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MULTI-AUTHOR REVIEW

Regulation of necrotic cell death: p53, PARP1 and cyclophilin D-overlapping pathways of regulated necrosis?

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Abstract In contrast to apoptosis and autophagy, necrotic cell death was considered to be a random, passive cell death without definable mediators. However, this dogma has been challenged by recent developments suggesting that necrotic cell death can also be a regulated process. Regulated necrosis includes multiple cell death modalities such as necroptosis, parthanatos, ferroptosis, pyroptosis, and mitochondrial permeability transition pore (MPTP)-mediated necrosis. Several distinctive executive molecules, particularly residing on the mitochondrial inner and outer membrane, amalgamating to form the MPTP have been defined. The c-subunit of the F1F0ATP synthase on the inner membrane and Bax/Bak on the outer membrane are considered to be the long sought components that form the MPTP. Opening of the MPTP results in loss of mitochondrial inner membrane potential, disruption of ATP production, increased ROS production, organelle swelling, mitochondrial dysfunction and consequent necrosis. Cyclophilin D, along with adenine nucleotide translocator and the phosphate carrier are considered to be important regulators involved in the opening of MPTP. Increased production of ROS can further trigger other necrotic pathways mediated through molecules such as PARP1, leading to irreversible cell damage. This review examines the roles of PARP1 and cyclophilin D in necrotic cell death. The hierarchical role of p53 in regulation and integration of key components of signaling pathway to elicit MPTP-mediated necrosis and ferroptosis is explored. In the context of recent insights, the indistinct role of necroptosis signaling in tubular necrosis after ischemic kidney injury is scrutinized. We conclude by discussing the participation of p53, PARP1 and cyclophilin D and their overlapping pathways to elicit MPTP-mediated necrosis and ferroptosis in acute kidney injury.

Keywords Regulated necrosis · Necroptosis · Bax · PARP1 · Cyclophilin D · p53

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Three modes of cell death

As important biologic processes, apoptotic cell death and autophagy are not only essential in normal development and homeostasis but also important in the pathogenesis of certain diseases. Autophagy is the basic catabolic mechanism that involves elimination of unnecessary or dysfunctional cellular components to preserve cellular homeostasis in baseline conditions and in response to stress. Autophagy also contributes to regulated cell death, during embryonic development in *Drosophila melanogaster* and in the death of cancer cells exposed to chemotherapeutic agents, hypoxia, or specific autophagy-inducing peptides [1, 2]. The stress factors, mechanisms and regulation and the specific subcellular compartments contributing to autophagy are elegantly reviewed elsewhere [3, 4] and are not discussed further. Apoptosis is ATP dependent and is characterized by cell and organelle shrinkage, membrane blebbing, chromosome condensation, apoptotic body formation and phagocytosis. Apoptosis is generally not associated with inflammation and is considered as a less harmful type of cell death. The apoptotic mode of cell death has been comprehensively reviewed by

several investigators [5–8] and is not the topic of this review. The third mode of cell death, necrosis, is ATP independent and has its unique morphological characteristics such as increased cell or organelle volume (oncosis), mitochondrial swelling, rupture of plasma membrane (cellular leakage), and consequent inflammation. Tissue detection of necrosis is usually defined in a negative fashion by excluding other types of cell death, such as apoptosis and autophagic cell death. Although semiquantification of tissue necrosis is possible based on histology, its gross quantification remains a challenge.

Mitochondria, Bcl2 family and cell death

Mitochondria, the principal energy source of the cell, are pivotal in necrotic and apoptotic cell death. Apoptosis is a highly orchestrated mode of cell death in which caspase-mediated executive canonical pathways have been investigated and revealed in detail. The extrinsic and intrinsic pathways of apoptosis are executed through triggering of a cascade of events leading to activation of executioner caspases [9]. The extrinsic pathway is activated primarily through inflammatory response and can occur independent of mitochondria [10]. The intrinsic pathway is dependent on mitochondrial release of cytochrome *c* (Cyt *c*) from mitochondria through the mitochondrial outer membrane permeability pore (MOMP), which is regulated by the Bcl2 family of proteins [9].

The 22 member Bcl2 family consists of pro-and anti-apoptotic proteins [10]. The primary function of the Bcl2 family is to regulate pore formation in the mitochondrial outer membrane thereby promoting MOMP and MPTP. Bax and Bak are the main proapoptotic family members involved in MOMP formation which is opposed by anti-apoptotic Bcl2-subfamily members [11]. In general, it is the equilibrium of concentrations of the pro-and anti-apoptotic members present in a cell that decides the fate of the cell [12]. The pore formation is also regulated by another subfamily of proapoptotic proteins termed BH3-only proteins, comprising of Bid, Bnip3 and PUMA among others. BH3 members either favor pore formation by binding to Bax and/or Bak or bind to anti-apoptotic members to block their binding to Bax and Bak [13, 14]. During apoptosis, Bax/Bak permeabilize the outer mitochondrial membrane (OMM) to release Cyt *c* to activate the caspase cascade and elicit apoptosis. The intricacies of the intrinsic and extrinsic apoptotic signaling pathways are elegantly reviewed elsewhere [15, 16] and will not be further discussed. Necrosis, however, involves the formation and opening of a pore connecting both the inner and outer membrane of mitochondria to form MPTP. Recent data indicate that Bax and Bak are also involved in the MPTP formation that drives the necrotic characteristics, mitochondrial swelling and rupture [17].

Multiple underlining signaling pathways have been defined in different cell death modalities of regulated necrosis, including MPTP-mediated necrosis, necroptosis, parthanatos, ferroptosis, and oxytosis [18]. On the one hand, these processes are characterized by their unique aspects of cell death mechanisms, but on the other, they also share some common signaling pathways and crosstalk between biochemical and molecular events [19]. Defining the role and molecular regulation of these different modes of necrotic cell death in specific disease states may help us to understand the disease pathogenesis and more importantly develop targeted therapeutic strategies.

MPTP-mediated necrotic cell death

Although necrotic cell death was considered as passive, blocking the functions of several molecules including cyclophilin D (CypD), PARP1 and RIP1 kinase are shown to inhibit the classic morphological characteristics of necrosis [8, 18]. These studies provide unequivocal evidence that necrosis is regulated and novel strategies to block necrosis could be developed. Necrotic cell death associated with acute ischemic injury including myocardial ischemia, stroke, acute liver, kidney and lung injury is primarily due to MPTP formation [20]. Upon MPTP formation, cytoplasmic water and solutes $\leq$1.5 kDa in size move osmotically into the mitochondrial matrix, resulting in organelle swelling and eventual rupture and catastrophic energy failure, key events in necrotic cell death [21]. ROS and Ca^{2+} increase the probability of opening the MPTP, whereas adenine nucleotides (i.e., ADP and ATP) inhibit pore formation [22, 23].

