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RIPK3 regulates p62–LC3 complex formation via the caspase-8-dependent cleavage of p62

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ABSTRACT

RIPK3 is a key molecule for necroptosis, initially characterized by necrotic cell death morphology and the activation of autophagy. Cell death and autophagic signaling are believed to tightly regulate each other. However, the associated recruitment of signaling proteins remains poorly understood. p62/sequestosome-1 is a selective autophagy substrate and a selective receptor for ubiquitinated proteins. In this study, we illustrated that both mouse and human RIPK3 mediate p62 cleavage and that RIPK3 interacts with p62, resulting in complex formation. In addition, RIPK3-dependent p62 cleavage is restricted by the inhibition of caspases, especially caspase-8. Moreover, overexpression of A20, a ubiquitin-editing enzyme and an inhibitor of caspase-8 activity, inhibits RIPK3-dependent p62 cleavage. To further investigate the potential role of RIPK3 in selective autophagy, we analyzed p62–LC3 complex formation, revealing that RIPK3 prevents the localization of LC3 and ubiquitinated proteins to the p62 complex. In addition, RIPK3-dependent p62–LC3 complex disruption is regulated by caspase inhibition. Taken together, these results demonstrated that RIPK3 interacts with p62 and regulates p62–LC3 complex formation. These findings suggested that RIPK3 serves as a negative regulator of selective autophagy and provides new insights into the mechanism by which RIPK3 regulates autophagic signaling.

1. Introduction

Receptor-interacting protein kinase 3 (RIP3/RIPK3) is a critical regulator of necroptosis. Necroptosis is a programmed necrotic cell death process driven by a defined molecular pathway. Necroptosis was originally observed under caspase inhibition in vitro [1,2] and confirmed in caspase-8-deficient mice [3,4]. Importantly, necroptosis can also occur in the absence of caspase inhibition in various situations [5,6], and it has an important pathophysiological role in pancreatitis [7,8], inflammatory bowel disease [9,10], and many other disorders. RIPK3 has an active kinase domain in its N-terminus that is conserved in other RIPKs and is required for necroptosis [7,11]. RIPK3 forms an intracellular complex with RIPK1 to assemble the necrosome [12]. RIPK3 also associates with the RIPK1/FADD/caspase-8 complex [7]. Recent studies have identified the mixed lineage kinase domain-like protein as an interacting partner of RIPK3 [13].

Autophagy is the common name for lysosome-based degradation of cytosolic cargos [14]. Autophagy is considered to be a nonselective, bulk process. A small portion of the cytoplasm is engulfed by an isolation membrane, which results in the formation of an autophagosome. Microtubule-associated protein 1 light chain 3 (LC3) is a marker of the autophagosome. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE). In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome [15]. Selective autophagy ensures the recognition and removal of various cytosolic cargos [16]. Selective autophagy is mediated by selective autophagy receptors, such as p62 (also known as sequestosome-1) and NBR1. p62 is a stress-inducible intracellular protein

Abbreviations: UBA domain, ubiquitin-associated domain; LIR, LC3-interacting region; LRS, LC3 recognition sequence; PLA, proximity ligation assay; TNT, in vitro translation.

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known to regulate various signal transduction pathways involved in cell survival and death [17]. By directly binding to LC3 via its LC3-interacting region (LIR) motif, p62 becomes a selective autophagy receptor, transporting ubiquitinated protein aggregates to the autophagosome. As p62 was identified as one of the specific substrates degraded through the autophagy–lysosomal pathway [18,19], the total cellular expression of p62 can be used to monitor autophagic flux [15]. However, recent studies indicated that p62 expression can be also regulated by transcription and protein cleavage [20,21]. In addition, p62 can suppress autophagy [22]. Although autophagy modulates the levels of p62 protein, there are potential limitations of evaluating autophagic flux by p62.

Dying cells often display an accumulation of autophagosomes and hence adopt a morphology called autophagic cell death. However, in many cases, autophagic cell death is called cell death with autophagy rather than cell death by autophagy [23]. Necroptosis is originally characterized by necrotic cell death morphology and the activation of autophagy [5], and interplay between autophagy and RIPK1-dependent necroptotic cell death has been reported [24]. However, how necroptotic and autophagic signaling proteins are recruited remains poorly understood. Given the potential importance of RIPK3 in necroptosis and the potential importance of p62 in autophagy, we investigated whether RIPK3 can regulate p62.

