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commentary online data

SUPPLEMENTARY ONLINE DATA

Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes

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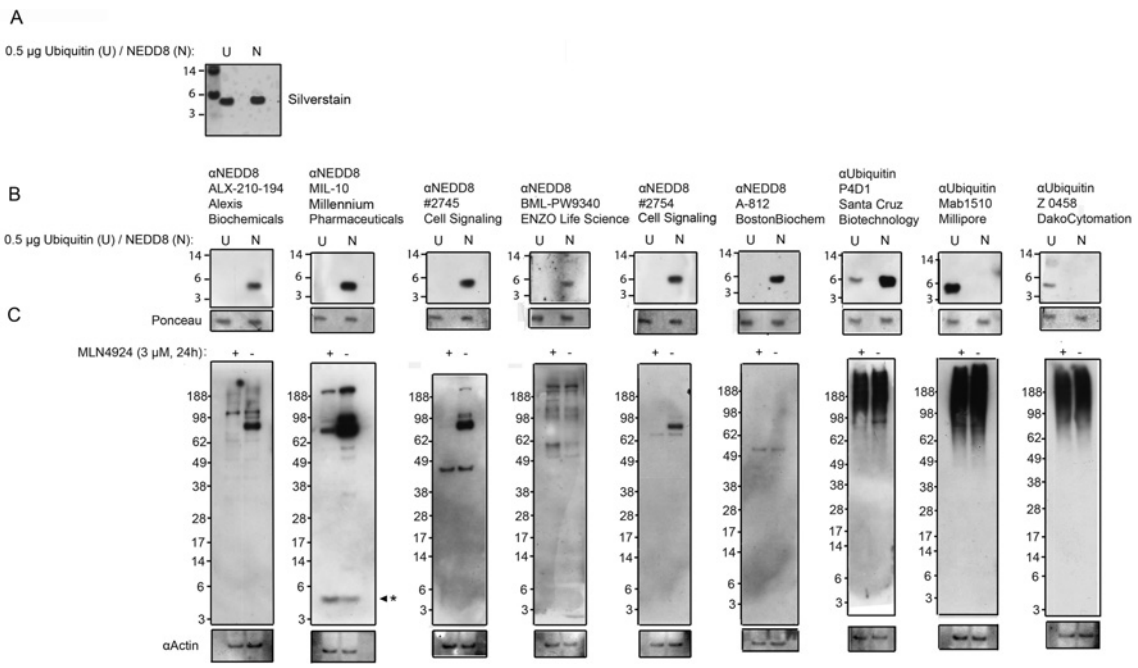

Figure S1 Evaluation of anti-NEDD8 and -ubiquitin antibodies

(**A**) Silver stain of NEDD8 and ubiquitin amounts used. (**B**) A panel of anti-NEDD8 and anti-ubiquitin antibodies were tested for specificity on 0.5 μg of purified NEDD8 (N) or ubiquitin (U). All tested antibodies were specific for their respective target, with the exception of the P4D1 anti-ubiquitin antibody from Santa Cruz, which cross-reacts heavily with NEDD8. (**C**) The same panel of antibodies was tested on U2OS whole cell lysates. Cells were either treated with 3 μM MLN4924 or DMSO (vehicle) to control for antibody specificity. The non-commercial MIL-10 anti-NEDD8 antibody (provided by Millennium Pharmaceuticals) was found to be the most potent, and also was the only option that detected free NEDD8 using these settings. Molecular masses in kDa are shown to the left-hand side of the Western blots.

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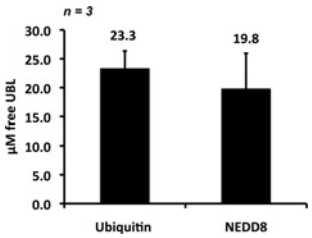

Figure S2 Measurement of endogenous free ubiquitin and NEDD8 concentrations in MCF7 cells

Measurements were performed under non-reducing conditions. Roughly equimolar concentrations of both UBLs were detected. Error bars indicate ± S.D.

