Supporting Information

Gliotoxin suppresses NF-κB activation by selectively inhibiting linear ubiquitin chain assembly complex (LUBAC)

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Supplemental Data

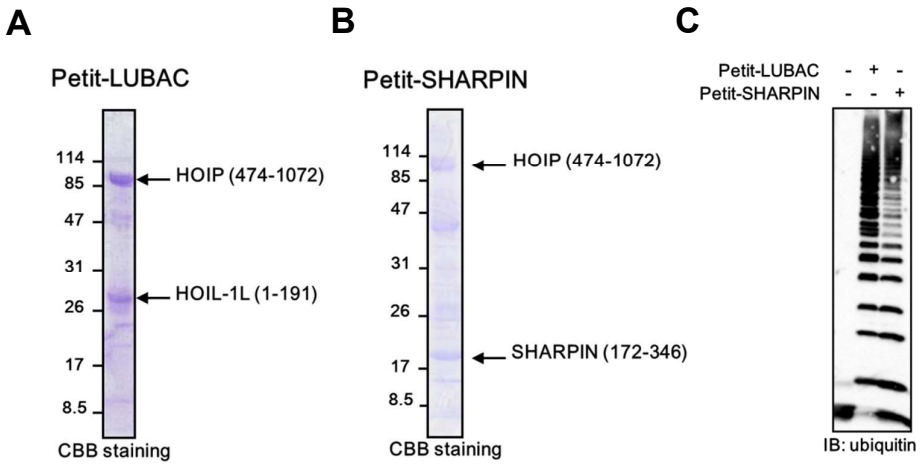

Figure S1. Petit-LUBAC and Petit-SHARPIN catalyze linear ubiquitin chain elongation.

(A, B) Petit-LUBAC and Petit-SHARPIN were expressed in *E. coli* BL21. Purified proteins were electrophoresed and stained with Coomassie Brilliant Blue (CBB).

(C) *In vitro* ubiquitylation assay of Petit-LUBAC and Petit-SHARPIN. Petit-LUBAC and Petit-SHARPIN were incubated with E1, UbcH5c (E2) and ubiquitin. Samples were probed with anti-ubiquitin antibody.

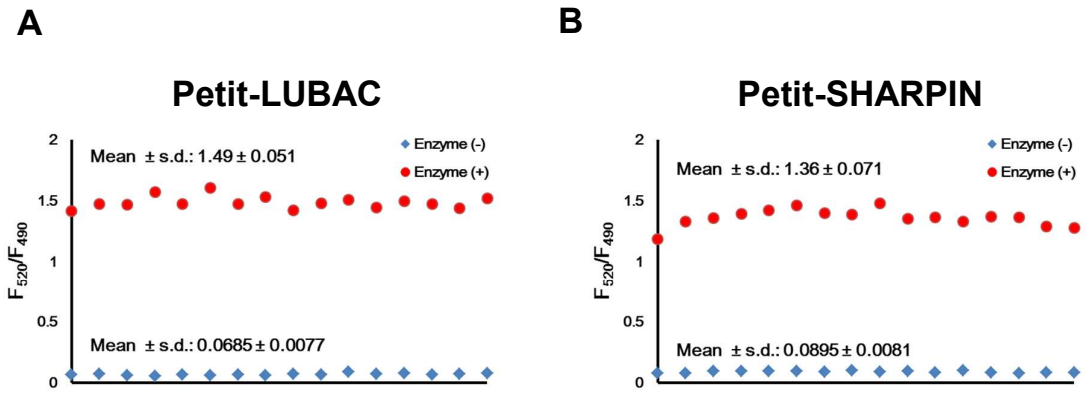

Figure S2. Z' value determination of time-resolved FRET-based ubiquitylation assay.

(A, B) F_{520}/F_{490} was measured after ubiquitylation reaction mediated by Petit-LUBAC (A) and Petit-SHARPIN (B). 16 replicate reactions were performed and then, mean value and standard deviation of each sample were calculated.

	Fold change'	Z'
Petit-LUBAC	21.7	0.88
Petit-SHARPIN	15.2	0.81

Table S1. Fold changes of F_{520}/F_{490} caused by each enzymatic reaction and calculated Z' values.

