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Supplemental Information

The Tumor Suppressor Hace1 Is a Critical Regulator of TNFR1-Mediated Cell Fate

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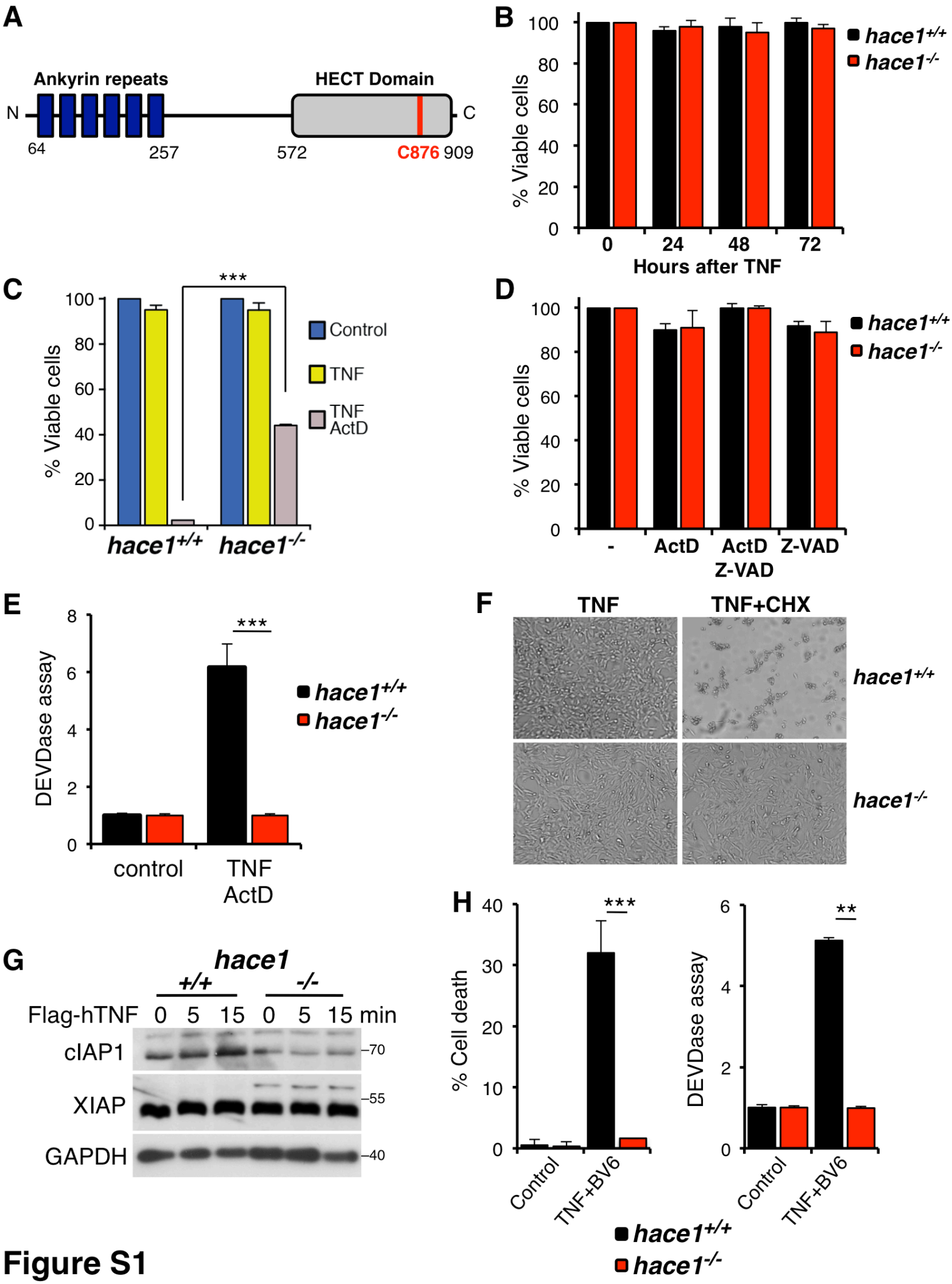
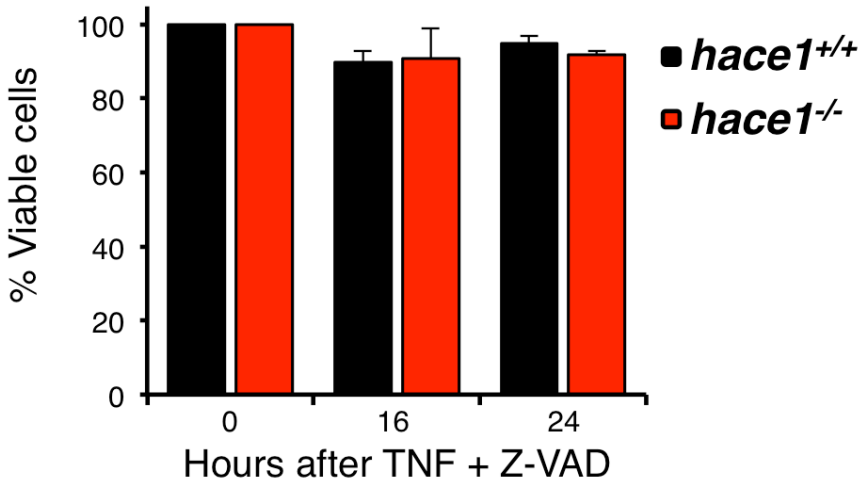

A
Ankyrin repeats
HECT Domain
N
C
64
257
572
C876
909
B
% Viable cells
hace1+/+
hace1-/-
0
24
48
72
Hours after TNF
C
***
% Viable cells
Control
TNF
TNF ActD
hace1+/+
hace1-/-
D
% Viable cells
hace1+/+
hace1-/-
-
ActD
ActD Z-VAD
Z-VAD
E
***
DEVDase assay
hace1+/+
hace1-/-
control
TNF ActD
F
TNF
TNF+CHX
hace1+/+
hace1-/-
G
hace1
+/+
-/-
Flag-hTNF 0 5 15 0 5 15 min
cIAP1
XIAP
GAPDH
70
55
40
H
***
% Cell death
**
DEVDase assay
Control
TNF+BV6
Control
TNF+BV6
hace1+/+
hace1-/-

Figure S1

A


100
80
60
40
20
0
% Viable cells
0
16
24
Hours after TNF + Z-VAD
hace1+/+
hace1-/-

