



## Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018

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# Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018

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## Abstract

Over the past decade, the Nomenclature Committee on Cell Death (NCCD) has formulated guidelines for the definition and interpretation of cell death from morphological, biochemical, and functional perspectives. Since the field continues to expand and novel mechanisms that orchestrate multiple cell death pathways are unveiled, we propose an updated classification of cell death subroutines focusing on mechanistic and essential (as opposed to correlative and dispensable) aspects of the process. As we provide molecularly oriented definitions of terms including intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death, autophagy-dependent cell death, immunogenic cell death, cellular senescence, and mitotic catastrophe, we discuss the utility of neologisms that refer to highly specialized instances of these processes. The mission of the NCCD is to provide a widely accepted nomenclature on cell death in support of the continued development of the field.

## Introduction

For a long time, cell death has been dismissed by biologists as an inevitable and, hence, spurious consequence of cellular life. A large body of experimental evidence accumulating over the past decades, however, has unveiled and characterized in ever greater detail a set of genetically encoded mechanisms for targeted elimination of superfluous, irreversibly damaged, and/or potentially harmful cells [1–4]. Intriguingly, regulated cell death (RCD) is not unique to multicellular life forms, a setting in which RCD has an obvious advantage for organismal homeostasis in both physiological and pathological settings [5–9], but is also found (in simplified variants) among unicellular eukaryotes living (at least for part of their life cycle) in colonies (such as several yeast species and *Dictyostelium discoideum*) [10–15], and at least in some prokaryotes (e.g., *Escherichia coli*) [16]. In striking contrast with accidental cell death (ACD)—the instantaneous and catastrophic demise of cells exposed to severe insults of physical (e.g., high pressures, temperatures, or osmotic forces), chemical (e.g., extreme pH variations), or mechanical (e.g., shear forces) nature—RCD relies on a dedicated molecular machinery, implying that it can be modulated (i.e., delayed

or accelerated) by pharmacological or genetic interventions [5, 17].

Although the underlying molecular mechanisms exhibit considerable overlap (see below), RCD is involved in two diametrically opposed scenarios. On the one hand, RCD can occur in the absence of any exogenous environmental perturbation, hence operating as a built-in effector of physiological programs for development or tissue turnover [6, 18]. These completely physiological forms of RCD are generally referred to as programmed cell death (PCD). On the other hand, RCD can originate from perturbations of the intracellular or extracellular microenvironment, when such perturbations are too intense or prolonged for adaptive responses to cope with stress and restore cellular homeostasis [5]. Importantly, stress-driven RCD also constitutes a strategy for the preservation of a biological equilibrium, hence resembling adaptive stress responses. However, while adaptive stress responses operate at the cellular level (which—by extension—promotes the maintenance of homeostasis at the level of organism or colony), RCD directly operates at the level of the organism or colony in spite of cellular homeostasis [5]. Such a homeostatic function not only reflects the elimination of useless or potentially dangerous cells, but also the ability of dying cells to expose or release molecules that alert the organism or colony about a potential threat. Such danger signals are

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commonly referred to as damage-associated molecular patterns (DAMPs) or alarmins [19–22].

Cell death manifests with macroscopic morphological alterations. Together with the mechanisms whereby dead cells and their fragments are disposed of, such morphotypes have historically been employed to classify cell death into three different forms: (1) type I cell death or apoptosis, exhibiting cytoplasmic shrinkage, chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), and plasma membrane blebbing, culminating with the formation of apparently intact small vesicles (commonly known as apoptotic bodies) that are efficiently taken up by neighboring cells with phagocytic activity and degraded within lysosomes; (2) type II cell death or autophagy, manifesting with extensive cytoplasmic vacuolization and similarly culminating with phagocytic uptake and consequent lysosomal degradation; and (3) type III cell death or necrosis, displaying no distinctive features of type I or II cell death and terminating with the disposal of cell corpses in the absence of obvious phagocytic and lysosomal involvement [23, 24]. Of note, this morphological classification is still extensively employed, irrespective of multiple limitations, and caveats. Starting from 2005, the Nomenclature Committee on Cell Death (NCCD) gathered on a regular basis (1) to address the issues related to the use of a nomenclature of cell death based on morphological grounds; (2) to precisely define major cell death modalities on a genetic, biochemical, pharmacological, and functional (rather than morphological) basis; (3) to distinguish essential (causal) from accessory (correlative) aspects of the death process; and (4) to identify consensus criteria for the identification of dead cells with irreversible plasma membrane permeabilization or complete cellular fragmentation [17, 25–28].

As the field continues to progress and novel signaling pathways that orchestrate RCD are still being characterized, we propose here an updated classification of cell death modalities centered on molecular and essential aspects of the process (Fig. 1 and Box 1). A major focus will be placed on the signal transduction modules involved in the initiation, execution, and propagation of cell death, as well as on the pathophysiological relevance of each of the main types of RCD.

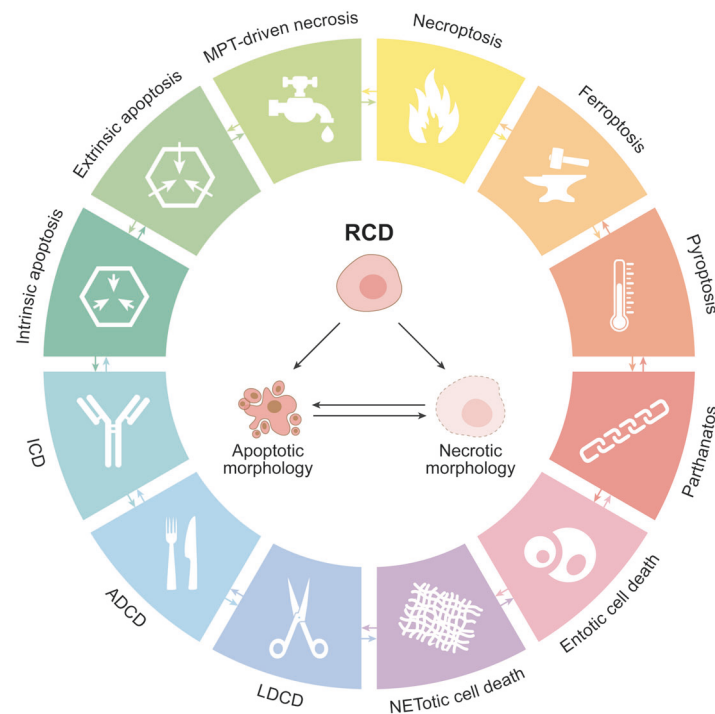
## Intrinsic apoptosis

Intrinsic apoptosis is a form of RCD initiated by a variety of microenvironmental perturbations including (but not limited to) growth factor withdrawal, DNA damage, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) overload, replication stress, microtubular alterations or mitotic defects [29–34]. Apoptotic cells retain plasma membrane integrity and metabolic activity (to some degree)

as the process proceeds to completion, which—in vivo—allows for the rapid clearance by macrophages or other cells with phagocytic activity (a process commonly known as efferocytosis) [35]. Importantly, intrinsic (and extrinsic, see below) apoptosis and consequent efferocytosis are not always immunologically silent, as previously thought (see below) [36, 37]. In vitro, end-stage apoptosis is generally followed by complete breakdown of the plasma membrane and the acquisition of a necrotic morphotype (secondary necrosis), unless cultured cells display phagocytic activity [38], a process that has recently been linked to the pore-forming activity of gasdermin E (GSDME; best known as DFNA5) [39].

The critical step for intrinsic apoptosis is irreversible and widespread mitochondrial outer membrane permeabilization (MOMP) [40, 41], which is controlled by pro-apoptotic and anti-apoptotic members of the BCL2, apoptosis regulator (BCL2) protein family, a group of proteins sharing one to four BCL2 homology (BH) domains (i.e., BH1, BH2, BH3, and BH4) [29, 42, 43]. In response to apoptotic stimuli, MOMP is mediated by BCL2 associated X, apoptosis regulator (BAX), and/or BCL2 antagonist/killer 1 (BAK1; best known as BAK), both of which contain four BH domains and a conserved transmembrane domain [44–46]. Together with BOK, BCL2 family apoptosis regulator (BOK) [47], BAX and BAK are the only BCL2 family members characterized so far in mammalian cells for their ability to form pores across the outer mitochondrial membrane (OMM) and possibly other intracellular membranes [29, 42, 43]. In physiological conditions, BAX continuously cycles between the OMM and the cytosol, where it exhibits a quiescent monomeric or inactive dimeric conformation [48–50]. In contrast, BAK constitutively resides at the OMM, where it inserts within the lipid bilayer via its hydrophobic C-terminal  $\alpha 9$  helix upon interaction with voltage dependent anion channel 2 (VDAC2) [51–54]. Of note, some degree of BAK retrotranslocation from the OMM to the cytosol has been documented [55]. Upon induction of apoptosis, BAX retrotranslocation ceases as the mitochondrial pools of BAX and BAK undergo direct or indirect activation (see below) by pro-apoptotic BH3-only proteins [48, 56–59].

These pro-apoptotic members of the BCL2 protein family (which contain a single BH3 domain) are activated transcriptionally or post-translationally as specific organelles or cellular compartments experience perturbations of homeostasis, de facto operating as cellular transducers of stress signaling [60–63]. Some BH3-only proteins—such as BCL2 binding component 3 (BBC3; best known as p53-upregulated modulator of apoptosis, PUMA), BCL2 like 11 (BCL2L11; best known as BCL2-interacting mediator of cell death, BIM), and phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1; best known as NOXA)—are



**Fig. 1** Major cell death subroutines. Mammalian cells exposed to unrecoverable perturbations of the intracellular or extracellular microenvironment can activate one of many signal transduction cascades ultimately leading to their demise. Each of such regulated cell death (RCD) modes is initiated and propagated by molecular mechanisms that exhibit a considerable degree of interconnectivity. Moreover, each type of RCD can manifest with an entire spectrum of

morphological features ranging from fully necrotic to fully apoptotic, and an immunomodulatory profile ranging from anti-inflammatory and tolerogenic to pro-inflammatory and immunogenic. *ADCD*: autophagy-dependent cell death, *ICD*: immunogenic cell death, *LDCD*: lysosome-dependent cell death, *MPT*: mitochondrial permeability transition.

mainly activated by transcriptional upregulation, while others—such as BH3 interacting domain death agonist (BID)—mostly undergo post-translational activation [64–70]. BID, BIM, PUMA, and NOXA share the ability to physically (but transiently) interact with the mitochondrial pool of BAX and/or BAK (hence being known as “activators”) to promote a series of conformational changes [59, 64, 67, 71–74] culminating with the dissociation of the core and latch domains of BCL2 effectors [75–77]. The current view is that activated BAX and BAK form homodimers (also heterodimers in specific settings), resulting in the release of BH3-only proteins and further dimer-by-dimer oligomerization [76–83]. Oligomerization ultimately leads to the assembly of a toroidal lipidic pore that alters mitochondrial permeability and causes profound rearrangements of the mitochondrial ultrastructure [78, 84–86]. In line with this model, it has recently been shown that (1) BAX can form rings or linear/arc-shaped oligomers that perforate the OMM [84, 85], and (2) MOMP proceeds upon the formation of pores (impinging on OMM curvature stress), which can vary in size depending on the number of BAX dimers recruited [87].

MOMP is antagonized by anti-apoptotic members of the BCL2 family, including BCL2 itself, BCL2 like 1 (BCL2L1;

best known as BCL-X<sub>L</sub>), MCL1, BCL2 family apoptosis regulator (MCL1), BCL2 like 2 (BCL2L2; best known as BCL-W), and BCL2 related protein A1 (BCL2A1; best known—in human—as BFL-1) [29, 42, 43]. These pro-survival proteins contain all four BH domains, are generally inserted into the OMM or the ER membrane through their  $\alpha 9$  helix, and mainly exert anti-apoptotic functions by directly binding pro-apoptotic members of the BCL2 family, an activity that generally—but not always—depends on a hydrophobic binding groove formed by BH1, BH2, and BH3 domains [88–94]. In addition, some anti-apoptotic BCL2 family members have been proposed to promote cellular survival by: (1) regulating Ca<sup>2+</sup> homeostasis at the ER [95–99]; (2) promoting bioenergetic metabolism upon interaction with the F<sub>1</sub>F<sub>0</sub> ATP synthase [100–104]; and (3) contributing to the regulation of redox homeostasis [105–109]. However, the importance of these functions has been challenged by the generation of cell lines that lack all major anti-apoptotic and pro-apoptotic BCL2 family members [93]. Thus, most pro-survival BCL2 family members inhibit BAX and BAK by preventing their oligomerization and pore-forming activity either directly, upon physical sequestration at the OMM, or indirectly, following the sequestration of BH3-only activators [29, 64, 79, 110]. Of note, in physiological conditions, some

### Box 1 Operational definitions

**Accidental cell death (ACD).** Virtually instantaneous and uncontrollable form of cell death corresponding to the physical disassembly of the plasma membrane caused by extreme physical, chemical, or mechanical cues.

**Anoikis.** Specific variant of intrinsic apoptosis initiated by the loss of integrin-dependent anchorage.

**Autophagy-dependent cell death.** A form of RCD that mechanistically depends on the autophagic machinery (or components thereof).

**Autosis.** A specific instance of autophagy-dependent cell death that critically relies on the plasma membrane  $\text{Na}^+/\text{K}^+$ -ATPase.

**Cell death.** Irreversible degeneration of vital cellular functions (notably ATP production and preservation of redox homeostasis) culminating in the loss of cellular integrity (permanent plasma membrane permeabilization or cellular fragmentation).

**Cellular senescence.** Irreversible loss of proliferative potential associated with specific morphological and biochemical features, including the senescence-associated secretory phenotype (SASP). Cellular senescence does not constitute a form of RCD.

**Efferocytosis.** Mechanism whereby dead cells and fragments thereof are taken up by phagocytes and disposed.

**Entotic cell death.** A type of RCD that originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes.

**Extrinsic apoptosis.** Specific variant of RCD initiated by perturbations of the extracellular microenvironment detected by plasma membrane receptors, propagated by CASP8 and precipitated by executioner caspases, mainly CASP3.

**Ferroptosis.** A form of RCD initiated by oxidative perturbations of the intracellular microenvironment that is under constitutive control by GPX4 and can be inhibited by iron chelators and lipophilic antioxidants.

**Immunogenic cell death.** A form of RCD that is sufficient to activate an adaptive immune response in immunocompetent hosts.

**Intrinsic apoptosis.** Type of RCD initiated by perturbations of the extracellular or intracellular microenvironment, demarcated by MOMP, and precipitated by executioner caspases, mainly CASP3.

**Lysosome-dependent cell death.** A type of RCD demarcated by primary LMP and precipitated by cathepsins, with optional involvement of MOMP and caspases.

**Mitochondrial permeability transition (MPT)-driven necrosis.** Specific form of RCD triggered by perturbations of the intracellular microenvironment and relying on CYPD.

**Mitotic catastrophe.** Oncosuppressive mechanism for the control of mitosis-incompetent cells by RCD or cellular senescence. Per se, mitotic catastrophe does not constitute a form or RCD.

**Mitotic death.** Specific variant of RCD (most often, intrinsic apoptosis) driven by mitotic catastrophe.

**Necroptosis.** A modality of RCD triggered by perturbations of extracellular or intracellular homeostasis that critically depends on MLKL, RIPK3, and (at least in some settings) on the kinase activity of RIPK1.

**NETotic cell death.** A ROS-dependent modality of RCD restricted to cells of hematopoietic derivation and associated with NET extrusion.

**Parthanatos.** A modality of RCD initiated by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophe coupled to AIF-dependent and MIF-dependent DNA degradation.

**Programmed cell death (PCD).** Particular form of RCD that occurs in strictly physiological scenarios, i.e., it does not relate to perturbations of homeostasis and hence does not occur in the context of failing adaptation to stress.

**Pyroptosis.** A type of RCD that critically depends on the formation of plasma membrane pores by members of the gasdermin protein family, often (but not always) as a consequence of inflammatory caspase activation.

**Regulated cell death (RCD).** Form of cell death that results from the activation of one or more signal transduction modules, and hence can be pharmacologically or genetically modulated (at least kinetically and to some extent).

anti-apoptotic BCL2 proteins, such as BCL- $\text{X}_\text{L}$ , exert a protective role by promoting the retrotranslocation of BAX and (to a lesser degree) BAK from the mitochondria to the cytoplasm, thus limiting their mitochondrial pool [48, 55, 111]. Evidence from T cells and platelets suggests that such retrotranslocation occurs in vivo, resulting in the physiological inhibition of BAK by BCL- $\text{X}_\text{L}$  [112]. Importantly, some BH3-only proteins including BCL2 associated agonist of cell death (BAD), Bcl2 modifying factor (BMF), or harakiri, BCL2 interacting protein (HRK) promote MOMP in the absence of a physical interaction with BAX or BAK. These BH3-only proteins, which are sometimes referred to as “sensitizers” or “inactivators” bind to anti-apoptotic BCL2 family members and hence limit their availability to sequester BAX, BAK, or BH3-only activators [58, 93].

Different BH3-only proteins have been suggested to preferentially bind specific anti-apoptotic BCL2 family members (e.g., BID, BIM, and PUMA potently bind all anti-apoptotic BCL2 family members; BAD preferentially

interacts with BCL2, BCL- $\text{X}_\text{L}$ , and BCL-W; NOXA preferentially inhibits MCL1; and HRK preferentially inhibits BCL- $\text{X}_\text{L}$ ) [57, 113, 114]. In vitro results suggest that the distinction between sensitizers and activators may be much less rigid than previously thought [79, 114–117]. However, overexpression of BH3-only sensitizers induces minimal apoptosis in cells lacking BID, BIM, PUMA, and NOXA [64], suggesting that BH3-only activators function downstream of BH3-only sensitizers. Of note, the interaction between anti-apoptotic and pro-apoptotic BCL2 family members has major therapeutic implications, with BCL2 representing the pharmacological target of the FDA-approved BH3 mimetic venetoclax (also known as ABT-199) and other molecules with a similar mechanism of action that are currently under development (e.g., the MCL1 inhibitor S63845) [118, 119]. Indeed, venetoclax kills chronic lymphocytic leukemia (CLL) cells by mimicking the activity of BH3-only proteins [120]. Recently, a mechanism of resistance to BH3 mimetics

has been ascribed to the tight association between BCL-X<sub>L</sub> and BH3-only activators at subcellular membranes [121, 122]. The relevance of this mechanism for CLL patients under venetoclax treatment, however, remains to be elucidated.

