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CLEARING THE FINAL HURDLES TO MITOCHONDRIAL APOPTOSIS: REGULATION POST CYTOCHROME *C* RELEASE

*P. Monian, X. Jiang**

Cell Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

In mammalian cells, the mitochondrial pathway of apoptosis plays a key role in various biological processes and has been extensively studied. One of the signature features of this pathway is permeabilization of the outer mitochondrial membrane (MOMP) and thus release of cytochrome c into the cytoplasm to trigger subsequent activation of executioner caspases. Because MOMP is associated with loss of mitochondrial function, it has long been believed to represent an irreversible commitment to cell death. However, emerging data over the last decade has indicated that induction of MOMP alone is not always sufficient to fully commit cells to death. As such, it becomes important to understand how apoptosis is regulated post-MOMP. Here we recount evidence investigating if and how cells can survive MOMP, and why this might have important physiological consequences. Furthermore, we review recent progress made in understanding how the pathway is regulated beyond MOMP and cytochrome c release. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

***Key Words*: apoptosis, regulation, cytochrome c release, mitochondrial outer membrane permeability.**

Apoptosis is one of the most fundamental processes to life. Essential to several processes ranging from normal development to regulation of the immune system and tissue homeostasis, apoptosis is conserved across all metazoans [1–3]. Deregulated apoptosis has been implicated in a variety of pathological conditions including cancer, neurodegenerative disorders and autoimmune diseases [4]. In mammalian cells, there are two major apoptotic pathways. The cell intrinsic pathway is characterized by mitochondrial outer membrane permeabilization (MOMP), a process regulated by the Bcl-2 family of proteins [5]. Following MOMP, several proteins in the intermitochondrial membrane space diffuse out into the cytosol. One such crucial factor, cytochrome *c*, binds to the cytosolic protein Apaf-1, triggering the formation of a heptameric caspase-9 activation complex, the apoptosome. Active caspase-9 then directly cleaves and activates the executioner caspases-3 and -7, leading to a series of morphological changes and ultimate apoptotic cell death [6, 7]. In the cell extrinsic pathway, binding of so-called "death-ligands" to their cognate receptors, triggers the recruitment of specific adaptor molecules such as FADD or TRADD which in turn induce dimerization of initiator caspases-8/10 [8]. The induced proximity activates caspase-8, which directly cleaves and activates downstream executioner caspases-3/7. Importantly, caspase-8 is also capable of activating the mitochondrial pathway of apoptosis by directly cleaving the BH3-only protein Bid. Truncated Bid localizes to the mitochondrial membrane and activates Bax, ultimately leading to MOMP [9–11]. Intriguingly, there exists a unique dichotomy relating to the functional significance of the crosstalk between the two pathways. In type I cells, direct processing of executioner caspases by caspase-8 is sufficient for robust death-receptor induced apoptosis. In type II cells however, amplification of the apoptotic pathway by caspase-8 mediated activation of Bid, and subsequent MOMP is essential for apoptosis.

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*Correspondence: Tel: 212-639-6814, fax: 212-794-4342
E-mail: jiangx@mskcc.org

Abbreviations used: APIP – Apaf-1 interacting protein; BIR3 – baculovirus IAP repeat 3; CARD – caspase recruitment domain; CAS/CSE1L – cellular apoptosis susceptibility protein; LTD – long-term depression; MOMP – permeabilization of the outer mitochondrial membrane; NAIP – neuronal apoptosis inhibitory protein; PHAP1 – putative HLADR-associated protein 1; ProT – prothymosin alpha; tRNA – transfer RNA; XIAP – X-linked inhibitor of apoptosis.

IS MOMP TRULY THE "POINT OF NO-RETURN" FOR CELL DEATH?

It has long been held that MOMP is a "point of no-return" for cell death, i.e., cells die following MOMP, irrespective of caspase activation [12–14]. However, given our ever-expanding understanding of the mitochondrial apoptotic pathway, this prevailing school of thought has increasingly been called into question. For instance, a deficiency in integral components of the pathway, like Apaf-1 or caspase-9, does not just delay, but completely inhibits developmental cell death in several cases [15–19]. In fact, deletion of Apaf-1 or caspase-9 can substitute for inactivation of p53 in myc-driven transformation of cells [20]. These observations strongly suggest that loss of mitochondrial membrane integrity without downstream caspase activation might not be sufficient to commit cells to death. Furthermore, it is now known that cytochrome *c* release is accompanied by release of additional factors like Smac/Diablo and Omi/HtrA2 which relieve caspase inhibition by members of the IAP family of proteins [21–24]. If MOMP were indeed the universal "point of no-return" for cell death, it would imply that such regulation of caspase activity beyond MOMP is unessential for cell death. However, differences in levels of the endogenous caspase-3 inhibitor XIAP not only modulate the cell intrinsic pathway, but can also have a profound effect on apoptosis induced by death ligands, i.e., the type I *versus* type II cells phenotype. Specifically, owing

