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To cite this version:

Francesco Napoletano, Olga Baron, Peter Vandenabeele, Bertrand Mollereau, Manolis Fanto. Intersections between Regulated Cell Death and Autophagy. Trends in Cell Biology, 2019, 29, pp.323 - 338. 10.1016/j.tcb.2018.12.007. hal-03484944

HAL Id: hal-03484944 <https://hal.science/hal-03484944v1>

Submitted on 20 Dec 2021

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Intersections between regulated cell death and autophagy

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- Word count: 3807 (main text)

ABSTRACT

In multicellular organisms, cell death is an essential aspect of life. Over the past decade, the spectrum of different forms of regulated cell death has expanded dramatically with relevance in several pathologies such as inflammatory and neurodegenerative diseases. This has been 27 paralleled by the growing awareness of the central importance of autophagy as a stress response that influences decisions of cell life and cell death. Here, we first introduce criteria and methodologies for correct identification of the different regulated cell death forms. We then discuss how the autophagy machinery is directly associated with specific cell death forms and dissect the complex interactions between autophagy and apoptotic and necrotic cell death. This highlights how the balance of the relationship between other cell death pathways and autophagy presides over life and death in specific cellular contexts.

Keywords: Cell Death, Apoptosis, Necrosis, Autophagy.

REGULATED CELL DEATH, DIVERSITY AND IMPLICATIONS FOR MULTICELLULAR LIFE

An intrinsic requirement for multicellularity is the ability to deal with the damage and removal of individual cells, while preserving, or promoting, the existence and function of the whole organism. Cell death forms have typically been defined based on morphological analysis of the dying cells and are considered Regulated Cell Death (RCD, opposed to accidental cell death; see Glossary) because of the concomitant or subsequent identification of specific molecular pathways that trigger and execute distinctive cell death morphologies. While RCD is not exclusive to multicellular organisms, it is here that it acquires heightened importance. The essential roles of RCD are diverse, ranging from shaping fully functional organs and body parts during development to orchestrating adaptive or maladaptive responses to prolonged internal or external harmful perturbations of cellular homeostasis. When cellular stress responses cannot cope with such perturbations, they lead to cell death as an adaptation to a sensed failure to reinstate cellular homeostasis, or, in the maladaptive scenario, as an unwanted by-product of the attempt to restore the required equilibrium.

Engaging a specific form of RCD or another acquires fundamental importance in the economy of organismal function, as cell death forms differ in their ability to signal to neighbouring cells the presence of existing threats and to raise appropriate responses (inflammatory or not) via the release or exposure of Damage Associated Molecular Patterns, alarmins, chemokines and cytokines. The well characterized cell death modality known as apoptosis is the prototypical form of RCD (Fig. 1). Conversely, necrosis (Fig. 1) has long been considered exclusively an accidental process triggered by physicochemical insults. However, recent research has led to the establishment of regulated necrosis (RN) as a non-apoptotic form of RCD [1]. Moreover, autophagy, the catabolic pathway centred on lysosomal degradation of bulk or selected cargo has also been recognised as a key regulator and, in some instances, executor of cell death [2]. Thus, the exploration of the autophagy-lysosome system has further expanded the spectrum of non-apoptotic cell death forms (Fig. 1). All of these cell death forms exhibit some degree of non-accidental processes that are subject to molecular regulation and specificity. However, it remains an open issue whether these forms of non-apoptotic cell death ever achieve a type of highly sophisticated regulation as that of apoptosis. In addition, it is unclear whether they develop into programmed cell death (PCD) that is absolutely required for the normal physiology of an organism [Box 1] or are triggered by external or internal pathological events.

A major ontological issue raised by RCD diversity is what can be defined as cell death, as opposed to non-lethal cell degeneration. Whether a precise demarcation line can be drawn is debatable, however, in the continuum between cell stress, stress responses and cell damage two milestones are reached when the integrity of the plasma membrane and/or of the genome have been significantly compromised, to a point that is incompatible with the ability of the cell to maintain itself and this results in the emergence of specific death morphologies. How the diversity of RCD is achieved and the role that autophagy plays in cell death and in the definition of specific RCD forms merit detailed consideration. Comprehensive reviews of the different cell death forms, of their intersections and of autophagy can be found elsewhere [3-5]. Here, we focus on the interplay between select forms of RCD, as defined in the recent community guidelines [3], and autophagy. We dedicate specific attention to the demonstrated *in-vivo* relevance of these events for physiopathology, in specific cellular contexts that highlight the factors that bias towards different cell death forms.