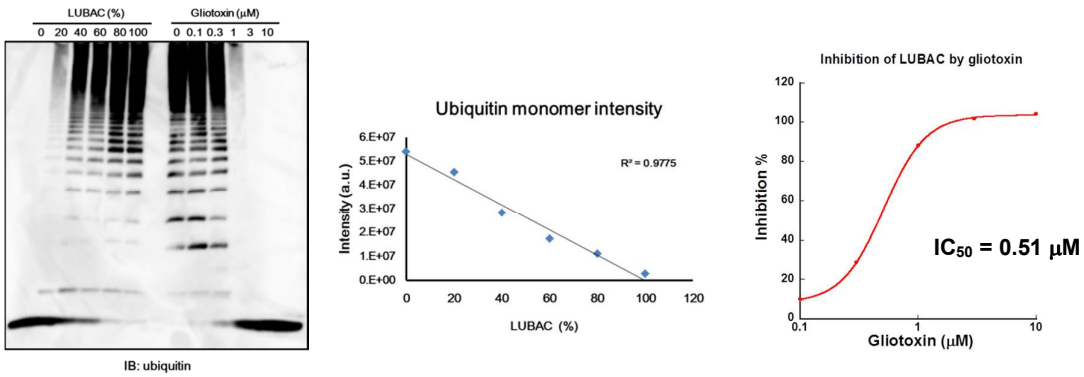

Figure S3. Quantification of LUBAC enzymatic activity.

Linear ubiquitylation assay was performed at various concentrations of LUBAC and gliotoxin (left). The standard curve (middle) was drawn by plotting the ubiquitin monomer intensity against LUBAC (%). This was used to quantify gliotoxin-mediated inhibition (%) of LUBAC and to estimate the IC_{50} (right).

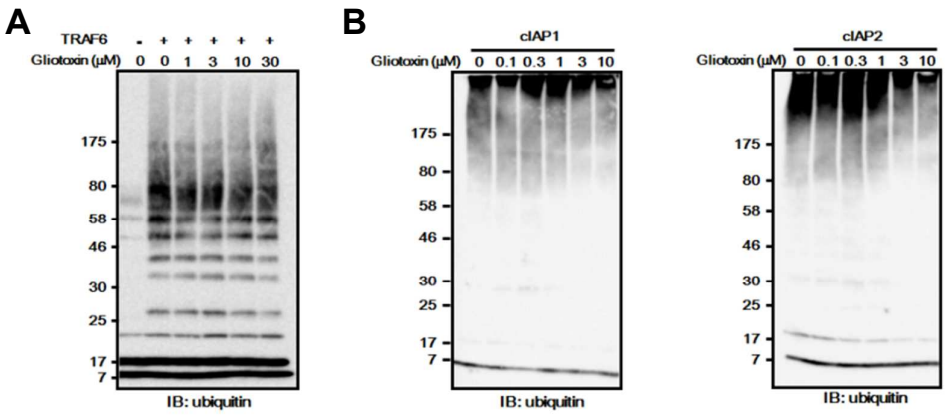

Figure S4. Inhibition assay of RING type E3-mediated polyubiquitin chain elongation by gliotoxin.

(A, B) *In vitro* ubiquitylation assay of RING type E3s, TRAF6 (A) and cIAP1/2 (B). E3s were incubated with E1, Ubc13/Uev1a (A) or UbcH5c (B) as an E2 and ubiquitin in the presence of the indicated concentration of gliotoxin. Gliotoxin failed to inhibit either TRAF6 or cIAP1/2-mediated ubiquitin chain elongation.

