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NEDD8 Overexpression Results in Neddylation of Ubiquitin Substrates by the Ubiquitin Pathway

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Ubiquitin and ubiquitin-like proteins use unique E1, E2, and E3 enzymes for conjugation to their substrates. We and others have recently reported that increases in the relative concentration of the ubiquitin-like protein NEDD8 over ubiquitin lead to activation of NEDD8 by the ubiquitin E1 enzyme. We now show that this results in erroneous conjugation of NEDD8 to ubiquitin substrates, such as p53, Caspase 7, and Hif1α, demonstrating that overexpression of NEDD8 is not appropriate for identification of substrates of the NEDD8 pathway.

The best-established role for NEDD8 is the activation of cullin-RING E3 ubiquitin ligases (CRLs), and under endogenous expression conditions, cullins are the most abundant NEDD8 substrates (Fig. 1a). After NEDD8 overexpression, however, an extensive ectopic neddylation pattern is detectable, which depends on the activity of the ubiquitin-activating enzyme UBE1 (Fig. 1a and b; see Refs. 1–3). To determine if this pattern represents NEDD8 conjugation to ubiquitin substrates, we directly tested UBE1-dependent neddylation of the known ubiquitin targets p53 and Caspase 7. These proteins have also previously been reported to be substrates of NEDD8,[4,5] but as their identification was performed with overexpressed NEDD8, we were expecting that their reported neddylation was UBE1 dependent.

Indeed, under these conditions, both p53 and Caspase 7 were NEDD8 modified in a UBE1-dependent manner (Fig. 1c and d). This suggests that the ubiquitin E1, E2, and E3 enzymes transfer NEDD8 and that inhibition of the NEDD8 pathway should not affect their modification. Consistently, treatment with the NEDD8 E1 inhibitor MLN4924 did not alter neddylation of p53 and Caspase 7 (Fig. 1c and d). As NEDD8 is a direct regulator of CRL E3 ubiquitin ligases, we predicted that if neddylation proceeded through ubiquitin enzymes, inhibition of both UBE1 and the NEDD8 pathway should reduce neddylation of a CRL substrate. Indeed, the CRL2 substrate HIF1α was clearly neddylated upon NEDD8 overexpression, and inactivation of either the NEDD8 or the ubiquitin pathway led to a drastic reduction of neddylated HIF1α (Fig. 1e).

These results do not exclude that p53, HIF1α, and Caspase 7 are substrates for NEDD8 enzymes at endogenous NEDD8 levels, but they demonstrate that overexpression of NEDD8 will result in neddylation by ubiquitin enzymes. During NEDD8 purification, this effect will likely mask any real substrates of the NEDD8 pathway and overexpression should thus not be used to identify targets of the bona fide NEDD8 enzymes. Previously reported substrates identified by overexpression should also be reexamined. Whether similar

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Abbreviations used: CRL, cullin-RING E3 ubiquitin ligase; UBE, ubiquitin-activating enzyme.

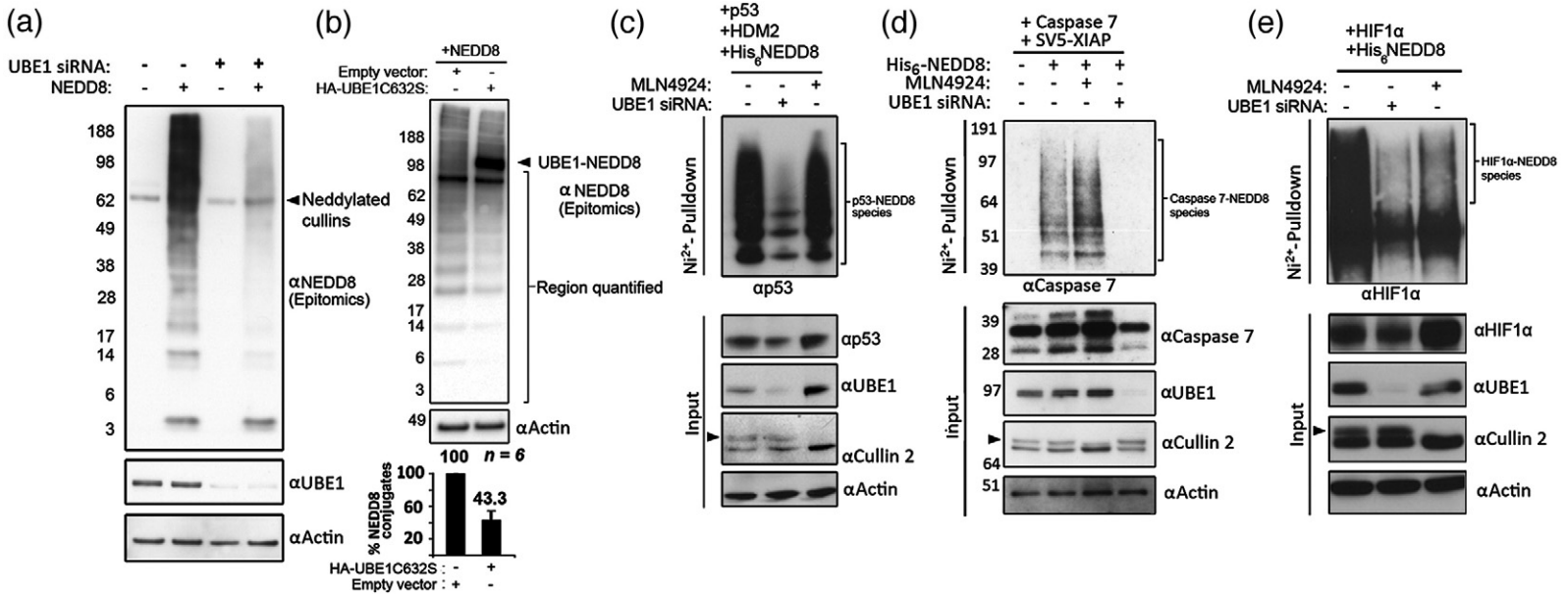

Fig. 1. (a) Overexpression of NEDD8 leads to a long-range molecular weight neddylation pattern, which is dependent on the ubiquitin-activating enzyme UBE1. UBE1 siRNA (small interfering RNA) strongly reduces the NEDD8 conjugates without affecting cullin neddylation (arrowhead). (b) Expression of a UBE1C632S dominant-negative mutant demonstrates that NEDD8 is activated by UBE1, as it traps NEDD8 on the active site of the enzyme by forming a stable oxy-ester. This reduces the NEDD8 modification pattern (quantification in lower panel). (c and d) Overexpression of His_6-NEDD8 with p53 (c) or Caspase 7 (d) and their respective E3 ligases (HDM2 and XIAP) leads to their UBE1-dependent neddylation, as shown by denaturing Ni^{2+} pulldown, where Ube1 siRNA strongly reduces their modification. Treatment with the NEDD8 E1 (NAE) inhibitor MLN4924 has no effect on p53 or Caspase 7 neddylation. (e) HIF1α is neddylated after expression of His_6-NEDD8, and this neddylation is dependent on both UBE1 and NAE1.

issues pertain to other ubiquitin-like proteins remains to be determined.

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Supplementary Data

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