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E3 ligase CHIP controls necroptosis via regulating RIPK3 and RIPK1 protein levels

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Abbreviations: RIPK3; Receptor-interacting protein kinase 3, RIPK1; Receptor-interacting protein kinase 1, CHIP; C-terminus HSC70-interacting protein, TNF; Tumor necrosis factor, MLKL; Mixed lineage kinase domain-like, MEF; Mouse embryonic fibroblast

ABSTRACT

Necroptosis is a well-known form of caspase-independent cell death. Necroptosis can be triggered by various extrinsic stimuli including death ligands in the presence of receptor-interacting protein kinase 3 (RIPK3), which is a key mediator of necroptosis induction. Our recent studies revealed that an E3 ligase, C-terminus HSC-70 Interacting Protein (CHIP), functions as an inhibitor of necroptosis. *CHIP*$^{-/-}$ mouse embryonic fibroblast cells showed higher sensitivity to necrotic stimuli than wild-type mouse embryonic fibroblast cells. Deleterious effects of CHIP knockout were retrieved by *RIPK3* depletion. We found that CHIP negatively regulates RIPK3 and RIPK1 by ubiquitylation- and lysosome-dependent degradation. In addition, *CHIP*$^{-/-}$ mice showed postnatal lethality with intestinal defects, which were rescued by crossing with RIPK3$^{-/-}$ mice. These results suggest that CHIP is a negative regulator of RIPK1 and RIPK3, leading to the inhibition of necroptosis.

The cell death pathway can be divided into caspase-dependent and -independent cell death. Necroptosis, a form of programmed necrosis, is an extensively studied caspase-independent cell death pathway, which is thought to be an alternative form of the cell death pathway developed in the host to fight against viruses and bacteria, escaping from apoptosis. Necroptosis is triggered by the activation of death receptors, including tumor necrosis factor receptor, Toll-like receptors or interferon receptors. Once activated, death receptors recruit a variety of signaling molecules to form primary complexes such as TNF complex I/II or death-inducing signaling complex. When caspase-8 activity is low or inhibited, necrosome complexes are formed, which are composed of phosphorylated receptor-interacting protein kinase 1 (RIPK1) and RIPK3. Activated RIPK3 induces the phosphorylation of mixed lineage kinase domain-like protein (MLKL), which leads to the oligomerization of MLKL. Oligomerized MLKL moves to the plasma membrane and directly or indirectly disrupts membrane integrity. While MLKL can be regarded as the final effector molecule of necroptosis, other unknown factors affecting these final steps cannot be excluded. In contrast to apoptosis, necroptosis induces the release of damage-associated molecular patterns, such as high-mobility group box 1, which leads to inflammation by activating innate immune cells. Recent studies showed that several disease models related to apoptosis such as ischemia-reperfusion injury and neurodegeneration are also associated with necroptosis. While more detailed *in vivo* studies are necessary, these observations indicate that necroptosis involves functionally integrated pathways causing a variety of human diseases.

Our study revealed that C-terminus HSC70 interacting protein (CHIP) functions as a negative regulator of necroptosis. CHIP is known as an UBOX-containing E3 ligase with tetratricopeptide, a chaperone binding motif. CHIP regulates various tumor suppressor proteins such as p53 and phosphatase and tensin homology, thus protecting cells from apoptosis. However, the function of CHIP in extrinsic or non-apoptotic cell death signaling remains unclear. Since hyperactivation of necroptosis is linked to the disruption of tissues or lethality, we evaluated whether non-apoptotic cell death is activated in *CHIP*$^{-/-}$ mice. To test this, we first employed *CHIP* knockout mouse embryonic fibroblasts (MEFs) to study necroptosis.

Based on our results, *CHIP*$^{-/-}$ MEFs showed higher sensitivity to necroptosis and promoted necrosome complex formation in response to necrotic stimuli. Western blotting data indicated that *CHIP*$^{-/-}$ MEFs showed higher RIPK1 and RIPK3 protein levels and stabilities compared to wild-type MEFs. We also showed that CHIP degrades RIPK1 and RIPK3 and directly ubiquitylates RIPK1 and RIPK3 under exogenous and endogenous conditions. Finally, CHIP-mediated ubiquitylation of RIPK1 and RIPK3, which occur at lysine 571, 604, and 627 of RIPK1 and lysine 55 and 363 of RIPK3, leads to the lysosomal localization of RIPK1 and RIPK3 (Fig. 1).

