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

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Ubiquitin and UBL (ubiquitin-like) modifiers are small proteins that covalently modify other proteins to alter their properties or behaviours. Ubiquitin modification (ubiquitylation) targets many substrates, often leading to their proteasomal degradation. NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) is the UBL most closely related to ubiquitin, and its best-studied role is the activation of CRLs (cullin-RING ubiquitin ligases) by its conjugation to a conserved C-terminal lysine residue on cullin proteins. The attachment of UBLs requires three UBL-specific enzymes, termed E1, E2 and E3, which are usually well insulated from parallel UBL pathways. In the present study, we report a new mode of NEDD8 conjugation (NEDDylation) whereby the UBL NEDD8 is linked to proteins by ubiquitin enzymes *in vivo*. We found that this atypical NEDDylation is independent of classical NEDD8 enzymes, conserved from yeast to mammals, and triggered by an increase in the NEDD8 to ubiquitin ratio. In cells, NEDD8 overexpression leads to this type of NEDDylation by increasing the concentration of NEDD8, whereas proteasome inhibition has the same effect by depleting free ubiquitin. We show that bortezomib, a proteasome inhibitor used in cancer therapy, triggers atypical NEDDylation in tissue culture, which suggests that a similar process may occur in patients receiving this treatment.

Key words: bortezomib, MG132, MLN4924, neural-precursor-cell-expressed developmentally down-regulated 8 (NEDD8)-activating enzyme (NAE), proteasome, ubiquitin-activating enzyme.

INTRODUCTION

Ubiquitin is a 76 amino acid protein that regulates various cellular processes. It is best studied for its role in proteasome-dependent protein degradation, but has also been shown to have proteasome-independent functions in many signalling events [1]. The activity of ubiquitin is connected to its covalent conjugation to other cellular proteins, where its C-terminus forms isopeptide bonds with ε-amino groups of lysine residues. A three-step enzymatic cascade is required to link ubiquitin to substrates [2]. ATP-dependent activation of ubiquitin by either one of two ubiquitin-activating enzymes (E1s), UBE1 or UBA6, initiates the process [3]. Afterwards, ubiquitin is transferred to the active-site cysteine of one of approximately 39 E2 enzymes [4] and then linked to thousands of cellular substrates by the actions of a large array of E3 ubiquitin ligases [1]. Often, ubiquitin is not only conjugated as a single moiety, but rather forms chains of multiple ubiquitin molecules linked via isopeptide bonds between the C-terminal glycine residue of ubiquitin and one of the seven internal lysine residues of the preceding ubiquitin in the chain. The signal transduced by ubiquitin chains will vary depending on which chain type is assembled. The best-studied chains are the ones formed on Lys^{48}, which in most cases trigger ubiquitin-dependent degradation of the substrate by the proteasome [5]. Lys^{11}-linked chains seem to have a similar effect, whereas Lys^{63}-linked and linear chains are involved in signal transduction processes [6]. In addition to ubiquitin, there are at least 17 UBL (ubiquitin-like) molecules in the cell [7], most of which are also conjugated to other cellular proteins. NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) is the ubiquitin-like modifier most closely related to ubiquitin, with a high level of sequence homology (58 % identity and 80 % similarity), as well as a very similar three-dimensional structure [8]. NEDD8 utilizes a distinct set of enzymes for conjugation to proteins [9]. NAE (NEDD8-activating enzyme) is formed by a heterodimer of the two proteins APPBP1 and UBA3. After activation, NEDD8 is transferred to one of the two NEDD8-conjugating enzymes Ube2M [9] or Ube2F [10], after which it is linked to its substrates via E3 enzymes. The only NEDD8-specific E3 complex identified to date is a ligase consisting of the two E3s Dcn1 and Rbx1 [11–13], whereas all other suggested NEDD8 ligases seem to have dual activity for NEDD8 and ubiquitin.

The NEDD8 substrate spectrum appears to be far more limited than the one for ubiquitin. The best-studied and most abundant NEDD8 substrates are the cullin proteins. Cullins are scaffolds for the largest class of ubiquitin E3 ligases, termed CRLs (cullin-RING ubiquitin ligases). Cullin NEDDylation activates CRLs by triggering structural changes and by counteracting the association of the CRL inhibitor Cand1 [14,15]. In recent years, various non-cullin NEDD8 substrates have also been identified, including p53, Mdm2 (murine double minute 2), p73, L11, BCA3 (breast cancer-associated gene 3), EGFR (epidermal growth factor receptor), VHL (von Hippel–Lindau protein), HIF1α (hypoxia-inducible factor 1α), XIAP (X-linked inhibitor of apoptosis) and caspase 7 [16–23]. Proteomic approaches to identify NEDD8 substrates have in addition been undertaken [24–26]. The effect of NEDD8

Abbreviations used: BCA3, breast cancer-associated gene 3; CHO, Chinese-hamster ovary; CRL, cullin-RING ubiquitin ligase; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HA, haemagglutinin; HRP, horseradish peroxidase; LDS, lithium dodecyl sulfate; NAE, NEDD8-activating enzyme; NEDD8, neural-precursor-cell-expressed developmentally down-regulated 8; siRNA, small interfering RNA; SUMO, small ubiquitin-like modifier; TCA, trichloroacetic acid; UBL, ubiquitin-like; WT, wild-type.

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conjugation for a particular protein differs; reported consequences include effects on stability, transcriptional activity and subcellular localization [16,22,27].

In the present study we report an unexpected crosstalk between the NEDD8 and ubiquitin pathways. We show that increases in the free NEDD8 to ubiquitin ratio trigger activation of NEDD8 by the ubiquitin E1 enzyme in cells (atypical NEDDylation), which probably leads to a global attenuation of ubiquitin-dependent protein degradation. The physiological significance of this effect remains unclear; however, the approved anti-cancer drug bortezomib causes atypical NEDDylation in cultured cells due to depletion of free ubiquitin, which possibly affects therapeutic outcome. Our results emphasize that understanding substrate and UBL specificity within these pathways is important for evaluating potential drugs, but also needs to be taken into consideration when defining the NEDDylated and ubiquitylated proteomes.

EXPERIMENTAL

MLN4924 synthesis

((1*S*,2*S*,4*R*)-4-{4-[(1*S*)-2,3-dihydro-1*H*-inden-1-ylamino]-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl}-2-hydroxycyclopentyl)methyl sulfamate, the compound that is MLN4924 (referred to throughout as such), was synthesized as described previously [28].

Cell culture and transfection

U20S, HEK (human embryonic kidney)-293 and HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium; GIBCO) supplemented with 10 % FBS (fetal bovine serum; GIBCO) and 100 units/ml penicillin/100 μg/ml streptomycin (Intvitrogen). CHO (Chinese-hamster ovary) TS41 cells were grown at 32 °C in F-12K (GIBCO) supplemented with 10 % FBS (Gibco) and penicillin/streptomycin (Invitrogen). MG132 and bortezomib were from Sigma–Aldrich and LC Laboratories respectively. All plasmid transfections were performed using Lipofectamine™ LTX PLUS (Invitrogen), following the manufacturer's instructions. For pCMV5-NEDD8/NEDD8ΔGG overexpressions, unless stated otherwise, 1 μg of plasmid was transfected per six-well plate, containing approximately 1.5×10^5 cells. For HA (haemagglutinin)–UBE1 immunoprecipitations, approximately 1×10^6 cells per 100 mm dish were co-transfected with 5 μg of pCMV HA-UBE1WT/HA-UBE1C632S (where WT is wild-type) and 5 μg of untagged pCMV5-NEDD8. All UBE1 and UBE1L2 siRNA (small interfering RNA) transfections were performed using Dharmacon ON-TARGET plus SMARTpool siRNA oligos at a final concentration of 20 nM and Lipofectamine™ RNAiMAX (Invitrogen), according to the manufacturer's instructions. All UBE1 and UBA6 (UBE1L2) knockdowns were performed 48 h prior to plasmid transfections, and for a total of 72 h.

