

## Review Article

# Neddylation and deneddylation in cardiac biology

Sridhar Kandala<sup>1</sup>, Il-man Kim<sup>1</sup>, Huabo Su<sup>1,2</sup>

<sup>1</sup>Vascular Biology Center, Medical College of Georgia, Georgia Regents University, Augusta, GA, USA; <sup>2</sup>Department of Pharmacology and Toxicology, Medical College of Georgia, Georgia Regents University, Augusta, GA, USA

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**Abstract:** Neddylation is a post-translational protein modification that conjugates a ubiquitin-like protein NEDD8 to target proteins. Similar to ubiquitination, neddylation is mediated by a cascade of three NEDD8 specific enzymes, an E1 activating enzyme, an E2 conjugating enzyme and one of the several E3 ligases. Neddylation is countered by the action of deneddylases via a process termed deneddylation. By altering the substrate's conformation, stability, subcellular localization or binding affinity to DNA or proteins, neddylation regulates diverse cellular processes including the ubiquitin-proteasome system-mediated protein degradation, protein transcription, cell signaling etc. Dysregulation of neddylation has been linked to cancer, neurodegenerative disorders, and more recently, cardiac disease. Here we comprehensively overview the biochemistry, the proteome and the biological function of neddylation. We also summarize the recent progress in revealing the physiological and pathological role of neddylation and deneddylation in the heart.

**Keywords:** Post-translational modification, NEDD8, neddylation, deneddylation, cardiac disease

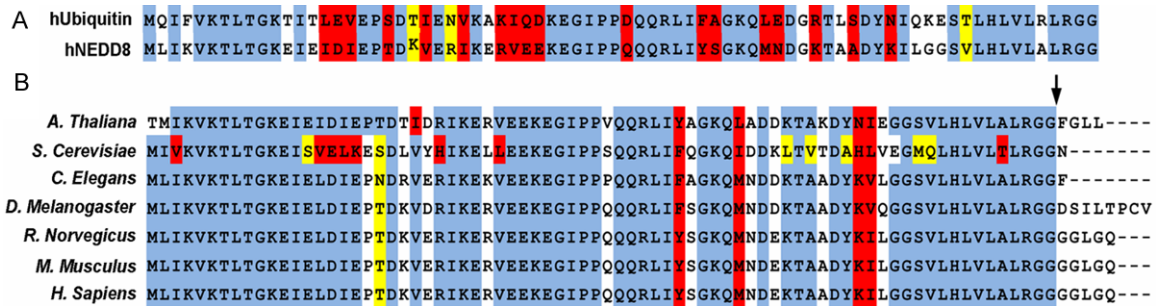
## Introduction

Protein post-translational modifications alter the target protein's conformation, stability, subcellular localization, or binding affinity to protein or DNA partners, thereby largely expanding the functional diversity and dynamics of the proteome. Eukaryotic proteins can be covalently modified by chemical groups (phosphate, acetyl, methyl etc.) or certain proteins. The first and most studied protein-based modification is ubiquitination, which conjugates a 76-amino-acid protein ubiquitin to target protein via an ATP-dependent enzymatic pathway consisting of an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase [1]. Although ubiquitination was first linked to signal the modified proteins for proteasomal degradation, it has since been shown to control many other cellular processes including receptor internalization, assembly of multiprotein complexes, regulation of enzyme activity, DNA repair, cell signaling, and vesicle trafficking [2, 3].

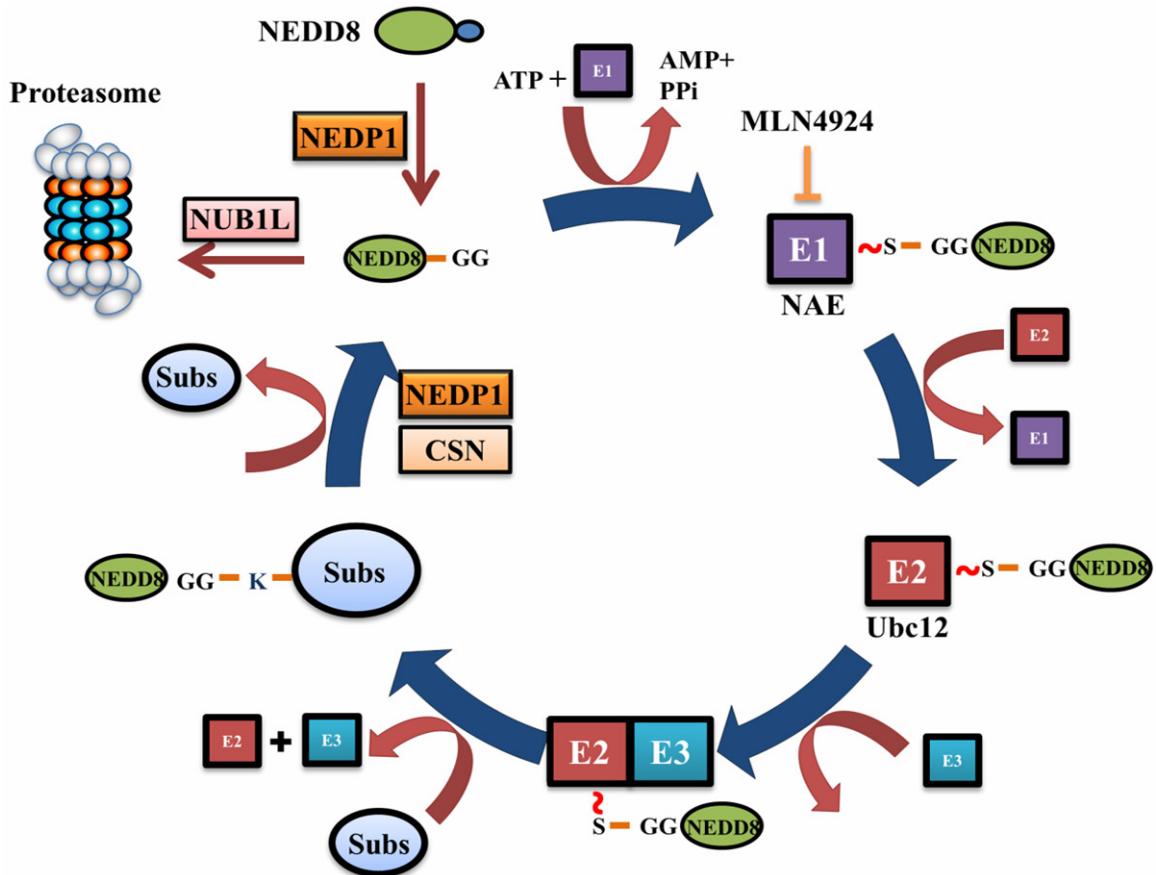
Following the discovery of ubiquitin, more than a dozen of ubiquitin-like proteins have been uncovered to utilize ubiquitination-analogous but functionally distinct process to modify pro-

