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Biochemical and Structural Consequences of NEDD8 Acetylation

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Similar to ubiquitin, the ubiquitin-like protein NEDD8 is not only conjugated to other proteins but is itself subject to posttranslational modifications including lysine acetylation. Yet, compared to ubiquitin, only little is known about the biochemical and structural consequences of site-specific NEDD8 acetylation. Here, we generated site-specifically mono-acetylated NEDD8 variants for each known acetylation site by genetic code expansion. We show that, in particular, acetylation of K11 has a negative impact on the usage of NEDD8 by the NEDD8-conjugating enzymes UBE2M and UBE2F and that this is likely due to electrostatic and steric effects resulting in conformational changes of NEDD8. Finally, we provide evidence that p300 acts as a position-specific NEDD8 acetyltransferase.

Introduction

Ubiquitin (Ub) and the ubiquitin-like protein NEDD8 (neural precursor-cell expressed developmentally downregulated protein 8) are best known for their potential to regulate the function/activity of target proteins by being covalently conjugated to these via isopeptide bond formation.[1] The covalent attachment of Ub ("ubiquitylation") and NEDD8 ("neddylation") involves the concerted action of three classes of enzymes, E1 activating enzymes, E2 conjugating enzymes, and E3 protein ligases, with the E3 ligases mediating the substrate specificity of the conjugation machinery.[1c,2] Since Ub and NEDD8 can serve as their own substrates, target proteins can be modified not only by single moieties of Ub and NEDD8 but also by so-called Ub/NEDD8 chains.[1c,3] Ub and NEDD8 contain several lysine residues (7 and 9, respectively) and at least in the case of Ub, any of these can be used for chain formation.[4] Thus, an astounding number of different types of Ub chains and potentially NEDD8 chains exist, which at least in part, target respectively modified proteins for different fates.[1a,5] The finding that Ub and NEDD8 are not only used to modify proteins but are subject to posttranslational modifications themselves, including phosphorylation and acetylation, has added yet another level of complexity to the functional landscape of Ub/NEDD8. Notably, phosphorylation of S65 of Ub has been shown to be involved in the activation of the E3 ligase PARKIN and thus in mitophagy.[6] NEDD8 is closely related to Ub at the amino acid sequence level and *inter alia* contains a serine residue at position 65 that can be phosphorylated.[7] Indeed, we recently reported that similar to phosphorylated Ub, S65-phosphorylated NEDD8 can activate PARKIN and that the phosphorylated forms of Ub and NEDD8 have overlapping, yet distinct interactomes.[8]

While our knowledge about the functional consequences of Ub/NEDD8 phosphorylation is still limited, even less is known about the consequences of acetylation of Ub/NEDD8. Acetylation and ubiquitylation/neddylation often compete for the same lysine residues, and thus it is not surprising that acetylation of certain lysine residues of Ub interferes with the formation of distinct Ub chains.[9] Whether this is also the case for NEDD8, is currently unknown. In fact, there is only one report on potential consequences of NEDD8 acetylation showing that unanchored (i.e. free) acetylated NEDD8 trimers act as regulators of poly(ADP-ribose) polymerase 1 (PARP-1).[10]

The lack of knowledge about the consequences of Ub/NEDD8 acetylation is mainly due to the notions that intracellular levels of acetylated Ub/NEDD8 variants are low, though detectable, and that the enzymes involved in Ub/NEDD8 acetylation are not known. To circumvent this shortcoming, we recently applied the genetic code expansion technology to generate all possible mono-acetylated Ub variants.[9a] With these, we could show that acetylation of distinct lysine residues has a significant impact on Ub conformation and that p300 acts as a position-specific acetyltransferase for Ub.[9a] Here, we extended our efforts to NEDD8. We show that NEDD8 acetylated at K11 is poorly used by the E2 enzymes UBE2F and UBE2M, while the effect on the activity of NAE, the E1 activating enzyme for NEDD8, appears to be negligible. This deficiency of K11-acetylated NEDD8 is likely explained by structural rearrangements induced by K11 acetylation, as shown by NMR spectroscopic analysis. Finally, we provide evidence that p300 can act as a position-specific acetyltransferase of NEDD8 *in vitro* and in cells.