B

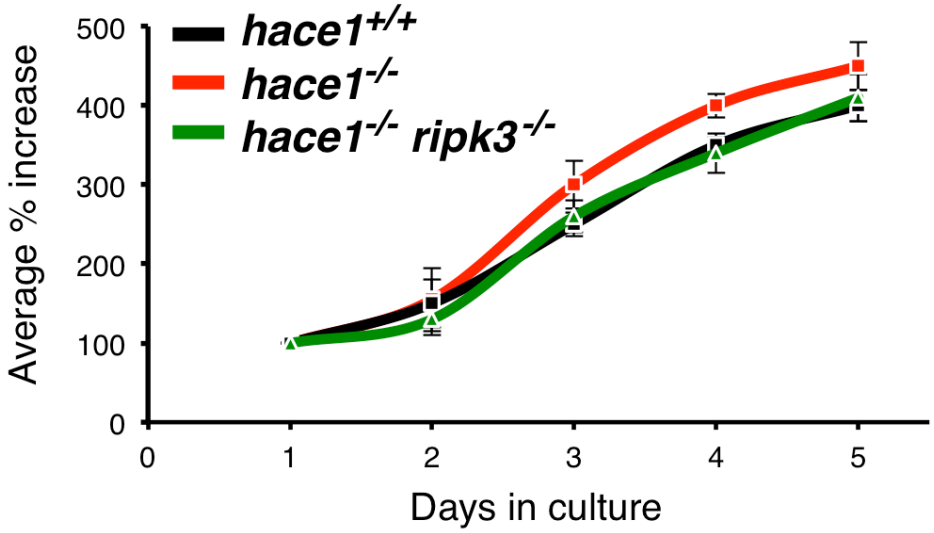

500
400
300
200
100
0
Average % increase
0
1
2
3
4
5
Days in culture
hace1+/+
hace1-/-
hace1-/- ripk3-/-

Figure S2

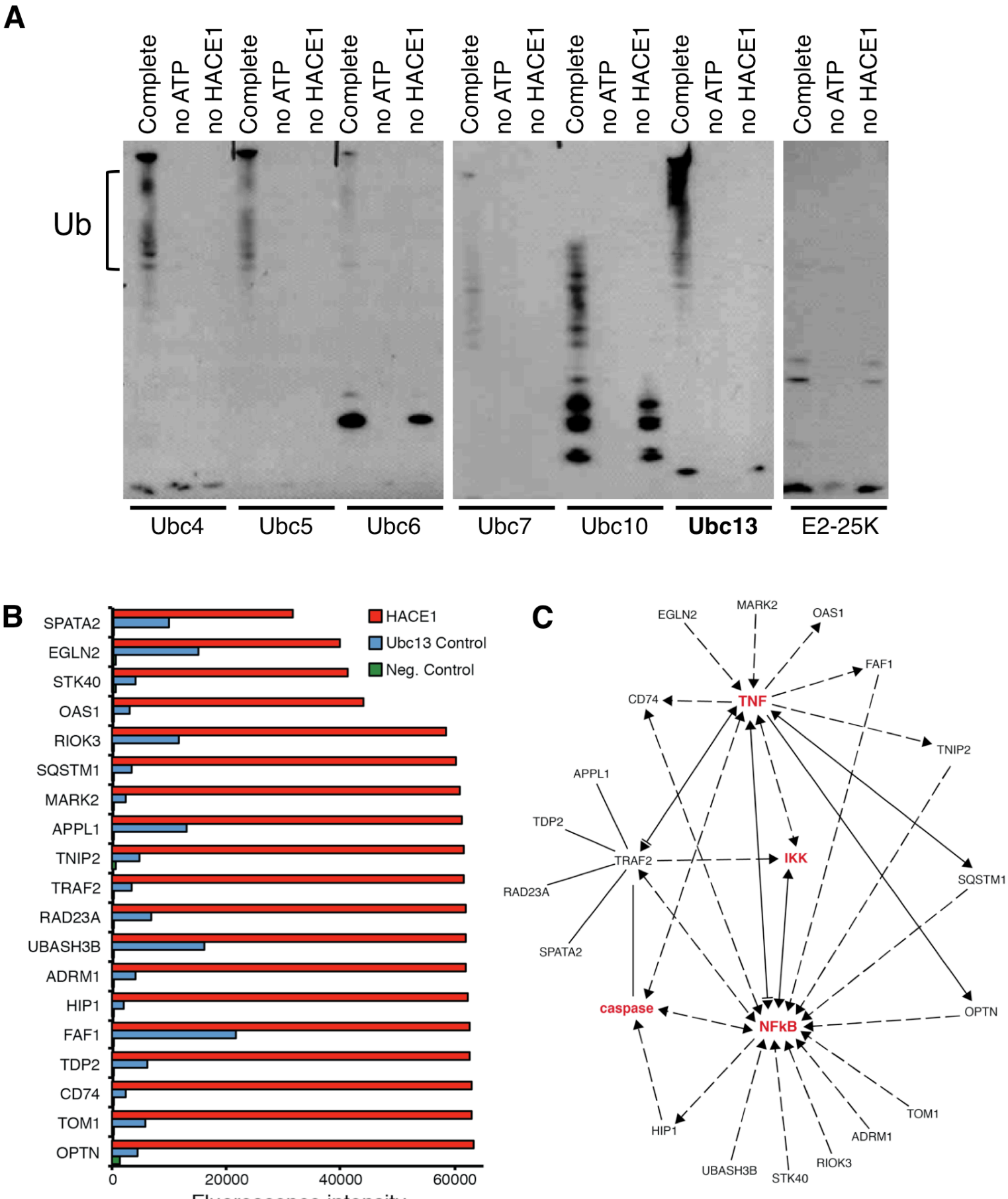

A
Complete
no ATP
no HACE1
Ub
Ubc4
Ubc5
Ubc6
Ubc7
Ubc10
Ubc13
E2-25K
B
HACE1
Ubc13 Control
Neg. Control
SPATA2
EGLN2
STK40
OAS1
RIOK3
SQSTM1
MARK2
APPL1
TNIP2
TRAF2
RAD23A
UBASH3B
ADRM1
HIP1
FAF1
TDP2
CD74
TOM1
OPTN
0
20000
40000
60000
Fluorescence intensity
C
EGLN2
MARK2
OAS1
FAF1
CD74
TNF
TNIP2
APPL1
TDP2
TRAF2
IKK
SQSTM1
RAD23A
SPATA2
caspase
NFkB
OPTN
HIP1
TOM1
ADRM1
UBASH3B
STK40
RIOK3