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ROBUST EXPERIMENTAL IDENTIFICATION OF RCD FORMS AS A PRE-REQUISITE FOR THE DETERMINATION OF INTERSECTIONS IN CELL DEATH

The diversity of non-apoptotic RCD forms raises the question of when, where and how these different forms of cell death are used in physiological or pathological contexts (Table 1) [6, 7]. A propaedeutic topic to such questions is that of correct identification of the RCD forms, without which any attempt at defining rules is grounded in unsafe territory. For instance, RN was neglected for many years, and the realization that apoptosis was in fact overestimated, came as a surprise for the research community. The lack of systematic studies on RN was due in part to the extensive use of TUNEL as the main tool to detect apoptosis, which however detects also RN [8, 9], combined with the scarcity of systematic genetic testing and proper tools to detect RN. A similar issue was highlighted in the debate on the role of autophagy in cell death, the extensive use of electron microscopy and steady-state assays displaying an increase in autophagosomes did not discriminate contexts in which autophagy was hyperactive from others in which it was blocked at the stage of clearance of autophagosomes. Experimentally, whereas apoptosis can be detected by TUNEL/activated Caspase 3 co-labelling, we propose that non-apoptotic RCD forms can be identified through a 3-step analysis (Fig. 2). In detail, extensive guidelines exist on autophagy assays and their interpretation [10, 11], while here we provide a brief list of assays for RN (see Box 2).

Finally, regarding the experimental analysis of cell death, a cautionary note must be issued on the use of inhibitors or genetic mutants. In many, if not most, cases, inhibition of one form of cell death will not lead to cell survival, rather to the activation of an alternative form of cell death. It is therefore essential that this does not confound the initial identification of the original cell death form and does not lead to erroneous conclusions regarding the requirement for some factors or pathways.

MECHANISM AND OUTCOME OF AUTOPHAGY INTERACTIONS WITH OTHER RCD FORMS

Macro-autophagy (referred to as autophagy *tout-court* here), from the Greek for "self-eating", leads to lysosomal degradation of diverse cellular substrates, from proteins to whole organelles. First discovered in yeast, subsequent work in this and other models has led to the reconstruction of the assembling and lysosomal digestion of the autophagy-specific organelle, the autophagosome (Fig. 3) [12]. Autophagy serves several functions within cells, from resistance to starvation to healthy housekeeping and quality control for defective proteins and organelles. In addition, the autophagy machinery is involved in extensive cross-talk with several, if not all, forms of RCD. It is therefore important to examine the interactions of autophagy with the different cell death mechanisms and how these may determine the form of cell death executed in different contexts.

Mutual inhibition between autophagy and apoptosis

Autophagy interactions with RCD (Fig. 4) have been primarily elucidated in the interplay with apoptosis, where substantial cross-talk has emerged in particular through the role of Beclin1. Beclin1, Atg6 in invertebrates and yeast, plays a key role in the nucleation of the phagophore in a complex with the PI3Kinase Vps34 and other proteins [13]. However, Beclin1 is also a haplo-insufficient tumour suppressor [14] and a key interactor of the anti-apoptotic Bcl2 proteins [15]. The formation of the Beclin1/Bcl2 complex is sufficient to inhibit autophagy, interfering with the formation of the Beclin1/Vps34 complex [16, 17]. In addition, during apoptosis, caspases cleave Beclin1, inactivate its pro-autophagy function [18], while its carboxy-terminal fragment re-localises to the mitochondria stimulating the pro-apoptotic release of Cytochrome C [19]. This provides a further shut down mechanism to restrict the pro-survival function of autophagy and an amplifying loop enhancing apoptosis. Beclin1 also interacts with NDR kinases that regulate the balance between autophagy and apoptosis by activating either pathway depending on the input of the RalB GTPase [20]. Beside Beclin1, other autophagy proteins regulate the balance between autophagy and apoptosis. Atg12 and Atg3 inhibit apoptosis [21], while Atg5 cleavage generates a pro-apoptotic fragment [22]. Furthermore, MST1, a pro-apoptotic kinase, has been reported to phosphorylate Beclin1 and inactivate autophagy in cardiomyocytes [23]. However, MST kinases and their downstream pathways are rather required for autophagy processes in most other contexts including autophagic cell death of the *Drosophila* salivary glands, neuronal homeostasis and autophagic clearance of bacteria [24-26], suggesting a context-dependent role for MST kinases. Another prominent tumour suppressor, p53, a regulator of autophagy and apoptosis [27] switches between the two processes through the transcriptional regulation of DRAM proteins, which are required for p53-induced apoptosis [28]. DRAM proteins are lysosomal factors that stimulate autophagy [29] and lysosomal membrane permeabilization [30], with the ability of mediating both cell survival and cell death. In *Drosophila*, expression of ∆Np53 (p53A) but not of p53 (p53B), induces photoreceptor apoptosis with elevated levels of the cleaved effector caspase, Death caspase-1 (Dcp-1), and inhibition of the autophagy flux [31]. Interestingly, the concomitant expression of ∆Np53 and inhibition of initiator or effector caspases restores a functional autophagy. This demonstrates that both initiator and effector caspases are actively inhibiting the autophagic flux. Cathepsin B also provides a critical switch through the degradation of Disabled2, a TGF-β transcriptional target. Degradation of Disabled2 promotes autophagy, whereas high levels of Disabled2 induce apoptosis via stabilization of the pro-apoptotic Bim [32]. Overall, these studies indicate that when the apoptosis machinery is engaged it exerts and an efficient inhibitory control on autophagy and can also exploit autophagy proteins to promote apoptosis.

