Supplementary information regarding Supplementary Figure S12

CLIPR-59 does not regulate internalization and expression of TNFR1 on the cell surface.

Previous studies have demonstrated that CLIPR-59 modulates transferrin uptake suggesting that CLIPR-59 may regulate endosomal dynamics [1]. Thus, we wondered whether CLIPR-59 altered TNFR1 signaling by affecting ligand-dependent receptor internalization or expression on the cell surface [2]. To determine the function of CLIPR-59 on TNFR1 expression, HeLa cells treated with siNS or siCLIPR-59 were labeled with biotin-conjugated TNF-α (Bio-TNF) or soybean trypsin inhibitor (Bio-STI, as a negative control for binding specificity) with streptavidin-FITC (STP-FITC) at 4℃ (non-internalizing condition), and expression of TNFR1 on cell surface was assessed by flow cytometry. CLIPR-59 knock down did not alter the percentage of FITC-positive cells exposed to Bio-TNF-α/STP-FITC, compared with siNS treatment (Supplementary Fig.S12**A**). Thus CLIPR-59 knock down failed to modify TNFR1 cell surface expression.

Next, we determined whether siCLIPR-59 treatment altered ligand-dependent TNFR internalization. siCLIPR-59 or siNS-transfected HeLa cells were labeled with Bio-TNF/STP-FITC at 4℃, as described above. After labeling, cells were incubated at 37℃ to allow TNF-α/TNFR1 internalization. Fluorescence of these labeled cells was analyzed by confocal laser scanning microscopy. At 4℃ (Supplementary Fig.S12**B**-**D**, **H**, **N** and **T**) or after 10 min of incubation at 37 ℃ (Supplementary Fig.S12**J**,**P** and **V**), FITC-labeled TNF-α/TNFR1 complex was detected largely on the cell surface of siNS-treated HeLa cells. After 30 min at 37℃, FITC-labeled TNF-α/TNFR1 endocytic vesicles were observed inside the cells (Supplementary Fig.S12**E**-**G**, **L**, **R** and **X**). Similarly, siCLIPR-59-transfected HeLa cells also internalized TNF-α/TNFR1

complexes at 30 min after 37℃ incubation (Supplementary Fig.S12**I**, **O**, **U**, **K**, **Q**, **W**, **M**, **S** and **Y**). Altogether, these results suggest that CLIPR-59 knock down does not significantly impact either cell surface expression or ligand-dependent internalization of TNFR.

Supplementary Material and Methods

Plasmid construction

The cytoplasmic regions of human DR6(370-655aa), hFas(191-335a.a.), hTNFR1(234-455a.a.), hDR3(233-426a.a.), hDR4(265-468a.a.), hDR5(234-440a.a.), full-length Caspase-8, RIP1 or FADD were fused to the GAL4 DNA-binding domain in pGBKT7 vector (Clontech, Palo Alto, CA). The cDNA encoding full-length human CLIPR-59 was fused to GAL4-trans activation domain in pGADT7 vector (Clontech). Expression plasmid for HA-CLIPR-59WT or FADD was constructed by inserting cDNA fragments fusing the N-terminal HA tag into the mammalian expression vector pcDNA3 (Invitrogen). The CLIPR-59ΔC construct was obtained by joining the upstream BstXI site (from HA-CLIPR-59) to the BamHI site of plasmid after filling ends with T4 polymerase. Expression plasmid for GFP-CLIPR-59WT and its mutants, ΔMTB, ΔC60, ΔA0 and CC534AA were described previously [1]. The cDNA encoding CYLD was generated by PCR, and the fragment was cloned into pcDNA3.1 myc/his ver.B plasmid (Invitrogen). Expression plasmids for FLAG-tagged DR3 were constructed by inserting cDNA fragments lacking coding sequence for the signal peptide into the mammalian expression vector pFLAG-CMV-1. Expression plasmid for human CD40 - human DR6 chimeric protein (hCD40-DR6) was constructed by joining the extracellular region of human CD40 (1-182 a.a.) with the transmembrane region and cytoplasmic

region of human DR6 (348-655 a.a.) at the SalI site. The cDNA encoding the extracellular region of human CD40 (1-182 a.a.) was subcloned into pCI/neo (Promega, Madison, WI) from SK(-)-hCD40/EC, which was kindly provided by Dr. JI. Inoue. HA–ubiquitin and its mutants (K63R or K48R) in a bacterial expression vector were kindly gifts from Dr. KI. Nakayama. For the mammalian expression experiments, we transferred a single copy of the cDNA into the pcDNA3.1 (+) vector (Invitrogen). Expression plasmid for FLAG-tagged A20 protein was kindly provided by Dr. S. Hatakeyama. Expressing plasmids for FLAG-tagged TNFR1, HA-tagged TRADD were kindly provided by Dr. N. Inohara. The deleted mutants of TNFR1, del 234 (deleted 234-358a.a.) or del DD (deleted 359-438a.a.), and point mutant of RIP1, which was replaced lysine to arginine at position 377, were generated by PrimeSTAR Mutagenesis Basal Kit (Takara Bio inc. Shiga, Japan), according to manufactures. The plasmids for siRNA-resistant human RIP1 (RIP1-R) wild type or mutated K377R were generated by PrimeSTAR Mutagenesis Basal Kit using the following primer set (forward,5'-GCACAGCAGCGGCCGTATGAAAACTTTCAGAATACAGAGGGA−3' ; reverse,5'-CTGAAAGTTTTCATACGGCCGCTGCTGTGCAAAAGGGTCATGG−3'). Horizontal bar indicates mutated site for preventing siRNA silencing.

Cell culture

HeLa cells and mouse hybridoma cell line clone G28-5 were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). HeLa, HEK293T or NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 10% FCS at 37℃ in 5% CO_2. Clone G28-5 were cultured in serum free medium (CD-Hybridoma Medium, Invitrogen, Carlsbad, CA) supplemented with 2% GLUTAMAX I (Invitrogen). Immune-susceptible TC-1 P0 or immune-resistant TC-1 P3 cells was kindly

provided by Dr. T.C.Wu (Johns Hopkins). These cells were maintained in RPMI1640 cell culture medium supplemented with 10% FCS at 37℃ in 5% CO_2.