2. Materials and methods

2.1. Cell culture and reagents

HEK293T cells were cultured in DMEM (Sigma) with fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C. Mouse p62 cDNA was kindly provided by Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science). Mouse p62 cDNA was cloned into the p3XFLAG-CMV-10 expression vector (Sigma). Mouse Myc-RIPK3 and mouse Flag-A20 expression vectors were kindly provided by Dr. Averil Ma (UCSF). Human RIPK3 cDNA was amplified by RT-PCR from the total RNA of Jurkat cells and then cloned into pCMV-3tag-2C plasmids (Agilent Technologies). The pan-caspase inhibitor Z-VAD-FMK was purchased from Peptide Institute. The caspase-8 inhibitor Z-IETD-FMK was purchased from R&D SYSTEMS.

2.2. DNA and siRNA transfection

HEK293T cells were transiently transfected empty plasmid and/or expression plasmids using TransIT-LT1 (Mirus) as indicated by the supplier. OnTarget Plus SMARTpool siRNA oligonucleotides specific for human caspase-8 and nontargeting siRNA (control siRNA) were purchased from ThermoFisher Scientific. HEK293T cells seeded on 6-well plates were firstly transfected with 150 pmol of siRNA using Lipofectamine RNAiMAX (Invitrogen); after 8 h, p62 and RIPK3 expression plasmids were transfected using TransIT-LT1, according to the manufacturer's protocol.

2.3. Immunoblotting and immunoprecipitation

Cells were incubated in lysis buffer [either 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% CHAPS, 10% glycerol, 2 mM NEM, Halt protease, and phosphatase inhibitor cocktail (Pierce) or 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 2 mM NEM, and protease inhibitors] on ice for 20 min and centrifuged at 14,000×*g* for 20 min. For Flag immunoprecipitation, cell lysates were incubated with anti-Flag M2 beads (Sigma) for 3 h at 4 °C. Samples were resolved on NuPage precast 4–12% Bis–Tris gels (Invitrogen) and transferred to a PVDF membrane. The following antibodies and reagents were used for immunoprecipitation and immunoblotting studies: anti-β-actin and anti-Flag (SIGMA); anti-LC3 (PD014, MBL), anti-A20 (5630, Cell Signaling), anti-Ub (P4D1), and anti-Myc (A-14) (Santa Cruz).

2.4. In situ proximity ligation assay (PLA)

To detect protein interactions in HEK293T cells, the Duolink PLA in situ kit (SIGMA–ALDRICH, 92101) was used, according to the manufacturer's instructions. To analyze the interaction between RIPK3 and p62 (Fig. 2), the primary antibodies were rabbit anti-c-Myc antibody (A-14, Santa Cruz) and mouse anti-Flag antibody (F3165, SIGMA). To analyze the interaction between LC3 and p62 (Fig. 4B), the primary antibodies were rabbit anti-Flag antibody (F7425, SIGMA) and mouse anti-LC3 antibody (M152-3, MBL). As a control, the primary antibody was mouse control IgG (Vector Laboratories, I-2000). Images were acquired with a confocal laser microscope (FV10i, Olympus) using a ×60 oil-immersion objective lens.

3. Results

3.1. RIPK3 mediates p62 cleavage

To investigate the potential roles of RIPK3 in regulating autophagy, we introduced Myc-mRIPK3 and Flag-p62 into HEK293T cells. HEK293T cells do not express any detectable endogenous human RIPK3 [7]. Immunoblotting analyses were performed with extracts from HEK293T cells with or without Flag-p62. A band corresponding to ~62 kDa was detected with anti-Flag antibody. This result indicated that the ~62-kDa band was the full-length p62 protein. Surprisingly, ~45-kDa and ~35-kDa bands were also observed using the same anti-Flag antibody in the presence of mouse RIPK3 (Fig. 1A). This result suggested that the ~45-kDa and ~35-kDa fragments were the products of p62 cleavage (Fig. 1A). As we performed this analysis in human HEK293T cells, we then investigated whether this p62 cleavage is specific for mouse RIPK3. We cloned human RIPK3 into an expression vector, and then human RIPK3 was transfected with Flag-p62 into HEK293T cells. Similar observations were also made using human RIPK3. Again, the ~45-kDa and ~35-kDa bands were detected in the presence of human RIPK3 (Fig. 1B). Taken together, these results suggested that RIPK3 mediates p62 cleavage in HEK293T cells.