Vice-versa, autophagy is very efficient at inhibiting apoptosis, and autophagy activation is protective *in-vivo* in models of neurodegeneration [33-36]. Several non-exclusive mechanisms have been proposed to explain this effect. An efficient way of limiting apoptosis is the activation of mitophagy that specifically recognizes and eliminates damaged mitochondria. Animal and cellular models of Parkinson's Disease (PD) accumulate leaky mitochondria that induce oxidative stress contributing to the elimination of dopaminergic neurons by apoptosis [37, 38]. Similarly, autophagy can inhibit apoptosis by degrading factors like the BH3 domain-containing protein PUMA, which promotes Mitochondrial Outer Membrane Permeabilization (MOMP) [39]. Another proposed mechanism for the inhibition of apoptosis by autophagy during neurodegeneration is the elimination of activated caspases (Fig. 3) that accumulate in autophagic vacuoles in dystrophic neurites, as shown in Alzheimer's Disease (AD) mouse models and in human brains of AD patients [40]. Finally, a mechanism through which autophagy intersects with apoptosis in a different context offers particularly intriguing links with neurodegenerative diseases involving aggregation-prone proteins. Pro-apoptotic amyloid structures such as those assembled by the TIR-domain-containing adapter-inducing interferon- β (TRIF) protein in innate immunity are degraded by autophagy, inhibiting cell death [41].

In some instances, autophagy has been associated with facilitation of apoptosis rather than repression. For instance, autophagy can degrade the pro-survival factor Caveolin1 and trigger apoptosis in astrocytes cultured *in-vitro* [42] and can lead to activation of Caspase 8 [43, 44]. Autophagosomes indeed act as scaffolds for the intracellular death-inducing signalling complex (iDISC) activation of Caspase 8 in apoptosis [45]. These studies suggest a more complex relationship between autophagy and apoptosis. A most revealing investigation shows that this relationship may be influenced by the level of the autophagy flux [46]. Within the same population of BJAB B-cell lymphoma cells, those with a high autophagy flux degrade efficiently Fap-1, an inhibitor of Fas-mediated apoptosis, sensitizing the cells to Fas-ligand induced apoptosis. Interestingly this effect was specific for Fas, as treatment with TNF-related

apoptosis-inducing ligand (TRAIL) resulted in the opposite, with cells at low flux experiencing a greater induction of apoptosis [46].

- In conclusion, it emerges that the relation between autophagy and apoptosis is mostly one of mutual repression, albeit further complexity is revealed by the particular effects reported in some contexts, in association with specific pathways.
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Autophagy and necrosis: a multifaceted relationship

Autophagy is also highly interconnected with necroptosis (Fig. 4). Autophagy directly degrades RIPK1 to inhibit necroptosis [47] and Atg16L was shown to inhibit necroptosis in intestinal cell by promoting autophagy-dependent mitochondrial homeostasis [48]. *Vice-versa* RIPK1 inhibits autophagy through ERK phosphorylation of the TFEB transcription factor [49] and RIPK3 promotes p62 cleavage by Caspase 8, resulting in the inhibition of LC3-p62 interactions during autophagy [50]. However, in other settings autophagy and mitophagy have been shown to be required for necroptosis, including providing a scaffolding surface for the necrosome [51-53]. Components of the autophagy machinery can even determine a switch between alternative RCD forms. For instance, p62 acts as a scaffold for the activation of RIPK1 by recruitment of the latter to a complex with other Atg proteins. This triggers necroptosis in murine prostate cells mutant for MAP3K7, while p62 knock-down or mutants in the p62 ZZ domains fail to allow activation of necroptosis, causing a switch to apoptosis in these cells [54]. Despite a limited amount of studies, the relationship between autophagy and necroptosis appears more complex than that between autophagy and apoptosis. This may reflect aspects of integration between RN and Autophagic Cell Death (ACD) and/or an influence of the autophagy-lysosome system in the switch between apoptosis and necroptosis. Investigations on the intersection of autophagy and the other RN sub-forms, especially ferroptosis and mitochondria-dependent necrosis, may provide additional insights in this respect. For instance, autophagy has been shown to promote ferroptosis by degrading ferritin, leading to higher accumulation of iron [55]. Another possible intriguing connection between ferroptosis and autophagy may be revealed through the additional function as an oxidative stress sensor of the p62/SQSTM1 autophagy receptor [56], which however promotes survival in this case. Thus, also the relationship between autophagy and ferroptosis appears to incorporate both facilitating and inhibitory drives.

In conclusion, the autophagy-lysosome system seems to be a most decisive factor in influencing decisions at the cross roads between cell life and cell death. Among stress-coping processes, autophagy appears to be the one most directly linked to cell death mechanisms, deserving special consideration in the cell death field.

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AUTOPHAGY-ASSOCIATED CELL DEATH IN DEVELOPMENT AND PATHOLOGY

In addition to its modulatory role for other RCD forms, the autophagy machinery has been associated with the execution of specific forms of cell death triggered either by hyper-activation of the autophagy flux or by its inhibition.

