

Non-apoptotic functions of apoptosis-regulatory proteins

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During the past two decades, apoptotic cell death has been the subject of an intense wave of investigation, leading to the discovery of multiple gene products that govern both its induction and execution. In parallel, it has progressively become evident that most, if not all, proteins that had initially been discovered for their essential role in apoptosis also mediate a wide range of non-apoptotic functions. On the one hand, apoptotic regulators and executioners are involved in non-lethal physiological processes as diverse as cell cycle progression, differentiation, metabolism, autophagy and inflammation. On the other hand, pro-apoptotic proteins can control other modalities of programmed cell death, in particular regulated necrosis. In this review, we summarize the unconventional roles of the apoptotic core machinery from a functional perspective and discuss their pathophysiological implications.

Keywords: AIF; apoptosome; BCL-2; caspase; death receptor; mitochondrial membrane permeabilization

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See Glossary for abbreviations used in this article.

Introduction

Apoptosis is a tightly regulated cell death modality that manifests with specific morphological features [1]. For some time, such a morphological uniformity has misled researchers to believe that most, if not all, instances of apoptosis would be executed by the same molecular mechanisms—with a prominent role for caspases, a peculiar class of cysteine proteases—and would have similar consequences at the organismal level (and hence fail to elicit inflammation), irrespective of the initiating stimulus. During the past two decades, along with an

increasingly more refined understanding of apoptosis and its pathophysiological implications, it has become clear that the apparent morphological uniformity of this cell death mode conceals a consistent degree of biochemical and functional heterogeneity [2].

According to accepted models, apoptotic cell death can result from the activation of either of two major molecular cascades. The 'extrinsic pathway' transduces extracellular pro-apoptotic signals through plasma membrane receptors, most often resulting in the activation of the caspase cascade. The 'intrinsic pathway' monitors the intracellular microenvironment and relays this information to mitochondria, where the decision between life and death is taken. If pro-apoptotic signals, such as those dispatched by damaged DNA or in response to oxidative stress, predominate, mitochondrial membranes become permeabilized, leading to the execution of apoptosis through both caspase-dependent and caspase-independent mechanisms. MMP can be initiated at the OM by the pore-forming activity of pro-apoptotic members of the BCL-2 protein family such as BAX and BAK, a process that is referred to as MOMP [3]. Alternatively, MMP can originate from an abrupt increase in the permeability of the IM to small solutes, leading to osmotic swelling of the mitochondrial matrix and the consequent breakdown of the OM [4]. This latter phenomenon, which is known as MPT, has been ascribed to the activity of the PTPC—a multiprotein complex assembled at the junctions between the IM and OM [5]. However, the existence of the PTPC as a pre-assembled complex, its precise molecular identity and its true relevance for apoptotic signalling remain a matter of debate (see below). Of note, the signalling modules for extrinsic and intrinsic apoptosis are not entirely disjointed as, in some instances, the activation of extrinsic apoptosis can be relayed to MMP, which amplifies and accelerates the process [2].

An exhaustive compendium of the molecules and biochemical processes that are involved in the regulation and execution of apoptosis goes beyond the scope of this review and can be found elsewhere [4,6]. Here, we analyse the unconventional roles of the apoptotic core machinery—that is, the functions of pro- and anti-apoptotic molecules in non-apoptotic settings—and discuss their pathophysiological relevance.

Differentiation

Various components of the apoptotic machinery, in particular caspases, become activated during, and are required for, the terminal

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differentiation of cell types as diverse as haematopoietic, epithelial, sperm, muscle and trophoblast cells. These functions of the apoptotic apparatus are exerted at the cellular level and do not entail the induction of cell death, implying that they must be conceptually discriminated from the roles that apoptotic molecules play as bona fide cell death regulators during morphogenesis and tissue homeostasis.

Initially, caspases—in particular, caspase-3, -9 and -14—were thought to participate only in differentiation programmes that exhibit some of the morphological signs of apoptosis, as they involve the degradation of entire organelles and portions of the cells (for example, the maturation of erythrocytes, platelets, keratinocytes and the lens epithelium; [7]). However, the chemical or genetic inhibition of caspase-3 arrests the differentiation of erythroid precursors at an early stage, well before chromatin condensation and subsequent nuclear extrusion occur [8]. Moreover, caspase-8 can mediate the maturation of macrophages [9] and the differentiation of the placental trophoblast [10], two processes that are not associated with morphological features of apoptosis. Along similar lines, caspase-6 negatively regulates the differentiation of plasma cells, an effect that might be related to its ability to control the cell cycle of B-cell progenitors [11]. There are at least three distinct, non-exclusive mechanisms that might explain why caspase activation leads to apoptosis in some cases and to differentiation in others: (i) spatial restriction, as exemplified by the activation of caspase-9 during thrombopoiesis, which only occurs in perinuclear, granular structures [12]; (ii) temporal restriction, as illustrated by the transient wave of caspase-3 activation that occurs during early erythropoiesis [8]; and (iii) substrate specificity, as demonstrated in several models of cellular differentiation [9,13]. Interestingly, such specificity might result from the presence of chaperone proteins, such as HSP70, that protect differentiation-relevant proteins, like the transcription factor GATA-1, from caspase-mediated proteolysis during erythropoiesis [13]. Similarly, during monocyte differentiation, the protein acinus is cleaved by activated caspase-3, while poly(ADP-ribose) polymerase is not processed [8].

