Cancer Cell
Review

NEDD8 Pathways in Cancer, Sine Quibus Non

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DOI 10.1016/j.ccr.2011.01.002

There are 17 known ubiquitin-like proteins (UBLs) from nine phylogenetically distinct classes (NEDD8, SUMO, ISG15, FUB1, FAT10, Atg8, Atg12, Urm1, and UFM1) that have been identified to conjugate to substrates in a manner analogous to ubiquitin. NEDD8 is one of the most studied UBLs and shares the highest amino acid similarity to ubiquitin. Here, we review the current knowledge of the NEDD8 conjugation cascade derived from functional studies in genetic model organisms, structural insights from crystallographic studies, biochemical studies identifying a growing list of NEDD8 substrates with oncogenic implications, and attempts to pharmacologically target the NEDD8 pathway in cancer.

The NEDD8 Conjugation Cascade

In 1992, a subtractive cloning screen between a cDNA library derived from mouse neural precursor cells and mouse adult mRNA identified ten neural precursor cell-expressed, developmentally downregulated (NEDD) genes (Kumar et al., 1992). The eighth gene discovered, NEDD8, shared approximately 60% amino acid identity with ubiquitin and similarly conjugated to substrates (Kamitani et al., 1997).

Analogous to ubiquitylation, the NEDD8 conjugation cascade or neddylation involves E1, E2, E3, and deneddylating enzymes (Figure 1) (Liakopoulos et al., 1998; Osaka et al., 1998). Like ubiquitin, NEDD8 is first synthesized as a precursor that is processed at the conserved C-terminal Gly76 residue by the hydrolase activity of deneddylating enzymes exposing a glycine-glycine motif that serves as the attachment site for target substrates (Kamitani et al., 1997). NEDD8-specific proteases able to hydrolyze the NEDD8 C-terminus include NEDP1 (also known as DEN1 and SENP8) and UCH-L3, which can also process ubiquitin precursors (for review, see Rabut and Peter, 2008; Xirodimas, 2008). The exposed C-terminal glycine of NEDD8 is adenylated by the E1 NEDD8-activating enzyme (NAE), which is composed of NAE1 (APP-BP1) and Uba3 heterodimer, in an ATP-dependent reaction and transferred to E1 cysteine side chain via thiolester linkage (for review, see Pan et al., 2004). Activated NEDD8 is subsequently transferred to the E2 NEDD8-conjugating enzyme, Ubc12, forming another thiolester linkage. Ube2f is another NEDD8 E2, which preferentially promotes neddylation of Cul5 (Huang et al., 2009). An E3 NEDD8 ligase then transfers NEDD8 to the ε-amino group of lysyl residue on substrates forming an isopeptide bond. NEDD8 E3s contain really interesting novel gene (RING) finger domains, which include Rbx1 and Rbx2 (also known as ROC1 and ROC2, respectively), MDM2, c-CBL, and SCF^{FBX011} (Abida et al., 2007; Kamura et al., 1999; Oved et al., 2006; Xirodimas et al., 2004) with the sole exception being DCN1 (Kurz et al., 2005, 2008). Interestingly, DCN1 does not require any of its cysteines for its catalytic activity; rather DCN1 in conjunction with the yeast Rbx1 homolog, Hrt1, functions synergistically as a dual NEDD8 E3 ligase to promote ligation to Cdc53 (Scott et al., 2010). There are five DCN1-like proteins in humans termed DCNL1-5, of which DCNL1-3 have been shown to promote Cul3 neddylation (Meyer-Schaller et al., 2009).

The types of ubiquitin chains linked to a given substrate play crucial roles in dictating the functional outcome. However, the types of NEDD8 chains are only beginning to be elucidated. Recently, Jones et al. discerned from mass spectrometry analyses that Lys11, Lys22, Lys48, and Lys60 can be linked to form NEDD8 chains in vivo (Jones et al., 2008). Poly-NEDD8 chain formation is thought to be mediated by a build-up of NEDD8 on Ubc12 catalytic cysteine prior to transfer to Cul1 (Ohki et al., 2009). NEDD8 can also be linked to ubiquitin chains in vitro (Whitby et al., 1998). Ubiquitin has also emerged in proteomic studies identifying NEDD8 substrates, although the prevalence of such mixed ubiquitin-NEDD8 chains appears minor and their function is not known (Jones et al., 2008; Xirodimas et al., 2008).

Neddylation is a reversible process. The COP9 signalosome (CSN) is a zinc metalloprotease and the most studied of the NEDD8 deconjugating enzymes. CSN is an 8 subunit complex, where CSN5 subunit possesses the catalytic activity (Lyapina et al., 2001; Schwechheimer et al., 2001). NEDP1 was recently identified as another NEDD8-specific protease (Rabut and Peter, 2008; Xirodimas, 2008). However, compared to CSN, NEDP1 exhibits three orders of magnitude less activity in cleaving neddylated Cul1 (Yamoah et al., 2005). Interestingly, deletion of *NEDP1* ortholog in *Schizosaccharomyces pombe* and *Drosophila melanogaster* did not result in accumulation of neddylated Cul1 or Cul3, although a significant accumulation of yet-identified neddylated substrates was observed, suggesting roles for NEDP1 in the regulation of cullin-independent substrates (Chan et al., 2008; Zhou and Watts, 2005). Additional proteases with dual NEDD8 and ubiquitin protease activity have been identified (see Figure 1; for review, see Rabut and Peter, 2008; Xirodimas, 2008).