Death driven by autophagy activation

ACD has been characterised in invertebrate models and has a marked importance in *Drosophila* metamorphosis. As this process is essential for the physiology and development of *Drosophila*, this form of cell death acquires characteristics of PCD. It contributes, either independently or in combination with caspases, for the elimination of the midgut and salivary glands [2, 57, 58]. Detailed analysis in the midgut highlights a selective requirement for the autophagy initiation complex genes Atg1, Atg13 and Atg17, as well as the upstream Tor pathway. Downstream, Atg8a is essential, but Atg3, Atg4a and Atg7, which are required for Atg8a processing and lipidation, are apparently dispensable [59], suggesting the existence of parallel conjugation pathways and, perhaps, of cell specific requirements for autophagy-related pathways. In the salivary glands, there is a clear requirement for Atg1, Atg8a and, differently from the midgut, for Atg3 and Atg7 [57]. In addition, ACD in the salivary glands requires mir-14, multiple inositol-1,4,5 trisphosphate (IP3) signalling pathway components [60], as well as the engulfment receptor Draper [61]. The association between vast organismal remodelling and 247 ACD raises the question of whether ACD is the selective RCD form for metamorphosis and, potentially, for other hormonal-led body maturation events. ACD has been reported in mammalian cells [62, 63], associated with induction of cell senescence [64], upon anticancer treatment [65, 66], as a response to hypoxia [67] and often upon inhibition of apoptosis [68, 69] or in apoptosis-incompetent Bax/Bak mutants *in-vitro* and *in-vivo* [70, 71]. Recent evidence however suggests that fibroblasts mutant for *Bax/Bak1* are resistant to ACD and that this can be restored by rescuing lysosomal membrane permeability with monomeric Bax or with a lysosomotropic detergent [72]. This revealing study prompts us to suggest that rather than undergoing proper ACD, Bax/Bak1 deficient cells display, at least in some instances, Lysosome Dependent Cell Death (LDCD) [3], which requires lysosomal membrane

- permeabilization. It is presently unclear, albeit unlikely, whether the latter is also required in ACD as described in *Drosophila* metamorphosis.
- *In-vivo* an Atg7-dependent form of cell death has recently been described in mouse model of heart failure [73], while a sub-form of ACD in mammals, referred to as autosis, and requiring ATG genes but not lysosomal degradation, has been observed *in-vivo* after cerebral hypoxic insults [74] and in mammary gland reabsorption [75]. The latter context is particularly interesting because describing a hormonal-driven large body overhaul that is equipollent with insect metamorphosis. However, the cell death observed during mammary re-absorption may be LDCD [76] and the role of autophagy would be required for clearance.
- Open questions in ACD relate to the implications of the requirement for selected Atg genes [59], arguing for aspects of non-canonical autophagy, and to the caspases that often act in parallel to autophagy [24] and restrict autophagic cell death [68, 69]. Whether this frequent interplay with caspases represents a parallel apoptotic pathway in which autophagy is a subsequent (and potentially also essential for death) clearance step remains to be established.
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Death by autophagy impairment

Whereas ACD progresses through hyper-active autophagy, autophagy is more commonly a pro-survival stress response that becomes of paramount importance in long-lived cells that do not maintain homeostasis through cell division [77]. As such, impairment of the autophagy-lysosome system has been reported extensively in neurodegeneration and is a hallmark of several pathologies such as AD or PD [78, 79]. This has been also associated with apoptotic cell death [36, 40, 80]. Failure to maintain the pro-survival function of autophagy is therefore a key event in determining neuronal cell death by apoptosis. However, this scenario deserves further consideration since significant *in-vivo* evidence for non-apoptotic RCD in neurodegeneration has been provided, also alongside apoptosis [40, 81-84] and the inhibition of autophagy in the nervous system was also shown to lead to a proposed novel form of cell death, named karyoptosis [85].

