

# Inhibition of NEDD8 and FAT10 ligase activities through the degrading enzyme NEDD8 ultimate buster 1: A potential anticancer approach (Review)

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**Abstract.** The capabilities of tumour cells to survive through deregulated cell cycles and evade apoptosis are hallmarks of cancer. The ubiquitin-like proteins (UBL) proteasome system is important in regulating cell cycles via signaling proteins. Deregulation of the proteasomal system can lead to uncontrolled cell proliferation. The Skp, Cullin, F-box containing complex (SCF complex) is the predominant E3 ubiquitin ligase, and has diverse substrates. The ubiquitin ligase activity of the SCF complexes requires the conjugation of neural precursor cell expressed, developmentally down-regulated 8 (NEDD8) to cullin proteins. A tumour suppressor and degrading enzyme named NEDD8 ultimate buster 1 (NUB1) is able to recruit HLA-F-adjacent transcript 10 (FAT10)- and NEDD8-conjugated proteins for proteasomal degradation. Ubiquitination is associated with neddylation and FAT10ylation. Although validating the targets of UBLs, including ubiquitin, NEDD8 and FAT10, is challenging, understanding the biological significance of such substrates is an exciting research prospect. This present review discusses the interplay of these UBLs, as well as highlighting their inhibition through NUB1. Knowledge of the mechanisms by which NUB1 is able to downregulate the ubiquitin cascade via NEDD8 conjugation and the FAT10 pathway is essential. This will provide insights into potential cancer therapy that could be used to selectively suppress cancer growth.

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## 1. Overview of ubiquitin-proteasome system (UPS) and ubiquitin-like proteins (UBL) pathway as a therapeutic target

The UPS is important in regulating protein homeostasis in human cells through programmed degradation. The degradation of regulatory proteins can affect cell-cycle regulation, cell proliferation, intracellular signaling, DNA repair and apoptosis (1-4). As UPS degradation is a major clearance system associated with proteolysis within the cells, its deregulation can cause the pathogenesis of cancer and other diseases through the inappropriate loss of regulatory proteins or unintentional activation of certain specific signalling cascades. For instance, cancerous cells develop when cell-cycle controls break down, leading to unregulated cell proliferation. Cancer cells can also evade apoptosis induced by a number of different cellular stresses. Hence, an interruption to the normal regulation of the UPS could lead to abnormal cell proliferation.

The 26S proteasome is a protease complex capable of degrading polyubiquitinated proteins. The 26S complex is composed of a barrel-shaped 20S proteasome core with a 19S regulatory particle at either or both of its ends. The 20S proteasome contains the enzymatic active sites, whilst the 19S regulatory particle helps to control access of ubiquitin-like protein (UBL)-conjugated substrates to the core. There are three proteasome active sites within the 20S core, namely the caspase-like ( $\beta$ 1), trypsin-like ( $\beta$ 2) and chymotrypsin-like ( $\beta$ 5) domains. These sites use an N-terminal threonine as the catalytic amino-acid residue (5,6).

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Table I. First- and second-generation proteasome inhibitors.

Drug	Company	Status
First generation		
Bortezomib	Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA)	FDA-approved for multiple myeloma and relapsed mantle cell lymphoma
Second generation		
Carfilzomib (Kyprolis)	Onyx Pharmaceuticals, Inc. (San Francisco, CA, USA)	FDA-approved for multiple myeloma
Oprozomib (ONX0912)	Onyx Pharmaceuticals, Inc. (San Francisco, CA, USA)	Phase I
Ixazomib citrate (MLN9708)	Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA)	Phase I/II
Marizomib (NPI-0052)	Nereus Pharmaceuticals (San Diego, CA, USA)	Phase I
Delanzomib (CEP-18770)	Cephalon, Inc. (Frazer, PA, USA)	Phase I
Calpeptin (IPSI-001)	Lanospharma Laboratories Co., Ltd. (Chongqing, China)	Phase I
ONX0914	Onyx Pharmaceuticals (San Francisco, CA, USA)	Phase I

FDA, Food and Drug Administration.

Ubiquitin (Ub) and UBLs share certain common structural elements, such as a three-dimensional structure called the Ub or  $\beta$ -grasp fold (7). The UBLs are a group of proteins encompassing neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), small ubiquitin-like modifier 1/2/3, interferon-stimulated gene 15 (ISG15), HLA-F-adjacent transcript 10 (FAT10), autophagy-related protein (ATG)8 and ATG12, which conjugate to their targets in a manner similar to that of ubiquitination (8). Ub is a highly conserved protein of 76 amino acids that are able to attach to other proteins in a reversible fashion. There are three vital structural domains within Ub: i) the  $\beta$ -grasp fold, commonly found in all UBLs; ii) a C-terminal tail; and iii) seven lysine residues that correspond to polyubiquitin-linked chains (6). NEDD8 has distinctive functions in cells due to its structural differences, which mediate specialised interactions with target proteins compared with Ub. The crystal structure of NEDD8 is analogous to that of Ub, with the exception of two surface regions (9,10). NEDD8 was initially discovered in fetal mouse brain (11) and can be found predominantly in adult tissues (11-13). NEDD8 and the neddylation pathway enzymes are overexpressed in human cancers (13-15). FAT10 is a 165-amino acid protein that comprises two Ub-like domains with 29% identity and 36% homology to Ub at its N and C-termini, respectively (16). The protein is known to be involved in apoptosis, immune responses and cancer (16-18). Fig. 1A shows the domain structure of Ub, NEDD8 and FAT10.