Other apoptotic proteins that influence cell differentiation include APAF-1 and cytochrome c which underlie the activation of caspase-9 upon spatiotemporally restricted MMP also in non-apoptotic settings [7]. In addition, AIF, a caspase-independent cell death effector, and CRADD, an adaptor protein that transduces DNA-damage-elicited signals, both control the differentiation of adipocytes [14,15], though the underlying molecular mechanisms remain obscure. Of note, transgenic mice engineered for the overexpression of a dominant-negative variant of the TNFR1 functional interactor FADD or bearing a T-cell-specific *Fadd* gene knockout (the whole-body knockout is lethal, see below) exhibit retarded thymocyte development and reduced numbers of peripheral T cells [16,17]. It remains elusive whether such effects on T-cell differentiation are accounted for by the inactivation of the apoptotic or non-apoptotic functions of FADD.

Inflammation and immunity

One of the earliest recognized unconventional functions of the apoptotic apparatus is represented by the death-receptor-mediated activation of NF- κ B-regulated inflammation [18]. Many other components of the apoptotic machinery, including several caspases, might participate in inflammatory and immune responses.

Ligand-bound death receptors, in particular TNFR1, have the potential to trigger a wide range of cellular responses ranging from cell death, through extrinsic apoptosis or regulated necrosis, to

Glossary

AIF	apoptosis-inducing factor
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
ANT	adenine nucleotide translocase
APAF-1	apoptotic peptidase activating factor 1
ATR	ataxia telangiectasia and Rad3 related
BAK	BCL-2 antagonist/killer
BAX	BCL-2-associated X protein
BCL	B-cell lymphoma
BID	BH3 interacting domain death agonist
BIM	BCL-2 interacting mediator of cell death
BNIP3	BCL-2/adenovirus E1B 19 kDa interacting protein 3
CDK2	cyclin-dependent kinase 2
CDKN1	cyclin-dependent kinase inhibitor 1
cIAP	cellular IAP
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
CrmA	cytokine response modifier A
DIABLO	direct IAP binding protein with low pI
ENDO G	endonuclease G
EPO	erythropoietin
ER	endoplasmic reticulum
FADD	FAS-associated death domain protein
FGF2	fibroblast growth factor 2
HSP70	heat-shock 70 kDa protein
IAP	inhibitor of apoptosis protein
Ig	immunoglobulin
IFN γ	interferon γ
IL	interleukin
IM	inner mitochondrial membrane
IMS	mitochondrial intermembrane space
IRE1 α	inositol-requiring enzyme 1 α
LATS1	large tumour suppressor, homologue 1
MEF	mouse embryonic fibroblast
MMP	mitochondrial membrane permeabilization
MOMP	mitochondrial outer membrane permeabilization
MPT	mitochondrial permeability transition
MW	molecular weight
NF- κ B	nuclear factor- κ B
NOD	nucleotide-binding oligomerization domain containing
NLRP	NLR family, pyrin domain containing
OM	outer mitochondrial membrane
PRR	pattern recognition receptor
PTPC	permeability transition pore complex
RIG-I	retinoic acid-inducible gene 1
RIPK	receptor-interacting protein kinase
ROS	reactive oxygen species
Smac	second mitochondrial activator of caspases
STAT1	signal transducer and activator of transcription 1
TCR	T-cell receptor
TLR4	Toll-like receptor 4
TNF α	tumour necrosis factor α
TNFR1	TNF α receptor 1
TRADD	TNFR1-associated death domain protein
TRAFs	TNFR-associated factors
VDAC	voltage-dependent anion channel
XIAP	X-linked IAP

NF- κ B activation. Depending on the cell type and specific context, NF- κ B can transactivate genes with anti-apoptotic functions, such as *BCL-2*, or drive the production of pro-inflammatory mediators including TNF α and IFN γ [19]. Thus, it is not surprising that most, if not all, TNFR1 interactors, including caspase-8 and -10 [20], cIAP-1 and -2 (first characterized for their ability to bind, and hence