teins, including NEDD8 (neural precursor cell expressed, developmentally down-regulated 8), SUMO-1, SUMO-2, SUMO-3, ISG15, UFM1, FAT10, URM1, Atg12, Atg8, FUB1, and HUB1 [4]. Among these ubiquitin-like proteins, NEDD8 has the highest identity (58%) to ubiquitin [5] (**Figure 1A**). NEDD8 was first cloned from mouse brain in 1993 and is ubiquitously expressed in various tissues and cell types [5], with highest expression in cardiac and skeletal muscles [6]. NEDD8 is evolutionarily highly conserved (83% identity between human and *Arabidopsis* and 100% identity among rat, mouse and human) (**Figure 1B**), suggesting its conserved function in eukaryotic cells. Like ubiquitination, protein modification by NEDD8, termed neddylation, requires NEDD8 specific E1 activating enzyme, E2 conjugating enzyme and E3 ligases. Neddylation can be reversed by a set of NEDD8 specific proteases (deneddylase) via a process called deneddylation. With quickly expanding knowledge on the functional consequence of NEDD8 modification on target proteins, neddylation has emerged as a critical regulatory process controlling ubiquitination, protein transcription, signaling transduction, mitochondria turnover, autophagy, cell

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**Figure 1.** NEDD8 is a ubiquitin-like protein highly conserved among species. A. The amino acid sequences of human ubiquitin and NEDD8 are aligned. B. The amino acid sequences of NEDD8 precursor from various species are aligned. The conserved amino acids and the amino acids with identical properties, as well as those with weakly similar properties, are represented in light blue, dark red and yellow, respectively. Arrow points to the site cleaved by NEDD8 protease. The exposed C-terminal glycine is to be fused with the lysine residue of the substrate. *A. thaliana*, *Arabidopsis thaliana*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *C. elegans*, *Caenorhabditis elegans*; *D. melanogaster*, *Drosophila melanogaster*; *R. norvegicus*, *Rattus norvegicus*; *M. musculus*, *Mus musculus*; *H. sapiens*, *Homo sapiens*.



**Figure 2.** Protein modification by NEDD8. NEDD8 is translated into a precursor form that has to be processed by NEDD8 protease such as NEDP1 before NEDD8 can be attached to target proteins. Mature NEDD8 is conjugated to target proteins in an ATP-dependent manner by a serial reactions catalyzed by E1 (NAE), E2 (Ubc12) and E3 sequentially. With the help of these enzymes, the C-terminal glycine of NEDD8 forms an isopeptide bond with the lysine of target protein. An adenosine sulfamate analogue MLN4924 binds to the ATP-binding site in NAE and forms an irreversible MLN4924-NAE adduct, thus inhibiting the activation of neddylation. Deneddylases such as CSN and NEDP1 deconjugate NEDD8 from the neddylated proteins, freeing the substrate and NEDD8. NUB1L can specifically bind to and direct NEDD8 to the proteasome for degradation.

death, etc. Moreover, dysregulation of neddylation has been linked to a broad spectrum of pathological conditions ranging from tumorigenesis [7], neurodegeneration [8], inflammation [9-11], immunodeficiency [12], and heart failure [13, 14]. In this review, we summarize the current understanding on the biology of neddylation and deneddylation, the NEDD8 proteome and involved biological processes, and highlight the influence of neddylation in cardiac function under physiological and pathological conditions.

### The biochemistry of the NEDD8 pathway

#### *NEDD8 maturation*

NEDD8, in yeast or plants also known as RUB1 (related to ubiquitin), is first produced as a non-conjugatable 81-amino-acid precursor in mammals [5]. Maturation of NEDD8 requires cleavage of its C-terminal additional five amino acids to expose glycine 76, which forms an isopeptide bond with the lysine residue on the substrate. The hydrolysis is catalyzed by NEDD8 proteases NEDP1 (NEDD8-specific protease 1, also known as DEN1 and SENP8) and UCH-LC3 [15, 16]. NEDP1 is a NEDD8 specific protease, while UCH-L3 is also able to process ubiquitin precursors. UCH-L3 knockout mice are viable and neddylation of proteins were accumulated in UCH-L3 null cells [17], suggesting that UCH-LC3 is not essential for NEDD8 processing in vivo. It remained to be understood why NEDD8 and other ubiquitin-like proteins are not directly produced as a mature form. One possibility is that this strategy prevents unprocessed precursors from entering into the conjugation system, offering additional regulatory mechanism for the conjugation of ubiquitin and ubiquitin-like proteins to target substrates.

#### *Neddylation*

Similar to ubiquitination, conjugation of mature NEDD8 to target proteins is executed by an E1-E2-E3 multienzyme cascade (**Figure 2**). Neddylation begins with the activation of mature NEDD8 by the E1 NEDD8 activating enzyme (NAE), a heterodimer of NAE1 (also known as APP-BP1) and UBA3, through forming a NAE-S~NEDD8 thioester-bond in an ATP-dependent manner [18]. Activated NEDD8 is then transferred to the E2 NEDD8 conjugating enzyme Ubc12, creating another thioester

bond [18]. An E3 NEDD8 ligase transiently interacts with NEDD8-charged E2 and subsequently transfers NEDD8 to the substrate through forming an isopeptide bond between the C-terminal glycine of NEDD8 and a lysine residue on the substrate. The discharged E2 is then released from the E3, allowing another charged E2 to associate with the E3 to initiate next round of NEDD8 transfer.

