Supporting Online Material

Materials and Methods

Plasmid, antibodies and reagents

For recombinant expression in *E. coli*, CHBP and Cif DNA (*1*) were cloned into pGEX-6P (GE Healthcare). cDNAs for p27, UbcH5c, UbcH6 and Ubc12, NEDD8, LC3b, SUMO1 and SUMO2 were amplified from reverse-transcribed human cDNA. UbcH5c, UbcH6 and Ubc12 were inserted into pET28 (Novagen), and p27, NEDD8, LC3b, SUMO1 and SUMO2 were cloned into pGEX-6p by the standard PCR-based strategy. pGEX-gp78c and pET28-ube2g2 (*2*) were kindly provided by Dr. Yihong Ye (The National Institute of Diabetes and Digestive and Kidney Diseases, Maryland). pGEX-4T3/pET15b-GST-HA-Roc1-Flag-Cul1-324-776 and bacterial expression constructs for Ubc13/Uev1was generous gifts from Dr. Zhenqiang Pan (Mount Sinai School of Medicine, New York) and Dr. Zhijian Chen (University of Texas Southwestern Medical Center, Texas), respectively. cDNA for GRAIL that lacks the signal sequence and transmembrane region, kindly provided by Dr. Garrison Fathman (Stanford University, California), was cloned into pGEX-6p. pGEX-CAND1 construct was previously described (*3*). Flag-Nrf2 (1-97) bacterial expression plasmid was constructed by ligating the human Nrf2 gene fragment encoding residues 1-97 with an N-terminal Flag tag into the pSUMO expression plasmid. pET22b-PA and pET15b-LFN used for anthrax lethal factor-mediated protein delivery were obtained from Addgene Inc. CHBP was inserted into pET15b-LFN to generate pET15b-LFN-CHBP for recombinant expression. UbΔGG and NEDD8ΔGG were cloned by PCR and inserted into pCS2 with an N-terminal Flag tag. The short-lived GFP reporter plasmids, Ub^{G76V}-GFP, Ub-R-GFP and Ub-M-GFP,

were also obtained from Addgene Inc. For expression in *B. thailandensis*, CHBP, together with 200-bp upstream promoter-containing sequence, was cloned into a broad-host range vector pME6032 (kindly provided by Dr. Dieter Haas at Université de Lausanne, Switzerland). All the mutations were generated by QuickChange Site-Directed Mutagenesis Kit (Stratagene). All the plasmids were verified by DNA sequencing.

Antibodies for GFP (sc-8334), p65 (sc-372), p21 (sc-397), ubiquitin (P4D1, sc-8017), Cul3 (sc-8556), Nrf2 (sc-13032), RhoA (26C4, sc-418), RhoB (sc-180), RhoC (sc-26480), Cdc42 (sc-8401), and Rac1 (sc-217) were purchased from Santa Cruz; Flag (M2) and actin antibodies were from Sigma; Cul1 and p27 antibodies were from ZYMED. PINK1 antibody was from Novus Biologicals, LLC (BC100-494). HIF-1α antibody was purchased from BD Transduction Laboratories. Mcl-1 antibody and HRP-conjugated p53 antibody were kindly provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center). MOAP1 antibody was a gift from Dr. Victor C. Yu (National University of Singapore). Wild-type ubiquitin and NEDD8 proteins were from Boston Biochem and Sigma, respectively. ATP was purchased from Promega. All other chemicals were Sigma-Aldrich products unless noted.

Mammalian cell culture, transfection, protein delivery and immunoprecipitation

HeLa and 293T cells obtained from American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 10% FBS (GIBCO) and 2 mM L-glutamine at 37°C in a 5% CO_2 incubator. Transfection was carried out by using standard calcium phosphate precipitation method or Lipofectamine 2000 (Invitrogen). For anthrax lethal factor (LF) and protective antigen (PA)-mediated protein delivery into mammalian cells, 5 μg of highly purified LFN-

CHBP (a single band on a Coomassie-stained SDS gel) and 1 μg of PA were pre-mixed and added into 1-ml cell culture medium (per well) in a 6-well plate. For immunoprecipitation, cells were lysed in buffer A (25 mM Tris-HCl [pH 7.4], 300 mM NaCl and 1% Triton X-100) supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals). Cell lysates were incubated with anti-Flag beads for 2 h with agitation. Beads were then washed three times with buffer A and twice with buffer B (25 mM Tris-HCl [pH 7.4] and 300 mM NaCl). Flag-tagged proteins were eluted into buffer B supplemented with 500 μg/ml Flag peptide (Sigma) for 1 h at room temperature (RT).

Cell cycle analysis, fluorescence staining, qRT-PCR analysis and luciferease assays

To determine the cell cycle distribution, synchronized HeLa cells were infected with EPEC strains harboring Cif or indicated Cif mutants for 1 h. 21 h after infection, cells were fixed and permealized in cold 70% ethanol followed by propidium iodide (PI) staining. DNA content was measured on Cell Lab Quanta SC flow cytometer (Beckman Coulter). For BrdU assays, BrdU (sigma) was directly added into the cell culture medium to achieve the final concentration of 10 μM. After 1 h incubation, HeLa cells were fixed in 70% cold ethanol for 30 min on ice and then were incubated in 2M HCl /0.5% Triton X-100 for 30 min at RT to denature the DNA into single-stranded molecules. Cells were subsequently incubated in 1 ml of Na2B4O7.10H2O (pH 8.5) to neutralize the acid and stained by anti-BrdU (BD) and Alexa 546 goat anti-mouse antibody. Rhodamine-phalloidin (Invitrogen) staining of actin stress fibers in infected/transfected HeLa cells and p65 staining of NF-κB activation in TNFα-treated HeLa cells were performed as previously described (*4-5*). To measure the mRNA level of CRL substrates, quantitative real-time PCR (qRT-PCR) assays were performed as described (*5*) using the primers as

follows: Nrf2, forward, 5'-CTTTTGGCGCAGACATTCCC-3', reverse, 5'-GACTGGGCTCTCGATGTGAC-3', HIF-1α, forward, 5'-GCCACATCATCACCATATAG -3', reverse, 5'-CAAAGCGACAGATAACACG-3', p27, forward, 5'-AAAATGTTTCAGACGGTTCCC-3', reverse, 5'-CATTCCATGAAGTCAGCGATA-3'. Dual luciferease reporter assays of TNFα-induced NF-κB pathway activation were performed as previously described (*4*).

