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Mass spectrometry-based proteomics strategies to define protein NEDDylation

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LIST OF PUBLICATIONS

This thesis is based on the manuscripts IV, and V. Previous contributions are listed in the manuscripts I and II. The publication III is a contribution to a book chapter.

- I. Lang, Valérie et al. (2014) “Tetramerization-Defects of p53 Result in Aberrant Ubiquitylation and Transcriptional Activity.” *Molecular Oncology* 8.5: 1026–1042.
- II. Rodriguez, M S et al. (2014) “The RING Ubiquitin E3 RNF114 Interacts with A20 and Modulates NF- κ B Activity and T-Cell Activation.” *Cell Death and Disease* 5.8: e1399.
- III. Mata-Cantero, Lydia., Lobato-Gil, Sofia et al. (2015) “The Ubiquitin-Proteasome System (UPS) as a Cancer Drug Target: Emerging Mechanisms and Therapeutics.” *Stress Response Pathways in Cancer: From Molecular Targets to Novel Therapeutics*. Dordrecht: Springer Netherlands, 225–264.
- IV. Maghames, Chantal M.; Lobato-Gil, Sofia et al. (2017) *A Cullin-Ring Ligase Independent Role of NEDD8 in Stress-Induced Nuclear Protein Aggregation and Proteotoxicity*. (Submitted)
- V. Lobato-Gil, Sofia et al. (2017) Large-scale identification of specific NEDDylation substrates and sites by MS-based proteomics. (Manuscript in preparation)

AUTHOR CONTRIBUTION

In Chapter I, results presented were done in collaboration with Chantal Maghames at CRBM, France. Chantal performed the microscopy analysis and some immunoblots for MS validation. In Chapter II, the optimized MS workflow was conducted by Jan Heidelberger as a part of a collaboration with Petra Beli’s lab at IMB, Germany.

RESUMEN

Las modificaciones post-traduccionales reguladas por la ubiquitina y la familia de la ubiquitina están implicadas en diversas funciones vitales en las células eucariotas. Entre todas las proteínas de la familia de la ubiquitina, NEDD8 es la que comparte mayor similitud con la ubiquitina tanto en su secuencia como en su estructura. A pesar de esta similitud, NEDD8 tiene su propio conjunto de enzimas que resulta en una distinta cascada de conjugación. Las culinas, forman parte de la familia de ligasas de ubiquitina CRLs (Culina-RING) y representan los sustratos mejor caracterizadas de NEDD8, por ello, la mayoría de los estudios sobre NEDDilación se han enfocado principalmente en la regulación y la degradación de proteínas ubiquitiladas. Recientemente, otros sustratos relevantes de NEDD8 han sido identificados lo cual sugiere que la NEDDilación está vinculada en muchos más procesos biológicos esenciales para la célula. De hecho, NEDD8 responde a situaciones de estrés celular en comunicación cruzada con la ubiquitina, sin embargo, todavía se desconoce el papel de la NEDDilación en respuesta al estrés. La importancia de la NEDDilación se ha sustentado gracias su inhibidor, MLN4924, usado exitosamente en el tratamiento de ciertos tipos de cáncer. En consecuencia, la identificación de nuevos sustratos de NEDD8 potencialmente ayudará a ampliar nuestra comprensión sobre los mecanismos por el cual su inhibición induce efectos anti-tumorales. No obstante, el conocimiento de las proteínas NEDDiladas está muy limitado debido, en gran parte, a que las recientes técnicas en espectrometría de masa no permiten distinguir entre la NEDDylación y la ubiquitilación ya que ambas proteínas liberan la misma señal doble glicina (GG) después de la digestión con tripsina del péptido modificado. En este trabajo nos enfocamos en el desarrollo de nuevas estrategias aplicadas en proteómica para resolver el problema que supone la identificación a gran escala de las proteínas NEDDiladas. Con nuestros avances hemos logrado no solo distinguir sustratos específicos de NEDD8 sino también los sitios de modificación. Además, por medio de métodos de cuantificación en combinación con espectrometría de masas, hemos podido caracterizar el papel de la NEDDilación en situaciones de conjugación canónica y atípica explorando así más allá de su papel homeostático como regulador de las CRLs.

ABSTRACT

Protein ubiquitination is an important post-translational modification (PTM) that, together with other members of the ubiquitin family (UBLs), is essential for the regulation of nearly every aspect of cellular homeostasis. Among all UBLs, NEDD8 shares the highest sequence and structure identity with ubiquitin. Despite this similarity, a set of NEDD8-specific proteins ensures its own conjugation pathway for downstream effects. Cullins represent the main NEDDylation substrates. Their modification by NEDD8 results in the activation of ubiquitination by favoring alternative conformations of the cullin-RING ligases (CRLs). For a long time, cullins were considered the only NEDD8 substrates but recently other physiologically relevant NEDD8 substrates have been reported. This suggests that NEDDylation controls a more diverse spectrum of biological processes. Indeed, a global increase in NEDDylation through the ubiquitin machinery has been observed under proteotoxic stress. However, the role of NEDD8 crosstalk with ubiquitin under stress conditions remains unknown. Moreover, the importance of protein NEDDylation has been supported by the success of its E1 enzyme inhibitor, MLN4924, in the treatment of certain types of cancer. Therefore, the identification of NEDD8 targets and regulators shows a strong potential to define pathways through which the inhibitors exert their anti-tumour effects. Nevertheless, there is still a limited knowledge of the non-cullin NEDDylation substrates. Identification of specific NEDD8 targets became a particular challenge due to knowledge that the overexpression of exogenous NEDD8 can trigger NEDDylation by the ubiquitin E1 enzyme UBE1. Furthermore, the current proteomics cannot distinguish between NEDD8 and ubiquitin modification, as both molecules provide the same diglycine signature on modified lysines after trypsin digestion. To overcome these technical barriers, here we developed mechanisms to distinguish between the two modes of NEDD8 activation. We created stable cell lines that express a NEDD8 transgene at endogenous levels. Also, we performed mass spectrometry (MS)-based proteomics approaches which resulted in the novel proteome-wide identification of specific NEDD8 conjugates and modification sites upon different cellular conditions. First, by using SILAC-based quantitative proteomics, we could define

the dynamic of NEDD8 under proteotoxicity and homeostasis. Secondly, by using a mutant form of NEDD8 in protease NEDP1-depleted cells we were able to identify up to 1,000 genuine NEDDylation sites. In conclusion, this work presents evidence of the broader role of NEDD8 besides CRLs regulation and provides a powerful resource to further investigate the apparent complexity of UBLs crosstalk during protein modification.

ABBREVIATIONS

6His	6 times polyhistidine tag
Ala	Alanine (A)
Apc2	component of the anaphase promoting complex/cyclosome APC/C
APPBP1	Amyloid beta Precursor Protein Binding Protein 1 (synonym NAE1)
Arg	Arginine (R)
Asn	Asparagine (N)
Atg	autophagy-related ubiquitin-like modifier
Cas9	CRISPR associated protein 9
COP9	Constitutive photomorphogenesis 9
CRISPR	Clustered regularly interspaced short palindromic repeats
CRL	Cullin RING ubiquitin ligases
Cys	Cysteine (C)
DEN1	Deneddylase-1 (synonym NEDP1 and SENP8)
DMP	Dimethyl pimelimidate
DUBs	Deubiquitinating enzymes
ER	Endoplasmic reticulum
ESI	electrospray ionization
FACS	Fluorescence-activated cell sorting
FAT10	HLA-F-adjacent transcript 10
FDR	False Discovery Rate
FUBI	Fau ubiquitin-like protein
Gly	Glycine (G)
GO	Gene Ontology
GST	Glutathione S-transferase
HS	Heat shock
HUB1	homologous to ubiquitin
ICAT	isotope-coded affinity tag
IF	Immunofluorescence
Ile	Isoleucine (I)
ISG15	IFN-induced 15-kDa protein
iTRAQ	isobaric tags for relative and absolute quantification
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knock-out
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
Lys	Lysine (K)
m/z	Mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MLN4924	NEDD8-activating enzyme inhibitor (Millenium pharmaceuticals)
MS	Mass spectrometry
NAE	NEDD8 Activating Enzyme
NAE1	NEDD8-activating enzyme E1 regulatory subunit (synonym APPBP1)
NEDD8	Neural-Precursor-Cell-Expressed Developmentally Down-regulated 8
NEDP1	NEDD8 Processing enzyme (synonym SENP8 and DEN1)
Ni-NTA	Nickel-nitrilotriacetic acid
NUB1	NEDD8 ultimate buster-1
ON	Overnight
PARC	p53 cytoplasmic anchor protein
PQC	Proteins quality control
PTM	Post-translational modification
RBX1	RING-box protein 1

RING	Really Interesting New Gene
RPs	Ribosomal proteins
rRNA	Ribosomal RNA
SCX	Strong cation exchanged
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis
SENp8	Sentrin-specific protease 8 (synonym NEDP1 and DEN1)
SILAC	Stable Isotope Labelling with Amino acids in Cell culture
SIMs	SUMO.interacting motifs (SIMs)
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
STUBLs	SUMO-targeted ubiquitin ligases
SUMO	Small Ubiquitin-like Modifier
TMT	tandem mass tags
TUBEs	Tandem Ubiquitin Binding Entities
Ub	Ubiquitin
UBA	Ubiquitin-Associated Domain
UBA3	NEDD8-activating enzyme E1 catalytic subunit
UBE1	Ubiquitin-activating enzyme E1
UBE2F	NEDD8-conjugating enzyme UBE2F
UBE2M	NEDD8-conjugating enzyme Ubc12
UBL	Ubiquitin-like protein
UCHL3	Ubiquitin carboxyl-terminal hydrolase isozyme L3
UFD	Ubiquitin-fold domain
UFM1	Ubiquitin-fold modifier 1
UPS	Ubiquitin-proteasome system
URM1	Ubiquitin-related modifier-1
USP21	Ubiquitin carboxyl-terminal hydrolase 25
WB	Western blotting
WCE	Whole cell extract

INTRODUCTION

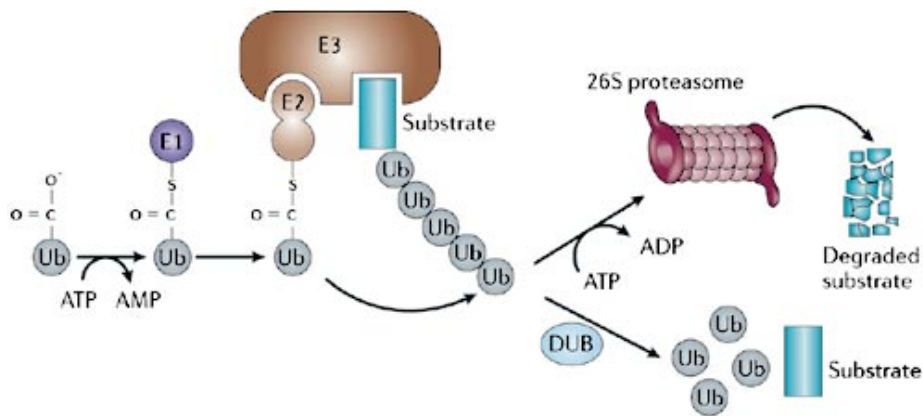
Besides water, proteins are the most abundant molecules in biology. After their biosynthesis by the ribosomes, the majority of proteins are converted into a folded compact structure that enables a proper function. These processes are highly organized given that a well-functioning cell relies on a efficient regulated proteome. (Alam, Siddiqi, Chturvedi, & Khan, 2017). Moreover, all proteins in the cell can be covalently modified by small molecules as phosphate, methyl or acetyl groups, but also by entire proteins. These called post-translational modifications (PTMs) are dynamically reversible and determine different structure, stability, activity, localisation and interactions with other cellular molecules without the need for changes in *de novo* protein synthesis (Jensen, 2006). Protein modification by ubiquitin and ubiquitin-like proteins (UBLs) have emerged as crucial PTMs for the regulation of almost every cellular process (Cipolla, Maffia, Bertoletti, & Sabbioneda, 2016).

UBIQUITIN AND UBIQUITIN-LIKE PROTEINS

Ubiquitin is a well conserved small 76 amino acid protein (8.5 kDa) firstly described as an ubiquitous abundant protein found in all eukaryotic organisms (Schlesinger, Goldstein, & Niall, 1975). In the human genome ubiquitin is produced by four different genes: UBB, UBC, UBA52 and RPS27A. Since the discovery of ubiquitin in 1975, protein modification by ubiquitin was further characterized in the early 1980 by Irwin A. Rose, Avram Hershko, and Aaron Ciechanover as a new mechanism for energy-depending intracellular proteolysis. This ubiquitin proteolytic model consists of the covalent attachment of ubiquitin to proteins to target them for proteasomal degradation and its apparent importance was recognized by the attribution of the Nobel prize in Chemistry in 2004 (Kimura & Tanaka, 2010; Wilkinson, 2005). Nowadays, we know that ubiquitin is the founding member of a large family of proteins which contain the same β -grasp fold, the so-called ubiquitin-like proteins (UBLs) which their is a signal to regulate proteins by modifying their structure, function and/or localization (van der Veen & Ploegh, 2012).

Ubiquitination

Ubiquitination is the best understood reversible post-translational modification, which is well related to a significant number of biological processes apart of its essential proteasome-dependent proteolytic role, including cell cycle progression, transcriptional regulation, endocytosis, DNA repair, signal transduction and transcription. Therefore, deregulation fo this pathway has been associated with several pathological conditions such as developmental abnormalities, cancer, neurodegenerative disorders, inflammation and immune response (Hoeller et al., 2006). The ubiquitination process consists of the covalent attachment of ubiquitin to the ϵ -amino group of lysine residues (Lys) of target proteins via its carboxyl-terminal-glycine residue, forming an isopeptide linkage in an ATP-dependent reaction. This attachment to substrates is catalysed by a hierarchical set of three types of enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes (Hershko & Ciechanover, 1998). Given that ubiquitin itself contains seven lysines (Lys6, 11, 27, 29, 33, 48, and 63), it can modify to other ubiquitin molecules which enables the formation of polyubiquitin chains with different linkages signal which drives to distinct functional outcomes for the tagged proteins (see **Scheme 1**). As a result, the intracellular ubiquitin exists in a dynamic equilibrium among three essential forms (i) either as a free monomer, (ii) a substrate-conjugated monoubiquitin or polyubiquitin, or (iii) an unanchored ubiquitin chains (Kimura & Tanaka, 2010). Deubiquitinating enzymes (DUBs) are ubiquitin-specific proteases which not only can reverse the ubiquitination process but also carry the activation of the ubiquitin proproteins and the ubiquitin recycle from trapped thiol-ester intermediates and polyubiquitin chains. The first two steps of the ubiquitination cascade is triggered by few E1 and E2 enzymes but it is estimated that approximately 600 E3s and 100 DUBs exist in mammalian cells. (Reyes-Turcu, Ventii, & Wilkinson, 2009).



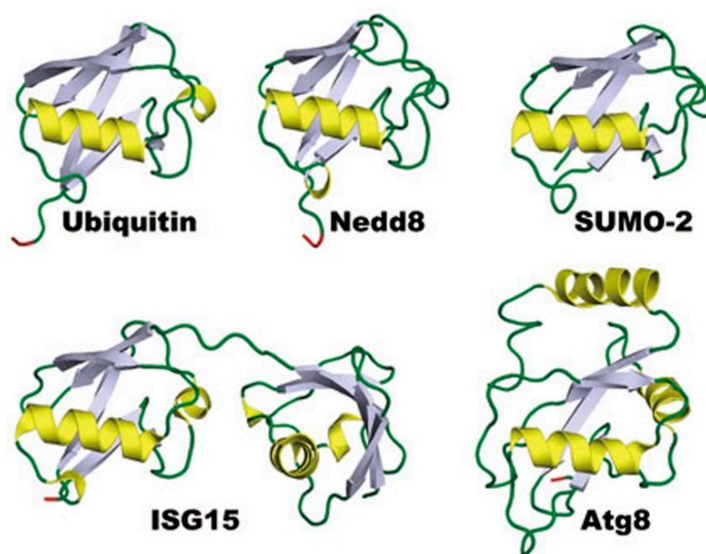
Scheme 1. Ubiquitin conjugation

Ubiquitin (Ub) is a highly conserved 8 kDa protein that becomes covalently attached to lysine residues of target proteins in an inducible and reversible manner. This occurs through a three-step process involving three different types of enzymes. Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating enzyme (E1), and is then transferred to a ubiquitin-conjugating enzyme (E2) through a thioester bond. A ubiquitin-protein ligase (E3) specifically attaches ubiquitin to the ϵ -amino group of a lysine residue in the target protein¹. Although only a few E1 enzymes are known, humans have more than 20 different E2s. E3 ligases are primarily responsible for substrate recognition. To provide specificity about 500–1,000 different E3 ligases exist in humans⁶. After the attachment of a Lys48-linked polyubiquitin chain to a substrate it is degraded in the 26S proteasome; the attached ubiquitin moieties can be recycled. Ubiquitylation reactions are reversible by de-ubiquitylating enzymes (DUBs), of which several types are known at present (Hoeller et al., 2006).

Ubiquitin like-proteins

In addition to ubiquitin, mammalian cells encode nearly 20 ubiquitin-like proteins (UBLs). Main members of the Ubl family include SUMO-1 to -4 (small ubiquitin-like modifier 1 to 4), NEDD8 (neural-precursor cell expressed developmentally down-regulated 8), FUBI (Fau ubiquitin-like protein), HUB1 (homologous to ubiquitin 1); ISG15 (interferon-stimulated gene); FAT10 (HLA-F-adjacent transcript 10), URM1 (Ubiquitin-related modifier-1); UFM1 (Ubiquitin-fold modifier 1); Atg8 and Atg12 (autophagy-related ubiquitin-like modifier 8 and 12). Most of these proteins, do not share a high sequence similarity with ubiquitin (except for NEDD8), but they all present a similar three-dimensional structure (the “ubiquitin fold” or β -grasp fold) (see **Scheme 2**) and an analogous modification enzymatic cascade. Although all these proteins are structurally related, each one plays

different roles in the regulation of protein degradation, subcellular localization, activity of the modified protein, among other functions. In this work, we are mainly focused on the ubiquitin-like protein NEDD8 therefore its process and regulation is further discussed in detail in this introduction (Kerscher, Felberbaum, & Hochstrasser, 2006; van der Veen & Ploegh, 2012).



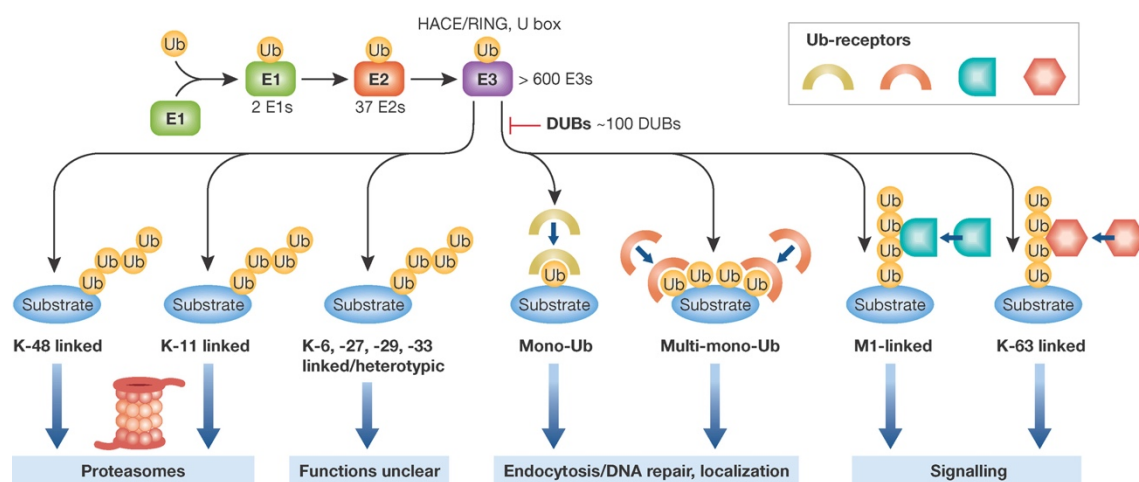
Scheme 2. Ubiquitin-like proteins structure

Structural comparison depicting the conserved β -grasp fold of ubiquitin and Ubl proteins (PDB codes: 1UBQ, 1NDD, 1WM3, 1Z2M], and 2KWC). ISG15 has tandem ubiquitin folds. The C-terminal glycine of ubiquitin and Ubls is colored red, except for SUMO2 because it was not ordered in the crystal structure (Ronau, Beckmann, & Hochstrasser, 2016).

The complex ubiquitin code

Ubiquitin can covalently modify protein substrates by different modes. The attachment of a single ubiquitin to one target lysine (on the protein substrate) is denominated mono-ubiquitination; and the attachment of several ubiquitin molecules on several lysines (on the same target protein) is called multiple mono-ubiquitination. As mentioned above, ubiquitin itself has seven internal lysines where other ubiquitin molecules can be attached and form different poly-ubiquitin chains through lysines K6, K11, K27, K29, K33, K48 and K63 (Ikeda & Dikic, 2008). Moreover, a linear type of ubiquitin chain can be formed through the

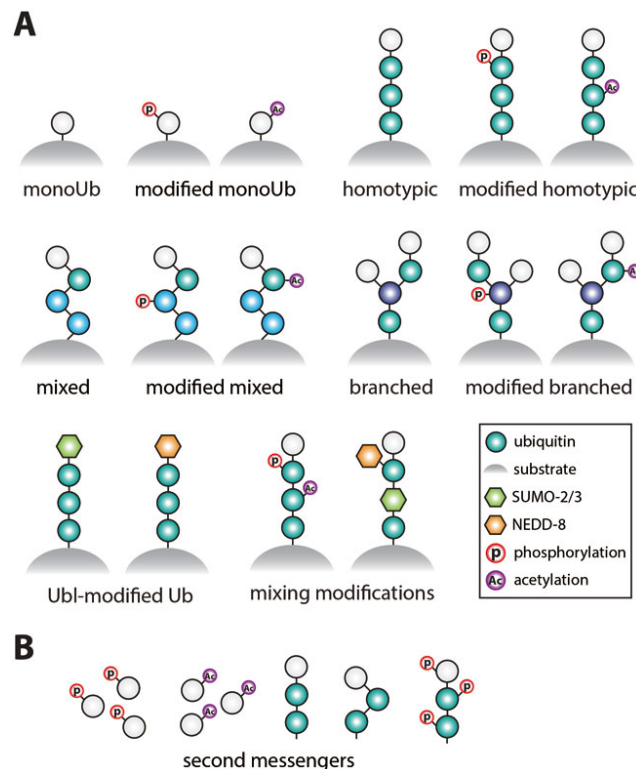
conjugation of the C-terminal Gly76 of the proximal ubiquitin to the N-terminal methionine residue of a distal ubiquitin. This linear topology chain is catalyzed by a multi-subunit E3 ligase complex termed LUBAC (linear ubiquitin chain assembly complex) (Kirisako et al., 2006). Depending on the type of ubiquitin modification and the type of linkage within the chains, the final distinct signals create the so-called “ubiquitin code” (**Scheme 3**). This ubiquitin code provides diversity in the ubiquitination signal which can be associated with many different cellular processes. For example, the best studied poly-ubiquitin chain is the one formed through K48 linkages which generally targets the modified proteins for proteasomal degradation but also the K11 chains have been associated to proteolysis (Ikeda, Crosetto, & Dikic, 2010). Mono-ubiquitination has been associated with endocytosis and DNA repair processes whereas the multiple mono-ubiquitination is linked to cell surface receptors with subsequent lysosomal degradation signal or for the recycling to the cell surface (K Haglund, Di Fiore, & Dikic, 2003). Ubiquitin chains through lysine K63 are correlated with proteasome-independent functions, such as vesicle trafficking, DNA repair and signal transduction (Kaisa Haglund & Dikic, 2005).



Scheme 3. Principles of ubiquitin signalling

Ubiquitylation is mediated by the sequential activity of a set of enzymes including activating (E1), conjugating (E2) and ligating (E3) enzymes. This leads to the conjugation of monoubiquitin or polyubiquitin chains of different lengths and linkages to target proteins. Depending on the different types of ubiquitin chains, proteins are subsequently degraded via the proteasome or participate in various cellular functions including signalling, DNA repair or endocytosis. See text for more details (Fulda, Rajalingam, & Dikic, 2012)

Unquestionably, the ubiquitin code is more complex than expected. There are new ways the ubiquitin modifications can be classified. For example, mono-ubiquitination can occur with an unmodified or a modified ubiquitin, chains can be from one linkage type (homotypic) or different linkage type (heterotypic) (Komander & Rape, 2012). Furthermore, chains of one linkage type can be extended by a second type, forming a mixed non-branched structure but when a ubiquitin molecule in a chain is ubiquitinated at various lysines this forms a “branched” or “forked” structure. Moreover, it has been recently shown that ubiquitin can be also modified by other UBLs including SUMO and NEDD8, and other PTMs such as phosphorylation and acetylation (Swatek & Komander, 2016). These alternative modifications increase the potential for unlimited number of combinations. Besides, non-attached (or unanchored) ubiquitin or ubiquitin chains exist in cells which leads to second-messenger-like functions (Swatek & Komander, 2016; Xia et al., 2009) (**Scheme 4**).



Scheme 4. New complexity in the ubiquitin code

(A) Conceptual representation of some of the possible ubiquitin-, Ubl (NEDD8, SUMO2/3)- and chemical modifications of ubiquitin. (B) Unanchored ubiquitin and

ubiquitin chains, with or without modifications, can function as second messengers in cells (Swatek & Komander, 2016).

Ubiquitin binding motifs as decoders

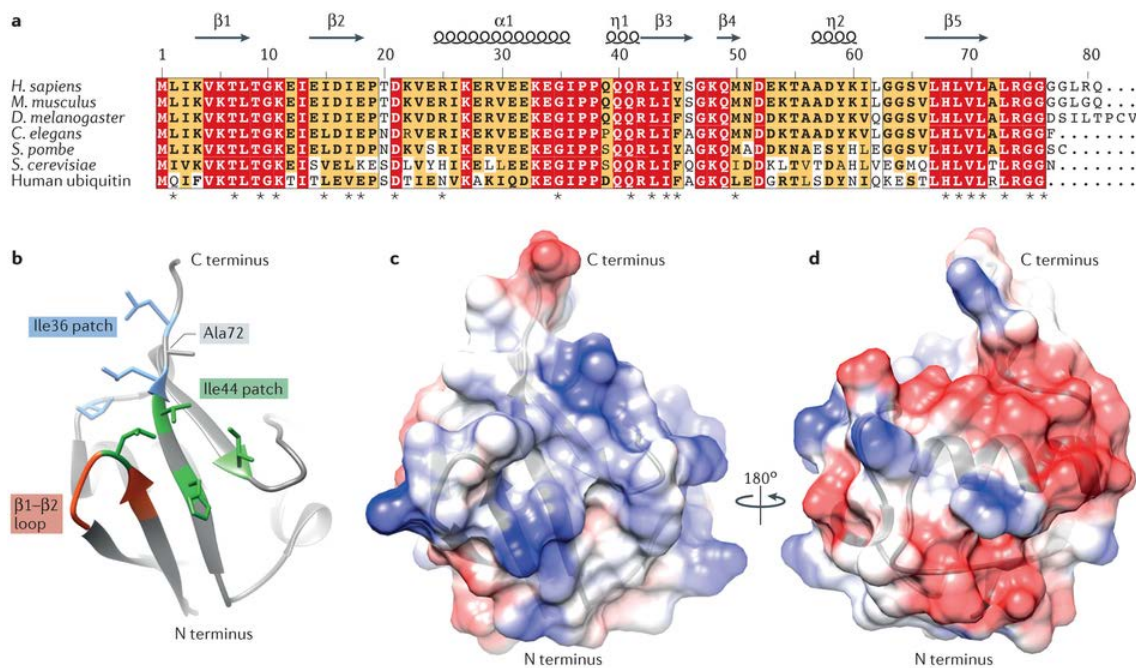
Ubiquitin modification of substrates is decoded by specific receptors containing ubiquitin-binding domains (UBDs). Several types of UBDs have been identified that differ in their structure and mode of interaction with ubiquitin. UBDs can be classified into four major subfamilies: those containing single or multiple α -helices, zing-fingers, a pleckstrin-homology (PH) fold and ubiquitin-conjugating-like structures. Despite their differences, they all bind non-covalently to mono and polyubiquitin to decode and translate the ubiquitin signal into the corresponding cellular fate (Ikeda et al., 2010). Most UBDs bind monoUbiquitin via the hydrophobic patch around Isoleucine 44. The binding affinities of individual UBDs for Ubiquitin are normally low. Nevertheless, in the cell, this affinity of interaction can be increased by the presence of several UBDs in the same receptor or even multiple Ubiquitin-binding surfaces within a single UBD. In fact, most Ubiquitin-receptors are known to contain different UBDs (Husnjak & Dikic, 2012). In addition to their interaction with monoubiquitin, many UBDs present high selectivity for precise Ubiquitin-linkages. This selectivity is ensured either by the recognition of the linkage between two Ubiquitin or by the detection of the spatial distribution of the Ubiquitin moieties (Dikic, Wakatsuki, & Walters, 2009). Among the different types of UBDs, the most common have single or multiple α -helices, like the Ubiquitin-associated domain (UBA) and the ubiquitin-interacting motif (UIM). In fact, UBA domains are found in many effectors of the Ubiquitin-proteasome system like the shuttle factors Rad23, PLIC1-2 (DSK2) that deliver Ubiquitinated substrates to the 26S proteasome (Funakoshi et al., 2002; Hofmann and Bucher, 1996; Rao and Sastry, 2002). In addition, P62 that ensures the degradation of Ubiquitinated substrates by the autophagic pathway also contains a UBA domain (Isogai et al., 2011). UBA domains normally show preference for K48-linked chains (Raasi and Pickart, 2003; Wilkinson et al., 2001). An example of other Ubiquitin-interacting proteins with UBDs is Rpn10/S5 α and Rpn13; both are intrinsic receptors of the proteasome containing

UIM and the Pleckstrin-homology (Pleckstrin-like receptor for Ubiquitin (PRU)) domains respectively (Husnjak et al., 2008; Riedinger et al., 2010). The DNA-double strand break response protein (RAP80) harbours two UIM domains and interacts preferentially with K63-linked chains due to the length of the linker region between these two UIMs (Sims and Cohen, 2009). To increase the diversity of the Ubiquitin signalling, a Ubiquitin-Like Fold (UBL) can be also found either in Ubiquitin-Like Molecules that share the same Ubiquitin fold and can be conjugated to substrates, or as a part of a multidomain structure in proteins that cannot be conjugated to substrates. As an example of the second type of proteins, shuttle factors use their UBDs to bind Ubiquitinated substrates and then interact with the proteasome via their UBL domains, thus allowing substrate transfer for degradation (Miller and Gordon, 2005; Welchman et al., 2005).

PROTEIN NEDDYLATION

Among all UBLs identified to date, NEDD8 (neural precursor cell expressed developmentally downregulated protein 8) shares the highest amino acid identity (60%) and homology (80%) with ubiquitin (T. K. Kamitani, Kito, Nguyen, & Yeh, 1997). NEDD8 is a conserved, mostly nuclear protein that consist of 81 amino acid residues which was first discovered as one of the ten NEDD genes that are down-regulated in mouse brain during development (Kumar, Tomooka, & Noda, 1992; Whitby, Xia, Pickart, & Hill, 1998). NEDDylation showed to be essential for the viability of most model organisms with a particular exception of *S.cerevisiae* (Rabut & Peter, 2008). As expected from the high level of sequence conservation, the structure of NEDD8 closely resembles that of ubiquitin. Nevertheless, the small differences in their sequences/structures can mediate distinct interactions that allow their specific functions in cells. In the sequence level, biochemical analyses have demonstrated that Ala72 of NEDD8 (Arg72 in ubiquitin), performs a key role in preventing the interaction of NEDD8 with the ubiquitin E1 enzyme (Whitby et al., 1998). In the overall structure level, NEDD8 and ubiquitin conserved several important properties. For example, two surface-exposed hydrophobic patches on ubiquitin (Ile44 and Ile36 patches) have been shown to

be essential for mediating protein–protein interactions although both are conserved in the NEDD8 structure. The largely conserved region in NEDD8 known as the β 1– β 2 loop is another functional important region conserved in ubiquitin. However, the intermediary polar and charged surfaces are less conserved between ubiquitin and NEDD8. These last features presumably influence their distinct functions, leading to specific NEDD8-binding proteins for downstream effects (Enchev, Schulman, & Peter, 2014; Whitby et al., 1998) (see **Scheme 5**)

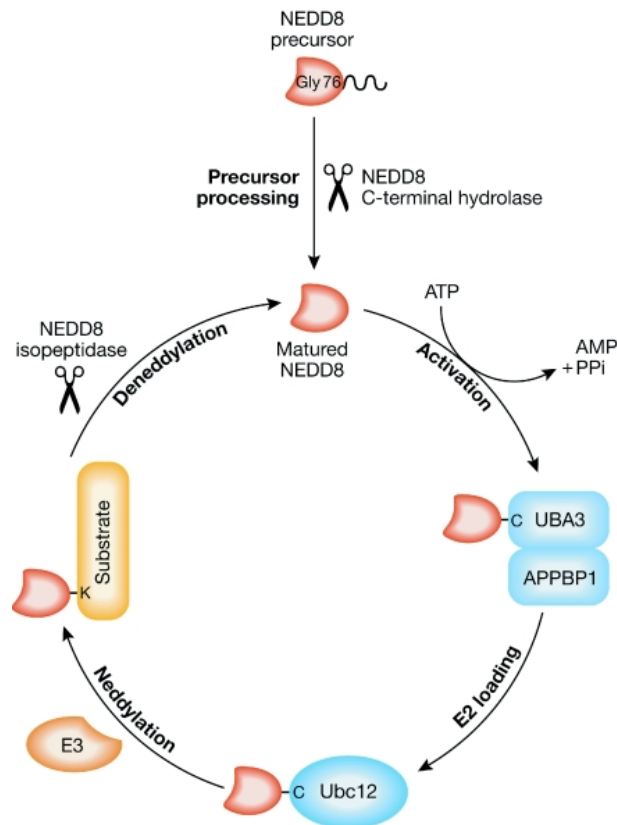


Scheme 5. NEDD8 structure

a) Structure-based multiple sequence alignment of NEDD8 (neural precursor cell expressed developmentally downregulated protein 8) from indicated species (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*) and of human ubiquitin, highlighting identical residues (red) and similar residues (yellow). Asterisks signify residues found to be essential for NEDD8 function in the fission yeast *Saccharomyces pombe*64. b) Structural representation of the NEDD8 hydrophobic patches, which contribute most of the known interfaces for interaction with binding partners. The side chains contributing to the Ile36 patch are shown in blue, the residues of the Ile44 patch in green and the β 1– β 2 loop in red. These interfaces are conserved in ubiquitin. Ala72, which is responsible for discrimination between NEDD8 and ubiquitin by the respective E1 enzymes, is also shown. c, d) Structural representations of the NEDD8-specific charged surface patches. Acidic patches are depicted in red and basic surfaces in blue on two views of NEDD8, rotated 180° around the y axis. These surfaces might be responsible for interactions that discriminate between NEDD8 and ubiquitin (Enchev et al., 2014).