to enhanced expression of XIAP in type II cells, MOMP-accompanied release of XIAP inhibitors Smac and Omi is essential for apoptosis induced by death ligands [25]. Thus, the regulation of proteins beyond MOMP has a significant effect on the cell extrinsic pathway of apoptosis as well. In this regard, it is worth noting that small molecules targeting anti-apoptotic Bcl-2 family proteins as well as IAPs are emerging as attractive agents for cancer therapy (see reviews by Almagro & Vucic [26], and Weyhenmeyer et al. [27] in this issue). Coupling such agents to traditional genotoxic regiments that induce caspase activation will likely maximize their therapeutic potential.

How might cells survive an insult to the mitochondrial membrane? It has been suggested that enhanced expression of GAPDH could mediate clonogenic survival following MOMP, provided caspase activation was inhibited [28]. Although the mechanism for this remains unclear, the data suggest GAPDH could mediate its effects by enhancing glycolysis and autophagy. Additionally, recent work has shown that such a cellular recovery following MOMP is dependent on the ability of the cell to repopulate its mitochondrial network through division of the few intact mitochondria that are able to maintain membrane integrity [29]. What intrinsic properties of individual mitochondria within the same cell cause them to be differentially affected by the same apoptotic stimulus remains an unanswered question. It is tempting to speculate that conditions such as individual mitochondrial localization relative to other cellular organelles, expression of anti-apoptotic Bcl-2 family members, and lipid composition of the membrane could be responsible for this effect.

WHY IS POST-MOMP REGULATION OF APOPTOSIS IMPORTANT?

Upon first glance, it seems counter-intuitive that a cell would commit precious resources to regulating apoptosis after the integrity of the mitochondrial membrane has been compromised. However, the additional nodes of regulation indeed have important physiological consequences. One obvious benefit to having additional regulation of apoptosis beyond MOMP is to ensure that a certain threshold of cytochrome *c*-mediated caspase activation needs to be achieved prior to a complete commitment to cell death. Such a safeguard against "accidental MOMP" is particularly relevant in the instance of post-mitotic cells, especially ones with poor regenerative potential. Indeed, long-lived cells such as cardiomyocytes and sympathetic neurons are particularly resistant to apoptosis induced by cytochrome *c* microinjection, likely due to markedly low Apaf-1 levels [30–32]. In both cell types, XIAP plays an important role in maintaining a high threshold of cytochrome *c* release needed to activate apoptosis. Such an intricate regulation of apoptosis beyond MOMP protects the longevity of these cells.

Caspases have been reported to have non-apoptotic functions as well. For instance, caspase-3 plays an essential role in the terminal differentiation of various cell types including myoblasts, lens cells, epidermal keratinocytes, and neural stem cells [33–37]. Cytochrome *c*-mediated caspase-3 activation also plays a significant role in long-term depression (LTD) in hippocampal neurons, a process essential for normal brain development and function [38]. Consequently, this process can be blocked by over-expression of Bcl-xL or XIAP, and caspase-3 knockout mice are deficient in their ability to undergo receptor-dependent LTD. Interestingly, both lens cell development and NMDA receptor-stimulated LTD are accompanied by detectable levels of cytochrome *c* release and caspase-3 activity which is significantly lower than that associated with apoptosis [39]. Rather than inducing cell death, the primary function of the mitochondrial pathway in these scenarios is to mediate such essential processes, through caspase-3 dependent cleavage of specific substrates. It is likely that concomitant regulation of caspase activity by pro-survival molecules like IAPs, survivin, Bcl-2, etc is required for these and other non-apoptotic functions of caspase-3 including regulation of B cell proliferation, dendritic cell maturation, forebrain development, etc. [40–42]. Thus, the ability to survive MOMP has functional relevance in proliferating cells as well.