Figure S3

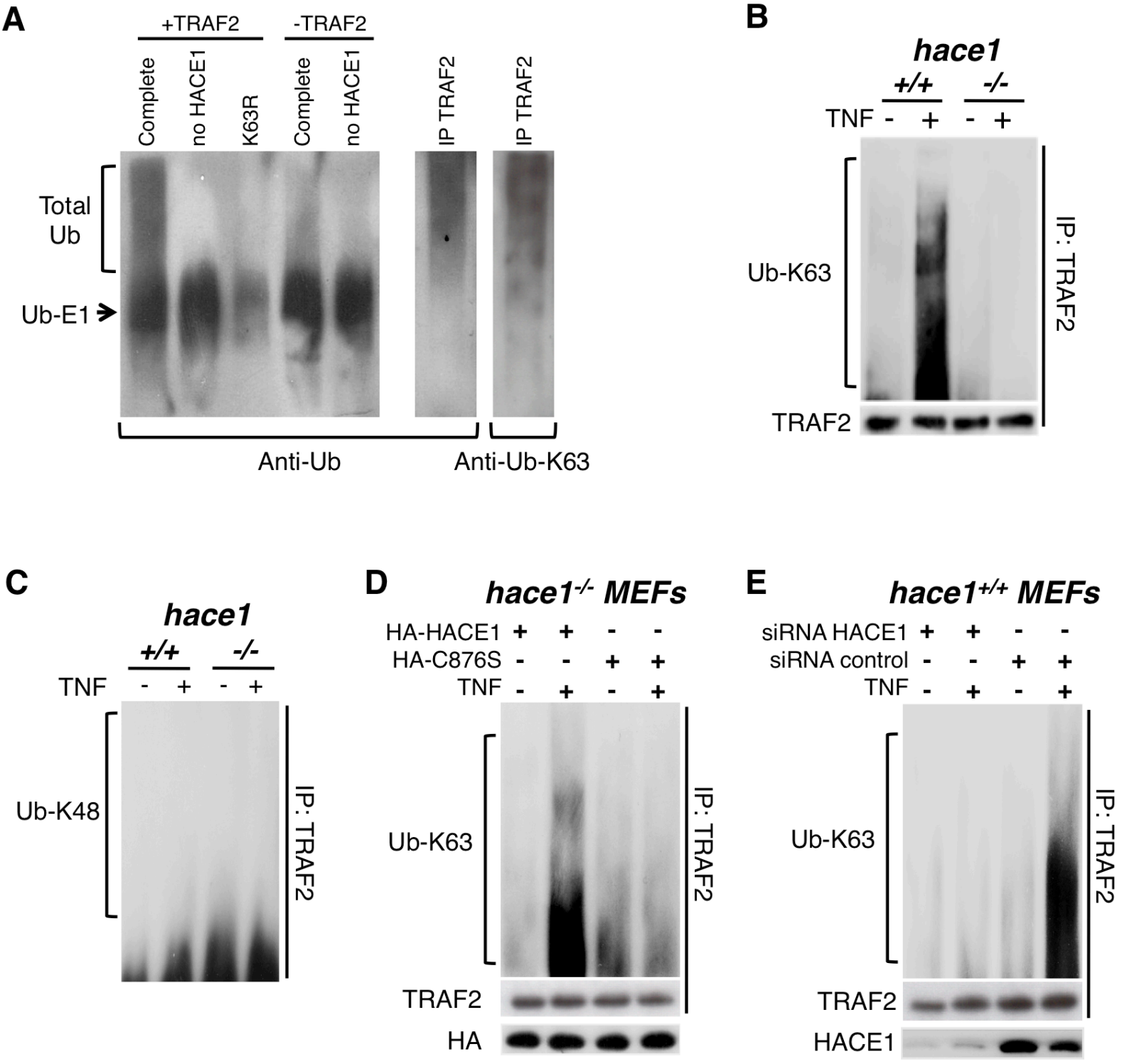

A
+TRAF2
-TRAF2
Complete
no HACE1
K63R
Complete
no HACE1
IP TRAF2
IP TRAF2
Total Ub
Ub-E1
Anti-Ub
Anti-Ub-K63
B
hace1
+/+
-/-
TNF - + - +
Ub-K63
TRAF2
IP: TRAF2
C
hace1
+/+
-/-
TNF - + - +
Ub-K48
IP: TRAF2
D
hace1-/- MEFs
HA-HACE1 + + - -
HA-C876S - - + +
TNF - + - +
Ub-K63
TRAF2
HA
IP: TRAF2
E
hace1+/+ MEFs
siRNA HACE1 + + - -
siRNA control - - + +
TNF - + - +
Ub-K63
TRAF2
HACE1
IP: TRAF2

