Supporting Information

Supporting Figure

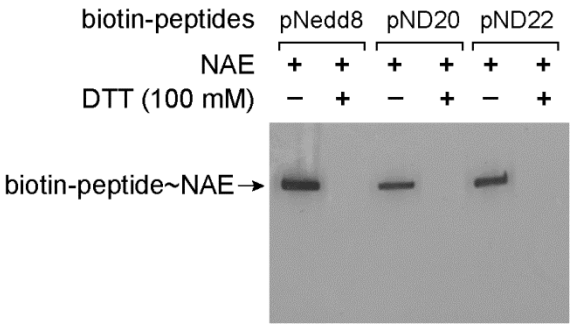

Figure S1. Cleavage of peptide~NAE thioester conjugates with dithiothreitol (DTT). Biotin-conjugated peptides were reacted with NAE in the presence of ATP to form thioester conjugates. Peptide loading reactions treated with and without 100 mM DTT were analyzed by SDS PAGE and Western blotting probed with a streptavidin-HRP conjugate.

Supporting Table

Table S1. DNA sequences encoding the C-terminal peptides of the Nedd8 clones selected by phage display.

Nedd8 clones (C-terminal peptide sequence)	Encoding DNA sequence
wt Nedd8 (^{70}VLALRGG^{76})	5'- GTG TTG GCT CTG AGA GGA GGA -3'
ND1 (^{70}VLQWFGG^{76})	5'- GTG TTG CAG TGG TTT GGA GGA -3'
ND4 (^{70}VRLWFGG^{76})	5'- GTG AGG TTG TGG TTT GGA GGA -3'
ND20 (^{70}VILTFGG^{76})	5'- GTG ATT TTG ACT TTT GGA GGA -3'
ND22 (^{70}VRLMFGG^{76})	5'- GTG AGG TTG ATG TTT GGA GGA -3'

Table S2. Primers used in this study.

Name	Sequence (5' – 3')
Keya1	CAGGAAACAGTATTCATGGCTAGCTCCCCTATACTAGGTTATTGG
Keya2	CATGAATACTGTTTCCTG
Keya3	CCAGACTACGCTAGCCTAATTAAAGTGAAGACG
Keya4	CTCGAGTGCGGCCGCTCATCCTCCTCCTCTCAG
Keya5	CTCGAGTGCGGCCGCTCATCCMNNMNNMNNMNNMNNCACCAGGTGAA GGAC
Jun13	ACTTTATGCTTCCGGCTCGTATGT
Jun14	AATCAAAATCACCGGAACCAGAGC
Bo167	CAA ATT GAT AAG TAC TTG
Bo168	GGAGGATCCGCGGCCGCTTATCCTCCAAACCACTGCAACACCAGGTGAA GGACTGA ACC
Bo169	GGAGGATCCGCGGCCGCTTATCCTCCAAACCACAACCTCACCAGGTGAA GGACTGA ACC
Bo170	GGAGGATCCGCGGCCGCTTATCCTCCAAAAGTCAAAATCACCAG GTGAAGGACTGAACC
Bo171	GGAGGATCCGCGGCCGCTTATCCTCCAAACATCAACCTCACCAG GTGAAGGACTGAACC

Experimental Section

General

Unless otherwise indicated, all reagents were obtained from commercial sources and used without further purification. All solutions and buffers for phage selection were sterilized by either autoclaving or filtration. Kits for isolating DNA plasmids, and Ni-NTA agarose resin for protein purification were from Qiagen. Restriction endonucleases were from New England Biolabs. Taq DNA polymerase was from Promega. Biotin-CoA was prepared following a published procedure.[1] HPLC purification of peptides and biotin conjugated peptides were carried out on a POLARIS BioInert Gradient LC System (Varian, Walnut Creek, CA, USA) with a reverse phase Nucleodur C-18 column of 250 mm in length, 21 mm i.d. and 10 mm particle size (Phenomenex, Torrance, CA, USA). Peptides were analysed by MALDI-TOF spectra acquired with a Voyager DE PRO MALDI mass spectrometer (PerSpective Biosystems, Framingham, MA, USA). Oligonucleotides were supplied by Integrated DNA Technologies and their sequences are listed in Table S2.

