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1 Intersections between regulated cell death and autophagy

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23 ABSTRACT

24 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 25 in several pathologies such as inflammatory and neurodegenerative diseases. This has been 26 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 28 29 methodologies for correct identification of the different regulated cell death forms. We then 30 discuss how the autophagy machinery is directly associated with specific cell death forms and 31 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 32 presides over life and death in specific cellular contexts. 33

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35 Keywords: Cell Death, Apoptosis, Necrosis, Autophagy.

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37 REGULATED CELL DEATH, DIVERSITY AND IMPLICATIONS FOR 38 MULTICELLULAR LIFE

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40 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 41 42 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; 43 44 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 45 46 exclusive to multicellular organisms, it is here that it acquires heightened importance. The 47 essential roles of RCD are diverse, ranging from shaping fully functional organs and body parts 48 during development to orchestrating adaptive or maladaptive responses to prolonged internal or external harmful perturbations of cellular homeostasis. When cellular stress responses 49 cannot cope with such perturbations, they lead to cell death as an adaptation to a sensed failure 50 to reinstate cellular homeostasis, or, in the maladaptive scenario, as an unwanted by-product of 51 the attempt to restore the required equilibrium. 52

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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

57 release or exposure of Damage Associated Molecular Patterns, alarmins, chemokines and cytokines. The well characterized cell death modality known as apoptosis is the prototypical 58 form of RCD (Fig. 1). Conversely, necrosis (Fig. 1) has long been considered exclusively an 59 accidental process triggered by physicochemical insults. However, recent research has led to 60 the establishment of regulated necrosis (RN) as a non-apoptotic form of RCD [1]. Moreover, 61 autophagy, the catabolic pathway centred on lysosomal degradation of bulk or selected cargo 62 63 has also been recognised as a key regulator and, in some instances, executor of cell death [2]. 64 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 65 non-accidental processes that are subject to molecular regulation and specificity. However, it 66 remains an open issue whether these forms of non-apoptotic cell death ever achieve a type of 67 highly sophisticated regulation as that of apoptosis. In addition, it is unclear whether they 68 develop into programmed cell death (PCD) that is absolutely required for the normal 69 physiology of an organism [Box 1] or are triggered by external or internal pathological events. 70

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A major ontological issue raised by RCD diversity is what can be defined as cell death, as 72 73 opposed to non-lethal cell degeneration. Whether a precise demarcation line can be drawn is 74 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 75 76 been significantly compromised, to a point that is incompatible with the ability of the cell to 77 maintain itself and this results in the emergence of specific death morphologies. How the 78 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 79 80 cell death forms, of their intersections and of autophagy can be found elsewhere [3-5]. Here, 81 we focus on the interplay between select forms of RCD, as defined in the recent community 82 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 83 factors that bias towards different cell death forms. 84

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88 ROBUST EXPERIMENTAL IDENTIFICATION OF RCD FORMS AS A PRE89 REQUISITE FOR THE DETERMINATION OF INTERSECTIONS IN CELL DEATH

90 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]. 91 A propaedeutic topic to such questions is that of correct identification of the RCD forms, 92 without which any attempt at defining rules is grounded in unsafe territory. For instance, RN 93 was neglected for many years, and the realization that apoptosis was in fact overestimated, 94 came as a surprise for the research community. The lack of systematic studies on RN was due 95 96 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 97 98 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 99 in autophagosomes did not discriminate contexts in which autophagy was hyperactive from 100 101 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 102 that non-apoptotic RCD forms can be identified through a 3-step analysis (Fig. 2). In detail, 103 extensive guidelines exist on autophagy assays and their interpretation [10, 11], while here we 104 provide a brief list of assays for RN (see Box 2). 105

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.

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114 MECHANISM AND OUTCOME OF AUTOPHAGY INTERACTIONS WITH OTHER 115 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.