The roles of UPS and UBL conjugation pathways in normal cell function and in disease has prompted the search for inhibitors that are able to selectively disrupt pathway function. Proteasome inhibitors have been synthesised to halt the function of the proteasomal activities. As mentioned, the proteasome has three active sites ( $\beta$ 1,  $\beta$ 2 and  $\beta$ 5), which utilise N-terminal threonine as the catalytic amino acid residue (7). All UPS inhibitors were developed to covalently modify this threonine residue in order to block the enzyme's kinetics. The therapeutic value of UPS inhibition has been

demonstrated with the proteasome inhibitor bortezomib (Velcade®; Millennium Pharmaceuticals, Inc., Cambridge, MA, USA). Bortezomib is used in the treatment of patients suffering from multiple myeloma (19,20) and mantle cell lymphoma (21).

As the first clinical proteasome inhibitor to target the UPS, bortezomib was approved in 2003. Several second-generation proteasome inhibitors are currently in development, such as carfilzomib, oprozomib, ixazomib citrate, marizomib and delanzomib. In comparison with UPS inhibition, blocking of UBL pathways may provide a more specific effect by targeting the substrate proteins. UPS inhibition is a common step in blocking the degradation of a broader range of substrates. For instance, inhibition of E1-activating enzymes may be achieved through covalent inactivation (e.g. PYR-41) (22) and adduct formation (e.g. MLN4924) (23). Blocked E2 interactions offer a more selective inhibition (e.g. synthetic peptide UBC12N26). Recent research has frequently focussed on targeting deubiquitinating enzymes (DUBs), as this class of proteins are capable of reversing the action of the Ub conjugation cascade. Table I summarises the current clinical development of second-generation proteasome inhibitors, and Table II lists the E1/2/3 inhibitors. Furthermore, a NEDD8-activating enzyme (NAE) inhibitor, MLN4924, which targets the NEDD8 pathway, appears to be a potentially important anticancer strategy (24). The development of DUB inhibitors is more recent compared with that of the proteasome and E1/2/3 inhibitors. To the best of our knowledge, no DUB inhibitors have entered clinical trials.

*NEDD8 ultimate buster 1 (NUB1), a NEDD8- and FAT10-degrading enzyme, and approaches to anticancer therapy.* NUB1 is an interferon (IFN)-inducible protein of 69 kDa, composed of 601 amino acids. It also has a splice variant, NUB1L, which possesses an extra 14 amino acids that encode an additional Ub-associated (UBA) domain (Fig. 1B). NUB1 proteins can recruit FAT10- and NEDD8-conjugated proteins to the proteasome for degradation and negatively

Table II. A summary of small molecule inhibitors targeting E1s, E2s and E3s.

Drug	Target	Company	Status
PYR-41	E1	Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA)	N/A
MLN4924	E1	Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA)	Phase II
CC0651	E2	N/A	N/A
NSC697923	E2	N/A	N/A
Nutlin	E3	Roche Products Limited (Pharmaceuticals) (Welwyn Garden City, UK)	Phase I
MI-773	E3	Sanofi S.A. (Gentilly, France)	Phase I
CGM097	E3	Novartis International AG (Basel, Switzerland)	Phase I

N/A, not applicable.

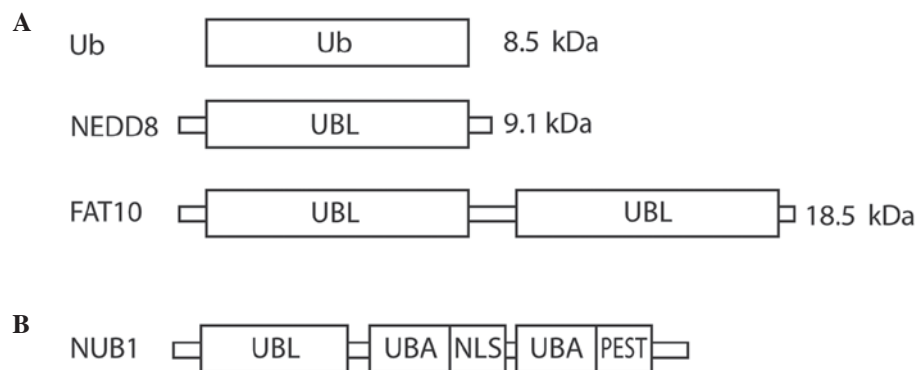


Figure 1. Structural representation of (A) Ub, NEDD8 and FAT10, and (B) NUB1. Ub, ubiquitin; NEDD8, neural precursor cell expressed, developmentally down-regulated 8; FAT10, HLA-F-adjacent transcript 10; NUB1, NEDD8 ultimate buster 1; UBL, ubiquitin-like domain; UBA, ubiquitin-associated; NLS, nuclear localisation signal; PEST, proline-enriched glutamic acid, serine and threonine domain.

regulate the NEDD8-conjugation system (25-29). The NUB1 proteins have been observed in various types of cancer cells, including cervical adenocarcinoma, rectal adenocarcinoma, neuroblastoma, malignant lymphoma and renal cell carcinoma (RCC) (26). Upregulated NUB1 expression has been linked to IFN $\alpha$ -induced antimitogenic actions. Additionally, NUB1 has demonstrated anticancer properties in RCC cell lines, where it was involved in apoptosis and S-phase transition through its action on p27 and cyclin E (30,31). Upregulation of NUB1 effectively inhibits the proliferation of IFN $\alpha$ -resistant RCC cells (31).

