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Review

Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012

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In 2009, the Nomenclature Committee on Cell Death (NCCD) proposed a set of recommendations for the definition of distinct cell death morphologies and for the appropriate use of cell death-related terminology, including 'apoptosis', 'necrosis' and 'mitotic catastrophe'. In view of the substantial progress in the biochemical and genetic exploration of cell death, time has come to switch from morphological to molecular definitions of cell death modalities. Here we propose a functional classification of cell death subroutines that applies to both *in vitro* and *in vivo* settings and includes extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophe. Moreover, we discuss the utility of expressions indicating additional cell death modalities. On the basis of the new, revised NCCD classification, cell death subroutines are defined by a series of precise, measurable biochemical features.

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Abbreviations: AIM2, absent in melanoma 2; AIF, apoptosis-inducing factor; BID, BH3-interacting domain death agonist; cIAP, cellular inhibitor of apoptosis protein; CrmA, cytokine response modifier A; CYTC, cytochrome *c*; $\Delta\psi_m$, mitochondrial transmembrane potential; DAPK1, death-associated protein kinase 1; DCC, deleted in colorectal carcinoma; DD, death domain; DIABLO, direct IAP-binding protein with low pI; DISC, death-inducing signaling complex; EGFR, epidermal growth factor receptor; ENDOG, endonuclease G; FADD, FAS-associated protein with a death domain; GMCSF, granulocyte-macrophage colony-stimulating factor; HTRA2, high temperature requirement protein A2; IL, interleukin; MOMP, mitochondrial outer membrane permeabilization; mTOR, mammalian target of rapamycin; NCCD, Nomenclature Committee on Cell Death; PAR, poly(ADP-ribose); NETs, neutrophil extracellular traps; PARP, PAR polymerase; PP2A, protein phosphatase 2A; PS, phosphatidylserine; RIP, receptor-interacting protein kinase; RNAi, RNA interference; ROS, reactive oxygen species; SMAC, second mitochondria-derived activator of caspases; ROCK1, RHO-associated, coiled-coil containing protein kinase 1; SQSTM1, sequestosome 1; TAB, TAK1-binding protein; TAK1, TGFβ-activated kinase 1; tBID, truncated BID; TG, transglutaminase; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; TNFR, TNFα receptor; TRADD, TNFR-associated DD; TRAF, TNFR-associated factor; TRAIL, TNFα-related apoptosis-inducing ligand; TRAILR, TRAIL receptor; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-YVAD-fmk, *N*-benzyloxycarbonyl-Tyr-Val-Ala-DL-Asp-fluoromethylketone
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Preface

Since the first descriptions of programmed cell death mechanisms, which date back to the mid-1960s,[1–3] several attempts have been made to classify cell death subroutines based on morphological characteristics. Thus, in 1973 Schweichel and Merker[4] proposed a classification of several cell death modalities, including 'type I cell death' associated with heterophagy, 'type II cell death' associated with autophagy and 'type III cell death', which was not associated with any type of digestion, corresponding to apoptosis, autophagic cell death and necrosis, respectively.[5,6]

Even though deep insights into the molecular pathways that regulate and execute cell death have been gained and biochemical assays for monitoring cell death-related phenomena have become laboratory routine, the scientific community has not yet adopted a systematic classification of cell death modalities based on biochemical rather than morphological criteria. Nonetheless, there has been a tendency to dichotomize cell death events into either of two mutually exclusive groups. Thus, caspase-dependent, tolerogenic, programmed and physiological cell death instances have been contrasted to their caspase-independent, immunogenic, accidental and pathological counterparts, respectively.[7]

The Nomenclature Committee on Cell Death (NCCD) has formulated two subsequent rounds of recommendations in 2005 and 2009, in *Cell Death and Differentiation*.[6,8] Therein, unified criteria for the definition of cell death morphotypes were proposed and guidelines on the use of cell death-related terminology were given. The mission of the NCCD, as formulated previously, is 'to provide a forum in which names describing distinct modalities of cell death are critically evaluated and recommendations on their definition and use are formulated, hoping that a non-rigid, yet uniform nomenclature will facilitate the communication among scientists and ultimately accelerate the pace of discovery'.[8] In line with this mission statement and following recent breakthroughs in cell death research that have invalidated the notion that necrosis would represent a merely accidental cell death mode (see below),[9–12] the NCCD believes that the time has become appropriate for a novel systematic classification of cell death based on measurable biochemical features.

Pros and Cons of Morphological *Versus* Biochemical Classifications of Cell Death

The very first catalogs of cell death[4] necessarily relied on morphological traits, because the biochemical tests that are available nowadays for assessing the cell demise[13] were only developed decades later. Nevertheless, morphological classifications have dominated the cell death research scene even after the introduction of biochemical assays into the laboratory routine. Several economical, methodological, educational and theoretical reasons can be invoked to explain why the scientific community has clung to a conservative, morphological classification of cell death modalities. First, while conventional light microscopy is available in all cell biology laboratories, this is not the case for more sophisticated equipment (e.g., fluorescence readers for monitoring caspase activity). Second, virtually all cell biologists are familiar with

the observation of cell cultures under the microscope before any sort of experimental intervention, a routine practice that has certainly contributed to the persistence of morphological classifications. Third, it has been assumed for a long time that some degree of morphological uniformity would represent the activation of identical or at least similar lethal signaling cascades. Only recently has it become clear that apparently similar cell death morphotypes most often hide a great degree of functional, biochemical and immunological heterogeneity.[5,8,14] Moreover, it should always be remembered that the presence of specific morphological features is not sufficient to establish a causal link between a given process and cellular demise.

Biochemical methods for assessing cell death have many advantages over morphological techniques in that they are quantitative, and hence less prone to operator-dependent misinterpretations. However, these methods also have major drawbacks and must be interpreted with caution, especially when single parameters are being investigated.[13,15] Thus, it should always be kept in mind that single biochemical readouts cannot be used as unequivocal indicators of a precise death modality, for a variety of reasons. First, a cell death pathway that is often associated with a particular biochemical process may be normally executed in the absence of this process. Thus, at least *in vitro*, caspase activation is not a strict requirement for multiple cases of apoptosis (see below).[16,17] Similarly, phosphatidylserine (PS) exposure, which is widely considered as an early marker of apoptotic cell death,[18] reportedly does not occur in autophagy-deficient cells succumbing to apoptosis.[19] Second, a 'specific' cell death-related phenomenon may occur along with the execution of another cell death mode. For instance, excessive generation of reactive oxygen species (ROS) and reactive nitrogen species has been associated with several cases of apoptosis,[20–22] yet it also occurs during regulated necrosis.[23,24] Along similar lines, PS exposure is not a prerogative of apoptotic cell death, as it also constitutes an early feature of parthanatos and netosis (see below).[25,26] Third, a cell death-associated biochemical process can develop at a sublethal or transient level, which does not lead to the cell demise. Thus, while full-blown mitochondrial outer membrane permeabilization (MOMP) constitutes a point-of-no-return of intrinsic apoptosis (see below),[20] limited extents of MOMP (i.e., concerning a fraction of the mitochondrial pool) and the consequent (localized) activation of caspase-3 have been shown to participate in several cell death-unrelated programs such as the differentiation of megakaryocytes and granulocytes.[27]

Definition of 'Extrinsic Apoptosis'

The term 'extrinsic apoptosis' has been extensively used to indicate instances of apoptotic cell death that are induced by extracellular stress signals that are sensed and propagated by specific transmembrane receptors.[28–30] Extrinsic apoptosis can be initiated by the binding of lethal ligands, such as FAS/CD95 ligand (FASL/CD95L), tumor necrosis factor α (TNFα) and TNF (ligand) superfamily, member 10 (TNFSF10, best known as TNF-related apoptosis inducing ligand, TRAIL), to various death receptors (i.e., FAS/CD95, TNFα receptor 1