Confirming the essential role of BCL2 family members for MOMP and the high degree of overlap between the machineries responsible for stress-driven RCD and PCD, the co-deletion of *Bax* and *Bak1* not only renders a large panel of cell types profoundly resistant to diverse lethal stimuli [74], a phenotype that in some settings can be exacerbated by the co-deletion of *Bok* [123], but also causes perinatal lethality in mice as a consequence of severe developmental defects [124]. Along similar lines, *Bcl2l1l*<sup>-/-</sup>*Bmf*<sup>-/-</sup> as well as *Bid*<sup>-/-</sup>*Bcl2l1l*<sup>-/-</sup>*Bbc3*<sup>-/-</sup> mice die prematurely or display severe developmental defects, respectively [68, 125]. However, transformed cells lacking all major BH3 activators (i.e., BID, BIM, PUMA, and NOXA) can still undergo apoptosis in response to DNA-damaging agents or downregulation of BCL2, BCL-X<sub>L</sub>, and MCL1 [64]. This observation is in line with the notion that BAX and BAK can self-activate in the absence of anti-apoptotic BCL2 family members and pro-apoptotic BH3 proteins (according to a relatively slow kinetics) [64, 93]. Perhaps, BAX and BAK can even be activated independently of BH3-only proteins by the concerted action of the prolyl isomerase peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (PIN1) and either tumor protein p53 (TP53; best known as p53) [126–128] or ATR serine/threonine kinase (ATR) [129, 130], several proteins containing BH-like motifs [131], as well as by detergents, heat, pH changes, or specific monoclonal antibodies [132]. That said, the actual pathophysiological relevance of non-canonical BAX and BAK activation remains to be formally established. Both anti-apoptotic and pro-apoptotic BCL2 proteins are also subjected to tight transcriptional and post-translational regulation, involving (but not limited to) proteasomal degradation, phosphorylation, and subcellular (re)localization [48, 108, 133–138]. Finally, it is becoming increasingly evident that mitochondrial size and shape [139–141] as well as lipid composition [142, 143] can influence the likelihood of mitochondria to undergo irreversible MOMP. These observations exemplify the number of factors involved in MOMP at the level of single mitochondria. Of note, active BAX and BAK have also been proposed to (1) permeabilize ER membranes, especially in response to reticular stress, leading to release of luminal ER chaperones into the cytosol [30, 144]; and (2) favor the activation of type 1 inositol trisphosphate receptors at the ER, resulting in the cytosolic leak of Ca<sup>2+</sup> ions and consequent mitochondrial Ca<sup>2+</sup> uptake [96, 145]. However, the actual relevance of ER permeabilization for intrinsic apoptosis remains to be elucidated. That said, the contact sites

between mitochondria and the ER (which are commonly known as mitochondria-associated ER membranes) appear to regulate a plethora of cellular processes that influence RCD or its immunological consequences, including (but not limited to) ER stress signaling, the transfer of Ca<sup>2+</sup> ions from the ER to mitochondria, and inflammatory reactions [146–148].

As for BOK, it has been proposed that this BCL2 protein contributes to the regulation of ER homeostasis, as demonstrated by its prominent localization at the ER membrane [149] and the defective apoptotic response of *Bok*<sup>-/-</sup> cells to some ER stressors [150]. Moreover, BOK has recently been shown to induce MOMP in the absence of BAX and BAK and independently of other BCL2 family members [47, 151, 152]. In particular, BOK appears to be constitutively active and to be antagonized by an ER-associated degradation pathway rather than by anti-apoptotic BCL2 proteins [47]. BOK is also regulated by a mechanism involving the binding to inositol 1,4,5-trisphosphate (IP<sub>3</sub>) receptors, which reportedly limits its proteasomal degradation [153]. Of note, *Bok*<sup>-/-</sup>, *Bax*<sup>-/-</sup>*Bok*<sup>-/-</sup> as well as *Bak1*<sup>-/-</sup>*Bok*<sup>-/-</sup> mice display no obvious abnormalities (except for persistence of primordial follicle oocytes in aged *Bax*<sup>-/-</sup>*Bok*<sup>-/-</sup> females) [154, 155], implying that physiological functions of BOK can be compensated for by BAK and/or BAX.

MOMP directly promotes the cytosolic release of apoptogenic factors that normally reside in the mitochondrial intermembrane space [40, 44, 156]. These mitochondrial proteins include (but are not limited to) cytochrome c, somatic (CYCS), which usually operates as an electron shuttle in the mitochondrial respiratory chain [157–160], and diablo IAP-binding mitochondrial protein (DIABLO; also known as second mitochondrial activator of caspases, SMAC) [161–163]. The release of CYCS and SMAC to the cytosol is favored by mitochondrial cristae remodeling [164], which relies on the oligomerization and activation of OPA1, mitochondrial dynamin like GTPase (OPA1) [165], possibly preceded by the BAX-dependent and BAK-dependent activation of OMA1 zinc metallopeptidase (OMA1) [166, 167], and/or dynamin 1 like (DNM1L; best known as DRP1) [168]. Accordingly, nitric oxide (NO) has been shown to precipitate the release of apoptogenic factors from mitochondria upon direct nitrosylation of DRP1 (at least in some settings) [169–171]. The cytosolic pool of CYCS binds to apoptotic peptidase activating factor 1 (APAF1) and pro-caspase 9 (CASP9) in a deoxyATP-dependent manner to form the supramolecular complex known as apoptosome, which is responsible for CASP9 activation [160]. Recently, the structure of the apoptosome from multiple organisms including humans has been characterized at atomic resolution [172–174]. These studies revealed that the autocatalytic maturation of CASP9 within

the apoptosome occurs through generation of CASP9 homodimers and CASP9-APAF1 heterodimers/multimers upon association of their respective caspase recruitment domains (CARDs) [175–178].

Activated CASP9 can catalyze the proteolytic activation of CASP3 and CASP7, which are widely perceived as the enzymes responsible for cell demolition during intrinsic (and extrinsic, see below) apoptosis in mammalian cells (and hence are commonly known as executioner caspases) [179, 180]. Cytosolic SMAC precipitates apoptosis by associating with members of the inhibitor of apoptosis (IAP) protein family, including X-linked inhibitor of apoptosis (XIAP) [162, 163, 181]. To acquire apoptogenic activity, SMAC must undergo a proteolytic maturation step that unleashes its latent IAP-binding domain, which is catalyzed by the inner membrane peptidase (IMP) complex [182] and perhaps by the inner mitochondrial membrane (IMM) protease presenilin associated rhomboid like (PARL) [183]. XIAP is the only IAP protein family member that counteracts the apoptotic cascade by stably binding to and hence physically blocking caspases [184, 185]. Conversely, baculoviral IAP repeat containing 2 (BIRC2; best known as c-IAP1) and BIRC3 (best known as c-IAP2) mostly do so as they (1) drive the upregulation of potent anti-apoptotic factors such as CASP8 and FADD like apoptosis regulator (CFLAR; best known as c-FLIP) [186]; (2) promote caspase inactivation by virtue of their E3 ubiquitin ligase activity [187–195]; (3) ubiquitinate receptor interacting serine/threonine kinase 1 (RIPK1) and hence trigger pro-survival NF- $\kappa$ B signaling [196–198]; and (4) perhaps promote SMAC degradation at mitochondria through a mechanism that depends on BCL2 proteins [199]. Of note, MOMP eventually leads to the dissipation of the mitochondrial transmembrane potential ( $\Delta\psi_m$ )—mostly as a consequence of the respiratory impairment imposed by the loss of CYCS—and hence to the cessation of  $\Delta\psi_m$ -dependent mitochondrial functions (including ATP synthesis and some forms of protein import) [200–203]. Intriguingly, BAK and BAX may not always be required for proapoptotic stimuli to promote CYTC release and consequent caspase activation, even in conditions in which mitochondrial permeability transition (MPT; see below) is disabled [204, 205]. This may suggest the existence of another—presently unidentified—mechanism for MOMP, possibly involving specific lipids like ceramide [206, 207]. The actual pathophysiological relevance of this potential mechanism remains obscure.

The catalytic activity of executioner caspases precipitates cellular demise and is responsible for many of the morphological and biochemical correlates of apoptosis, including DNA fragmentation [208], phosphatidylserine (PS) exposure [209, 210], and the formation of apoptotic bodies [211, 212]. CASP3 favors DNA fragmentation by

catalyzing the proteolytic inactivation of DNA fragmentation factor subunit alpha (DFFA; best known as ICAD), hence unleashing the catalytic activity of DFFB (best known as CAD) [213–215]. Recent experimental evidence demonstrates that CASP3 promotes PS exposure by activating proteins involved in PS externalization, such as the phospholipid scramblases [216–218], or inactivating factors that mediate PS internalization, such as phospholipid flippases [219–221]. Thus, in response to apoptotic stimuli, active CASP3 reportedly cleaves (1) XK related protein 8 (XKR8), which interacts with basigin (BSG) or neuropilin (NPTN) to form a phospholipid-scrambling complex responsible for PS exposure [216, 217], and (2) ATPase phospholipid transporting 11A (ATP11A) and ATP11C, resulting in inhibition of their flippase activity and PS exposure, as demonstrated by absent or reduced PS translocation on the cell surface of cells expressing a non-cleavable ATP11C or developing erythrocytes from *Atp11a*<sup>−/−</sup> mice [219–221]. That said, PS exposure may not universally accompany intrinsic (and extrinsic) apoptosis [222–224].

Of note, a large body of evidence suggests that executioner caspases precipitate intrinsic apoptosis, once a hitherto poorly defined point-of-no-return has been trespassed, but are not essential for it [17]. Accordingly, blocking post-mitochondrial caspase activation by genetic means or with specific pharmacological inhibitors, such as to *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone (Z-VAD-fmk) and (3S)-5-(2,6-difluorophenoxy)-3-[[[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarboxyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate (Q-VD-OPh), generally delays (but does not prevent) intrinsic apoptosis in vitro and in vivo (at least in the mammalian system), as it promotes a switch to other types of RCD [17, 225]. In addition, when MOMP affects a limited number of mitochondria, the consequent sublethal activation of caspases does not precipitate RCD but promotes genomic instability [226]. Finally, at least some cells exposed to transient apoptotic stimuli appear to survive MOMP affecting a limited number of mitochondria and the partial activation of executioner caspases by a hitherto poorly characterized process called anastasis (most likely constituting a robust adaptive response upstream of the boundary between cellular life and death) [226–228]. Altogether, these observations suggest that CASP3 and CASP7 mediate a facilitating, rather than indispensable, role in RCD (for an extensive discussion on this topic, please refer to ref [17]). This said, executioner caspases can positively or negatively regulate the emission of multiple DAMPs from dying cells, including immunostimulatory [229] as well as immunosuppressive [230] factors. Thus, pharmacological agents targeting executioner caspases may be unable to mediate bona fide cytoprotection, but may

efficiently switch RCD modality. Interestingly, although CASP6 has long been considered as an executioner caspase based on its homology with CASP3 and CASP7, recent data on substrate specificity suggest that CASP6 may actually be involved in RCD initiation [179, 231, 232]. Additional investigation is required to elucidate the function of CASP6 in mammalian cells.

A specific variant of intrinsic apoptosis elicited by the loss of integrin-dependent attachment to the extracellular matrix is commonly known as anoikis [233, 234]. As such, anoikis is demarcated by MOMP and precipitated by the activation of executioner caspases, notably CASP3 [233]. At least in some settings, detachment from the extracellular matrix triggers MOMP upon activation of the BH3-only proteins BIM and BMF [137, 235]. Since anoikis prevents anchorage-independent proliferation and attachment to an improper matrix, it is generally considered as an oncosuppressive process [234, 236]. Accordingly, cancer cells need to acquire at least some degree of resistance to anoikis to initiate and progress through the so-called “metastatic cascade” [237–239]. Neoplastic cells can evade anoikis upon activation of mitogen-activated protein kinase 1 (MAPK1; best known as ERK2) caused by cellular aggregation and consequent epidermal growth factor receptor (EGFR) stabilization mediated by erb-b2 receptor tyrosine kinase 2 (ERBB2) [237, 240], or degradation of the negative ERK2 regulator BRCA1-associated protein (BRAP), which is favored by coiled-coil domain containing 178 (CCDC178) [241]. Once activated, ERK2 reportedly supports anoikis resistance by promoting the cytosolic sequestration of BIM in complex with dynein light chain LC8-type 1 (DYNLL1; best known as LC8) and beclin 1 (BECN1) [138, 238], or the transactivation of integrin subunit alpha 6 (*ITGA6*) via a mechanism dependent on KRAS [242].

Additional strategies that limit the sensitivity of malignant cells to anoikis encompass (but are not limited to): (1) activation of anti-apoptotic BCL2 proteins, including MCL1 stabilization as induced by fibroblast-derived insulin like growth factor-binding proteins (IGFBPs) [243] and increase in BCL2 expression levels as imposed by hepatitis B virus X protein [244]; (2) epigenetic silencing of adhesion-related genes, such as SHC adaptor protein 1 (*SHC1*) upon overexpression of the hematopoietic transcription factor IKAROS family zinc finger 3 (IKZF3; also known as AIOLOS) [245]; (3) perturbation of ITG-protein tyrosine kinase 2 (PTK2; best known as FAK) signaling, which usually suppresses anoikis [246–249]; (4) activation of the so-called “epithelial-to-mesenchymal transition” (EMT), which is associated with multiple signal transduction and metabolic modules for RCD resistance [242, 250, 251]; (5) targeting of Yes associated protein 1 (YAP1) by *miR-200a* or via a platelet-dependent mechanism [252, 253]; (6) increased antioxidant responses driven by the

activating transcription factor 4 (ATF4)-mediated upregulation of heme oxygenase 1 (HMOX1) [254]; (7) autophagy activation [254, 255]; (8) upregulation of the molecular chaperone crystallin alpha B (CRYAB; also known as HSPB5) [256]; (9) signaling via AMPK and proliferation and apoptosis adaptor protein 15 (PEA15), which favors anchorage-independent cell growth [257]; (10) upregulation of matrix metalloproteinases (MMPs) by a mechanism involving the epidermal growth factor (EGF)-driven autocrine production of angiopoietin like 4 (ANGPTL4) [258]; (11) expression and phosphorylation of signal transducer and activator of transcription 3 (STAT3) [259]; and (12) rewiring of central carbon metabolism toward NADPH synthesis, resulting in improved redox homeostasis [260, 261]. That said, it has become evident that the adaptation of cancer cells to the loss of attachment involves multiple processes beyond (but presumably highly interconnected to) anoikis resistance [234 262–264], suggesting that multiple barriers need to be overcome for the metastatic cascade to be initiated.

The NCCD proposes to define intrinsic apoptosis as a form of RCD initiated by perturbations of the intracellular or extracellular microenvironment, demarcated by MOMP and precipitated by executioner caspases, mainly CASP3 (Box 1).

## Extrinsic apoptosis

Extrinsic apoptosis is an RCD modality initiated by perturbations of the extracellular microenvironment [265–268]. Extrinsic apoptosis is mostly driven by either of two types of plasma membrane receptors: (1) death receptors, whose activation depends on the binding of the cognate ligand(s), and (2) dependence receptors, whose activation occurs when the levels of their specific ligand drop below a specific threshold [267 269–271].

Death receptors include (but are not limited to): Fas cell surface death receptor (FAS; also known as CD95 or APO-1), and TNF receptor superfamily member 1A (TNFRSF1A; best known as TNFR1), 10a (TNFRSF10A; best known as TRAILR1 or DR4), and 10b (TNFRSF10B; best known as TRAILR2 or DR5) [269, 270, 272, 273]. As a general rule, death receptor ligation allows for the assembly of a dynamic multiprotein complex at the intracellular tail of the receptor, such as so-called “death-inducing signaling complex” (DISC), “complex I”, and “complex II”, which operate as molecular platforms to regulate the activation and functions of CASP8 (or CASP10, in a limited number of settings) [274–276]. In the case of FAS and TRAILRs, the cognate ligands—namely, FAS ligand (FASLG; also known as CD95L or APO-1L) and TNF superfamily member 10 (TNFSF10; best known as TRAIL), respectively—stabilize

performed receptor homotrimers to induce a conformational change at their intracellular tails that enables the death domain (DD)-dependent association of the adapter Fas associated via death domain (FADD) [277–282]. In turn, FADD drives DISC assembly by promoting the death effector domain (DED)-dependent recruitment of CASP8 (or CASP10) and multiple isoforms of c-FLIP. In contrast, TNFR1 signaling involves the association of TNFRSF1A associated via DD (TRADD), which acts as an adaptor for the assembly of complex I, generally consisting of TNF receptor associated factor 2 (TRAF2), TRAF5, c-IAP1, c-IAP2, RIPK1, and the linear ubiquitin chain assembly complex (LUBAC), a supramolecular entity consisting of SHANK associated RH domain interactor (SHARPIN), RANBP2-type, and C3HC4-type zinc finger containing 1 (RBCK1; best known as HOIL-1), and ring finger protein 31 (RNF31; best known as HOIP) [283–287]. Of note, the glycosylation state of some death receptors (e.g., FAS) has been shown to impact on the sensitivity of T lymphocytes to extrinsic apoptosis, hence influencing the termination of inflammatory responses [288–290]. The relevance of death receptor glycosylation for extrinsic apoptosis in other cell types has not been investigated in detail.