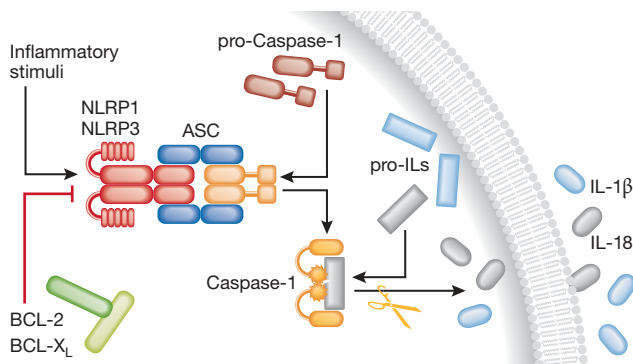


Fig 1 | Pro-inflammatory functions of caspase-1. Several stress conditions can trigger the assembly of the inflammasome, a multiprotein platform for the activation of caspase-1 consisting of specific PRRs such as NLRP1 and NLRP3 and the adaptor protein ASC. Active caspase-1 catalyses the proteolytic processing of pro-IL-1 β and pro-IL-18 followed by the secretion of mature IL-1 β and IL-18 into the extracellular milieu. BCL-2 and BCL-XL inhibit IL-1 β and IL-18 secretion by binding to NLRP1. ASC, apoptosis-associated speck-like protein containing a CARD; BCL, B-cell lymphoma; IL, interleukin; NLRP, NLR family, pyrin domain containing; PRR, pattern recognition receptor.

inhibit, active caspase-3; [21]), TRADD, FADD and RIPK1 [22], influence inflammatory responses as they are required for optimal NF- κ B activation. In addition, TRADD, RIPK1 and FADD participate in the intracellular signalling pathways elicited by PRRs such as RIG-I and TLR4 [23–25]. Similarly, XIAP, another member of the IAP family, is required for the full-blown activation of NF- κ B by cytosolic PRRs of the NOD family [26], presumably due to its capacity to bind directly to RIPK2 [27]. Finally, a nuclear pool of TRADD reportedly binds to STAT1, thereby altering its DNA-binding activity in response to IFN γ [28].

Thus, several components of the extrinsic apoptotic pathway are involved in inflammation and innate immunity. This also applies to some caspases: caspase-1 and -5, which are responsible—within the supramolecular complex known as the inflammasome—for the proteolytic maturation of the pro-inflammatory cytokines IL-1 β and IL-18 (Fig 1; [29,30]); caspase-11, which was originally described as an obligate co-activator of caspase-1 [31]; and caspase-12, which might be important for the attenuation of septic responses [32], as well as to the BH3-only protein BID, which can interact with cytosolic PRRs of the NOD family to facilitate the production of pro-inflammatory cytokines in response to NOD agonists [33]. Recent data suggest that the IL-1 β -deficient phenotype of *Casp1*^{−/−} mice described in 1995 [34,35] might be partly due to the concomitant, unwarranted deletion of *Casp11* [36]. Accordingly, caspase-11 seems to be required for the caspase-1-mediated production of IL-1 β by macrophages responding to bacterial products, but not to ATP and monosodium urate [36].

The regulation of intrinsic apoptosis also impinges on immune functions. The BCL-2 protein family has a key role in both the induction (BH3-only members) and the regulation (pro- and anti-apoptotic multidomain members) of both MOMP- and MPT-driven MMP [37]. Moreover, BCL-2 and its anti-apoptotic homologue BCL-X_L inhibit the inflammasome by interacting physically with NLRP1 [38,39]. The deletion of *Endog*, which codes for a mitochondrial endonuclease that participates in caspase-independent

apoptosis [4], was initially thought to be embryonic-lethal in mice [40], owing to the unwarranted concomitant deletion of an adjacent gene. Subsequent studies with proper knockout models revealed that *Endog*^{−/−} mice are viable and develop into adulthood with no obvious abnormalities [41]. B cells isolated from *Endog*^{−/−} mice are deficient in Ig class switch, due to an impaired generation of double-strand breaks in the switch regions of Ig genes [42]. Although this aspect has not yet been investigated, it is tempting to speculate that *Endog*^{−/−} mice might display increased susceptibility to some infectious diseases and a reduced propensity to develop autoimmune and allergic diseases.

Cell cycle control

Several constituents of the apoptotic apparatus, including components of both the extrinsic and the intrinsic pathway, have been shown to influence cell cycle progression, either by modulating proliferation in a rather general fashion or by controlling specific cell cycle phases (Fig 2).

Executioner caspases including caspase-3 and -6 reportedly restrain the proliferation of B cells [11,43], perhaps linked to changes in the expression of cell cycle inhibitors such as CDKN1 or to alterations in the requirements for B cells to trespass the G₀–G₁ boundary. In addition, caspase-3 might cause the release of prostaglandin E2 by tumour cells succumbing to chemotherapy, in turn stimulating the proliferation of residual, therapy-resistant, cancer cells [44]. Such a caspase-3-mediated autocrine circuit might have important therapeutic implications, as demonstrated by the fact that caspase-3 activation inversely correlates with disease-free and overall survival in cohorts of head and neck carcinoma, and breast cancer patients, respectively [44]. Intriguingly, the contribution of caspases to cell cycle regulation might be phylogenetically ancient, as suggested by the critical role of *Leishmania major* metacaspase in the correct segregation of the nucleus and the kinetoplast [45].

