDD8 and SUMO have been subjected to a very different evolutionary pressure, which have led to a clear pattern for the SUMO recognition by a specific set of binding partners, while maintaining a strong overlap in the recognition of NEDD8 versus ubiquitin. This “ambiguity” reflects the biological need to maintain a high degree of identity between ubiquitin and NEDD8, with few well-defined differences. But it also leaves open the question of whether neddylation is always functionally distinct from ubiquitination.

Despite the limited understanding of the NEDD8 interaction network, recent advances support the assumption that, similarly to ubiquitin, specific NEDD8-binding proteins recognize neddylated substrates acting as downstream effectors (**Table 3**). As shown, in most cases these proteins recognize non-cullin NEDD8-conjugated substrates or NEDD8-containing chains. In some cases they interact with NEDD8 only when conjugated to cullins, and, in a few cases, they also recognize free NEDD8.

Besides the enzymes of the neddylation pathway that has been previously discussed, the first NEDD8-binding protein to be identified was the negative regulator of the NEDD8 pathway NUB1 and its splicing variant NUB1L [135–138]. Following overexpression, NUB1 and NUB1L, which have both a nuclear localization signal, mainly localize in the nucleus [135]. Tanaka and collaborators characterized the NEDD8-binding sites in the two splicing variants: these signals localize at the C-terminal end of the amino acid chain and respond to the consensus sequence A(X4)L(X10)L(X3)L. Moreover, Leu to Ala substitutions abolish the interaction of NUB1 and NUB1L with NEDD8 in vitro as well as the capability of NUB1 to down-regulate NEDD8 conjugates. Another NEDD8 interactor, identified a short time later, was UBXN7 that belongs to the UBA-UBX family of substrate adaptors [139]. The UBA domain recruits proteasome substrates by interacting with the ubiquitin chains conjugated on the target protein, while the UBX takes contact with the p97

Interactor	Description	NEDD8-binding interface	Domain-binding interface	Ref.
NUB1/ NUB1L	Adaptor protein negatively regulates Nedd8 and neddylated substrates	Ile44 patch (predicted)	Consensus sequence A(X4) L(X10)L(X3)L	[135–138]
UBXD7	Involved in the degradation of misfolded or damaged proteins	Ile44 patch	UIM	[139, 140]
RNF168	RING E3 ligase involved in DNA double-strand break repair	NEDD8 chains	MIU2	[145]
TRIM40	Negative regulator of inflammation and carcinogenesis	Unknown	Unknown	[143]
DNMT3b	DNA methyltransferase that functions in de novo methylation	Unknown	Residues 532–583 of mouse DNMT3b	[142]
HGS	Adaptor involved in the endosomal sorting of membrane receptors	Ile44 patch (predicted)	UIM	[64]
TRIAD1	RBR ligase	Unknown	UBA-like domain	[147]
HHARI	RBR ligase	Unknown	UBA-like domain	[147]
hHR23a, UBQLN1 and Ddi1	Non-proteasomal ubiquitin receptors and shuttles	Ile44 patch	UBA2 (RAD23), UBA (UBQLN1), UBA (Ddi1)	[141]
BRAP2 (or RNF52)	Cytoplasmic protein, which may regulate nuclear targeting BRCA1 and other proteins with a nuclear localization signal, in the cytoplasm	Unknown	Multiple domains in BRAP2 show NEDD8-binding properties	[72]
AHR	Transcription factor localized in the cytoplasm that moves to the nucleus upon ligand binding and stimulates transcription of target genes involved in the response to planar aromatic hydrocarbons. Activated AhR is exported from the nucleus for degradation in the cytosol by the ubiquitin/proteasome pathway	Unknown	Unknown	[144]
SMURF1	HECT E3-ligase belonging to the NEDD4 family	Ile-patch	Consensus sequence L(X7) R(X5)F(X) ALQ.	[102]
SMURF2	HECT E3-ligase belonging to the NEDD4 family	Ile-patch	Consensus sequence L(X7) R(X5)F(X) ALQ.	[102]
NEDL1	HECT E3-ligase belonging to the NEDD4 family	Unknown	Unknown	[102]
NEDL2	HECT E3-ligase belonging to the NEDD4 family	Unknown	Unknown	[102]
Rpt6	Regulatory particle base subunit of the proteasome	Unknown	Unknown	[116]



