RESEARCH ARTICLE

Necroptosis Interfaces with MOMP and the MPTP in Mediating Cell Death

Jason Karch¹, Onur Kanisicak¹, Matthew J. Brody¹, Michelle A. Sargent¹, Demetria M. Michael¹, Jeffery D. Molkentin¹,²

¹ Department of Pediatrics, Cincinnati Children’s Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio, United States of America, ² Howard Hughes Medical Institute, Cincinnati, Ohio, United States of America

Abstract

During apoptosis the pro-death Bcl-2 family members Bax and Bak induce mitochondrial outer membrane permeabilization (MOMP) to mediate cell death. Recently, it was shown that Bax and Bak are also required for mitochondrial permeability transition pore (MPTP)-dependent necrosis, where, in their non-oligomeric state, they enhance permeability characteristics of the outer mitochondrial membrane. Necroptosis is another form of regulated necrosis involving the death receptors and receptor interacting protein kinases (RIP proteins, by Ripk genes). Here, we show cells or mice deficient for Bax/Bak or cyclophilin D, a protein that regulates MPTP opening, are resistant to cell death induced by necroptotic mediators. We show that Bax/Bak oligomerization is required for necroptotic cell death and that this oligomerization reinforces MPTP opening. Mechanistically, we observe mixed lineage kinase domain-like (MLKL) protein and cofilin-1 translocation to the mitochondria following necroptosis induction, while expression of the mitochondrial matrix isoform of the antiapoptotic Bcl-2 family member, myeloid cell leukemia 1 (Mcl-1), is significantly reduced. Some of these effects are lost with necroptosis inhibition in Bax/Bak1 double null, Ppif-/-, or Ripk3-/- fibroblasts. Hence, downstream mechanisms of cell death induced by necroptotic stimuli utilize both Bax/Bak to generate apoptotic pores in the outer mitochondrial membrane as well as MPTP opening in association with known mitochondrial death modifying proteins.

Introduction

During apoptosis the Bcl-2 family members Bax and Bak form hetero- and homo- oligomers within the mitochondrial outer membrane leading to release of cytochrome c and other apoptosis inducing proteins [1]. During regulated necrosis the mitochondrial permeability transition pore (MPTP) opens due to increased reactive oxygen species (ROS) and calcium in the matrix of the mitochondria. The MPTP is an inner mitochondrial membrane event that when opened leads to mitochondrial depolarization and if sustained, organelle rupture [2]. Although the actual pore forming component of the MPTP remains an area of ongoing investigation,
cyclophilin D (CypD) is a known regulator of MPTP opening and necrotic cell death [2]. Indeed, Ppif+/− (CypD encoding gene) cells are resistant to necrotic stressors such as calcium overload induced by ionomycin, ROS induced by H2O2, or in vivo in response to ischemic injury [3, 4]. However, loss of the Ppif gene does not protect from apoptotic-induced cell death, such as with staurosporine stimulation [3, 4].

Bax and Bak1 (encodes Bak protein) gene deficient cells are resistant to both apoptosis and regulated necrosis [5–7]. While Bax and Bak are responsible for apoptotic cell death by inducing mitochondrial outer membrane permeability (MOMP) and cytochrome c release, recently it was shown that they are also required for MPTP-dependent necrotic cell death by generating a level of permeability in the outer mitochondrial membrane in their non-oligomerized forms [6]. Bax/Bak were also recently implicated as necessary mediators of another type of cell death known as necroptosis, although the mechanism underlying this observation was not described [8, 9], which we have investigated here. Necroptosis is a specific form of regulated necrosis that occurs in response to caspase inhibition and simultaneous death receptor stimulation, such as through the tumor necrosis factor-α (TNFα) receptor [10]. Upon TNFα receptor stimulation caspase 8 becomes activated causing inhibition of receptor interacting protein kinase 1 (RIP1) [11]. If caspase 8 is inhibited during death receptor stimulation, RIP1 is activated where it binds to RIP3 and causes its activation and subsequent necrotic cell death [12, 13]. One known substrate of the RIPs that is required for necroptosis is mix lineage kinase domain-like (MLKL), as Mlkl null cells are resistant to necrototic cell death [14, 15], although how this mechanistically leads to cell death is not understood. Morphologically, necroptosis shares similar features with MPTP-dependent necrosis, such as mitochondrial swelling and early plasma membrane rupture [10].