FADD and caspase-8 are required for the proper entry of activated lymphocytes into the S phase of the cell cycle [46,47]. T cells expressing a dominant-negative variant of FADD exhibit limited proliferation rates in response to TCR stimulation, correlating with defects in Ca²⁺ signalling [47], reduced phosphorylation of the S6 kinase, impaired expression of cyclin E and activation of CDK2 [46]. Many mitochondrial components of the apoptotic apparatus can participate in the regulation of the cell cycle. For instance, HTRA2—a mitochondrial protease that, upon MMP, promotes both caspase-dependent and -independent apoptosis—reportedly cleaves LATS1 in non-apoptotic circumstances, thereby generating LATS1 fragments that inhibit the G₁–S transition [48]. Along similar lines, the ectopic overexpression of DIABLO (another mitochondrial activator of caspases that is released after MMP) has been demonstrated to arrest leukaemic cells at the G₁–S boundary [49]. This contrasts with the observation that mice lacking the murine orthologue of DIABLO (*Smac*) are viable and normally develop into fertile adults [50], casting some doubts on the actual pathophysiological relevance of the apoptosis-unrelated roles of DIABLO. Finally, ENDOG not only seems to be required for DNA recombination and repair in both mammalian and yeast cells [51], but also might be necessary for proliferation, as ENDOG-depleted cells accumulate at the G₂–M transition [52].

Both BCL-2 and BCL-X_L function as negative regulators of the cell cycle, through several mechanisms. BCL-2 delays the G₁–S transition by (i) inhibiting CDK2 [53]; (ii) upregulating CDKN1 [54];

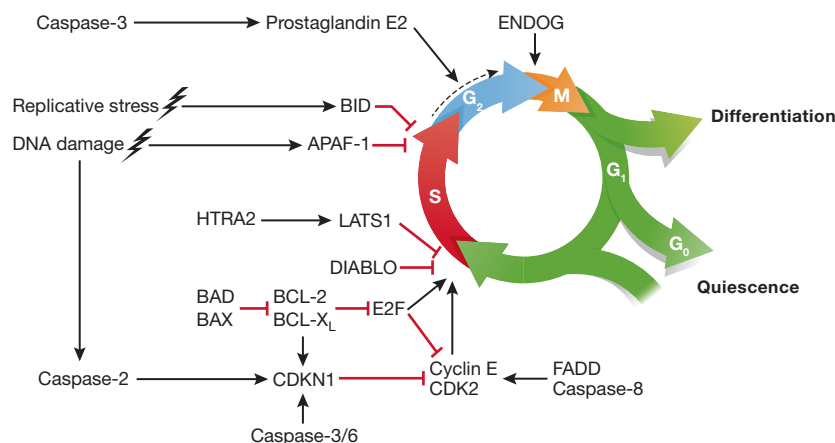


Fig 2 | Cell cycle control by apoptotic regulators. Several pro- and anti-apoptotic proteins have been shown to influence cell cycle progression. This can occur in a rather generalized fashion (as exemplified by the caspase-3-dependent release of prostaglandin E2 by dying cells) or involve specific cell cycle phases (as exemplified by the fact that cells overexpressing DIABLO are selectively arrested at the G₁-S boundary). Moreover, apoptotic regulators can control the cell cycle under physiological circumstances (as suggested by the fact that ENDOG-deficient cells accumulate at the G₂-M transition in the absence of any other stimulus) or in response to stress (as exemplified by caspase-2 and APAF-1, the depletion of which affects the cell cycle arrest induced by DNA damage). APAF-1, apoptotic peptidase activating factor 1; BAX, BCL-2-associated X protein; BCL, B-cell lymphoma; BID, BH3 interacting domain death agonist; CDK2, cyclin-dependent kinase 2; CDKN1, cyclin-dependent kinase inhibitor 1; DIABLO, direct IAP binding protein with low pI; ENDOG, endonuclease G; FADD, FAS-associated death domain protein; LATS1, large tumour suppressor, homologue 1.

and/or (iii) interfering with the transcriptional activity of E2F [54,55]. Similar cell cycle inhibitory functions have been ascribed to BCL-X_L [56,57], while pro-apoptotic BCL-2-like proteins such as BAX and BAD stimulate cell cycle progression [53,56]. Of note, the cell cycle regulatory functions of anti-apoptotic BCL-2 family members seem to be evolutionarily conserved [57,58] and mediated by a specific protein domain that does not participate in the regulation of apoptosis [59].