Figure S4

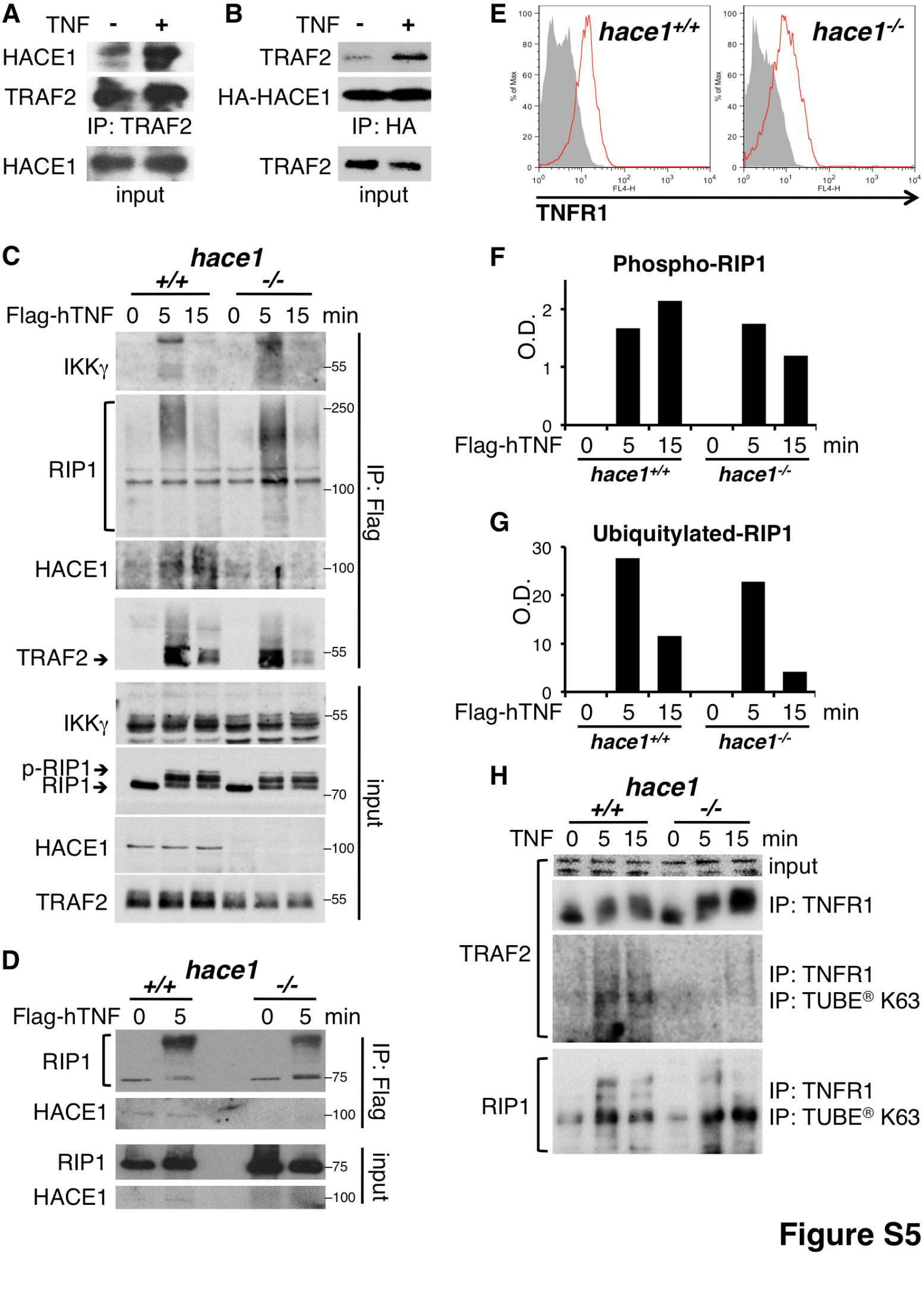
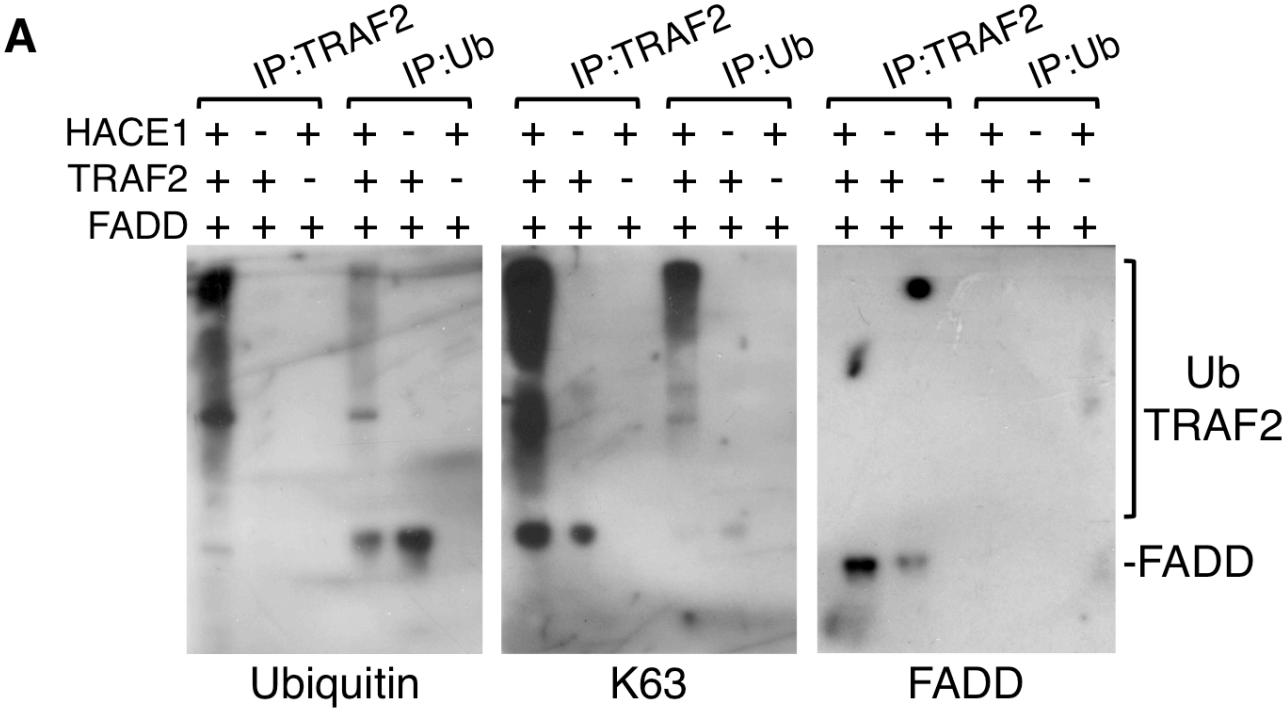
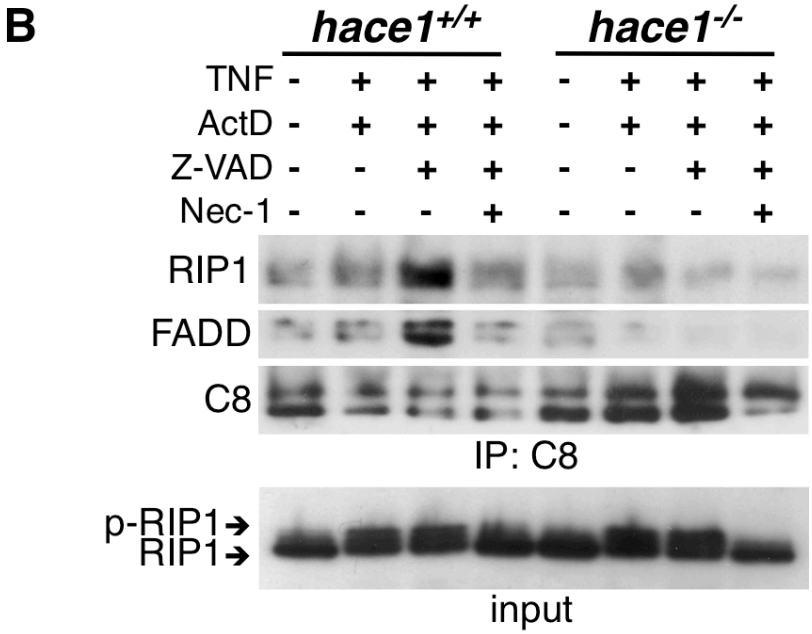

A
TNF - +
HACE1
TRAF2
IP: TRAF2
HACE1
input
B
TNF - +
TRAF2
HA-HACE1
IP: HA
TRAF2
input
E
hace1+/+
hace1-/-
% of Max
FL4-H
TNFR1
C
hace1
+/+
-/-
Flag-hTNF 0 5 15 0 5 15 min
IKKγ
RIP1
HACE1
TRAF2
IP: Flag
p-RIP1
RIP1
input
F
Phospho-RIP1
O.D.
Flag-hTNF 0 5 15 0 5 15 min
hace1+/+
hace1-/-
G
Ubiquitylated-RIP1
O.D.
Flag-hTNF 0 5 15 0 5 15 min
hace1+/+
hace1-/-
D
hace1
+/+
-/-
Flag-hTNF 0 5 0 5 min
RIP1
HACE1
IP: Flag
RIP1
HACE1
input
H
hace1
+/+
-/-
TNF 0 5 15 0 5 15 min
input
IP: TNFR1
TRAF2
IP: TNFR1
IP: TUBE® K63
RIP1
IP: TNFR1
IP: TUBE® K63