The NEDDylation cascade

Despite this similarity, NEDD8 has its own set of enzymes to ensure a distinct conjugation pathway. UCHL3 (ubiquitin carboxyl-terminal hydrolase isozyme L3) is a cysteine (Cys) protease that removes the C-terminal extensions of both ubiquitin and NEDD8 precursors. A second Cys protease, known as NEDP1/DEN1/SEN8, has high specificity for NEDD8 and not for ubiquitin. Both enzymes can expose the NEDD8 diglycine motif through which the E1 complex APPBP1-Uba3, also known as NEDD8-activating enzyme (NAE), the E2s UBE2M (Ubc12) or UBE2F (NEDD8-conjugating enzymes) and multiple NEDD8-E3s ligases ensures the covalent modification of NEDD8 through an isopeptide bond to a Lys residue in the target protein. Most of the reported NEDD8 E3 ligases have shown dual specificity for NEDD8 and ubiquitin, such as the p53-negative regulator MDM2, c-CBL, SMURF1, IAPs, and RBX1/2. The only clear exception is DCN1 which is a NEDD8-specific ligase known to enhance cullin NEDDylation. The reversible de-NEDDylation process is carried out either by the multi-component COP9 signalosome, the protease NEDP1 or the ubiquitin protease USP21 (Enchev et al., 2014; Rabut & Peter, 2008; D. Xirodimas, 2008) (see **Scheme 6**). The COP9 signalosome complex, a zinc metalloproteinase, has a particular low affinity for NEDD8 but is highly specific to de-NEDDylate cullins. The protease USP21 was reported to be able to deconjugate both ubiquitin and NEDD8 showing more specificity towards ubiquitin. Interestingly, NEDP1 has an insignificant activity when it comes to deconjugating a single NEDD8 from cullin substrates. However, NEDP1 is more active in the de-NEDDylation of hyper-NEDDylated cullins, resulting in mono-NEDDylated substrates, which indicates that it might prevent the formation of poly-NEDD8 chains and that it is implicated in the regulation of cullin-independent substrates (Chan et al., 2008; Cope & Deshaies, 2003; Ye et al., 2011). Besides the de-NEDDylating enzymes, the protein levels of NEDD8 and NEDDylated substrates are also negatively regulated by cellular factors such as NUB1 (NEDD8 ultimate buster-1). NUB1 can interact with NEDD8 and promote the proteasomal degradation of NEDD8 and its protein conjugates (T. Kamitani, Kito, Fukuda-Kamitani, & Yeh, 2001).



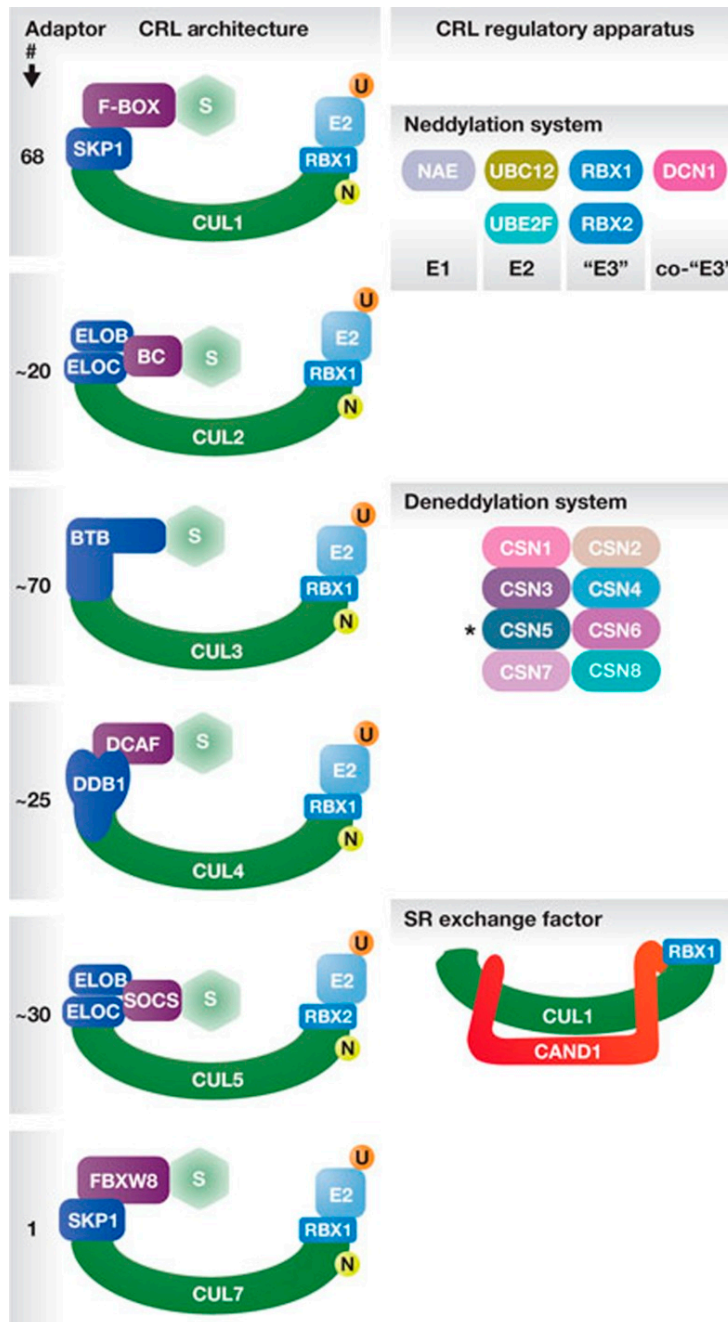
Scheme 6. Neddylation pathway

Schematic representation of the main steps of the neddylation pathway, including NEDD8 precursor processing, activation by the E1 (UBA3–APPBP1 heterodimer), loading onto the E2 (Ubc12), conjugation to a substrate by an E3 and recycling of NEDD8 by an isopeptidase. APPBP1, APP binding protein 1; NEDD8, neural precursor cell expressed developmentally downregulated protein 8; UBA3, ubiquitin-like modifier activating enzyme 3; Ubc, ubiquitin-conjugating (Rabut & Peter, 2008)

NEDD8 substrates

The best characterized substrates for NEDD8 are the cullin family members. Therefore, the NEDDylation targets are essentially classified in two groups: cullins and non-cullins. The cullin family is composed by Cul1, 2, 3, 4A, 5 and 7. Moreover, PARC (p53 cytoplasmic anchor protein) and Apc2 (component of the anaphase promoting complex/cyclosome APC/C) proteins possess the cullin homology domain. All cullins are NEDDylated except for Apc2 which lacks the lysine consensus motif for NEDDylation and still the NEDDylation of Cul7 is

unclear (Jones et al., 2008; Pan, Kentsis, Dias, Yamoah, & Wu, 2004). Cullins are scaffold proteins necessary for the dynamic assembly of the ubiquitin ligase complexes called Cullin-RING ligases (CRLs). Different types of CRLs can be formed using the distinct cullins. The essential complex consists of a cullin protein that interacts with a RING finger protein (RBX1 or RBX2) at its C-terminus and with a substrate receptor (directly or via an adaptor) at its N-terminus. The RBX1/2 proteins catalyse the ubiquitination of the CRL substrates by its binding with the ubiquitin-loaded E2. The NEDDylation of cullins enhance the ubiquitination process of CRLs by favouring alternative conformations of the cullin C-terminal domain and consequently of the bounded RBX protein (Lydeard, Schulman, & Harper, 2013) (see **Scheme 7**). The regulation of this CRL-NEDDylated mechanism is mainly regulated by the Cop9 signalosome (CSN) complex and the exchange factor CAND1 (cullin-associated NEDD8-dissociated protein 1). The CSN complex mediated the deNEDDylation of cullins so then CAND1 can bind to the cullin protein which triggers the substrate-specific receptors exchange (Zemla et al., 2013).



Scheme 7. Architecture of human cullin–RING E3 ubiquitin ligase system

The number of human SRs for each CRL complex is indicated on the left. CUL4A and CUL4B are represented as a single CRL. The CRL regulatory apparatus is composed of the neddylaton system, the deneddylaton system and the SR-exchange factor. UBC12 is the neddylaton E2 for RBX1-based CRL complexes, whereas UBE2F is the E2 for CRL5–RBX2. DCN1 is a co-E3 for both RBX1 and RBX2. The CSN deneddylates CRLs. CAND1 is a CRL exchange factor that interacts with both the amino- and carboxy-terminal regions of the cullin–RING complex. CSN subunits are indicated, an asterisk indicates that CSN5 is the catalytic subunit. CRL, cullin–RING E3 ubiquitin ligase; CSN, COP9 signalosome; N, NEDD8; S, substrate; SR, substrate receptor; U, ubiquitin (Lydeard et al., 2013).

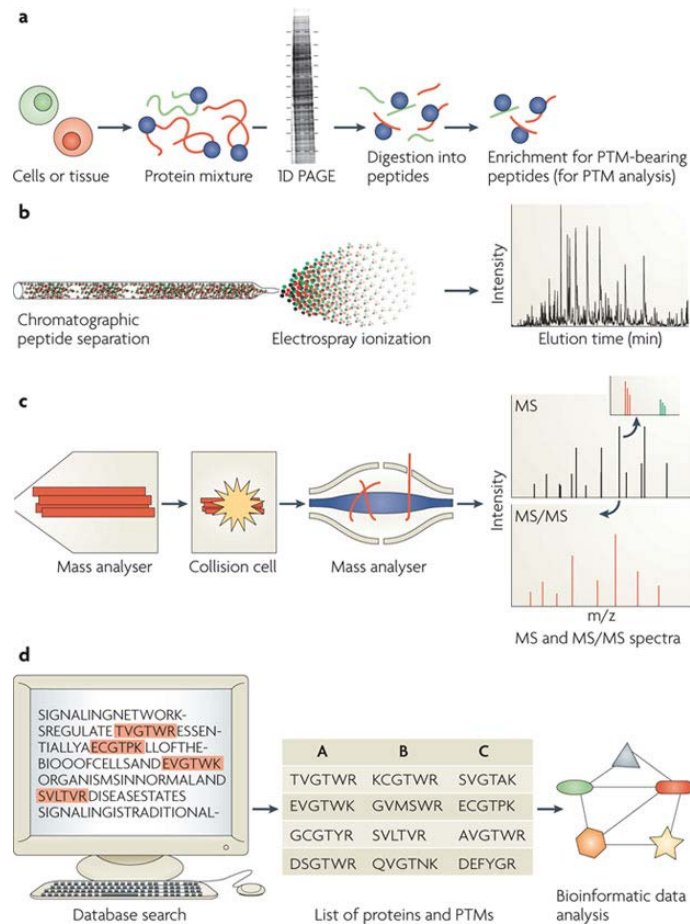
Proteomic approaches have identified several non-cullin NEDD8 substrates that participate in many important cellular processes such as: p53, p73, BCA3, pVHL, EGFR, Mdm2 and ribosomal proteins suggesting that the NEDD8 proteome is more diverse than previously thought.(Jones et al., 2008; D. P. Xirodimas et al., 2008). The NEDDylation of p53 and ribosomal proteins are some of the best characterized non-cullin substrates. NEDDylation of p53 is ensured by the ubiquitin and NEDD8 E3 ligase Mdm2 but also by FBXO11, a member of the F-box protein family and a component of the cullin1 RING ligase. The NEDDylation increases the stability of p53 but inhibits its transcriptional activity (Abida, Nikolaev, Zhao, Zhang, & Gu, 2007; D. P. Xirodimas, Saville, Bourdon, Hay, & Lane, 2004). Even that p53 is a common substrate for both NEDD8 and ubiquitin, it is differentially controlled by these two modifiers. A fusion of NEDD8 to the C-terminus of p53 causes its nuclear localisation while a ubiquitin fusion relocalises it to the cytosol (Brooks & Gu, 2006). In addition, NUB1 was shown to inhibit p53 NEDDylation, stimulate its monoUbiquitination and thus mediate its cytoplasmic localisation (Liu and Xirodimas, 2010). Moreover, Tip60 acetyltransferase was shown to exclusively blocks Mdm2-mediated p53 NEDDylation and not ubiquitination (Dohmesen et al., 2008). Mdm2 also NEDDylates TAp73 (p53 homologue) causing an inhibition of its activity. But in contrast to p53, NEDDylated p73 localizes to the cytosol (Watson et al., 2006). Ribosomal proteins (RPs) have emerged as important targets of NEDD8 (Xirodimas et al., 2008). For example, NEDDylation of RPL11 by Mdm2 increases its stability and mediates its nucleolar localization. However, upon nucleolar stress caused for example by low doses of Actinomycin D, NEDDylation of RPL11 decreases, thus allowing its re- localization into the nucleoplasm. By binding Mdm2, RPL11 represses Mdm2-mediated ubiquitination of p53, therefore ensuring p53 stabilization and transcriptional activation in response to stress (Mahata et al., 2012; Sundqvist et al., 2009).

MASS SPECTROMETRY- BASED PROTEOMICS

The human genome can generate hundreds of thousands of different gene products. Nevertheless, the result of allelic variations, mRNA splicing, protein processing and post-translational modifications (PTMs) make proteomes significantly more complex than genomes and transcriptomes in their molecular heterogeneity and diversity. Site-specific covalent modifications, such as phosphorylation, acetylation and ubiquitination, can regulate protein structure and function in real-time dynamic manner. In general, PTMs creates a molecular code that delivers protein conformation, cellular location, macromolecular interactions and activities, depending on the cell type, the tissue and the environmental conditions. These reversible modifications are implicated in a wide variety of cellular processes, including transcription, replication, cell-cycle progression, and responses to DNA damage. Therefore, protein PTMs has become an important study for the biological and biomedical areas (Jensen, 2006; Vertegaal, 2011). The goal of large-scale proteome analysis (proteomics) is to be able to elucidate this molecular and functional plasticity of proteins by mapping and dynamically quantifying PTMs. The rapid evolution of proteomics is due in large part to many improvements in mass spectrometry (MS) during the past 10 years (Parker, Warren, & Mocanu, 2010). MS-based proteomics englobes the advances in many technical areas such as affinity enrichments, protein quantitation, the availability of gene and genome sequence databases, the discovery and development of protein ionization methods and bioinformatic analysis (bioinformatics) (Aebersold & Mann, 2003; Jensen, 2006). Given the ability of MS-based proteomics to precisely identify and quantify complex proteomes from samples, nowadays it becomes the indispensable tool from molecular, cellular and systems biology. The advances include the characterization of protein-protein interactions, large-scale identification of substrates and modification sites and the generation of quantitative protein profiles from different organelles within human cells or diverse species (Aebersold & Mann, 2003).

MS overview and workflow

The basic of mass spectrometers is to measure mass. The measurement of the mass is dependent of its gas-phase ions behaviour in an electromagnetic field which provides information on the protein identity, its chemical modification, and its structure. A mass spectrometer consists in three main components: (i) an ion source from charged species, (ii) a mass analyser that separate ionized analytes based on their mass (mass-to-charge ratio, m/z), and (iii) a detector that register the number of ions at each m/z to give a measurable signal (Aebersold & Mann, 2003; Zee & Garcia, 2012). The first m/z measurement of the ionized peptides is called the MS1 step. Then, each ion is sequentially isolated, fragmented (chemical bonds break) and measured again to produce a final “fragmentation spectra” or also called tandem mass spectrum (MS2 or MS/MS) (Henrich & Gavin, 2015). There are two main ionization techniques: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). ESI technique is immediately linked to liquid-based separation tools, such as chromatography and electrophoresis, due to its ability to ionize peptides from a solution. In contrast, MALDI uses laser pulses to vaporise and ionize the samples in a dry crystalline matrix. Therefore, MALDI-MS is frequently applied for the analysis of relatively simple peptide mixtures, whereas liquid-chromatography ESI-MS procedure (LC-MS) are preferred for complex samples (Aebersold & Mann, 2003). Current MS-based proteomics is mainly using a general workflow for the proteome and PTM analysis in complex samples. This “shotgun” proteomics approach basically consist in four sections: (i) proteome extraction, enzymatic digestion and specific enrichment (ii) peptide separation and ionization by liquid chromatography, (iii) mass spectra fragmentation and (iv) mass spectrum analysis (Choudhary & Mann, 2010; Sylvestersen, Young, & Nielsen, 2013) (see **Scheme 8**)



Scheme 8. Typical workflow for proteome and PTM analysis using shotgun proteomics

a | Proteins extracted from organs, tissues or cells are separated by one-dimensional polyacrylamide gel electrophoresis (1D PAGE) and 'in-gel digested' into peptides using proteases such as trypsin. The peptides containing specific post-translational modifications (PTMs) can be enriched using different approaches. Non-modified peptides are used to identify and quantify total cellular proteins. b | Purified peptides are separated on a miniaturized reverse phase chromatography column with an organic solvent gradient. Peptides eluting from the column are ionized by electrospray at the tip of the column, directly in front of the mass spectrometer (known as on-line coupling). c | The electrosprayed ions are transferred into the vacuum of the mass spectrometer. In the mass spectrometry (MS) mode, all ions are moved to the orbitrap mass analyser, where they are measured at high resolution (top mass spectrum). The first mass analyser then selects a particular peptide ion and fragments it in a collision cell. The inset in the MS panel indicates the stable isotope labelling by amino acids in cell culture (SILAC) ratio of one of the peptides. The MS/MS spectrum can be obtained in the ion trap mass analyser at low resolution or in the orbitrap at high resolution. For modified peptides, the peptide mass will be shifted by the mass of the modification, as will all fragments containing the modification, allowing the unambiguous placement of the PTM on the sequence. d | The mass and list of fragment masses for each peptide are scanned against protein sequence databases, resulting in a list of identified peptides and proteins.

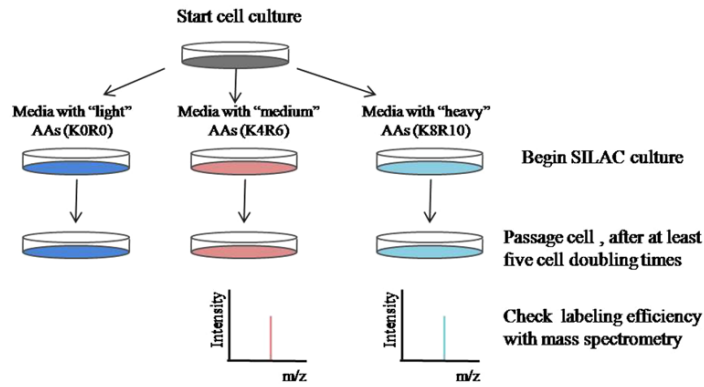
These lists of proteins and their quantitative changes are the basis for biological discovery (Choudhary & Mann, 2010).

SILAC-quantitative proteomics

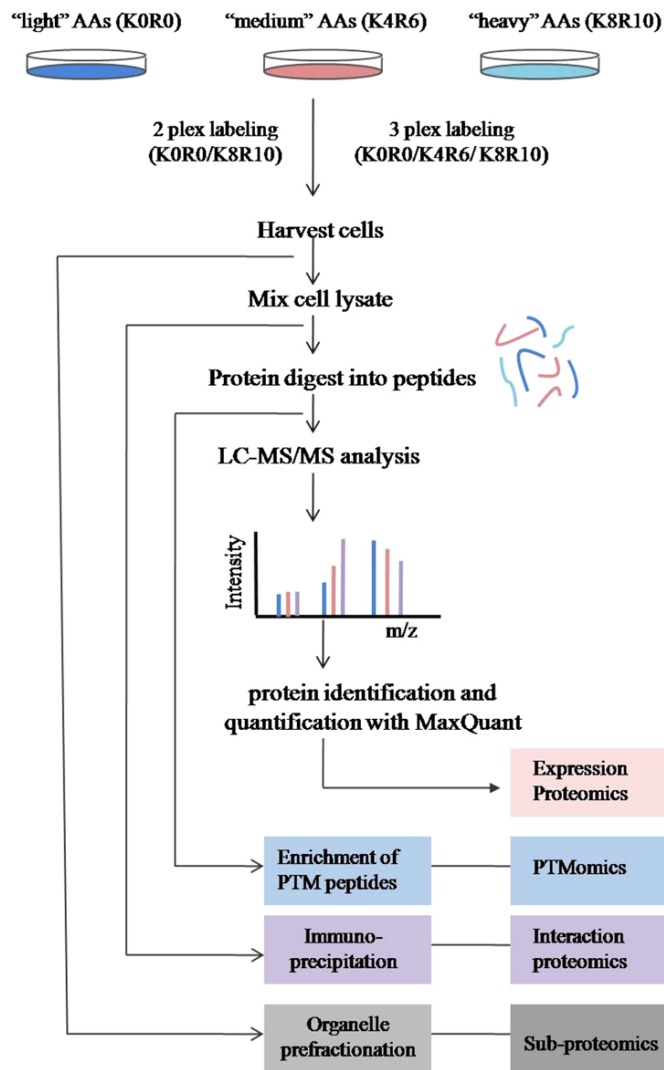
Proteome quantification has become the best strategy to understand post-translational modification dynamics. Accurate quantitative MS-based proteomics techniques are essential due to the inherent nonquantitative aspect of MS. The limitations of MS quantitation come from the several physicochemical properties of the proteolytic resulted peptides such as size, charge and hydrophobicity which give rise to variable mass spectrometric responses. Therefore, in MS it is not possible to compare the relative abundance changes from different experimental samples. This issue can be overcome by the introduction of stable isotopes into the proteins which reduce the variability from sample injection and ion fragmentation by mass spectrometers in different MS runs and hence, allows the quantification from different samples in the same MS analysis (X. Chen, Wei, Ji, Guo, & Yang, 2015). There several procedures for MS-based quantitative proteomics which can be grouped in three categories: (i) chemical/enzymatic labelling, (ii) metabolic labelling, and (iii) label-free. Chemical/enzymatic labelling methods includes isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tags (TMT), dimethyl-labeling, and ¹⁸O labelling whereas for the metabolic labelling procedures is used biological the incorporation of stable isotope labels such as SILAC (stable isotope labeling by amino acids in cell culture) and ¹⁵N labeling. In contrast, label-free methods compare samples by measuring the ion intensity changes or by spectrum counting which consist of counting the number of fragment spectra of the peptides identified of a particular protein (X. Chen et al., 2015; Hennrich & Gavin, 2015). The SILAC technique was first used in 2002 (S.-E. Ong et al., 2002) and, so far, this method posses the most advantages for quantitation over the other labeling approaches. SILAC can be used for study extensive sample processing such as subcellular fractionation, affinity purification or enrichment of peptides with PTMs. The major disadvantage of SILAC is that because of the limited labeling combinations available, few different cellular states can be compared.

Nevertheless, so far is the best method used for quantitative proteomics because its great compatibility, quantitative reliability, and practical implementation (S. E. Ong, 2012). The SILAC workflow can be divided in two phases: (i) the adaptations phase and, (ii) the experimental phase (see **Scheme 9**). As a general view, SILAC metabolically incorporates stable isotope labelled amino acids, such as ^{13}C or ^{15}N -labeled arginine or lysine, into the entire cells proteome. This incorporation is driven to the innate proteins metabolism happening during cell culturing. Thus, the basic incorporation method give rise to two populations, (i) the “light” medium containing amino acids with the natural isotop, and (ii) the “heavy” medium containing the isotope labelled amino acids. For a complete labeling (at least >95% labeling efficiency), at least five cell division cycles are needed for mammalian cells but this can vary within cell lines depending on the rate of protein synthesis, degradation, and turnover. Once cells are totally incorporated with the respective isotopes then cells are experimentally manipulated and mixed resulted protein extracts in equal amounts for further enzymatic digestion. The digested peptides are analysed with LC-MS/MS for protein quantification which is based on the ratio of “heavy” isotope-labeled peptides to the “light” unlabeled peptides. The intensities of the light and heavy signals enable the quantitative comparison of the proteins relative abundance in the proteome sample mixture (X. Chen et al., 2015; Mann, 2006)

A Adaptation phase



B experimental phase



Scheme 9. Workflow for quantitative proteomic experiments using SILAC

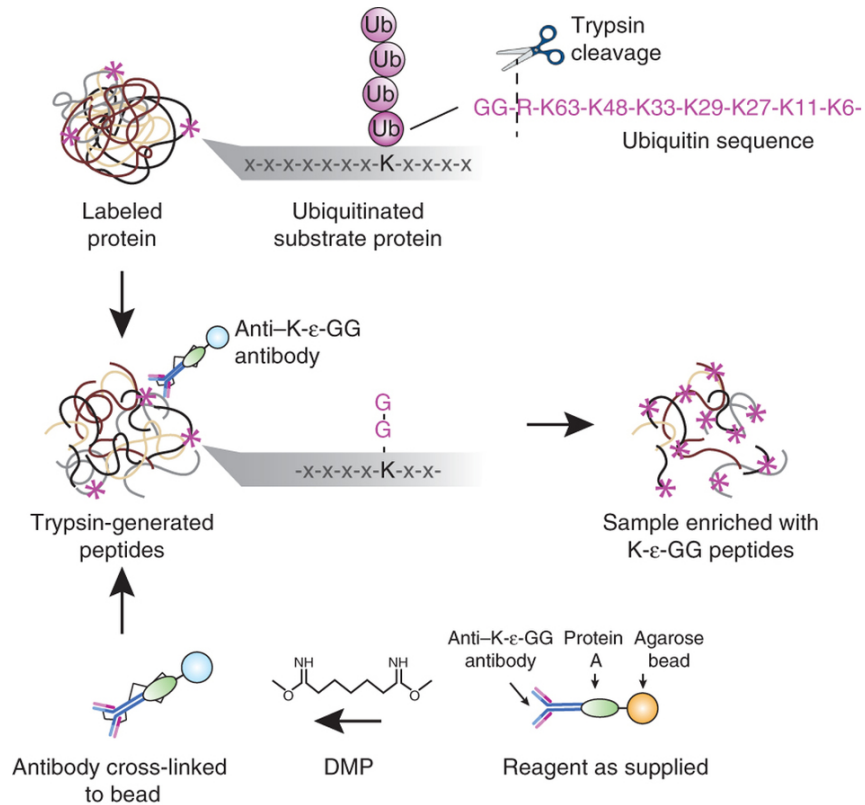
The SILAC experiment consists of two phases: an adaptation phase (A) and an experimental phase (B). (A) During the adaptation phase, cells are grown in light and heavy SILAC media for several cell divisions until full incorporation of the heavy amino acids in the growing cells. The degree of SILAC amino acids incorporation can be

evaluated by MS analysis. Depending on the study design, a triple strategy using light, medium, and heavy labeling can be used. (B) During the experimental phase, after the full incorporation of SILAC amino acids was confirmed, the cells populations are experimentally manipulated. Subsequently, the cells populations or protein lysates are mixed depending on the study. For sub-proteome analysis, cells populations are combined for organelle prefractionation; and for expression proteomics, interaction proteomics or PTMomics, the extracted protein lysates are mixed. After digestion of the SILAC-labeled proteins into peptides, peptides are then analyzed with LC-MS/MS. The identification and quantification of peptides is accomplished with quantitation software such as MaxQuant. In case of investigating protein-protein interaction, protein complexes are immuno-precipitated from the mixture of SILAC-labeled cell lysates. For PTMomics analysis, SILAC labeling peptides are subject to a fractionation and an enrichment step to improve identification of PTM-peptides (X. Chen et al., 2015).

Proteomics for ubiquitinated sites

The ubiquitin remnant profile is the most efficient proteomic strategy use for the large-scale identification of ubiquitination sites. Trypsin digestion is the proteolytic step typically used for the MS identification of ubiquitinated residues. This digestion is able to cleave all the ubiquitin molecule with an exception for the last two C-terminal glycine residues of ubiquitin which remains covalently linked to the lysine residue of the target protein. Therefore, this tryptic digestion releases a signature called the diglycine remnant or di-Gly motif. The diglycine remnant itself gives a peptide mass shift of ~114 Da which can also be detected in the MS/MS spectrum as a mass difference corresponding to the diglycine modification. Indeed, the first large-scale ubiquitination analysis was performed using this 114 Da mass shift approach and allowed the identification of around 100 ubiquitination sites in yeast (Peng et al., 2003). This finding give rise to the rapid improvement of the MS instrumentation by speed and sensitivity and therefore up to 1000 sites where achieved using this approach (Shi et al., 2011). The disadvantage of this strategy was that even with the enrichment of ubiquitinated proteins by using an epitope tagged ubiquitin reduces the complexity of the analyzed sample, the amount of peptides possessing the diglycine remnant was predominantly low. In consequence, the studies were mainly supported by the sensitivity and capability of the mass spectrometers. An important innovation of the ubiquitination proteomics was done in 2010 by Xu et al. during the

development of the monoclonal antibody GX41 that presented a great specificity for the diglycine adducts on the targeted lysines. Then, the workflow for the analysis consisted in the trypsin proteolysis of the sample, enrichment of the diglycine-modified peptides by using the specific diglycine lysine antibodies for the further analysis by LC-MS/MS (**Scheme 10**). This new enrichment advantage together with the advances in high resolution mass spectrometry allowed the first proteome-wide identification of thousands of endogenous ubiquitination sites in human cells (Kim et al., 2011; S. a. Wagner et al., 2011). Some of the limitations of the antibody enrichment is its low sensitivity for identified lower expressed modified proteins as is mainly identifying more abundant proteins. Moreover, antibodies itself also exhibits slight peptide sequence preferences and this can create a great variation between studies. In fact, this was the case for the analyses done by Kim et al, and Wagner et al. where even they identified more than 23, 000 ubiquitination sites by using the same diglycine antibody, only 4300 sites were common between experiments. Moreover, the two databases also presented different preferences for certain amino acids adjacent to the identified lysine site (Sylvestersen et al., 2013). Interestingly, this antibody-sequence preference observation was confirmed with a recent study that compared two different diglycine antibodies in murine tissues (Sebastian a. Wagner et al., 2012). Nevertheless, the novel techniques in the discovery of ubiquitination sites reveal its importance and demonstrated that ubiquitination is occurring abundantly in the cell in a similar way as the phosphorylation and acetylation.



Scheme 10. Enrichment of K-ε-GG peptides using anti-K-ε-GG antibody

After the antibody has been chemically cross-linked to a protein A bead using DMP, peptides are individually enriched for K-ε-GG peptides using the anti-K-ε-GG antibody, which recognizes the di-glycyl remnant remaining on modified lysine residues after trypsin digestion (Udeshi, Mertins, Svinkina, & Carr, 2013).

MATERIALS AND METHODS

Reagents

- gRNA-NEDP1-pCas9-GFP vector (Sigma-Aldrich)
- Lipofectamine RNAiMAX (Invitrogen)
- Lysyl endopeptidase (Endoproteinase Lys-C; Wako)
- MG132 (Viva Bioscience)
- MLN4924 (Millenium Pharmaceuticals - Takeda)
- Nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen)
- Protease Inhibitor Cocktail Tablets EDTA-free (Roche)
- PTMScan® ubiquitin branch motif (K-ε-GG) immunoaffinity beads (K-ε-GG antibody conjugated to protein A agarose beads; Cell Signaling Technology)
- Sequencing Grade Modified Trypsin (Promega)
- SILAC labelled amino acids (Euriso-Top)
- siRNA On-TARGETplus SMARTpools (Dharmacon)

Primary antibodies

Name	Species	Dilution	Source
NEDD8 (Y297)	Rabbit	1:150 (IF) 1:2000 (WB)	GeneTex
SUMO-2/3	Mouse	1:200 (IF) 1:2000 (WB)	In house
Ubiquitin (FK2)	Mouse	1:150 (IF)	Viva Bioscience
Ubiquitin	Rabbit	1:2000 (WB)	DAKO
GAPDH (C65)	Mouse	1:1000 (WB)	Abcam

H2A	Rabbit	1:1000 (WB)	Abcam
RPL7	Rabbit	1:2000 (WB)	Abcam
α -tubulin	Mouse	1:1000 (WB)	Cell Signaling
p53 (DO-1)	Mouse	1:1000 (WB)	In house
PCNA (PC10)	Mouse	1:1000 (WB)	Millipore
NEDP1	Sheep	1:1000 (WB)	In house
APPBP1	Mouse	1:1000 (WB)	Abnova
H2B	Rabbit	1:1000 (WB)	Millipore

Cell culture

With the exception of H1299 lung carcinoma cells cultured in RPMI medium the rest of cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and standard antibiotics (Penicillin, 50 U/ml and Streptomycin 50 μ g/ml) in 5% CO₂ at 37°C in a humidified incubator. Cells stably expressing 6His-NEDD8^{WT} or 6His-NEDD8^{R74K} were cultured in the presence of 2.5 μ M puromycin for selection.