In vitro NEDD8/ubiquitin activation/transfer assays

His_6–UBE1 (6 pmol; Ubiquigent) was added to 20 μl of reaction buffer (50 mM Hepes, pH 7.5, 100 mM $MgCl_2$ and 2 mM MgATP) containing 2.5 μM ubiquitin E2 (Ubiquigent). For E1 activation assays, E2 enzymes were left out. The reaction was started by addition of either 2 nmol of purified ubiquitin or 2 nmol of purified NEDD8, incubated at 30 °C and stopped after 30 min by addition of reducing or non-reducing 3× Laemmli buffer.

Immunoprecipitations

HA immunoprecipitations were performed under denaturing conditions. Cells were lysed in 1 % SDS, 5 mM EDTA, 10 mM iodoacetamide, 15 units/ml DNase I (Qiagen) and 1× Complete™ protease inhibitor cocktail (Roche). Lysis was performed on ice, followed by immediate heating of the samples to 95 °C, after which lysates were diluted 10-fold with 20 mM Tris/HCl, pH 8, 137 mM NaCl, 10 % glycerol, 1 % Nonidet P-40, 2 mM EDTA, 10 mM iodoacetamide and 1× Complete™ protease inhibitor cocktail. DNA was fragmented by passing lysates through a syringe. Lysates were precleared for 1 h rotating at 4 °C with control agarose beads (Pierce, 26150), after which lysates were incubated with anti-HA beads (Sigma–Aldrich, A2095). Immunprecipitation was performed at 4 °C for 1 h with rotation. Beads were washed, and bound proteins were eluted by addition of low pH buffer (50 mM glycine, pH 2.8). Eluted samples were split into two, and either reducing or non-reducing 3× Laemmli buffer supplemented with 8 M urea was added 1:1.

Western blotting and antibodies

Anti-NEDD8 antibodies used were: rabbit ALX-210-194 (Alexis Biochemical), rabbit MIL-10 (Millennium Pharmaceuticals), rabbit #2745 (Cell Signaling Technology), rabbit #2754 (Cell Signaling Technology), rabbit BML-PW9340 (ENZO Lifesciences) and rabbit A-812 (BostonBiochem). Anti-ubiquitin antibodies used were: mouse P4D1 (Santa Cruz Biotechnology), mouse MAB1510 (Millipore) and rabbit Z0458 (DakoCytomation). All of the above antibodies were used at a dilution of 1:3000, with the exception of MIL-10, which was used at 1:10000. Rabbit anti-UBE1 Ab34711 (Abcam), anti-UBE1L2 antibody (Abcam) and rabbit anti-actin Ab1801-100 were all used at 1:3000. Mouse anti-HA HA.11 16B12 (Covance) and anti-HA HRP (horseradish peroxidase) clone HA-7 (Sigma–Aldrich) were used at 1:2000. Anti-FLAG HRP (Sigma–Aldrich) was used at 1:2000. The goat anti-mouse 170-5046 and goat anti-rabbit 170-5047 secondary antibodies (Bio-Rad) were used at 1:5000. Western blotting was performed using Amersham™ Hybond™ ECL (enhanced chemiluminescence) nitrocellulose membranes (GE Healthcare) with 5 % non-fat dried skimmed milk powder/2 % BSA blocking agent and standard laboratory techniques.

PP_i exchange and kinetic thioester experiments

[^{32}P]PP_i and [α-^{32}P]ATP were obtained from PerkinElmer. Bovine ubiquitin was purchased from Sigma (U6253). NEDD8 was generated in an untagged form in a pDEST vector and was expressed in *Escherichia coli*. N-terminal His_6-tagged E1 enzymes (UBE1 and NAE) were expressed in Sf9 insect cells and purified as described previously [30]. Mouse monoclonal anti-FLAG M2 antibody was purchased from Sigma (F1804). Alexa Fluor® 680-labelled secondary antibodies were purchased from Invitrogen (A21109 for anti-rabbit antibody and A21057 for anti-mouse antibody). The ATP–PP_i exchange assays were performed using an improved protocol developed by Bruzzese et al. [30]. The final reaction mixture of 50 μl contained 2.5–20 nM UBE1 or NAE, 0.6 μM ubiquitin or 0.2 μM NEDD8 for UBE1 reactions, 0.16 μM NEDD8 for NAE reactions, 100 μM ATP, 0.5 mM PP_i, ~50 c.p.m./pmol [^{32}P]PP_i, 10 mM $MgCl_2$ and 1 mM TCEP [tris-(2-carboxyethyl)phosphine], in 1× E1 buffer (50 mM Hepes, pH 7.5, 25 mM NaCl, 0.05 % BSA and 0.01 % Tween 20). Reactions were initiated by adding E1 enzymes and the reaction mixtures were incubated at 37 °C. At various time points,

the reaction was quenched with 5 % (w/v) TCA (trichloroacetic acid) containing 10 mM PP_i. The quenched reaction mixtures were transferred to a Schleicher & Schuell Minifold-I Dot-Blot System (Whatman; 10447900) with activated charcoal filter paper (Whatman; 10320163) pre-rinsed in 2 % TCA and 10 mM PP_i, which was then washed for 3×5 min in the same solution. The charcoal filter paper blots were air dried, exposed to an imaging plate for 1 h and visualized using a phosphorimager (Fujifilm FLA-7000; GE Healthcare). Samples from each time point were analysed in duplicate. The spot intensities were converted into the quantity of ATP using a standard curve generated with [α-^{32}P]ATP.

Rate of UBE1-S–NEDD8 and NAE (UBA3) thioester formation

For UBE1, the reaction mixture contained 1.5 μM UBE1, 1 μM NEDD8, 100 μM ATP in 10 mM $MgCl_2$ and 50 mM Hepes, pH 7.5, and was incubated at 37 °C. For NAE, the reaction mixture contained 1 μM NAE, 5 μM NEDD8, 25 μM ATP in 5 mM $MgCl_2$ and 20 mM Hepes, pH 7.5. At designated time points, the reaction mixture was quenched with LDS (lithium dodecyl sulfate) sample loading buffer (Invitrogen; the 4× solution contained 40 % glycerol, 4 %, LDS 4 % Ficoll-400, 0.8 M triethanolamine-Cl pH 7.6, 0.025 % Phenol Red, 0.025 % Coomassie Blue G250 and 2 mM EDTA). The quenched samples were analysed by SDS/PAGE under non-reducing conditions. Samples from each time point were analysed in duplicate. The SDS/PAGE gels were transferred to a 0.2 μm-pore-size Immobilon-P PVDF membrane (Millipore; ISEQ20200) and probed with rabbit anti-NEDD8 or mouse anti-FLAG antibodies. Alexa Fluor® 680-labelled secondary antibody was then used and the intensities of protein bands were quantified on a Li-Cor Odyssey Imaging System (Li-Cor Biosciences).

