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(ABIN1 は、直鎖状ユビキチン鎖を認識すること
で NF- κ B 活性化を減衰させる刺激誘導
性オートファジーレセプターである)

新川 雄高

ABIN1 is a signal-induced autophagy receptor that attenuates NF- κ B activation by recognizing linear ubiquitin chains

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Abstract

Linear ubiquitin chains play pivotal roles in immune signaling by augmenting NF- κ B activation and suppressing programmed cell death induced by various stimuli. A20 binding inhibitor NF- κ B 1 (ABIN1) binds to linear ubiquitin chains and attenuates NF- κ B activation and cell death induction. Although interactions with linear ubiquitin chains are thought to play a role in ABIN1-mediated suppression of NF- κ B and cell death, the underlying molecular mechanisms remain unclear. Here, we show that upon stimulation by Toll-like receptor (TLR) ligands, ABIN1 is phosphorylated on Ser 83 and functions as a selective autophagy receptor. ABIN1 recognizes components of the MyD88 signaling complex via interaction with linear ubiquitin chains conjugated to components of the complex in TLR signaling, which leads to autophagic degradation of signaling proteins and attenuated NF- κ B signaling. Our current findings indicate that phosphorylation and linear ubiquitination also play a role in down-regulation of signaling via selective induction of autophagy.

Abbreviations

Linear ubiquitin chain assembly complex, LUBAC; Toll-like receptors, TLRs; A20 binding inhibitor NF- κ B 1, ABIN1; Ubiquitin binding domain in ABIN and NEMO, UBAN; TNFAIP3 interacting protein 1, TNIP1; LC3-interacting regions, LIRs; Myeloid differentiation primary response protein 88, MyD88; Interleukin-1 receptor-associated kinase-like 1/2/4, IRAK1/2/4

Introduction

The ubiquitin conjugation system was identified initially as an energy-dependent protein degradation system [1]; however, the system is now recognized as one of the most sophisticated reversible post-translational modification mechanisms [2]. Ubiquitin, a small regulatory protein, is conjugated to other proteins in the form of (mainly) polymeric chains [3]. Multiple types of ubiquitin chains are present in cells, and the type of ubiquitin chain appears to determine the manner in which it regulates the interaction between the conjugated protein and specific binding proteins [2]. Previously, we examined the function of unique linear ubiquitin chains in detail [4, 5]. Linear ubiquitin chains, which are generated specifically by the linear ubiquitin chain assembly complex (LUBAC) ubiquitin ligase, are involved in signal-induced activation of NF- κ B and suppression of programmed cell death, including apoptosis and necroptosis [6]. The ubiquitin chains exert their functions via recognition by specific binding proteins. For example, recognition of linear chains conjugated to NF- κ B essential modifier (NEMO) by another NEMO molecule plays a crucial role in activating the I κ B kinase (IKK) complex, and in subsequent activation of NF- κ B [7]. The ubiquitin binding domain of NEMO (called UBD in ABIN and NEMO (UBAN)) recognizes linear di-ubiquitin linkages (linear di-Ub) [8, 9]. Several proteins harbor the UBAN domain, and most recognize linear di-Ub with high affinity [10]. Optineurin, the causative agent of glaucoma and amyotrophic lateral sclerosis (ALS) [11], functions as a selective autophagy receptor to deliver clients to autophagosomes; it does this by recognizing linear ubiquitin chains conjugated to clients via its UBAN domain [12, 13].

A20 binding inhibitor NF- κ B1 (ABIN1), also called TNFAIP3 interacting protein 1 (TNIP1), interacts specifically with linear di-ubiquitin via its UBAN domain and

72 suppresses activation of NF- κ B [10, 14]. Moreover, genome-wide association studies
73 have identified a role for ABIN1 in systemic lupus erythematosus (SLE) and psoriasis
74 [15-17]. Some ABIN1-null mice exhibit lupus-like glomerulonephritis, as do mice
75 expressing an ABIN1 mutant defective in linear ubiquitin [14, 18]. These data indicate
76 that the linear ubiquitin binding activity of ABIN1 plays a role in the pathogenesis of SLE.
77 ABIN1 also suppresses NF- κ B activation in response to signaling via Toll-like receptors
78 (TLRs) [14]. Indeed, TLRs, including TLR7, are thought to play pathogenic roles in SLE
79 [19]. However, the mechanism underlying ABIN1-mediated suppression of TLR-induced
80 NF- κ B activation is unclear.

81 The aim of this study was to identify the precise mechanism underlying suppression of
82 NF- κ B activation upon TLR signaling. We found that phosphorylated ABIN1 functions
83 as an autophagy receptor that recognizes the ubiquitin chains on proteins within the
84 MyD88 signaling pathway, thereby marking them for autophagic degradation. The
85 subsequent reduction in the amounts of signaling molecules suppresses activation of the
86 NF- κ B signaling pathway.

87 **Materials and methods**

88 *Cell culture*

89 Mouse embryonic fibroblast (MEFs; ATG7 (+/+, -/-)) cells were a kind gift from Dr.
90 Komatsu (Juntendo University, Tokyo, Japan). ABIN1 null MEF cells, and RAW 264.7
91 cells lacking ATG5 or ABIN1 were generated using the CRISPR/Cas9 system. MEFs and
92 human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's
93 medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU ml⁻¹ penicillin, and
94 100 µg ml⁻¹ streptomycin. RAW 264.7 cells were grown in DMEM containing 10% FBS,
95 antibiotics, 55 µM 2-mercaptoethanol (2-ME, M7522; Sigma-Aldrich), and 1% non-
96 essential amino acids (MEM-NEAA, 11140-050; Gibco) at 37°C/7.5% CO₂.

97

98 *Transfection and retroviral expression*

99 Transfection of plasmids to HEK 293T or RAW 264.7 and MEFs were performed using
100 FuGENE HD (Promega) or a NEPA21 electroporator (NEPA GENE, Chiba, Japan),
101 respectively. For retroviral expression, Plat-E packaging cells were transfected with a
102 pMXs-IRES-blasticidin vector encoding ABIN1 components, as described previously [7].
103 The resultant viruses were used to infect ABIN1 KO MEFs. Stably transfected cells were
104 selected using puromycin, G-418, or blasticidin.

105

106 *Plasmids, antibodies, and reagents*

107 The following proteins were generated from the amplified ORF of mouse ABIN1:
108 UBAN-WT (aa 465–525) and UBAN-D485N (aa 465–525). Mutants of ABIN1 (D485N,
109 F84A, F137A, FF84/137AA, S83A, S83D) were generated by two-step PCR; cDNAs
110 were ligated into the appropriate epitope-tag sequences and then cloned into p3×FLAG-

neo and pMXs-myc-IRES-Bsr vectors. Next, cDNA fragments encoding mouse ABIN1-UBAN (aa 465–525) were inserted into pGEX-6p-1 between the *EcoRI* and *NotI* sites (GE Healthcare). All antibodies and reagents used in the study are listed in the supplementary file (Tables S1 and S2).

Generation of knockout cells using CRISPR/Cas9

Synthesized sgRNA oligos targeting ABIN1 (GTTTCCCGTGGTGGCGGTGG) and ATG5 (GCCTCAACCGCATCCTTGGA) were phosphorylated, annealed, and inserted into the BbsI sites of pSpCas9(BB)-2A-GFP (pX458) (#48138, Addgene) [20] and pSpCas9(BB)-2A-Puro (pX459) (#48139, Addgene) [20], and into the BsmBI sites of LentiCRISPRv2 (#52961, Addgene) [21]. Plasmids pX458 and pX459 were transfected into RAW 264.7 and MEFs, respectively, by electroporation using a NEPA21 electroporator (NEPA GENE). After 24 h, GFP-expressing RAW 264.7 cells transfected with pX458 were purified using a FACSaria III cell sorter (BD Biosciences). MEF cells transfected with pX459 were selected for 2 days by exposure to puromycin (3 µg/ml). Isolated colonies were verified as ABIN1 KO and ATG5 KO cells by immunoblotting with anti-ABIN1 and anti-ATG5 antibodies and by genomic PCR using the following primers:

ABIN1 typing_Fwd, 5'-AGGCAGAGAACCATGACCACCTTGG-3' and

ABIN1 typing_Rev, 5'-ACATGGTTTCTGAGCATCTTGGAAG-3'; and

ATG5 typing_Fwd, 5'-ATAATGAAATGTGGCATGCTGT-3' and

ATG5 typing_Rev, 5'-AGTTACCATTCTGCAGTCCCAT-3'.

To produce lentivirus, LentiCRISPRv2 encoding a sgRNA sequence targeting ABIN1 and ATG5, along with psPAX2 (#12260, Addgene) and pMD2.G (#12259, Addgene)

plasmids, were co-transfected into 293 T cells using PEIMAX (24765-1, Polysciences). After 2 days, MEFs were infected with lentivirus in the presence of polybrene (10 µg/ml, TR-1003-G; Millipore), followed by cultivation for 2 days with puromycin (3 µg/ml).

Prime editing

To construct sgRNA-expressing plasmids, complementary oligos representing the target sequences were annealed and cloned into pFYF1320 (Addgene #47511). To construct pegRNA-expressing plasmids, complementary oligos representing the target sequences, an sgRNA scaffold, and 3' extensions were annealed and cloned into the pU6-pegRNA-GG-acceptor (Addgene #132777). The oligos are listed in Table S3.

MEFs were transfected with 750 ng of the PE2-encoding plasmid (Addgene #132775), 250 ng of the pegRNA-encoding plasmid (Addgene #132777), 100 ng of the sgRNA-encoding plasmid (Addgene #47511), and 50 ng of a plasmid expressing a puromycin resistance gene. Transfected cells were then cultured for 2 days with puromycin (3 µg/ml). pCMV-PE2 was a gift from David Liu (Addgene plasmid #132775) [22]; pFYF1320 EGFP Site#1 was a gift from Keith Joung (Addgene plasmid #47511) [23]; and the pU6-pegRNA-GG-acceptor was a gift from David Liu (Addgene plasmid #132777) [22].

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Next, Dnase-treated RNA (400 ng) was reverse-transcribed to cDNA using the high-capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and an ABI ViiA7 Real-Time PCR system (Applied Biosystems). The primers are listed in Table S4. All gene expression levels were

normalized against expression of *Actb* (encoding β -actin).