The cytosolic adaptor APAF-1 is involved in the regulation of the cell cycle in response to DNA damaging agents, as demonstrated by the fact that APAF-1-depleted human cancer cells fail to accumulate in the S phase upon cisplatin- and irradiation-induced DNA damage [60] and that the absence of APAF-1 facilitates DNA-damage-induced chromosomal instability [61]. *Bid*^{-/-} murine cells have also been ascribed with defects in the DNA damage response [62], but these results have been questioned by subsequent studies [63]. A nuclear pool of BID might be required for the proper activation of ATR in response to replicative stress [64]. Moreover, caspase-2—a DNA-damage-responsive caspase that can catalyse the proteolytic activation of BID [65]—appears to operate, independently of its catalytic functions and of any of its known interactors, as a translational regulator of CDKN1, mediating cytoprotective cell cycle arrest following DNA damage [66]. These observations suggest that components of the apoptotic machinery regulate the cell cycle not only in physiological settings but also in the context of stress responses.

Metabolism and autophagy

Some pieces of the molecular apparatus for apoptosis, notably those that operate in close connection with mitochondria, have been discovered to modulate various aspects of the bioenergetic and biosynthetic metabolism of the cell.

Due to its essential role as an electron shuttle between complexes III and IV of the mitochondrial respiratory chain, cytochrome c,

which upon MMP drives the apoptosome-mediated activation of caspase-9 [67], is the most representative metabolism-relevant component of the apoptotic machinery (Fig 3; [68,69]). AIF, a phylogenetically ancient protein of the IMS, exhibits NADH oxidase enzymatic activity [70]. Besides its role as a caspase-independent cell death effector [71], mammalian AIF is required for the assembly or stabilization of respiratory complex I (Fig 3; [70]), and the whole-body knockout of *Aif1* results in early embryonic death [72]. Yeast cells lacking *aif1* proliferate slowly when cultured on non-fermentable energy sources and are characterized by a respiratory defect in complex III [70]. Similarly, *Drosophila melanogaster* larvae lacking the fly orthologue of AIF manifest respiratory deficits in complexes I and IV, resulting in premature death by day 8 after egg laying [73]. In humans, distinct loss-of-function mutations of AIF either cause prenatal ventriculomegaly linked to decreased activities of respiratory complexes I and IV [74] or a severe mitochondrial encephalomyopathy that manifests postnatally [75]. In mice, the muscle- and liver-specific deletion of *Aif1* can result in increased glucose tolerance, enhanced insulin sensitivity, reduced fat mass and resistance against obesity induced by high caloric intake [76]. Altogether, these observations underscore an evolutionarily conserved, pathophysiologically relevant role of AIF in the regulation of mitochondrial metabolism.

Another point at which apoptotic cell death and metabolism intersect is represented by the PTPC [4]. By assuming a high conductance state, the PTPC mediates MPT (thus initiating MMP) in response to selected intracellular stress conditions, such as the overgeneration of ROS and cytosolic Ca^{2+} overloads [4]. In addition, most, if not all, structural components and functional interactors of the PTPC mediate important metabolic functions. For example, ANT catalyses the exchange of ATP with ADP between the mitochondrial matrix and the cytosol [77]. VDAC operates as a voltage-regulated channel for the transport of anionic solutes across the outer mitochondrial membrane [78]. Cyclophilin D functions as a peptidyl-prolyl *cis-trans* isomerase,

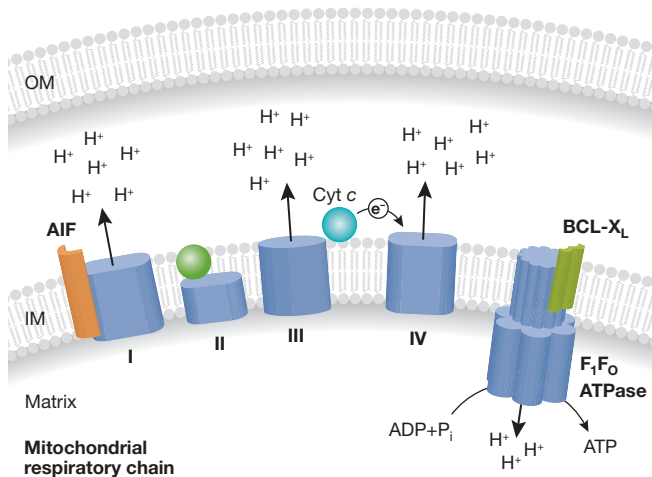


Fig 3 | Metabolic roles of BCL-X_L, cytochrome *c* and AIF. Both cytochrome *c* and AIF are essential for the function of the respiratory chain, and thus for mitochondrial ATP generation through the F₁F₀ ATPase. In particular, cytochrome *c* mediates the transport of electrons between complex III and complex IV, while mammalian AIF is critical for the structural stability of complex I. BCL-X_L binds to the F₁F₀ ATPase, thereby stimulating its enzymatic activity. AIF, apoptosis-inducing factor; BCL, B-cell lymphoma; IM, inner mitochondrial membrane; OM, outer mitochondrial membrane.