(TNFR1) and TRAIL receptor (TRAILR)1–2, respectively.[28] Alternatively, an extrinsic pro-apoptotic signal can be dispatched by the so-called 'dependence receptors', including netrin receptors (e.g., UNC5A-D and deleted in colorectal carcinoma, DCC), which only exert lethal functions when the concentration of their specific ligands falls below a critical threshold level.[30]

One prototypic signaling pathway leading to extrinsic apoptosis is elicited by FAS ligation. In the absence of FASL, FAS subunits spontaneously assemble at the plasma membrane to generate trimers, owing to the so-called pre-ligand assembly domain (PLAD).[31] Ligand binding stabilizes such trimers while inducing a conformational change that allows for the assembly of a dynamic multiprotein complex at the cytosolic tail of the receptor. This occurs owing to a conserved sequence of 80 residues that is shared by all death receptors, the so-called 'death domain' (DD).[32,33] Proteins recruited at the DD of FAS include receptor-interacting protein kinase 1 (RIPK1, best known as RIP1); FAS-associated protein with a DD (FADD); multiple isoforms of c-FLIP;[34,35] cellular inhibitor of apoptosis proteins (cIAPs), E3 ubiquitin ligases that also inhibit apoptosis owing to their ability to interfere with caspase activation;[36] and pro-caspase-8 (or -10).[37–41] The resulting supramolecular complex, which has been dubbed 'death-inducing signaling complex' (DISC), constitutes a platform that regulates the activation of caspase-8 (or -10).[38,42]

Of note, TNFR1-like proteins also require TNFR-associated DD (TRADD) for recruiting FADD and caspase-8, whereas FAS and TRAILR1/2 do not,[29] pointing to the existence of subgroups of death receptors with specific signaling properties. Similarly, the DDs of some death receptors, for instance, TNFR1, recruit several other proteins that are not found at FADD-assembled DISCs, including TNFR-associated factor 2 (TRAF2) and TRAF5.[43] In this specific context, RIP1 is polyubiquitinated by cIAPs,[44] allowing for the recruitment of transforming growth factor β (TGFβ)-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3, which together can stimulate the canonical activation pathway for the multifunctional transcription factor NF-κB.[45] Thus, death receptor activation not always entails the transduction of a lethal signal. This is particularly true for TNFR1, which has been shown to mediate cellular outcomes as different as proliferation and (distinct modalities of) cell death (see below). Irrespective of these variations, both FAS- and TNFR1-elicited signaling pathways appear to be subjected to a consistent degree of regulation upon receptor compartmentalization. A detailed discussion of these aspects goes largely beyond the scope of this paper and can be found in Schutze et al.[29]

In some cell types including lymphocytes (which have been dubbed 'type I cells'),[46,47] active caspase-8 directly catalyzes the proteolytic maturation of caspase-3, thereby triggering the executioner phase of caspase-dependent apoptosis in a mitochondrion-independent manner.[48] In other cells such as hepatocytes and pancreatic β cells ('type II cells'),[46,47,49] caspase-8 mediates the proteolytic cleavage of BH3-interacting domain death agonist (BID), leading to the generation of a mitochondrion-permeabilizing fragment (known as truncated BID, tBID).[49–51] Thus, while type I cells undergo extrinsic apoptosis irrespective of any contribution by mitochondria

(tBID and MOMP can occur in these cells, but they are dispensable for the execution of extrinsic apoptosis), type II cells succumb from the activation of death receptors while showing signs of MOMP, including the dissipation of mitochondrial transmembrane potential ($\Delta\psi_m$) and the release of toxic proteins that are normally retained within the mitochondrial intermembrane space (IMS).[20] Among these, cytochrome c (CYTC) drives – together with the cytoplasmic adaptor protein APAF1 and dATP – the assembly of the apoptosome, another caspase-activating multiprotein complex (see below).[52] The actual contribution of caspase-10, a close homolog of caspase-8, to extrinsic apoptosis remains unclear. Thus, whereas several reports indicate that caspase-10 is recruited at the DISC and gets activated in response to death receptor signaling,[41,53] it seems that caspase-10 cannot functionally substitute for caspase-8.[53] Moreover, it has recently been suggested that caspase-10 might be required for the lethal signaling cascade ignited by death receptors in the presence of caspase inhibitors (see below).[54]

The molecular routes by which dependence receptors are connected to the rapid activation of executioner caspases, in particular caspase-3, have only recently begun to emerge. Thus, in the absence of their ligands, some dependence receptors like Patched and DCC appear to interact with the cytoplasmic adaptor protein DRAL to assemble a caspase-9-activating platform.[55] Another dependence receptor, UNC5B, responds to the withdrawal of netrin-1 by recruiting a signaling complex that includes protein phosphatase 2A (PP2A) and death-associated protein kinase 1 (DAPK1).[56] This multiprotein interaction would lead to the PP2A-mediated dephosphorylation of DAPK, in turn unleashing its multifaceted pro-apoptotic potential.[57]

As a note, there are several other transmembrane proteins that – at least under selected circumstances – can transduce lethal signals in response to ligand binding, including (although presumably not limited to) CD2,[58] CD4,[59] TNFRSF8/CD30,[60,61] TNFRSF5/CD40,[60] CD45,[62] CXCR4[59] and class I/II MHC molecules.[63] Similar to TNFR1, most of these proteins have dual functions: depending on the cellular context and triggering stimulus they can engage either pro-survival or pro-death signals. However, the molecular cascades triggered by these receptors are complex and often poorly elucidated, in particular with regard to their dependency on caspases.

On the basis of these considerations, we propose the following operational definition of extrinsic apoptosis. Extrinsic apoptosis is a caspase-dependent cell death subroutine, and hence can be suppressed (at least theoretically) by pan-caspase chemical inhibitors such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) or by the overexpression of viral inhibitors of caspases like cytokine response modifier A (CrmA).[64] Extrinsic apoptosis would feature one among three major lethal signaling cascades: (i) death receptor signaling and activation of the caspase-8 (or -10)→caspase-3 cascade; (ii) death receptor signaling and activation of the caspase-8→tBID→MOMP→caspase-9→caspase-3 pathway; or (iii) ligand deprivation-induced dependence receptor signaling followed by (direct or MOMP-dependent) activation of the caspase-9→caspase-3 cascade (Table 1 and Figure 1).