Competition assay to estimate the affinity of NEDD8 for UBE1

In the competition experiment using the ATP–PP_i exchange assay, the reaction mixtures containing 0.5 nM UBE1, 0.6 μM N-terminal FLAG-tagged ubiquitin, 1 mM ATP, 0.5 mM PP_i (~100 c.p.m./pmol [^{32}P]PP_i) and various amounts of NEDD8 in 1× E1 buffer were incubated at 37 °C for 30 min, quenched and analysed as described above. To monitor competition of NEDD8 in UBE1-S–ubiquitin thioester assays, reaction mixtures containing 50 nM UBE1, 0.8 μM ubiquitin, 1 mM ATP, 10 mM $MgCl_2$ and various amounts of NEDD8 (up to 184 μM) in 1× E1 buffer were incubated at 37 °C for 15 min and quenched with the LDS loading buffer. The UBE1 thioester levels were analysed using non-reducing SDS/PAGE and Western blot analysis Li-Cor imaging as described above.

Quantification of NEDD8 and ubiquitin

For NEDD8 quantifications, U2OS cells were transfected with the indicated plasmids 24 h prior to harvest. For ubiquitin and NEDD8, cells were treated with 30 μM MG132 or 3 μM MLN4924 for 4 h as indicated. Cells were harvested by trypsination and counted, followed by immediate lysis in reducing or non-reducing Laemmli buffer, supplemented with 8 M urea. A minimum of three independent repeats was performed for all points. Quantification of NEDD8 and ubiquitin was performed by Western blot analysis using standard curves based on known amounts of purified protein. Data acquisition and densitometric analysis was performed using the ChemiDoc™ XRS system and Image Lab software respectively (Bio-Rad). Estimations of NEDD8 and ubiquitin levels were based on interpolation within linear ranges.

Saccharomyces cerevisiae experiments and strains

For overexpression of Rub1, pRD54-HA-Rub1 or pRD54-HA-Rub1ΔGG was transformed into *S. cerevisiae* S288c using one-step transformation, and expression was induced by addition of 2 % final concentration galactose. Extracts were prepared using the TCA precipitation protocol, and resuspended in 3× Laemmli buffer supplemented with 8 M urea. The yeast deletion strains ubc12Δ, rub1Δ and uba3Δ were obtained from the Euroscarf haploid knockout collection. Double deletion mutants were generated by PCR-based knockout methods. Strain uba1-204 and the isogenic WT were from Professor Raymond Deshaies (Division of Biology, California Institute of Technology, Pasadena, CA, U.S.A.). The TRP1 N-end rule reporter strains were obtained from Professor Daniel Finley (Department of Cell Biology, Harvard Medical School, Boston, MA, U.S.A.). Growth assays were performed by serial dilution and spotting on to tryptophan-deficient glucose or galactose agar plates as indicated.

RESULTS

UBE1 activates NEDD8 *in vitro*

To better characterize the NEDDylated proteome, it is importnt to understand the pathways mediating the modification. Owing to the high similarity between ubiquitin and NEDD8, we explored whether ubiquitin enzymes can NEDDylate proteins by quantifying the potential for UBE1 to activate NEDD8 *in vitro* [8]. In endpoint activation assays, we detected thioester formation between UBE1 and ubiquitin or NEDD8, but not with the more distantly related UBL SUMO1 (small ubiquitin-like modifier 1) (Figure 1A). Furthermore, UBE1 could transfer NEDD8 to 28 ubiquitin E2 enzymes (Figure 1B and Supplementary Table S1 at http://www.BiochemJ.org/bj/441/bj4410927add.htm), but not the SUMO E2 Ube2I (Ubc9) or the two NEDD8 E2s Ube2M (Ubc12) and Ube2F. Thus UBE1 is able to activate NEDD8 and feed it into the ubiquitin pathway.

We next determined the kinetics of the UBE1–NEDD8 charging reaction, using PP_i exchange assays [30]. The UBE1–NEDD8 reaction was approximately 100-fold less efficient than the activity of NEDD8 with NAE and 200-fold less efficient than the activity of ubiquitin with UBE1 (Figure 1C). We further measured the rate of UBE1–NEDD8 thioester formation using a stop-flow instrument and Western blotting. The observed rate of $k_{obs} = 8.1 \pm 1.2 \times 10^{-3}\ s^{-1}$ (Figure 1D) was 380-fold less efficient than thioester formation of NAE (UBA3) with NEDD8 (Figure 1E). Thus, even though NEDD8 can be activated by UBE1, the reaction is slow and ubiquitin is a significantly better substrate.

In vitro UBE1 activates NEDD8 and ubiquitin in parallel

In the cell, ubiquitin and NEDD8 would probably simultaneously compete for UBE1. To determine if in this situation NEDD8 could still be activated, we next performed an *in vitro* competition assay using thioester formation as readout with fixed amounts of UBE1 (100 nM) and ubiquitin (0.8 μM) and increasing amounts of NEDD8 (Figure 1F). Consistent with the less efficient activation of NEDD8 by UBE1, only minor competition with ubiquitin at high concentrations of NEDD8 (>100 μM) was detectable (Figure 1F). However, UBE1–NEDD8 thioester formation was apparent at a concentration of ~6 μM NEDD8, albeit at a low level. Thus activation of NEDD8 by UBE1 can occur *in vitro* even in the presence of ubiquitin, but requires at least a 7-fold excess of NEDD8. Both activities may thus also take place in parallel in the cell, in situations where the levels of free NEDD8 exceed that of free ubiquitin.