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Results and Discussion

Generation of Site-Specifically Acetylated NEDD8 Variants

Published data indicate that, at least, 6 out of 9 lysine residues of NEDD8 are subject to acetylation in cells.[10–11] However, besides the notion that acetylation of four of these (K11, K22, K33, K48) are involved in the regulation of PARP-1,[10] the functional consequences of acetylation of these residues remain unknown. To obtain first insights into potential consequences, we applied the genetic code expansion technology to generate homogeneous populations of site-specifically mono-acetylated NEDD8 variants by incorporating acetyllysine (AcK) at the known acetylation sites (Figure 1A and in the Supporting Information S1). For purification of the six NEDD8 xAcK variants (x stands for the position of the acetylated lysine residue), we made use of the fact that NEDD8 is synthesized as a precursor protein with a C-terminal extension in cells and converted to the mature form by protease cleavage.[8,12] Thus, we equipped NEDD8 with a C-terminal His_6-tag allowing the purification of the different variants via affinity chromatography and subsequently the removal of the His_6-tag by addition of recombinant UCHL3 (Figure S1). Homogeneity of the purified NEDD8 variants and quantitative incorporation of AcK were confirmed by SDS-PAGE (Figure S1) and MALDI-MS analyses (Figure S2).

Performance of Acetylated NEDD8 Variants in Auto-Neddylation Assays

The two E2 NEDD8-conjugating enzymes, UBE2M (aka UBC12) and UBE2F (aka NCE2), are known for their capacity to neddylate themselves (auto-neddylation).[10,13] Thus, we determined whether the different NEDD8 AcK variants behave differently in E2 auto-neddylation assays (Figure 1B). To do so, we incubated the respective NEDD8 xAcK variants with either UBE2F (Figure 1C) or UBE2M (Figure 1D) in the presence of the NEDD8 activating enzyme NAE, a heterodimer consisting of APPBP1 and UBA3,[14] under standard neddylation conditions (Supporting Information). This showed that the NEDD8 AcK variants do not significantly differ from unmodified NEDD8 in their potential to be used by the two E2 enzymes for auto-neddylation, except for NEDD8 11AcK that is less efficiently used (see also Figure S3). To determine whether this negative effect is caused at the level of NAE or at the E2 level, we performed time-resolved ATP cleavage sensor (TRASE) assays, which allow to monitor NAE-mediated ATP hydrolysis in real time, which is an essential step in NEDD8 activation.[15] In this assay, NEDD8 11AcK behaved similar to unmodified NEDD8 (Figure 2A), indicating that NEDD8 11AcK is inefficiently used by the E2 s rather than by E1. This interpretation is supported by the finding that in TRASE assays addition of UBE2F or UBE2M stimulated NAE-mediated activation of unmodified NEDD8 more efficiently than that of NEDD8 11AcK, as measured by the rate of ATP hydrolysis (Figures 2B, 2C). In reactions with either UBE2F or UBE2M, a decrease in NAE activity of approx. 35 % and 45 %, respectively, is observed with NEDD8 11AcK in comparison to the reactions with unmodified NEDD8 (Figure 2D).

Structural Consequences of NEDD8K11 Acetylation

The observation that acetylation of K11 interferes with E2 auto-neddylation is reminiscent to results we recently obtained with Ub, insofar as acetylation of K11 of Ub had a negative impact on E3-mediated auto-ubiquitylation.[9a] As for Ub, the positively charged amino group of the side chain of K11 of NEDD8 forms a salt bridge with E34,[16] which cannot form upon charge neutralization of the side chain by acetylation. To provide evidence that the salt bridge between K11 and E34 is important for NEDD8 function, we generated NEDD8 variants, in which K11 was replaced by arginine to preserve the positive charge or by glutamine that is frequently used as AcK surrogate. Indeed,