Based on existing studies, Ca^{2+}, the most noted mediator of permeability transition, enters the matrix through the mitochondrial calcium uniporter complex (MCU/MCUR1), although the target of this ion is not defined [24, 25]. Calcium entry is driven by the highly negative membrane potential ($\Delta\psi$) [22, 23]. Calcium overload can lead to the opening of MPTP, which is inhibited by cyclosporin A (CsA). CsA is a well-known calcineurin inhibitor, but also is a CypD inhibitor. Thus, CypD was considered as one of the downstream players in calcium-induced cell death [26]. Convincing evidence obtained in CypD knockout mice suggests that its deletion can prevent necrosis associated with ischemia/reperfusion injury in the heart [27] and brain [28] and kidney [29]. However, it should be emphasized that even in the absence of CypD, the MPTP can still open in response to a strong enough stimulus such as higher [Ca^{2+}] and oxidative stress [21, 27, 28]. Based on these data, CypD was accepted as a regulator but not necessarily a component of the MPTP pore [30].

Cyclophilin D belongs to the polypeptidyl-prolyl cis–trans isomerase family. CypD is located in the mitochondrial matrix and can interact with mitochondrial inner membrane proteins to induce the opening of the MPTP. Although numerous molecular constituents of the MPTP have been suggested to be required for pore formation, only CypD has held up to genetic scrutiny [31, 32]. Until the past decade, the model for the MPTP included the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane (IMM) with the voltage-dependent anion channels in the OMM, together forming a continuous channel across the intramembranous space under the control of CypD. However, genetic deletion studies showed that the ANT and voltage-dependent anion channels are dispensable for pore formation [31, 32]. Nevertheless, mitochondria from ANT null mice had twice the Ca^{2+} retention level from that of normal suggesting that ANT is a potential pore regulator. Deletion of various isoforms of voltage-dependent anion channel (VDAC) in mice also had no effect on MPTP formation and necrosis [31]. These studies disproved the original model of MPTP that was proposed to be one contiguous pore composed of VDAC on the OMM and ANT on the IMM regulated by CypD. Another candidate that was proposed to be the IMM component was the phosphate carrier (PiC) (*SLC25A3* gene). In the initial studies, the PiC carrier satisfied the requirements to be the IMM component including that it has the ability to form a pore, bind to CypD and can induce MPTP opening. However, recent studies using genetic deletion of the PiC did not affect the permeability transition, although mice with cardiac-specific PiC knockout were partially resistant to reperfusion injury and mitochondria isolated from the heart tissue were less sensitive to MPTP opening [33]. These data suggest that like CypD and the ANT, PiC may not be a component of MPTP but it plays a regulatory role in MPTP opening [34].

The recent search for the IMM component of the MPTP is converging on the F1/F0 ATP Synthase (Complex V) of the electron transport chain [35, 36]. F1/F0 ATP Synthase can physically interact with CypD and can form the permeability transition pore in a Ca^{2+}-dependent manner [37]. This binding also decreases the catalytic activity of ATP synthase, which can be restored by CsA, an inhibitor of CypD. However, the mechanism by which the F1/F0 ATP synthase transforms from a catalytic conformation into a channel is still under intense investigation. A possibility is that, when matrix Ca^{2+} level increases, the Mg^{2+} in the catalytic site will be replaced. Together with the action of CypD, the conformation change of F1/F0 ATP synthase ensues, leading to persistent MPTP opening and irreversible damage to the cells. In support of F1/F0 ATP synthase involvement of MPTP formation, gene silencing of the c-subunit of the F1/F0 ATP synthase inhibited pore formation. Further, the c-subunit by itself is capable of pore formation in proteoliposomes [38]. The CypD binding subunit of the F1/F0 ATP synthase is identified to be the oligomycin sensitivity-conferring protein (OSCP), the oligomycin-sensitive component that triggers MPTP formation by dimerization of the F1/F0 ATP synthase [36]. It is proposed that the F1 β-subunit of the F1/F0 ATP synthase inhibits the c-subunit and the inhibitory F1 subunit must be removed from the F0 ATP synthase to induce MPTP formation [38]. This hypothesis is in agreement with the role of Ca^{2+}-induced swelling, which induces release of the c-subunit from the F1, whereas CsA and ADP blocked this release.

Mitochondrial ATP synthasome

Classically, the ATP synthase, PiC and ANT are viewed as separate entities existing in the membrane. However, recent electron microscopy evidence shows that they co-localize as an ATP synthase/PiC/ANT complex, termed the ATP synthasome [39, 40]. The ATP synthasome is a very large complex with molecular mass of at least 0.7 million and is comprised of 17 subunit types and >30 total subunits. Although genetic ablation of ANT and PiC demonstrated that they are not required for MPTP formation, there is evidence that they could still regulate pore activity. Because of the close association between these components in the ATP synthasome, it is likely that may interact and/or cooperate to facilitate the MPTP formation by the c-subunit of the F1/F0 ATP synthase [41]. In addition, both ANT and PiC can bind CypD which may be an alternate or complementary mechanism by which CsA or cypD may regulate MPTP [34].

The lack of evidence for VDAC to form the MPTP also resulted in an intense search for potential OMM components of the MPTP. Although Bax and Bak were considered as proapoptotic molecules, several reports since 1998 indicated a role for Bcl2 family members, including Bax and Bak, in the regulation of MPTP. Bax was reported to be required for MPTP formation and mitochondrial membrane permeabilization [42] while Bcl2 overexpression prevented MPTP-dependent mitochondrial swelling [43]. Cell death induced by overexpression of Bax can be inhibited by CsA, which desensitizes CypD [44]. In 2012, Whelan et al. found that in response to Ca^{2+} challenge, mitochondria isolated from *Bax/Bak* double-knockout mouse embryonic fibroblasts were resistant to swelling and loss of membrane potential [$\Delta\psi$]. Importantly, these findings suggest that both *Bax* and *Bak* genes are required components of MPTP [45]. Following this discovery, in 2013, Karch et al. showed that loss of *Bax/Bak* reduced both permeability and conductance of the OMM, without altering inner membrane MPTP function; further, loss of

Bax/Bak resulted in resistance to mitochondrial Ca^{2+} overload and necrosis [17]. Reconstitution with *Bax* mutants that cannot oligomerize and form apoptotic pores can still enhance outer membrane permeability, permit MPTP-dependent mitochondrial swelling, and restore necrosis [17]. These data suggest that the MPTP is an IMM-regulated process, although in the absence of *Bax/Bak,* the OMM resists swelling and prevents organelle rupture, which prevents necrosis [17].