The involvement of mitochondria, Bax/Bak or the MPTP as downstream mediators of necroptosis is a point of contention in the literature [16]. Some studies have implicated Bax/Bak and the MPTP in playing an important role in necroptosis [8, 9, 17], while other studies have suggested no involvement [18, 19]. Here, we show that Bax/Bak are required mediators of necroptosis through a mechanism involving MOMP, which then secondarily enhances MPTP-dependent cellular necrosis. Furthermore, we show that MLKL and cofilin-1 translocate to the mitochondria following a necroptotic stimuli. Cofilin-1 translocation to the mitochondria is known to induce MPTP opening and cell death [20–22]. Necroptosis also causes a decrease in the mitochondrial matrix isofrom of myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic Bcl-2 family member that leads to mitochondrial dysfunction when deleted [23]. A model is proposed whereby the upstream regulators of necroptosis feed into known regulators of mitochondrial dependent cell death, such as Bax/Bak and the MPTP, thus suggesting that mitochondrial dysfunction is required for effective necroptosis.

Materials and Methods

Animal models

Ppif+/− mice were described previously [3]. For the pancreatitis model, male mice 6–8 weeks old received intraperitoneal injections of 50 μg/kg caerulein (Sigma) or vehicle control every hour for 6 hours and sacrificed by CO2 inhalation 2 hours following the last injection (n = 5 each group). The pancreases were fixed in 4% paraformaldehyde, sectioned, and stained with H&E to determine damaged areas by microscopy.

Ethics statement

All animal experimentation was approved by the Office of Research Compliance and Regulatory Affairs and by the Cincinnati Children’s Hospital Institutional Animal Care and Use
Committee (Protocol Number: 2E11104). No surgical procedures were used nor were other procedures used that caused suffering. No human subjects were used.

Tissue culture and analysis of cell death and viability
All cells were cultured in IMDM medium supplemented with 10% bovine growth serum, antibiotics, and nonessential amino acids. DKO MEFs stably expressing WT Bax or Bak or mutant Bax were described previously [24, 25]. Clonal cell lines were created by plating 0.5 cells/well onto a 96-well plate. After 10 days cells were expanded and western blots were performed to determine the expression level of Bax or Bak. Ppif null MEFs were described previously [6]. Rip3k null MEFs were described previously [26]. At 80% confluence, MEFs were treated with 200 nM staurosporine for 24 hr, 20 μM ionomycin for 24 hr, or 10 ng/mL TNFα and 50 μM caspase inhibitor zVAD-fmk for 24 hr. In some experiments, cells were pretreated with 20 μM ABT-737, or 10 μM Gossypol. Cell death was determined by the Muse Count & Viability assay (Millipore). Briefly, cells were trypsinized and washed twice and incubated with Muse Count & Viability reagent. The cells were then quantified on a Muse cell analyzer (Millipore) at 5,000 counts per sample.

Mitochondrial isolation and analyses
Liver mitochondria were isolated by homogenization with a Teflon homogenizer followed by differential centrifugation. Mitochondrial calcium uptake was measured with Calcium Green-5N (Invitrogen) as previously described [6]. Briefly, mitochondria were isolated in MS-EGTA buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.4). Mitochondria (200 μg) were pretreated with 1 μg tBID or vehicle (boiled tBID) for 15 minutes at 37°C and then were incubated in KCl buffer (125 mM KCl, 20 mM HEPES, 2 mM MgCl2, 2 mM KH2PO4, and 40 μM EGTA, pH 7.2) containing 200 nM Calcium Green-5N, 7 mM pyruvate, and 1 mM malate. After a baseline read was recorded, mitochondria were treated with 1 pulse of 250 μM CaCl2. Fluorescence was quantified using a Synergy 2 Multi-Mode Microplate Reader (BioTek). Mitochondria from MEFs were isolated by homogenization with a glass homogenizer followed by differential centrifugation. For some experiments the cytoplasmic fraction was preserved and subjected to 3 additional high speed (>14000 g) centrifugations to further purify the cytoplasmic proteins.