The molecular mechanisms regulating CASP8 activity upon death receptor stimulation have been extensively investigated. In particular, CASP8 maturation involves a cascade of events initiated by the binding of CASP8 to FADD at the DISC. This interaction enables the assembly of a linear filament of CASP8 molecules (depending on their DEDs) that facilitates homodimerization and consequent activation by autoproteolytic cleavage [291–295]. A key role in this setting is mediated by c-FLIP, which is a catalytically inactive close relative of CASP8 [296, 297]. Compelling evidence indicates that the short variant of c-FLIP (c-FLIP<sub>S</sub>) and its long counterpart (c-FLIP<sub>L</sub>) inhibit and activate CASP8, respectively, by modulating CASP8 oligomerization [298–301]. Active CASP8 reportedly cleaves c-FLIP<sub>L</sub> [302] and heterodimeric complexes of CASP8 with c-FLIP<sub>L</sub> (but not c-FLIP<sub>S</sub>) are endowed with limited enzymatic activity that favors CASP8 oligomerization and consequent activation [301]. c-FLIP isoforms and CASP8 seem to be recruited at the DISC to comparable levels [303], supporting the notion that elevated expression levels of c-FLIP<sub>L</sub> inhibit, rather than activate, extrinsic apoptosis possibly by disrupting CASP8 maturation [301, 304]. Of note, *CFLAR* (the gene encoding c-FLIP) is under direct transcriptional control by NF- $\kappa$ B, which largely contributes to pro-survival TNFR1 signaling in specific circumstances (see below) [287, 296, 305]. The enzymatic activity of CASP8 appears to be controlled by additional post-translational mechanisms, including (but not limited to): (1) phosphorylation at Y380, which inhibits the autoproteolytic activity of CASP8 upon FAS activation

[306], (2) phosphorylation at T273, which is catalyzed by polo like kinase 3 (PLK3) at the DISC and promotes CASP8 apoptotic functions [307], and (3) deubiquitination, which decreases CASP8 activity and interrupts extrinsic apoptosis [302].

The execution of extrinsic apoptosis driven by death receptors follows two distinct pathways. In so-called “type I cells” (e.g., thymocytes and mature lymphocytes) the CASP8-dependent proteolytic maturation of executioner CASP3 and CASP7 suffices to drive RCD, which cannot be inhibited by the transgene-driven overexpression of anti-apoptotic BCL2 proteins, the co-deletion of *Bax* and *Bak1*, or the loss of BID [308, 309]. Conversely, in “type II cells” (e.g., hepatocytes, pancreatic  $\beta$  cells, and a majority of cancer cells), in which CASP3 and CASP7 activation is restrained by XIAP [310], extrinsic apoptosis requires the proteolytic cleavage of BID by CASP8 [70, 311, 312]. This leads to the generation of a truncated form of BID (tBID), which translocates to the OMM [313, 314] via a mechanism that, at least upon FAS stimulation, reportedly depends on the binding of modulator of apoptosis 1 (MOAP1) to the alleged BID receptor mitochondrial carrier 2 (MTCH2) [315, 316]. At the OMM, tBID operates as a BH3-only activator to engage BAX/BAK-dependent MOMP-driven and consequent CASP9-driven RCD. Although human CASP10 shares some degree of substrate specificity with CASP8 [317] and possibly contributes to extrinsic apoptosis in primary T cells [318], rodents including mice and rats lack a functional *Casp10* gene, and the precise role of this caspase in death receptor-driven apoptosis in humans and other CASP10-proficient species remains a matter of controversy [319–321]. A recent study shows that—following FAS activation—CASP10 causes the dissociation of CASP8 from the DISC, thereby promoting cell survival [319]. FAT atypical cadherin 1 (FAT1) appears to mediate similar anti-apoptotic function by limiting the association of CASP8 with the DISC [322].

A large body of evidence demonstrates that death receptor ligation does not necessarily culminate in RCD. In particular, TNFR1 activation can have diverse outcomes depending on multiple variables, such as the post-translational modification status of RIPK1, which has a direct impact on the assembly of pro-survival vs. pro-death signaling complexes [323–325]. Thus, following tumor necrosis factor (TNF) stimulation, RIPK1 is recruited at complex I in a TRADD-independent manner, followed by RIPK1 polyubiquitination by c-IAP1, c-IAP2, and LUBAC [196, 324, 326–329]. Polyubiquitinated RIPK1 promotes cell survival and inflammation by acting as a scaffold for the sequential recruitment of TGF- $\beta$  activated kinase 1/ MAP3K7-binding protein 2 (TAB2), TAB3, and mitogen-activated protein kinase kinase kinase 7 (MAP3K7; best known as TAK1), which can drive mitogen-activated

protein kinase (MAPK) signaling or I $\kappa$ B kinase (IKK)-dependent NF- $\kappa$ B activation [283, 287 330–333]. Moreover, the phosphorylation of RIPK1 by TAK1, the IKK complex or mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2; best known as MK2) appears to alter its ability to interact with FADD and CASP8, hence preventing the variants of TNF-induced RCD that depends on RIPK1 kinase activity and favoring RIPK1-independent TRADD-, FADD-, and CASP8-driven apoptosis [285 334–336]. Conversely, in the presence of so-called “SMAC mimetics” (which de facto operate as IAP inhibitors) [337], RIPK1 is deubiquitinated by CYLD lysine 63 deubiquitinase (CYLD), favoring its release from complex I and its association with FADD and CASP8 in the cytosol to form complex II, which drives extrinsic apoptosis [338]. Complex II formation also requires TRAF2 ubiquitination by HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1) [339]. To add a further layer of complexity, the proteasomal degradation of TRAF2 appears to be prevented (at least in hepatocytes) by RIPK1, independently of its kinase activity [340, 341]. Of note, TNFR1 can also activate alternative RCD modalities, such as necroptosis (see below).

Death receptor signaling can also lead to NF- $\kappa$ B activation, generally resulting in cell survival associated with a robust inflammatory response [272, 342]. The ability of some death receptors including TNFR1 to promote NF- $\kappa$ B activation over CASP8 activation appears to depend on the degree of receptor oligomerization (i.e., trimerization vs. higher-order multimerization) [343], the scaffolding (i.e., non-enzymatic) functions of CASP8, and the consequent assembly of TNFR1-like complexes containing RIPK1 and LUBAC [272, 286, 344]. Upon TRAILR activation, LUBAC reportedly ubiquitinates both CASP8 and RIPK1 while promoting the recruitment of IKK to complex I [286], which also explains the requirement of LUBAC for the inhibition of TNF-induced cell death [345]. In line with this notion, TNF alpha-induced protein 3 (TNFAIP3; best known as A20) inhibits CASP8 activation downstream of TRAILRs in glioblastoma cells, owing to its ability to polyubiquitinate RIPK1 [346, 347]. A recent study suggests that the ability of TRAILR2 to dispatch pro-survival rather than pro-apoptotic signals may depend on its preferential localization outside of lipid rafts [348]. It remains to be demonstrated whether the same also applies to other death receptors.

The family of dependence receptors consists of approximately 20 members, including: (1) the netrin 1 (NTN1) receptors DCC netrin 1 receptor (DCC), unc-5 netrin receptor A (UNC5A), UNC5B, UNC5C, and UNC5D; (2) the neurotrophin receptor neurotrophic receptor tyrosine kinase 3 (NTRK3); and (3) the sonic hedgehog (SHH) receptor patched 1 (PTCH1) [267, 349,

350]. Intriguingly, dependence receptors promote cell survival, proliferation and differentiation in physiological conditions (when their cognate ligands are normally available), but activate distinct (and not completely elucidated) lethal signaling cascades (generally impinging on caspase activation) once ligand availability falls below a specific threshold level [350]. Thus, in the absence of their respective ligands: (1) DCC is cleaved by CASP3 and this promotes its association with adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) and CASP9, resulting in the activation of the CASP9-CASP3 cascade [350, 351]; (2) PTCH1 interacts with the cytosolic adaptor four and a half LIM domains 2 (FHL2; best known as DRAL), hence favoring the assembly of a CASP9-activating complex consisting of caspase recruitment domain family member 8 (CARD8; also known as TUCAN) and neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase (NEDD4) [352–354]; (3) UNC5B enables the protein phosphatase 2 (PP2A)-mediated activating dephosphorylation of death associated protein kinase 1 (DAPK1), which is known to promote p53-dependent RCD [355–357]; and (4) UNC5D and NTRK3 are subjected to CASP3 cleavage generating intracellular fragments that translocate either into the nucleus to trigger the E2F transcription factor 1 (E2F1)-driven expression of pro-apoptotic genes (as in the case of UNC5D) or at mitochondria to activate CASP9 upon MOMP (as in the case of NTRK3) [358, 359].

Dependence receptor-driven RCD has been involved in multiple pathophysiological settings, and exerts robust oncosuppressive functions [350]. Accordingly, neoplastic cells often escape from dependence receptor-mediated RCD by (1) upregulating the expression of their cognate ligands such as NTN1 [360–362]; (2) inactivating, downregulating, or losing gene(s) encoding specific dependence receptors, including DCC, UNC5C, and NTRK3 [350 363–369]; or (3) silencing signal transducers operating downstream of dependence receptors—such as DAPK1—via epigenetic mechanisms [370]. That said, whether the actual pathophysiological relevance of dependence receptor signaling stems from the initiation of extrinsic apoptosis remains to be formally established. Of note, in specific cell types, some members of the toll-like receptor (TLR) protein family including toll like receptor 3 (TLR3) have also been suggested to trigger RCD by a mechanism that involves toll like receptor adaptor molecule 1 (TICAM1; best known as TRIF), and ultimately impinges on CASP8 activation [371, 372]. However, it remains unclear whether TLR3 and other TLRs actually initiate a private RCD program that directly engages CASP8, or whether they promote RCD upon the activation of an NF- $\kappa$ B-dependent autocrine/paracrine signaling pathway involving TNF.

We propose to define extrinsic apoptosis as a type of RCD initiated by perturbations of the extracellular microenvironment that are detected by plasma membrane receptors, propagated by CASP8 (with the optional involvement of MOMP), and precipitated by executioner caspases, mainly CASP3 (Box 1).

## MPT-driven necrosis

MPT-driven necrosis is a form of RCD initiated by specific perturbations of the intracellular microenvironment such as severe oxidative stress and cytosolic  $\text{Ca}^{2+}$  overload, which generally manifests with a necrotic morphotype [373, 374]. The term MPT refers to an abrupt loss of the impermeability of the IMM to small solutes, resulting in rapid  $\Delta\psi_m$  dissipation, osmotic breakdown of both mitochondrial membranes, and RCD [373, 374].

At the biochemical level, MPT-driven necrosis has been proposed to follow the opening of the so-called “permeability transition pore complex” (PTPC), a supramolecular complex assembled at the junctions between the IMM and OMM [103, 374]. The composition, regulation, and precise mechanism of action of the PTPC are still under intense investigation and matter of a vivid debate [373, 375]. To date, peptidylprolyl isomerase F (PPIF; best known as cyclophilin D, CYPD) is the only protein whose in vivo requirement for MPT induction has been formally validated with robust genetic tools (although there is consensus around the notion that CYPD does not constitute the pore-forming unit of the PTPC) [376–379]. Accordingly, pharmacological inhibitors of CYPD including cyclosporin A (CsA) [376 379–381], sanglifehrin A (SfA) [382, 383], and JW47 [384] limit MPT-driven necrosis and confer protection in multiple rodent models of disease in which oxidative stress and cytosolic  $\text{Ca}^{2+}$  overload constitute major etiological determinants (e.g., neuronal, cardiac, and renal ischemia/reperfusion). Along similar lines, CYPD degradation through a mechanism initiated by the overexpression of HCLS1 associated protein X-1 (HAX1) abolishes MPT-driven necrosis and limits the demise of cardiomyocytes experiencing ischemia/reperfusion in vivo [385]. Nonetheless, a large randomized clinical study completed in 2015 (the CIRCUS trial) failed to confirm previous findings from 2008 [386] on the cardioprotective effects of cyclosporine administered before percutaneous coronary intervention to patients with acute myocardial infarction [387]. Although multiple caveats linked to the methods employed to measure infarct size and the use of a specific pharmacological CsA formulation can be invoked to explain the negative results of the CIRCUS trial [388], the elevated interconnectivity of RCD subroutines (notably, intrinsic apoptosis and MPT-driven necrosis) may have played a key role in this setting.

At odds with CYPD, several other proteins that had previously been hypothesized to mediate a non-redundant role within the PTPC turned out to be dispensable for MPT in vivo, based on relatively robust genetic models [373]. Thus, an inducible cardiomyocyte-specific deletion of solute carrier family 25 member 3 (*Slc25a3*, which codes for the inorganic phosphate carrier) in mice does not affect the ability of mitochondria to undergo MPT in vitro, as it establishes partial PTPC desensitization in cellula and slightly mitigates cardiac injury upon ischemia/reperfusion in vivo (~10% reduction in ischemic area over area at risk) [389]. Similar findings have been obtained for distinct isoforms of the IMM integral protein adenine nucleotide translocator (ANT) and the OMM protein VDAC. In particular, the concurrent knockout or knockdown of *Slc25a4* and *Slc25a5*, which encode ANT1 and ANT2, respectively [390], or that of *Vdac1*, *Vdac2*, and *Vdac3* [391, 392] fails to prevent the induction of MPT by oxidative stress or  $\text{Ca}^{2+}$  overload. However, mitochondria isolated from *Slc25a4*<sup>-/-</sup>*Slc25a5*<sup>-/-</sup> mouse livers are desensitized to  $\text{Ca}^{2+}$ -driven MPT to a similar extent than mitochondria exposed to CsA [390]. Moreover, *Slc25a31* encodes another ANT isoform (i.e., ANT4), that (at least in some mouse tissues) may compensate for the absence of ANT1 and ANT2 [393, 394]. These results reflect a consistent degree of genetic and functional redundancy among the components of the molecular machinery for MPT [373].

Several lines of evidence suggest that the mitochondrial  $\text{F}_1\text{F}_0$  ATPase mediates a non-redundant role within the PTPC. Initially, the c-ring of the  $\text{F}_1\text{F}_0$  ATPase [395–398] as well as  $\text{F}_1\text{F}_0$  ATPase dimers [399] have been proposed to constitute the long-sought PTPC pore-forming unit. A specific interaction between CYPD and the lateral stalk of the  $\text{F}_1\text{F}_0$  ATPase, as well as the ability of  $\text{Ca}^{2+}$  ions (which are potent MPT inducers) to bind to ATP synthase, H<sup>+</sup>-transporting, mitochondrial F1 complex, beta polypeptide (ATP5B) [100], lend further support to this interpretation [395, 400, 401]. However, very recent findings seem to exclude the possibility that the  $\text{F}_1\text{F}_0$  ATPase constitutes the pore-forming component of the PTPC [402–405]. First, it seems unlikely for c-rings (which exist as pores across the IMM) to lose their lipid plugs in relatively physiological conditions [402]. Second, mitochondria from human cells lacking all the genes coding for the c subunit of the  $\text{F}_1\text{F}_0$  ATP synthase, i.e., ATP synthase, H<sup>+</sup>-transporting, mitochondrial Fo complex subunit C1 (subunit 9; *ATP5G1*), subunit C2 (subunit 9; *ATP5G2*), and subunit C3 (subunit 9; *ATP5G3*), reportedly retain the ability to undergo MPT in response to  $\text{Ca}^{2+}$  overload [403]. Finally, cells lacking ATP synthase, H<sup>+</sup>-transporting, mitochondrial F1 complex, O subunit (ATP5O; best known as OSCP), or the membrane domain of the b subunit of the  $\text{F}_1\text{F}_0$  ATP synthase (encoded by *ATP5F1*) appear to preserve normal PTPC activity [405].

That said, the implication of the  $F_1F_0$  ATPase or components thereof in MPT-driven necrosis remains a matter of intensive investigation. An RNA interference (RNAi)-based screening identified SPG7, paraplegin matrix AAA peptidase subunit (SPG7) as an essential component of the PTPC acting as part of VDAC-containing and CYPD-containing hetero-oligomers [406]. Despite the availability of *Spg7*<sup>-/-</sup> mice, the actual involvement of SPG7 in MPT-derived necrosis in vivo remains to be validated.

Several physical or functional PTPC interactors have been shown to regulate MPT-driven necrosis. These include: (1) pro- and anti-apoptotic BCL2 family members such as BAX, BAK, and BID [407–410], as well as BCL2 and BCL-X<sub>L</sub> [411–414]; (2) DRP1, which appears to promote PTPC opening in response to chronic  $\beta$  adrenergic receptor stimulation, via a mechanism that relies on DRP1 phosphorylation by calcium/calmodulin dependent protein kinase II (CAMK2G; best known as CaMKII) [415]; and (3) p53, which participates in MPT-driven necrosis upon physical interaction with CYPD [416]. The latter interaction has been shown to participate in the pathogenesis of ischemic stroke in mice [416]. Its pathophysiological relevance in humans, however, remains to be elucidated. Recent findings lend additional support to the relevance of tight  $Ca^{2+}$  homeostasis at the mitochondrial level for cellular and organismal fitness. Thus, perturbing the activity of the IMM  $Ca^{2+}$  uniporter, consisting of mitochondrial calcium uniporter (MCU), single-pass membrane protein with aspartate-rich tail 1 (SMDT1; also known as EMRE), mitochondrial calcium uptake 1 (MICU1) and MICU2, reportedly affects mouse survival and liver regeneration after partial hepatectomy by promoting mitochondrial  $Ca^{2+}$  overload and MPT-driven necrosis [417]. Along similar lines, the loss of mitochondrial *m*-AAA proteases of the IMM, which regulate the assembly of the IMM  $Ca^{2+}$  uniporter, induces mitochondrial  $Ca^{2+}$  overload, PTPC opening, and neuronal cell death [418]. Adult mice subjected to the cardiomyocyte-specific deletion of *Mcu* are protected against cardiac ischemia/reperfusion as a consequence of MTP inhibition [419]. Moreover, the inducible cardiomyocyte-specific deletion of solute carrier family 8 member B1 (*Slc8b1*, which encodes a mitochondrial potassium-dependent sodium/calcium exchanger) in mice reportedly provokes sudden lethality owing to heart failure imposed by MTP-regulated necrosis upon mitochondrial  $Ca^{2+}$  overload [420]. Finally, rap guanine nucleotide exchange factor 3 (RAPGEF3; best known as EPAC1) appears to trigger PTPC opening by increasing mitochondrial  $Ca^{2+}$  levels through interaction with VDAC1, heat shock protein family A (Hsp70) member 9 (HSPA9; best known as GRP75), and inositol 1,4,5-trisphosphate receptor type 1 (ITPR1; best known as IP<sub>3</sub>R1), and the knockout of *Rapgef3* protects mice against myocardial ischemia/

reperfusion injury [421]. However, EPAC1 activation with bicarbonate reportedly decreases mitochondrial  $Ca^{2+}$  uptake, stimulates ATP production, and inhibits multiple forms of RCD including MPT-driven necrosis in rat cardiomyocytes [422]. The precise reasons underlying this apparent discrepancy remain to be elucidated.