The structure of the neddylation enzymes is hierarchical. One or two E1s and E2s face towards a presumably huge and yet unknown number of E3s, which targets numerous substrates. NAE is the only dedicated NEDD8 E1. An adenosine sulfamate derivative MLN4924 can irreversibly form a NEDD8-MLN4924 adduct at the ATP-binding site of NEDD8, which prevents the binding of NAE to NEDD8, therefore specifically and potently inhibiting the activation of neddylation [19]. Besides NAE, the ubiquitin E1 activating enzyme UBE1 can also function as an atypical NEDD8 E1 to mediate the attachment of NEDD8 to the ubiquitin chain [20]. So far, only two E2s are identified, Ubc12 and UBE2F. In vivo, Ubc12 and UBE2F showed distinct patterns of cullin neddylation: Ubc12 activates neddylation of Cullins1-4 while UBE2F preferably activates neddylation of cullin 5 [21]. In yeast, DCN1 (defective in cullin neddylation 1 protein) is a bona fide E3 NEDD8 ligase by serving as a scaffold for the interaction of its cullin substrates and the NEDD8 E2 Ubc12 [22, 23]. In mammalian cells, there are 5 DCN1-like proteins (DCNL), of which DCNL1 and DCNL3 facilitates cullin 1 and cullin 3 neddylation respectively [24, 25]. The precise in vivo contributions of each DCNL protein to cullin neddylation remain to be defined. Other ubiquitin ligases such as MDM2, c-Cbl, parkin and IAP, can also function as the E3 NEDD8 ligases to promote the neddylation of various cellular proteins [26-30]. It is still mysterious how these ligases distinguish signals for neddylation from ubiquitination on the same substrate.

Proteins can be modified by one or multiple NEDD8 in trans, or by a chain of NEDD8 through extending on its own lysine residue. At least four of the nine lysines of NEDD8 (Lys 11, 22, 48, and 60) can be used for NEDD8 chain extension [31]. The NEDD8 chain is first built up on the E2 Ubc12 and subsequently transferred to the substrate [32]. Supporting the existence of polyneddylation, high-molecular-weight NED-

D8-immunoreactive species were often observed when affinity purification of NEDD8 targets was carried out from cells expressing tagged NEDD8 [29, 33-35]. It is well-accepted that the length and linkage types of the ubiquitin chain on a given substrate dictate the fate of the ubiquitinated proteins. In contrast, the biological significance of polyneedylation remains to be elucidated.

### *Deneddylation*

Neddylation is dynamically regulated and counter balanced by the actions of deneddylases, termed deneddylation. A number of deneddylases are capable of removing NEDD8 from the modified proteins, including the COP9 signalosome (CSN), NEDP1, USP21, Ataxin-3, UCH-L1, and UCH-L3 [7]. Among these enzymes, only CSN and NEDP1 are NEDD8-specific, whereas the rest are also known to regulate deubiquitination [36, 37].

CSN, a zinc metalloprotease containing 8 subunits from CSN1 to CSN8, is the best characterized deneddylase [38]. CSN was originally discovered in *Arabidopsis* and subsequently found to be evolutionarily conserved from plants to mammals. CSN is repeatedly demonstrated to remove NEDD8 from cullin proteins [38]. Loss of CSN function in mice accumulates neddylated cullins and various neddylated proteins with unknown identities, suggesting that CSN is also able to remove NEDD8 from non-cullin proteins [13, 14]. The isopeptidase activity of CSN is conferred by the metalloenzyme-containing CSN5, but requires all 8 subunits to form a holo-complex. Loss of any CSN subunits disrupts the complex formation and impairs the deneddylation activity [39-43].

The cysteine protease NEDP1 is another specific deneddylase with no activity on processing ubiquitin and SUMO. Mutation of its cysteine to alanine suffices to abolish the protease activity [44]. Besides processing NEDD8 precursor, NEDP1 also removes NEDD8 from NEDD8 conjugates. Although NEDP1 was shown to efficiently deconjugate NEDD8 from neddylated cullin 1 and cullin 3 in vitro, deficiency of NEDP1 in *Drosophila* does not accumulate neddylated cullin 1 and cullin 3 [44-46]. Surprisingly, silencing of NEDP1 blunts stress-induced neddylation of cullin 1 and 2 in cultured mammalian cell lines, suggesting that NEDP1 may be neces-

sary for cullin neddylation [9, 47]. Probing its deneddylation activity in a loss-of-function mouse model, which so far is still lacking, will help explain the discrepancy. Despite its inconclusive role in cullin neddylation, NEDP1 appears to control the deneddylation of many non-cullin proteins such as p53, MDM2, Tap73, BCA3, and E2F1 [28, 35, 48-50]. Interestingly, CSN interacts with NEDP1 in *Aspergillus nidulans* and human cells and may target NEDP1 for proteasomal degradation [51]. Whether these two deneddylases coordinately regulate deneddylation in mammal remains to be determined.

### *NUB1L (NEDD8 ultimate buster 1 long), a negative regulator of neddylation*

NUB1L is an interferon (IFN)-inducible protein that negatively regulates neddylation [52-56]. Overexpression of NUB1L in culture cells decreases the levels of NEDD8 and neddylated proteins, which is abrogated by proteasome inhibition [56, 57]. It was first postulated that NUB1L directs neddylated proteins to the proteasome for degradation. This notion is now challenged by the observation that proteasome inhibition induces atypical neddylation in UBE1-dependent manner [20, 58, 59]. Therefore, the recovery of neddylated proteins by proteasome inhibition in NUB1L-overexpressed cells could be due to activation of atypical neddylation, rather than blockade of proteasomal degradation of neddylated proteins. Indeed, NUB1L specifically interacts with NEDD8, but not ubiquitin, and facilitates the transfer of NEDD8 to the proteasome for degradation through coordinating with P97-UFD1-NPL4 complex [60, 61]. Thus, NUB1L suppresses neddylation by promoting the proteasomal degradation of NEDD8.

NUB1L is highly conserved among species [62]. Human *NUB1L* gene encodes two functionally nondifferentiable transcript variants, with the short form absent in other species. Structurally, NUB1L possesses a ubiquitin-like (UBL) domain, three ubiquitin-associated (UBA) domains and interacts with the 19S proteasome subunit Rpn10 and Rpn1 via its UBL domain [62, 63]. Despite the similarity of NEDD8 and ubiquitin in sequence and structure, NUB1L binds to NEDD8 through its C-terminal sequence but not UBA domains [57]. Additionally NUB1L recognizes and promotes the degradation of another ubiquitin-like protein FAT10, whose substrates and function remain poorly defined [64].