Expression and purification of recombinant proteins

Recombinant proteins were produced in *E. coli* BL21 (DE3) strain unless noted. Protein expression was induced at RT for 6-12 h with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after OD600 reached 0.6-1.0. Affinity purification of GST or His-tagged proteins was performed using Glutathione Sepharose (GE Healthcare) and Ni-NTA agarose (Qiagen), respectively, following the manufactures' instruction. GST tag was removed from CHBP/Cif by overnight on-column PreScission digestion at 4°C. Periplasmic purification of PA follows a previously published protocol (*6*) and GST-HA-Roc1/His-Flag-Cul1-324-776 complex were also purified essentially as described (*7*). Expression and purification of 3xFlag-RhoA was previously described (*5*). The Cul3/Roc1 complex was produced in Rosetta (DE3) (Novagen) strain harboring the pCool-mRoc1-hCul3 plasmid. Expression was induced at 15°C by IPTG. The Cul3/Roc1 complex was purified by sequential Ni-NTA and glutathione-based affinity chromatography. The expression of Keap1 was also carried out at 15°C in Rosetta (DE3) strain harboring the pCool-mKeap1 plasmid. Following the glutathione affinity purification, GST-Keap1 was on-column digested by the TEV protease to remove the GST tag. His_6-SUMO-Flag-Nrf2 (1-97) protein was produced in BL21 (DE3) at 22°C

and purified by Ni-NTA agarose. The SUMO tag was removed by on-column digestion using the Ulp1 protease. GST-CAND1 protein was expressed in Rosetta (DE3) strain transformed with the pGEX-CAND1 plasmid at 37°C.

Bacterial strains and infection

Two *Enteropathogenic E. coli* (EPEC) strains were used in this study. EPEC E22 strain (kindly provided by Dr. Edgar C. Boedeker, University of New Mexico) bears a full-length functional Cif while EPEC E2348/69 strain naturally harbors a truncated and non-functional Cif (*8*). The E2348/69 strain, when complemented with a Cif-expressing plasmid, behaves identically as the E22 strain in inducing the Cif-dependent cytopathic effect of cell cycle arrest and stress fibers formation (*8*). Procedures of EPEC infection of HeLa cells have been described before (*1*). *B. thailandensis* E264 was obtained from ATCC and cultured as described (*9*). *B. thailandensis* was transformed by electroporation with indicated CHBP-expressing plasmids (pME6032-CHBP) or a control empty vector. For infection, a single colony of *B. thailandensis* was used to inoculate 2 ml of LB containing 25 μg/ml tetracycline and 50 μg/ml polymyxin B. The bacterial culture was shaked until OD600 reached 1.5-2 before infection.0. 1 mM IPTG was added 1 h before infection to induce the production of CHBP. 293T cells seeded in the 6-well plate were infected by *B. thailandensis* at MOI of 50:1. Bacteria attachment was facilitated by centrifugation at 800 x g for 5 min at RT. The cells were co-incubated with bacteria for 2 h at 37°C in a 5% CO_2 incubator and then washed twice with PBS. Infected cells were further cultured for 12 h in fresh medium containing 500 μg/ml kanamycin to kill the bacteria prior to harvest.

***In vitro* and *in vivo* ubiquitination assays**

Ubiquitin ligation catalyzed by gp78c and Ube2g2 was carried out as described (*2*). Briefly, 60 nM E1, 200 nM His-Ube2g2 and 300 nM of GST-gp78c were incubated with 10 μM of ubiquitin at 37°C for the indicated time in a buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM MgCl2, 2 mM ATP and 0.1 mM DTT. CHBP or CHBP C156S mutant protein was added into the reaction mixture as indicated. Reaction was stopped by adding equal volume of 2 × SDS loading buffer. Other *in vitro* ubiquitination assays were performed as follows. 20 nM E1, 500 nM UbcH5c, and 0.5 μg of GST-HA-Roc1/His-Flag-Cul1-324-776 for Cul1-mediated ubiquitination or 20 nM E1, 1 μM UbcH6, and 600 nM GST-GRAIL for GRAIL-catalyzed ubiquitin chain synthesis were incubated with ubiquitin in a 30-μl reaction at 37°C for 1 h in a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM MgCl2 and 0.6 mM DTT. *In vitro* ubiquitination reactions using IpaH (a ubiquitin-ligase effector from *Shigella flexneri*) and LubX (a U-box ubiquitin ligase from *Legionella pneumophila*) as the E3 ligase were performed as previously described (*10-11*). For the two-step ubiquitination assay, 60 nM E1 and 200 nM His-Ube2g2 were incubated with 10 μM ubiquitin for 15 min at RT in a buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM MgCl2 and 2 mM ATP. 50 mM EDTA was then added to the reaction mixture, incubated for another 5 min at RT, and then cooled on ice. Recombinant CHBP was added into the reaction mixture and incubated at 37°C. 300 nM of GST-gp78c was added either concurrently with CHBP or 20 min afterwards to initiate ubiquitin chain formation. Reaction mixtures were subjected to SDS-PAGE, native PAGE or immunoblotting analyses as indicated.

To prepare neddylated Cul3 CRL complex and also check effects of NEDD8 deamidation on cullin neddylation, 4 μg of purified Cul3/GST-ROC1 complex was

subjected to a 60-μl neddylation reaction (37°C for 20 min) containing 20 ng of NEDD8 E1, 600 ng of His-UbcH12, 4 μg of His-NEDD8-WT (or His-NEDD8-Q40E) buffered with 50 mM Tris-HCl, pH7.4, 5 mM $MgCl_2$, 0.6 mM DTT, 2 mM ATP and 0.1 mg/ml BSA. The reaction mixture was analyzed by anti-Cul3 immunoblotting to check neddylation efficiency, or subjected to glutathione affinity purification. To assay Nrf2 ubiquitination, intact or the neddylated Cul3/GST-ROC1 complex immobilized onto glutathione beads was added into a 30-μl reaction mixture containing 30 ng of ubiquitin E1, 300 ng of His-UbcH5c, 3 μg of ubiquitin, 2.5 μg of Keap1 and 0.5 μg of Flag-Nrf2 (1-97) in the ubiquitination buffer (50 mM Tris-HCl, pH7.4, 5 mM $MgCl_2$, 0.6 mM DTT and 2 mM ATP). The reaction was carried out at 37°C for 1 h and stopped by adding 30 μl of denature buffer I (50 mM Tris-HCl, pH7.4, 0.5 mM EDTA and 1% SDS). After boiling at 95°C for 10 min, the denatured mixture was diluted by 20 folds in denature buffer II (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate and 0.5% SDS) followed by anti-Flag immunoprecipitation. The immunoprecipitates were washed five times with denature buffer II, eluted in 1X SDS loading buffer, and analyzed by anti-Flag and anti-ubiquitin immunoblotting.

In vivo ubiquitination was performed essentially as previously described (*5*). Briefly, HeLa cells infected with indicated EPEC strains were treated with 25 mM MG132 for 4 h. Cells were lysed in 1% SDS buffer. Following centrifugation, the lysates were diluted by 10-fold with a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5% NP-40 and 0.5% sodium deoxycholate. The diluted lysates were pre-cleared by protein G Sepharose and subjected to overnight immunoprecipitation using either Nrf2, p27, or a

control antibody. After extensive wash, the immunoprecipitates were subjected to immunoblotting analysis.

