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## Can Cycles of Neddylolation and Deneddylolation Provide Points for Possible Therapeutic Intervention?

Nadía Nocera

### *Introduction*

The process of ubiquitination serves as an important signaling mechanism in many biological processes such as protein trafficking, DNA repair, protein-protein interactions and proteolysis.<sup>1</sup> Ubiquitin is a small polypeptide that is covalently linked to the lysine residue of target proteins by a multienzymatic system consisting of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating) enzymes. E3 ligases include cullin-based ubiquitin ligases, in which the cullin acts as a scaffold for the assembly of a multisubunit ubiquitin ligase complex that contains a RING-box protein and a cullin-specific substrate adaptor protein. Cullin3 (Cul3) forms a complex, which controls cyclins, transcription factors, and cellular pathways.<sup>1</sup> All cullins require an attachment of the ubiquitin homologue neural-precursor-cell-expressed and developmentally down regulated 8 (Nedd8) at a specific lysine residue near its C terminal end to activate its ubiquitin ligase function. After protein is tagged with ubiquitin, it is targeted to the proteasome, where it is degraded.

Because these components in the cell cycle (E1, E2, E3, Cullins, Nedd8, etc.) are essential in controlling proteolysis, null function or increased production of any of these proteins may lead to unregulated cellular processes and possibly to tumorigenesis. Knowing the functional details of these interactions could lead to clues for therapeutic targeting.

### *What is Nedd8 and what is its function?*

Cullin family proteins organize ubiquitin ligase (E3) complexes to target numerous cellular proteins, such as those involved in cell proliferation and proteasomal degradation. Cullins directly interact with Roc1, a Ring finger protein. The Cullin-Roc1 complex comprises the core module of a series of ubiquitin E3 ligases, which confer substrate specificity and therefore regulate the degradation process.<sup>2</sup> Cullin family proteins; Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5, have been shown to be modified by Nedd8 (a ubiquitin-like protein) in mammalian cells.<sup>3</sup> Neddylolation of cullins is critical to cullin function and is required to facilitate processive transfer of ubiquitin from E2 to E3 to the target protein.<sup>4</sup>

Nedd8 is a highly conserved, 81- residue protein that is attached to cullins by a process termed neddylation.<sup>1</sup> Neddylation occurs through the action of a neddylation cascade similar to that used in the ubiquitin system. The first step in neddylation is the formation of a thiol-ester bond via the C-terminal glycine residue of Nedd8 with APP-BP1/Uba3, a heterodimeric E1-activating enzyme.<sup>1</sup> The process is completed by the formation of an isopeptide bond, linking the carboxyl-end of Nedd8 Gly-76 to the  $\epsilon$ -amino group of a conserved cullin lysine residue.<sup>5</sup> Neddylation results in mononeddylation of cullin substrates.

Ubiquitination activities of cullin-RING ligases (CRLs) require neddylation to control their E3 ligase activity. Studies focusing on the relationship between neddylation and E3 ligase func-

tion<sup>6,7</sup> suggest that Nedd8 plays a direct role in the activation of the E3 ligase function in ubiquitination. Inactivation of the CRL ligase activity requires the COP9 signalosome (CSN) that removes Nedd8 from cullins, a process called deneddylation.<sup>8</sup> Although the significance of Nedd8 in cullin complex activation has been established, it is not yet clear what the mechanism of Nedd8 action is.

### ***Neddylation and deneddylation provide means to maintain homeostasis***

It has been found that deneddylation by CSN protects cullins from degradation and that Nedd8-conjugated cullins are unstable and depleted *in vivo*.<sup>8</sup> CSN has been implicated in a wide range of biological processes including plant photomorphogenesis, yeast mating pathways, signal transduction, the regulation of DNA repair, and cell cycle regulation.<sup>9</sup> CSN inhibits ligase activity and negatively regulates the cell cycle by promoting deneddylation of cullins.

The regulation of Cul1 and Cul3 by neddylation and deneddylation was examined by generating CSN-null mutants of *D. melanogaster*.<sup>8</sup> Cul1 and Cul3 were found to be depleted, as shown in Western blots with lysates prepared from CSN-null larvae and CSN double-stranded RNA (dsRNA) treated S2 cells. The depletion was primarily due to the absence of unneddylated Cul1 and Cul3.<sup>8</sup> Although this study showed that neddylated cullins were degraded in the absence of CSN, the protective role of CSN remains debated. A different demonstrated that although the CSN complex was inactivated, both the percentage of neddylated cullins in cells, and the cullin substrates themselves, increased.<sup>7</sup> Further research is required to elucidate the role of cullins.

Cul1 was found to accumulate in *D. melanogaster*, with the Nedd8-null allele present in the eye and wing discs—indicating that Nedd8 may have a role in down regulating the levels of Cul1 and Cul3 proteins.<sup>8</sup> This suggests the efficient degradation of neddylated cullins, unless the conjugated Nedd8 is removed by CSN. It therefore appears that neddylation and deneddylation provide a means to maintain normal cellular levels of activated CRLs and prevent excessive ubiquitin ligase activity.<sup>8</sup>

### ***Neddylation and deneddylation may provide points for therapy***

Because of the apparent role of neddylation in the function of cullin, blocking this process may provide some real therapeutic benefit in cancer patients, by promoting cell death or cell cycle arrest in excessively proliferating cells. Furthermore, because of the requirement that cullins undergo deneddylation in addition to neddylation, blocking Nedd8 removal could severely interfere with cell viability. Specifically, the inhibition of cullin deneddylation through small molecule inhibitors would be expected to lead to defects in the cell's ability to ubiquitinate numerous cullin-based E3 targets—ultimately leading to defects in cell proliferation.<sup>10</sup> However, it remains to be determined whether cancer cells have a greater rate of deneddylation, as compared to normally proliferating cells. If research reveals this to be the case, there could be a therapeutic window for small molecule inhibitors of the CSN protease.