Cloning

Plasmid pGEX-GST-AppBp1-Uba3[2] was provided by Professor Brenda A. Schulman of the St. Jude Children's Hospital (Tennessee, USA). The plasmid co-expressed the GST-APPBP1 fusion and Uba3 so that a functional NAE could be assembled and purified as a heterodimer of the two subunits. [2] To construct the expression plasmid of PCP-NAE fusion, we replaced the GST gene in pGEX-GST-AppBp1-Uba3 with the gene encoding the peptidyl carrier protein (PCP). [1] To do this, an NheI restriction site was introduced at the 5' of the GST gene in pGEX-GST-AppBp1-Uba3 by PCR amplifying the GST gene with overlapping primers Keya1 and Keya2. After the NheI site was introduced, the GST gene was deleted by double digestion with the NheI and NotI. The PCP gene was PCR amplified, digested with the same pair of restriction enzymes, and ligated into the plasmid yielding pGEX-PCP-APPBP1-Uba3 for the expression of PCP-NAE.

Plasmid pGEX-NEDD8 was provided by Professor Ning Zheng of the University of Washington (Seattle, USA). It expresses Nedd8 with an N-terminal GST fusion. To express HA-Nedd8 with a N-terminal HA tag fused to Nedd8, the Nedd8 gene was PCR-amplified from the plasmid with primers Keya3 and Keya4, double-digested by NheI and NotI, and ligated into the expression plasmid pET28a.

To express the Nedd8 variants from phage selection, the genes of ND1, ND4, ND20 and ND22 in the pJF3H phagemid vector were amplified by PCR. A common upstream PCR primer Bo167 was used that inserted a ScaI restriction site at the 5' end of the Nedd8 gene. The downstream PCR primers of ND1, ND4, ND20 and ND22 were Bo168, Bo169, Bo170 and Bo171, respectively. These primers inserted a NotI site at the 3' end of the encoding sequences of the Nedd8 variants. The amplified PCR fragments were digested with ScaI and NotI and cloned into the pGEX-4T-1 vector for the expression of Nedd8 variants with a GST tag at their N-termini.

Expression of NAE and Nedd8

PCP-NAE and HA-Nedd8 were expressed as 6×His tagged proteins following similar protocols. Briefly, the expression plasmid was transformed into BL21(DE3) competent cells. An overnight

culture inoculated with a single colony of the transformed cell was prepared and was used to inoculate Lysogeny Broth (LB) (1 L) supplemented with ampicillin (100 μg/mL). The culture was shaken at 37 °C until the cell density reached an OD_{600} in the range of 0.6-0.8. Protein expression was induced by the addition of IPTG to a final concentration of 1 mM in the culture media. The culture was continued to grow overnight at 15 °C. On the next day, cells were harvested by centrifugation at 5,000 rpm (revolution per minute) (2,795 g) in an SLA-3000 rotor (Sorvall) for 10 min, resuspended in lysis buffer (50 mM Tris base, 500 mM NaCl, 5 mM imidazole, pH 8.0) and lysed with a French Press (Thermo Scientific). The resulting crude suspension was centrifuged at 12,000 rpm to remove the pelleted cell debris. The supernatant of the lysate was supplied with 1 mL Ni-NTA agarose and the mixture was rocked gently at 4°C for 2 hours. The slurry was transferred to a gravity column, and the lysate was drained by gravity flow. The resin in the column was washed once with lysis buffer (15 mL), twice with wash buffer (15 mL) (50 mM Tris base, 500 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with elution buffer (5 mL) (50 mM Tris base, 500 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was dialyzed overnight at 4 °C against storage buffer (1L, 25 mM Tris base, pH 8.0, 150 mM NaCl and 0.5 mM DTT) followed by a second dialysis the next day with fresh buffer for another 3 hours. All proteins purified were assayed by electrophoresis on 4-15% SDS Tris polyacrylamide gels to confirm the size and purity of NAE and HA-Nedd8. Solutions of the purified proteins were aliquoted and stored at -80 °C.

Nedd8 variants from phage selection were cloned into the pGEX-4T-1 plasmids for expression as GST fusions. The expression plasmids were transformed into BL21(DE3) pLysS chemical competent cells (Invitrogen) and plated on LB-agar plates with ampicillin (100 μg/ml). Protein expression and purification followed the protocol provided by the vendors of the pGEX expression system (GE Healthcare).

Ubc12 was expressed as a fusion protein with a C-terminal 6×His tag following an established protocol. [2] The CRL complex cullin3-Rbx1 [3] was kindly provided by Professor Ning Zheng's lab at the University of Washington.