## Neddylaton in the heart

**Table 1.** Neddylaton targets

Neddylated protein	E1/E2/E3	NEDD8 Protease	Modified Sites	Consequence of Neddylaton	Ref
Regulation of UPS					
Cullins	NAE/Ubc12/DCN1	CSN	C-terminal lysine	Induces the assembly of CRLs	[65, 74]
Parkin	NAE/-/-	-	K76	Increases ubiquitin ligase activity	[33, 34]
Mdm2	-/Ubc12/Mdm2	NEDP1	-	Increases the stability	[27, 50]
Smurf1	-/Ubc12/Smurf1	-	Multiple lysines	Increases ubiquitin ligase activity	[76]
XIAP	-/Ubc12/IAP	NEDP1	-	?	[30, 126]
Ubiquitin	UBE1/-/-	CSN?	K29	Terminate ubiquitin chain extension?	[20, 79]
Regulation of transcription factors					
E2F1	NAE/-/-	NEDP1	Multiple lysines	Decreases the stability and transcriptional activity	[83, 84]
HuR	-/-/Mdm2	NEDP1	K283, 313, 326	Enables nuclear localization and stabilization, increases transcriptional activity	[26]
HIF1 $\alpha$	NAE/-/-	-	-	Increases protein stability and activity	[69]
AICD/APP	-	-	Multiple lysines	Inhibits the transcriptional activity	[127]
RCAN1	-	-	K96, 104, 107	Increases protein stability	[78]
BCA3	-	NEDP1	Multiple lysines	Increases its binding to p65; recruits SIRT1	[49]
BRAP2	-	-	K432	Promotes NF- $\kappa$ B nuclear translocation and transcription activity	[128]
P73	SMC/-/Mdm2	NEDP1	K321,327, 331	Promotes cytoplasmic localization; inhibits transcription activity	[28]
Regulation of p53 signaling					
P53	NAE/-/Mdm2 (FBOX11)	NEDP1	K370, 372, 373	Inhibit the transcriptional activity	[27, 50, 90]
L11	-	NEDP1	-	Increases stability; enhances P53 activity	[67]
S14	-/-/HDM2	NEDP1	-	Increases stability; enhances P53 activity	[92]
Regulation of receptor-mediated signaling					
EGFR	NAE/Ubc12/c-Cbl	-	Multiple lysines	Reduces its stability	[72]
TGF $\beta$ RII	NAE/Ubc12/c-Cbl	-	K556, 567	Stabilizes TGF $\beta$ RII, promotes TGF $\beta$ signaling	[66]
Regulation of cell death effectors					
Caspase7?	NAE/Ubc12/IAP1	NEDP1	K142 and others	Inhibits caspase activity	[30, 126]
RIPK1	-/-/cIAP1	-	-	-	[30]
Caspase1?	NAE/-/-	-	-	Activates catalytic activity	[129]
Others					
pVHL	-	-	K159	Inhibits its binding to cul2; Promotes fibronectin matrix assembly	[70, 71]
PINK1	NAE/-/-	-	-	Stabilizes its 55kD cleaved form	[33]
SHC	NAE/Ubc12/-	-	K3	Forms ZAP70-SHC-Grb2 complex; activates Erk	[11]
Histone H4	-/Ubc12/RNF111	-	N-terminal lysines	Alters the chromatin orientation for DNA repair	[130]

### Neddylated proteins and their biological functions

#### *Neddylation targets*

In contrast to a wide spectrum of proteins subjected to ubiquitin modification, the identities of the NEDD8 targets are poorly understood. Identification of NEDD8 targets is challenging owing to the relative low steady-state abundance of endogenous neddylation targets and the antagonization of neddylation by deneddylases. In general, a bona fide NEDD8 target should meet at least a few of the criteria below: 1) the target protein is covalently modified by NEDD8 in vivo at its one or multiple lysine residues. This can be revealed by mass spectrometry analysis and/or mutagenesis analysis; 2) the neddylation should occur at endogenous condition in vivo and can be reconstituted in vitro; and 3) the neddylation relies on specific neddylation enzymes and/or is countered by deneddylases in vivo. Modulation of these enzyme activities, either by genetic or pharmacological means, should alter the neddylation status.

The NEDD8 proteome is quickly expanding. The first- and best-studied NEDD8 targets are cullin proteins that scaffold the assembly of cullin-RING ubiquitin ligases (CRLs) [65]. Besides cullins, an increasing list of cellular proteins are recently validated as NEDD8 substrates (**Table 1**) [26, 27, 30, 33-35, 49, 66-72]. Importantly, many of these proteins such as Mdm2, p53, HIF1 $\alpha$ , parkin, PINK1, and TGF- $\beta$  receptor II (TGF- $\beta$ RII) are known to regulate various cellular pathways important to cardiomyocyte survival and function, urging the needs to determine the impact of their neddylation in cardiomyocytes. The NEDD8 proteome is further enlarged by the finding that NEDD8 can be added into the existing ubiquitin chain [20], suggesting that any ubiquitinated proteins could be NEDD8 targets. By affinity purification of tagged-NEDD8 modified proteins followed by mass spectrometry analysis, several proteomics studies have identified a vast number of potential NEDD8 targets that regulate diverse cellular pathways involving DNA repair, replication, transcription and chromatin remodeling [31, 73]. It should be pointed out that the diglycine addition to the substrate upon trypsin digestion is common to NEDD8, ubiquitin and ISG15 modifications in mass spectrometry

analysis. Other peptidase such as Lys-C has been suggested to distinguish NEDD8 from other ubiquitin-like protein modification by mass spectrum [20].

Like any other post-translational modification, neddylation influences protein function by changing the target protein's conformation, half-life, subcellular distribution or DNA/protein binding partners. Based on the identified NEDD8 targets, we highlight a selection of studies from several cellular pathways, which are known to play crucial roles in cardiac physiology and pathology, to demonstrate the diverse functions of neddylation.

