OTUD1 deubiquitylase regulates NF- κ B- and KEAP1-mediated inflammatory responses and reactive oxygen species-associated cell death pathways

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21 Deubiquitylating enzymes (DUBs) regulate numerous cellular functions by
 22 removing ubiquitin modifications. We examined the effects of 88 human DUBs
 23 on linear ubiquitin chain assembly complex (LUBAC)-induced NF-κB activation,
 24 and identified OTUD1 as a potent suppressor. OTUD1 regulates the canonical
 25 NF-κB pathway by hydrolysing K63-linked ubiquitin chains from NF-κB signalling
 26 factors, including LUBAC. OTUD1 negatively regulates the canonical NF-κB
 27 activation, apoptosis, and necroptosis, whereas OTUD1 upregulates the
 28 interferon (IFN) antiviral pathway. The N-terminal intrinsically disordered region
 29 of OTUD1, which contains an EGTE motif, is indispensable for KEAP1-binding
 30 and NF-κB suppression. OTUD1 is involved in the KEAP1-mediated antioxidant
 31 response and reactive oxygen species (ROS)-induced cell death, oxoapoptosis. In
 32 *Otud1*^{-/-}-mice, inflammation, oxidative damage, and cell death were enhanced in
 33 inflammatory bowel disease, acute hepatitis, and sepsis models. Thus, OTUD1
 34 is a crucial regulator for the inflammatory, innate immune, and oxidative stress
 35 responses and ROS-associated cell death pathways.

The protein ubiquitylation system, catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), generates monoubiquitylations and various types of polyubiquitylations *via* seven internal Lys (K) residues (*i.e.*, K6, K11, K27, K29, K33, K48, and K63) and the N-terminal methionine (M1). Moreover, hybrid- and branched-ubiquitin chains, as well as chemically-modified ubiquitins, have been recently identified. These diverse ubiquitin linkages participate in numerous cellular functions, in the so called “ubiquitin code”¹.

Deubiquitylating enzymes (DUBs) function as erasers in the ubiquitin code². Approximately 100 DUBs have been identified in the human genome and classified into seven subfamilies: ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase (UCH), ovarian tumour protease (OTU), Josephins, motif interacting with ubiquitin (MIU)-containing novel DUB (MINDY)³, zinc finger with UFM1-specific peptidase domain protein (ZUFSP)⁴, and JAB1/MPN/MOV34 metalloenzymes (JAMM/MPN⁺). The USP, UCH, OTU, Josephins, MINDY, and ZUFSP subfamilies are cysteine proteases, whereas the JAMM/MPN⁺ proteins are zinc metalloproteases.

The linear ubiquitin chain assembly complex (LUBAC), composed of HOIL-1L (also known as RBCK1), HOIP (RNF31), and SHARPIN, is an E3 complex that specifically generates the M1-linked linear polyubiquitin chain^{5, 6}. Upon stimulation by

proinflammatory cytokines, such as TNF- α , LUBAC is recruited to the TNF receptor (TNFR), and conjugates M1-ubiquitin chains to NF- κ B-essential modulator (NEMO) and receptor-interacting serine/threonine-protein kinase 1 (RIP1). The M1-ubiquitin chain functions as a scaffold to form the TNFR signalling complex I and recruit the canonical I κ B kinase (IKK), composed of IKK α , IKK β , and NEMO⁷. The recruited IKK molecules are activated by a *trans*-phosphorylation mechanism, leading to the activation of NF- κ B signalling. Moreover, the LUBAC activity is crucial for anti-apoptosis, and genetic defects and polymorphisms of LUBAC are associated with various disorders⁶.

At present, the K11-, K63-, and M1-ubiquitin chains are known to be involved in TNFR signalling complex I⁸, and DUBs, such as Cezanne (OTUD7B), OTULIN, CYLD, and A20, are reportedly involved in NF- κ B regulation⁹. Cezanne¹⁰ and OTULIN¹¹, OTU family DUBs, selectively hydrolyze K11- and M1-ubiquitin chains, respectively. Moreover, OTULIN and CYLD bind to the N-terminal PUB domain of HOIP, which regulates LUBAC activity^{12, 13}. In contrast, A20, an OTU family DUB, does not cleave linear polyubiquitin chains, and instead it binds to the M1-ubiquitin chain through the C-terminal zinc finger 7, resulting in the suppression of NF- κ B activation¹⁴. In addition to the homotypic linear ubiquitylations, complex ubiquitylations such as M1/K63-hybrid chain¹⁵ and HOIL-1L mediated ester-bound monoubiquitylation¹⁶ have

been identified in LUBAC-mediated NF- κ B activation. However, the DUBs that physiologically regulate LUBAC functions have remained elusive.

In this study, we examined the effects of 88 human DUBs on LUBAC-mediated NF- κ B activation, and identified an OTU-family DUB, OTUD1 (also known as DUBA7)¹⁷, as the most potent down-regulator. OTUD1 reportedly regulates tumour progression¹⁸, cell death^{19, 20}, innate immune responses²¹⁻²³, YAP signalling²⁴, autoimmune disease²⁵, iron transport²⁶, and inhibition of colonic inflammation²⁷, whereas the regulatory mechanism of OTUD1 on the NF- κ B pathway has not been revealed.

80 **Results**

81 **OTUD1 is a negative regulator of canonical NF- κ B signalling.** To
 82 comprehensively explore the DUBs involved in LUBAC-mediated NF- κ B activation, we
 83 prepared 88 human DUB cDNAs and analysed their effects by a luciferase assay in
 84 HEK293T cells ([Fig. 1a](#)). Taking the NF- κ B activity induced by the expression of
 85 LUBAC alone as 100%, its co-expression with 16 DUBs, such as USP10, USP32, USP9Y,
 86 and so on, upregulated the NF- κ B activity. In contrast, 34 DUBs significantly
 87 downregulated the LUBAC-induced NF- κ B activation. Among them, USP2, USP13,
 88 OTULIN, OTUB1, A20, OTUD6A, OTUD2, and OTUD1 showed potent (>95%)
 89 inhibitory effects. Since OTUD6A, OTUD2, and OTUD1 had stronger inhibitory effects
 90 than those of the known LUBAC-suppressive DUBs, such as CYLD, OTULIN, and A20,
 91 we performed another NF- κ B luciferase assay by expressing 10-fold higher amounts of
 92 LUBAC subunits than those in [Fig. 1a](#). As a consequence, OTUD6A, OTUD2, and
 93 OTUD1 dose-dependently suppressed the LUBAC- and/or TNF- α -induced NF- κ B
 94 activation ([Fig. 1b](#), [Extended Data Fig. 1a, 1b](#)). Since OTUD1 showed the most potent
 95 inhibitory effect, we focused on investigating its physiological functions.

96 Human OTUD1 (481 a.a.) is composed of an unknown N-terminal region (a.a. 1-290),
 97 an OTU-type Cys protease domain (a.a. 291-446), which contains the active center

Cys320, and a ubiquitin-interacting motif (UIM, a.a. 457-481) (Fig. 1c)¹⁷. Although the N-terminal region lacks homology with known domains, it shares 17% identity and 67% similarity with the amino acid sequence of the hypothetical protein Rv1157c of *Mycobacterium tuberculosis* (NP_215673.1) (Extended Data Fig. 1c), although its physiological significance remains unknown. Interestingly, the region contains an abundance of Ala (63/290 residues; 21.7%), Pro (49/290 residues; 16.9%), and Gly (28/290 residues; 9.6%). Therefore, we named the region as the Ala-, Pro-, and Gly-rich region (APGR).

To identify the critical region of OTUD1 in NF- κ B inhibition, we constructed various mutants of OTUD1 and performed luciferase assays (Fig. 1c, 1d, Extended Data Fig. 1d). The OTUD1-Wt strongly suppressed the LUBAC- and TNF- α -induced NF- κ B activities, and the catalytically inactive mutant of OTUD1 (CA) showed partial NF- κ B suppression. The combined mutation with the catalytically inactive APGR-deletion (Δ APGR-CA) completely abolished the NF- κ B suppressive effect of OTUD1. These results suggested that both the catalytic activity and the APGR play a role in NF- κ B suppression.

The protein disorder prediction by DICHOT²⁸ suggested that APGR is an intrinsically disordered low complexity domain (Fig. 1e). Moreover, the endogenous and transiently expressed FLAG-tagged OTUD1 (calculated molecular weight: 51 kDa) in HEK293T

cells eluted in broad fractions in a gel filtration analysis, mainly at approximately 330-240 kDa ([Fig. 1f](#)). In contrast, FLAG-ΔAPGR (calculated molecular weight: 22 kDa) predominantly eluted at monomer fractions of <44 kDa. These results suggested that the low complexity APGR functions as a multiple protein-interaction site.

OTUD1 preferentially cleaves K63-linked ubiquitin chain. OTUD1 primarily cleaves K63-linked diubiquitin¹⁷, but it also cleaves K11- and K48-chains at higher concentrations²⁹. The DUB protein array analysis showed that OTUD1 preferentially cleaves the K63-ubiquitin chain, followed by the K6- and K48-chains³⁰. However, several cellular analyses suggested that OTUD1 cleaves K33-linked polyubiquitin chains in SMAD7¹⁸, and atypical K6-, K11-, and K29-linked polyubiquitin chains from IRF3²¹. Our biochemical analyses reconfirmed that the transiently expressed and immunoprecipitated full-length FLAG-OTUD1-Wt in HEK293T cell lysates, but not the catalytically inactive mutant (FLAG-OTUD1-CA), effectively converted K63-linked diubiquitin to monoubiquitin, among the 8 types of diubiquitins tested *in vitro* ([Extended Data Fig. 1e](#)). Moreover, recombinant GST-OTUD1 completely hydrolysed the K63-linked polyubiquitin chain, and processed a long K48-linked polyubiquitin chain to tetraubiquitin ([Extended Data Fig. 1f](#)). However, OTUD1 did not cleave di- and poly-M1

chains, indicating that OTUD1 downregulates the NF- κ B activation independently of the cleavage of a LUBAC-generated linear ubiquitin chain, and principally by hydrolysing the K63-linked ubiquitin chain.