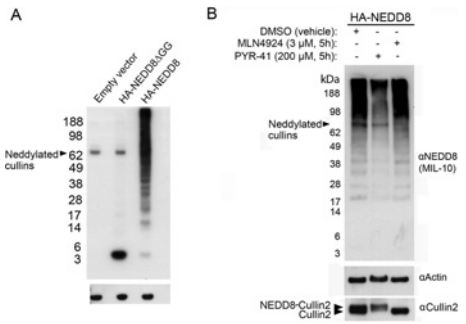

Figure S3 A typical NEDDylation is not induced by overexpression of NEDD8ΔGG, and reduced by treatment with PYR-41

(**A**) Mature NEDD8 and NEDD8ΔGG were overexpressed in U2OS cells. Widespread NEDDylation is apparent after mature NEDD8 overexpression, but not after NEDD8ΔGG overexpression. (**B**) Treatment with the UBE1 inhibitor PYR-41, but not MLN4924 or DMSO, reduces atypical NEDDylation after NEDD8 overexpression. Molecular masses in kDa are shown to the left-hand side of the Western blots.

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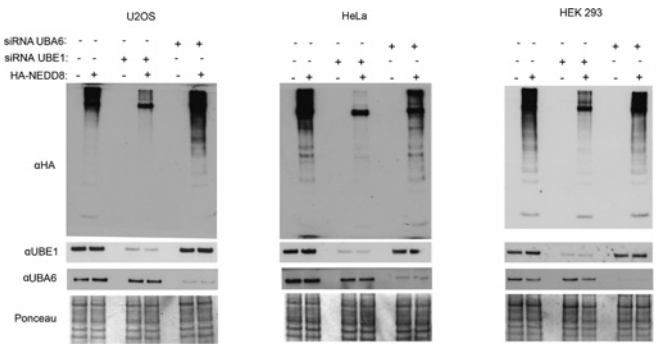

Figure S4 Assessment of contribution of UBA6 to NEDD8 smears and cell type dependence

HeLa, HEK-293 and U2OS cell lines were tested. Knockdown of UBE1 or UBA6 (UBE1L2) was performed 48 h prior to transfection with NEDD8, and whole cell lysates were analysed for NEDD8 by Western blotting.

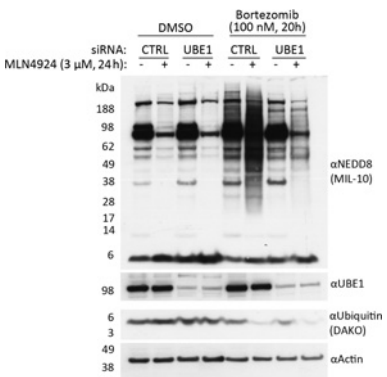

Figure S5 A low concentration of bortezomib induces atypical NEDDylation

U2OS cells were treated with bortezomib (100 nM, 20 h) or DMSO (vehicle) and siRNA to UBE1 or MLN4924 as indicated. NEDDylation induced by bortezomib treatment is independent of NAE, whereas it is diminished upon ubiquitin E1 siRNA. Molecular masses in kDa are shown to the left-hand side of the Western blot.

Table S1 Human E2 enzymes tested for capacity to accept NEDD8 from UBE1 *in vitro*

+ or − indicates whether the E2 could or could not accept NEDD8 from UBE1 respectively.

E2	NEDD8 chargable
Ube2A	+
Ube2B	+
Ube2D1	+
Ube2L3	+
Ube2I	−
Ube2L6	+
Ube2N	+
Ube2D4	+
Ube2C	+
Ube2G1	+
Ube2K	+
Ube2E3	+
Ube2D2	+
Ube2Q2	+
Ube2E1	+
Ube2H	+
Ube2s	+
Ube2N/V1	+
Ube2G	+
Ube2Q	+
Ube2E2	+
Ube2T	+
Ube2M	−
Ube2F	−

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