Reagents and antibodies

Anti-HA (clone 3F10), anti-myc (clone 9E10), anti-GFP (clone 7.1+13.1) antibodies (Abs) were purchased from Roche (Mannheim, Germany). Anti-FLAG-M2 Ab and anti-FLAG-M2 conjugated agarose beads were from Sigma. Anti-TNFR1 Ab (cloneH-5) was from Santa Cruz (Santa Cruz, CA). Anti- Caspase 8 (clone 1C12) Ab was from Cell Signaling Technology (Beverly, MA). Anti- RIP1 (clone 38/RIP), anti-FADD (clone 1/FADD) and anti-TRADD (clone 37) Abs were from BD Transduction laboratories (San Jose, CA). anti-pan cIAP (315301) was from R&D systems (Minneapolis, MN). Anti-tubulin Ab (clone 236-10501) was from Molecular Probes (Eugene, OR). Anti-CYLD polyclonal Ab (ab33929) was from Abcam (Cambridge, MA). FLAG-tagged recombinant human TNF-α was from Alexis (Lausen, Switzerland). Affinity purified anti-CLIPR-59 was produced by Immuno-Biological Laboratories (Gunma, Japan) using a synthetic peptide corresponding to sequence of human CLIPR-59 (GGSTDSPGDSVGAKKVH). Anti-human CD40 mouse Ab (clone G28-5) was purified from the culture medium of mouse hybridoma cell (clone G28-5) by protein G sepharose column (HiTrap Protein G HP, Amersham Biosciences , Sunnyvale, CA). Human recombinant TNF-α was obtained from Strathmann Biotec (Hamburg, Germany). Anti-Ubiquitin, Lys48- and Lys63-specific Ab (clone Apu2 and Apu3, respectively) were from Millipore (Bedford, MA). Smac mimetic, compound 3 was provided as a kindly gift by Dr. Xiaodong Wang [3].

RT-PCR

Cellular RNA was isolated with the TRIzol reagent (Invitrogen) and reverse-transcribed using MMLV reverse transcriptase and randam hexamer primer. The resultant cDNA was then used in conventional PCR reactions, and products were analyzed by gel electrophoresis with ethidium bromide staining. Quantitative real-time PCR measurements were performed with a *Mx3000* Multiplex Quantitative PCR System (Stratagene) by using SYBR® *Premix Ex Taq*™ II (Perfect Real. Time) (TaKaRa, Otsu, Japan). Primer sequences for detecting human and mouse CLIPR-59 gene expression were shown below.

For human CLIPR-59 (+444 to +575bp)

forward ; 5'-CCGAGCTGTTTGCCATTGT-3',

reverse ; 5'-GGAGCAGTGTCATGTCGGTCA-3'.

For mouse CLIPR-59 (+565 to +802bp)

forward ; 5'-GTGAACTCCACCTGCAGTGAC-3',

reverse ; 5'-GTGGCACAGCCTCTTCTAGGA-3'.

β-Gal-based Apoptosis assay

β-Gal-based apoptosis assay was performed as described previously [4]. Briefly, HeLa cells were cultured in 6-well tissue culture plates and transfected with various plasmids (total amount of DNA was adjusted to 6 μg using empty plasmid) with 1 μg/well β-Gal expression plasmid (pSV-β-Galactosidase, Promega) using metafectene (Biontex Laboratories, GmbH, Munich, Germany) according to manufactures. After 19-24 hr, cells were fixed and stained with X-gal and the extent of apoptosis was measured by counting morphologically apoptotic blue cells and determining their percentage among the total number of blue transfected cells (at least 300 cells per sample).

Detection of the protein expression on cell surface and ligand-dependent internalization of TNF/TNFR

To detect expression of TNF/TNFR on cell surface, HeLa cells were transfected with siNS or siCLIPR-59 as described in above. At 72 hr after transfection, expression of TNF/TNFR on the surface of these cells was analyzed by flow cytometry using TNF-α biotin conjugate (Fluorokine, R&D systems). Briefly, these cells were washed twice with ice-cold PBS, scraped and resuspended in ice-cold PBS ($4x10^6$cells / ml), respectively. Cell suspensions (25μl) were reacted with 10 μl of biotinylated TNF-α (Bio-TNF) or soybean trypsin inhibitor (Bio-STI) reagent in 1.5 ml tubes, and incubated at 4℃ for 60 min. After incubation, 10 μl of avidin-FITC reagent was added to each tube and the reaction mixtures were incubated for a further 30 min at 4℃ in the dark. Cells were then washed twice with 2ml ice-cold of 1xRDF1 buffer, and resuspended in 500μl of 1xRDF1 buffer for flow cytometric analysis.

To determine the ligand-dependent internalization of TNF/TNFR, HeLa cells transfected with siNS or siCLIPR-59 ($3x10^4$cells) were seeded on poly-L-lysine -coated Lab-Tek II chamber glass slide (Nalge Nunc, Roskilde, Denmark). The following day, cells were washed twice with ice-cold PBS and reacted with 100 μl of Bio-TNF reagent diluted (x10) with PBS for 60 min at 4℃. After incubation, each cells were treated with 10 μl of avidin-FITC reagent and incubated for a further 30 min at 4℃ in the dark. After incubation at 4℃, cells were returned 37℃ for induction of internalization. At various times after incubation at 37℃, cells were washed twice with 500 μl of ice-cold 1xRDF1 buffer and fixed with ice-cold 4% paraformaldehyde in PBS for 20 min. Samples were then mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Inc.

Burlingame, CA) and fluorescent images were analyzed by confocal laser scanning microscopy (Fluoview FV1000, Olympus, Tokyo, Japan).

Doxycycline dependent shRNA production

Doxycycline-inducible TC1-P0 cell was generated using Knockout Single Vector Inducible RNAi System (Clontech). TC1-P0 cells were electroporated with a construct (pSingle-tTS-shRNA) containing an shRNA directed against mouse CLIPR-59 (Targeted sequence was described in [5]). To induce shRNA expression, cells were cultured in RPMI-1640 containing 2 μg/ml doxycycline and 10% tetracycline free fetal calf serum during 3 days.

Supplementary Figure Legends

Figure S1. The specificity of knockdown by siRNA treatment for CLIPR-59 or CYLD gene expression.

To determine the specificity of siRNA treatment which was used in the present study, HeLa cells were transfected with gene specific siRNA for CLIPR-59 (siCLIPR-59), CYLD (siCYLD) or control (siNS). At 72 hr after the transfection, cells were lysed and analyzed by SDS-PAGE and immunoblot using the indicated antibodies.

Figure S2. Time course analysis for the effects of knock down of CLIPR-59 expression on the cell survival under TNF-α stimulation.

HeLa cells transfected with siRNA for CLIPR-59 gene specific (siCL) or control (siNS), were stimulated by TNF-α in the presence or absence of CHX (10 μg/ml) for the indicated periods. Cell viability was assessed by the method as described in Fig.2**B.** Error bar means ± std dev; n=3.

Figure S3. CLIPR-59 should play its role at upstream of Caspase-8 in TNF-α signaling.