Finally, post-MOMP regulation of apoptosis may have important implications in oncogenesis. Presumably, inhibiting caspase activity downstream of MOMP would confer upon cells the ability to survive apoptosis induced by various forms of chemotherapy, and thus impart significant oncogenic potential. Indeed, tumors often evolve mechanisms to inhibit caspase activity, most notably through upregulation of XIAP and survivin, or repression of Apaf-1 [43–45]. It is likely that many of the other proteins involved in post-MOMP regulation of apoptosis could also impact tumorigenesis in a similar manner. Thus, understanding how apoptosis is regulated beyond MOMP is important and will be discussed here.

REGULATION OF APOPTOSOME FORMATION

Under normal conditions, Apaf-1 is present in the cytosol in a monomeric, auto-inhibitory state. Binding of cytochrome *c* to Apaf-1 is the first step towards relieving this repression and triggering the formation of the apoptosome [46, 47]. Transfer RNA (tRNA) and intracellular K^+ are thought to inhibit this interaction by directly binding cytochrome *c* at physiological concentrations [48–50]. Additionally, independent studies have identified several proteins that regulate apoptosome formation through diverse mechanisms. For instance, Aven directly binds Apaf-1 and inhibits its oligomerization [51]. APIP (Apaf-1 interacting protein) competes with caspase-9 for Apaf-1 binding [52]. Although the exact mechanism remains unclear, the oncoprotein prothymosin alpha (ProT) potently inhibits Apaf-1 oligomerization [53]. The redox state of cytochrome *c* also influences caspase activation *in vitro*; oxidized cytochrome *c* stimulates, while re-

duced cytochrome *c* inhibits caspase activation [54, 55]. However, the exact mechanism underlying the modulation of the pro-apoptotic potential of cytochrome *c* in this manner remains unclear, and it should be noted that independent experiments have found reduced cytochrome *c* to be proficient at caspase activation as well [56].

Following cytochrome *c* binding, Apaf-1 undergoes a conformational change accompanied by nucleotide exchange, driving formation of the apoptosome [57]. Although Apaf-1 contains a bound nucleotide in its monomeric state, following cytochrome *c* binding, exchange of the bound nucleotide is a required step in the path to apoptosome formation. Based on *in vitro* studies, the absence of nucleotide exchange results in the formation of irreversible Apaf-1 aggregates, thus blocking downstream caspase activation [58]. This process of nucleotide exchange can be enhanced by a combination of three proteins: putative HLADR-associated protein 1 (PHAP1), Hsp70 and the cellular apoptosis susceptibility protein (CAS/CSE1L), thus driving apoptosome formation and subsequent cell death [59]. Consistently, PHAP1 has previously been characterized as a tumor suppressor in several different tumorigenesis models [60–63]. Furthermore, mutational analysis has demonstrated that the apoptotic activity of PHAP1 is required for its tumor-suppressive function [64]. Similarly, knockdown of CAS has been shown to inhibit cell death induced by a variety of apoptotic stimuli [59, 65–68]. Intriguingly, CAS has also been reported to have an essential function in mitosis and deletion of CAS in mice leads to embryonic lethality [69, 70]. Consistently, CAS expression is upregulated in highly proliferating tissue as well as several cancer cell lines. Furthermore, amplification and/or overexpression of CAS have been observed in several human tumors including melanoma, glioblastoma, ovarian, endometrial, liver, breast, prostate, and colon cancers among others [71–77]. These observations imply both putative tumor-suppressive and oncogenic roles for CAS, suggesting that perhaps CAS could have a janus-like function: playing an essential role in cell proliferation on one hand, and promoting apoptosis on the other.

Counteracting the effect of PHAP1, Hsp70 and CAS, intracellular levels of calcium inhibit apoptosome formation by binding and locking Apaf-1 in a "closed" conformation that is resistant to nucleotide exchange [78]. Nitric oxide is also thought to hinder Apaf-1 oligomerization, although the exact mechanism of inhibition remains unclear [79].

