A mass spectrometry-based strategy for mapping modification sites for the Ubiquitin-like modifier NEDD8

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Abstract

The identification of modification sites for Ubiquitin and Ubiquitin-like modifiers is an essential step in the elucidation of controlled processes. The Ubiquitin-like modifier NEDD8 is an important regulator of plethora of biological processes both under homeostatic and proteotoxic stress conditions. Here, we describe a detailed protocol for proteome-wide identification of NEDDylation sites. The approach is based on the use of cell lines stably expressing the $NEDD8^{R74K}$ mutant. Digestion of samples with Lysyl endopeptidase, generates peptides with a di-Glycine remnant only from proteins modified with $NEDD8^{R74K}$ but not with Ubiquitin or ISG15. The isolation of these peptides with anti-di-Glycine antibodies (K-ε-GG) allows the identification of NEDDylation sites by liquid chromatography-tandem mass spectrometry.

Key words: Ubiquitin-like modifiers, NEDD8, di-Glycine remnant, mass spectrometry

1. Introduction

The family of Ubiquitin and Ubiquitin-like modifiers (Ubls) are key components of cell signaling and cellular mechanics. By covalently and reversibly modifying target proteins, they generate a vast repertoire of subtle but crucial variations within the proteome that affect many, if not all, cellular processes (Williamson et al. 2013; Oh et al. 2018). The Ubl NEDD8 is the closest homologue to Ubiquitin amongst the members of the Ubl family. Upon processing and exposure of the C-terminal di-Glycine motif by the NEDD8 processing enzyme NEDP1 (SENP8, DEN1), conjugation of NEDD8 on targets (NEDDylation) is mechanistically similar to Ubiquitin conjugation, depending on E1-activating, E2-conjugating and E3-ligase enzymes. The UBA3/APPBP1 heterodimer is the NEDD8-activating enzyme (NAE), UBE2M and UBE2F are the E2 enzymes and several E3ligases have been reported. The above-described mode of protein NEDDylation, defined as canonical, operates under homeostatic conditions and the Cullin family of proteins represent the main but not sole target for NEDD8 (Enchev et al. 2015; Abidi and Xirodimas 2015). Modification of NEDD8 is kept in a dynamic equilibrium through the action of deconjugating (deNEDDylating) enzymes. While enzymes with dual specificity for Ubiquitin/NEDD8 deconjugation have been reported, the COP9 signalosome and NEDP1, display high specificity as deNEDDylating enzymes that control the modification of Cullin and non-Cullin substrates, respectively (Enchev et al. 2015; Abidi and Xirodimas 2015).

In addition to the canonical pathway, NEDD8 is also activated and conjugated on substrates using enzymes of the Ubiquitin system. This mode of conjugation defined as atypical is observed under conditions of proteotoxic stress, including heat shock, oxidative stress, proteasome inhibition and results in the modification of non-Cullin targets. A key characteristic of the response is the formation of hybrid NEDD8-Ubiquitin, NEDD8-SUMO-2 chains that regulate protein degradation (see below). However, the mechanism(s) for processing/deconjugation of such hybrid chains remain unclear (Vijayasimha and Dolan 2021; Meszka et al. 2022).

The most well-studied, but clearly not the sole, function for protein NEDDylation is the control of protein stability. Through modification of the Cullin family of proteins, NEDD8 activates the Ubiquitin E3-ligase activity of the Cullin-Ring-Ligases (CRLs) that mediate the degradation of almost 20% of the human proteome (Soucy et al. 2009; Harper and Schulman 2021). Modulation of protein stability by NEDDylation is also reported for non-Cullin NEDD8 substrates. Here, NEDDylation can either promote or compromise substrate degradation, through competition with Ubiquitin for substrate modification, formation of hybrid NEDD8-Ubiquitin chains (atypical NEDDylation conditions) that may compromise targeting of the substrate to the proteasome, or regulation of non-CRLs E3-ligases, such as SMURF1/2, Parkin or Mdm2 (Xirodimas et al. 2004; Choo et al. 2012; Xie et al. 2014; Shu et al. 2016; Vijayasimha and Dolan 2021; Meszka et al. 2022). Non-proteolytic functions for protein NEDDylation have been reported, including control of transcriptional activity, subcellular localization, receptor endocytosis, apoptosis induction,

nucleolar signaling, neuronal maturation and synaptic plasticity (Enchev et al. 2015; Abidi and Xirodimas 2015; Meszka et al. 2022).