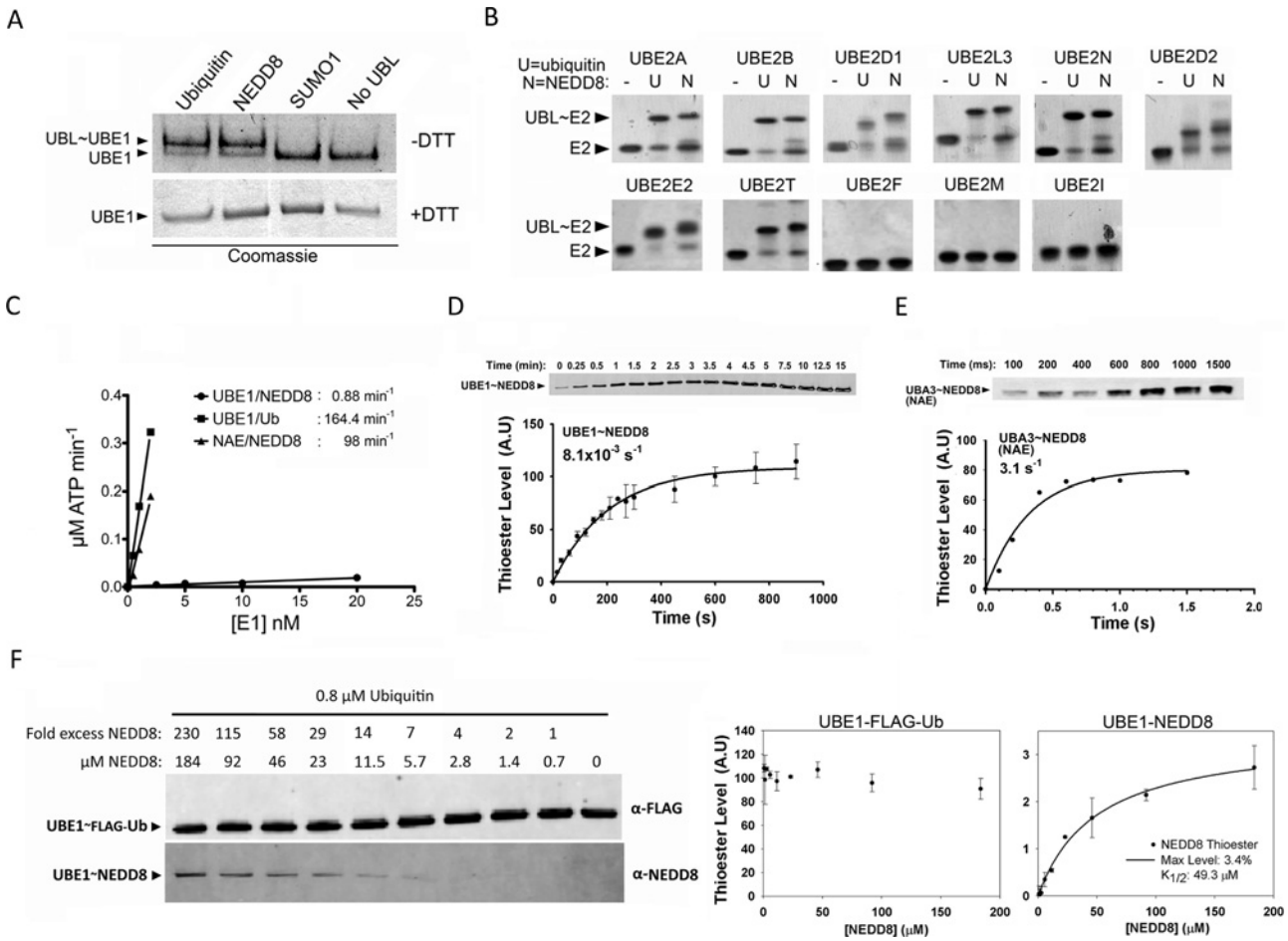

Figure 1 Assessment of the capacity of UBE1 to activate NEDD8 *in vitro*

(**A**) Coomassie Blue-stained gel of UBE1 charging reaction with either ubiquitin, NEDD8 or SUMO1. A bandshift under non-reducing conditions [– DTT (dithiothreitol)] indicates successful charging. (**B**) UBE1 can transfer both NEDD8 and ubiquitin to a panel of ubiquitin E2s, but not to the NEDD8 E2s Ube2M and Ube2F or the SUMO E2 Ube2I. (**C**) Quantification of ATP–PP_i exchange reaction monitoring the specific activity of UBE1 towards ubiquitin and NEDD8 and NAE towards NEDD8. The activity of UBE1 with NEDD8 is roughly 100- and 200-fold slower than NAE with NEDD8 and UBE1 with ubiquitin respectively. (**D** and **E**) Quantification of the rate of NEDD8 thioester formation on UBE1 and NAE (UBA3). Thioester formation of NAE with NEDD8 is 380-fold faster than thioester formation of NEDD8 with UBE1. (**F**) Thioester competition assay determining the capacity of NEDD8 to form a thioester intermediate with UBE1 (50 nM) when competing with ubiquitin (0.8 μM). A 7-fold excess of NEDD8 is minimally required for parallel activation of both UBLs. A.U., absorbance units; Ub, ubiquitin.

Free NEDD8 and ubiquitin are present at approximately equimolar amounts in cells

To estimate whether such a situation could occur, we next measured the cellular concentration of free ubiquitin and NEDD8 using MIL-10 NEDD8 and Z0458 ubiquitin antibodies (for antibody specificity data, see Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410927add.htm). The measurements were performed under non-reducing conditions to preserve thioesters on the E1, E2 and E3 enzymes. We used standard curves based on known protein amounts ($R^2 > 0.98$) to estimate protein content in cell extracts by interpolation (Figures 2A and 2B). Assuming an average cellular volume of 1 pl, the concentration of free NEDD8 in U2OS cells was $\sim$29 μM and that of free ubiquitin was $\sim$25.6 μM (Figures 2C and 2D). Free NEDD8 and ubiquitin are thus present at roughly equimolar amounts; similar values were reproduced using another cell line (MCF7; see Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410927add.htm). According to the *in vitro* results, this concentration would not allow activation of NEDD8 by UBE1; however, relatively small changes in the concentration of either UBL could be sufficient to trigger the process.

NEDD8 overexpression triggers UBE1-dependent NEDDylation

We next overexpressed an HA-tagged form of NEDD8 in U2OS cells, to test whether increases in the relative concentration of NEDD8 over ubiquitin causes UBE1-dependent NEDDylation. Overexpression increased the cellular concentration of free NEDD8 to 173 μM (Figure 2C), which translated into an increase in the ratio of free NEDD8 to ubiquitin from 1:1 to 6.8:1 (Figure 3A). This corresponds roughly to the minimal amount required for activation by UBE1 *in vitro* (Figure 1F). Moreover, overexpression of HA–NEDD8ΔGG, a non-conjugatable form of NEDD8 from which the two C-terminal glycine residues were deleted, increased the concentration of free NEDD8 to 415 μM. This result suggests that roughly 60 % of the overexpressed NEDD8 is converted into conjugates, possibly by UBE1 (Figure 2C).

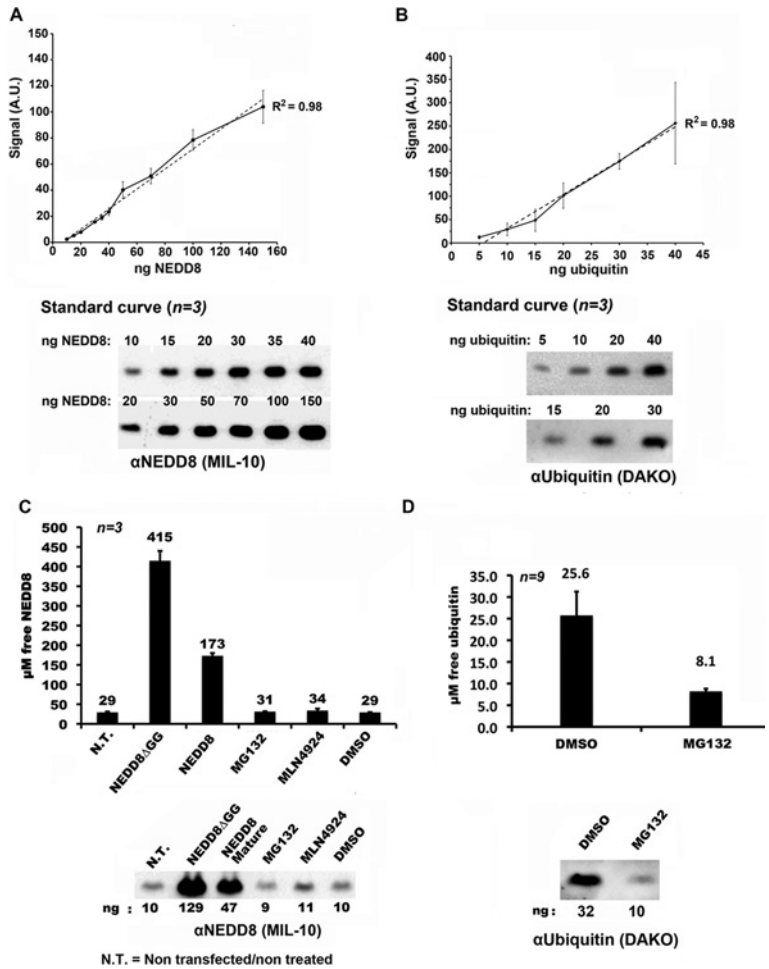

Figure 2 Intracellular concentrations of endogenous free NEDD8 and ubiquitin

(**A** and **B**) Purified NEDD8 and ubiquitin was used to construct standard curves. Data points represent means ± S.D. Lower panels show representative images of standards. A.U., absorbance units. (**C**) Endogenous intracellular concentrations of free NEDD8 (μg) in U2OS cells under the indicated conditions, as well as intracellular concentration of overexpressed mature NEDD8 and NEDD8ΔGG. A representative Western blot is shown in the bottom panel, with estimated amounts of free NEDD8 (ng) calculated by interpolation. (**D**) Endogenous levels of non-conjugated ubiquitin were evaluated under MG132 (30 μM, 4 h) and control (DMSO) conditions. Data points in (**C**) and (**D**) represent means ± S.D.