Karyoptosis, which is based on the nucleophagy machinery [86], was reported in invertebrate and mammalian models of neurodegeneration with nuclear accumulation of misfolded proteins, as well as in cells in cultures, and can be triggered by chronic inhibition of autophagy [87]. BafilomycinA1 (an inhibitor of autophagosome/lysosome fusion and a potent inducer of karyoptosis *in-vitro*) induces cell death without Caspase 3 activation and PARP1 cleavage [88]. Importantly, some of the milestone descriptions of the neurodegenerative effects of Atg5 and Atg7 knock-out in mice [89, 90] are consistent with the presence of karyoptosis in these mutants. Moreover, on the basis of the morphological descriptions given (cell shrinkage, distorted nuclei, accumulation of lysosomal compartments such as lipofuscin and plasmalemmal blebbing), karyoptosis may represent the Caspase 3-negative cell death form observed in models for AD [40] and in polyglutamine diseases [82, 91, 92]. A feature of karyoptosis, nuclear distortion, is shared by another form of caspase-independent RCD, identified first in *C.elegans* development, Linker Cell Death (LCD) [93, 94]. LCD displays characteristics of PCD as it is essential for the normal organism development and number of evolutionary conserved genes and processes have been identified as promoting this form of RCD, including the ubiquitin proteasome system [95]. However, it is not established whether autophagy and nucleophagy are involved in LCD, and thus what the overlap may be between LCD and karyoptosis. Specific *in-vivo* and *in-vitro* hallmarks of karyoptosis are cytoplasmic re-localisation of LaminB1 associated with LC3 as a consequence of nucleophagy [86], alongside senescence-associated DNA damage, as well as the excretion of LaminB1 and the p62 receptor [87] in exopher-like structures [96]. The reliance of karyoptosis on the LaminB1- LC3 nucleophagy machinery activated in senescence [86] suggests that karyoptosis may as well represent progression of a form of terminal senescence into non-apoptotic cell death. The association of the key karyoptosis molecule, LaminB1, with other RCD forms has been minimal. Caspase 6 has been shown to cleave LaminB1 in some apoptotic contexts [97, 98] and LaminB1 released in exophers in karyoptosis is in a cleaved form [87]. However, the released LaminB1 fragment in karyoptosis appears different from the one generated by Caspase 6 and the involvement of Caspase 6 and other proteases in karyoptosis is presently unclear. LaminB1 knock down also promotes a switch from necrosis to apoptosis during 5-fluoro-2'- deoxyuridine treatment [99], however, in this case apoptosis was identified merely based on cell shrinkage, a feature also shared by karyoptosis. Finally, karyoptosis appears to be more easily detected *in-vitro* in slowly dividing cells, and we speculate that this form of cell death may have a greater relevance in long-lived non-dividing cells, paralleling the heightened importance of autophagy in homeostasis of these cells. In particular, karyoptosis may represent a prominent form of neuronal cell death in aggressive neurodegenerative diseases with a clear impairment of autophagy accompanied by significant accumulation of nuclear misfolded proteins. Several open questions in the relationship between karyoptosis and autophagy remain. What are the initiating stimuli for the expulsion of LaminB1 and how is its association with LC3 regulated? What components of the autophagy machinery are directly involved in mediating the degradation of the nucleus? Is this a canonical autophagy process?

Besides karyoptosis, failures in the autophagy-lysosome system can also be mechanistically linked to LDCD. However, the key question regarding the precise mechanism that executes cell death is whether this is linked to autophagy for death and/or clearance, as suggested in a model of retinitis pigmentosa [84]. While in some cases the cell death evolves into an apoptotic morphology, non-apoptotic modalities are also activated and have rather necrotic aspects [100, 101]. We argue that under the current LDCD umbrella definition, separate sub-forms of cell death are probably gathered. Full characterisation of the role, if any, that autophagy play in the execution of cell death may clarify the relationship between LDCD sub-forms and the other autophagy-associated cell death forms.

In summary, the autophagy machinery is directly involved in determining some specific cell death morphologies. Whether by hyperactivation or by inhibition of the autophagy flux, autophagy-associated forms of cell death seem to display a distinctive link with clearance of cellular content. This association raises a number of issues on whether clearing represents a distinct, and subsequent, process from cell death *per se*, and will require additional clarifying studies in a number of cases. However, this self-clearing activity, either through lysosomal digestion or through excretion in the extracellular space, may as well constitute an integral part of the killing process and be the distinctive feature of autophagy-associated forms of cell death.

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Concluding remarks

The field of cell death has been revitalized with the emergence of new forms of cell death and the understanding of their role in pathologies. The emerging picture is that the two key principles that underlie this diversity are those of context and balance. Different cellular contexts bias towards the cell death form activated. The point of balance between pro-death and pro-life pathways is also placed differently in varying cellular contexts, and it determines the relative importance that homeostatic processes, notably autophagy, have in shaping the cell death process. In-depth knowledge of the essential role for autophagy in modulating, and in some contexts mediating, cell death is likely to be vital for the informed design of any future therapeutic strategies aiming at controlling cell death in human pathologies (see Outstanding Questions).

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GLOSSARY

Autophagy: a process of self-eating of proteins and organelles through formation of a dedicated engulfing vesicle called the autophagosome followed by its degradation inside the lysosome. It serves as a quality control for proteins and organelles, as well as a starvation resistance mechanism.

Caspases: cysteine-dependent aspartate-directed proteases are a family of proteases playing essential roles in the activation of apoptosis but also in signalling events that do not lead to cell death.

Ferroptosis: a RN sub-form dependent on reduced levels of glutathione levels, leading to diminished activity of glutathione peroxidase 4 (GPX4), an enzyme that reduces peroxidized lipids to lipid alcohols. Lipid peroxidization occurs spontaneously due to iron-dependent Fenton reactions, which led to its name.

Karyoptosis: a proposed new form of autophagy-associated cell death based upon the degeneration of the nucleus (from the Greek words karyon - i.e. nucleus - and apoptosis - i. e. falling apart) due to the expulsion of LaminB1 upon direct interaction with LC3, followed by the excretion of the nuclear content in large microvescicles or exophers.

Lysosome Dependent Cell Death: a RCD form that is caused by Lysosomal membrane permeabilization leading to the leakage of the lysosomal content into the cytosol that can evolve in cell death with necrotic, apoptotic or apoptosis-like features and is associated with a block in the autophagy flux.

Neurodegenerative diseases: a group of pathologies (e.g. Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis etc…) caused by the progressive degeneration of parts of the nervous system, including extensive neuronal cell death, and that are caused either by hereditary genetic mutations or by sporadic events.