**Table 1** Functional classification of regulated cell death modes

	Main biochemical features	Caspase dependence	Examples of inhibitory interventions[a]
Anoikis	Downregulation of EGFR Inhibition of ERK1 signaling Lack of β1-integrin engagement Overexpression of BIM Caspase-3 (-6,-7) activation	++	BCL-2 overexpression Z-VAD-fmk administration
Autophagic cell death	MAP1LC3 lipidation SQSTM1 degradation	−−	VPS34 inhibitors AMBRA1, ATG5, ATG7, ATG12 or BCN1 genetic inhibition
Caspase-dependent intrinsic apoptosis	MOMP Irreversible $\Delta\psi_m$ dissipation	++	BCL-2 overexpression Z-VAD-fmk administration
Caspase-independent intrinsic apoptosis	Release of IMS proteins Respiratory chain inhibition	−−	BCL-2 overexpression
Cornification	Activation of transglutaminases Caspase-14 activation	+	Genetic inhibition of TG1, TG3 or TG5 Genetic inhibition of caspase-14
Entosis	RHO activation ROCK1 activation	−−	Genetic inhibition of metallothionein 2A Lysosomal inhibitors
Extrinsic apoptosis by death receptors	Death receptor signaling Caspase-8 (-10) activation BID cleavage and MOMP (in type II cells) Caspase-3 (-6,-7) activation	++	CrmA expression Genetic inhibition of caspases (8 and 3) Z-VAD-fmk administration
Extrinsic apoptosis by dependence receptors	Dependence receptor signaling PP2A activation DAPK1 activation Caspase-9 activation Caspase-3 (-6,-7) activation	++	Genetic inhibition of caspases (9 and 3) Genetic inhibition of PP2A Z-VAD-fmk administration
Mitotic catastrophe	Caspase-2 activation (in some instances) TP53 or TP73 activation (in some instances) Mitotic arrest	−−	Genetic inhibition of TP53 (in some instances) Pharmacological or genetic inhibition of caspase-2 (in some instances)
Necroptosis	Death receptor signaling Caspase inhibition RIP1 and/or RIP3 activation	−−	Administration of necrostatin(s) Genetic inhibition of RIP1/RIP3
Netosis	Caspase inhibition NADPH oxidase activation NET release (in some instances)	−−	Autophagy inhibition NADPH oxidase inhibition Genetic inhibition of PAD4
Parthanatos	PARP1-mediated PAR accumulation Irreversible $\Delta\psi_m$ dissipation ATP and NADH depletion PAR binding to AIF and AIF nuclear translocation	−−	Genetic inhibition of AIF Pharmacological or genetic inhibition of PARP1
Pyroptosis	Caspase-1 activation Caspase-7 activation Secretion of IL-1β and IL-18	++	Administration of Z-YVAD-fmk Genetic inhibition of caspase-1

Abbreviations: ATG, autophagy; BCN1, beclin 1; $\Delta\psi_m$, mitochondrial transmembrane potential; CrmA, cytokine response modifier A; DAPK1, death-associated protein kinase 1; EGFR, epidermal growth factor receptor; ERK1, extracellular-regulated kinase 1; IL, interleukin; MAP1LC3, microtubule-associated protein 1 light chain 3; MOMP, mitochondrial outer membrane permeabilization; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PAR, poly(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PP2A, protein phosphatase 2A; ROCK1, RHO-associated, coiled-coil containing protein kinase 1; SQSTM1, sequestosome 1; TG, transglutaminase; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-YVAD-fmk, *N*-benzyloxycarbonyl-Tyr-Val-Ala-DL-Asp-fluoromethylketone. [a]For classification purposes, pharmacological and genetic interventions should be considered inhibitory when they truly reduce the incidence of cell death, but not when they only provoke a shift between different cell death modalities or when they change the morphology of cell death. For further details, please refer to the 'Notes of Caution' and 'Concluding Remarks' sections

Definition of caspase-dependent and caspase-independent 'intrinsic apoptosis'. The apoptotic demise of cells can be triggered by a plethora of intracellular stress conditions, including DNA damage, oxidative stress, cytosolic Ca^{2+} overload, mild excitotoxicity (related to glutamate receptor overstimulation in the nervous system), accumulation of unfolded proteins in the endoplasmic reticulum (ER) and many others. Although the signaling cascades that trigger intrinsic apoptosis are highly heterogeneous as far as the initiating stimuli are concerned, they are all wired to a mitochondrion-centered control mechanism.[20] Frequently, along with the propagation of the pro-apoptotic signaling cascade, anti-apoptotic mechanisms are also engaged, in an attempt to allow cells to cope with stress. In this scenario, both pro- and anti-apoptotic signals converge at mitochondrial membranes, which become permeabilized when the former predominate over the latter.[20] MOMP can start at the outer mitochondrial
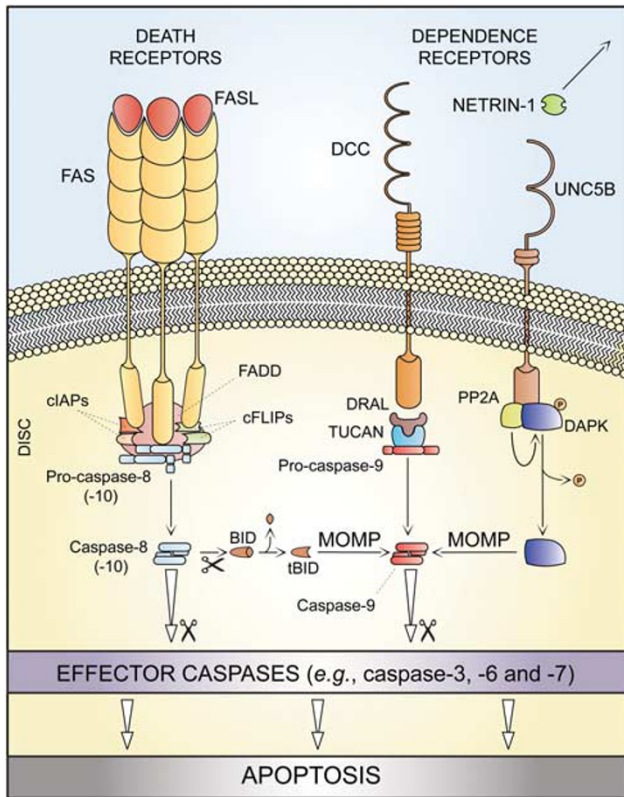
Figure 1 Extrinsic apoptosis. Upon FAS ligand (FASL) binding, the cytoplasmic tails of FAS (also known as CD95, a prototypic death receptor) trimers recruit (among other proteins) FAS-associated protein with a death domain (FADD), cellular inhibitor of apoptosis proteins (cIAPs), c-FLIPs and pro-caspase-8 (or -10). This supramolecular platform, which has been dubbed 'death-inducing signaling complex' (DISC), controls the activation of caspase-8 (-10). Within the DISC, c-FLIPs and cIAPs exert pro-survival functions. However, when lethal signals prevail, caspase-8 gets activated and can directly trigger the caspase cascade by mediating the proteolytic maturation of caspase-3 (in type I cells) or stimulate mitochondrial outer membrane permeabilization (MOMP) by cleaving the BH3-only protein BID (in type II cells). Extrinsic apoptosis can also be ignited by dependence receptors like DCC or UNC5B, which relay lethal signals in the absence of their ligand (netrin-1). In the case of DCC and UNC5B, the pro-apoptotic signaling proceeds through the assembly of a DRAL- and TUCAN- (or NLRP1-) containing caspase-9-activating platform or by the dephosphorylation-mediated activation of death-associated protein kinase 1 (DAPK1) by UNC5B-bound protein phosphatase 2A (PP2A), respectively. DAPK1 can mediate the direct activation of executioner caspases or favor MOMP. tBID, truncated BID

membrane owing to the pore-forming activity of pro-apoptotic members of the BCL-2 protein family such as BAK and BAX or can result from a phenomenon (the so-called mitochondrial permeability transition, MPT) that originates at the inner mitochondrial membrane due to the opening of a multiprotein complex known as the permeability transition pore complex (PTPC).[65,66] Irrespective of the precise biochemical and physical mechanisms through which it develops, irreversible MOMP affecting most mitochondria within a single cell has multiple lethal consequences: (i) the dissipation of the $\Delta\psi_m$, with cessation of mitochondrial ATP synthesis and $\Delta\psi_m$-dependent transport activities; (ii) the release of toxic proteins from the IMS into the cytosol, as this applies to CYTC, apoptosis-inducing factor (AIF), endonuclease G (ENDOG), direct IAP-binding protein with

low pI (DIABLO, also known as second mitochondria-derived activator of caspases, SMAC) and high temperature requirement protein A2 (HTRA2); and (iii) the inhibition of the respiratory chain (favored by the loss of CYTC), eliciting or aggravating ROS overproduction and hence activating a feed-forward circuit for the amplification of the apoptotic signal.[20]