Construction of Nedd8 library for phage display

The Nedd8 mutant library was generated by randomizing the C-terminal sequence of Nedd8 covering residues ^{71}LALRG^{75}. Primer Keya5 bound to the 3' of the Nedd8 gene and replaced the native codons of Nedd8 residues 71-75 with the NNK codon. The Nedd8 gene in pET28a-Nedd8 was PCR amplified with primers Keya3 and Keya5. The PCR product was digested with restriction enzymes NheI and NotI, and ligated into the pJF3H phagemid. [4-5] After ligation, the phagemid library was transformed into SS320 cells [6] and the transformed cells were plated out on LB-agar plates supplemented with ampicillin (100 μg/mL). The cells were allowed to grow on the plate for overnight. The resulting colonies were scraped off and the phagemid DNA in the cell was extracted with a DNA maxiprep kit (Qiagen). Random clones were also picked from the plate to sequence the phagemid DNA so the quality of the phagemid library could be confirmed.

Preparation of the phage library of Nedd8

The phagemid library of Nedd8 was transformed into SS320 supercompetent cells infected with M13KO7 helper phage. The transformed cells were allowed to grow for 1 hour at 37 °C before being

added to 2xYT medium (100 mL) supplemented with ampicillin (100 μg/mL) and kanamycin (50 μg/mL). The culture was allowed to grow overnight at 37 °C. On the next day, the supernatant of the cell culture was collected by centrifuging the culture for 10 min at 5,000 rpm (2,795 g) in an SLA-3000 rotor. The clear supernatant was poured into a 500 mL centrifuge bottle containing polyethylene glycol-8000 (PEG-8000) (100 mL, 20% (w/v)) and NaCl (2.5 M). After thorough mixing, the phage solution was incubated on ice for 45 min and centrifuged at 9,000 rpm (9,055 g) in a SLA-3000 rotor for 20 min to isolate the pellets of phage particles. The residual PEG mix in the centrifuge bottle was drained and the phage pellet was resuspended in TBS buffer (2 mL, 20 mM Tris HCl, 150 mM NaCl, pH 7.5). The phage solution was subjected to an additional 10 min centrifugation at 13,000 rpm (11,336 g) to remove cell debris. The phage solution was stored at 4˚C before selection.

Biotin labeling of PCP-NAE and immobilization of NAE on the streptavidin plate

Sfp phosphopantetheinyl transferase was used to label PCP-NAE with biotin. [1] Labeling reaction (100 μL) was set up containing PCP-NAE (5 μM), biotin-CoA (5 μM), Sfp (0.3 μM) in a reaction buffer (50 mM HEPES, 10 mM $MgCl_2$, pH 7.5). The reaction was allowed to proceed for 1 hour at 30 °C, and then BSA (100 μL, 3% (w/v)) was added. 100 μL of the reaction mixture was distributed to a 96-well plate coated with streptavidin. The solution was incubated in the plate well for 1 hour at room temperature so that biotin labeled NAE could bind to the plate. The plate was then washed three times with TBS buffer to remove unbound enzyme before the selection reaction.

Selection of the Nedd8 library displayed on phage with NAE

To select for Nedd8 variants that can be activated by NAE, phage library in the reaction mixture (100 μL) with a final concentration of ATP (5 mM), $MgCl_2$ (10 mM), and BSA (3%) in TBS was added to each well of the streptavidin plate coated with biotin-labeled NAE. For the first round of reaction, the reaction mixture (900 μL) containing the phage library was prepared and evenly distributed to nine NAE coated wells. For subsequent rounds of selections, the reaction conditions were made more stringent by gradually decreasing the amount of NAE immobilized in each well, by decreasing the concentration of the phage in the reaction mixtures, and by decreasing the time of the reaction from 1 hour to 5 minutes. In parallel with the selection reactions, control reactions containing the same concentration of phage but lacking either ATP or NAE were also set up. After the reactions, the wells were washed extensively with TBS-T (0.05 % (v/v) Tween 20, 0.05 % (v/v) Triton X-100 in TBS) for 30 times and TBS for another 30 times. Phage bound to the plate due to the formation of thioester conjugates between NAE and Nedd8 variants displayed on phage surface were eluted by the addition of 100 μL elution solution (20 mM dithiothreitol (DTT) in TBS) to each well. The elution mixture from the plate was combined and added to freshly grown XL1-Blue cells (10 mL) with an OD_{600} around 0.6-1. Phage infection of the *E. coli* cells was allowed to proceed for an hour at 37˚C. The culture was then plated out on multiple LB-agar plates supplemented with ampicillin (100 μg/mL) and glucose (2% (w/v)) followed by overnight incubation at 37°C. On the next day, cells were harvested from the agar plates and the phagemid DNA was extracted with a DNA miniprep kit. The numbers of phage particles eluting from the selection and control reactions were also measured by titering the phage after infecting the XL1-Blue cells. For subsequent rounds of selections, the phagemid DNA from the previous round was used to transform SS320 competent cells infected by M13KO7 helper phage. A new generation of the Nedd8 library was then prepared for the next round of selection.