Regulated Cell Death: A loss of plasma membrane and/or nuclear genome integrity that leads to the inability of the cell to sustain itself as an organised system and evolves in specific death morphologies through the direct action of identified molecular pathways and effectors.

Oxidative stress: the damage resulting from accumulation of highly reactive chemical radicals, mainly produced either during mitochondria respiration or as a result of iron metabolism.

Acknowledgments

We apologise to all colleagues whose work we were unable to cite due to restrictions in the list of references. This work was supported by grants from the MRC (NIRG-G1002186) the NC3R (NC/L000199/1) Ataxia UK (A43), Alzheimer Research UK (PPG2017B-018)and Motor Neurone Disease Association (855-791) to MF and by ANRs Ire1-PD (13-ISV4-0003) and LipidinRetina (12-BSV1-0019), the ARC foundation, la Ligue contre le Cancer, Rhône, France Parkinson, Fondation de France (2012-00034435) and FINOVI (2017-01 26-A011) grants to BM. FN was supported by a European Union FP7/AIRC - Associazione Italiana Ricerca sul Cancro Reintegration Grant (iCARE N°17885) and a University of Trieste FRA 2018 Starting Grant. Research in P. Vandenabeele's unit is further supported by Belgian grants (EOS 30826052 MODEL-IDI), Flemish grants (Research Foundation Flanders: FWO G.0C31.14N, FWO G0E04.16N, G.0C76.18N, G.0B71.18N, Methusalem grants - BOF16/MET_V/007), 'Foundation against Cancer (FAF-F/2016/865) and VIB. MF is an invited scientist of the École des Neurosciences Paris Île de France.

Box 1. Regulated Necrosis as physiologically Programmed Cell Death

Many forms of regulated cell death arise in pathological situations, during neurodegeneration 412 or infection and therefore are not programmed for the normal physiology of an organism but are rather a response to a loss of homeostasis due to internal or external stimuli. However, in 414 some specific contexts, non-apoptotic RCD forms also display programmed characteristics. We discuss Autophagic Cell Death and Linker Cell Death in the main text and illustrate here 416 an example for RN.

Similar to apoptosis, studies in model organisms have proven fundamental to reveal the physiological relevance of RN. This is exemplified by the mitochondria-dependent necrotic death of the *Drosophila* male germ cells that maintains tissue homeostasis and prevents hyperplasia [102] and in which p53-dependent transcription is activated selectively in *Drosophila* necrotic male germ cells. Interestingly, *Drosophila* male germ cell RN requires the mitochondrial factor Debcl, a pro-death BCL2 homologue, and a non-catalytic function of the initiator apoptotic Caspase 9 (Dronc in *Drosophila*), also emerging for other non-apoptotic processes [103-106]. In addition, *Drosophila* male germ cell RN requires the function of lysosomal regulators (Deep Orange, a VPS18 homologue, and Carnation, a VPS33 homologue, 426 both part of the HOPS complexes that promote vesicular docking and fusion) and of lysosomal enzymes that can be released upon lysosomal membrane permeabilization (Dnase-II, 428 Cathepsin-D). The formers are important for autophagy [107, 108] and the latter occurs also during Lysosome Dependent Cell Death (LDCD). *Drosophila* male germ cell RN may therefore represent an ancient form of RCD including the features of mitochondria-dependent apoptotic (primordial Caspase 9 and Debcl function) and necrotic RCD forms, and perhaps 432 also of LDCD. These observations also suggest that key regulators of mitochondria-dependent RCD pathways, including Bcl-2 proteins, initiator caspases and p53, may have evolved a pro-apoptotic role on top of a primordial pro-necrotic function. Since several RN genes are 435 evolutionarily conserved and a mitochondria-lyososome RN axis has been observed as early as in *Dictyostelium*, it is tempting to propose that RN may predate the appearance of apoptosis. This pathway may represent a quality control system to eliminate damaged/unfit germ cells 438 whose genome integrity is compromised, which could be harmful for the offspring and also 439 may pose a risk of accumulating tumorigenic lesions. Alternatively, germ cell RN could be a homeostatic mechanism selected to maintain proper size of the developing germ cell population during spermatogenesis.

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Box 2. Guidelines for the detection of regulated necrosis

Morphological features - Loss of membrane integrity and often cellular and organelle swelling (oncosis, from the greek onkos, *i.e.* swelling) are typically observed in necrotic cells [109] by 446 transmission electronic microscopy (TEM) [110]. To demonstrate that necrotic features observed by TEM are not secondary to apoptosis, time course analysis is required.