Immunoprecipitation, immunoblotting, and deubiquitination analysis

Lysates were prepared by incubating cells in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), a phosphatase inhibitor cocktail (Nacalai Tesque), and a protease inhibitor cocktail (Sigma-Aldrich)) at 4°C for 20 min. Lysates were clarified by centrifugation at 15,000 rpm for 20 min at 4°C. Lysates were boiled in SDS sample buffer, separated on SDS-PAGE gels, and transferred to PVDF transfer membranes (Millipore). The membranes were blocked in 5% (w/v) skim milk in TBS/0.1% Tween-20 and then incubated overnight at 4°C with appropriate primary antibodies. A secondary antibody was used to detect proteins by enhanced chemiluminescence (Thermo Fisher Scientific). Densitometric quantification of the bands on the immunoblot was performed using Image Lab software version 5.2.1 (Bio-Rad Laboratories). Values were normalized to those of the loading controls, and then normalized for brightness to yield relative intensity values. For immunoprecipitation, cell lysates were pre-cleared for 1 h at 4°C. Next, 1 μ l of the appropriate primary antibody was added to the cleared lysate for 1 h under rotation. Next, 20 μ l rmp-Protein A-Sepharose beads (GE17-5138-01; GE Healthcare) was added to precipitate the immune complexes, followed by six washes with wash buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% Triton X-100, and two washes with TBS. Finally, the bead pellet was resuspended in 15 μ l of 2 $\times$ sample buffer and boiled at 95°C for 5 min and subjected to SDS-PAGE. For de-ubiquitination analysis, USP2cc (1 μ g) in 20 μ l buffer containing 20 mM Tris-HCl (pH 7.5) and 5 mM DTT was added to the immunoprecipitated beads or to 30 μ g cell lysate, followed by incubation at 30°C for 60 min. For phosphatase

183 treatment, cell lysates were incubated at 30°C for 60 min with 10 units of CIP in buffer
184 containing 1 mM MgCl₂ and subjected to SDS-PAGE.

185
186 *GST pull-down assay*

187 GST-UBAN fusion protein was purified as described previously [24]. Next, 10 µg
188 purified protein was immobilized onto GST beads and combined with 1.0 µg M1-/K63-
189 /K48-linked di-ubiquitin chains in 300 µl pull-down buffer (20 mM Tris-HCl, 150 mM
190 NaCl, 1 mM DTT, 0.1% Triton X-100). The proteins were then incubated on a rotating
191 platform at 4°C for 2 h. After three washes with buffer, the proteins were diluted with
192 SDS sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting.

193
194 *Tandem ubiquitin binding entity (TUBE) assay*

195 Halo-tagged linear ubiquitin chain-specific tandem ubiquitin binding entity (M1-specific
196 TUBE) was purified as described previously [25, 26]. To measure linear ubiquitination
197 in MEFs, 3 mg cell lysate plus 10 mM N-ethylmaleimide were incubated at 4°C for 3 h
198 with 2 µg M1-specific TUBE and 20 µl equilibrated Magne HaloTag beads (Promega) in
199 buffer comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100. The
200 precipitates were washed five times with 1% Triton-X100 in TBS, boiled in SDS sample
201 buffer, and analyzed by immunoblotting.

202
203 *Immunofluorescence analysis and microscopy*

204 MEFs and RAW 264.7 cells stimulated with LPS as indicated in Figure legends in the
205 presence of DMSO, Bafilomycin A1 (BafA), or E64d and pepstatin A (E64d/PepA) were
206 fixed for 15 min at room temperature in 2% formalin solution buffered with PBS. Next,

cells were washed three times with PBS, followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were subsequently blocked for 1 h at room temperature with 10% FBS in PBS/0.005% azide and then incubated overnight at 4°C with the indicated primary antibodies. Cells were then washed three times with PBS and incubated for 45 min at room temperature with secondary antibodies. After washing, cells were mounted in SlowFade Diamond Antifade Mountant with DAPI (S36964; Invitrogen) on microscopic glass supports. Confocal images were acquired under an Olympus FLUOVIEW FV1000 confocal laser scanning microscope using a Plan Apo N ×60 /1.42 objective lens (Olympus). Image processing was carried out using ImageJ software.

Mass spectrometry analysis

ABIN1 KO RAW 264.7 cells and stably expressing 3×FLAG-ABIN1 WT or D485N were subjected to mass spectrometry analyses. Three biological replicates were prepared for each condition. Each sample was prepared by culturing cells in two dishes (4.0×10^7 cells/15 cm dish). On the next day, cells were stimulated for 3 h with 100 ng/ml LPS. ABIN1 was immunoprecipitated for 2 h at 4°C with 30 µl anti-FLAG M2 magnetic beads (M8823, Sigma-Aldrich), followed by four washes with lysis buffer and two washes with TBS. Immunoprecipitated samples were eluted by incubation at 90°C for 5 min in 100 µl PTS buffer (12 mM sodium deoxycholate (SDC), 12 mM sodium *N*-lauroylsarcosinate (SLS), and 0.1 M Tris-HCl, pH 9.0) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Proteins were reduced by exposure to 10 mM DTT for 30 min and alkylated by treatment with 50 mM iodoacetamide for 30 min in the dark. The samples were diluted 5-fold with 50 mM ammonium bicarbonate (ABC). Each sample

was split into three tubes for protein digestion with Lys-C (FUJIFILM-Wako) and trypsin (Promega), chymotrypsin (Promega), or Glu-C (Promega) (0.3 μ g each). Protein digestion was performed overnight at 25°C. On the next day, SDC and SLS were separated from the digested peptides by addition of ethyl acetate, followed by acidification with 0.5% (final concentration) trifluoroacetic acid, as described elsewhere [27]. The peptides were desalted using StageTip [28] with SDB-XC Empore disk membranes (GL Sciences).

Nano-scale reversed-phase liquid chromatography coupled with tandem mass spectrometry (nanoLC/MS/MS) was performed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) connected to a Thermo Ultimate 3000 RSLCnano pump and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a self-pulled analytical column (150 mm length $\times$ 100 μ m i.d.) [29] packed with ReproSil-Pur C18-AQ materials (3 μ m; Dr. Maisch GmbH, Ammerbuch, Germany). The mobile phases comprised (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% ACN. Peptides were eluted from the analytical column at a flow rate of 500 nl/min, with the following gradient: 5–10% B for 5 min, 10–40% B for 60 min, 40–99% B for 5 min, and 99% for 5 min. The Orbitrap Fusion Lumos instrument was operated in the data-dependent mode, with a full scan in the Orbitrap followed by MS/MS scans for 3 sec using higher-energy collisional dissociation (HCD). The applied voltage for ionization was 2.4 kV. The full scans were performed at a resolution of 120,000, a target value of 4×10^5 ions, and a maximum injection time of 50 ms. The MS scan range was m/z 300–1,500. The MS/MS scans were performed at a resolution of 15,000, a target value of 5×10^4 , and a maximum injection time of 100 ms. The isolation window was set to 1.6, and the normalized HCD collision energy was 30. Dynamic exclusion was applied for 20 sec.

255

256 *Processing of mass spectrometry data*

257 All raw data files were analyzed and processed by MaxQuant (version 1.6.5.0) [30], and
258 the database search was performed with Andromeda [30], which is a peptide search
259 engine integrated into the MaxQuant environment. Searches were conducted against a
260 mouse UniProt database (version 2019-4; 54,185 protein entries) spiked with common
261 contaminants and enzyme sequences. Search parameters included two missed cleavage
262 sites and variable modifications such as methionine oxidation; protein N-terminal
263 acetylation; deamidation of glutamine and asparagine residues; and phosphorylation of
264 tyrosine, serine, and threonine residues. Cysteine carbamidomethylation was set as a
265 fixed modification. The enzyme was set as trypsin/P (cleaves after lysine, and arginine if
266 followed immediately by a proline), chymotrypsin (cleaves after tyrosine, tryptophane,
267 phenylalanine), or Glu-C (cleaves after glutamic acid) and asp-C (cleaves after aspartic
268 acid). The peptide mass tolerance was 4.5 ppm, and the MS/MS tolerance was 20 ppm.
269 The false discovery rate (FDR) was set to 1% at the peptide spectrum match (PSM) level
270 and protein level. The ‘match between runs’ function was employed. For label-free
271 protein quantification (related to Fig. 4C: heatmap), a minimum of two unique peptide
272 ions per protein group was used. Only proteins quantified in at least two out of the three
273 replicates under at least one condition were used for further analysis; missing values were
274 imputed from a normal distribution of log₂ LFQ intensity using a default setting (width,
275 0.3; down shift, 1.8) in Perseus [31]. Proteins whose abundance differed significantly
276 between conditions were identified by multiple sample ANOVA at a permutation-based
277 FDR cut-off of 0.05. Log₂ LFQ intensities were further z-transformed (only for proteins
278 showing a significant change), and hierarchical clustering was performed to analyze and

visualize the data (see Fig. 4C: heatmap). All necessary information regarding proteomic analyses, including protein and peptide lists, peak lists, and MS2 spectra, were deposited in a publicly accessible repository (jPOST) (see Data availability).

Statistical analyses

Statistical analysis of the qPCR data was conducted using paired-Student's t-tests (*p < 0.05; **p < 0.005). Data are expressed as the mean $\pm$ SD and are representative of at least three independent experiments. Statistical analysis of immunofluorescence data was conducted using a one-tailed unpaired-Student's t-test (*p < 0.01, **p < 0.001). Error bars represent the mean $\pm$ SEM.

Results

Phosphorylation-dependent delivery of ABIN1 to lysosomes via autophagy

ABIN1 inhibits activation of NF- κ B via recognition of ubiquitin chains through its UBAN domain [10]. To dissect the molecular mechanism underlying ABIN1-mediated suppression of NF- κ B, we stimulated monocytic RAW 264.7 cells and MEFs with lipopolysaccharide (LPS), a ligand for TLR4. As shown in Fig. 1A and B, slower migration of ABIN1 was observed as early as 30 min after LPS stimulation, followed by reduced expression over time. The slower migrating ABIN1 was phosphorylated, as evidenced by the finding that the band disappeared upon phosphatase (CIP) treatment (Fig. 1C). In addition, ABIN1 was degraded via lysosomes upon phosphorylation. Treatment with bafilomycin A1 (BafA; an inhibitor of lysosomes), but not with the proteasome inhibitor MG132, effectively increased the amount of slower migrating ABIN1 (Fig. 1D). Macroautophagy (autophagy) is one of the major pathways that delivers cytoplasmic proteins to lysosomes [32]. To examine involvement of autophagy in ABIN1 degradation, we knocked out an autophagy factor, ATG5, from RAW 264.7 cells using the CRISPR/Cas9 system. We found substantial accumulation of phosphorylated ABIN1 in ATG5-null RAW 264.7 cells upon LPS stimulation (Fig. 1E). This was also the case for MEFs from mice lacking ATG7, another autophagy factor (Fig. 1F). Moreover, immunofluorescence analyses revealed that ABIN1 levels increased, and that ABIN1 co-localized with LC3 (a marker for autophagosomes) in cells treated with BafA (Fig. 1G). Collectively, these results indicate that LPS triggers phosphorylation of ABIN1 and its subsequent degradation via autophagy.