CRISPR/CAS9

U2OS and HCT116 cell lines were transfected with 6 μ g of gRNA-NEDP1-pCas9-GFP vector (Sigma-Aldrich), respectively. 24 hrs transfection cells were subjected to fluorescence-activated cell sorting (FACS) to isolate cell populations with significantly increased frequencies of Cas9-induced modifications (sorted into fractions with low, medium, and high GFP expression levels). Cell clones were tested for NEDP1 depletion by immunoblot analysis. The best NEDP1 KO clones were selected for the experiments.

Generation of stable cell lines by lentivirus based approach

6His-NEDD8^{WT} and 6His-NEDD8^{R74K} respectively were cloned into lentivirus vector. 293T cells were seeded in 10cm cell-culture dishes at 5×10^6 cells per plate in DMEM with 10% FBS. Next day, 1.5 μ g of transfer plasmid (lentiviral 6His-NEDD8^{WT} or 6His-NEDD8^{R74K}) was mixed with 1 μ g of packaging construct (pCMV) and 1 μ g of envelope plasmid (pMDG). Transfections were performed using Fugene6 HD (Roche) transfection reagent according to the manufacturer's instructions. Medium was replaced with DMEM with 10% FBS the next day. Supernatant was harvested 2 and 3 days post-transfection, filtered through a 0.45 μ m pore-size filter (Sartorius, Minisart), and stored at -80°C.

For generating stable cell lines, HCT116 and U2OS cells, respectively, were seeded in 6-well plate at 40×10^4 cells per well in DMEM with 10% FBS. 200 μ l of the lentiviral supernatant was added to the cells in the presence of 10 μ g/ml polybrene. Medium was replaced with DMEM with 10% FBS the next day. 3 days post-infection, puromycin was added to the medium at 5 μ g/ml. The stable cell line was maintained in the medium with 2.5 μ g/ml of puromycin and the expression of 6His-NEDD8 was tested monthly.

Transfections

Cells were seeded in 6-well plates or 10cm dishes to the desired confluency. 5nM of siRNA was transfected with Lipofectamine RNAiMAX according to manufacturer's instructions. Non-target siRNA was used in control transfections. For the SILAC experiment transfections were performed in 6-well plates and 6hrs later cells were combined and reseeded in 10cm dishes. Cells were harvested 48hrs post-transfection.

Subcellular fractionation

After the appropriate treatment, cells in 10cm culture dish were washed twice with ice cold PBS, then scrapped into 1ml PBS. 100 μ l of cells were pelleted at

13000rpm for 1min and lysed with the appropriate volume of 2xSDS loading buffer. The remaining 900µl of cells were pelleted at 10800rpm for 20s. Cell pellet was resuspended in 300µl buffer A (10mM HEPES-KOH pH 8.0, 10mM KCl, 1.5mM MgCl₂) with protease inhibitor and 10mM iodoacetamide. Cells were lysed by adding Triton-X100 at a final concentration of 0.1% for 1min at 4°C, then spun for 5min at 1300g 4°C. The supernatant (cytoplasmic fraction) was mixed with equal volume of 2xSDS. The pellet (nuclear fraction) was washed 3 times with buffer A, then resuspended with 300µl of buffer B (20mM HEPES-KOH pH 8.0, 300mM NaCl, 2mM EDTA and 1%NP40) and incubated 30min on ice. Lysates were sonicated on ice, 8x30s with 50% amplitude (Branson Digital Sonifier) and centrifuged at 13000 rpm for 15min. The supernatant (nucleoplasmic fraction) was mixed with equal volume of 2xSDS, and the pellet (pellet fraction) was washed 3 times with buffer B then resuspended in 2xSDS.

BCA protein assay

Protein concentration was measured by BCA Protein Assay (Thermo scientific). Reagent A and reagent B were mixed in 50:1 volume ratio. 8µl of each sample/or the lysis buffer (Blank) were added to 1ml of the BCA mix and incubated at 60°C in a water bath until the color became light purple (30min). Tubes were left to cool down back to room temperature and the absorbance was measured by spectrophotometer at OD562. The protein concentration was determined from a standard curve using BSA.

Western blot analysis

Proteins were resolved in sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis (SDS-PAGE) gels and transferred onto PVDF membrane using the Bio-Rad Mini Trans-Blot apparatus. Membranes were blocked in 5% milk solution (PBS with 0.1% Tween-20 and 5% skimmed milk) for 1hr at room temperature with gentle agitation. Membranes were incubated with the primary antibodies overnight at 4°C. Primary antibodies were diluted in TBS 0.1% Tween-20 with 3% BSA and 0.1% NaN₃. Membranes were washed 3x10min with PBS 0.1% Tween-20 and then incubated with the appropriate secondary antibody

(Sigma Aldrich) for 1hr at room temperature (5% milk). After incubation, membranes were washed 2x15min with PBS 0.1% Tween-20 followed by 2x5min with PBS. Detection was performed with ECL Western Blotting Detection Reagents and membranes were exposed to X-ray Medical Film before being developed.

Synchronization and cell cycle analysis

Cells were pre-treated with 500nM MLN4924 for 15 hrs or arrested in G1/S phase prior to ultraviolet irradiation at the indicated intensity. For synchronization, cells were treated with 2.5 mM thymidine (Sigma) for 18 h, released from the G1/S block into fresh supplemented DMEM for 8 h and then treated again with 2.5 mM thymidine for 18 h. After release from the second block, cells were exposed to UV damage. Fluorescence Activated Cell Sorting (FACS) was used to analyse the cell-cycle distribution at the indicated times after UV release. Cells were harvested in PBS, fixed in 70% ethanol and pelleted before resuspended in 1ml PBS containing RNase A (10µg/ml, Invitrogen) propidium iodide (20µg/ml, Sigma, P4170) for 30min. incubation at room temperature. Samples were analysed using a Becton Dickinson FACScan operated by the CELLQuest software.

Immunofluorescence microscopy

Cells were seeded on round coverslips 24hrs before treatment. After the HS/MG132 treatment, cells were washed twice with warm PBS and fixed with 4% formaldehyde for 10min. Cells were washed three times (5min) with warm PBS, and permeabilized with 1% Triton X-100 in PBS for 10min. Cells were washed 3x10min with PBS before blocking with 0.05% Tween-20 + 1% Goat serum (from where) in PBS for 1hr. After blockage, cells were incubated with the appropriate primary antibody diluted in 0.05% tween-20 + 1% Goat serum in PBS for 1hr at room temperature (or overnight at 4°C). After 3x10min washes with 0.05% Tween-20 in PBS, cells were incubated with corresponding secondary antibodies diluted in 0.05% Tween-20 + 1% Goat serum in PBS for 1hr at room temperature in the dark. Samples were washed 3x10min with 0.05% tween-20 in PBS, and

then stained with DAPI (1/20000) for 20s at room temperature in the dark. Slides were washed 3x with PBS, mounted with Vectashield Mounting Medium (H-1000, Vector), sealed, and viewed under the microscope Leica DM6000 or Leica SP5-SMD using metamorph software. The images were analyzed by ImageJ64 software.

Ni-NTA pull-down

After the indicated treatment, cells were washed twice with ice cold PBS and scraped in 1ml PBS. 200µl of each sample were pelleted at 14000rpm for 1min and lysed with 2xSDS. The 800µl left were lysed in 6ml 6M GmCl lysis buffer [6 M Guanidinium-HCl, 10mM Tris-HCl (pH 8), 0.1M Na₂HPO₄/NaH₂PO₄, pH 8] supplemented with 10mM Imidazole and 10mM β-mercaptoethanol. 60µl of Ni-NTA-agarose beads were added to the lysates and incubated overnight at 4°C. Beads were pelleted by centrifuging at 2000rpm-5min at 4 C then were washed once with 750 µl of 6M GmCl lysis buffer, twice with 8M Urea pH 8 [8M Urea, 100 mM Na₂HPO₄/NaH₂PO₄ (pH 8), 10 mM Tris-HCl (pH 8)] and three times with 8M Urea pH 6.3 [8M Urea, 100 mM Na₂HPO₄/NaH₂PO₄ (pH 6.3), 10 mM Tris-HCl (pH 6.8)], all wash buffers were supplemented with 0.1% Triton X-100 and 10mM β-mercaptoethanol. For each wash, beads were spun at 5000rpm-1min. After the last wash, 100µl of elution buffer (2xSDS with 250mM Imidazole) was added, samples were heated at 95°C for 2min, incubated for 20min at room temperature and spun at 14000rpm for 10min. Supernatants were collected and analyzed by western blot.

Ni-NTA pull-down from pellet fraction

Cells stably expressing 6His-NEDD8 were subjected to a subcellular fractionation after the appropriate treatment as previously described. Pellet fractions were washed 3 times with the buffer B and then resuspended in 1ml of 8M Urea, 100 mM Na₂HPO₄/NaH₂PO₄ (pH 8), 10 mM Tris-HCl (pH 8) and sonicated 4×30s at 30% amplitude (Branson Digital Sonifier). Insoluble particles were eliminated by centrifugation at 14000rpm-10min at 4°C. 100µl of each collected supernatant were mixed with equal volume of 2xSDS loading buffer. The rest was diluted up

to 6ml in the 8M Urea pH 8 buffer with 10mM Imidazole and 10mM β -mercaptoethanol. The purification was then performed using 60 μ l of Ni-NTA agarose beads as previously described.

Ni-NTA pull-down for mass spectrometry analysis

After the indicated treatment, cells stably expressing 6His-NEDD8 or 6His-NEDD8^{R74K} were washed twice with ice cold PBS and scraped directly in 6M GmCl lysis buffer. DNA was disrupted by sonication 10x30s at 41% amplitude (Branson Digital Sonifier). Insoluble particles were removed by centrifugation at 15000rcf for 1h at 15°C. Protein extract was filtered with 0.2- μ m sterile filters and protein concentration was determined by BCA assay. Pre-washed Ni-NTA agarose beads were added to the lysate previously supplemented with 20mM Imidazole and 10mM β -mercaptoethanol for incubation at 4°C for 16hrs. Beads were washed once with 1 ml of 6M GmCl lysis buffer, twice with 8M Urea pH 8, three times with 8M Urea pH 6.3 and three times with 8M Urea pH 8. All buffers were supplemented with 20mM Imidazole and 10mM β -mercaptoethanol. Proteins were eluted in three sequential steps in elution buffer pH 8 [8M Urea, 100 mM Na₂HPO₄/NaH₂PO₄ (pH 8), 10 mM Tris-HCl (pH 8), 250mM Imidazole and 10mM β -mercaptoethanol]. For each elutions the beads were pre-incubated 10min in elution buffer. Proteins from lysate were submitted to 3 successive purifications/elutions process by adding new pre-washed beads. The protein concentration was determined by BCA assay. A fraction of the elutions were analyzed by western blotting. Selected elutions were subjected to in-solution digestion with the indicated endoproteinases. Peptides were desalted prior to mass spectrometry analysis.

In-solution digestion and desalting of the peptides

Proteins in 8M urea were supplemented with ammonium bicarbonate to 50mM. Reduction and alkylation were performed with 5mM DTT (45 min, RT) and 55mM iodoacetamide (20 min, RT in the dark), respectively. Samples were then diluted to 6M Urea concentration using 50mM ammonium bicarbonate. 1:50 (wt/wt) ratio

of Lys-C was added to the samples for digestion overnight at 25°C. Subsequently, resulted samples were diluted to 2M Urea concentration with 50mM ammonium bicarbonate. Digestion with trypsin (1:50) was performed overnight, at 25°C, still and in the dark. In-solution digested peptides were desalted using OMIX C18 reversed chromatography StageTips (Agilent) following manufacture's instructions. StageTips were kept at 4°C before eluted for mass spectrometry analysis.

SILAC-mass spectrometry analysis

Cells were labeled in SILAC DMEM either with light (Lys0/Arg0) or heavy (Lys8/Arg10) amino acids supplemented with dialyzed FBS and standard antibiotics. In the experiments for 6His-NEDD8 conjugates analysis, cells grown in light medium were left at 37°C whereas cells in heavy medium were heat-shocked at 43°C for 1h or pre-NEDP1 knockdowned by CRISPR/Cas9 approach. Respective lysates were mixed 1:1 prior to Ni-NTA purification appropriate for MS analysis as previously described.

For pellet fraction analysis, cells grown in light medium were left at 37°C whereas cells in heavy medium were heat-shocked at 43°C for 2hrs. In the experiment of NEDD8 knockdown and MLN4924 treatment, control (light) and siNEDD8 transfected cells (heavy) or control siRNA transfected (light) and MLN4924 treated cells (heavy) were all heat-shocked at 43°C for 2hrs. For each condition, cells were collected in PBS, counted and mixed 1:1. Cell fractionation was followed and the pellet was resuspended in 2xSDS sample buffer. 50µg of protein were run for 15min on 4-12% precast NuPAGE and coomassie blue stained. Lanes were cut in 2 gel pieces and in gel trypsin digestion was performed as described in (Sun et al., 2006).

Peptides were analyzed online by nano-flow HPLC-nanoelectrospray ionization using a Qexactive Plus mass spectrometer (Thermo Fisher Scientific) coupled to a nano- LC system (U3000-RSLC, Thermo Fisher Scientific). Desalting and preconcentration of samples were performed on-line on a Pepmap® precolumn (0.3 × 10 mm; Dionex). A gradient consisting of 0–40% B in A for 140 min (A:

0.1% formic acid, 2% acetonitrile in water, and B: 0.1% formic acid in acetonitrile) at 300 nl/min, was used to elute peptides from the capillary reverse-phase column (0.075 × 150 mm, Pepmap®, Dionex). Data were acquired using the Xcalibur software (version 4.0). A cycle of one full-scan mass spectrum (375–1,500 m/z) at a resolution of 70,000 (at 200 m/z), followed by 12 data-dependent MS/MS spectra (at a resolution of 17,500, isolation window 1.2 m/z) was repeated continuously throughout the nanoLC separation. Raw data analysis was performed using the MaxQuant software (version 1.5.5.1) with standard settings. Used database consist of Humanentries from Uniprot (reference proteome UniProt 2017_03) and 250 contaminants (MaxQuant contaminant database). Relative proteins quantifications were calculated on the median SILAC ratios.

Sample preparation and mass spectrometry analysis (IMB, Germany)

HCT116 NEDP1 depleted cells stably expressing 6His-NEDD8^{R74K} were washed with PBS and lysed in modified RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% Na-deoxycholate and supplemented with protease inhibitors] then incubated for 15 min on ice. Lysate was sonicated for 3x30s at 30% amplitude (Branson Digital Sonifier) and cleared by centrifugation at 13,000rpm for 15 min. Supernatants were combine and measured for protein concentration using BCA assay. Around 200 mg of proteins were precipitated overnight in 4xVol cold acetone at -20°C. Precipitated proteins were re-dissolved in denaturing buffer [6M Urea and 2M thiourea in 10mM HEPES at pH 8]. Cysteines were reduced with 1 mM dithiothreitol for 45 min and alkylated with 5.5 mM chloroacetamide for 30 min in the dark. Proteins were subsequently proteolysed in 1:100 and 1:500 with endoproteinase Lys-C (Wako Chemicals) overnight. Peptides were purified using reversed-phase Sep-Pak C18 cartridges (Waters), and dissolved in immunoprecipitation buffer [10 mM sodium phosphate and 50 mM sodium chloride in 50 mM MOPS at pH 7.2]. Di-glycine-modified peptides were immuno-enriched using PTMScan® Ubiquitin Branch Motif (K-ε-GG) beads. Peptides were incubated for 4 h at 4°C on a rotation wheel. The beads were washed three times in ice-cold immunoprecipitation buffer followed by three washes in water. Immunoenriched peptides were eluted with 0.15%

trifluoroacetic acid in H₂O, fractionated in three or six fractions using micro-column-based strong-cation exchange chromatography (SCX) and desalted on reversed phase C18 StageTips as described previously (Rappsilber, Mann, & Ishihama, 2007).

Peptide fractions were analyzed on a quadrupole Orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific) equipped with a UHPLC system (EASY-nLC 1000, Thermo Scientific). Peptide samples were loaded onto C18 reversed phase columns (15 cm length, 75 µm inner diameter, 1.9 µm bead size) and eluted with a linear gradient from 8 to 40% acetonitrile containing 0.1% formic acid in 2 hours. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS₂ acquisition. Survey full scan MS spectra (m/z 300 – 1700) were acquired in the Orbitrap. The 10 most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation (HCD). An ion selection threshold of 5,000 was used. Peptides with unassigned charge states, as well as with charge states less than +2 were excluded from fragmentation. Fragment spectra were acquired in the Orbitrap mass analyzer.

Raw data files were analyzed using MaxQuant (development version 1.5.2.8). Parent ion and MS₂ spectra were searched against a database containing 88,473 human protein sequences human protein sequences obtained from the UniProtKB released in December 2013 using Andromeda search engine. Spectra were searched with a mass tolerance of 6 ppm in MS mode, 20 ppm in HCD MS₂ mode, strict trypsin specificity and allowing up to 3 miscleavages. Cysteine carbamidomethylation was searched as a fixed modification, whereas protein N-terminal acetylation, methionine oxidation modification of cysteines (mass difference to cysteine carbamidomethylation) and di-glycine-lysine were searched as variable modifications. Site localization probabilities were determined by MaxQuant using the PTM scoring algorithm. The dataset was filtered based on posterior error probability (PEP) to arrive at a false discovery rate of below 1% estimated using a target-decoy approach. Di-glycine lysine modified peptides with a minimum score of 40 and delta score of 6 are reported and used for the analyses.

Bioinformatic analysis

For consensus sequence analysis, identified sites for NEDDylation with localization probability above 0.9 were analyzed. pLogo software (O'Shea et al., 2013) was used to overlay sequence windows to generate a consensus sequence. IceLogo software (Colaert, Helsens, Martens, Vandekerckhove, & Gevaert, 2009) was used to generate Heatmaps in a similar manner as in pLogo sequence generation. All amino acids shown as enriched or depleted are significant with $P < 0.05$.

STRING network analysis was performed using the online STRING database (Szklarczyk et al., 2015), using NEDDylated proteins as input. Protein interaction enrichment was performed based on the amount of interactions in the networks, as compared with the randomly expected amount of interactions, with both variables directly derived from the STRING database output. Visualization of the interaction network was performed using Cytoscape version 3.4.0 (Shannon et al., 2003).

For NEDDylation, SUMOylation and ubiquitination site comparative overlap analysis, all NEDDylation sites identified for NEDP1 KO cells in IMB were analyzed. All MS/MS-identified ubiquitination sites and SUMOylation sites were extracted from PhosphoSitePlus (www.phosphosite.org) (Hornbeck et al., 2012). For each data set, sites from mouse proteins were removed. Perseus software was used to generate a matrix where all proteins from all PTMs were cross-referenced to each other.

CHAPTER I

DECODING THE ATYPICAL AND CANONICAL NEDD8 PROTEOME

BACKGROUND

UBLs share structure and sequence similarity to ubiquitin. As with ubiquitin conjugation cascade, specific E1, E2, and E3 enzymes ensure the covalent link of each UBL to the lysine residue of substrates (Hochstrasser, 2009; van der Veen & Ploegh, 2012). Among all UBLs identified so far, NEDD8 is the most highly related to ubiquitin at the sequence and secondary structure levels with 60 % identity and 80% homology, respectively (T. K. Kamitani et al., 1997). As well as between NEDD8 and ubiquitin itself, the E1 enzymes of NEDD8 (NAE) and ubiquitin (UBE1) share high extent of homology. Nevertheless, critical residues, along with structural, biophysical and thermodynamic properties, ensures the respective E1 enzymes discriminate between NEDD8 and ubiquitin for their specific modifications (Bohnsack & Haas, 2003; Burch & Haas, 1994; Kitahara et al., 2006; Souphron et al., 2008). Despite these features, it seems that the specificity between NEDDylation and ubiquitination pathways only occurs during cellular homeostasis. Recent studies have revealed that under cellular stress conditions, such as proteasome inhibition, heat shock or oxidative stress, global protein NEDDylation increase due to the activation by the ubiquitin enzyme UBE1 rather by the NEDD8 enzyme NAE (Hjerpe, Thomas, & Kurz, 2012; Leidecker, Matic, Mahata, Pion, & Xirodimas, 2012). Interestingly, this response of NEDD8 conjugation by the ubiquitin pathway leads to the formation of poly-NEDD8 chains and new incorporation of mixed NEDD8-ubiquitin chains into the "ubiquitin code" (Kim et al., 2011; Leidecker et al., 2012; Singh et al., 2012) (see **Figure 1**). The NEDD8 response to stress is reversible and cell recovery is accomplished once stress is alleviated (Leidecker et al., 2012). In principle, UBE1 has less affinity to activate NEDD8. Thus, part of the mechanisms that induce the "atypical

NEDDylation" by UBE1 is when the NEDD8: ubiquitin balance is affected either upon depletion of free ubiquitin or by the artificial increase in the levels of NEDD8 through overexpression (Hjerpe, Thomas, Chen, et al., 2012; Leidecker et al., 2012; Whitby et al., 1998). Remains an open question whether the NEDD8 conjugation through the ubiquitination machinery and subsequent formation of hybrid NEDD8-ubiquitin chains has a physiological outcome. This crosstalk could represent either a stress signal that is detected by specific cellular factors, an alteration of the recognition of typical ubiquitin chains, or NEDD8 only replaces ubiquitin when free ubiquitin levels fall. (Abidi & Xirodimas, 2015; Lane, 2012; Singh et al., 2012; Singh, Sundar, & Fushman, 2014). Although under homeostasis the concentrations of free NEDD8 and ubiquitin are almost equal, is evident the increase of NEDDylation levels observed in many types of cancers, cancer-derived cell lines, and certain cell types. (Enchev et al., 2014; Hjerpe, Thomas, Chen, et al., 2012). Unquestionably, the study of the role of atypical NEDDylation and crosstalk between NEDD8 and ubiquitin pathways is critical to clarify what are the biological functions or repercussion within the cells.

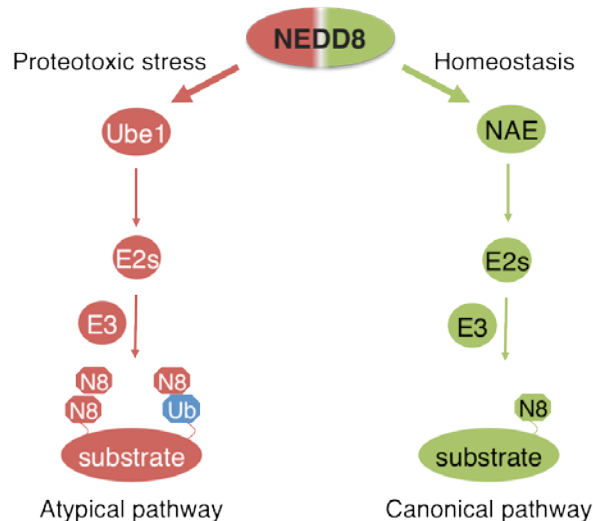


Figure 1. Modes of NEDD8 conjugation

Activation of NEDD8 by NEDD8-activating enzyme (NAE) defines the canonical NEDDylation pathway under homeostatic conditions. Proteotoxic stress causes an increase in protein NEDDylation that depends on the activation of NEDD8 by the ubiquitin E1 enzyme UBE1. This leads to the formation of poly-NEDD8 and/or hybrid NEDD8–ubiquitin chains on target proteins (Abidi & Xirodimas, 2015).

The function of NEDD8 in homeostasis involves mainly the regulation of Cullin-RING ligases (CRLs) through the modification of cullins. The family of cullin proteins is the best-characterized target for NEDD8. Cullins are scaffold proteins which under NEDDylation enhance the ubiquitination activity of the CRLs complex and, therefore, regulates the degradation of substrates by the ubiquitin-proteasome system (UPS) (reviewed in Abidi & Xirodimas 2015). Besides cullins, recently additional relevant targets for NEDDylation have been identified such as p53, ribosomal proteins, and histones. These non-cullin substrates are involved in critical biological processes including cell cycle regulation, transcription, protein synthesis, DNA damage response, and apoptosis. (Abidi & Xirodimas, 2015; Rabut & Peter, 2008; M. Wang et al., 2011; Watson, Irwin, & Ohh, 2011; D. Xirodimas, 2008). Despite the obvious importance of NEDDylation, there is still a limited knowledge on its substrates. NEDDylation is a reversible process. Two main enzymes have shown great specificity for NEDD8 deconjugation: the multi-component COP9 signalosome and the protease NEDP1 (also called DEN1 or SEPN8). The COP9 signalosome (CSN) complex is a zinc metalloprotease, which has low affinity for NEDD8 itself but more specifically promotes deNEDDylation of cullins (Biol et al., 2014; Enchev et al., 2012; Lingaraju et al., 2014; Lyapina et al., 2001; C. Zhou et al., 2001). In contrast, NEDP1 is an NEDD8-specific protease that binds selectively to NEDD8, and not only can deconjugate NEDD8 from substrates but also catalyzes the processing of precursor NEDD8 to expose the C-terminal di-glycine motif necessary for its further activation (Gan-Erdene et al., 2003; Mendoza et al., 2003; Rabut & Peter, 2008; Shen et al., 2005; Wu et al., 2003; D. Xirodimas, 2008). Compared with the CSN complex, NEDP1 is inefficient at deNEDDylating mono-NEDDylated cullins, however, is more effective in deNEDDylating poly-NEDDylated cullins (Wu et al., 2003). Interestingly, the depletion of NEDP1 orthologs in *S. pombe* and *D. melanogaster* did not result in accumulation of NEDDylated cullins but the accumulation of new potential NEDDylated substrates. These studies suggested the great possible role of NEDP1 in the regulation of non-cullin substrates (Chan et al., 2008; Watson et al., 2011; L. Zhou & Watts, 2005). It is apparent that as for atypical NEDDylation, the role of the canonical NEDD8 pathway beyond cullins remains unclear. To elucidate the different biological outcomes of protein NEDDylation under homeostasis or stress status, in this work we developed

approaches that differentiate and determine the NEDD8 proteome from the canonical and atypical pathways through mass spectrometry analysis.

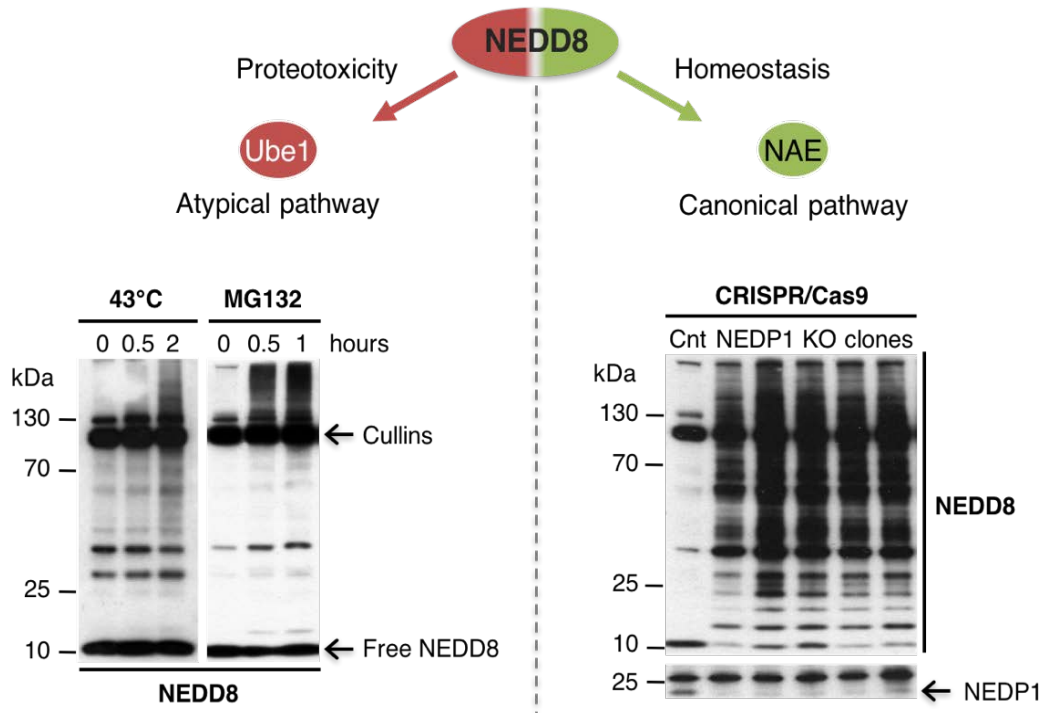
RESULTS

Mechanisms that differentiate between atypical and canonical NEDDylation

NEDD8 protein conjugation can be triggered by two distinct pathways depending on the cellular status. It is well established that heat shock (HS), proteasome inhibition, and oxidative stress are some of the proteotoxic stimuli that induce global protein NEDDylation by the atypical Ube1 activation. In contrast, NEDDylation through the canonical NAE activation occurs under homeostatic conditions by mainly modifying cullins for the regulation of CRLs. The role of NEDD8 upon proteotoxicity remains unclear and, still little is known about the non-cullin substrates of NEDD8 through the canonical pathway. The large-scale identification of the NEDDylome is challenging due to the low intrinsic expression of NEDD8 in homeostasis and, due to the consequence of exogenous NEDD8 overexpression to perturb the NEDD8: ubiquitin balance. Therefore, special care is needed in order to characterize and distinguish between both possible NEDDylation pathways. In this work, we determined different mechanisms to differentiate both ways of NEDD8 activation. Supported by the notion that the de-NEDDylating enzyme NEDP1 essentially regulates non-cullin substrates, here we established NEDP1 knockout (NEDP1 KO) cell lines using the recent CRISPR/Cas9 system (Ran et al., 2013). As expected, the depletion causes a dramatic increase on protein NEDDylation and, consequently, enables the identification of potential canonical NEDD8 substrates besides cullins. Therefore, while heat-shocked at 43°C or treated cells with the proteasomal inhibitor MG132 causes a progressive accumulation of atypical NEDDylated substrates, the NEDP1 depleted cells accumulate canonical NEDD8 substrates (**Figure 2A**). In addition, to avoid NEDD8: ubiquitin imbalance in the cell, we generated stable cell lines expressing polyhistidine (6His)-tagged NEDD8 (6His-NEDD8) using a lentivirus based approach that provides low-level expression of the transgene similar to endogenous NEDD8 levels (Dull et al., 1998; Naldini et al., 1996).

Consequently, stable cell lines of the transgene guaranty reliable NEDDylation response. Then, specific 6His-NEDD8 conjugates can be highly enriched by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography for further analysis (Figure 2B).