Figure S5


A
IP:TRAF2
IP:Ub
HACE1
TRAF2
FADD
Ub
TRAF2
-FADD
Ubiquitin
K63
FADD


B
hace1+/+
hace1-/-
TNF
ActD
Z-VAD
Nec-1
RIP1
FADD
C8
IP: C8
p-RIP1
RIP1
input

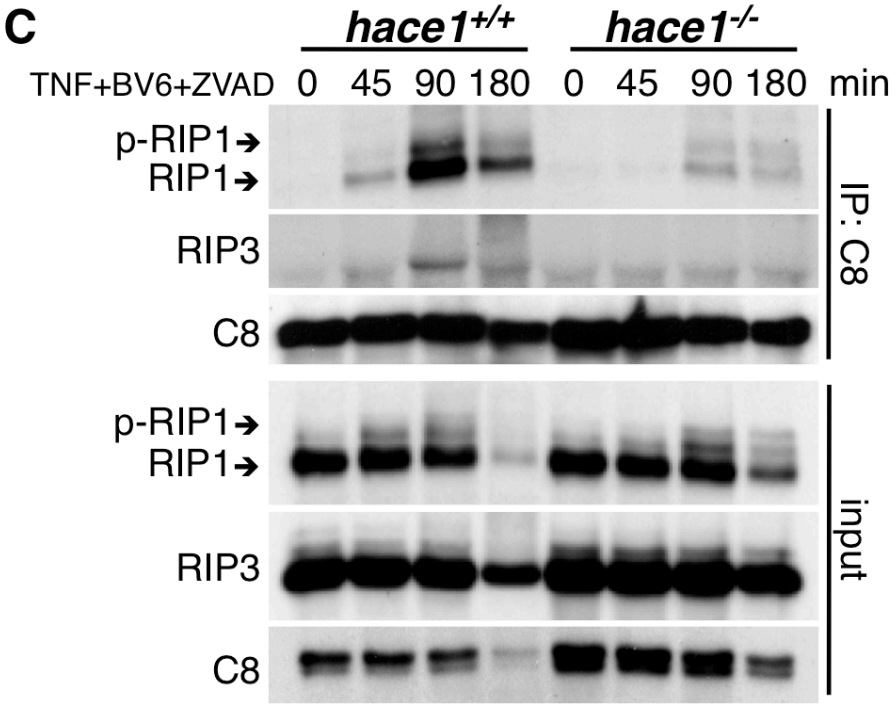

C
hace1+/+
hace1-/-
TNF+BV6+ZVAD
0 45 90 180 0 45 90 180 min
p-RIP1
RIP1
RIP3
C8
IP: C8
p-RIP1
RIP1
RIP3
C8
input

Figure S6

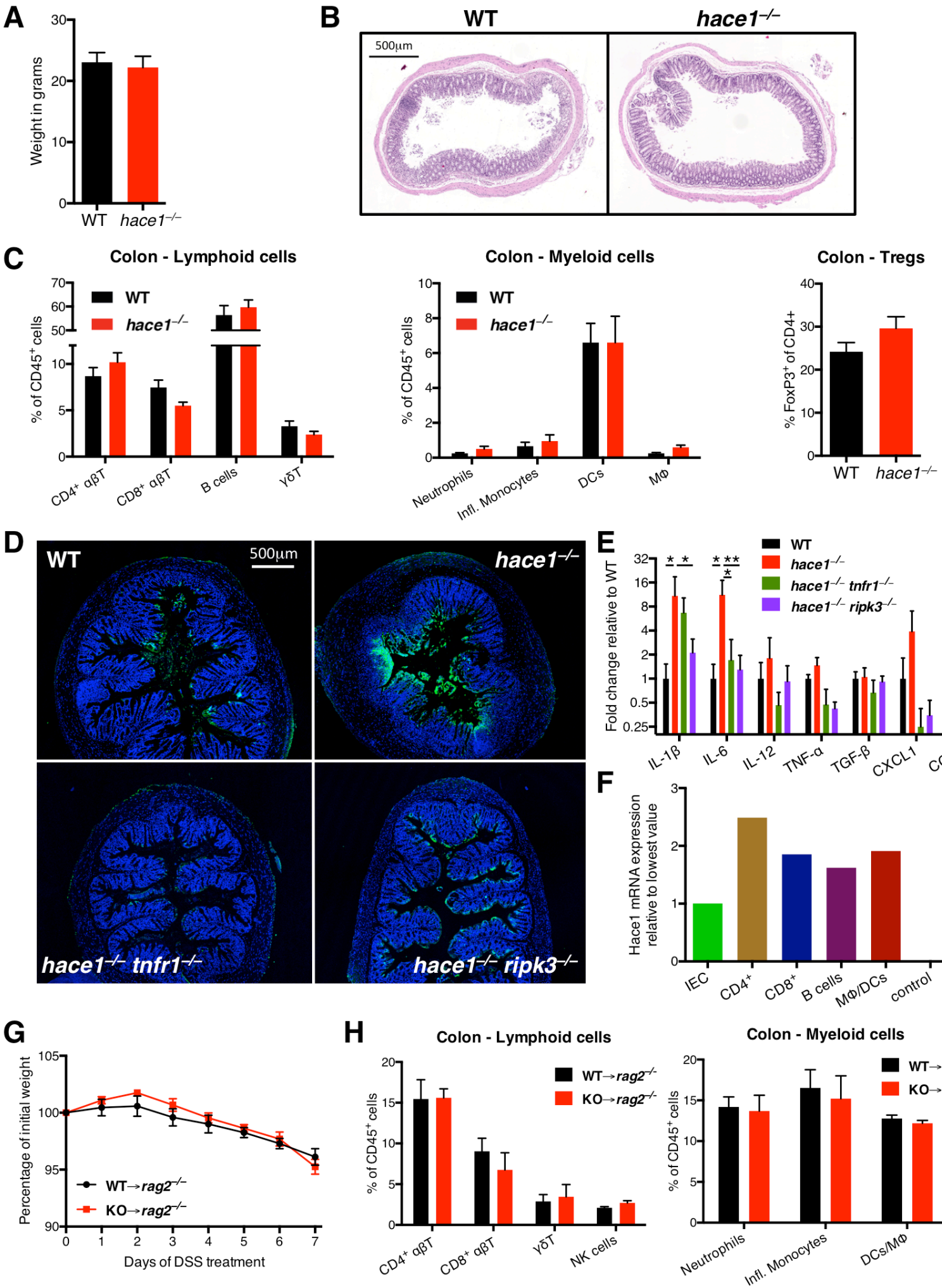

A
Weight in grams
WT
hace1−/−
B
WT
hace1−/−
500µm
C
Colon - Lymphoid cells
% of CD45+ cells
CD4+ αβT
CD8+ αβT
B cells
γδT
Colon - Myeloid cells
Neutrophils
Infl. Monocytes
DCs
MΦ
Colon - Tregs
% FoxP3+ of CD4+
D
500µm
hace1−/− tnfr1−/−
hace1−/− ripk3−/−
E
Fold change relative to WT
hace1−/− tnfr1−/−
hace1−/− ripk3−/−
IL-1β
IL-6
IL-12
TNF-α
TGF-β
CXCL1
CCL2
F
Hace1 mRNA expression relative to lowest value
IEC
CD4+
CD8+
B cells
MΦ/DCs
control
G
Percentage of initial weight
WT→rag2−/−
KO→rag2−/−
Days of DSS treatment
H
Colon - Lymphoid cells
Colon - Myeloid cells
NK cells
Neutrophils
DCs/MΦ

Figure S7

Supplemental Information

Supplemental Figure Legends

Figure S1 (related to Figure 1 and 2).