REGULATION OF CASPASE-9 ACTIVATION

Following nucleotide exchange and the accompanying conformational changes, the N-terminal caspase recruitment domain (CARD) on Apaf-1 is exposed allowing for a homotypic interaction with procaspase-9, which also contains a CARD domain. This induced proximity triggers dimerization and subsequent activation of caspase-9, which then directly activates caspase-3 [80–82]. Interestingly, apoptosome mediated activation of caspase-9 leads to robust auto-processing, which serves to decrease caspase-9 affinity for the apoptosome as well as its catalytic activity [83]. It can be argued that this "molecular timer" model of caspase-9 activation serves as another node of apoptotoic regulation following MOMP: intracellular concentrations of caspase-9, rate of procaspase-9 auto-processing, and rate of cleaved caspase-9 dissociation from the apoptosome together help set the pace of caspase-3 activation, and subsequent cell death. Genome-wide analysis in *D. melanogaster* revealed that procaspase 9 levels are subject to regulation by Tango 7 (human orthologue PCID1). Consistently, knockdown of PCID1 leads to decreased expression of procaspase-9, and is sufficient to inhibit cell death [84]. It is also worth noting that PCID1 is commonly repressed in pancreatic cancer [85]. Several CARD domain-containing proteins also impact the apoptosome-caspase 9: TUNCAN binds caspase-9 and inhibits its recruitment to apoptosome, while HCA66 and NAC/DEFCAP counteract this effect by binding Apaf-1 and enhance the amount of caspase-9 recruited or retained in the apoptosome [86–89]. It is likely that the cumulative effects of such protein-protein interactions determine the potency of caspase-9 activation.

The master suppressor of apoptosis, X-linked inhibitor of apoptosis (XIAP) also potently regulates caspase-9 activation. Following recruitment to the apoptosome and auto-processing, a N-terminal four amino acid neo-epitope becomes exposed on the small subunit of caspase-9. Structural and biochemical analysis have revealed that the baculovirus IAP repeat 3 (BIR3) domain of XIAP binds to this tetrapeptide sequence and prevents caspase-9 dimerization [90, 91]. Thus, XIAP sequesters processed caspase-9 in an inactive monomeric state, putting the breaks on cell death. Following apoptotic insult, the mitochondrial protein Smac is released into the cytosol where it relieves this impediment by competing for binding the BIR3 domain of XIAP, thus permitting caspase-9 dimerization [90, 92]. Omi/HtrA2 is another protein released from the mitochondria of apoptotic cells and following processing, binds and inhibits the XIAP-caspase interaction through a similar mechanism [23, 24, 93–95]. It should be noted that Omi additionally possesses a serine-protease activity that also contributes to its pro-apoptotic function.

Finally, caspase-9 activation is subject to regulation by multitude of protein kinases. Phosphorylation at Thr125 by Akt, CDK1-cyclin B1, ERK1/2, and DYRK1A inhibits the cleavage, and subsequent activation of procaspase 9 [96–100]. Although the exact mechanism underlying this inhibitory phosphorylation remains unresolved, it is clear that it has important physiological implications. For instance, mitotic arrest caused by chemotherapeutic agents like taxol, induces apoptosis in a caspase-9 dependent manner, which can be accelerated by a phospho-deficient mutation

at Thr125 [97]. Phosphorylation at the same residue by DYRK1A has been shown to play an important role in development of retinal cells in mice [101]. Given the importance of these kinases in other signaling pathways such as response to growth factors, cellular stresses, development, and cell cycle progression, such a node of regulation serves to intimately couple apoptosis to these diverse cellular processes. Other documented inhibitory kinases for caspase 9 include PKCz (Ser144), PKA (Ser99, Ser183, and Ser195) and CK2 (Ser348) [102–104]. Although some of these phosphorylation sites are dispensable, it is likely that they play significant regulatory roles in response to specific apoptotic stimuli. Caspase-9 processing can also be stimulated by phoshphorylation at Tyr153 by c-Abl, especially in response to genotoxic stress [105].

REGULATION OF CASPASE-3/7 ACTIVITY

The final cog in the wheel of mitochondria-mediated apoptosis is activation of the executioner caspase-3/7. In contrast to apical caspases, executioner caspases are present as inactive dimers under basal conditions, and require cleavage of the catalytic domain for activation [106, 107]. In the case of the intrinsic pathway, this need for cleavage is satisfied by activated caspase-9.