Short-lived GFP reporter assay

The activity of the general ubiquitin-proteasome pathway was determined by stabilities of the short-lived ubiquitin-GFP fusion proteins (*12*). Ub^{G76V}-GFP has non-cleavable ubiquitin variant (G76V) fused with GFP, and the N-terminal ubiquitin forms the anchor for polyubiquitin chain that targets the fusion protein for degradation through the ubiquitin-fusion degradation pathway. Fusion proteins expressed from Ub-R-GFP (Ub-arginine-GFP) and Ub-M-GFP (Ub-methionine-GFP) are cleaved by endogenous deubiquitinating enzymes (DUBs). The resulting R-GFP is recognized by a specific ubiquitin ligase in the N-end rule pathway and therefore rapidly degraded, while M-GFP is resistant to ubiquitination and is a long-lived protein. 1.4×10^6 HeLa cells were plated in 10-cm dishes 24 h before transfection with Ub^{G76V}-GFP, Ub-R-GFP or Ub-M-GFP reporter plasmids. Cells were split into 6-well plates 5 h post-transfection and cultured overnight before delivery of recombinant LFN-CHBP. As a control, 50 μM MG132 was added to the culture medium at the same time. At time indicated, cells were harvested by direct addition of 1 × SDS loading buffer and the steady level of transfected GFP reporter constructs were analyzed by immunoblotting.

***In vitro* deamidation of ubiquitin or NEDD8**

3 μg of ubiquitin or NEDD8 were incubated with indicated amounts of recombinant CHBP or Cif at 37°C for 20 min. The reaction mixtures were then load onto 8% native PAGE gel buffered by 375 mM Tris-HCl (pH 8.8). Proteins on the native gel were

directly visualized by Coomassie blue or silver staining as indicated or transferred to the PVDF membrane for immunoblotting analysis.

Mass spectrometric analysis

MALDI-TOF mass spectrometry was initially employed (*13*) to analyze CHBP-incubated entire ubiquitin molecule as well as triptic ubiquitin peptides. The results showed that there were no mass changes greater than 5 dalton at the whole protein level and a Gln-40-contaning ubiquitin peptide had a one-dalton mass increase after CHBP treatment. ESI mass spectrometry was then performed to confirm the MAIDI data and also to further reveal the nature of the one-dalton mass increase. Briefly, protein bands on the SDS-PAGE gel were de-stained and in-gel digested with sequencing grade trypsin (10 ng/μL trypsin, 50 mM ammonium bicarbonate, pH 8.0) overnight at 37°C. Peptides were extracted with 5% formic acid/50% acetonitrile and 0.1% formic acid/75% acetonitrile sequentially and then concentrated to ~ 20 μl. The extracted peptides were separated by a analytical capillary column (50 μm × 10 cm) packed with 5 μm spherical C18 reverse phase material (YMC, Kyoyo, Japan). An Agilent 1100 binary pump was used to generate HPLC gradient as follows: 0%-5% B in 5 min, 5%-40% B in 25 min, 40%-100% B in 15 min (A, 0.1 M acetic acid in water; B, 0.1 M acetic acid/70% acetonitrile). The eluted peptides were sprayed into a QSTAR XL mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with a nano-ESI ion source. The mass spectrometer was operated in information-dependent mode with one MS scan followed by three MS/MS scans for each cycle. The mass range of MS scan was from m/z 400 to 2000. Database searches were performed on an in-house Mascot server (Matrix Science Ltd., London, UK). Methionine oxidation was set as variable modification.

Supporting figures

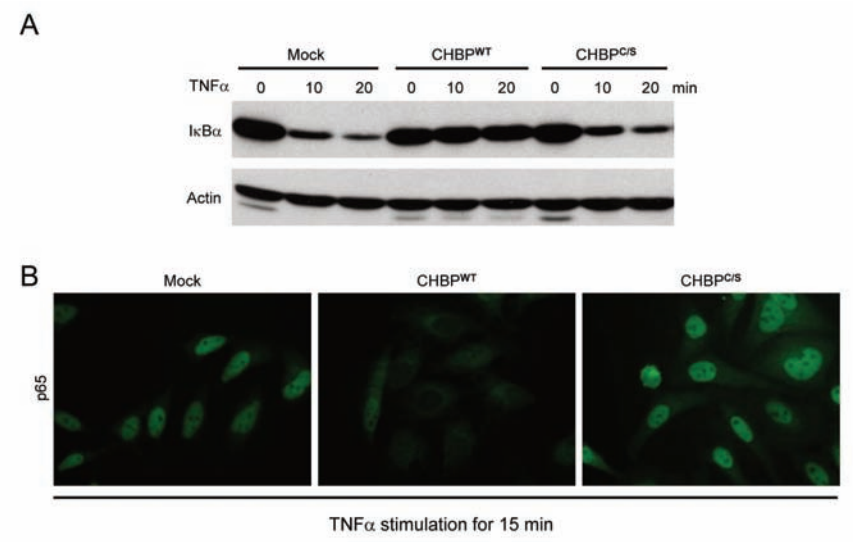

Fig. S1. Effects of CHBP on TNFα-induced IκBα degradation and NF-κB nuclear translocation. Purified recombinant CHBP protein was delivered into HeLa cells using the anthrax lethal factor system. Cells were treated with TNFα for indicated time to induce NF-κB pathway activation. Shown in (A) are anti-IκBα and anti-actin immunoblots of the total cell lysates. Immunofluorescence staining of the p65 subunit of NF-κB is shown in (B). $CHBP^{WT}$ and $CHBP^{C/S}$ denote wild-type and the catalytic cysteine mutant CHBP, respectively.

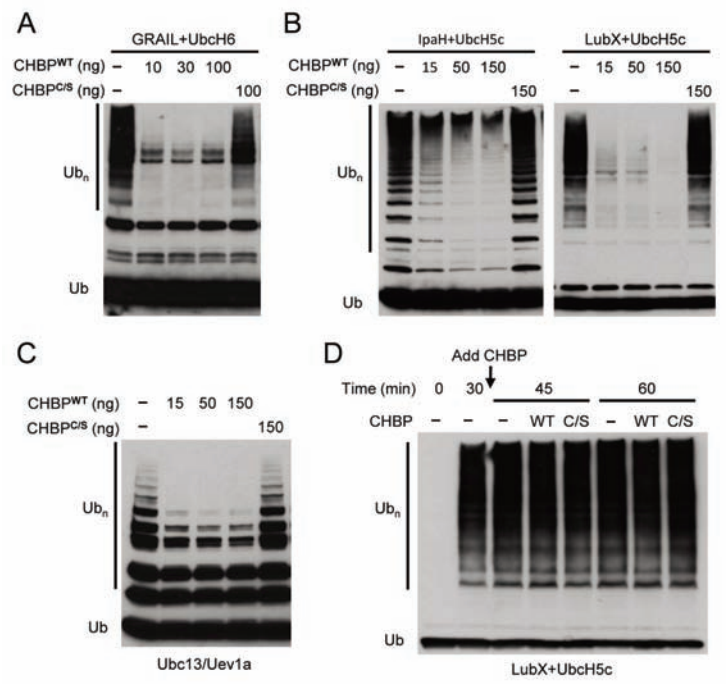

Fig. S2. Effects of recombinant CHBP on *in vitro* synthesis of or synthesized ubiquitin chains mediated by multiple E3/E2 enzyme pairs. *In vitro* ubiquitination reactions using GRAIL/UbcH6 (A), IpaH/UbcH5c (the left panel in (B)), LubX/UbcH5c (the right panel in (B) and (D)), Ubc13/Uev1a (C) were carried out in the presence of indicated amounts of CHBP protein except for (D), in which CHBP was added after the ubiquitination reaction has been stopped at the 30-min time point. $CHBP^{WT}$ and $CHBP^{C/S}$ denote wild-type and the catalytic cysteine mutant CHBP, respectively. IpaH is a ubiquitin-ligase effector from *Shigella flexneri* and LubX is a U-box ubiquitin ligase from *Legionella pneumophila*. Reactions were subjected to immunoblotting analysis for ubiquitin chain (Ub_n) formation.