NUB1 protein has been reported to play a role in Huntington's disease (32) and congenital amaurosis (33). In cancers, NUB1 is an attractive candidate for inhibition of p27<sup>KIP1</sup> and p21<sup>CIP1</sup> via the regulation of the Skp, Cullin, F-box-containing (SCF)<sup>SKP2</sup> ligase activity (31). The upregulated p21<sup>CIP1</sup> in NUB1-knockdown cancer cells is thought to be promising in directing the cells to senescence. NUB1 protein was reported to be a tumour suppressor as it exerts growth inhibition during its overexpression; upon IFN $\alpha$  treatment, overexpressed NUB1 induced apoptosis in IFN $\alpha$ -resistant A498 cells (31). However, its general lack of enzymatic activities makes NUB1 less suitable for small molecule inhibition (32). Thus, the low-molecular-weight proteins FAT10 and NEDD8 could be key to developing novel strategies in anticancer therapy, as they interact with NUB1 (34). The current review focuses on the relevance of NUB1 protein in NEDD8 and FAT10 conjugation in cancers, and the potential for targeting it as a novel therapeutic approach.

## 2. The NEDD8-conjugation (neddylation) pathway and the effect of neddylation on transcription factors

The UBL enzymatic cascade scheme that results in UBL conjugation and protein degradation involves several distinct steps. Each step requires different classes of enzyme, as shown in Table III.

Neddylation is a post-translational modification process that conjugates NEDD8 to its target proteins, in a process that is analogous to that observed for ubiquitination. However, the neddylation process uses a distinct E1 and E2 enzyme reaction scheme (5,8,11,35,36) (Table III). Fig. 2A summarises the NEDD8 conjugation and deconjugation steps (34,37). The C-terminal glycine of NEDD8 is adenylated by an E1 NEDD8-activating enzyme (NAE), a heterodimer composed of amyloid- $\beta$  precursor protein-binding protein 1 (APPBP1) and Ub-like modifier-activating enzyme (UBA) 3. NEDD8 is covalently conjugated to the NAE via a thioester linkage (38). The activated NEDD8 is consecutively transferred to the E2 NEDD8-conjugation enzyme and then to the specific substrates (i.e. cullin proteins) via an isopeptide bond (39). The RING-box protein (RBX) 1/ROC1, mouse double minute 2 homolog (MDM2), F-box protein 11 and c-Cbl proteins are neddylated in the same way (39).

There are two NEDD8-specific E2-conjugating enzymes, namely ubiquitin conjugating enzyme E2 (UBE2)M (also known as UBC12) and UBE2F. These E2 enzymes act to

Table III. Overview of the enzymatic cascade involved in UBL conjugation.

UBL	Ub	NEDD8	FAT10 and Ub
E1-activating enzymes	UAE	APPBP1-UBA3 heterodimer	UBA6
E2-conjugating enzymes	UBCs	UBE2M and UBE2F	USE1
E3 ligases	Ub E3 ligases	NEDD8 E3 Ligases	N/A
Substrates	1,000s	200s	N/A

UBL, ubiquitin-like protein; Ub, ubiquitin; NEDD8, neural precursor cell expressed, developmentally down-regulated 8; FAT10, HLA-F-adjacent transcript 10; UAE, ubiquitin-activating enzymes; APPBP1, amyloid-β precursor protein-binding protein 1; UBA, ubiquitin-like modifier-activating enzyme; UBCs, Ub-conjugating enzymes; UBE2, ubiquitin-conjugating enzymes E2; USE1, UBA6-specific E2 enzyme; N/A, not applicable.