HACE1 is essential for TNFR1-induced apoptosis.

(A) Schematic overview of the structure of HACE1. The C876 residue responsible for catalytic activity is highlighted in red. **(B)** *Hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs were treated with TNF (10ng/ml) for the indicated time points. Cell viability was determined in triplicates (mean values +/- SD). **(C)** *Hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs were treated with TNF (10ng/ml) and ActD (1μg/ml) for 24h. Cell viability was determined in triplicates (mean values +/- SD). **(D)** *Hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs were treated with ActD (1μg/ml) and Z-VAD (5μg/ml) alone or in combination for 8 hours. Cell viability was determined in triplicates (mean values +/- SD). **(E)** DEVDase assay to determine apoptosis in untreated (control) and TNF (10ng/ml) plus ActD (1μg/ml) stimulated *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs. Activation of Caspase-3 to monitor apoptosis was determined in sextuplicate cultures (mean values +/- SD) 2.5 hrs after addition of TNF plus ActD. **(F)** *Hace1*$^{-/-}$ MEFs do not undergo cell death in response to TNF plus cycloheximide (10ng/ml TNF+10μg/ml CHX). Representative images are shown for *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs treated for 8 hrs with either TNF alone (10ng/ml) or the apoptotic trigger TNF+CHX. **(G)** cIAP and XIAP protein levels as detected by Western Blot in *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs treated with human TNF alone (10ng/ml). **(H)** Percent cell death and Caspase activation (DEVDase) assay of untreated (control) and TNF plus BV6 treated *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs. Mean values (+/- SD) from sextuplicate cultures are shown.

Figure S2 (related to Figure 4).

TNF plus Z-VAD treatment, growth curves.

(A) *Hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs are treated with TNF (10ng/ml) plus Z-VAD (5μg/ml) for the indicated time points. Cell viability was determined in triplicates (mean values +/- SD). **(B)** Growth curve measurements of *hace1*$^{+/+}$, *hace1*$^{-/-}$ and *hace1*$^{-/-}$ *ripk3*$^{-/-}$ MEFs. Cells were trypsinized and counted using a Casy Counter ® every day for 5 days. Mean values (+/- SD) were determined in quadruplicate cultures.

Figure S3 (related to Figure 5).

Identification of Hace1 ubiquitylation targets.

(**A**) HACE1 E3 ligase activity was determined by *in vitro* ubiquitylation reactions using the indicated E2 enzymes. Complete indicates 100nM recombinant His-HACE1, 1μM of the respective E2 enzyme, 100μg/ml HA-ubiquitin, 200nM ubiquitin E1 enzyme and ATP (1mM). Reactions were incubated at 30°C for 1 hr. Control reactions in the absence of ATP (no ATP) or the absence of His-Hace1 (no HACE1) are shown. (**B**) Ubiquitylation levels of 19 identified HACE1 ubiquitylation targets, measured as fluorescence intensity. Data from background control, Ubc13 alone, and

Ubc13 plus recombinant HACE1 are shown (t-test, for all $p<0.001$). (**C**) Ingenuity pathway analysis showing the relationship of the ubiquitylation targets with TNF and NFκB signaling. Solid and dashed lines depict direct and indirect interactions, respectively. Arrows indicate activation whereas blunted lines indicate inhibition. Lines without arrows indicate physical interaction.

Figure S4 (related to Figure 5).

Hace1 mediates TNF-induced K63-linked ubiquitylation of TRAF2.

(A) *In vitro* HACE1 ubiquitylation reactions (complete = HACE1, Ubc13, E1, ubiquitin, and ATP) in the presence of 100mM recombinant TRAF2 (+ TRAF2) or absence of TRAF2 (- TRAF2) as substrate. Reactions in the absence of HACE1 (no HACE1) or in the presence of ubiquitin-K63-mutant (K63R) are shown as controls. Ubiquitination reaction was performed at 30ºC for 1 hr. TRAF2 was then immunoprecipitated from the complete reaction and probed for either total ubiquitin or using an antibody that specifically detects ubiquitin K63 branched chains. **(B)** TRAF2 was immunoprecipitated from *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs treated with TNF (10ng/ml) (+) or left untreated (-). Immunoblotting was performed using an antibody specific to the ubiquitin K63-chain. **(C)** TRAF2 was immunoprecipitated from *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs treated with TNF (+) or left untreated (-) as described in Figure S7B. Western blotting was performed using an antibody specific to ubiquitin K48-linked chains. TRAF2 control levels are shown in Figure S7B. **(D)** *hace1*$^{-/-}$ MEFs re-expressing either full length wild type HACE1 or its ligase-dead form C876S were stimulated with TNF (10ng/ml) or left untreated. TRAF2 was immunoprecipitated and total TRAF2 and TRAF2 K63 ubiquitylation (poly-Ub-K63) analyzed by Western blot. Levels of wild type and C876S HACE1 were determined using anti-HA antibody. **(E)** *hace1*$^{+/+}$ MEFs were transfected with HACE1 specific siRNA or with scrambled siRNA. Following TNF (10ng/ml) stimulation, TRAF2 was immunoprecipitated and analyzed as in Figure S7B-7D. HACE1 and TRAF2 levels were determined using specific antibodies.

Figure S5 (related to Figure 5).

Complex I formation, TNFR1 surface expression and quantification of phospho- and ubiquitylated-RIP1 kinase.

(A) Immunoprecipitation of TRAF2 from lysates of *hace1*$^{+/+}$ MEFs with (+) and without (-) TNF (10ng/ml) stimulation. Endogenous HACE1 interacting with TRAF2 was detected using specific antibodies. **(B)** Wild type MEFs overexpressing HA-tagged HACE1 were stimulated with TNF (10ng/ml) (+) or left untreated (-). HACE1 was immunoprecipitated using an anti-HA antibody and immunoprecipitates probed with antibodies to detect HA-HACE1 and TRAF2. Total TRAF2 is shown to control for the input. **(C)** Complex I was immunoprecipitated as described in Figure 5A. Flag-IPs and input lysates were probed with antibodies to IKKγ, RIP1 kinase, TRAF2, and HACE1. RIP1 kinase phosphorylation (p-RIP1) is indicated. **(D)** Cells were stimulated with Flag-hTNF for the indicated time. Immediately before Flag-IP, Flag-hTNF was added to the lysates of untreated samples to allow analysis of the TNFR1 signaling complex also in unstimulated cells. Flag-IPs and input lysates were probed with antibodies against RIP1 and HACE1. **(E)** FACS histograms showing surface

expression levels of TNFR1 (red lines) in *hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs. Background staining using an isotype control is shown in grey. **(F-G)** Quantification of the phosphorylated (F) and ubiquitylated RIP1 kinase (G) after TNF treatment as shown in Figure 5A**. (H)** Serial immunoprecipitation of K63-ubiquitylated proteins from the TNFR1 complex. Western blots were performed to detect the presence of ubiquitylated TRAF2; ubiquitylated RIP1 kinase is shown as control.