#### *Regulation of the ubiquitin proteasome system (UPS)*

Majority of the intracellular proteins are degraded through the UPS, which consists of two steps: ubiquitination of the target protein and the degradation by the proteasome. Ubiquitin ligases specifically recognize the target proteins and catalyze the ubiquitination, thereby controlling the specificity of UPS-mediated proteolysis. In recent years, there has been growing appreciation of the crosstalk between ubiquitination and neddylation. The NEDD8 pathway appears to impact UPS function at least through regulating ubiquitin ligase activity, antagonizing ubiquitination or controlling ubiquitin chain extension.

First, neddylation fine-tunes the activity of CRLs, the largest known class of ubiquitin ligases [65, 74]. CRLs are typically composed of one or seven cullin family members (Cul-1, -2, -3, -4A, -4B, -5, -7), an E2 interacting RING finger protein such as Rbx1 or Rbx2, a F-box interacting adaptor protein such as Skp1, and a substrate-recognizing F-box protein such as Skp2. By pairing individual cullin with different F-box proteins, CRLs control the degradation of a vast number of cellular proteins and, not surprisingly, involve in many aspects of biological processes. In general, neddylation of cullin at their conserved C-terminal lysine residue induces conformational change of cullin, which dislodges the CRL inhibitory protein CAND1 from cullin and facilitates simultaneous binding of Rbx1 and Skp1 to cullin. Furthermore, Rbx1 recruits charged ubiquitin E2 while Skp1 associates with Skp2, which further recruits the substrate to be ubiquitinated. Therefore, neddylation of cullins triggers the assembly of functional



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modified by NEDD8 and such modification affects their ubiquitin ligase activities [27, 33, 34, 76]. For instance, Smurf1 is a HECT ubiquitin ligase that plays a crucial role in multiple processes including cell cycle progression, cell proliferation, differentiation, maintenance of genomic stability and metastasis [77]. Smurf1 catalyzes its own neddylation on multiple lysine sites and requires an active site at cysteine 426 for the neddylation. Smurf1 neddylation is repressed by neddylation inhibitor MLN4924 or silencing of Uba3 and Ubc12 respectively. Neddylation of Smurf1 enhances its association with ubiquitin E2 and augments the ubiquitin ligase activity of Smurf1 [76]. In human colorectal cancers, the elevated expression of Smurf1, NEDD8, NAE1 and Ubc12 correlates with cancer progression.

Third, neddylation of a number of cellular proteins such as PINK1, HuR and RCAN1 were shown to antagonize ubiquitination and increase their stabilities [33, 66, 78]. For example, HuR is an oncogenic RNA-binding protein that regulates cell proliferation and survival. HuR is modified by NEDD8 at its lysine 283, 313 and 326, which is mediated by NEDD8 E3 ligase Mdm2. Neddylation of HuR suppresses its ubiquitination, increases its stability and promotes its nuclear localization [26]. It remains unclear whether NEDD8 competes with ubiquitin for a common lysine target to stabilize the modified protein.

Finally, proteins can be simultaneously modified by both NEDD8 and ubiquitin upon proteasome inhibition and other stresses, in a form of mixed ubiquitin-NEDD8 chain or “in trans” on different sites on the target [20, 79]. In yeast and human cells, NEDD8 forms NEDD8-ubiquitin heterodimer with ubiquitin and can be extended on existing ubiquitin chain, possibly acting as a chain terminator [79]. In vitro, ubiquitin shuttle proteins and receptors recognize NEDD8-ubiquitin heterodimer with equivalent binding affinity to ubiquitin-ubiquitin homodimer [79]. Proteomic studies revealed the interaction of neddylated proteins with proteasome components [31]. Given the sequence and structure similarity between ubiquitin and NEDD8, it is possible that upon (proteotoxic) stress, NEDD8 functions as a substitute for ubiquitin and caps the ubiquitin chain, therefore preventing excessive ubiquitin chain extension and depletion of ubiquitin pool, which otherwise would be catastrophic to the cells. On the other hand, the heterologous ubiquitin-NEDD8 chains

can be cleaved by CSN and 26S proteasome in vitro [79], suggesting that the proteins with mixed ubiquitin-NEDD8 chain may be still degraded by the proteasome, maybe at a less efficient rate compared to those modified with homologous ubiquitin chain. The functional consequence of such mixed ubiquitin-NEDD8 chain is to be determined.

### *Regulation of protein transcription*

A number of transcription factors are found to be modified by NEDD8, which leads to either activation or suppression of their transcription activity. E2F1 is an important transcription factor that lies downstream of retinoblastoma protein, promotes apoptosis and suppresses cell proliferation [80]. E2F1 deficiency has been shown to suppress cardiomyocyte cell death and protect the heart against ischemia-reperfusion injury in mice [81, 82]. Neddylation of E2F1 decreases its stability and inhibits the interaction with its transcription cofactor MCPH1, leading to reduced transcription activity. Moreover, overexpression of NEDP1 reduces neddylated E2F1 and promotes its transcription activity, while silencing of NEDP1 does the opposite [83, 84]. Another example is HIF1 $\alpha$ , a transcription factor that functions as a master regulator of oxygen homeostasis. HIF1 $\alpha$  are protective to the pathogenesis of ischemic heart disease and pressure-overload heart disease [85]. Neddylation of HIF1 $\alpha$  stabilizes and activates HIF1 $\alpha$  at baseline and upon hypoxia, which is attenuated by silencing of NAE1 [69].

Neddylation can also indirectly regulate gene expression by modulation of the regulator of transcription factor. Calcineurin-NFAT signaling promotes the nuclear translocation of transcription factor NFAT and is necessary and sufficient to induce pathological cardiac hypertrophy [86]. RCAN1 is a feedback inhibitor of calcineurin by directly binding to the catalytic subunit of calcineurin and inhibiting its activity [87]. Neddylation of RCAN1 increases its stability by inhibiting ubiquitination and promotes its nuclear translocation and binding to calcineurin, leading to suppression of NFAT activity in HEK293 cells [78].