A



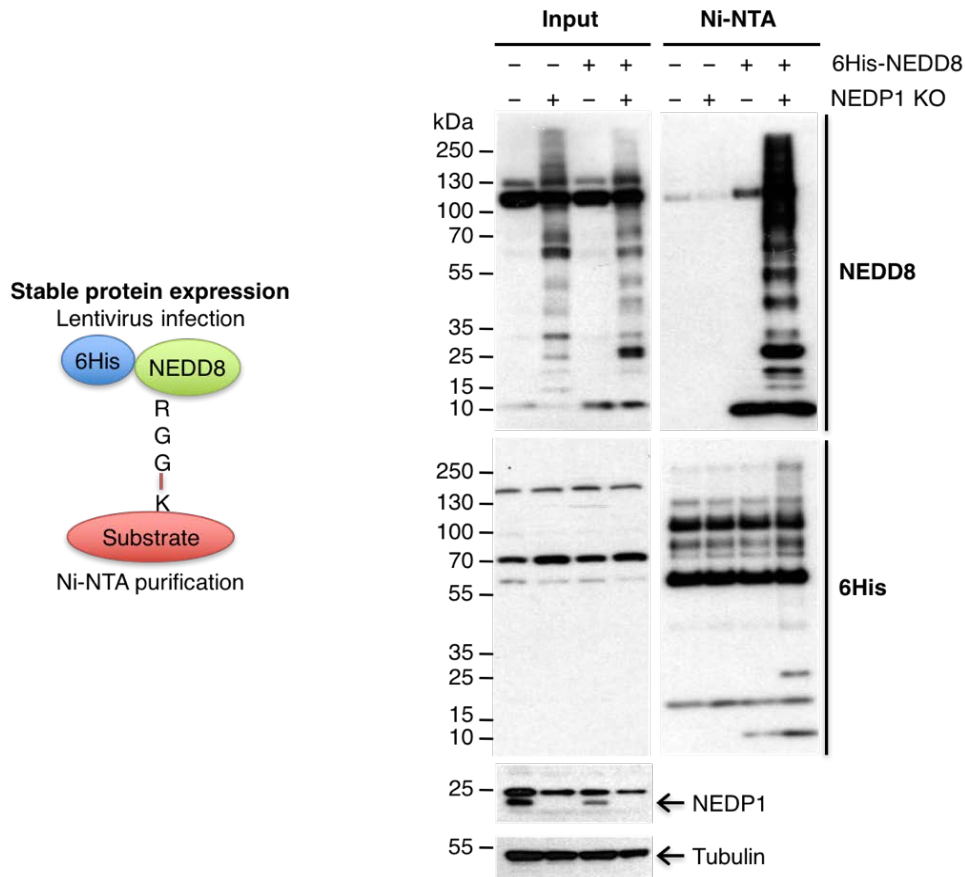
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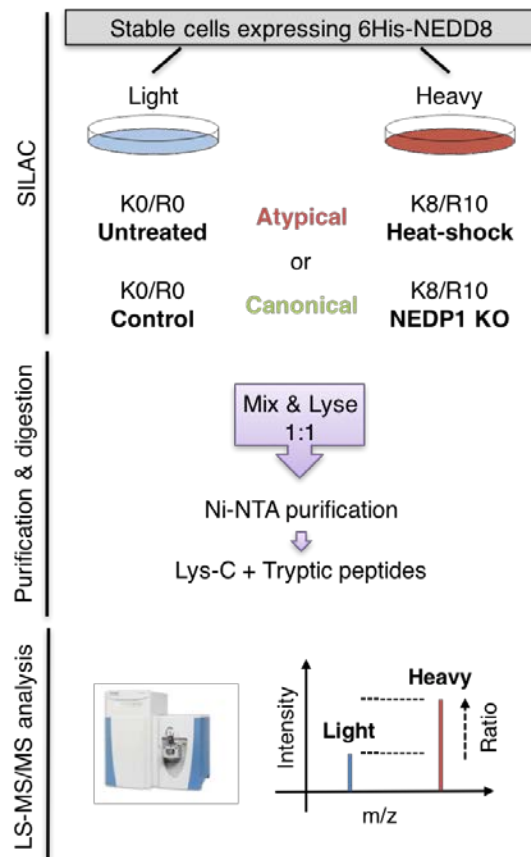
Figure 2. Approaches to distinguish atypical and canonical NEDD8 proteome by MS analysis

(A) Overview of the mechanisms that potentially discriminates between the atypical and canonical NEDD8 proteome. Either progressive heat-shock at 43°C or treatment with MG132 (30 μM) in the indicated times causes proteotoxicity which triggers an increase of atypical NEDDylation through Ube1 activation. Instead, NEDP1 depleted clones (NEDP1 KO), resulted from a CRISPR/Cas9 strategy, causes an accumulation of canonical NEDD8 substrates through NAE activation. Western blot analysis showed for NEDD8 and NEDP1 antibodies respectively. **(B)** Left: schematic representation of a 6His-NEDD8 target purified by Ni-NTA pulldown from lentiviral infected cells. Right: Control or NEDP1 depleted HCT116 cells were either infected or not with lentivirus for stable expressing 6His-NEDD8 construct. NEDD8 conjugates were enriched using Ni-NTA chromatography. Immunoblotting analysis from total cell lysates and Ni-NTA elution samples confirmed the low-level expression of 6His-NEDD8 and showed high-specificity enrichment of their conjugates.

Having established the mechanisms to differentiate between the two types of NEDDylation, furthermore, we applied the SILAC strategy in combination with mass spectrometry to study the dynamics of NEDD8 modification under the atypical and canonical pathway. HCT116 stable cell line expressing 6His-NEDD8

were metabolically labeled with “light” lysine/arginine (K0/R0) for control conditions and with “heavy” (K8/R10; + 18 Da shift) for heat shock (43°C, 1h) stress stimuli or for NEDP1 KO status, respectively. Subsequently, the respective “light” and “heavy” cell lysates were mixed in equal amount (1:1). 6His-NEDD8 conjugated proteins were enriched by Ni-NTA affinity under denaturing conditions by three successive purification/elution processes (**Figure 3A**). Selected elution fractions with higher NEDDylation patterns were pulled together, either two samples from heat shocked cells (E1s of second and third bindings) and three samples from NEDP1 depleted cells (E1 of first binding with E2s of second and third bindings) respectively. (**Figure 3B**) Lys-C and trypsin endoproteases were used for protein digestion followed by peptide purification using OMIX C18 reversed chromatography for the final mass spectrometry analysis (LC-MS/MS) (**Figure 3A**).

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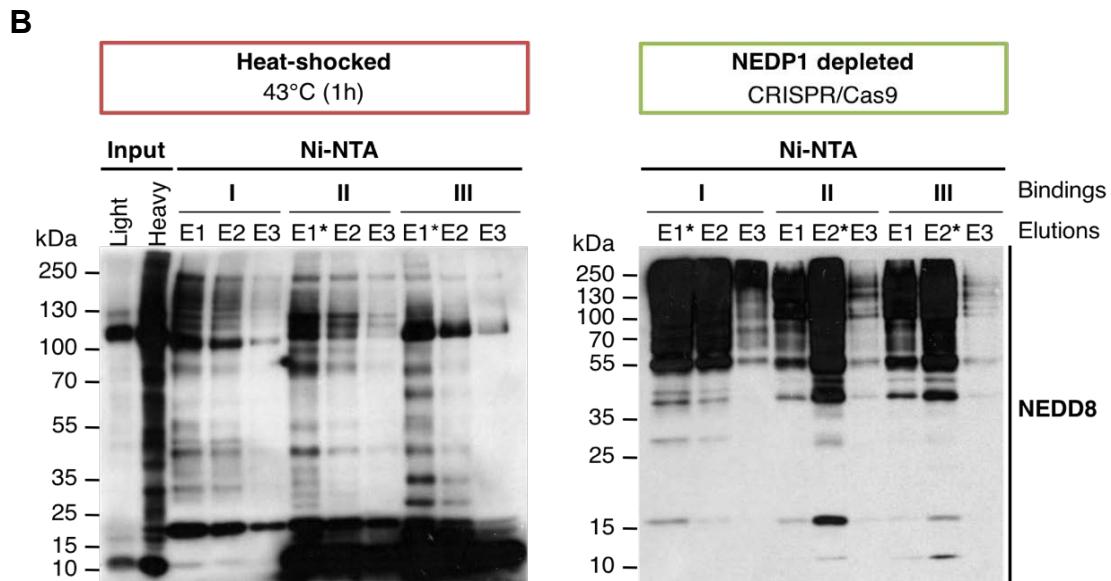


Figure 3. SILAC based quantitative proteomics to monitor atypical and canonical NEDDylation pathway

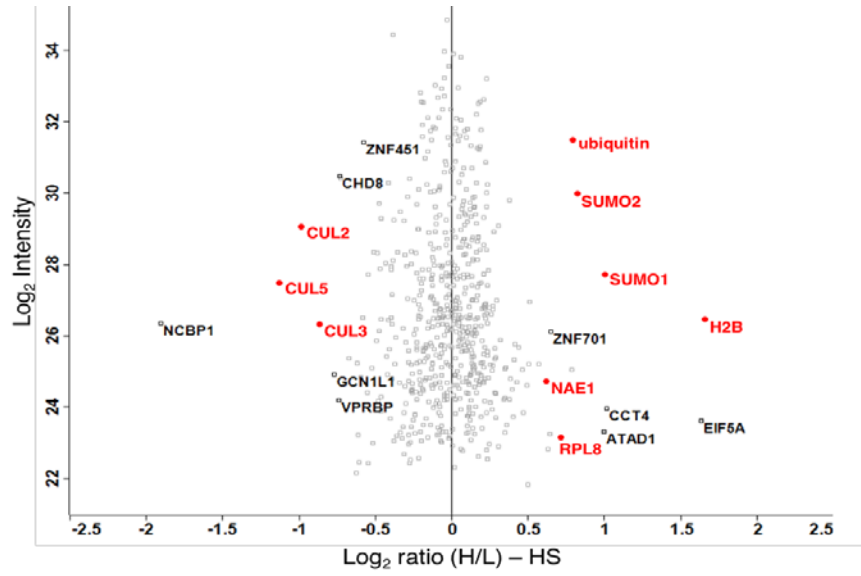
(A) Overview of the workflow strategy used to characterize the NEDDylation dynamic upon the atypical and canonical pathway, respectively. (B) Western blot analysis from elution samples after consecutive Ni-NTA enrichments of the respective heat-shocked or NEDP1 depleted 6His-NEDD8 conjugates previously labeled and mixed. (*) Chosen elution fractions for MS analysis.

NEDDylation of ubiquitin and SUMO as part of the proteotoxic stress response

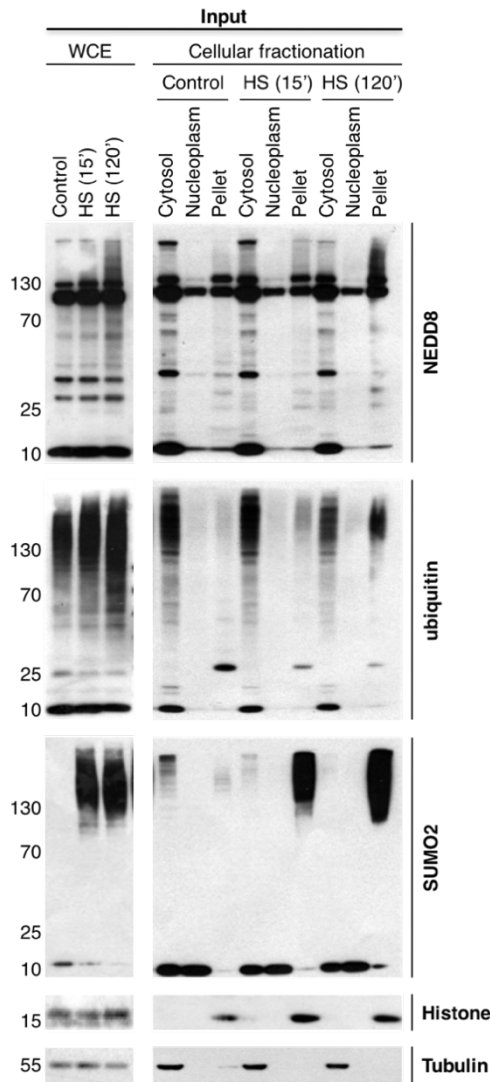
Following the SILAC quantitative analysis, we could characterize the atypical NEDD8 modification during HS-induced proteotoxicity. As expected, we identified ubiquitin as one of the proteins highest increased in NEDDylation upon heat shock but, interestingly, also SUMO1/2, Histone 2, NAE1 and ribosomal proteins exhibited high levels of enrichment in NEDD8 modification. This data confirmed the previously described NEDD8-ubiquitin mixed chains formation under proteotoxic stress (Leidecker et al., 2012) and suggest that the NEDDylation of the small ubiquitin-like modifier SUMO (formation of NEDD8-SUMO mixed chains) might be also a crucial part of the NEDD8 response. Furthermore, we found cullins as some of the proteins decreasing on NEDD8 modification, possibly indicating a compromised in their basal modification to release free NEDD8 for modifying different proteins during the stress. Another striking observation was the identification of NAE1 (regulatory subunit of the NAE

enzyme) as a substrate for NEDD8 during HS which might be crucial, together with the free NEDD8/ubiquitin ratio, for the unusual behavior of NEDD8 to use the ubiquitin pathway (**Figure 4A**). Previously in the laboratory, we observed that the accumulation of NEDDylated proteins upon HS proteotoxicity is characterized by a progressive translocation of the modified substrates from a cytosolic soluble fraction after mild HS (15 min) into an insoluble pellet fraction after prolonged HS (2hrs), leading to a possible aggregation of NEDDylated substrates after prolonged stress. By performing subcellular fractionation separating soluble (cytosol, nucleoplasm) from insoluble (pellet) fractions after mild and prolonged HS, we could detect a parallel increase response of protein NEDDylation, ubiquitination and SUMOylation into the insoluble fractions (**Figure 4B**). In addition, we confirmed the co-existence of NEDD8, ubiquitin and SUMO2 on the same substrates by using biochemical Ni-NTA isolation of the 6His-tagged NEDDylated substrates from the insoluble pellet fractions. As predicted, the isolated NEDDylated substrates were enriched with ubiquitin but also with SUMO2, thus confirming our SILAC data for NEDDylation upon heat shock stress response (**Figure 4C**). Interestingly, when cells were subjected to a severe proteotoxic stress, overnight MG132 treatment, nuclear co-localization of NEDD8/ubiquitin (**Figure 4D**) and NEDD8/SUMO2 (**Figure 4E**) was detected by immunofluorescence analysis which appeared as bright ring-like structures. Further analyses confirmed the formation of these nuclear ring-like NEDD8 aggregates and their high co-localization with ubiquitin (**Figure 4F**). In conclusion, these observations suggest that the increase in NEDDylation upon proteotoxic stress causes the progressive accumulation of NEDD8 conjugates as nuclear aggregates together with ubiquitin and SUMO.

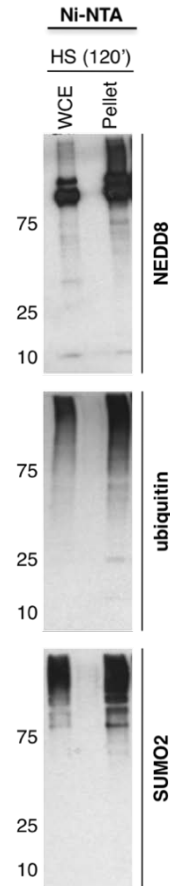
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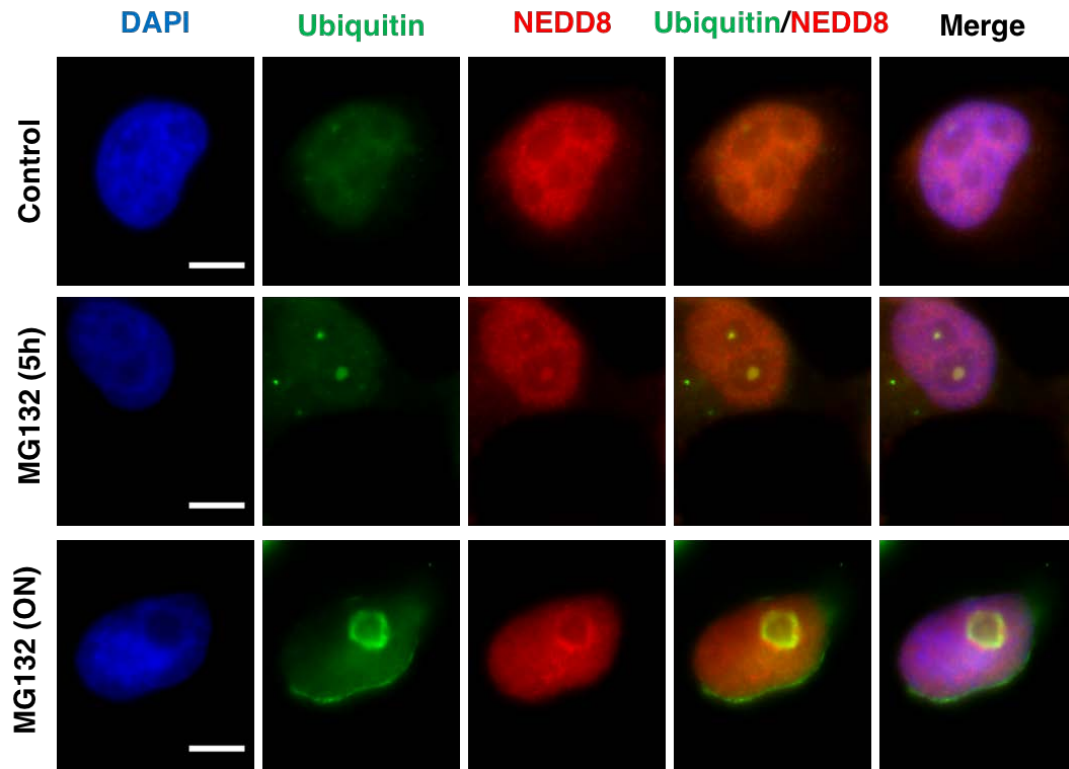
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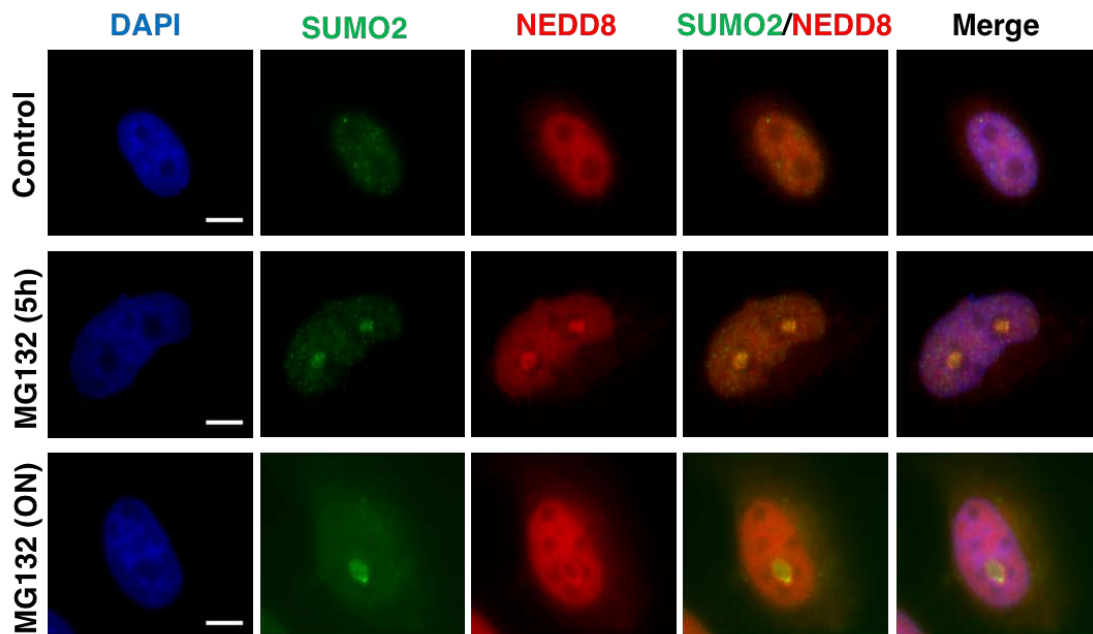
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E



F

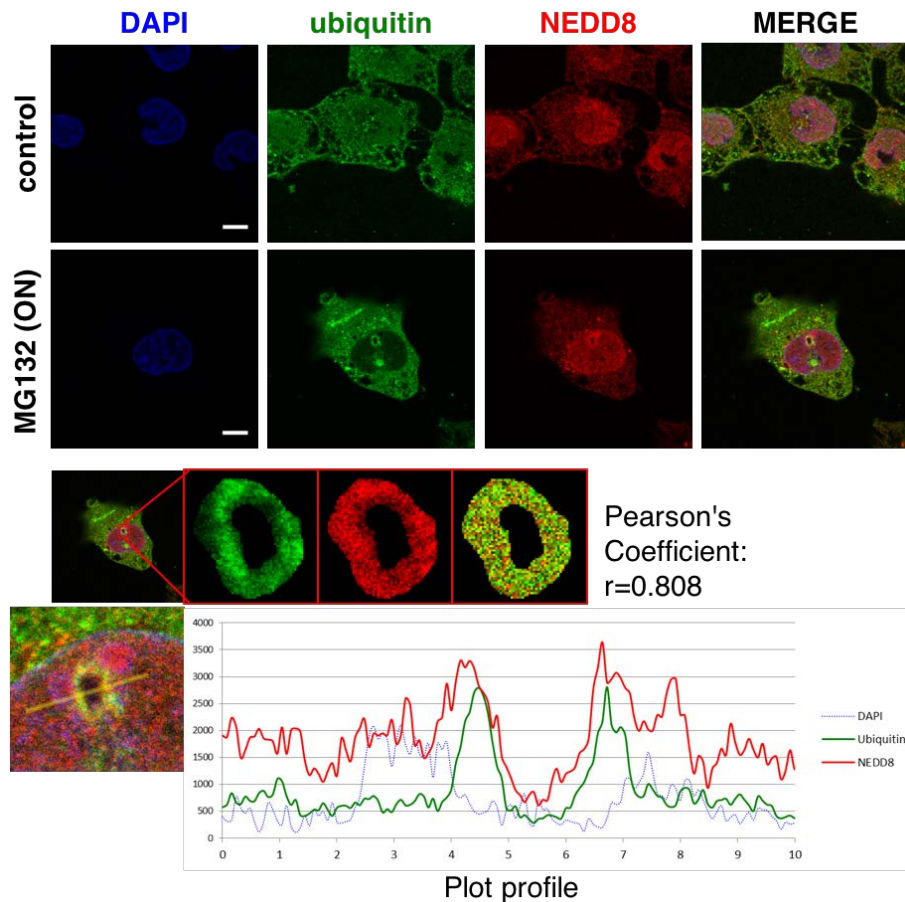


Figure 4. NEDD8, ubiquitin and SUMO2 co-exist on the same substrates upon proteotoxicity as nuclear ring-like aggregates

(A) Scatter-plot of the SILAC 6His-NEDD8 purification under HS stress. Log₂ ratios of heavy to light (H/L) mass-to-charge (m/z) peak correlated to the intensity of NEDDylated peptides under heat shock at 43°C for 1 hour. Ubiquitin, SUMO1/2, histone and ribosomal proteins increased whereas cullin family members decreased on NEDDylation in response to the stress. **(B)** Stable U2OS cells expressing 6His-NEDD8 were either left at 37°C (control) or heat-shocked at 43°C for the indicated times. Cells were subjected to subcellular fractionation. Western blot analysis showed a progressive accumulation of NEDDylated, ubiquitinated and SUMOylated proteins in the insoluble pellet fraction after prolonged HS (120'). Tubulin was used as cytoplasmic marker and histone as nuclear marker. **(C)** Western blots of the Ni-NTA pull-downs of the whole cell extract (WCE) or insoluble pellet fractions of stable cells heat-shocked at 43°C for 120' indicating a high enrichment of ubiquitin and SUMO2 as part of the NEDDylated substrates. H1299 cells were pre-treated with MG132 (25µM-5h) or (5µM-ON), then fixed and stained for NEDD8 (red) with either Ubiquitin (green) **(D)** or SUMO2 (green) **(E)**. Nuclei were stained with DAPI (blue). Scale bars, 10µm. Pictures were acquired with a Leica DM6000 using metamorph software. **(F)** U2OS cells were either untreated or treated with MG132 (5µM-ON) before staining for NEDD8 (red) and ubiquitin (green). Nuclei were stained with DAPI (blue). The co-localization between NEDD8 and ubiquitin in the ring-like structures was analyzed as described previously (Bailly et al., 2016; Bolte & Cordelieres, 2006).

Ribosomal proteins aggregate in response to proteotoxicity promoted by NEDD8

As indicated above, prolonged periods of HS induce the formation of insoluble aggregates with a parallel aggregation and accumulation of NEDD8, ubiquitin and SUMO1/2. To further characterize the composition of protein aggregation induced by HS, we performed SILAC quantitative proteomics on the aggregates. H1299 cells were labelled with either light (K0R0) or heavy medium (K8R10) and either left at 37°C (light) or heat-shocked (heavy) at 43°C for 2hrs. Cells were mixed 1:1 ratio before subcellular fractionation and isolation of the insoluble pellet fraction, which was resuspended in 2xSDS buffer and sonicated. After in gel-trypsin digestion peptides were identified and quantified by mass spectrometry as described in methods (**Figure 5A**). Approximately 1600 proteins significantly increased in the formation of aggregates upon prolonged HS stress in which, consistent with our previous data, ubiquitin, NEDD8 and SUMO1/2 showed highly accumulation (**Figure 5B**). Another distinguished observation was that among all the group of proteins which increased in the aggregates (above Log₂ ratio 1), ribosomal proteins (RPs) were the most abundant with approximately 10% of the total number and 20% of the related overrepresented pathway (**Figure 5C-D**).

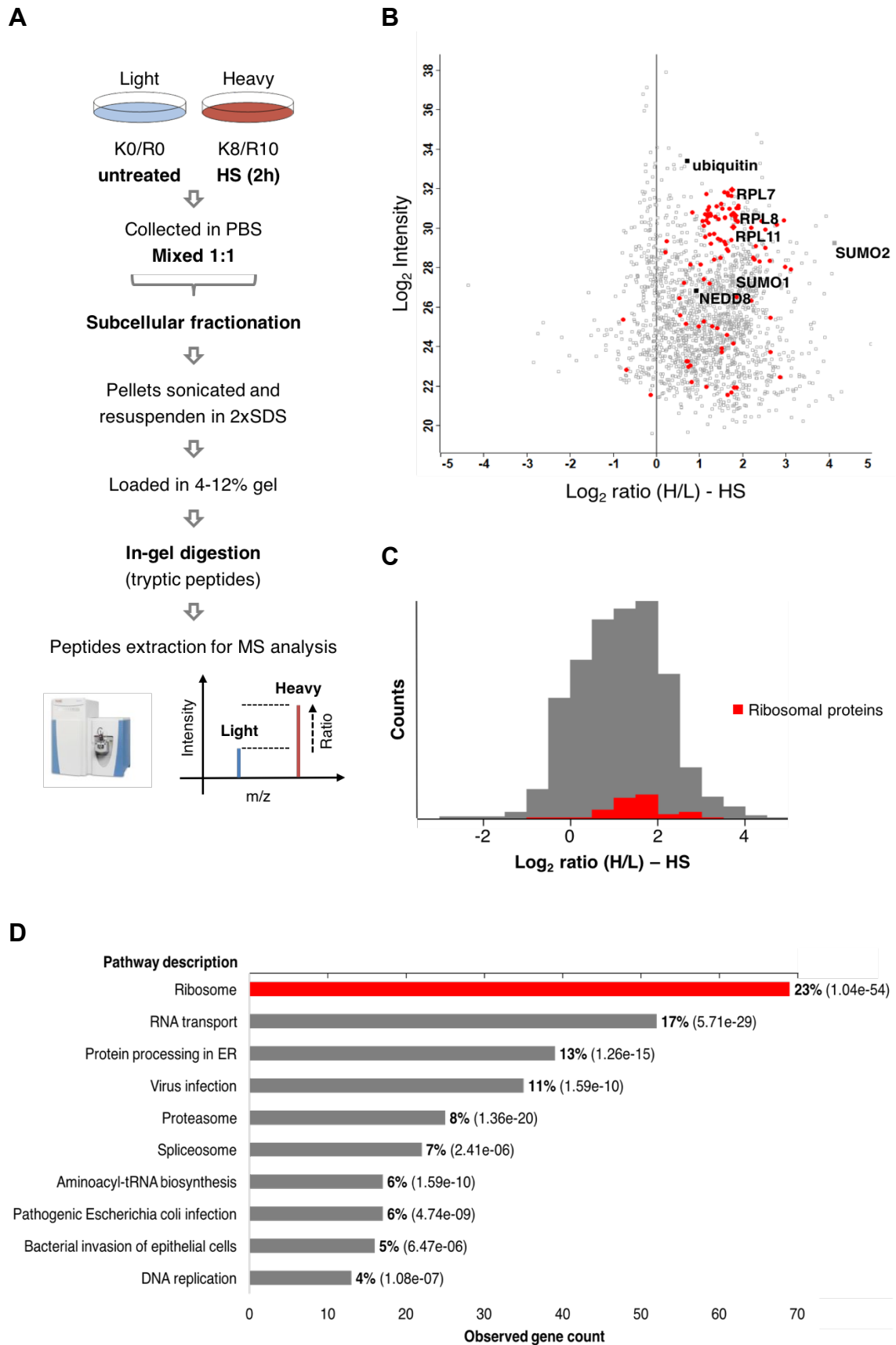


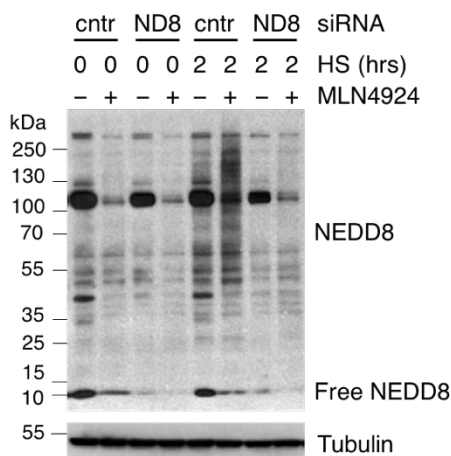
Figure 5. HS-induced aggregates are constituted mainly by NEDD8, ubiquitin, SUMO and ribosomal proteins

(A) Schematic overview of the SILAC quantitative proteomics strategy for investigate the protein composition of aggregates produced upon HS. **(B)** Scatter plot of the HS SILAC on aggregates indicate the Log_2 ratios (H/L) correlated with the intensity of peptides. Protein aggregation increase upon prolonged HS (2h) with a notably parallel accumulation of NEDD8, ubiquitin, SUMO1/2, and ribosomal proteins. The red dots represent the ribosomal proteins. Same results obtained from a duplicate experiment. **(C)** Histogram representation of the HS SILAC on aggregates correlating the Log_2 ratios (H/L) with the protein counts. ~970 proteins were enriched with SILAC ratio above 1 on which approximately 10 % (~90) corresponded to ribosomal proteins (colored in red). **(D)** STRING database analysis and bar graph representation of the KEGG pathways overrepresented on the proteins identified with H/L SILAC ratio >1. The top 10 highest-enriched pathway descriptions are presented on the graph indicating the percentage corresponded to the total number of gene counts observed and their respective False Discovery Rate (FDR). The ribosome pathway (red bar) was the most significant overrepresented with 23% of the total observed gene count.