We propose to define MPT-driven necrosis as a form of RCD triggered by perturbations of the intracellular microenvironment and relying on CYPD (Box 1).

## Necroptosis

Necroptosis is a form of RCD initiated by perturbations of the extracellular or intracellular microenvironment detected by specific death receptors, including (but not limited to) FAS and TNFR1 [423–427], or pathogen recognition receptors (PRRs), including TLR3, TLR4, and Z-DNA binding protein 1 (ZBP1; also known as DAI) [428–430]. It is now clear that necroptosis (which generally manifests with a necrotic morphotype) not only mediates adaptative functions upon failing responses to stress, but also participates in developmental safeguard programs (to ensure the elimination of potentially defective organisms before parturition), as well as in the maintenance of adult T-cell homeostasis (de facto serving as a PCD subroutine, at least in specific settings) [2 431–433].

At the molecular level, necroptosis critically depends on the sequential activation of RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL) [434, 435]. Upon necroptosis initiation by TNFR1, RIPK3 is activated by RIPK1 (provided that CASP8 is inactive, see below) through a mechanism involving the physical interaction between their respective RIP homotypic interaction motif (RHIM) domains and RIPK1 catalytic activity [436–438]. Accordingly, chemical inhibitors of RIPK1 including necrostatin-1 (Nec-1) and derivatives (e.g., Nec-1s) robustly inhibit TNFR1-driven necroptosis, in vitro and in vivo [425, 427]. Alternatively, RIPK3 can be activated following the RHIM-dependent interaction with (1) TRIF upon either TLR3 activation by double-stranded RNA (dsRNA) within endosomes, or TLR4 activation by lipopolysaccharide (LPS) or various DAMPs at the plasma membrane [428]; or (2) ZBP1, which operates as a sensor for cytosolic DNA-promoting type I interferon (IFN) synthesis and NF- $\kappa$ B activation [439–441]. Active RIPK3 catalyzes the phosphorylation of MLKL, resulting in the formation of MLKL oligomers (most likely trimers or tetramers) that translocate to the plasma membrane, where they bind specific phosphatidylinositol phosphate species by a roll-over mechanism and hence trigger plasma membrane permeabilization [435 442–453].

Although the essential contribution of MLKL to necroptosis has been confirmed by genetic studies [435] as well as by pharmacological (i.e., inhibition of MLKL with necrosulfonamide, NSA) interventions [442], the precise mechanism through which MLKL executes necroptosis is not completely understood. Recent studies ascribe to the heat shock protein 90 kDa alpha family class A member 1 (HSP90AA1; best known as HSP90) a specific and non-redundant role in MLKL oligomerization and translocation [454, 455]. Moreover, it has also been reported that MLKL oligomerization promotes a cascade of intracellular events involving (1)  $\text{Ca}^{2+}$  influx, which is presumably mediated by the MLKL target transient receptor potential cation channel subfamily M member 7 (TRPM7) [449]; and (2) PS exposure, which seems to be directly operated by MLKL [456]. This is followed by the formation of PS-exposing plasma membrane bubbles whose breakdown and release is negatively regulated—in conditions of limited MLKL activation—by the antagonistic activity of the endosomal sorting complex required for transport (ESCRT)-III machinery [456, 457]. Once localized at the plasma membrane, MLKL reportedly activates cell-surface proteases of the ADAM family, which can promote the shedding of plasma membrane-associated proteins [458], or form  $\text{Mg}^{2+}$  permeant channels [459]. Of note, active MLKL also appears to translocate to the nucleus, but the relevance of this phenomenon for necroptosis remains to be investigated [460]. Previous data supporting the involvement of PGAM family member 5, serine/threonine protein phosphatase, mitochondrial (PGAM5)- and DRP1-driven mitochondrial fragmentation in necroptosis [461] have been conclusively invalidated [435, 446 462–466], confirming that necroptotic signaling can proceed normally independent of mitochondria. Of note, the core components of necroptosis are poorly conserved across the animal kingdom, as some species lack *RIPK3* and/or *MLKL* [467]. Moreover, a few non-canonical instances of pseudonecroptotic RCD involving MLKL (but not *RIPK3*) [468] or *RIPK3* (but not *MLKL*) [469] have been described. These observations reinforce the notion that the signaling pathways leading to RCD display a hitherto incompletely understood degree of interconnectivity.

Death receptor (in particular TNFR1) engagement is the trigger for *RIPK3* activation best characterized so far. As mentioned above, the biological outcome of TNFR1 signaling spans from cell survival and activation (i.e., cytokine secretion) to multiple subroutines of RCD, depending on a variety of cell-intrinsic (e.g., expression levels of the proteins involved in the process) and cell-extrinsic (e.g., intensity and duration of TNF stimulation) factors [283]. In particular, the activation of *RIPK3* downstream of TNFR1 ligation relies on the formation of a *RIPK1*-containing and *RIPK3*-containing amyloid-like

signaling complex commonly known as necrosome [436, 470], wherein first *RIPK1* and then *RIPK3* undergo a series of trans-phosphorylation or auto-phosphorylation events that are required for MLKL recruitment and necroptosis activation [437, 438, 442, 471]. Major negative regulators of the necrosome include: (1) STIP1 homology and U-box containing protein 1 (STUB1; also known as CHIP), which promotes *RIPK1* and *RIPK3* ubiquitination followed by lysosomal degradation [472, 473]; (2) A20, which inhibits necrosome assembly by deubiquitinating *RIPK3* [473, 474]; (3) protein phosphatase,  $\text{Mg}^{2+}/\text{Mn}^{2+}$  dependent 1B (PPM1B), which prevents MLKL recruitment to the necrosome by dephosphorylating *RIPK3* [475]; and (4) aurora kinase A (AURKA), which mediates inhibitory function upon physical interaction with *RIPK1* and *RIPK3* [476]. *RIPK3* activation also depends on its physical association with a HSP90-containing and cell division cycle 37 (CDC37)-containing co-chaperone complex [477]. In addition, the assembly of the necrosome upon TNFR1 stimulation impinges on two conditions: (1) pharmacological or genetic *CASP8* inactivation [478, 479], and (2) *RIPK1* deubiquitination-dependent phosphorylation (at least in some settings), which can be favored by exogenously provided SMAC mimetics, ensuring the release of *RIPK1* from complex I (see above) [334, 335, 480, 481].

As for the first condition, compelling experimental findings demonstrate that the concerted activity of *CASP8*, *FADD*, and c-FLIP<sub>L</sub> tonically inhibits necroptosis [432, 466, 478, 479 482–484]. Thus, the embryonic lethality imposed on mice by the loss of *Casp8* or *Fadd* can be rescued by concurrent ablation of *Ripk3* or *Mkl1*, even though these double knockout animals generally display lymphoproliferative and/or systemic autoimmune disorders as adults [432, 466, 484, 485]. Of note, *Cflar*<sup>−/−</sup> mice require the concomitant knockout of *Ripk3* and *Fadd* to develop into adulthood, which underscores the inhibitory role of c-FLIP in both necroptosis and extrinsic apoptosis reported above [483, 486]. Along similar lines, the concurrent deletion of *Ripk3* averts perturbations of cutaneous and intestinal homeostasis imposed by the tissue-specific ablation of *Fadd* or *Casp8* [483, 487, 488]. Moreover, the proliferative defects of *Casp8*<sup>−/−</sup> or *Fadd*<sup>−/−</sup> T cells can be rescued by the administration of the *RIPK1* inhibitor Nec-1 or the concomitant ablation of *Ripk3* [489]. Necroptosis is also tonically inhibited by c-IAPs, owing to their ability to ubiquitinate *RIPK1* [490–493]. Accordingly, necroptosis relies on the deubiquitinating activity of CYLD [338], which is also a proteolytic target of *CASP8* [494–496]. Finally, some components of the TNFR1 signaling cascade reportedly regulate necroptosis either in a negative manner, by catalyzing the inhibitory phosphorylation of *RIPK1* (e.g., the IKK complex and MK2) [335, 336] and constitutively interacting with (and thus preventing the

activation of) MLKL (e.g., TRAF2) [497], or in a positive manner, by favoring the activating phosphorylation of RIPK1 or RIPK3 upon prolonged activation (e.g., TAK1) [334, 498]. In this context, CYLD also contributes to necroptosis by deubiquitinating—and hence suppressing the anti-necroptotic activity of—TRAF2 [497].

That said, mounting evidence indicates that necroptosis driven by several stimuli—in some circumstances even TNFR1 activation—does not necessarily rely on RIPK1. Thus, in contrast to *Ripk3*<sup>−/−</sup> mice that are viable and fertile, the *Ripk1*<sup>−/−</sup> genotype causes perinatal lethality [482], which cannot be prevented by the ablation of *Ripk3*, *Casp8*, or *Fadd* alone, but can be rescued by the co-deletion of *Ripk3* and *Casp8*, *Fadd* or *Tnfrsf1a* [482, 499–501]. Moreover, *Ripk1*<sup>−/−</sup> cells display increased sensitivity to necroptosis and/or extrinsic apoptosis induced by a set of innate immune stimuli [499]. Conditional knockout mouse models demonstrate the key role of RIPK1 for the preservation of intestinal and cutaneous homeostasis and survival [502, 503]. In particular, mice lacking *Ripk1* in intestinal epithelial cells display increased rates of spontaneous CASP8-driven apoptosis and develop severe inflammatory lesions leading to premature death, a detrimental phenotype that can be prevented by co-deleting *Fadd* or (to a lesser degree) *Tnfrsf1a* [502]. Likewise, the absence of *Ripk1* from keratinocytes promotes spontaneous necroptosis and consequent cutaneous inflammation, which can be prevented by the co-deletion of *Ripk3*, *Mkl1*, or *Zbp1* but not *Fadd* [440, 502]. Collectively, these results suggest that (at least in some settings) RIPK1 can inhibit (rather than activate) RIPK3-dependent necroptosis and/or CASP8-dependent extrinsic apoptosis [504]. At least in some settings, this reflects the major role of RIPK1 in NF-κB activation [505–507].

Intriguingly, the pro-survival role of RIPK1 in development seems to be independent of both its kinase activity and RIPK3 binding, as demonstrated by the fact that mice genetically engineered to express a kinase-dead variant of RIPK1 (e.g., RIPK1<sup>K45A</sup>) are viable and fertile [447, 499, 508]. Moreover, it has recently been reported that the autophagic receptor optineurin (OPTN) [509] actively regulates the proteasomal turnover of RIPK1, as the loss of OPTN induces axonal degeneration via RIPK1-dependent necroptosis [510]. Inhibitor of nuclear factor kappa B kinase subunit gamma (IKBKG; best known as NEMO) also prevents RIPK1-driven intestinal inflammation and epithelial cell death, although the underlying molecular mechanisms remain poorly understood [511]. Finally, when catalytically inactive or inhibited by specific pharmacological agents such as Nec-1, RIPK1 (and, at least under certain circumstances, RIPK3) reportedly contributes to specific forms of CASP8-dependent apoptosis (see above) [335, 336, 446, 447, 481, 512–516]. The current view ascribes the opposing roles of RIPK1 (and—at

least in part—RIPK3) in promoting or inhibiting RCD to its kinase-dependent vs. kinase-independent (i.e., scaffolding) functions, respectively [4, 517].

As mentioned above, RIPK3 can be activated by proteins involved in innate immunity to invading pathogens including TRIF and ZBP1 [428, 439]. Thus, in the absence of CASP8 activity, stimulation of TLR3 or TLR4 by their respective ligands promotes necroptosis upon the interaction between TRIF and RIPK3 and the consequent activation of MLKL [428]. Accordingly, the synthetic TLR3 ligand polyinosinic-polycytidylic acid (polyI:C) or the co-administration of low-dose LPS and the caspase inhibitor Z-VAD-fmk trigger necroptosis in dendritic cells (DCs) [518] or microglial cells [519], respectively. In this context, IFN alpha and beta receptor subunit 1 (IFNAR1) and IFN gamma receptor 1 (IFNGR1) also appear to have pro-necroptotic functions [520–523]. Thus, *Ifnar1*<sup>−/−</sup> macrophages are resistant to RCD induced by LPS or polyI:C in the context of caspase inhibition, which would otherwise trigger a necroptotic process relying on TRIF and tonic IFN-stimulated gene factor 3 (ISGF3) signaling [523]. Genetic studies demonstrate that the lethality imposed to mice by the *Ripk1*<sup>−/−</sup>*Tnfrsf1a*<sup>−/−</sup> genotype is delayed (but not prevented) by the co-deletion of *Ticam1* or *Ifnar1* [482]. Moreover, *Ripk1*<sup>−/−</sup> cells are more sensitive to necroptosis induced by polyI:C or type I IFN [482]. However, *Tnfrsf1a*<sup>−/−</sup>*Ripk1*<sup>−/−</sup>*Ripk3*<sup>−/−</sup> mice develop into adulthood, suggesting the existence of additional RIPK3 activators [482].

Recently, the mechanism underlying ZBP1-mediated necroptosis and its regulation by RIPK1 has been elucidated [440, 441]. ZBP1 acts at the initial steps of necroptosis by mediating the sequential activation of RIPK3 and MLKL. Moreover, mice expressing a variant of RIPK1 mutated in the RHIM domain die perinatally, a phenotype that can be rescued by concurrent *Ripk3*, *Mkl1*, or *Zbp1* (but not *Ticam1*) deletion, as well as by the knock-in of catalytically inactive RIPK3 or RIPK3 mutated in the RHIM domain [440, 441]. This suggests that the RHIM of RIPK1 acts as an inhibitor of ZBP1-driven necroptosis, most likely because it prevents the interaction between ZBP1 and RIPK3. Further investigation is required to clarify the mechanisms of ZBP1 activation in this context and its relevance for development and homeostatic tissue regulation. Importantly, multiple components of the molecular machinery for necroptosis—including ZBP1, RIPK3, MLKL, and TNFR1 (mainly via NF-κB)—impinge on the control of the so-called “inflammasome”, a supramolecular platform for the activation of CASP1 and consequent secretion of mature interleukin 1 beta (IL1β; best known as IL-1β) and IL18 [524–529]. Discussing in detail these links—which exemplify the complex interconnection between RCD signaling and inflammatory responses—goes beyond the scope of this review [4, 8, 530–532].

In summary, we propose to define necroptosis as a type of RCD triggered by perturbations of extracellular or intracellular homeostasis that critically depends on MLKL, RIPK3, and (at least in some settings) on the kinase activity of RIPK1 (Box 1).

## Ferroptosis

Ferroptosis is a form of RCD initiated by specific perturbations of the intracellular microenvironment, notably severe lipid peroxidation, which relies on ROS generation and iron availability [533–536]. The molecular mechanisms precipitating ferroptosis have begun to emerge [534], and (so far) ferroptotic RCD has been linked to toxic lipid peroxide accrual [537, 538]. Ferroptosis occurs independently of caspases, necrosome components and CYPD, and the molecular machinery for autophagy [539], manifests with a necrotic morphotype (with a predominance of mitochondrial alterations encompassing shrinkage, an electron-dense ultrastructure, reduced/disappeared cristae, and ruptured OMM) [374], and is potentially associated with a consistent release of immunostimulatory DAMPs [540, 541]. Interestingly, BCL2 has been suggested to limit the physiological demise of neuron progenitors failing to differentiate via a mechanism that (1) does not depend on BAX and caspases, and (2) can be suppressed by ferroptosis inhibitors [542]. The actual implication of BCL2 in the regulation of ferroptosis, however, remains to be firmly established.

Some of the molecular circuitries regulating the initial steps of ferroptosis have been recently unveiled by employing (1) specific ferroptosis-inducing agents, including erastin [543, 544], RSL3 [543, 544], and FIN56 [545]; and (2) specific ferroptosis-inhibiting agents, including ferrostatins [539, 546] and lipoxstatins [547]. In particular, the reduced glutathione (GSH)-dependent enzyme glutathione peroxidase 4 (GPX4)—which is directly targeted by RSL3—has emerged as the main endogenous inhibitor of ferroptosis by virtue of its ability to limit lipid peroxidation by catalyzing the GSH-dependent reduction of lipid hydroperoxides to lipid alcohols [547–550]. In line with this notion, erastin triggers ferroptosis by (indirectly) affecting the catalytic cycle of GPX4 via a mechanism that involves the inhibition of the cystine/glutamate antiporter system  $x_c^-$  and consequent decrease in intracellular cysteine (which derives from cystine reduction in the cytoplasm) and GSH (which is synthesized from cysteine) [539, 548, 549, 551]. Accordingly, depleting GSH with *L*-buthionine sulfoximine (BSO)—an inhibitor of the glutamate–cysteine ligase complex—can induce ferroptotic RCD (at least in some cases) [547]. Moreover, the toxicity of high extracellular glutamate may depend (at least in part) on the activation of

ferroptosis through cysteine imbalance [534, 538, 552]. Of relevance for cancer therapy, the pronounced addiction of triple-negative breast carcinoma to glutamine relates (at least in part) to its ability to drive cystine uptake via  $x_c^-$ , implying that  $x_c^-$  may constitute a therapeutic target in this setting [553, 554]. Moreover, the FDA-approved tyrosine kinase inhibitor sorafenib can trigger ferroptosis in distinct cellular models by depleting GSH upon system  $x_c^-$  inhibition [551 555–557], while altretamine (an FDA-approved alkylating agent) has been recently identified as a potential inhibitor of GPX4 by a regulatory network genome-wide system strategy [558]. Thus, the antineoplastic effects of sorafenib and altretamine may partially stem from the activation of ferroptosis. Notably, the demise of neurons caused by inhibition of  $x_c^-$  was initially referred to as oxytosis, oxidative glutamate toxicity, or excitotoxicity, and was linked to alterations in intracellular  $Ca^{2+}$  homeostasis [559–561]. It remains unclear to which extent oxytosis can be mechanistically discriminated from ferroptosis and MPT-driven necrosis in diverse cellular contexts.