### *Regulation of p53 signaling*

The tumor suppressor p53 plays an important role in apoptosis, autophagy and angiogenesis in the heart and has been linked to a number of cardiac disorders including cardiac hypertro-



## Neddylation in the heart

**Table 2.** Roles of components of the NEDD8 pathway in embryonic development

Components	Phenotypes	Mechanisms	Species	Ref
<b>NEDD8/Rub1</b>				
Rub1 & 2 mutant	Embryonic lethal or retarded growth	-	<i>Arabidopsis</i>	[131]
NEDD8 mutant	Embryonic lethal	Defects in cell proliferation and survival	<i>Drosophila</i>	[132]
NEDD8 KD	Embryonic lethal	-	<i>C. elegans</i>	[133]
<b>NEDD8 conjugation enzymes</b>				
ULA1* KD	Embryonic lethal	-	<i>C. elegans</i>	[133]
Uba3 KO	Embryonic lethal	Apoptosis and cell cycle arrest	Mouse	[98]
Ubc12 KD	Embryonic lethal	-	<i>C. elegans</i>	[133]
SCCRO KO	Viable	Compensation by other paralogues?	Mouse	[134]
SCCRO O/E	Embryonic lethal	-	Mouse	[99]
<b>Deneddylase</b>				
NEDP1 & CSN double mutant	Impaired multicellular development	-	<i>A. nidulans</i>	[51]
CSN4 & 5 mutant	Larval lethal	Defective oogenesis, hypersensitive to DNA damage	<i>Drosophila</i>	[135]
CSN8 mutant	Larval lethal	Defective oogenesis	<i>Drosophila</i>	[136]
CSN2 KO	Embryonic lethal	Cell cycle arrest	Mouse	[39]
CSN3 KO	Embryonic lethal	-	Mouse	[40]
CSN5 KO	Embryonic lethal	Impaired proliferation, apoptosis	Mouse	[41]
CSN6 KO	Embryonic lethal	-	Mouse	[42]
CSN8 KO	Embryonic lethal	-	Mouse	[43]

Abbreviations used in the table: KO, knockout; KD, knockdown; O/E overexpression; \*Uba3 homologous in *C. elegans*; *C. elegans*, *Caenorhabditis elegans*; *A. nidulans*, *Aspergillus nidulans*.

phy, dilated cardiomyopathy, diabetic cardiomyopathy and cardiac ischemia reperfusion injury [88]. Mounting evidence suggests that neddylation regulates p53 signaling at different levels. First, p53 is the direct neddylation target. Neddylation of p53 prevents the nuclear translocation of p53 and inhibits its transcriptional activity. In contrast, neddylation-resistant mutants retain the transcriptional activity [27]. Consistently, overexpression or downregulation of NAE1 inhibits or enhances p53 activity respectively [89]. Second, FBXO11 and Mdm2 promote p53 neddylation by acting as E3 NEDD8 ligases [27, 90]. The stability of Mdm2 and FBXO11 are directly or indirectly controlled by neddylation. Neddylation of Mdm2 increases its stability and NEDP1-induced deneddylation results in destabilization of Mdm2 [50]. FBXO11 is an F-box protein and a component of the Skp1-Cullin1- F-box ubiquitin ligase. As mentioned above, the stability of F-box protein is often affected by neddylation of cullins [91]. Third, ribosome proteins activate p53 upon nucleolar stress by binding to and inhibiting Mdm2. Ribosome protein L11 and S14 are identified as NEDD8 targets [68, 92]. Neddylation of L11 and S14 controls their stability and subcellular localization, leading to p53 activation [67, 68, 92].

### Regulation of receptor internalization

The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is crucial to cardiac

development and pathological hypertrophy [93, 94]. EGFR can be directly modified by multiple NEDD8 molecules. Neddylation of EGFR is stimulated by EGF and enhances its ubiquitination, resulting in downregulation of EGFR. These effects are attenuated by either overexpression of dominant-negative mutant of Ubc12 or silencing of NEDD8 [72]. Therefore, neddylation of EGFR blunts its downstream signaling. In contrast, neddylation of TGFβ receptor II (TGFβRII) has positive effect on its downstream signaling [29]. TGFβRII is essential to TGFβ-mediated signaling and is critical to pathological cardiac remodeling [95]. Neddylation of TGFβRII occurs at its lysine556 and lysine567 at plasma membrane, and requires NEDD8 E2 Ubc12 and NEDD8 E3 c-Cbl, a ligase with dual function for both ubiquitination and neddylation. Neddylation of TGFβRII inhibits ubiquitination and subsequent degradation of TGFβRII by regulating receptor compartmentalization. Furthermore, a c-Cbl mutant identified from leukemia patients has defective neddylation activity, which may account for the low TGFβ responsiveness in human leukemia cells [29].

### Regulation of mitochondria turnover

Mitochondria are cellular fuel plants critical for matching the energy demands of eukaryotic cells and precise control of mitochondria quality has to be achieved to prevent cellular damage. Defective mitochondria turnover has been

linked to many forms of cardiac pathological conditions [96]. Although direct evidence is lacking, a couple of recent studies have suggested the potential roles of neddylation in mitochondria turnover [17, 18]. Parkin and PINK1 are known to be important to mitochondria turnover and are implicated in Parkinson's disease. It is generally believed that upon mitochondrial depolarization, the mitochondrial localized protein kinase PINK1 phosphorylates parkin, which leads to the translocation of parkin from cytoplasm to depolarized mitochondria, whereby parkin functions as a ubiquitin ligase to promote the ubiquitination of mitochondrial proteins and thus autophagic degradation of damaged mitochondria [97]. Both parkin and PINK1 can be neddylated [33, 34]. Neddylation of parkin elevates its ubiquitin ligase activity, which results from enhanced association of parkin with ubiquitin E2 enzyme and the substrates. Neddylation of PINK1 leads to stabilization of a PINK1 fragment with unknown functional significance [33]. Moreover, expression of NAE1 partially rescues abnormalities induced by PINK1 knockdown in *Drosophila*, while neurotoxin MPP(+) suppresses the neddylation of parkin and PINK1 in HEK293 cells [33, 34], suggesting that neddylation of parkin could be protective against stress. Further studies to determine the consequence of neddylation of parkin and PINK1 on mitochondria turnover are clearly warranted.