Electron and fluorescent microscopy
Electron microscopy was performed on WT, DKO, and Ppif null MEFs. Prior to fixation, cells were treated for 2 or 12 hrs with TNFα and zVAD-fmk or vehicle. Samples were then fixed in glutaraldehyde and cedacode, embedded in epoxy resin, sectioned, and counterstained with uranyl acetate and lead citrate. Fluorescent microscopy was performed on WT MEFs. Prior to fixation, cells were treated with TNFα and zVAD-fmk or vehicle for 3 hours. Samples were then fixed in 4% paraformaldehyde and subjected to immunocytochemistry for MLKL (EMD Millipore) and SAMM50 as a mitochondrial marker (Sigma-Aldrich).

Western blotting
MEFs or isolated mitochondria were homogenized in RIPA buffer containing protease inhibitor cocktails (Roche). After protein concentrations were measured SDS sample buffer was added and western blots were performed. For the Bak oligomerization assay WT and DKO MEFs were treated with TNFα and zVAD-FMK for 6 hours. Mitochondria were isolated as described above and suspended in tris-buffered saline containing 1% DDM and 1% digitonin. After the protein
concentrations were assessed NativePAGE sample buffer (Life Technologies) was added and a western blot was performed. The following antibodies were used: Bax (Santa Cruz), Bak (Millipore), CypD (Abcam), RIP1 (Santa Cruz), RIP3 (Abcam), gapdh (Fitzgerald), BID (R&D Systems), Bcl-2 (Santa Cruz), Mcl-1 (Rockland), MLKL (EMD Millipore), coflin-1 (Santa Cruz), complex V/III (MitoProfile Total OXPHOS, MitoSciences), and αtubulin (Santa Cruz).

Statistical Tests
Statistical significance was determined by ANOVA and Newman-Keuls pairwise comparisons for multivariate experiments and t-test for experiments with 2 groups.

Results
Necroptosis does not occur in Bax/Bak1 DKO MEFs
We first evaluated the ability for Bax/Bak1 double knock-out (DKO) mouse embryonic fibroblasts (MEFs) to die by necroptosis. Wildtype (WT) MEFs showed high levels of killing induced by the classical necroptotic inducers, TNFα in the presence of the pan caspase inhibitor zVAD-fmk, which was inhibited by the RIP1 blocking drug necrostatin-1 (Fig 1A). Similar to protection from apoptosis and MPTP-dependent necrosis, Bax/Bak1 DKO MEFs were also highly resistant to necroptotic cell death (Fig 1A). Electron microscopy showed that WT MEFs treated with TNFα and zVAD-fmk displayed hallmarks of necrotic cell death; swollen mitochondria (arrows) at early time points followed later by plasma membrane rupture, with the absence of apoptotic morphologic features (Fig 1B). However, DKO MEFs displayed no signs of necrotic death or mitochondrial swelling or any other ultrastructural features of disorganization (Fig 1B).

To safeguard against an epiphenomenon associated with the clonally derived DKO MEFs we generated 6 additional clonal DKO lines with replacement of either Bax or Bak at low, medium and high levels of protein expression (Fig 1C and 1D). Importantly, all DKO lines expressed RIP1 and RIP3 at comparable levels to the DKO parent cell line, although RIP3 was slightly downregulated in cell lines with Bak reconstitution (Fig 1C and 1D). With respect to cell death, restoration of Bax or Bak in the DKO parent cell line produced a dosage-dependent rescue of apoptosis due to staurosporine, necrosis due to ionomycin or necroptosis due to TNFα and zVAD-fmk (Fig 1E and 1F). This strategy negates any potential secondary issues associated with the DKO MEF parent line and shows that Bax/Bak are required for necroptotic cell death.