Autophagy receptors often interact with ubiquitin-like modifier LC3 via LC3-interacting regions (LIRs) to deliver proteins to autophagosomes [33]. Two amino acid

314 sequences resembling LIRs were found in ABIN1 (Fig. 2A). To validate whether these
315 LIR-like motifs interact with LC3s, we introduced mutations at Phe84 or Phe137 (both
316 of which are critical N-terminal and C-terminal LIR-like motif residues, respectively) to
317 Ala (F84A and F137A, respectively). The finding that ABIN1 F84A, but not F137A,
318 abolished interaction with LC3a (Fig. 2B) suggests that N-terminal LIR interacts with
319 LC3. Treatment with E64d and pepstatin A (E64d/PepA), both lysosomal protease
320 inhibitors, did not overtly increase the amount of ABIN1 F84A, and immunofluorescence
321 analyses revealed that expression of ABIN1 F84A did not overlap with the lysosomal
322 protein Lamp1 upon LPS stimulation (Fig. 2C and D). Since phosphorylation of ABIN1
323 triggered autophagic degradation of the protein (Fig. 1), we tried to identify the ABIN1
324 residues in LPS-stimulated RAW 264.7 cells that were phosphorylated. To do this, we
325 immunopurified FLAG-tagged ABIN1 from RAW 264.7 cells lacking ABIN1 (RAW
326 264.7 ABIN1 KO cells) expressing 3×FLAG-tagged ABIN1. These cells were cultivated
327 in the presence or absence of LPS for 3 h (Fig. 3A), and anti-FLAG-immunoprecipitates
328 were subjected to mass spectrometry to identify phosphorylated residues (Fig. 3B and
329 S1A). As illustrated in Fig. 3C, phosphorylation of several residues including those
330 located adjacent to the functional LIR of ABIN1 (aa. 84–87) were induced upon LPS
331 stimulation (see Fig. S1 for the MS/MS spectra of the corresponding phosphopeptides).
332 Among these phosphorylation of Ser73, Ser83, Ser118, Ser182, Ser279, Ser441, and
333 Ser448 were confirmed to upregulated by LPS (i.e., we observed their phosphorylation in
334 the majority of samples). Since phosphorylation of the Ser residue located at the -1
335 position N-terminal to the LIR increases the affinity of some autophagy receptors for LC3
336 [13, 34], we focused on Ser83, which is conserved during evolution (Fig. 2A). We found
337 that phosphorylation of Ser83 was augmented upon LPS stimulation (Fig. S1B and C).

Substitution of Ser83 with Ala (S83A) rendered ABIN1 unable to bind to LC3a, whereas ABIN1 harboring phosphor-mimetic substitution of Ser83 with Asp (S83D) interacted with LC3a efficiently (Fig. 3D). This was supported by results showing that E64d/PepA did not overtly increase the amount of ABIN1 S83A in cells stimulated with LPS (Fig. 3E), whereas treatment with E64d/PepA did increase the amount of ABIN1 S83D in the absence of LPS stimulation more potently than WT ABIN1 (Fig. 3F). Moreover, immunofluorescence analyses revealed that ABIN1 S83D co-localized with the lysosomal protein, Lamp1, in the absence of LPS stimulation (Fig. 3G), whereas ABIN1 S83A did not overtly colocalize with Lamp1 in LPS-treated MEFs (Fig. 3H). These results indicate that ABIN1 functions as an autophagy receptor upon phosphorylation.

ABIN1 delivers MyD88 signaling pathway components to the autophagic pathway in the linear ubiquitination-dependent manner

The UBAN domain of ABIN1 recognizes linear ubiquitin chains, for which Asp485 within ABIN1 UBAN is critical [14, 24]. We confirmed that ABIN1 WT, but not an ABIN1 mutant in which Asp485 was substituted for Asn (D485N), recognizes linear di-ubiquitin specifically (Fig. 4A). We did this by introducing FLAG-tagged ABIN1 WT or D485N into RAW 264.7 ABIN1 KO cells to identify proteins that interacted with the ABIN1 UBAN domain. Then, proteins associated with ABIN1 WT or ABIN1 D485N were immunopurified using anti-FLAG magnetic beads after cultivation for 3 h in the presence or absence of LPS (this is because LPS induces linear ubiquitination) (Fig. 4B). Mass spectrometry analyses revealed that MyD88 and IRAK1, both of which are components of the MyD88 activation complex (MyDDosome) [35], were associated specifically with ABIN1 WT isolated from LPS-treated RAW 264.7 cells (Fig. 4C). The

amount of ABIN1 D485N mutant in LPS-stimulated cells was comparable to that of ABIN1 WT (Fig. 4D).

Since ABIN1 in MEF cells stimulated with Pam3CSK4 (a ligand for TLR1 and TLR2) was phosphorylated and degraded (Fig. 5A) [36], we examined ABIN1 KO MEFs reconstituted with ABIN1 WT or D485N (Fig. 5B). Upon stimulation with Pam3CSK4, the amount of MyD88 and IRAK1 that was associated with ABIN1 WT appeared to be very small (Fig. 5C). We realized that the apparent amount of a protein can be underestimated when the protein has undergone heavy post-translational modifications; this is because heavy post-translational modifications affect the mobility of proteins in SDS-PAGE gels. Indeed, treatment with USP2cc, which cleaves virtually all ubiquitin chains, and phosphatase revealed that larger amounts of MyD88 and IRAK1 were associated with ABIN1 WT than with D485N (Fig. 5C). Both K63 and linear ubiquitin chains are generated upon stimulation with TLR ligands [37]. Therefore, to examine whether components of the MyDDosome interact with ABIN1 in a linear ubiquitination-dependent manner, cells were treated with HOIPIN-8, a specific inhibitor of LUBAC-mediated linear ubiquitination [38], prior to stimulation with Pam3CSK4. HOIPIN-8 effectively suppressed linear ubiquitination and abolished binding of ABIN1 to IRAK1 and MyD88 in a dose-dependent manner (Fig. 5D and E), which clearly indicated that ABIN1 interacted with components of the MyDDosome in Pam3CSK4 stimulated MEFs in a linear ubiquitination-dependent manner. Since ABIN1 is a signal-dependent autophagy receptor (Fig. 1), we next examined the roles of ABIN1 in regulating components of the MyDDosome upon Pam3CSK4 stimulation. The amount of IRAK1 fell upon stimulation with Pam3CSK4. Deletion of ABIN1 reversed this, whereas loss of ATG7 further increased the amount of IRAK1 (Fig. 5F). These results clearly indicate

that proteins comprising the MyDDosome are destined for degradation via ABIN1-dependent selective autophagy upon Pam3CSK4 stimulation, and that this process occurs in a linear ubiquitination-dependent manner although involvement of other autophagy receptor cannot be ruled out.

Involvement of ABIN1-mediated autophagy in suppression of signal-induced NF- κ B activation

ABIN1 inhibits NF- κ B activation induced by various stimuli [10, 14], and we confirmed herein that loss of ABIN1 augments Pam3CSK4-mediated phosphorylation and degradation of I κ B α , both hallmarks of NF- κ B activation (Fig. 6A). We also found that loss of ABIN1 enhanced Pam3CSK4-mediated transcription of NF- κ B target genes (Fig. 6B). To further examine the involvement of ABIN1-mediated autophagy in suppression of signal-induced NF- κ B activation, we stimulated ABIN1-null MEFs reconstituted with mutant or WT ABIN1 with Pam3CSK4. ABIN1 WT suppressed Pam3CSK4-mediated induction of an NF- κ B target gene, *ccl5*, in ABIN1 null MEFs, whereas ABIN1 mutants defective in autophagy (F84A) or linear ubiquitin recognition (D485N) failed to suppress *ccl5* expression (Fig. 6C); this implies that linear ubiquitination-dependent autophagy underlies ABIN1-mediated NF- κ B suppression. It was noteworthy that the amount of ABIN1 introduced by the retroviral expression system was much higher than that of endogenous ABIN1 (Fig. 6D). To probe the involvement of ABIN1-mediated autophagy in a physiological setting, we used the CRISPR-mediated base-editing system to generate MEF clones in which endogenous ABIN1 is replaced by the autophagy-defective F84A mutant (Fig. 6E) [22]. We found that the Pam3CSK4-induced decrease in ABIN1 was reversed by the F84A mutation (Fig. 6F). The F84A mutation in ABIN1 also increased,

and then maintained, the amount of mRNA encoding NF- κ B target genes, including *ccl5* and *mcp1* (Fig. 6G). Collectively, these results strongly indicate that linear ubiquitination-mediated autophagy is involved in ABIN1-mediated suppression of signal-induced NF- κ B activation.

Suppression of TNF- α -mediated NF- κ B activation and cell death by ABIN1-mediated autophagy

ABIN1 also suppresses TNF- α -mediated NF- κ B activation [39, 40]. As shown in Fig. 7A, ABIN1 was phosphorylated and expressed in lower amounts in MEFs stimulated with TNF- α (as observed in cells stimulated with Pam3CSK4) (Fig. 5A); this reduction in ABIN1 expression was substantially reversed in autophagy-defective ATG7 KO cells (Fig. 7A), indicating that ABIN1 is phosphorylated and degraded via autophagy upon TNF- α stimulation. We then examined the role of ABIN1 in TNF- α -mediated NF- κ B activation. We found that loss of ABIN1 augmented TNF- α -mediated phosphorylation and degradation of I κ B α (Fig. 7B). More importantly, the amount of phosphorylated I κ B α increased again 30 min after stimulation, suggesting sustained TNF- α stimulation (Fig. 7B). Expression of transcripts encoding NF- κ B target genes was also augmented by deletion of ABIN1 from MEFs (Fig. 7C). To further validate the role of ABIN1-mediated autophagy in TNF- α -signaling, we stimulated ABIN1 F84A MEFs with TNF- α . The reduction in ABIN1 F84A was less than that of ABIN1 WT upon TNF- α stimulation, confirming that ABIN1 is subjected to autophagy upon stimulation with TNF- α (Fig. 7D). As shown in Fig. 7E and F, TNF- α -mediated phosphorylation and degradation of I κ B α , as well as expression of transcripts of NF- κ B target genes, were augmented and sustained in ABIN1 F84A MEFs, suggesting involvement of ABIN1-mediated autophagy in

434 suppressing NF- κ B activation by various stimuli. A previous study showed that loss of
435 ABIN1 renders cells hypersensitive to TNF- α -induced cell death [40]. Since activation
436 of NF- κ B suppresses apoptosis, we treated cells with TNF- α and cycloheximide to avoid
437 the effects of NF- κ B activation, and then examined involvement of ABIN1-mediated
438 autophagy in TNF- α -induced cell death. We found that replacing ABIN1 WT with
439 autophagy-defective F84A augmented cleavage of caspase 3, a hallmark of apoptosis (Fig.
440 7G), which indicates that ABIN1 suppresses programmed cell death by functioning as an
441 autophagy receptor for proteins marked by linear ubiquitin chains, and that this process
442 is phosphorylation-dependent.