Structural Insight into the NEDD8 Machinery

E1 activating enzymes for ubiquitin and ubiquitin-like proteins (UBLs) possess adenylation domain that binds ATP and ubiquitin or UBL, a catalytic Cys domain possessing the cysteine for thiolester linkage, and an ubiquitin fold domain (UFD) that binds

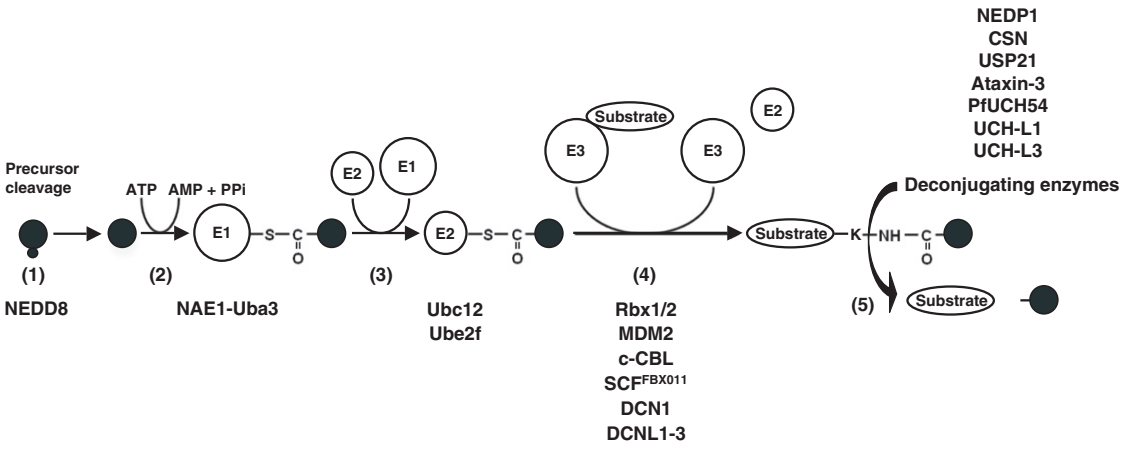

Figure 1. General Overview of the NEDD8 Conjugation Pathway

(1) NEDD8 is synthesized as a precursor that is processed at a conserved C-terminal gly(76) residue by the hydrolase activity of deneddylating enzymes, which include NEDP1 and UCLH3, exposing a glycine-glycine motif that serves as the attachment site for target substrates. (2) The exposed C-terminal glycine of NEDD8 is adenylated by an activating (E1) enzyme in conjunction with ATP and transferred to a cysteine side chain of an E1 via a thiolester linkage. (3) Activated NEDD8 is subsequently transferred to a conjugating (E2) enzyme forming another thiolester linkage. (4) A ligase (E3) transfers NEDD8 to the ε-amino group of a substrate lysyl residue resulting in the formation of an isopeptide bond. (5) Covalent modification of substrates can be reversed by the action of deneddylating enzymes, which include CSN, NEDP1, USP21, Ataxin-3, PfUCH54, UCH-L1, and UCH-L3.

E2 (for review, see Schulman and Harper, 2009). The catalytic mechanisms governing activation of ubiquitin and subsequent transfer to an E2 enzyme is best characterized for the ubiquitin E1, Uba1. In brief, following E1-mediated adenylation of the ubiquitin C-terminus, the E1 catalytic cysteine attacks the ubiquitin adenylate forming a thiolester intermediate (Ciechanover et al., 1981, 1982). Uba1 subsequently loads an additional ubiquitin molecule by catalyzing the adenylation of a second ubiquitin (Haas and Rose, 1982; Haas et al., 1982). Occupancy of ubiquitin-AMP in the adenylation domain was found to stimulate ubiquitin transthiolation from the E1 to E2 enzymes, demonstrating the crosstalk between the adenylation active site and the E1-E2 transfer (Pickart et al., 1994). Finally, structural overlap between E1 and E3 binding sites found in E2s result in mutually exclusive E1-E2 and E2-E3 interactions, ensuring progression of the E1-E2-E3 cascade (Eletr et al., 2005).