Necroptosis is dependent on the oligomerization Bax/Bak
Induction of necroptosis with TNFα and zVAD-fmk induced Bak oligomers in WT MEFs, suggesting a partial apoptotic-like mechanism (Fig 2A). In addition, DKO MEFs that stably expressed the oligomerization-dead mutant form of Bax (Bax 63–65A) were resistant to TNFα and zVAD-fmk induced necroptosis, again showing a more apoptotic mode of action for how Bak/Bak might induce cell death, but distinct from regulated necrosis where Bax monomers are sufficient for MPTP-dependent cell death (Fig 2B). As further proof of this mechanistic distinction we examined the effects of the BH-3 mimetics ABT-737 and gossypol on TNFα and zVAD-fmk induced cell death in DKO MEFs. As we previously showed [6], gossypol, but not ABT-737, restored ionomycin-induced necrosis but not staurosporine-induced apoptosis in the DKO MEFs (Fig 2C). Gossypol had no effect on DKO MEFs treated with TNFα and zVAD-fmk, similar to staurosporine (Fig 2C). These results indicate that necroptosis requires some level of Bax and Bak oligomerization to produce cell death, and that simply increasing
the permeability characteristics of the outer membrane with gossypol is not sufficient to restore cell death induced by TNFα and zVAD-fmk. Finally, the fact that ABT-737 had no effect on necroptosis in DKO MEFs rules out compensation by any sort of anti-apoptotic effect of Bcl-2 family members in the absence of Bax and Bak at the outer membrane. Thus, necroptosis requires Bax/Bak through a MOMP mechanism of action.

Necroptosis does not occur efficiently in Ppif null MEFs

We previously showed that Bax and Bak can also influence necrotic cell death through the MPTP [6], hence here we employed Ppif<sup>−/−</sup> SV40 transformed MEFs and Ppif<sup>−/−</sup> mice to assess
the involvement of the MPTP as a putative downstream necroptotic effector pathway. We first verified that the necroptotic regulators RIP1 and RIP3 were equally expressed in Ppif−/− MEFs, as were Bax and Bak (Fig 3A). Ppif−/− MEFs are partially resistant to necrosis induced by ionomycin or H2O2, but not against apoptosis induced by staurosporine [3, 4, 6]. Ppif−/− MEFs were also partially resistant to necroptosis induced by TNFα and zVAD-FMK compared with WT MEFs (Fig 3B). Furthermore, like the DKO MEFs, Ppif−/− MEFs lacked the ultrastructural signs of necrotic cell death observed in WT MEFs after TNFα and zVAD (Figs 3C, versus 1B). To examine if this result could be translated to an in vivo setting we treated WT and Ppif−/− mice with caerulein to cause necroptotic cell death in the pancreas. Caerulein treatment generates a model of necroptosis, supported by the observation that both Ripk3 and Mlkl null mice are resistant to cell death induced by this agent [13][15]. Remarkably, Ppif−/− mice were resistant to caerulein-induced cell death in the pancreas compared to WT mice (Fig 3D). To more carefully address the potential mechanism here we treated isolated liver mitochondria with and without tBID to induce Bax/Bak oligomerization/MOMP, and then subjected them to a calcium stress. Calcium uptake in the mitochondria treated with tBID was inhibited suggestive of greater MPTP opening when compared to mitochondria without tBID given the same amount of calcium (Fig 3E). These results suggest that the Bax/Bak oligomerization that occurs during