Interactor	Description	NEDD8-binding interface	Domain-binding interface	Ref.
SMC1	Cohesins are required for nuclear division; they prevent premature separation of sister chromatids	Unknown	Unknown	[116]
DNA-PK	DNA-dependent protein kinase belonging to the PI3/PI4 kinase family, required for DNA double-strand break repair and recombination	Unknown	Unknown	[116]
KHNYN	Unknown function	Negatively charged surface centered on residues 31-EEKE-34	CUBAN domain	[119]

**Table 3.**  
*NEDD8 interacting proteins.*

ATPase of the proteasome. UBXN7 also contains a UIM motif, which interacts with neddylated CUL2 and sequesters it, thus negatively regulating the ubiquitin ligase activity of the CRL complex. Accordingly, UBXN7 overexpression causes the accumulation of HIF1 $\alpha$  in its non-ubiquitinated form, suggesting the involvement of the UIM-NEDD8 interaction in the processivity of the CRL ubiquitin ligase [139]. Subsequently, den Besten and colleagues reported that the replacement of NEDD8 with ubiquitin on CUL2 does not affect the interaction with UBXN7, indicating that this recognition is rather context dependent [140].

The non-proteasomal ubiquitin receptors hHR23a, UBQL1, and Ddi1 have been shown to interact non-covalently with NEDD8 [141]. These shuttle proteins deliver ubiquitinated cargoes from the cytoplasm and the nucleus to the proteasome. Their structures contain an N-terminal ubiquitin-like (UBL) domain that binds to the proteasome and a C-terminal UBA domain that binds to ubiquitin chains. It has been suggested that, under stress conditions in which heterologous chains are formed by the enzymes of the ubiquitin pathway in order to compensate the ubiquitin depletion, these chains would be recognized by the shuttles and processed by the proteasome. Since mixed chains are shorter than the polyubiquitin chains, due to the less efficiency in NEDD8 chain extension, the formation of the heterologous chains would counteract the further depletion of free ubiquitin within cells.

A heterogeneous group of proteins that non-covalently bind NEDD8 substrates includes the DNA methyltransferase DNMT3b [142], the RING-ligase TRIM40 [143], the HECT ligases NEDL1 and NEDL2 [102], the DNA-dependent protein kinase DNA-PK [116], cohesin SMC1 [116], the transcription factor AHR [144], the cytoplasmic retention factor for nuclear proteins BRAP2 [72], and a regulatory particle of the proteasome Rpt6 [116]. All of them have been identified by using yeast two-hybrid screenings or the immunoprecipitation of overexpressed tagged NEDD8. Moreover, with the exception of DNMT3b and BRAP2 for which an attempt, although not conclusive, to identify the NEDD8-binding region has been carried out, neither the identification of the NEDD8 recognition surface nor its function has been well established. This lack of knowledge does not allow the identification of a putative NEDD8 recognition module. Nevertheless, these works represent documented examples linking NEDD8 to the recruitment of effectors in processes like proteasome degradation, DNA methylation, transcription, and DNA damage repair.

A second class of NEDD8-interacting proteins comprises proteins in which a typical UBD has been shown to mediate NEDD8 binding with similar efficiency, thus leaving open the issue of whether NEDD8 and ubiquitin are effectively distinguished. These proteins exert downstream signaling effects by binding to NEDD8 chains and/or neddylated proteins. The motif interacting with ubiquitin (MIU) 2 domain of RNF168 binds NEDD8 chains and mediates the recruitment of the E3 ligase on sites of DNA damage, thus allowing the RNF168-mediated polyubiquitination of  $\gamma$ -H2AX [145]. The UIM motif of hepatocyte growth factor-regulated Tyr kinase substrate (HGS) promotes the recruitment of the activated EGF receptor, which is ubiquitinated and neddylated on several lysine targets in the cytoplasmic tails [64].