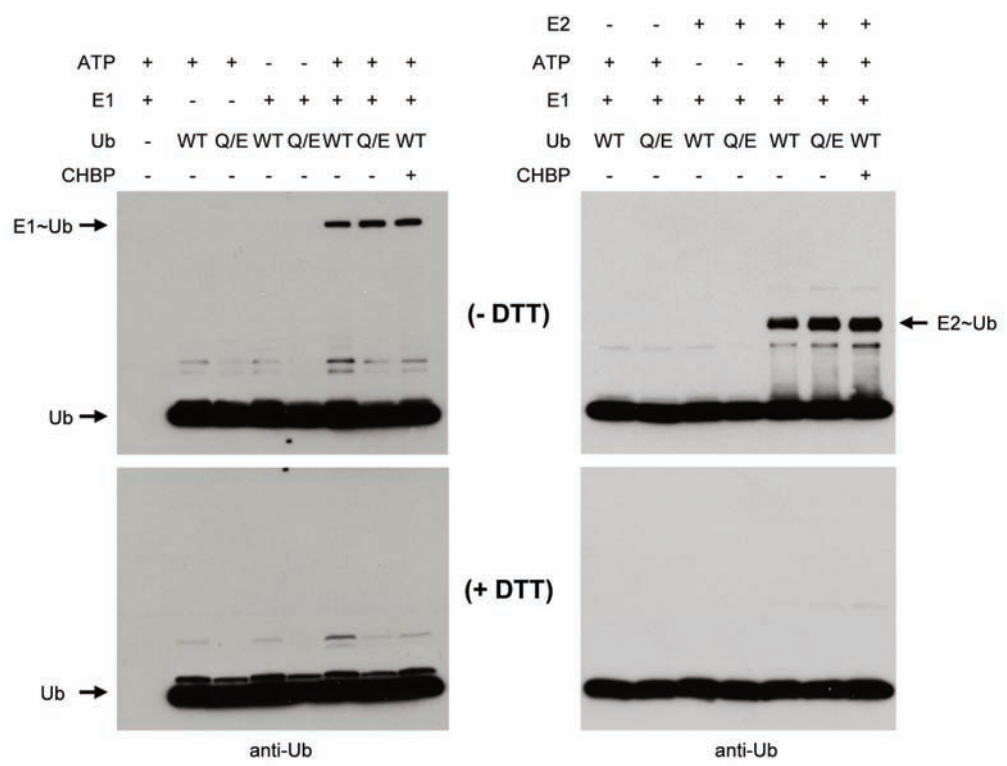

Fig. S3. Effects of CHBP on ubiquitin charge onto E1 and E2. Ubiquitin charge reactions (Left, E1 charge; Right, E2 charge) were performed with wild-type (WT) ubiquitin in the presence/absence of purified CHBP protein or with ubiquitin Q40E mutant (Q/E). The E2 enzyme used is UbcH5c. Reactions were subjected to non-reducing (-DTT, upper panels) or reducing (+DTT, lower panels) SDS-PAGE followed by anti-ubiquitin immunoblotting analysis.

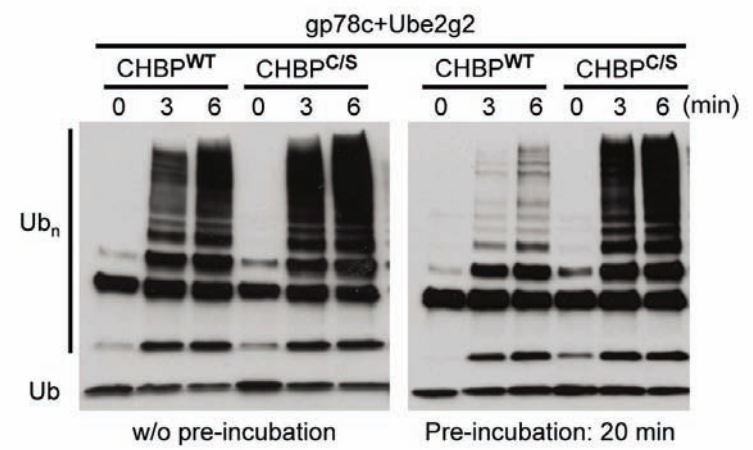

Fig. S4. Effects of CHBP incubation with pre-charged E2 on subsequent E3-catalyzed ubiquitin-chain synthesis. Ube2g2 was charged with ubiquitin and the charge reaction was stopped by excess amounts of EDTA. To initiate the ubiquitin chain synthesis, gp78c was added into the reaction concurrent with (left panel) or 20-min after (right panel) addition of CHBP protein. Ubiquitination reactions stopped at the indicated time points were subjected to immunoblotting analysis for ubiquitin chain (Ub_n) formation.

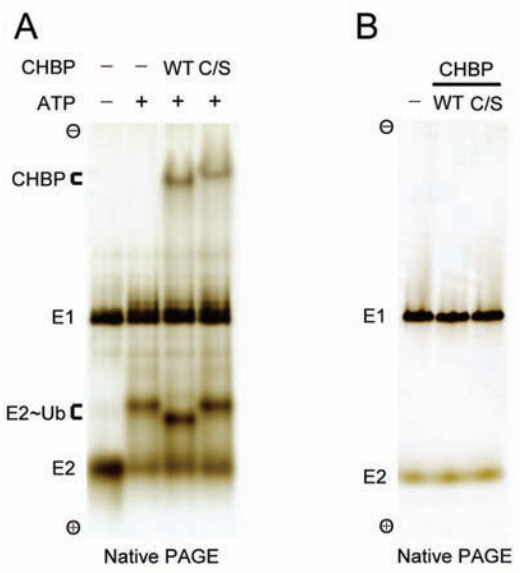

Fig. S5. Native PAGE analysis of CHBP-incubated E2~Ub thioester (A) and E2 hydrolyzed from CHBP-incubated E2~Ub thioester (B). Ube2g2 was charged with ubiquitin (A) or GST-ubiquitin (B) and the charge reaction was stopped by excess amounts of EDTA. The reaction was then incubated with recombinant CHBP before subjected to native PAGE (pH-8.8) analysis in (A). In (B), the reaction was further subjected to glutathione bead precipitation. The beads were eluted with DTT and the elution was subjected to native PAGE analysis. Shown in (A) and (B) are the silver stained native gels with the identity of each band marked at the left. Free ubiquitin was invisible due to its insensitivity to silver staining on the native gel.