However, the saga on the identity of the molecular components of the MPTP continues as a new model comprising of Spastic paraplegia 7 (SPG7), its homologue AFG3L, and VDAC1 was recently proposed. SPG7 is a mitochondrial AAA-proteinase located in the IMM. Immunoprecipitation studies demonstrated that SPG7 can interact with CypD and VDAC1 and deletion of SPG7 protects cells from Ca^{2+}- or oxidant-induced MPTP opening, as observed in CypD knockout cells [46]. However, the model has its shortcomings as (1) like CypD, SPG7 deletion failed to protect cells from high Ca^{2+}-induced MPTP opening; (2) there is no evidence that SPG7 can actually form a channel and (3) VDAC isoforms are not essential components of the MPTP. It is likely that SPG7, like CypD, ANT and PiC, may be a regulator of the pore complex and not a component of the pore. Further studies are needed to delineate the precise role of SPG7 in MPTP formation.

Regulation of MPTP

The interaction of a multichaperone complex comprising heat-shock protein 60 (Hsp60), Hsp90, and tumor necrosis factor receptor-associated protein-1 with CypD [47] to regulate MPTP has been previously established in tumor cells [48]. A recent report by Lam et al. demonstrates that a newly identified partner of Hsp90, the hematopoietic substrate-1-associated protein x-1 (HAX-1) is involved in the regulation of CypD in the heart. HAX-1 binds to CypD and interferes with its binding to Hsp-90, rendering CypD ubiquitination and degradation, resulting in protection against MPTP opening and cell death [49].

PARP1-mediated necrosis

The highly conserved Poly-(ADP-ribose) polymerase (PARP) family consists of 18 members [50]. Poly(ADP-ribose) (PAR) polymerase 1 (PARP1), the most studied member is an important nuclear enzyme that regulates protein functions by poly(ADP-ribosyl)ation and gene expression as a transcription cofactor [51]. PARP1 catalyzes the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) and conjugates PAR onto various proteins as well as to PARP1 itself, thus leading to a variety of physiologic processes including up- or down-regulation in protein function, conformational changes and promotion of protein–protein interactions [52, 53]. Additionally, the role of PARP1 as a transcriptional regulator is confirmed by genetic or pharmacological inhibition, demonstrating its influence on the expression of inflammatory genes including nuclear factor-κB (NFκB) [54–56], tumor necrosis factor-α (TNF-α) [57, 58], interleukin-1-β (IL1-β) [57, 59], IL-6 [58, 59], intracellular adhesion molecule-1 (ICAM-1) [59, 60], and toll-like receptor (TLR4) [60]. Alternatively, activation of PARP1 is required for DNA repair [61] and in the presence of DNA single- or double-strand breaks, PARP1 transfers the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD^+) to various nuclear proteins and to PARP1 itself. Excessive activation of PARP1, such as in the setting of ischemia reperfusion injury (IRI), can lead to glycolytic inhibition [62], depletion of NAD^+, and consequent depletion of ATP [55]. Glycolysis is dependent on NAD^+ and its depletion could lead to inhibition of ATP production through glucose metabolism. PARP1 can also inhibit crucial enzymes in glucose metabolism, including GAPDH [62] and glucose phosphatase (hexokinase) [63] via poly(ADP-ribosylation). This will further exacerbate ATP depletion and impair cellular viability, particularly in cells that are highly dependent on glycolysis. Previous reports from us and others have demonstrated that PARP1 inhibition or gene deletion is protective against ischemia–reperfusion [62, 64, 65], diabetes [66], and ureteral obstruction [67].

An alternative mechanism by which PARP1-mediated cell death occurs is by parthanatos, where nuclear-to-mitochondrial translocation of poly(ADP-ribose) (PAR) triggers translocation of apoptosis-inducing factor (AIF) from mitochondria to nucleus. During translocation into the nucleus, AIF recruits an unidentified endonuclease, to mediate a caspase-independent cell death by inducing chromatin condensation and fragmentation into large fragments (~50 kb) [68] that are characteristics of parthanatos [69, 70]. When fibroblasts were pretreated with PARP inhibitors (DHIQ, DPQ) or if fibroblasts were isolated from PARP1-knockout mice, cell death was suppressed and AIF remained in mitochondria [71]. A later study in a cell-free system, provided further proofs for the role of PAR in AIF-mediated cell death signaling [72]. Recent data identified AIF as a high affinity PAR-binding protein with three putative PAR-binding domains [73]. AIF mutants with modified PAR-binding fragments failed to interact with the polymer or showed reduced PAR binding [74]. These data suggest that direct binding of pure or protein-bound PAR to AIF is necessary to induce AIF translocation but the exact mechanism responsible for AIF release in response to PAR binding is not yet understood.

Despite the strong evidence for AIF-mediated cell death, the lack of AIF translocation in cell death models characterized by DNA damage and PARP also has been reported [75]. These data demonstrate that parthanatos is dispensable in certain cell types or in response to certain cell death inducers, while it is the predominant pathway in other PARP1-dependent models of cell death, suggesting that parthanatos is context dependent.

Although the energy catastrophe theory and parthanatos have been suggested to explain the mechanism of PARP1-induced cell death, recent studies implicated several specific molecules including RIP1, JNK, Bax, and MPTP and their signaling pathways in PARP1-mediated necrosis. In an earlier study, Xu et al. [76] proposed that PARP1 activation is mediated through the pro-necrotic kinase RIP1. RIP1 activates JNK1, which induced necrosis through MPTP. A more recent study has also implicated RIP1 and JNK as part of PARP1-mediated necrosis [77]. A role for JNK activation in PARP1-induced necrosis is corroborated by other groups [78]. However, these findings were challenged because inhibition of PARP1 failed to block TNFα-induced necrosis and that inhibition of RIP1 or RIP3 could not prevent PARP1-induced necrosis [79]. Consistent with this finding, RIP1 deficiency or acute inhibition of RIP1, either with siRNA or the RIP1 inhibitor necrostatin, also failed to prevent PARP1-induced necrosis. Further, the protective effects of RIP1 and PARP1 are additive in oxidative stress-induced necrosis suggesting that they represent separate necrotic pathways [79].