### **Neddylation and deneddylation are essential for embryonic development**

Emerging evidence has revealed a critical role of the intact NEDD8 pathway in embryonic development. Disruption of either neddylation or deneddylation caused lethality at an early embryonic development stage in most model organisms (**Table 2**). Given the enormous capacity of neddylation in regulating CRL activity and that CRLs mediate the degradation of most cell cycle and survival regulators, it is anticipated that the observed phenotypes are more or less associated with aberrant cell cycle progression and prevalent cell death. Indeed, the Uba3-deficient mouse embryos displayed defective blastocyst formation and hatching at as early as embryonic day (E)3.5 and eventually died at the periimplantation stage [98]. Massive apoptosis and accumulation of cyclin E and CDK are evident in Uba3-deficient embryos at

E6.5. Furthermore, suppression of deneddylation by germline deletion of any CSN subunit (CSN2, CSN3, CSN5, CSN6 and CSN8) in mice all results in early embryonic lethality [39-43]. CSN5 null embryos survived to the blastocyst stage with relatively smaller size, and died quickly after implantation without undergoing gastrulation. Increase of p27, p53, cyclin E and massive apoptosis were observed in the CSN5 null embryos, suggesting that defective cell cycle progression and apoptosis are at least in part attributable to the development arrest [41]. Interestingly, an attempt to generate transgenic mice overexpressing the E3 NEDD8 ligase SCRRO (DCNL1) has not been successful [99], raising the possibility that enhancement of neddylation may not be desired to embryonic development. In mice, cardiogenesis starts at around E7.5 and the development continues up to postnatal day 7 (P7) [100]. The early embryonic lethality of these knockout mice precludes the examination of the specific role of neddylation in cardiac development. Conditional deletion of neddylation or deneddylation enzymes in cardiac progenitor cells will be helpful to address this question.

### **Impact of neddylation and deneddylation in cardiac disease**

The importance of the neddylation in health and disease has begun to be understood, especially in the field of cancer. Neddylation is hyperactivated in several cancer cell lines [76]. Expression of NEDD8 E3 ligase SCCRO is oncogenic in mice [99]. The deneddyase CSN has also been closely linked to tumorigenesis [75]. Moreover, the specific and potent NAE inhibitor MLN4924 was shown to effectively suppress tumor growth in animal models and in clinical trials [7, 101, 102]. Targeting neddylation is now emerging as a novel anticancer therapeutic strategy.

Dysfunction of neddylation is also implicated in neurodegenerative disorders [8]. Accumulation of NEDD8 was frequently found in neuronal and glial inclusions from a number of neurodegenerative diseases [103]. NUB1L, the negative regulator of neddylation, promotes the degradation of mutant Huntington and synphilin-1 [55, 104], prevents tau aggregation [105], and incorporates into the inclusion bodies in diseased brains [106-108], implying its potential role in protein quality control.

## Neddylation in the heart

Our knowledge with respect to the importance of neddylation in other tissues, including the heart, is lagging behind. This is largely due to the lack of animal models in which neddylation/deneddylation is genetically manipulated in a tissue-specific manner. To this end, we have recently created a mouse model in which the eighth subunit of CSN (*Csn8*) gene is specifically deleted in cardiomyocytes [13, 14, 109]. Using this mouse model, we have obtained the first line of evidence for the impact of neddylation in cardiac homeostasis.

### *Denedylase CSN is essential for the integrity of the structure and function of postnatal and adult heart*

CSN8 is the smallest and least conserved CSN subunit. Using Cre-loxP strategy, we were able to specifically ablate *Csn8* gene in mouse cardiomyocytes at both perinatal and adult stage. Loss of CSN8 in neonatal mouse hearts disrupts the formation of CSN complex and accumulates neddylated cullins and non-cullin proteins, confirming an essential role of CSN in deneddylation [13]. The CSN8-deficient hearts are indistinguishable from the littermate controls at 1 week of age but develop hypertrophy by 2 weeks of age, which quickly progresses into dilated cardiomyopathy with significantly reduced contractility and relaxation at 3 weeks of age. The neonatal CSN8-deficient mice eventually die of heart failure at around 4 weeks of age [13]. Similarly, loss of CSN8 in adult mouse hearts also greatly impairs CSN deneddylation activity and causes rapid heart failure and premature death [14]. These findings provide compelling evidence that CSN-mediated deneddylation is indispensable for the maintenance of cardiac structure and function.

### *CSN controls UPS-mediated proteolysis in the heart*

Insufficient proteasomal proteolysis is being recognized as an important pathogenic factor to various forms of cardiac disease [110, 111]. Although CSN has been long viewed as a UPS regulator due to its ability to control CRL activity and its structural similarity to the 19S proteasome lid [112], the *in vivo* experimental evidence is still lacking. By probing the UPS function with a reporter mouse, it was found that loss of CSN8 accumulated the UPS surrogate substrate in the hearts, suggesting severe impairment of UPS function. Consistently, mas-

sive ubiquitinated and oxidized proteins as well as protein aggregates were evident in CSN8-deficient mouse hearts [13, 14]. Several chaperone proteins such as Hsp25, Hsp90 and  $\alpha$ -Crystallin B (CryAB) were upregulated in CSN8-deficient hearts, presumably due to adaptive response to proteotoxic stress [13]. Together, these data indicate that CSN-mediated deneddylation is critical for proteasomal proteolysis in the heart and that the compromised UPS function can be attributed to the severe cardiac phenotype of CSN8-deficient mice.

Persistent neddylation of cullins led to destabilization of CRL components and accumulation of their substrates in non-cardiac cells [38, 91]. In the heart, loss of CSN8 reduces the expression of several F-box proteins but surprisingly does not affect the abundances of their substrates, suggesting that CRL dysfunction does not likely account for the impairment of UPS function. Instead, CSN deficiency increases proteasome abundance and proteasomal peptidase activities [13, 14]. Based on the accumulation of ubiquitinated proteins and contrasting elevated proteasomal peptidase activities, it is speculated that CSN dysfunction may uncouple the ubiquitination with the subsequent degradation of misfolded proteins in cardiomyocytes.