Start-End	Sequence	Mr (expt)	Mr (calc)
1-11	MQIFVKTLTGK	1264.71	1264.72
7-27	TLTGKTITLEVEPSDTIENVK	2287.14	2287.22
12-27	TITLEVEPSDTIENVK	1786.87	1786.92
12-29	TITLEVEPSDTIENVKAK	1986.02	1986.05
12-33	TITLEVEPSDTIENVKAKIQDK	2470.31	2470.32
28-42	AKIQDKEGIPPDQQR	1722.83	1721.91
30-42	IQDKEGIPPDQQR	1523.73	1522.77
30-48	IQDKEGIPPDQQRLIFAGK	2153.15	2152.16
43-48	LIFAGK	647.19	647.40
49-63	QLEDGRTLSDYNIQK	1178.85	1178.88
49-72	QLEDGRTLSDYNIQKESTLHLVLR	2827.50	2827.48
55-63	TLSKYNIQK	1080.53	1080.55
55-72	TLSDYNIQKESTLHLVLR	2129.17	2129.15
55-74	TLSDYNIQKESTLHLVLRLR	2398.31	2398.33
64-72	ESTLHLVLRL	1066.59	1066.61

CHBP-treated Ub

Start-End	Sequence	Mr (expt)	Mr (calc)
1-11	MQIFVKTLTGK	1264.71	1264.72
7-27	TLTGKTITLEVEPSDTIENVK	2287.17	2287.22
12-27	TITLEVEPSDTIENVK	1786.91	1786.92
12-29	TITLEVEPSDTIENVKAK	1986.01	1986.05
12-33	TITLEVEPSDTIENVKAKIQDK	2470.27	2470.32
28-42	AKIQDKEGIPPDQQR	1721.90	1721.91
30-42	IQDKEGIPPDQQR	1522.75	1522.77
34-42	EGIPPDQQR	1038.50	1038.51
43-54	LIFAGKQLEDGR	1345.72	1345.74
49-63	QLEDGRTLSDYNIQK	1178.83	1178.88
49-72	QLEDGRTLSDYNIQKESTLHLVLR	2827.57	2827.48
55-63	TLSKYNIQK	1080.51	1080.55
55-72	TLSDYNIQKESTLHLVLR	2129.12	2129.15
55-74	TLSDYNIQKESTLHLVLRLR	2398.28	2398.33
64-72	ESTLHLVLRL	1066.58	1066.61

Untreated Ub

Fig. S6. Mass spectrometry analysis of CHBP-treated (left) and untreated (right) ubiquitin. Sequences of all tryptic peptides identified by the mass spectrometry are shown along with their theoretic [Mr (calc)] and experimentally determined [Mr (expt)] molecular mass. Start and end residue numbers of each peptide are listed at the left of the sequence. Three overlapping peptides showing one-dalton mass increase upon CHBP treatment are colored in red.

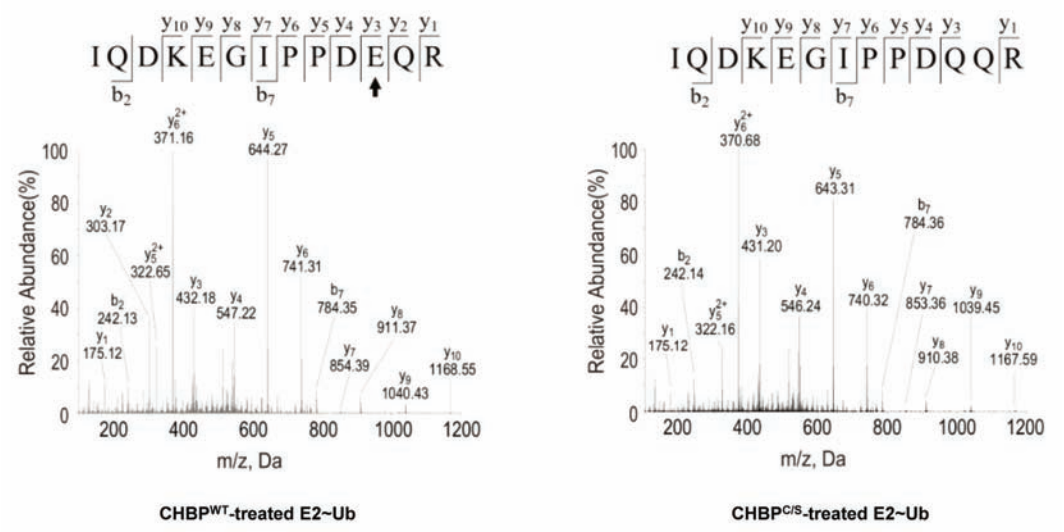

Fig. S7. Electrospray ionization (ESI) tandem mass spectrometry (MS/MS) spectrum of a Gln-40-containing tryptic peptide from CHBP-treated E2~Ub thioester. b and y ions are marked in the spectrum. The amino acid sequence of the peptide is shown on top of the spectrum. The fragmentation patterns that generate the observed b and y ions are illustrated along the peptide sequence. The arrow in the left panel marks the residue (Gln-40) showing one-dalton mass increase in wild-type CHBP ($CHBP^{WT}$) treated ubiquitin (left) compared with that in the catalytic cysteine mutant ($CHBP^{C/S}$) treated ubiquitin (right).

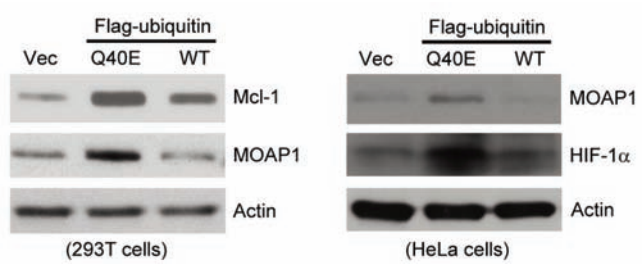

Fig. S8. Effects of ectopic expression of ubiquitin Q40E on steady levels of substrates of the ubiquitin-proteasome pathway. 293T (left panels) or HeLa cells (right panels) were transfected with Flag-ubiquitin or Flag-ubiquitin Q40E expression plasmid. Endogenous levels of indicated proteins were shown by immunoblotting of total cell lysates using specific antibodies.