126

127 Mutual inhibition between autophagy and apoptosis

Autophagy interactions with RCD (Fig. 4) have been primarily elucidated in the interplay with 128 129 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 130 131 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 132 proteins [15]. The formation of the Beclin1/Bcl2 complex is sufficient to inhibit autophagy, 133 interfering with the formation of the Beclin1/Vps34 complex [16, 17]. In addition, during 134 apoptosis, caspases cleave Beclin1, inactivate its pro-autophagy function [18], while its 135 carboxy-terminal fragment re-localises to the mitochondria stimulating the pro-apoptotic 136 release of Cytochrome C [19]. This provides a further shut down mechanism to restrict the pro-137 survival function of autophagy and an amplifying loop enhancing apoptosis. Beclin1 also 138 interacts with NDR kinases that regulate the balance between autophagy and apoptosis by 139 140 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 141 142 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 143 144 inactivate autophagy in cardiomyocytes [23]. However, MST kinases and their downstream pathways are rather required for autophagy processes in most other contexts including 145 autophagic cell death of the Drosophila salivary glands, neuronal homeostasis and autophagic 146 clearance of bacteria [24-26], suggesting a context-dependent role for MST kinases. Another 147 148 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 149 required for p53-induced apoptosis [28]. DRAM proteins are lysosomal factors that stimulate 150 autophagy [29] and lysosomal membrane permeabilization [30], with the ability of mediating 151 both cell survival and cell death. In *Drosophila*, expression of $\Delta Np53$ (p53A) but not of p53 152 (p53B), induces photoreceptor apoptosis with elevated levels of the cleaved effector caspase, 153 Death caspase-1 (Dcp-1), and inhibition of the autophagy flux [31]. Interestingly, the 154 concomitant expression of $\Delta Np53$ and inhibition of initiator or effector caspases restores a 155

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 proapoptotic 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.

163 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 164 have been proposed to explain this effect. An efficient way of limiting apoptosis is the 165 activation of mitophagy that specifically recognizes and eliminates damaged mitochondria. 166 Animal and cellular models of Parkinson's Disease (PD) accumulate leaky mitochondria that 167 induce oxidative stress contributing to the elimination of dopaminergic neurons by apoptosis 168 [37, 38]. Similarly, autophagy can inhibit apoptosis by degrading factors like the BH3 domain-169 containing protein PUMA, which promotes Mitochondrial Outer Membrane Permeabilization 170 (MOMP) [39]. Another proposed mechanism for the inhibition of apoptosis by autophagy 171 during neurodegeneration is the elimination of activated caspases (Fig. 3) that accumulate in 172 autophagic vacuoles in dystrophic neurites, as shown in Alzheimer's Disease (AD) mouse 173 models and in human brains of AD patients [40]. Finally, a mechanism through which 174 autophagy intersects with apoptosis in a different context offers particularly intriguing links 175 176 with neurodegenerative diseases involving aggregation-prone proteins. Pro-apoptotic amyloid structures such as those assembled by the TIR-domain-containing adapter-inducing interferon-177 178 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 179 180 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]. 181 Autophagosomes indeed act as scaffolds for the intracellular death-inducing signalling 182 complex (iDISC) activation of Caspase 8 in apoptosis [45]. These studies suggest a more 183 complex relationship between autophagy and apoptosis. A most revealing investigation shows 184 that this relationship may be influenced by the level of the autophagy flux [46]. Within the 185 same population of BJAB B-cell lymphoma cells, those with a high autophagy flux degrade 186 efficiently Fap-1, an inhibitor of Fas-mediated apoptosis, sensitizing the cells to Fas-ligand 187 induced apoptosis. Interestingly this effect was specific for Fas, as treatment with TNF-related 188

apoptosis-inducing ligand (TRAIL) resulted in the opposite, with cells at low flux experiencinga 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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195 Autophagy and necrosis: a multifaceted relationship

Autophagy is also highly interconnected with necroptosis (Fig. 4). Autophagy directly 196 197 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 198 199 RIPK1 inhibits autophagy through ERK phosphorylation of the TFEB transcription factor [49] 200 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 201 been shown to be required for necroptosis, including providing a scaffolding surface for the 202 necrosome [51-53]. Components of the autophagy machinery can even determine a switch 203 between alternative RCD forms. For instance, p62 acts as a scaffold for the activation of RIPK1 204 205 by recruitment of the latter to a complex with other Atg proteins. This triggers necroptosis in 206 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]. 207 208 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 209 210 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 211 212 on the intersection of autophagy and the other RN sub-forms, especially ferroptosis and 213 mitochondria-dependent necrosis, may provide additional insights in this respect. For instance, 214 autophagy has been shown to promote ferroptosis by degrading ferritin, leading to higher accumulation of iron [55]. Another possible intriguing connection between ferroptosis and 215 autophagy may be revealed through the additional function as an oxidative stress sensor of the 216 p62/SQSTM1 autophagy receptor [56], which however promotes survival in this case. Thus, 217 also the relationship between autophagy and ferroptosis appears to incorporate both facilitating 218 219 and inhibitory drives.