**Fig 2. Bax/Bak oligomerization is required for necroptosis.** A, Western blot for Bak from extracts of WT and Bax/Bak1 DKO MEFs treated with and without TNFα and zVAD-FMK (TNF-Z) for 6 hours. In order to detect Bak oligomerization the extracts were processed under non-reducing conditions. Asterisks show Bak oligomers, and con. represents control conditions without stimulation. B, Quantitation of dead cells in WT and oligomerization dead Bax (Bax 63–65A) stably expressing DKO MEFs treated with TNFα and zVAD-FMK with and without necrostatin. C, Quantitation of dead cells in DKO MEFs pretreated with and without ABT-737 (ABT, in orange) or gossypol (goss., in green) and then treated with the necroptotic inducer TNFα and zVAD-FMK (TNF-Z), the apoptotic inducer staurosporine (Staur.), or the necrotic inducer ionomycin (Iono.). All assays were performed in duplicate and averaged from three independent experiments. *p<0.01 vs control; #p<0.01 vs WT treatment.

doi:10.1371/journal.pone.0130520.g002
necroptosis can sensitize a cell to die by MPTP-dependent cell death simply by increasing the permeability of the outer mitochondrial membrane.

Upstream necroptotic regulators signal through mitochondrial death modifiers

It is known that RIP1, RIP3 and MLKL are the integral regulators of necroptotic cell death but it is unclear how these effectors might connect downstream to the MOMP or the MPTP. To address this issue we isolated mitochondria from WT, Ripk3 null, Bax/Bak1 DKO and Ppif null MEFs treated with and without TNFα and zVAD-fmk and probed for known regulators of necroptosis and the MOMP and MPTP (Fig 4A). We found that upon treatment of TNFα and zVAD-fmk the expression of MLKL increases in the mitochondrial fraction (Fig 4A). To confirm this result we performed immunocytochemistry for MLKL and SAMM50 (mitochondrial marker), which showed greater translocation to the mitochondria in WT MEFs treated with TNFα and zVAD-fmk compared with vehicle stimulation (Fig 4B). However, necroptosis-induced translocation of MLKL to the mitochondria was not observed in the Ripk3−/− MEFs.

Fig 3. MPTP opening is required for necroptosis. A, Western blots for CypD, Bax, Bak, RIP1, RIP3, and gapdh (control) from extracts of WT and Ppif−/− MEFs. B, Quantitation of dead cells in WT and Ppif−/− MEFs treated with TNFα and zVAD-FMK with and without necrostatin. All assays were performed in duplicate and averaged from three independent experiments. *p<0.01 vs untreated; # p<0.01 vs WT treated. C, Transmission electron microscopy in Ppif−/− MEFs treated with and without TNFα and zVAD-FMK for 2 hours (Early) and 12 hours (Late) at 2 different magnifications. D, Images of pancreas stained with H&E from WT and Ppif−/− mice treated with caerulein to induce necroptosis-mediated pancreatitis (200X magnification). E, Calcium uptake capacity assay with membrane impermeable calcium indicator dye Calcium Green-5N in purified mitochondria from WT liver pretreated with tBID (red) or vehicle (black). The calcium addition is labeled with an arrowhead. Fluorescence reduces as the mitochondria take up the calcium from the solution; three independent experiments were performed, although a representative trace is shown.

doi:10.1371/journal.pone.0130520.g003

MOMP and MPTP Mediate Necroptosis

PLOS ONE | DOI:10.1371/journal.pone.0130520 June 10, 2015 7/12
suggesting that this event required RIP3 activity (Fig 4A). Less MLKL translocation to the mitochondria was also observed in Bax/Bak1 DKO MEFs with TNFα and zVAD-fmk (Fig 4A).