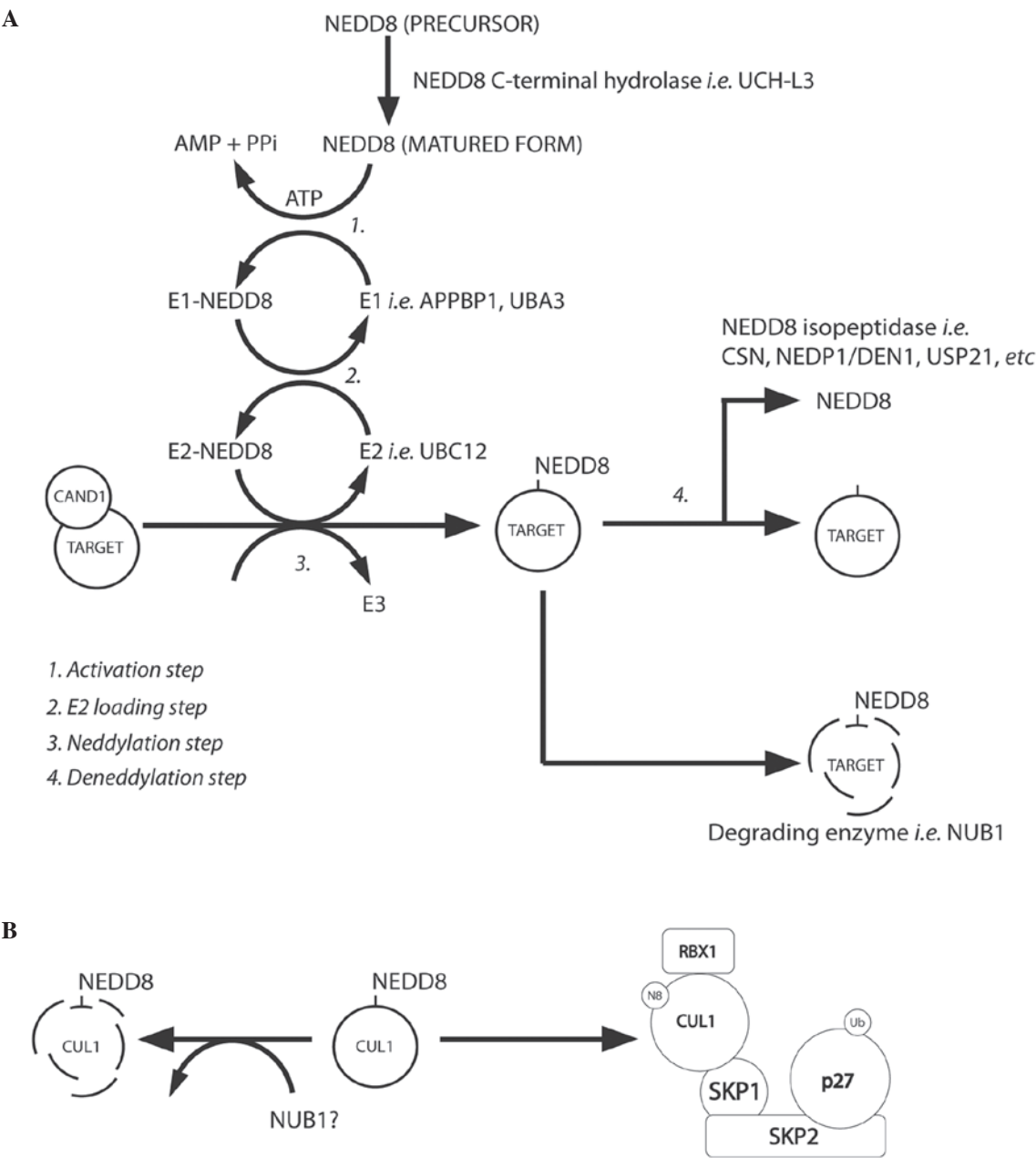


Figure 2. (A) NEDD8 conjugation pathway. Schematic summary of the main steps of the neddylation pathway [modified from Rabut and Peter, 2008 (37); Tanaka *et al.*, 2012 (34)]. (B) Neddylation of CUL1 locks the SCF complex with phosphorylated p27 and cyclin E [as suggested by Bornstein *et al.*, 2006 (30) and Tanaka *et al.*, 2012 (34)]. CAND1, cullin-associated and neddylation-dissociated 1; AMP, adenosine monophosphate; PPi, anion P2O7<sup>4-</sup>; ATP, adenosine triphosphate; UCH-L3, ubiquitin C-terminal hydrolase isozyme L3; APPBP1, amyloid-β precursor protein binding protein 1; DEN1, deneddylase 1, CSN, COP9 signalosome; NEDP1, NEDD8-specific protease 1; NUB1, NEDD8 ultimate buster 1; USP21, ubiquitin specific peptidase 21, CUL1, cullin 1; SKP1, S-phase kinase-associated protein 1; SKP2, S-phase kinase-associated protein 2; RBX1, ring-box 1, E3 ubiquitin protein ligase; SCF, Skp, cullin, F-box-containing complex.



transfer NEDD8 to its target protein through E3 enzymes. It has been reported that all NEDD8 E3 enzymes can function as Ub E3 enzymes. The predominant NEDD8 E3 ligases are the RING subunits RBX1 and RBX2 (38,40-43). Meanwhile, the non-RBX family NEDD8 E3 ligases include c-CBL, ring finger protein 111, MDM2 and inhibitor of apoptosis 1 (44).

Of the numerous NEDD8 substrates, neddylation has been best described in the cullin family (45). In this mechanism, NEDD8 from the E2 cysteine active site is transferred onto a lysine residue in the N-terminus of the target proteins (Fig. 2A) (46). Cullin neddylation is further mediated by defective in cullin neddylation protein 1-like proteins (44). It was reported that RING E3 ligases could neddylate the same substrate on multiple lysine residues (44). However, the interaction between non-RBX RING E3 ligase and E2 enzymes remains to be elucidated.

NEDD8-conjugated substrates are deneddylated by various proteins that include COP9 signalosome (CSN), NEDD8-specific protease 1 (NEDP1/DEN1) and ubiquitin specific peptidase 21 (37,47-51). Neddylation may be inhibited by cullin-associated and neddylation-dissociated 1 through its direct binding to cullins (52,53). NEDD8 and neddylated substrates are recruited by NUB1 for proteasomal degradation (25,26) (Fig. 2A). In the G1-S-phase transition, Bornstein *et al* (30) demonstrated how neddylated cullin 1 cooperatively activates the SCF<sup>SKP2</sup> Ub ligase complex, which results in p27 degradation (Fig. 2B). Furthermore, previous studies found that cullin neddylation increased the Ub E3 ligase activity of the SCF complex (Fig. 3) (46,54).