Discussion

ABIN1 suppresses NF- κ B activation and programmed cell death induced by various stimuli [14, 40]. In this study, we show that phosphorylated ABIN1 reduced the amounts of components comprising the MyDDosome, including IRAK1, by recognizing linear ubiquitin chains conjugated to these MyDDosome components via its UBAN domain (Fig. 5F). Although ABIN1 is shown to be degraded via autophagy [41, 42], the mechanism underlying autophagic degradation of ABIN1 has not been dissected. We found that ABIN1 functions as a selective autophagy receptor upon phosphorylation of Ser83, which is located N-terminal to the LIR (aa 84–87) (Fig. 2) [33]. Studies show that phosphorylation of the serine residue located at the -1 position N-terminal to the LIR of several autophagy receptors, such as BNIP3, NIX, and Optineurin, augments interaction with LC3 family proteins [18, 34]. Thus, ABIN1 is another member of the phosphorylation-dependent selective autophagy receptor family. However, ABIN1 appears distinct from other receptors because it delivers signaling molecules to lysosomes, whereas most other phosphorylation-dependent autophagy receptors are involved in autophagy of mitochondria (mitophagy) or invading pathogens (xenophagy) [18]. Both phosphorylation and ubiquitination, along with formation of large protein complexes, play crucial roles in transmitting signals [43]. Binding of TLRs to ligands induces formation of the MyDDosome, in which several kinases (including IRAK1 and IRAK4) play crucial roles [35]. The TRAF6 ubiquitin ligase is recruited to the MyDDosome via interaction with IRAK1 and IRAK2; this generates K63 chains, which triggers recruitment of LUBAC. LUBAC conjugates linear ubiquitin chains to the components of the MyDDosome (such as IRAK1), which leads to activation of NF- κ B [44]. Thus, TLR signaling is transmitted by the coordinate functions of phosphorylation

and ubiquitination. Our current findings indicate that phosphorylation and ubiquitination are also involved in down-regulating signaling via selective autophagy. It is well established that selective autophagy receptors recognize cargos via ubiquitin chains [33]. Since ABIN1 functions as an autophagy receptor upon phosphorylation, it seems reasonable that it down-regulates MyD88 signaling by inducing autophagic degradation of the MyDDosome via recognition of linear ubiquitin chains. We also found that ABIN1 inhibits NF- κ B activation induced by TNF- α . Although not shown, it seems plausible that ABIN1 reduces the amount of signaling molecules involved in TNF- α signaling by recognizing linear ubiquitin chains; indeed, receptors such as receptor-interacting protein kinase 1 (RIPK1) are linearly ubiquitinated.

With respect to TNF- α -signaling, ABIN1 also restricts TNF- α -induced programmed cell death, as well as suppressing NF- κ B activation [40]. Because we found that TNF-induced cell death was enhanced in ABIN1 F84A MEF cells, the autophagy receptor function of ABIN1 is requisite for suppression of cell death (Fig. 5F). Oshima et al. reported that the amount of complex-II, which is a complex comprising RIPK1, Fas-associated death domain protein (FADD), and caspase 8, was significantly greater in ABIN1-deficient cells [40]. Thus, ABIN1 might suppress cell death by delivering complex-II to autophagy via recognition of linear chains on FADD, which is linearly ubiquitinated [45]. However, further analyses are needed to clarify the precise mechanism underlying ABIN1-mediated suppression of TNF- α -induced cell death.

Optineurin is an autophagy receptor with a UBAN domain [13]. Moreover, phosphorylation at the -1 position N-terminal to the LIR augments interaction with LC3 family modifiers. Thus, both Optineurin and ABIN1 function as a phosphorylation-induced autophagy receptor that delivers linear ubiquitinated cargo proteins for

autophagy [13]. Indeed, Optineurin interacts with linear ubiquitin and suppresses TNF- α -induced NF- κ B activation [46, 47]. However, Optineurin also delivers Salmonella to xenophagy in a linear ubiquitination-dependent manner [13], and NF- κ B activation in mice lacking Optineurin is not overtly perturbed [48]. Thus, it is of interest to know whether ABIN1 and Optineurin coordinately suppress NF- κ B activation or display distinct physiological functions, even though both are phosphorylation- and linear ubiquitination-dependent autophagy receptors.

Interestingly, genome-wide association studies identified *ABIN1* as a susceptibility gene for SLE or psoriasis [16, 17]. The physiological functions of ABIN1 have been probed using several mouse models. The results show that ABIN1-null mice exhibit either embryonic lethality caused by augmented cell death or have lupus-like glomerulonephritis [18, 40]. Moreover, mice expressing an ABIN1 mutant defective for linear ubiquitin binding (ABIN1 D485N) also exhibit lupus-like glomerulonephritis [14], which indicates a role for linear ubiquitin binding proteins in protecting mice from lupus nephritis.

Mounting evidence suggests involvement of TLR7 in initiating and progressing SLE [49, 50]. In particular, TLR7-mediated activation of various transcription factors, including interferon regulatory factors (IRF)3, IRF5, and IRF7, in plasmacytoid dendritic cells stimulated by single-stranded RNA is thought to play a crucial role in disease pathogenesis [51]. SLE-like phenotypes of ABIN1(D485N) knock-in mice were rescued by crossing with MyD88 KO mice, or with knock-in mice expressing catalytically inactive mutants of IRAK1 or IRAK4. Our finding that ABIN1 attenuates signaling elicited by the TLR-MyD88 pathway supports the notion that ABIN1-mediated attenuation of TLR signaling plays crucial roles in preventing SLE [52].

516 In summary, we show here that ABIN1 is a novel selective autophagy receptor that
517 suppresses TLR-MyD88 signaling by mediating the degradation of the MyDDosome
518 complex, which might offer a new perspective on the pathogenesis of SLE.

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Author contributions

Y. S. and K. Im: data acquisition; Y. F., K. S., and Y. I: analysis and interpretation of data; K. O., A. M., and K. Iw: conceived and designed the project; Y. S., K. Im., and K. Iw: drafted the article.

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Data accessibility

The data that supports the findings of this study are available in the supplementary material of this article. In addition, the supporting data are available from the corresponding author (kiwai@mcp.med.kyoto-u.ac.jp) upon reasonable request.

The raw MS data and analysis files have been deposited with the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the jPOST partner repository (<https://jpostdb.org>) [53], with the data set identifier PXD024864/JPST001110.

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Figure legends

Figure 1. Phosphorylation-dependent delivery of ABIN1 to lysosomes via autophagy.

(A) RAW 264.7 or (B) MEF cells were stimulated with LPS (100 ng/ml or 10 µg/ml, respectively) for the indicated times, and lysates were subjected to immunoblotting as indicated. (C) RAW 264.7 cells were stimulated for 60 min with 100 ng/ml LPS, and lysates were dephosphorylated with CIP prior to immunoblotting as indicated. (D) RAW 264.7 cells were treated for 2 h with 100 ng/ml LPS together with 100 nM Bafilomycin A1 (BafA) or 5 µM MG132, followed by immunoblotting as indicated. (E) wild-type (WT) or ATG5KO RAW 264.7 cells were stimulated with 100 ng/ml LPS. (F) WT and ATG7KO MEFs were stimulated with 10 µg/ml LPS. Lysates were immunoblotted as indicated (E, F). (G) ABIN1 KO MEFs stably expressing myc-ABIN1 were stimulated for 2 h with 10 µg/ml LPS with or without 100 nM BafA, followed by staining with an antibody specific for ABIN1 (green) or LC3 (magenta), and quantification of the number of ABIN1(green) and LC3 (magenta) positive speckles per cell. Bars, 20 µm. Error bars indicate the SEM. **p < 0.001, one-tailed, unpaired t test.

Figure 2. Amino acids 84–87 form the LC3-interacting region (LIR) of ABIN1.

(A) Alignment of the region containing the ABIN1 LIR across species. Two LIR-like motifs are highlighted in yellow. (B) Immunoprecipitation of ABIN1 mutants. Anti-HA-immunoprecipitates from lysates of HEK 293T cells expressing HA-LC3a together with 3×FLAG tagged ABIN1 WT or mutants harboring mutations in ABIN1 LIR-like regions, and lysates were immunoblotted as indicated. (C) ABIN1KO MEF cells stably expressing myc-ABIN1 WT or the F84A mutant were pretreated with 10 ng/ml E64d/PepA (E/P) for 1 h and then stimulated for 2 h with LPS. (D) ABIN1 KO MEF cells stably expressing

myc-ABIN1 WT or the F84A mutant were stimulated for 2 h with 10 µg/ml LPS and 10 ng/ml E64d/PepA, followed by staining with an antibody specific for ABIN1 (green) or Lamp1 (magenta), and quantification of the number of ABIN1 WT or F84A (green) and Lamp1 (magenta) positive speckles per cell. Bars, 20 µm. Error bars indicate the SEM. *p < 0.01, one-tailed, unpaired t test.

Figure 3. Phosphorylation of residues N-terminal to LIR augments ABIN1 autophagy.

(A) Schematic description of the MS strategy used to detect phosphorylation sites in ABIN1. (B) An MS spectrum of phospho-Ser83 ABIN1 in LPS-stimulated cells (see also Fig. S1B and C showing that phosphorylation of Ser83 in ABIN1 was induced by LPS treatment). (C) Schematic representation of the architecture of the ABIN1 domain in the identified phosphorylation sites. (D) Phosphorylation of Ser 83 augments interaction between ABIN1 and LC3. Anti-HA-immunoprecipitates of lysates from HEK 293T cells co-expressing 3×FLAG tagged ABIN1 WT, or the indicated mutants and HA-LC3a, were immunoblotted as indicated. (E) ABIN1 KO MEF cells stably expressing myc-ABIN1 WT or S83A mutant were stimulated for 3 h with LPS after pretreatment with or without 10 ng/ml E64d/PepA for 1 h and quantitation of the amount of myc-ABIN1 WT or S83A. (F) ABIN1 KO MEFs stably expressing myc-ABIN1 WT or mutant S83D were treated with or without 10 ng/ml E64d/PepA for 4 h. Lysates were immunoblotted as indicated and quantitation of the amount of myc-ABIN1 WT or S83D. (G) ABIN1 KO MEF cells stably expressing myc-ABIN1 WT or S83D were treated with 10 ng/ml E64d/PepA, for 4 h and quantification of the number of ABIN1 WT or S83D (green) and Lamp1 (magenta) positive speckles per cell. (H) ABIN1 KO MEF cells stably expressing myc-ABIN1 WT

or S83A were pretreated with 10 ng/ml E64d/PepA for 1 h and then stimulated with 10 μ g/ml LPS for 3 h, followed by quantification of the number of ABIN1WT or S83A (green) and Lamp1 (magenta) positive speckles per cell. Bars, 20 μ m Error bars indicate the SEM. * $p < 0.01$, one-tailed, unpaired t test. (G, H).

Figure 4. Identification of proteins that interact with ABIN1 in a linear ubiquitination-dependent manner.