Recently, intestinal epithelial cell-specific knockout of Fas-associated death domain or caspase-8 was shown to induce massive cell death and inflammatory phenotypes in mouse intestinal epithelial cells. These phenotypes were rescued by treatment with necrostatin-1, a necroptosis inhibitor, or crossing with RIPK3 knockout mice, indicating the critical role of necroptosis in intestinal defects. Our analysis showed that *CHIP*$^{-/-}$ mice died within 4–8 weeks with a high rate of cell death in the intestinal epithelium, which is a typical phenotype of necroptotic animal models. These phenotypes of *CHIP*$^{-/-}$ mice were rescued by crossing with *RIPK3*$^{-/-}$ mice. There was no change in cleaved-caspase-3 protein levels in the intestines of *CHIP*$^{-/-}$ mice, indicating that intestinal defects in *CHIP*$^{-/-}$ mice occur in an apoptosis-independent manner. RIPK1 and RIPK3 protein levels were increased in the intestines of *CHIP*$^{-/-}$ mice, suggesting that CHIP deficiency can lead to the activation of necroptosis by up-regulating the RIPK1 and RIPK3 proteins. Although further analyses of organs in *CHIP*$^{-/-}$ mice related to necroptosis are required, our data suggest that CHIP negatively regulates necroptosis in the intestinal epithelium by ubiquitylation-mediated lysosome-dependent degradation of RIPK1 and RIPK3.

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Figure legend

Fig. 1. CHIP inhibits necroptosis by ubiquitin- and lysosome- dependent RIPK1 and RIPK3 degradation.

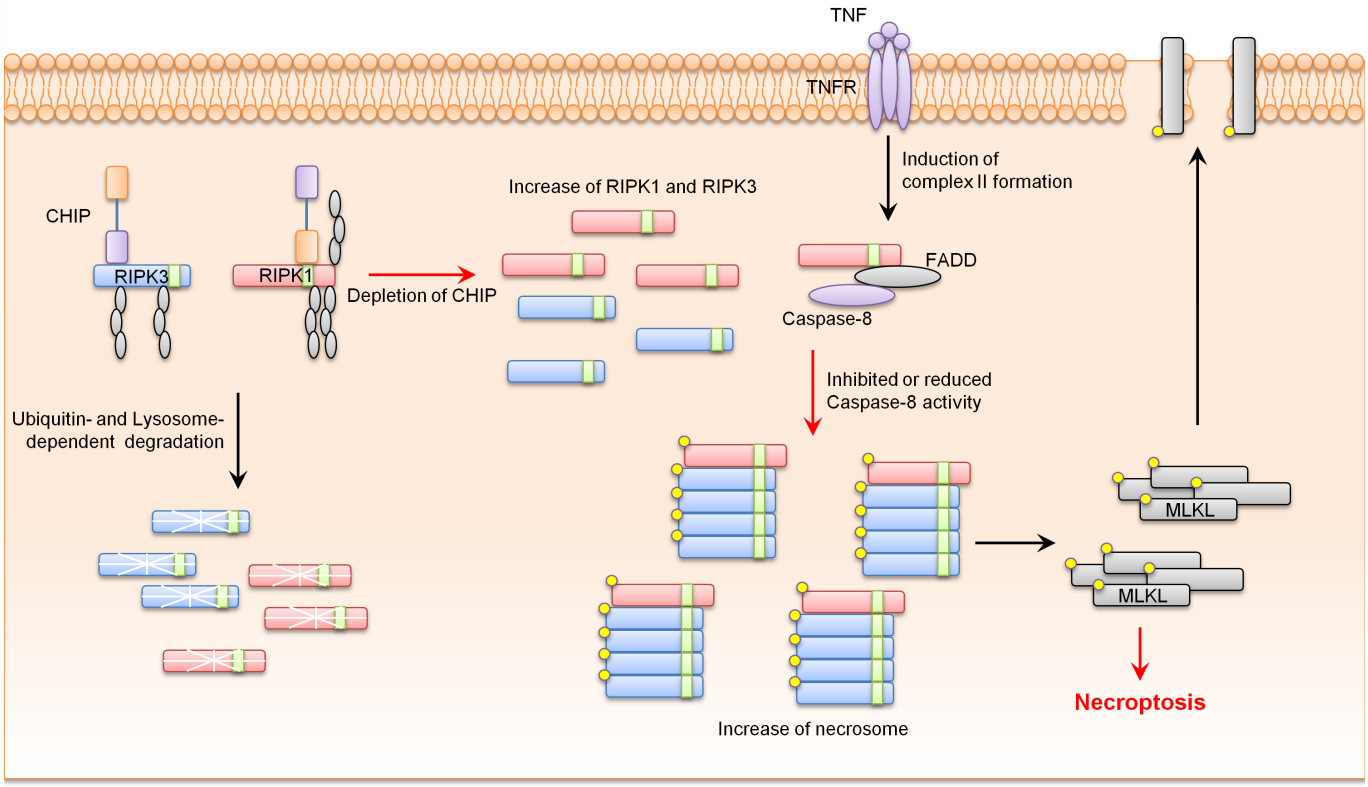

Fig. 1