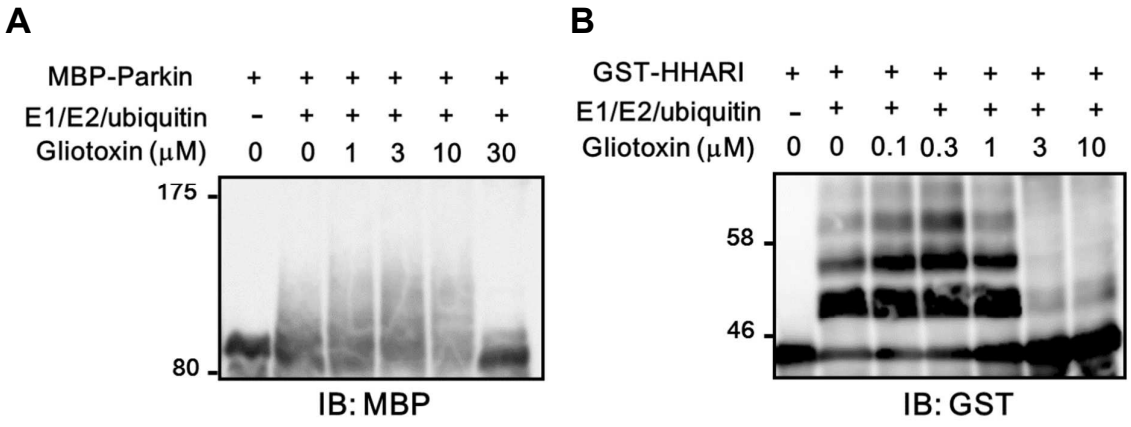

Figure S5. Inhibition of RING-IBR-RING (RBR) type E3-mediated polyubiquitin chain elongation by gliotoxin.

(A) *In vitro* ubiquitylation assay of RBR type E3, Parkin. MBP-fused Parkin was incubated with or without E1, UbcH5c (E2) and ubiquitin in the presence of the indicated concentration of gliotoxin. Samples were probed with anti-MBP antibody.

(B) *In vitro* ubiquitylation assay of RBR type E3, HHARI. GST-fused HHARI was incubated with or without E1, UbcH5c (E2) and ubiquitin in the presence of the indicated concentration of gliotoxin. Samples were probed with anti-GST antibody.

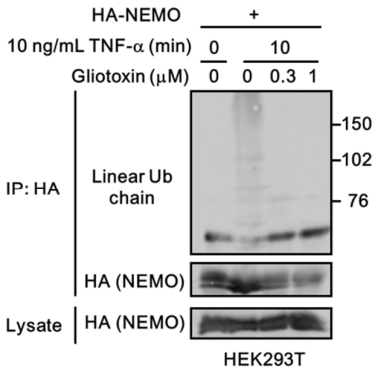

Figure S6. Immunoprecipitation of HA-NEMO under denaturing conditions.

HA-NEMO-expressing HEK293T cells were lysed with PBS containing 1% SDS followed by heating to remove proteins bound non-covalently to NEMO. Linear ubiquitin chain conjugated covalently to NEMO was decreased by treatment with gliotoxin.