(A) GST-pulldown assay. Purified linear Lys63-linked or Lys48-linked di-ubiquitin (di-Ub) was incubated with GST, GST-ABIN1 UBAN-WT, or D485N mutant and then pulled down with glutathione beads, followed by immunoblotting as indicated. (B) Anti-ABIN1 immunoprecipitates from WT or ABIN1 KO RAW 264.7 cells stimulated with or without 100 ng/ml LPS for 3 h were immunoblotted as indicated. (C) Heatmap showing differential expression of proteins interacting with ABIN1 WT, KO, or D485N mutant in the presence or absence of LPS. Proteins whose abundance differed significantly between conditions were identified by multiple sample ANOVA tests at a permutation-based FDR cut-off of 0.05. (D) ABIN1 KO RAW 264.7 cells stably expressing 3 $\times$ FLAG-ABIN1 WT or D485N mutant were stimulated with or without 100 ng/ml LPS for 3 h, followed by immunoblotting as indicated.

Figure 5. ABIN1 delivers components of the MyD88 signaling pathway for autophagy in a linear ubiquitination-dependent manner.

(A) WT and ATG7 KO MEFs were stimulated with 100 ng/ml Pam3CSK4 for the indicated times and then immunoblotted as shown. (B) ABIN1 KO MEFs stably

expressing myc-ABIN1 WT or D485N were stimulated for 30 min with 100 ng/ml Pam3CSK4 and then immunoblotted as indicated. (C) Anti-ABIN1-immunoprecipitates from ABIN1 KO MEFs or cells stably expressing myc-ABIN1 WT or the D485N mutant treated with or without 100 ng/ml Pam3CSK4 for 30 min, followed by deubiquitination/dephosphorylation by USP2cc/CIP and immunoblotting as indicated. (D) ABIN1 KO MEF cells stably expressing myc-ABIN1 were treated for 30 min with or without 100 ng/ml Pam3CSK4 after pretreatment with 0, 10, 30 μ M HOIPIN-8 for 30 min. Cell lysates were pulled down with M1-TUBE and immunoblotted as indicated. (E) Anti-ABIN1 immunoprecipitates from ABIN1 KO MEFs or those stably expressing myc-ABIN1 were treated for 30 min with or without 100 ng/ml Pam3CSK4 after pretreatment with 0, 10, 30 μ M HOIPIN-8 for 30 min and then deubiquitinated/dephosphorylated by USP2cc/CIP. Finally, lysates were immunoblotted as indicated. (F) WT, ABIN1KO or ATG7 KO MEF cells were stimulated with 100 ng/ml Pam3CSK4 for the indicated times. Lysates were treated with USP2cc and CIP, followed by immunoblotting as indicated.

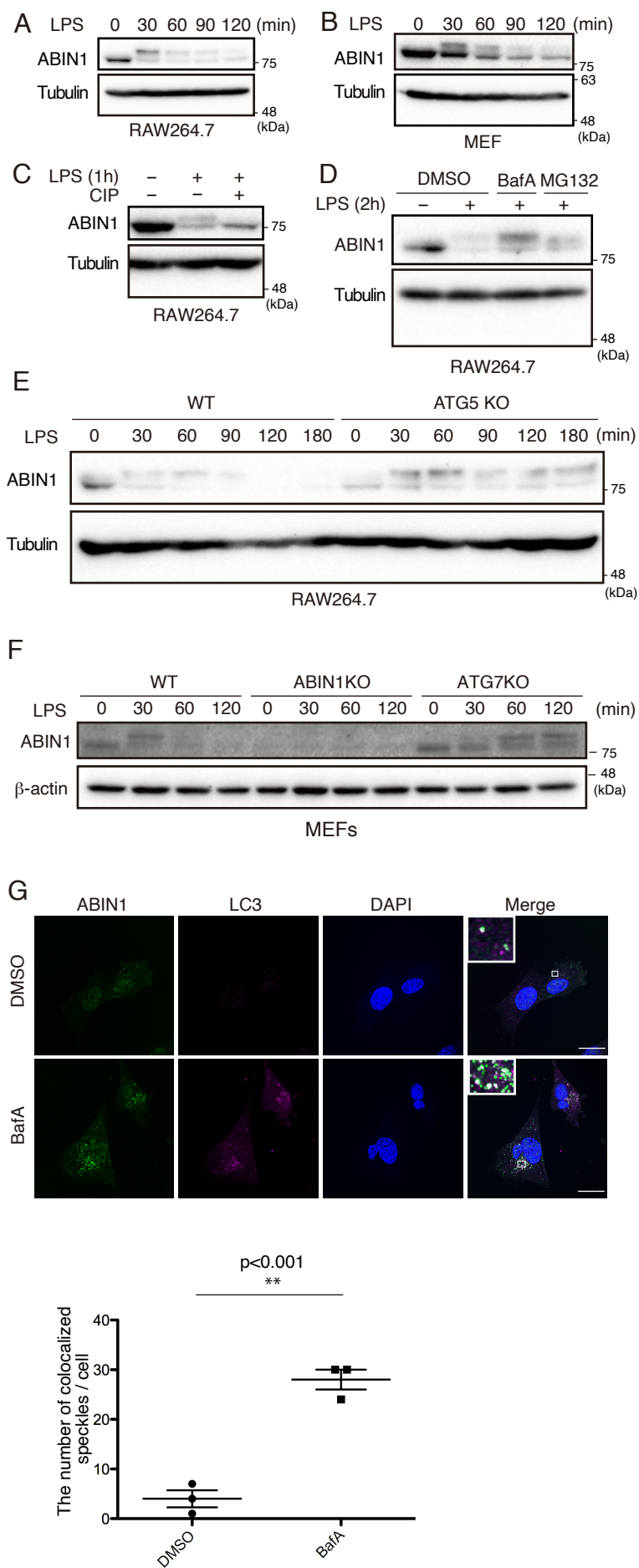
Figure 6. Involvement of ABIN1-mediated autophagy in suppression of signal-induced NF- κ B activation.

(A) WT or ABIN1 KO MEFs were stimulated with Pam3CSK4 for the indicated periods, followed by immunoblotting as indicated. (B) Quantitative PCR analyses of the indicated genes in WT or ABIN1 KO MEFs generated by the Lenti-CRISPR system were stimulated with 100 ng/ml Pam3CSK4 for the indicated times. The results are representative of three independent experiments. (C) Quantitative PCR analysis of *cc15* expression in WT and ABIN1 KO MEF cells, or in ABIN1 KO MEFs stably expressing

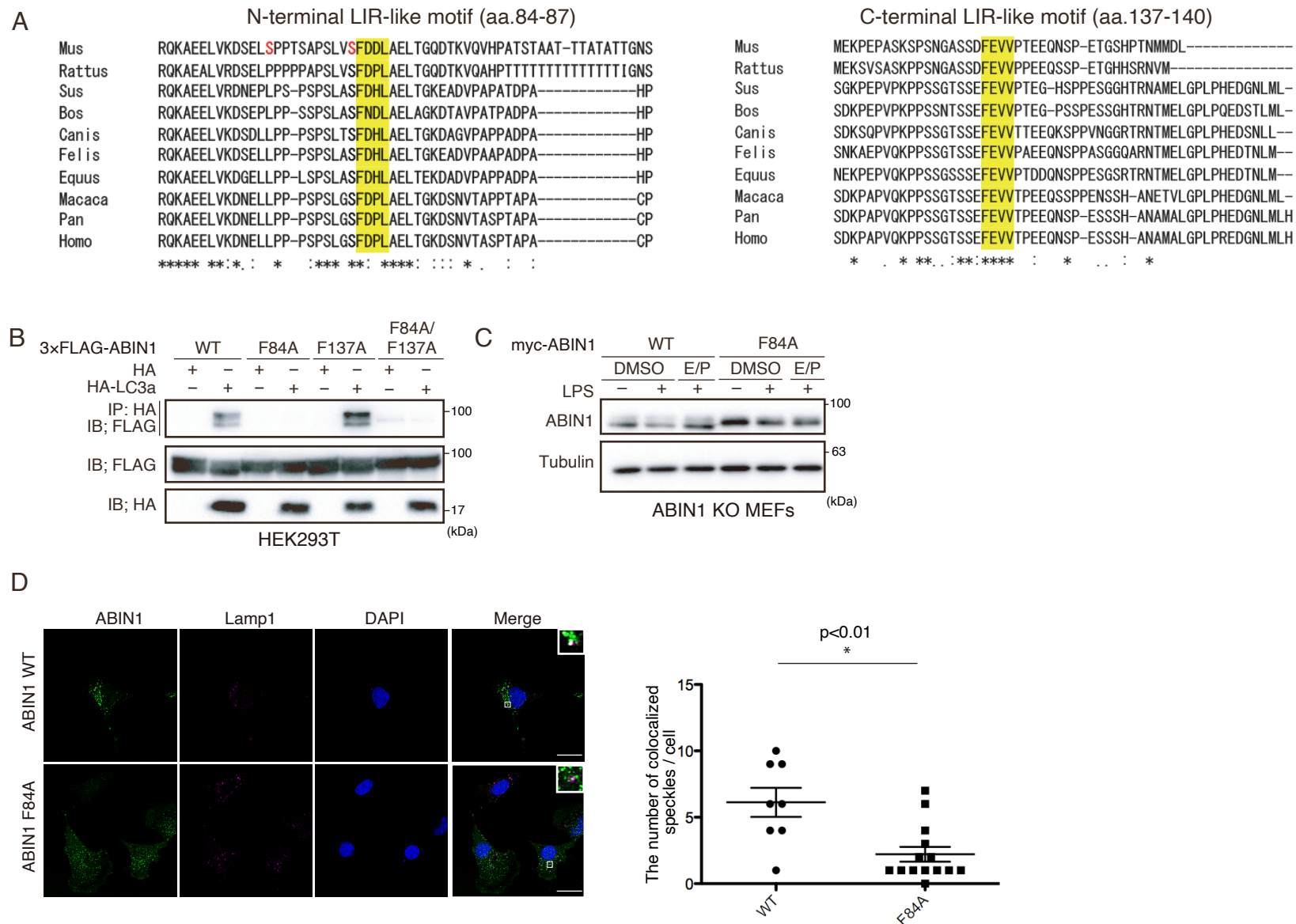
myc-ABIN1 WT, F84A, or D485N, stimulated with 100 ng/ml Pam3CSK4 for the indicated times. (D) WT or ABIN1 KO MEFs stably expressing wild-type myc-ABIN1 were stimulated for 2 h with 10 µg/ml LPS with or without 100 nM BafA, followed by immunoblotting as indicated. (E) Sanger sequencing of MEF cells expressing ABIN1 WT or the F84A mutant endogenously. (F) WT or ABIN1 F84A MEFs were stimulated with 100 ng/ml Pam3CSK4, followed by immunoblotting as indicated. (G) Quantitative PCR analyses of the indicated genes in WT or ABIN1 F84A MEFs stimulated with 100 ng/ml Pam3CSK4 for the indicated times. (B, C, G) The results are representative of three independent experiments and are expressed as the mean and SD. * $p < 0.05$; ** $p < 0.005$.