OTUD1 down-regulates inflammatory cytokine-induced canonical NF- κ B

activation. The *OTUD1* gene is composed of a single exon in both human and mouse. To further clarify the physiological function of OTUD1, we constructed *OTUD1*-knockout (*OTUD1*^{-/-}) HeLa and HEK293T cells and *Otud1*^{-/-}-mice by the CRISPR/Cas9 technique ([Extended Data Fig. 2a, 2b](#)). The genetic depletion of *OTUD1* in HEK293T cells showed no effect on the expression of LUBAC subunits and NF- κ B signalling factors, such as p105 and I κ B α ([Extended Data Fig. 2c](#)). Moreover, *Otud1* is ubiquitously expressed in various mouse tissues ([Extended Data Fig. 2d](#)).

To examine the cellular functions of OTUD1, we analysed the TNF- α -induced canonical NF- κ B pathway. The genetic ablation of *OTUD1* in HEK293T cells enhanced the TNF- α -induced NF- κ B luciferase activity ([Extended Data Fig. 2e](#)). Upon TNF- α stimulation, multiple proteins, such as RIP1, TNF receptor-associated protein with a death domain (TRADD), IKK complex, and LUBAC, are recruited by TNFR to form signalling

complex I^{8, 31}. In *OTUD1*^{-/-}-HeLa cells, TNF- α -induced phosphorylation of I κ B α , a hallmark of NF- κ B activation, was upregulated as compared to that in the parental cells (Fig. 2a). Moreover, the FLAG-TNF- α precipitation indicated that polyubiquitylation and RIP1 and LUBAC recruitment were enhanced in *OTUD1*^{-/-}-cells, suggesting that OTUD1 is involved in the regulation of TNFR complex I (Fig. 2a). TANK-binding kinase 1 (TBK1) and IKK ϵ reportedly prevent TNF-induced cell death by RIP1 phosphorylation³². Importantly, the recruitment and phosphorylation of TBK1 and IKK ϵ were reduced in the TNFR signalling complex I from *OTUD1*^{-/-} cells (Extended Data Fig. 2f). Thus, OTUD1 reciprocally regulates TNF- α -mediated NF- κ B activation and TBK1/IKK ϵ -mediated signalling in HeLa cells. We further investigated the effect of the genetic ablation of *OTUD1* on TNF- α -induced K63-ubiquitylation, by using a tandem ubiquitin binding entity (TUBE)³³. After TNF- α stimulation, the K63-polyubiquitylations of NEMO and RIP1 were enhanced in *OTUD1*^{-/-}-HeLa cells (Fig. 2b), indicating that OTUD1 regulates K63-ubiquitylation in complex I. Indeed, the expression of NF- κ B target genes was enhanced in TNF- α -treated *OTUD1*^{-/-}-HeLa cells (Extended Data Fig. 2g).

Similarly, upon stimulation with IL-1 β , the phosphorylation of NF- κ B factors, such as p105, p65, and I κ B α , and the subsequent degradation of I κ B α , were enhanced in *OTUD1*^{-/-} cells as compared to the parental cells (Fig. 2c). In contrast, the phosphorylation of JNK,

a MAP kinase, was not affected in *OTUD1*^{-/-}-cells. When K63-polyubiquitinated proteins were captured from cell lysates by K63-TUBE, enhanced high molecular weight smear migrations of interleukin-1 receptor-associated kinase 1 (IRAK1), HOIP, and SHARPIN were detected in IL-1 β -treated *OTUD1*^{-/-}-HeLa cells (Fig. 2d). In contrast, the M1-TUBE analysis indicated that the linear ubiquitylation of these factors was not increased (Fig. 2e), suggesting that OTUD1 physiologically regulates the IL-1 β -induced K63-deubiquitylation of IRAK1 and LUBAC. After IL-1 β -treatment, the mRNA (*IL-6*, *ICAM1*, and *BIRC3*) and protein (VCAM and I κ B ζ) levels of NF- κ B targets were enhanced in *OTUD1*^{-/-}-cells, as compared to those in parental cells (Extended Data Fig. 2h, 2i). Interestingly, the expression level of OTUD1 gradually increased after IL-1 β stimulation, suggesting that OTUD1 is an NF- κ B target gene.

To further investigate the effect of OTUD1 on NF- κ B signalling, we performed an RNA sequencing (RNA-seq) analysis, and compared the transcripts in IL-1 β -treated parental and *OTUD1*^{-/-}-HeLa cells. A principal component analysis clearly demonstrated that the *OTUD1*-deficiency affected the transcription of a set of genes (Fig. 2f). The heatmap, molecular signature database (MSigDB), and KEGG pathway analyses revealed the significant upregulation of inflammatory responses and NF- κ B-related factors in IL-1 β -stimulated *OTUD1*^{-/-}-HeLa cells (Fig. 2g, 2h, Extended Data Fig. 3a). Collectively,

these results indicated that OTUD1 is a negative regulator for the IL-1 β -induced canonical NF- κ B activation pathway.

We further examined the role of *Otud1* using splenic B cells from Wt- and *Otud1*^{-/-} mice, and confirmed that the *Otud1*-deficiency did not affect the CD40- or B cell receptor-mediated induction of NF- κ B target genes, such as *Tnfaip3* (A20) and *Bcl-xl* (Extended Data Fig. 3b, 3c). Furthermore, lymphotoxin β -mediated non-canonical NF- κ B activation, which is demonstrated by the intranuclear translocation of p52 and RelB, was not affected in *Otud1*^{-/-}-MEFs (Extended Data Fig. 3d). Thus, *Otud1* does not play a crucial role in B cells and the non-canonical NF- κ B activation pathway.

Otud1 activates IFN antiviral signalling. To further investigate the effect of *Otud1* on innate immune responses, we examined the antiviral pathway using *Otud1*^{+/+}- and *Otud1*^{-/-}-MEFs. Upon stimulation with poly(dA:dT), which activates the IFN antiviral pathway³⁴, the phosphorylation of TBK1 and IRF3 and the expression of IRF3-target genes, such as *Isg15* and *Isg56*, were reduced in *Otud1*^{-/-}-MEF cells as compared to the *Otud1*^{+/+}-MEFs (Fig. 3a, 3b). These results indicated that in contrast to NF- κ B signalling, *Otud1* functions as a positive-regulator in the IFN antiviral pathway. Similarly, the TLR3 ligand poly(I:C)-induced phosphorylation of IRF3 and the expression of IRF3-target

genes were decreased in *Otud1*^{-/-}-bone marrow derived macrophages (BMDMs) and MEFs (Fig. 3c, 3d, Extended Data Fig. 4a). Upon infection with Sendai virus (SeV), a single-stranded RNA virus, *Otud1*^{-/-}-MEFs and -BMDMs showed reduced expression of IRF3-target genes, such as *interferon β* (*Ifnb*), *Isg15*, *Isg56*, and *Cxcl10*, as compared to *Otud1*^{+/+}-cells (Fig. 3e, 3f). Indeed, an RNA-seq analysis of poly(I:C)-treated *Otud1*^{+/+}- and *Otud1*^{-/-}-MEF cells revealed that the *Otud1*-deficiency downregulated the transcription of a set of IFN genes (Fig. 3g, 3h). MSigDB and KEGG pathway analyses demonstrated that the *Otud1*-deficiency affected the expression of IFN and the RIG-I-like receptor signalling pathway after poly(I:C) stimulation (Fig. 3i, Extended Data Fig. 4b). Collectively, these results indicated that Otud1 is a positive regulator for the type I IFN antiviral pathway.

OTUD1 suppresses TNF-α-induced apoptosis and necroptosis. When TNFR signalling complex I fails to induce NF-κB-mediated cell survival under conditions where protein synthesis is inhibited by cycloheximide, TNF-α stimulation induces cell death with the formation of a second complex, named complex II, which is composed of Fas-associated death domain protein (FADD) and caspase 8^{35, 36}. A pan-caspase inhibitor (ZVAD) suppresses apoptosis, whereas it induces another programmed necrosis,

necroptosis. The kinase activity of RIP1 subsequently phosphorylates RIP3 and then mixed lineage kinase domain-like pseudokinase (MLKL), which is indispensable for necroptosis³⁷, and therefore, the RIP1 kinase inhibitor, necrostatin-1 (Nec-1), suppresses necroptosis³⁸.

After an 8 h treatment with TNF- α +cycloheximide (TC), the trypan blue-positive dead cells were significantly increased in *OTUD1*^{-/-}-HeLa and HEK293T cells, which both lack RIP3 expression (Fig. 4a). The cell survival analysis by an xCELLigence real-time cell analyser indicated that *OTUD1*^{-/-}-HEK293T cells are more sensitive to cell death than the parental cells after TC-treatment (Fig. 4b). Indeed, the cleavages of PARP, caspase 3, and caspase 8, hallmarks of the extrinsic apoptotic pathway, were enhanced in TC-treated *OTUD1*^{-/-}-HEK293T cells as compared to those in parental cells (Fig. 4c, 4d). When cell lysates were immunoprecipitated by an anti-FADD antibody, effective complex II formation, as shown by the enhanced association of caspase 8 with FADD, was detected in *OTUD1*^{-/-}-HEK293T cells (Fig. 4d). These results suggested that OTUD1 has an inhibitory effect on the TNF- α -induced apoptosis.

We next investigated the involvement of Otud1 in necroptosis, using MEFs. In the presence of TC with ZVAD (TCZ), the *Otud1*^{-/-}-MEFs died more rapidly than the *Otud1*^{+/+}-MEFs (Fig. 4e). The phosphorylation of MLKL and RIP3 was enhanced in

Otud1^{-/-}-MEFs (Fig. 4f), and TCZ-induced cell death in *Otud1*^{-/-}-MEFs was rescued by Nec-1 (Fig. 4g). Interestingly, the polyubiquitylation of RIP3, but not RIP1, was enhanced in *Otud1*^{-/-}-MEFs (Fig. 4h, left panel), indicating that RIP3 is a possible endogenous substrate of Otud1. When K63-ubiquitinated proteins were pulled down by K63-TUBE, the smeared migration of RIP1 was slightly suppressed upon stimulation with TCZ in *Otud1*^{-/-}-MEFs (Fig. 4h, middle panel). In contrast, the M1-ubiquitylation of RIP3 was not detected in either MEFs (Fig. 4h, right panel). These results indicated that Otud1 is a critical regulator in TNF- α -mediated necroptosis pathways through the removal of K63-ubiquitin chains from RIP3 under steady state conditions.

OTUD1 regulates KEAP1-mediated oxidative stress response and cell death.