Upon examination of an array of Bcl-2 family members we found only 1 to be changed during necroptosis. The mitochondrial matrix isoform of Mcl-1, which is normally thought to be protective [23], was mostly lost upon necroptosis but this loss was inhibited by deletion of Ripk3 suggesting that necroptosis can have a direct effect on mitochondrial death regulatory proteins (Fig 4A). However, deletion of Bax/Bak1 or Ppif did not antagonize the loss of Mcl-1 from the mitochondrial matrix (Fig 4A). Another protein that translocated to the mitochondria in WT MEFs and not in Ripk3−/− MEFs upon TNFα and zVAD-fmk treatment was cofilin-1 (Fig 4A). Cofilin-1 is an actin depolymerase, but in times of stress it translocates to the mitochondria where it can lead to mitochondrial dysfunction [20–22]. For example, isolated mitochondria treated with recombinant oxidized cofilin-1 induces MPTP dependent mitochondrial swelling [22]. Here we observed that cofilin-1 translocation did not occur in the Bak/Bak1 DKO or Ppif null MEFs following treatment of TNFα and zVAD-fmk, suggesting cofilin-1 translocation is downstream of mitochondrial dysfunction and that it could also participate in necroptosis.
Discussion

Bax/Bak lead to two permeability states of the outer mitochondrial membrane, a low permeability state produced by inactive monomeric Bax/Bak and a high permeability state created by active oligomeric Bax/Bak that is responsible for MOMP and the release of cytochrome c. In addition, the lower permeability state induced by Bax/Bak monomers is sufficient to allow mitochondrial swelling and rupture with ionomycin treatment, leading to a type of regulated necrotic cell death through the MPTP. Here, we show that necroptosis involves the mitochondria in mediating cell killing, but through a primary mechanism involving Bax/Bak oligomerization in the outer mitochondrial membrane that in turn increases the sensitivity of MPTP opening to any given calcium or ROS stimuli, which then produces the characteristic necrotic signature of the cells as visualized by electron microscopy. Therefore, necroptosis is a hybrid of apoptosis and necrosis as it utilizes both mitochondrial MOMP and MPTP in mediating how the cell actually dies (Fig 5). Furthermore, we show that the upstream regulators of the necroptotic pathway impact the protein content of the matrix isoform of Mcl-1, which could participate as a triggering event of mitochondrial dysfunction (Fig 5).

Here we show that mitochondrial dysfunction is a necessary downstream mechanism whereby necroptosis produces cell death, as Ppif or Bax/Bak1 null MEFs maintain mitochondrial integrity and are protected from cell death with TNFα and zVAD-fmk treatment. These results are consistent with a recent manuscript showing that ablation of the mitochondria through treatment of CCCP and Parkin overexpression has no effect on necroptosis while
severely blunting apoptosis, as apoptosis requires cytochrome-c release and significant ATP to proceed [18]. Hence, maintenance of mitochondrial function in the face of necrotic stimuli serves to protect the cell from death, as in either Ppif or Bax/Bak1 DKO null MEFs. These results explain some of the ongoing controversy in the necroptosis field in regards to mitochondrial involvement [16]. In the case of necroptosis, death receptor activation in the presence of caspase inhibition leads to necrosis [27] that others have subsequently shown utilizes a RIP-dependent mechanism of cell killing [12, 13]. However, it remains unclear and controversial how RIP pathway activation directly leads to cell killing. Here we suggestively linked the necroptotic RIP effectors to these 2 processes by showing MLKL and cofilin-1 translocation to mitochondria as well as showing depleted matrix Mcl-1 following necroptosis induction, effects that are inhibited in Ripk3 null MEFs.

One inconsistency with our proposed model is based on the observation that caspase 8 deficient mice, which die during embryogenesis due to aberrant necroptotic cell death, are not rescued by simultaneous deletion of the Ppif gene [18]. In contrast, embryonic lethality associated with caspase 8 deficiency is fully rescued by deletion of Ripk3 in mice [28]. Our data show that MOMP occurs during necroptosis and that Ppif null cells are resistant to death induced by TNFα and zVAD-fmk. During this treatment all caspase activity is blocked with zVAD-fmk while in caspase 8 null mice other caspases remain functional. Since the MOMP presumably takes place downstream of RIP activation, death receptor stimulation in the absence of caspase 8 and CypD protein may result in apoptotic cell death in vivo as mitochondrial function is preserved and all other caspases are unaffected. Another reason why the Ppif caspase 8 double null mice may not be viable is that the MPTP may still be engaging in the absence of CypD, as loss of this protein only desensitizes opening and extreme stimuli can still cause necrosis [29].