Schulman and colleagues have elucidated many of the molecular mechanisms that mediate the E1-E2-E3 cascade reaction for the NEDD8 pathway, and identified specific molecular determinants that distinguish the ubiquitin and NEDD8 conjugation cascades (for review, see Schulman and Harper, 2009). First, Uba1 and the NEDD8 activating enzyme component, Uba3, differ in their ability to interact with ubiquitin and NEDD8, respectively, due to amino acid dissimilarity at residue 72 (Ala in NEDD8 and Arg in ubiquitin) (Walden et al., 2003). The selectivity is attributed to a unique Arg in Uba3, which repels Arg72 of ubiquitin. Second, the selectivity of NEDD8 cascade is also dictated by interactions of the N-terminal sequence of the E2 catalytic domain and the UFD of Uba3. A unique 26 amino acid N-terminal extension of Ubc12 docks in a groove exclusive to Uba3, further ensuring pathway specific E1-E2 interactions (Huang et al., 2005a). Interestingly, when the NAE1/Uba3 heterodimer is doubly loaded with NEDD8 linked by a thiolester bond and an adenylated NEDD8, the UFD undergoes a striking conformational change that unmasks a cryptic Ubc12 binding site, and as a result, promotes E2 binding when the E1 is ready to transfer NEDD8 (Huang et al., 2007). Thus, the transfer of NEDD8 to Ubc12 may eliminate the second binding site, which would promote Ubc12-NEDD8 dissociation (Schulman and Harper, 2009). Finally, as demonstrated for ubiquitin and SUMO E1 and E2 enzymes, the NEDD8 E1 and E3 binding sites overlap on Ubc12, which ensures progression of the E1-E2-E3 cascade (Huang et al., 2005a).

Biological Roles of NEDD8 from Model Organisms and Links to Cancer

An intact NEDD8 pathway is required for viability in *S. pombe*, *D. melanogaster, Caenorhabditis elegans,* and mice (for review, see Pan et al., 2004). Inactivation of the NEDD8 pathway in the ts41 CHO cell line possessing a temperature-sensitive mutation in the *SMC* gene (the hamster ortholog of human *NAE1*) caused multiple rounds of S-phase DNA replication without intervening mitosis (Handeli and Weintraub, 1992; Hirschberg and Marcus, 1982). RNAi-mediated knockdown of NED-8, or NED-8 E1 and E2 enzymes in *C. elegans* caused hypersensitivity to ENU-induced apoptosis in germ cells (Gao et al., 2008), and other developmental abnormalities including defects in cytoskeleton regulation (Jones and Candido, 2000; Kurz et al., 2002). In the plant, *Arabidopsis thaliana*, NEDD8 E2 ortholog *RCE1* mutants had a reduced growth phenotype (Dharmasiri et al., 2003).

A general elevation in the level of NEDD8 conjugation has been observed in oral squamous cell carcinoma cell lines where NEDD8 pathway inhibition decreased cell proliferation (Chairatvit and Ngamkitidechakul, 2007). The components of cullin-RING ligases (CRLs) are overexpressed, amplified or mutated in several human cancers, and many CRLs regulate the activity of numerous players in tumorigenesis (see Table 1) (for review, see Guardavaccaro and Pagano, 2004). Several newly discovered NEDD8 substrates are established tumor suppressors or oncoproteins, including VHL, p53 and MDM2 (Table 1). Furthermore, the general inhibitor of NEDD8 pathway, MLN4924, was recently shown to possess tumor-inhibiting properties (Soucy et al., 2009).

Table 1. NEDD8 Substrates Implicated in Tumorigenesis

Substrates	Function of NEDD8 Modification	Cancer Implications
Cul1	Increases CRL activity	CRL1 or SCF complexes degrade a multitude of substrates. For example, SCF^{Skp2} degrades inhibitors of cell cycle progression and is amplified in a number of human cancers; $SCF^{\beta\text{-}Trcp}$ promotes degradation of the IκB family of NF-κB inhibitors, and elevated levels of β-Trcp has been reported in tumors and often associated with poor prognosis; SCF^{Fbw7} complex primarily mediates degradation of oncoproteins Cyclin E, Notch and c-myc, and Fbw7 is a bona fide tumor suppressor that is frequently mutated in human cancer. Notably, more than 350 potential CRL1 substrates have been identified using global protein stability (GPS) profiling technology.
Cul2	Increases CRL activity	Cul2 neddylation required for the tumor suppressor VHL function of mediating HIFα degradation via the ECV complex.
Cul3	Increases CRL activity	CRL3 complexes have roles in regulating cytokinesis, oxidative stress response pathways, oncogenic Wnt-β-catenin and Hedgehog signaling pathways.
Cul4	Increases CRL activity	CRL4 complexes regulate DNA replication and nucleotide excision repair (NER), and Cul4A is amplified in breast cancer.
Cul5	Increases CRL activity	Adenoviral proteins have been identified that utilize CRL5 complexes to promote degradation of tumor suppressor proteins to promote propagation of the infection cycle.
Cul7	—	Cul7 possesses an antiapoptotic function through its ability to regulate p53, and Cul7 is overexpressed in non-small cell lung carcinoma.
PARC	—	PARC negatively regulates p53 function by promoting cytoplasmic localization in neuroblastoma cells.
p53/p73/ BCA3/ VHL	Regulates transactivation function and protein-protein interactions	Neddylation regulates transactivation function of the p53 family, the ability of BCA3 to regulate NF-κB, and the regulation pVHL-fibronectin interaction.
MDM2, L11	Increases protein stability	MDM2 and L11 stability is regulated by NEDD8.
EGFR	Increases receptor lysosomal sorting	c-Cbl mediates EGFR endocytosis by promoting ubiquitylation and neddylation of EGFR, and c-Cbl mutations are observed in a number of human cancers.