NEDD8 is negatively regulated by NUB1, which links the UBLs to the 26S proteasome for further UPS degradation. Reports have described that NUB1 is able to recruit NEDD8 and NEDD8-conjugated proteins to the proteasome for degradation, and this may modulate the cell-cycle profile in response to stresses (34). The capability of NEDD8 to activate the Ub E3 ligase-SCF complex (by covalent binding to cullins) adds further complexity to the ubiquitination machinery (11,55-59). Therefore, validation of NEDD8 targets would allow identification of genuine NEDD8 substrates.

**Challenges in identifying physiological neddylation targets.** Hjerpe *et al* (45) demonstrated that NEDD8 and Ub cascades are independent of one another during normal cellular homeostasis. NEDD8 conjugation onto Ub substrates through the Ub cascade has a spurious role in normal physiological conditions. The single amino acid change in the C-terminus of NEDD8 compared to Ub, from Arg72 to Ala72, confers the specificity between these two UBLs (44). This ensures that the correct UBL is passed to the appropriate E2 enzyme, E3 enzyme and the substrate respectively (Table III). However, when NEDD8 is in excess, the NEDD8 E1 enzyme UBA1 can activate NEDD8, which is then transthiolated to Ub E2 enzymes. This phenomenon results in the neddylation of Ub-specific substrates (10,45). NEDD8 can form NEDD8 chains or mixed Ub-NEDD8 chains (39,60). An increase of NEDD8 over Ub, as a result of cellular stresses, cellular diversity or pathological conditions, could exert different effects on neddylated substrates (44). This raises concerns, since the majority of research performed to date to identify neddylated substrates in cells relies on the overexpression of NEDD8; as this would cause an imbalance

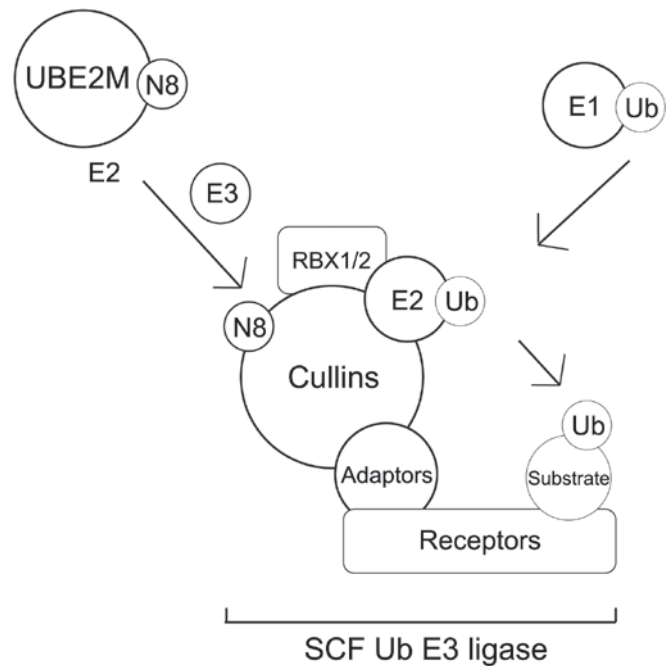


Figure 3. NEDD8 conjugation and ubiquitination pathway through cullin proteins. UBE2M, ubiquitin-conjugating enzyme E2M; RBX1/2, ring-box 1; Ub, ubiquitin; NEDD8, neural precursor cell expressed, developmentally down-regulated 8; SCF, Skp, cullin, F-box-containing complex.

between cellular NEDD8 and Ub levels, it could result in the aberrant neddylation of proteins via the Ub pathway (45).

Enchev *et al* (44) therefore revised and proposed a set of criteria to define the search for physiological neddylation targets: A neddylation substrate must demonstrate the covalent attachment of NEDD8 through the carboxyl-terminal glycine to the lysine residue of the substrates; and the neddylation must be detected under homeostatic conditions under endogenous NEDD8 levels and substrate expression. The NAE inhibitor MLN4924 should be incorporated into the study, as it blocks cullin neddylation but not ubiquitination (44). It remains optional to examine the possible NEDD8 E2 and E3 enzymes (44,45). It is also advisable to look at the regulation and biological consequences of neddylation (44). In endogenous protein experiments, immunoprecipitation with specific antibodies is a recommended approach (44). Genome editing techniques, such as a CRISPR/cas9 approach, may be used to introduce affinity-tagged versions of a particular gene product (44). The NEDD8 substrate should also be confirmed using mass spectrometry, using LysC protease as the cleavage enzyme, as it can discriminate between Ub, NEDD8 and ISG15 conjugates (61). Mass spectrometry can also be used to determine the site of the neddylated Lys residue, and the type of NEDD8 chains that are formed. The neddylated Lys residue needs further study if it is also targeted by Ub. The relative abundance of Ub, NEDD8 and FAT10 must be examined for its physiological relevance (44). A mutant form of the substrate that can no longer be neddylated must also be included to serve as a negative experimental control (44).