Many questions remain as to how TNFα and zVAD-fmk treatment leads to mitochondrial dysfunction following RIP activation, although it is clear that mitochondrial preservation is protective during a necroptotic stress. The role of MLKL at the mitochondria needs to be further elucidated since this protein is required for necroptosis and it translocates to the mitochondria following a necroptotic stress. Furthermore, cofilin-1 translocation and matrix Mcl-1 depletion are two intriguing mechanisms that may be initiating events leading to mitochondrial dysfunction during necroptosis, as both events are known to cause mitochondrial dysfunction. Overall our results support the hypothesis that mitochondrial dysfunction is a critical step required for cell death due to upstream necroptotic signaling. Thus, necroptosis is a unique form of cell death that incorporates downstream mechanistic effectors of apoptotic (MOMP) and necrotic (MPTP) pathways.

Author Contributions

Conceived and designed the experiments: JK JDM. Performed the experiments: JK OK MJB MAS DMM. Analyzed the data: JK JDM. Contributed reagents/materials/analysis tools: MJB. Wrote the paper: JK JDM.

References

1. Green DR, Kroemer G. The Pathophysiology of Mitochondrial Cell Death. Science. 2004; 305 (5684):626–9. doi: 10.1126/science.1099320 PMID: 15286356
2. Halestrap AP. What is the mitochondrial permeability transition pore? J Mol Cell Cardiol. 2009; 46(6): 821–31. Epub 2009/03/07. doi: 10.1016/j.yjmcc.2009.02.021 PMID: 19265700.
3. Baines CP, Kaiser RA, Purcell NH, Blair NS, Oainska H, Hambleton MA, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature. 2005; 434(7033): 658–62. Epub 2005/04/01. doi: 10.1038/nature03434 PMID: 15800627.
4. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature. 2005; 434(7033):652–8. Epub 2005/04/01. doi: 10.1038/nature03317 PMID: 15800626.

5. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 2001; 292(5517):727–30. Epub 2001/04/28. doi: 10.1126/science.1059108 PMID: 11326099; PubMed Central PMCID: PMC3049805.

6. Karch J, Kwong QJ, Burr AR, Sargent MA, Elrod JW, Peixoto PM, et al. Bax and Bak function as the outer membrane component of the mitochondrial permeability pore in regulating necrotic cell death in mice. Elife. 2013; 2. doi: eARTN e00772. doi:10.7554/eLife.00772. ISI:000328625400001.

7. Karch J, Molkentin JD. Regulated Necrotic Cell Death The Passive Aggressive Side of Bax and Bak. Circ Res. 2015; 116(11):1800–9. PMID: 25999420

8. Irrinki KM, Mallilankaraman K, Thapa RJ, Chandramoorthy HC, Smith FJ, Jog NR, et al. Requirement of FADD, NEMO, and BAX/BAK for Aberrant Mitochondrial Function in Tumor Necrosis Factor Alpha-Induced Necrosis. Mol Cell Biol. 2011; 31(18):3745–58. doi: 10.1128/mcb.05303-11 PMID: 21746883

9. Tischner D, Manzl C, Soratco C, Villunger A, Kruminsnabel G. Necrosis-like death can engage multiple pro-apoptotic Bcl-2 protein family members. Apoptosis. 2012; 17(11):1197–209. doi: 10.1007/s10495-012-0756-8 PMID: 22971741

10. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ. 2008; 16(1):3–11. doi: 10.1038/cdd.2008.150 PMID: 18846107

11. Lin Y, Devin A, Rodriguez Y, Liu Z-g. Cleavage of the death domain kinase RIP by Caspase-8 prompts TNF-induced apoptosis. Genes Dev. 1999; 13(19):2514–26. PMID: 1052396

12. Cho Y, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. Cell. 2009; 137(6):1112–23. doi: 10.1016/j.cell.2009.05.037 PMID: 19524513

13. He S, Wang L, Miao L, Wang T, Du F, Zhao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α. Cell. 2009; 137(6):1100–11. doi: 10.1016/j.cell.2009.05.021 PMID: 19524512

14. Sun L, Wang H, Wang Z, He S, Chen S, Liao D, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell. 2012; 148(1):213–27.

