A cytosolic heat shock protein 90 and cochaperone CDC37 complex is required for RIP3 activation during necroptosis

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Receptor-interacting protein kinase 3, RIP3, and a pseudokinase mixed lineage kinase-domain like protein, MLKL, constitute the core components of the necroptosis pathway, which causes programmed necrotic death in mammalian cells. Latent RIP3 in the cytosol is activated by several upstream signals including the related kinase RIP1, which transduces signals from the tumor necrosis factor (TNF) family of cytokines. We report here that RIP3 activation following the induction of necroptosis requires the activity of an HSP90 and CDC37 cochaperone complex. This complex physically associates with RIP3. Chemical inhibitors of HSP90 efficiently block necroptosis by preventing RIP3 activation. Cells with knocked down CDC37 were unable to respond to necroptosis stimuli. Moreover, an HSP90 inhibitor that is currently under clinical development as a cancer therapy was able to prevent systemic inflammatory response syndrome in rats treated with TNF- α . HSP90 and CDC37 cochaperone complex-mediated protein folding is thus an important part of the RIP3 activation process during necroptosis.

HSP90 | CDC37 | RIP3 | necrosis | kinase

Necroptosis is a form of necrotic cell death that is executed by a defined biochemical pathway. The key signaling molecule of this pathway is the protein kinase receptor interacting protein kinase 3 (RIP3) (1–3). RIP3 is expressed in a cell type-specific manner, with pronounced expression in hematopoietic cells including lymphocytes and macrophages, as well as in endothelial cells that line the gastric-intestinal tract (2). A variety of upstream signaling molecules have been shown to transduce signals to RIP3 to trigger necrosis. These include the close relative RIP1, which signals the activation of the tumor necrosis factor (TNF) family of cytokines; TRIF, which activates RIP3 upon the activation of toll-like receptors; and DAI, which causes necrosis upon viral infection (4, 5). All of these upstream proteins contain RIP homotypic interaction motif (RHIM) domains, through which they interact with a similar domain in RIP3. The activation of RIP3 is marked by the phosphorylation of the serine 227 site of human RIP3; this causes the formation of amyloid-like structures that can be observed as dots under a light microscope (6, 7). This serine phosphorylation is required for the interaction of RIP3 with its substrate MLKL (7).

MLKL is a pseudokinase that contains an N-terminal helix bundle of four α -helices that is connected to its C-terminal kinase-like domain through a two-helix linker (8). The protein typically exists in an inactive monomer form in live cells. Upon binding to RIP3 through its kinase-like domain, human MLKL is phosphorylated at two adjacent sites: threonine 357 and serine 358 (7). These phosphorylations drive MLKL toward an oligomeric state that allows MLKL to translocate from the cytosol to the plasma and intracellular membranes by binding to phosphoinositol phosphates and cardiolipin (9–13). The oligomer MLKL either directly disrupts membrane structures or indirectly damages membrane structures through calcium and/or sodium channel-mediated ion influxes, resulting in necrotic cell death.

RIP3–MLKL-mediated cell death is an important component of antiviral responses of host animals (1, 4, 14, 15). This process can also be activated by the host immune system in response to tissue injury. Such a response may have detrimental effects to the host animal through secondary, immune-inflicted multitissue damage (4). Blocking necroptosis could therefore improve the outcome of diseases with such an element. For example, a lethal systematic inflammatory response syndrome induced by TNF- α injection could be mitigated when the animals are lacking such a response (16).

Cellular kinases are often associated with heat shock proteins, a protein family that is elevated upon stresses (17). HSP90 and its cochaperones are such an important part of kinase activation that small molecule inhibitors have been developed to treat cancers in which the aberrant activation of kinases drives cancer cell growth. Inhibition of HSP90 in those cancer cells selectively drives the overexpressed or mutated active kinase toward degradation, thereby curbing cancer cell growth (18). Such strategies have been advanced in more than 60 clinical trials for a variety of cancer indications over the last 10 years.

In the course of our research into necroptosis, we noticed that RIP3 kinase is normally associated with the HSP90–CDC37 cochaperone complex. In this report, we present biochemical and genetic evidence to support the assertion that this cochaperone complex is required for RIP3 activation and the induction of necroptosis. This finding should have significant implications for the ongoing clinical trials that are based on HSP90 inhibitors.