Moubarak and colleagues reported that PARP1-induced necrosis was dependent on activation of the calcium-activated protease calpain, which in turn induced Bax translocation to the mitochondrion where it elicited the release and nuclear translocation of AIF [80]. A critical role for calpain in mediating PARP1-induced cytotoxicity also was supported by several other independent studies [[77, 81], [82]]. However, Calpain-mediated cleavage of AIF was later shown to be dispensable for mitochondrial AIF release [83]. Further, Ca^{2+} chelation had no effect on mitochondrial-to-nuclear AIF translocation evoked by PARP1 activators of cell death [84]. Collectively, data so far suggest that while RIP1 is dispensable, JNK is an important mediator of PARP1-induced necrosis [84].

A notable finding is that Bax/Bak might also be important for PARP-induced cell death. In the Bax/Bak null cells, DNA alkylating agent-induced cell death is reduced, indicating that even preserving the normal function of mitochondrial outer membrane can protect cells from PARP-induced cell death. Bcl-2 overexpression also protected cells from DNA alkylating agent-induced cell death [80]. It is hypothesized that Bax/Bak deletion stabilizes the MOMP, whereby ATP depletion by PARP1 overactivation may be prevented or slowed and thus prevents mitochondrial damage. This premise is supported by the finding that when Bax/Bak depleted cells were forced to depend on glycolytic energy, they became more susceptible to DNA alkylating agent-induced cell death [85].

p53-dependent necrosis

The tumor suppressor protein p53 is a stress-responsive transcription factor because of its ability to transactivate multiple target genes in response to diverse stress conditions, including oxidative stress, genotoxic damage, oncogene activation, and hypoxia. Increased expression/activity of these p53 targets could stimulate prominent biological functions including cell cycle arrest, apoptosis, senescence, metabolism, and autophagy modulation [80, 81]. In addition, recent studies revealed a role for p53 in regulating necrotic cell death by activating independent signaling pathways that include induction of mitochondrial outer and inner membrane permeability, altered mitochondrial dynamics and PARP1 activation.

p53 and MOMP

The MOMP is required for the execution of necrotic cell death as it forms part of MPTP. Although Bax and Bak were considered as proapoptotic molecules, emerging evidence clearly demonstrates that Bax and Bak are integral components of the MOMP of the MPTP. p53 can control the MOMP by (1) regulating the expression and function of Bax and Bid at the transcriptional level; (2) direct interaction with proapoptotic Bax/Bak and antiapoptotic Bcl2 to modulate the pore activity and (3) suppressing Bcl2 expression via activation of microRNAs (miR). However, the role of p53 in regulating Bcl2 family members to elicit necrosis remains undefined.

As discussed earlier, Bax/Bak in their non-oligomerized form induced a low level of permeability of the outer mitochondrial membrane that is distinct from its mode of releasing Cyt *c* in apoptosis, and permits necrosis through the MPTP [17]. However, the role of p53 in regulating Bax/Bak is context dependent. For example, muscle-specific deletion of Bax, but not p53, significantly reduced skeletal muscle necrosis and dystrophic pathology in a mouse model of muscular dystrophy [86]. On the other hand, our recent data demonstrated significant decrease in necrosis p53 KO ischemic kidneys possibly through diminished expression of PARP1, Bax and Bid [87]. A recent study also showed that p53 can translocate to mitochondria to interact with CypD and induce the opening of MPTP regardless of Bax/Bak-activated MOMP [88].

p53, MPTP and mitochondrial dynamics

It was shown that in mitochondria isolated from Bax/Bak double-knockout mice, oxidative stress triggers the p53 translocation to the mitochondria and induces the formation of a CypD–p53 complex that promotes MPTP opening and necrosis [89]. These results were supported by independent studies demonstrating p53–CypD interaction in MPTP-dependent necrosis in different cell models, including neuronal [90], pancreatic [91], and osteoblast cells [92]. The translocation of p53 to the mitochondria is shown to be dependent on dynamin-related protein (Drp1), a mitochondrial fission protein. Drp1 mediates the mono-ubiquitination of p53 by MDM2 to facilitate its translocation to induce necrosis in the setting of oxidative stress [93]. Inhibition of Drp1 by the Drp1 peptide inhibitor P110 prevented p53 association with the mitochondria and reduced brain infarction in rats subjected to brain ischemia/reperfusion injury [93]. Although these results provide a novel mechanism by which CypD is activated to trigger MPTP, a few questions about the universal nature of this interaction in MPTP remain to be answered. For example, the formation of MPTP in mitochondria lacking p53 and the absence of p53 involvement in calcium-induced MPTP opening are intriguing [94].

p53 and PARP1 activation

In response to cellular stress such as DNA damage, oncogene activation or hypoxia, the p53 tumor suppressor is activated and stabilized [95, 96]. The final outcome of p53 activation depends largely on the initial stress, the level of p53 expression and activation and the cellular milieu, in which different factors that modulate various signaling pathways, to elicit apoptosis, autophagy, cell cycle arrest or necrosis [97, 98]. PARP1 is also activated by various agents capable of inducing DNA breaks, including reactive oxygen and nitrogen species, ionizing radiation and alkylating agents such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) [61]. As discussed earlier, PARP1 activation could lead to necrotic cell death by different mechanisms. However, the initial response to DNA damage by both P53 and PARP1 is to repair the damaged DNA and thus maintain genomic integrity [99, 100]. Can these molecules act synergistically to carry out their functions and possibly regulate necrosis?

The interaction between p53 and PARP1 were reported almost 18 years ago. In aging cells undergoing telomere shortening [101], PARP1 binding was shown to be critical for p53 activation and function. p53 and PARP1 interaction was also demonstrated in cells undergoing apoptosis in response to DNA damage, independent of poly(ADP-ribosyl)ation of p53 [102]. Interestingly, in DNA-damaged neurons, PARP1 regulated p53 transcription activity by poly(ADP-ribosyl)ation [103], which was further supported by studies in irradiation-induced DNA-damaged cell lines, where pharmacological or genetic deletion of PARP1 modulated p53 activation [104, 105].

