Regulation of cancer-related pathways by protein NEDDylation and strategies for the use of NEDD8 inhibitors in the clinic

Naima Abidi and **Dimitris P Xirodimas**

Centre de Recherche de Biochimie Macromoléculaire, UMR5235, 1919 Route de Mende, Montpellier 34293, France

Correspondence should be addressed to D P Xirodimas
Email
dimitris.xirodimas@crbm.cnrs.fr

Abstract

Post-translational modification of proteins with ubiquitin and ubiquitin-like molecules (UBLs) controls a vast if not every biological process in the cell. It is not surprising that deregulation in ubiquitin and UBL signalling has been implicated in the pathogenesis of many diseases and that these pathways are considered as major targets for therapeutic intervention. In this review, we summarise recent advances in our understanding of the role of the UBL neural precursor cell expressed developmentally downregulated-8 (NEDD8) in cancer-related processes and potential strategies for the use of NEDD8 inhibitors as chemotherapeutics.

Key Words
- molecular biology
- oncology

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The neural precursor cell expressed developmentally downregulated-8 pathway and its cross-talk with ubiquitin

Neural precursor cell expressed developmentally down-regulated-8 (NEDD8) is a ubiquitin-like molecule (UBL) sharing ~60% amino acid identity with ubiquitin. NEDD8 is attached to its substrates in a manner similar to that described for ubiquitination, resulting in the formation of an isopeptide bond linking the terminal carboxyl group of NEDD8 with the ε-amino group of a lysine residue of the substrate (Kumar *et al.* 1993, Kamitani *et al.* 1997, Lammer *et al.* 1998, Liakopoulos *et al.* 1998, Osaka *et al.* 1998, Pozo *et al.* 1998).

The NEDDylation enzymatic cascade is composed of the key enzymatic activities named E1, E2, E3 and deconjugating enzymes. The NEDD8 E1-activating enzyme (NAE) is a heterodimer of APPBP1 and UBA3 corresponding to the N-terminal and C-terminal of the single polypeptide of the ubiquitin E1 respectively (Leyser *et al.* 1993, Walden *et al.* 2003*a*,*b*). UBE2M (UBC12) and UBE2F are the E2-conjugating enzymes, where multiple E3-ligases promote the conjugation of NEDD8 to its targets (Gong & Yeh 1999, Huang *et al.* 2009). With the exception of SMURF1 all identified E3-ligases that promote NEDD8 conjugation belong to the RING family of E3s, including the cullins-associated RBX1/2, the p53-negative regulator MDM2, c-CBl and the transcriptional co-activator TFB3 (Skowyra *et al.* 1999, Kamura *et al.* 1999*a*,*b*, Xirodimas *et al.* 2004, Oved *et al.* 2006, Yang *et al.* 2007*a*,*b*, Rabut *et al.* 2011, Xie *et al.* 2014). The DCN1 protein co-operates with RBX1 to enhance cullin NEDDylation (Kurz *et al.* 2008, Scott *et al.* 2011). While NAE is regarded as the only and specific E1 enzyme for NEDD8, biochemical studies provided evidence that the ubiquitin E1 enzyme UBE1 can activate NEDD8 *in vitro*, however with much lower efficiency compared with ubiquitin (Whitby *et al.* 1998, Hjerpe *et al.* 2012*a*). It has been unclear whether this level of cross-talk between NEDD8 and ubiquitin pathways

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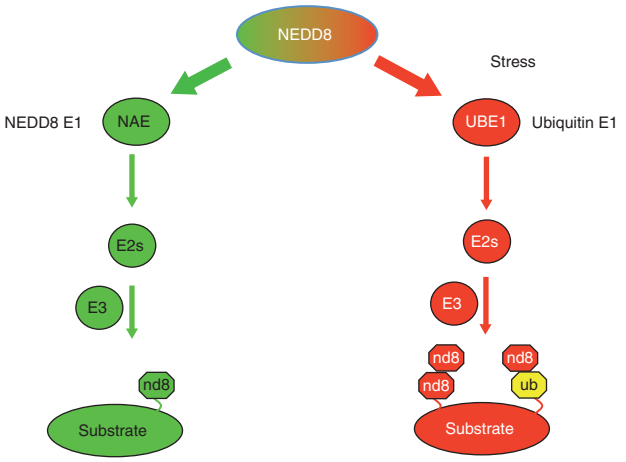

Figure 1
Modes of NEDD8 conjugation. Activation of NEDD8 by NEDD8-activating enzyme (NAE) defines the canonical NEDDylation pathway under homeostatic conditions. Proteotoxic stress causes an increase in protein NEDDylation that depends on the activation of NEDD8 by the ubiquitin E1 enzyme UBE1. This leads to the formation of poly-NEDD8 and/or hybrid NEDD8–ubiquitin chains on target proteins.

operates *in vivo*. The role of NEDD8 in homeostasis has been established mainly through regulation of Cullin-RING-ligases (CRLs; see below). More recent studies have shown that the NEDD8 pathway responds to cellular stress including proteasome inhibition, heat shock and oxidative stress (Xirodimas *et al*. 2008, Kim *et al*. 2011, Leidecker *et al*. 2012, Hjerpe *et al*. 2012*a*). Under such stress conditions, a global increase in protein NEDDylation is observed, which does not depend on NAE but rather on the ubiquitin E1 enzyme UBE1. The detection of a thioester bond between NEDD8 and UBE1 provided evidence for the activation of NEDD8 by UBE1 *in vivo* (Leidecker *et al*. 2012, Hjerpe *et al*. 2012*a*). Proteomic studies designed to discriminate between NEDDylation and ubiquitination sites *in vivo* identified branched peptides, which strongly indicate the formation of poly-NEDD8 and mixed NEDD8–ubiquitin chains upon stress conditions (Xirodimas *et al*. 2008, Leidecker *et al*. 2012, Singh *et al*. 2012; Fig. 1). A similar increase in protein NEDDylation was observed in the brains of hibernating ground squirrels; however, it is not known whether the increase in NEDDylation is UBE1 dependent (Lee *et al*. 2012). A well-known phenomenon upon the above described stress conditions is the depletion of free ubiquitin, which has been considered as a stress signal (Finley *et al*. 1987, Finley & Chau 1991). It appears that this observed decrease in ubiquitin levels is at least part of the mechanism for the activation of NEDD8 by UBE1 (Leidecker *et al*. 2012, Hjerpe *et al*. 2012*a*). This was also supported by the observation that the artificial increase in the levels of NEDD8 by overexpression could lead to NEDD8 conjugation by the ubiquitin pathway (Hjerpe *et al*. 2012*b*). Critical issues to be resolved are whether the hybrid NEDD8–ubiquitin chains create a new signal that is specifically detected by cellular factors, alter the recognition of ubiquitin chains by cellular machineries such as the proteasome, or indeed upon depletion of ubiquitin, NEDD8 conjugation acts as a backup mechanism functionally replacing ubiquitin (Singh *et al*. 2012, 2014).