Significance

Necroptosis is a form of programmed necrotic cell death controlled by receptor-interacting protein kinase 3, RIP3. RIP3 exists in live cells as a latent form in the cytosol and is activated by death-inducing cytokines such as tumor necrosis factor. We report here that RIP3 activation requires Heat Shock Protein 90, HSP90, and its cochaperone protein CDC37. HSP90 inhibitors, currently under investigation in clinical trials for a variety of cancer indications, are capable of inhibiting necroptosis at concentrations below the clinically achieved concentration in patients' sera. Since the components of the necroptosis pathway are being pursued as potential drug targets for many degenerative diseases, the finding described here revealed that there are already clinical stage molecules available to inhibit necroptosis.

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Results

Latent RIP3 Associates with the HSP90/CDC37 Cochaperone Complex.

To analyze RIP3-associated proteins during the induction of necroptosis, we performed immunoprecipitation of an HT29 cell line stably expressing HA-Flag-tagged RIP3. The immune complexes from cells in normal conditions and cells in the process of necroptosis (induced by the combination of TNF- α , a Smac mimetic, and the pan-caspase inhibitor Z-VAD-FMK) were analyzed side by side with SDS/PAGE analysis. The protein bands of interest from this analysis were subsequently identified by mass spectrometry. As demonstrated previously and shown again in Fig. 1A, RIP3 was upshifted in a gel following the induction of necroptosis, and more MLKL was recruited to RIP3 in the necroptosis cell samples (2, 7). Interestingly, a protein band below the 100-kDa marker and another band below the 55-kDa marker were also among the proteins that were coprecipitated with RIP3, although the levels of these proteins did not change as the cells underwent necroptosis. These two proteins were identified as HSP90 and one of its cochaperones, CDC37.

The identities of HSP90 and CDC37 were confirmed by Western blotting analysis, as shown in Fig. 1B. Both HSP90 and CDC37 were coprecipitated with RIP3, and these associations were independent of necroptosis induction (lanes 1–3). On the other hand, the association between RIP3 and RIP1 only occurred during necroptosis (lane 2), and the RIP3–RIP1 interaction was prevented by the presence of the RIP1 kinase inhibitor necrostatin-1 (Nec-1, lane 3) (19).

HSP90 and CDC37 Cochaperone Activity Is Required for the Induction of Necroptosis.

To investigate the functional significance of the association of RIP3 with the HSP90–CDC37 complex, we tested six different HSP90 inhibitors during necroptosis induction. These inhibitors included the natural product geldanamycin (GA), which competitively binds to the ATP-binding pocket of HSP90, and its less toxic derivatives, 17-allylamino-17-desmethoxygeldanamycin (17AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17DMAG), as well as three nongeldanamycin-based resorcinol-containing compounds: STA9090, AT13387, and BIIB021. As shown in Fig. 1C, all of these inhibitors can potentially block necroptosis at 0.2- μ M concentrations, similar to the effects of a 10 μ M dosage of Nec-1. A dose titration has shown that 17AAG is able to completely inhibit necroptosis, even at a 100-nM concentration (Fig. S1). Such concentrations are well below those reached in patients' sera who were given 17AAG or 17DMAG (20, 21).

The inhibition of necroptosis by chemical inhibitors of HSP90 was recapitulated by the knockdown of CDC37. The knockdown of CDC37 completely blocked necroptosis (Fig. 1D, Left), but did not affect the levels of other proteins in the pathway, including RIP1, RIP3, MLKL, or HSP90 (Fig. 1D, Right).

The HSP90–CDC37 Complex Mediates RIP3 Activation During Necroptosis.

To test at which step of the necroptosis pathway the HSP90–CDC37 exerts its effect, we used monoclonal antibodies that specifically recognize either the phosphorylated serine 227 of human RIP3, or serine 358 of human MLKL, to monitor the activation of these proteins. As is shown in Fig. 2A, the induction of necroptosis specifically induced upshift of both RIP1 and RIP3 (lane 3). More specifically, the phosphorylation of RIP3 serine 227 and MLKL serine 358 were elevated (lane 3). Treatment with 17AAG decreased the basal level of phosphorylated serine 227 of RIP3, but the protein levels of RIP1, RIP3, MLKL, as well as those of HSP90 and CDC37, were not affected (lanes 1–4). Cotreatment of 17AAG with necroptosis inducers completely prevented the two phosphorylation events from occurring (lane 4). Similar results were obtained with the CDC37 knockdown cells, although the basal level of RIP3 serine 227 phosphorylation did not seem to be affected (Fig. 2B, lanes 1–4).