Figure S6 (related to Figure 5).

Formation of complex II and the RIP1/RIP3 kinase necrosome.

(A) *In vitro* HACE1 ubiquitylation reactions [Ubc13, E1, ubiquitin, and ATP in the presence of 100mM recombinant HACE1 and TRAF2 (+) or absence of HACE1 or TRAF2 (-)] were immunoprecipitated using anti-TRAF2 and anti-Ubiquitin antibodies. Western blots were probed with antibodies to detect total ubiquitin and K63-linked ubiquitin chains. Recombinant FADD was added to the completed ubiquitylation reaction before TRAF2 immunoprecipitation and then probed with anti FADD antibody. **(B)** *Hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs were treated with TNF, ActD, Z-VAD and Nec-1 followed by immunoprecipitation of Caspase-8 (C8) to detect its interactions with RIP1 and FADD. Phosphorylated RIP1 (p-RIP1) kinase is indicated in the loading control. **(C)** *Hace1*$^{+/+}$ and *hace1*$^{-/-}$ MEFs were treated with the necroptotic trigger TNF (10ng/ml), BV6 (1μM), plus Z-VAD (5μg/ml) for the indicated time points followed by immunoprecipitation of Caspase-8 to assay for the formation of the Caspase-8/RIP1/RIP3 necrosome. Input lysates were assayed for the indicated proteins. Phosphorylated RIP1 (p-RIP1) is indicated.

Figure S7 (related to Figure 6).

Analysis of naïve, DSS-treated and bone marrow chimeric mice.

(**A**) Total body weight and (**B**) representative H&E-stained transversal colon sections of untreated WT and *hace1*$^{-/-}$ mice. (**C**) Characterization of the immune compartment in the colonic lamina propria: lymphoid cells (left panel), myeloid cells (central panel) and FoxP3+ regulatory T cells (right panel). 5 untreated mice per group were used per cohort. Values show mean values ± SEM. (**D**) DAPI (blue) and TUNEL (green) staining of transversal colon sections from DSS-treated WT, *hace1*$^{-/-}$, *hace1*$^{-/-}$ *tnfr1*$^{-/-}$ and *hace1*$^{-/-}$ *ripk3*$^{-/-}$ mice. (**E**) Expression of inflammatory mediators in the colon of WT, *hace1*$^{-/-}$, *hace1*$^{-/-}$ *ripk3*$^{-/-}$ and *hace1*$^{-/-}$ *tnfr1*$^{-/-}$ mice after 7 days of treatment with DSS (2.5%) as determined by qPCR. Two-way ANOVA with Bonferroni post-test: *P < 0.05; **P < 0.01; ***P < 0.001. (**F**) Expression of Hace1 mRNA in the indicated cell populations as detected by qPCR. Intestinal epithelial cells (IEC) were defined as CD45^{-} live cells. (**G-H**) Bone marrow chimeric mice were subjected to DSS treatment for 7 days. Mice were weighed daily and sacrificed for analysis on day 7. (**G**) Changes in body weight. (**H**) Percentages of lymphocyte subpopulations and myeloid cell populations were determined by flow cytometry. Cells from the colonic lamina propria were isolated on day 7 after DSS challenge and analyzed by flow cytometry. Values show averages ± SEM. n=5 per group. Neutrophils were defined as CD11b^{+} Gr-1^{+} Ly6G^{+}, inflammatory monocytes as CD11b^{+} Gr-1^{+} Ly6G^{-}, and DC/MΦ as CD11b$^{+/-}$ CD11c$^{lo/+}$ Gr-1^{-}.

Supplemental Experimental Procedures

Western Blot Antibodies. Western blots were performed using the following specific antibodies: anti-β-Actin (100M4789, Sigma), anti-GAPDH (3683, HRP-conjugated, Cell Signaling Technology), anti-cleaved Caspase-3 (9661, Cell Signaling Technology), anti-cleaved Caspase-8 (9748, Cell Signaling Technology), anti-Caspase-8 (for IP we used clone C20 6136, Santa Cruz Biotech; for Western blots the clone ALX-804-447, ENZO Life Sciences – Alexis), anti-cJun (Cell Signaling Technology, 9165), anti-phospho-cJun (9164, Cell Signaling Technology), anti-ERK1/2 (9102, Cell Signaling Technology), anti-phospho-ERK1/2 (9106, Cell Signaling Technology), anti-HACE1 (TA310566, Origene), anti-IκBα (4814, Cell Signaling Technology), anti-IKKα (CST2682, Bioké), anti-P38 (8690, Cell Signaling Technology), anti-phospho-P38 (9216, Cell Signaling Technology), anti-P65 NFκB (6956, Cell Signaling Technology), anti-phospho-P65 NFκB (3033, Cell Signaling Technology), anti-RIP1 kinase (sc-133102, Santa Cruz Biotech; 610459, BD Biosciences), anti-RIP3 (R4277, Sigma), anti-TNFR1 (sc-8436, Santa Cruz Biotech; for IP we used clone sc-12746), anti-TRAF2 (4724, Cell Signaling Technology; for serial IP we used clone sc-876, Santa Cruz Biotech), anti-FADD (sc-5559, Santa Cruz Biotech), anti-TRADD (sc-7868, Santa Cruz Biotech), anti-Ubiquitin (sc-8017, Santa Cruz Biotech), anti-Ubiquitin Lys-48 (05-1307, Millipore), anti-Ubiquitin Lys-63 (05-1308, Millipore), anti-MLKL (ab196436, Abcam) and anti-phospho-MLKL (MABC604, Millipore). Immunoprecipitations were performed according to the manufacturer's protocol. Serial IPs were performed after dissolving the first immunoprecipitated complex either in 6M urea (for TUBE-IP) or in 1% SDS (for TRAF2 IP) and subsequent dilution 1:25 (urea) and 1:10 (SDS) and re-immunoprecipitated as described in the figure legends. Cell numbers were determined and adjusted using a CASY counter (Roche Innovatis). All Western blot quantifications were calculated from scanned.tiff file using the ImageJ analysis software developed by NIH. Duo-Set ELISAs to detect Interleukin-6 concentrations in cell supernatants were performed according to manufacturer specifications (R&D Systems).