3.2. RIPK3 forms a complex with p62

p62 regulates various signal transduction pathways and interacts with many molecules [17]. To understand how RIPK3 mediates p62 cleavage, we investigated whether RIPK3 binds to the p62 complex. We used PLA to detect whether Myc-RIPK3 interacts with Flag-p62. In this assay, a pair of oligonucleotide-labeled secondary antibodies (PLA probes) generates an individual fluorescent signal when bound to two primary antibodies in close proximity [25]. Although no PLA foci were detected in the presence of Flag-p62 or Myc-RIPK3 alone, many PLA foci were observed in the presence of both Flag-p62 and Myc-RIPK3 (Fig. 2). When we used control mouse IgG instead of mouse anti-Flag antibody, PLA foci were not observed even in the presence of both Flag-p62 and Myc-RIPK3. These data illustrated that Flag-p62 and Myc-RIPK3 interact in situ.

3.3. p62 is cleaved by caspase-8

Self-oligomerization of p62 is essential for its localization to the autophagosome formation site [26]. p62 also interacts with

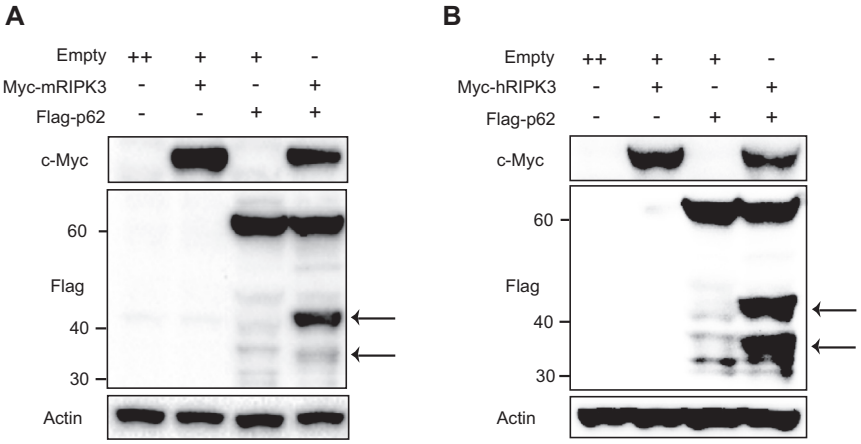

Fig. 1. Receptor-interacting protein kinase 3 (RIPK3) mediates p62 cleavage. (A) HEK293T cells were transfected with Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids. (B) HEK293T cells were transfected with Myc-tagged human RIPK3 and/or Flag-tagged p62 expression plasmids. Thirty-six hours post-transfection, cells were harvested. Flag, c-Myc, and actin antibodies were used for immunoblotting. The resultant cleavage products are indicated by arrows. The positions of the molecular mass markers are indicated in kDa at the left of the panels. Data are representative of two independent experiments.

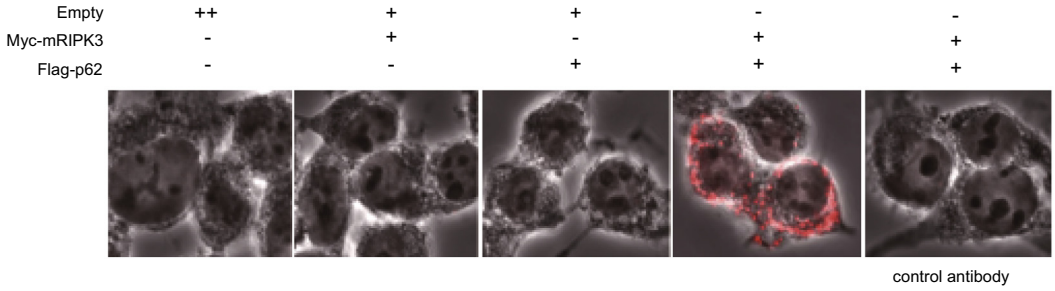

Fig. 2. Receptor-interacting protein kinase 3 (RIPK3) forms a complex with p62. HEK293T cells were transfected with Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids. Thirty-six hours post-transfection, the Duolink proximity ligation assay (PLA) demonstrated the close proximity of Flag-p62 and Myc-mouse RIPK3. Rabbit anti-c-Myc and mouse anti-Flag antibodies were used for PLA. Mouse IgG was used as a control. Data are representative of two independent experiments.

signaling molecules through several domains. The ubiquitin-associated (UBA) domain is capable of interaction with ubiquitinated proteins (Fig. 3A). p62 was identified as one of the specific substrates through the autophagy–lysosomal pathway. This degradation is mediated by an interaction with LC3 though the LIR domain [27]. Human p62 has several potential cleavage sites. TNT reaction assays revealed that human p62 is cleaved by rCaspase-6, rCaspase-8, and rCalpain [21]. A schematic representation of the domains of mouse p62 is presented in Fig. 3A. The putative cleavage site and LIR domain are highly conserved between human and mouse p62.