Fig. 1. (A) Identification of HSP90 and CDC37 as RIP3 interacting components. HT29-HA-Flag-RIP3 cell was treated as indicated. The cells were then harvested, and whole-cell extracts were sequentially immunoprecipitated with anti-Flag and anti-HA antibodies as described in *Materials and Methods*. The peptide-eluted RIP3-associated complexes were then analyzed by SDS/PAGE followed by silver staining. The indicated protein bands were identified by mass spectrometry. S, Smac mimetic; T, TNF- α ; Z, z-VAD. The final concentrations of 20 ng/mL TNF- α , 100 nM Smac mimetic, and 20 μ M z-VAD were used to induce necrosis. Identical concentrations of these necrosis-inducing agents were used in subsequent experiments unless otherwise stated. (B) The effect of necrostatin-1 (Nec-1) on the HSP90/Cdc37/RIP1/RIP3 interaction. HT29-RIP3 cells were treated with the indicated stimuli for 6 h. The immunoprecipitation was done as described in *Materials and Methods*. (C) Inhibition of HSP90 blocks RIP3-mediated necrosis. The effect of different HSP90 inhibitors on necrosis was evaluated using Cell-Titer Glo assay. The number of surviving cells was normalized to the control cells that were treated with DMSO. Nec-1 was used as a positive control. The data are represented as the mean $\pm$ SD of triplicate wells. (D) Knocking down of Cdc37 blocks T/S/Z induced necrosis. HT29 and HT29-shRNA-Cdc37 cells were treated with the indicated stimuli for 24 h. The number of surviving cells was determined by measuring ATP levels (Left). The data are represented as the mean $\pm$ SD of triplicate wells. Expression of necrosis-related protein was examined (Right).

Fig. 2. (A) Inhibition of HSP90 blocks T/S/Z induced phosphorylation of RIP3 and MLKL. HT29 cells were treated with the indicated stimuli for 6 h and then harvested. Aliquots of 20 μ g whole-cell lysates were subjected to SDS/PAGE followed by Western blot analysis of the indicated protein. (B) Knocking down of Cdc37 blocks T/S/Z induced phosphorylation of RIP3 and MLKL. HT-29 cells and HT29-RIP3-shCdc37 cells were treated with the indicated stimuli for 6 h and then harvested. Aliquots of 20 μ g whole-cell lysates were subjected to SDS/PAGE followed by Western blot analysis of phosphorylated RIP3 (p-S227-RIP3) and MLKL (p-MLKL), RIP3, and MLKL, RIP1, HSP90, Cdc37, and β -actin. (C) Inhibition of HSP90 blocks the formation of RIP3 punctae. Nec-1 (10 μ M), NSA (1 μ M), 17AAG (Tanesprimycin, 0.2 μ M), and GA (0.2 μ M) were used. HT29-RIP3 cells were treated with the indicated stimuli for 12 h. The distribution of RIP3 (green) was detected by immunofluorescence as described in *Materials and Methods*. (Scale bars, 10 μ m.)

Upon activation, RIP3 forms amyloid-like structures that can be observed as aggregated dots in cells (2, 6). Indeed, the induction of necroptosis caused the appearance of bright aggregated dots when HT29 cells expressed a Flag-fusion form of RIP3 (Fig. 2C, T/S/Z). As reported previously by Sun et al. (7), the MLKL inhibitor necrosulfonamide (NSA) blocked necroptosis without affecting the appearance of these dots (T/S/Z + NSA), whereas Nec-1 prevented the formation of RIP3 aggregates (T/S/Z + Nec-1). Consistent with the observation that HSP90 inhibitors block necroptosis before RIP3 activation, cotreatment with 17AAG or GA completely prevented the formation of the RIP3 dots (Fig. 2C, last two rows). Similar results were obtained by knocking down CDC37 (Fig. S24).