Based on the broad role of NEDD8 in promoting protein degradation through activation of CRLs and the success of proteasome inhibitors in the clinic, targeting the NEDD8 pathway was predicted as an attractive therapeutic approach for the treatment of cancer (Soucy et al. 2010). This notion is also supported from the observation that protein NEDDylation is upregulated in several types of cancer, including hepatocellular carcinoma, lung adenocarcinomas, squamous-cell carcinoma (Abidi and Xirodimas 2015; Delgado et al. 2018). The small molecule inhibitor of NAE, MLN4924/Pevonedistat is tested in more than 40 clinical trials, from phase 1 to phase 3, for the treatment of different forms of leukaemia (Acute Myeloid Leukemia), non-small cell lung cancer or melanoma (clinicaltrials.gov, as of the day 17.12.2021).

A critical information for the elucidation of the regulated processes by Ubiquitin/Ubls is the identification of sites of modification on substrates. The development of antibodies that specifically recognize the di-Glycine signature generated on modified lysines after trypsin digestion, has been a revolution in the field, as it allows the enrichment of modified peptides and the identification of modification sites at the proteome-wide level (Xu et al. 2010; Wagner & Beli et al. 2011; Kim et al. 2011; Ordureau et al. 2015). However, a key handicap of these tools is that they cannot discriminate between Ubiquitin, NEDD8 or ISG15 modification, as all these molecules provide a di-Glycine signature on modified proteins after trypsin digestion. For protein NEDDylation this issue was resolved with the use of a NEDD8 mutant ($NEDD8^{R74K}$) in combination with LysC digestion (Figure 1). In this case, only proteins modified with the $NEDD8^{R74K}$ mutant will generate peptides with di-Glycine signatures. The use of the anti-di-Glycine antibodies allows the enrichment of these peptides and the specific identification of NEDDylation sites at proteome-wide level. This approach has been successfully applied for the characterization of the NEDD8 proteome under conditions of canonical and atypical NEDDylation or upon deletion of the NEDP1 deNEDDylating enzyme that causes the accumulation of NEDD8 conjugates (Vogl et al. 2020; Lobato-Gil et al. 2021). Here, we describe a detailed protocol for the proteome-wide identification of NEDDylation sites using the above-described approach.

2. Materials

All solutions should be prepared with Milli-Q water of 18.2 MΩ.cm resistivity at 25 °C.

2.1. Common solutions and equipment

1. Acetic acid.
2. Acetonitrile (ACN).
3. Chloroacetamide (CAA).
4. Dithiothreitol (DTT).
5. Formic acid.
6. NuPAGE LDS Sample Buffer (4x) (Thermo Fisher Scientific).
7. Methanol.
8. Trifluoroacetic acid (TFA).
9. NanoDrop 2000 spectrophotometer.
10. Sonicator with a micro tip.
11. Thermomixer.
12. Vacuum concentrator.

2.2. Cell culture

1. Generation of NEDP1 knockout cell lines by CRISPR/Cas9 and cells lines stably expressing His6-NEDD8^{R74K} at endogenous levels were previously described in (Bailly et al. 2019; Lobato-Gil et al. 2021).
2. Dulbecco's phosphate-buffered saline (DPBS).
3. Cell culture media.
4. Fetal bovine serum (FBS).
5. Antibiotics for cell culture (penicillin, streptomycin).
6. L-glutamine.