Indeed, the NEDDylation pattern of cells overexpressing NEDD8 was vastly different from non-overexpressing cells. Endogenous NEDD8 formed very few conjugates, which by molecular mass corresponded to NEDDylated cullins and thioesters with the NEDD8 E2 enzymes and E1 (Figure 3B). Treatment with MLN4924 abolished this NEDDylation, demonstrating the dependence on the classical NEDD8 pathway. NEDD8-overexpressing cells, however, displayed numerous NEDD8 substrates covering almost the entire molecular mass range of the gel (Figure 3C). Expression of the non-conjugatable form of NEDD8 (HA–NEDD8ΔGG) did not result in this extensive NEDDylation pattern (Supplementary Figure S3A at http://www.BiochemJ.org/bj/441/bj4410927add.htm), demonstrating that this atypical NEDDylation represents conjugation of NEDD8 to proteins. Furthermore, treatment with MLN4924 had no affect on this type of NEDDylation (Figure 3C). Rather, siRNA to the ubiquitin E1 enzyme UBE1 (Figure 3C), but not UBA6 (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/441/bj4410927add.htm), strongly reduced its appearance. Importantly, cullin NEDDylation was unaffected by down-regulation of the ubiquitin-activating enzyme and this phenomenon was also observed in other cell lines (Figure 3C and Supplementary Figure S4). Treatment with the UBE1 inhibitor PYR-41 [31] also diminished atypical NEDDylation (Supplementary Figure S3B), suggesting that it is indeed mediated by the ubiquitin E1 enzyme.

MG132 treatment lowers free ubiquitin levels and triggers UBE1-dependent NEDDylation

Next, we wanted to test if increasing the relative concentration of free NEDD8 to ubiquitin by lowering the levels of free ubiquitin also triggers atypical NEDDylation. To efficiently reduce the free ubiquitin levels, we exposed cells to the proteasome inhibitor MG132, which leads to the accumulation of ubiquitin in high molecular mass conjugates [32]. MG132 treatment reduced the free ubiquitin concentration to 8.1 μM, whereas free NEDD8 was unaffected (Figures 2C and 2D). Consequently, the NEDD8 to ubiquitin ratio increased to 3.6:1, approximately half the minimal amount required to trigger UBE1-dependent NEDDylation *in vitro* (Figure 1F). Nevertheless, this increase was sufficient to trigger widespread UBE1-dependent NEDDylation (Figure 3D). We concluded that both increases in NEDD8 levels and decreases in free ubiquitin levels can trigger UBE1-dependent NEDDylation, and that this system is probably more sensitive to lower ubiquitin levels than to excess NEDD8.

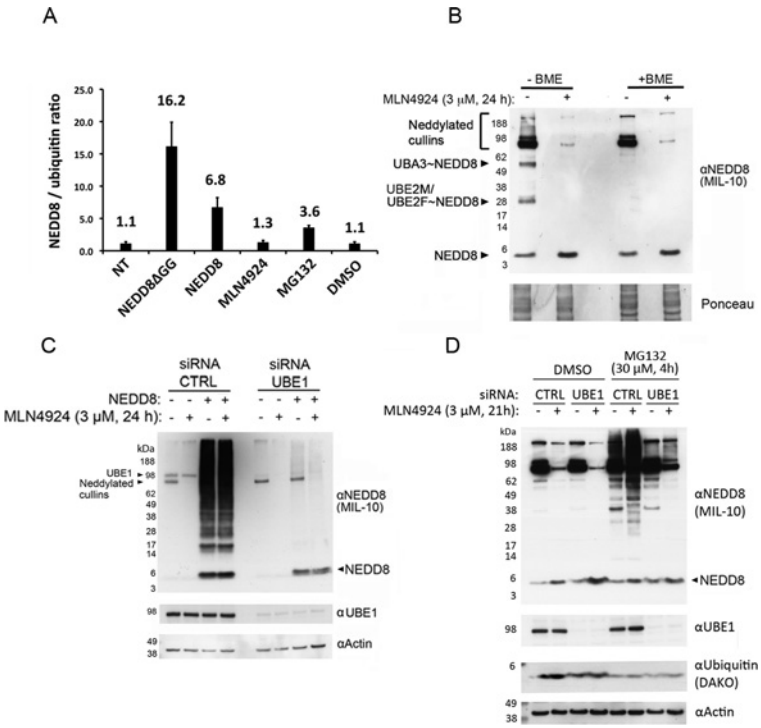

Figure 3 Perturbing the NEDD8 to ubiquitin ratio leads to UBE1-dependent NEDDylation

(**A**) Ratios of free NEDD8 to ubiquitin under the indicated conditions, and after expression of different forms of NEDD8. Error bars represent ± S.D. (**B**) Pattern of endogenous NEDD8 conjugates in U2OS cells under non-reducing [− BME (2-mercaptoethanol)] and reducing conditions (+ BME). Main bands correspond by molecular mass to neddylated cullins and thioesters with the NEDD8 E2s and E1. (**C**) NEDD8 overexpression induces strong ectopic NEDDylation (lanes 3 and 4). This type of NEDDylation is resistant to MLN4924 treatment, but strongly reduced by down-regulation of UBE1 by siRNA. UBE1 is detected in the anti-NEDD8 antibody blot due to a prior Western blot using UBE1 antibody. (**D**) MG132 treatment induces atypical NEDDylation. U2OS cells were treated with 30 μM MG132 for 4 h, and with siRNA to UBE1 and MLN4924 as indicated. Widespread UBE1-dependent NEDDylation is apparent after MG132 treatment. Molecular masses in kDa are shown to the left-hand side of the Western blots. NT, non-transfected.

Genetic alleles of NEDD8 pathway enzymes do not affect atypical NEDDylation

As MLN4924 treatment only results in transient inhibition of NAE, we next verified our results using two genetic approaches to inactivate the enzyme. First, we overexpressed NEDD8 in a cell line carrying a temperature-sensitive allele of the NEDD8 E1 (CHO ts41) [33]. Consistent with our previous results, overexpression of NEDD8 induced atypical NEDDylation at the permissive temperature (32 °C), which was unaffected by a shift to the restrictive temperature (39 °C), even though cullin NEDDylation was strongly decreased (Figure 4A).

Next, we turned to *S. cerevisiae*, a model system in which the NEDD8 pathway is not essential. Endogenous expression of yeast HA–NEDD8 (scNEDD8) revealed that under these conditions the major substrates for NEDDylation are the cullins (Figure 4B), whereas overexpression of scNEDD8, but not of scNEDD8ΔGG, induced atypical NEDDylation similar to mammalian cells (Figure 4C).

Importantly, deletion of the scNEDD8 E1 *uba3* or the single E2 *ubc12* had no effect on atypical NEDDylation, whereas cullin NEDDylation was absent (Figure 4D). These yeast strains do not carry functional NEDD8 enzymes, proving unequivocally that atypical NEDDylation is independent of the classical NEDD8 E1 and E2. Instead, atypical NEDDylation in yeast was abolished by a temperature-sensitive allele of the ubiquitin E1 enzyme Uba1 (*uba1-204)* [34], strongly suggesting that in yeast atypical NEDDylation is also mediated by ubiquitin enzymes (Figure 4E).