See text for details.

NEDD8 Substrates: Cullins, Tumor Suppressors, and Oncoproteins

The cullin family is composed of Cul1, 2, 3, 4A, 4B, 5, and 7, and PARC and Apc2, a component of the anaphase promoting complex/cyclosome (APC/C), contain a cullin homology domain (for review, see Petroski and Deshaies, 2005). The first and best-characterized NEDD8 substrates are the cullins, which are structurally related proteins that function as molecular scaffolds of CRLs. PARC is also subject to neddylation, but Apc2, which lacks the purported C-terminal NEDD8 consensus sequence (IVRIM**K**MR) found in cullins, is not (Pan et al., 2004; Skaar et al., 2007). CRLs are composed of RING finger protein Rbx1 or Rbx2, a substrate recognition subunit that binds directly or via an adaptor protein to the N-terminus of cullins (Figure 2).

Rbx1 and Rbx2 in conjunction with Ubc12 and Ube2f, respectively, promote NEDD8 modification of cullins (Huang et al., 2009; Kamura et al., 1999). Cullin neddylation has been shown to increase the ubiquitylation activity of CRLs. For example, Cul1 neddylation increased SCF-mediated degradation of IκBα and p27 (for review, see Pan et al., 2004). Cullin neddylation promotes conformational changes that increase binding of Rbx1 to ubiquitin E2s, reduce the distance between E2 and the substrate recognition component, thus bringing ubiquitin closer to its target, and increase ubiquitin chain extension by allowing greater E2 access to the nascent polyubiquitin chain (Duda et al., 2008; Pan et al., 2004; Saha and Deshaies, 2008; Yamoah et al., 2008). Neddylation also abrogates binding of CAND1, which associates with unneddylated cullins to inhibit CRL activity by preventing cullin binding to adaptor and substrate recognition components (Liu et al., 2002; Zheng et al., 2002). CSN-mediated deneddylation has the reverse effect of activating CRL-based E3s, and the CSN also recruits the deubiquitinating enzyme Ubp12/USP15 to prevent ubiquitin chain assembly by CRLs (Lyapina et al., 2001; Schwechheimer et al., 2001; Zhou et al., 2003). However, dynamic cycling of NEDD8 conjugation and deconjugation are critical in maintaining CRL activity, as deletion of any CSN subunit or CAND1 leads to decreased CRL function (for review, see Petroski and Deshaies, 2005). Recently, Wolf and colleagues demonstrated that only a subset of F-box proteins (FBP) requires CSN for protection against autocatalytic destruction, and CAND1 does not play a role in the regulation of adaptor stability but regulates FBP complex formation. Upon substrate-induced neddylation, CRLs are subjected to regulation by CSN. Following substrate degradation, CSN deneddylates cullin to permit CAND1 regulation of CRL (Schmidt et al., 2009). Through the use of general NAE inhibitor MLN4924 and a quantitative mass spectrometry based methodology, Harper and colleagues recently demonstrated that prolonged global dennedylation does not convert CRL complexes to cullin-CAND1 complexes (Bennett et al.,