While these reports established a role for PARP1 in regulation of p53 activity, a reciprocal regulation of PARP1 by p53 was described by Montero et al. [106] in ROS-induced cell death. Genetic deletion of p53 in mouse embryonic fibroblasts (MEFs), human breast or colorectal cancer cells, conferred increased resistance to ROS and PARP-mediated necrotic cell death [106]. PARP activity at baseline and after ROS stimulation was reduced in the absence of p53. The mechanism by which p53 regulates PARP1 activation and necrosis, in the setting of ROS-induced DNA damage, remains to be defined. Interestingly, inhibition of PARP1-mediated necrosis led to p53-mediated caspase activation and apoptosis. p53 can also induce necrotic cell death in response to ROS-induced DNA damage, through the activation of the lysosomal cysteine protease cathepsin Q [107]. Further, as discussed above, p53 can translocate to the mitochondrial matrix and bind to cyclophilin D to induce the opening of the MPTP to elicit necrosis.

p53 and ferroptosis

Ferroptosis is a type of iron-dependent cell death characterized by accumulation of lipid peroxides [108]. Morphologically, ferroptosis is characterized by the presence of small mitochondria with condensed membrane densities, and is not associated with chromatin condensation, plasma membrane rupture, swelling of cytoplasmic organelles, or the formation of cytoplasmic vesicles/vacuoles [108]. Lipid peroxidation and ferroptosis are inhibited physiologically by antioxidant mechanisms including glutathione peroxidase 4 (GPX4), an enzyme, whose function depends on the glu/cys antiporter in the plasma membrane known as system Xc^-. SLC7A11 (xCT), together with SLC3A2 (4F2hc), encodes the heterodimeric amino acid transport system Xc^-, which mediates cystine–glutamate exchange. Decreased cystine transport results in reduced intracellular glutathione (reduced form) levels and increased production of ROS [108]. A recent report shows that p53 activation suppresses transcription of SLC7A11, leading to reduced cystine uptake, intracellular glutathione (GSH) and consequent increased ROS levels. Thus, in p53-activated cells, sensitivity to ROS-induced ferroptosis is increased. The effect of p53 on ferroptosis was independent of the ability of p53 to induce cell cycle arrest or apoptosis as a p53 mutant ($p53^{KR}$) lacking these functions retained the capacity to induce ferroptosis [109]. Further, erastin-induced ferroptosis was not prevented by inhibitors of autophagy, apoptosis or necroptosis suggesting that regulation of cystine uptake by p53 to promote ferroptosis is a

distinct mechanism [109]. However, it should be noted that this study did not exclude the possibility that cyclophilin D could be a downstream responder after p53 activation, even though MPTP-driven necrosis and ferroptosis are reported to be distinct mechanisms [108, 110].

Regulation of necrosis in acute kidney injury

Acute kidney injury (AKI) is a clinical syndrome with a rapid decline in glomerular filtration rate (GFR) over a period of hours to days, leading to retention of metabolic waste and disruption of fluid, electrolytes and acid–base balance. A leading cause of AKI is ischemia/reperfusion injury (IRI), which results from compromised perfusion to renal tissue. AKI has multiple contributing factors including low blood pressure, cisplatin nephrotoxicity, radiocontrast agent-induced injury, sepsis and various antibiotics used in the clinics. Given the numerous causes of AKI, it occurs in approximately 30 % of *all* patients admitted into intensive care units and is associated with very high mortality rates (50–80 %) [111–113]. It also develops in approximately one-third of patients who undergo cardiac surgery [114–116]. Further, for patients who undergo kidney transplants, post-transplant acute tubular necrosis, another cause of AKI, often results in delayed graft functioning and is one of the strongest predictors for the recurrence of end-stage kidney disease [117]. Even when patients regain normal, or near normal, kidney function after acute episodes, they still carry significant risks for long-term loss of renal function and development of chronic kidney disease [118]. Thus, given the increasing frequency of AKI, its related morbidity and mortality and the increasing financial burden, effective treatment options are critically needed. Despite decades of effort to alter the course of AKI, there is no approved effective therapy for this syndrome, implying that this syndrome involves several pathogenic factors which are not fully understood.

Pathogenesis of ischemic acute kidney injury

It has been determined that pathophysiologic abnormalities of IRI are characterized by changes in renal hemodynamics, tubular injury and cell death, and tissue inflammation [119, 120]. Ischemia/reperfusion to the kidney parenchyma leads to many secondary effects including disruption of cellular energy metabolism, production of ROS, and DNA damage. These secondary effects lead to activation of the nuclear repair enzyme PARP1 and the transcription factor p53. Further, activation of these molecules can initiate inflammatory signaling, intrinsic pathways that induce necrosis through opening of the MPTP, and apoptotic tubular cell death through MOMP [22, 56]. Although discussion of the role of apoptosis in full detail is beyond the purpose of this review, there are certainly some connections between these two types of cell death, possibly through participation of Bax/Bak and/or p53 signaling pathways.

As discussed earlier, although the MPTP is an inner membrane regulated process, in the absence of Bax/Bak, the outer membrane resists swelling and prevents organelle rupture to prevent necrotic cell death [17, 45]. The role of Bax/Bak in regulating or contributing to the MPTP and necrosis in AKI has not been studied, although some evidences suggest that Bax/Bak can induce non-apoptotic cell death. Dong and colleagues dissected the roles of Bax and Bak during ischemic AKI using several gene knockout mouse models. The major outcomes of the study were that global knockout of Bax increased inflammatory response and was not protective against ischemic AKI; however, conditional knockout of Bax from proximal tubules was renoprotective [121]. On the other hand, global knockout of Bak protected against ischemic AKI possibly through the maintenance of mitochondrial dynamics and integrity. Single knockouts for Bax or Bak blocked kidney tubular apoptosis during ischemic AKI without affecting tubular necrosis. These data suggest that distinct mechanisms exist for these two forms of cell death and deletion of both Bax and Bak may be required to prevent necrosis [121]. The effect of simultaneous deletion of Bax and Bak on necrosis in AKI remains to be defined, although their dual deletion is required to prevent apoptosis in obstructive nephropathy [122].