NEDD8 is present on the active-site cysteine residue of UBE1 from cells

To unequivocally prove that NEDD8 is activated by UBE *in vivo* it is necessary to detect NEDD8 on its active-site cysteine residue. We thus co-expressed an untagged version of NEDD8 with HA–UBE1 or HA–UBE1 where the catalytic cysteine residue has been mutated to serine (HA–UBE1C632S; OXY). This mutant UBE1 can accept the UBL, but forms a non-reducible oxyester with the modifier. After denaturing immunoprecipitation of HA–UBE1 WT or OXY from cells, we detected a NEDD8 reactive band co-migrating with HA–UBE1 under non-reducing conditions (Figure 5A). Under reducing conditions, this NEDD8–UBE1 thioester was strongly diminished, coinciding with the appearance of free NEDD8 (Figure 5A). For the UBE1 OXY mutant, however, the reduction did not occur, demonstrating that NEDD8 resides on the active site of the E1 enzyme. Furthermore, although free NEDD8 clearly falls off the E1 enzyme under reducing conditions, additional high molecular mass species of NEDD8 can also be seen (Figure 5A). We currently have no explanation for this, but it is tempting to speculate that they are formed prior to activation by UBE1 and represent forms of NEDD8 much more efficiently activated by UBE1.

Finally, to test if endogenous NEDD8 is also in principle available for activation by the ubiquitin-activating enzyme, we immunoprecipitated HA–UBE1 from cells that had not been co-transfected with NEDD8. A reducible interaction of endogenous NEDD8 with HA–UBE1 was indeed detectable (Figure 5B), demonstrating that NEDD8 is in principle available to UBE1 and

that under endogenous conditions low UBE1 levels are limiting to the reaction. Taken together, these experiments demonstrate that, given the right conditions, NEDD8 can be directly activated by UBE1 in cells.

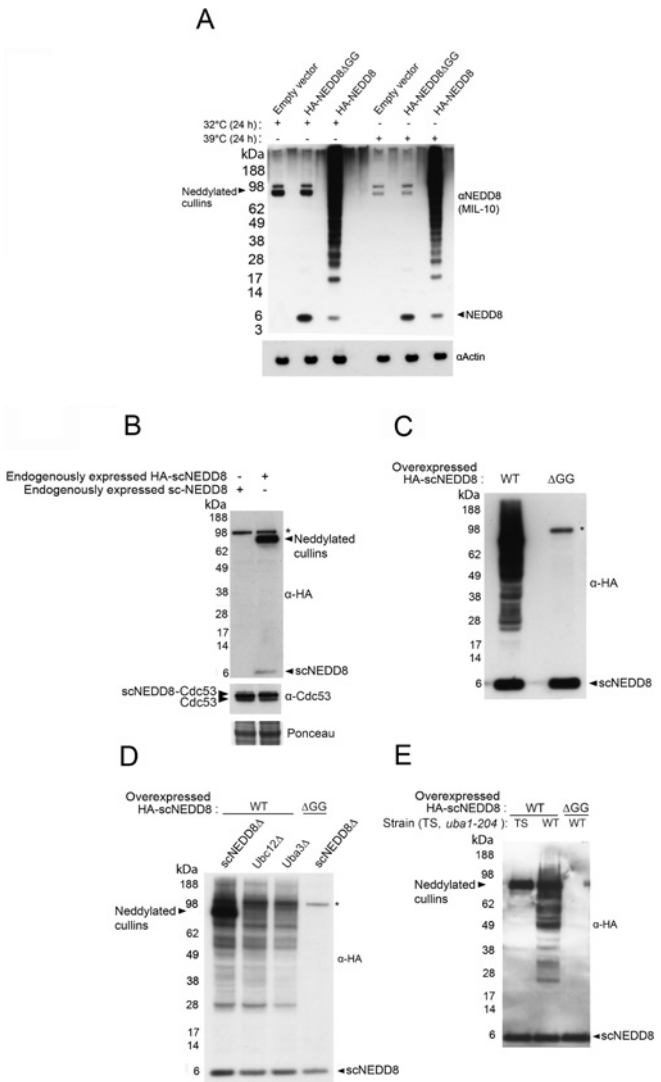

Figure 4 Genetic alleles of the NEDD8 pathway do not affect atypical NEDDylation

(**A**) Mature NEDD8 and NEDD8ΔGG were overexpressed in CHO TS41 cells harbouring a temperature-sensitive allele of NAE, and incubated at the permissive (32°C) and restrictive (39°C) temperature, as indicated. A shift to the restrictive temperature does not affect atypical NEDDylation, suggesting that NEDD8 conjugates are independent of NAE. Importantly, cullin NEDDylation is strongly reduced at the restrictive temperature. Expression of NEDD8ΔGG does not induce atypical NEDDylation, indicating that smears observed upon mature NEDD8 expression correspond to conjugates to proteins. (**B**) Atypical NEDDylation by UBE1 is conserved in *S. cerevisiae*. A Western blot using an anti-HA antibody of cells expressing endogenous levels of untagged or HA-tagged scNEDD8 is shown. The bands detected correspond (by molecular mass) to NEDDylated cullin proteins. The asterisk denotes the antibody cross-reacting band. (**C**) HA–scNEDD8 forms ectopic conjugates upon overexpression (GAL promoter), whereas the ΔGG version does not. (**D**) Deletions of the scNEDD8 E2 Ubc12 and E1 Uba3 affect cullin NEDDylation but not atypical NEDDylation, showing that atypical NEDDylation is strictly independent of the NEDD8 enzymes. (**E**) Overexpression of scNEDD8 in a temperature-sensitive *UBA1* mutant (*uba1–204*) at the restrictive temperature diminishes atypical NEDDylation, indicating that the ubiquitin E1 enzyme is mediating the formation of atypical NEDD8 conjugates. Molecular masses are shown in kDa to the left-hand side of the Western blots.

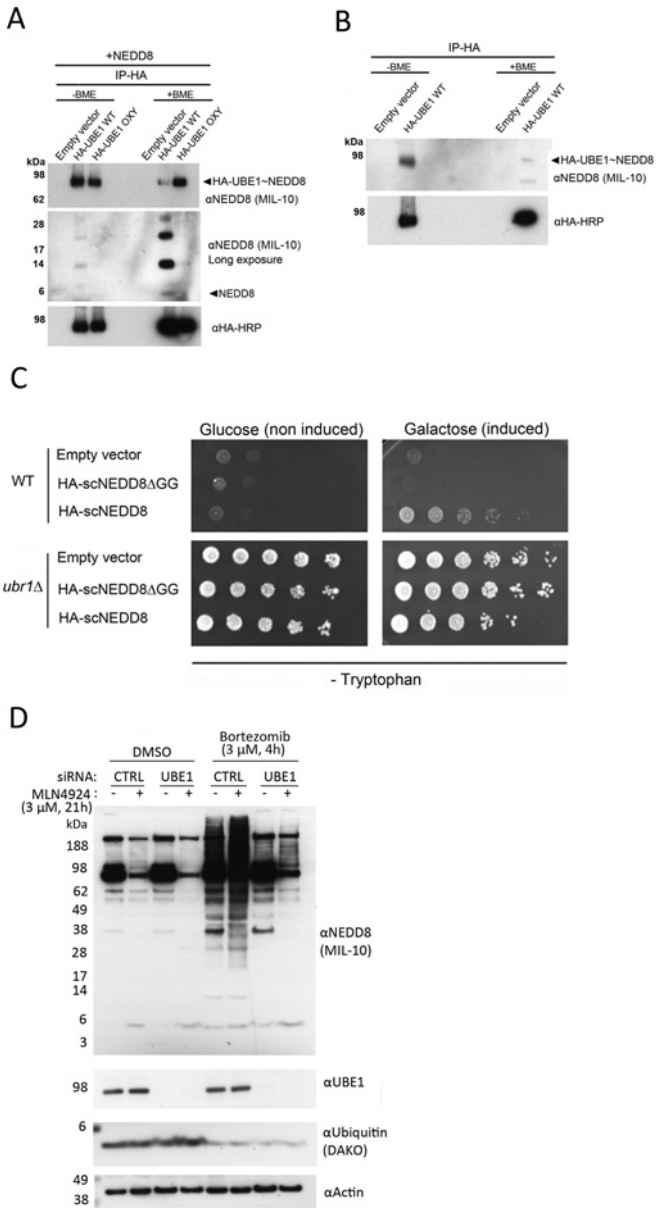

Figure 5 NEDD8 is present on the UBE1 catalytic cysteine *in vivo*, and NEDD8 overexpression attenuates proteasomal degradation of a reporter substrate