2.3. Cell treatment and lysis

1. Modified RIPA buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.1% sodium deoxycholate.
2. Phosphatase inhibitor stock solutions (100x): 500 mM β-glycerophosphate (Sigma Aldrich), 500 mM sodium fluoride (Sigma Aldrich), 100 mM sodium orthovanadate (Sigma Aldrich).
3. Cysteine protease inhibitor N-ethylmaleimide (NEM) solution 1 M (100x) (Sigma Aldrich).
4. Protease inhibitors cocktail (Sigma Aldrich).
5. Sodium chloride (NaCl) 5 M.

6. Quick Start Bradford 1x Dye Reagent.

2.4. Protein precipitation and digestion

1. Acetone.
2. Denaturation buffer: 6 M urea, 2 M thiourea in 10 mM HEPES-NaOH, pH 8.0.
3. Lysyl endopeptidase (LysC; Wako): 0.5 µg/µL in 50 mM ammonium bicarbonate, pH 8.0.

2.5. Peptide purification

1. Sep-Pak C_{18} Classic Cartridge (30 mg sorbent per cartridge, 55-105 µm particle size) (Waters).
2. 10 mL syringe.

2.6. Di-Glycine modified peptide immuno-enrichment

1. PTMScan® Ubiquitin Remnant Motif (K-ε-GG) (#5562, Cell Signaling Technology).
2. Immunoprecipitation buffer (10x): 500 mM MOPS pH 7.2, 500 mM NaCl, 100 mM sodium phosphate (Na_2HPO_4) in water.
3. Supplemented immunoprecipitation buffer: immunoprecipitation buffer (1x), 150 mM NaCl, 0.5% IGEPAL CA-630 in water.

2.7 Micro Tip-Based Strong Cation Exchange Chromatography (Micro-SCX)

1. Cation 47 mm Extraction Disk (3M, Empore).
2. SCX wash buffer: 40% ACN, 0.1% TFA
3. SCX stock buffers: 40 mM acetic acid, 40 mM boric acid, 40 mM phosphoric acid. Adjust pH to 4.0, 5.0, 6.0, 7.0, 8.5 and 11.0.
4. SCX elution buffers: 60% SCX stock buffer, 40% ACN.
5. Buffer A': 3% ACN, 1% TFA.

2.8 Desalting and Concentration of Peptides

1. C_{18} 47 mm Extraction Disk (3M, Empore).
2. Buffer A: 0.1% formic acid.
3. Buffer B: 80% ACN, 0.1% formic acid.
4. C_{18} elution buffer: 50% ACN, 0.1% formic acid.
5. 96-well plate.

2.9 Analysis of Peptides by LC-MS/MS

1. Reprosil-Pur Basic C_{18}, 1.9 µm (Dr. Maisch HPLC GmbH).

2. 30 cm fused silica emitter, 360 μm outer diameter, 75 μm inner diameter, 8 μm laser-pulled tip (PicoTip) (New Objective, Inc.).
3. Pressure injection cell.
4. EASY-nLC 1000 liquid chromatograph (Thermo Fisher Scientific).
5. Hybrid Quadrupole-Orbitrap mass spectrometer: Q Exactive Plus (Thermo Fisher Scientific).

3. Methods

3.1. Cell treatment and lysis

1. Treat cells with the stimuli of choice according to your experimental setup (Figure 2(a); *see* **Note 1** and **Note 2**).
2. Wash cells twice with ice-cold DPBS. In case of suspension cells, centrifuge cells and wash them twice with ice-cold DPBS. Following steps should be performed on ice.
3. Add ice-cold modified RIPA buffer freshly supplemented with protease and phosphatase inhibitors to the cells (~500 µL lysis buffer per $1x10^7$ cells).
4. Collect cells using a cell scraper in a 15/50 mL tube (adherent cells) and incubate lysates on ice for 15 minutes.
5. Add 1:10 volume of 5 M NaCl to cells harvested in lysis buffer and sonicate the lysates 3x30s, in order to shear released DNA (after sonication, lysates should lose viscosity) (*see* **Note 3**).
6. Pellet cell debris by high-speed centrifugation (16,900 x *g*) at 4 °C for 15 minutes (*see* **Note 4**).
7. Measure protein concentration before precipitation (*see* **Note 5**).