To further examine the physiological roles of OTUD1, proteins interacting with FLAG-OTUD1 in HEK293T cells were analysed by immunoprecipitation-mass spectrometry. We identified KEAP1 as the most prominent interactor of OTUD1 (Fig. 5a, Extended Data Fig. 5a). KEAP1 regulates the proteasomal degradation of the NRF2 transcription factor, as a component of the Cullin 3 (CUL3)-RING E3 complex³⁹. Moreover, KEAP1 is involved in excess reactive oxygen species (ROS)-induced cell death, named oxoapoptosis, with the apoptosis-inducing factor mitochondria associated 1 (AIFM1) and the

mitochondrial Ser/Thr protein phosphatase PGAM5⁴⁰. Indeed, peptides derived from AIFM1, CUL3, and PGAM5 were identified in FLAG-OTUD1 co-precipitates (Fig. 5a, Extended Data Fig. 5a). We detected the endogenous association of KEAP1 and OTUD1 (Fig. 5b), and the K63-polyubiquitylation of KEAP1 was enhanced in *Otud1*^{-/-}-MEFs (Fig. 5c). Moreover, we determined that the N-terminal APGR of OTUD1 interacts with the C-terminal Kelch and CTR of KEAP1 (Fig. 5d, 5e). Importantly, we found that the human and mouse OTUD1 proteins contain ETGE and the similar DTGE sequence, respectively, in APGR, which plays a crucial role in NRF2 for KEAP1-binding⁴¹ (Fig. 5f). Indeed, the deletion of the ETGE motif from OTUD1 abolished the KEAP1-binding ability (Fig. 5g). Upon H₂O₂-treatment, ROS production was upregulated in *Otud1*^{-/-}-MEFs as compared to *Otud1*^{+/+}-MEFs (Fig. 5h), resulting in the enhanced expression of antioxidant genes, such as *Hmox-1* (Ho-1) and *Nhe2l2* (Nrf2) at the early phase (~3 h) (Fig. 5i). However, a prolonged treatment with H₂O₂ caused drastic cell death in *Otud1*^{-/-}-MEFs, suggesting that the excessive ROS production subsequently induces oxelptosis (Fig. 5j).

Interestingly, the restoration of OTUD1-Wt, but not the catalytically inactive mutant (CA), rescued ROS resistance and cell viability (Fig. 5k, 5l), suggesting that the DUB activity of OTUD1 is necessary to resist oxelptosis. OTUD1 directly bound with KEAP1, and PGAM5 co-precipitated with OTUD1 only in the presence of KEAP1

([Extended Data Fig. 5b](#)). Although the co-precipitation of endogenous AIFM1 with FLAG-OTUD1 was detected by the MS analysis ([Fig. 5a](#), [Extended Data Fig. 5a](#)), the co-precipitation of exogenous OTUD1 with AIFM1 was not detected in the presence of KEAP1 and PGAM5 ([Extended Data Fig. 5b](#)). Whereas, in *Otud1*^{-/-}-MEFs, increased intranuclear AIFM1 was detected concomitantly with the reduced mitochondrial AIFM1 ([Extended Data Fig. 5c](#)), which may affect mitochondrial oxidative phosphorylation and ROS generation²⁰. These results indicated that OTUD1 physiologically binds and regulates the K63-ubiquitylation of KEAP1 under basal conditions and is important for the efficient antioxidative stress response, ROS production, and oxeiptosis.

In addition to H₂O₂-treatment, the ROS levels were also upregulated in *Otud1*^{-/-}-MEFs upon apoptotic (TC) and necroptotic (TCZ) stimuli, although TNF- α alone showed no effects on ROS production ([Extended Data Fig. 5d](#)). Since the enhanced ROS production was cancelled by the restoration of *Otud1*-Wt, but not -CA, in TC- or TCZ-treated *Otud1*^{-/-}-MEFs, the catalytic activity of *Otud1* is necessary to regulate TNF- α -induced cell death pathways ([Extended Data Fig. 5e](#)). Collectively, these results indicated that OTUD1 activity regulates ROS-associated cell death pathways, such as TNF- α -induced apoptosis and necroptosis, as well as KEAP1-mediated oxeiptosis.

OTUD1 suppresses inflammatory and oxidative stress responses *in vivo*. A

recent report showed that OTUD1 inhibits colonic inflammation *in vivo*²⁷. We also confirmed that significant shortening of the colon, and higher diarrhea and fecal blood scores were observed in a DSS-administrated ulcerative colitis-like inflammatory bowel disease (IBD) model of *Otud1*^{-/-}-mice (Extended Data Fig. 6a, 6b). The middle and distal colon regions in DSS-treated *Otud1*^{-/-}-mice showed more severe damage, characterized by multiple focal dropouts of entire crypts, inflammatory cell infiltration, and edema, as compared with the DSS-treated *Otud1*^{+/+} mice (Fig. 6a). Histologic scores of colitis were significantly higher in the middle and distal colon of DSS-treated *Otud1*^{-/-}-mice, as compared with DSS-treated *Otud1*^{+/+} mice (Fig. 6b). Moreover, the expressions of NF-κB target genes such as *Tnf* and *Il1b* were upregulated in the distal colon from *Otud1*^{-/-}-mice (Fig. 6c). Importantly, we detected the increased oxidative DNA damage and cell death in the middle and distal colon of DSS-treated *Otud1*^{-/-}-mice, as evidenced by increased numbers of 8-OHdG-positive cells and TUNEL-positive cells in the colon epithelium, respectively (Fig. 6d, 6e). In addition to the IBD-like phenotype, DSS-treated *Otud1*^{-/-}-mice also showed splenomegaly with increased TUNEL-positive splenocytes (Extended Data Fig. 6c, 6d). These results indicated that Otud1 suppresses the inflammatory and oxidative damage responses in an *in vivo* colitis model.

313 To further clarify the pathophysiological role of *Otud1*, we adopted an
 314 LPS/GalN-induced acute hepatitis model. The LPS/GalN-treated *Otud1*^{-/-}-mice showed
 315 significantly shorter survival, remarkably increased hepatocellular death, and increased
 316 caspase 3 activation ([Fig. 6f, 6g, 6h](#)), indicating that *Otud1* protects against acute hepatitis.

317 In contrast to LPS/GalN, LPS challenge alone induces systemic inflammation
 318 and sepsis. When mice were intraperitoneally administered 20 mg kg⁻¹ LPS, we found
 319 that the survival times of *Otud1*^{-/-}-mice were significantly shorter as compared to those
 320 of *Otud1*^{+/+}-mice, indicating that *Otud1*^{-/-}-mice were sensitive to LPS-induced systemic
 321 inflammation and sepsis ([Extended Data Fig. 6e](#)). Histopathological findings in the livers
 322 are shown in [Extended Data Table 1](#) and [Extended Data Fig. 6f](#). In male mice, mild fatty
 323 changes characterized by increased microvacuoles in the cytoplasm of hepatocytes were
 324 observed in all LPS-treated *Otud1*^{+/+}- and *Otud1*^{-/-}-mice, but not in the *Otud1*^{+/+} and
 325 *Otud1*^{-/-} controls ([Extended Data Fig. 6f, upper panels](#)). Hydropic degeneration in
 326 midzonal and centrilobular hepatocytes was observed in 2 of the 6 LPS-treated male
 327 *Otud1*^{+/+}-mice ([Extended Data Table 1](#)), but not in the *Otud1*^{+/+} controls. No hydropic
 328 degeneration was observed in the male *Otud1*^{-/-}-mice regardless of LPS treatment.
 329 Notably, in the female mice, the incidence of hepatocellular death (necrosis and/or
 330 apoptosis), predominantly localized in the midzonal area, was increased in the LPS-

treated *Otud1*^{-/-}-mice (5/6, 83.3%) as compared with the LPS-treated *Otud1*^{+/+}-mice (2/6, 33.3%), albeit without statistical significance ([Extended Data Table 1](#), [Extended Data Fig. 6f](#), [lower panels](#)). This suggested that the female *Otud1*^{-/-}-mice exhibited higher susceptibility to LPS-induced hepatotoxicity than their wild-type counterparts. The incidence of hydropic degeneration of hepatocytes was comparable between the LPS-treated *Otud1*^{+/+} (4/6, 66.7%) and *Otud1*^{-/-}-mice (3/6, 50%). Inflammatory cell infiltration was also observed in all LPS-treated mice, without apparent gender and genotype differences. In addition, the AST activities were significantly elevated in LPS-treated *Otud1*^{-/-}-mice, as compared to the LPS-treated *Otud1*^{+/+}-mice ([Extended Data Fig. 6g](#)). These results indicated that Otud1 is a crucial regulator of innate immune responses, and the genetic ablation of *Otud1* causes aberrant signal transduction resulting in septic shock.

OTUD1 is associated with prognosis of kidney cancer. Finally, to investigate the involvement of OTUD1 in human diseases, we analysed cancer databases. We found that low levels of *OTUD1* expression are significantly associated with a poor prognosis in renal clear cell carcinoma patients ([Fig. 7a](#)). Importantly, the expressions of OTUD1 and KEAP1 were significantly correlated ($P = 4.7 \times 10^{-9}$, $R = 0.54$) in the normal kidney cortex; however, the positive correlation was lost in the tumour kidney cortex ([Fig. 7b](#)).

In contrast, the expression levels of OTUD1 and NF- κ B1 (p105/p50) were correlated in both the normal and tumour kidney cortexes. Thus, we concluded that the orchestration of OTUD1 with KEAP1 functions to suppress kidney cancer in humans, and therefore, OTUD1 is a critical regulator to maintain homeostasis.

Discussion

We have identified that USP2, USP13, OTULIN, OTUB1, A20, OTUD6A, OTUD2, and OTUD1, among 88 human DUBs, exert potent inhibitory effects on LUBAC-induced NF- κ B activation (Fig. 1a). Indeed, USP2 reportedly down-regulates the NF- κ B pathway by removing ubiquitin chains generated by TRAF2 and TRAF6⁴²⁻⁴⁴, and the genetic ablation of *USP13* enhances the NF- κ B and IFN antiviral pathways by deubiquitylating STING⁴⁵. OTULIN and A20 are well-known down-regulators of the LUBAC-mediated NF- κ B pathway⁹. OTUB1 affects NF- κ B signalling by associating with TRAF3, TRAF6, and c-IAP-1^{46, 47}, and OTUD2 interacts with TRAF6 during IL-1 β -induced NF- κ B activation⁴⁸. Thus, most of the listed DUBs are known as regulators for the innate immune and NF- κ B pathways.