(**A**) HeLa cells transfected with the indicated siRNA were stimulated with TNF-α (10ng/ml) plus CHX (10μg/ml). At 3hr after stimulation, cells were lysed and analyzed by immuno blotting using the indicated antibodies. (**B**) HeLa cells were transfected with siCLIPR-59 or siNS. At 48 hr after the transfection, cells were transfected with expression plasmids along with β-Gal expression vector. At 24 hr after the 2nd transfection, apoptosis was assessed as described in *Materials and Methods*. Error bar means ± std dev; n=3. Asterisk indicates $p<0.05$ by unpaired t-test.

Figure S4. Effect of CLIPR-59 knock down on K63 linked ubiquitination of receptor associated RIP1 in TNF signaling

HeLa cells transfected with control siRNA(NS), siCLIPR-59-1, siCLIPR-59-2 or siCLIPR-59-3 were stimulated with TNF-α after MG132 pre-treatment. At 10min after the stimulation, K63 linked ubiquitination on RIP1 associated with TNFR1 was assessed as described in Fig.4**B**. Ratio of the intensity of smear bands at high molecular weight (>210 kDa, blue box) in each samples compared with that in siNS control was shown (upper).

Figure S5. Interaction of CLIPR-59 with CYLD is required for its de-ubiquitinating activity.

HEK293T cells were transfected with plasmids for expressing mutants of CLIPR-59, (schematically shown in (**A**)), and a plasmid encoding myc tagged CYLD. At 24 hr after the transfection, cells were lysed and analyzed as described in Fig.1**B** (**B**), and de-ubiquitinating activities of these mutants of CLIPR-59 were assessed as described in Fig.5**B** (**C**). This assay was done

under non-reducing conditions, for assessing the relationship between the interaction of the mutants with RIP1 and de-ubiquitinating activity. (**D**) 293T cells were transfected with the combination of plasmids for expressing HA-CLIPR-59, FLAG-RIP1 and myc-CYLD. At 24 hr after the transfection, cells were stimulated with TNF-α (100ng/ml) for the indicated periods. These cells were lysed and the lysates were immunoprcipitated with anti-HA Ab in the presence of phosphatase inhibitor cocktail. The precipitated proteins were analyzed as described in Fig.1**B**. (**E**) HeLa cells (10^8cells) treated with siNS (N) or siCLIPR-59 (C) were stimulated with TNF-α (100 ng/ml) and CHX (10 μg/ml). At 3 hr after stimulation, the cells were lysed and the lysates were immunoprecipitated by anti-RIP1 specific Ab. The precipitated proteins were analyzed by immunobloting using the indicated Abs.

Figure S6. Functional relationship betwen CLIPR-59 and CYLD in TNF-α signaling.

(**A**) HeLa cells transfected with siNS or siCYLD were seeded in 6 well culture plate. After overnight incubation, cells were re-transfected with the expression vector plasmid for GFP alone or GFP-tagged CLIPR-59. The cells were stimulated with TNF-α (20 ng/ml, 6hr) in the presence of CHX (10 μg/ml). Percentage of dead cells in transfected cells was assessed as described in Fig.5**C**. Data are shown as mean ± std dev; n=3. Asterisk indicates $p<0.05$ by unpaired t-test. (**B**) HeLa cells, transfected with the combination of expression vector plasmids and siRNA for the indicated genes, were stimulated with TNF-α (100ng/ml) in the presence of CHX (10 μg/ml). At 3hr after the stimulation, cells were lysed and analyzed as described in Fig.1**B**.

Figure S7. Dose dependent effect of Smac mimetic, Compond-3 on the

induction of cell death by TNF-α stimulation in HeLa cells.

HeLa cells seeded in 96 well culture plate, were pretreated with the indicated amount of Compound 3. At 4hr after treatment, cells were stimulated with TNF-α (100 ng/ml, 6hr). Viability of these cells was assessed as described in Fig.2**B** (Error bar means ± std dev; n=3).

Figure S8. Effects of siRNA treatment against CLIPR-59 and CYLD on the ubiquitination on RIP1 mediated by TNF-α stimulation in the presence of smac mimetic, Compound 3, or the absence of cIAP1+2 proteins.

HeLa cells transfected with siNS, siCLIPR-59 or CYLD were treated with Compound 3 (200nM, 4hr) or siRNA against sIAP1 + 2 (48hr). After the treatment, cells were stimulated with TNF-α (100ng/ml, 3hr). After the stimulation, cells were lysed and analyzed as described in Fig.1**B**. Protein expression level of cIAP1 and 2 of these cells were also analyzed in Fig.5**G**.

Figure S9. Reduction of CLIPR-59 gene expression correlates with sensitivity to apoptosis induced by TNF-α in immune-resistant TC-1 P3 cells.

(**A**) CLIPR-59 mRNA expression level in the immune-susceptible TC-1 P0 (P0) cells and immune-resistant TC-1 P3 (P3) cells was assessed by quantitative real-time PCR. The data were represented as ratio to the mean of the level in TC-1 P0 cells. Error bar means ± std dev; n=3. (**B**) Expression level of CLIPR-59 protein in P0 and P3 cells was assessed by immunoblotting using anti-CLIPR-59 or tubulin antibody (Ab). (**C**) P0 or P3 cells were stimulated by TNF-α (20 ng/ml) in the presence or absence of CHX (10 μg/ml). At 3 hr (for detecting Caspase-8 activity) or 4.5 hr (for Caspase-3) after stimulation, the activity of Caspase-8 (**C**), or Caspase-3 (**D**) was assessed as described in

Materials and Methods. Error bar means ± std dev; n=3. (**E**) P3 cells were transfected with vector plasmids encoding GFP alone or GFP-tagged CLIPR-59. At 24 hr after transfection, the cells were stimulated with the indicated concentrations of TNF-α and CHX (10 μg/ml). The percentages of dead cells in transfected cells were assessed using Flowcytometry. Error bar means ± std dev; n=3. (**F**) P0 cells transfected with doxycycline dependent shRNA against mouse CLIPR-59 producing plasmid, were cultured in the presence (closed column) or absence (opne column) of doxycycline in 96 well white wall culture plate (2μg/ml, 3days). After the incubation, cells were stimulated with TNF-α (10ng/ml) and CHX (10μg/ml). At 3hr after the stimulation, Caspase-8 activity was assessed as described in **C**. Error bar means ± std dev; n=3. The effect of shRNA production on the protein expression level of CLIPR-59 was assessed by immunoblotting using anti-CLIPR-59 Ab (right panel).

Figure S10. Model depicting role of CLIPR-59 in TNF-α signaling.