assisting the folding of proteins within the mitochondrial matrix [79]. The peripheral benzodiazepine receptor regulates the flux of cholesterol across mitochondrial membranes, thus controlling steroidogenesis [80]. Hexokinase converts glucose into glucose-6-phosphate, the starting block of glucose metabolism [81]. Finally, creatine kinase catalyses the ATP-dependent transformation of creatine into phosphocreatine, constituting a highly diffusible intracellular energy store [82]. Murine hepatocytes lacking *Ant1* and *Ant2* respond almost normally to inducers of MPT [83]. Similarly, *Vdac1*^{-/-} *Vdac2*^{-/-} *Vdac3*^{-/-} MEFs undergo MPT that is indistinguishable from their normal counterparts [84]. These observations suggest that the PTPC, or at least some of its constituents, might be dispensable for MPT-driven cell death. However, many PTPC components exist in multiple, at least in part, functionally redundant isoforms, which complicates the generation of reliable gene knockout models [7]. One notable exception is provided by cyclophilin D, which, unexpectedly, is fully dispensable for embryonic and post-embryonic development but required for the induction of necrotic cell death in response to several distinct triggers, *in vitro* and *in vivo* [85]. Thus, the actual pathophysiological relevance of both the apoptotic and non-apoptotic functions of the PTPC remain to be elucidated.

Both pro- (for example, BAX) and anti-apoptotic (for example, BCL-2 and BCL-X_L) BCL-2 family members operate at the ER to modulate the release of Ca²⁺ transients [86], which has direct implications for mitochondrial bioenergetics and for the function of a plethora of cytosolic Ca²⁺-regulated enzymes [87]. At the IM of neurons, BCL-X_L interacts physically with and stimulates the F₁F₀ ATPase, the enzymatic complex that exploits the electrochemical gradient generated by respiratory complexes I–IV to synthesize ATP (Fig 3; [88]). In addition, anti-apoptotic proteins from the BCL-2 family bind to and inhibit the essential autophagic modulator

Beclin 1 [89]. Conversely, multiple BH3-only proteins including BAD, BNIP3, BIM, BID and PUMA stimulate autophagy, owing to their ability to competitively displace Beclin 1 from inhibitory interactions with anti-apoptotic BCL-2 proteins [89]. Thus, by modulating autophagy, BCL-2 family members influence the maintenance of organellar homeostasis as well as the preservation of ATP and metabolic substrates during stress responses [90]. In addition, both pro- and anti-apoptotic BCL-2 proteins participate in the regulation of mitochondrial morphology and dynamics [91]. This intimate connection has important implications not only for apoptosis [92], but also for the bioenergetic functions of mitochondria in a variety of pathophysiological scenarios [93].

Regulation of non-apoptotic cell death

Recently, non-apoptotic instances of programmed cell death, in particular regulated necrosis, have spurred great interest [94]. Although the molecular characterization of this cell death sub-routine is only in its infancy, it has become increasingly clear that the molecular machineries for apoptosis and regulated necrosis are interconnected in a complex network of reciprocal control.

One of the best-characterized pathways of regulated necrosis, which has been named necroptosis, is elicited by the ligation of TNFR1 in conditions in which caspases, in particular caspase-8, are inhibited [94]. Necroptosis relies on the activation and mutual interaction of two homologue kinases, RIPK1 and RIPK3, and is executed by a bioenergetic catastrophe that involves, among other processes, ROS overgeneration and enhanced glycogenolytic and glutaminolytic fluxes [94]. As early as in 1998, when the existence of regulated necrosis was first intuited, the inhibition of caspases by Z-VAD-fmk or by the overexpression of the viral serpin CrmA was found to enhance the sensitivity of murine fibrosarcoma cells to TNF α -induced cell death [95]. This discovery ignited an intense wave of research that, throughout the following decade, led to the discovery of the molecular mechanisms whereby multiple components of the apoptotic apparatus inhibit non-apoptotic cell death modalities, in particular regulated necrosis.

In line with the original observations by Vercammen and colleagues [95], caspase-8 has been shown to tonically inhibit regulated necrosis by mediating the proteolytic cleavage of RIPK1 and RIPK3 [96,97]. *Casp8*^{-/-} mice do not survive embryogenesis beyond day 11.5 [98]. Strikingly, this embryonic lethal phenotype fully depends on RIPK3, as *Casp8*^{-/-} *Ripk3*^{-/-} mice develop into fertile adults [99]. Of note, *Casp8*^{-/-} *Ripk3*^{-/-} mice accumulate abnormal T cells in peripheral lymphoid organs, thus far resembling *Fas*^{-/-} mice [99]. These results suggest that caspase-8 is required for the proper development of the T-cell compartment by virtue of its apoptotic functions, whereas it is indispensable for embryonic development owing to its role as an anti-necrotic factor (Fig 4).