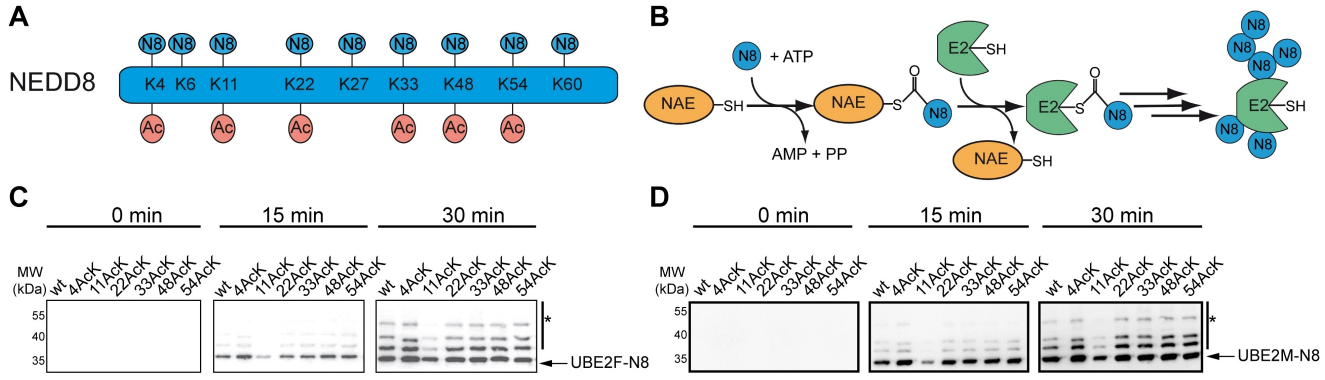
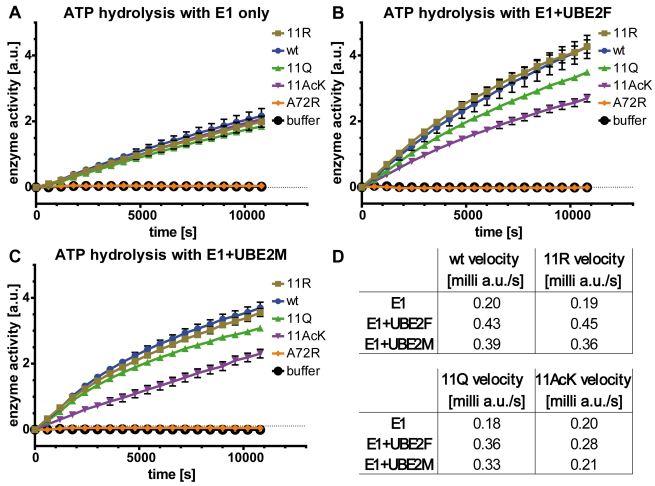


Figure 1. Acetylated NEDD8 Variants and Their Performance in E2 Auto-Neddylation Assay. A, Lysine residues of NEDD8 used for NEDD8 chain formation (N8) and acetylation (Ac) in cells. B, Scheme of the auto-neddylation reaction of NEDD8 conjugating enzymes (E2) with NEDD8 (N8) and NEDD8 activation enzyme (NAE). C and D, NEDD8 variants acetylated at K4 (4AcK), K22 (22AcK), K33 (33AcK), K48 (48AcK) or K54 (54AcK) resemble unmodified NEDD8 (wt) in their efficiency to be used by UBE2F (C) and UBE2M (D) for auto-neddylation, while at K11 acetylated NEDD8 (11AcK) is inefficiently used. Auto-neddylation reactions were performed as described in the Supporting Information and started by addition of the respective NEDD8 variants or unmodified NEDD8. Reactions were stopped at the times indicated. All reactions were analyzed by SDS-PAGE followed by western blotting and immunodetection of NEDD8. Running positions of the mono-neddylated and multi-neddylated forms of the E2 enzymes are indicated by an arrow and an asterisk, respectively.



	wt velocity [milli a.u./s]	11R velocity [milli a.u./s]
E1	0.20	0.19
E1+UBE2F	0.43	0.45
E1+UBE2M	0.39	0.36

	11Q velocity [milli a.u./s]	11AcK velocity [milli a.u./s]
E1	0.18	0.20
E1+UBE2F	0.36	0.28
E1+UBE2M	0.33	0.21

Figure 2. Charge Neutralization by K11 Acetylation of NEDD8 Affects UBE2F and UBE2M but not the NEDD8 E1 Activation Enzyme. A–C, ATP hydrolysis by the NEDD8 activating enzyme E1 is monitored by TRASE assay. The NEDD8 K11 to arginine (11R), K11 to glutamine (11Q), K11 acetylated (11AcK) variants or unmodified NEDD8 (wt) were added to a FRET-able ATP analog. Reactions without NEDD8 (buffer) and with a NEDD8 variant A72R (A72R) that is not used by E1[14b] served as additional controls. The mixtures were incubated with either the E1 enzyme alone (A) or E1 enzyme plus UBE2F (B) or E1 enzyme plus UBE2M (C). ATP hydrolysis was measured every 10 min over a time course of 270 min. The continuous lines show regression fits preserving color coding. D, The reaction velocity of the E1 activities from A–C are shown in milli activity units per second. The SD of three independent experiments is indicated in A–C.