(**A**) Untagged mature NEDD8 was co-expressed with WT HA–UBE1 as well as with HA–UBE1(C632S; OXY), which forms a non-reducible oxyester with the UBL. Immunoprecipitation reveals reducible thioester (WT) and non-reducible oxyester (OXY) adducts with NEDD8. In reducing conditions, free NEDD8 can be seen to fall off the WT HA–UBE1, but not the oxyester mutant, demonstrating that NEDD8 resides on the catalytic cysteine residue of this enzyme. Higher molecular mass forms of NEDD8 are also apparent after reduction from UBE1 WT, possibly corresponding to forms of NEDD8 more efficiently activated by UBE1. (**B**) The same experiment as described in (**A**) was performed for WT HA–UBE1, but without simultaneous NEDD8 overexpression, to investigate if endogenous NEDD8 would be available for activation upon UBE overexpression. A reducible thioester adduct with endogenous NEDD8 is apparent after immunoprecipitation (IP), demonstrating that endogenous NEDD8 can be activated when UBE1 is available. (**C**) scNEDD8 overexpression attenuates degradation of a Trp1 reporter substrate. Serial dilution of Trp1 reporter yeast carrying galactose-inducible HA–scNEDD8 or HA–scNEDD8ΔGG. Induction of HA–scNEDD8 restores growth on − TRP plates, indicating stabilization of the Trp1 reporter. Deletion of the yeast N-end rule E3 ligase Ubr1 stabilizes Trp1 and results in loss of tryptophan auxotrophy. (**D**) Bortezomib treatment induces atypical NEDDylation. U2OS cells were treated with 3 μM bortezomib or DMSO (vehicle) for 4 h, and with siRNA to UBE1 and MLN4924 as indicated. Widespread UBE1-dependent NEDDylation is apparent after bortezomib treatment. Molecular masses in kDa are shown to the left-hand side of the Western blots. CTRL, control.

Atypical NEDDylation interferes with ubiquitin-dependent protein degradation in yeast

On the basis of the extensive atypical NEDDylation pattern seen on Western blots, it is probable that activation by UBE1 allows NEDD8 to proceed through the ubiquitin pathway on to many substrates. We thus asked next if this would functionally interfere with the system. To test this possibility, we used a yeast reporter strain for proteasomal activity, which cannot grow on tryptophan-deficient medium due to constitutive proteasomal degradation of the Trp1 protein by the N-end rule E3 ubiquitin ligase Ubr1 [35]. If proteasomal degradation is impaired, for example by deletion of Ubr1, Trp1 is stabilized and growth restored (Figure 5C). Similarly, growth of this strain was partially restored upon scNEDD8 overexpression, but not upon overexpression of scNEDD8ΔGG (Figure 5C), indicating that atypical NEDDylation indeed interferes with the ubiquitin–proteasome system, possibly by linking NEDD8 to otherwise ubiquitylated substrates.

Bortezomib treatment induces atypical NEDDylation

Interference with proteasomal degradation by direct inhibition of the proteasome is also the mechanism of action of the anti-cancer drug bortezomib. This drug may thus induce atypical NEDDylation, similar to what we observed with MG132. To test this possibility, we exposed cells to 3 μM bortezomib and followed the NEDDylation pattern by Western blot analysis. Significant Ube1-dependent NEDDylation was indeed detectable, concomitant with a drop in free ubiquitin levels (Figure 5D). We furthermore observed similar results at a low concentration of bortezomib over a longer period of treatment (100 nM; see Supplementary Figure S5 at http://www.BiochemJ.org/bj/441/bj4410927add.htm). Although experiments with cultured cells are not directly comparable with drug administration *in vivo*, the treatment with lower levels of bortezomib should more accurately reflect the situation in patients. Our results thus indicate that atypical NEDDylation may also occur in patients treated with bortezomib. Whether this process contributes to the efficacy of the drug, however, remains to be determined.

DISCUSSION

Atypical NEDDylation appears to result in the modification of a large set of proteins. It is unclear whether substrate specificity exists or if NEDD8 indiscriminately modifies many, if not all, ubiquitin substrates. Given that all of the ubiquitin E2 enzymes we tested were charged with NEDD8 *in vitro*, it is likely that most ubiquitin substrates would be modified.

Consequences for the NEDD8 proteome

Previous years have seen a rapid expansion of the reported NEDDylated proteome. The best-characterized non-cullin NEDD8 substrate is p53 [16,37], but other proteins have also been described to be NEDDylated, including BCA3, EGFR and caspase 7 [19,20,23]. Many of these substrates have also been shown to be ubiquitylated, and in most cases examined ubiquitylation and NEDDylation require the same E3 enzyme. The effect of NEDD8 on these substrates varies. For p53, it seems to alter its transcriptional profile, whereas for others there is no obvious effect. In the case of EGFR, for example, NEDD8 and ubiquitin seem to be equally good at inducing receptor internalization [20]. What all of these substrates have in common is that their initial characterization and identification was mainly based on overexpression of a tagged form of NEDD8. Although still requiring experimental verification, on the basis of our results it is possible that in those cases NEDDylation was independent of the NEDD8 pathway, and instead was mediated by ubiquitin enzymes. This issue equally pertains to the interpretation of large-scale MS data that was aimed at identifying the NEDDylated proteome after overexpression of tagged NEDD8 [24]. Additional work is now required to verify if these reported NEDDylated proteins are indeed substrates of NEDD8 under endogenous conditions or whether they only become substrates when the levels of NEDD8 or ubiquitin are changed.

Alterations of the pool of free NEDD8 and ubiquitin

The results from the present study demonstrate that an altered NEDD8 to ubiquitin ratio results in atypical NEDDylation. Particularly, ubiquitin depletion appears to more readily trigger atypical NEDDylation than increased NEDD8 levels. The observation that increased expression of UBE1 is sufficient to activate endogenous NEDD8 may be able to explain this difference, as it suggests that the availability of UBE1 is limiting for the reaction in cells. Possibly, the drop in ubiquitin levels upon MG132 treatment frees up UBE1, making it available for activation of NEDD8 in the absence of competing ubiquitin. This raises the possibility that atypical NEDDylation is important as a response to ubiquitin depletion. For example, in many neurodegenerative diseases, cells accumulate ubiquitin conjugates [38] and it is conceivable that under these conditions free ubiquitin is depleted enough for atypical NEDDylation to occur. NEDD8 has indeed been found in protein aggregates from many neurological diseases, including Parkinson's and Alzheimer's [39]. It remains to be seen, however, if any of these conditions will lead to atypical NEDDylation *in vivo* and, if so, what the physiological consequences would be.