Prior to TNF-α stimulation, CLIPR-59 interacts with TNF receptor (TNFR) 1 at cell membranes. Ligation of receptors recruits RIP1 and promotes ubiquitination of RIP1. The protein complex containing CLIPR-59 and RIP1 subsequently dissociates from receptor and recruits CYLD, a de-ubiquitinating enzyme for RIP1. Recruitment of CYLD in this complex removes K63-linked ubiquitin chains from RIP1, facilitating the interaction of Caspase-8 and FADD with RIP1. The formation of the protein complex, containing Caspase-8, FADD, RIP1 and CLIPR-59, activates pro-apoptotic Caspase cascade.

Figure S11. Involvement of CLIPR-59 and CYLD in Complex-I.

HeLa cells (1 x 10^8 cells / sample) were stimulated by FLAG-tagged TNF-α plus CHX (10μg/ml) or medium alone (-) during the indicated periods. The cells

were lysed and subjected into co-IP assay using anti-FLAG Ab conjugated agarose beads. After 4 times washes, the precipitated proteins were analyzed as described in Fig.1**B**.

Figure S12. Effect of knockdown for CLIPR-59 gene expression on the cell surface distribution or ligand-dependent internalization of TNF/TNFR.

(**A**) Effect of knockdown treatment for CLIPR-59 expression on cell-surface expression level of TNFR at intact state. HeLa cells were transfected with siNS or siCLIPR-59. At 72hr after the transfection, expression level of TNFR on cell surface was determined as described in *Materials and Methods*. (**B**-**Y**) Effect of knockdown treatment for CLIPR-59 expression on the ligand dependent internalization of TNF-α/TNFR1. HeLa cells, transfected with siNS or siCLIPR-59 were labeled by Bio-TNF with STP-FITC complex at 4℃. At 30min after incubation at 4℃, cells were replaced at 37℃ for inducing the ligand dependent internalization during the indicated periods. After incubation at 37℃, cells were fixed and mounted with mounting medium containing DAPI. Fluorescent signals of FITC and DAPI in siNS (**B**-**G**, **H**, **N**, **T**, **J**, **P**, **V**, **L**, **R** and **X**) or siCLIPR-59(**I**, **O**, **U**, **K**, **Q**, **W**, **M**, **S** and **Y**) transfected HeLa cells were detected with conforcal laser microscopy.

Figure S13. Effect of knockdown for CLIPR-59 expression on the induction of apoptosis by TNF-α in several TNF-α sensitive cell lines.

Various TNF-α sensitive cell lines, HeLa (human cervical cancer cells), A549 (human alveolar basal epithelial cells) or HT1080 cells (human fibrosarcoma cells) transfected with siNS or siCLIPR-59, were stimulated with TNF-α (10ng/ml) plus CHX (10μg/ml) (closed column) or CHX alone (open column) in 96 well white wall culture plate. At 4.5 hr after the stimulation,

Caspase-3 activity in these cells was assessed as described in Fig.2**G** (Error bar means ± std dev; n=3).

References

1. Perez F, Pernet-Gallay K, Nizak C, Goodson HV, Kreis TE, Goud B. CLIPR-59, a new trans-Golgi/TGN cytoplasmic linker protein belonging to the CLIP-170 family. *Journal of Cell Biology* 2002; **156**(4)**:** 631-642.

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Supplementary Figures

Fig.S1

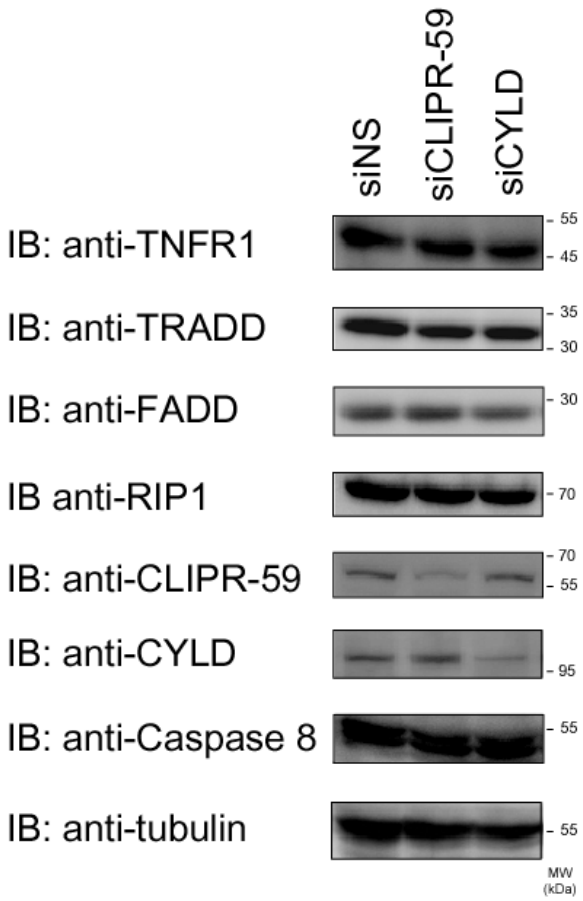
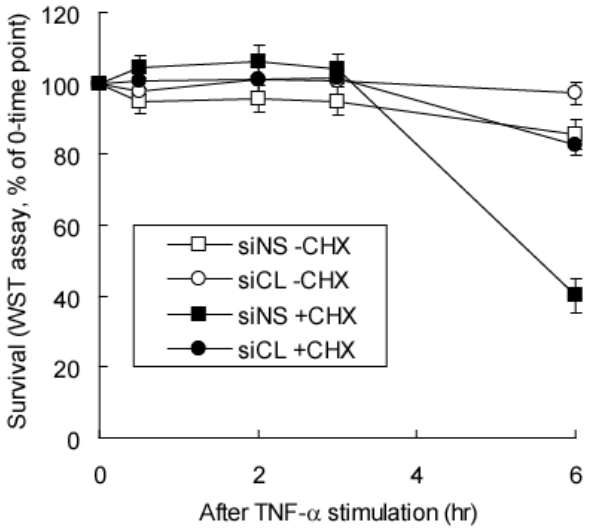