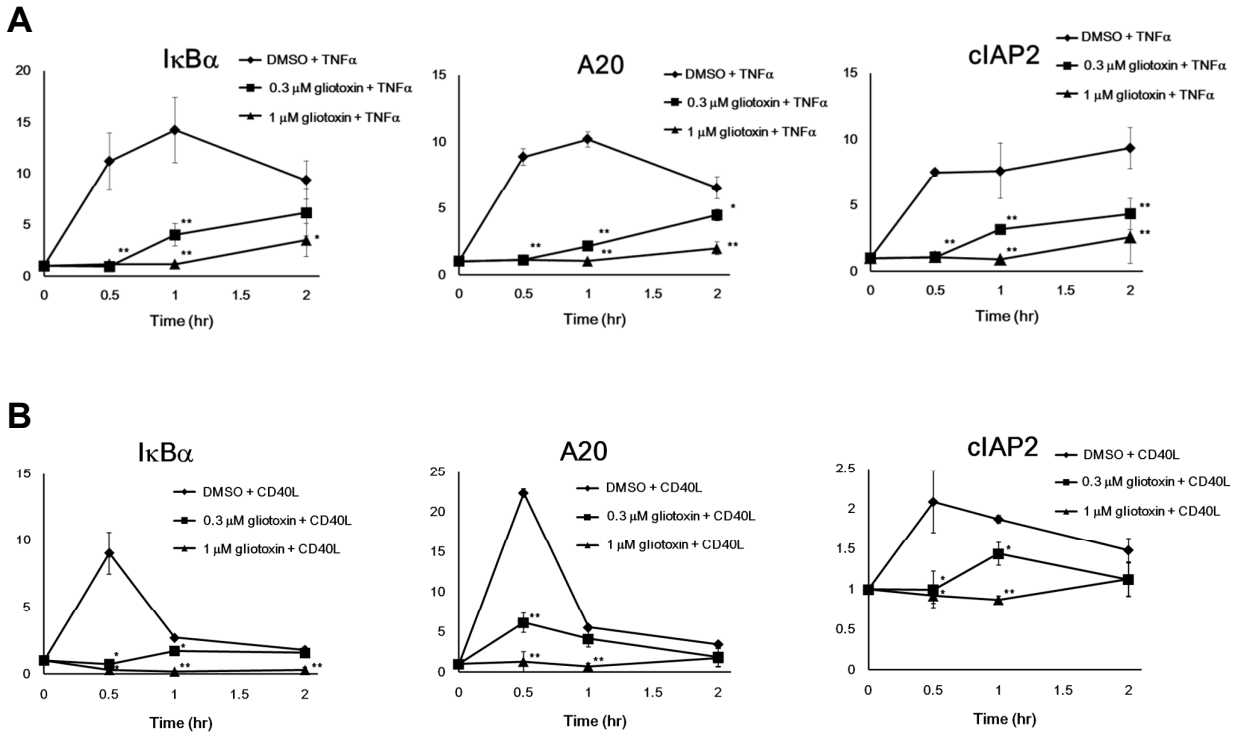

Figure S7. Suppression of signal-induced transcriptional activation of NF-κB-targeted genes by gliotoxin in Jurkat cells.

(A) Jurkat cells were stimulated with TNF-α for the indicated periods and the abundance of IκBα, A20 and cIAP2 mRNAs was evaluated by qRT-PCR. **P* < 0.05, ***P* < 0.01, error bars show s.d. (n = 4 each).

(B) Bal17.2 cells were stimulated with CD40L for the indicated periods and the abundance of IκBα, A20 and cIAP2 mRNAs was evaluated by qRT-PCR. **P* < 0.05, ***P* < 0.01, error bars show s.d. (n =4 each).

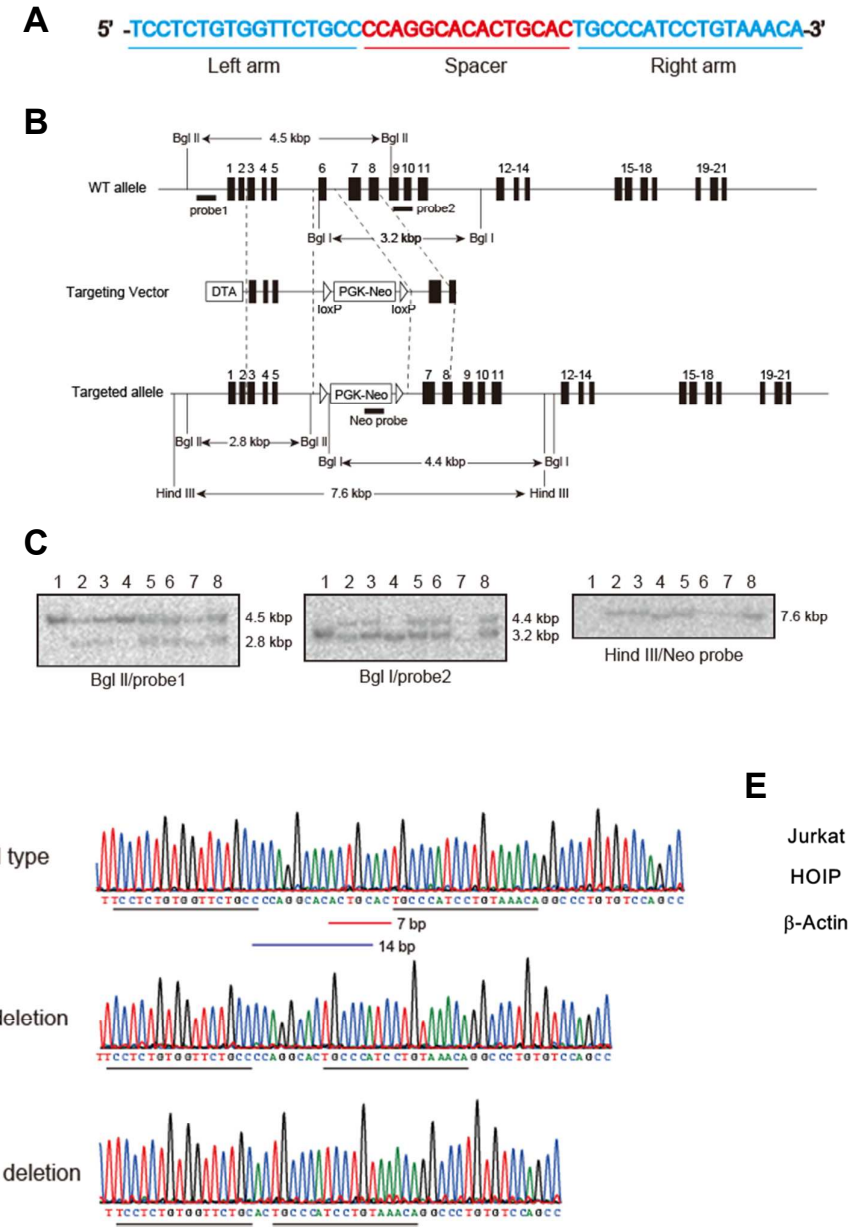
A
5' -TCCTCTGTGGTTCTGCCCCAGGCACACTGCACTGCCCATCCTGTAAACA-3'
Left arm
Spacer
Right arm
B
Bgl II
4.5 kbp
Bgl II
WT allele
1 2 3 4 5
6
7 8
9 10 11
12-14
15-18
19-21
probe1
probe2
Bgl I
3.2 kbp
Bgl I
Targeting Vector
DTA
PGK-Neo
loxP
loxP
Targeted allele
1 2 3 4 5
PGK-Neo
7 8
9 10 11
12-14
15-18
19-21
Neo probe
Bgl II
2.8 kbp
Bgl II
Bgl I
4.4 kbp
Bgl I
Hind III
7.6 kbp
Hind III
C
1 2 3 4 5 6 7 8
4.5 kbp
2.8 kbp
Bgl II/probe1
1 2 3 4 5 6 7 8
4.4 kbp
3.2 kbp
Bgl I/probe2
1 2 3 4 5 6 7 8
7.6 kbp
Hind III/Neo probe
D
Wild type
7 bp
14 bp
7 bp deletion
14 bp deletion
E
Jurkat
HOIP
WT
null
HOIP
β-Actin