NEDDylation is a reversible process. The COP9 signalosome is a zinc metalloprotease, which has minimal affinity for NEDD8 but specifically promotes deNEDDylation of cullins (Lyapina *et al*. 2001, Zhou *et al*. 2001, Enchev *et al*. 2012, Birol *et al*. 2014, Lingaraju *et al*. 2014). NEDP1 also called as DEN1 or SENP8 due to its sequence similarity to Sumo-specific proteases is a NEDD8-specific protease that can deconjugate NEDD8 from its substrates and it also catalyses the processing of NEDD8 to expose the C-terminal di-glycine motif before its activation by the NAE (Gan-Erdene *et al*. 2003, Mendoza *et al*. 2003, Wu *et al*. 2003, Shen *et al*. 2005, Rabut & Peter 2008, Xirodimas 2008). COP9 and NEDP1 are regarded as specific deNEDDylating enzymes. UCH-L3 (Yuh1 in *Saccharomyces cerevisiae*) is a protease with dual specificity, which can hydrolyse the C-terminus of ubiquitin and NEDD8, and knockout of Uch-L3 in mice causes elevation of NEDD8 protein levels (Wada *et al*. 1998, Linghu *et al*. 2002, Kwon *et al*. 2004). The USP21 deubiquitinating enzyme was also shown to deconjugate NEDD8 upon overexpression in human cell lines, but further structural and biochemical studies have shown a rather specific activity of USP21 towards ubiquitin, so the mechanism behind the effect of USP21 on NEDDylation *in vivo* is still unclear (Gong *et al*. 2000, Ye *et al*. 2011). The Epstein–Barr-virus-encoded member of proteases BPLF1 was shown that in addition to its activity in processing ubiquitin it can also process NEDD8 and de-conjugate NEDD8 from cullins. The de-NEDDylating activity of BPLF1 is required for efficient DNA re-replication to allow synthesis and production of virus DNA (Gastaldello *et al*. 2010). Additional reported proteases with dual activity towards NEDD8 and ubiquitin include ataxin and the parasite hydrolase PfUCH54 (Artavanis-Tsakonas *et al*. 2006, Ferro *et al*. 2007).

Substrates for NEDD8

The family of cullin proteins is the most established target for NEDD8. In humans, it is composed of seven cullins

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(Cul1, 2, 3, 4A, 4B, 5 and 7), whereas PARC (CUL9) and APC2 (component of the anaphase promoting complex APC) contain a cullin-homology domain (Hochstrasser 1998, Pan *et al*. 2004, Skaar & Pagano 2009, Schreiber *et al*. 2011, Watson *et al*. 2011, Chang *et al*. 2014). All cullins are modified with NEDD8, while modification of CUL7 is still controversial (Pan *et al*. 2004, Skaar *et al*. 2007). Cullins are scaffold proteins of multicomponent complexes named CRLs that control the stability of a rapidly growing list of proteins with diverse functions including cell cycle regulation, signalling, DNA repair, the response to hypoxia and oxidative stress, centrosome duplication cycle and cytoskeleton dynamics (Watson *et al*. 2011, Zhao & Sun 2013). The role of cullin NEDDylation is to enhance the activity of the CRLs and subsequent ubiquitination and degradation of the regulated substrates. CRLs control the stability of a vast variety of targets with established roles in cell cycle progression (p21, p27 and cyclin D/E), DNA replication (CDT1), the oxidative response (NFR2) and the response to hypoxia (HIF1a) (Freed *et al*. 1999, Karin & Ben-Neriah 2000, Kondo & Kaelin 2001, Ohh *et al*. 2002, Bloom *et al*. 2003, Hu *et al*. 2004, Li & Kong 2009). A study has identified hundreds of potential CRL targets, where functional inactivation of cullins was combined with genetic and proteomic approaches, displaying the diversity of CRLs to control protein stability (Emanuele *et al*. 2011).

Different models exist for the role of NEDD8 in the regulation of CRL function, including cullin dimerisation, dissociation of cullins from its negative regulator CAND1, conformational changes that bring the E3-RING ligases RBX1/2 in close proximity to the substrate protein, stabilisation of the active CRL state, or control the CRL binding with other E3-ligases and components of the p97 pathway (Duda *et al*. 2008, Saha & Deshaies 2008, Merlet *et al*. 2009, Deshaies *et al*. 2010, Duda *et al*. 2011, Bandau *et al*. 2012, den Besten *et al*. 2012, Kelsall *et al*. 2013, Pierce *et al*. 2013, Wu *et al*. 2013, Zemla *et al*. 2013).

While cullins represent the major substrates for NEDD8, additional targets for NEDDylation have been identified. These include transcription factors and co-regulators, signalling receptors, components of the protein synthesis and apoptotic machineries, E3-ligases, histones (Rabut & Peter 2008, Xirodimas *et al*. 2008, Wang *et al*. 2011, Watson *et al*. 2011). Many of the non-cullin NEDD8 targets are also established ubiquitin substrates and the E3-ligases that promote NEDDylation are also ubiquitin E3-ligases (Rabut & Peter 2008, Xirodimas 2008). Recent structural studies have revealed an exquisite mechanism that ensures the preferential modification of cullins with NEDD8. It relies on the coordinated action of NEDD8, the RBX1/DCN1 E3-ligases and the substrate itself to preferentially position the NEDD8-loaded UBC12 for cullin modification (Scott *et al*. 2014). The mechanistic insights revealed in these studies will be important for the determination and characterisation of the pathway used for NEDDylation of non-cullin targets (see below) (NAE or UBE1 NEDD8 activation) and to also understand the mechanism of NEDD8 conjugation by components of the ubiquitin pathway under stress conditions.