Fig.S2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60
40
20
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2
4
6
After TNF-α stimulation (hr)
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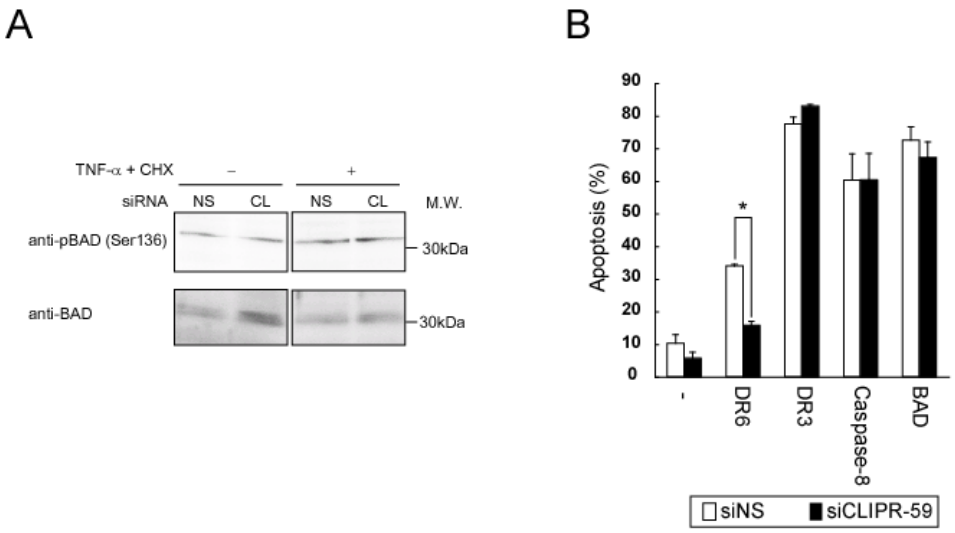
siCL -CHX
siNS +CHX
siCL +CHX

Fig.S3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30kDa
B
Apoptosis (%)
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80
70
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10
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*
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DR3
Caspase-8
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Fig.S4

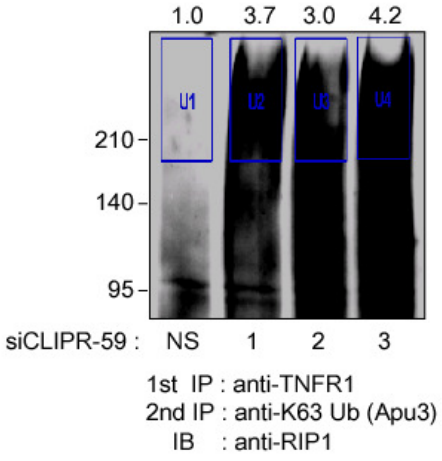
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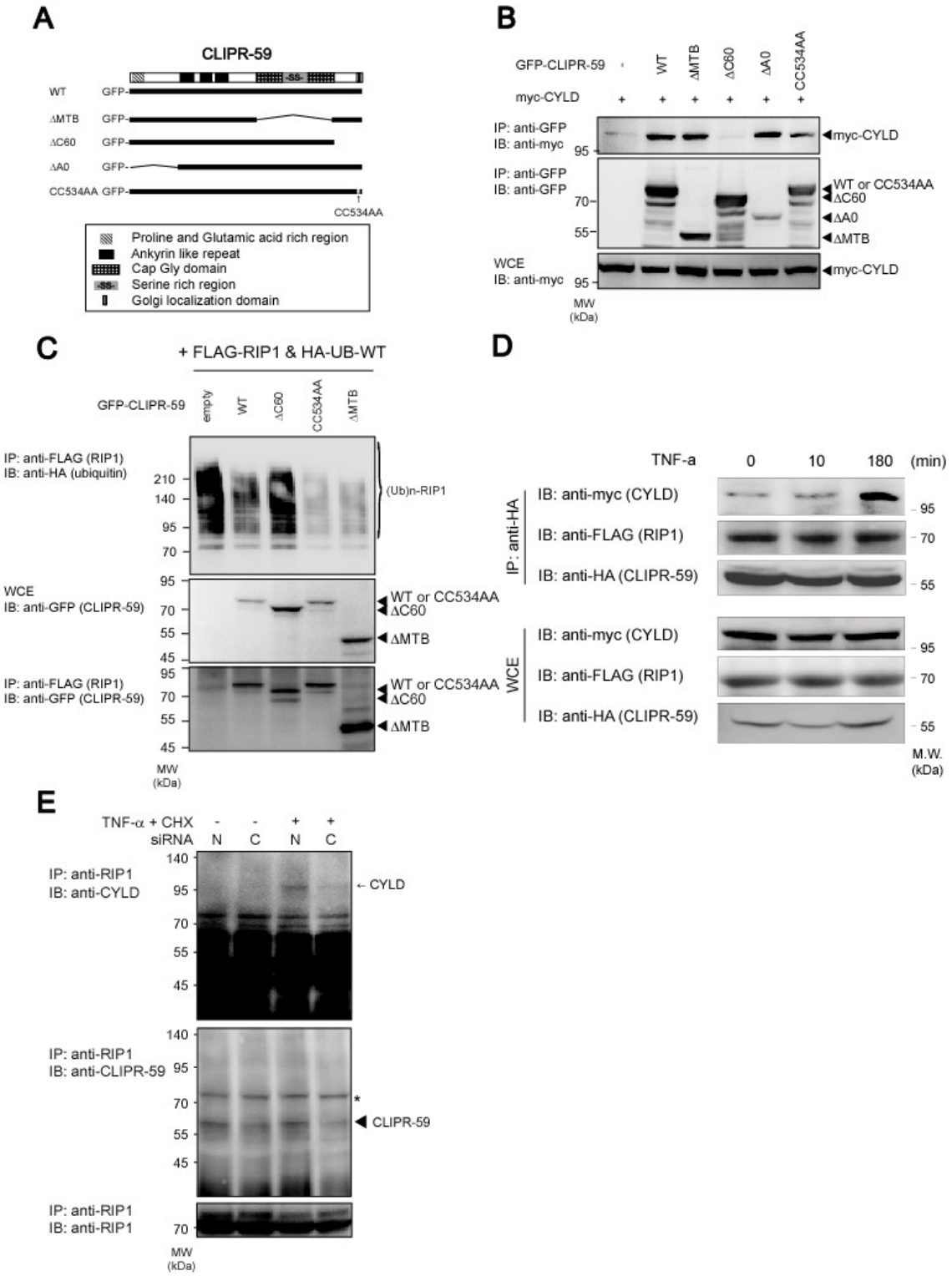
Fig.S5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myc-CYLD
WT or CC534AA
ΔC60
ΔA0
ΔMTB
95
70
55
MW
(kDa)
C
+ FLAG-RIP1 & HA-UB-WT
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140
95
70
55
45
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(kDa)
D
TNF-a
0
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180
(min)
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N
C
N
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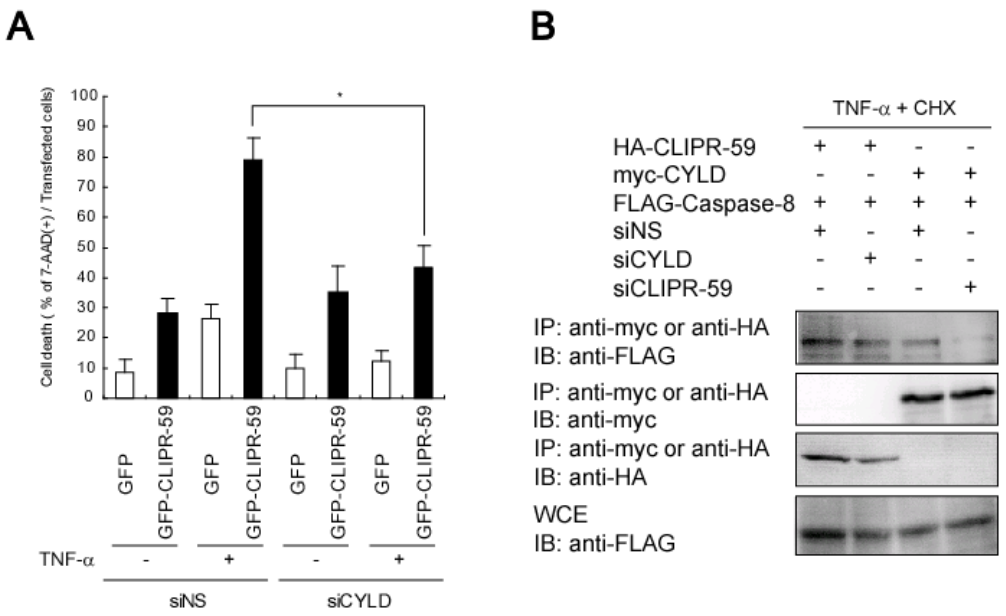
(kDa)