Figure S8. Generation of HOIP-null Jurkat cells.

(A) TALEN binding sites in exon 6 of human *HOIP* gene.

(B) Schematic representation of the targeting strategy. Structures of the human HOIP genomic allele, the targeting vector, and the targeted allele are shown. Black boxes denote the coding exons. The probes used for Southern blot analysis are shown as bars.

(C) Southern blot analysis of targeted Jurkat clones. Genomic DNA from wild-type (lane 1) and seven targeted Jurkat clones (lane 2-8) were analyzed by means of Southern blotting using the indicated restriction enzymes and probes.

(D) DNA sequences of TALEN-mediated deletions of untargeted alleles of the clone positive for homologous recombination. One correctly targeted clone (lane 6 in C) carries one allele with a 7 bp deletion and one allele with a 14 bp deletion. The wild-type sequence is shown above with TALEN binding sites underlined in black and the sequences of the 7 bp deletion in red and the 14 bp deletion in blue.

(E) Expression of HOIP in WT or HOIP-null Jurkat cells

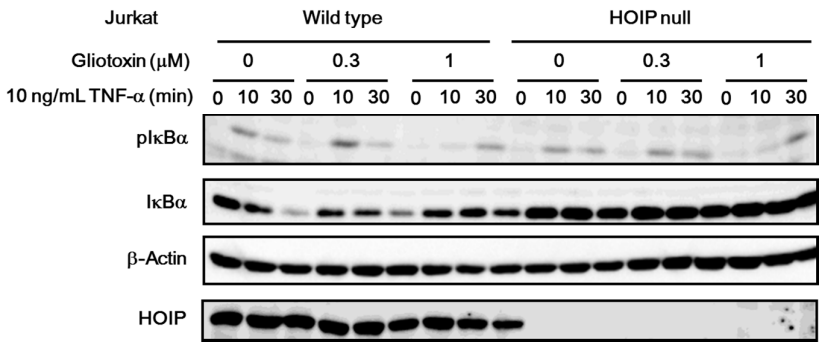

Figure S9. Gliotoxin inhibits NF-κB activation in HOIP-null Jurkat cells.