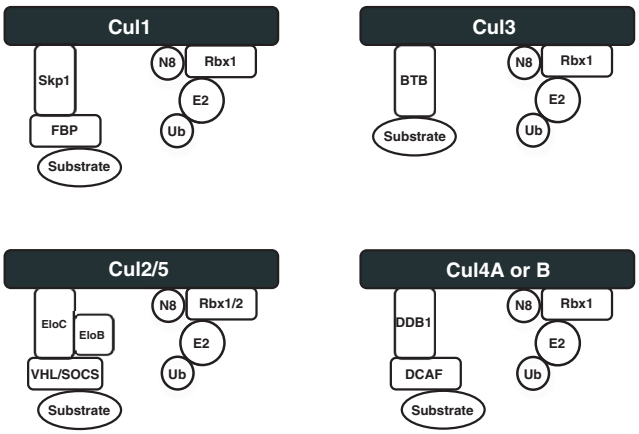

Figure 2. The Cullin RING Finger E3 Ligase Complexes
The best characterized cullin RING ligase (CRL) is the Skp1-Cul1-F-box protein (SCF) complex, where Cul1 functions as a scaffolding protein in which its N-terminus interacts with the adaptor subunit, Skp1, and its C terminus with the Rbx1 RING finger protein that associates with an E2 ubiquitin conjugating enzyme to promote substrate ubiquitylation. Skp1 interacts with an F-box protein (FBP) substrate recognition component of the SCF. Both Cul2 and Cul5 interact with Elongin B and C adaptor proteins and Rbx1 or Rbx2, respectively. Cul2 and Cul5 associate with VHL and suppressor of cytokine signaling (SOCS) box protein, respectively, that function as the substrate recognition subunit. Cul3 interacts with Rbx1and broad complex, Tramtrack and Bric-a-Brac (BTB) protein that mediates both substrate specificity and binding to Cul3. Cul4 interacts with Rbx1 and the damaged DNA binding (DDB1) adaptor protein, and a DDB1 and Cul4-associated factor (DCAF) substrate recognition protein. There are approximately 110 F-box proteins, 50 SOCS/BC box proteins, 450 BTB, and 90 DCAF known or predicted human proteins, and as a result, a multitude of CRLs can be potentially assembled from the 7 human cullin family members to target a myriad of substrates for degradation (for review, see Jackson and Xiong, 2009 and Petroski and Deshaies, 2005).

2010). The study indicates that irrespective of their neddylation status, most cullins are bound to adaptors whereas a small fraction is associated with CAND1, suggesting that the abundance of adaptor modules, rather than cycles of neddylation and CAND1 binding, drives global CRL network organization (Bennett et al., 2010).

Neish and colleagues discovered enteric bacteria-induced ROS production from colonized epithelial cells inactivates the catalytic cysteine residue of Ubc12 resulting in decreased Cul1 neddylation and inhibition of the NF-κB pathway due to stabilization of the $SCF^{\beta\text{-Trcp}}$ substrate and NF-κB inhibitor, IκB-α (Collier-Hyams et al., 2005; Kumar et al., 2007). A recent study identified an additional mechanism of bacteria-induced inhibition of ubiquitin/NEDD8 conjugation via the bacterial effector deamidase, CHBP, which promotes deamidation of Gln40 of NEDD8, resulting in abolishment of CRL activity (Cui et al., 2010).

Cul1-Based CRLs

The three most studied Cul1-CRLs (CRL1) or Skp1-Cul1-F-box protein (SCF) complexes with cancer implications are SCF^{Skp2}, $SCF^{\beta\text{-Trcp}}$, and SCF^{Fbw7}. SCF^{Skp2} degrades inhibitors of cell cycle progression p130 (retinoblastoma-related "pocket protein") and the cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$, $p57^{Kip2}$, and $p21^{Cip1}$ (for review, see Guardavaccaro and Pagano, 2004). Tumor suppressor BRCA2, TOB1, FOXO1, and RASSF1A have also been identified as SCF^{Skp2} substrates (Hiramatsu et al., 2006; Huang et al., 2005b; Moro et al., 2006; Song et al., 2008). $SCF^{\beta\text{-Trcp}}$ promotes degradation of IκB family of NF-κB inhibitors and pro-apoptotic BH3-only tumor suppressor protein, BimEL (Dehan et al., 2009; Guardavaccaro and Pagano, 2004). In addition, overexpression of β-Trcp in the mammary epithelia of female mice promotes the development of carcinomas in approximately 40% of these mice (Kudo et al., 2004). Conversely, SCF^{Fbw7} primarily mediates degradation of Cyclin E, Notch and c-*myc* oncoproteins (for review, see Guardavaccaro and Pagano, 2004). Fbw7 is located on chromosome 4q32 where genetic aberrations are frequent and Fbw7 mutations have been detected in human cancer (for review, see Welcker and Clurman, 2008). Studies from a number of model organisms have demonstrated roles for Fbw7 in the negative regulation of cell cycle, development and tumorigenesis (Matsuoka et al., 2008; Moberg et al., 2001; Tetzlaff et al., 2004). Thus, Cul1 assembles CRLs that have tumor suppressor or oncogene functions depending on the respective substrate recognition components. Notably, there are approximately 110 F-box proteins and as a result a number of SCF complexes can be formed to regulate a plethora of substrates (Petroski and Deshaies, 2005). Recently, Elledge and colleagues applied global protein stability (GPS) profiling technology and identified more than 350 potential CRL1 substrates, highlighting the importance of cullin-mediated regulation in a broad range of cellular functions (Yen and Elledge, 2008).

Cul2-based CRL, ECV

Cul2 forms a CRL2 complex with the tumor suppressor von Hippel-Lindau (VHL) protein, germline mutation of which causes VHL disease characterized by the development of multiple tumors in numerous organs upon the loss of the remaining wild-type allele in a susceptible cell. Biallelic inactivation of *VHL* is also responsible for the development of the vast majority of sporadic clear-cell renal cell carcinomas (RCC), the most common form of kidney cancer (for review, see Kaelin and Ratcliffe, 2008).