To study how RIPK3 mediates p62 cleavage, we first tested whether caspases induce p62 cleavage. We introduced Myc-mRIPK3 and Flag-p62 into HEK293T cells and treated the cells with caspase inhibitors. Although Myc-mRIPK3 expression might be enhanced by caspase inhibition, p62 cleavage was blocked completely by the addition of a pan-caspase inhibitor (Z-VAD-FMK) and was concentration-dependently inhibited by a caspase-8 inhibitor (Z-IETD-FMK) (Fig. 3B). These data indicated that caspases, especially caspase-8, might be involved in the regulation of p62 cleavage. However, the proper final concentration of inhibitors may vary between experiments, and high concentrations of inhibitors results in non-specific effects. Next, to test whether caspase-8 is required for p62 cleavage, we silenced caspase-8 with siRNA oligos in HEK293T cells. Caspase-8 expression was efficiently silenced in HEK293T compared with actin expression (Fig. 3C). Whereas the levels of RIPK3 in HEK293T cells might be enhanced after caspase-8 silencing, p62 cleavage was inhibited under the condition of caspase-8 deficiency (Fig. 3C). These data imply that p62 is cleaved by caspase-8. The ubiquitin-editing enzyme A20 binds to caspase-8, and overexpression of A20 inhibits caspase-8 activity in HEK293T cells [28]. To further confirm the role of caspase-8, we transfected mouse A20 together with Myc-mRIPK3 and Flag-p62. Although full-length p62 expression was not influenced by A20 expression, cleaved bands disappeared in an A20 expression-dependent manner (Fig. 3D). These data indicated that A20 may regulate RIPK3-dependent p62 cleavage though the inhibition of caspse-8 activity. Taken together, p62 is cleaved by caspase-8 under RIPK3 expression.

3.4. RIPK3 regulates complex formation between p62 and LC3

In addition to the binding capacity to LC3, p62 is also considered a receptor facilitating the delivery of ubiquitinated cargos to deliver them to the autophagosomes as a result of the presence of the UBA domain of p62 [17]. Therefore, we next examined whether p62 cleavage regulates p62 complex formation. We transfected Flag-p62 into HEK293T cells and pulled down the p62 complex with anti-Flag antibody (Fig. 4A). Flag-p62 bound to LC3-II and poly-ubiquitinated protein. Consistent with previous reports, p62 bound to LC3-II. Putative p62 cleavage sites are located upstream of the LC3 binding site and ubiquitin binding site. Based on predictive algorithms, the ~45-kDa fragments of p62 lacked ubiquitin binding sites, and the ~35-kDa fragments lacked ubiquitin binding sites and an LC-3 binding site. When we transfected Myc-mRIPK3 together with Flag-p62, we confirmed Myc-mRIPK3 in association with Flag-p62 by both the PLA and immunoprecipitation. Moreover, p62–LC3 and p62–polyubiquitin complex formation was prevented by the presence of RIPK3. These data illustrated that RIPK3 regulates p62 complex formation by p62 cleavage.