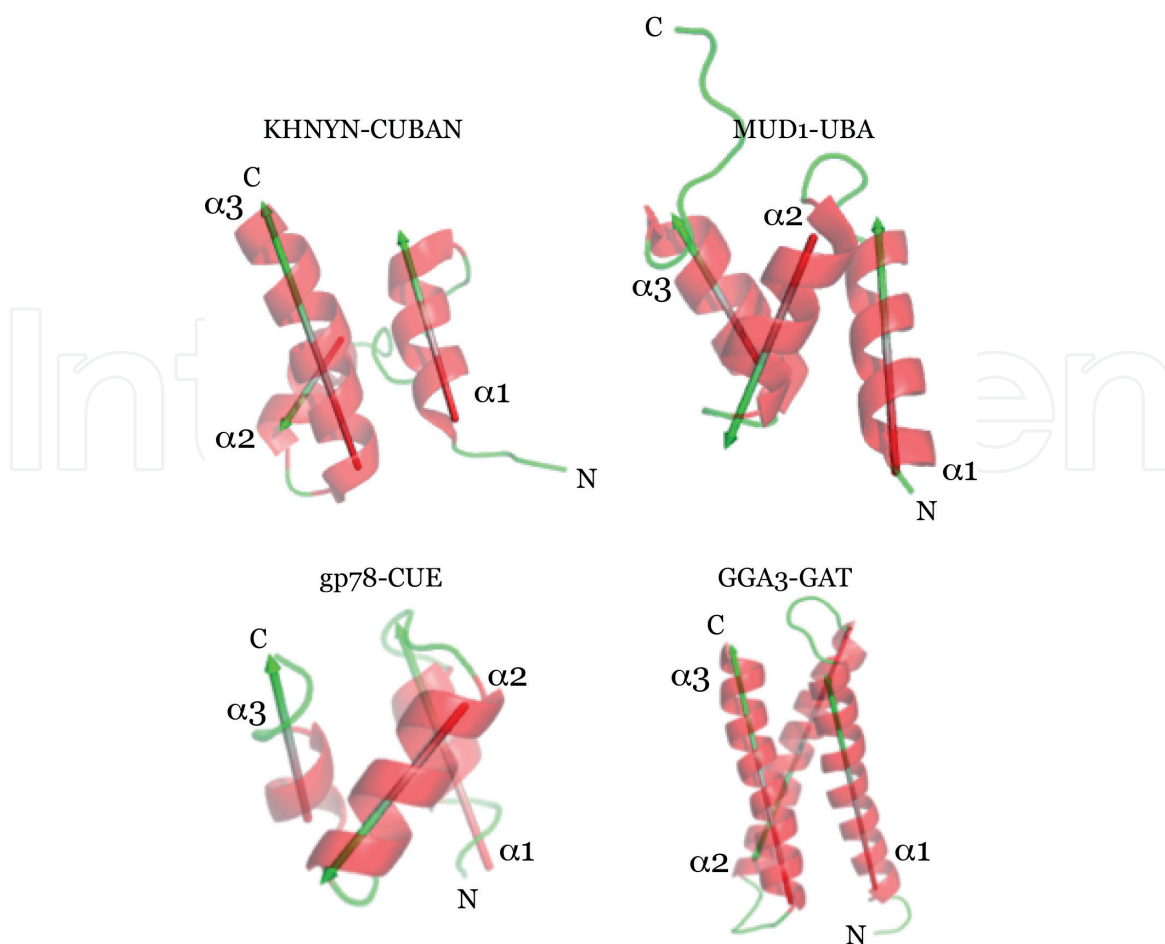
In the recent years, the HECT E3-ligases SMURF1 and SMURF2 have been demonstrated to require the direct interaction with free NEDD8 in order to promote their ubiquitin ligase activity toward both substrates and the enzyme itself. Interestingly, each ligase contains two NEDD8-binding sites, located, respectively, in the N-lobe and C-lobe, that are not embedded in a known UBD. Both sites conform to the consensus sequence L(X7)R(X5)F(X)ALQ [102]. Importantly, the NEDD8-binding sites in both ligases are clearly distinct from the ubiquitin-binding sites (the “exosites”) that map in the N-lobe and recognize the Ile44 patch of ubiquitin [146]. This spatial separation supports the notion that, despite being closely associated and functionally interconnected, the non-covalent interactions with ubiquitin and NEDD8 play roles that are rather complementary more than simply overlapping.