3.2. Protein precipitation and digestion

1. Add 4 volumes of ice-cold acetone to precipitate the proteins. Incubate at -20 °C overnight (*see* **Note 6**).
2. Pellet the precipitated proteins by centrifugation at 1,000 x *g* for 5 minutes.
3. Dissolve completely the precipitated proteins in denaturation buffer (*see* **Note 7**).
4. Add DTT to a final concentration of 1 mM and incubate at room temperature for 45 minutes with shaking.
5. Add CAA to a final concentration of 5.5 mM and incubate at room temperature for 30 minutes, in the dark, with shaking (*see* **Note 8**).
6. Add LysC in an enzyme to total protein ratio of 1:100 and digest overnight at room temperature with shaking.
7. Add TFA to a final concentration of 1% to acidify peptides and stop the enzymatic reaction.
8. Incubate the samples at 4 °C for at least 30 minutes to allow precipitates to form.
9. Clarify the solution by centrifuging acidified peptide solution at 4,000 x *g* for 10 minutes and transfer supernatant to a new tube.

3.3. Peptide purification

1. Attach a C_{18} cartridge to a 10 mL syringe and wash it once with 5 mL of 100% ACN.
2. Wash C_{18} cartridge three times with 5 mL 0.1% TFA in water.
3. Load clarified peptide solution onto the C_{18} cartridge.

4. Wash the C_{18} cartridge three times with water (*see* **Note 9**).

3.4. Di-Glycine modified peptide immuno-enrichment

1. Elute the peptides from the C_{18} cartridge with 4 mL 50 % ACN in water (*see* **Note 10**).
2. Quantify peptide amount by NanoDrop spectrophotometer (A280).
3. Add 100 µL of immunoprecipitation buffer (10x) to each 1 mL of the eluate and SpeedVac-dry the sample until the total volume reaches 1 mL.
4. Clarify the peptide solution by centrifugation for 10 minutes at 16,900 x *g* and transfer supernatant to a fresh tube.
5. Wash the PTMScan® Ubiquitin Branch Motif (K-ε-GG) immunoaffinity beads (one vial per replicate) twice with ice-cold immunoprecipitation buffer (1x) (centrifugation steps from now on at 1,000 x *g*/45 seconds/4 °C).
6. Incubate clarified peptide solution with beads for 4 hours at 4 °C in a rotation wheel.
7. Spin down the beads and collect flowthrough (*see* **Note 11**).
8. Wash beads twice with 1 mL of ice-cold supplemented immunoprecipitation buffer.
9. Wash beads twice with 1 mL of ice-cold immunoprecipitation buffer (1x).
10. Wash beads twice with 1 mL of water and dry them with a syringe.
11. Elute peptides 4 times with 100 µL 0.15% TFA at room temperature with gentle shaking and after each step spin down beads and transfer supernatant to a fresh tube (*see* **Note 12**).

3.5. Micro Tip-Based Strong Cation Exchange Chromatography (Micro-SCX)

The micro tip-based strong cation exchange chromatography protocol is based on the following publications (Rappsilber et al. 2007; Wiśniewski et al. 2009; Weinert et al. 2013) (*see* **Note 13**).