In between the selection rounds, colonies from the previous selection were randomly picked from the LB-agar plate and individually grown in an overnight culture of LB (5 mL) containing glucose (2% (w/v)) and ampicillin (100 μg/mL). The phagemid harboring the gene of the selected Nedd8 variant was extracted from the cultivated cells using a Qiagen miniprep kit. The phagemid was sequenced with Jun13 or Jun14 primers to reveal the identities of the selected Nedd8 clones.

Biotin conjugation of the peptides

Heptameric peptides with the C-terminal sequences of wt Nedd8, and Nedd8 variants from phage selection were ordered from EZBiolab (Carmel, Indiana, USA). The peptides were further purified by HPLC to be more than 95% pure. The procedure of biotin conjugation to the N-terminal amino group of the peptides was as follows. Peptide (2 mg, 2500 μmol) was dissolved in a solution of 50 mM sodium phosphate buffer (pH 7.0, 300 μL) and DMSO (300 μL). The peptide solution was added to a solution of N-hydroxysuccinimidyl-6'-(biotinamido)- 6-hexanamido hexanoate (NHS-LC-LC-biotin, Pierce) (4.4 mg, 7.750 μmol) to initiate the conjugation reaction. The reaction was allowed to proceed by stirring overnight at room temperature. The reaction mixture was purified by HPLC with a gradient of acetonitrile (5-85 %) in TFA/water (0.1 %) at a flow rate of 10 mL/min over the course of 25 minutes. The purified biotin-peptide conjugates were lyophilized, and their identities were confirmed via MALDI-TOF mass spectrometry (positive mode): biotin-pNedd8 (VLALRGG), calculated: 1138.06 Da (MH^+), found: 1137.78 Da; biotin-pND20 (VILTFGG), calculated: 1159.54 Da (MH^+), found: 1260.49 Da; biotin-pND22 (VRLMFGG), calculated: 1231.66 Da (MH^+), found: 1131.78 Da.

ATP-PP_i exchange assay

The initial velocities of NAE-catalyzed Nedd8 and peptide activation were measured by the ATP-PP_i exchange assay. Reactions (50 μL) for the assay included varying concentrations of Nedd8 proteins or Nedd8-mimicking peptides and NAE (0.05 μM), Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), and ATP (1 mM). The reactions were initiated by the addition of sodium [^{32}P] pyrophosphate (4.6 Ci/mol, 1 mM). The reactions were incubated at room temperature and quenched at various time points by adding suspension of activated charcoal (0.5 mL, 1.6 % (w/v)), tetrasodium pyrophosphate (0.1 M), and perchloric acid (0.35 M)) to each reaction. The charcoal was pelleted by centrifugation and was washed with trichloroacetic acid (1 mL, 2% (w/v)) for three times. Finally the charcoal pellets were resuspended in water (0.5 mL) and the suspension was added to Ultima Gold LSC-cocktail (PerkinElmer) (3.5 mL). The radioactivity bound to charcoal was determined by liquid scintillation counting. To measure the kinetics of the activation of wt Nedd8 and variants by NAE, initial velocities of ATP-PP_i exchange were determined at varying concentrations of Nedd8 from 0.05 μM to 5.0 μM. To measure the kinetics of the activation of Nedd8-mimicking peptides by NAE, the concentrations of the peptides were varied from 10 μM to 800 μM. The kinetic data were fitted to the Michaelis-Menten equation with the data analysis software Origin.

Western blot analysis

To assay the transfer of wt Nedd8 and Nedd8 variants to cullin 3 through the NAE-Ubc12 cascade, wt Nedd8 (5 μM) or variants with a GST tag were incubated with NAE (1 μM), Ubc12 (1 μM) and cullin-Rbx1 complex (1 μM) in the presence of ATP (1 mM), $MgCl_2$ (10 mM), and DTT (50 μM) in

the TBS buffer for 1 hour at room temperature. Reaction mixtures (20 μL) were separated on a 4-15 % SDS-PAGE gel (Bio-Rad). After electrophoresis, the protein bands were electroblotted onto a piece of polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with BSA (3% in TBS buffer) for 1 hour followed by incubation with an anti-GST antibody (Santa Cruz Biotechnology) (1:500 dilution of a stock concentration of 200 μg/mL) in BSA (3%) in TBS for 1 hour. The membrane was washed 5 times with TBS-T and five times with TBS. After washing, the membrane was incubated with an anti-mouse antibody - horseradish peroxidase (HRP) conjugate (Pierce) (1:10,000 dilution in TBS with 3% BSA) for 1 hour. The membrane was again washed 5 times with TBS-T buffer and 5 times with TBS buffer followed by detecting GST-Nedd8 and GST-Nedd8 conjugated cascade enzymes with the ECL luminescent detection kit (GE Healthcare).