OTUD1 was identified as the most potent inhibitor for LUBAC-induced NF- κ B activation (Fig. 1). OTUD1 was initially found as a biomarker of thyroid cancer⁴⁹ that reportedly deubiquitinates and stabilizes the p53 tumour suppressor⁵⁰. OTUD1 also upregulates the expression of p21 and Mdm2, thus accelerating apoptosis⁵⁰, and cleaves K33-linked polyubiquitin chains from the TGF- β pathway inhibitor SMAD7, which is involved in the repression of breast cancer metastasis¹⁸. Furthermore, OTUD1 is induced by RNA viruses, which gives rise to the upregulation of the intracellular amounts of Smurf1 through deubiquitylation⁵¹. Since Smurf1 leads to the ubiquitylation-dependent degradation of MAVS, TRAF3, and TRAF6, subsequently suppressing IFN production, the genetic deficiency of *Otud1* in mice resulted in the production of more antiviral cytokines and resistance to RNA virus infection⁵¹. A recent report showed that OTUD1 removes the K63-linked polyubiquitin chain from YAP, which is conjugated by SCF^{Skp2}, and thus regulates the Hippo pathway and tumourigenesis²⁴. Furthermore, OTUD1 reportedly down-regulates the IFN antiviral pathway by removing the K6-linked ubiquitin chain from IRF3²¹, and quite recently OTUD1 was found to suppress IBD by removing the K63 ubiquitin chain from RIP1²⁷. Collectively, these findings indicate that OTUD1 functions as a crucial regulator of cancer progression, antiviral host defense responses, and inflammatory responses.

In this study, we initially characterized the N-terminal APGR as a disordered low complexity region (Fig. 1). APGR is required to suppress canonical NF- κ B activation, exhibits a broad elution profile in gel filtration, and possesses an interaction site for KEAP1 (Figs. 1, 5). Therefore, APGR seems to play a crucial role in the physiological function of OTUD1.

After confirming that OTUD1 preferentially hydrolyzes K63-linked ubiquitin chains, we found that the K63-polyubiquitylations of NF- κ B signalling factors, such as IRAK1, LUBAC subunits (HOIP and SHARPIN), NEMO, and RIP1, were increased upon inflammatory cytokine-stimulation in *OTUD1*-knockout cells, suggesting that these proteins are potential substrates of OTUD1 upon stimulation (Figs. 2, 7c). Since the ablation of *OTUD1* did not affect B cell receptor- and noncanonical NF- κ B activation (Extended Data Fig. 3), OTUD1 principally regulates the inflammatory cytokine-induced canonical NF- κ B pathway by hydrolysing K63 ubiquitin chains.

Although the TNF- α -induced NF- κ B activation was upregulated in *OTUD1*^{-/-}-cells, the activation of TBK1 and IKK ϵ was suppressed (Extended Data Fig. 2), and in contrast to the previous report²¹, we determined that IRF3-mediated IFN antiviral signalling was down-regulated by the deficiency of *Otud1* (Fig. 3). Since TBK1 activation and IRF3 phosphorylation were suppressed in poly(dA:dT)- and poly(I:C)-treated *Otud1*^{-/-}-MEFs,

OTUD1 seems to be primarily involved in the upstream TBK1/IKK ϵ activation (Fig. 7c), but not the downstream atypical K6-ubiquitylation of IRF3²¹, in the type I IFN production pathway.

The ubiquitylation and deubiquitylation of RIP1/RIP3 regulate the NF- κ B activation, apoptotic, and necroptotic pathways⁵², and DUBs such as CYLD⁵³, A20⁵⁴, and OTUB1⁵⁵ are involved in the regulation of the K63 ubiquitylation of RIP1/RIP3. In this study, we showed that the genetic deficiency of *OTUD1* accelerated the TNF- α -induced apoptotic and necroptotic cell death pathways, and the basal K63 ubiquitylation of RIP3 was enhanced in *Otud1*^{-/-}-MEFs (Figs. 4, 7c), suggesting that RIP3 is an endogenous substrate of OTUD1 under physiological conditions. Although PELI1 and TRIM25 reportedly function as E3s to K48-linked ubiquitylation at Lys363 and Lys501 in RIP3, respectively, to induce proteasomal degradation^{56, 57}, the E3s that catalyse the K63-ubiquitylation of RIP3 have not identified, although they may be involved in the regulation of human health and disease.

Importantly, we identified OTUD1 as a physiological interactor with KEAP1 through the ETGE motif in APGR (Figs. 5, 7c). KEAP1 senses ROS *via* critical Cys residues, and protects against oxidative and electrophilic stress as a component of the KEAP1-CUL3-RBX1 E3 complex to induce the proteasomal degradation of NRF2³⁹. The

DLG and ETGE motifs in the Neh2 domain of NRF2 are crucial for KEAP1-binding⁴¹, and the C-terminal Kelch domain of KEAP1 binds NRF2 as well as OTUD1. We showed that the K63-ubiquitylation of basal KEAP1 was upregulated in *Otud1*^{-/-}-MEFs, indicating that Otud1 is physiological regulator of KEAP1. At present, several E3s, such as TRAF6⁵⁸, TRIM25⁵⁹, CKIP1, and SMURF1⁶⁰, reportedly ubiquitylate NRF2-KEAP1. Therefore, OTUD1 may antagonise these E3s. Furthermore, since KEAP1 also binds with the phosphorylated ubiquitin-binding autophagy receptor protein p62/SQSTM1 through the KIR motif and regulates autophagy⁶¹, OTUD1 may regulate p62-KEAP1-mediated autophagy and related diseases, such as cancer progression.

In the presence of excess amounts of ROS, KEAP1 induces oxelptosis, a caspase- and RIP3-independent apoptosis-like cell death, through binding with PGAM5 and dephosphorylating the evolutionally conserved Ser116 in AIFM1⁴⁰. Moreover, upon TNF- α -induced necroptosis, PGAM5 binds necrosome, composed of RIP1-RIP3-MLKL, which mediate the activation of the mitochondrial fission factor Drp1 and mitochondrial fragmentation⁶². A recent report found that OTUD1 is involved in caspase-dependent and -independent apoptosis by facilitating the intranuclear translocation of AIFM1 and the degradation of MCL1²⁰. The deubiquitylation of K244 in AIFIM1 by OTUD1 affects the mitochondrial structure and oxidative phosphorylation. We have shown that OTUD1 is a

regulator of the KEAP1-PGAM5-AIFM1 axis, which affects ROS generation and cell death pathways upon oxidative and inflammatory responses (Figs. 5, 7c, Extended Data Fig. 5).

Free radicals, such as superoxides, hydroxyl radicals, peroxynitrite, and so on, induce oxidative damage, which causes genotoxicity, oxidations of proteins, lipids, and nucleotides, and cell death. Together, KEAP1-NRF2 and NF- κ B co-operate the oxidative stress and inflammatory responses, and dysfunctions of these systems are associated with various disorders, such as neurodegeneration and inflammatory diseases^{63, 64}. This study has demonstrated that OTUD1 is a central regulatory factor in these signal pathways, and the genetic ablation of *OTUD1* is associated with inflammatory bowel disease, hepatitis, sepsis, and kidney cancer (Figs. 6, 7, Extended Data Fig. 6)^{21, 27}. Therefore, OTUD1 is a critical drug discovery target for the treatment of these diseases.

Methods

Reagents and plasmids. The following reagents were obtained as indicated: human TNF- α and IL-1 β (BioLegend), poly(dA:dT) and high molecular weight poly(I:C) (Invivogen), LPS from *E. coli* O111:B4 (Invivogen), cycloheximide (Sigma-Aldrich),

recombinant mouse CD40 ligand (R&D Systems), α -IgM (Southern Biotech), zVAD-FMK (ZVAD, Peptide Institute, Inc.), and necrostatin-1 (ENZO Life Sciences). The open reading frames of cDNAs, including 88 human DUBs, were amplified by reverse transcription-PCR. Mutants of these cDNAs were prepared by the QuikChange method, and the entire nucleotide sequences were verified. The cDNAs were ligated to the appropriate epitope sequences and cloned into the pcDNA3.1 (Invitrogen) and pGEX-6P-1 (GE Healthcare) vectors. For lentiviral transduction, pCSII-CMV-RfA-IRES-Blast (RIKEN BioResource Research Center) was used.

Antibodies. The following antibodies were used for immunoblotting. Catalogue numbers and dilutions are indicated in parentheses. P-I κ B α (#9246; 1:1,000), I κ B α (#4812; 1:1,000), P-p105 (#4806; 1:1,000), p105 (#13586; 1:1,000), P-p65 (#3033; 1:1,000), p65 (#8242; 1:1,000), p100/p52 (#4882; 1:1,000), P-RelB (#4999; 1:1,000), RelB (#4922; 1:1,000), P-JNK (#4668; 1:1,000), JNK (#5668; 1:1,000), P-IRF3 (#4947; 1:1,000), IRF3 (#4302; 1:1,000), P-TBK1 (#5483; 1:1,000), TBK1 (#3504; 1:1,000), P-IKK ϵ (#8766; 1:1,000), IKK ϵ (#2690; 1:1,000), I κ B ζ (#9244; 1:1,000), caspase 3 (#9662; 1:1,000), cleaved caspase 3 (#9661; 1:1,000), caspase 8 (#9746; 1:1,000), caspase 8 (#4790; 1:1,000), cleaved caspase 8 (#9496; 1:1,000), PARP (#9542; 1:1,000), P-RIP1

472 (#31122; 1:1,000), P-RIP3 (#57220; 1:1,000), RIP3 (#15828; 1:1,000), P-MLKL
 473 (#37333; 1:1,000), MLKL (#37705; 1:1,000), IRAK1 (#4504; 1:1,000), CYLD (#8462;
 474 1:1,000), OTULIN (#14127; 1:1,000), KEAP1 (#8047; 1:1,000), AIFM1 (#5318,
 475 1:1,000), Tom20 (#42406, 1:1,000), Lamin A/C (#4777, 1:1,000), and GST (#2622,
 476 1:1,000) were obtained from Cell Signaling. HOIL-1L (sc-393754; 1:250), TNFR1 (sc-
 477 7895; 1:1,000), VCAM (sc-8304; 1:1,000), and β -actin (sc-47778; 1:250) were purchased
 478 from Santa Cruz Biotechnology. HOIP (ab125189; 1:1,000), NEMO (ab178872; 1:3,000),
 479 and K63-linkage specific ubiquitin (ab179434; 1:1,000) were purchased from Abcam.
 480 RIP1 (610458; 1:1,000) and FADD (610399; 1:1,000) were from BD Transduction
 481 Laboratories. OTUD1 (NBP1-90484, Novus Biologicals; 1:1,000), tubulin (CLT9002,
 482 Cedarlane; 1:5,000), linear ubiquitin (Millipore, clone LUB9, MABS451; 1:1,000),
 483 SHARPIN (14626-1-AP, Proteintech; 1:5,000), KEAP1 (60027-1-Ig, Proteintech;
 484 1:2,000), PGAM5(28445-1-AP, Proteintech; 1:1,000), DYKDDDDK (1E6, HRP-
 485 conjugate, Wako; 1:10,000), Myc (M192-7, HRP-conjugate, MBL; 1:20,000), and HA
 486 (11867423001, Roche; 1:2,000) were also used. To stimulate the lymphotoxin β receptor
 487 (LT β R), a 10:1 mix of the 4H8 WH2 (Adipogen, AG-20B-0008) and 3C8 (eBiosciences,
 488 16-5671-82) monoclonal antibodies was used. For immunoprecipitation, Myc (sc-40,

Santa Cruz Biotechnology; 1 μ g), HA (16B12, BioLegend; 1 μ g) and OTUD1 (NBP1-90484, Novus Biologicals; 3 μ g) were used.