Wild-type or HOIP-null Jurkat cells were stimulated with TNF-α as indicated and NF-κB activation was evaluated in terms of phosphorylation and degradation of IκBα.

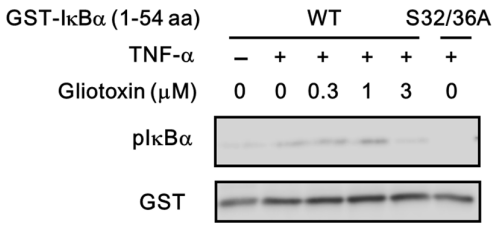

Figure S10. Gliotoxin inhibits IKKβ-mediated phosphorylation of GST-IκBα *in vitro*.
Jurkat cells were stimulated with TNF-α and activated IKK complex was separated with anti-NEMO antibody. IKKβ-mediated phosphorylation activity was diminished by addition of 3 μM gliotoxin. S32/36A IκBα was used as a control that is not phosphorylated by IKKβ.

Table S2. Sequences of primers used in real-time PCR.

Primer	Sequence, 5'-3'
Human IκBα F	TCCACTCCATCCTGAAGGCTAC
Human IκBα R	CAAGGACACCAAAAGCTCCACG
Human A20 F	CTCAACTGGTGTCGAGAAGTCC
Human A20 R	TTCCTTGAGCGTGCTGAACAGC
Human cIAP2 F	GCTTTTGCTGTGATGGTGGACTC
Human cIAP2 R	CTTGACGGATGAACTCCTGTCC
Human β-actin F	CACCATTGGCAATGAGCGGTTC
Human β-actin R	AGGTCTTTGCGGATGTCCACGT
Mouse IκBα F	GCCAGGAATTGCTGAGGCACTT
Mouse IκBα R	GTCTGCGTCAAGACTGCTACAC
Mouse A20 F	AGCAAGTGCAGGAAAGCTGGCT
Mouse A20 R	GCTTTCGCAGAGGCAGTAACAG
Mouse cIAP2 F	GGACATTAGGAGTCTTCCCACAG
Mouse cIAP2 R	GAACACGATGGATACCTCTCGG
Mouse β-actin F	CATTGCTGACAGGATGCAGAAG
Mouse β-actin R	TGCTGGAAGGTGGACAGTGAGG

Supporting Materials and Methods

Preparation of purified proteins and antibodies. Petit-LUBAC, Petit-SHARPIN, GST-TNF-α (77-233 aa), GST-IκBα (1-54 aa), GST-HHARI (177-393 aa), MBP-cIAP1/2, His_6-Ubc13, His_6-Uev1a, MBP-Parkin and MBP-HOIP (699-1072 aa)-His_6 were expressed in *Escherichia coli* BL21 and purified with appropriate affinity resins, such as Ni-NTA Agarose (Qiagen), Glutathione Sepharose 4 Fast Flow (GE Healthcare) or Amylose Resin (New England Biolabs). His_6-TRAF6 and LUBAC, which is composed of HOIL-1L, SHARPIN and His_6-tagged HOIP, were expressed using BacPAK6 baculovirus expression system (BD Biosciences) and purified with Ni-NTA Agarose. Antibodies were purchased or supplied as indicated below: anti-ubiquitin (Santa Cruz sc-8017), anti-NEMO for immunoprecipitation (Santa Cruz sc-8330) and for immunoblotting (BIOZOL MBL-K0159-3), anti-IκBα (Cell Signaling Technology #9246), anti-pIκBα (Cell Signaling Technology #4812), anti-β-actin (Santa Cruz sc-47778), anti-RelA (Santa Cruz sc-109), anti-LaminB (Abcam 16048), anti-RIP1 (BD Biosciences 610458), anti-TNFR1 (Santa Cruz sc-8436), anti-HA (Santa Cruz sc-805), anti-MBP (Santa Cruz sc-13564) and anti-GST (Santa Cruz sc-459). Anti-HOIP, anti-HOIL-1L, anti-SHARPIN and anti-linear ubiquitin chain antibodies were described previously [1,2]