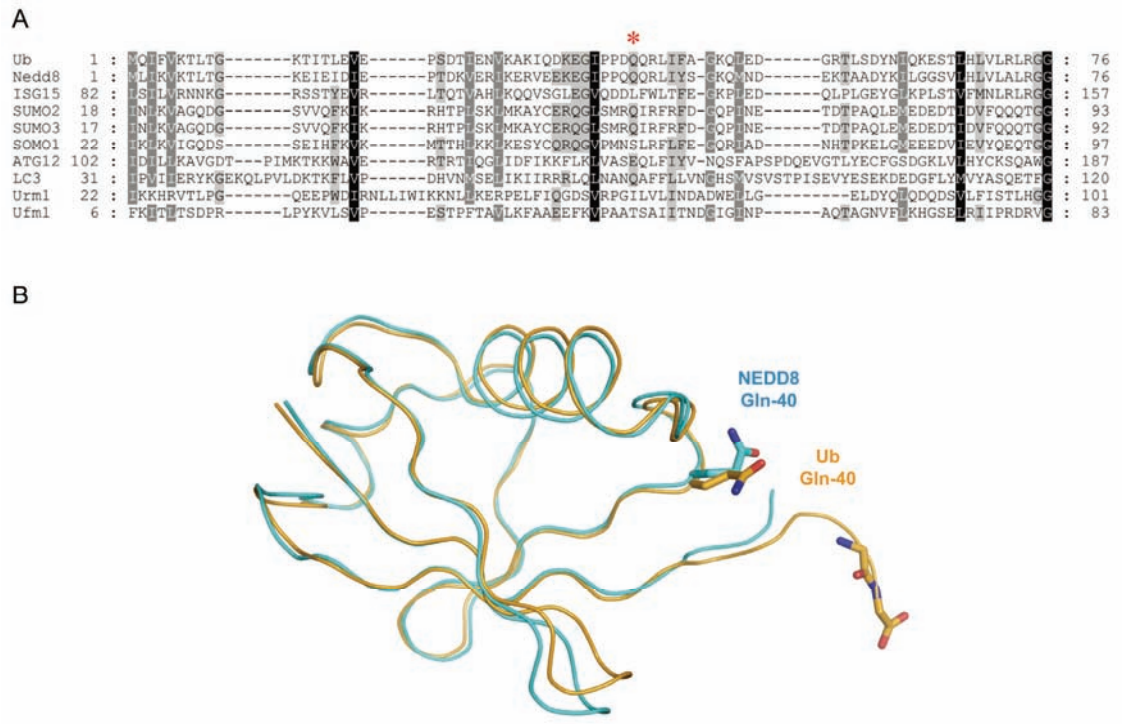

Fig. S9. Multiple sequence alignment of the ubiquitin-like protein family and structural positions of Gln-40 in ubiquitin and NEDD8. (A) Sequences of indicated human ubiquitin-like proteins were aligned according to their secondary structures. NEDD8 has about 80% sequence similarity with ubiquitin and other ubiquitin-like proteins are generally not similar to ubiquitin at the primary sequence level. The position of Gln-40 in ubiquitin is marked by the red asterisk (*). (B) Superimposition of three dimensional structures of ubiquitin (PDB ID 1UBQ) and NEDD8 (PDB ID 1NDD), colored in orange and cyan, respectively. The conserved Gln-40 are colored accordingly and shown in sticks.

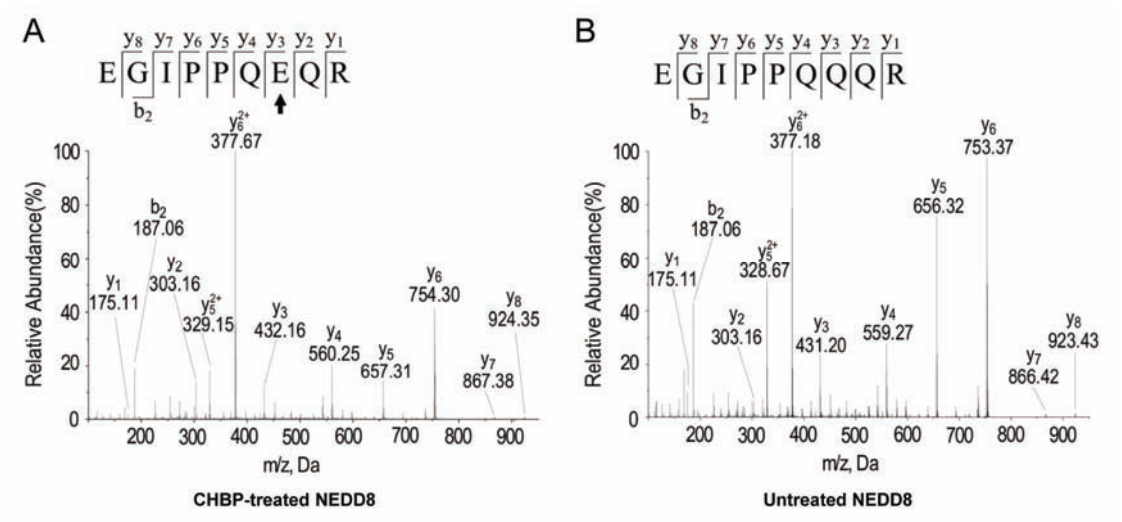

Fig. S10. Electrospray ionization tandem mass spectrometry spectrum of a Gln-40-containing tryptic peptide from CHBP-treated (left) and untreated (right) NEDD8. b and y ions are marked in the spectrum. The amino acid sequence of the peptide is shown on top of the spectrum. The fragmentation patterns that generate the observed b and y ions are illustrated along the peptide sequence. The arrow in the left panel marks the residue that shows one-dalton mass increase after CHBP treatment and is converted from glutamine (Gln-40) into glutamic acid.

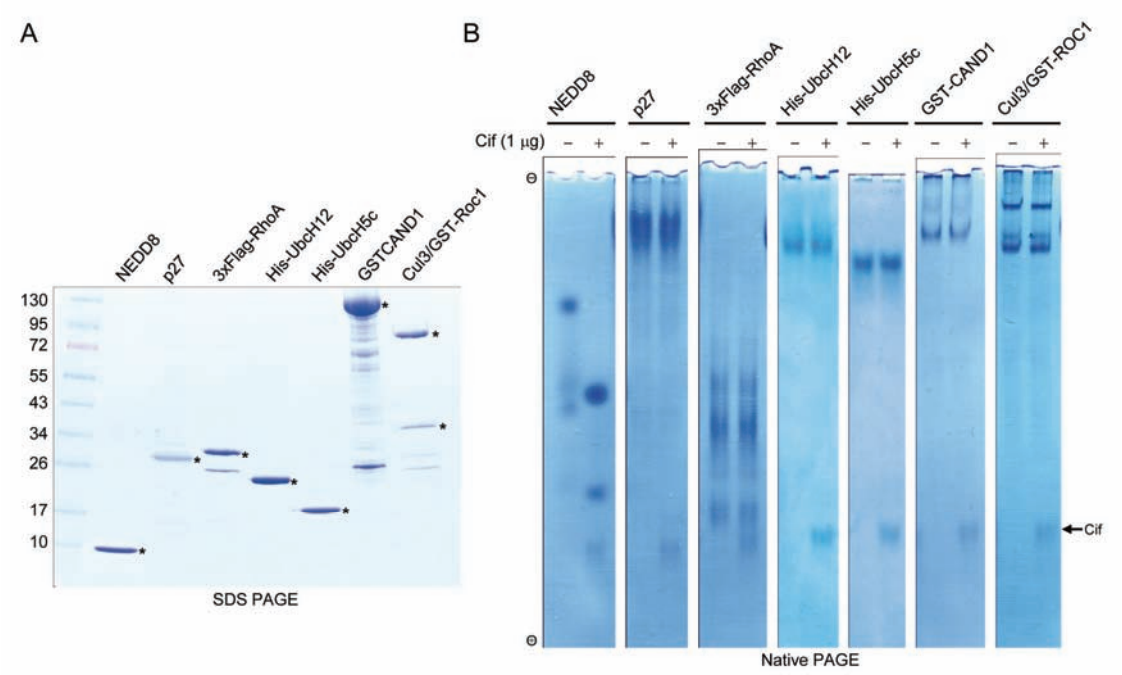

Fig. S11. Native PAGE analysis of effects of Cif incubation on NEDD8 and related proteins in the cullin pathway. Indicated purified proteins shown on the SDS-PAGE in (A) were incubated with recombinant Cif (1 μg). Samples were loaded on native PAGE gels stained by Coomassie blue as shown in (B).