Fig.S6


A
B
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90
80
70
60
50
40
30
20
10
0
*
GFP
GFP-CLIPR-59
GFP
GFP-CLIPR-59
GFP
GFP-CLIPR-59
GFP
GFP-CLIPR-59
TNF-α
-
+
-
+
siNS
siCYLD
TNF-α + CHX
HA-CLIPR-59 + + - -
myc-CYLD - - + +
FLAG-Caspase-8 + + + +
siNS + - + -
siCYLD - + - -
siCLIPR-59 - - - +
IP: anti-myc or anti-HA
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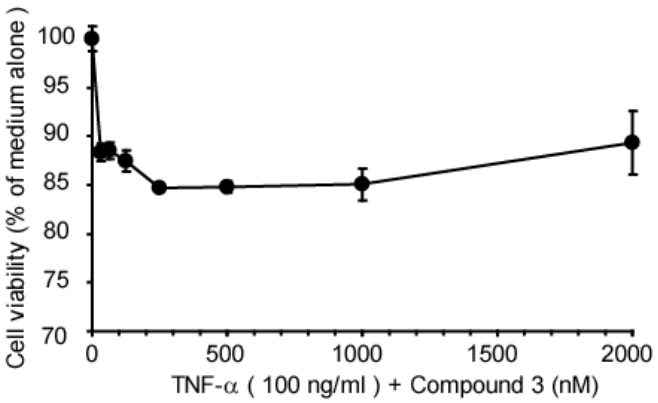
Fig.S7


Cell viability (% of medium alone)
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95
90
85
80
75
70
0
500
1000
1500
2000
TNF-α (100 ng/ml) + Compound 3 (nM)

Fig.S8

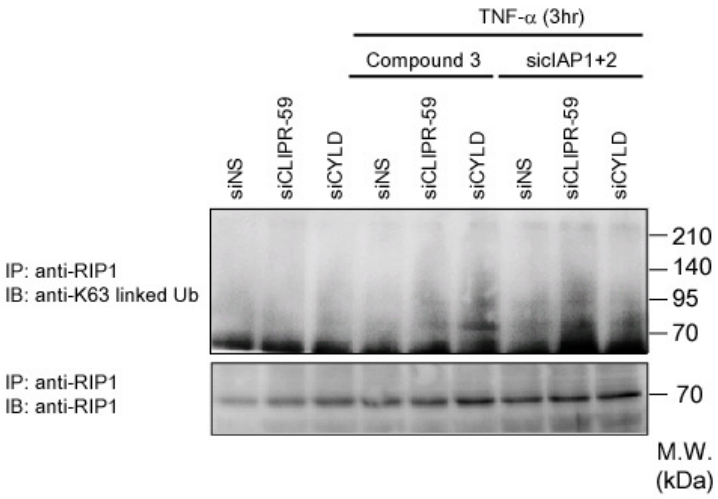

TNF-α (3hr)
Compound 3
sicIAP1+2
siNS
siCLIPR-59
siCYLD
siNS
siCLIPR-59
siCYLD
siNS
siCLIPR-59
siCYLD
IP: anti-RIP1
IB: anti-K63 linked Ub
IP: anti-RIP1
IB: anti-RIP1
210
140
95
70
70
M.W.
(kDa)