To assay the transfer of biotin conjugated peptides to NAE, Ubc12 and cullin, biotin labeled peptide (5 μM), NAE (1 μM), Ubc12 (1 μM), and cullin-Rbx1 (1 μM) were incubated with ATP (1 mM), $MgCl_2$ (10 mM) and DTT (50 μM) in TBS buffer for 1 hour at room temperature. The reaction mixtures were assayed on an SDS-PAGE gel. The blot of the gel was blocked with BSA (3% in TBS) for 1 hour followed by incubation with streptavidin-HRP conjugate (Pierce) (1:10,000 dilution of 1 mg/mL stock in TBS with 3% BSA) for 1 hour. The blot was washed 5 times with the TBS-T buffer and 5 times with TBS buffer followed by detection with the ECL luminescent detection kit.

To assay DTT cleavage of the peptide~NAE conjugates, biotin labeled peptide (5 μM) was allowed to react with NAE (1 μM) in the presence of ATP (1 mM), $MgCl_2$ (10 mM) and DTT (50 μM) in TBS buffer for 1 hour at room temperature. Before loading on the SDS-PAGE gel, DTT was added to half of the reaction to a final concentration of 100 mM and samples were boiled at 100 ˚C for 15 minutes. Both DTT treated and untreated samples were analyzed by SDS-PAGE and Western blotting probed with a streptavidin-HRP conjugate.

Inhibition of Nedd8 transfer through the NAE-Ubc12 cascade by the Nedd8-mimicking peptides

To assay the inhibition of Nedd8 transfer to cullin by the peptides, varying concentrations of the peptides at 5, 10, 20 and 50 μM were pre-incubated with NAE (0.5 μM), Ubc12 (0.5 μM) and cullin-Rbx1 (1 μM) for 1 hour in the TBS buffer containing ATP (1 mM), $MgCl_2$ (10 mM) and DTT (50 μM). HA-Nedd8 was added to a final concentration of 5 μM and the Nedd8 transfer reaction was allowed to proceed for 15 min before SDS-PAGE and Western blot analysis. To probe for HA-Nedd8 conjugated proteins, the blot was developed by sequential incubation with BSA (3% in TBS buffer, pH 7.5) containing a mouse anti-HA antibody (Santa Cruz Biotechnology) (1:500 dilution of 200 μg/mL stock), and an anti-mouse antibody - HRP conjugate (Pierce) (1:10,000 dilution) for 1 hour, respectively.

Measuring the IC_{50} values of the peptides inhibiting the formation of Nedd8~NAE conjugate

Peptides pNedd8, pND20 and pND22 of varying concentrations in the range of 0 to 900 μM were incubated with HA-Nedd8 (1 μM) and NAE (0.3 μM) in reactions (50 μL) containing $MgCl_2$ (10 mM), ATP (1 mM) and TBS buffer. The reaction was allowed to proceed for 15 minutes at room temperature. Reaction mixtures were analyzed by SDS-PAGE and Western blots probed with the anti-HA antibody. The intensities of the protein bands corresponding to HA-Nedd8~NAE conjugates were quantified with the Molecular Imager (Bio-Rad FX Pro Plus) and the associated software. [7] The

ratio of the amount of Nedd8~NAE formed in the reactions with and without the peptide inhibitors were calculated and plotted against the concentrations of the peptides. The IC_{50} values were calculated by the Origin program as the concentration of the peptide inhibitors that afforded 50% inhibition for the formation of Nedd8~NAE conjugate.

Modeling of the interaction of the C-terminal peptides of the Nedd8 variants with NAE

Models of the complex structures between NAE and Nedd8 variants were generated on the basis of the Nedd8-NAE-ATP triple complex (PDB ID 1R4N) [8] by replacing the native residues 71-75 of Nedd8 with the residues of the Nedd8 variants from phage selection. *In sil*ico site directed mutagenesis and optimization of the side chain and occasional main chain interactions were carried out with the program COOT. [9]

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