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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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227 AUTOPHAGY-ASSOCIATED CELL DEATH IN DEVELOPMENT AND228 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.

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233 Death driven by autophagy activation

ACD has been characterised in invertebrate models and has a marked importance in Drosophila 234 metamorphosis. As this process is essential for the physiology and development of Drosophila, 235 this form of cell death acquires characteristics of PCD. It contributes, either independently or 236 in combination with caspases, for the elimination of the midgut and salivary glands [2, 57, 58]. 237 Detailed analysis in the midgut highlights a selective requirement for the autophagy initiation 238 complex genes Atg1, Atg13 and Atg17, as well as the upstream Tor pathway. Downstream, 239 240 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 241 242 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, 243 244 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 245 engulfment receptor Draper [61]. The association between vast organismal remodelling and 246 ACD raises the question of whether ACD is the selective RCD form for metamorphosis and, 247 248 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 249 treatment [65, 66], as a response to hypoxia [67] and often upon inhibition of apoptosis [68, 250 69] or in apoptosis-incompetent Bax/Bak mutants in-vitro and in-vivo [70, 71]. Recent 251 evidence however suggests that fibroblasts mutant for Bax/Bak1 are resistant to ACD and that 252 this can be restored by rescuing lysosomal membrane permeability with monomeric Bax or 253 254 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, 255 Lysosome Dependent Cell Death (LDCD) [3], which requires lysosomal membrane 256

- 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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272 Death by autophagy impairment

Whereas ACD progresses through hyper-active autophagy, autophagy is more commonly a 273 274 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-275 276 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 277 278 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 279 280 further consideration since significant in-vivo evidence for non-apoptotic RCD in 281 neurodegeneration has been provided, also alongside apoptosis [40, 81-84] and the inhibition 282 of autophagy in the nervous system was also shown to lead to a proposed novel form of cell death, named karyoptosis [85]. 283

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 291 mutants. Moreover, on the basis of the morphological descriptions given (cell shrinkage, 292 distorted nuclei, accumulation of lysosomal compartments such as lipofuscin and plasmalemmal blebbing), karyoptosis may represent the Caspase 3-negative cell death form 293 observed in models for AD [40] and in polyglutamine diseases [82, 91, 92]. A feature of 294 295 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 296 297 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 298 299 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 300 301 LCD and karyoptosis. Specific *in-vivo* and *in-vitro* hallmarks of karyoptosis are cytoplasmic 302 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 303 p62 receptor [87] in exopher-like structures [96]. The reliance of karyoptosis on the LaminB1-304 LC3 nucleophagy machinery activated in senescence [86] suggests that karyoptosis may as 305 well represent progression of a form of terminal senescence into non-apoptotic cell death. The 306 307 association of the key karyoptosis molecule, LaminB1, with other RCD forms has been 308 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 309 310 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. 311 312 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 313 314 cell shrinkage, a feature also shared by karyoptosis. Finally, karyoptosis appears to be more 315 easily detected *in-vitro* in slowly dividing cells, and we speculate that this form of cell death 316 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 317 a prominent form of neuronal cell death in aggressive neurodegenerative diseases with a clear 318 impairment of autophagy accompanied by significant accumulation of nuclear misfolded 319 proteins. Several open questions in the relationship between karyoptosis and autophagy remain. 320 What are the initiating stimuli for the expulsion of LaminB1 and how is its association with 321 LC3 regulated? What components of the autophagy machinery are directly involved in 322 323 mediating the degradation of the nucleus? Is this a canonical autophagy process?

324 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 325 cell death is whether this is linked to autophagy for death and/or clearance, as suggested in a 326 model of retinitis pigmentosa [84]. While in some cases the cell death evolves into an apoptotic 327 328 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 329 330 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 331 332 autophagy-associated cell death forms.