Cell culture and transfection. HEK293T, HeLa (ATCC), and MEF cells were cultured in DMEM, containing 10% fetal bovine serum, 100 IU ml⁻¹ penicillin G, and 100 μ g ml⁻¹ streptomycin, at 37°C under a 5% CO₂ atmosphere. Bone marrow-derived macrophages (BMDM) were prepared basically as described⁶⁵. Briefly, bone marrow cells were isolated from the femurs and tibias of B6 mice. After reticulocyte removal, the residual cells were cultured in 10% FCS/RPMI, supplemented with 5% of L-929 conditioned medium. The floating cells were removed, and the attached cells were passed every 2 days. The resultant adherent cells were used as macrophages on day 7. To obtain splenic B cells, suspensions of mouse splenocytes were prepared from freshly removed mouse spleens, and erythrocytes were depleted with ACK lysing buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2), separated by BD IMag anti-mouse CD45R/B220 particles (BD Biosciences), and then maintained in RPMI containing 10% FBS, 55 μ M 2-mercaptoethanol, and antibiotics, at 37 °C under a 5% CO₂ atmosphere. Plasmid transfection into HEK293T cells was performed using Lipofectamine 2000 (Thermo Fisher Scientific) or polyethylenimine (PEI). To stimulate the cells, poly(dA:dT)

or poly(I:C) was transfected using Lipofectamine 2000 (Thermo Fisher Scientific). For the stable expression of FLAG-tagged Otud1 in *Otud1*^{-/-}-MEFs, lentiviral infection followed by selection with 5 µg ml⁻¹ blasticidin was performed.

Luciferase assay. HEK293T cells were cultured in 6-well or 24-well plates, and co-transfected with the pGL4.32 [*luc2P*/NF-κB-RE/Hygro] vector (Promega) and the phRL-TK *Renilla* Luciferase control reporter vector (Promega). At 24 h after transfection, the cells were lysed and the luciferase activity was measured with a GloMax 20/20 luminometer (Promega), using the Dual-Luciferase Reporter Assay System (Promega).

Screening for DUBs. HEK293T cells cultured in 6-well plates were transfected with pcDNA3.1 plasmids encoding DUB (1 µg), HA-HOIP (30 ng), HOIL-1L-HA (15 ng), and HA-SHARPIN (10 ng), with NF-κB and control luciferase reporters. After 24 h, the luciferase activity was measured as described above. Taking the NF-κB-luciferase activity induced by LUBAC alone as 100%, the relative residual activity in the presence of the respective DUBs was calculated.

Gel filtration analysis. Cells were suspended in 50 mM Tris-HCl, pH 7.5, containing

1 mM MgCl₂, 1 mM DTT, 1 mM PMSF, and a protease inhibitor cocktail (Complete EDTA-free, Roche), and homogenized by a Dounce homogenizer. After adding an equal volume of buffer containing 300 mM NaCl, lysates were centrifuged at 100,000 g for 30 min. The supernatant was fractionated on an ÄKTA pure 25 chromatography system using a Superdex 200 HR (10/30) column (Cytiva), which was equilibrated with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. The column was calibrated by the elution profile of a Gel Filtration HMW Calibration Kit (Cytiva).

Preparation of recombinant OTUD1. The pGEX-6P-1 expression vector, which encodes glutathione *S*-transferase (GST)-fused OTUD1-Wt, was expressed in *E. coli* BL21(DE3) (Novagen), and the recombinant protein was prepared as described previously⁶⁶.

***In vitro* DUB assay.** For the *in vitro* DUB assay with diubiquitins, HEK293T cells were transfected with FLAG-OTUD1-Wt or catalytically inactive FLAG-OTUD1-CA and lysed in lysis buffer, containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1 mM DTT. The cell lysates were immunoprecipitated by a rabbit anti-FLAG antibody (Sigma-Aldrich, F7425) and protein A beads (Cytiva). After a wash with lysis

buffer, the beads were reacted with 0.5 µg of M1-, K6-, K11-, K27-, K29-, K33-, K48- or K63-linked diubiquitins (Boston Biochem) in 20 mM Tris-HCl, pH 7.5, containing 1 mM DTT, for 30 min at 37 °C. For the *in vitro* deubiquitylation assay with polyubiquitin chains, the M1-, K48-, or K63-linked polyubiquitin chains (2.0 µg), prepared as described previously¹⁴, were treated with 0.6 µM recombinant GST-OTUD1-Wt in a total volume of 20 µl of 20 mM Tris-HCl, pH 7.4, containing 1 mM DTT, for the indicated time periods at 37 °C.

Immunoprecipitation, SDS-PAGE, and immunoblotting. Cells were lysed with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 2 mM PMSF, and complete protease inhibitor cocktail (Sigma). Immunoprecipitation was performed using appropriate antibodies followed by Protein G agarose beads (Cytiva) at 4°C with gentle rotation. Immunoprecipitates were washed five times with the lysis solution. Samples were separated by SDS-PAGE and transferred to PVDF membranes. After blocking, the membranes were incubated with the appropriate primary antibodies, followed by an incubation with HRP-conjugated secondary antibodies. The chemiluminescent images were obtained with an LAS4000 imaging analyzer (GE Healthcare) or a Fusion Solo S imaging system (Vilber).

561

562 **Construction of *OTUD1*-knockout cells.** To generate *OTUD1*-knockout HeLa and
 563 HEK293T cells, the gRNA cloning vector (Addgene; #41824) targeting 5'-
 564 CCGagaccggtgagtgccagccc-3' and 5'-taccggagtgctggccaTGG-3' (Capital letters
 565 indicate PAM-sequence) in the coding sequence of human *OTUD1*, and the pCAG-hCas9
 566 expression vector (Addgene; #51142) were transfected using Lipofectamine 3000
 567 (Thermo Fisher). After 96 h, cells were cloned by limiting dilution to obtain single cell
 568 clones. The clones were validated by *AgeI* or *NcoI* digestion, sequenced with PCR-
 569 amplified fragments to confirm mutations, and subjected to immunoblotting with an anti-
 570 OTUD1 antibody.

571

572 **Construction of *Otud1*-knockout mice.** C57BL/6J mice were used as the source of
 573 embryos for micromanipulation and subsequent breeding trials. A mixture of sgRNAs
 574 targeting the 5'-UTR of *Otud1*: 5'-attcgcggtcctgacgcggCGG-3' (Capital letters indicate
 575 PAM-sequence) and 3'-UTR of *Otud1*: 5'-gaaccacacgaactctaattAGG-3' (Capital letters
 576 indicate PAM-sequence), and Cas9 protein was injected into the cytoplasm of one cell-
 577 stage fertilized embryos. Zygotes that survived were transferred into the oviducts of
 578 pseudopregnant foster mothers. The heterozygous mutant mice were intercrossed to

produce the homozygous mutant mice. For PCR primers to detect the wild-type *Otud1* allele, we used 5'-GTATTTGGCTCAGTTGGCTC-3' and 5'-TTCATACCGTGAGACATCAG-3' oligo DNAs to detect the 450 bp band. For the *Otud1* deleted allele, we used 5'-ATCCGCGCGCTGCACTCCTG-3' and 5'-TTCATACCGTGAGACATCAG-3' oligo DNAs to detect the 440 bp band (which was 1,960 bp in the wild-type allele). C57BL/6J mice were kept in a specific pathogen-free, temperature-controlled facility at the Graduate School of Medicine, Osaka City University. Mice were euthanized according to the guidelines of the Animal Experiment Committee of Osaka City University, and all efforts were made to minimize the suffering of the animals. MEFs were prepared from littermate embryos (E12.5) of Wt- and *Otud1*-KO mice, transfected with an SV40 T-antigen containing pEF321-T plasmid, and then immortalized.