The HSP90–CDC37 Complex Is Required for RIP3 to Interact with RIP1.

Because RIP3 cannot be activated without the HSP90–CDC37 complex, we further investigated if this is due to its failure to bind to RIP1, which is the upstream kinase in this pathway. We therefore generated cell lines from the parental HT29 cells in which either Flag-tagged RIP3 or Flag-tagged RIP1 was expressed.

These cells were then induced to undergo necroptosis in the presence or absence of 17AAG. Interaction between the two proteins was probed by immunoprecipitation with an anti-Flag antibody, followed by Western blotting with antibodies against RIP1 or RIP3. As is shown in Fig. 3A, RIP3 was normally associated with the HSP90–CDC37 complex; upon necroptosis induction, RIP3 was associated with RIP1 (lane 3). Treatment with 17AAG disrupted the association between RIP3 and the HSP90–CDC37 complex (lane 2) and prevented RIP3 from associating with RIP1 during necroptosis (lane 4). Similarly, 17AAG caused disassociation of the HSP90–CDC37 complex from RIP1 and prevented RIP1 from binding to RIP3 (Fig. 3B). The levels of those proteins were not affected by the presence of the HSP90 inhibitor 17AAG (input).

We confirmed the effect of the HSP90–CDC37 complex on the interaction of RIP1 and RIP3 during the induction of necroptosis by knocking down CDC37 (Fig. 3C and Fig. S2B). Interestingly, unlike with HSP90 inhibitors, knockdown of CDC37 did not affect HSP90 binding to RIP3 (Fig. 3C, lanes 3 and 4), whereas the interaction between RIP1 and RIP3 did not occur under necroptosis conditions (Fig. 3C, lanes 2 and 4).

Because RIP1 is degraded with long-term inhibition of HSP90 (22), we therefore sought to check the direct effect of HSP90 inhibitors on RIP3-dependent necroptosis. First, we checked the effect of HSP90 inhibitors on LPS/Smac-mimetic/z-VAD-induced necrosis, in which RIP1 is not required (4, 5). As expected, in human monocyte cell line THP-1, TNF α /Smac-mimetic/z-VAD-induced necroptosis is blocked by HSP90 inhibitor (Fig. 3D, Left). As shown in Fig. 3D, LPS/Smac-mimetic/z-VAD-induced necroptosis in human monocyte cell line THP-1 is also attenuated by inhibition of HSP90 with 17AAG. Furthermore, we engineered a U2OS human osteosarcoma cancer cell line in which Flag-tagged RIP3 fusion with two AP20187 binding domains (FKBPv) is inducibly expressed when doxycycline (Dox) is added to the culture medium (diRIP3-U2OS cell) (Fig. S2C). Cell death was induced when RIP3-(FKBPv)₂ was polymerized and activated by adding dimerizer compound AP20187. The cell death induced by RIP3 polymerization is mainly dependent on activation of RIP3 but not on RIP1. Indeed, polymerized RIP3-induced cell death is not blocked by Nec-1. In diRIP3-U2OS cells, inhibition of HSP90 blocked AP20187-induced serine 227 phosphorylation of RIP3 and subsequent cell death, whereas RIP3 level was not affected (Fig. 3E).

HSP90 Inhibitors Attenuate Necroptosis in Vivo. To confirm that HSP90 inhibitors are indeed able to block necroptosis in vivo, we tested primary macrophages from mice for their response to 17DMAG, a second generation HSP90 inhibitor that is currently being tested in clinical trials. To our surprise, 17DMAG was totally ineffective in preventing necroptosis in mouse macrophages (Fig. 4A). In fact, the presence of 1 μ M 17DMAG exacerbated necroptosis induced by the combination of TNF- α , a Smac mimetic, and z-VAD-FMK in mouse macrophages (Fig. 4A). In contrast, treatment with Nec-1 completely prevented necroptosis in mouse macrophages under the same conditions. Consistent with such observations, no association of HSP90 with RIP3 was observed in a mouse cell line (Fig. S3).

This enhancement of necroptosis by 17DMAG was not observed in macrophages isolated from rats. Similar to what was observed in human cells, 17DMAG (at the same concentration used in mouse macrophages) was able to completely block TNF- α , Smac mimetic, and z-VAD(T/S/Z)-induced necroptosis (Fig. 4B) and attenuate LPS/Smac-mimetic/z-VAD-induced necroptosis in primary macrophages isolated from rats (Fig. 4C).