Caspase-independent DNA fragmentation - The Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is very sensitive to detect 3'hydroxyl (3'OH) termini of cleaved 450 | DNA strand. This is typically generated by type I DNAses, such as caspase activated-DNAses 451 (CAD) that execute apoptosis. However, 3'OH ends can be produced also by dephosphorylation 452 \int of 3'PO₄ ends generated by caspase-independent type II DNAses, typically through activity of 453 acid phosphatases released upon lysosomal membrane permeabilization in necrotic cells [111]. 454 Phosphatase pre-treatment has been used to allow/enhance labelling of necrotic cells with 455 TUNEL [110, 112]. To distinguish necrosis from apoptosis using TUNEL, co-labelling with 456 an anti-activated Caspase 3 antibody is required. As caspase activation precedes chromatin 457 cleavage during apoptosis, a time course analysis is recommended. Alternatively, the Apoptag 458 ISOL Dual Fluorescence assay uses Topoisomerase I and T4 DNA Ligase for oligonucleotide-459 mediated differential labelling of 3'OH and 3'PO₄ ends.

Membrane permeability - Plasma/nuclear membrane integrity is commonly detected monitoring intracellular/nuclear diffusion of membrane impermeable dyes, typically DNA intercalators such as propidium iodide (PI). *In-vivo*, the use of PI to detect primary necrosis is 463 greatly simplified by the fact that apoptotic cells are eliminated by phagocytosis and do not undergo secondary necrosis, an artefact common in cell culture [102, 113]. For multiple fluorescence staining, dyes with narrower emission spectra than PI, such as 7- 466 aminoactinomycin D (7-AAD) are required.

467 A common method to discriminate apoptosis and necrosis in dissociated/cultured mammalian 468 cells is coupling PI staining with detection of phosphatidylserine (PS), which is required for 469 engulfment of apoptotic cells, using Annexin-V and flow cytometry [110, 114]. However it is 470 recommended to combine this method with the use of specific inhibitors of apoptosis and 471 | necrosis, as cultured necrotic cells have been reported to transiently appear Annexin-V⁺/PI⁻, in 472 | some conditions [115].

473 Quantifying leakage of intracellular components *in-vivo* is challenging, though successful in 474 cell culture, typically monitoring the chromatin protein high mobility group box 1 (HMGB-1) 475 [116]. However, release of HMGB1-containing microparticles has been reported in apoptotic 476 cells $[117-119]$.

477 *Mitochondria swelling* - During mPT-dependent necrosis, Ca^{++} influx leads to osmotic 478 Swelling of mitochondria, which can be measured by absorbance of purified mitochondria with 479 light scattering at 540nm [72]. Atomic force microscopy has been also recently employed 480 [120].

481 *RNA loss* - Spectral properties of Acridine Orange (AO), a membrane-permeable nucleic acid-482 binding dye, have been recently exploited to distinguish between apoptotic and necrotic cells 483 [121].

484 *Loss of lysosomal integrity* – Lysosomal membrane is permeabilized in many RN paradigms 485 and can be efficiently detected by enzymatic activity of released lysosomal hydrolases, 486 visualization of fluorescent dextran release from lysosomes, immunocytochemistry for 487 cathepsins into the cytosol, and translocation of galectins to lysosomes [122].

488

489 **Table 1. Forms of regulated cell death and their physiological and pathological relevance**

490

491 **FIGURE LEGENDS**

492 **Figure 1. Overview of the morphological changes in different forms of Regulated Cell** 493 **Death.**

In this and in all figures, we apply a colour code: apoptosis and molecules/events influencing apoptosis are in blue, autophagy and molecules/events that influence autophagy are in green, regulated necrosis and molecules/events influencing it are in red. The central cell with healthy nucleus, with an intact genome, healthy mitochondria and a homeostatic autophagy flux can undergo cell death through different modalities. Apoptosis is characterised at morphological level by cell shrinkage and cell and nuclear fragmentation into self-contained large apoptotic bodies. In addition, mitochondria may degenerate and become permeabilised, and autophagy may be inhibited. Regulated necrosis is instead characterised by cell and nucleus swelling, cytoplasm granulation, plasma and nuclear membranes rupture releasing conspicuous cell content. In addition, some organelles such as mitochondria and lysosomes may become permeabilised. Autophagic cell death is characterised by accumulation of autophagosomes and large auto-lysosomes in which the whole cell content is engulfed and digested. Karyoptosis is characterised by cell shrinkage, nuclear shape alteration, accumulated undigested autophagosomes and LaminB1 in the cytoplasm often co-localising with autophagic markers. Finally, large macrovesicles or exophers with LaminB1 and other cellular content bleb from the plasma membrane.

510

Figure 2. A 3-step process for the identification of specific RCD sub-forms.

In Step1, detection of apoptosis (blue) can be demonstrated by a TUNEL and activated Caspase 3 co-labelling and can be additionally confirmed through Annexin V/Propidium Iodide staining and by measuring the DEVDase activity in cultured cells. Further assays the can confirm apoptosis using different set ups and technologies. These include visualization of chromatin condensation by Transmission Electron Analysis (TEM), and quantification of caspase-dependent PARP1 cleavage in protein extracts. Depending on the set-up, apoptosis can be confirmed by pharmacological or genetic inhibition of key components for instance suppression by effector caspase inhibitors (e.g. zVAD) and through downregulation or mutations in required factors (e.g. Apaf-1 or Caspase 8). A cautionary note should be applied when using inhibitors, siRNA or introduced mutations, since in most cases these may cause a switch to a different RCD form and this must be clearly distinguished from the original RCD form in a wt condition.