Figure 7. ABIN1-mediated autophagy suppresses TNF- α -mediated NF- κ B activation and cell death.

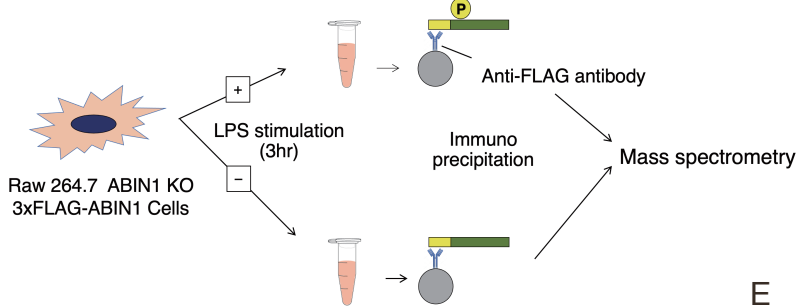
(A) WT and ATG7 KO MEF cells were stimulated with 10 ng/ml TNF- α for the indicated times, followed by immunoblotting as depicted. (B) WT or ABIN1KO MEF cells were stimulated with 10 ng/ml TNF- α for the indicated times, followed by immunoblotting as depicted. (C) Quantitative PCR analyses of the indicated genes in WT or ABIN1 KO MEF cells stimulated with 10 ng/ml TNF- α for the indicated times. (D, E) WT or ABIN1 F84A MEF cells were stimulated with 10 ng/ml TNF- α for the indicated times, followed by immunoblotting as depicted. (F) Quantitative PCR analyses of the indicated genes in WT or ABIN1 F84A MEFs stimulated with 10 ng/ml TNF- α for the indicated times. (C, F) The results are representative of three independent experiments and expressed as the mean and SD. * $p < 0.05$. (G) WT or ABIN1 F84A MEFs were treated with 20 ng/ml CHX and 0, 5, or 10 ng/ml TNF- α prior to immunoblotting of cell lysates as indicated.



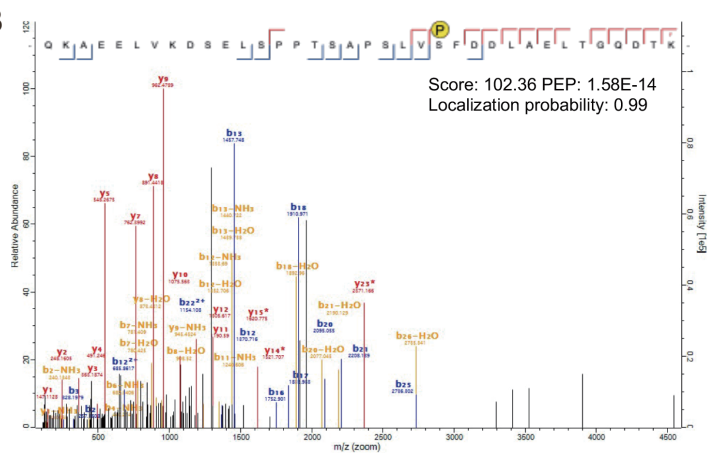
Shinkawa et al. Figure1



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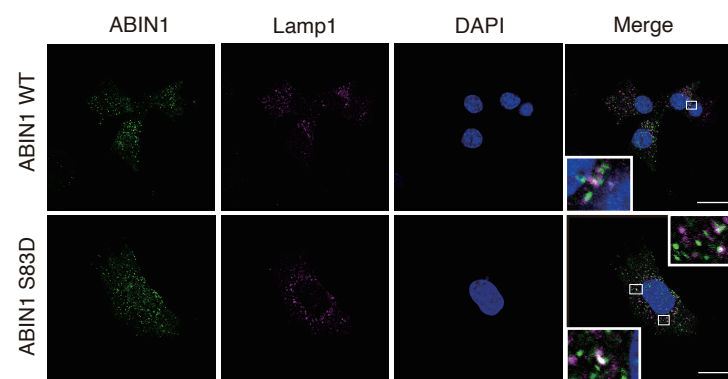
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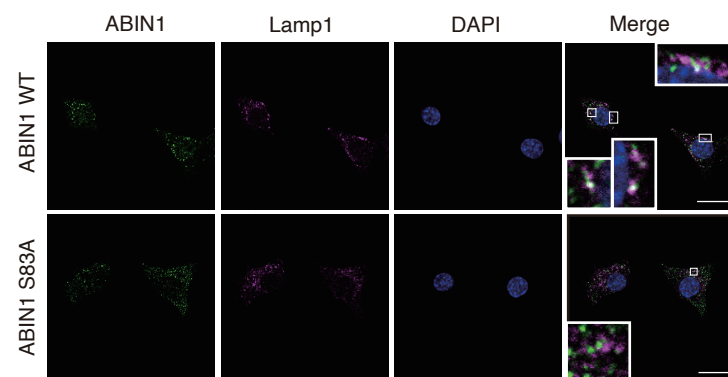
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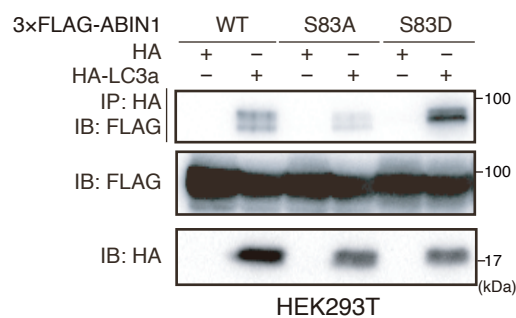
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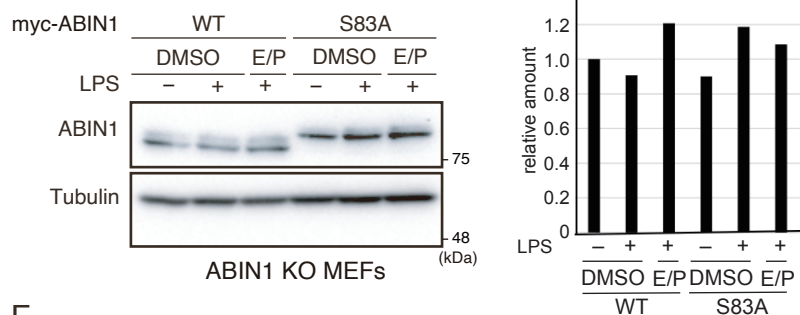
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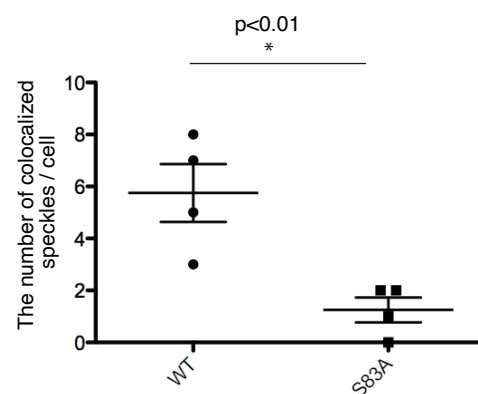
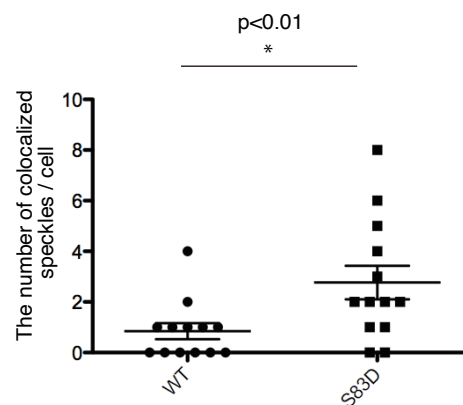
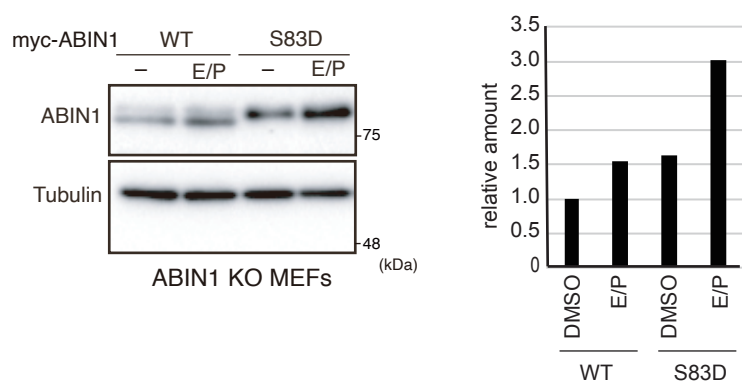
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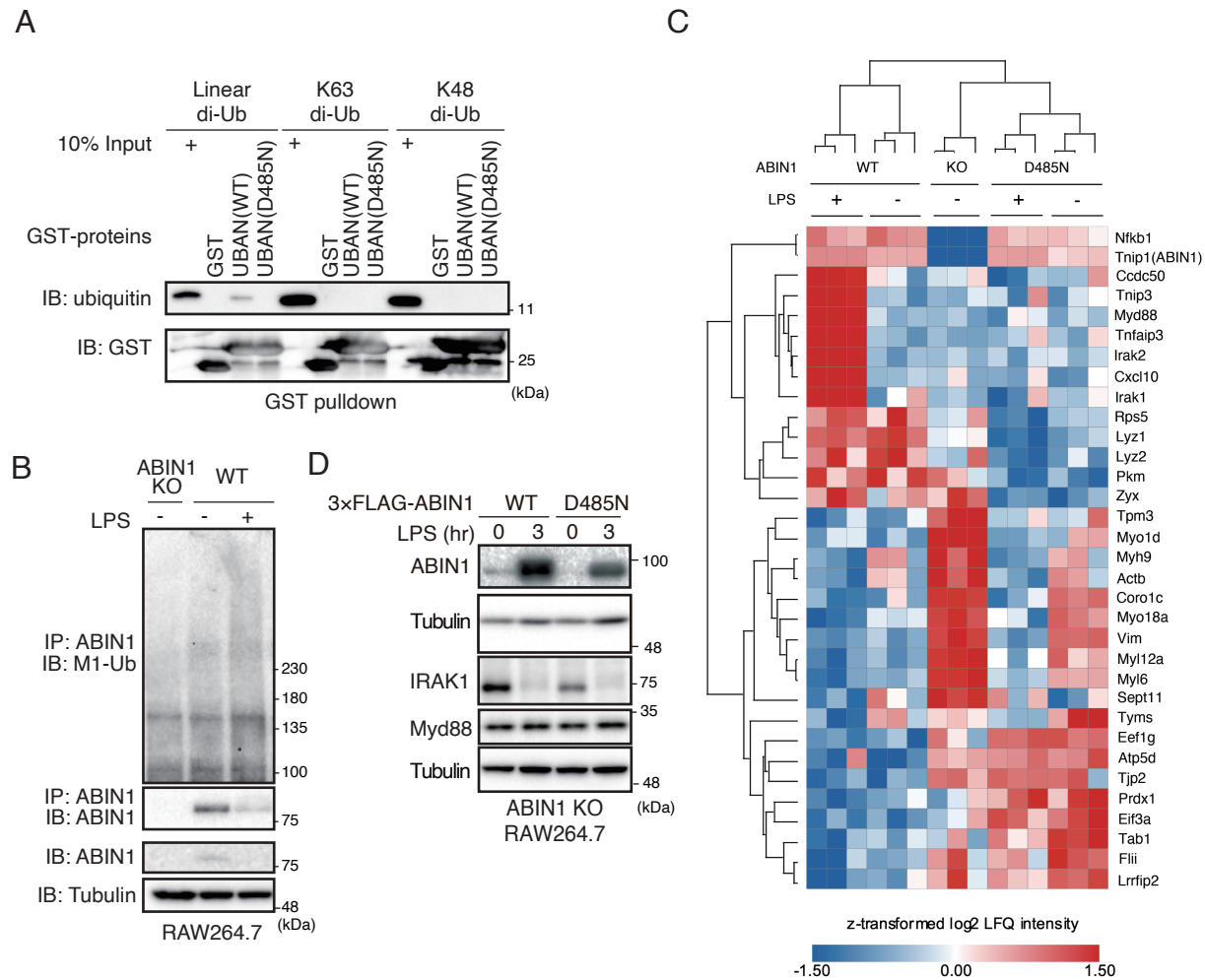