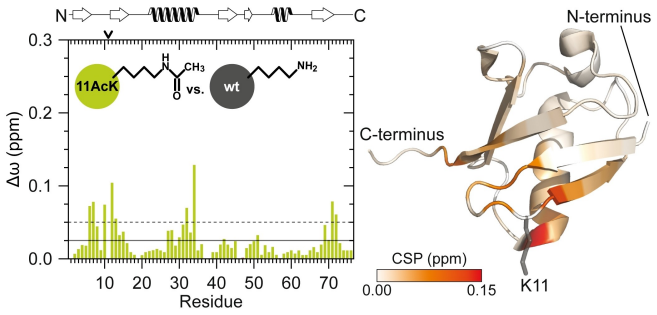


Figure 3. Site-Specific Acetylation of NEDD8 at K11 Induces a Steric Clash in the Central α-Helix. Weighted chemical shift perturbation (CSP, Δω) mappings of backbone amide resonances are shown comparing NEDD8 11AcK variant (11AcK) with unmodified NEDD8 (wt). The corresponding CSP plot is depicted on the left for NEDD8 11AcK, and significant Δω values are highlighted on the right on the NMR solution structure of unmodified NEDD8 (PDB ID 2KO3)[17] presented in cartoon mode. The cutoff values in the plot were calculated by taking the mean (horizontal solid line) and the mean plus one standard deviation (horizontal dashed line), respectively, considering all Δω values. Secondary structural elements of NEDD8 are schematically illustrated on the top of the plot. The position of K11 is indicated by an arrow on the top of the CSP plot and the corresponding side chain is drawn as a stick model in the shown right structure.

NEDD8 11R performed similar to unmodified NEDD8 in TRASE assays (Figure 2) and auto-neddylation assays (Figure S4).

Furthermore, NEDD8 11Q performed somewhere between unmodified NEDD8 (i.e. it was less efficiently used than unmodified NEDD8 but more efficiently than NEDD8 11AcK), indicating that the negative effect of K11 acetylation is not solely due to charge neutralization.

The results obtained so far are in line with the notions that acetylation of K11 causes structural rearrangements in NEDD8 and that as we have shown for Ub 11AcK, these are induced by charge neutralization and by steric hindrance due to the extension of the length of the lysine side chain.[9a] To prove this hypothesis, we prepared isotopically labeled forms of NEDD8 11AcK and unmodified NEDD8 for comparative analysis by high-resolution NMR spectroscopy. We acquired corresponding two-dimensional ^{1}H—^{15}N heteronuclear single quantum coherence (HSQC) NMR spectra (Figure S5) and determined chemical shift perturbations (CSPs) of backbone amide resonances (Figure 3). Thereby, alterations in the chemical environment of corresponding amide proton and nitrogen spins as consequence of the conformational changes induced by acetylation of K11 can be monitored on a residue-by-residue basis. The analysis revealed that NEDD8 11AcK experiences pronounced acetylation-dependent perturbations in the region next to the actual acetylation site as well as the very C-terminus. In addition, significant perturbations are apparent at the C-terminal end of NEDD8's central α-helix spanning residues 30–34, which is in direct contact with the K11 side chain. Especially these perturbations are likely due to a steric clash of the acetylated side chain of K11 and the α-helix.

Thus, we conclude that electrostatic (disruption of the salt bridge between K11 and E34) as well as steric effects are involved in the structural rearrangement of NEDD8 induced by K11 acetylation.

p300 Acetylates NEDD8

An important prerequisite to study the functional consequences of acetylation of a protein of interest in cells is the identity of the enzymes involved in modulating the acetylation status of the protein (in other words, which acetyltransferases and deacetylases are involved in NEDD8 acetylation). As a first step into this direction, we studied whether p300 can act as acetyltransferase for NEDD8, for two reasons. Firstly, p300 is known to have an acetylation preference for lysine residues located directly after a glycine residue.[18] NEDD8 contains two of such sites, K11 and K48. Secondly, the p300-dependent acetylome was determined in proteomics studies using mouse embryo fibroblasts, in which p300 was knocked out by genetic means or p300 activity was enhanced by activation via CBP112 treatment.[11b] Our analysis of this data revealed that the acetylation status of K48, but not of K22 and K54 correlates with the acetyltransferase activity of p300 (Figure 4A).