Overexpression of NEDD8 and the aberrant activation of the neddylation pathway and cullin-RING Ub ligase (CRL) activity can drive the progression of cancers (4,13), inflammatory and autoimmune diseases (7). Mainstream research

focuses on the effects of CRL inhibition, neddylation and deneddylation. The small-molecule NAE inhibitor MLN4924 is undergoing clinical trials. MLN4924 is an analog of adenosine monophosphate that competitively binds to the enzymatic pocket of NAE. This small molecule therefore inhibits neddylation and CRL activity. MLN4924 treatment causes DNA replication by stabilising chromatin licensing and DNA replication factor 1, a DNA replication licensing factor and CRL substrate. MLN4924-treated cells accumulate DNA damage due to DNA repair failure, leading to apoptosis (62) or senescence (19). Neddylation is able to inhibit the transcriptional activity of the tumour suppressors p53 and p73, and to stabilise Hu-antigen R (63), cell division cycle 6 and hypoxia-inducible factors (64). One of the important outcomes of MLN4924 treatment is that it causes the cancer cells to undergo apoptosis and senescence (44).

*Transcriptional regulation via the neddylation of transcription factors.* Several studies have suggested that neddylation of transcription factors can lead to the suppression of their transcriptional activity (44,65).

*E2F transcription factor 1 (E2F1).* Neddylation of E2Fs reduces their transcriptional activity (66,67). E2F1 was shown to be neddylated in the DNA-binding domain and its protein levels reduced following neddylation (67). DEN1 deneddylates E2Fs and consequently activates E2F-mediated transcription. DNA damage promotes the expression of DEN1, which subsequently deneddylates E2F and causes its stabilisation (68). Neddylation specifically regulates a subset of E2F target genes; for example, E2F1 deneddylation upon DNA damage triggers the transcription of proapoptotic factors (66).

*p53 and p73.* p53 acts by inhibiting cell cycle progression or triggering senescence or apoptosis (33). It is inhibited by the RING-domain E3 ligase MDM2, which targets ubiquitinated p53 for degradation. MDM2 is able to neddylate p53 and inhibit its transcriptional activity (33). The neddylated p53 is further recruited by NUB1, leading to its inactivation (69). p73 that is neddylated by MDM2 undergoes cytoplasmic relocalisation and downregulation of transcriptional activity (70). In addition, the Ub E3 ligase SKP1-CUL1-F-box protein 11 ( $SCF^{Fbox11}$ ) may neddylate p73 and downregulate its transcriptional activities (71).

*Nuclear factor  $\kappa$ B (NF- $\kappa$ B).* When extracellular signaling is absent, NF- $\kappa$ B is distributed in the cytoplasm and inhibited by inhibitor of NF- $\kappa$ B (I $\kappa$ B) family members. Upon stimulation by proinflammatory cytokines [such as tumour necrosis factor (TNF)], I $\kappa$ B kinases (IKKs)  $\alpha$ -,  $\beta$ - and  $\gamma$  phosphorylate I $\kappa$ B, which is then ubiquitinated and targeted for degradation by  $SCF^{\beta TrCP}$ . Under the same conditions, IKK $\gamma$  was reported to be neddylated and degraded by the proteasome, which reduces NF- $\kappa$ B activation and inhibits NF- $\kappa$ B activity in gastrointestinal neoplasia (72). Therefore, neddylated IKK $\gamma$  may exert a tumour suppressor function.

*Amyloid precursor protein intracellular domain (AICD).* Amyloid precursor protein is cleaved by secretase to become amyloid- $\beta$  peptide and AICD. AICD is a component of a transcription factor complex with amyloid- $\beta$  (A4) precursor protein-binding family B member 1 (FE65) and

TAT-interactive protein 60 (73). Neddylated AICD blocks its interaction with FE65 and prevents the formation of the transcription factor complex, thereby reducing its transcriptional activity (2,74). Thus, neddylated AICD inhibits the transcription of downstream targets.

### 3. The FAT10-conjugation (FAT10ylation) pathway and its function

FAT10 was discovered by Sherman Weissman in 1996 (5). Due to the poor solubility of the protein at high concentrations, the structure of FAT10 protein was only recently defined (75). FAT10 consists of two  $\beta$ -grasp fold domains connected by a short linker (75). FAT10 protein was found to be expressed predominantly in immune tissue, including the thymus, lymph nodes and spleen (76-78). Its expression is stimulated by proinflammatory cytokines, namely IFN $\gamma$  and TNF $\alpha$  (79). FAT10 protein is found in mature dendritic cells and it demonstrates oncogenic characteristics; ectopic expression of FAT10 causes malignant transformation and promotes tumour growth (80), and it is known to be upregulated in several tumour types, including liver and colon tumours (18,81).

FAT10 shares the same E1 and E2 enzymes with the Ub conjugation pathway. The FAT10 E1 enzyme UBA6 is able to activate Ub and FAT10 (29,82-84). The adenylation and transthiolation reactions of FAT10 are kinetically slower than those for Ub. UBA6 protein is thought to be the only FAT10 E1 enzyme in cells, since UBA6 knockdown can effectively abolish the formation of FAT10-conjugates *in vitro* (84,85). Similarly, UBA6-specific E2 enzyme (USE1) is the only UBA6-specific E2 enzyme discovered to be involved in FAT10 conjugation, although it also functions in a similar fashion to the conjugation of Ub (83). USE1 may only bind to activated Ub from UBA6, not UBE1 (83).