The mammalian 19S proteasome has an intrinsic "deubiquitinase" (DUB) subunit RPN11 to couple ubiquitination and proteasome degradation of the substrate. RPN11 removes ubiquitin from the substrate before it is translocated to the 20S proteasome to be hydrolyzed [113]. The release of ubiquitin prevents it from degradation, minimizing the fluctuations in ubiquitin pools. Thus, RPN11 is required for economic and efficient degradation of the substrates. It is recently found that NEDD8 can incorporate into and cap the ubiquitin chain, particularly under stress conditions [20, 58]. Interestingly, CSN interacts with proteasome *in vivo* and cleave off NEDD8 from the ubiquitin chain *in vitro* [79]. Therefore, it is possible that CSN may facilitate proteasomal degradation of misfolded proteins by decapping NEDD8 from the mixed NEDD8-ubiquitin chain, in a way similar to processing of ubiquitin chain by RPN11.

### *CSN regulates autophagic proteolysis in the heart*

Autophagy is a metabolic process with bulk protein degradation capacity [114]. It begins with

membrane nucleation, which further elongates and sequesters cytoplasmic contents including protein aggregates, damaged organelles and invading pathogens, to form autophagosome with characteristic double membranes. The autophagosome is then fused with lysosome to form autolysosome, whereby the sequestered contents are degraded by lysosomal enzymes. It becomes apparent that both excessive activation and suppression of autophagy contribute to the development of cardiac diseases [114, 115].

Neddylation appears to regulate autophagy in the heart. CSN8 deficiency increases the abundance of autophagosomes, which is due to blockade of autophagosome clearance but not activation of autophagy [14, 109]. The compromised autophagosome degradation is at least in part caused by a defect in the fusion of autophagosome with lysosome. Downregulation of Rab7, a critical protein for autophagosome maturation, likely accounts for the defective autophagosome maturation in CSN8-deficient heart [109]. These findings indicate that CSN-mediated deneddylation is crucial for autophagic degradation and that impaired autophagic function likely contributes to cardiac dysfunction in CSN8-deficient mice.

Additionally, neddylation also seems to control the initiation of autophagy, which is known to be repressed by activation of mTOR signaling. In cancer cells, administration of NAE inhibitor MLN4924 inactivates CRL and accumulates a mTOR inhibitory protein Deptor, leading to suppression of mTOR activity and activation of autophagy [116]. Consistently, silencing of a CRL component ROC1 also accumulates Deptor, inhibited mTOR activity and induced autophagy [117].

### *Neddylation and necrosis in the heart*

Necrosis is a major form of cell death that is morphologically distinct from apoptosis [118]. Recognition of necrosis as a regulatory process has reignited cardiac researchers' enthusiasm to understand how necrosis is regulated in the heart [119]. In CSN8-deficient mouse hearts, massive necrotic but not apoptotic cardiomyocytes were observed, suggesting that necrosis may be an underlying mechanism of cardiac dysfunction [13, 14, 109]. The necrotic cardiomyocytes were accumulated with autophagic

vesicles in CSN8-deficient hearts, raising a possibility that the defective autophagy is causative to necrotic cell death [109]. Indeed, inhibition of autophagic degradation is associated with necrosis and cardiac disease [120, 121]. In contrast to the absence of prevalent apoptosis in the heart, loss of CSN8 in hepatocytes induces massive apoptosis and impaired liver function [122, 123], suggesting that CSN8-deficiency induced cell death may be cell-type specific.

Necrosis can occur in a highly regulated and genetically controlled manner. Recent studies have identified RIPK1-RIPK3 signaling as an underlying mechanism of TNF $\alpha$ -induced regulated necrosis [118]. Pharmacological or genetic inhibition of RIPK1-RIPK3 signaling has been shown to protect the heart against cardiac insults [124, 125]. Interestingly, RIPK1 seems to be modified by NEDD8, which is mediated by IAP, a novel NEDD8 ligase [30]. It will be interesting to investigate whether CSN regulates necrosis through modulating RIPK1-RIPK3 pathway in the heart.

### **Concluding remarks and future perspectives**

Over the past decades, we have achieved a better understanding of the functional significance of neddylation and its association with diseases. Existing evidence strongly indicate that neddylation impacts profoundly on many basic cellular processes and that fine-tuning of neddylation is critical for the maintenance of cardiac structure and function. With assuming equal prominence to the ubiquitination and other ubiquitin-like protein modification, it is anticipated that virtually no complex cellular process will be untouched by neddylation. Therefore, a deeper understanding of the role of neddylation in cardiac development and pathological remodeling is needed for the development of novel therapeutic strategy to treat cardiac disease. In our opinion, future studies may be extended and directed to the following areas, using a combination of biochemical, proteomics, pharmacological and genetic approaches.

First of all, the association of dysregulation of neddylation with cardiac diseases remains to be further established. So far, no clinical evidence is available regarding the association of mutations within the NEDD8 networks with any

inherited cardiomyopathies. Also, the functional status of neddylation/denoddylation in different forms of cardiac disorders remains to be defined, both in patients and in animal models.

Second, novel gain-of-function and loss-of-function mouse models awaits to be established to specifically target the major components of the NEDD8 pathway in the heart, including NAE, Ubc12, UBE2F, DCNL1/2/3/4/5, NEDP1 and NUB1L. Creation and characterization of these mice will not only provide us the entire picture of physiological functions of neddylation/denoddylation in the heart, but also differentiate distinct versus redundant functions between family members. These mice will also be valuable tools to dissect the impact of neddylation in cardiac pathological remodeling.

Third, efforts need to be directed to investigate cellular mechanisms by which neddylation controls the integrity of cardiomyocyte structure and function. Despite the observations on the involvement of neddylation in the UPS, autophagy and necrosis, the exact molecular mechanisms are to be elucidated. Considering the wide spectrum of NEDD8 targets in cardiomyocytes, novel processes regulated by neddylation remain to be discovered.

Finally, our understanding of protein neddylation will be strongly improved by systematic, unbiased and proteome-wide studies to identify NEDD8 targets in the heart, at both baseline and in response to physiological/pathological stimuli. This is relevant for better understanding of deregulation of neddylation in cardiac diseases and for the development of drugs that target components of neddylation signaling to battle cardiac diseases.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Huabo Su, Vascular Biology Center, Georgia Regents University, 1459 Laney Walker Blvd, Augusta, GA 30912, USA. Tel: 706-721-9152; Fax: 706-721-9799; E-mail: HSU@gru.edu

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