Fig.S9

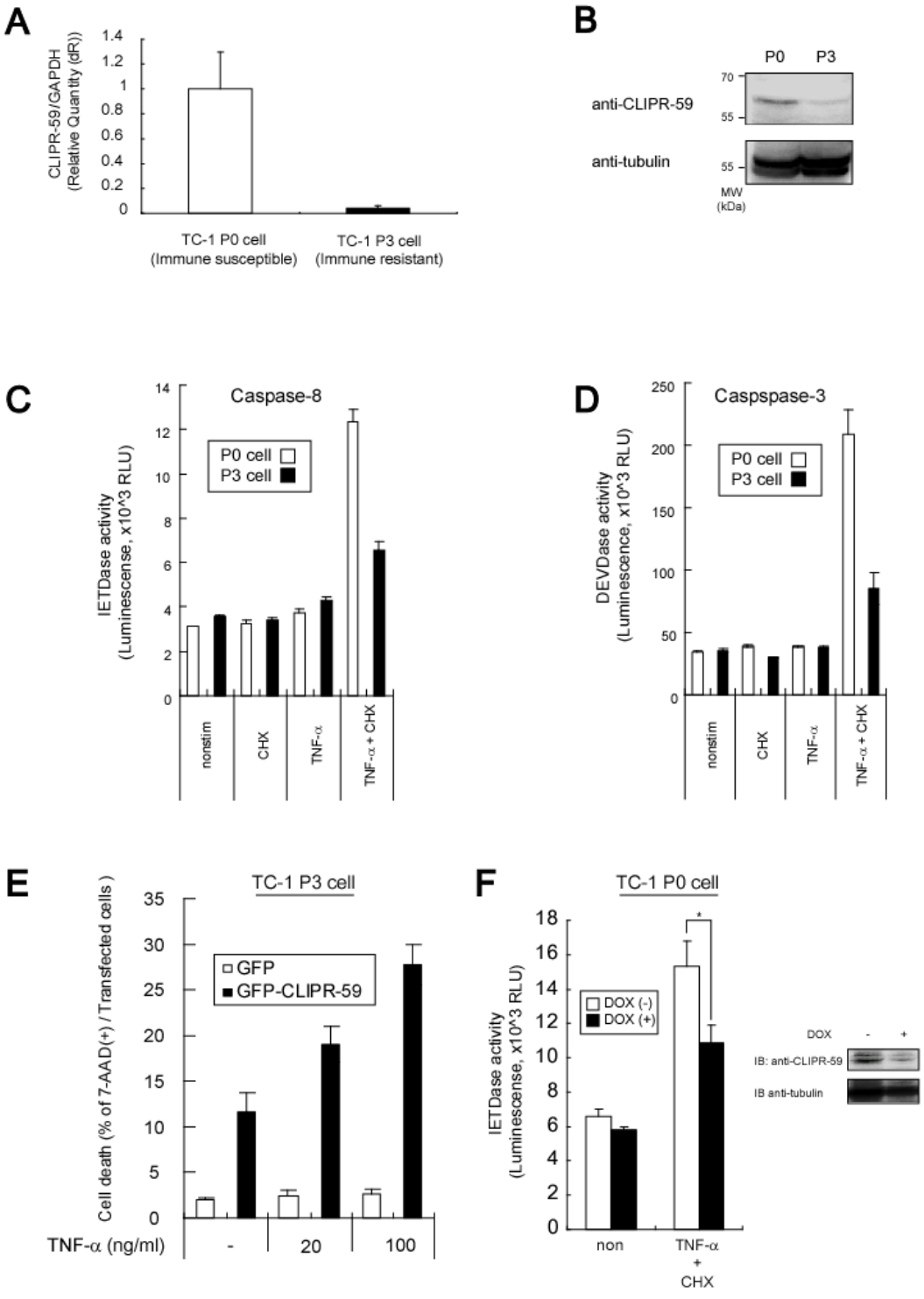

A
CLIPR-59/GAPDH (Relative Quantity (dR))
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TC-1 P0 cell (Immune susceptible)
TC-1 P3 cell (Immune resistant)
B
P0
P3
anti-CLIPR-59
anti-tubulin
70
55
55
MW (kDa)
C
Caspase-8
IETDase activity (Luminescense, x10^3 RLU)
P0 cell
P3 cell
14
12
10
8
6
4
2
0
nonstim
CHX
TNF-α
TNF-α + CHX
D
Caspspase-3
DEVDase activity (Luminescence, x10^3 RLU)
P0 cell
P3 cell
250
200
150
100
50
0
nonstim
CHX
TNF-α
TNF-α + CHX
E
TC-1 P3 cell
Cell death (% of 7-AAD(+) / Transfected cells)
GFP
GFP-CLIPR-59
35
30
25
20
15
10
5
0
TNF-α (ng/ml)
-
20
100
F
TC-1 P0 cell
IETDase activity (Luminescense, x10^3 RLU)
DOX (-)
DOX (+)
*
18
16
14
12
10
8
6
4
2
0
non
TNF-α + CHX
DOX
-
+
IB: anti-CLIPR-59
IB anti-tubulin

Fig.S10

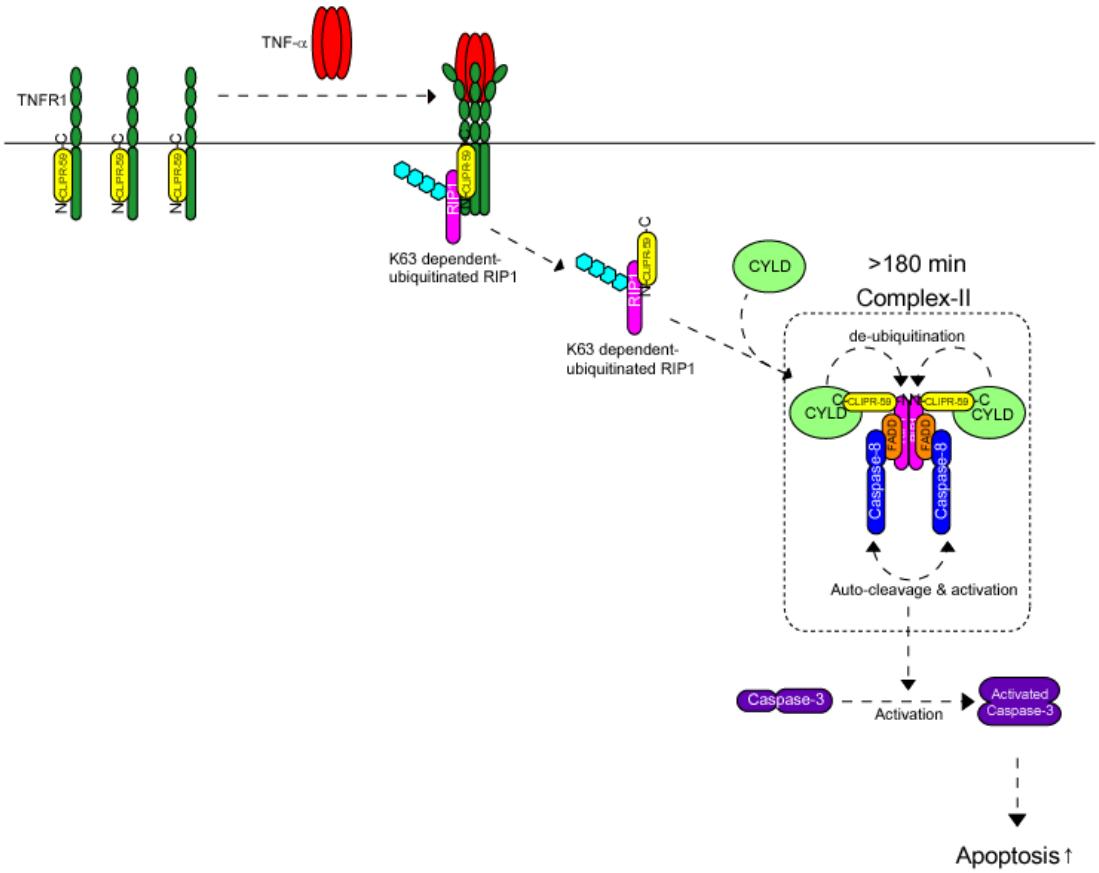
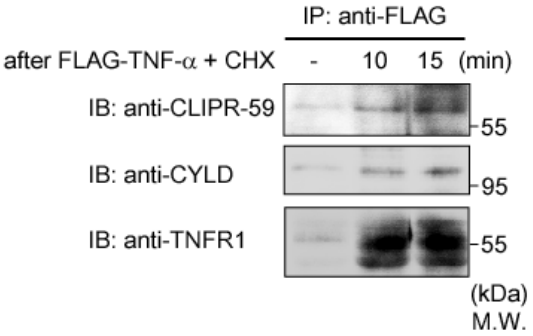

TNF-α
TNFR1
C
N
CLIPR-59
RIP1
K63 dependent-
ubiquitinated RIP1
K63 dependent-
ubiquitinated RIP1
CYLD
>180 min
Complex-II
de-ubiquitination
CYLD
CLIPR-59
FADD
Caspase-8
Auto-cleavage & activation
Caspase-3
Activation
Activated
Caspase-3
Apoptosis↑

Fig.S11


IP: anti-FLAG
after FLAG-TNF-α + CHX
-
10
15
(min)
IB: anti-CLIPR-59
55
IB: anti-CYLD
95
IB: anti-TNFR1
55
(kDa)
M.W.