Little is currently known about FAT10, and research to identify possible FAT10 E3 ligases and deconjugating enzymes is ongoing. One study demonstrated that ectopically expressed FAT10 was not degraded over time, suggesting the possibility that a group of FAT10-deconjugating enzymes may not exist (86). It is believed that FAT10 is capable of promoting its own proteasomal-dependent degradation without the aid of deconjugating enzymes (86). FAT10-conjugated proteins were found to have a reduced half-life, similar to that observed for Ub-conjugated proteins (21). Conversely, it was demonstrated that FAT10-conjugated p62 accumulated under proteasome inhibition (79).

The interferon-inducible protein NUB1 interacts with FAT10 non-covalently (25), and significantly accelerates the degradation of FAT10 by the proteasome (25). NUB1 binds to the proteasome subunit S5a (28), and also to FAT10 via its three C-terminal UBA domains (Fig. 4) (87). NUB1 is also able to interact with the von Willebrand A (VWA) domain of RPN10 (S5a), one of the subunits of the 26S proteasome (25,28). The degradation of FAT10 is accelerated further by NUB1 splicing variant, NUBIL, which is able to bind to regulatory particle non-ATPase (RPN)10 in addition to the 19S regulator subunit, RPN1 (S2) (87).

The 26S proteasome subunit Rpn10 (S5a) is the docking site for FAT10, NUBIL and polyubiquitin. Ub interaction motifs 1 and 2 of Rpn10 are bound by lysine 48-linked polyubiquitin

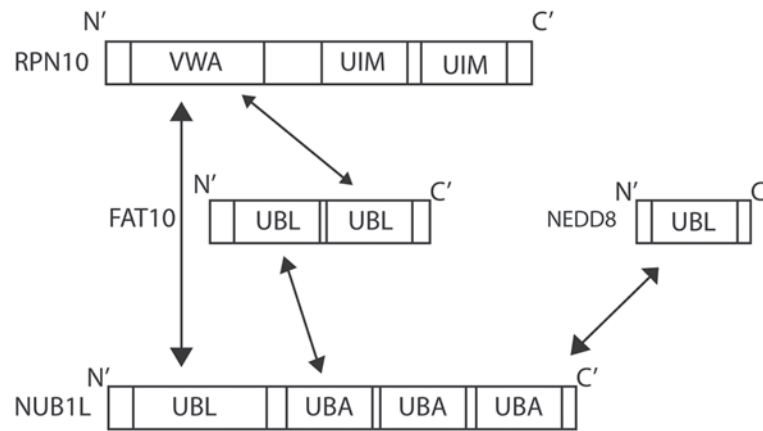


Figure 4. Domain structure of NUB1L, FAT10 and RPN10 and their interacting domains [Hipp *et al* (87); Kamitani *et al* (25); Tanji *et al* (28)]. RPN10, regulatory particle non-ATPase 10; FAT10, HLA-F-adjacent transcript 10; NUB1L, NEDD8 ultimate buster 1 long isoform; UBL, ubiquitin-like domain; UBA, ubiquitin-associated; UIM, ubiquitin-interacting motif; VWA, von Willebrand A motif.

chains. FAT10 is able to target substrate proteins to the proteasome independently of poly-FAT10ylation. FAT10 interacts directly with the VWA domain of RPN10, and no ubiquitination is required (28). The co-expression of NUB1L has been shown to accelerate the degradation of FAT10, suggesting a preference for proteasomal degradation (28).

**Substrates of FAT10 conjugation.** The biological function of FAT10 remains poorly understood. FAT10 overexpression has been demonstrated to induce apoptosis in mouse fibroblasts (67), HeLa cells (68) and renal tubular epithelial cells (70). FAT10 is synergistically induced by IFN $\gamma$  and TNF- $\alpha$ , which leads to the induction of apoptosis (25). Several FAT10-interacting proteins have been identified, and are summarised in the following paragraphs.

The inflammatory mediator leucine-rich repeat Fli-I-interacting protein 2 (LRRFIP2) is covalently modified by FAT10 (6). LRRFIP2 positively regulates the activity of NF- $\kappa$ B in the inflammatory response mediated by toll-like receptor (TLR)4 (6). FAT10ylation of LRRFIP2 hinders its recruitment to the plasma membrane, which results in the inhibition of lipopolysaccharides (LPS)/TLR4-mediated NF- $\kappa$ B activation (6). This consequently leads to the reduced expression of NF- $\kappa$ B-responsive genes, including apoptosis inhibitors (6). Overexpression of FAT10 can induce apoptosis, causing FAT10<sup>NULL</sup> mice to be hypersensitive to LPS challenge due to NF- $\kappa$ B inhibition (33). However, FAT10 was observed to protect leukocytes in the spleen, thymus and bone marrow from apoptosis in a mouse model (33). In another study, the colon cancer cell line HCT116 was protected from TNF- $\alpha$ -induced apoptosis in the presence of FAT10 (88). The induction of apoptosis by FAT10 is therefore cell type-specific; however, the mechanisms involved remain unknown.