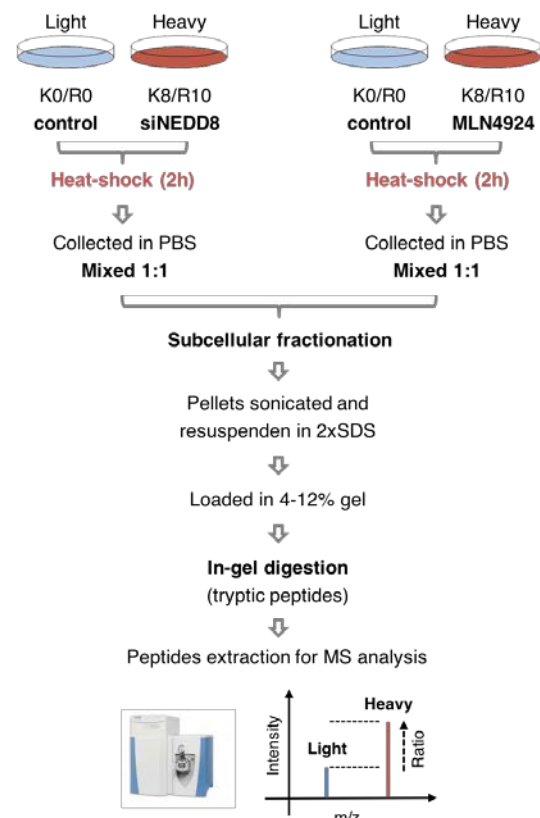
The SILAC quantification on aggregates upon HS revealed their general protein composition which clearly showed the high accumulation of NEDD8, SUMO1/2, ubiquitin and RPs. We then addressed the potential role of NEDD8 in the protein aggregation induced by HS. Previous studies in the laboratory established that while short-term (36-48 hrs) knockdown of NEDD8 completely blocks the stress-induced NEDD8 response, it does not have a crucial impact on cullin NEDDylation which is the major homeostatic target of NEDD8. In contrast, treatment with MLN4924, a specific inhibitor of the canonical NAE enzyme, has no effect on the stress-induced NEDDylation but almost fully blocks cullin NEDDylation (**Figure 6A**) (Leidecker et al., 2012). Based on that notion, we applied the above experimental settings to determine by SILAC quantitative proteomics the effect of NEDD8 itself on the composition of the HS-induced aggregates, and to distinguish between any potential related effects by cullin NEDDylation and CRL function. H1299 cells labelled with either light (R0K0) or heavy medium (R10K8) were divided in two sets. The first set of cells was transfected with control (light) or with NEDD8 (heavy) siRNAs. For the second set, control siRNA transfected cells were either treated with DMSO (light) or with MLN4924 (heavy). After 48hrs of transfection or MLN4924 treatment, cells were heat-shocked at 43°C for 2 hrs, collected in PBS, counted and then mixed 1:1 ratio (control with siNEDD8) and (control-DMSO with MLN4924). This was followed by a subcellular fractionation and mass spectrometry analysis as above

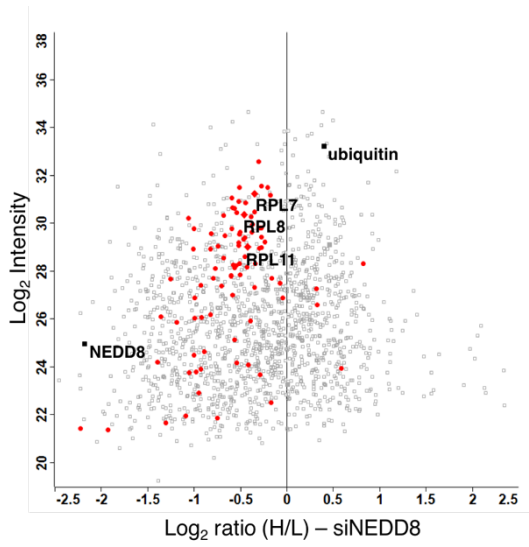
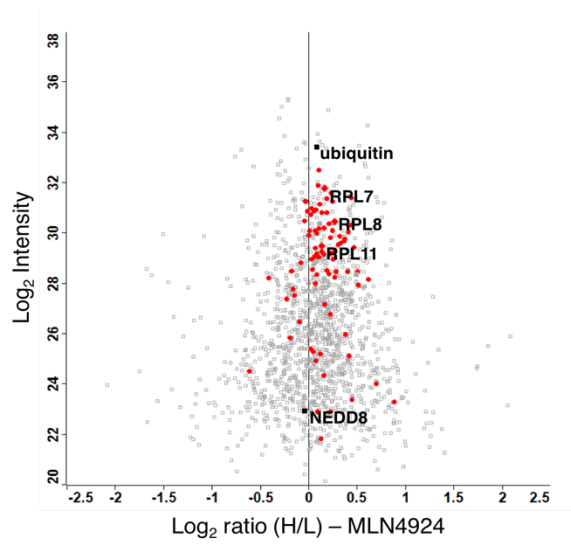
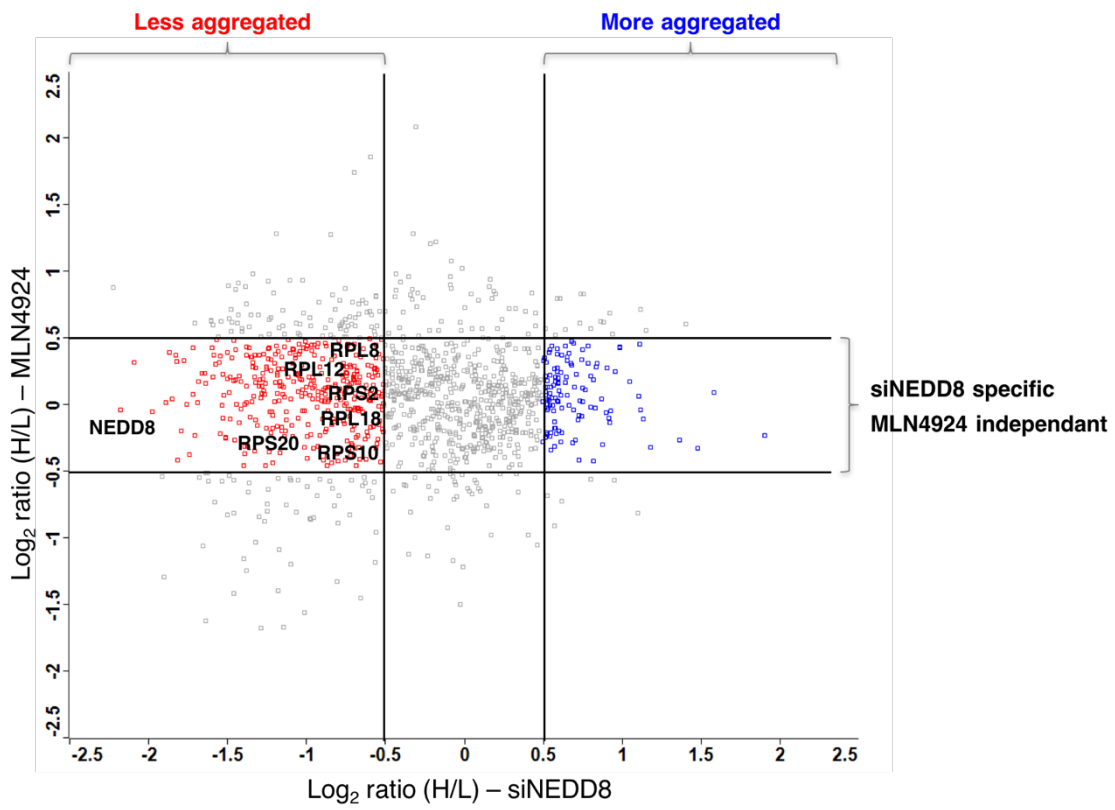
(**Figure 6B**). The SILAC quantitative results identified proteins that increase or decrease in the aggregates during NEDD8 depletion but the most profound effect of NEDD8 knockdown was the general reduction of protein aggregation itself (~40% decreased of total proteins). In particular, reduced aggregation was observed for a large number of ribosomal proteins (~50 below Log₂ ratio 0.5) (**Figure 6C**). Importantly, these effects were specific for NEDD8 knockdown and not observed upon inhibition of the canonical pathway and CRL function by MLN4924 (**Figure 6D**). Additionally, by comparing the siNEDD8 with the MLN4924 data, we obtained the proteins which aggregate less and more specifically by the NEDD8 depletion (**Figure 6E**). The absence of NEDD8 significantly reduced the aggregation of approximately 350 proteins (75%) in compare to the ~115 proteins which aggregated more (25%). Interestingly, the protein composition of the less aggregated proteins was highly overrepresented with the ribosome related pathway (**Figure 6F**). Thus, this data indicates that atypical NEDDylation induces the aggregation of a specific subset of proteins (mainly RPs) which consequently affects the composition of the HS-induced aggregates.

A



B



C**D****E**

F

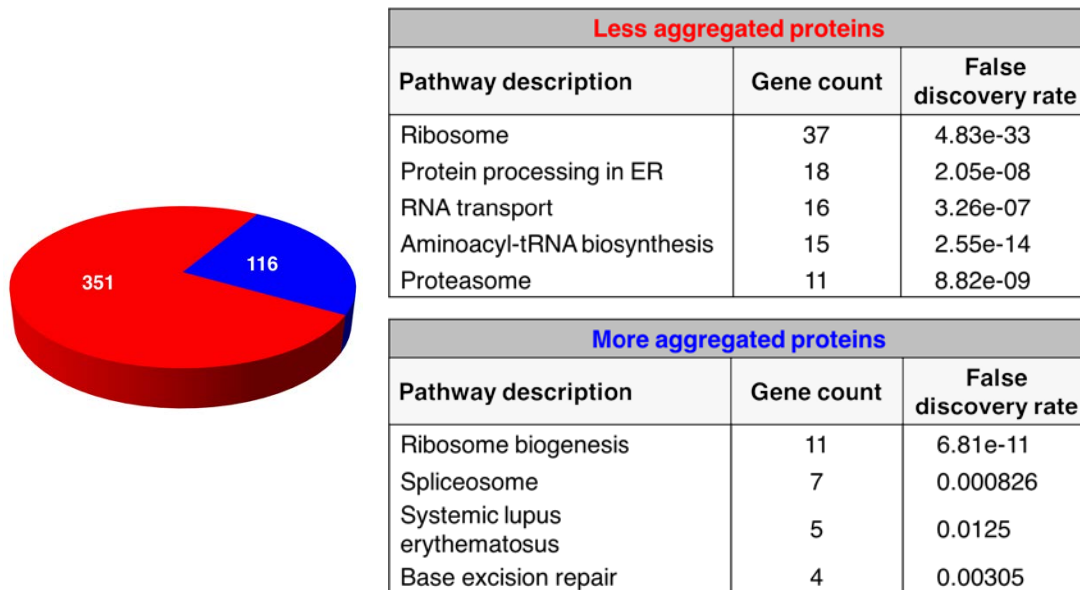


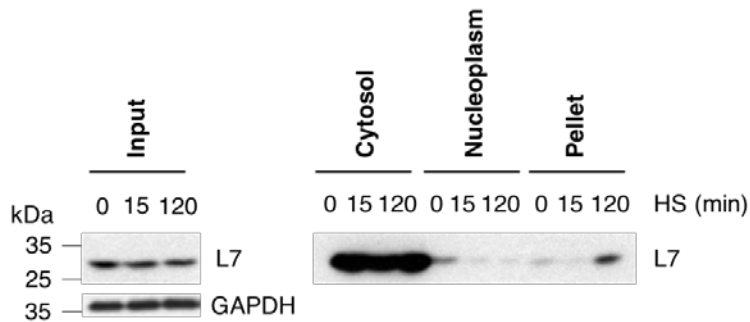
Figure 6. Role of NEDD8 in the HS-induced aggregation of ribosomal proteins

(A) Mechanism used to discriminate between canonical and atypical NEDD8 response upon HS. H1299 cells were transfected with control or NEDD8 siRNA or treated with MLN4924 (200 nM) for 48 hrs before were heat-shocked or not as indicated. Cell extracts were used for western blotting for the indicated proteins. MLN4924 decreased only the canonical NEDDylation whereas siNEDD8 only affected the atypical NEDD8 response (B) Workflow overview of the SILAC proteomics strategy to determine the role of canonical and atypical NEDD8 pathway on protein composition of the HS induced aggregates. Scatter plots indicating the Log₂ ratios (H/L) of SILAC quantification effect during NEDD8 knockdown (C) and MLN4924 treatment (D). Ribosomal proteins were significantly affected by the depletion of atypical NEDDylation (siNEDD8). Same results were obtained from duplicate experiments. (E) Scatter plots comparing siNEDD8 with MLN4924 SILAC data. Proteins indicated in red and blue were either less or more aggregated upon HS during NEDD8 knockdown (inhibition of atypical NEDDylation) but not during MLN4924 treatment (inhibition of canonical NEDDylation). (F) Left: Pie chart of the number of proteins less aggregated (red) and more aggregated (blue) selected from the analysis in E. Right: Table indicating the highest KEGG pathways overrepresented in the less and more aggregated proteins respectively from STRING database analysis.

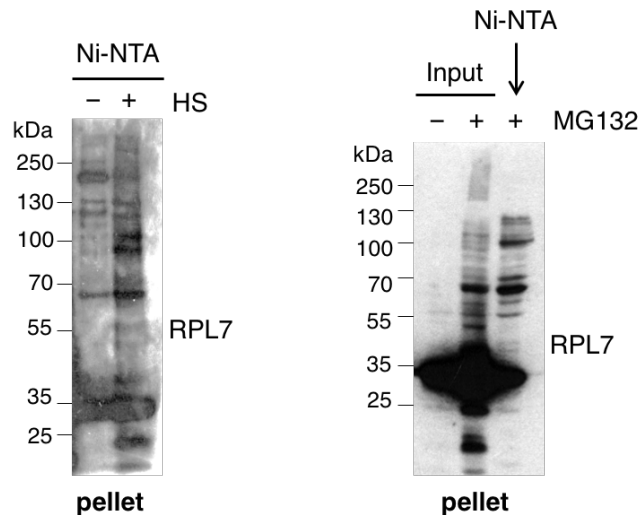
Given that the atypical NEDDylation induce the aggregation of RPs, we assumed that NEDD8 is directly modifying the aggregated RPs upon proteotoxicity. First, we reanalyzed the cellular extracts from **Figure 4B** and blotted against the ribosomal protein RPL7, which was identified in our proteomic analysis and previously reported to be NEDDylated (D. P. Xirodimas et al., 2008). As expected, the ribosomal protein accumulates in the insoluble pellet fraction after prolonged

HS, confirming similar response and co-localization with NEDD8, ubiquitin, and SUMO2 (**Figure 7A**). Secondly, to determine the NEDDylation of RPs in the aggregates, HCT116 cells stably expressing 6His-NEDD8 were heat-shocked or treated with MG132, then subjected to a subcellular fractionation. Pellet fractions were solubilized with 8M urea, and sonicated. 6His-tagged NEDDylated proteins were isolated by Ni-NTA pull down and blotted against the ribosomal protein RPL7. We could detect a polyNEDDylation of RPL7 upon proteotoxic stress, which validate a direct modification of NEDD8 triggered by the atypical NEDDylation pathway (**Figure 7B**). Finally, by immunofluorescence analysis we identified that after severe proteotoxic stress (MG132-ON), RPL7 accumulates within the nuclear ring-like structures stained with NEDD8 and ubiquitin (**Figure 7C-D**). As well as for RPL7, the ribosomal protein RPL11 which was also identified in our data, presented the same results (data not shown). In conclusion, we confirmed the direct NEDDylation of some aggregated RPs upon proteotoxic stress which appeared surrounded by the nuclear ring-like structures.

A



B



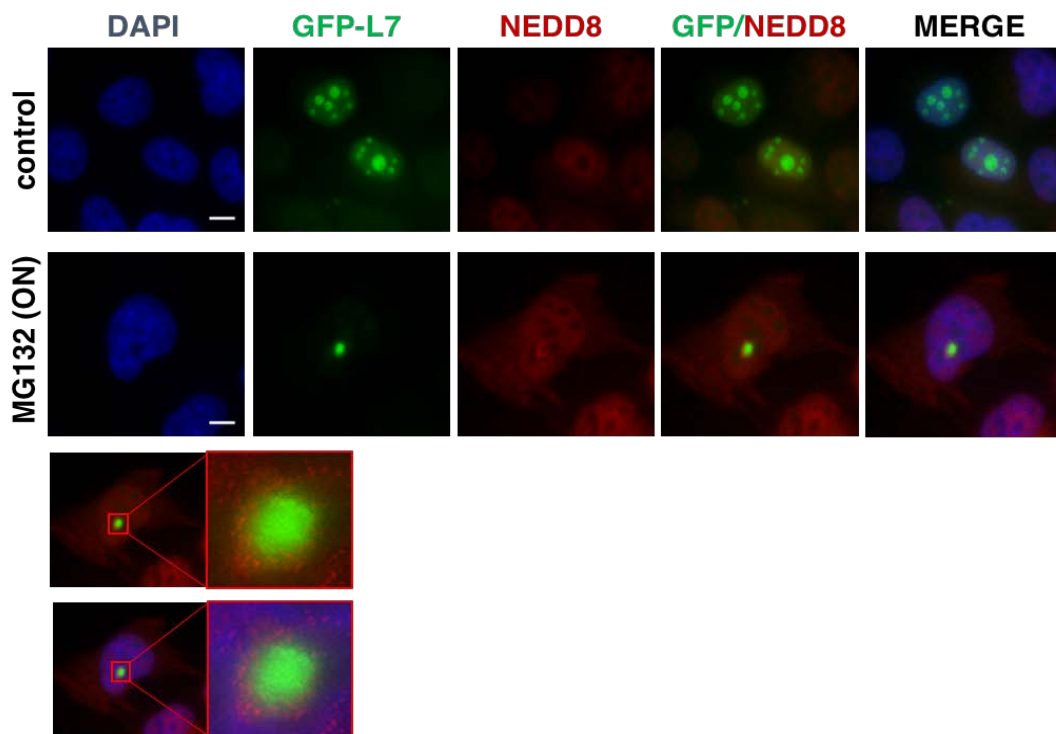
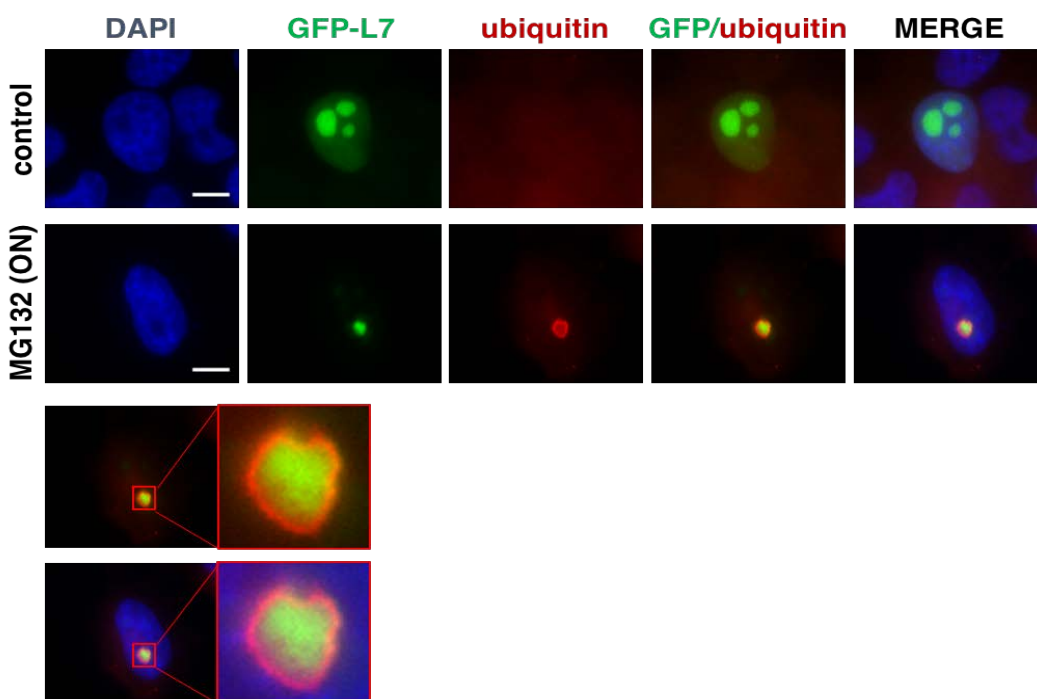
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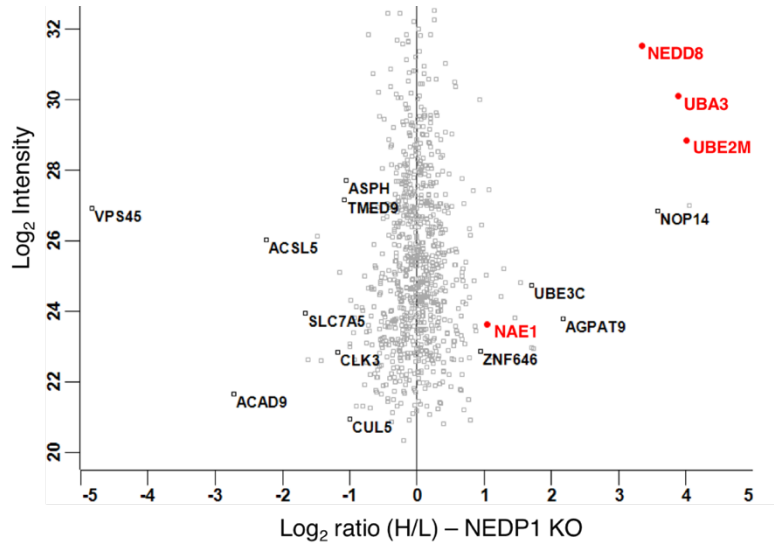
Figure 7. NEDDylation of aggregated ribosomal protein upon proteotoxic stress
(A) Cellular extracts from the experiment in Figure 4B were immunoblotted against RPL7 which accumulated in the pellet fraction after HS for 2 h. This analysis confirmed a co-localization with NEDD8, ubiquitin and SUMO2. **(B)** Stably HCT116 cell line expressing 6His-NEDD8 were heat-shocked at 43°C (left) or treated with MG132 at 25 μ M (right) for

2 h, and then subjected to subcellular fractionation. NEDDylated substrates from pellet fractions were solubilized and enriched by Ni-NTA pull-down as described in methods. Western blot analysis validated the NEDDylation of RPL7 upon proteotoxicity. HCT116 cells were transfected with RPL7-GFP and 48 hrs later were treated or not with MG132 (5 μ M-ON). After fixation cells were stained for RPL7-GFP (green) with either NEDD8 (**C**) or ubiquitin (**D**) (red). Enlarged insets represent the NEDD8/ubiquitin ring-like structures surrounding the aggregated RPL7. Nuclei were stained with DAPI. Scale bars indicate 10 μ m. Pictures were acquired with Leica DM6000 using metamorph software.

Canonical NEDDylation controls NEDD8 enzymes and polyNEDD8 chain formation

NEDD8 is mainly modifying the cullin family members during cellular homeostasis. However, it is possible that many other proteins are indeed NEDDylated through NAE activation, but their modification may exist transiently or in low levels during this physiological condition. The enzyme NEDP1 is one of the proteases showing greatest specificity for NEDD8 and, interestingly, previous results have demonstrated its high potential to regulate non-cullin substrates (Chan et al., 2008; D. P. Xirodimas et al., 2008). Therefore, here we developed NEDP1 KO cell lines which successfully increased the accumulation of new potential NEDDylated proteins (see **Figure 2**). To further study the dynamic modification of canonical NEDDylation regulated by NEDP1, we performed SILAC quantitative proteomics for the NEDP1 depleted cells stably expressing 6His-NEDD8. Different elutions from the consecutive Ni-NTA purifications were selected and analyzed separately by MS (see **Figure 3**). Two replicates with three elution samples each was analyzed (6 samples in total) from which we obtained similar results. As expected, our SILAC quantitative analysis detected NEDD8 itself as one of the substrates highly NEDDylated when NEDP1 is deleted, confirming its crucial role to process polyNEDD8 chains. A comparable observation was previously obtained in the preference of NEDP1 to process hyper-NEDDylated rather than mono-NEDDylated CUL1 *in vitro*. (Wu et al., 2003). Moreover, components of the NEDD8 conjugation pathway such as NAE1, UBA3 and UBE2M were significantly increased for NEDDylation upon NEDP1 depletion (**Figure 8A**). Certainly, functional protein association analysis showed that among all the proteins identified with Log₂ ratio above 1 were principally

enriched for the protein NEDDylation process itself (NEDD8 and the canonical enzymes) but also for other biological processes such as protein localization, protein transport and cellular metabolism (**Figure 8B**). In contrast, no biological process was significantly enriched among the proteins with less NEDDylation upon NEDP1 depletion. Nevertheless, we could observe in one of the elution samples analysis that CUL5 decreased for NEDDylation in the absence of NEDP1 with a Log₂ ratio of -1 (**Figure 8A**). This observation suggests as it happened during stress conditions that the formation of polyNEDD8 chains can compromise the free NEDD8 availability for regulating cullins. Furthermore, we could validate the modification of the E1 regulatory subunit NAE1 (APPBP1) with NEDD8 coordinated by NEDP1 (**Figure 8C**). This modification was also reported during the depletion of NEDP1 ortholog in *Arabidopsis thaliana* (Mergner, Heinzlmeir, Kuster, & Schwechheimer, 2015) which confirms its NEDDylation controlled by NEDP1 and provided evidence of the importance of its functional regulation by NEDD8. Interestingly, the E1 catalytical subunit UBA3 and the E2 enzyme UBE2M appeared to have even more rates of NEDDylation moderated by NEDP1 than the regulatory subunit APPBP1 (see **Figure 8A**). Unquestionably, the function of NEDP1 in maintaining a particular NEDD8 pathway activity in the cells is still to be clarified and therefore would be an important area for further research. In conclusion, our data proposed that the cullin-independent canonical NEDDylation controlled by NEDP1 is mainly involved in the protein NEDDylation process itself. Clearly, SILAC quantitative analysis is a powerful tool to determine the dynamic of a specific process. However, it cannot provide a broader global identification of proteins. Consequently, to elucidate the identity and function of additional NEDD8 conjugates, the next section of this work is focused on the large-scale identification of NEDD8 targets either regulated by the atypical activation but especially from the canonical pathway.

A**B**

Biological process (GO)		
Pathway description	Gene count	FDR
Protein neddylation	4	1.28e-05
Protein localization	16	0.00151
Macromolecule localization	17	0.0021
Protein transport	13	0.00431
Cellular metabolic process	33	0.00922

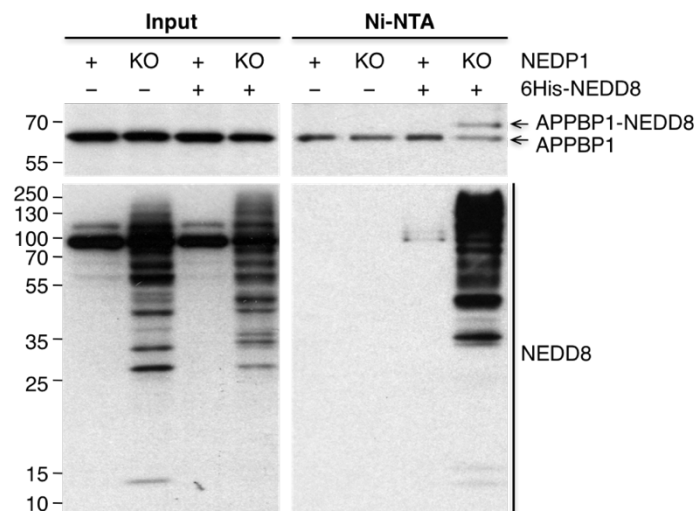
C

Figure 8. NEDD8 controls its canonical protein NEDDylation by NEDP1 regulation

(A) Scatter-plot of the SILAC 6His-NEDD8 purification under NEDP1 depletion. Log₂ ratios of heavy to light (H/L) mass-to-charge (m/z) peak correlated to the intensity of NEDDylated peptides. NEDD8 itself, as well as NEDD8 enzymes such as NAE1, UBA3, and UBE2M were highly increased in the absence of NEDP1. **(B)** Table representation indicating the highest biological processes (GO) overrepresented in the more NEDDylated proteins in NEDP1 KO cells (ratio >1) from STRING database analysis. **(C)** HCT116 lysates from control or NEDP1 depleted cells either stably expressing 6His-NEDD8 or not were subjected to Ni-NTA purification in denaturing conditions and immunoblotted against APPBP1 (NAE1). Covalent conjugation of APPBP1 by NEDD8 is observed only in 6His-NEDD8 conjugates from NEDP1 KO cells.

CHAPTER II

IDENTIFICATION OF NEDD8 SUBSTRATES AND MODIFICATION SITES

BACKGROUND

Post-translational modifications (PTMs) are crucial for various signaling pathways as well for the maintenance of cellular protein homeostasis. The large-scale determination of PTMs have generated valuable data into many biological functions, but most of their analysis have encountered many obstacles over an extended period due to a lack of suitable methods (Mann & Jensen, 2003). In fact, several PTMs, such as ubiquitination, were discovered by chance during studies on single proteins or cellular processes by using standard molecular and biochemical techniques (Hershko, 1996; Mann & Jensen, 2003). Nowadays, the use of quantitative mass spectrometry (MS) to investigate cell proteome PTMs has provided successful results, not only in the broad identification of modified proteins but also in the dynamic mapping of their modification sites. Nevertheless, large-scale identification of post-translational modification sites still faces analytical challenges due to the inability of current mass spectrometers to detect all peptide sequences generated by proteome digestion. Besides, PTMs are primarily exhibited at substoichiometric levels which considerably impede their analysis and identification (Choudhary & Mann, 2010; Parker et al., 2010; Sylvestersen et al., 2013; Zee & Garcia, 2012). Protein ubiquitination is the covalent attachment of ubiquitin by its C-terminus linked to an ϵ -amino group of the Lys residue of a substrate. This linkage is an important PTM involved in numerous cellular processes, including the well-studied regulation of protein degradation through the ubiquitin-proteasome system (UPS) (Hershko & Ciechanover, 1998). However, the analysis of global ubiquitination has been particularly challenging because of its inherent large mass (~8 kDa) and its rapid modification turnover (Peng et al., 2003). Therefore, over the last decade, many

technical advances have been developed to improve the enrichment and final detection of ubiquitinated proteins and modification sites by mass spectrometry-based proteomics (reviewed in Heidelberger et al., 2016; Sylvestersen et al., 2013). The shotgun (or bottom-up) proteomics approach commonly involves trypsin enzymatic digestion which cleaves after lysine and arginine residues. Given that the C-terminal sequence of ubiquitin is Leu73-Arg74-Gly75-Gly76, the tryptic peptide of a ubiquitinated protein releases two useful features. First, the Gly-Gly (diglycine) remnant signature attached to the modified lysine which is no longer recognized by trypsin. The second is the extra ~114Da mass shift on the modified peptide corresponding to the diglycine motif on the lysine which is further used as search criteria for MS detection of precise sites of ubiquitination (Kirkpatrick, Denison, & Gygi, 2005; Komander, 2009; Peng et al., 2003). Both the improvement in high-resolution mass spectrometry and the generation of Lys- ϵ -GlyGly (K- ϵ -GG) antibodies, which specifically recognize and enrich diglycine modified peptides, allowed the first proteome-wide detection of more than 10,000 endogenous ubiquitination sites in human cells (Kim et al., 2011; S. a. Wagner et al., 2011; Xu, Paige, & Jaffrey, 2010). However, the modification by the ubiquitin-like proteins NEDD8 and ISG15 also results in a K- ϵ -GG motif after trypsin digestion. Therefore, one limitation of the trypsin remnant profile is its incapacity to distinguish between ubiquitinated, NEDDylated and ISG15ylated sites (Udeshi et al., 2013). Despite the lower expression levels of NEDD8 in the cell, it has been shown that up to 6% of identified di-Gly remnants could be derived from NEDDylation under homeostasis (Kim et al., 2011). Cullins represent the main NEDD8 substrates by its ligation to a conserved Lys in their C-terminal's winged helix-binding (WHB) domain (Duda et al., 2008; Pan et al., 2004). Consequently, NEDDylation is considered to only modify and regulate Cullin-RING ligases (CRLs) (Lydeard et al., 2013). Indeed, recent structural studies have revealed the specific mechanism that ensures the preferential modification of cullin with NEDD8 by the multifunctional RING E3, RBX1 (Scott et al., 2014). However, novel additional targets and modification sites for NEDD8 have been identified which include physiologically important proteins such as transcription factors, signaling receptors, components of the protein synthesis and apoptotic regulators (Abidi & Xirodimas, 2015; Rabut & Peter, 2008; M. Wang et al., 2011; Watson et al., 2011; D. Xirodimas, 2008). These findings suggest that a more diverse

spectrum of biological processes is controlled by NEDDylation. The reported NEDDylation sites in non-cullin substrates have been identified by both mass spectrometry and mutagenesis methods. Nevertheless, the majority of these novel reported NEDD8 targets are also substrates for ubiquitination and, their NEDDylation is triggered by ubiquitin E3-ligases. Hence, the current thinking is that only the members of the cullin family are the genuine physiological NEDDylation targets (Enchev et al., 2014). Abnormal upregulation of global NEDDylation is found in several types of cancer (Chairatvit & Ngamkitidechakul, 2007; Lee & Zhou, 2010; H. Li et al., 2014; L. Li et al., 2014; T. Li, Guan, Huang, Hu, & Zheng, 2014; Salon et al., 2007; X. Wang et al., 2014). This pattern gives evidence of the importance of NEDD8 modification in the normal physiology of cells. In effect, the significance of protein NEDDylation has been supported by the success of the MLN4924 inhibitor in the treatment of certain types of cancer which is currently in Phase II clinical trials (Nawrocki, Griffin, Kelly, & Carew, 2012). MLN4924's action in blocking protein NEDDylation downstream of NAE for potential anti-tumor effects has been focused on the resulted CRL inhibition and accumulation of CRL targets (T A Soucy et al., 2009; Teresa A Soucy, Dick, Smith, Milhollen, & Brownell, 2010; Swords et al., 2010). Considering that many of the reported non-cullin NEDD8 targets include not only cell cycle, transcriptional and apoptotic regulators but also tumor suppressors and DNA damage response mediators, supports the notion that also non-cullin NEDD8 conjugation might have key roles in the therapeutic efficacy of MLN4924 in the clinic (Abidi & Xirodimas, 2015; Enchev et al., 2014; Watson et al., 2011). Although protein NEDDylation is emerging as an important regulator of several cancer-associated pathways, there is still a limited knowledge of the non-cullin substrates controlled by NEDD8. The current experimental MS approaches cannot determine large and authentic specific targets and modification sites for NEDD8 due to its basal conjugation levels in homeostatic conditions, but as well for its high similarity and cross-talk with ubiquitin. In this work, we aimed to determine criteria strategies to distinguish between NEDD8 and ubiquitin conjugation to define NEDD8 proteome besides cullins with a wide specific profile of modifications sites for NEDDylation.

RESULTS

Developing a point mutant NEDD8 strategy to distinguish between NEDD8 and ubiquitin substrates and modification sites by MS analysis

The current proteomic studies cannot distinguish between NEDD8, ISG15, and ubiquitin modification, as those molecules provide the same signature on the modified lysine after trypsin digestion. In the same manner of as the analysis of ubiquitination sites, the use of immunoaffinity purification with a diGly-Lys-specific antibody has been adapted for the broad identification of SUMOylation sites, the conjugation by the small ubiquitin-like modifier SUMO (Tammsalu et al., 2014). Here, we developed a method based on this mutation strategy to guarantee a site-specific assessment of NEDDylation. Our approach consists of a tagged point mutant of NEDD8 with Arg74 mutated to Lys. Thereby, digestion with Lys-C provides a unique diglycine signature on modified lysines which specifically identify NEDDylation sites on substrates (**Figure 9A**). The method involves the establishment of stable cell lines expressing polyhistidine (6His) – tagged NEDD8 mutant (6His-NEDD8^{R74K}) by a lentivirus infection approach. 6His-NEDD8^{R74K} conjugated proteins can be purified under denaturing conditions by Ni-NTA chromatography and subsequently cleaved with the endoproteinase Lys-C. A di-Gly Lys-specific antibody is then used to enrich NEDD8^{R74K} remnant-containing peptides for mass spectrometry analysis (LC-MS/MS) (**Figure 9B**). Previously in the laboratory, we generated similar systems with NEDD8 wild type using the lentivirus-based approach which provides low-level expression of the transgene similar to endogenous NEDD8 levels (see **Figure 2B**). Importantly, we have tested and confirmed that the NEDD8^{R74K} construct also expresses as endogenous NEDD8 in homeostatic conditions. NEDD8^{R74K} is negatively affected to modify cullins by the treatment with the inhibitor MLN4924 and instead, not altered by the knockdown of the ubiquitin-activating enzyme UBE1, indicating that the activation of NEDD8 mutant during homeostasis is UBE1 independent but NAE specific. Moreover, we examined the accumulation of 6His-NEDD8^{R74K} conjugates upon proteotoxic stress and NEDP1 depletion. By comparing with the endogenous NEDD8 and 6His- NEDD8^{WT} we could observe

the vast ability of the mutant to increase the NEDDylation levels in the NEDP1 KO cells. Nevertheless, the response of NEDD8^{R74K} to proteasome inhibition and, most notably, heat shock was not as abundant as the dramatic NEDP1 dependent accumulation (**Figure 9C**). This observation indicates that the NEDD8^{R74K} mutant is less efficient to modify substrates by the atypical UBE1 dependent pathway. Even in general the increase of NEDDylation is less dramatic during proteotoxic stress than during NEDP1 depletion (see **Figure 2A**), another explanation could be that the mutation of NEDD8 in the residue 74 might affect the recognition and further activation by the UBE1 enzyme. Likewise, the residue 72 on NEDD8 and ubiquitin seems to be crucial for the discrimination and specific activation of the distinct E1 enzymes (Walden et al., 2003). In conclusion, our NEDD8 mutation strategy enables the specific identification of NEDDylation sites by differentiating between the ISG15 and ubiquitin modification sites based on the diglycine remnant released under Lys-C digestion.