Development of NEDD8 inhibitors-MLN4924

The success of Bortezomib in the clinic highlighted the potential of blocking protein degradation as therapeutic approach. Inhibition of NEDDylation was predicted to satisfy this criterion as NEDD8 can promote diverse protein degradation through activation of CRLs. In addition, expression of NAE, UBC12 and global NEDDylation are found upregulated in a variety of cancers, including lung adenocarcinomas and squamous-cell carcinomas (Chairatvit & Ngamkitidechakul 2007, Salon *et al*. 2007, Li *et al*. 2014*a*,*b*, *c*). Suppression of NEDDylation by either knockdown of NAE or expression of dominant-negative mutants of UBC12 reduced growth rates in the oral carcinoma cell line HSC4 (Chairatvit & Ngamkitidechakul 2007). Cullins and CRL components are found overexpressed in several types of cancers (Lee & Zhou 2010, Wang *et al*. 2014). The well-established examples include the Cul1 F-box adaptor protein FBW7, which is found mutated in 6% of all cancers, but in some cases of leukaemias or gastrointestinal cancers the mutation rate can be up to 30% (Welcker & Clurman 2008). In many cases mutations in FBW7 exist within the substrate binding region, preventing degradation of key regulators of cell proliferation, including cyclin E as the best characterised FBW7's substrate (Koepp *et al*. 2001, Welcker *et al*. 2003, Tetzlaff *et al*. 2004). Increase in the levels of CUL3 and CUL4A was also associated with tumour progression in breast cancers, but the mechanism for this upregulation is currently unclear (Haagenson *et al*. 2012). Increase in mRNA levels of CUL7 was also observed in non-small cell lung carcinoma. Interestingly, CUL7 was proposed to block the apoptotic function of p53 and to cooperate with MYC for anchorage-independent growth, providing insights for the oncogenic properties of CUL7 (Kim *et al*. 2007). The gene for the human homologue of the NEDD8 E3-ligase DCN1, DCUN1D1/RP42/SCCRO, is localised in chromosome 3, which gets amplified particularly in squamous cell carcinoma. Overexpression

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of SCCRO was related with transcriptional activation of GLI1, a key regulator of the hedgehog pathway. Targeting the expression of SCCRO or GLI1 in SCCRO overexpressing cells by short hairpin shRNA induces an apoptotic response, providing a possible functional insight for the oncogenic properties of SCCRO (Sarkaria *et al*. 2006). Collectively, there is a strong correlation between dysfunction in CRL activity and tumourgenesis.

Through an initial screen of chemical libraries and additional medicinal chemistry, MLN4924 was developed as a first in class inhibitor of NAE and the NEDD8 pathway (Soucy *et al*. 2009). It is an adenosine sulfamate analogue and its action of inhibition is based on a substrate-assisted mechanism (Brownell *et al*. 2010). NEDD8 and MgATP bind to NAE where NEDD8 is adenylated, before it reacts with the catalytic cysteine in UBA3 to form a NEDD8 thioester bond. A second round of NEDD8 adenylation allows the thioster-linked NEDD8 to be transferred to UBC12 or UBE2F (Walden *et al*. 2003*a*). It is during this round that MLN4924 competes for MgATP binding on NAE and is able to attack the thioester-linked NEDD8. The resultant NEDD8–MLN4924 covalent adduct is unable to be transferred on the E2s and subsequently blocks NEDD8 conjugation (Brownell *et al*. 2010). The IC_{50} of MLN4924 for NAE is single nanomolar compared with micromolar scale for UBE1 or other E1-activating enzymes (>1.5 μM), displaying the specificity of MLN4924 towards NAE (Soucy *et al*. 2009). MLN4924 was shown to be effective in reducing growth and inducing apoptosis in a variety of tumour cell lines and tumour xenografts, suggesting a broad anti-tumour spectrum for NEDD8 inhibitors (Soucy *et al*. 2010). MLN4924 has entered cancer clinical trials and more details can be found in http://clinicaltrials.gov/ct2/results?term=MLN4924&Search=Search)

Importantly, recent studies have demonstrated that NAE is the key target of MLN4924 *in vivo*. In tumour cell lines and xenografts, treatment-emerging resistance was observed for MLN4924. Sequencing in the resistant lines identified heterologous mutations in UBA3 (predominantly A171T) within the MgATP-binding cleft where MLN4924 also binds (Milhollen *et al*. 2012, Toth *et al*. 2012, Xu *et al*. 2014). These data strongly suggest that the observed biological effects in MLN4924-treated cells are primarily due to NAE inhibition.

Effects of NEDD8 inhibition through CRLs inactivation

Treatment of several tumour cell lines with MLN4924 produced a rapid (within 5 min) decrease in cullin NEDDylation and increase in several known CRL targets, indeed validating MLN4924's action in blocking protein NEDDylation downstream of NAE (Soucy *et al*. 2009). Although many CRL targets are involved in cell cycle regulation at different phases, the predominant effect of MLN4924 is an initial S-phase arrest. This is due to accumulation of CDT1, a replication licensing factor that is degraded by $CUL4^{Cdt2}$- or $CUL1^{Skp2}$-based CRLs to allow entry into mitosis (Zhong *et al*. 2003, Higa *et al*. 2006, Jin *et al*. 2006, Sansam *et al*. 2006, Senga *et al*. 2006, Kim & Kipreos 2007). In the absence of CDT1 degradation, the resulting DNA re-replication in the absence of mitosis causes the induction of the DNA damage response and apoptosis (Kim & Kipreos 2007, Soucy *et al*. 2009). A different mechanism for MLN4924-induced apoptosis was proposed in activated B-cell-like diffuse large B-cell lymphoma (DLBCL). It is due to lack of degradation of IκBα and subsequent inhibition of NFκB, a pathway, which DLBCL growth depends on (Milhollen *et al*. 2010, Swords *et al*. 2010, Duncan *et al*. 2012).