Mitochondria-mediated necrosis in AKI

The MPTP-dependent necrosis is a common type of cell death in ischemia–reperfusion injury because of the associated mitochondrial Ca^{2+} overload and ROS production [20]. As discussed earlier, ROS or Ca^{2+} are two of the major inducers of MPTP-mediated necrosis via activation of CypD. Accordingly, genetic deletion of CypD in mice led to significant protection against renal ischemia/reperfusion injury (IRI) as demonstrated by increased renal function and morphological protection [29]. CypD-deficient mice have less tubular necrosis as compared with wild-type mice after the IRI [29] and these data have been subsequently bolstered by others [123]. Interestingly, in an in vitro hypoxia/reoxygenation injury model of the renal tubules, the protective effect of cyclosporine A, an inhibitor of MPTP, was overcome by accumulation of non-esterified fatty acids [124]. It is suggested that the MPTP does not contribute to the initial bioenergetic deficit produced by hypoxia/reperfusion, but the deficit predisposes to subsequent development of the MPT [124]. In this regard,

our data indicate that the initial ATP depletion may be due to targeted glycolytic inhibition in the ischemic proximal tubules [62, 125] (discussed below). Recently, an interaction between cyclophilin D and glycogen synthase kinase 3β is shown to exert protection from diclofenac-induced ROS production and tubular cell necrosis [126].

PARP1-mediated necrosis in AKI

We reported that pharmacological inhibition of PARP1 protects rats and mice kidneys from ischemic injury [127, 128]. We have shown that in ischemic kidneys, PARP1 expression and activity significantly increased specifically in the S_3 segments of the proximal tubule. PARP1 inhibition protects from IRI by improving renal function and tissue morphology, attenuated ATP depletion, leukocyte infiltration and activation of inflammatory molecules. An interesting observation from these studies is that PARP1 inhibition blocked necrosis but had no effect on apoptosis. These results suggest that necrosis and inflammation are primary mediators of ischemic renal injury [127, 128]. Similarly, we showed that PARP1 deficiency reduced cisplatin-induced kidney dysfunction, oxidative stress, and tubular necrosis, but not apoptosis [56]. Moreover, neutrophil infiltration, activation of nuclear factor-κB, c-Jun N-terminal kinases, p38 mitogen-activated protein kinase, and upregulation of pro-inflammatory genes were all abrogated by PARP1 deficiency. We demonstrated evidence for a PARP1/Toll-like receptor 4/p38/tumor necrosis factor-α axis following cisplatin injury suggesting that PARP1 activation is a primary signal and its inhibition/loss protects against cisplatin-induced nephrotoxicity [56]. Collectively, these data suggest that targeting PARP1 may offer a potential therapeutic strategy for both cisplatin and IRI-mediated AKI by preventing necrosis and inflammation.

Although the energy catastrophe theory and parthanatos have been suggested to explain the mechanism of PARP1-induced cell death, our studies demonstrated that PARP1 plays a critical role in energy depletion after AKI. In the ischemic kidneys, lack of oxygen leaves the glycolytic metabolism as the main pathway for ATP production in proximal straight tubules (PST), thick ascending limbs and collecting ducts located in the severely hypoxic outer medullary region. However, glycolytic capacity in proximal straight tubules (PST) is selectively inhibited under ischemic conditions accounting for the incurred selective damage [129–132]. It was suggested that the differences in glucose utilization are not due to a difference in the distribution of glycolytic enzyme activities but due to a differential regulation of hemodynamic factors [133, 134]. Our data, however, indicate that PARP1 inhibits glycolysis specifically in the proximal tubule S_3 segments by poly(ADP-ribosyl)ation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Decreased activity of GAPDH makes proximal tubular cells vulnerable to necrotic cell death when subjected to ischemic injury. These data indicate that poly(ADP-ribosyl)ation of GAPDH and the subsequent inhibition of anaerobic respiration exacerbate ATP depletion and induce necrosis selectively in the proximal tubule cells after IRI.

p53-mediated necrosis after IRI

Several studies have investigated the pathological role of p53 in IRI but the results yielded different outcomes. For example, in 2003, Kelly et al. first showed that after IRI, the expression of p53 is increased in the medulla, and inhibition of p53 reduced renal injury [135]. Likewise, in 2009, Molitoris et al. showed that p53-targeted siRNA attenuated ischemic AKI [136]. However, Dagher et al. showed the p53-inhibitor pifithrin-α actually increased long-term renal fibrosis after IRI [137]. Sutton et al. further evidenced that p53 deficiency exacerbated the IRI [138]. These conflicting results may reflect different experiment protocols, severity of injury and most likely, the global inhibition of p53 in cells, including inflammatory cells. We reported that after IRI, proximal tubule-specific p53-KO mice showed significantly reduced levels of plasma creatinine and blood urea nitrogen (BUN), and improved renal morphology compared to WT mice [87]. Quantitative studies indicated that necrosis is significantly reduced in the PTs after IRI. Although the mechanism by which p53 deletion results in decreased necrosis is not defined, our results demonstrated that the expression level of PARP is attenuated in p53 KO compared to wild-type mice ischemic kidneys. These data suggest that PARP1 expression is regulated by p53 in ischemic kidneys and that this may be a potential mechanism by which p53 regulates necrosis. Further, in the p53-KO proximal tubule (PT) Bax and Bid levels were decreased in ischemic kidneys. These data suggest that specific deletion of p53 in the PT decreases the expression of potential components of MOMP and thus could inhibit both apoptosis and necrosis after IRI.

Like PARP1, p53 plays a major role in ATP depletion selectively in the S_3 segments of the proximal tubule. p53 induces the expression and activation of Tp53-induced glycolysis and apoptosis regulator (TIGAR) selectively in proximal tubules after ischemia–reperfusion injury. The activation of TIGAR inhibits the rate-limiting, phosphofructokinase-1 activity and glucose 6-phosphate dehydrogenase (G6PD) activity [125, 139, 140]. Therefore, it makes the proximal tubules more susceptible to ischemia and cell death in the settings of severe ischemia–reperfusion injury. Thus, TIGAR activation, along with PARP activation [62], could be key mechanisms involved in the cellular regulation of selective inhibition of glycolysis in the ischemic kidney proximal tubules.

Cross talk between defined necrotic pathways

Is MPTP required for PARP1-induced necrosis?