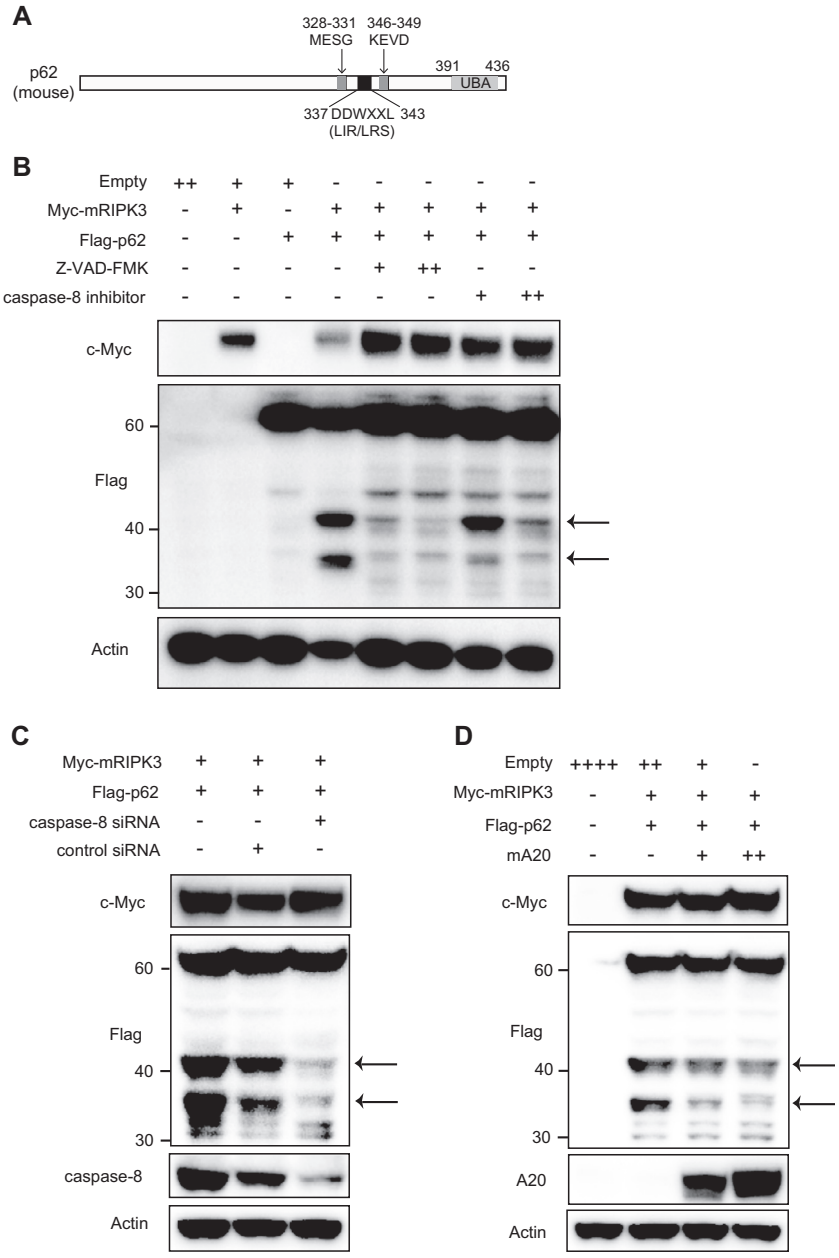

Fig. 3. p62 is cleaved by caspase-8. (A) Domain structure of mouse p62. Microtubule-associated protein 1 light chain 3 (LC3) recognizes p62 through the LC3-interacting region (LIR)/LC3 recognition sequence (LRS) domain. p62 interacts with ubiquitinated proteins via the ubiquitin-associated domain. Potential caspase-8 cleavage sites in mouse p62 were mapped to its C-terminus. Cleavage sites are based on predictive algorithms [21]. (B) HEK293T cells were transfected with Myc-tagged mouse receptor-interacting protein kinase 3 (RIPK3) and/or Flag-tagged p62 expression plasmids. Cells were treated with Z-VAD-FMK (20 or 50 nM) or caspase-8 inhibitor (10 or 50 nM) for 24 h. (C) HEK293T cells were transfected with a nontargeting control siRNA or siRNAs against human caspase-8. Eight hours later, Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids were transfected. After 36 h, cells were lysed and analyzed by immunoblotting. (D) HEK293T cells were transfected with Myc-tagged mouse RIPK3, Flag-tagged p62, and/or mouse A20 expression plasmids. Thirty-six hours post-transfection, cells were harvested. All data are representative of three independent experiments.

To confirm these observations in situ, we perform PLA using Flag and LC-3 antibody. We detected the Flag-p62–LC3 complex in situ in HEK293T cells. We observed decreased numbers of PLA foci in the presence of RIPK3 (Fig. 4B). This result is consistent with a previous report demonstrating that the colocalization of LC3 with the p62 mutant (lacking both LIR and the UBA domain) was profoundly inhibited [26]. In addition, RIPK3-dependent disruption of p62–LC3 complex is restored by caspase inhibition. These data indicated that p62 cleavage regulates p62–LC3 complex formation. Taken together, RIPK3 regulates the association of p62 with LC3 through caspase-8 activity.