A

Endogenous

Ubiquitin:
 MQIFVKLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLLIFAGKQLEDGRTLSDYNIQK|ESTLHLVLRLLGG

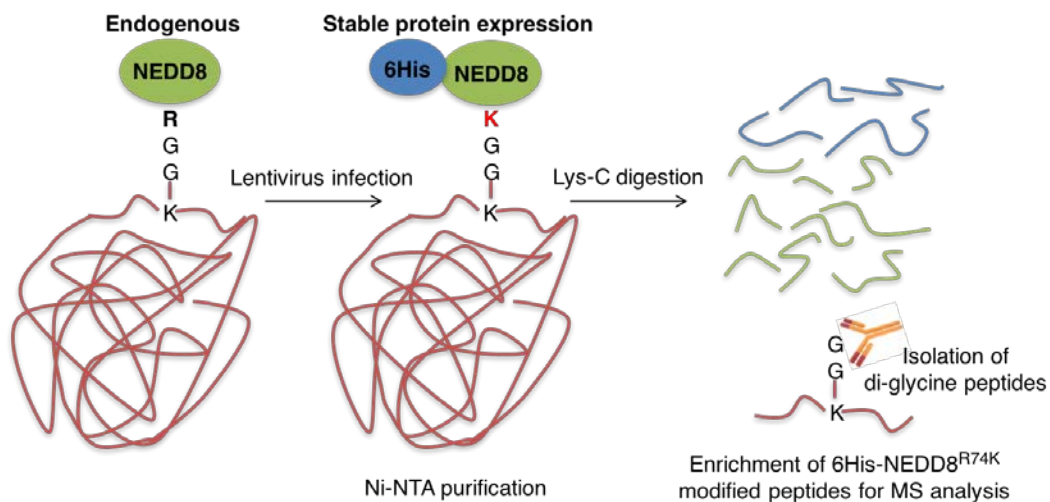
ISG15:
 MGWDLTVK....YEVRLTQTVAHLKQQVSGLEGVQDDLFWLTFFEGKPLEDQLPLGEYGLK|PLSTVFMNLRLLGG

NEDD8:
 MLIKVKLTGKKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLLIYSGKQMNDEKTAADYK|ILGGSVLHLVLRLLGG

Mutagenesis

NEDD8^{R74K}:
 MLIKVKLTGKKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLLIYSGKQMNDEKTAADYK|ILGGSVLHLVLRLLGG

B



C

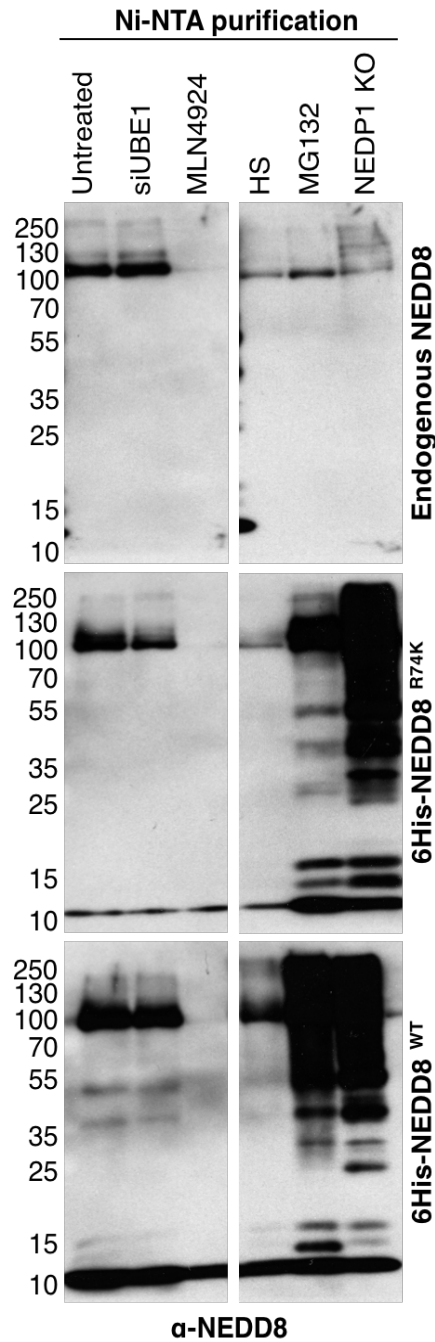


Figure 9. NEDD8 point mutation strategy to identify specific substrates and modification sites by MS analysis

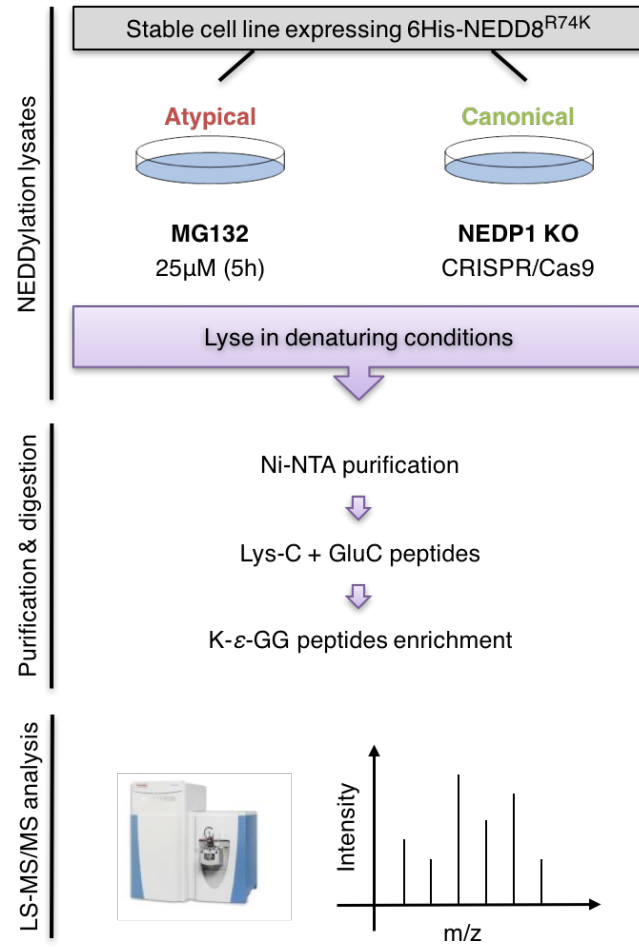
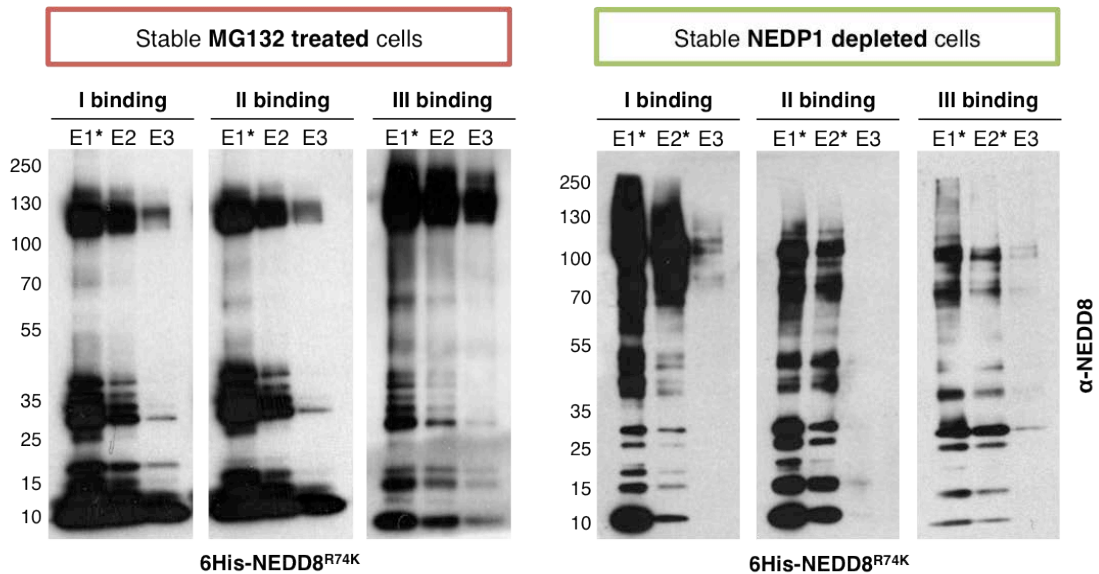
(A) Sequence alignment of matured human ubiquitin, ISG15, and NEDD8 which terminate in a diglycine motif. In contrast to the endogenous protein, a point mutation of NEDD8 in Arg74 to Lys provides a unique GG signature on modified lysine residues upon digestion with Lys-C. The sequence of the predicted peptide after Lys-C digestion is underlined and highlighted in bold. Lys-C cleavable C-terminal lysine residue is colored in red. (B) Lentivirus infection provides stable protein expression of 6His-NEDD8^{R74K} mutant construct in cells. The biochemical purification of 6His-NEDD8^{R74K}

conjugates allows to distinguish and identify specific NEDD8 covalently modified lysines on substrates by MS analysis. **(C)** Comparison of the NEDDylation response under various conditions in whether parental non-lentivirus infected HCT116 cells, stable expressing 6His-NEDD8^{WT} or 6His-NEDD8^{R74K}. The lysates were either unstressed (Control, 37°C), UBE1 knock downed (siRNA), MLN4924 treated (16hrs at 1µM), heat-shocked (1h at 43°C), MG132 treated (25µM, 5hrs) or NEDP1 depleted (CRISPR-Cas9 approach). Ni-NTA purifications of the respective cell lines were immunoblotted against NEDD8.

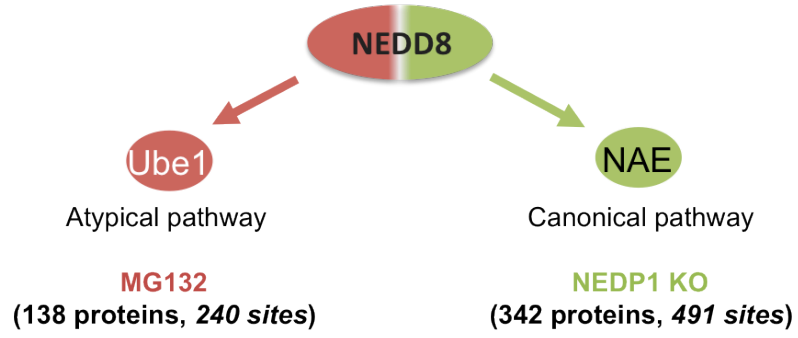
The capacity of the mutation approach to obtain specific NEDD8 substrates and modification sites

To obtain a NEDDylation site spectrum from the atypical and canonical pathway we analyzed NEDD8^{R74K} substrates and sites from different conditions. For the atypical NEDDylation (UBE1 dependant), we treated a 6His-NEDD8^{R74K} stable cell line with MG132 (25µM, 5 hours) to cause proteasome inhibition. In contrast, for the study of canonical NEDDylation (NAE dependant), we analyzed a NEDP1 KO stable cell line developed previously in the laboratory by the CRISPR/Cas9 approach (see **Figure 2**). Cells were lysed using denaturing conditions and 6His-NEDD8^{R74K} conjugates were purified through Ni-NTA columns. Proteins from respective lysates were subjected to three successive purification/elution processes, to recover a maximum of NEDDylated proteins (set of three elutions per Ni-NTA binding) (**Figure 10A**). For further analysis by Cell Signaling Technology service (CST, United States), we selected and combined three samples for the MG132 elutions (E1 from each binding) and six samples from the NEDP1 KO elutions (E1 and E2 from each binding). The CST facility performed the following 3 steps: (i) LysC and additional GluC digestion to help truncate long substrate peptides, (ii) the Lys-ε-GlyGly immunoprecipitation to enrich peptides containing diGly-Lys, (iii) the final LC-MS/MS analysis by using specific parameters for the identification of low-abundance peptides from post-translational modifications (PTMScan). (**Figure 10B**). By using this novel strategy, we were able to identify specific targets and modification sites for NEDD8. Clearly, we acquired more NEDD8 substrates and modification sites under NEDP1 KO condition (342 proteins / 491 sites) than after MG132 treatment (138

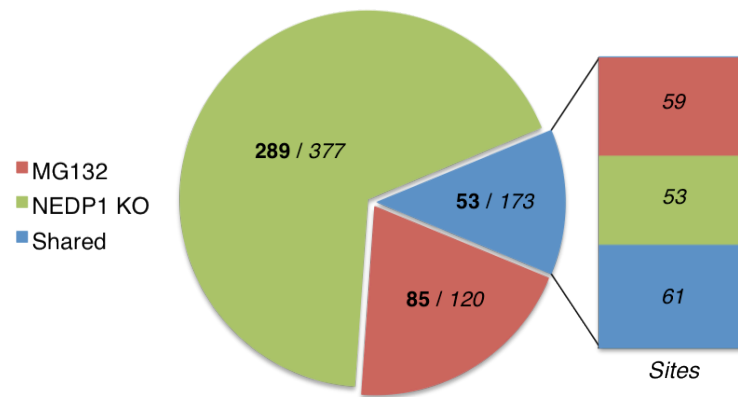
proteins / 240 sites), probably due to the lower rate response of the mutant by the atypical NEDDylation as observed in the immunoblotting analysis. 53 proteins were identified in both conditions which indicate that general NEDDylation independently of the pathway (atypical or canonical) might regulate common proteins (**Figure 10C**). Interestingly, not all of them shared the same modification sites (**Figure 10D-E**) but more accurate comparative analyses are needed in order to address these differences observed in NEDD8 site modifications within proteins. Furthermore, to characterize the cellular processes in which the proteins of our NEDD8^{R74K} data set are involved, derived from both MG132 treated and NEDP1 depleted cells, we performed a gene ontology (GO) enrichment analysis. The analysis showed functional annotations of proteins statistically overrepresented in biological processes including protein NEDDylation itself, chromatin organization, gene expression regulation, cellular biogenesis, RNA processing and DNA damage response (**Figure 10F**). This observation suggests that the majority of the identified proteins fulfill their function in the nuclear cellular compartment which correlates with the statement that overall NEDD8-conjugated proteins appeared to reside predominantly in the nucleus (T. K. Kamitani et al., 1997). Importantly, these findings are coherent with our previous quantitative results on the proteins regulated by the atypical and canonical NEDDylation.

A**B**

C



D



E

Protein	MG132	NEDPI KO
PCNA	K254	K164, K168
H2B	K6, K47, K117, K121	K6, K121
COPS7A	K20	K20, K23
ENO1	K81, K228, K420, K434	K71
FABP5	K50	K61
XRCC5	K565	K532, K603
DCUN1D1	K11	K11, K24, K171
PTMA	K103	K15

F

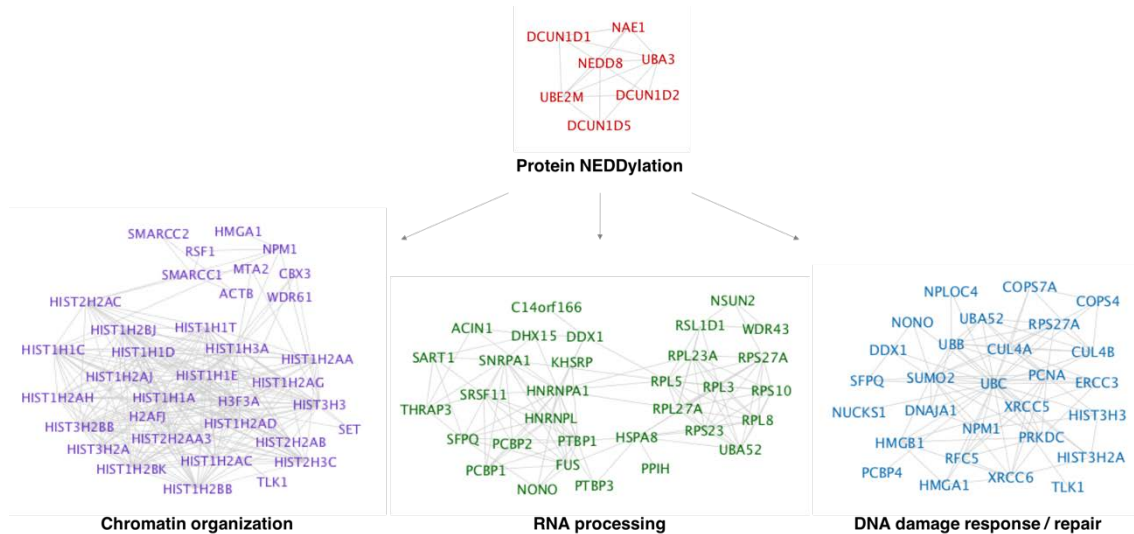


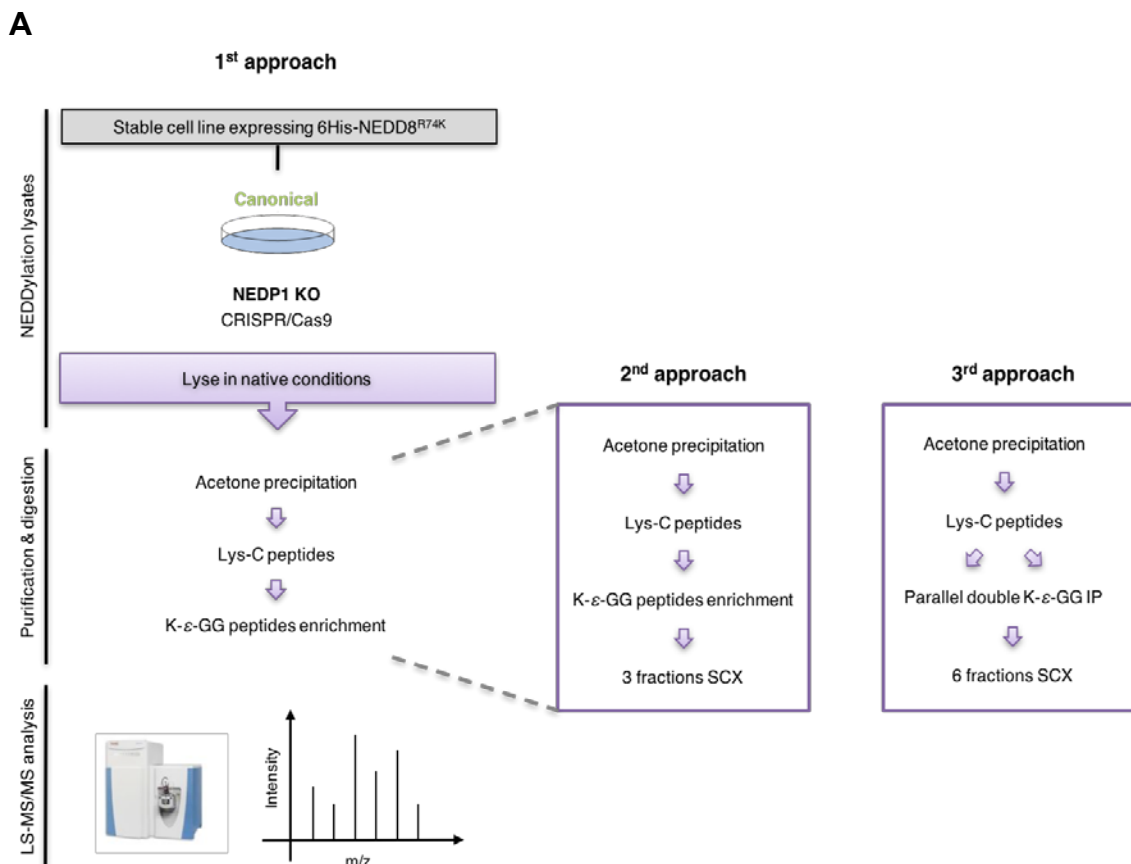
Figure 10. NEDD8 mutation approach enables broad identification of substrates and modification sites

(A) Overview of MS-based proteomics workflow for NEDD8 substrates and sites identification. Stable cells expressing 6His-NEDD8^{R74K} after proteasome inhibition with MG132 (atypical pathway) or NEDP1 depletion (canonical pathway) were lysed using denaturing conditions for further Ni-NTA biochemical purification, Lys-C and GluC digestion, diGly-Lys peptide enrichment and final MS analysis. (B) Western blots show the consecutive Ni-NTA elution samples from respective 6His-NEDD8^{R74K} lysates after three successive purification/elution processes. (*) Chosen elution fractions for MS analysis. (C) PTMScan analysis identified around 240 sites in 138 proteins for atypical NEDDylation after MG132 treatment and 491 sites in 342 proteins for canonical NEDDylation under NEDP1 knockdown on which 53 proteins were common for both conditions. (D) LC-MS/MS results identified around 289 proteins / 377 sites specific for NEDP1 KO cells and 85 proteins / 120 sites specific for MG132 treated cells, respectively. 53 proteins were found in both conditions but, the total 173 sites designated among these proteins were 53 sites specific for NEDP1 KO, 59 sites for MG132 and 61 sites were common for both conditions. (E) Table shows examples of prevalent proteins found in both conditions with differences in their modifications sites by NEDD8. PCNA, Proliferating cell nuclear antigen. H2B, Histone H2B type. COPS7A, COP9 signalosome complex subunit 7a. ENO1, Alpha-enolase. FABP5, Fatty acid-binding protein. XRCC5 (also Ku80), X-ray repair cross-complementing protein 5. DCUN1D1, DCN1-like protein 1. PTMA, Prothymosin alpha. (F) NEDD8 modifies highly interconnected functional networks of proteins. STRING database analysis and Cytoscape visualization shows that putative NEDD8 substrates are significantly overrepresented in Gene Ontology (GO) biological processes including chromatin organization, RNA processing and DNA damage response and repair. 41.82 and 4.80e-06 for protein NEDDylation, 6.38 and 5.55e-23 for chromatin organization, 3.04 and 9.05e-05 for RNA processing, 4.12 and 1.46e-05 for DNA damage response/repair are the respective fold enrichment and p-values associated with GO parameters in each biological process term.

Improved workflow led to an increase of NEDD8 site identification

Given the success of the NEDD8^{R74K} strategy, we developed a workflow optimization to further increase the number of identified sites. This work was performed in collaboration with Dr. Petra Beli's laboratory from the Institute of Molecular Biology (IMB) in Mainz, Germany. The previous protocol was adapted by first analyzing the NEDP1 KO depleted cells which show a dramatic increase of protein NEDDylation related to the canonical pathway. We changed the preparation of lysates from denaturing conditions to native conditions for subsequent acetone precipitation. Instead of performing Ni-NTA biochemical purification of 6His-NEDD8^{R74K} substrates, proteome from lysates was directly digested with Lys-C, and the resulted peptides were submitted to K-ε-GG immunoprecipitation for di-glycine enrichment to the final LC-MS/MS analysis using specific parameters for PTMs mapping (**Figure 11A**). This first workflow approach (Experiment 1), gave us a total identification of ~130 NEDD8 position sites within ~100 proteins. Secondly, we performed another approach (Experiment 2) to enhance the identification by decreasing the complexity of Lys-C digested peptides by fractionating them with strong cation-exchange chromatography (SCX) using three different consecutive elution buffers (pH 4, 7 and 11 respectively). Fortunately, this second approach increased the number of identified sites for NEDD8^{R74K}-modified proteins (~270 sites within ~180 proteins) by almost two-fold. In a third approach (Experiment 3), we used double the amount of starting proteome material for Lys-C digestion and splitted in two separate diGly enrichments. The resulted diglycine containing peptides were combined and then fractionated into six different SCX fractions (elution buffers with pH 4, 5, 6, 7, 8.5 and 11 respectively). Indeed, the final LC-MS/MS analysis from this experiment showed us that we could enrich by nearly more than 5 times the NEDD8 identification profile from ~130 at the beginning to ~740 sites within ~410 proteins. Finally, we repeated the 3rd optimized approach (Experiment 4) not only to estimate the reproducibility of the based enrichment workflow but also to extend the number of total unique NEDD8 identification sites. The duplicate could identify around 520 sites in ~300 conjugated proteins of which 243 sites (24%) and 209 proteins (40%) were common between replicates but, importantly,

around 280 sites were specific to this new result. The comparison of the duplicates indicates that the degree of reproducibility can fluctuate from deviations in the manipulation of samples to reactants and mass spectrometer efficacy. In general, by matching all the analyses together from the consecutive optimizations, we successfully improve the strategy by identifying for the first time an extensive total of ~1,100 NEDDylated sites in ~530 proteins (**Figure 11B**). From this, we identified a total of ~4,500 protein groups of which ~530 (11%) contained the ~1,100 unique Lys-diGly motifs for NEDDylation. To ensure accurate data, we observed that 87% of our identifications (around 908 sites in 494 proteins) had a localization probability above 0.75. In conclusion, this study confirms that the double parallel antibody-based enrichment in combination with NEDD8^{R74K}-modified peptide fractionation increase the identification of NEDDylated sites in substitution of the nickel affinity purification step performed previously.



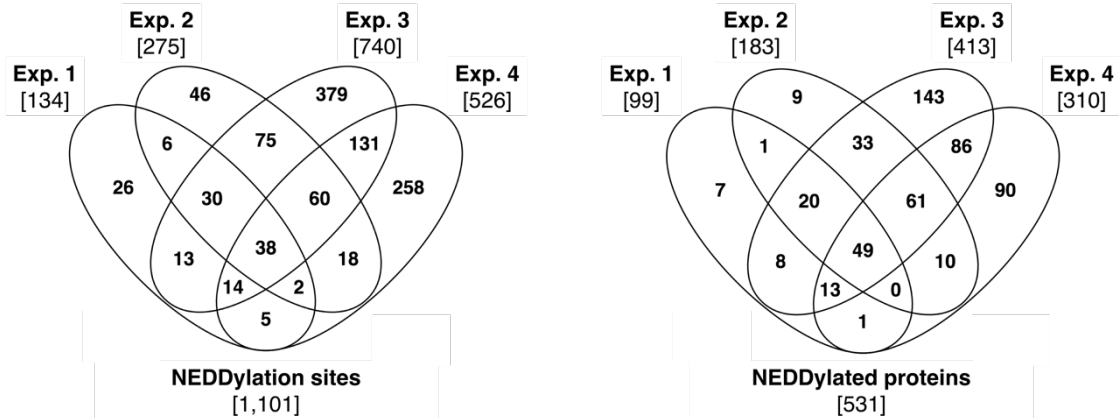
B

Figure 11. Workflow optimization of NEDD8^{R74K} strategy identified over 1,000 sites upon NEDP1 depletion

(A) Schematic overview of the NEDD8^{R74K} workflow optimizations and their individual MS results. For the first approach (Exp. 1), lysates from stable HCT116 cell line expressing 6His-NEDD8^{R74K} were prepared in native conditions instead of denaturing conditions. 6His-NEDD8^{R74K} conjugates were not purified by nickel affinity chromatography but, in contrast, the whole proteome from lysates was precipitated with acetone. The subsequent procedures consisted in the digestion with Lys-C endopeptidase and the diGly-based immunoprecipitation enrichment of NEDDylated peptides for the final high-resolution LC-MS/MS analysis. In the second approach (Exp. 2) the resulted enriched peptides from K-ε-GG immunoprecipitation (IP) were separated in three different pH-based fractions from strong cation- exchange (SCX) chromatography. For the third approach (Exp. 3), the Lys-C peptides were divided and subjected into two parallel IP for K-GG motif enrichment and further distribution into six eluted fractions from SCX columns. A final replicate from 3rd approach (Exp. 4) was performed. **(B)** Venn diagram representations show the number of NEDDylated sites and proteins discovered in relation to the different workflow approaches. Over 1,000 NEDDylation sites in 530 proteins were identified in total.

NEDD8 modifies in multiple sites and regulate functionally interconnected proteins

Fortunately, our prior analysis led us the identification of more than 1, 000 sites for NEDD8 which therefore can be used to compare with established mass spectrometry analysis for other PTMs such as for Ubiquitination and SUMOylation sites. For example, recent Ubiquitin and SUMO lysine modification analyses have demonstrated their ability to modify proteins in multiple sites (I A Hendriks et al., 2014; Ivo A Hendriks, D'Souza, Chang, Mann, & Vertegaal, 2015;

Kim et al., 2011; Tammsalu et al., 2014; Xu et al., 2010). Normally, NEDDylated proteins have shown the same reduce electrophoretic mobility as Ubiquitinated and SUMOylated proteins profiles which may indicate that conjugates also contain multiple modifications sites for NEDD8. In this study, we observed that approximately 40% (~230) of total NEDD8^{R74K} identified proteins had more than one NEDDylation site, confirming a multisite modification of NEDD8 on proteins (**Figure 12A**). Examples of >10 multisited proteins found in our data were BCLAF1 (Bcl-2-associated transcription factor 1), AKAP12 (A-kinase anchor protein 12), TCOF1 (Treacle protein) and AHNAK (Neuroblast differentiation-associated protein AHNAK) which share a large molecular weight above 100 kDa, explaining the high frequency of modification. Moreover, we analyzed the Gene Ontology (GO) functional annotations of 494 NEDD8^{R74K}-modified proteins with GlyGly site localization probability above 0.75 of the new optimized data set derived from NEDP1 KO cells. Related with our previous data, the GO annotation for KEGG pathways revealed statistical overrepresentation of proteins involved in complexes that regulate gene expression, translation, protein folding, actin cytoskeleton organization, mitotic cell cycle regulation and DNA replication, recombination and repair (**Figure 12B**). Accordingly, several ribosomal, annexin and heat shock proteins, RNA helicases, ribonucleoproteins and zinc finger proteins were frequently identified in our analysis with various NEDD8 modifications sites. These results suggest that NEDD8 and NEDP1 directly regulate different groups of functionally related proteins apart from cullins. In fact, cullin family members were not part of the significant substrates identified in our data. Among all the identified sites with great localization probability parameters, we only found the modification of the consensus reported Lys712 on Cullin-3 (Bennett, Rush, Gygi, & Harper, 2010; Pan et al., 2004). The low abundance identification of cullin proteins in this study can be explained by the limited role of NEDP1 to de-NEDDylate cullins (Chan et al., 2008). Another reason could be the minimal NEDD8 modification levels for cullin sites in comparing to its number of lysine residues (e.g. 68 lysines within 776 sequence length of Cullin-1), or by the possible formation of inaccurate MS-detectable peptides after Lys-C digestion.

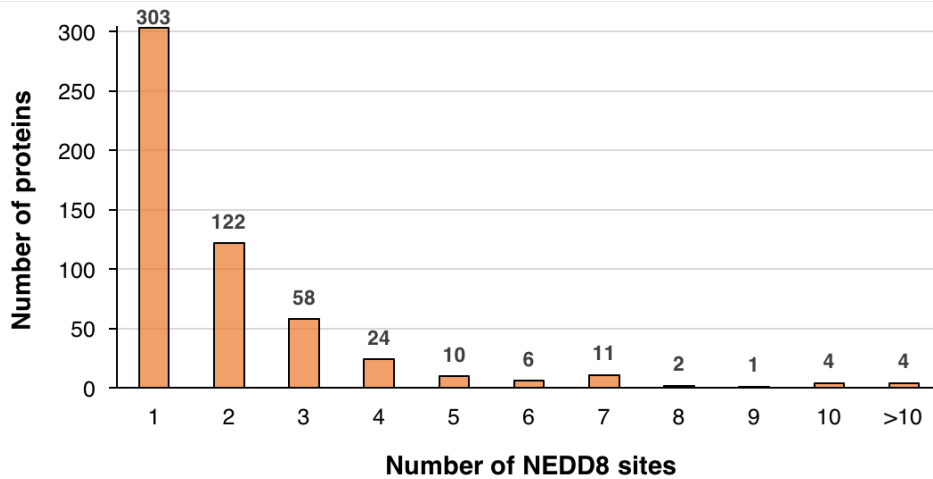
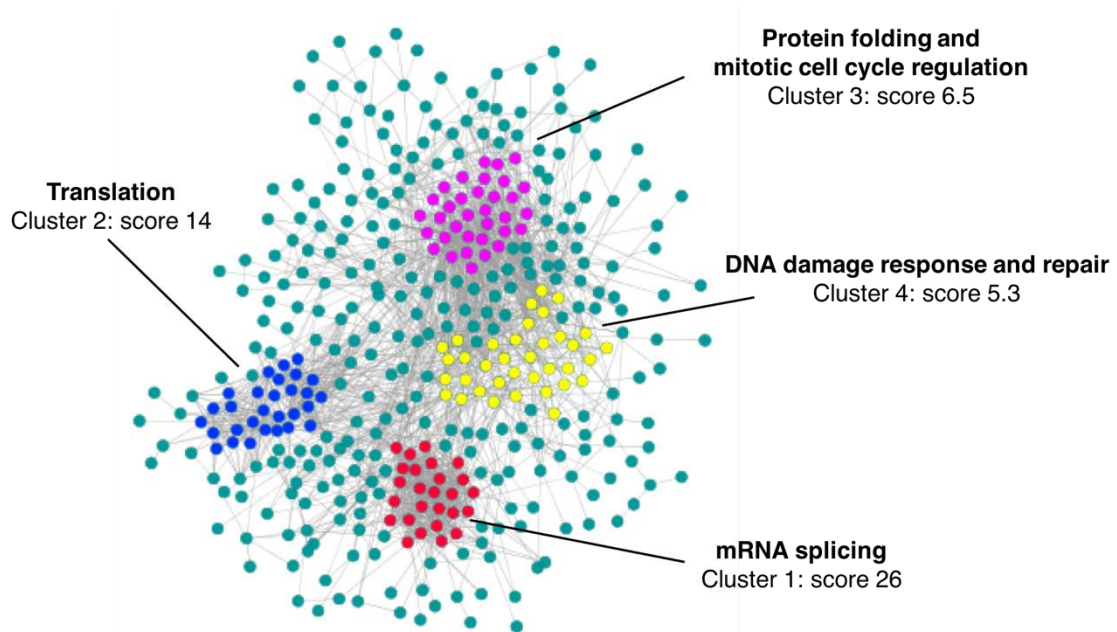
A**B**

Figure 12. Evidence for multisite modifications by NEDD8 on highly interconnected functional networks of proteins

(A) Graph of the number of NEDD8^{R74K}-modified sites per protein in NEDP1 depleted cells. **(B)** Overview of STRING network analysis and Cytoscape visualization of all identified NEDDylated proteins which had site localization probability above 0.75. STRING interaction confidence of 0.7 or greater was applied for data settings. MCODE was used to extract the most highly interconnected functional clusters from the network. The four highest-scoring MCODE clusters are indicated in different colors with their respective GO functional annotation.