To corroborate the data obtained in cells, we incubated unmodified NEDD8 with recombinant p300 and acetyl-CoA *in vitro*. As control, reactions in the absence of either p300 or acetyl-CoA were performed. Reaction products were analyzed by mass spectrometry via parallel reaction monitoring (PRM) (Figure 4B; Figure S6). Indeed, we found that p300 acetylates K48 of NEDD8. In addition, K11 was acetylated by p300, though apparently less efficiently than K48. Thus, we conclude that

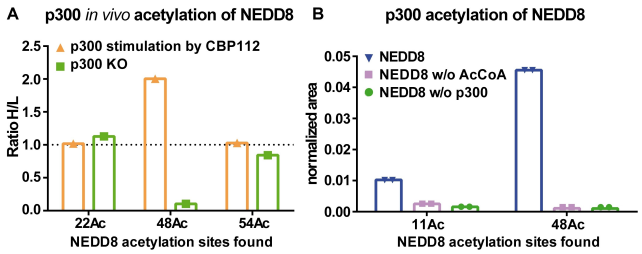


Figure 4. Acetylation of NEDD8 by p300. A, Quantification of the respective NEDD8 acetylation levels from data generated by Weinert et al.[11b] upon p300/CBP knockout (p300 KO) and by activation of acetyltransferase activity of p300/CBP via compound CBP112 (CBP112) in mouse embryonic fibroblasts. In this report, SILAC-based quantitative MS was applied to identify CBP/p300 regulated acetylation sites. In brief, after cell lysis and sonication, acetone precipitated proteins were digested with trypsin and acetylated peptides were enriched. In each experiment, heavy (H) labels represent respective perturbations and light (L) labels the control. The corrected ratio of H to L (Ratio H/L) is plotted for the respective perturbations and the indicated NEDD8 acetylation sites. Values less than 1 indicate decreased acetylation and values greater than 1 indicate increased acetylation of the respective site upon perturbation. Data points (square and triangle) indicate the mean of three independent analyses. B, Monomeric NEDD8 is acetylated at K48 and K11 by p300 in presence of acetyl-CoA (NEDD8) *in vitro*, while only a background signal can be observed in the absence of either acetyl-CoA (NEDD8 w/o AcCoA) or p300 (NEDD8 w/o p300). For quantification, the respective product ions (MS2-level) of the indicated respective acetylated NEDD8 peptide precursors were used and normalized against a highly abundant peptide of NEDD8. Data points (triangle, square and dot) of two independent measurements are shown.

p300 acts as a *bona fide* acetyltransferase for NEDD8 both *in vitro* and in cells.

Conclusions

The knowledge about the functional consequences of post-translational modification of NEDD8 is limited. Here, we show that acetylation interferes with the usage of NEDD8 by UBE2M and UBE2F in a position-specific manner, i.e. NEDD8 acetylated at K11 is inefficiently used by the two E2 enzymes for auto-neddylation, whereas acetylation of the other lysine residues studied had no significant effect. Furthermore, by substitution experiments and structural analysis by NMR spectroscopy, we provide strong evidence that this functional impairment of NEDD8 11AcK is caused by electrostatic and steric effects. Finally, our study provides first insights into the enzymes involved in NEDD8 acetylation by showing that the acetyltransferase p300 acetylates NEDD8 at K11 and K48. Intriguingly, these acetylation sites were also found in unanchored NEDD8 trimers that act as regulators of PARP-1 activity upon oxidative stress.[10] Thus, the ability to generate site-specifically acetylated NEDD8 variants at will provides the basis for further elucidating the proteins and pathways that are affected/regulated by NEDD8 acetylation, e.g. by identification of the interactomes of the differently acetylated NEDD8 variants or by studying their subcellular localization in the absence and presence of distinct stress stimuli. Similarly, it will be interesting to explore multiple acetylated NEDD8 variants and to study the potential interplay between NEDD8 phosphorylation and acetylation. For the latter it will be necessary to further develop systems allowing the simultaneous as well as site-specific incorporation of two different non-canonical amino acids into a protein of interest.

Supporting Information

This Article is accompanied by a Supporting Information. The authors have cited additional references within the Supporting Information.[19]

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Acetylation · Genetic code expansion · NEDD8 · NMR spectroscopy · Protein modifications

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