Based on these observations, we selected rat as an animal model to test the effects of the HSP90 inhibitor 17DMAG in a model of TNF- α -induced systemic inflammatory response syndrome (SIRS). As shown in Fig. 4D, rats with i.v. injection of

Fig. 3. (A) The effect of HSP90 inhibitor on the interaction of RIP3-HSP90/CDC37/RIP1. HT29-Flag-RIP3 cells were treated with the indicated stimuli for 6 h. Then the cell extracts were prepared and used for immunoprecipitation with an anti-Flag antibody as described in *Materials and Methods*. The immunocomplexes were then analyzed by Western blotting using antibodies as indicated. Aliquots of 20 μg whole-cell lysates (input) were subjected to SDS/PAGE. (B) The effect of HSP90 inhibitor on the interaction of RIP1-HSP90/CDC37/RIP3. HT29-Flag-RIP1 cells were treated with the indicated stimuli for 6 h. Then the cell extracts were prepared and used for immunoprecipitation with an anti-Flag antibody as described in *Materials and Methods*. The immunocomplexes were then analyzed by Western blotting using antibodies as indicated. Aliquots of 20 μg whole-cell lysates (input) were subjected to SDS/PAGE. (C) Knocking down of Cdc37 blocks T/S/Z induced interaction of RIP3 and RIP1. HT29-Flag-RIP3 cell and HT29-Flag-RIP3-shCdc37 cell were treated with the indicated stimuli for 6 h. Then the cell extracts were prepared and used for immunoprecipitation with an anti-Flag antibody as described in *Materials and Methods*. The immunocomplexes were then analyzed by Western blotting using antibodies as indicated. Aliquots of 20 μg whole-cell lysates were used as input. (D) Inhibition of HSP90 attenuates TNF- α /S/Z and LPS/S/Z induced necrosis in human monocytic cell THP-1 cell lines. The cells were treated with T/S/Z (Upper) or LPS/S/Z (Lower) plus the indicated concentrations of 17AAG. Cell viability was determined by measuring ATP levels. The data are represented as the mean $\pm$ SD of triplicate wells. (E) Inhibition of HSP90 blocks polymerized RIP3-induced necrosis. The diRIP3-U2OS cells were induced by Dox overnight and then treated with different compounds for 2 h before the AP20187 was added. After 10 h, cell death was determined by measuring ATP levels (Left). The data are represented as the mean $\pm$ SD of triplicate wells (Left). Inhibition of HSP90 blocks polymerized RIP3 autophosphorylation. The diRIP3-U2OS cells were induced by Dox overnight and then treated with different compounds for 2 h before the AP20187 was added. After 10 h, the cells were then harvested, and the whole-cell extracts were analyzed by Western blotting to check RIP3, p-S227-RIP3, and β -actin (Right).

TNF- α died between 5–9 h after the injection. Coinjection with Nec-1 largely prevented animal death. Coinjection of 17DMAG also significantly delayed animal death. The histological analysis of internal organs of those animals showed significant reductions in inflammation of the small and large intestine, lung, spleen, and kidney, when animals received HSP90 inhibitor 17DMAG (Fig. 4E).

Discussion

RIP1 and RIP3 Are Clients of the HSP90 and CDC37 Cochaperone Complex. The RIP1 and RIP3 kinases transduce signals from the TNF receptor family of cytokine receptors to cause necrotic cell death. Both kinases are latent in the cytosol before RIP1 engagement with the activated receptors mediated by the interaction between its death domain and that of the receptors (23, 24). Activated RIP1 binds to and activates RIP3 through their

respective RHIM domains. One marker of RIP3 activation is the autophosphorylation of serine 227 (human RIP3), which can be specifically recognized by the rabbit monoclonal antibody reported here (Fig. 24). This phosphorylation event renders RIP3 able to bind its downstream substrate MLKL (7).