Induction of apoptosis is not the sole mechanism responsible for the growth suppressive effects of MLN4924. Treatment of several tumour cell lines with low doses of MLN4924 (>100 nM) induces irreversible senescence in a p21-dependent but p53- and Rb-independent manner (Jia *et al*. 2011). In addition, inhibition of NEDDylation by MLN4924 activates autophagy, a process of intracellular proteolysis that delivers cytoplasmic components to lysosomal degradation (Luo *et al*. 2012*a*,*b*, Yang *et al*. 2012*a*,*b*, Zhao *et al*. 2012, Hurley & Schulman 2014, Schreiber & Peter 2014). Autophagy can act both as tumour suppressor and as a survival signal in established tumours (Yang *et al*. 2011). The activation of autophagy by MLN4924 is due to accumulation of Deptor and HIF1α, substrates of $Cul1^{\beta TrCP}$ and $CUL2^{VHL}$ ligases (Zhao *et al*. 2012). The downstream effect is the inhibition of the mTORC1 pathway, which supressess autophagy. The activation of autophagy was observed in multiple tumour cell lines treated with MLN4924 and importantly, autophagy induction protected cells against the apoptotic effects of MLN4924 (Zhao *et al*. 2012). More recently, MLN4924 has been shown to inhibit tumour angiogenesis and tumourgenesis in melanoma or $KRAS^{G12D}$-driven lung tumours in mice model systems. At least partially, this is due to accumulation of the cell cycle inhibitor p27 (Tan *et al*. 2013) or inhibition of the NFκB and mTOR pathways respectively (Li *et al*. 2014*a*,*b*,*c*). Similar anti-tumour effects of MLN4924 were observed in both *in vitro* and *in vivo* model systems of pancreatic cancer, resulting in suppression of tumour growth and metastasis (Yao *et al*.

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2014). In this system, the anti-tumourgenic effects of MLN4924 are due to the accumulation of RhoA, a member of the Rho GTPase family that is involved in the control of cellular migration (Chen *et al.* 2009, Leck *et al.* 2010). The assessment for the potential clinical effects of MLN4924 has so far been focussed on CRL inhibition and accumulation of CRL targets (Soucy *et al.* 2010, Swords *et al.* 2010). Another emerging concept is that inhibition of cullin NEDDylation can also induce anti-apoptotic or tumourgenic responses (see also below) that could modulate the efficacy of MLN4924 in clinic. For example, MLN4924 causes the stabilisation of proteins such as the NRF2 transcription factor, which controls the induction of anti-oxidant genes. NRF2 degradation is mediated by a $CUL3^{Keap1}$ CRL and is prevented in ~30% of lung cancers either due to mutation in *KEAP1* or *NRF2* (Itoh *et al.* 1999, Cullinan *et al.* 2004, Lee & Zhou 2010).

Inhibition of NEDDylation of non-cullin targets

Many of the reported non-cullin NEDD8 targets include key cell cycle regulators and tumour suppressors. The Von-Hipple-Lindau (VHL) and p53 tumour suppressors were identified as the first non-cullin substrates (Stickle *et al.* 2004, Xirodimas *et al.* 2004). VHL is a component of a Cul2-based E3-ligase that controls the stability of HIF1α upon hypoxic conditions (Ohh 2006). NEDDylation of VHL promotes its binding to fibronectin and prevents the incorporation of VHL within a CRL2 complex (Stickle *et al.* 2004, Russell & Ohh 2008). The interaction of VHL with fibronectin is important in tumour progression, as all VHL tumour-derived mutants are deficient in fibronectin interaction (Russell & Ohh 2008). This predicts that inhibition of NEDDylation would predispose cells to increased tumourgenesis through lack of interaction of VHL with fibronectin. The studies also proposed a role of NEDD8 as regulatory switch that can selectively control the VHL incorporation either within a CRL2 complex or to bind to fibronectin, defining two distinct functional outcomes (Fig. 2).

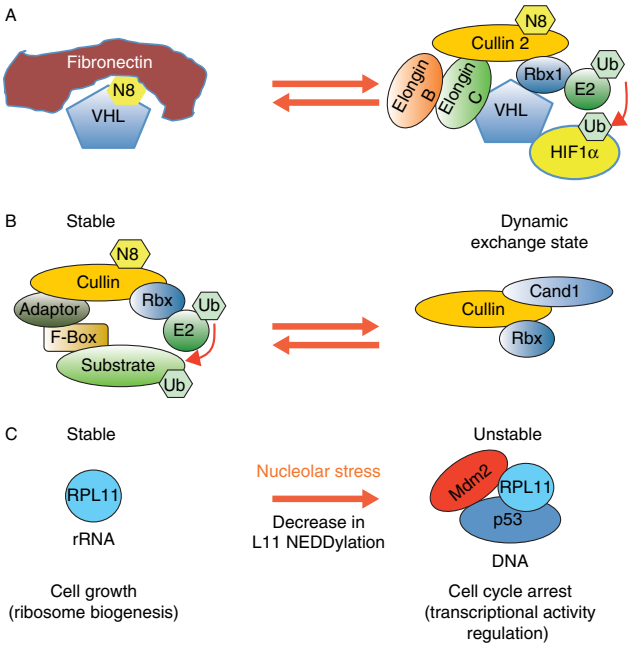

Figure 2
NEDD8 controls protein complex formation. (A) NEDDylation of Von-Hippel-Lindau (VHL) promotes binding of VHL to fibronectin excluding its incorporation within a CRL2 complex. (B) Modification of cullins with NEDD8 stabilises the active Cullin-RING-ligase (CRL) complex containing an F-box protein bound to its substrate. DeNEDDylation allows the reshuffling of the complex (dynamic state) through binding of CAND1 that acts as an exchange factor allowing the incorporation of new F-box proteins and substrates. (C) Under homeostatic conditions ribosomal proteins (RPL11) are associated with rRNA for ribosome biogenesis. Upon nucleolar stress, decrease in RPL11-NEDDylation allows the incorporation of RPL11 in complexes at transcriptional sites controlling gene expression.