Thus, intrinsic apoptosis results from a bioenergetic and metabolic catastrophe coupled to multiple active executioner mechanisms. Upon MOMP, cytosolic CYTC participates with APAF1 and dATP in the formation of the apoptosome, which triggers the caspase-9→caspase-3 proteolytic cascade.[52,67] AIF and ENDOG relocate to the nucleus, where they mediate large-scale DNA fragmentation independently of caspases.[68–71] SMAC/DIABLO and HTRA2 inhibit the anti-apoptotic function of several members of the IAP family, thereby derepressing caspase activation.[72–74] In addition, HTRA2 exerts caspase-independent pro-apoptotic effects by virtue of its serine protease activity.[75,76] These mechanisms present a considerable degree of redundancy, as demonstrated by the fact that the knockout or genetic inhibition of single IMS proteins not always affects the execution of intrinsic apoptosis.[77] Moreover, the relative contribution of these processes to intrinsic apoptosis varies in distinct physiological, pathological and experimental scenarios. Thus, while ENDOG appears to be dispensable for intrinsic apoptosis in mammalian models,[77] Nuc1p, the yeast ortholog of ENDOG, has an important role during the apoptotic response of *Saccharomyces cerevisiae* to chronological aging in non-fermentable carbon sources (which potentiate mitochondrial respiration).[71] DRONC, the ortholog of caspase-9 in *Drosophila melanogaster*, is required for many forms of developmental cell deaths and apoptosis induced by DNA damage *in vivo*.[78] Conversely, caspase activation seems to have a prominent role in a limited number of instances of stress-induced intrinsic apoptosis *in vitro*, as demonstrated by the fact that – in contrast to extrinsic apoptosis – chemical and/or genetic inhibition of caspases rarely, if ever, confers long-term cytoprotective effects and truly prevents cell death. In this context, caspase inhibition only delays the execution of cell death, which eventually can even exhibit morphological features of necrosis.[16,17,79]

In view of these observations, we suggest to define 'intrinsic apoptosis' as a cell death process that is mediated by MOMP and hence is always associated with (i) generalized and irreversible $\Delta\psi_m$ dissipation, (ii) release of IMS proteins into the cytosol (and their possible relocalization to other subcellular compartments) and (iii) respiratory chain inhibition. We propose to differentiate between caspase-dependent and caspase-independent intrinsic apoptosis based on the extent of cytoprotection conferred by (pharmacological or genetic) inhibition of caspases (Table 1 and Figure 2). This distinction is particularly relevant *in vivo*, as in some[78,80] (but not all)[81] instances of developmental cell death, caspase inhibition has been shown to provide stable cytoprotection. *In vitro*, in the long run, caspase-independent mechanisms, be they active (e.g., AIF, ENDOG) or passive (e.g., ATP depletion), tend to prevail over caspase inhibition and to kill cells even in instances of intrinsic apoptosis that would have normally been rapidly executed by the caspase cascade.
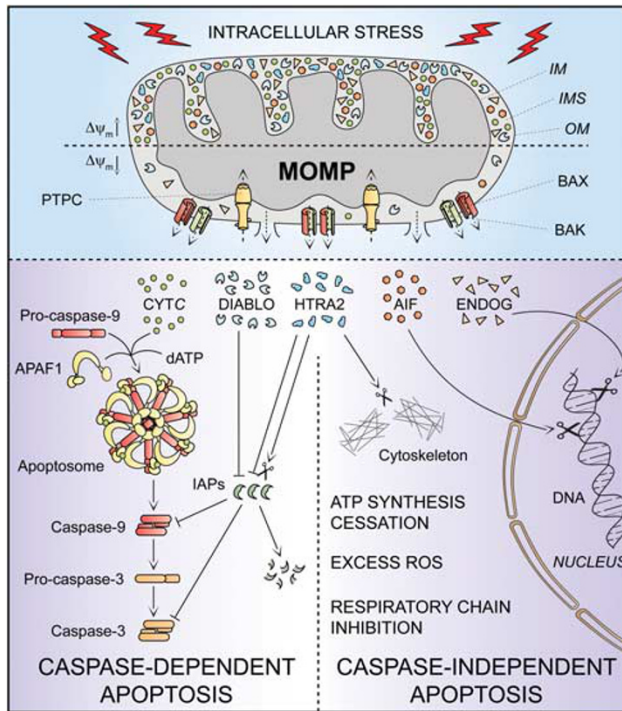
Figure 2 Intrinsic apoptosis. In response to multiple intracellular stress conditions (e.g., DNA damage, cytosolic Ca^{2+} overload), pro-survival and pro-death signals are generated and converge to a mitochondrion-centered control mechanism. When lethal signals prevail, mitochondrial outer membrane permeabilization (MOMP) occurs and leads to mitochondrial transmembrane potential ($\Delta\psi_m$) dissipation, arrest of mitochondrial ATP synthesis and $\Delta\psi_m$-dependent transport activities. Moreover, the respiratory chains gets uncoupled, leading to reactive oxygen species (ROS) overgeneration, and proteins that are normally confined within the mitochondrial intermembrane space (IMS) are released into the cytosol. Among these, cytochrome c (CYTC) drives – together with the cytoplasmic adaptor protein APAF1 and dATP – the assembly of the so-called apoptosome, a multiprotein complex that triggers the caspase-9 → caspase-3 proteolytic cascade. Direct IAP-binding protein with low pI (DIABLO, also known as second mitochondria-derived activator of caspases, SMAC) and high temperature requirement protein A2 (HTRA2) facilitate caspase activation by sequestering and/or degrading several members of the inhibitor of apoptosis protein (IAP) family. On the contrary, apoptosis-inducing factor (AIF) and endonuclease G (ENDOG) function in a caspase-independent manner by relocating to the nucleus and mediating large-scale DNA fragmentation. Of note, the serine protease HTRA2 also contributes to caspase-independent apoptosis by cleaving a wide array of cellular substrates (including cytoskeletal proteins). IM, mitochondrial inner membrane; OM, mitochondrial outer membrane; PTPC, permeability transition pore complex

Thus, *in vitro*, intrinsic apoptosis appears to entail a caspase-dependent and a caspase-independent phase, whose relative contribution to the execution of cell death might be estimated by the extent of short-term (24–48 h) cytoprotection conferred by caspase inhibitors.

Definition of 'Regulated Necrosis'

For a long time, necrosis has been considered as a merely accidental cell death mechanism and was defined by the absence of morphological traits of apoptosis or autophagy.[8,82] Owing to the work of several laboratories,[9–12,83] it is now clear that necrosis can occur in a regulated manner, and that

necrotic cell death has a prominent role in multiple physiological and pathological settings.[23] Several triggers can induce regulated necrosis, including alkylating DNA damage, excitotoxins and the ligation of death receptors, at least under selected circumstances.[9,11,12,84,85] Indeed, when caspases (and in particular caspase-8) are inhibited by genetic manipulations (e.g., by gene knockout or RNA interference, RNAi) or blocked by pharmacological agents (e.g., chemical caspase inhibitors), RIP1 and its homolog RIP3 are not degraded and rather engage in physical and functional interactions that ultimately activate the execution of necrotic cell death.[9,11,12,23]

Regulated necrosis can be further characterized with regard to its dependence on specific signaling modules, and should be named consequently. For instance, cases of regulated necrosis that exhibit RIP1 activation (which can be measured by enzymatic assays or by monitoring RIP1 phosphorylation on S161)[11,86] and that can be suppressed by RIP1 inhibitors including necrostatin-1[10,86,87] should be labeled 'RIP1-dependent regulated necrosis'. Of note, RIP3-dependent, but RIP1-independent instances of regulated necrosis have recently been identified,[12,88] and these are insensitive to necrostatins.