VHL is the substrate-recognition component of CRL2 called ECV (Elongin BC/Cul2/VHL), which targets hypoxia-inducible factor α subunit (HIFα) in an oxygen-dependent manner (Kaelin and Ratcliffe, 2008). Similar to Cul1, Cul2 neddylation increases E3 function of ECV (Ohh et al., 2002). Unexpectedly, VHL was identified as the first tumor suppressor targeted for neddylation and thereby expanding the substrate repertoire of NEDD8 beyond cullins (Stickle et al., 2004). VHL neddylation prohibited the engagement of Cul2 while promoting VHL association with fibronectin, which was necessary for the tumor suppressor activity of VHL (Russell and Ohh, 2008).

Cul3-Based CRLs

Cul3-CRL complexes (CRL3s) have been implicated in the degradation of Cyclin E (Singer et al., 1999). Studies from *C. elegans* showed that Cul3 interacts with several BTB protein subunits, including Mel-26 that targets Mei-1, an essential component of the mitotic spindle, for degradation (Furukawa et al., 2003; Geyer et al., 2003; Kurz et al., 2002; Pintard et al., 2003; Xu et al., 2003). Furthermore, BTB subunits KLHL9-KLHL13 form a complex with Cul3 to remove Aurora B from mitototic chromosomes promoting completion of cytokinesis in human cells (Sumara et al., 2007). In *Drosophila*, Cul3 regulates the stability of Cubitus interuptus (Ci), which is a known regulator

of the oncogenic Hedgehog signaling pathway (Ou et al., 2002). KLHL12-Cul3 negatively regulates Dishevelled, which is often overexpressed in non-small cell lung cancer and is a positive regulator of the oncogenic Wnt-β-catenin pathway (Angers et al., 2006; Uematsu et al., 2003). CRL3s are also known to regulate oxidative stress response pathways. The BTB subunit KEAP1 (also known as INRF2) interacts with Cul3 to promote the degradation of the transcription factor, NRF2 (for review, see Niture et al., 2010). Following the generation of reactive oxygen species (ROS) in response to a number of stress-inducing agents, NRF2 dissociates from KEAP1 to transactivate genes possessing an antioxidant response element (ARE) that protect against oxidative stress. Therefore, NRF2 plays a role in protecting against DNA damaging agents as demonstrated from *Nrf2 null* mice that have increased incidence of carcinogen-induced tumors (Niture et al., 2010). However, somatic mutations of KEAP1 and NRF2 have been observed in a number of cancers that allow NRF2 to escape KEAP1-mediated regulation resulting in NRF2-mediated constitutive induction of cytoprotective enzymes that provide cancer cells with a survival advantage to anticancer treatments (Shibata et al., 2008).

Cul4-Based CRLs

Cul4A and Cul4B are two closely related paralogs in mammals. DDB1-Cul4^{CDT2} promotes degradation of *C. elegans* polymerase η, *Drosophila* E2F1, human p21, and the replication factor CDT1 that is degraded during S-phase and in response to DNA damage (for review, see Jackson and Xiong, 2009). RNAi-mediated inactivation of *cul-4* in *C. elegans* results in cell proliferation accompanied by massive levels of DNA re-replication, which is rescued by reduction of CDT-1 levels (Zhong et al., 2003). Mutations in the DCAF protein DDB2 are responsible for the heritable human disorder Xeroderma pigmentosum (XP) subtype E phenotype, which is characterized by defects in nucleotide excision repair (NER) and susceptibility to skin cancer (Chu and Chang, 1988). DDB1-Cul4^{DDB2} mediates monoubiquitylation of core histones H2A, H3 and H4, which are thought to initiate NER (Kapetanaki et al., 2006). DDB1-Cul4^{DDB2} also regulates polyubiquitylation of the NER protein, XPC, which is thought to promote its DNA binding affinity (Sugasawa et al., 2005) and DDB2 itself is negatively regulated by autoubiquitylation (Chen et al., 2001; Nag et al., 2001). Additional Cul4-CRL complexes (CRL4s) that mediate degradation of cancer relevant proteins include tuberous sclerosis 2 (TSC2) by DDB1-Cul4^{FBW5}, merlin by DDB1-Cul4^{VprBP}, ERα by DDB1-Cul4^{AhR}, and c-Jun by DDB1-Cul4$^{hDET1\text{-}hCOP1}$ (for review, see Jackson and Xiong, 2009). An oncogenic role for Cul4 is further supported by the fact Cul4A is located on 13q34, a region frequently amplified in human tumors including breast cancer (Chen et al., 1998; Melchor et al., 2009), and Cul4A deficient mice are resistant against UV-induced skin tumors (Liu et al., 2009).