Similarly to their *Casp8*^{-/-} counterparts, neither *Fadd*^{-/-} nor *Ripk1*^{-/-} mice are viable. *Fadd*^{-/-} mice die *in utero* at embryonic day 15.5, displaying widespread necrosis and signs of cardiac failure and abdominal haemorrhage [100,101]. *Ripk1*^{-/-} mice appear normal at birth but succumb at 1–3 days of age, manifesting extensive apoptosis in the lymphoid and adipose compartments [102]. Notably, *Fadd*^{-/-} *Ripk1*^{-/-} double knockout mice are born at Mendelian frequencies, yet fail to survive beyond 3 weeks of age [101]. Moreover, lymphocytes isolated from *Fadd*^{-/-} *Ripk1*^{-/-} mice maintain some of the developmental defects that characterize their *Ripk1*^{-/-} counterparts [101]. Thus, while Fadd seems to be dispensable for the phenotypic

manifestations of the *Ripk1* knockout, at both the organismal and cellular levels, *Ripk1* is required for the embryonic lethality induced by the absence of *Fadd* (Fig 4). The intestinal epithelial-cell-specific knockout of *Fadd* has also been associated with spontaneous enteritis and colitis in mice, a pathology that failed to develop if *Ripk3* was simultaneously knocked out [103]. Similarly, the absence of *Fadd* in epidermal keratinocytes drives *Ripk3*-dependent necrosis and skin inflammation in mice [104]. Taken together, these observations demonstrate that the anti-necrotic activities of apoptotic regulators are particularly relevant *in vivo*, in both developmental and pathological scenarios.

Others

In addition to the structured functional profiles discussed above, some apoptotic regulators and executioners exert highly specific, often cell-type-dependent and context-dependent, activities, as illustrated here by a few examples.

Caspase-1 has been shown to mediate the proteolysis-independent secretion of multifunctional proteins including pro-IL-1 α and FGF2 [105]. In neurons, excessive but non-apoptotic caspase-3 activity triggers the calcineurin-mediated dephosphorylation and subsequent removal of AMPA-type receptors, leading to the degeneration of dendritic spines and alterations in glutamatergic signalling [106]. BCL-2 family members including BAK and BAX stimulate the unfolded protein response by interacting physically with the ER stress sensor IRE1 α [107]. FADD participates in the induction of neuroplasticity and in the development of the abstinence syndrome [108]. HTRA2 is important for the maintenance of neuronal homeostasis, and loss-of-function mutations of *HTRA2* have been associated with familial variants of Parkinson disease [109]. Finally, signals transduced by the death receptor FAS have been implicated in the paracrine crosstalk between the implanting embryo and endometrial cells [110]. Thus, the unconventional functions of the apoptotic apparatus are relevant in a plethora of distinct pathophysiological settings.

Concluding remarks

As we have discussed in this review, during the past two decades the molecular machinery for apoptosis has been demonstrated to mediate a wide array of non-apoptotic effects. These range from general bioenergetic functions, such as those mediated by cytochrome *c*, the depletion of which is incompatible with mammalian life, to more specific, often cell-type-restricted activities. These observations suggest that the apoptotic machinery has not evolved 'from scratch' but rather by taking advantage of pre-existing proteins with non-lethal functions. In this hypothetical scenario, one might wonder which proteins would have been selected by evolution to mediate apoptosis. On the basis of our current knowledge of the apoptotic apparatus, it is tempting to speculate that two types of proteins have mainly been co-opted to acquire functions in cell death regulation. First, proteins with peculiar structural features and/or that are normally segregated in non-accessible cell compartments. This is the case with IMS proteins such as cytochrome *c* and AIF, which receive their prosthetic groups—haem and FAD, respectively—and acquire their final conformation only once their precursors have been imported into mitochondria. Second, proteins that play a role in the management of stress. These factors—including BCL-2 family members, which regulate autophagy and the unfolded protein response, APAF-1, which controls DNA damage responses, and others (see above)—might have initially evolved as stress-response proteins