### ***E1, E2 and Nedd8 form a complex***

Recently, a study conducted by Huang and colleagues<sup>11</sup> found a unique N-terminal sequence on the E2 protein that helps form a complex to stabilize E1 and Nedd8. In this complex,

three E1 domains pack to generate a large central groove, which cradles ATP, molecules of Nedd8, and E2 substrates together. E1 activates Nedd8 through adenylation and forms a bond with Nedd8, transferring the protein to E2. E2 then transfers Nedd8 to E3, which joins Nedd8 with Cul1. NEDD8 is in the center of the complex, with its C terminus tethered within a channel focused on the thioester bond.<sup>11</sup> A network of charged and polar side-chains contacts E1's catalytic cysteine and Nedd8's C terminus. Mutational analyses showed that these residues contribute to E1, E2 and Nedd8 complex formation.<sup>11</sup> It was also demonstrated that deleting the tail from E2 significantly hinders the ability of E2 to transfer Nedd8 to E3, thereby decreasing the transfer of Nedd8 to Cul1.<sup>12</sup> Therefore, a decrease in the transfer of Nedd8 to Cul1 would lead to increased stability and negative regulation of the cell cycle.

The discovery of this unique E2 tail is very intriguing for researchers because it may provide one way to target the process of neddylation in cancer treatment. Scientists now know the exact shape and function of the E2 tail, and the E1 groove within which it fits. Novel drugs that are designed to disrupt the tail, the groove, or both might block the ability of the Nedd8 pathway to accelerate the replication of cancer cells.<sup>12</sup>

### ***Research reveals a new substrate for Nedd8***

Although it is known that neddylation plays an important role in ubiquitin-mediated proteolysis by modification of cullins, it was found that cullins are not the only substrates targeted for Nedd8 modification. In a study focusing on the neddylation of a breast cancer associated protein, it was found that BCA3 (breast cancer associated protein 3), a non-cullin protein, is also a Nedd8 substrate.<sup>13</sup> BCA3 has recently been found to be over-expressed in both breast and prostate cancers. Although BCA3 does not have an inherent relationship to cancer, it can act as a tumor suppressor when modified by Nedd8.<sup>13</sup>

A yeast two-hybrid screen was performed in a human placental cDNA library using SENP8 (a Nedd8-specific protease) as bait—an interacting plasmid encoding BCA3 was identified.<sup>13</sup> BCA3 was tagged and was found to be modified by Nedd8. It thus appears that neddylation may occur through Nedd8's association with eleven lysine residues on BCA3 because when these residues were replaced by arginine, neddylation did not occur.

In the cell, BCA3 is localized within the nucleus. It has been reported to be a Kyo-T2 binding protein, which was shown to regulate the DNA binding protein Recombination Signal Binding Protein-Jk (RBPJk) and to participate in transcription regulation of NFkB (nuclear factor kappa B).<sup>14</sup> NFkB is a family of proteins that turn on genes involved in apoptosis and cell proliferation. When NFkB is over expressed, it can protect cells from undergoing apoptosis, so the more NFkB that is expressed, the more resistant a cell is to death.<sup>15</sup>

In the study focusing on the neddylation of breast cancer associated protein, investigators examined whether BCA3 could act as a transcription regulator of NFkB, as well as whether the neddylation of BCA3 is required for its transcriptional inhibitory activity.<sup>13</sup> To investigate this, several lysine residues on BCA3 were “mutated”, whereby they were replaced with an arginine. One mutant had a single lysine mutated, while in two other mutants contained ten lysine replacements. Of interest, researchers found that each of these mutants inhibited NFkB activation, with the exception of a BCA3 mutant in which all 11 lysine residues had been replaced. The

latter mutant was also unable to undergo neddylation, demonstrating that BCA3 must be neddylation to inhibit NFkB activation.<sup>13</sup> The same study revealed that BCA3 binds to p65, one of the two proteins that make up NFkB, in order to regulate NFkB. Therefore, Nedd8-modified BCA3 binds to p65 and recruits a histone deacetylase (SITR1) to suppress NFkB-mediated transcription.<sup>13</sup>

The aforementioned study describes a cancer-promoting (or demoting) pathway.<sup>13</sup> Interfering with this pathway may provide a possible way to diminish the number of factors that promote tumorigenesis. With further study, researchers may soon be able to design drugs that block the removal of Nedd8 from BCA3, or alternatively, promote the addition of Nedd8 to BCA3. By increasing the amount of Nedd8-modified BCA3, there would be a decrease in NFkB. Decreasing NFkB would render cancer cells less resistant to chemotherapy and more able to undergo apoptosis.

### **Conclusion**

Through their control of cullins, cycles of neddylation and deneddylation have proven to be important processes in the cell cycle. Neddylation of cullins activates their ubiquitin ligase activity, subsequently allowing cullins to control the cell cycle via the ubiquitination of cellular proteins involved in cell proliferation. In contrast, the inactivation of cullins is achieved by deneddylation through the COP9 signalosome. Another substrate for Nedd8 is the BCA3 protein, found to be a tumor suppressor when modified by Nedd8. When Nedd8 is removed from BCA3, oncogenes are no longer suppressed, resulting in resistance to apoptosis and excessive cell proliferation. Because of Nedd8's critical roles in the cell cycle and modification of tumor suppressor genes, developing a way to control cycles of neddylation and deneddylation could prove to be an effective cancer therapy.

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