NEDD8 and transcriptional activity regulation

p53 and TAp73

An emerging role for NEDDylation of non-cullin targets is the control of transcriptional activity. Several studies have implicated the NEDD8 pathway as regulator of the p53 tumour suppressor and its homologue TAp73. Direct NEDDylation of p53 and TAp73 inhibits their transcriptional activity (Xirodimas *et al.* 2004, Watson *et al.* 2006). The MDM2 E3-ligase, which is a common negative regulator for p53 and TAp73, promotes NEDDylation through direct binding to the substrates (Xirodimas *et al.* 2004, Watson *et al.* 2006). FBX011, an F-box protein and a component of CUL1-CRL preferentially promotes p53 NEDDylation but not ubiquitination and inhibits p53 transcriptional activity (Abida *et al.* 2007). The required lysines for p53 NEDDylation (K370, K372, K373 for MDM2 and K320, K321 for FBX011) are also reported sites for p53 ubiquitination, suggesting mutual exclusion or possibly cooperation between NEDD8 and ubiquitin for controlling p53 function (Xirodimas *et al.* 2004). Despite the close sequence similarity between NEDD8 and ubiquitin, these molecules differentially control p53 localisation. A fusion of C-terminal p53 to NEDD8 causes a nuclear p53 localisation, whereas a similar fusion with ubiquitin is found exclusively in the cytoplasm (Brooks & Gu 2006, Carter *et al.* 2007). Evidence for a physiological

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role for such differential role of NEDD8 and ubiquitin comes from studies on NEDD8 Ultimate Buster 1 (NUB1) and the TIP60 acetyltransferase. NUB1 was identified as a NEDD8 interacting protein and contains two UBA domains (Kamitani *et al.* 2001, Kito *et al.* 2001). Expression of NUB1 decreases p53 NEDDylation and promotes p53 mono-ubiquitination, which creates a nuclear export signal and cytoplasmic localisation of p53 (Liu & Xirodimas 2010). *TIP60* expression causes a preferential decrease in p53 NEDDylation but not ubiquitination (Dohmesen *et al.* 2008). The localisation of TAp73 is also controlled by NEDD8, but in contrast to p53 it promotes its cytoplasmic localisation (Watson *et al.* 2006). Whether NEDD8 specifically controls binding of p53 and TAp73 with nuclear or cytoplasmic anchoring factors is not yet known.

Another level of control of p53 function was revealed through the identification of ribosomal proteins as NEDD8 targets (Xirodimas *et al.* 2008). Ribosomal proteins have emerged as critical regulators of the p53 pathway. Under normal growth conditions ribosomal proteins rapidly enter the nucleolus as part of the ribosome biogenesis process for the production of the small and large subunits of the ribosome. However, conditions that mainly block transcription in the nucleolus, including nutrient starvation or treatment with low doses of the chemotherapeutic drug actinomycin D, cause the so-called nucleolar or ribosomal stress. This is manifested by the relocalisation of ribosomal proteins from the nucleolus to the nucleoplasm. Under these conditions, ribosomal proteins are able to bind and inhibit the activity of MDM2, which under unstressed conditions targets p53 for degradation (Zhang & Lu 2009, Boulon *et al.* 2010, Golomb *et al.* 2014, Vlatkovic *et al.* 2014). NEDDylation of RPL11 and RPS14 was shown to control the above signalling event (Sundqvist *et al.* 2009, Zhang *et al.* 2014). Nucleolar stress conferred by actinomycin D causes a decrease in RPL11 NEDDylation that allows RPL11 relocalisation from the nucleolus to the nucleoplasm and p53 activation. The MDM2 E3-ligase and the myeloma overexpressed 2 (MYEOV2) proteins were shown as cellular factors that promote or decrease RPL11-NEDDylation respectively (Sundqvist *et al.* 2009, Ebina *et al.* 2013). Similarly to RPL11, MDM2 promotes NEDDylation of RPS14 and it is required for its nucleolar localisation (Zhang *et al.* 2014). The human coilin-interacting nuclear ATPase protein (hCINAP), a protein essential for Cajal body formation, directly binds to RPS14 and inhibits RPS14 NEDDylation due to the recruitment of NEDP1 (Zhang *et al.* 2014). Mechanistically, at least for RPL11, the observed relocalisation upon stress is accompanied with the recruitment of RPL11 to the transcription sites of p53-regulated genes, facilitating the recruitment of the p300 transcriptional co-activator and p53 activation (Mahata *et al.* 2012).

The models derived from the studies on VHL and cullin NEDDylation propose that NEDD8 controls the substrate incorporation within different complexes or stabilises the active state of a complex respectively (Russell & Ohh 2008, Pierce *et al.* 2013). Similarly, NEDDylation may also control the differential incorporation of ribosomal proteins either within the ribosome biogenesis pathway (unstressed conditions) or within transcription factor complexes (nucleolar stress conditions) (Fig. 2). Inhibition of protein NEDDylation may cause the re-organisation of multicomponent complexes with distinct functional outcomes (Fig. 2).

Several cullins have also been implicated in negatively regulating p53 stability and function, either through direct binding to p53 (CUL7, PARCc) and cytoplasmic sequestration, through binding to MDM2 (CUL4A/B) or viral proteins (CUL2, 5) that facilitate p53 degradation (Querido *et al.* 2001, Nikolaev *et al.* 2003, Ali *et al.* 2004, Nag *et al.* 2004, Andrews *et al.* 2006, Banks *et al.* 2006, Sato *et al.* 2009, Thirunavukarasou *et al.* 2014).

The key biological outcome of all the above regulatory processes is the inhibition of p53 function by the NEDD8 pathway. Initial studies suggested that the biological effect of NEDD8 inhibition by MLN4924 is independent of the p53 status (Soucy *et al.* 2009). However, further studies showed that the p53 pathway could affect the biological outcome of NEDD8 inhibition in tumour cells (Lin *et al.* 2010, Blank *et al.* 2013). Interestingly, knockdown of p53 in MCF7 breast cancer cells facilitated the apoptotic response to MLN4924 treatment, suggesting that p53 activation may indeed protect cells against the apoptotic effect induced by NEDD8 inhibition (Lin *et al.* 2010). It is therefore important to establish the mechanism for the cytoprotective effect of p53, as inhibitors of p53 function could promote the MLN4924-induced apoptotic effect in tumours containing WT p53, which account for 50% of all cancer cases (Muller & Vousden 2014).