Mitotic arrest-deficient 2 (MAD2), a spindle assembly checkpoint protein, binds to FAT10 protein non-covalently (33). In prometaphase, overexpressed FAT10 in HCT116 cells was found to reduce the localisation of MAD2 at the kinetochore (88,89). Ren *et al* (88) reported that TNF- $\alpha$ -induced upregulation of FAT10 also delocalised MAD2 from kinetochores in a similar way and accelerated cell mitosis. The mis-segregation of chromosomes was shown to be abolished when FAT10 levels were

reduced by siRNA (89). Hence, FAT10 is considered to cause mis-segregation of chromosomes during cell division (88,89).

FAT10 has been found to be highly expressed in colorectal, ovarian, hepatocellular and uterine carcinomas, suggesting that FAT10 expression may promote oncogenesis (89). A study found that 72% of hepatocellular carcinoma and 53% of colon carcinoma tissues overexpressing FAT10 also expressed the IFN $\gamma$ /TNF- $\alpha$ -dependent immunoproteasome subunit low molecular mass protein 2 (90), suggesting that the pro-inflammatory cytokine response may be responsible for FAT10 overexpression in carcinoma tissues.

Autophagy adaptor p62 or sequestosome-1 protein can regulate aggresome formation, which protects cells from aggregation-prone protein-induced toxicity (79). FAT10ylated p62 tends to be proteasomally degraded (79). A previous study revealed that FAT10 expression induced by pro-inflammatory cytokines leads to a decrease in endogenous p62 (79). FAT10 was found to be transported by histone deacetylase 6 along microtubules into aggresomes, causing p62 degradation (79). Under pathological conditions, p62 is localised in aggresomes along with the aggregated proteins found in neuronal diseases, including Alzheimers (91). The impact of FAT10 on P62-induced pathogenesis remains unresolved. However, there is no evidence that FAT10ylated p62 has a role in autophagic pathways (91).

#### 4. NEDD8 and FAT10 pathway perspectives

The SCF Ub E3 ligases have been shown to be deregulated in various cancers; this results in unlimited cell proliferation and carcinogenesis via accumulation of their substrate proteins (34). Consequently, the E3 ligases are the subject of research into potential strategies for anticancer therapy (34). It is believed that the NEDD8-Ub-SCF complexes and the NEDD8-FAT10-degrading enzyme NUB1 are potential candidates for therapy (Fig. 2B) (34).

The search for neddylation targets requires further experimental validation. The conventional ectopic overexpression of UBLs is thought to lead to false positive conjugation of substrates (45). However, genome editing techniques, such as CRISPR-Cas9 technology, could overcome this, as it permits the neddylation substrates to be examined endogenously (44).



In addition to NEDD8 and Ub chain formation, proteomic studies have reported phosphorylation, acetylation and succinylation sites on NEDD8. The functional significance of this observation remains unknown. There is a general lack of information on non-cullin protein neddylation under homeostatic conditions. Furthermore, the physiological relevance of several reported NEDD8 substrates, including p62/sequestosome, remains unknown. Whether neddylation is functionally distinct from ubiquitination is a question that remains unresolved. For example, polyneddylation and polyubiquitination at DNA damage sites or in response to other stress conditions are functionally redundant, and NEDD8 and Ub may be recognised by the same interaction motifs. However, in certain circumstances, such as the neddylation or ubiquitination of TGF $\beta$ RII, these two modifications can elicit distinct biological responses. Efforts are clearly needed to identify and characterise NEDD8-interacting domains and proteins.

NUB1 proteins cause the degradation of FAT10- and NEDD8-conjugated targets. Their expression regulates NEDD8- and FAT10-based signalling in response to cellular stresses (34,45). However, the structural mechanisms of NUB1 protein and its clinical relevance in UBL pathways remain to be explored. Hosono *et al* (31) found that overexpression of NUB1 inhibits cell growth, and the same study demonstrated lower NUB1 mRNA expression in IFN $\alpha$ -sensitive 4THUR cells. The same study highlighted that NUB1 is not induced in IFN $\alpha$ -resistant cells, although transiently expressed NUB1 sensitised the same cells and induced apoptosis (31). Therefore, killing IFN $\alpha$ -resistant cells by increasing NUB1 activity is a potential strategy (31).

FAT10 research is still in an early stage and the biological consequences of FAT10ylation are poorly described. De-FAT10ylating enzymes are under active investigation as drug targets in the pharmaceutical industry at present, based on the fact that a number of putative FAT10 targets are oncogenes or inhibitors of apoptosis. Future works should focus on the FAT10-modulated proteasome system and mechanisms of cytokine-induced reactions.

## 5. Conclusion

Experimental studies have demonstrated that negative regulation of the Ub, NEDD8 and FAT10-conjugation pathways have great potential in the context of cancer suppression. SCF complexes are often deregulated in cancer and could be modulated through manipulation of cullin neddylation. Neddylation and FAT10ylation inhibitors have recently been developed as a novel class of anticancer agent. These compounds are expected to exhibit better specificity for cancer cells and have reduced toxicity. Degrading enzymes, such as CSN and NUB1/NUBIL, are attractive candidates for the inhibition of Ub, NEDD8 and FAT10-ligase activities (34). These are expected to provide new strategies in anticancer therapy.

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