Recent evidence indicates that ferroptosis involves the preferential oxidation of specific phosphatidylethanolamine-containing polyunsaturated fatty acids (PUFAs) such as arachidonic and adrenic acid [562]. In line with a critical requirement for oxidizable PUFAs, genetic and/or pharmacological inhibition of acyl-CoA synthetase long chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), both of which are involved in the incorporation of long PUFAs into cellular membranes, protects cells against ferroptosis (at least in some settings) [562–565]. Lipid hydroperoxides can be formed by auto-oxidation or via enzymatic reactions catalyzed by lipoxygenases (LOXs) or cyclooxygenases (COXs). In the context of ferroptosis, PUFA peroxidation seems to be mainly regulated by the mutually antagonistic activity of LOXs (which directly catalyze lipid peroxidation) and GPX4 (which indirectly inhibits it) [550, 566]. Although arachidonate 15-lipoxygenase (ALOX15) was initially thought to play a major role in this setting, the deletion of *Alox15* fails to rescue the renal phenotype imposed by the *Gpx4*<sup>−/−</sup> genotype (see below) [547], suggesting that multiple LOXs are involved in PUFA peroxidation and consequent ferroptosis in some mouse tissues. Accordingly, oxidized PUFAs accumulate upon GPX4 inactivation and this can result in PUFA fragmentation and ferroptosis [539, 547]. This lethal cascade can be prevented by antioxidant agents such as ferrostatin-1 (Fer-1), lipoxstatin-1 (Lip-1) as well as by vitamin E, coenzyme Q<sub>10</sub> and their analogs, all of which efficiently limit lipid peroxidation by operating as ROS scavengers [539, 547, 550, 562 567–569]. Of note, the catalytic sites of LOXs contain di-iron centers [570]. This may explain: (1) the ferroptosis-inhibiting effect of iron depletion by either chelators [539, 543, 548] or

phosphorylase kinase catalytic subunit gamma 2 (*PHKG2*) knockdown [566], and (2) the ferroptosis-promoting effect of increased intracellular iron availability consequent to import by the circulating iron carrier transferrin (TF) [571, 572], degradation of ferritin (a cellular iron storage complex) by a specific autophagic mechanism known as ferritinophagy [573, 574], disruption of iron homeostasis induced by nanoparticles [541], or administration of a bioavailable iron form [575]. Alternatively, the critical dependency of ferroptosis on iron can also be ascribed to the ability of this heavy metal to promote non-enzymatic lipid oxidation via lysosomal Fenton reactions [538, 572, 576, 577].

Additional ferroptosis regulators described so far include: (1) the mevalonate pathway component farnesyl-diphosphate farnesyltransferase 1 (FDDT1; best known as SQS) [545]; (2) the transsulfuration pathway enzyme cysteinyl-tRNA synthetase (CARS) [578]; (3) heat shock protein family B (small) member 1 (HSPB1; best known as HSP27) [579] and heat shock protein family A (Hsp70) member 5 (HSPA5) [580]; (4) glutaminolysis [571]; (5) components of the MAPK signaling pathway [539, 581]; (6) the nuclear factor, erythroid 2 like 2 (NFE2L2; best known NRF2) signaling pathway [582]; (7) metallothionein 1G (MT1G) [583]; (8) dipeptidyl peptidase 4 (DPP4) [584]; (9) Fanconi anemia complementation group D2 (FANCD2) [585]; and (10) CDGSH iron sulfur domain 1 (CISD1; also known as mitoNEET) [586]. Elucidating the precise role of these proteins or signaling pathways in ferroptosis requires further investigation.

Accumulating evidence demonstrates that the pro-survival functions of GPX4 contribute to development and homeostatic tissue maintenance. *Gpx4*<sup>-/-</sup> mice display embryonic lethality with complete penetrance [547, 550, 587, 588]. Moreover, the inducible or tissue-specific ablation of *Gpx4* in mice provokes a variety of pathological conditions, including acute renal or hepatic injury [547, 563, 589], neurodegeneration [550, 590, 591], and defective immunity to infection [567], all of which can be prevented or mitigated by ferroptosis-inhibiting strategies. A similar protective effect is observed in GPX4-independent models of renal ischemic or toxic injury [540, 592], Parkinson disease [593], and other human pathologies [594]. Moreover, ferroptosis appears to operate as a bona fide oncosuppressive mechanism [548, 595–598]. It has been proposed—but remains to be formally established—that part of the multipronged oncosuppressor functions of p53 may derive from the transcriptional down-regulation of components of system  $x_c^-$ , which would impinge on specific post-translational modifications of p53 [596, 598]. Accordingly, the ability of ATF4 to upregulate system  $x_c^-$  and stabilize GPX4 (upon *HSPA5* transactivation) is causally involved in some models of oncogenesis and chemoresistance to ferroptosis induction [580, 599]. Along

similar lines, parts of the oncogenic effects of NRF2 activation driven by cancer-associated mutations in kelch like ECH associated protein 1 (*KEAP1*) may derive from the upregulation of system  $x_c^-$  [582]. Conversely, p53 appears to inhibit ferroptosis in colorectal cancer cells, at least in part by inhibiting DPP4 activity in a transcription-independent manner [584]. Of note, malignant cells with a mesenchymal phenotype (which are generally more resistant to treatment) reportedly acquire an accrued dependency on GPX4 activity, which can be exploited therapeutically [600]. Recently, a ferroptosis-like RCD subroutine has been described in plants responding to moderate heat stress, supporting some degree of evolutionary conservation and the relevance of ferroptosis for organismal homeostasis [601]. In this context, it is worth noting that the pharmacological inhibition of ferroptosis, but not necroptosis or apoptosis, protects tissues such as renal tubules from ischemia/reperfusion injury [540]. The precise role of ferroptosis in development and tissue homeostasis, however, remains to be fully elucidated.

We propose to define ferroptosis as a form of RCD initiated by oxidative perturbations of the intracellular microenvironment that is under constitutive control by GPX4 and can be inhibited by iron chelators and lipophilic antioxidants (Box 1).

## Pyroptosis

Pyroptosis is a form of RCD triggered by perturbations of extracellular or intracellular homeostasis related to innate immunity (e.g., pathogen invasion) manifesting with specific morphological feature [602]. These include a peculiar form of chromatin condensation that differs from its apoptotic counterpart, as well as cellular swelling culminating with plasma membrane permeabilization [602]. The term pyroptosis was originally coined by Cookson and Brennan to define a particular type of RCD partially resembling apoptosis but dependent on inflammatory CASP1 (and hence linked to pyrexia) [603], and several names including pyronecrosis have been introduced since to define partially related processes [604, 605]. Initially, pyroptosis was thought to be relevant only for the demise of monocytes or macrophages undergoing canonical CASP1 activation [606, 607]. However, recent findings indicate that pyroptosis (1) can be also driven by several other caspases including CASP3 [608], (2) can also occur in cell types other than cells from the monocytic lineage [609], (3) has a major role in innate immunity against intracellular pathogens [602], and (4) is etiologically involved in pathological conditions such as lethal septic shock (at least as induced by LPS) [610, 611].

At a molecular level, pyroptosis generally relies on the activation of one or more caspases, including CASP1,

CASP3, murine CASP11, and its human homologs CASP4 and CASP5, depending on the initiating stimulus [612, 613]. Thus, pyroptosis is often (if not always) associated with IL-1 $\beta$  and IL18 secretion, and hence mediates robust pro-inflammatory effects [614, 615]. A large body of evidence indicates that cytosolic LPS from invading Gram-negative bacteria is a major trigger of pyroptosis. In particular, it has been shown that CASP11 is responsible for the CASP1-independent death of macrophages responding to Gram-negative bacterial infection [616–619]. Moreover, *Casp11* deletion protects mice against a challenge with cytosol-invasive bacteria [612, 620], as well as against systemic LPS administration and consequent pyroptosis-dependent endotoxic shock [616, 621]. Further experimental observations confirmed that CASP11, CASP4, and CASP5 trigger pyroptosis upon sensing cytosolic LPS [609, 622–624], in monocytes as well as in other cell types [609, 625]. In particular, LPS-induced pyroptosis involves the physical interaction of LPS (or its lipid moiety) with the CARD domain of CASP11, CASP4, or CASP5, a highly specific binding resulting in caspase oligomerization and consequent activation [609]. Thus, CASP11, CASP4, and CASP5 act as bona fide PRRs for cytosolic LPS. Once activated beyond a specific threshold, inflammatory caspases precipitate pyroptosis by catalyzing the proteolytic cleavage of GSDMD [621, 626, 627]. However, at least in some cell types including DCs, CASP11 activation can drive IL-1 $\beta$  secretion in the absence of cell death [628].

In line with a critical role of GSDMD, *Gsdmd*<sup>−/−</sup> macrophages are resistant to LPS-induced and Gram-negative bacteria-induced pyroptosis, and *Gsdmd*<sup>−/−</sup> mice survive doses of LPS that induce lethal endotoxic shock in their wild-type counterparts [621, 626]. Recent findings demonstrate that GSDMD is constitutionally auto-inhibited by the binding of its C-terminal repressor domain (GSDMD-C, or RD) to its N-terminal pore-forming domain (GSDMD-N, or PFD). On pyroptosis induction, inflammatory caspases relieve this inhibition by catalyzing the proteolytic cleavage of the interdomain loop, which promotes the release of the pyroptotic inducer GSDMD-N [621, 629]. Cleaved GSDMD-N acquires the ability to translocate to the inner leaflet of the plasma membrane (or the bacterial plasma membrane), where it binds with high specificity to selected phosphoinositides (or cardiolipin) [629, 630]. Membrane targeting enables GSDMD-N oligomerization, generating a pore that is responsible for rapid plasma membrane permeabilization [629–633]. Of note, the GSDMD pore has recently been characterized at the ultrastructural level, consisting of 16 symmetric protomers with an inner diameter of ~10–14 nm [629].

Active CASP1 can also cleave GSDMD, suggesting that microbe-associated molecular patterns (MAMPs) other than cytosolic LPS as well as DAMPs usually stimulating

canonical inflammasome signaling can initiate pyroptosis [626, 634–636]. CASP1-driven pyroptosis limits the spreading of intracellular bacteria by (1) killing the host cell [637], and (2) generating so-called “pore-induced intracellular traps (PITs)”, which essentially are dead macrophages that can be efficiently disposed of (together with the living bacteria they trap) by efferocytosis [638]. Supporting the critical role of CASP1-driven pyroptosis for innate immune responses against invading bacteria, *Nlrp4*<sup>−/−</sup> mice (which are unable to normally activate CASP1) succumb to low amounts of otherwise innocuous environmental opportunists (e.g., *Chromobacterium violaceum*) [639]. Instances of CASP1-dependent but GSDMD-independent RCD have also been reported, including the demise of macrophages experiencing prolonged canonical inflammasome activation [621, 636]. Of note, additional substrates of inflammatory caspases have been hypothesized to participate in pyroptosis. Experiments with knockout mice suggest that the endotoxic shock caused by systemic LPS administration involves the CASP11-dependent cleavage not only of GSDMD, but also of pannexin 1 (PANX1) channels, leading to ATP release in the extracellular space and consequent activation of purinergic receptor P2X<sub>7</sub> (P2RX7), which further impinges on the collapse of ionic gradients and inflammasome signaling [640]. Most likely, however, these findings reflect the presence of an inactivating passenger mutation in *Casp11* specifically affecting transgenic mice generated from 129/Sv-derived embryonic stem cells [641]. Moreover, CASP1 has been proposed to drive pyroptosis by causing mitochondrial damage upon cleavage of parkin RBR E3 ubiquitin protein ligase (PRKN; best known as PARKIN) [642]. However, contrasting observations have been reported [528], and the actual pathophysiological relevance of the latter mechanism remains to be established.

Accumulating evidence indicates that the (shared) N-terminal domains of other members of the gasdermin family, including GSDMA, GSDMB, GSDMC, GSDME/DFNA5, and GSDMA3 (which is encoded by the mouse—but not the human—genome), resemble those of GSDMD as they display pore-forming and pyroptotic activity [39, 613, 626, 629]. Although the mechanisms underlying the activation of GSDMA remain unknown, two recent studies demonstrate the existence of an instance of pyroptotic RCD dependent on GSDME-N/DFNA5-N that can be elicited by multiple challenges, including TNF, various DNA-damaging chemotherapeutic agents, and/or infection with the vesicular stomatitis virus [39, 613]. In this setting, CASP3 is responsible for the proteolytic cleavage of GSDME/DFNA5, which precipitates in pyroptosis rather than apoptosis [39, 613]. As GSDME/DFNA5 is often silenced in malignant cells but expressed by their normal counterparts, the activation of GSDME/DFNA5 by CASP3 contributes to the side effects of multiple chemotherapeutic

agents, at least in mice [613]. Interestingly, GSDME/DFNA5 has also been involved in the acquisition of a necrotic phenotype by cells undergoing CASP3-driven apoptosis in vitro (in the absence of proficient phagocytosis) [39], further demonstrating the elevated degree of interconnectivity that exists between distinct types of RCD. The identification of multiple gasdermin family members as key factors in the late steps of pyroptosis, as well as the characterization of CASP3 as an activator of GSDME/DFNA5, expanded the relevance of this RCD form (and its definition, see below) well beyond inflammatory settings [613, 626, 629]. Of note, type I IFN and IFN gamma (IFNG) also contribute to pyroptosis by promoting: (1) the transactivation of *CASP11*, through an IFNAR1-dependent or IFNGR1-dependent mechanism initiated by TLR4 or IL18 signaling [620, 643, 644]; (2) TLR7, cyclic GMP-AMP synthase (CGAS), transmembrane protein 173 (TMEM173; best known as STING), DExD/H-box helicase 58 (DDX58; best known as RIG-I), or mitochondrial antiviral signaling protein (MAVS) signaling upon bacterial or viral infection [525, 645]; or (3) the expression of guanylate-binding proteins and an IFN-inducible GTPase commonly known as IRGB10 (official name Gm12250), which increase cytosolic LPS levels by mediating the lysis of vacuoles containing Gram-negative bacteria [646–648].

Further underscoring the complexity of the interaction between inflammation and pyroptosis [649], CASP11 can also be upregulated by a complement cascade dependent on carboxypeptidase B1 (CPB1) acting downstream of TLR4 and IFNAR1 activation [650]. Moreover, cytosolic LPS promotes the secretion of inflammatory cytokines by a mechanism involving CASP11 activation, followed by GSDMD cleavage, loss of K<sup>+</sup> ions, and consequent activation of CASP1 by the NLR family pyrin domain containing 3 (NLRP3) inflammasome, in vitro (but perhaps not in vivo) [616, 620, 621, 626 651–655].

We propose to define pyroptosis as a form of RCD that critically depends on the formation of plasma membrane pores by members of the gasdermin protein family, often (but not always) as a consequence of inflammatory caspase activation. The NCCD discourages the use of alternative terms including pyronecrosis (Box 1).

## Parthanatos

Parthanatos is a form of RCD driven by the hyperactivation of a specific component of the DNA damage response (DDR) machinery, namely, poly(ADP-ribose) polymerase 1 (PARP1). Notably, parthanatos appears to occur not only as a consequence of severe/prolonged alkylating DNA damage, but also in response to oxidative stress, hypoxia, hypoglycemia, or inflammatory cues [656–658]. In this

context, reactive nitrogen species (RNS) including NO stand out as major triggers for PARP1 hyperactivation, especially in neurons [659–661]. PARP1 hyperactivation mediates cytotoxic effects as it causes (1) NAD<sup>+</sup> and ATP depletion, which ultimately results in a bioenergetic and redox collapse, and (2) the accumulation of poly(ADP-ribose) polymers and poly(ADP-ribosyl)ated proteins at mitochondria, ultimately causing  $\Delta\psi_m$  dissipation and MOMP [656, 657 662–665].

One of the key processes of parthanatos is the binding of poly(ADP-ribose) polymers to apoptosis inducing factor mitochondria associated 1 (AIFM1; best known as AIF). This promotes the release of AIF into the cytosol and its translocation into the nucleus, where it mediates large-scale DNA fragmentation and chromatin condensation [656, 658 664–667]. Further corroborating a key role for poly(ADP-ribosyl)ation in parthanatos, the poly(ADP-ribose)-degrading protein ADP-ribosylhydrolase like 2 (ADPRHL2; also known as ARH3) and the poly(ADP-ribose)-binding protein ring finger protein 146 (RNF146; best known as IDUNA) prevent AIF release and consequent RCD as they decrease poly(ADP-ribose) levels and availability, respectively [662, 668]. Moreover, specific pharmacological inhibitors of PARP1 efficiently delay parthanatos in multiple cell types, in vitro and in vivo [1, 669]. Parthanatotic DNA fragmentation occurs independently of apoptotic caspases and endonuclease G (ENDOG) [670], a mitochondrial nuclease that precipitates RCD by a mechanism involving its release followed by translocation to the nucleus (at least in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*) [671–674]. Indeed, the actual contribution of ENDOG to RCD in mammals [675] has been questioned by the generation of *Endog*<sup>−/−</sup> mice, whose cells display normal sensitivity to multiple lethal triggers [676, 677]. That said, it seems that the catalytic activity of CPS-6 (the homolog of ENDOG in *C. elegans*) is boosted upon interaction with WAH-1 (the worm homolog of AIF) [678].