K63- and M1-TUBE pulldowns. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with 2 mM PMSF, protease inhibitor cocktail, and 10 mM N-ethylmaleimide, and centrifuged at 20,000 g for 20 min at 4 °C. The cleared lysates (4 mg) were diluted 10-fold with 1% Triton X-100 buffer (50 mM Tris-HCl, pH 7.5, 150

mM NaCl, 1% Triton X-100), and then incubated with 30 µl of K63-Tandem Ubiquitin Binding Entity (TUBE)-Agarose (LifeSensors, UM401), or 2.4 µg of K63-TUBE-Biotin (LifeSensors, UM304) or M1-TUBE-Biotin (LifeSensors, UM306) with 30 µl of Dynabeads M-280 Streptavidin (Veritas), overnight at 4°C. The precipitates were washed five times with 1% Triton X-100 buffer, boiled in SDS sample buffer, and then subjected to immunoblotting analysis.

qPCR. Cell lysis, reverse-transcription, and qPCR were performed with a SuperPrep Cell Lysis RT Kit for qPCR (TOYOBO) and Power SYBR Green PCR Master Mix (Life Technologies), according to the manufacturers' instructions. Quantitative real-time PCR was performed with a Step-One-Plus PCR system (Applied Biosystems) by the $\Delta\Delta CT$ method, using the following oligonucleotides: human *IL-6* sense, 5'-AGCCACTCACCTCTTC-3', and human *IL-6* anti-sense, 5'-GCCTCTTTGCTGCTTT-3'; human *ICAM1* sense, 5'-GTGGTAGCAGCCGCAGT-3' and human *ICAM1* anti-sense, 5'-TTCGGTTTCATGGGGGT-3'; human *BIRC3* sense, 5'-AGATGAAAATGCAGAGTCATCAAT-3' and human *BIRC3* anti-sense, 5'-CATGATTGCATCTTCTGAATGG-3'; and human *GAPDH* sense, 5'-

614 AGCAACAGGGTGGTGGAC-3' and human *GAPDH* anti-sense, 5'-
615 GTGTGGTGGGGGACTGAG-3'.

616 For analyses with mouse-derived immune cells or tissues, total mRNA was
617 extracted with an RNeasy Mini Kit (QIAGEN), and then transcribed to cDNA with
618 ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO), according to the
619 manufacturers' instructions. Quantitative real-time PCR was performed with Power
620 SYBR Green PCR Master Mix (Life Technologies) and a Step-One-Plus PCR system by
621 the $\Delta\Delta CT$ method, using the following oligonucleotides: mouse *Ifnb1* sense: 5'-
622 CCCTATGGAGATGACGGAGA-3' and mouse *Ifnb1* anti-sense: 5'-
623 CTGTCTGCTGGTGGAGTTCA-3'; mouse *Isg15* sense: 5'-
624 GGAACGAAAGCGGCCACAGCA-3' and mouse *Isg15* anti-sense: 5'-
625 CCTCCATGGGCCTTCCCTCGA-3'; mouse *Isg56* sense: 5'-
626 CCCTATGGAGATGACGGAGA-3' and mouse *Isg56* anti-sense: 5'-
627 CTGTCTGCTGGTGGAGTTCA-3'; mouse *Cxcl10* sense, 5'-
628 CCAAGTGCTGCCGTCATTTTC-3' and mouse *Cxcl10* anti-sense, 5'-
629 GGCTCGCAGGGATGATTCAA-3'; mouse *Tnfaip3* sense, 5'-
630 GCTTTCGCAGAGGCAGTAACAG-3' and mouse *Tnfaip3* anti-sense, 5'-
631 AGCAAGTGCAGGAAAGCTGGCT-3'; mouse *Bcl-xl* sense, 5'-

632 GGTGAGTCGGATTGCAAGTT-3' and mouse *Bcl-xl* anti-sense, 5'-
633 GCTGCATTGTTCCCGTAGAG-3'; mouse *Hmox-1* sense, 5'-
634 AAGCCGAGAATGCTGAGTTCA-3' and mouse *Hmox-1* anti-sense, 5'-
635 GCCGTGTAGATATGGTACAAGGA-3'; mouse *Nfe2l2* sense, 5'-
636 TCTTGGAGTAAGTCGAGAAGTGT-3' and mouse *Nfe2l2* anti-sense, 5'-
637 GTTGAACTGAGCGAAAAAGGC-3'; mouse *Tnf* sense, 5'-
638 TAGCCAGGAGGGAGAACAGA-3' and mouse *Tnf* anti-sense, 5'-
639 TTTTCTGGAGGGAGATGTGG-3'; mouse *Il-1b* sense, 5'-
640 CCCTGCAGCTGGAGAGTGTGGA-3' and mouse *Il-1b* anti-sense, 5'-
641 TGTGCTCTGCTTGTGAGGTGCTG-3'; and mouse *Gapdh* sense, 5'-
642 TTTGGCATTGTGGAAGGGCTCAT-3' and mouse *Gapdh* anti-sense, 5'-
643 CACCAGTGGATGCAGGGATGATGT-3'.

644

645 **Analyses of TNFR complexes I and II.** The TNFR complex I analysis was
646 performed as described previously¹⁴. Briefly, parental and *OTUD1*^{-/-}-HeLa cells were
647 stimulated for the indicated times with 1 µg ml⁻¹ FLAG-tagged TNF-α, and then lysed in
648 1 ml lysis buffer, containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100,
649 and complete protease inhibitor cocktail. After centrifugation at 20,000 g for 15 min, the

supernatants were immunoprecipitated with 40 μ l anti-FLAG M2 beads (Sigma) overnight at 4°C. For the TNFR complex II analysis, parental and *OTUD1*^{-/-}-293T cells were treated with 10 ng ml⁻¹ TNF- α and 10 μ g ml⁻¹ CHX. The cell lysates were then immunoprecipitated with an anti-FADD antibody (Santa Cruz; sc-271748) and immunoblotted.

RNA-seq. Parental and *OTUD1*^{-/-}-HeLa cells stimulated with 1 ng ml⁻¹ IL-1 β for 1 and 3 h, and *Otud1*^{+/+}- and *Otud1*^{-/-}-MEFs stimulated with 10 μ g ml⁻¹ poly(I:C) for 2 and 4 h, were lysed, and the total RNA was extracted using a RNeasy Mini kit (Qiagen, 74104) according to the manufacturer's instructions. The mRNA template library was constructed with a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina), and the sequencing analysis was performed by Macrogen Corp. (Korea), using a NovaSeq 6000 sequencer (Illumina) and an S4 Reagent Kit. The data were processed by a Multidimensional Scaling Analysis and a Hierarchical Clustering Analysis by the Euclidean algorithm with the Complete Linkage program.

Cell survival assay. The number of viable cells was measured with a CellTiter-Glo Luminescent Cell Viability Assay (Promega), which quantifies the ATP content, and a

trypan blue exclusion assay. The cell viability was also continuously monitored as an impedance-based cell index, using an xCELLigence RTCA S16 instrument (ACEA Biosciences Inc.). For the experiments, the parental and *OTUD1*^{-/-}-HEK293T cells (40,000 cells/well) or MEF cells (20,000 cells/well) were seeded in an E-Plate VIEW 16 (ACEA Biosciences, Inc.). The next day, the cells were stimulated with TC or TCZ, and the cell index was continuously monitored every 15 min. The data were analysed with real-time cell analysis (RTCA) software, and normalized against the cell indices at the time of TC or TCZ treatment.

ROS measurement. Intracellular ROS levels were measured using an ROS Assay Kit -Highly Sensitive DCFH-DA (Dojindo), which is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of an oxidant, according to the manufacturer's instructions. Briefly, cells were cultured in 96-well black plates (clear bottom) and treated as indicated. After treatment, the cells were washed with HBSS (Gibco), and incubated with the DCFH-DA solution for 30 min at 37°C under a 5% CO₂ atmosphere. ROS production was analysed with a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) with excitation and emission wavelengths at 490 and 520 nm, respectively.

686

687 **Identification of OTUD1-associated proteins by LC-MS/MS.** An empty vector
 688 or a FLAG-tagged OTUD1 expressing vector was transfected into HEK293T cells in 10-
 689 cm dishes. At 24 h after transfection, the cells were washed with HEPES-saline (20 mM
 690 HEPES-NaOH, pH 7.5, 137 mM NaCl) and lysed with 20 mM HEPES-NaOH, pH 7.5,
 691 150 mM NaCl, 1% Triton X-100, and complete protease inhibitor cocktail (Sigma). After
 692 centrifugation at 20,000 g, 4°C for 10 min, the supernatants were incubated with anti-
 693 FLAG M2 Magnetic Beads (M8823, Sigma) for 1 h at 4°C with gentle rotation. The beads
 694 were collected using a magnetic stand and washed four times with the 1% Triton X-100
 695 lysis buffer, and then another three times with 50 mM ammonium bicarbonate. Proteins
 696 on the beads were digested with 200 ng trypsin/Lys-C mix (Promega, V5072) for 16 h at
 697 37°C. The digests were reduced, alkylated, acidified with trifluoroacetic acid (Wako,
 698 206–10731; TFA), and desalted using GL-Tip SDB (GL Sciences, 7820–11200). The
 699 eluates were evaporated in a SpeedVac concentrator and then dissolved in 0.1% TFA and
 700 3% acetonitrile (ACN). The LC-MS/MS analysis of the resultant peptides was performed
 701 on an EASY-nLC 1200 UHPLC connected to an Orbitrap Fusion mass spectrometer
 702 through a nanoelectrospray ion source (Thermo Fisher Scientific). The peptides were
 703 separated on a 75 µm inner diameter × 150 mm C18 reversed-phase column (Nikkyo

Technos) with a linear 4–32% ACN gradient for 0–100 min, followed by an increase to 80% ACN for 10 min and a final hold at 80% ACN for 10 min. The mass spectrometer was operated in the data-dependent acquisition mode with a maximum duty cycle of 3 s. MS1 spectra were measured with a resolution of 120,000, an automatic gain control (AGC) target of 4e5, and a mass range from 375 to 1,500 m/z . HCD MS/MS spectra were acquired in the linear ion trap with an AGC target of 1e4, an isolation window of 1.6 m/z , a maximum injection time of 35 ms and a normalized collision energy of 30. Dynamic exclusion was set to 20 s. Raw data were directly analysed against the SwissProt database restricted to *Homo sapiens*, using Proteome Discoverer version 2.4 (Thermo Fisher Scientific) with the Mascot search engine. The search parameters were as follows: (a) trypsin as an enzyme with up to one missed cleavage; (b) precursor mass tolerance of 10 ppm; (c) fragment mass tolerance of 0.6 Da; (d) carbamidomethylation of cysteine as a fixed modification; and (e) acetylation of protein N-terminus and oxidation of methionine as variable modifications. Peptides and proteins were filtered at a false discovery rate (FDR) of 1%, using the Percolator node and the Protein FDR Validator node, respectively. Label-free quantification was performed based on the intensities of precursor ions, using the Precursor Ions Quantifier node. Normalization was performed such that the total sum of abundance values for each sample over all peptides was the same.

722

723 **PRM analysis.** To quantify KEAP1, AIFM1, CUL3, and PGAM5, two or three
 724 peptides/protein were measured by parallel reaction monitoring (PRM), an MS/MS-based
 725 targeted quantification method using high-resolution MS. The LC-MS/MS analysis was
 726 performed on an EASY-nLC 1200 UHPLC connected to a Q Exactive Plus mass
 727 spectrometer through a nanoelectrospray ion source (Thermo Fisher Scientific). Targeted
 728 HCD MS/MS scans were acquired by a time-scheduled inclusion list at a resolution of
 729 70,000, an AGC target of 2e5, an isolation window of 2.0 m/z , a maximum injection time
 730 of 300 ms, and a normalized collision energy of 27. Time alignment and relative
 731 quantification of the transitions were performed using the Skyline software.