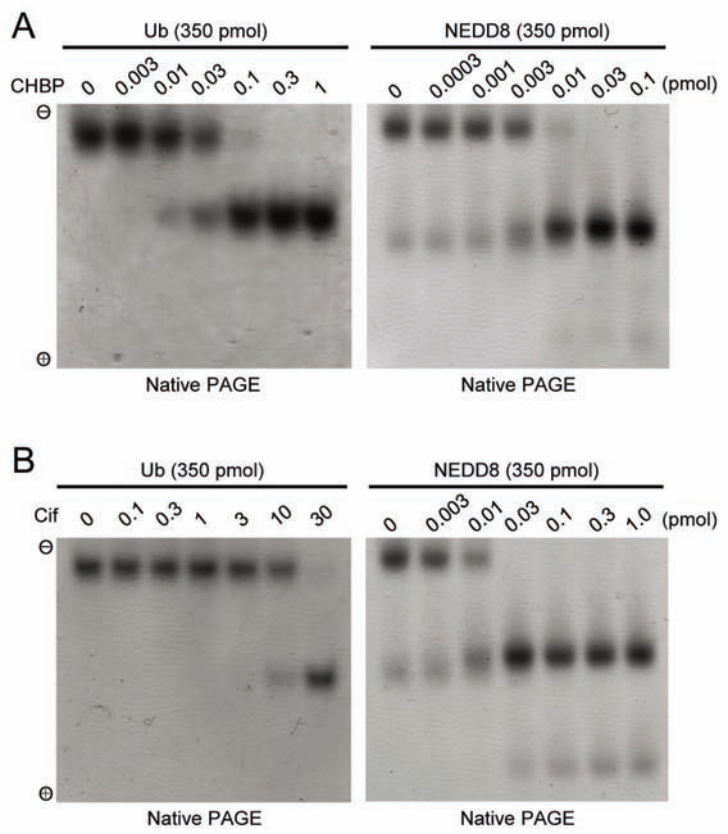

Fig. S12. Enzyme-titration measurements of the deamidase activity of CHBP/Cif towards ubiquitin and NEDD8. 350 pmol (3 μg) of ubiquitin (left panels) or NEDD8 (right panels) were subjected to 20-min incubation with indicated amounts of purified CHBP (A) or Cif (B) in a 20-μl reaction. The whole reaction mixtures were loaded onto a native gel and Coomassie blue stained gels are shown. Results of quantitative analysis are summarized in Fig. 3A.

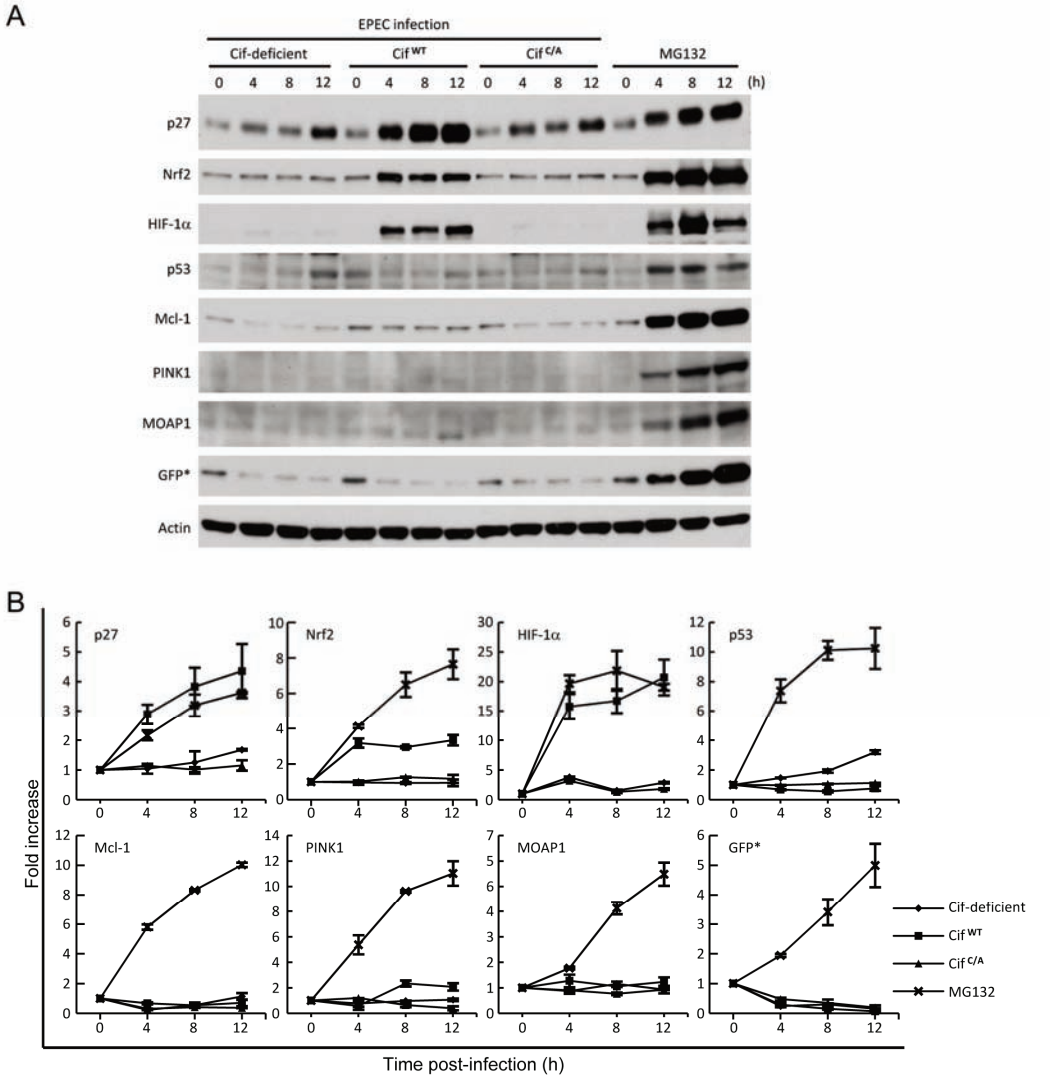

Fig. S13. Effects of Cif infection on steady levels of CRL and non-CRL substrates. Shown in (A) are results from an independent repeat experiment of that shown in Fig. 3C. Shown in (B) is the quantitative analysis of raw data presented in (A) and Fig. 3C.