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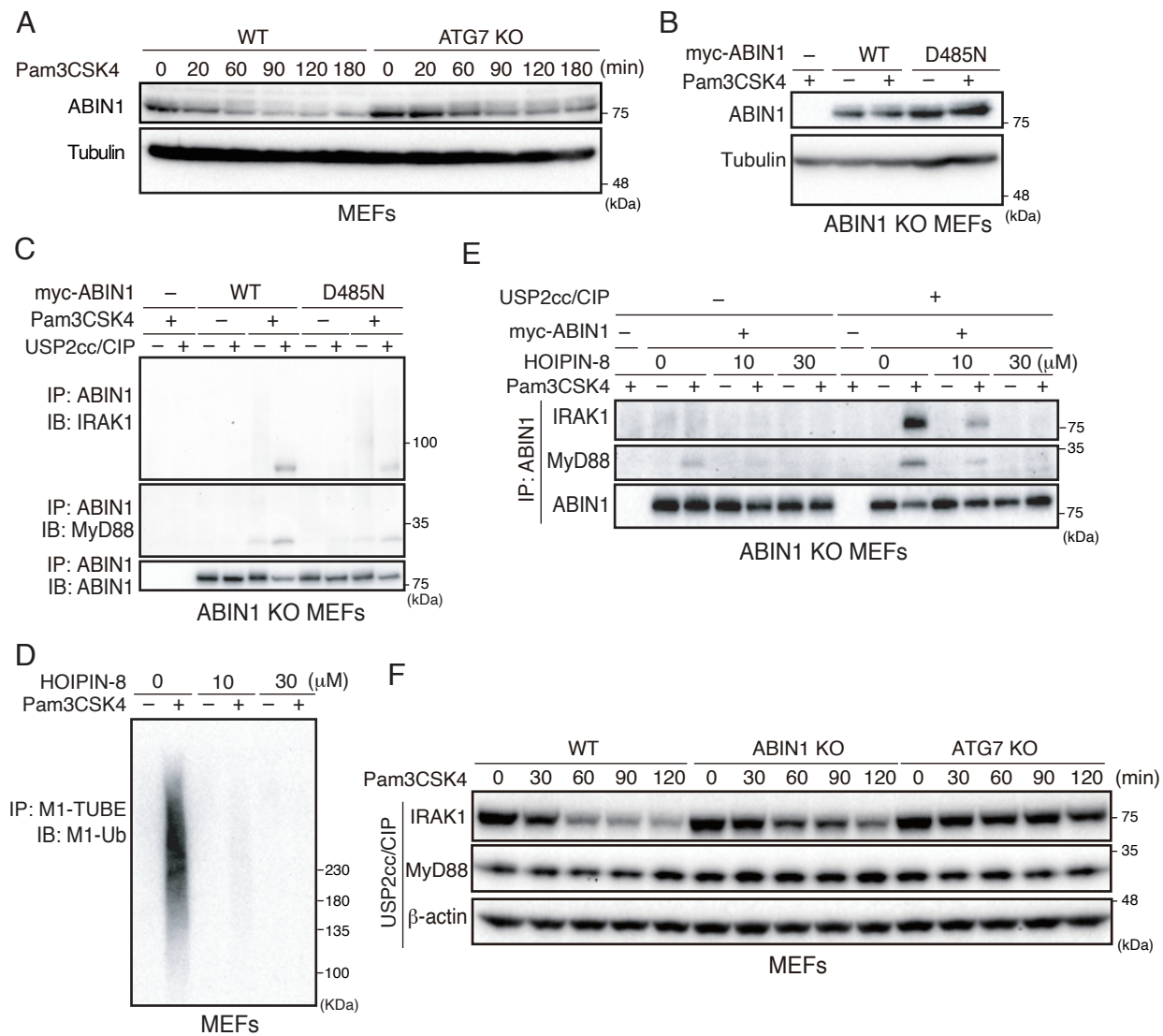


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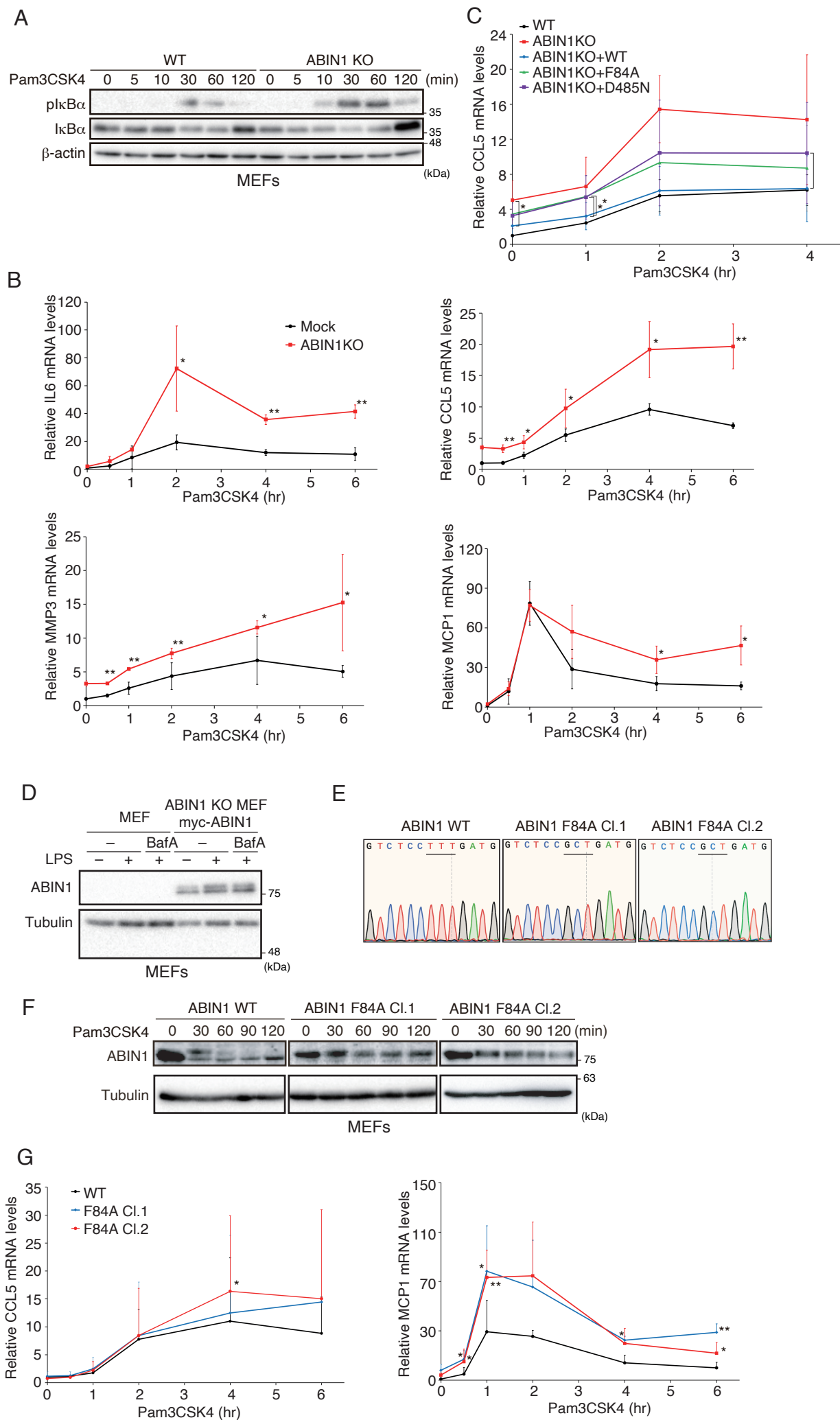