732

733 **Sendai virus infection.** MEFs and BMDMs were infected with Sendai virus (SeV)
 734 strain Cantell at a cell infectious unit (CIU) of 10 for 1 h. The washed cells were then
 735 incubated for the indicated times.

736

737 **Dextran sulfate sodium (DSS)-induced IBD model.** To analyse the DSS-induced
 738 colitis model, 8-week old Wt- and *Otud1*^{-/-}-C57/BL/6 background male mice were used.
 739 Each mouse was weighed once a day and monitored for the appearance of bloody and

loose stools. Mice were sacrificed after a 7 day administration of 2.5% DSS (MW 36,000-50,000, MP Biomedicals) in drinking water. The stool consistency (diarrhea) and visible fecal blood were scored separately on a scale of 0 to 3, as described⁶⁷

Acute hepatitis model mice. LPS with D-galactosamine (GalN)-induced acute hepatitis model mice were prepared by intraperitoneal injections of LPS (10 $\mu\text{g kg}^{-1}$) and GalN (800 mg kg^{-1} , Nacalai Tesque) in 200 μl PBS into 8-week old Wt- and *Otud1*^{-/-}-C57/BL/6 background mice. For the survival test, mice were injected with LPS/GalN and monitored every 15 min for 10 h. For immunoblotting or histopathologic examination, mice were sacrificed at 5 h after injection, and liver tissues were lysed with RIPA buffer or fixed in a 4% paraformaldehyde (PFA) solution.

Sepsis model mice. LPS (20 mg kg^{-1}) was intraperitoneally injected into 8-week old Wt- and *Otud1*^{-/-}-C57/BL/6 background mice, and their survival was monitored.

Histopathological analysis. In the DSS-induced colitis model, colons and spleens were excised. Colons were distended in 0.9% saline and opened longitudinally, and then cut into three segments (proximal, middle, and distal colon). A transverse section of the

spleen was made at the largest extension of the organ. After fixation in a 4% PFA solution, colon and spleen specimens were embedded in paraffin and processed for hematoxylin/eosin (H&E) and immunohistochemical staining. The histologic scores of colitis were evaluated using a quantitative evaluation form, as described⁶⁸.

In the sepsis model or acute hepatitis model, a total of 5 sections of liver tissue (one section each from the left lateral lobe, left middle lobe, right middle lobe, right lateral lobe and caudate lobe) were fixed in a 4% PFA solution, embedded in paraffin, and processed for H&E and immunohistochemical staining.

Immunohistochemical analysis. Paraffin sections (4- μ m thickness) were deparaffinized and dehydrated through a graded ethanol series. 8-Hydroxy-2-deoxyguanosine (8-OHdG) staining was performed using a Histofine MOUSESTAIN kit (Nichirei, 414321) and an anti-8-OHdG (8-hydroxy-2'-deoxyguanosine) antibody (JaICA, MOG-100P) diluted 1:50. To detect cell death (necrosis and/or apoptosis), TUNEL staining was performed using an ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, S7100), according to the manufacturer's instructions. TUNEL-positive cells in the spleens were counted in six 40x fields of each specimen, and quantified as the number of positive cells per field. Hepatocellular death was scored on a semiquantitative

scale of 1 to 3, using the following criteria: 1, $\leq 1\%$ TUNEL-positive cells; 2, $>1\%$ but $\leq 5\%$ TUNEL-positive cells; 3, $>5\%$ TUNEL-positive cells. Microscopic image capture was performed using an Olympus BX53 microscope equipped with a DP72 digital microscope camera and a DP2-BSW image acquisition system (Olympus Corp., Tokyo, Japan).

Gene expression database analysis. The correlation of patient survival and the level of OTUD1 gene expression, and the OTUD1 gene comparison in normal or tumour kidney cortex, were analysed online using GEPIA (<http://gepia.cancer-pku.cn/>). For the analysis of gene expression correlation, TCGA tumour (KIRC), TCGA normal (KIRC), and GTEx (Kidney-Cortex) were selected.

Statistics. One-way ANOVA followed by a post-hoc Tukey HSD test, *t*-test, Mann-Whitney test, and Log-rank test of Kaplan-Meier survival curve were performed using the GraphPad Prism 8 software. For all tests, a *P* value of less than 0.05 was considered statistically significant.

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Author contributions

D.O., K.S., H.T., and K.K. performed biochemical and cell biological experiments, M.G. performed histochemical analyses, H.K. conducted mass spectrometric analyses, and M.S. and S.H. participated in the mouse analyses. H.W., D.T., T.S., and F.T. conceived the project. D.O., M.G., H.K., K.S., and F.T. wrote the manuscript and all of the authors participated in commenting on the manuscript.

Conflict of interest

The authors declare no competing interests.

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Fig. 1 OTUD1 suppresses canonical NF-κB activation through the catalytic activity and the N-terminal region. a, Screening for DUBs that regulate LUBAC-mediated NF-κB activation. Effects of 88 human DUBs on LUBAC-induced NF-κB activation were analysed by a luciferase assay. **b**, Dose-dependent inhibition by OTUD1 on LUBAC- and TNF-α-induced NF-κB activation. Effects of increasing amounts (0.1, 0.3, and 1.0 μg) of OTUD1 were examined with co-

981 expression of LUBAC or 6 h treatment with 10 ng ml⁻¹ TNF-α in HEK293T cells.

982 **c**, Domain structure of wild-type (Wt) and mutants of OTUD1. APGR: Ala-, Pro-,

983 and Gly-rich region; OTU: ovarian tumour protease; UIM: ubiquitin-interacting

984 motif. **d**, Effect of OTUD1 mutants on the LUBAC-induced NF-κB activity. The

985 relative NF-κB activity induced in the presence of Wt or various mutants of

986 OTUD1, and expression levels of OTUD1 and LUBAC subunits are shown. **a**, **b**,

987 **d**, Data are shown as mean ± SD by ANOVA post-hoc Tukey test (*n* = 3 or 4). *:

988 *P*<0.05, **: *P*<0.01, ***: *P*<0.001, ****: *P*<0.0001, NS: not significant. **e**, The N-

989 terminal APGR region is disordered. Intrinsically ordered and disordered

990 segments of OTUD1 were analysed by DICHOT²⁸

991 (<https://idp1.force.cs.is.nagoya-u.ac.jp/dichot/>). **f**, OTUD1 eluted in the high

992 molecular weight fractions. Gel filtration analyses of lysates prepared from

993 parental and *OTUD1*^{-/-} cells, and FLAG-OTUD1-Wt- and FLAG-ΔAPGR-

994 expressing HEK293T cells were performed using a Superdex 200 column.

995 Concentrated fractions were subjected to immunoblotting with the indicated

996 antibodies. *: non-specific signal.

Fig. 2 OTUD1 downregulates inflammatory cytokine-induced canonical NF-κB activation. **a**, The enhanced ubiquitylation and recruitment of NF-κB

1000 signalling factors to TNFR complex I. Parental and *OTUD1*^{-/-}-HeLa cells were
1001 stimulated with 1 µg ml⁻¹ FLAG-TNF-α for the indicated periods, and cell lysates
1002 and anti-FLAG immunoprecipitates were subjected to immunoblotting with the
1003 depicted antibodies. **b**, K63-linked polyubiquitylation of NF-κB activators is
1004 enhanced in *OTUD1*^{-/-} cells. Parental and *OTUD1*^{-/-}-HeLa cells were stimulated
1005 with 20 ng ml⁻¹ TNF-α for the indicated periods, and then pulled down by K63-
1006 TUBE. Samples were subjected to immunoblotting with the indicated antibodies.
1007 **c**, The enhanced IL-1β-induced NF-κB activation in *OTUD1*^{-/-} cells. Parental and
1008 *OTUD1*^{-/-} cells were stimulated with 1 ng ml⁻¹ IL-1β, and analysed as in **a**. **d**, **e**,
1009 Alterations of K63- and M1-linked ubiquitylation of NF-κB activators in *OTUD1*^{-/-}-
1010 HeLa cells. Parental and *OTUD1*^{-/-} cells were stimulated with 1 ng ml⁻¹ IL-1β for
1011 the indicated periods, and pulled down by K63-TUBE (**d**) or M1-TUBE (**e**).
1012 Samples were immunoblotted with the indicated antibodies. **f**, **g**, RNA-seq
1013 analysis. Parental and *OTUD1*^{-/-}-HeLa cells were stimulated with 10 ng ml⁻¹ IL-
1014 1β for 1 and 3 h. The cells were lysed, and subjected to a transcriptome-wide
1015 expression analysis using their extracted total RNA. The principal component
1016 analysis (**f**) and heatmap analysis of significantly varied 34 mRNAs, using the
1017 cut off value of 10 (**g**) were performed. **h**, The enhanced inflammatory responses

1018 in *OTUD1*^{-/-}-HeLa cells. Taking the cut off value of 3.0, pathway analyses of 175
1019 up-regulated genes were performed by MSigDB Hallmark 2020, and are listed in
1020 ascending order of *P*-value.

Fig. 3 OTUD1 upregulates type I IFN antiviral pathway. **a**, The poly(A:T)-mediated IFN antiviral pathway is suppressed in *Otud1*^{-/-}MEFs. *Otud1*^{+/-} and *Otud1*^{-/-}MEF cells were stimulated with 1 μ g ml⁻¹ poly(A:T) for the indicated periods, and the cell lysates were immunoblotted with the indicated antibodies.

b, The expression of IRF3-target genes is suppressed in poly(A:T)-treated *Otud1*^{-/-}-MEF cells. MEFs were stimulated with 1 µg ml⁻¹ poly(A:T) for the indicated periods, and the mRNA levels were assessed by qPCR. **c**, The suppressed poly(I:C)-mediated antiviral response in *Otud1*^{-/-}-MEFs. *Otud1*^{+/-}- and *Otud1*^{-/-}-MEFs were stimulated with 10 µg ml⁻¹ poly(I:C) for the indicated periods, and cell lysates were immunoblotted with the indicated antibodies. **d**, Reduced poly(I:C)-mediated antiviral response in *Otud1*^{-/-}-BMDMs. BMDMs from *Otud1*^{+/-}- and *Otud1*^{-/-}-mice were stimulated with 10 µg ml⁻¹ poly(I:C) for the indicated periods, and a qPCR analysis was performed. **e**, **f**, Sendai virus (SeV)-induced antiviral response is suppressed in *Otud1*^{-/-}-cells. *Otud1*^{+/-}- and *Otud1*^{-/-}-MEFs (**e**) or BMDMs (**f**) were infected with SeV at a CIU of 10 for 1 h, incubated for the indicated periods, and then subjected to qPCR analyses. **b**, **d**, **e**, **f**, Data are shown as mean ± SD by ANOVA post-hoc Tukey test (*n* = 3). *: *P*<0.05, **: *P*<0.01, ***: *P*<0.001, ****: *P*<0.0001, NS: not significant. **g-i** Suppressed transcription of IRF3-target genes in *Otud1*^{-/-}-MEFs. *Otud1*^{+/-}- and *Otud1*^{-/-}-MEFs were stimulated with 10 µg ml⁻¹ poly(I:C) for 2 and 4 h, and RNA-seq analyses were performed. **g**, A principal component analysis of the RNA-seq analysis. **h**, Taking the cutoff value of 10, significantly varied mRNAs are shown by the

1044 heatmaps. i, Taking the cutoff value of 3.0, pathway analyses of 179 down-
1045 regulated genes were performed by MSigDB Hallmark 2020, and listed in
1046 ascending order of *P*-value.