The term 'necroptosis' has recently been used as a synonym of regulated necrosis, but it was originally introduced to indicate a specific case or regulated necrosis, which is ignited by TNFR1 ligation and can be inhibited by the RIP1-targeting chemical necrostatin-1 (Table 1 and Figure 3).[10] The NCCD encourages scientists and authors of scientific publications to prefer the use of general *versus* specific nomenclature (see below). However, 'necroptosis' can be used to indicate RIP1- and/or RIP3-dependent regulated necrosis, provided that this expression is explicitly defined at its first appearance and used consistently thereafter.

Definition of 'Autophagic Cell Death'

On the basis of morphological features, the term 'autophagic cell death' has widely been used to indicate instances of cell death that are accompanied by a massive cytoplasmic vacuolization, which often (although not always) indicates increased autophagic flux.[5,8] Although originally this expression did not imply any functional consideration, scientists have quickly adopted the term 'autophagic cell death' and used it to imply that autophagy would actually execute the cell demise.[89] This applies to at least two very distinct settings. First, autophagy has been shown to mediate physiological cell death *in vivo*, during the developmental program of *D. melanogaster*.[90–92] Second, autophagy appears to be responsible for the death of some cancer cells (especially when they lack essential apoptotic modulators like BAX and BAK or caspases)[93,94] that respond to a selected panel of chemotherapeutic agents *in vitro*.[95,96] Nonetheless, in most known cases, autophagy constitutes a cytoprotective response activated by dying cells in the attempt to cope with stress, and its inhibition accelerates, rather than prevents, cell death.[97]

Several methods may be used to determine whether the autophagic pathway is activated above baseline levels in the context of the cellular demise. Discussing the advantages and
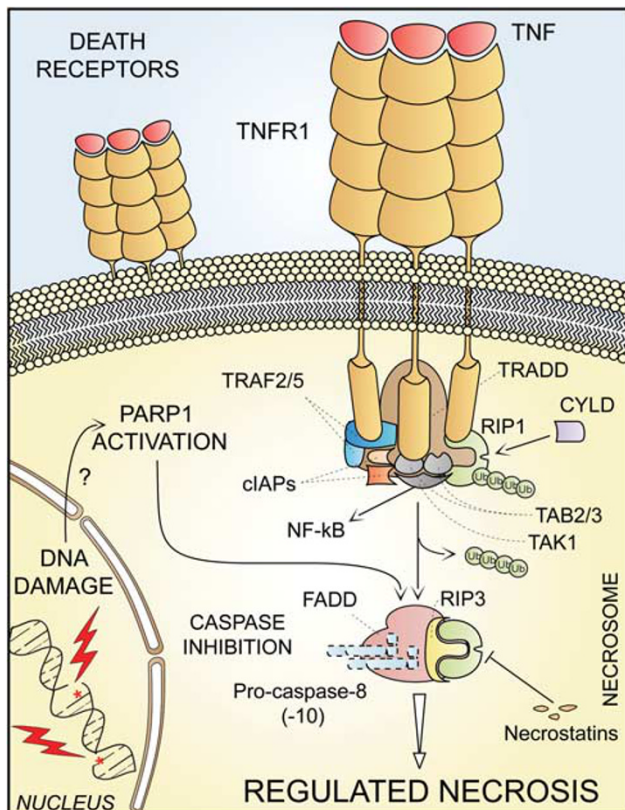
Figure 3 Regulated necrosis. Upon tumor necrosis factor α (TNFα) binding, the cytoplasmic tails of TNF receptor 1 (TNFR1, a prototypic death receptor) trimers recruit TNFR-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIP1), cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2) and TRAF5. Within the so-called complex I, RIP1 is polyubiquitinated by cIAPs, thereby providing a docking site for the recruitment of transforming growth factor β (TGFβ)-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3 (which together deliver a pro-survival signal by activating the transcription factor NF-κB). In some pathophysiological and experimental settings, and in particular when caspase-8 is absent or when caspases are inhibited by pharmacological agents, cylindromatosis (CYLD)-deubiquitinated RIP1 engage in physical and functional interactions with its homolog RIP3, ultimately activating the execution of necrotic cell death. Regulated necrosis can also be induced by alkylating DNA damage (possibly by the overactivation of poly(ADP-ribose) polymerase 1, PARP1). In some (but not all) instances, regulated necrosis requires the kinase activity of RIP1, that is, it can be blocked by the RIP1-targeting compounds necrostatins. FADD, FAS-associated protein with a death domain

pitfalls of these techniques is beyond the scope of this article, and several excellent reviews on methods for monitoring the autophagic flux have recently been published.[15,98,99] Nevertheless, it should be remembered that steady-state methods do not provide any reliable estimation of autophagic activity, as they are intrinsically unable to discriminate between enhanced rates of autophagy (increased on-rate) and situations in which the late steps of the pathways are blocked (decreased off-rate).[15,99]

From a purely morphological perspective, the term 'autophagic cell death' is highly prone to misinterpretation and hence must be used with caution.[89] On the contrary, we suggest to reintroduce the term 'autophagic cell death' based on biochemical and functional considerations, to indicate a cell

death instance that is mediated by autophagy, that is, that can be suppressed by the inhibition of the autophagic pathway by chemicals (e.g., agents that target VPS34) and/or genetic means (e.g., gene knockout/mutation or RNAi targeting essential autophagic modulators like AMBRA1, ATG5, ATG12 or beclin 1[100,101] (Table 1 and Figure 4). As some ATG proteins may have autophagy-independent functions and may even be converted from pro-autophagy to pro-death proteins by proteolytic cleavage (e.g., ATG5 and ATG6),[102–104] it may be advisable to interrogate possible cases of autophagic cell death by knocking down at least two distinct essential autophagic proteins. On the basis of our definition, all cases of cell death that exhibit markers of autophagy such as the lipidation of microtubule-associated protein 1 light chain 3 (better known as LC3/Atg8) or an increased degradation of autophagic substrates like seques-tosome 1 (SQSTM1), but cannot be blocked by autophagy inhibition should not be classified as autophagic cell death.

Definition of 'Mitotic Catastrophe'

During the past decade, several attempts have been made to delineate the molecular pathways leading to mitotic catastrophe.[5,8,105,106] Occasionally, researchers restrictively employ the term 'mitotic catastrophe' for cell death occurring in mitosis.[106,107] More frequently, mitotic catastrophe refers to cases of cell death that are triggered by aberrant mitosis and executed either during mitosis or in the subsequent inter-phase.[5,8] Recently, it has been proposed that mitotic catastrophe might not even constitute a *bona fide* cell death executioner mechanism, but an oncosuppressive pathway that precedes and is distinct from, yet operates through, cell death or senescence.[106,108]

After aberrant mitosis, cells frequently exhibit gross nuclear alterations (e.g., micro- and multinucleation), which have been used as morphological markers for the detection of mitotic catastrophe.[8] However, apoptotic and necrotic traits have also been detected in such cells, either concomitant with or following multinucleation.[109,110] Thus, end-point techniques are intrinsically unsuitable for assessing mitotic catastrophe, as they cannot reconstruct the sequence of events that have lead to cell death. To circumvent this issue, novel methods relying on high-throughput video microscopy or time-lapse fluorescence microscopy are under development.[99,111–113] Several processes were originally associated with and were then shown to be dispensable for (at least some instances of) mitotic catastrophe. These include, but are not limited to, the activation of the DNA damage-responsive protease caspase-2,[114] of the tumor suppressor TP53[109,115] and of other members of the TP53 family, including the TP73 variant TAp73.[116,117]