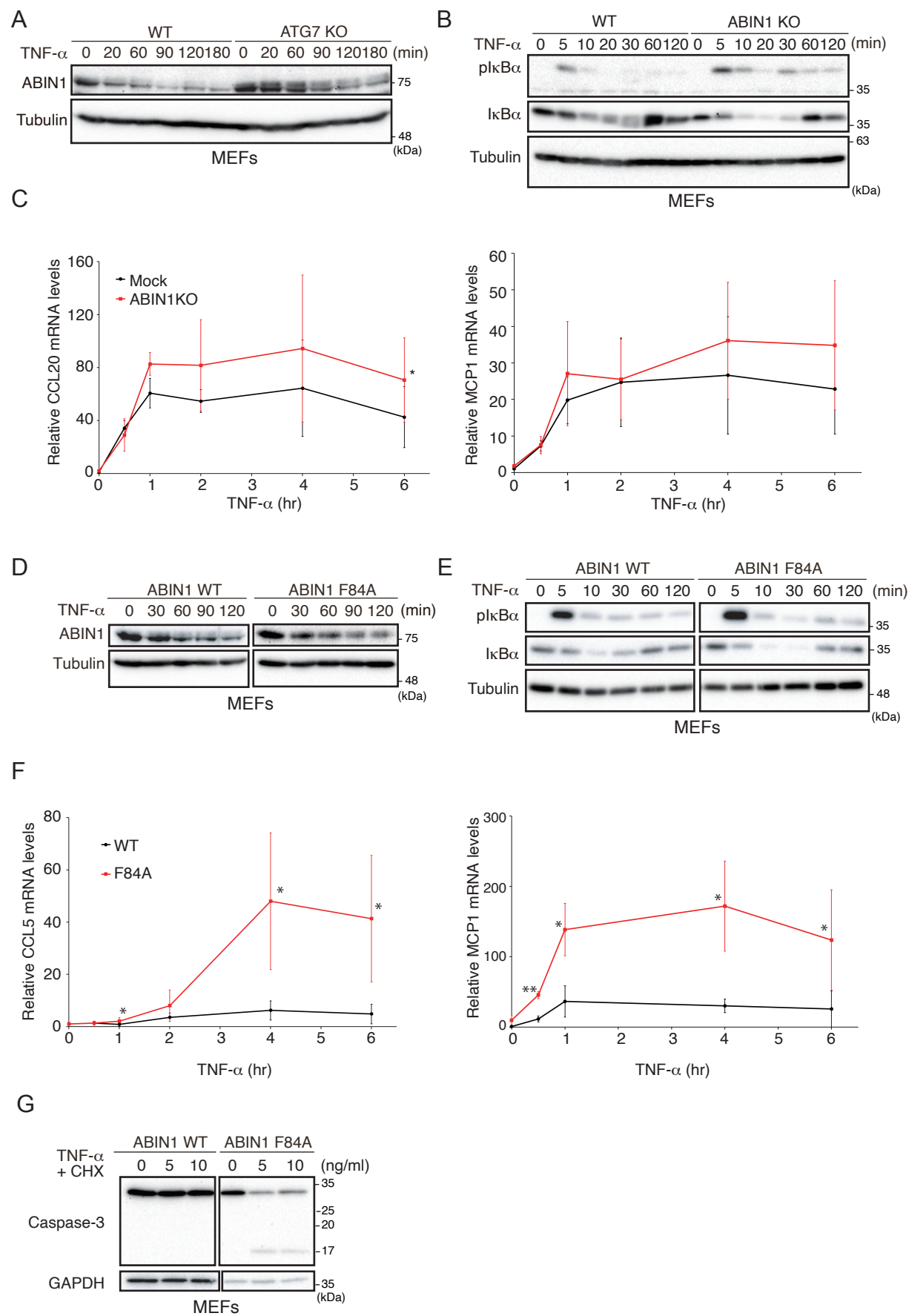
Shinkawa et al. Figure 4



Shinkawa et al. Figure 5



Shinkawa et al. Figure 6



Shinkawa et al. Figure 7

Supplemental Information

ABIN1 is a signal-induced autophagy receptor that attenuates NF- κ B activation by recognizing linear ubiquitin chains

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Supporting information Table S1. List of antibodies used for western blot analysis and immunofluorescence analysis.

Antibody	Catalog no.	Dilution	Company
FLAG-tag	PM020	1:2000	MBL
HA-tag (Y11)	sc-805	1:2000	Santa Cruz Biotechnology
HA-tag (Tana-2)	M180-3	1:2000	BML
ABIN1	37-6100	1:2000, 1:200 (IF)	Invitrogen
ATG5	#12994	1:2000	Cell Signaling Technology
LC3	PM036	1:2000, 1:1000 (IF)	MBL
LAMP1	sc-19992	1:100 (IF)	Santa Cruz Biotechnology
Linear ubiquitin, clone LUB9		1:2000	in-house
Ubiquitin (P4D1)	sc-8017	1:2000	Santa Cruz Biotechnology
IRAK1	#4504	1:2000	Cell Signaling Technology
MyD88	AF3109	1:2000	R&D systems
I κ B α	#9242S	1:2000	Cell Signaling Technology
Phospho-I κ B α (Ser32/36)	#9246	1:2000	Cell Signaling Technology
Caspase-3	#9662S	1:2000	Cell Signaling Technology
GST	sc-459	1:2000	Santa Cruz Biotechnology
GAPDH	sc-365062	1:5000	Santa Cruz Biotechnology
Actin, clone AC74	A5316	1:5000	Sigma-Aldrich
Tubulin	CLT9002	1:5000	CEDARLANE
HRP conjugated anti-rabbit IgG antibody	Cat#NA934V	1:5000	Amersham Pharmacia
HRP conjugated anti-mouse IgG antibody	#7076S	1:5000	Cell Signaling Technology
HRP conjugated anti-goat IgG antibody	sc-2056	1:5000	Santa Cruz Biotechnology
Goat anti-Mouse IgG (H+L) highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	#A-11029	1:2000 (IF)	Thermo Fisher Scientific
Goat anti-Rabbit IgG (H+L) highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	#A-32733	1:2000 (IF)	Thermo Fisher Scientific

Supporting information Table S2. List of reagents.

Reagents	Catalog no.	Company
Lipopolysaccharide from Escherichia coli 055:B5	L4005	Sigma-Aldrich
Pam3CSK4	tlrl-pms	Invivogen
mTNF-alpha	410-MT	R&D systems
CHX	239764	Calibiochem
Bafilomycin A1	S1413	Selleck
E64d	4321-v	Peptide
Pepstatin A	4397	Peptide
MG132	3175-v	Peptide
DMSO	67-68-5	Nakarai
Alkaline Phosphatase, Calf Intestinal	M290S	New England Bio Labs
USP2cc		in-house
HOIPIN-8	2972	Axon Medchem

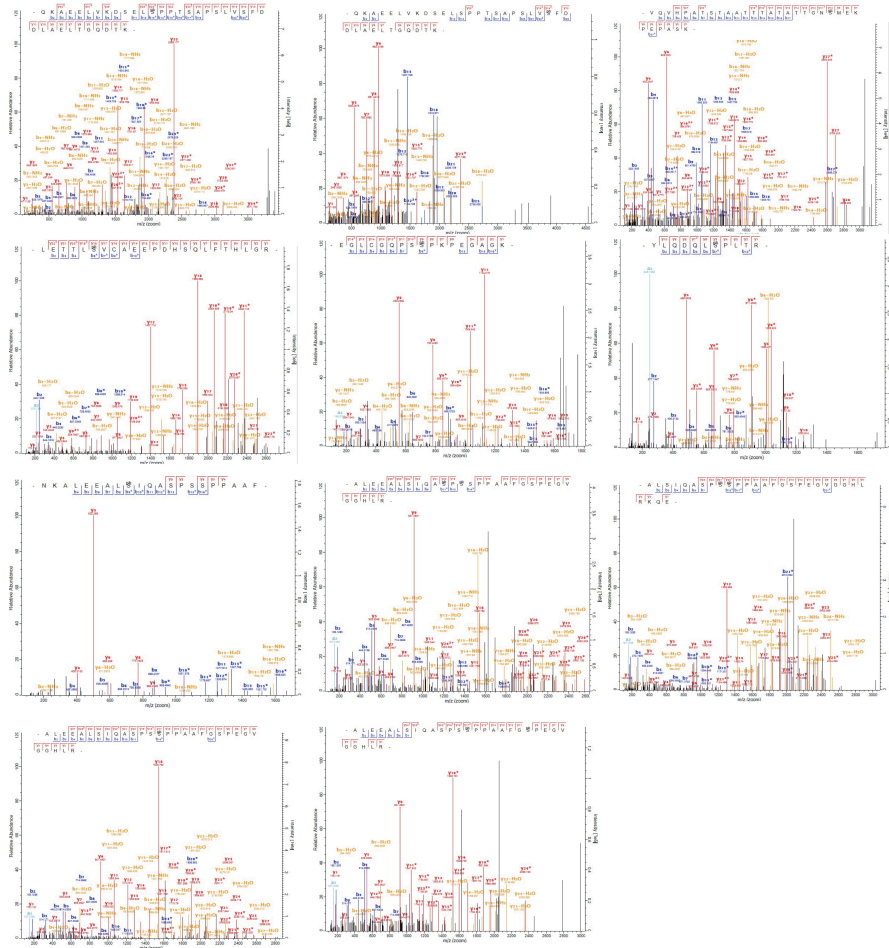
Supporting information Table S3. List of primers used for prime editing.

pegRNA scaffold	
	agagctagaa atagcaagtt aaaataaggc tagtccgta tcaactgaa aaagtggcac cgagtcg
Spacer sequence:	
	Sense: caccGAGCTCAGCCAGGTCATCAAgtttt
	Antisense: ctctaaaacTTGATGACCTGGCTGAGCTC
3' extention:	
	Sense: gtgcTTGGTCTCCGCTGATGACCTGGCTGA
	Antisense: aaaaTCAGCCAGGTCATCAGCGGAGACCAA

Supporting information Table S4. List of primers used for qRT-PCR.

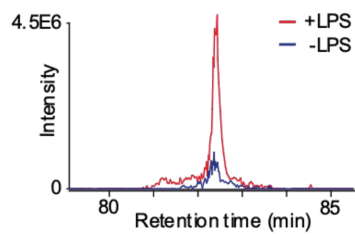
Gene		Sequence
IL-6	Forward	5'-TACCACTTCACAAGTCGGAGGC-3'
	Reverse	5'-CTGCAAGTGCATCATCGTTGTTC -3'
Ccl5	Forward	5'-GCTGCTTTGCCTACCTCTCC-3'
	Reverse	5'-TCGAGTGACAAACACGACTGC-3'
Mmp3	Forward	5'-TGTCCCGTTTCCATCTCTCTC-3'
	Reverse	5'-TGGTGATGTCTCAGGTTCCAG-3'
Ccl20	Forward	5'-GCCTCTCGTACATACAGACGC-3'
	Reverse	5'-CCAGTTCTGCTTTGGATCAGC-3'
Mcp1	Forward	5'-CCGGCTGGAGCATCCACGTGT-3'
	Reverse	5'-TGGGGTCAGCACAGACCTCTCTCT-3'
Actb (encoding b-actin)	Forward	5'- CATTGCTGACAGGATGCAGAAG -3'
	Reverse	5'- TGCTGGAAGGTGGACAGTGAGG -3'

A



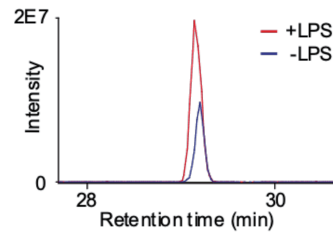
B

ABIN1 phosphopeptide (pS83)
QKAEELVKDSELSPTSPAPSLVpSFDDLAELTGQDTK (+4)
XIC: m/z 982.2265 +3ppm



C

ABIN1 unmodified peptide
GVAGQQQASVMASK (+2)
XIC: m/z 681.3457 +3ppm



Shinkawa et al.Ex.Figure.1

Supplemental information Figure S1. Supporting data for MS analyses.

A, MS/MS spectra of ABIN1 phosphopeptides with high-confidence phosphorylation sites.

B, MS-based quantification of a phosphorylated peptides containing pS83 in the presence (red) and absence (blue) of LPS stimulation. *C*, MS-based quantification of an unmodified peptide in the presence/absence of LPS stimulation.