Both RIP1 and RIP3 are clients of the HSP90–CDC37 cochaperone complex. Although it has been previously reported that RIP1 can be better expressed and purified when coexpressed with CDC37, and an HSP90 inhibitor caused the down-regulation of the RIP1 protein level (22, 25, 26), it is now clear that not only are both latent RIP1 and RIP3 associated with the HSP90–CDC37 complex, their activation during necroptosis is critically dependent on the activity of such a chaperone complex. Interventions with either chemical HSP90 inhibitors or CDC37 knockdown blocked RIP1–RIP3 interaction, RIP3 autophosphorylation, and the ensuing necroptosis.

Fig. 4. (A) Inhibition of HSP90 enhances T/S/Z-induced necrosis in mouse macrophage cells. The cells were treated with T/S/Z plus the indicated concentrations of 17AAG or GA for 12 h. Cell viability was determined by measuring ATP levels. The data are represented as the mean $\pm$ SD of triplicate wells. (B) Inhibition of HSP90 blocks T/S/Z-induced necrosis in rat macrophage cells. The cells were treated with T/S/Z plus the indicated concentrations of 17DMAG for 4 h. Cell viability was determined by measuring ATP levels. The data are represented as the mean $\pm$ SD of triplicate wells. (C) Inhibition of HSP90 blocks LPS/S/Z-induced necrosis in rat macrophage cells. The cells were treated with LPS/S/Z plus the indicated concentrations of 17DMAG for the indicated time. Cell viability was determined by measuring ATP levels. The data are represented as the mean $\pm$ SD of triplicate wells. (D) Inhibition of HSP90 attenuates TNF- α -induced SIRS in a rat model. Rats aged 8–9 wk were injected i.v. with murine TNF- α (5 mg/kg, $n = 8$), TNF- α and 17DMAG (5 mg/kg, $n = 8$), TNF- α and Nec-1 (6 mg/kg, $n = 7$), or saline ($n = 5$). Body temperature was measured with a lubricated rectal thermometer. Rats with a temperature below 25 $^{\circ}$ C were killed for ethical reasons. (E) Inhibition of HSP90 and RIP1 attenuates TNF- α -induced tissue damage in the rat model. Rats aged 8–9 wk were injected in the tail vein with murine TNF- α , TNF- α and 17DMAG, TNF- α and Nec-1, or saline. Six hours after the last injection, rats were killed and the tissue sections stained with H&E. Each image is representative of at least three rats.

Short-term inhibition of HSP90 may cause conformational change of RIP3, which renders its susceptibility to cleavage by proteinase K (Fig. S4A). Similar to other kinase clients of the HSP90–CDC37 chaperone complex, prolonged inhibition of HSP90 leads to the ubiquitin-mediated degradation of RIP3. As shown in Fig. S4B, the presence of 17AAG decreased the level of RIP3 in HT29 cells and increased the level of polyubiquitinated RIP3 (Fig. S4C). The presence of the proteasome inhibitor MG132 prevented a drop in RIP3 levels and further increased the amount of polyubiquitinated RIP3 (Fig. S4C, lanes 3 and 6).

The Inhibition of Necroptosis by HSP90 Inhibitors Has Clinical Implications.

The inhibition of necroptosis by HSP90 inhibitors that are currently in clinical trials raises several interesting possibilities. The

systematic exposure of such inhibitors intended to block cancer-driving kinases should attenuate the necroptosis pathway in humans, which will result in the following consequences: patients should be carefully monitored for viral infection, because necroptosis is important for antiviral responses; and cancer cell death through necroptosis will be compromised, and this will complicate the interpretation of trial outcomes.

One interesting yet unexpected finding from our study is that the HSP90–CDC37 effect on RIP3 in human cells is conserved in rat, but not in mice. The necroptosis pathway thus seems to be more deviated between mouse and human than between human and rat. Human MLKL does not interact with mouse RIP3, but does interact with the rat RIP3 and therefore is phosphorylated by rat RIP3 (Fig. S5 B and C). Furthermore, rat but not mouse

RIP3 is also a client of the HSP90–CDC37 complex. Unlike apoptosis, for which the core components including Bcl-2-like, Apaf-1, and caspase are conserved from human to *Drosophila* and *Caenorhabditis elegans*, the necroptosis pathway seems more evolutionarily divergent and does not exist in the latter two model organisms. Our study suggests that rat is a more relevant model for the study of necroptosis for human diseases.