In view of recent results from several laboratories indicating that mitotic aberrations can induce cell senescence,[118–120] and that cell death can be either apoptotic or necrotic,[8] we have recently proposed a novel definition and categorization of mitotic catastrophe based on purely functional considera-tions.[108] Thus, mitotic catastrophe would not constitute a 'pure' cell death executioner pathway, but an oncosuppres-sive mechanism that: (i) is initiated by perturbations of the mitotic apparatus (i.e., chromosomes and the complex
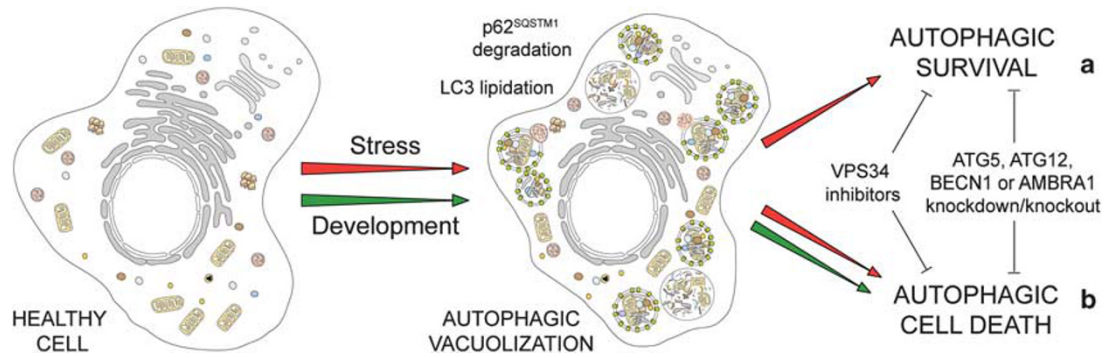
Figure 4 Autophagic cell death. In response to stress and during development, eukaryotic cells often activate autophagy, a mechanism whereby organelles and portion of the cytoplasm are sequestered in double-membraned vesicles (autophagosomes) that are delivered to lysosomes for degradation. Stress-induced autophagy most often exerts cytoprotective functions and favors the re-establishment of homeostasis and survival (**a**). In this setting, pharmacological or genetic inhibition of autophagy accelerates cell death. On the contrary, these interventions frequently inhibit developmental cell death, indicating that autophagy also constitutes a lethal mechanism that mediates 'autophagic cell death' (**b**)
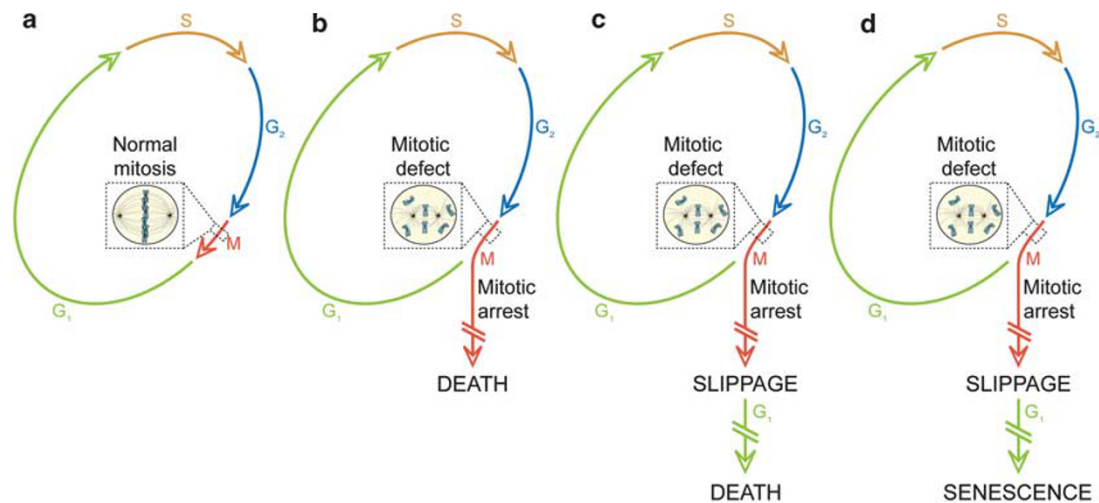
Figure 5 Mitotic catastrophe. (**a**) In the absence of chemical and genetic perturbations of the mitotic apparatus (including chromosomes and the molecular machinery that ensures their faithful segregation), cells progress through the different phases of the cell cycle to generate a diploid offspring. On the contrary, if chromosomal defects or problems affecting the mitotic machinery are sensed during the M phase, cells become arrested in mitosis due to the activation of mitotic catastrophe (**b**–**d**). These cells can undergo different fates: they can die without exiting mitosis (**b**), reach the G_1 phase of the subsequent cell cycle (through a phenomenon that is known as mitotic slippage) and then die (**c**), or exit mitosis and undergo senescence (**d**). Irrespective of this diversity of outcomes, mitotic catastrophe can be defined as an oncosuppressive mechanism that precedes and is distinct from, but operates through, cell death and senescence

machinery that ensure their faithful segregation); (ii) is initiated during the M phase of the cell cycle; (iii) is paralleled by some degree of mitotic arrest; and (iv) ultimately triggers cell death or senescence (Table 1 and Figure 5). It remains an open conundrum whether the duration of the mitotic arrest truly influences[121] or not[111] the cell fate after mitotic catastrophe. However, it appears that the crosstalk between TP53- and mammalian target of rapamycin (mTOR)-relayed signals might (at least partially) determine cell senescence.[122,123] In line with our definition, the DNA damage-induced signaling that is initiated at the G_2/M transition by the G_2 checkpoint (which often, although not always, is mediated by members of the TP53 family)[124,125] does not constitute a case of mitotic catastrophe. Moreover, instances of mitotic arrest that are followed by the re-establishment of homeostasis and resumed proliferation cannot be considered as events of mitotic

catastrophe, even when they lead to the gain or loss of chromosomes, and hence to the generation of aneuploid cells.[108]

Tentative Definition of Other Cell Death Modalities

Anoikis. Literally meaning 'the state of being homeless', this term of ancient Greek derivation was introduced by Frisch and Francis in 1994 to describe the apoptotic response of adherent cells due to the absence of cell-to-matrix interactions.[126] The survival of non-transformed adherent cells does indeed depend on signals transduced by integrins and by some growth factor receptors (such as the epidermal growth factor receptor (EGFR)) upon interaction with the extracellular matrix (ECM).[127] As the resistance to anoikis of epithelial cancer cells sustains invasiveness and metastatic

potential, great efforts have been undertaken to precisely characterize the underlying molecular cascades.[128] On the basis of biochemical and functional considerations, anoikis may currently be defined as an adherent cell-restricted lethal cascade that is ignited by detachment from the matrix and that is characterized by (i) lack of β1-integrin engagement, (ii) downregulation of EGFR expression, (iii) inhibition of extracellular-regulated kinase 1 (ERK1) signaling, and (iv) overexpression of the BCL-2 family member BIM (Table 1).[127,129] It should be noted that in most, if not all, instances, the cell death program ignited by anoikis is executed by the molecular machinery for intrinsic apoptosis (see above).[127]