XIAP exerts its anti-apoptotic effect on executioner caspases as well, albeit through mechanisms different from its inhibition of caspase-9. Crystal structures have revealed that a "linker" region immediately N-terminal to the BIR2 domain of XIAP interacts with the substrate-binding grove of caspase-3/7, occluding binding of substrates [108–111]. The BIR2 domain itself is thought to play an auxiliary role in caspase inhibition, through contact with the small subunit of the activated caspase, which stabilizes interaction of the "linker" region with the catalytic site [111, 112]. As with caspase-9, Smac can relieve XIAP-inhibition of caspase-3, but through a different mechanism. While Smac competes with caspase-9 for the same binding site on XIAP, i.e. the BIR3 domain, the Smac-BIR3 interaction alone is insufficient to relieve inhibition of caspase-3 by XIAP. Rather, one of the N-termini of Smac protein (which is a homodimer) first binds to the BIR3 domain of XIAP (sufficient to relief inhibition of caspase-9); this interaction then anchors a subsequent interaction of the second N-terminus of Smac with the BIR2 domain of XIAP. This latter interaction is responsible for disrupting inhibition of caspase-3 by the "linker" region of XIAP [113]. XIAP also possess a RING finger domain though which it can ubiquitinate and target caspase-3/7 for proteasomal degradation [114]. However, the contribution of this E3 ligase activity of XIAP to its pro-apoptotic function remains unclear. Another member of the IAP family, neuronal apoptosis inhibitory protein (NAIP) plays an important regulatory role in neuronal apoptosis by directly inhibiting caspase-3/7 activation through its BIR2 and BIR3 domains [115–117].

CONCLUDING REMARKS

The long held view that MOMP is the final barrier to cell death has evolved quickly over the last decade. While, MOMP is still likely to be the point of no-return in the majority of scenarios, it is important to realize that this is not universally true and the exceptional cases have significant physiological consequences. We have discussed known details of how the mitochondrial pathway of apoptosis is regulated beyond cytochrome *c* release (summarized in the Figure), and despite the vast increase in our knowledge on this subject, several unanswered questions remain. For instance, experiments suggest that different nodes of regulation are largely cell-type and stimuli specific. How does the nature of the apoptotic insult determine which nodes of regulation play critical roles in determining cell fate? Furthermore, how do factors like GAPDH and possibly others stimulate recovery of mitochondrial integrity following MOMP and inhibition of caspase activation? Can the ability to survive MOMP be sufficient to impart oncogenic potential to a single cell? Answering these questions and others will help further our understanding of this critical pathway. Furthermore, this knowledge will serve to improve the design of small-molecule compounds that inhibit and/or accelerate apoptosis, and such drugs could ultimately have broad impacts across the treatment of various pathological conditions including cancer, infectious diseases, and autoimmune disorders.

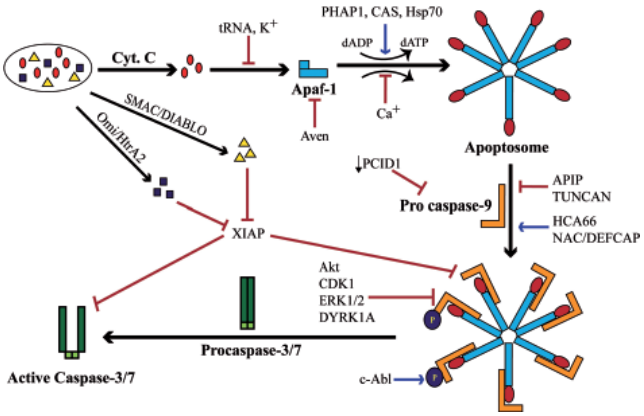

Figure. Post-MOMP regulation of apoptosis. Following release from the mitochondria, cytochrome *c* binds Apaf-1 and triggers the formation of a heptameric wheel-like complex, the apoptosome, which recruits and activates caspase-9. Proteins like Aven, physiological levels of nucleotides like tRNA and ATP, as well as intracellular K^+ can all inhibit this process by directly inhibiting the interaction between Apaf-1 and cytochrome *c*. Formation of the apoptosome also requires nucleotide exchange on Apaf-1, a process stimulated by a combination of three proteins: PHAP1, Hsp70, and CAS, and inhibited by intracellular Ca^{2+}. Recruitment of procaspase-9 to the apoptosome is antagonized by APIP and TUNCAN, and stimulated by HCA66 and NAC/DEFCAP. Furthermore, downregulation of PCID1 causes concomitant decrease in procaspase-9 levels. Direct phosphorylation at Thr125 by Akt, CDK-cyclin B1, ERK1/2, and DYRK1A inhibits caspase-9 activity through unclear mechanisms. Conversely, phosphorylation at Tyr153 stimulates activiation. XIAP mediated inhibition of caspase-9 and caspase-3 activity occurs through distinct mechanisms, and in both cases, this repression is relieved by SMAC/DIABLO and Omi/HtrA2, which are also released from the mitochondria following MOMP. See text for more details

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