Finally, it has been recently shown that two RING-between-RING E3 ligases, TRIAD1 and HHARI, engage a UBA-like domain to recruit different NEDD8-conjugated cullins [147]. Despite interacting with different CRL complexes, the outcome of both interactions is the same: the release of the auto-inhibited state and the subsequent stimulation of their E3-ligase activity, which also results in their own proteasomal degradation. Concluding, these observations reveal a novel role of NEDD8 in recruiting additional E3 ligases to existing CRL E3 ubiquitin ligase complexes.

## **9. CUBAN: a novel domain showing a preference for NEDD8**

Recently, the characterization of several Ubl-binding domains has been performed by using the phage display approach [119]. By panning a human brain phage-displayed cDNA library, a number of ubiquitin- and NEDD8-binding domains were identified. In particular, panning with NEDD8 identified five putative binding partners, including the UBA domains of the ubiquitin shuttles RAD23 and UBQLN1. Both proteins have been previously shown to interact with NEDD8 ([141] and **Table 3**), and they have been suggested to be involved in the recognition of mixed ubiquitin-NEDD8 chains by the proteasome under stress conditions. Moreover, while the binding affinity for ubiquitin of UBA domain of RAD23 is sensibly higher than NEDD8, the corresponding domain in UBQLN1 shows a similar affinity for both molecules.

Even though the approach did not provide evidence for a protein domain strictly specific for NEDD8 (since all NEDD8-binding proteins are also ubiquitin interactors), it allowed the identification of a novel domain in the KHNYN (KH domain- and NYN domain-containing) protein, which has unique features compared to the previously characterized binding domains. The first peculiarity of this domain is to show a clear preference for NEDD8. Indeed, it interacts with monomeric NEDD8 with a binding affinity that has been evaluated to be around  $24 \pm 2 \mu\text{M}$ , a measure significantly higher than that detected for ubiquitin and that is comparable to the highest affinity for ubiquitin observed in some CUE and UBA domains [126]. Secondly, in addition to showing a clear preference for NEDD8 over monomeric



**Figure 4.** Ribbon representation of CUBAN (KHNYN), UBA (MUD1), CUE (gp78), and GAT (GGA3) domains. Arrows indicate the relative spatial distributions of the three helices ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ). N- and C-terminal ends are indicated.

ubiquitin, this domain—both isolated and in the context of the full-length protein—promotes the interaction with neddylated cullins, the reason for which it has been dubbed CUBAN, for cullin-binding domain associating with NEDD8. The investigation by NMR spectroscopy showed structural elements that are common in other UBDs belonging to the most populated category represented by a three-bundle helix, such as CUE and UBA. Nevertheless, unique features of CUBAN domain reveal the presence of a novel three-alpha-helix bundle domain, which is characterized by an unusually extended loop1. Moreover, by comparing the folding of the CUBAN domain with other known ubiquitin-binding domains, it can be observed that the central helix in CUE and UBA domains is located in front of the plane formed by helices 1 and 3, in CUBAN as well as in the GAT domain projected on the opposite side (**Figure 4**).

By studying the protein complex between NEDD8 and CUBAN, a third relevant feature emerged: CUBAN, although requiring the presence of an integral hydrophobic patch in NEDD8, binds to residues in the second  $\beta$ -strand (Ile13 and Glu14) and in the C-terminal end of helix  $\alpha$ -1 (31-Glu-Glu-Lys-Glu-34) of NEDD8, indicating that the interaction is more electrostatic than hydrophobic. Accordingly, positively charged residues (His651, Arg652, Arg659, and Arg664) mapping in turn1, helix  $\alpha$ 2, and turn2 characterize the binding interface in CUBAN. Interestingly, the molecular details of this interaction are reminiscent of the electrostatic interaction between the acidic residues Glu31 and Glu32 in NEDD8 and the linker of RBX1 [148], suggesting that distinct interactors require the recognition of the same binding surface. A fourth consideration regarding CUBAN is that this domain also binds di-ubiquitins and this interaction disrupts the association with free NEDD8.