Detection of cell viability. In order to calculate cell viability, treated and control untreated cells were trypsinized at the indicated time points, harvested and stained with trypan blue to exclude dead cells. Cell numbers were determined using a hemocytometer. In addition, cells were washed twice with PBS, directly fixed on the Petri dish with 4% PFA and stained with crystal violet. All images were taken using a light microscope (Zeiss). Time lapse images of apoptotic and necroptotic MEFs were taken using the Live Cell Observer Axiovert 200M (Zeiss). Cells were left in the microscope 37ºC heated chamber for 1 hr prior the treatments with TNF and ActinomycinD or cycloheximide and phase contrast images taken before and every 2 hrs after treatment. The fluorogenic DEVDase substrate assay was carried out following previously described protocols (Vercammen et al., 1998). MEFs were seeded in 6-plicates in a 96 wells plate at a density of 15×10^3 cells per well. The next day, after exposure to various treatments, the cells were lysed in 100μl of Caspase lysis buffer. Caspase activity was measured by incubating 50μl of the supernatant with 50 μl of 2 X cell-free system buffer containing 20 mM Hepes, pH 7.4, 440 mM mannitol, 136 mM sucrose, 4 mM NaCl, 5 mM KH_2PO_4, 4 mM $MgCl_2$, 20 mM dithiothreitol, and 100 μM Ac-DEVD-MCA (Peptide, Scientific Marketing Associate,

3171-V). Release of fluorescent aminomethylcoumarin was measured at 2 min intervals by fluorometry using the Fluostar Omega (BMG Labtech) (excitation at 360 nm and emission at 460 nm). The maximal rate of increase in fluorescence was calculated as ΔF/min.

***In vitro* E3 ligase assays and protoArrays.** To identify substrates for HACE1, a ProtoArray with ~9,000 human proteins spotted on a nitrocellulose-coated glass slide (Invitrogen) was probed with an *in vitro* ubiquitylation reaction containing 200 nM His-Uba1, 1 mM Ubc13, and 100 μg/ml HA-ubiquitin (Boston Biochem) in transport buffer (20 mM HEPES, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA pH 7.3) using different recombinant His-HACE1 concentrations (0, 50 and 100 nM). Detection of HA-ubiquitin tagged proteins was done using a fluorescence reader with anti-HA (Ha.11 Clone 16B12, Covance) and Alexa Fluor 647 conjugated rabbit anti-mouse IgG antibodies (Invitrogen). His-Uba1 and His-HACE1 (using full lengths mouse HACE1) were expressed in E. coli BL21 gold (Novagen) and Rosetta RIL (Stratagene), respectively. Proteins were purified according to standard protocols followed by size exclusion chromatography (Superdex 200, GE Healthcare) in transport buffer (20 mM HEPES, 110 mM KOAc, 2 mM Mg(OAc)2, 1 mM EGTA pH 7.3, supplemented with 1 mg/mL each of aprotinin, leupeptin, pepstatin and 1 mM DTT). All ubiquitin E2s were expressed as Thrombin-cleavable GST-fusion proteins in E. coli BL21 gold (Novagen). After binding to GST-sepharose, the E2s were cleaved with Thrombin (Novagen) and subsequently purified via size exclusion chromatography (Superdex 200, GE Healthcare). HA-ubiquitin was purchased from Boston Biochem. Ubiquitylation reactions were performed in transport buffer supplemented with 0.05% (v/v) Tween, and 0.2 mg/mL ovalbumin grade VI (Sigma) in the presence of ATP.

Cell preparation and flow cytometric analysis. Single cell suspension from thymus, spleen and lymph nodes using 70μm pore size strainers. For the isolation of colonic lamina propria cells, the colons were excised, flushed with PBS and cut longitudinally. The epithelial layer was removed by incubation of the tissue in PBS supplemented with 5mM EDTA and 1mM DTT followed by vigorous agitation. The remaining tissue was then finely minced and digested in IMDM containing 300units/ml of Collagenase IV and 200units/ml of DNaseI (both enzymes from Worthington). After digestion, cells were passed through a 70μM cell strainer. For flow cytometric analysis, cells were stained with the fixable viability dye eFluor780 (eBioscience), then incubated with Fc receptor blocking antibody and subsequently with fluorescently labeled antibodies. For intracellular stainings, cells were fixed using the FoxP3 fixation/permeabilization solution (eBioscience), incubated with antibodies diluted in Permeabilization buffer (eBioscience), washed and acquired using a BD LSR Fortessa. The following antibodies were used: anti-FoxP3 FITC (clone FJK-16s), anti-TCRβ PE-Cy5 (clone H57-597), anti-TCRγδ APC (clone GL3), anti-CD19 BV785 (clone 6D5), anti-CD4 BV711 (clone GK1.5), anti-CD8α BV605 (clone 53-6.7), anti-NK1.1 BV421 (clone PK136), anti-Gr-1 PE (clone RB6-8C5), anti-CD11b PerCP-Cy5.5 (clone M1/70), anti-CD11c APC (clone N418), anti-CD45 PE-Cy7 (clone 30-F11), anti-Ly6G BV510 (clone 1A8). All antibodies from Biolegend or eBioscience.

Induction of colitis and colon cancer. Acute colitis was induced with 2.5% (w/v) DSS (molecular mass 36-50 kDa; MP Biologicals) dissolved in drinking water ad libitum for the experimental days 0-7 followed by normal drinking water until the end of the experiment. Fresh DSS solutions were administered at day 2 and day 4. Body weight, diarrhea, and occult blood (Hemoccult) were determined daily. Diarrhea scores were: 0 = no diarrhea; 1 = mild diarrhea; 2 = severe watery diarrhea; 3 = mild diarrhea with blood; 4 = severe watery diarrhea with blood. Colon length was determined at the end of the experiments. Histopathologies were assessed on paraffin sections using H&E staining (Hashimoto et al., 2012). For induction of colon cancer, mice received a single injection of AOM at day 0 (10mg/kg; i.p.). DSS (2% w/v) was added to in the drinking water at day 5 followed by normal drinking water for the next 14 days. The DSS-water cycles were repeated 3 times. Colonoscopies were performed as previously described (Welz et al., 2011). In order to clean the bowel, mice were treated 24 hours prior to colonoscopies with the following solution: 13.5 g glucose, 2.9 g sodium citrate (trisodium citrate, dihydrate), 2.6 g sodium chloride, 1.5 potassium chloride and tap water to 1 liter.

Generation of bone marrow chimeric mice. *rag2*$^{-/-}$ mice were lethally irradiated by two sequential exposures to a radiation source (2x600 rad, with an interval of 4 hours). Irradiated mice were reconstituted by intravenous injection of freshly harvested bone marrow cells from *hace1*$^{+/+}$ or *hace1*$^{-/-}$ mice and recipient mice were subsequently treated for 5 weeks with antibiotics (Baytril, 0.2mg/ml in drinking water). Experiments were performed after 8 weeks.

Supplemental References

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