Macrophage migration inhibitory factor (MIF) has emerged as the main nuclease precipitating parthanatos in a recent screening for AIF-binding proteins [679]. Thus, cytosolic AIF reportedly promotes the translocation of MIF into the nucleus, where MIF precipitates parthanatos by catalyzing DNA cleavage. Accordingly, MIF depletion or specific mutations in its nuclease domain confer protection against parthanatos in vitro and in vivo (in an experimental model of focal cerebral ischemia) [679]. Another protein involved in parthanatos is hexokinase 1 (HK1), whose binding to poly(ADP-ribose) polymers inhibits glycolysis to cause a bioenergetic collapse that precipitates RCD [680, 681]. Recently, a non-canonical, AIF-independent instance of parthanatos, presumably centered on the impairment of energetic metabolism, has been

proposed to contribute to the demise of retinal epithelial cells and consequent retinal degeneration [682]. Interestingly, some authors suggest a certain degree of interconnectivity between the parthanatotic and the necroptotic machineries. Thus, upon induction of necroptosis by TRAIL or  $\beta$  lapachone (an ortho naphthoquinone with antineoplastic activity) [683], activated RIPK1 and RIPK3 appear to stimulate the enzymatic activity of PARP1 and hence promote ATP depletion and/or AIF release [684, 685]. This interpretation may not hold true in all experimental settings [686].

Parthanatos reportedly contributes to various pathological conditions, including some cardiovascular and renal disorders, diabetes, cerebral ischemia, and neurodegeneration [534, 656 687–690]. Accordingly, PARP1 inhibition by pharmacological or genetic interventions mediates robust cytoprotective effects in multiple animal models of disease [1, 669]. However, further experiments are required to clarify the actual role of parthanatos in the etiology of these (and possible other) pathologies and the true therapeutic benefits of parthanatos-inhibiting agents.

The NCCD proposes to define parthanatos as a form of RCD initiated by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophe coupled to AIF-dependent and MIF-dependent DNA degradation (Box 1).

## Entotic cell death

Entosis is a form of cell cannibalism that occurs in healthy and malignant mammalian tissues, involving the engulfment of viable cells by non-phagocytic cells of the same (homotypic) or a different (heterotypic) type [691, 692]. Often (but not always), internalization is followed by the demise of internalized cells (which are commonly referred to as “entotic cells”) [691–693].

Entosis is mainly triggered by the detachment of epithelial cells from the extracellular matrix and consequent loss of integrin signaling [263, 694], although alternative mechanisms have been reported. These include: (1) the deregulated expression of myosins during the formation of cell-to-cell contacts [695]; and (2) differences in the mechanical properties [696] or responses to metabolic stress [697] of cancer cells competing for proliferation. Moreover, a recent study suggests the existence of a specific form of entosis occurring in cancer cells during mitosis (entotic mitosis), which is driven by aberrant mitotic rounding (and thus reduced adhesion) in conditions of cell division cycle 42 (CDC42) depletion or upon exposure to antimetabolic agents [698].

The current model proposes that the internalization of entotic cells occurs through cell invasion rather than by phagocytosis [691]. Accordingly, the uptake of entotic cells

is an integrin-independent process promoted by the formation of junctions between engulfing and entotic cells that involve the adhesion proteins cadherin 1 (CDH1; also known as E-cadherin) and catenin alpha 1 (CTNNA1) [694, 699]. Actomyosin chains accumulate at the cortex of internalizing cells (at the pole opposite to the cell-to-cell contact site), via a mechanism that depends on the localized activity of ras homolog family member A (RHOA), Rho associated coiled-coil containing protein kinase 1 (ROCK1), ROCK2, and diaphanous related formin 1 (DIAPH1), and results in a contraction that promotes engulfment [695 699–701]. Actin drives entosis by promoting pro-invasive (non-apoptotic) cortical plasma membrane blebbing upon activation of a signaling pathway that involves myocardin-related transcription factor (MRTF) and serum response factor (SRF) culminating with ezrin (EZR) upregulation [702]. The regulation of microtubule dynamics by AURKA has also been attributed a role in cell invasion [703], but the relevance of AURKA signaling for entosis awaits experimental confirmation. In line with the actomyosin-dependent cell-in-cell invasion model, the administration of exogenous CDH1 promotes entosis among CDH1-deficient breast cancer cells, whereas forced overexpression of RHOA or ROCK1 plus ROCK2 enables the internalization of entotic cells by epithelial CDH1-expressing cells [701]. In addition, hyperactivation of contractile myosin induces entotic cell-in-cell invasion via a mechanism involving the activation of RHOA, ROCK1, and ROCK2 [695]. Intriguingly, competition in the tumor system can occur via an entotic process whose outcome is dictated by the activation of KRAS proto-oncogene, GTPase (KRAS), and Rac family small GTPase 1 (RAC1) signaling, which confers an advantage to engulfing cells by favoring myosin downregulation [696]. In this context, it has recently been demonstrated that, in conditions of glucose withdrawal, cells displaying high 5' AMP-activated protein kinase (AMPK) activity succumb to entosis, underscoring a possible function of this process for nutrient recovery by cells with comparatively lower AMPK activity (which a priori are metabolically fitter) [697].

Once engulfed, entotic cells are often eliminated by a RCD subroutine that occurs independently of BCL2 proteins and caspases [694, 704], but relies (at least in part) on a specific autophagy-related process commonly known as LC3-associated phagocytosis (LAP) [509, 705, 706]. In this context, some (but not all) components of the macroautophagy apparatus, including microtubule associated protein 1 light chain 3 beta (MAP1LC3B; best known as LC3), autophagy related 5 (ATG5), ATG7, and phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3; best known as VPS34) are recruited to the cytosolic side of entotic cell-containing vesicles and promote their fusion with lysosomes (in the absence of bona fide autophagosome

formation) [704]. Eventually, the lysosomal degradation of internalized entotic cells generates nutrients that are recovered by engulfing cells, via a mechanism that reportedly involves phosphoinositide kinase, FYVE-type zinc finger containing (PIKFYVE) [704, 707, 708].

Entotic cell death has been documented in several human neoplasms, presumably operating as an oncosuppressor mechanism [694, 695, 701, 709, 710]. Thus, abrogation of entosis by a chemical ROCK inhibitor reportedly favors the anchorage-independent growth of malignant cells [694]. However, entotic invasion has also been suggested to favor aneuploidization and polyploidization (which promote tumor progression) [711–713] through a mechanism involving cytokinesis failure of engulfing cells [714, 715]. A potential role for entosis in development and tissue homeostasis has recently been proposed. Thus, in the course of mammalian embryo implantation, trophoblast cells reportedly eliminate uterine luminal epithelial cells upon entosis [716]. Moreover, the spermatozoa of hibernating Chinese soft-shelled turtle appear to be degraded within Sertoli cell by entotic cell death [717]. Further experiments are required to elucidate the actual role of entosis in the pathophysiology of mammalian organisms. Importantly, entosis does not always lead to the death of invading cells within the lysosome. Thus, at least in some circumstances, entotic cells remain viable and even proliferate inside host cells or upon escape [716].

On the basis of this consideration, we propose to define entotic cell death as a form of RCD that originates from actomyosin-dependent cell-in-cell internalization and is executed by lysosomes (Box 1). In the absence of precise experimental determination of terminal cell fate, we recommend to use the term entosis to refer to the internalization process only.

## NETotic cell death

The term “NETotic cell death” refers to a rather controversial type of RCD initially characterized in neutrophils for being associated with the extrusion of a meshwork of chromatin-containing and histone-containing fibers bound to granular and cytoplasmic proteins known as neutrophil extracellular traps (NETs), a process commonly referred to as NETosis [718, 719]. NETs, which are produced in response to various microbial and sterile activators or upon stimulation of specific receptors including (but not limited to) TLRs, de facto constitute a stable extracellular platform for trapping and degrading microbes [718 720–723]. Several reports demonstrate that a considerable fraction of the nucleic acids contained in NETs is of mitochondrial, rather than nuclear, origin [724–728]. Besides having antimicrobial effects, NETs reportedly contribute to the

etiology of some human pathologies, including diabetes and cancer [729–731]. Of note, NET-like structures can be released by cells other than neutrophils, including mast cells [732], eosinophils [733], and basophils [734]. Importantly, NET extrusion per se does not necessarily result in cellular lysis [722, 724, 735].

Although the precise molecular mechanisms underlying NET generation are not fully elucidated, both NETotic cell death and NET extrusion in the absence of RCD appears to rely on the activity of NADPH oxidases [724, 736, 737]. NETotic cell death has been suggested to result from a signaling pathway that involves Raf-1 proto-oncogene, serine/threonine kinase (RAF1), mitogen-activated protein kinase kinases (MAP2Ks), and ERK2, culminating with NADPH oxidase activation and consequent ROS generation [736, 738, 739]. According to this model, intracellular ROS would drive NETotic cell death (1) by triggering the release of elastase, neutrophil expressed (ELANE), and myeloperoxidase (MPO) from neutrophil granules to the cytosol, followed by their translocation to the nucleus, and (2) by promoting the MPO-dependent proteolytic activity of ELANE [740]. Once activated, the cytosolic pool of ELANE would catalyze the proteolysis of F-actin, followed by an impairment of cytoskeleton dynamics [741]. Alongside, the nuclear pool of ELANE would promote the degradation of histones (and possibly of the nuclear envelope) and, in conjunction with MPO, chromatin decondensation [737 740–742]. This would culminate with the extrusion of chromatin fibers intermixed with cytoplasmic and nuclear components, ultimately leading to plasma membrane rupture and RCD [736]. That said, recent findings indicate that ROS drive NET extrusion by a mechanism that requires an intact cytoskeleton [743]. Moreover, ELANE is dispensable for NET formation in the course of deep vein thrombosis (in mice) [744]. Peptidyl arginine deiminase 4 (PADI4; also known as PAD4) has also been proposed to participate in chromatin dispersion [745], but its actual involvement remains a matter of debate and appears to depend on the initiating stimulus [746, 747]. Finally, NETotic cell death has been proposed to depend (at least in part) on components of the necroptotic apparatus, based on the fact that the administration of chemical RIPK1 or MLKL inhibitors (i.e., Nec-1 or NSA, respectively) as well as the *Ripk3*<sup>−/−</sup> genotype appeared to inhibit NET extrusion and neutrophil lysis [748]. However, *Ripk3*<sup>−/−</sup> neutrophils as well as neutrophils exposed to NSA were fully proficient in NET formation in another study [749]. These apparently contradicting findings call for additional studies to address the precise contribution of necroptosis to NET extrusion and NETotic cell death.

We propose to define NETotic cell death as a ROS-dependent modality of RCD restricted to cells of hematopoietic derivation and associated with NET extrusion

(Box 1). That said, it is clear that NET can be formed and extruded by fully viable neutrophils, eosinophils, and basophils. Thus, the NCCD recommends to avoid the use of the term NETosis when no experimental evidence in support of cell death (vs. NET extrusion only) is available. Moreover, we discourage the use of alternative terms proposed to describe this process, including ETosis.

## Lysosome-dependent cell death

Lysosome-dependent cell death is a subroutine of RCD initiated by perturbations of intracellular homeostasis and demarcated by the permeabilization of lysosomal membranes. Lysosome-dependent cell death is relevant for several pathophysiological conditions, including inflammation, tissue remodeling (e.g., mammary gland involution after lactation), aging, neurodegeneration, cardiovascular disorders, and intracellular pathogen response [750–752]. Moreover, a type of RCD that is highly reminiscent of lysosome-dependent cell death, which has been dubbed “germ cell death”, appears to play a critical role in the physiological elimination of a fraction of emerging male germ cells (at least in *D. melanogaster*) [753–756].

At a biochemical level, lysosome-dependent cell death proceeds upon lysosomal membrane permeabilization (LMP), resulting in the release of lysosomal contents, including proteolytic enzymes of the cathepsin family, to the cytoplasm [750]. The molecular mechanisms operating upstream of LMP are not fully elucidated. In some circumstances, LMP appears to occur downstream of MOMP as a result of apoptotic signaling, de facto constituting an epiphenomenon of intrinsic apoptosis [757–759]. In other experimental settings, however, lysosomes are permeabilized before mitochondria [752, 760, 761], via a mechanism that optionally involves BAX recruitment to the lysosomal membrane followed by the activation of its pore-forming activity [762–765]. More commonly, ROS play a prominent causal role in LMP, not only as the H<sub>2</sub>O<sub>2</sub>-driven luminal production of hydroxyl radicals by Fenton reactions destabilizes the lysosomal membrane upon lipid peroxidation [766, 767], but also as ROS reportedly favor the activation of lysosomal Ca<sup>2+</sup> channels [768]. Primary LMP has been documented in vitro in cells responding to specific pro-apoptotic stimuli, including the administration of lysosomotropic agents such as *L*-leucyl-*L*-leucine methyl ester, ciprofloxacin, and hydroxychloroquine, TRAIL signaling, and viral infection [760, 761, 765, 769–772], as well in an animal model of Parkinson's disease [764]. The p53 effector DNA damage regulated autophagy modulator 1 (DRAM1) [773] provides a major contribution to lysosome-dependent cell death in HIV1-infected T cells by linking LMP to MOMP [770]. Additional LMP triggers include

lysosomotropic agents (e.g., sphingosine), calpains, and ROS [751]. Moreover, STAT3 reportedly promotes lysosome-dependent cell death during the involution of mammary gland post-lactation as it upregulates the expression of cathepsin B (CTSB) and CTSL, while downregulating their endogenous inhibitor serine (or cysteine) peptidase inhibitor, clade A, member 3G (SERPINA3G; best known as SPI2A) [774, 775].

Cytosolic cathepsins usually precipitate RCD by catalyzing the proteolytic activation or inactivation of several substrates, including BID, BAX, anti-apoptotic BCL2 family members, and XIAP [776–778], hence engaging a feed-forward amplification circuitry of the lethal cascade involving MOMP and caspases. Moreover, primary lysosomal dysfunction may negatively affect the mitochondrial network as a consequence of impaired mitophagic responses (which normally target damaged or dysfunctional mitochondria to lysosomes for degradation) [779, 780]. In aged neutrophils, LMP also allows for the release of proteinase 3 (PRTN3) from cytotoxic granules, where it promotes RCD by catalyzing the proteolytic activation of CASP3 [781]. Of note, lysosome-dependent cell death does not necessarily involve MOMP and caspases, and does not necessarily manifest with an apoptotic morphotype [782]. Moreover, CTSL appears to play a key role in the regulation of autophagic adaptation vs. RCD in cells responding to the LMP inducer resveratrol [783, 784]. These observations suggest that LMP and lysosome-dependent cell death are intimately interconnected with adaptive responses to stress and other RCD subroutines.

Lysosome-dependent cell death can be retarded in vitro and in vivo by inhibiting LMP or blocking cathepsin activity via pharmacological or genetic means [750, 752]. Commonly employed cathepsin-targeting molecules include endogenous protease inhibitors (cystatins and serpins), as well as various pharmacological agents specific for cysteine cathepsins (e.g., E64D and Ca-074-Me) or aspartyl cathepsins (e.g., pepstatin A) [785–787]. Moreover, under physiological conditions, lysosomal membranes can be stabilized by altering lysosomal cholesterol content [788] or by boosting endogenous activity of heat shock protein family A (Hsp70) member 1A (HSPA1A; best known as HSP70) [789, 790]. In line with this notion, the administration of recombinant HSP70 or the HSP70-inducing agent arimiclomol reverts lysosomal abnormalities in cellula as well as in murine models of various lysosomal storage disorders [789, 791]. Of relevance for cancer therapy, cancer cells may present an increased sensitivity to lysosomotropic agents and are generally vulnerable to LMP, which supports the clinical development of LCD-inducing agents [752, 792–795].

We propose to define lysosome-dependent cell death as a form of RCD demarcated by primary LMP and precipitated

by cathepsins, with optional involvement of MOMP and executioner caspases (Box 1).

## Autophagy-dependent cell death

Autophagy-dependent cell death is a type of RCD that relies on the autophagic machinery or components thereof [509 796–798]. Proficient autophagic responses (which are under tight transcriptional and post-translational regulation) [509 799–805] most often operate at the hub of adaptation to stress, hence mediating cytoprotective (rather than cytotoxic) effects [806–811]. Thus, blocking autophagy with pharmacological or genetic interventions generally accelerates (rather than delays) the demise of cells responding to stress, and permanent or transient endogenous defects in autophagy have been associated with embryonic lethality, developmental defects, and multiple pathological disorders, including (but not limited to) neurodegeneration, cancer, and cardiovascular disorders [812–817]. However, in a number of developmental and pathophysiological settings, the molecular machinery for autophagy etiologically contributes to cellular demise [796, 798 818–820]. Thus, the term autophagy-dependent cell death does not refer to settings in which the autophagic apparatus (or components thereof) is activated alongside (rather than precipitates) RCD [821] or it favors the engagement of other RCD modalities, such as (1) ferroptosis, which is promoted by the autophagic degradation of ferritin (ferritinophagy) [573, 574]; (2) FAS-driven extrinsic apoptosis, which is enhanced by the autophagic degradation of protein tyrosine phosphatase, non-receptor type 13 (PTPN13; best known as FAP1) [822], and (3) necroptosis, which is favored by a necrosome-scaffolding function of the autophagy apparatus [823–825], as well as by the autophagic degradation of c-IAP1 and c-IAP2 [826].