It must be pointed out that ubiquitin chains follow the principle of the higher avidity of binding, which is mediated through multivalent interactions that largely increase binding affinity [149]. Since these contact sites are in close proximity, it is not surprising that the di-ubiquitin competes out monomeric NEDD8, at least when analyzing the isolated domain. Nevertheless, it cannot be excluded that once NEDD8 is conjugated to cullins, KHNYN could interact with the CRL complex in a way that hampers the access of ubiquitin chains to the CUBAN domain. More important, more than being specific, the CUBAN domain demonstrates a discriminating capability, since it has gained structural features that clearly make it capable, differently from other ubiquitin-binding domains, to select and distinguish NEDD8 from its closer relative. This feature, which is the result of the evolution of two distinct binding sites, suggests that the interaction with NEDD8 and ubiquitin must be finely tuned, similarly to what has been shown for RBX1, which shares with CUBAN both the dual specificity toward ubiquitin and NEDD8 and the recognition of an electrostatic surface on the Ubl molecule.

Such discriminating capability is also revealed by the evidence that the A72R mutation in the C-terminal tail of NEDD8 partially affects binding of CUBAN to NEDD8, suggesting that the acquisition of ubiquitin features interferes with the ability of this domain to bind its specific target in KHNYN. On the contrary, the same mutation strongly favors the interaction with those UBDs showing an *in vitro* weak binding toward NEDD8, a consequence of the evolutionary conserved features mediating the ubiquitin recognition by the majority of UBDs. Therefore, among the residues that are divergent in NEDD8 and ubiquitin, Ala72 is responsible for the weak binding of NEDD8 to the UBDs that can potentially recognize both posttranslational modifications.

The NEDD8-binding region identified in KHNYN maps to the carboxyl-terminal end (aa 627–678). Interestingly, the full-length KHNYN is also consistently ubiquitinated in cells, and such covalent modification is abrogated in the absence of the CUBAN domain. The protein localizes both in the nucleus and the cytoplasm and contains a putative nuclear localization signal, and it has been found to be associated to membranes [150]. Interestingly, an evolutionary related protein called NEDD4-binding protein 1 (N4BP1) has been identified in the same panning experiment and shares with KHNYN the presence of a domain interacting with ubiquitin at the very carboxyl-terminal end. Despite the high level of identity (about 40%), the divergences are sufficient to determine a marked difference in their binding preferences, as highlighted by the evidence that only KHNYN can recognize monomeric and conjugated NEDD8 [119]. Both proteins contain an N-terminal evolutionary conserved KH domain (K homology), which is present in a wide variety of nucleic acid-binding proteins where it can function in RNA recognition, followed by the NYN domain (N4BP1, YacP nucleases), with predicted ribonuclease activity [151, 152]. The comprehension of the regulatory mechanisms that associate RNA recognition and degradation with the recruitment of activated cullin RING-ligase complexes will help in clarifying the biological functions and the cellular processes that are regulated by this protein.

## **10. Concluding remarks**

Our understanding of the complexity within the NEDD8 pathway and how it cross-reacts with ubiquitin is rapidly growing. Numerous signaling pathways, such as apoptosis, DNA damage, and nucleolar stress signaling, are clearly emerging as biological contexts in which neddylation plays a key role. Nevertheless, an important question that needs to be further investigated is whether neddylation

is always functionally distinct from ubiquitination. Current knowledge indicates that the two posttranslation modifications must be closely interconnected, as also testified by the evidence that NEDD8 has evolutionary gained few, but key, features that allow it to be distinguished from ubiquitin while maintaining a considerable degree of identity that is much higher than other Ubls. Moreover, the identification of novel NEDD8-binding motifs that are always close to ubiquitin-binding sites, for example, in SMURF1/2, RBX1 and KHNYN, would suggest that these interactions act cooperatively rather than in a mutually exclusive manner. A complete understanding of how these interactions are modulated and the mechanisms controlling the NEDD8 pathway will help in identifying new potential NEDD8 targets and inhibitors which can pave the way to clinical developments in the treatment of several diseases.

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## **Conflict of interest**

I have no conflict of interest to declare.

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