Possible functions of UBE1-dependent NEDDylation

Atypical NEDDylation appears to slow proteasome-mediated degradation of substrates. Given the probable lack of substrate specificity, this would result in a global attenuation of protein degradation, which could be its primary function. The mechanism underlying this effect may be that NEDD8 is a poor substitute for ubiquitin in the ubiquitin proteasome system. As UBE1 will activate NEDD8 and ubiquitin in parallel, chains formed on substrates possibly consist of both UBLs, resulting in mixed chains. NEDD8 itself is a very poor substrate for ubiquitylation *in vitro* [8,40], suggesting that the addition of NEDD8 to a ubiquitin moiety would slow chain extension and effectively terminate chains akin to what has been suggested for SUMO1 and SUMO chains [41]. Terminating chains before they reach a critical length for proteasome recognition could be one way of slowing degradation. Furthermore, one could imagine that proteasome-bound deubiquitinating enzymes less efficiently process NEDD8, which could also slow degradation of a substrate.

It is also possible that under ubiquitin-depletion stress, atypical NEDDylation serves as a generalized response to preserve a pool of free ubiquitin by terminating or slowing ubiquitin chain elongation on substrates. The effect we saw in yeast on stability of the Trp1 reporter could in this case just be a secondary consequence of an attempt of the cell to preserve free ubiquitin. Although highly speculative, these options are attractive possibilities, but require further investigations for verification.

Implications for therapeutic interventions

Irrespective of its physiological role, atypical NEDDylation will probably occur in therapeutic settings where the intracellular ubiquitin to NEDD8 ratio is affected. On the basis of the results from the present study, it is conceivable that not only bortezomib, but also other proteasome inhibitors currently in development, such as MLN9708, carfilzomib or CEP-18770, will lead to induction of atypical NEDDylation. The consequences of this are currently not possible to predict, but it is tempting to speculate that bortezomib-induced NEDDylation of ubiquitin substrates has an impact on the therapeutic outcome of this and similar drugs. For example, it may further slow the degradation of p53 and/or other tumour suppressors. Although speculative, atypical NEDDylation may indeed persist even after the direct inhibition of the proteasome has worn off, effectively prolonging the inhibition of protein degradation.

AUTHOR CONTRIBUTION

Roland Hjerpe, Yann Thomas, Jesse Chen and Thimo Kurz designed the research, performed the experiments and analysed the data. Aleksandra Zemla and Siobhan Curran performed the experiments and analysed the data. Natalia Shpiro synthesized MLN4924. Lawrence Dick designed the research and analysed the data. Roland Hjerpe and Thimo Kurz co-wrote the paper. Lawrence Dick and Thimo Kurz supervised the research.

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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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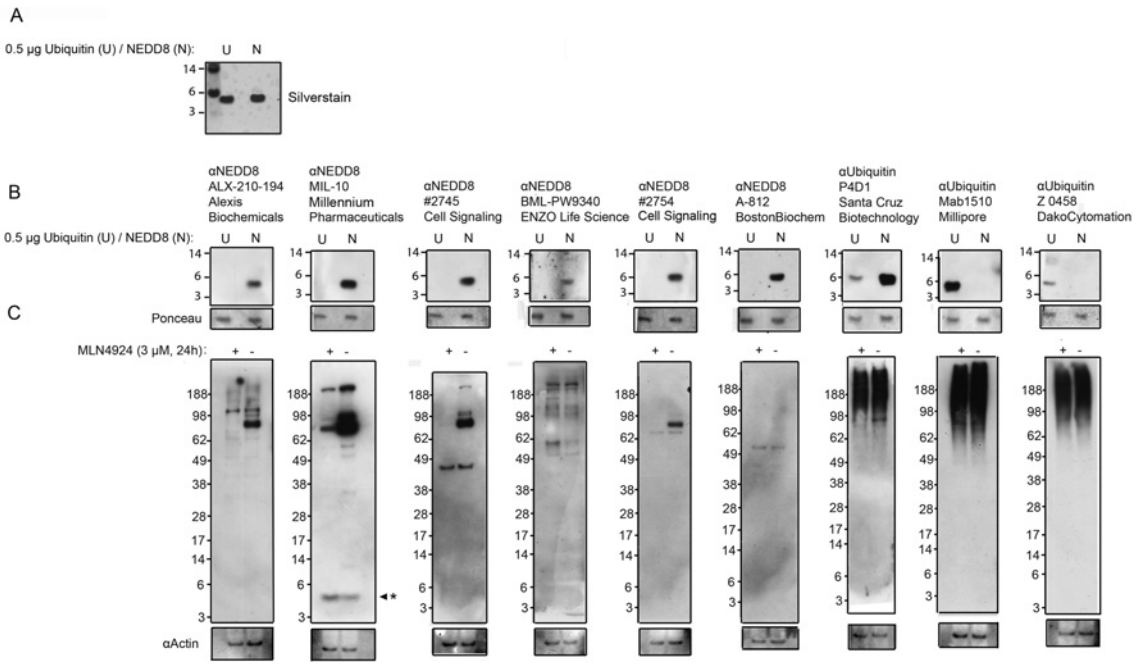

Figure S1 Evaluation of anti-NEDD8 and -ubiquitin antibodies

(**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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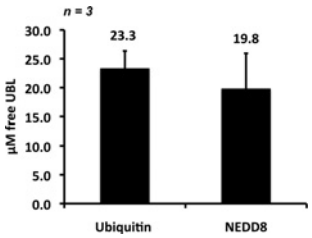

Figure S2 Measurement of endogenous free ubiquitin and NEDD8 concentrations in MCF7 cells

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

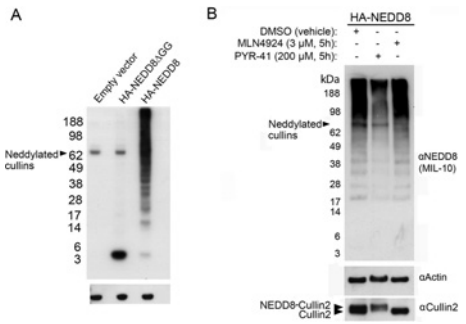

Figure S3 A typical NEDDylation is not induced by overexpression of NEDD8ΔGG, and reduced by treatment with PYR-41

(**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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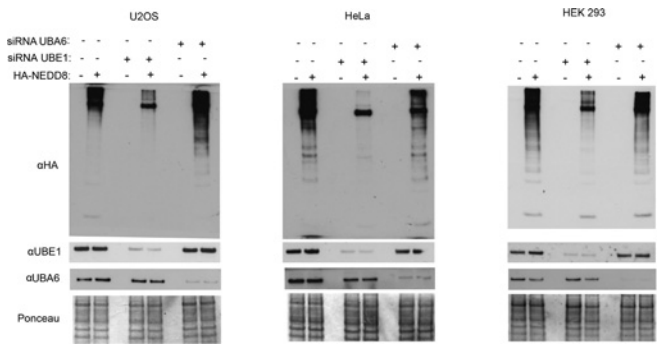

Figure S4 Assessment of contribution of UBA6 to NEDD8 smears and cell type dependence

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.

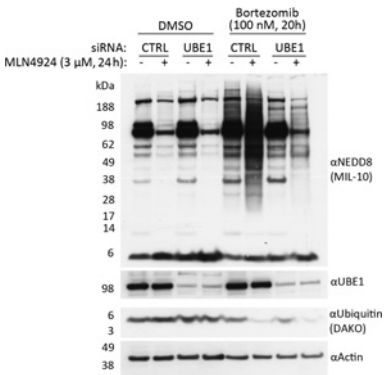

Figure S5 A low concentration of bortezomib induces atypical NEDDylation

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.

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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	−