E2F1

The theme of transcriptional repression by NEDD8 was further expanded with studies on the NEDDylation of the transcription factor E2F1. The E2F family of eight members of transcription factors has an essential role in cell cycle progression and the G1 to S phase transition. E2F can also control apoptosis as in response to DNA damage

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induces expression of proapoptotic genes such as p73, *APAF1*, caspases (Biswas & Johnson 2012). NEDDylation represses the transcriptional activity of E2F1, which is relieved upon DNA damage and the action of the NEDP1 deNEDDylating enzyme (Loftus *et al*. 2012, Aoki *et al*. 2013). This allows the interaction of E2F1 with the co-activator Microcephalin (MCHP1) that is required for p73 induction (Aoki *et al*. 2013). Interestingly, the site of E2F1 NEDDylation (K185) is also methylated, providing another example of a possible interplay between NEDDylation and additional post-translational modifications (Loftus *et al*. 2012).

NFκB

NEDD8 also controls the function of NFκB by modifying the regulators of the pathway. The NEDDylation of the breast cancer-associated protein 3 (BCA3) inhibits NFκB activity in the nucleus through recruitment of the SIRT1 deacetylase (Gao *et al*. 2006). More recent studies have shown that the TRIM40 and BRCA1-associated protein 2 (BRAP2) E3-ligases could impact on the activity of NFκB. TRIM40, a member of the Tripartite motif (TRIM) family of RING finger proteins, was found to interact with NEDD8 in a yeast two-hybrid screen (Noguchi *et al*. 2011). A role for TRIM40 in controlling NFκB function was then revealed, as TRIM40 interacts with IKKγ, a component of the IKK complex that activates NFκB. TRIM40 was shown to promote NEDDylation of IKKγ and to repress NFκB function. K63-linked or linear poly-ubiquitination activates IKK and NFκB and therefore TRIM40-mediated NEDDylation of IKKγ may oppose this activation signal (Noguchi *et al*. 2011). As TRIM40 expression is significantly reduced in gastrointestinal carcinomas, it will be interesting to determine the level of IKKγ NEDDylation in these tumours. BRAP2 was also identified as a NEDD8 interacting protein in a yeast two-hybrid screen and to also bind CUL1 within the $Cul1^{\beta Trcp}$ complex that activates NFκB. Interestingly, BRAP2 is NEDDylated in a lysine that is within a motif similar to that of cullin NEDDylation (Takashima *et al*. 2013). The role of NEDD8 in BRAP2 function is not clear. However, overexpression or knockdown of BRAP2 leads to inhibition of NFκB nuclear translocation and activation, suggesting that BRAP2-NEDDylation or other post-translational modifications may control BRAP2 complex formation with components of the NFκB pathway (Takashima *et al*. 2013). Depending on cellular conditions NFκB can act as an oncogene or tumour suppressor (Perkins 2012). Differential regulation of components and complex formation within the NFκB pathway by post-translational modifications such as NEDDylation could provide the required elements for such functional switch. As our knowledge on NFκB function as an oncogene or tumour suppressor is growing, appropriate cancer model systems will be available to test the effect of NEDD8 inhibitors on NFκB functional switch and tumour suppression.

Membrane receptors

An opposing function of NEDD8 in controlling signalling through transmembrane tyrosine receptors was proposed. The epidermal-growth-factor-receptor (EGFR) and the transforming growth factor β type II receptor (TβRII) initiate signalling events upon binding to their respective ligands that control a plethora of biological processes related to cancer, including cell proliferation, fate determination and apoptosis (Massaguéet *al*. 2000). Receptor turnover is controlled by ubiquitination and in the case of EGFR and TBRII, the c-CBL E3-ligase promotes ubiquitination and degradation of the receptors (Oved *et al*. 2006, Zuo *et al*. 2013). As a common emerging function of RING E3-ligases, c-CBl can also promote NEDDylation of both receptors but with opposing outcomes. Upon ligand stimulation, c-CBL promotes EGFR NEDDylation, which further enhances EGFR ubiquitination and clathrin-mediated endocytosis for lysosomal degradation (Oved *et al*. 2006). However for TBRII, c-CBL-mediated NEDDylation at K556 and K567 protects the receptor from ubiquitination. This is due to the preferential targeting of the receptor for endocytosis to EEA1-positive early endosomes rather than to caveolin-positive compartments, where TBRII is ubiquitinated and degraded (Zuo *et al*. 2013). c-*CBL* mutations have been identified in leukaemia patients and one such identified mutation (H398L) severely impaired the activity of c-CBL to NEDDylate TBRII but also to ubiquitinate EGFR (Zuo *et al*. 2013).

The anti-diglycine antibody that enriches for peptides modified either by ubiquitin, NEDD8 or the Ubl ISG15 has been used in a recent proteomic approach to identify new targets for the Fanconi anaemia core complex (FANC) E3-ligase. The studies confirmed that fanconi anaemia complementation group D2 protein (FANCD2) is a major target for the FANC core complex (see also below). In addition, di-glycine peptides derived from the chemokine membrane receptor CXCR5 were significantly reduced in cells deficient for FANCA (one of the FANC partners) compared with cells corrected for FANCA expression. Further analysis showed that CXCR5 is NEDDylated but

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not ubiquitinated on lysine 339. The role of CXCR5 NEDDylation is to promote the membrane localisation of the receptor and is required for cell motility, a process controlled by CXCR5 in B-lymphocytes upon its ligand binding (Renaudin *et al*. 2014).

Histone modification and DNA damage response

There is also evidence to support a non-cullin role of NEDD8 in controlling the DNA damage response and apoptosis. Modification of histone 4 (H4) and histone H2A with NEDD8 is involved in the DNA damage response (Ma *et al*. 2013, Li *et al*. 2014*a*,*b*,*c*). RNF111 and RNF168 RING E3-ligases are involved in a complex way to control NEDDylation of H4 and H2A. DNA damage causes an increase in H4 NEDDylation mediated by RNF111, and a decrease in H2A-NEDDylation, which is physiologically mediated by RNF168. Under these conditions, RNF168 recognises the NEDD8 chains on H4, whereas the decrease in H2A-NEDDylation allows its ubiquitination. Both events are required for the subsequent recruitment of γ-H2AX and BRAC1 to sites of DNA damage (Ma *et al*. 2013, Li *et al*. 2014*a*,*b*,*c*). This intricate regulation of histone NEDDylation and ubiquitination may represent another example of cooperation between these modifiers to reorganise complex formation and allow, for example, the redistribution of E3-ligases such as RNF168 from H2A to H4.