The genetics and pathophysiological significance of autophagy-dependent cell death is now well established [818–820]. Thus, whereas the genetic inactivation of caspases in the midgut of developing *D. melanogaster* has no consequences, mutations or deletions in essential autophagy-related (*Atg*) genes suppress midgut tissue degradation [827–829]. Along similar lines, the complete removal of larval salivary glands from *D. melanogaster* larvae undergoing metamorphosis requires the apoptotic as well as the autophagic machinery [830–832]. In both these developmental scenarios, autophagy-dependent cell death is preceded by growth arrest and is controlled by ecdysone, a steroid hormone that is critically required in *Drosophila* to undergo the larva-to-pupa transition and subsequent metamorphosis into an adult fly [830, 833]. The autophagic machinery also precipitates germ cell and ventral cord neuron RCD during *C. elegans* development [834], and

perhaps contributes to embryonic development in mammals, as suggested by the fact that *Atg5* ablation in apoptosis-deficient (i.e., *Bax*<sup>−/−</sup>*Bak*<sup>−/−</sup>) mice further delays interdigital web clearance, aggravates cerebral abnormalities (at least in the C57BL/6 background), impairs negative selection of autoreactive thymocytes, and increases the resistance of some cell types to multiple stressors [835, 836].

Of note, the molecular machinery of autophagy-dependent cell death and adaptive autophagy exhibit some differences (at least in *D. melanogaster*) [837, 838]. For example, the autophagy-dependent degradation of the midgut tissue proceeds independently of *Atg7*, *Atg3*, and several other *Atg* genes that are required for starvation-induced autophagy in the fat body, but relies on ubiquitin activating enzyme 1 (Uba1) [837, 838]. Moreover, the developmental degradation of *Drosophila* salivary glands by autophagy requires the activity of: (1) Utx histone demethylase (Utx), which contributes to the transcriptional regulation of apoptosis and autophagy genes [839]; (2) *miR-14*, which specifically activates autophagy-dependent cell death by modulating multiple IP<sub>3</sub>-driven signaling pathways upon targeting inositol 1,4,5-triphosphate kinase (IP3K2) [840]; (3) Ras-like protein A (Rala), which ignites autophagy-dependent cell death upon Notch activation [841]; (4) Draper (Drpr), which is thought to promote the engulfment of dying salivary gland cells [842]; and (5) macroglobulin complement-related (Mcr), which promotes autophagy-dependent cell death at least in part by triggering Drpr signaling [843]. Of note, the apoptotic and autophagic machineries are highly interconnected during developmental cell death [796]. Thus, during *Drosophila* oogenesis apoptotic proteins, including effector caspases, regulate autophagy-dependent cell death [844], while the autophagic apparatus acts upstream of DNA fragmentation by promoting IAP degradation and caspase activation [845, 846]. In this context, the autophagic apparatus also drives the developmental clearance of apoptotic cells [796].

Autophagy-dependent cell death also appears to contribute to the pathogenesis of some human disorders. The neuron-specific deletion of *Atg7* confers robust neuroprotection in a mouse model of severe neonatal hypoxia-ischemia by preventing neuronal RCD [847]. Along similar lines, abolishing autophagy by pharmacological means or by genetically depleting *ATG5* or *BECN1*, prevents the neurotoxicity of cocaine in cultured neurons [848]. Recently, a signalome-wide RNAi-based screen identified glucosylceramidase beta (GBA) as a positive regulator of autophagy-dependent cell death in human cells, presumably linked to the ability of GBA to convert glucosylceramide to ceramide (and glucose) [849]. However, additional investigation is required to elucidate the molecular mechanisms whereby GBA drives autophagy-dependent cell death.

Finally, the long non-coding RNA autophagy-promoting factor (APF) has been implicated in the pathogenesis of myocardial infarction owing to its ability to indirectly promote the expression of ATG7 [850]. Autosis is a specific variant of autophagy-dependent cell death that relies on the plasma membrane  $\text{Na}^+/\text{K}^+$ -ATPase [851]. Corroborating the physiological relevance of this process, the administration of  $\text{Na}^+/\text{K}^+$ -ATPase inhibitors such as cardiac glycosides, confers neuroprotection in a rat model of neonatal hypoxia-ischemia [851].

In summary, autophagy-dependent cell death can be defined as a form of RCD that depends on the autophagic machinery (or components thereof) (Box 1). To avoid confusion, this term should be consistently avoided (1) in the absence of robust experimental evidence mechanistically linking RCD to (ideally more than one) components of the autophagy apparatus, as well as (2) when pharmacological or genetic manipulations of the molecular machinery for autophagy impact on other RCD subroutines.

## Immunogenic cell death

Immunogenic cell death (ICD) is a functionally peculiar form of RCD that is sufficient to activate an adaptive immune response specific for endogenous (cellular) or exogenous (viral) antigens expressed by dying cells [852, 853]. ICD can be initiated by a relatively restricted set of stimuli, including viral infection, some FDA-approved chemotherapeutics (e.g., anthracyclines, bortezomib), specific forms of radiation therapy, and hypericin-based photodynamic therapy [854–861]. These agents are able to stimulate the timely release of a series of DAMPs, whose recognition by PRRs expressed by innate and adaptive components of the immune system warns the organism of a situation of danger, resulting in the elicitation of an immune response generally associated with the establishment of immunological memory [853 862–864]. So far, six DAMPs have been mechanistically linked to the perception of RCD as immunogenic: (1) calreticulin (CALR) [865, 866], (2) ATP [867–869], (3) high-mobility group box 1 (HMGB1) [870–872], (4) type I IFN [854, 873, 874], (5) cancer cell-derived nucleic acids [864, 875], and (6) annexin A1 (ANXA1) [876].

In the course of ICD, CALR translocates from the ER, where it is involved in the maintenance of  $\text{Ca}^{2+}$  homeostasis [877], to the outer leaflet of the plasma membrane [865, 866]. CALR translocation occurs as an early ICD-associated event, i.e., it occurs before PS exposure [878], and is mediated (at least in the case of chemotherapy-driven ICD) by three sequential signal transduction modules: (1) an ER stress module, which involves the phosphorylation of eukaryotic translation initiation factor 2 subunit alpha

(EIF2S1; best known as eIF2 $\alpha$ ) by eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3; best known as PERK) coupled to a block in protein synthesis; (2) an apoptotic module, which involves the CASP8-dependent cleavage of B-cell receptor-associated protein 31 (BCAP31), BAX, and BAK; and (3) an exocytosis module, which involves the anterograde transport of CALR from the ER to the plasma membrane via the Golgi apparatus depending on vesicle-associated membrane protein 1 (VAMP1) and synaptosomal-associated protein 25 (SNAP25) [878–880]. Defects at any level of this cascade compromise the immunogenicity of RCD in vivo [878]. In most instances of ICD, CALR translocates to the cell surface together with protein disulfide isomerase family A member 3 (PDIA3; best known as known as ERp57) [881]. Cell surface-exposed CALR functions (1) as an “eat me” signal for phagocytosis by macrophages, neutrophils, and DCs, which is required for subsequent antigen cross-presentation to cytotoxic T cells; and (2) as a trigger for  $\text{T}_\text{H}17$  cell priming [882]. In line with a key role of CALR in the immunogenicity of RCD, the RNAi-mediated knock-down of CALR as well as natural defects in the CALR exposure pathway reportedly abolish the ability of dying cancer cells succumbing to anthracyclines to establish protective immunity in mice, whereas the exogenous provision of recombinant CALR confers immunogenic properties to otherwise non-immunogenic variants of RCD [865, 878, 881, 883, 884]. Of note, in some preclinical and clinical instances, the activity of surface-exposed CALR is antagonized by CD47, which operates as a “don’t eat me” signal as it inhibits phagocytosis by DCs and macrophages upon interaction with signal regulatory protein alpha (SIRPA) [885, 886]. Accordingly, while CALR exposure has positive prognostic value in patients with acute myeloid leukemia (AML) [887], increased CD47 levels on the surface of malignant cells correlate with dismal prognosis in subjects with AML, esophageal carcinoma, and ovarian cancer [888–891]. That said, CD47 appears to be required for the efficient phagocytic uptake of some murine cell lines undergoing RCD [892, 893]. The reasons underlying this apparent discrepancy remain to be elucidated.

Extracellular ATP not only operates as a “find-me” signal for macrophages and DC precursors upon binding to purinergic receptor P2Y G-protein coupled (P2RY2), but also mediates immunostimulatory effects by activating the canonical inflammasome upon binding to P2RX7 [867, 868 894–897]. In the context of ICD, ATP is released through a cascade of events occurring downstream of caspase activation and involving: (1) the autophagy-dependent accumulation of ATP within autolysosomes (the organelles forming by the fusion of autophagosomes or amphisomes with lysosomes), (2) the relocalization of lysosomal-associated membrane protein 1 (LAMP1) to the plasma membrane, (3)

ROCK1-dependent cellular blebbing, and (4) the opening of PANX1 channels [229, 898, 899]. Accordingly, pre-mortem autophagy is required for optimal ATP release in the course of ICD, and hence for cell death induced by several (but not all) ICD inducers to be perceived as immunogenic [867, 900, 901]. Moreover, overexpression of the ATP-degrading ectoenzymes ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1; best known as CD39) and 5' nucleotidase, ecto (NT5E; best known as CD73) efficiently lowers extracellular ATP levels in favor of adenosine accumulation, hence abolishing the immunogenicity of cell death [902]. CD39 is expressed at high levels on the surface of immune cells endowed with immunosuppressive properties, including CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T (T<sub>REG</sub>) cells, and this promotes tumor progression and spreading [903, 904].

Type I IFN is produced by cancer cells succumbing to ICD by a mechanism involving the detection of endogenous dsRNA by TLR3 [873], or double-stranded DNA (dsDNA) by cGAS [854, 905, 906]. Besides mediating broad immunostimulatory effects on immune cells expressing IFNAR1 [520, 907], type I IFN reportedly activates an autocrine/paracrine signaling pathway in malignant cells, culminating with the expression of a spectrum of IFN-stimulated genes (ISGs) that includes the chemoattractant for T cells C-X-C motif chemokine ligand 10 (CXCL10) [873]. Accordingly, defects in dsRNA or dsDNA detection imposed by genetic interventions, including the *Tlr3*<sup>-/-</sup> genotype and the transgene-driven overexpression of the three prime repair exonuclease 1 (TREX1), as well as the deletion of *Ifnar1* from cancer or host cells, abolish the immunogenicity of cell death triggered by various ICD inducers [854, 873]. Of note, cancer cell-derived nucleic acids do not mediate immunostimulatory functions only by autocrine/paracrine circuitries [908]. Rather, cancer cells succumbing to ICD release dsDNA and RNA molecules that can be efficiently taken up by DCs, neutrophils, and macrophages, resulting in the activation of a potent type I IFN response driven by multiple TLRs and the cGAS-STING pathway [864 909–911]. In line with this notion, the enzymatic degradation of extracellular nucleic acids considerably limits the immunogenicity of RCD [864, 875].

The molecular mechanisms underlying the ICD-associated release of HMGB1 and ANXA1 remain to be fully elucidated. Once secreted, the non-histone chromatin-binding protein HMGB1 mediates potent pro-inflammatory effects by binding to TLR2, TLR4, and advanced glycosylation end product-specific receptor (AGER; best known as RAGE) [912], although TLR4 seems to be the sole HMGB1 receptor relevant to perceive cell death as immunogenic [870 913–915]. In particular, the ligation of HMGB1 to TLR4 on DCs promotes antigen processing and cross-presentation, yet does not induce DC maturation,

which is mainly stimulated by RAGE [870, 916]. That said, it should be noted that biological activities of HMGB1 may flip from pro-inflammatory to anti-inflammatory depending on multiple variables including its oxidation state [917–923]. Extracellular ANXA1 reportedly acts as a DAMP and supports the activation of adaptive immune responses by engaging formyl peptide receptor 1 (FPR1) on DCs [876]. In line with these observations, RCD is not perceived as immunogenic when cancer cells are depleted of HMGB1 or ANXA1, as well as when the host lacks TLR4 or FPR1 [870, 876].

Of note, the immunogenicity of RCD is robustly suppressed by some caspases, notably CASP8 and CASP3, by a variety of mechanisms [7, 924]. These include: (1) the prominent ability of CASP8 to inhibit necroptosis (see above), which is generally associated with the establishment of robust inflammatory responses linked to NF- $\kappa$ B activation [496 925–928]; (2) the capacity of CASP3 to drive PS exposure (see above), which generally supports the phagocytic removal of dying and dead cells while delivering anti-inflammatory signals to macrophages and DCs [218, 219]; (3) the ability of CASP3 to boost the secretion of prostaglandin E<sub>2</sub> from dying cells, which has robust immunosuppressive effects [230, 929, 930]; and (4) the CASP3-dependent inhibition of type I IFN signaling elicited by mitochondrial DNA release upon MOMP [931, 932]. These observations suggest that specific caspase inhibitors may be harnessed to potentially boost the immunogenicity of RCD.

The NCCD proposes to define ICD as a type of RCD that is sufficient to activate an adaptive immune response in immunocompetent hosts (Box 1).

## Non-lethal processes

The molecular machinery for RCD is involved in several processes that have been mistakenly considered as bona fide instances of cell death over the past decades, including cellular senescence, mitotic catastrophe, and multiple cases of terminal differentiation.

### Cellular senescence

The term “cellular senescence” refers to a pathophysiological process by which the cells permanently lose their proliferative capacity while remaining viable and metabolically active [933–935]. Senescent cells exhibit specific morphological traits including flattening, intracellular vacuolization, cellular/nuclear enlargement, and altered chromatin structure. At the biochemical level, cellular senescence is often characterized by: (1) increased lysosomal galactosidase beta 1 (GLB1) activity; (2) inhibition of multiple

cyclin-dependent kinases (CDKs) and consequent dephosphorylation of various members of the retinoblastoma (RB) protein family—including RB transcriptional corepressor 1 (RB1), RB transcriptional corepressor like 1 (RB1L; best known as p107) and RB2L (best known as p130)—upon upregulation of cyclin dependent kinase inhibitor 1A (CDKN1A; best known as p21) [936] and/or the *CDKN2A* products p16 (a powerful inhibitor of CDK4 and CDK6) [937, 938] and ARF (an activator of p53) [939, 940]; (3) absence of proliferation markers, such as marker of proliferation Ki-67 (MKI67); (4) activation of the DDR machinery, generally as a consequence of telomere erosion; and (5) presence of so-called “senescence-associated heterochromatic foci” (SAHF) [934, 941]. Senescent cells secrete a variety of immunomodulatory and mitogenic cytokines, chemokines, growth, and MMPs [941–943]. Although such a senescence-associated secretory phenotype (SASP) appears to be involved in the immunological clearance of senescent cells, it also affects the biology of neighboring cells with an intact proliferative potential (and this has major implications for senescence-inducing anticancer agents) [941–945].

Waves of cellular senescence (followed by RCD) appear to contribute to developmental embryogenesis (although in a dispensable manner) [6, 946, 947] as well as to multiple pathophysiological processes in the adult, including tissue repair and regeneration, immunity, preservation of the stem cell compartment, and oncosuppression [948–957]. In particular, this failsafe cellular senescence reportedly occurs in response to (1) potentially carcinogenic events including oncogene activation or oncosuppressor gene inactivation; and (2) several sublethal cellular insults, including telomere shortening, DNA damage, mitochondrial dysfunction, defective/stalled DNA replication and epigenetic, lysosomal, mechanical, metabolic, mitotic, oxidative, or proteotoxic challenges [933, 958, 959]. Mounting evidence, however, indicates that senescent cells accumulate during organismal aging due to their increased generation coupled to inefficient clearance [935, 941, 960, 961]. Accordingly, chronic cellular senescence has been involved in natural aging, lifespan shortening, tissue deterioration, and the etiology of multiple age-related diseases, including atherosclerosis and osteoarthritis [935, 945, 948, 962–969]. Moreover, senescent cells have been implicated in the adverse effects of some chemotherapeutic regimens as well as in the recurrence of specific neoplasms, at least in mice [969]. Thus, senescence stands out as an attractive therapeutic target for extending healthy lifespan [941, 970]. In this context, one promising senolytic regimen relies on the elevated vulnerability of senescent cells to inhibitors of pro-survival BCL2 family members (in particular BCL-X<sub>L</sub>), reflecting the elevated dependence of senescent cells on these proteins for survival [971–973]. The role of acute

cellular senescence in multiple physiological processes, however, casts doubts on the actual feasibility of this approach [958, 968].

For these reasons, cellular senescence cannot be considered as a form of RCD (Box 1).

### Mitotic catastrophe

Mitotic catastrophe is a regulated oncosuppressive mechanism that impedes the proliferation and/or survival of cells that are unable to complete mitosis owing to extensive DNA damage, problems with the mitotic machinery, and/or failure of mitotic checkpoints [974, 975]. Mitotic catastrophe is morphologically defined by unique nuclear changes, including multinucleation and macronucleation (two potential consequences of chromosomal missegregation) as well as micronucleation (perhaps resulting from the persistence of lagging or acentric chromosomes) [974, 975]. Mitotic defects can derive from: (1) exogenous sources, including a large panel of xenobiotics that alter DNA replication, cell cycle checkpoints, chromosome segregation, and/or microtubular dynamics [976]; or (2) endogenous sources, such as high levels of DNA replication stress or mitotic stress caused by an aberrant ploidy or by deregulated expression/activity of factors involved in DNA replication or chromosome segregation [977, 978]. Of note, the primary alterations that drive catastrophic mitoses can originate in other phases of the cell cycles, including the S phase (e.g., a premature entry in mitosis caused by failure of the intra-S-phase checkpoint) [979, 980]. The precise molecular mechanisms through which mitotic alterations are sensed and trigger mitotic catastrophe cascade are unclear, but presumably involve p53 (at least in many cell types) [975]. A large body of experimental evidence suggests that—at least in specific experimental settings—mitotic catastrophe is precipitated by a signal transduction cascade that relies on CASP2 activation, often (but not always) triggering a variant of intrinsic apoptosis regulated by the BCL2 protein family and demarcated by MOMP [974, 981–984]. In line with a key role for CASP2 in the control of mitotic proficiency, the bone marrow of *Casp2*<sup>−/−</sup> mice accumulates aneuploid cells with aging [985], and *Casp2*<sup>−/−</sup> malignant cells exhibit increased levels of aneuploidy as compared to their wild-type counterparts [982, 986, 987]. Moreover, *Casp2*<sup>−/−</sup> mice are more susceptible than their wild-type littermates to oncogenesis in a multitude of experimental settings [982, 985–989]. In the absence of p53, however, mitotic defects appear to drive a necrotic variant of RCD independent of CASP2 signaling (at least in some settings) [990–992].