4. Discussion

We have identified a unique mechanism by which RIPK3 regulates p62–LC3 complex formation. Our results indicated that RIPK3 forms a complex with p62 and mediates p62 cleavage by caspase-8. These findings provide new insights into the mechanism by which necroptosis signaling regulates autophagic signaling.

To investigate the RIPK3-dependent mechanism, we performed co-transfection assays in human HEK293T cells. HEK293T can sufficiently mimic RIPK3-deficient cells with high transient transfection efficiency [8,29]. As the p62 amino acid sequence is highly

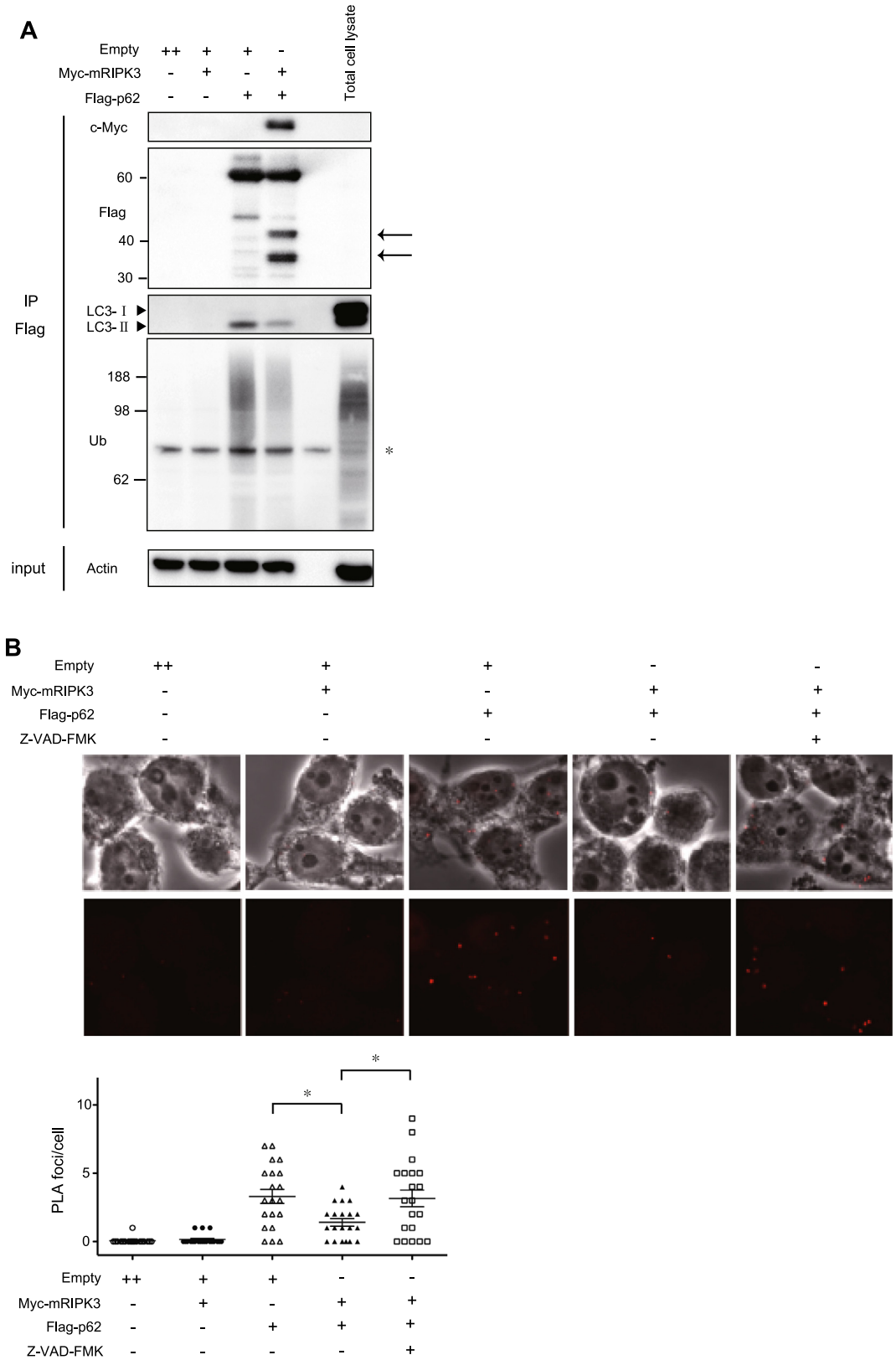

Fig. 4. Receptor-interacting protein kinase 3 (RIPK3) regulates the association of p62 with microtubule-associated protein 1 light chain 3 (LC3). (A) RIPK3 releases LC3 from p62. HEK293T cells were transiently cotransfected with mouse RIPK3 and/or p62 expression plasmids. Thirty-six hours post-transfection, protein extracts were immunoprecipitated (IP) with Flag antibody and immunoblotted for the indicated proteins. The total cell lysate of HEK293T cells was used as a positive control for immunoblotting. *: indicates a nonspecific band. (B) HEK293T cells were transfected with Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids. Thirty-six hours post-transfection, the Duolink proximity ligation assay (PLA) demonstrated the close proximity of Flag-p62 and LC-3. Rabbit anti-Flag and mouse anti-LC-3 antibodies were used for PLA. Mouse IgG was used as a control. Cells were treated with Z-VAD-FMK (50 nM) for 24 h. The numbers of PLA foci per cell are shown ($n = 20$ per group). The student's unpaired t-test was performed for the statistical analysis. $^{*}P \leqslant 0.05$. Data are representative of two independent experiments.

conserved between humans and mice, we used a mouse p62 expression vector. Thus, we could detect RIPK3-dependent p62 cleavage.

Human RIPK3 is cleaved at Asp328 by caspase-8 in HeLa or 293T cells under stimulation [29]. In contrast to the cleavage of p62, we could not detect obvious cleavage of RIPK3 in our study. A potential explanation is that we used HEK293T cells without stimulation. However, overexpression of RIPK3 mediates caspase-8-dependent p62 cleavage. Because a previous paper revealed that p62 associates with caspase-8 [28], RIPK3 may regulate p62–caspase-8 complex formation. In addition, there were some weak bands other than the ~45-kDa and ~35-kDa fragments (Figs. 1 and 3). These bands might be cleaved by other enzymes, such as caspase-6 [21]. Further investigation is required to clarify the mechanisms by which RIPK3 regulates p62 cleavage.