In vivo siRNA screen of individual deubiquitinating enzymes in the developing eye of *Drosophila*, expressing antagonists for the inhibitors of apoptosis (IAPs) identified three different deNEDDylating enzymes as potential apoptosis regulators. NEDP1 (DEN1, SENP8) was one of the identified enzymes and its knockdown suppressed apoptosis. Further characterisation showed that the apoptosis executors Drice and caspase 7 are NEDDylated by the E3-ligases DIAP and XIAP, respectively, inhibiting their function. NEDP1 can reverse this effect by removing NEDD8, facilitating the execution of apoptosis (Broemer *et al*. 2010). While subsequent studies challenged the direct NEDDylation of caspase 7, it appears that NEDD8 can suppress apoptosis through cullin-independent mechanisms using NEDP1 as a regulator of the process (Broemer *et al*. 2010, Nagano *et al*. 2012). The above recent studies highlight potential new roles for NEDD8 in controlling cancer-related processes such as the DNA damage response and induction of apoptosis.

The Hu antigen R (HuR) is an RNA-binding protein enhancing the stability of multiple mRNAs encoding proteins with important roles in cell cycle and proliferation or tumour cell growth. HuR is found overexpressed in a wide variety of tumours, and there is a close correlation between HuR overexpression and tumourgenesis. NEDDylation of HuR, mediated by the MDM2 E3-ligase, protects HuR stability. Inhibition of NEDDylation either by siRNA knockdown of NEDD8 or UBC12 causes a decrease in HuR levels in colon and human liver cancer cell lines that overexpress HuR. The effect of NEDD8 in HuR stability is related with HuR subcellular localisation as NEDDylation promotes HuR nuclear localisation and protection from degradation (Embade *et al*. 2012). Therefore, similarly to what is observed for ribosomal proteins, NEDD8, in some cases, can protect targets from degradation, potentially by competing for ubiquitination sites or altering the rates of proteasomal degradation of substrates that can be simultaneously NEDDylated and ubiquitinated.

E3-ligases

The studies on the NEDDylation of non-cullin substrates had so far identified RING E3-ligases as the mediators of NEDD8 conjugation (Rabut & Peter 2008, Xirodimas 2008). Recent evidence has strongly supported that the HECT E3-ligase Smad ubiquitination regulator factor 1 (SMURF1) can also promote NEDDylation (Xie *et al*. 2014). In a yeast two-hybrid screen using SMURF1 as bait, NEDD8 was identified as potential interactor. A strong correlation between the expression of SMURF1 and components of the NEDD8 machinery in colorectal cancers prompted the authors to test the interaction of SMURF1 with NEDD8 and UBC12. A series of biochemical and biological studies revealed that SMURF1 binds to UBC12 promoting auto-NEDDylation. Interestingly, the authors identified CYS426 as potential catalytic residue for auto-NEDDylation distinct from its ubiquitination catalytic site CYS699. Reminiscent to the role of NEDD8 in CRL activity regulation, SMURF1 NEDDylation increases its ubiquitin ligase activity towards substrates and to itself. The phenomenon is conserved in *S. cerevisiae*, as the SMURF1 homologue RSP5 is similarly controlled by NEDDylation. However, RSP5 uses the same catalytic site CYS777 both for NEDDylation and ubiquitination. Importantly, the NEDD8 catalytic mutant of SMURF1 (C426A) has lost the ability to promote tumour formation in both *in vitro* and *in vivo* colorectal xenograft models. The regulatory role of NEDD8 for SMURF1 activity may be particularly important for colorectal cancers where SMURF1 is found to be overexpressed (Xie *et al*. 2014).

Additional NEDD8 targets with implications in neurodegenerative diseases include the amyloid precursor protein

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(APP) intracellular domain (AICD), the E3-ligase Parkin and the Pink1 kinase. Processing of the transmembrane protein APP creates a C-terminal AICD fragment, which translocates into the nucleus to control transcription by the FE65 and TIP60 transcriptional co-regulators. NEDDylation of APP was shown to occur at the AICD fragment and inhibited its interaction with FE65 and TIP60 (Lee *et al*. 2008). Parkin a RING in between RING E3-ligase and the Pink1 kinase play important roles in mitochondria maintenance and are found mutated in familial forms of Parkinson's disease. Parkin was shown to be NEDDylated, which increased Parkin ubiquitin E3-ligase activity towards itself and to its substrates (Choo *et al*. 2012, Um *et al*. 2012). NEDDylation of PINK1 increased the stability of the PINK1 55 kDa fragment, a processed form of PINK1 that is found in the cytoplasm. Importantly, NEDDylation of Parkinson's Disease was shown in extracts from human brains of PD patients, and treatment of cells with PD-related neurotoxins decreased NEDDylation of both Parkin and Pink1 (Choo *et al*. 2012). These studies may have implications in understanding Parkin and Pink1 regulation in neurodegeneration. However, as a potential function of Parkin as a tumour suppressor is revealed, these findings could expand Parkin's regulatory mechanisms to cancer-related pathways (Picchio *et al*. 2004, Fujiwara *et al*. 2008, Alderton 2010).