Site modification crosstalk between NEDD8, ubiquitin and SUMO pathways

NEDDylation is known to crosstalk with ubiquitination during the proteotoxic stress response, proposing a competition of NEDD8 with Ubiquitin for acceptor lysines in target proteins (Leidecker et al., 2012). In this work, we observed that SUMO is also overlapping with NEDDylated and ubiquitinated proteins upon proteotoxicity (see **Figure 4**). However, the extent of this crosstalk is currently unclear. To investigate the potential overlap between NEDD8-acceptor lysines with ubiquitin but also with SUMO2 lysine modifications, we extracted all known human MS-identified ubiquitination and SUMOylations sites from PhosphoSitePlus (PSP) database and compared with our NEDD8^{R74K} data. 53% (~601) and 10% (~110) of the NEDD8^{R74K}-identified sites overlapped with the ubiquitin and SUMO2 modified sites respectively (**Figure 13A**). In total, 635 NEDDylated sites (55%) overlapped with ubiquitinated and SUMOylated sites in which 76 sites (12%) were common between the three modifiers, 525 sites (83%) were shared exclusively with known sites of ubiquitination and only 34 sites (5%) among the known SUMOylation sites. As expected, these observations suggest that ubiquitination and, less commonly, SUMOylation can occur on the same residues as NEDDylation.

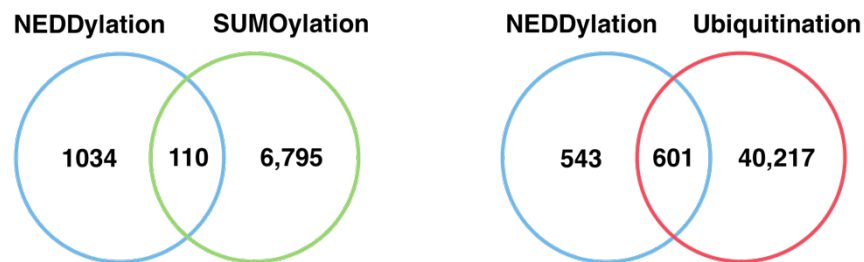
One of the main modification observed in every analysis of the NEDD8 R74K site identification in NEDP1 depleted cells was the NEDDylation of endogenous NEDD8, confirming the high regulation of NEDP1 in the formation of polyNEDD8 chains (see **Figure 8**). NEDD8 has 9 internal lysines in comparison to the 7 present in ubiquitin, which in principle indicates the potential of NEDD8 to form more diversity polyNEDD8 chain topology. Here, we were able to identify the modification of 7 lysines of NEDD8 (K6, K11, K22, K27, K33, K48 and K54) (**Figure 13B**). Of interest, NEDDylation of Lys6 and Lys11 were previously found in our laboratory under proteotoxic stress using a different MS strategy (Leidecker et al., 2012). Importantly, also in this study we observed the NEDDylation of ubiquitin under NEDP1 depletion by identifying the modification in all their lysines (K6, K11, K27, K29, K33, K48 and K63) (**Figure 13B**), this suggest that the regulation of mixed NEDD8-ubiquitin chains can be regulated by NEDP1 enzyme under stress conditions but also probably under homeostatic conditions.

Interestingly, we also found direct modification of SUMO2 by NEDD8 on lysines 11 and 33 (**Figure 13B**), confirming our previous observation of the formation of mixed NEDD8-SUMO chains in response to proteotoxic stress by heat shock (see **Figure 4**). However, the NEDD8 modification on Lys11 was observed only in our previous MS analysis under MG132 treatment (CST facility) whereas the modification on the Lys33 was only identified in the latest NEDD8 R74K strategy under NEDP1 depletion (IMB collaboration). We will need further comparative analysis to verify if the site of modification is specific depending on the stimuli in the cell. So far, the major site involved in chain formation on SUMO2 and SUMO3 is Lys11 (Tammsalu et al., 2014; Tatham et al., 2001). Nevertheless, the SUMOylation on Lys5, Lys7, Lys21, Lys33, Lys42 and Lys45 in SUMO2 has been recently reported (I A Hendriks et al., 2014; Ivo A Hendriks et al., 2015; Tammsalu et al., 2014). Additionally, novel strategies developed for SUMO2 sites identification have shown not only the SUMOylation on Ubiquitin (K6, K11, K27, K48 and K63) but also on NEDD8 (K48), suggesting a further complex nature of SUMO chain formation (**Figure 13B**). Besides, ubiquitination site analyses show that ubiquitin can modify all the lysines of NEDD8, SUMO2, and ubiquitin itself (as annotated by PhosphoSitePlus, **Figure 13B**). Unquestionably, these findings propose that the mixed-chain formation between ubiquitin and ubiquitin-like family members is more common than previously thought which suggest a higher complexity code of modification.

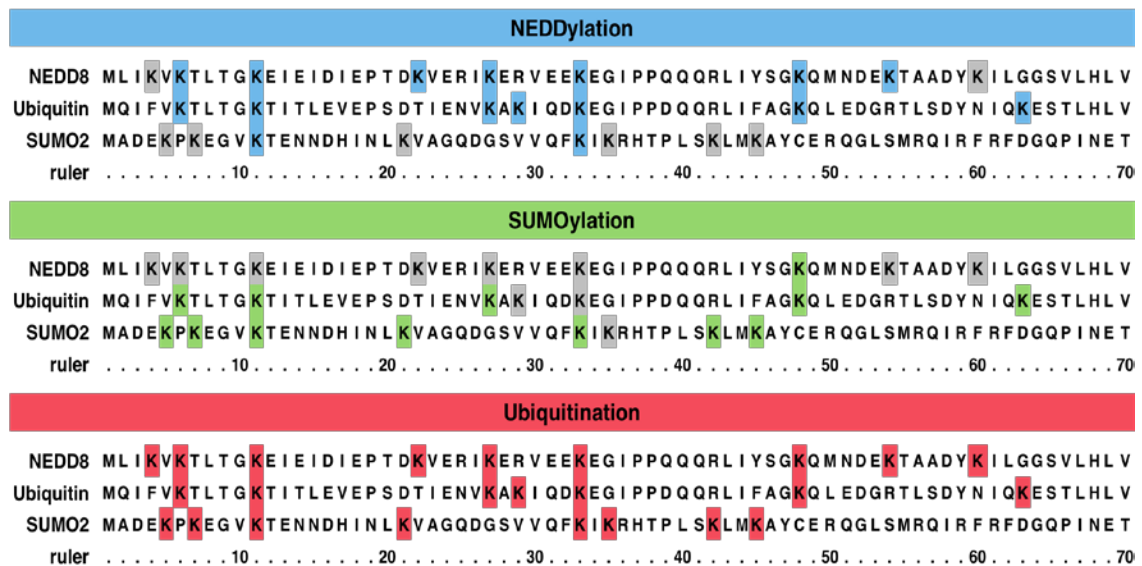
Crosstalk between NEDDylation and ubiquitination includes the direct regulation of enzymatic components of the respective pathways. For example, we could observe the modifications of UBA3, NAE1, UBE2M, UBE2F and DCN1-like proteins, which are members of the NEDD8 conjugation machinery. This observation suggests that they may undergo autoNEDDylation regulated by NEDP1 (see **Figure 8**). We also found the direct modification of the cellular factor NUB1, which, besides the de-NEDDylating enzymes, negatively regulates the protein levels of NEDD8 and NEDDylated substrates (T. Kamitani et al., 2001; Liu et al., 2013). Furthermore, we identified the modification of ubiquitin protein ligases, ubiquitin proteases and general regulators of the ubiquitination process, such as UBA1, USP5, RNF20, CAND1 and VCP (**Figure 13C-D**), indicating a broader crosstalk between NEDDylation and ubiquitination pathways apart from

cullins and proteotoxicity-associations. Evidence of SUMOylation of components of the ubiquitination machinery (I A Hendriks et al., 2014; Tammsalu et al., 2014) suggest that these ubiquitin family members directly regulate each other. However, until now no evidence of direct modification has been shown between enzymatic components for NEDDylation and SUMOylation which indicates a less interconnection between their pathways.

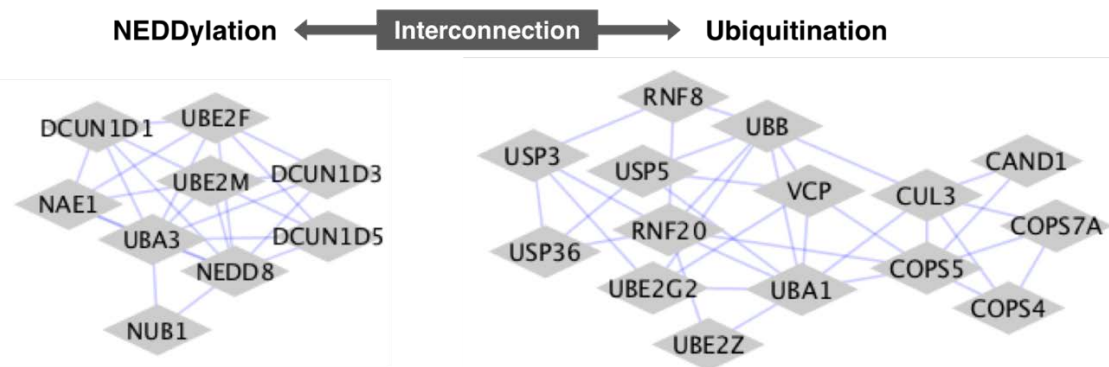
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B



C



D

NEDDylation-related network		Ubiquitination-related network	
Protein	Sites	Protein	Sites
DCUN1D1	K11, K171	CAND1	K14, K20, K60
DCUN1D3	K58	COPS4	K20
DCUN1D5	K8, K23	COPS5	K11
NAE1	K6	COPS7A	K20
NUB1	K159, K167	CUL3	K712
UBA3	K398, K409, K434, K458	RNF20	K716
UBE2F	K7, K9	RNF8	K266
UBE2M	K3, K8, K36, K45	UBA1	K43
		UBE2G2	K7
		UBE2Z	K113
		USP3	K145
		USP36	K666
		USP5	K360
		VCP	K18, K20

Figure 13. Crostalk between NEDD8, SUMO2 and ubiquitin modifications

(A) Venn diagrams showing the overlap of the identified NEDDylated lysines in this study with the respective ubiquitinated and SUMOylated lysine sites identified in other studies using MS as annotated in the PhosphoSitePlus database. (B) Sequence alignments of the reported and newly identified lysines (K) modified by NEDDylation (blue), SUMOylation (green) and Ubiquitination (red) on human NEDD8, SUMO2 and Ubiquitin, respectively. The lysines marked in grey are yet not identified as NEDDylated, SUMOylated or Ubiquitinated sites. The ruler indicates until 70-amino acid length of the sequence of the respective proteins. (C) NEDDylation and ubiquitination are highly interconnected. STRING-network analysis and Cytoscape visualization shows that potentially NEDD8 directly modifies and regulate proteins related to the NEDDylation pathway itself but also to more diverse members of the Ubiquitination pathway beyond cullins. (D) Table representation indicating the NEDDylated proteins and sites identified in our NEDP1 KO cells (localization probability above 0.75) to the respective NEDDylation or ubiquitination-related network.

Evidence of NEDDylation consensus motif on non-cullin substrates

Cullins are the best-defined NEDD8 substrates. NEDDylation on cullins (CUL1, 2, 3, 4A, 4B, 5, 7 and PARC), is known to occur on the carboxyl-terminal consensus motif (IVRIMKMR) (Bennett et al., 2010; Enchev et al., 2014; Pan et al., 2004) (**Figure 14A**). With the identification of new NEDD8 sites, our dataset provides an opportunity to obtain further insight into NEDDylation sequences to investigate a possible NEDD8 modification motif for non-cullin substrates. Here, we selected NEDP1 regulated NEDD8^{R74K} sites with a GlyGly- ϵ -Lys localization probability above 0.9 (758 sites) to examine the NEDDylated protein sequences using pLogo (O'Shea et al., 2013) and iceLogo (Colaert et al., 2009) for statistical analysis. Overall, both analyses revealed a significant overrepresentation of (i) Met six amino acids C-terminal to the conjugated Lys (position -6), (ii) of residues Ala or Asn at position -2, (iii) of the hydrophobic residue Leu at position -1, (iv) of residue Gly at position +1, (v) of residue Pro at position +2 and, (vi) the acidic residue Asp at position +5. In contrast, NEDDylated peptides were deficient in histidines, arginines and, most notably, cysteines (**Figure 14B**). This observation of reduced frequency of cysteines around modified lysines is also consistent with ubiquitinated and SUMOylated regions, which is proposed to prevent the formation of thioesters between the modifiers and target proteins (I A Hendriks et al., 2014). Interestingly, the NEDDylated regions were enriched with lysines and are therefore easily exposed for modification. The only particular similarity shared with the cullin-consensus motif is certain tendency to have a Lys residue in position +3. In conclusion, the sequence context analysis of NEDD8 modifications sites exhibit a potential existence of a consensus motif for cullin-independent NEDDylation.

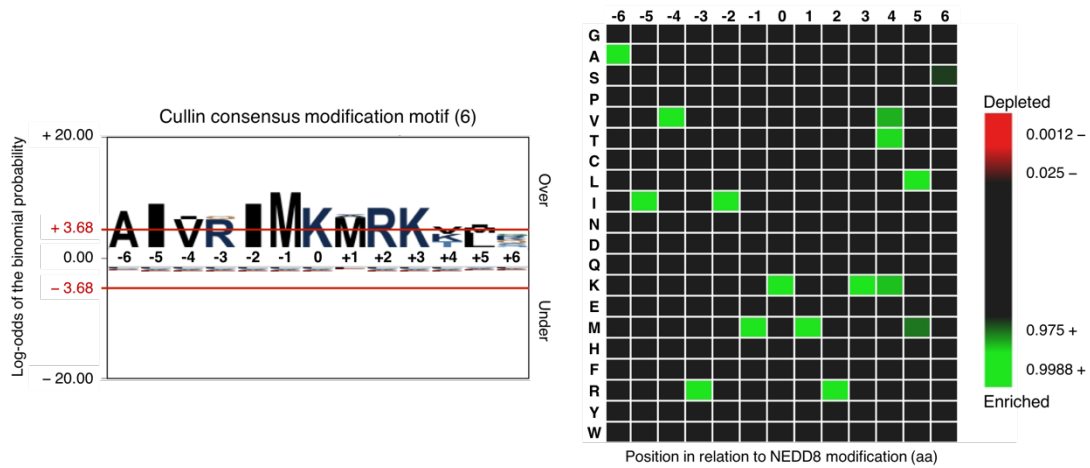
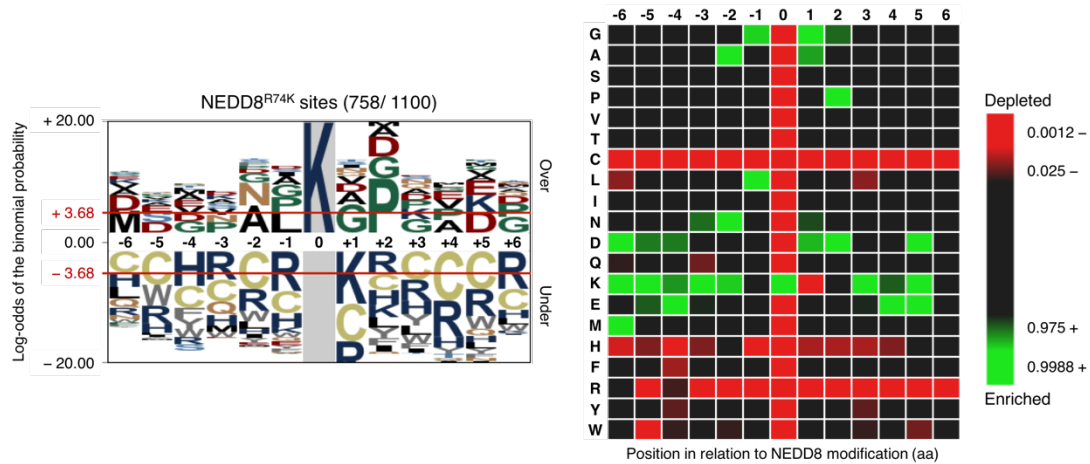
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Figure 14. Sequence analysis of NEDD8^{R74K} – modified peptides

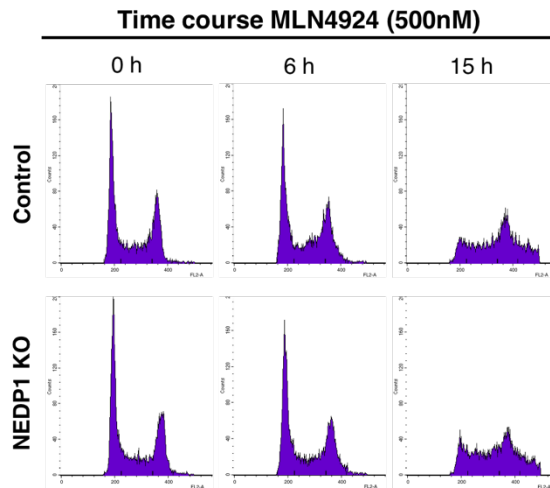
Sequence logo (pLogo) and heat-map graphs (iceLogo) of amino acid sequence conservation surrounding NEDD8-modified sites. **(A)** Sequence representation of the reported cullin consensus motif for NEDDylation from 6 members of the cullin family (CUL1, 2, 3, 4A, 4B and 5) **(B)** Sequence representation of the analysis of 758 NEDD8^{R74K}-modified sites (from the ~1,100 total identified) which had localization probability above 0.9. Threshold values of 3.68 ($P < 0.05$) are shown in red and marked with red horizontal lines for logo graphs.

Insight into the cullin-independent role of NEDD8 in DNA damage response

The functional annotation analysis of the total NEDDylated proteins found in this study upon NEDP1 depletion and proteotoxicity suggested that NEDD8 has a broader direct role, besides CRL regulation, in controlling a network of proteins involved in cellular processes such as cell cycle and DNA damage response (DDR) (see **Figure 10F and 12B**). It is well established that an accurate and efficient DDR requires the recruitment of post-translational modifications on a complex group of proteins. Ubiquitin and SUMO have important functions in the cellular response to DNA double-strand breaks (DSBs). Moreover, the crucial role of NEDD8 in DNA repair processes is now emerging (Brown & Jackson, 2015). Unquestionably, the irreversible NEDDylation inhibitor MLN4924 have shown how the prolonged inhibition of CRL activity and subsequent accumulation of CRL substrates, specially the stabilization of CDT1 through CRL4^{CDT2} inhibition, leads to S-phase arrest and DNA re-replication resulting in DNA damage and eventual cellular apoptosis (Blank et al., 2013; Lin, Milhollen, Smith, Narayanan, & Dutta, 2010). Proliferating cell nuclear antigen (PCNA) is an essential replication protein, which also coordinates multiple DNA damage response pathways. Exposing cells to DNA-damaging agents such as ultraviolet (UV) irradiation commonly results in the modification of PCNA by ubiquitin or ubiquitin-like modifiers including SUMO and ISG15, which eventually regulates the recruitment of different proteins to the stalled replication fork (Cipolla et al., 2016). Here, we identified PCNA as a potential protein directly modified by NEDD8. Based on this result, we tested if we could find specific differences in PCNA modification during UV-induced DDR in our NEDP1 depleted cells. First, we observed that NEDP1 KO cells are deficient in both mono and poly-modify PCNA during DNA damage response when cells are arrested in S-phase and having DNA re-replication from prolonged treatment (15 hours of 500nM) with the inhibitor MLN4924 (**Figure 15A**). After 6 hours of UV recovery, PCNA displayed high levels of poly-modification, while in NEDP1 depleted cells this modification was significantly decreased (**Figure 15B**). Secondly, when we blocked cells in G1/S-phase using double-thymidine treatment (2.5 mM) and, subsequently, expose cells to UV irradiation (10 J/m²) we obtained the same reduced modification pattern of PCNA in the NEDP1 depleted cells. The lower levels of mono and poly-modification of

PCNA were observed during the late DNA damage response (between 9 and 12 hours after exposure). This indicates a potential role in the repair process. Interestingly, NEDP1 expression increases during the response to DNA damage especially from 6 hours after UV irradiation which remains stable until 24 hours when cells are recovered from damage. (**Figure 16A**). In addition, FACS analysis demonstrated that the fraction of cells in G1 decrease drastically after 9 hours of exposure when compared to the control cells. In contrast, the fraction of NEDP1-depleted cells in G2 increase greater than in NEDP1 expressing cells, more notably, during the late DNA damage response. This observation suggests that the NEDP1-depleted cells resume the cell cycle faster than the wild-type cells, indicating also that NEDD8 might have a direct role in cell cycle checkpoint regulation after DNA damage (**Figure 16B-C**). In agreement with our findings, we validated that NEDD8 directly modifies PCNA and histone proteins (H2B) regulated by NEDP1, which are essential proteins in the DNA damage response after DSBs (**Figure 16D**). Further analyses are necessary in order to elucidate the function of NEDP1 in regulating the NEDDylation of CRL-independent substrates important for cellular stress responses such as during DNA damage.

A



B

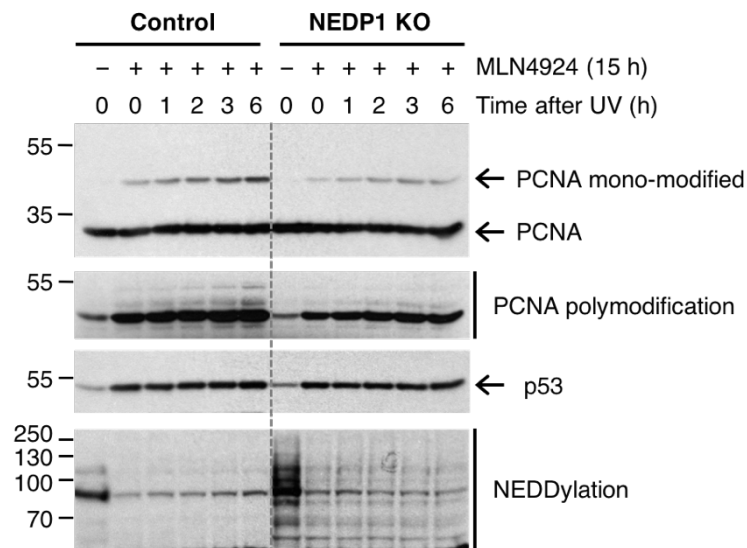
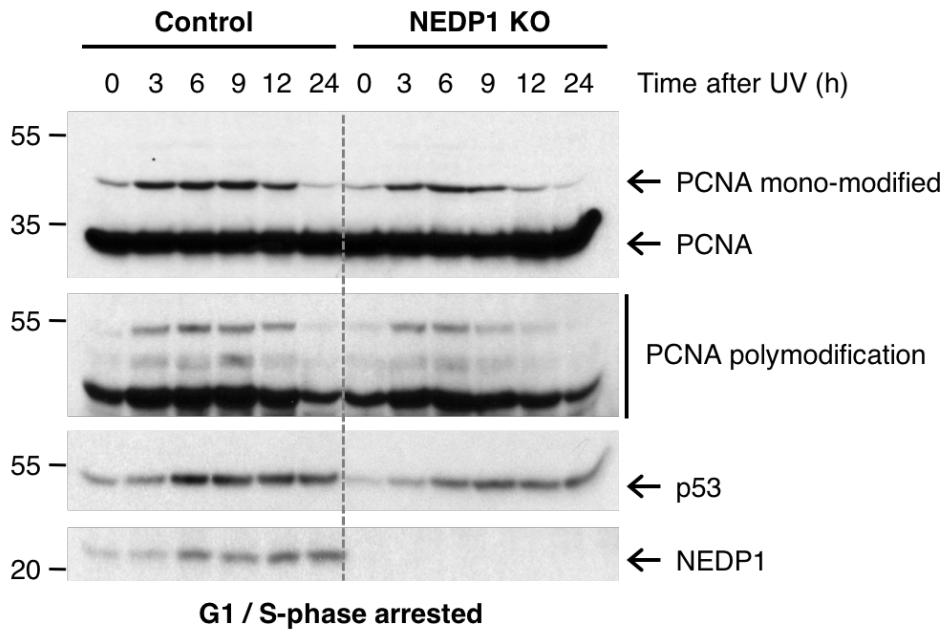


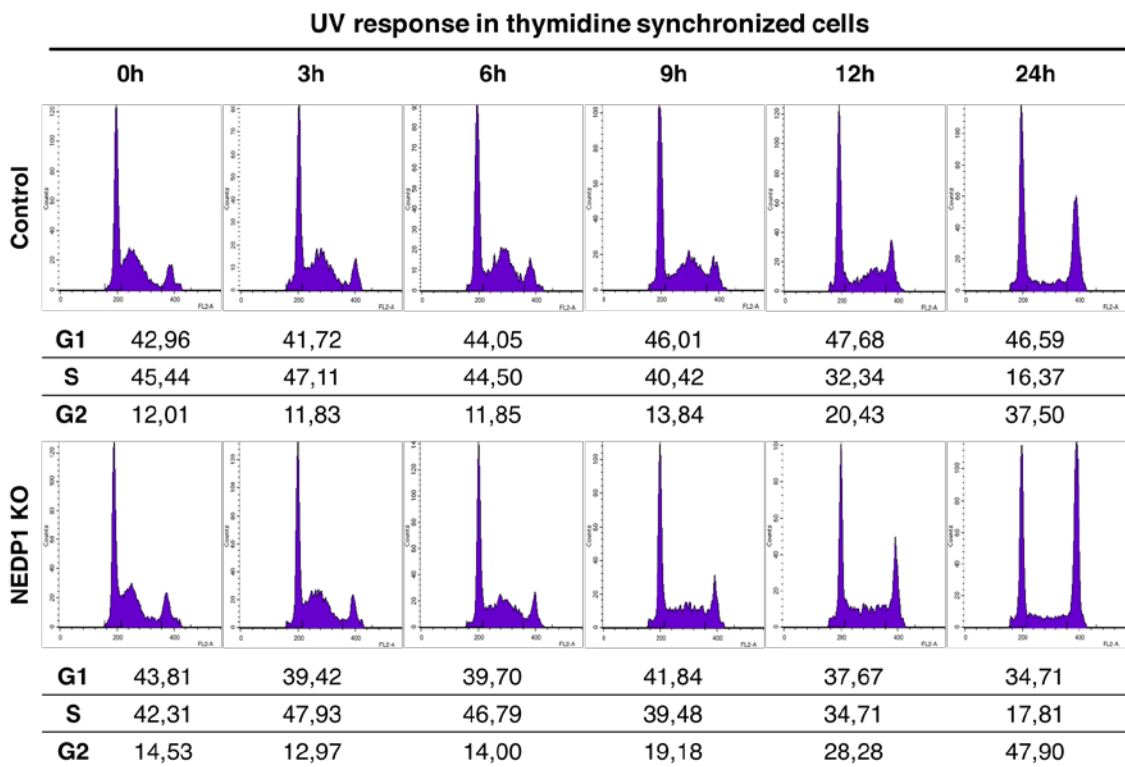
Figure 15. NEDP1 regulates the post-translational modification of PCNA during DNA damage response

(A) Flow cytometric analysis of DNA content of control and NEDP1 depleted HCT116 cells which shows the S-phase arrest and re-replication phenotypes resulting after 15 hours of MLN4924 treatment. **(B)** Control and NEDP1 depleted cells pre-treated with MLN4924 (500nM) for 15 hours and subsequently exposed to ultraviolet (UV) irradiation (15 J/m^2) were collected for the indicated times for immunoblot analysis with PCNA, p53 and NEDD8 antibodies respectively.

A



B



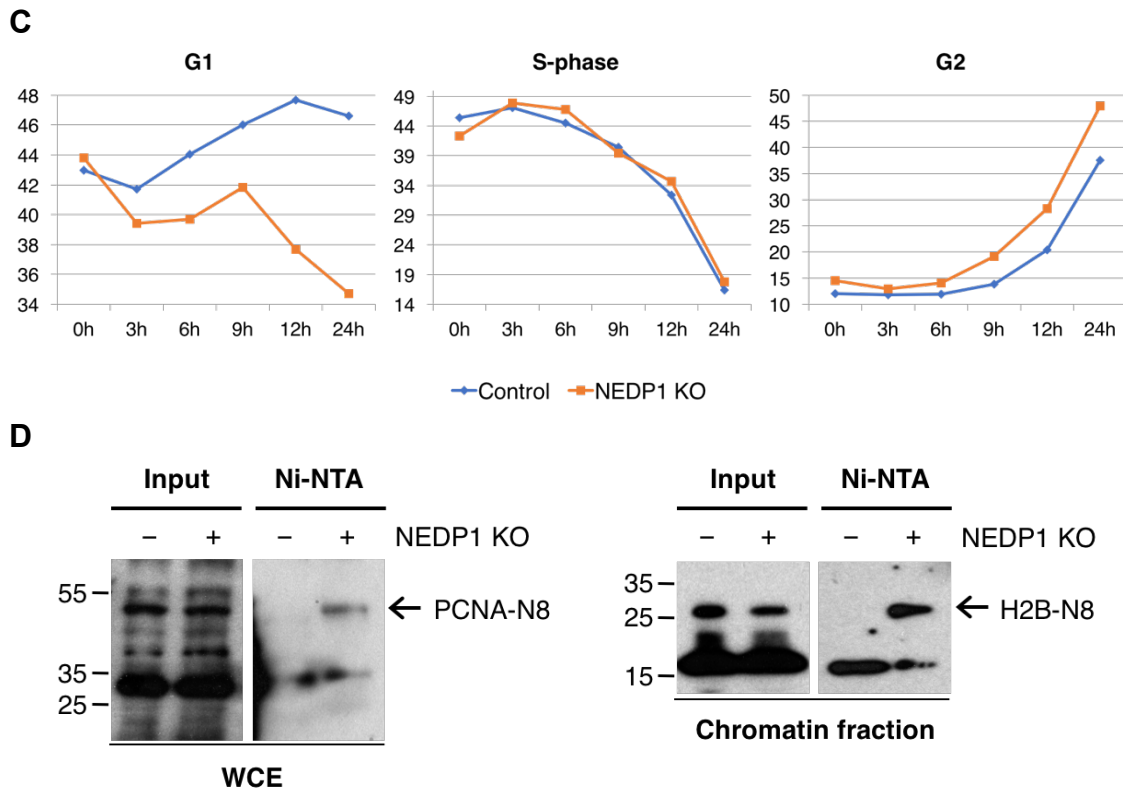


Figure 16. NEDP1 responds to DNA damage affecting cell cycle and the modification of PCNA in S-phase arrested cells

(A) Control and NEDP1 depleted HCT116 cells were double treated with thymidine (2.5 mM) and exposed to ultraviolet (UV) irradiation (10 J/m²). Cells recovering for the indicated times were collected and lysates were blotted for PCNA, p53 and NEDP1, respectively. (B) Propidium iodide-stained cells from each time point were analyzed by flow cytometry. (C) Schematic comparison of the percentage of cells in the respective cell cycle checkpoint (G1, S-phase and G2) from the different time points presented in the control and NEDP1 depleted cells after double-thymidine block and UV recovery. (D) Western blot analysis of whole cell extract (WCE) and chromatin extract from wild-type or NEDP1-depleted HCT116 cells stably expressing 6His-NEDD8^{WT} construct. 6His-pulldown (Ni-NTA) was performed, afterwards PCNA (left) and H2B (right) were detected for NEDD8 (N8) modification.