Materials and Methods

The following antibodies and reagents were used: anti-FLAG M2 monoclonal antibody and affinity gel (Sigma-Aldrich); Flag-HRP (Sigma-Aldrich, A8592); anti-MLKL polyclonal antibody (Sigma-Aldrich); anti-phospho-MLKL antibody (ab187091; Abcam); anti-RIP1 monoclonal antibody (Cell Signaling, 34935); anti-HSP90 (Proteintech Group, 13171–1-AP); anti-CDC37 antibody (Cell Signaling, 47935); and Alexa Fluor 488 goat anti-mouse IgG, IgA, and IgM(H+L) (Life Technologies). Anti-p-S227-RIP3 antibody was generated by Epitomics using phosphor-peptide (ELTPES(p)LVYEAV).

Cell Survival Assay. Cell survival assay was performed using the CellTiter-Glo Luminescent Cell Viability Assay kit. A CellTiter-Glo Assay (Promega) was performed according to the manufacturer's instructions. Luminescence was recorded with a Tecan GENios Pro plate reader.

Cell Culture and Stable Cell Lines. HT29-Flag-RIP3 and HT29-Flag-RIP1 cells were established as described (2, 27). For Cdc37-shRNA expression cells, HT-29 cells were transfected with Cdc37-shRNA construct and selected with 0.5 mg/mL puromycin (Calbiochem). The same strategy was used to establish the HT29-Flag-RIP3-shRNA-Cdc37 and HT29-Flag-RIP1-shRNA-Cdc37 cell lines. NIH 3T3-Flag-HA-mRIP3 cell was generated using NIH 3T3 cells with stable expression of Flag-HA-mRIP3. diRIP3-U2OS cell was generated using U2OS Tet-On cells with Tet-inducible expression of Flag-tagged RIP3 fusion with two AP20187 binding domains (FKBPv).

Flag-HA Tandem Pull-Down. Cell lysates was incubated overnight with 50 μ L of anti-Flag M2-agarose. After three washes with lysis buffer, the beads were eluted twice with 1 mL of lysis buffer containing 0.5 mg/mL 3 $\times$ Flag peptide for 4 h. The combined elution was incubated overnight with 30 μ L anti-HA

agarose. After five washes with lysis buffer, the beads were eluted with 100 μ L of lysis buffer containing 0.5 mg/mL HA peptide for 4 h. A total of 30 μ L of 4 $\times$ SDS loading buffer was added to the final elution, and the mixture was boiled down to 60 μ L and loaded into a 4–12% gradient gel (Invitrogen, NP0321). All of the procedures were performed at 4 $^{\circ}$ C unless otherwise stated. Silver staining was performed according to the manufacturer's instructions (SilverQuest silver staining kit, Invitrogen, LC6070).

Immunoprecipitation and Immunoblotting. The cells were cultured on 10-cm dishes and grown to confluence. Cells at 90% confluence were washed once with PBS and harvested by scraping and centrifugation at 800 $\times$ g for 5 min. The harvested cells were washed with PBS and lysed for 30 min on ice in the lysis buffer. Cell lysates were then spun down at 12,000 $\times$ g for 20 min. The soluble fraction was collected, and the protein concentration was determined by Bradford assay. Next, 1 mg of extracted protein in lysis buffer was immunoprecipitated overnight with anti-Flag (Sigma-Aldrich) at 4 $^{\circ}$ C. The immunoprecipitates were washed three times with lysis buffer. The beads were then eluted with 0.5 mg/mL of the corresponding antigenic peptide for 4 h or directly boiled in 1% SDS loading buffer.

Cell Transfection Immunofluorescent Staining. Transfection of cells with plasmid DNA was performed according to the procedures described previously (2). Cells were plated onto glass coverslips and treated as indicated. After two washes with cold PBS, the cells were fixed in 4% paraformaldehyde at room temperature for 30 min. The cells were permeabilized and incubated with blocking buffer (0.2% Triton X-100 and 3% BSA in PBS) for 15 min. The primary antibodies were diluted in blocking buffer, and the cells were immunolabeled at 4 $^{\circ}$ C overnight. The cells were washed three times with PBS followed by a 1-h incubation with a fluorescein-conjugated secondary antibody (Life Technologies). DAPI was used to stain the nuclei. Triplicate cultures were examined, and similar results were obtained in at least three independent experiments.

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