333

In summary, the autophagy machinery is directly involved in determining some specific cell 334 death morphologies. Whether by hyperactivation or by inhibition of the autophagy flux, 335 autophagy-associated forms of cell death seem to display a distinctive link with clearance of 336 cellular content. This association raises a number of issues on whether clearing represents a 337 distinct, and subsequent, process from cell death per se, and will require additional clarifying 338 studies in a number of cases. However, this self-clearing activity, either through lysosomal 339 340 digestion or through excretion in the extracellular space, may as well constitute an integral part 341 of the killing process and be the distinctive feature of autophagy-associated forms of cell death.

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344 Concluding remarks

345 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 346 347 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 348 349 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 350 death process. In-depth knowledge of the essential role for autophagy in modulating, and in 351 some contexts mediating, cell death is likely to be vital for the informed design of any future 352 therapeutic strategies aiming at controlling cell death in human pathologies (see Outstanding 353 Questions). 354

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357 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.

362

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

366

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.

371

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.

376

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

381

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.

386

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.

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391 Oxidative stress: the damage resulting from accumulation of highly reactive chemical 392 radicals, mainly produced either during mitochondria respiration or as a result of iron 393 metabolism.

394

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Box 1. Regulated Necrosis as physiologically Programmed Cell Death

Many forms of regulated cell death arise in pathological situations, during neurodegeneration
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
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
an example for RN.

Similar to apoptosis, studies in model organisms have proven fundamental to reveal the 417 418 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 419 420 hyperplasia [102] and in which p53-dependent transcription is activated selectively in 421 Drosophila necrotic male germ cells. Interestingly, Drosophila male germ cell RN requires the 422 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 423 424 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, 425 both part of the HOPS complexes that promote vesicular docking and fusion) and of lysosomal 426 enzymes that can be released upon lysosomal membrane permeabilization (Dnase-II, 427 Cathepsin-D). The formers are important for autophagy [107, 108] and the latter occurs also 428 during Lysosome Dependent Cell Death (LDCD). Drosophila male germ cell RN may 429 therefore represent an ancient form of RCD including the features of mitochondria-dependent 430 apoptotic (primordial Caspase 9 and Debcl function) and necrotic RCD forms, and perhaps 431 also of LDCD. These observations also suggest that key regulators of mitochondria-dependent 432 433 RCD pathways, including Bcl-2 proteins, initiator caspases and p53, may have evolved a proapoptotic role on top of a primordial pro-necrotic function. Since several RN genes are 434 evolutionarily conserved and a mitochondria-lyososome RN axis has been observed as early as 435 in *Dictyostelium*, it is tempting to propose that RN may predate the appearance of apoptosis. 436 437 This pathway may represent a quality control system to eliminate damaged/unfit germ cells whose genome integrity is compromised, which could be harmful for the offspring and also 438 439 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 440 441 during spermatogenesis.

442

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 transmission electronic microscopy (TEM) [110]. To demonstrate that necrotic features observed by TEM are not secondary to apoptosis, time course analysis is required. 447

Caspase-independent DNA fragmentation - The Terminal deoxynucleotidyl transferase dUTP 448 nick end labeling (TUNEL) is very sensitive to detect 3'hydroxyl (3'OH) termini of cleaved 449

DNA strand. This is typically generated by type I DNAses, such as caspase activated-DNAses
(CAD) that execute apoptosis. However, 3'OH ends can be produced also by dephosphorylation
of 3'PO₄ ends generated by caspase-independent type II DNAses, typically through activity of
acid phosphatases released upon lysosomal membrane permeabilization in necrotic cells [111].
Phosphatase pre-treatment has been used to allow/enhance labelling of necrotic cells with
TUNEL [110, 112]. To distinguish necrosis from apoptosis using TUNEL, co-labelling with
an anti-activated Caspase 3 antibody is required. As caspase activation precedes chromatin
cleavage during apoptosis, a time course analysis is recommended. Alternatively, the Apoptag
ISOL Dual Fluorescence assay uses Topoisomerase I and T4 DNA Ligase for oligonucleotidemediated 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 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 7aminoactinomycin D (7-AAD) are required.