1. Use a 17-gauge Hamilton syringe to cut out six disks from a cation 47 mm extraction disk and place them into a 200 µL pipette tip (SCX tip).
2. Wash SCX tip once with 50 µL methanol by centrifugation at 500 x *g* (*see* **Note 14**).
3. Wash SCX tip once with 50 µL SCX elution buffer corresponding to the lowest pH (4.0) and once with SCX elution buffer corresponding to the highest pH (11.0).
4. Wash SCX tip once with 50 µL wash buffer.
5. Load sample on the SCX tip by centrifugation at 500 x *g*.
6. Elute the peptides with 100 µL SCX elution buffer in six pH steps (4.0, 5.0, 6.0, 7.0, 8.5 and 11.0), from lowest to highest pH, by centrifugation at 500 x *g*. Collect eluates in separate tubes.
7. Vacuum concentrate the eluted peptides at 45 °C for 15 min to reduce the sample volume to ~50 µL.
8. Adjust pH to ~2 using Buffer A'.

3.6. Desalting and Concentration of Peptides

1. Use a 17-gauge Hamilton syringe to cut out two disks from a C_{18} 47 mm extraction disk and place them into a 200 µL pipette tip (C_{18} tip) (Rappsilber et al. 2007).
2. Wash C_{18} tips once with 25 µL methanol by centrifugation at 400–800 × *g*.
3. Wash C_{18} tips once with 25 µL Buffer B.
4. Wash C_{18} tips twice with 25 µL Buffer A.
5. Load acidified samples onto the C_{18} tips by centrifugation at 400–800 × *g*.
6. Wash C_{18} tips once with 50 µL Buffer A (see Note Peptide-loaded C_{18} tips can be stored for several months at 4 °C).
7. Elute the peptides directly into a 96-well plate by passing 50 µL C_{18} elution buffer through the C_{18} tips using a syringe.
8. Vacuum concentrate peptides at 45 °C for 25 min to reduce the sample volume to 4.5–5 µL.
9. Add 0.5–1 µL Buffer A to the sample.

3.7. Analysis of Peptides by LC-MS/MS

1. Pack a nanospray column (15 cm length, 75 µm inner diameter) with C_{18} reversed-phase chromatography material (1.9 µm bead size) using a pressure injection cell (Ishihama et al. 2002).
2. Use a nano-flow UHPLC system to load the peptide sample onto the C_{18} column.
3. Elute the peptides with a linear gradient from 8 to 40% ACN containing 0.1% FA in 2 h.
4. The mass spectrometer is operated in data-dependent mode, automatically switching between MS and MS^2 acquisitions (Michalski et al. 2011; Kelstrup et al. 2012).
5. MS spectra (m/z 300–1700) are acquired in the Orbitrap mass analyzer with a resolution of 70,000 at m/z = 200 after accumulation of ions to a target value of 3e6 estimated based on predictive automatic gain control from the previous full scan.
6. The 10 most intense ions are isolated using the quadrupole mass filter (maximum injection time 120 ms, isolation window 2.6 m/z, AGC target 1e5) and subsequently fragmented in the higher-energy C-trap dissociation (HCD) cell (Olsen et al. 2007).
7. MS^2 spectra are acquired in the Orbitrap mass analyzer with a resolution of 35,000 at m/z = 200.

3.8. Data Analysis

1. Analyze raw MS data using the MaxQuant software (Cox and Mann 2008).

2. Search MS^2 spectra against a database containing protein sequences obtained from the UniProtKB using the Andromeda search engine (Cox et al. 2011). Spectra are searched with a parent ion mass tolerance of 6 ppm, fragment ion mass tolerance of 20 ppm, strict LysC specificity and allowing up to three miscleavages. Cysteine carbamidomethylation is searched as fixed modification, whereas protein N-terminal acetylation, methionine oxidation modification of cysteines and di-Glycine-Lysine were searched as variable modifications.
3. Filter reverse hits and potential contaminants from the MaxQuant output table containing all identified di-Glycine-Lysine sites.
4. In case multiple biological replicates have been performed, identify significantly regulated di-Glycine-Lysine sites using appropriate statistical approaches (Tusher et al. 2001; Smyth 2004) (*see* **Note 15**).