DISCUSSION

Atypical NEDDylation: the NEDD8 response to proteotoxic stress

The NEDD8 proteome is mechanistically and functionally divided into two groups: the atypical (UBE1) and canonical (NAE) dependant. In this study, we developed SILAC-based proteomic strategies to characterize the global dynamic of the human NEDDylome according to these distinct modes of activation. We performed a lentiviral infection approach to stable express a 6His-NEDD8 transgene at endogenous levels, assuring the NEDD8: ubiquitin balance during homeostasis. In consequence, our studies on the NEDDylation state changes lead to a valid biological response. To investigate the atypical NEDDylation response, we applied hyperthermic stress and isolated the 6His-NEDD8 conjugates for MS analysis. We observed that heat shock strongly increased the atypical NEDD8 modification of ubiquitin and SUMO 1/2 modifiers. Hence, our findings suggest that the crosstalk between NEDD8, ubiquitin, and SUMO is the main phenomenon throughout proteotoxic stress. Consistent with the knowledge that NEDD8 is activated by the ubiquitin machinery during proteotoxicity leading to mixed NEDD8-ubiquitin chain formation (Hjerpe, Thomas, Chen, et al., 2012; Leidecker et al., 2012), it was not surprising to see the great increase of ubiquitin modification by NEDD8 upon heat shock (HS). However, SUMO1/2 proteins appeared even more NEDDylated upon this condition. This observation highly correlates with the fact that SUMO has a vital role for thermal stress response by substantially forming polySUMO chains (Častorálová et al., 2012; Golebiowski et al., 2009). Hence, our findings imply that mixed NEDD8-ubiquitin but also NEDD8-SUMO chain are extensively forming in proteotoxic conditions driven by atypical NEDDylation.

One of the interrogatives that are not entirely understood is about the precise cellular mechanism of atypical NEDDylation. Previous studies indicate that the ubiquitin-activating enzyme UBE1 has reduced kinetics to activate NEDD8 in vitro in comparison to the NEDD8-activating enzyme NAE (Whitby et al., 1998). Accordingly, the increased levels of NEDD8 over ubiquitin is enough to trigger

this activation in vivo (Hjerpe, Thomas, Chen, et al., 2012; Hjerpe, Thomas, & Kurz, 2012). Besides, it is well documented that the free ubiquitin pool is limited in the cell. Although ubiquitin is highly expressed, it seems that the majority is either conjugated or in the process of being conjugated for the regulation of proteolysis and other cellular processes (Dantuma, Groothuis, Salomons, & Neefjes, 2006; Dantuma & Lindsten, 2010). Thus, proteotoxic stress results in a dramatic depletion of the levels of free ubiquitin due to the increase in the accumulation of polyubiquitinated proteins. We assume that the ubiquitin limiting factor is the most important mechanism that initiates the NEDD8 atypical activation but also that there must be other determinants that cooperate for this response to occur. In the laboratory, we examined that the increase of NEDDylation upon proteotoxicity is not related to newly synthesized NEDD8 (data not shown). This observation indicates that upon proteotoxic stress the cell is changing the fate of the existent free NEDD8 pool by (i) increasing its levels, (ii) reducing the competition with ubiquitin for the E1 activating enzyme and finally, (iii) modifying substrates through the ubiquitin machinery. Interestingly, we also identified the NEDD8 E1 regulatory subunit NAE1 (APPBP1) as one of the NEDDylated substrates increasing during HS response. In contrast, members of the cullin family significantly decrease in NEDDylation upon HS. It appears that this thermal stress condition induces the autoNEDDylation of NAE1 which could result in a reduced activity of the NAE enzyme. Therefore, NEDD8 is not efficiently activated by its cognate pathway and compromises the basal modification of cullins. Probably, the limiting modification of cullins is increasing the levels of free NEDD8 but also is helping to diminish the ubiquitination of CRL substrates and in consequence, ubiquitin is used for other stress-related purposes. Likewise, we identified the increase in NEDDylation of the histone protein H2B upon heat shock. Our finding coincides with the reduction of the levels of monoubiquitinated histone H2A in thermally shocked cells observed in previous studies (Dantuma et al., 2006). Interestingly, similar results have been shown for canonical SUMO target proteins including RANGAP1 which are no longer SUMOylated in response to stress (I A Hendriks et al., 2014). Together with the induction of ubiquitin synthesis and the disassembly of ubiquitin chains (Crosas et al., 2006; Fornace Jr, Alamo Jr, Hollander, & Lamoreaux, 1989; Kimura et al., 2009), it seems that the atypical NEDDylation is another important

cooperative function for increasing ubiquitin availability when is highly demanded such as during proteotoxic stress. Given that the UPS dysfunction induced upon proteotoxicity is related exclusively for the ubiquitin-dependent substrates (Kelly, Vanslyke, & Musil, 2007; Salomons et al., 2009), the limitation of free ubiquitin has a significant impact on the cell response and recovery after stress. Furthermore, time is another ultimate critical factor to overcome stress. The proteotoxic stress-induced “replacement” of ubiquitin with NEDD8 might be a favorable cellular mechanism because (i) is a fast and efficient process which take advantage of the closest similarity of the modifiers, (ii) the cell saves energy by shifting the activation through one pathway machinery, (iii) is reversible and gives the possibility to recover from this atypical situation.

Outcome of the atypical NEDDylation into the ubiquitin code

Given that NEDD8 can cross the barrier of the ubiquitin enzymes during proteotoxic stress, this bears a fascinating enigma concerning the potential biological consequences. Either atypical NEDDylation is a new signal necessary for stress response factors, or simply NEDD8 is mimicking ubiquitination. The truth is that proteins which are usually ubiquitinated, suddenly they are instead getting NEDDylated and this potentially can change the signaling fate of the substrates. Significantly, in our SILAC results, we observed that NEDD8 is altering the topology of both polyubiquitin and polySUMO chains. This NEDD8 "editing" is a critical event giving the existence of adaptor proteins which use specific binding domains to recognize these modifications for essential cellular functions (Husnjak & Dikic, 2012). Therefore, the formation of mixed NEDD8-ubiquitin and NEDD8-SUMO chains are likely (i) affecting the rate of recognition, (ii) abolishing the binding or (iii) creating a new code for distinct downstream effects. A well-established link between different modification pathways and binding recognitions is the evidence of the ubiquitin-SUMO crosstalk. SUMO-targeted ubiquitin ligases (STUBLs) attach ubiquitin chains to proteins that are already polySUMOylated (Geoffroy & Hay, 2009; Prudden et al., 2007; Uzunova et al., 2007). The SUMOylation is a signal for the recruitment of the STUBLs through the binding affinity with SUMO-interacting motifs (SIMs), then the resulted ubiquitination mediates proteasome degradation of the modified

proteins. These type of ligases, such as RNF4, are implicated in several signaling pathways, including protein quality control and cellular responses to arsenic poisoning and DNA damage (Guo et al., 2014; Tatham et al., 2008; Yin et al., 2012). By having atypical NEDDylation, if NEDD8 modifies a SUMOylated protein prior the recognition by a particular STUBL thus is possible that it will be no longer a target for ubiquitination. Alternatively, if a ubiquitin ligase like the STUBLs adds mixed NEDD8-ubiquitin or polyNEDD8 chains instead of polyubiquitin chains, then the further recognition and degradation by the proteasome can be somehow affected or recognized by different cellular surface receptors. Indeed, it has been shown that NEDD8 binds more weakly to the proteasomal ubiquitin receptor Rpn10 (Singh et al., 2012). Moreover, several NEDD8-interacting proteins have been reported such as the NEDD8 Ultimate Buster 1 (NUB1) and its splicing variant NUB1L. NUB1 contains UBA domains that bind to NEDD8 and, therefore promotes the transfer of NEDDylated substrates for proteasomal degradation (T. Kamitani et al., 2001; Kito, Yeh, & Kamitani, 2001; Tanaka, Kawashima, Yeh, & Kamitani, 2003). However, unlike for ubiquitin and SUMO, not yet specific binding motifs for NEDD8 has been identified. In fact, some of the domains of NEDD8-interacting proteins have shown affinities for NEDD8 in the similar μM range as for ubiquitin (Enchev et al., 2014). Thus, our findings confirm the high crosstalk and complexity regarding the ubiquitin-like proteins modifications, especially between NEDD8 and ubiquitin, which remain poorly understood. Further studies are needed to examine enzymes, substrates, interfaces with distinct specificities and functions for a particular type of modification of the expanded ubiquitin code. Our SILAC approach on atypical NEDDylation provides reliable information on the outcome of NEDD8 proteome during stress. Nevertheless, it does not reveal the form of linkage of the target protein. Previous diglycine remnant-base quantitative proteomics upon proteasome inhibition have shown that this mixed NEDD8-ubiquitin modification is primarily occurring via the K48 linkage which correlates for proteasomal degradation (Kim et al., 2011). For future research, it will be interesting to explore the composition and form of linkages in chains topology leading by stress responses.

Role of NEDD8 in nuclear aggregation during proteotoxicity

Proteins face constant structural dynamic and their proper integrity is pivotal for cellular function and survival. Protein folding is intrinsically an error-prone process. Consequently, cellular stress conditions induce the accumulation of misfolded proteins which exposure of hydrophobic residues leads to the formation of insoluble aggregates (Alam et al., 2017; Balchin, Hayer-Hartl, & Hartl, 2016). Protein aggregation has been linked to both cytoprotective and toxic effects depending on the stress induction, the composition of the sequestered proteins and the type of aggregation ultimately formed. Therefore, the active organized control of aggregated proteins is necessary for the maintenance of proteome homeostasis (proteostasis) (Miller, Mogk, & Bukau, 2015). Efficient protein quality control (PQC) systems ensure proteostasis toward promoting refolding by chaperones or via proteolytic degradation either through the ubiquitin-proteasome system (UPS) or autophagy (B. Chen et al., 2012). Here, we characterized that upon proteotoxic stress NEDD8 promotes the translocation of aggregates from the cytosol to the nucleus together with ubiquitin and SUMO. Interestingly, by comparing from atypical and canonical NEDDylation, we found that atypical NEDD8-mediated nuclear aggregation is mainly composed of ribosomal proteins but also of proteins related to the endoplasmic reticulum, RNA transport, aminoacyl-tRNA biosynthesis and proteasomes. Because of the protein composition within these nuclear aggregates regulated by NEDD8, we could appreciate the link with protein synthesis and proteasome proteolysis. Indeed, protein synthesis is a complex multistep process highly coordinated and regulated by the UPS, involving diverse mechanisms in several subcellular compartments, from nucleolus in the nucleus to endoplasmic reticulum in the cytoplasm (Amm, Sommer, & Wolf, 2014). Ribosomes are the central factory for protein synthesis and the ribosome biogenesis itself involves the organized assembly of ribosomal RNA (rRNA) with almost 80 ribosomal proteins (RPs) (Kressler, Hurt, & Baßler, 2010). Moreover, a major challenge is that the cell needs equimolar amounts of rRNA and RPs for the precise production of ribosomal subunits to form the ribosomes. Studies suggest that substantial excess of RPs are produce over rRNA. Therefore, once synthesized, RPs are rapidly imported into the nucleus for degradation by the UPS which careful

regulation is essential for cell physiology (Rudra & Warner, 2004; Sung et al., 2016; Warner, 1999; Wójcik & DeMartino, 2003). Still is unclear how the cell manages to recognize and degrade the accurate number of unassembled RPs. Interestingly, SUMO proteins are known to be involve in controlling the rate of ribosome biogenesis (Finkbeiner, Haindl, & Muller, 2011). NEDDylation of RPs were identified previously in the laboratory (D. P. Xirodimas et al., 2008) and in this study, we could validate the direct NEDD8 modification of ribosomal proteins (RPL7 and RPL11) upon proteotoxicity. Given that atypical NEDDylation is mainly involved in the modification of inherent unstable newly synthesized proteins (data not shown), we hypothesize that during stress the majority of NEDD8 substrates are nuclear-misfolded proteins already destined for this compartment, such as the RPs. Furthermore, because UPS proteolytic capacity is importantly needed in stress situations, it is possible that NEDD8-mediated nuclear aggregation is avoiding nuclear and, most notably, cytosolic UPS impairment. The formation of mixed NEDD8-ubiquitin and NEDD8-SUMO chains by atypical NEDDylation can potentially alter the recognition and final processing of substrates by the proteasome. The remain question is: why NEDD8 specifically promote the nuclear aggregation of proteins implicated on ribosomes, translation, and proteasomes during the stress? According to the UPS “protective” hypotesis, we can expeculate for ribosomal proteins that their excess synthesis and degradation will potentially induce rapid and severe cellular proteotoxicity by overloading proteasomes. Then, after stress either they are (i) removed by nuclear proteasomes, (ii) exported to the cytosol for UPS or autophagic degradation or (iii) eliminated by disassembly of the nuclear envelope during mitosis. On the other hand, given the ring-like structure of the aggregates observed after prolonged stress, another possibility is that this “sequestration” of proteins protects them from severe damage and provides correct function once the cell is alleviated. The reversible mechanism of active re-solubilized aggregates is supported by recent studies which, interestingly, coincide with our finding of aminoacyl-tRNA synthetase related proteins aggregating upon heat stress (Wallace et al., 2015). This process reflects a powerful adaptation of eukaryotes to recover and survive from stresses such as thermal damage. Considering that the balance of ribosomal proteins is a key factor for proteostasis, their NEDDylation and further nuclear aggregation in response to stress can

represent a mechanism which ensures an effective cellular recovery. It makes sense as an evolutionary adaptation from stress to protect and reuse ribosomes and proteasomes for overcoming de novo biogenesis. Because the assembly of these complex types of machinery is massive in time and energy consumer, their up-regulation synthesis after stress will have substantial repercussions on the expression, synthesis, and regulation of other crucial proteins which were damaged or lost during the stress. Our studies do not offer knowledge on the mechanism of aggregation driven by NEDD8, ubiquitin, and SUMO but it gives new evidence of the identity of the induced aggregated proteins during stress for further study. We hypothesized that the intrinsic nuclear localization and low thermodynamic stability of NEDD8 over ubiquitin (T. K. Kamitani et al., 1997; Kitahara et al., 2006) are partially promoting the unfolding properties of the mixed NEDD8-ubiquitin chains and thus providing an auxiliary signal for the aggregation of nuclear proteins. These scenarios on atypical NEDDylation help to explain the purpose of most eukaryotes to conserve such similar proteins to ubiquitin such as NEDD8.

NEDP1 maintains the canonical NEDDylation for cellular proteostasis

It is evident that the homeostatic activation of NEDD8 by its genuine E1 enzyme (NAE) is mainly limited to the modification of cullins for the regulation of the cullin-RING ubiquitin ligases (CRLs). Also, the cellular proteostasis sustains a steady-state of the free NEDD8 pool. Given the dynamic role of NEDD8 to enter into the ubiquitin pathway, it is evident the importance to regulate the balance of NEDD8 and ubiquitin pool to maintain cellular proteostasis. In this study, we aimed to understand the role of canonical NEDD8 conjugation beyond cullins by analyzing the function of the specific protease NEDP1 due to its noted potential to regulate non-cullin NEDDylation targets. We determined the global NEDDylation changes derived from the depletion of NEDP1 by using SILAC quantitative proteomics in CRISPR/Cas9-derived knockout cells (NEDP1 KO). Our proteomic analysis revealed the significant function of NEDP1 to regulate the NEDDylation of NEDD8 itself, and components within the NEDD8 pathway including the E1 subunits NAE1 (APPBP1) and UBA3 (UBE1C), as well as the E2 enzyme UBE2M (Ubc12). Most of the proteins we identified were also found in recent NEDP1 KO

quantitative MS analysis of NEDD8 conjugates which confirms and strongly validate these novel NEDD8 substrates regulated by NEDP1 (Coleman et al., 2017). Unquestionably, polyNEDDylation together with the depletion of free NEDD8 is part of the main consequences of NEDP1 depletion. It is unclear the biological implications of the dramatic increase of polyNEDD8 conjugation given that the chain accumulation itself is not lethal and does not lead to a prominent cell phenotype. Therefore, according to the dynamic of atypical NEDDylation during stress and the evident role of NEDP1 in maintaining NEDD8 homeostasis, it appears that the unavailability of free NEDD8 due to the accumulation of polyNEDD8 chains will be strongly crucial for cell survival upon proteotoxic stress response. This NEDD8 modification of the enzymes is by an autoNEDDylation process given their conjugation observed in an E1-E2-E3 step independent manner *in vitro* (Mergner, Kuster, & Schwechheimer, 2017). Furthermore, it could be that the outcome of polyNEDD8 modification is either (i) to compete with other post-translational modifications, (ii) to interfere with protein-protein interactions, (iii) to alter proteins functionality or (iv) to give a signal for a precise function. For example, the autoNEDDylation of the E1 NAE subunits (NAE1 and UBA3) have shown decrease transfer efficiency of the activated NEDD8 to the E2 UBE2F in plant homologs by the progressive reduction of the NAE activity (Mergner et al., 2017). Additionally, the NEDD8 auto-modification of the E2 UBE2M relatively reduced its binding with the E3 NEDD8-ligase DCN1 which shows the repercussion for protein interactions (Coleman et al., 2017). Interestingly, another common identification was observed for the decreased of only one member of the cullin family (CUL5) in NEDP1-deficient cells which NEDD8 modification is almost exclusively triggered by the E2 enzyme UBE2F over UBE2M (Huang et al., 2009). However, the decrease of CUL5 modification was not due to the autoNEDDylation of UBE2F and resulted malfunction, but instead due to the decline in the total protein levels of the UBE2F enzyme (Coleman et al., 2017). This finding suggests a different proteolytic role of the autoNEDDylation of enzymes or the polyNEDD8 accumulation of other substrates controlled by NEDP1, but the exact mechanism will need to be elucidated. In contrast with recent observations in plants (Mergner et al., 2017), our quantitative NEDP1 KO experiment suggest a preference for NEDD8 to covalently conjugate UBA3 subunit than NAE1 subunit. Therefore, we will need to validate this finding *in vitro*

and to study the inherent functional relevance of this dynamic in vivo for human cells. All this data implies that NEDP1 recovers NEDD8 moieties from conjugated proteins and, most notably, from the autoNEDDylation of the NEDD8 conjugation machinery. By inhibiting the accumulation of NEDDylated substrates, NEDP1 is not only regulating the free NEDD8 pool which limitation can be critical in a cellular stress context, but also is coordinating the proper functionality of the NEDD8 machinery to maintain the steady-state NEDDylation during homeostasis. Similar recycling functions have been observed for some deubiquitinating enzymes (DUBs) during ubiquitin-dependant degradation by the 26S proteasome (Reyes-Turcu et al., 2009). Nevertheless, it is clear that even the autoNEDDylation of the NEDD8 machinery affects their conjugation activity throughout NEDP1 depletion, this enzymes misfunction does not lead to a dramatic effect on all cullins NEDDylation. Accordingly, NEDP1 KO does not mimic the substantial effect on NAE as the inhibitor MLN4924 which blocks the activation of all CRLs. This observation reflects a substrate-specific effect of NEDP1 KO as it was shown recently through the COP9 signalosome (CSN) inhibition which likewise only affected the stability of specific CRL substrates. Interestingly, this deNEDDylation-independent activity of some CRL complexes is due to a sustained auto-ubiquitination of certain substrate recognition modules (Schlierf et al., 2016). Furthermore, our SILAC analysis identified other NEDD8 pathway-independent proteins that increase in NEDP1 KO cells such as the E3 ubiquitin-ligase UBE3C, the nucleolar protein NOP14, the glycerol-3 phosphate acyltransferase 3 AGPAT9 (GPAT3) and the zinc finger protein ZNF646. These new potential non-cullin substrates for NEDD8 are mainly nuclear involved in subcellular translocation, transcription and UPS proteolysis which strongly correlates with our observations in the overall outcome of atypical NEDDylation. In conclusion, our findings on atypical and canonical NEDDylation provide broad evidence of the biology of NEDD8 conjugation besides CRL regulation which remains largely unexplored.

NEDDylome and its specific modification sites

System-wide proteomics is a powerful tool to study the outcome of post-translational modifications (Mann & Jensen, 2003). Nowadays, MS-based

proteomics and bioinformatics advantages have reported thousands of substrates and modification sites for ubiquitination and SUMOylation (Ivo A. Hendriks & Vertegaal, 2016; Kim et al., 2011; S. a. Wagner et al., 2011). With the aim to expand the knowledge of the role of NEDDylation, in this work we developed a MS-based strategy for the large-scale identification of novel substrates and sites from NEDD8 modification. By using a 6His-NEDD8 R74K mutant and the highly selective Lys- ϵ -GG antibodies, we were able to isolate and identify for the first time more than 1,000 specific NEDDylation sites in approximately 500 conjugates from human cells. Several of the NEDD8-conjugated proteins we identified were also found in previous and recent analyses using ectopically-expressed NEDD8 wild type (Jones et al., 2008; D. P. Xirodimas et al., 2008) or NEDD8 L73P mutant (Coleman et al., 2017), which strongly confirms our findings for NEDD8 proteome. The principal advantage of our strategy is that not only we could avoid artificial effects of exogenous NEDD8 overexpression (Hjerpe, Thomas, Chen, et al., 2012), but also the replacement of trypsin for Lys-C endoprotease provides us specific diglycine remnants from NEDD8 modifications thus excluding from ubiquitination and ISG15ylation. Therefore, these features allow us to detect many common and new conjugates and sites for NEDD8 which considerably extends the understanding of the NEDDylome. As a first approach, we used Ni-NTA affinity purification to isolate 6His-NEDD8 R74K conjugates prior diglycine immunoprecipitation either from MG132 treated cells or CRISPR/Cas9-NEDP1 depleted cells. Clearly, we perceived a weaker identification of substrates and sites from the conjugates upon proteasome inhibition than NEDP1 KO. The possibilities are either the atypical NEDDylation is limited to form mixed chains with ubiquitin and SUMO and modify certain substrates such as histones and ribosomal proteins, or the R74K mutation is affecting the recognition and further activation by the UBE1 enzyme. Previous mutagenesis analysis in NEDD8 and ubiquitin have shown the existence of key residues, most notably residue 72, to be critical for the activation and discrimination between the modifiers by their respective cognate E1 enzymes (Bohnsack & Haas, 2003; Walden et al., 2003; Whitby et al., 1998). This observation indicates that the structure is essential for E1 enzymes to recognize and activate the precise substrate. Even lysine and arginine residues have positive-charged side chains we do not know in which extent the differences in

their side chains might affect the final folding of the protein. Nevertheless, the mutation itself does not seem to affect the activation and final conjugation of substrates as we could identify multiple modification sites, especially in NEDP1 KO cells. Surprisingly, we identified many shared proteins within the atypical and canonical NEDD8 activation. Although specific substrates were found from proteasome inhibition and NEDP1 depletion, the proteome biological outcome was almost the same including processes such as chromatin assembly, RNA binding, and actin regulation which correlates with the implied role of NEDD8 in nuclear functions and protein transport. Similarly, among the shared conjugates, proteins were implicated in functions including chromatin organization, RNA processing, and DNA damage response as well as the NEDDylation pathway itself. Accordingly, our findings strongly suggest the role of NEDD8 in controlling these processes in both homeostasis and stress conditions. However, more differences appeared regarding the modifications sites within the shared proteins in both conditions. Therefore, this remark opens the question if NEDD8 compete with ubiquitin for the same substrates but not necessarily for the same sites of modification. Interestingly, concerning the modification of ubiquitin and SUMO modifiers, we identified the modification of ubiquitin in K63 only under NEDP1 depletion, in contrast, the modification of SUMO in K11 was found exclusively under proteasome inhibition. The disadvantage of our study is that we can not conclude these variations in the modification sites without an accurate quantitative analysis comparing both atypical and canonical NEDDylation. Hence, one of our proximal directions to investigate is with the aim to clarify this interrogation about NEDD8 and ubiquitin crosstalk.

System-wide analysis of the canonical NEDDylation substrates and sites

In our second approach for the NEDD8 R74K workflow, we performed stringent peptide purification instead of Ni-NTA isolation upon the conjugates from NEDP1 KO cells. Our central purpose was to broader examine on the apparent unknown canonical NEDDylation substrates besides cullins. This workflow optimization greatly increased the identification of NEDD8 sites for up to 1,000. The gain of substrates and site identification allowed us to explore the NEDDylation outcome in a system-wide level through bioinformatics. We could proof the ability of

NEDD8 to modify proteins in multiple sites as it was observed previously for ubiquitin and SUMO (Ivo A Hendriks et al., 2015; Kim et al., 2011; Tammsalu et al., 2014). Unfortunately, the GG remnant procedure is unable to determine whether the multiple modifications can occur simultaneously. Therefore, it exists the possibility that during crosstalk either the modifiers compete promiscuously for the lysine residues within substrates or that they modify a protein simultaneously in different sites. Furthermore, we performed protein interaction analysis to visualize the functional interconnectivity of NEDD8 substrates. Major protein correlation was observed in processes involve in mRNA splicing, translation, protein folding, cell cycle regulation and DNA damage response. Interestingly, the majority of these functions are also highly related to SUMOylated proteins. Indeed, whereas ubiquitin is throughout the cell, NEDD8 and SUMO are predominantly nuclear proteins (T. K. Kamitani et al., 1997; Saitoh & Hinchey, 2000). In consequence, it is likely that the occurrence of crosstalk for NEDD8 and SUMO during homeostasis is greater than expected as we observed under stress conditions. Importantly, about 50% of our NEDDylation site database overlapped with the ubiquitination and SUMOylation site databases annotated in PhosphositePlus (Hornbeck et al., 2012). As expected, the greater amount of the overlapped sites corresponded to the reported ubiquitination sites. Nevertheless, this ubiquitination identified sites are underestimated given that the tryptic GG remnant technique does not exclude if it was also a NEDDylation or ISG15ylation modification site. Then, our analysis could mean that within the ~50% sites overlapping with ubiquitination sites they can be certainly modified by both proteins or exclusively by NEDD8 but not by ubiquitin. Strikingly, the other ~50% of the identified NEDDylation sites not overlapping could represent unique NEDD8 modification sites. Likewise ubiquitin and SUMO proteins, it has been shown that NEDD8 is able to form chains (Leidecker et al., 2012; Singh et al., 2012). Another apparent limitation of the GG remnant analysis is that it does not report whether the target site was mono- or poly modified by the nine possible isopeptide-based chain linkages of NEDD8. This lack of information is an important issue since our data could confirm the ability of NEDD8 to modify multiple sites in NEDD8 itself, ubiquitin and SUMO for the viable formation of polyNEDD8 and mixed chains with different linkages. NEDD8 could be forming a proximal or distal modification within the chains. In fact, previous studies have

proposed that NEDD8 could act as a chain terminator for polyubiquitin chains due to the Lys60 conserved in NEDD8 but not ubiquitin which prevents the further ubiquitination of NEDD8 (Choi, Jeon, Ryu, & Cheong, 2009; Singh et al., 2012). The formation of mixed NEDD8-ubiquitin, NEDD8-SUMO, SUMO-ubiquitin chains together with the possibility to form them via distinct linkages, generate a more complex view of the ubiquitin code. Additionally, these chains are not excluded to crosstalk with other UBLs and also with other PTMs such as phosphorylation, acetylation, and methylation. Indeed, recent analyses have confirmed the high crosstalk between ubiquitination and SUMOylation with acetylation and phosphorylation (I A Hendriks et al., 2014; Ivo A Hendriks et al., 2017; Herhaus & Dikic, 2015). Thus, determining the mechanisms, functions and dynamic of the more complicated ubiquitin code is an important direction for future research.

We could reconfirm and distinguish the modification sites for all the NEDD8 conjugating enzymes yielding autoNEDDylation due to NEDP1 depletion. For example, we identified the modification of the E1 regulatory subunit NAE1 (APPBP1) at K6 and K341 and the E1 catalytic subunit UBA3 (UBE1C) at K398, K409, K434 and K458. Strikingly, these sites are already annotated in the PhosphositePlus database of diglycine identification for ubiquitination as many other sites, except for K458 in UBA3. This observation suggests that the modification sites might be related to NEDD8 conjugation and not ubiquitin as the tryptic GG remnant can not discriminate between both. Another possibility is that certainly all these sites can be modified by NEDD8 and ubiquitin, and that potentially NEDD8 can exclusively modify UBA3 at the C-terminal site K458. We could also identify the modification sites for the NEDD8 E2 conjugating enzymes UBE2M (Ubc12) at K3, K8, K36, K45 and UBE2F at K7 and K9. The UBE2M autoNEDDylation was recently identified and characterized both in vitro and in vivo. The analyses confirm the catalytic Cys residue-dependant NEDD8 covalent conjugation of UBE2M at its extreme N-terminus (K3) which is different from its Cys active site that forms the UBE2M~NEDD8 thioester intermediate (Coleman et al., 2017; Huang et al., 2007). As observed in previous structural studies, the N-terminus of UBE2M is the region required for the interaction with the hydrophobic binding pocket of DCN1 (Scott, Monda, Bennett, Harper, &

Schulman, 2011). This finding might explain the reason why the NEDDylation of UBE2M impede the DNC1-UBE2M complex conformation (Coleman et al., 2017). Similar results have been shown by the SUMOylation of the ubiquitin-conjugating enzyme E2 K (UBE2K) at Lys14 N-termini, which blocks the interactions and further conjugation activity with the ubiquitin E1 and E3 enzymes (Pichler et al., 2005). In fact, we could also see that NEDD8 modifies not only its conjugation machinery but also some enzymes of the ubiquitin machinery as well as ubiquitination regulators such as UBE1 (the E1 for atypical NEDDylation), UBE2Z, USP3, RNF8, CAND1, and VCP. As mentioned above, it is possible that the NEDDylation of proteins directly alters conformations and/or impede protein interactions as observed in these examples. In conclusion, our findings suggest that the crosstalk of NEDD8, ubiquitin and probably SUMO is not only limited to the regulation and competition of downstream substrates but also for the upstream enzymatic pathways.

Here we provide for the first time a large-scale specific modification site database for NEDDylation. It is well established that the NEDDylation on cullins occur in the consensus motif IVRIMKMR within C-terminal's winged helix-binding (WHB) (Duda et al., 2008; Pan et al., 2004). Therefore, we used our NEDDylation site database to investigate the existence of a sequence motif for NEDDylation besides the cullins consensus motif. This sequence analysis provided us a global scale picture of the fundamental amino acid nature of lysines utilized for NEDD8 modifications, but we could not estimate a precise motif for NEDDylation. Previous studies have predicted a sequence motif for ubiquitination (Kim et al., 2011), but as for NEDDylation its occurrence is not readily apparent as in the case for the basal SUMOylation consensus motif Ψ KXE-type which has been widely described as a required subject for the direct binding with the SUMO-conjugating enzyme UBC9 (Bernier-Villamor, Sampson, Matunis, & Lima, 2002). A particular consistency between our NEDDylation motif analysis with the SUMOylation and GG base ubiquitination analysis is the striking reduce of cysteines around the modified lysines which greatly confirm the physiological avoidance of cysteine thioester linkage formation. At least two possible reasons are compatible with the lower evidence of ubiquitination and NEDDylation consensus motif. First, it could be that both modifications occur promiscuously at

various lysine residues. Secondly, it might be that different modification motifs exist for certain groups of proteins as it has been observed for SUMOylation ((Ivo A. Hendriks & Vertegaal, 2016). Here, our NEDDylation sequence analysis revealed a similar ubiquitination preference for negative charge aminoacids surrounded the modified lysine residue, most notably for glycines and glutamic acids. Likewise, a slight reduced in positive charge residues such as histidine and arginine was observed. The most striking difference in the analysis is that in our NEDDylation motif we found a great increase in lysine residues which in the ubiquitination analyses was strongly diminished. Given that the polar and charged surfaces between ubiquitin and NEDD8 are the less conserved properties (Whitby et al., 1998), the motif differences will influence to discriminate in their potential specific modification sites, interactions and final functions. Unfortunately, because of the inability of the GG remnant approach to discriminate between NEDD8 and ubiquitin conjugation, it has not been determined a large-scale database for genuine ubiquitination sites. Clearly, separate specific databases for NEDDylation and ubiquitination sites will be necessary for future research on consensus motif. The sequences motif analysis definitely represents a key element to study the structural elements in lysine recognition for post-translational modifications and therefore gain a better prediction for NEDD8, ubiquitin and SUMO sites and targets.

PERSPECTIVES

In this work, we used the advantages of the depletion of NEDP1 protease together with the endogenous levels expression of a transgene NEDD8 R74K as a strategy which provided the broadest identification of specific NEDDylation substrates and sites. Nevertheless, certain limitations of our strategy can be improved. Unquestionably, further research in NEDDylation proteomics should be conducted to develop approaches to analyse endogenously expressed NEDD8 modifications. In fact, we are still lacking methods that allow to identify site-specific level of endogenous ubiquitin and SUMO which would provide a better reliable comparison of these UBLs modifications. An alternative for this could be to perform an endogenous NEDD8 R74K mutant by using the genome editing CRISPR/Cas9 approach. However, given that the NEDD8 mutation potentially can alter its activation and further conjugation (especially as observed during atypical NEDDylation), it will be important to avoid the use of NEDD8 mutants. One possibility to prevent the use of NEDD8 mutant but still ensure NEDD8 specificity is by adapting the protease-reliant identification technique which has been successfully used for the identification of SUMO modification sites, called PRISM (protease-reliant identification of SUMO modification) (Ivo A Hendriks et al., 2015). The disadvantage of this strategy is that even it can identify modification sites without a modifier mutation, still needs the transgene expression. Definitely, further studies should be conducted to the development of a highly selective antibodies recognizing the different NEDDylation and ubiquitination Lys-C remnant to perform a global analysis of a genuine endogenous human NEDDylome and ubiquitinome. In conclusion, here we provided a first large-scale dataset of potentially specific NEDDylated sites and substrates. We are confident that our resource will be useful knowledge for the NEDD8 and general ubiquitin-like proteins field.

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