In Step2, the RN (red) can be identified through its major hallmark, i.e. loss of membrane integrity associated with increased permeability. This can be identified by detection of Propidium Iodide incorporation - provided that secondary necrosis due to other forms of cell death, often observed in cultured cells, has been ruled out. Additional assays are discussed in Box 2. Specific RN sub-forms can be revealed in some set ups by the use of inhibitory compounds or mutations, although care should be applied to consider a possible switch to other 531 RCD forms. Necroptosis requires RIPK3 and/or MLKL, while the use of RIPK1 inhibitors (e,g, Necrostatin-1) should consider the existence of RIPK1-dependent apoptosis. mPT-dependent RN requires Cyclophilin-D, which can be inhibited by Cyclosporin-A, while parthanatos can be tested through PARP1 inhibitors or mutation. Germ cell necrosis is mostly revealed *in-vivo* through mutations in required mitochondrial factors (e.g. *Pink1*, *EndoG-*). Ferrostatin is a reliable inhibitor of ferroptosis.

In Step3, autophagy-associated cell death (green boxes) can be revealed by accumulation of autophagy markers (LC3) and receptors (p62). In addition, accumulation of all type of autophagic vacuoles can be visualised by TEM. Assessment of the autophagy flux is essential and can be achieved through combined use of Rapamycin and Bafilomycin A1 (Rap/Baf assays), and in some set ups with tandem fluorescent assays (mCherry-GFP-LC3) or in protein extracts though LC3 lipidation assays and GFP cleavage assays from GFP-LC3. Autophagic

cell death is specifically identified through an increased autophagy flux and for its requirement for at least a subset of Atg genes (e.g. Atg5 and Atg6). Karyoptosis is associated with decrease in the autophagy flux and DNA damage and is specifically identified by detection of 546 LaminB1+/LC3+ cytoplasmic puncta and, in cell cultures, of excreted LaminB1 and p62 in the microvescicular fraction in the medium.

Figure 3. Autophagy at a glance.

The autophagy cycle starts when the initiation complex constituted by the ULK1 (or Atg1)/Atg13/FIP200 (or Atg17) proteins is recruited to the sites of the Endoplasmic Reticulum in contact with mitochondria where the isolation membrane or phagophore will be detaching from. At these sites the nucleation complex containing Beclin1 (Atg6), the Vps34 class III phosphatidylinositide 3-kinase and additional adaptors is activated and promotes the formation of the phagophore. Thereafter the elongation is driven by the Ubiquitin-like cascades involving Atg5/Atg12 conjugation and LC3 (Atg8) cleavage and lipidation with Phosphoethanolamine (PE). The cargo recognition is determined through the interaction between autophagy receptors like p62/SQSTM1 and lipidated proteins of the LC3 family present on the membrane of the autophagosomes. Finally, upon autophagosome closure and maturation, the fusion with the lysosome is determined through the HOPS machinery, Rab 7 and a set of SNARE proteins.

Figure 4. Mechanisms of interactions between autophagy and select RCD pathways.

Same colour code used in Figures 1-3. Some molecules have multiple roles, which is represented by different colours. Apoptosis, triggered by extrinsic death receptors on the plasma membrane or by intrinsic BCL2-dependent release of Cytochrome C (CytC) from damaged mitochondria, is antagonised by autophagy via the degradation of caspases in autophagic vacuoles and of mitochondria through mitophagy. In some contexts, however, autophagosomes facilitate apoptosis by providing a scaffold for the activation of Caspase 8. Apoptosis antagonizes autophagy through Caspase 8 cleavage of the p62 receptor. Effector caspases inhibit autophagy by cleaving Atg6/Beclin1 and other pro-autophagic factors. Autophagy antagonizes necroptosis by degrading RIPK1 in some contexts, while in other contexts both autophagosomes and the p62 autophagy receptors promote necroptosis by helping through scaffolding the assembly of the RIPK1/RIPK3/MLKL necrosome. Autophagy also promotes Ferroptosis through degradation of Ferritin. NDR1, MST1/2 kinases and p53 act

- as molecular switches that allow control of apoptosis, autophagy or RN depending on context.
- MST1/2 kinases activate NDR1 kinase that either induces apoptosis or interacts with Beclin1
- promoting autophagy. MST1/2 also regulates directly autophagy and apoptosis through
- transcriptional regulation in the nucleus. p53 activates either autophagy, apoptosis or
- mitochondria-dependent RN in different contexts either via transcription or via cytoplasmic
- functions.
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Autophagic Cell Death Karyoptosis

Napoletano *et al.* Figure 1

Napoletano *et al.* Figure 2

Step1: Identify Apoptosis

Incorporation of propidium iodide (in absence of secondary necrosis) $\overline{}$ PI+

TUNEL / activated Caspase 3

Step2: Identify Necrosis

Step3: Measure Autophagy

Autophagic markers (LC3, p62) Autophagy flux assays (Rap/Baf)

mPT-dependent necrosis

Parthanatos

Germ cell necrosis

Ferroptosis

Napoletano *et al.* Figure 3

Napoletano *et al.* Figure 4