Potential strategies for the use of NEDD8 inhibitors in cancer therapy

The promising pre-clinical studies for MLN4924 have demonstrated the potential of NEDD8 inhibitors as chemotherapeutics (Table 1). They also provide the proof of principle to develop E1 inhibitors for other UBL E1 enzymes, such as SUMO or autophagy system, for which their role in cancer-related processes is well established. Common issues for compounds that enter into clinical trials include the potential development of resistance and their use as single agents or in combination therapy. The studies in tissue culture and xenograft model systems have indeed showed that resistance to MLN4924 is a highly anticipated outcome (Milhollen *et al*. 2012, Toth *et al*. 2012, Xu *et al*. 2014). These results are critical as not only demonstrate that the observed anti-tumour effects of MLN4924 depend primarily on its target, NAE, but also raise the necessity to develop new compounds that potentially will overcome resistance. Targeting the E2-conjugating enzymes or E3-ligases could be an interesting approach. For example, allosteric inhibitors for the ubiquitin E2-conjugating enzyme Cdc34, which is the primary ubiquitin E2 for CRLs, have been developed and similarly to NAE inhibitors they block CRL activity and cause accumulation of CRL targets (Ceccarelli *et al*. 2011). The unique N-terminus of the NEDD8 E2-conjugating enzyme UBC12, which provides an additional and specific interaction surface for NAE binding may also represent an interesting target to specifically block NEDD8 conjugation (Huang *et al*. 2004, Scott *et al*. 2011). Combination of MLN4924 with radiation or chemotherapeutics may also be an alternative or additional therapeutic approach. MLN4924 was shown to sensitise breast, pancreatic and lung cancer cells to ionising radiation. The combination dramatically increased the proportion of cells in the

Table 1 Potential combination approaches for MLN4924

Agent	Target	Tumour type	Targets of MLN4924 in combination therapy	References
Ionising radiation	DNA double strand breaks	Breast, pancreatic, lung cancer	p21 (breast cancer); wee1, cdt1 (pancreatic, lung cancers)	Wei *et al*. (2012), Yang *et al*. (2012*a*,*b*)
Cisplatin	DNA crosslinker	Ovarian cancer	FANCD2	Kee *et al*. (2012), Jazaeri *et al*. (2013)
Mitomycin C	DNA crosslinker	Melanoma	Re-replication, cdt1?	Garcia *et al*. (2014)
Non-genotoxic (TRAIL)	Death receptors	Head and neck squamous cell carcinoma	FLICE-inhibitory protein	Zhao *et al*. (2011)
Pifithrin	p53 inhibitor	Tumours with WT p53	?	Komarov *et al*. (1999), Lin *et al*. (2010), Blank *et al*. (2013)
Proteasome inhibitors	26S proteasome	?	?	Kim *et al*. (2011), Leidecker *et al*. (2012), Hjerpe *et al*. (2012*a*)
PYR-41	UBE1 (ubiquitin activating enzyme)	?	?	Yang *et al*. (2007*a*,*b*), Leidecker *et al*. (2012), Hjerpe *et al*. (2012*b*)
Autophagy inhibitors	Atg7?	?	?	Zhao *et al*. (2012)

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G2/M phase, which was associated with the observed sensitisation. Mechanistically, the effects appear to be due to the accumulation of CRL substrates cyclin-dependent kinase inhibitor p21 (breast cancer), or the wee1 kinase and the replication licensing factor CDT1 (pancreatic, lung cancers) (Wei *et al*. 2012, Yang *et al*. 2012*a*,*b*). In other studies, MLN4924 showed moderate activity in ovarian cancer cell lines as single therapy, but an additive effect was observed upon the combination of MLN4924 with the platinum-containing chemotherapeutic cisplatin. Importantly, the MLN4924–cisplatin combination was effective in ovarian cancer cell lines derived from patients who exhibited poor response to cisplatin alone, providing a potential new therapeutic approach for these cancers (Jazaeri *et al*. 2013). MLN4924 was shown to suppress cisplatin-induced mono-ubiquitinatination of FANCD2, a key step in the repair of DNA interstrand cross-linkages (ICLs) produced by cisplatin (Kee *et al*. 2012). These studies provide insights on the mechanism used for the observed sensitisation of MLN4924-treated cancer cells to DNA ICLs-inducing agents. Similar exciting results were obtained from the combination of MLN4924 with another DNA-alkylating chemotherapeutic, mitomycin C. In several tumour cell lines and in A375 melanoma xenografts, a synergy was observed between MLN4924 and mitomycin C, which is dependent on intact DNA damage and repair pathways, comprising ATR, BRAC1/2 and transcription coupled-nucleotide excision repair (TC-NER) (Garcia *et al*. 2014).

Another combination strategy for MLN4924 was proposed in head and neck squamous cell carcinoma (HNSCC) model systems. MLN4924 efficiently decreased the viability of several tested HNSCC cell lines as single agent, but its combination with the tumour necrosis factor-related apoptosis inducing signal (TRAIL/Apo2L) had a synergistic effect on induced apoptosis. At the molecular level, MLN4924 causes the degradation of the cellular FLICE-inhibitory protein (c-FLIP), a truncated form of caspase 8 that acts in a dominant-negative manner to block caspase 8 activation induced by TRAIL (Zhao *et al*. 2011). Combination of MLN4924 with non-genotoxic inducers of apoptosis may prove an attractive therapeutic approach, as non-genotoxic agents are generally less toxic to healthy cells compared with DNA damaging agents.

As inhibition of NEDDylation by MLN4924 can also induce responses that protect cells against treatment-induced apoptosis, inhibitors of such pathways may enhance the anti-tumour effects of MLN4924. Using p53 inhibitors such as pifithrin could prevent the observed cytoprotective effect of p53 activation upon MLN4924 treatment (Komarov *et al*. 1999). Similarly, inhibitors of the autophagy pathway may also sensitise cells to MLN4924. Such an approach will be also part of a growing concept for an extensive cross-talk between ubiquitin and UBLs that is observed in different cellular conditions. The NEDD8–ubiquitin cross-talk is at the level of CRL activation and protein ubiquitination and through activation of NEDD8 by the ubiquitin E1 enzyme UBE1. The later may be relevant should MLN4924 is combined with proteasome inhibitors in the clinic, as proteasome inhibition increases protein NEDDylation through UBE1 and is insensitive to MLN4924 treatment (Leidecker *et al*. 2012, Hjerpe *et al*. 2012*a*). This also raises the question whether MLN4924 should be combined with inhibitors of UBE1 such as PYR-41 or it will prove too toxic? (Yang *et al*. 2007*a*,*b*, Lane 2012).

Our knowledge on the complexity within the ubiquitin and UBL pathways and diversity of the regulated processes is rapidly growing. Protein NEDDylation is clearly emerging as an important regulator of several cancer-associated pathways with either pro- or anti-tumourgenic potential. A complete understanding of the spectrum of regulated processes and mechanisms of control of the NEDD8 pathway will help the potential use of NEDD8 inhibitors in the clinic as a single agent or in combination with other therapeutics.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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