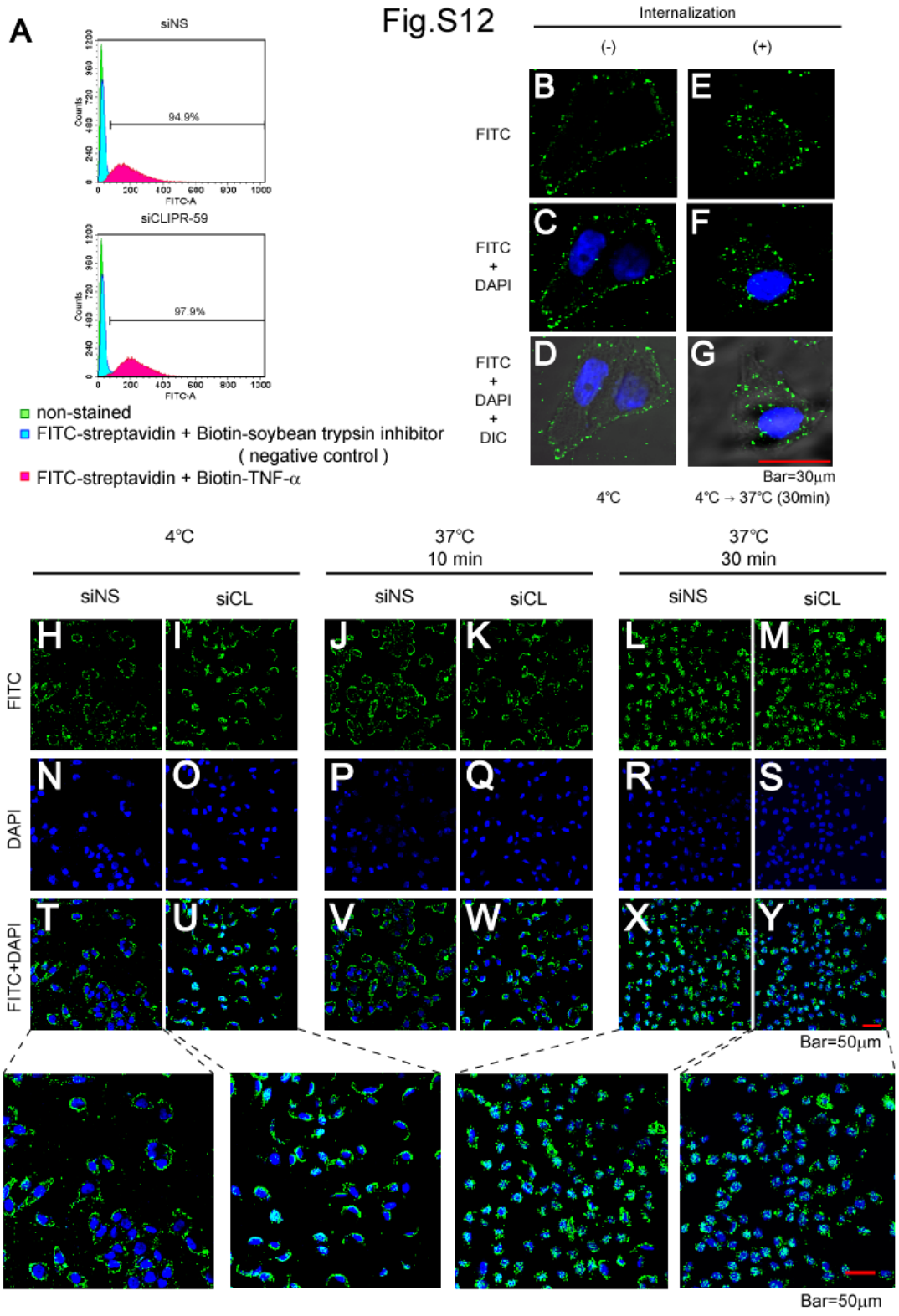
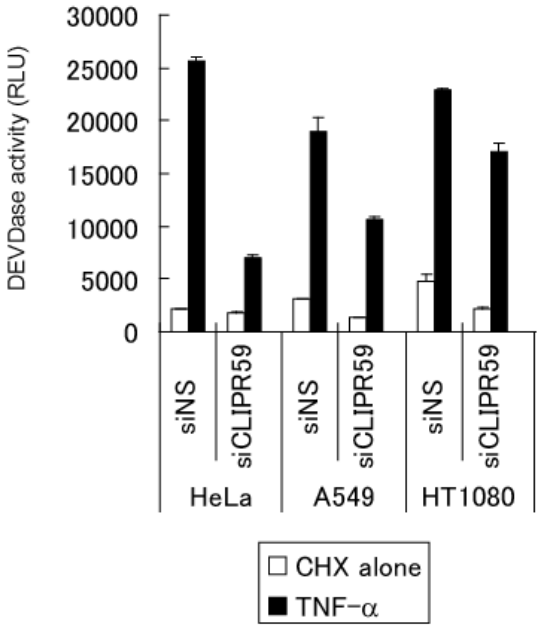


Fig.S12
A
siNS
94.9%
siCLIPR-59
97.9%
Counts
FITC-A
non-stained
FITC-streptavidin + Biotin-soybean trypsin inhibitor
(negative control)
FITC-streptavidin + Biotin-TNF-α
Internalization
(-)
(+)
FITC
FITC + DAPI
FITC + DAPI + DIC
B
C
D
E
F
G
Bar=30μm
4℃
4℃ → 37℃ (30min)
4℃
37℃ 10 min
37℃ 30 min
siNS
siCL
FITC
DAPI
FITC+DAPI
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Bar=50μm
Bar=50μm

Fig.S13


DEVDase activity (RLU)
30000
25000
20000
15000
10000
5000
0
siNS
siCLIPR59
siNS
siCLIPR59
siNS
siCLIPR59
HeLa
A549
HT1080
CHX alone
TNF-α