High-Throughput Screening. In the Petit-LUBAC screening system, enzymes and ubiquitins were mixed on the 384-well plate. The reaction components were: 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 0.5% Tween 20 (w/v), 0.1% DMSO (v/v), 2 μM each compound, 0.025 μM Tb-Ub, 0.05 μM Fl-Ub, 1.2 μM ubiquitin, 0.4 μg/mL E1, 3.5 μg/mL UbcH5c, 0.6 μg/mL Petit-LUBAC. In the Petit-SHARPIN screening system: 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 0.5% Tween 20 (w/v), 0.1% DMSO (v/v), 2 μM compound, 0.025 μM Tb-Ub, 0.1 μM Fl-Ub, 2.8 μM ubiquitin, 4 μg/mL E1, 5 μg/mL UbcH5c, 3.5 μg/mL Petit-SHARPIN. These reaction solutions were prepared in a volume of 15 μL. Enzymatic reactions were performed at room temperature for 2.5 hr and 5 hr in Petit-LUBAC and Petit-SHARPIN, respectively. 10 μL of 5 M guanidine-HCl was added to stop the reaction and F_{490} and F_{520} were measured by time-resolved detection to evaluate ubiquitin chain elongation. Small chemicals used in this screening were provided by the Open Innovation Center for Drug Discovery, The University of Tokyo. The small molecule library used in this screening is composed of structurally diverse compounds, including natural products. The integrity of these compounds was assessed by LC-MS.

***In vitro* ubiquitylation assay.** *In vitro* ubiquitylation reactions were performed as described below, 5 μg/mL E1, 10 μg/mL UbcH5c, 50 μg/mL each E3 and 150 μg/mL ubiquitin (SIGMA) were incubated in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP at 37 °C, together with cIAP1/2 for 1 hr, Petit-LUBAC, Petit-SHARPIN, HOIP (699-1072 aa), Parkin, HHARI (177-393 aa) for 2 hrs. In TRAF6-mediated ubiquitylation assay, 5 μg/mL E1, 2.5 μg/mL Ubc13 and Uev1a as an E2, 50 μg/mL TRAF6 and 150 μg/mL ubiquitin were incubated in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP at 37 °C for 1 hr. Ubiquitylation reaction products were probed with anti-ubiquitin antibody.

Isothermal titration calorimetry. ITC measurements were performed using an ITC 200 MicroCalorimeter (GE Healthcare). All assays were performed in 20 mM Tris-HCl buffer (pH 7.5) containing 5% DMSO (v/v) at 25 °C. In the sample cell, 20 μM of proteins were loaded and 500 μM of gliotoxin was filled into syringe. Data were analyzed with the Origin7 software (MicroCal) supplied by the manufacturer.

Cell culture. Bal17.2 and Jurkat cells were cultured in RPMI medium (SIGMA) containing 10% fetal bovine serum (v/v), 100 U/mL penicillin G, 100 μg/mL streptomycin and 60 μM 2-mercaptoethanol at 37 °C in humidified air containing 5% CO_2 (v/v).

NF-κB activation assay. Jurkat and Bal17.2 cells treated with DMSO or gliotoxin for 10 min were stimulated with 10 ng/mL of recombinant human TNF-α (Promega) or 100 ng/mL of recombinant mouse CD40L (R&D Systems) for the indicated times, respectively. Cells were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 (w/v), protease inhibitor cocktail (SIGMA), 2 mM PMSF, phosphatase inhibitor cocktail (Nacalai Tesque) and probed with anti-pIκBα and anti-IκBα antibodies. β-Actin was detected as an internal control. For fractionation of nucleus and cytoplasm, cells were separated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo) and nuclear translocation of RelA was evaluated by immunoblotting. LaminB was used as a loading control for nuclear fraction.

NEMO linear ubiquitylation assay in cells. Lysates from TNF-α-stimulated Jurkat and CD40L-stimulated Bal17.2 cells were incubated with anti-NEMO antibody on ice for 1 hr, followed by incubation with rmp Protein A Sepharose™ Fast Flow (GE Healthcare) at 4 °C for 1 hr. After intensive washing of the beads with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 (w/v), proteins conjugated to the beads were eluted with 2x SDS-PAGE sample buffer and probed with anti-linear ubiquitin chain and anti-NEMO antibodies.