Xu et al. [76] proposed that PARP1 activation is mediated through the pro-necrotic kinase RIP1-JNK1 axis, which induced necrosis through MPTP. Data from Alano et al. also suggested that PARP1-mediated NAD^+ depletion could lead to MPTP opening and necrosis. However, Dodoni et al. demonstrated that the alkylating agent, MNNG, could directly induce MPT independent of PARP1 suggesting that the MPT pore may in fact be upstream of PARP1 activation and NAD^+ depletion [141]. Further, inhibition of PARP1 failed to block TNFα-induced necrosis and inhibition of RIP1 or RIP3 could not prevent PARP1-induced necrosis [79]. In addition, the protective effects of RIP1 and PARP1 are additive in oxidative stress-induced necrosis suggesting that they represent separate necrotic pathways [79]. It is also noteworthy that although a role for MPT in PARP1-induced AIF translocation is suggested by two reports [77, 142], a recent study suggests that PARP1 and MPTP can induce necrosis independent of each other [84]. Collectively, these data suggest that PARP1 and CypD-mediated necrotic pathways may be independent of each other. It will be interesting to determine if mice doubly deficient for PARP1 and CypD additively protects against ischemia/reperfusion injury, which would clarify their independent roles in mediating necrosis.

Necroptosis, MPTP-mediated necrosis and ferroptosis in AKI

The signaling pathway of necroptosis has been reviewed in detail recently [143–145]. Briefly, necroptosis can be triggered by death receptors including tumor necrosis factor 1 (TNFR1) [146, 147], stimulation of Toll-like receptors (TLR3 or 4) [148, 149], signaling through interferon receptors [150], or recognition of intracellular viruses by the protein DAI [151]. These pathways can induce the association of RIPK1 with RIPK3 via receptor-interacting protein–homotypic interacting motif (RHIM) RHIM–RHIM domain interactions and phosphorylation of RIPK3, which leads to aggregation of phosphorylated RIPK3 and phosphorylation of MLKL by RIPK3 [152]. Necroptosis generally occurs only if pro-survival transcriptional and/or apoptotic pathways are compromised. Direct phosphorylation of RIPK3 by RIPK1 has not been demonstrated and it is proposed that oligomerization of RIPK3 driven by the RHIM domain of RIPK1 and RIPK3 leads to RIPK3 autoactivation [152]. Phosphorylation exposes the N-terminal portion of MLKL [153] to induce plasma membrane rupture and induce necroptosis, with release of DAMPs [154–156]. Thus, MLKL appears to be a key necroptotic effector, but exactly how it disrupts membranes is still not completely understood [157].

Contribution of necroptosis to ischemic injury in the kidney was demonstrated by the protective effect of necrostatin-1 (Nec-1), which was considered as an inhibitor of RIPK1 [158]. A protective role for Nec-1 in cisplatin and hypoxia injured tubular cells [159, 160] was also reported. Similarly, RIPK3-deficient mice were also shown to be protected against ischemic and cisplatin-induced AKI [161]. However, a recent study questions the specificity of Nec-1 since it might have off-target effects on ferroptosis [162]. A recent study demonstrated that deletion of FADD or caspase-8 or Nec-1 inhibition failed to protect isolated renal tubules from hypoxic injury [110]. Similarly, Nec-1 mediated protective effect in cyclosporin-mediated tubular damage [163] or contrast-mediated AKI [164] was not due to prevention of tubular cell death. These data suggested that the effect of genetic loss of RIPK3, FADD or caspase-8 or Nec-1 inhibition on reducing kidney ischemia–reperfusion injury may not be due to loss of necroptosis, but may be due to extratubular effects including vascular diameter changes and hemodynamic alterations [110]. Further, these data argue against necroptosis as the primary mode of regulated cell death in renal tubules.

Evidence that ferroptosis plays an important role in acute renal failure was demonstrated by Angeli et al. in Gpx4-deficient human renal proximal tubular epithelial cells [162]. Gpx4 catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, and lipid peroxides utilizing reduced glutathione and protects against oxidative stress. Gpx4 knockdown rendered cells succumb to ferroptosis-inducing agents, indicating a Gpx4-regulated ferroptotic machinery in the cells [162]. In a recent study, Linkermann et al. reported a significant role for iron-dependent ferroptosis in necrosis of renal tubules, in models of severe IRI and oxalate crystal-induced acute kidney injury [110]. Linkermann's group also reported that double knockout of cyclophilin D and RIPK3 provides stronger protection against prolonged ischemic injury than the respective single knockout mice [161]. These data suggested that cyclophilin D-induced necrosis and necroptosis might be two independent pathways, although both are important in ischemic renal injury. Pharmacological inhibition studies also demonstrated that the ability of the ferroptosis inhibitor ferrostatin (termed 16–86) to protect from renal IRI is superior to Nec-1 and to that of sanglifehrin A (SfA) [110]. A combination therapy with 16-86 and [Nec-1 + SfA] in a model of ultrasevere IRI (bilateral renal pedicle clamping for 50 min) reduced plasma levels of serum urea and serum creatinine, suggesting that a triple combination therapy with [Nec-1 + SfA] plus 16–86 is superior in the prevention of renal IRI compared with the double-combination therapy with [Nec-1 + SfA]. These data suggest that at least three independent pathways of

regulated necrosis may be involved in IR-mediated organ damage or that inhibition of overlapping elements with SfA and Nec-1 is incomplete [110].

Conclusion

Attempts to integrate different types of necrotic cell death into a universal process have been made, yet it seems only some terminal changes are overlapped. Adding to this puzzle, injured cells also manifest with necrotic cell death after inhibition of apoptotic and/or autophagic steps. How cell chooses one type of cell death over the other is not well understood. The current hypothesis is that inadequate energy production might be a reason why cell falls into necrotic death rather than apoptosis, based on the observation that apoptosis is ATP dependent, while necrosis is ATP independent. If this premise is true, then the health status of mitochondria as well as glycolytic capacity of the cells would be crucial points in deciding what mode of cell