Fig. 4 OTUD1 regulates TNF- α -induced apoptosis and necroptosis. a,

Increased TNF- α -induced cell death by the genetic ablation of OTUD1. Wt- and

OTUD1 $^{-/-}$ -HeLa and HEK293T cells were treated with or without 10 ng ml $^{-1}$ TNF-

α and 10 μ g ml $^{-1}$ CHX (TC), and trypan blue-positive cells were counted. **b,**

1052 Reduced cell viability of *OTUD1*-deficient cells. Wt- and *OTUD1*^{-/-}-HEK293T cells
1053 were treated with TC, and the cell proliferation was measured by xCELLigence
1054 real-time cell monitoring. **c**, Enhanced apoptosis in *OTUD1*^{-/-}-HEK293T cells. Wt-
1055 and *OTUD1*^{-/-}-HEK293T cells were treated with TC, and cell lysates were
1056 immunoblotted with the indicated antibodies. **d**, TNFR complex II formation was
1057 accelerated in *OTUD1*^{-/-}-HEK293T cells. Wt- and *OTUD1*^{-/-}-HEK293T cells were
1058 treated with TC as in **c**. The cell lysates and anti-FADD immunoprecipitates were
1059 then blotted with the indicated antibodies. **e**, Increased necroptosis in *Otud1*^{-/-}-
1060 MEFs. *Otud1*^{+/+}- and *Otud1*^{-/-}-MEFs were treated with 10 ng ml⁻¹ TNF-α, 10 μg ml⁻¹
1061 ¹ CHX, and 20 μM ZVAD (TCZ), and the cell proliferation was measured as in **b**.
1062 **f**, Increased phosphorylation of MLKL and RIP3 in TCZ-treated *Otud1*^{-/-}-MEFs.
1063 MEFs were treated with TC with or without ZVAD as indicated, and the cell
1064 lysates were immunoblotted with the depicted antibodies. **g**, Necrostatin-1 (Nec-
1065 1) rescued cell death. *Otud1*^{+/+}- and *Otud1*^{-/-}-MEFs were treated with TC, ZVAD,
1066 and/or 100 μM Nec-1 for 8 h, as indicated, and cell viability was analysed by a
1067 Celltiter Glo assay. **h**, Enhanced K63-ubiquitylation of RIP3 in *Otud1*^{-/-}-MEFs.
1068 MEFs were treated with TCZ for the indicated periods, and cell lysates and K63-
1069 or M1-TUBE precipitates were immunoblotted with the indicated antibodies. Data

1070 are shown as mean $\pm$ SD by ANOVA post-hoc Tukey test (**a**, **g**; $n = 3$) or *t*-test at
 1071 each time point (**b**, **e**; $n = 4$). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$,
 1072 NS: not significant.

Fig. 5 OTUD1 regulates KEAP1-mediated oxidative stress response and ROS-associated cell death. **a**, Identification of OTUD1-interacting proteins. FLAG-tagged OTUD1 was expressed in HEK293T cells and immunoprecipitated

1077 with an anti-FLAG antibody, and the co-immunoprecipitants were then analysed
1078 by MS. Proteins with an abundance ratio greater than 50, and values for CUL3
1079 and PGAM5 are shown. **b**, OTUD1 physiologically interacts with KEAP1.
1080 HEK293T cell lysates and anti-OTUD1 immunoprecipitates were subjected to
1081 immunoblotting with the indicated antibodies. **c**, OTUD1 regulates K63-
1082 ubiquitylation of KEAP1. Cell lysates and K63-TUBE precipitates from *Otud1*^{+/-}-
1083 and *Otud1*^{-/-}-MEFs were immunoblotted with the indicated antibodies. **d**, APGR
1084 of OTUD1 is required for KEAP1-binding. Wt- and mutants of Myc-OTUD1 were
1085 expressed with HA-KEAP1 in HEK293T cells. Cell lysates and anti-Myc-
1086 immunoprecipitates were immunoblotted with the indicated antibodies. **e**, The
1087 Kelch domain and the C-terminal region (CTR) of KEAP1 are responsible for
1088 OTUD1-binding. A similar analysis as in **d** was performed using Wt- and
1089 mutants of HA-KEAP1 and Myc-OTUD1. **f**, OTUD1 contains an ETGE motif in
1090 APGR. Localization of the ETGE motif in OTUD1, and amino acid sequence
1091 alignment with NRF2 are shown ⁴¹. h: human, m: mouse, c: chicken, z: zebrafish.
1092 **g**, The ETGE motif in OTUD1 is indispensable for KEAP1-binding. Myc-tagged
1093 Wt- or ETGE motif-deleted mutant of OTUD1 was expressed with HA-KEAP1-Wt
1094 as indicated, and cell lysates and anti-HA immunoprecipitates were

1095 immunoblotted with the depicted antibodies. **h**, Enhanced hydrogen peroxide-
1096 induced ROS generation in *Otud1*-deficient cells. *Otud1*^{+/+}- and *Otud1*^{-/-}-MEFs
1097 were treated with 0.3 mM H₂O₂ for the indicated periods, and the intracellular
1098 ROS levels were analysed by a DCFH-DA assay. **i**, Enhanced expression of
1099 NRF2 target genes in *Otud1*^{-/-}-MEFs. MEFs were treated with or without 0.3 mM
1100 H₂O₂ for 3 h, and a qPCR analysis was performed. **j**, Reduced cell viability in
1101 *Otud1*^{-/-}-MEFs under oxidative stress. MEFs were treated with 0.3 mM H₂O₂ for
1102 the indicated periods, and cell viability was analysed by Celltiter Glo assay. **k**,
1103 DUB activity of OTUD1 is necessary to suppress ROS production. OTUD1-Wt or
1104 active site mutant (CA) was restored into *Otud1*^{-/-}-MEFs, and ROS levels were
1105 analysed by a DCFH-DA assay after treatment with or without 0.3 mM H₂O₂ for 2
1106 h. **l**, DUB activity of OTUD1 protects cells from H₂O₂-induced death. A similar
1107 treatment as in **k** was performed with or without 0.1 mM H₂O₂ for 8 h, and cell
1108 viability was analysed by a Celltiter Glo assay. Data are shown as mean ± SD by
1109 *t*-test of each time point (*n* = 4, **h**, **j**) or ANOVA post-hoc Tukey test (*n* = 4, **i**, **k**, **l**).
1110 *: *P*<0.05, **: *P*<0.01, ***: *P*<0.001, ****: *P*<0.0001, NS: not significant.

Fig. 6 Enhanced inflammation, oxidative damage, and cell death in mouse

1113 **inflammatory disease models. a, b**, Colon damage in DSS-administered *Otud1*^{-/-}
1114 ^{-/-}-mice. *Otud1*^{+/-}- and *Otud1*^{-/-}-mice were treated with 2.5% DSS for 7 days, and
1115 then sacrificed. Representative images of H&E staining of distal colon (**a**) and
1116 histologic scores of colitis (**b**) (*n* = 6-7) are indicated. *Bars*; 100 μm. **c**, Increased
1117 expression of NF-κB target genes in DSS-treated *Otud1*^{-/-}-mice. qPCR analysis
1118 of NF-κB targets in distal colon from DSS-treated mice (*n* = 6). **d, e**, Increased
1119 oxidative DNA damage and cell death in DSS-administered *Otud1*^{-/-}-mice.
1120 Specimens as in **a** were stained for 8-OHdG (**d**) or TUNEL (**e**). *Bars*: 50 μm. **f**,
1121 *Otud1*^{-/-}-mice were labile for the LPS/GalN-induced acute hepatitis model. After
1122 injections of LPS (10 μg kg⁻¹) and GalN (800 mg kg⁻¹), the % survivals of *Otud1*^{+/-}-
1123 (*n* = 5) and *Otud1*^{-/-} (*n* = 6) mice were determined by the Kaplan-Meier method
1124 with the Log-rank test. **g**, Enhanced liver damage in *Otud1*^{-/-}-mice. H&E and
1125 TUNEL staining of livers from *Otud1*^{+/-}- and *Otud1*^{-/-}-mice after 5 h administration
1126 of LPS/GalN, and hepatocellular death scores (*n* = 5) are shown. *Bars*: 100 μm
1127 (*upper panels*) and 50 μm (*lower panels*). **h**, Increased caspase 3 activation in
1128 hepatocytes from *Otud1*^{-/-}-mice. After a 5 h treatment with LPS/GalN, livers were
1129 excised, lysed, and immunoblotted with the indicated antibodies. **b, c, g**, Data

1130 are shown as scatter plots and evaluated by Mann-Whitney test. *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$, NS: not significant.

1131 $P < 0.01$, ****: $P < 0.0001$, NS: not significant.

1138 *KEAP1* in kidney cancer. Correlations of expression levels with Pearson
 1139 correlations and *P*-values of *OTUD1* vs. *KEAP1* (*upper panels*) or *OTUD1* vs.
 1140 *NF-κB1* (*lower panels*) in normal (*left panels*) and tumour (*right panels*) kidney
 1141 cortex are shown. **c**, Schematic representation of OTUD1 functions in the
 1142 regulation of inflammatory and KEAP1-mediated oxidative stress responses,
 1143 and cell death pathways.