Cul5-Based CRLs

The suppressor of cytokine signaling (SOCS) family of proteins is the substrate recognition component of Cul5-CRL complexes (CRL5), and is comprised of 8 members (SOCS1-7, CIS) containing an N-terminal domain, a SH2 domain and a C-terminal SOCS box, which is composed of two regions; a BC box responsible for binding Elongins BC and a Cul5 box that directs engagement to Cul5 (Hilton, 1999). The SOCS box is also found in other proteins, such as the SPRY (repeats in SplA/Ryanodine receptor) domain-containing SOCS box (SSB) protein family (Wang et al., 2005). Notably, SOCS1 promotes proteasomal degradation of the TEL-JAK2 fusion oncoprotein associated with leukemia (Frantsve et al., 2001; Kamizono et al., 2001). Biallelic mutation of SOCS1 has been found in mediastinal lymphoma and ectopic expression of SOCS1 downregulates JAK2-STAT5 signaling (Melzner et al., 2005). Additionally, the promoter of SOCS3 promoter has been shown to be hypermethylated in lung cancer cell lines and primary tumor specimens resulting in reduced SOCS3 protein expression and elevated pSTAT3 expression (He et al., 2003). In addition, the human adenovirus serotype 5 (Ad5) products, E4orf6 and E1B55k, interact with CRL5 to promote p53 degradation, which requires an intact NEDD8 pathway (Querido et al., 2001). Kaposi's sarcoma-associated herpes virus (KSHV)-encoded latency-associated nuclear antigen (LANA) was found to use a similar mechanism to promote degradation of the tumor suppressors VHL and p53 in collaboration with CRL5 complex (Cai et al., 2006).

Cul7-Based CRLs and PARC

Fbxw8 is the only F-box protein known to interact with Cul7 and unlike other cullins, Cul7 does not appear to be modified by NEDD8 (Skaar et al., 2007). Nevertheless, Cul7 is overexpressed in non-small cell lung carcinoma and regulates Cyclin D1, insulin receptor substrate-1 (IRS-1), and p53 (Andrews et al., 2006; Kim et al., 2007; Okabe et al., 2006; Xu et al., 2008). The Cul7 homolog PARC is modified by NEDD8 and appears to function as a cytoplasmic anchor of p53 in neuroblastoma cells (Nikolaev et al., 2003). However, the functional consequence of PARC neddylation is yet to be determined.

MDM2, p53 Family, and Other NEDD8 Substrates

MDM2 is a RING finger E3 ligase amplified in human cancers that promotes ubiquitylation and degradation of p53. Xirodimas et al. (2004) demonstrated that MDM2 also promotes NEDD8 modification of p53, which attenuates its transactivation function. Dohmesen et al. (2008) subsequently provided evidence that Tip60 acetyl transferase, a known regulator of the MDM2-p53 axis, preferentially inhibited MDM2-mediated neddylation, but not ubiquitylation, providing insight into the molecular mechanisms differentially regulating these two processes. Interestingly, NEDD8-interacting protein, NUB1, was shown to decrease p53 neddylation and preferentially stimulate p53 mono-ubiquitylation, resulting in p53 nuclear export (Liu and Xirodimas, 2010). The F-box protein, FBX011, was identified as a p53-interacting protein that unexpectedly promoted p53 neddylation rather than ubiquitylation to inhibit p53 transactivation function (Abida et al., 2007). Notably, p53-NEDD8 fusion protein was shown to differentially regulate transactivation of known p53 target genes in comparison to wild-type p53, suggesting that neddylation may play a role in determining p53-target gene specificity (Carter and Vousden, 2008). MDM2 also promotes neddylation of the proapoptotic p53 family member, TAp73β, promoting accumulation of NEDD8-modified TAp73β in the cytoplasm to inhibit its transactivation function (Watson et al., 2006).

MDM2 itself is subjected to NEDD8 modification (Xirodimas et al., 2004), which was shown to significantly increase its protein stability (Watson et al., 2010). Conversely, chemotherapy-induced NEDP1 was shown to promote MDM2 deneddylation and destabilization resulting in p53 activation (Watson et al., 2010). p53 is also activated in the response to

ribosomal/nucleolar stress, such as following exposure to the chemotherapy agent, Actinomycin D (ActD), due to ribosomal protein-mediated inhibition of MDM2′s E3 ligase activity (for review, see Zhang and Lu, 2009). Ribosomal proteins were identified as NEDD8 substrates and NEDP1 was shown to regulate both the stability and localization of ribosomal protein L11 (Xirodimas et al., 2008). ActD-induced NEDP1 was associated with the relocalization of L11 from the nucleolus to the nucleoplasm, providing a signal for p53 activation (Sundqvist et al., 2009). However, MDM2-mediated neddylation of L11 is also required for its stabilization, illustrating a complex role of NEDD8 as a regulator of p53 nucleolar stress signaling (Sun et al., 2010; Sundqvist et al., 2009).

In addition, studies have also linked CRLs in the regulation of the p53 family. In *C. elegans* mutants of the F-box protein, FSN-1, were hypersensitive to ENU-induced germline apoptosis, which was rescued by loss of the p53 homolog *cep-1* (Gao et al., 2008). The human ortholog of FSN-1 was later shown to promote proteasomal degradation of p73 and the JFK F-box protein was found to promote p53 degradation via SCF complexes (Peschiaroli et al., 2009; Sun et al., 2009). Last, $SCF^{\beta\text{-Trcp}}$ was recently shown to regulate the stability of MDM2 (Inuzuka et al., 2010).