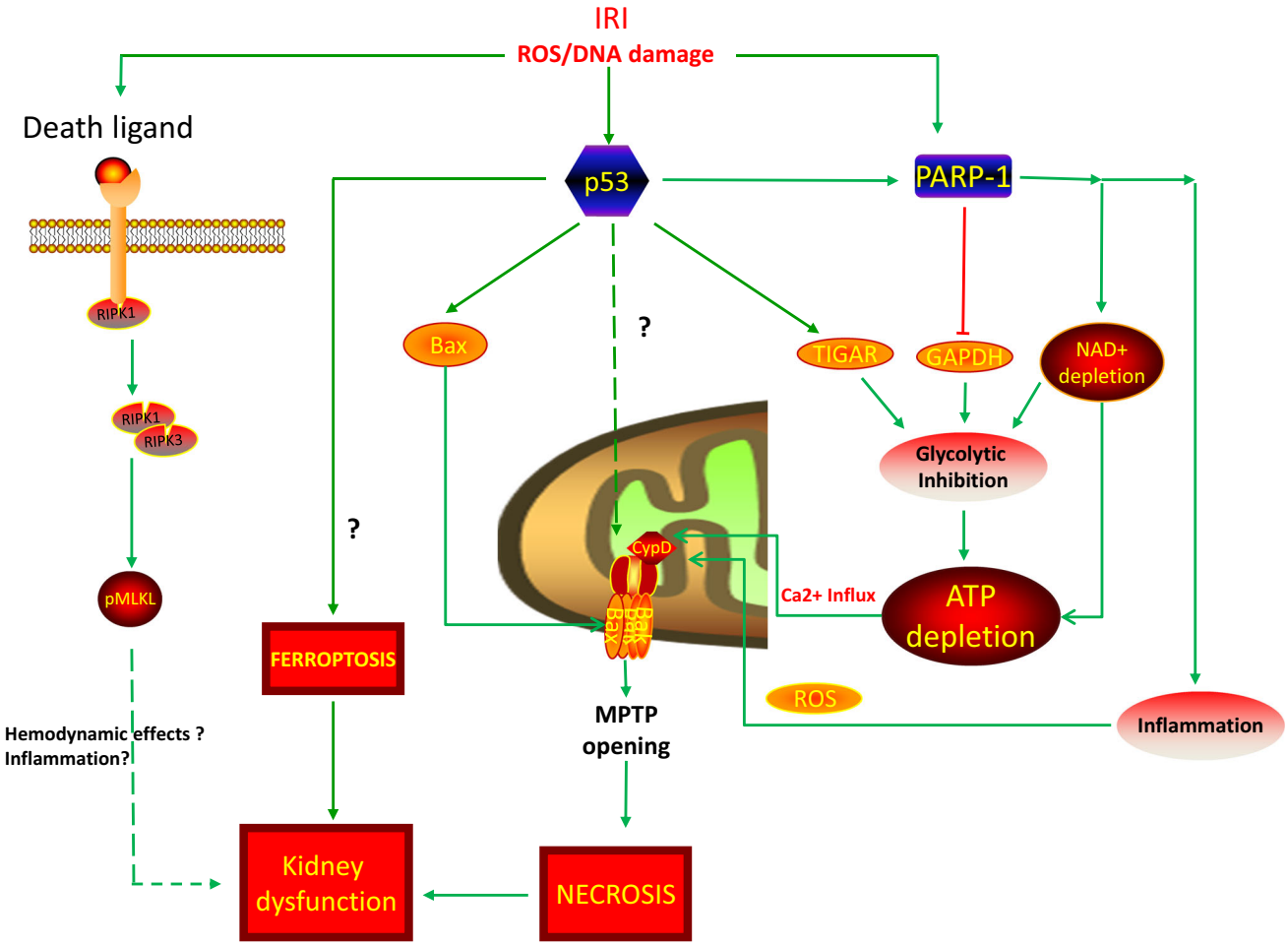

Fig. 1 A hypothetical scheme of the molecular hierarchy and cross talk characterizing regulated necrotic cell death in ischemic AKI. In the ischemic renal injury model, necrosis in proximal tubular cells is a common type of cell death. Initial injury results in DNA damage and rapid activation of p53 and PARP1. p53 induces the expression of Bax, which will facilitate the MOMP for necrosis [87]. Activated PARP1 will rapidly deplete intracellular NAD^+ and ATP, and simultaneously inhibit GAPDH, which reduces glycolytic capacity in proximal tubules [55, 62]. p53-induced TIGAR expression inhibits the rate limiting PFK and the glycolytic pathway [125, 139]. The severe ATP depletion from glycolytic inhibition and PARP1 activation shuts down ion homeostasis resulting in Ca^{2+} influx and uptake into mitochondria. PARP1 as a transcriptional cofactor induces several cytokines and promote infiltration of inflammatory cells to the injured renal parenchyma, all leading to increased ROS production [55]. ROS and Ca^{2+}, the most prominent mediators of permeability transition, increase the probability of MPTP opening via activation of CypD and the ATP synthasome complex [35]. Osmotic influx of water and solutes into the mitochondrial matrix leads to mitochondrial swelling and rupture of outer membrane, to elicit mitochondrial dysfunction and necrosis. Inhibition of ferroptosis can attenuate ischemic AKI. Although recent evidence suggests that p53 can mediate ferroptosis by regulating the expression of SLC7A11 [109], this pathway has not been tested in ischemic renal injury models. The contribution of necroptosis in proximal tubule cell death has recently been challenged and the mechanism by which RIP1 K blockade prevents renal injury remains to be elucidated [158]. Although, recent evidences suggest p53 translocation to mitochondrial matrix and activation of CypD [89], such a role for p53 is not established in kidney injury

death to develop. In clinical disorders such as IRI, it is unlikely that the different modes of cell death will exist in isolation. To develop efficacious treatment strategy, it is important to determine the relative contribution of each type of cell death and the key molecules and pathways that mediate specific forms of cell death.

Apoptosis plays an important role in different types of AKI and its inhibition has shown protective effects in experimental models. The recent findings that necrosis is significant in AKI and molecules including p53, PARP1, Bax/Bid, and CypD participate in necrotic cell death insinuate that integration of their signaling pathways may be required to elicit necrotic cell death in IRI. However, the hierarchy of their activation, physical interactions and cross talk between these molecules and how these molecules interact and integrate their functions to elicit necrosis in distinct AKI settings remain largely undefined. Based on the evidence presented in this review, a hypothetical scheme of the molecular hierarchy and cross talk characterizing necrotic cell death in ischemic AKI is presented in Fig. 1. Given the marked interest to pharmacologically target necrotic pathways in IRI, delineation of these pathways will provide key insights to our understanding of the pathophysiology of IRI and allow us to develop context-dependent therapeutic strategies to interfere with necrosis at precise signaling levels, without compensation from alternate pathways or potential systemic side effects. A key cellular organelle through which the above molecules may integrate their functions is mitochondria and hence maintaining mitochondrial health could prevent cell death. However, mitochondria are not involved in necroptosis, ferroptosis or PARP1-mediated ATP depletion, and hence combination therapies that may target multiple modalities of cell death may be required to completely prevent necrosis and maintain renal function.

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