4. Notes

1. Biological replicates should be performed to be able to significantly determine sites with di-Gly remnants derived from NEDD8 modification.
2. The recommended starting amount is at least 200 mg of total protein. Adapt size and number of dishes according to the cell line of choice.
3. High NaCl concentrations are used to facilitate the extraction of proteins that are associated to chromatin.
4. Knockout, knock-down, knock-in or inhibition of proteins or complexes should be also tested via western blotting.
5. The recommended starting amount is at least 200 mg of total protein. An aliquot of each sample (50-100 µg) can also be used for proteome analysis.
6. Acetone-precipitated proteins can be stored for several months at -20 °C.
7. Use 4 mL of denaturation buffer to dissolve the precipitated proteins under shaking (1000 rpm) at room temperature. Complete denaturation is required for following steps.
8. Alkylation of cysteines with CAA is required to prevent unspecific side-reactions.
9. C_{18} cartridges loaded with peptides can be stored at 4 °C for several months.
10. If more than one C_{18} cartridge is used, the eluates from all the cartridges can be combined after elution before SpeedVac-drying.
11. Saving the flowthrough for multiple rounds of immuno-enrichment can be performed for increase in depth of the analysis.
12. After the last elution step, spin down the tube containing the eluate fraction to completely remove any beads leftovers.
13. The Micro-SCX fractionation steps can be omitted. This will decrease the number of identified peptides.
14. Avoid drying SCX tips during the washing steps.
15. For functional association analysis the dataset is 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.

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Figure Legends

Figure 1: Strategy for proteome-wide identification of NEDD8 sites. Trypsin digestion generates di-Glycine remnant for NEDD8, Ubiquitin and ISG15, making it impossible to discriminate between NEDD8, Ubiquitin or ISG15-modified peptides. The mutant $NEDD8^{R74K}$ creates an extra site for LysC protease, which after digestion forms di-Gly remnants only in NEDD8-modified proteins, enabling the immuno-enrichment of di-Gly peptides and the identification of NEDDylation sites.

Figure 2: Workflow for the identification of NEDDylation sites by mass spectrometry-based proteomics. (a) Example for an experimental setup for identification of canonical and atypical NEDDylation sites. NEDP1 is a deNEDDylating enzyme that displays a high specific activity towards NEDD8-modified substrates, while other deNEDDylating enzymes have been reported to target both NEDD8 and Ubiquitin-modified substrates. NEDP1 knockout directly relates to changes in NEDD8 modification via the canonical pathway, meanwhile stresses such as proteasome inhibition via MG132 is linked to atypical NEDDylation pathway, in which modification of substrates rely mainly on Ubiquitin cascade enzymes (Lobato-Gil et al. 2021). (b) Step-by-step workflow of the described protocol with indicated time durations.

Ubiquitin Trypsin
...LEDGRTLSDYNIQKESTLHLVLRLR GG

NEDD8 Trypsin
...MNDEKTAADYKILGGSVLHLVLALR GG

ISG15 Trypsin
...EDQLPLGEYGLKPLSTVFMNLRLR GG

NEDD8^{R74K} Trypsin
...MNDEKTAADYKILGGSVLHLVLALK GG

Ubiquitin Lys-C
...LEDGRTLSDYNIQK ESTLHLVLRLRGG

NEDD8 Lys-C
...MNDEKTAADYK ILGGSVLHLVLALRGG

ISG15 Lys-C
...EDQLPLGEYGLK PLSTVFMNLRLRGG

NEDD8^{R74K} Lys-C
...MNDEKTAADYKILGGSVLHLVLALK GG

Ubiquitin (or NEDD8 and ISG15)

NEDD8^{R74K}

K

K

Lys-C digestion

...LRGG
K

GG
K

Di-Gly modified peptide immunoenrichment

...LRGG
K

GG
K

a)

CONDITIONS	1	2	3	4
NEDP1 knockout	●		●	
MG132 treatment		●		●
Stimulus of choice			●	●

b)