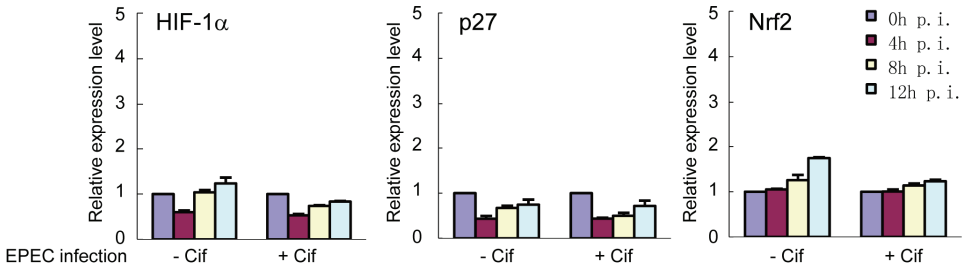

Fig. S14. Effects of Cif on mRNA levels of CRL substrates during EPEC infection. HeLa cells were infected with Cif-deficient EPEC strains (E2348/69) complemented with a vector (- Cif) or a Cif-expressing plasmid (+ Cif). mRNA levels of HIF-1α, p27 and Nrf2 in infected cells were measured by qRT-PCR analysis and normalized by those of actin. Shown are mean values ± SD (error bars) from duplicate determinations.

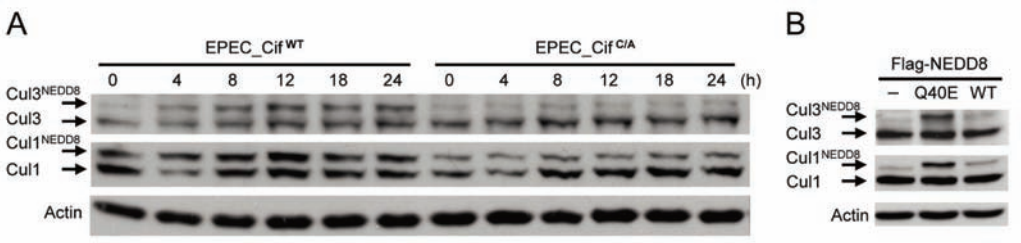

Fig. S15. Effects of Cif infection and ectopic expression of NEDD8 Q40E on the neddylation status of Cul1 and Cul3. In (A), HeLa cells were infected with EPEC strains expressing wild-type Cif (Cif$^{\mathbf{WT}}$) or the catalytic cysteine mutant (Cif$^{\mathbf{C/A}}$). In (B), HeLa cells were transfected with Flag-NEDD8 (Q40E or wild-type) expression plasmid. Levels of unmodified endogenous Cul1 and Cul3 as well as those of neddylated Cul1 and Cul3 (Cul1^{NEDD8} and Cul3^{NEDD8}) were shown by immunoblotting of the total cell lysates using the Cul1 or Cul3 specific antibody.

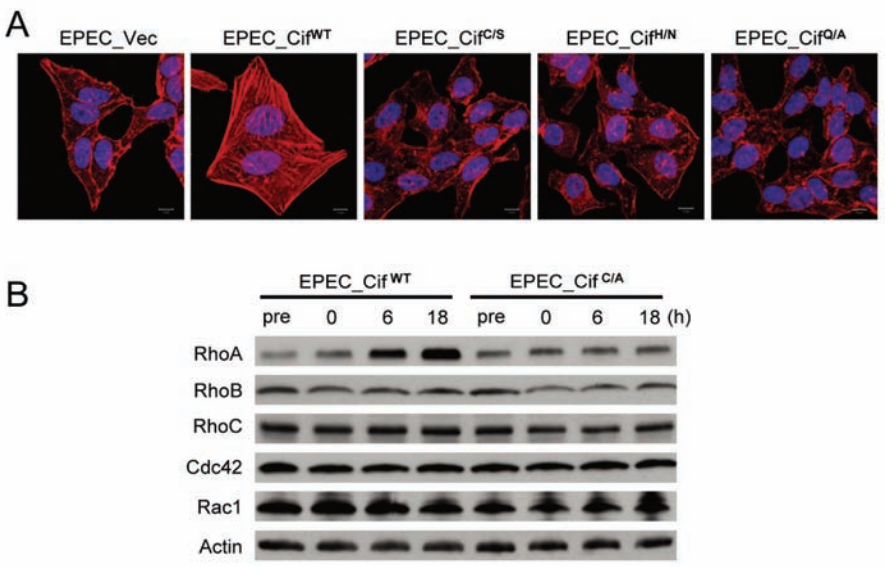

Fig. S16. Effects of mutations in Cif catalytic triad on stress fiber formation and steady levels of Rho family of small GTPases in EPEC-infected cells. HeLa cells were infected EPEC harboring wild-type Cif (Cif^{WT}) or indicated catalytic triad mutants ($Cif^{C/S}$, $Cif^{H/A}$ and $Cif^{Q/A}$) for indicated time durations (Pre, pre-infection). Fluorescence images of actin stress fibers stained by Rhodamine-phalloidin and DAPI-stained nuclei are shown in (A). Steady levels of indicated cellular proteins were show by immunoblotting of total cell lysates using their specific antibodies (B).

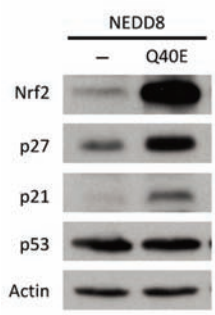

Fig. S17. Effects of ectopic expression of NEDD8 Q40E mutant on steady levels of cullin and non-cullin ubiquitination substrates. 293T cells were transfected with Flag-NEDD8 Q40E expression plasmid. Steady levels of indicated endogenous proteins were shown by immunoblotting of the total cell lysates using corresponding specific antibodies.

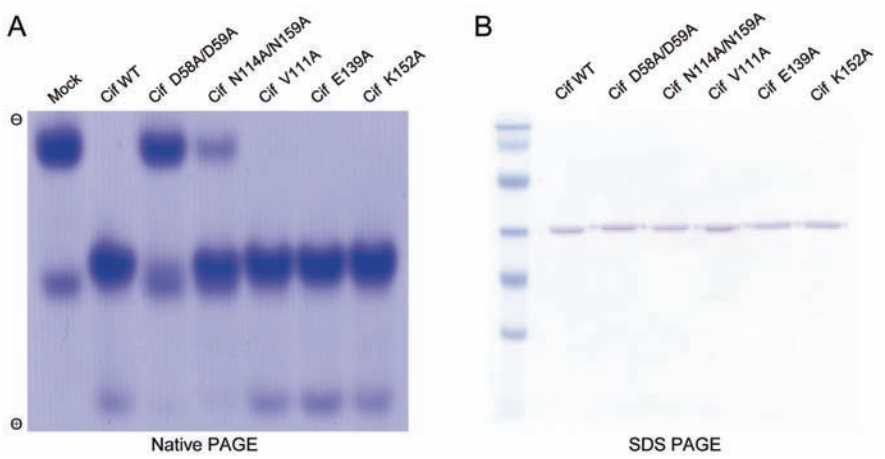

Fig. S18. Effects of mutations in the substrate-contacting surface in Cif on *in vitro* deamidation of NEDD8. 3 μg of NEDD8 was incubated with 0.5 ng of purified Cif or Cif mutants as indicated. Reactions were analyzed by native PAGE analysis and Coomassie blue stained native gel is shown (A). In (B), 1 μg of the mutants used in (A) was loaded on a SDS-PAGE gel stained by Coomassie blue.

Supporting references and notes

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