Although cleaved fragments of p62 disappeared upon caspase inhibition, full-length p62 expression was not obviously affected (Fig. 3B–D). However, we revealed that RIPK3-dependent disruption of the p62–LC3 complex was regulated by caspase inhibition. One potential reason is that cleaved fragments of p62 may prevent full-length p62 self-oligomerization [26]. In contrast to p62, Myc-RIPK3 protein expression may be enhanced by caspase inhibition in our systems (Fig. 3B–D). These results are consistent with a previous study reporting that RIPK3 protein was significantly overexpressed in the small intestine in caspase-8-deficient mice compared with the findings in the control littermate mice [9].

The total cellular expression levels of p62 can be used to monitor autophagic flux [15]. However, we demonstrated that p62 expression is regulated by some types of cleavage under RIPK3 expression. This may be an important mechanism of selective autophagy to control protein degradation during necroptosis.

Our data illustrated that RIPK3 interferes with p62–LC3 complex formation in the absence of Z-VAD treatment. These data indicated that RIPK3 constitutively attenuates selective autophagy, including the degradation of aggresomes, mitochondria, and invading bacteria [30]. When necroptosis is triggered by Z-VAD, death receptors, and other signals, RIPK3 induces downstream death signaling and enhances selective autophagy. On the contrary, a recent paper uncovered that p62 can suppress autophagy via mTORC1 activation [17,22,31]. These findings indicated that RIPK3 may regulate both selective autophagy and the induction of autophagy via the caspse-8-dependent cleavage of p62 under some conditions. However, to confirm this hypothesis, we need to analyze RIPK3 kinase-dead mutants under necroptotic stimulation. Because necroptosis can also occur in the absence of caspase inhibition, we should analyze RIPK3-dependent disruption of the p62–LC3 complex under pathophysiological conditions [32].

In conclusion, we demonstrated the functions of RIPK3 in regulating p62 cleavage by casapse-8. These functions are critical for p62–LC3 complex formation. In addition to a new molecular mechanism of p62 regulation, these studies provide critical insights into the regulation of necroptosis and autophagy.

Conflict of interest

The authors declare that they have no conflicts of interest.

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