In addition to cullins, VHL and the p53 family, the breast cancer-associated protein 3 (BCA3) was identified as a NEDD8 substrate by Yeh and colleagues, which was shown to suppress NF-κB-dependent transactivation through its ability to bind to NF-κB subunit p65 and to recruit histone deacetylase SIRT1 (Gao et al., 2006). c-Cbl was shown to promote NEDD8 modification of EGFR, which accelerated EGFR degradation via endocytosis (Oved et al., 2006). Finally, proteomic approaches have identified several potential NEDD8 substrates that play roles in mRNA splicing, chromatin remodeling, DNA repair and replication (Jones et al., 2008; Xirodimas et al., 2008).

Targeting the NEDD8 Pathway: A New Approach to Treat Cancer

Recently, the generation of MLN4924, a selective inhibitor of general NEDD8 conjugation of substrates, was reported (Soucy et al., 2009). MLN4924 is an adenosine sulfamate derivative and the final product of an iterative medicinal chemistry effort following the identification of its parent compound, N6-benzyl adenosine, in a high throughput screen for NAE inhibitors. MLN4924 is structurally related to AMP and forms a covalent adduct with NEDD8, which is catalyzed by the NAE in a process resembling the first NEDD8 adenylation step in the neddylation cascade reaction. As described above, the transthiolation reaction of UBL between E1-E2 enzymes occurs through a multistep process. First, E1 mediates adenylation of UBL C-terminus and the E1 catalytic cysteine subsequently attacks the adenylate to form a thiolester bond. E1 adenylates an additional UBL molecule and the occupancy of this second UBL-AMP promotes the formation of thiolester bond between the first UBL loaded and the E2. Interestingly, the formation of the NEDD8-MLN4924 adducts occurs through a mechanism that takes advantage of the reversibility of the E1 reaction cycle. Using a number of approaches, Brownell et al. (2010) demonstrated that MLN4924 adduct formation first requires ATP to form NEDD8-AMP, followed by the formation of the NAE-NEDD8 thiolester bond and the release of AMP. MLN4924 subsequently binds the AMP-vacated nucleotide-binding site of NAE where its sulfamate group acts as a nucleophile that attacks the thiolester bond between NEDD8 and NAE subunit, Uba3, forming the MLN4924 adduct. This study demonstrated that MLN4924 is a competitive ATP inhibitor of NAE and showed that the NEDD8-MLN4924 adduct once formed binds tightly and is stable, thereby inhibiting further NAE enzymatic activity.

Studies in HCT-116 colorectal cells showed that addition of MLN4924 inhibits the NEDD8 pathway as early as 5 min post-treatment where MLN4924-NEDD8 adducts are observed, while significantly decreasing Ubc12-NEDD8 thiolester-linked complexes and neddylated cullins (Brownell et al., 2010). Treatment of HCT-116 cells with MLN4924 resulted in S-phase defects, DNA damage and subsequent apoptosis (Soucy et al., 2009). Low doses of MLN4924 also inhibited tumor growth in xenograft assays. Notably, MLN4924-mediated inhibition of cullin neddylation resulted in increased total levels of the CRL substrates CDT1, p27 and NRF2 (Soucy et al., 2009).

MLN4924 was recently tested in the treatment of acute myeloid leukemia (AML) due to the fact that NEDD8 regulates proteins, namely targets of CRLs, vital for AML cell survival (Swords et al., 2010). MLN4924 was shown to have tumor growth inhibiting properties in AML cells in vitro and in xenograft assays, concomitant with increases in key CRL substrates, including IκBα. AML cells appears to undergo increased apoptosis in response to MLN4924 due to excessive generation of ROS mediated by a decrease in NF-κB-mediated transactivation of target genes with antioxidant functions, such as superoxide dismutase 2 (SOD2) (Swords et al., 2010). Given the number of oncoproteins and tumor suppressors that are targets of NEDD8 conjugation cascade or CRLs whose activity is regulated by NEDD8, MLN4924 may have promising anticancer activity for cancers where perturbation of these pathways are observed. Moreover, combination of NEDD8-mediated pathways rather than an individual pathway affected by MNL4924 should be carefully considered in predicting the utility of MLN4924 treatment in the context of specific cancers.

Unquestionably, our discovery of the complexity in NEDD8-dependent signaling pathways will continue to increase, which will provide better understanding of cancer-specific biology and afford new or perhaps unprecedented avenues of therapy.

ACKNOWLEDGMENTS

Our research was supported by funds from the Canadian Cancer Society (18460 and 20110 to M.O. and 18054 to M.S.I.). I.R.W. is a recipient of the Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship. M.O. and M.S.I. are Canada Research Chairs. We apologize to those whose work could not be cited due to space restrictions.

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