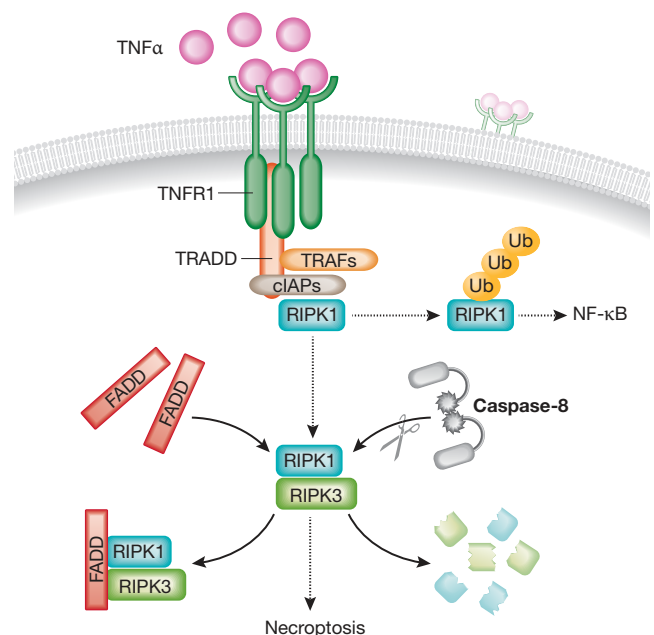


Fig 4 | Anti-necrotic functions of caspase-8 and FADD. In response to TNF α , the intracellular tails of TNFR1 trimers drive the assembly of a supramolecular complex including, among other factors, TRADD, TRAFs, cIAPs and RIPK1. Upon ubiquitination, RIPK1 can stimulate the canonical pathway of activation of the anti-apoptotic and pro-inflammatory transcription factor NF- κ B. Alternatively, deubiquitinated RIPK1 can interact with RIPK3 and trigger necroptosis. The pro-apoptotic proteins FADD and caspase-8, the latter by cleaving RIPK1 and RIPK3, tonically inhibit this pro-necrotic signalling cascade. cIAP, cellular inhibitor of apoptosis protein; FADD, FAS-associated death domain protein; NF- κ B, nuclear factor- κ B; RIPK, receptor-interacting protein kinase; TNF α , tumour necrosis factor α ; TNFR1, TNF α receptor 1; TRADD, TNFR1-associated death domain protein; TRAF, TNFR-associated factor.

and subsequently acquired the capacity to regulate the demise of severely damaged cells.

One particular type of stress that is intimately linked to cell death is represented by viral infection. On the one hand, viruses must divert the cell metabolism to their own advantage, while avoiding the premature death of their hosts. On the other hand, at late stages of viral infection, viruses often actively induce cell death, to facilitate the dissemination of new infectious particles. Viral genomes encode a plethora of apoptotic regulators, including anti-apoptotic orthologues of BCL-2 and inhibitors of caspases that operate similarly to IAPs [111]. Moreover, as viral genomes are sometimes very small, they might have favoured the selection of proteins with multiple functions. Thus, at least some of the proteins that regulate both lethal and vital aspects of the cellular metabolism might have evolved in viruses and have subsequently been acquired by host cells during host-pathogen co-evolution.

One intriguing question is how apoptotic regulators might get activated to mediate non-apoptotic functions without affecting cell death. In addition to what was mentioned above for caspases, there are many scenarios that might at least partly explain this. First, activation threshold: while generalized MMP—that is, MMP near-to-simultaneously involving most mitochondria within one single cell—is lethal, MMP affecting single mitochondria is a relatively

Sidebar A | In need of answers

One of the main questions that permeate cell death research since the discovery of the role of cytochrome c in intrinsic apoptosis is to which extent the different—death-related and death-unrelated—functions of apoptotic proteins contribute to the phenotypic effects of their pharmacological or genetic inhibition? The fact that most, if not all, proteins involved in the regulation and execution of apoptosis exert several non-apoptotic functions has provided an additional layer of complexity to cell death research, at least at two distinct levels. First, appropriate knockout models could not be generated for all proteins with a key role in metabolism at the organismal level, including cytochrome c and AIF. Second, the phenotypic manifestations of knockout models could not be appropriately interpreted for components of the apoptotic machinery that are dispensable for mammalian development, such as HTRA2. Appropriate genetic models in which the apoptotic and non-apoptotic functions of cytochrome c and AIF can be discriminated from one another have been generated [116,117], but these remain notable exceptions. Most often, the molecular determinants that underlie the pleiotropic functions of apoptotic proteins have not yet been entirely understood, de facto precluding similar developments. The generation of an in-depth molecular understanding of the apoptotic machinery should allow for the establishment of genetic models in which the multifaceted activities of apoptotic regulators are uncoupled, in turn providing deeper insights into the actual requirement for apoptosis in embryonic and post-embryonic development.

common process that entails their autophagic removal [90]. In this scenario, IMS proteins might be released in insufficient levels to trigger cell death, yet in amounts high enough to exert non-apoptotic functions. Second, post-translational modifications: several apoptotic regulators, including members of the BCL-2 protein family, are subjected to post-translation control, for instance being (de)activated upon (de)acetylation or (de)phosphorylation (for example, BAD and BCL-2; [112,113]), or as a result of de(oligomerization) reactions (for example, BAX; [114]). Thus, distinct sets of post-translational modifications might activate the same protein to mediate distinct effects. In cases in which apoptotic and non-apoptotic functions have been mapped to different domains of the same protein [39,59], post-translational modifications might simply determine the predominant activity by exposing or concealing the corresponding domains. Third, subcellular localization: multiple apoptotic regulators are localized, either physiologically or upon translocation in response to specific triggers, to distinct subcellular compartments. For instance, under physiological conditions, BCL-2 can be found to variable extents at the ER, at the OM and in the nucleus [115], whereas AIF can be detected in extra-mitochondrial sites only upon MMP [71]. In this scenario, the same protein might exhibit context-dependent activities, perhaps linked to localization-specific post-translational changes or interactors.

By identifying and thoroughly characterizing further non-apoptotic functions of apoptosis-regulatory proteins, future studies will surely provide additional insights into these questions (see also Sidebar A).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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