Of note, the ultimate fate of cells undergoing mitotic catastrophe seems to be dictated (at least in part) by the time spent under mitotic arrest and their capability to slip out of

aberrant mitoses [993]. Thus, while cells arrested in aberrant mitosis for prolonged periods often undergo intrinsic apoptosis, cells escaping the mitotic block and reaching interphase can undergo a similar fate, enter cellular senescence on activating specific cell cycle checkpoints mediated by p53, and/or Hippo signaling, or simply be outcompeted by their proliferating counterparts [978 994–1002]. Importantly, the abrogation of mitotic catastrophe is a key event during neoplastic transformation and progression, mainly as it allows for the generation and/or survival of polyploid and aneuploid cells [975], whereas cancer (stem) cells reportedly display increased sensitivity to mitotic defects [1003]. However, mitotic catastrophe appears to constitute a major mechanism of action of anticancer chemotherapeutics, mostly reflecting the increased resistance of neoplastic cells to the induction of intrinsic apoptosis [1004–1009]. Moreover, recent data indicate that cancer cells escaping mitotic catastrophe efficiently promote the secretion of type I IFN following the detection of cytosolic dsDNA by cGAS, potentially resulting in their elimination by immunological mechanisms [905, 906]. The latter observation lends further support to the notion that extracellular homeostasis in mammalian organisms is preserved by a plethora of mechanisms that are initiated at the cell-intrinsic level but only operate once cellular homeostasis is compromised.

Since mitotic catastrophe does not always result in RCD (but can also drive cellular senescence), it cannot be considered as a form of RCD per se. We propose the use of the term mitotic death to indicate the specific variant of RCD (most often intrinsic apoptosis) driven by mitotic catastrophe (Box 1).

## Terminal differentiation and others

Multiple components of the signal transduction cascades that regulate or precipitate RCD are involved in the terminal differentiation of a variety of cell types [157, 180 1010–1013], including (but presumably not limited to) neurons [1014–1017], granulocytes [1018], megakaryocytes [1019], erythroblasts [1020], osteoclasts [1021], sperm cells [1022], skeletal myocytes [1023], lens cells [1024], and the keratinized epithelium [1025]. The latter process, which is commonly known as cornification (or keratinization) and critically relies on CASP14 [1026, 1027] and multiple isoforms of transglutaminase [1025, 1028, 1029], has long been considered as a form of PCD [1025, 1030]. However, the NCCD suggests to keep PCD and terminal differentiation conceptually well discriminated from each other. Indeed, dead cells are disposed of (and hence cease to have a function) in the course of PCD. Conversely, when terminal differentiation involves cellular demise, as in the case of cornification, dead cells become integral part of a tissue (and hence mediate a specific physiological function).

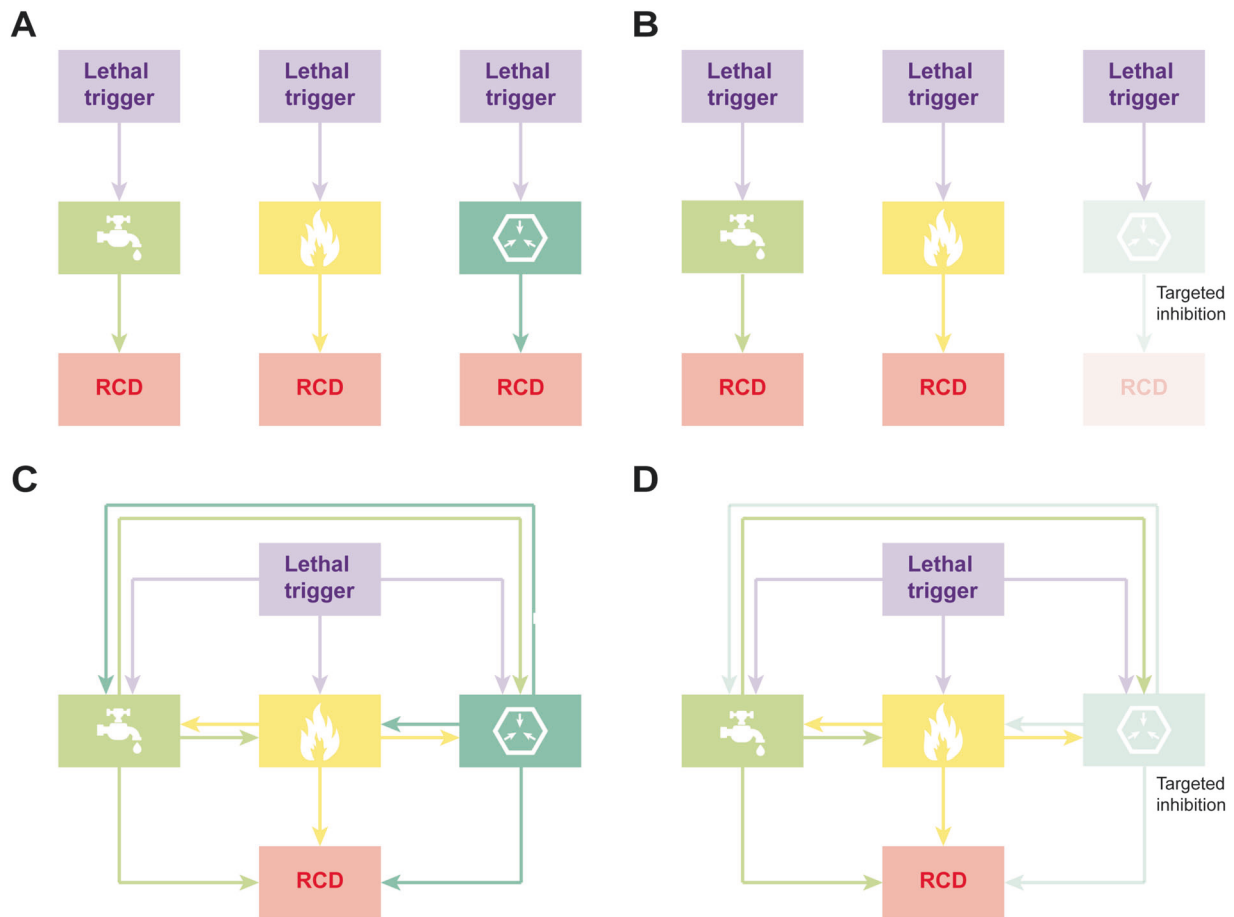
Along similar lines, the NCCD discourages the use of the term eryptosis, which has been coined to indicate the demise of erythrocytes exposed to stress [1031]. Irrespective of the unquestionable relevance of this process for human pathophysiology [1032–1034], it is indeed extremely complex from a conceptual standpoint to define the death of entities that—in physiological conditions—exist in a debatable state between life and death (such as erythrocytes and viruses).

## Concluding remarks

As amply discussed above, RCD plays a major role in development, tissue homeostasis, inflammation, immunity, and multiple pathophysiological conditions. On the one hand, RCD constitutes a primary etiological determinant in diseases associated with the irreversible loss of post-mitotic tissues (e.g., myocardial infarction, neurodegeneration) [687, 752, 851, 869, 1035, 1036]. On the other hand, defects in the signaling cascades that precipitate RCD are associated with pathologies characterized by uncontrolled cell expansion or accumulation (e.g., some autoimmune disorders, cancer) [44, 263, 267, 537 1037–1042]. Thus, RCD stands out as a major therapeutic target for the management of multiple human disorders [1, 2, 649, 1043].

Over the past two decades tremendous efforts have been dedicated to the development of cytoprotective strategies aimed at interrupting RCD signaling after the initiation of the process (a clinically relevant scenario for most ischemic disorders) [1044, 1045], with relatively deceiving results (despite multiple clinical trials, no drug based on this concept has ever been approved for use in humans by regulatory agencies) [387]. Conversely, the BCL2 inhibitor venetoclax is currently available for the treatment of CLL patients who fail to obtain clinical benefit from conventional therapies [1046], and several other molecules with a similar mechanism of action are currently in clinical development [118] (source <https://clinicaltrials.gov/>). Why does the specific activation of RCD (that should not be confounded with the alteration of normal cellular functions, although this also can lead to RCD) appear as a much simpler clinical objective than its inhibition?

Besides potential issues linked to the pharmacokinetics and pharmacodynamics of the compounds tested so far, this discrepancy likely reflects the high interconnectivity of the signaling modules involved in RCD control in mammalian organisms (which has begun to emerge only recently) [374 1047–1049]. Thus, while tilting the balance toward RCD appears as a relatively easy task, blocking it—once a hitherto poorly defined point-of-no-return has been trespassed—may require the simultaneous inhibition of several



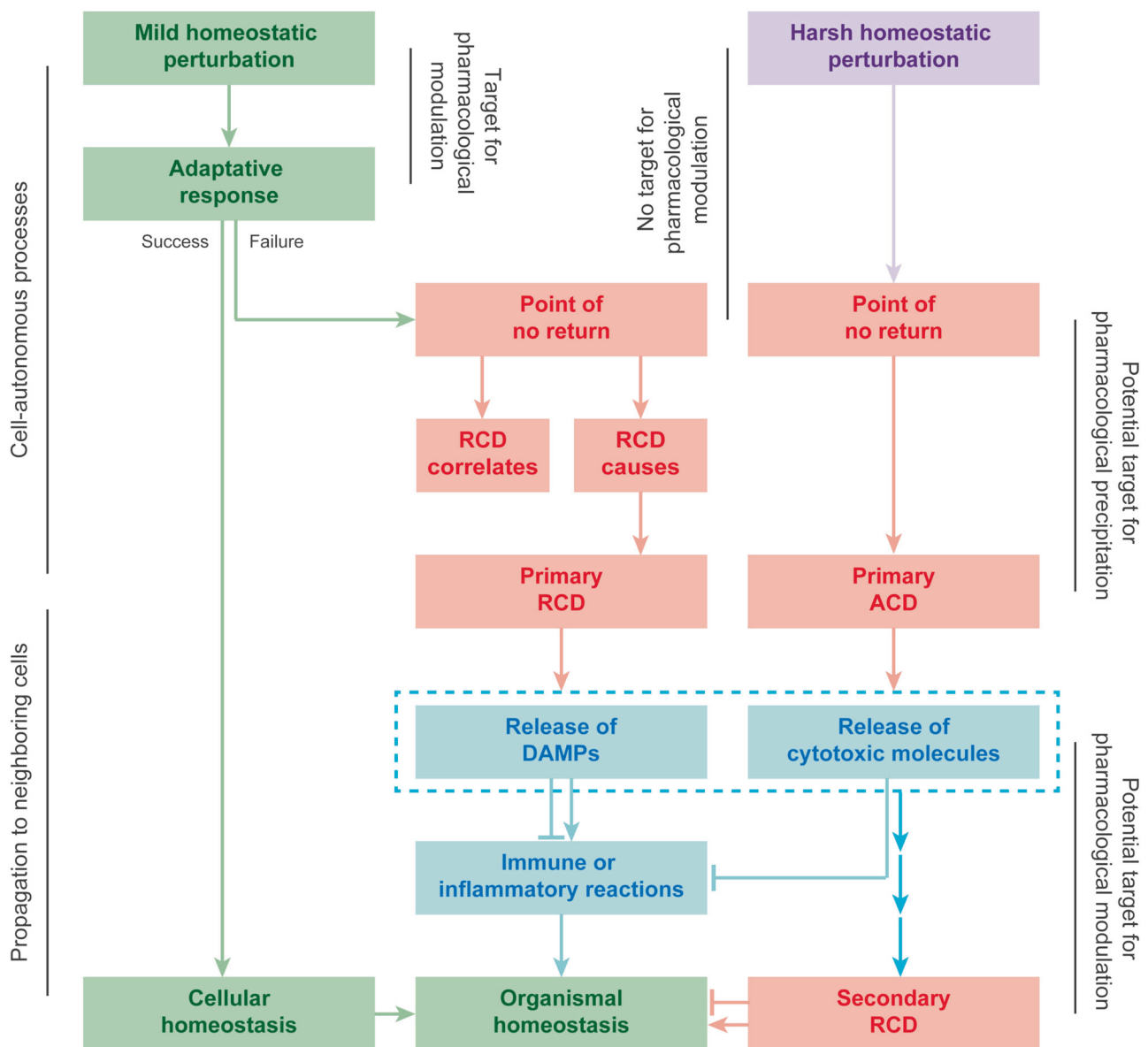
**Fig. 2** Interconnectivity of cell death from a therapeutic perspective. On the basis of the assumption that each regulated cell death (RCD) subroutine would operate in a virtually isolated manner (**a**), considerable efforts have been dedicated to the development of pharmacological agents that would interrupt RCD by operating on a single signal transduction module (**b**). It is now clear that the molecular

mechanisms underlying distinct RCD modalities exhibit a considerable degree of interconnectivity (**c**). This implies that robust cytoprotection may not be achieved by targeting a single RCD subroutine, but only upon the simultaneous inhibition of multiple signal transduction modules (assuming that these modules are the actual cause of cell death, and not simple epiphenomena of RCD signaling (**d**)).

signal transduction modules, and hence may be hardly achievable (Fig. 2). Moreover, the community has focused for a long time on specific enzymes that were thought to have a key causal role in RCD execution, but in a majority of scenarios only appear to accelerate (rather than causally determine) cellular demise (e.g., caspases) [17, 374]. Indeed, cell death in all its forms (including ACD) is ultimately associated with a bioenergetic and redox crisis that may constitute its actual cause [17, 374]. In this scenario, true cytoprotection may be achieved only by interventions that counteract such crisis or the causes (rather than the epiphenomena) thereof. Interestingly, one of the most rapid consequences of potentially lethal ATP depletion in *D. discoideum* is an abrupt nucleolar disorganization coupled to an irreversible block in ribosomal RNA and DNA synthesis [1050]. A similar process has also been observed in mammalian and plant cells succumbing to

multiple forms of RCD, perhaps suggesting that nucleolar stress plays a key role in RCD execution across different species [1051]. This possibility, however, remains to be formally addressed.

Only recently, it has become clear that the modality through which an individual cell succumbs to stress may have a major impact on how RCD affects the local and systemic microenvironment [36, 852, 1052]. This opened an entirely new therapeutic perspective for the field, involving two major approaches: (1) the development of approaches aimed at switching RCD modality, rather than increasing or limiting the incidence of RCD (which may be problematic in both directions) [856 1053–1056]; and (2) the development of agents that intercept DAMPs or regulate DAMP-dependent signaling pathways [20 1057–1059]. In this context, ACD may also constitute a therapeutic target. Indeed, although ACD occurs in a limited number of human



**Fig. 3** Causal vs. accessory aspects of cell death from a therapeutic perspective. Cells exposed to very harsh environmental conditions disassemble in a virtually instantaneous and uncontrollable manner, a process that is referred to as accidental cell death (ACD). Conversely, relatively mild perturbations of exogenous or endogenous origin promote adaptative stress responses aimed at the restoration of cellular homeostasis. If such responses fail, cells generally activate one or more of multiple, highly interconnected signal transduction modules that precipitate regulated cell death (RCD). ACD cannot be retarded by pharmacological or genetic interventions, and most (if not all) strategies conceived so far to block RCD in mammalian organisms fail to efficiently do so, at least in part owing to the elevated interconnectivity of the process. Conversely, some agents that de facto promote RCD by

primarily targeting the underlying molecular machinery (rather than by targeting normal cellular functions) are already available for use in the clinic. The events that follow primary cell death—including a secondary wave of RCD in neighboring cells established (directly or indirectly) by molecules released from the cells succumbing to the primary insult, as well as danger-associated molecular pattern (DAMP) signaling—may also be targets for pharmacological interventions. Finally, although altering quantitatively the percentage of cells succumbing to primary RCD remains challenging (especially when a hitherto poorly defined point-of-no-return of the process has been trespassed), favoring the use of specific signaling modules over others may have prominent effects on long-term disease outcome.

disorders (e.g., trauma, severe burns) and it cannot be pharmacologically inhibited (by definition), the molecules released by cells undergoing ACD may be blocked (at least theoretically) with specific interventions, and this may have a positive impact on long-term disease outcome [21 1060–

1062]. These observations exemplify the complexity of targeting primary RCD or ACD (the death of cells succumbing to primary environmental perturbations), secondary RCD (the death of cells succumbing to the microenvironmental conditions established, directly or

indirectly, by neighboring cells undergoing primary RCD or ACD), and RCD-driven or ACD-driven DAMP signaling for therapeutic purposes [1063] (Fig. 3).

In conclusion, targeting RCD holds great promise for the treatment of several human disorders and considerable efforts are being made to generate RCD modulators for clinical use, but additional studies are required to devise the most efficient strategies in that sense. We are confident that a correct, but flexible, use of the RCD-related terms defined herein will strongly support the progress of the field toward such an ambitious goal. To avoid confusion, it will be important to incorporate neologisms into the scientific literature only for novel RCD subroutines clearly relying on signal transduction modules and effector mechanisms that show little or no overlap with known types of RCD. Along these lines, we believe that terms mostly referring to morphological features of cellular demise and/or indicating considerable mechanistic overlap with well-established RCD forms, such as auto-schizis [1064, 1065], should be dismissed. The NCCD surmises that this is the only way for new cell death-related terms to acquire genuine utility and be broadly adopted by the scientific community.

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