Entosis. In 2007, Overholtzer *et al*.[130] introduced the term 'entosis' to describe a cell death mechanism linked to the 'cell-in-cell' phenotype that is frequently exhibited by non-phagocytic cells in clinical tumor samples. Of note, Mormone *et al*.[131] had previously reported a similar phenotype in lymphoblasts from patients with Huntington's disease, and had dubbed it 'cell cannibalism'.[131,132] Entosis would be provoked by the loss of ECM interaction, but would not entail the activation of apoptotic executioners, thereby constituting a cell death mode distinct from anoikis.[130] Recently, the RNAi-mediated downregulation of metallothionein 2A has been shown to promote the 'cell-in-cell' phenotype, pointing to a prominent role for adherens junctions in the entotic response.[133] Entosis reportedly proceeds in the absence of caspase activation, is insensitive to inhibition by BCL-2 and is paralleled by the activation of the small GTPase RHO and the RHO-associated, coiled-coil containing protein kinase 1 (ROCK1) in the engulfing cell.[130,134] Internalized cells appear virtually normal and later disappear, leading to the hypothesis that they are degraded by lysosomal hydrolases. In some instances, however, such cells have been shown to divide and eventually to be released, raising doubts on the inexorable fate of engulfed cells.[130] Conclusive cell fate experiments are urgently awaited to resolve this debate. Meanwhile, we propose to define entosis as an instance of cell death only when all the three following conditions are met. First, the engulfed cells should never exit the phagosome (as detectable by time-lapse microscopy or videomicroscopy) and should be degraded within the lysosome (implying that entosis can be blocked by lysosomal inhibitors). Second, the 'cell-in-cell' phenotype should arise from homotypic interactions (i.e., it should involve cells of the same type) and should not engage professional phagocytes. Third, the process should be insensitive to chemical and genetic interventions that normally block caspase-dependent and -independent intrinsic apoptosis (e.g., caspase inhibitors, BCL-2 overexpression) (Table 1).

Parthanatos. Coined after Thanatos, the personification of death in Greek mythology, the term 'parthanatos' has been introduced to indicate a particular cell death mode involving the DNA damage-responsive enzymes poly(ADP-ribose) polymerases (PARPs), and in particular PARP1, which alone accounts for more than 90% cellular PARP activity.[135] In physiological conditions, PARP1 cooperates with the DNA repair machinery to ensure genomic homeostasis upon mild DNA damage.[136] Conversely, PARP1 overactivation has several toxic consequences, including NAD$^+$ and ATP depletion, as well as the accumulation of mitochondriotoxic PAR, which favors $\Delta\psi_m$ dissipation and AIF release.[137–139] Of note, AIF has recently been shown to possess a high-affinity PAR-binding site, and the physical interaction between PAR and AIF appears to be required for parthanatos, both *in vitro* and *in vivo*.[140] Parthanatos have a role in multiple experimental and physiopathological scenarios, including stroke, diabetes, inflammation and neurodegeneration.[141] In line with the original definition of parthanatos, cell death instances should be considered as parthanatic when they depend on early PARP1 activation (i.e., they can be blocked by its chemical and/or genetic inhibition),[142] and exhibit NAD$^+$ plus ATP depletion paralleled by AIF-mediated chromatinolysis. Parthanatos constitutes a caspase-independent cell death pathway (Table 1),[141] and possibly represents – together with necroptosis – a particular case of regulated necrosis (see above).

Pyroptosis. The term 'pyroptosis' has been introduced in 2000 by Brennan and Cookson[143] to functionally describe the peculiar death of macrophages infected by *Salmonella typhimurium*. Several other bacterial triggers of this atypical cell death modality have been identified, including *Shigella flexneri*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Francisella tularensis* and the *Bacillus anthracis* toxin.[144–147] However, it has become clear that pyroptosis neither constitutes a macrophage-specific process nor a cell death subroutine that only results from bacterial infection.[148] Of note, pyroptotic cells can exhibit apoptotic and/or necrotic morphological features.[149]

The most distinctive biochemical feature of pyroptosis is the early, induced proximity-mediated activation of caspase-1.[150] The pyroptotic activation of caspase-1 can occur in the context of a multiprotein platform known as the inflammasome, which involves the adaptor protein ASC and NOD-like receptors (NLRs) or the cytosolic DNA sensor absent in melanoma 2 (AIM2).[147,151,152] Alternatively, caspase-1 can be activated by the so-called pyroptosome, a supramolecular assembly of ASC dimers.[153] In both cases, active caspase-1 catalyzes the proteolytic maturation and release of pyrogenic interleukin-1β (IL-1β) and IL-18.[154] Moreover, in some (but not all) instances, this is followed by caspase activation and cell death.[143] Active caspase-1 reportedly mediates the proteolytic activation of caspase-7 (rather than that of caspase-3),[155] suggesting that pyroptotic cell death might proceed via an unusual caspase-1 → caspase-7 cascade with limited (and perhaps caspase-1-independent) involvement of caspase-3.[156] Still, the molecular mechanisms determining why caspase-1 activation sometimes results in cytokine secretion without death and sometimes culminates in the cellular demise remain to be elucidated. We therefore propose to define pyroptosis as a caspase-1-dependent cell death subroutine (i.e., that can be suppressed by the genetic knockout/knockdown of caspase-1 or by caspase-1-specific blockers like *N*-benzyloxycarbonyl-Tyr-Val-Ala-DL-Asp-fluor-omethylketone (Z-YVAD-fmk)) that is associated with the

generation of pyrogenic mediators such as IL-1β and IL-18 (Table 1). It remains to be clarified whether pyroptosis truly constitutes a cell death subroutine on its own or whether it represents a particular case of caspase-dependent intrinsic apoptosis (see above).

Netosis. In response to several stimuli, neutrophils and eosinophils can release the so-called neutrophil extracellular traps (NETs), that is, microbicidal structures composed of nuclear chromatin, histones and granular antimicrobial proteins.[157,158] Upon the administration of granulocyte–macrophage colony-stimulating factor (GMCSF) or short-term stimulations with the complement fragment C5a, two rather physiological conditions, NETs are generated by viable cells, as demonstrated by several observations: (i) no cytosolic proteins are detected in NETs; (ii) NET-releasing cells fail to take up exclusion dyes; and (iii) NETs can be detected within 30–60 min after stimulation with IL-8 and lipopolysaccharide.[158,159] However, following non-physiological stimulation with phorbol-12-myristate-13-acetate, NETs are released by a subset of neutrophils undergoing a peculiar form of cell death,[160] which has been dubbed 'netosis'.[161]

Netotic cells exhibit massive vacuolization of the cytoplasm, rapid chromatin decondensation and breakdown of both the nuclear and granular membranes, which is required for proper NET formation.[160] Netosis is insensitive to caspase inhibitors and necrostatin-1,[162] further demonstrating that it constitutes a cell death subroutine distinct from apoptosis and regulated necrosis. However, the netotic process can be suppressed by pharmacological inhibition of NADPH oxidase (which is responsible for the oxidative burst occurring during neutrophil activation) or autophagy.[160,162] Of note, ROS appear to be required but insufficient for netosis to occur, suggesting that the autophagic component of netosis cannot be entirely attributed to the autophagy-promoting activity of ROS.[160,163] Histone citrullination (i.e., the conversion of positively charged arginine side chains into polar, but uncharged citrulline side chains) also appears to participate in netosis by mediating chromatin decondensation and NET formation.[164] Thus, genetic inhibition of the major histone-citrullinating enzyme, that is, peptidylarginine deiminase 4 (PAD4), interfered with NET release by HL-60 cells stimulated with Ca^{2+} ionophores or *Shigella flexneri*.[164] Importantly, cell death with netotic features has been observed in a subset of cytokine-primed neutrophils following ligation of Siglec-9 and CD44.[26,165] In response to these rather physiological stimuli, dying neutrophils reportedly do not release NETs.[26] Thus, whereas NET formation may be paralleled by netosis, this is not always the case: netotic cell death can occur in the absence of NET release and, *vice versa*, NET can be generated in the absence of cell death.

In view of these observations, netosis might be defined as a cell death subroutine that is: (i) restricted to granulocytic cells; (ii) insensitive to (and perhaps dependent on)[163] caspase inhibition; (iii) insensitive to necrostatin; (iv) dependent on NAPDH oxidase-mediated superoxide generation; and (v) dependent on (components of) the autophagic machinery (Table 1). As it stands, netosis shares biochemical features with both autophagic cell death and regulated necrosis. Further investigation is required to elucidate whether netosis

is a specific case of either these cell death subroutines or whether it constitutes a cell death mechanism *per se*.