Immunoprecipitation under denaturing conditions. HEK293T cells were transfected with HA-human NEMO-expressing vector and then stimulated with human TNF-α in the presence or absence of gliotoxin. After the stimulation, cells were lysed with PBS containing 1% SDS (w/v) and heated at 95 °C for 10 minutes. Lysates were sheared with a 25-gauge needle and centrifuged at 15,000 rpm. The supernatant was collected and incubated with anti-HA tag antibody on ice for 1 hr, followed by incubation with Protein A Sepharose™ at 4 °C for 1 hr. After intensive washing of the beads, proteins conjugated to the beads were eluted with 2x SDS-PAGE sample buffer and probed with anti-linear ubiquitin chain and anti-HA antibodies.

Real-time RT-PCR. Jurkat and Bal17.2 cells treated with DMSO or gliotoxin for 10 min were stimulated with 10 ng/mL of recombinant human TNF-α or 100 ng/mL of recombinant mouse CD40L for the indicated times, respectively. Then, total RNA was isolated with an RNeasy Minikit (Qiagen) and cDNA was generated by using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR was performed in the presence of SYBR® Green (Applied Biosystems) and relative abundance of cDNA was calculated by the comparative Ct method (ΔΔCt). β-Actin was used as an internal control. Primers used for real-time PCR were listed in **Supplementary Table S2**.

Flow cytometry. Jurkat cells treated with DMSO or gliotoxin for 10 min were stimulated with TNF-α. After staining with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's protocol, cells were analyzed with a BD FACSCanto™ II (BD Biosciences). Data were analyzed with FlowJo version 9.5.3.

Generation of HOIP-null Jurkat cells. The targeting vector was designed to place exon 6 with a *loxP*-flanked neomycin resistance gene. The selection of TALEN genome sites (left arm, TCCTCTGTGGTTCTGCC; right arm, TGCCCATCCTGTAAACA) in exon 6 of human HOIP gene and the construction of TALEN vectors were done by Cellectics Bioresearch. Jurkat cells (a human T lymphoblastoid cell line) were transfected with the targeting vector and two TALEN vectors and selected in the presence of 1000 μg/mL G418. Homologous recombination at the 5' end was identified by Southern blot analysis of *BglII*-digested genomic DNA using a 0.45-kbp fragment as a 5' external probe (probe 1). A single integration of the targeting vector and homologous recombination at the 3' end in the positive clones for homologous recombination at the 5' end were confirmed by Southern blot analysis of *HindIII*-digested genomic DNA using a 0.8-kbp fragment as a neo probe and *BglI*-digested genomic DNA using a 0.55-kbp fragment as a 3' external probe (probe 2), respectively. To identify TALEN-mediated indels in other alleles the TALEN targeting sites in correctly targeted clones were amplified by PCR using an appropriate primer set (forward: 5'-GGGTTTCTCCATGTTGGTCAGGCTGGTCTG-3' and reverse: 5'-GATGAGCACGGGATGGGTGTCCATGGAACAG-3'). PCR products were cloned into pCRII-TOPO-blunt (Life Technologies) and subjected to sequence analysis.

***In vitro* IKK assay.** Jurkat cells stimulated with 10 ng/mL of TNF-α for 10 min in the presence or absence of gliotoxin were lysed with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 (w/v) and NEMO was immunoprecipitated with anti-NEMO antibody and rmp Protein A Sepharose™. After intensive washing, beads were suspended in the buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$. The anti-NEMO immunoprecipitates were incubated with 5 μg/mL of WT or S32/36A GST-IκBα (1-54 aa) in 20 μL of reaction buffer composed of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM ATP at 30 °C for 30 min in the presence or absence of gliotoxin, followed by immunoblotting with anti-pIκBα antibody.

Supporting References

(1) Sasaki, Y., Sano, S., Nakahara, M., Murata, S., Kometani, K., Aiba, Y., Sakamoto, S., Watanabe, Y., Tanaka, K., Kurosaki, T., and Iwai, K. (2013) Defective immune responses in mice lacking LUBAC-mediated linear ubiquitination in B cells, *EMBO J. 32*, 2463-2476.
(2) Tokunaga, F., Nakagawa, T., Nakahara, M., Saeki, Y., Taniguchi, M., Sakata, S., Tanaka, K., Nakano, H., and Iwai, K. (2011) SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex, *Nature 471*, 633-636.