15. Wu J, Huang Z, Ren J, Zhang Z, He P, Li Y, et al. Mlik knockout mice demonstrate the indispensable role of Mlik in necroptosis. Cell Res. 2013; 23(8):994–1006. doi: 10.1038/cr.2013.91 PMID: 23835476

16. Marshall KD, Baines CP. Necroptosis: is there a role for mitochondria? Frontiers in physiology. 2014; 5.

17. Lim S, Davidson S, Mocanu M, Yellon D, Smith C. The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. Cardiovasc Drugs Ther. 2007; 21(6):467–9. PMID: 17965927

18. Tait SW, Oberst A, Quaratro G, Milasta S, Haller M, Wang R, et al. Widespread mitochondrial depletion via mitophagy does not compromise necroptosis. Cell reports. 2013; 5(4):878–85. doi: 10.1016/j.celrep.2013.10.034 PMID: 24268776

19. Linkermann A, Bräsen JH, Darding M, Jin MK, Sanz AB, Heller J-O, et al. Two independent pathways of regulated necrosis mediate ischemia–reperfusion injury. Proceedings of the National Academy of Sciences. 2013; 110(29):12004–9. doi: 10.1073/pnas.1305381110 PMID: 23818611

20. Chua BT, Volbracht C, Tan KO, Li R, Victor CY, Li P. Mitochondrial translocation of cofilin is an early step in apoptosis induction. Nature cell biology. 2003; 5(12):1083–9. PMID: 14634665

21. Wabnitz G, Goursot C, Jahraus B, Kirchgessner H, Hellwig A, Klemke M, et al. Mitochondrial translocation of oxidized cofilin induces caspase-independent necrotic programmed cell death of T cells. Cell death & disease. 2010; 1(7):e58.

22. Klamt F, Zdanov S, Levine RL, Pariser A, Zhang Y, Zhang B, et al. Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. Nature Cell Biology. 2009; 11(10):1241–6. doi: 10.1038/ncb1968 PMID: 19734890

23. Pericaiavalle RM, Stewart DP, Koss B, Lynch J, Milasta S, Batinha M, et al. Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. Nature cell biology. 2012; 14(6):575–83. doi: 10.1038/ncb2488 PMID: 22544066

24. Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, et al. Stepwise activation of BAX and BAK by IBI, BIM, and PUMA initiates mitochondrial apoptosis. Mol Cell. 2009; 36(3):487–99. Epub 2009/11/18. doi: 10.1016/j.molcel.2009.09.030 PMID: 19917256; PubMed Central PMCID: PMC3163439.

25. Hoppins S, Edlich F, Cleland MM, Banerjee S, McCaffery JM, Youle RJ, et al. The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Mol Cell. 2011; 41(2):150–60. Epub 2011/01/28. doi: 10.1016/j.molcel.2010.11.001 PMID: 21214086; PubMed Central PMCID: PMC3022713.
26. Li L, Chen Y, Doan J, Murray J, Molkentin JD, Liu Q. A TAK1 Signaling Pathway Critically Regulates Myocardial Survival and Remodeling. Circulation. 2014;CIRCULATIONAHA. 114.011195.

27. Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. The Journal of experimental medicine. 1998; 187(9):1477–85. PMID: 9565639

28. Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature. 2011; 471(7338):368–72. doi: http://www.nature.com/nature/journal/v471/n7338/abs/10.1038-nature09857-unlocked. html#supplementary-information. doi: 10.1038/nature09857 PMID: 21368762

29. De Marchi U, Basso E, Szabó I, Zoratti M. Electrophysiological characterization of the Cyclophilin D-deleted mitochondrial permeability transition pore. Mol Membr Biol. 2006; 23(6):521–30. PMID: 17127624