Cornification. Although cells belonging to the basal layer of the epidermis respond to insults, for instance UV irradiation, by undergoing apoptosis or necrosis, cells of the external layer continuously undergo a physiological cell death subroutine that has been dubbed 'cornification'.[166] This underlies the generation of the stratum corneum, a layer of dead keratinocytes (so-called 'corneocytes') containing an mixture of specific proteins (e.g., keratin, loricrin, SPR and involucrin) and lipids (e.g., fatty acids and ceramides) that confers to the skin structural stability, mechanical resistance, elasticity and water repellence.[166] Also known as 'keratinization' or 'cornified envelope formation', cornification is often viewed as a program of terminal differentiation similar to those underlying the maturation of other anucleated tissues (e.g., the lens epithelium, red blood cells).[167,168] All these processes are indeed associated with the (partial) activation of cell death executioners, notably caspases.[167–169] However, lens and red blood cells preserve the ability to succumb from stress-induced cell death,[170,171] whereas corneocytes do not, suggesting that only cornification constitutes a *bona fide* cell death program.

From a biochemical perspective, cornification is associated with the synthesis of the enzymes and substrates that are required for the generation of the stratum corneum. Enzymes include, although presumably are not limited to, caspase-14[172] and transglutaminase (TG)-1, -3 and -5, which catalyze crosslinking reactions.[173] Substrates include proteins (e.g., filaggrin, loricrin, SPR, involucrin and SP100), but also lipids, which are extruded into the extracellular space and covalently attached to cornified envelope proteins to ensure skin impermeability.[166] The skin of $Casp14^{-/-}$ mice presents an altered composition of the stratum corneum and is characterized by reduced hydration levels, increased water loss and high sensitivity to UV-induced DNA damage,[172] pointing to a critical role for caspase-14 in cornification.

On the basis of these considerations, cornification can be defined as cell death subroutine that: (i) is restricted to keratinocytes; (ii) is functionally linked to the generation of the stratum corneum of the epidermis; and (iii) can be altered, although not blocked, by the inhibition of transglutaminases or caspase-14 (Table 1).

Notes of Caution

There are several issues that should be taken into consideration for appropriately classifying cell death subroutines based on biochemical parameters.

(1) *Physiopathological relevance*: Here, we provide a functional classification that can be applied to both *in vitro* and *in vivo* observations. This said, cell death subroutines should be considered relevant from a physiopathological perspective only when they are shown to occur *in vivo* and to be critical either for embryonic/post-embryonic development or as an etiological determinant of disease. There probably is a plethora of genes that can influence cell death in physiopathologically relevant settings, but they are never tested under such conditions and most of them remain

therefore unidentified. Thus, *in vivo* studies constitute the ultimate tool to recognize the true importance of cell death signaling pathways and to understand their regulation.

(2) *Pharmacological modulators*: Chemical agents including the RIP1 inhibitor necrostatin-1, the pan-caspase inhibitor Z-VAD-fmk and the VPS34 inhibitors 3-methyladenine and wortmannin have been widely employed in cell death research, and surely contributed to important discoveries, for example, that of regulated necrosis.[10] However, most of these compounds lack adequate specificity to precisely define a cell death program. For instance, Z-VAD-fmk has repeatedly been shown to inhibit non-caspase proteases including calpains.[174–176] Similarly, 3-methyladenine affects multiple facets of cellular metabolism.[177] Thus, while tests with pharmacological inhibitors constitute useful starting points for experimentation, they cannot be employed as surrogates of genetic studies based on gene knockout or RNAi.

(3) *Specificity of signaling*: Most if not all proteins that ignite or mediate cell death subroutines have multiple – and sometimes cell-death-unrelated – functions.[27] Thus, whereas cytosolic CYTC activates the apoptosome,[52] in the mitochondria CYTC functions as an electron shuttle within the respiratory chain,[178] and its complete absence is incompatible with life.[179] RIP1 has a plethora of downstream targets that mediate different biological outputs, and not only is it involved in apoptotic and necrotic cell death,[23] but is also critical for pro-survival NF-κB signaling.[45] Along similar lines, the VPS34 complex regulates autophagy as well as other vesicle trafficking pathways including endocytosis.[180] Hence, results coming from either the ablation or genetic inhibition of pleiotropic modulators including (but not limited to) RIP1 and VPS34 should be interpreted with caution, as it is easy to underestimate the number of signaling cascades that have been affected by apparently highly specific perturbations.

(4) *Crosstalk between different cell death subroutines*: It should always be kept in mind that, in the vast majority of settings (in particular *in vivo*), cell death subroutines are neither isolated nor mutually exclusive signaling cascades. Most often, pro-survival pathways are engaged along with the propagation of lethal signals. Moreover, stress conditions can result in the activation of multiple lethal mechanisms, which can exhibit variable degrees of overlap. It is the crosstalk between pro-survival and pro-death pathways that determines if and by which subroutine the cell will eventually die. This level of complexity must be taken into account for the classification of cell death modalities, as the inhibition of one specific pathway often unveils the existence of backup mechanisms instead of truly blocking the cell demise.

(5) *'Programmed', 'regulated' and 'accidental' cell death*: We suggest to preserve the adjective 'programmed' for those physiological instances of cell death – irrespective of the modality by which they are executed – that occur in the context of embryonic/post-embryonic development and tissue homeostasis. 'Regulated' should be used to indicate cases of cell death – be they programmed or not – whose initiation and/or execution is mediated by a dedicated molecular machinery, implying that they can be inhibited by targeted pharmacological and/or genetic interventions. Finally, the expression 'accidental' should be employed to indicate cell death triggered by extremely harsh physical conditions (e.g.,

freeze–thawing cycles, high concentrations of pro-oxidants), which cannot be inhibited by pharmacological and/or genetic manipulations and usually exhibits morphological features of necrosis.

(6) *General versus specific nomenclature*: During the past decade, several neologisms have been introduced to indicate very specific signaling pathways that lead to cell death, including parthanatos, necroptosis, paraptosis, pyronecrosis and several others.[5,6,8] Although some of these expressions (e.g., necroptosis, parthanatos) have been used in a relatively homogeneous manner, others (e.g., paraptosis, pyronecrosis) have acquired a variety of connotations, facilitating confusion. For this reason, and in line with our mandate (i.e., to provide truly functional definitions of cell death subroutines), we encourage scientists and authors of scientific publications to (i) prefer the use of general terms that bear functional connotations to that of specific names, and (ii) to avoid the introduction of neologisms.

Concluding Remarks

Until now, the field of cell death research has been dominated by morphological definitions that ignore our relentlessly increasing knowledge of the biochemical features of distinct cell death subroutines. Here, the NCCD proposes a new classification of lethal signaling pathways based on biochemical and functional considerations. In this context, 'loss-of-function' and 'gain-of function' genetic interventions (e.g., RNAi, knockout models and plasmid-driven overexpression systems), as well as chemical inhibitors or activators of important signaling nodes, constitute irreplaceable tools to characterize cell death. During the process of functional characterization, great attention should be paid to ensure that genetic and chemical interventions truly modify the incidence of cell death (as assessed by clonogenic cell survival), rather than activate alternative lethal pathways. With these recommendations in mind and the appropriate tools at hand, researchers have the possibility to label cell death instances with functional and biochemical tags. Our belief is that this classification, if properly applied, will facilitate the understanding of scientific reports, stimulate the communication among scientists and ultimately accelerate the pace of cell death discovery.

Conflict of Interest

The authors declare no conflict of interest.

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