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

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

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

RNA loss - Spectral properties of Acridine Orange (AO), a membrane-permeable nucleic acidbinding dye, have been recently exploited to distinguish between apoptotic and necrotic cells
[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

RCD form	Examples in Physiology and	Experimental
	Pathology	conditions
Apoptosis	Elimination of supernumerary cells,	Mouse, Drosophila, C-
	Homoostasis of the immune system	troated with DNA
	Evidence of epoptosis in most	demoging agents EP
	Evidence of apoptosis in most	damaging agents, ER
	degenerative pathologies, diabetes, viral	stress inducers
	infections	
Necroptosis	Anti-viral defence [123-125]	Humal cell models of
	Axonal and dendritic degeneration	viral infection
	[126], Ischemia [127, 128]	
		Myocardial Ischemia in
		<i>Ripk3</i> and <i>Mlkl</i> mutant
		mice
		Cell cultures treated
		with TNF α and
		inhibitors of apoptosis
Mitochondria-	Germ Cell Death during Drosophila	Drosophila
dependent necrosis	spermatogenesis [102]	spermatogenesis
	mitochondrial permeability transition	Mouse middle cerebral
	(mPT)-dependent necrosis in brain,	artery occlusion +
	cardiac and renal ischemia [129, 130]	CypD inhibitors
	Parthanatos in Stroke, kidney ischemia	<i>parp1</i> knock out mice
	and retinal degeneration	I I I I I I I I I I I I I I I I I I I
Ferroptosis	Tumor suppression [131-133]	Mouse xenograft
	Glutamate neurotoxicity [134]	cancer models +
		Erastin or GPX4
	Sepsis, renal, hepatic ischemia [135]	inhibitors
	Mouse ischemia models [136]	
		Mouse <i>Gpx4</i> knock out
		-
Autophagic cell	Salivary gland and midgut elimination	Drosophila midgut and
death/autosis	in Drosophila [57, 58]	salivary glands
		J B
	Inhibition of caspases [68, 69]	Rat Ischemia

	Cerebral hypoxia-ischemia [74]	Cell cultures with oncogenic insults or apoptosis incompetent
Lysosomal	Cells deficient in apoptosis or treated	Cell cultures treated
Dependent Cell	with lysosomotropic drugs [72].	with lysosome-
Death	Mammary gland involution [75, 76]	destabilizing agents or calcium ionophores.
	Retinitis pigmentosa [137]	
Karyoptosis	Neurodegeneration [87]	Drosophila and mouse models of
	Tumour suppression and senescence [86]	neurodegeneration
		Cell cultures treated with autophagy inhibitors.

490

491 FIGURE LEGENDS

492 Figure 1. Overview of the morphological changes in different forms of Regulated Cell493 Death.

494 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, 495 496 regulated necrosis and molecules/events influencing it are in red. The central cell with healthy 497 nucleus, with an intact genome, healthy mitochondria and a homeostatic autophagy flux can undergo cell death through different modalities. Apoptosis is characterised at morphological 498 level by cell shrinkage and cell and nuclear fragmentation into self-contained large apoptotic 499 500 bodies. In addition, mitochondria may degenerate and become permeabilised, and autophagy may be inhibited. Regulated necrosis is instead characterised by cell and nucleus swelling, 501 cytoplasm granulation, plasma and nuclear membranes rupture releasing conspicuous cell 502 content. In addition, some organelles such as mitochondria and lysosomes may become 503 504 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 505 506 characterised by cell shrinkage, nuclear shape alteration, accumulated undigested 507 autophagosomes and LaminB1 in the cytoplasm often co-localising with autophagic markers. 508 Finally, large macrovesicles or exophers with LaminB1 and other cellular content bleb from 509 the plasma membrane.

510

511

512 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 513 514 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 515 apoptosis using different set ups and technologies. These include visualization of chromatin 516 condensation by Transmission Electron Analysis (TEM), and quantification of caspase-517 dependent PARP1 cleavage in protein extracts. Depending on the set-up, apoptosis can be 518 confirmed by pharmacological or genetic inhibition of key components for instance 519 suppression by effector caspase inhibitors (e.g. zVAD) and through downregulation or 520 mutations in required factors (e.g. Apaf-1 or Caspase 8). A cautionary note should be applied 521 522 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 523 524 form in a wt condition.

525 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 526 Propidium Iodide incorporation - provided that secondary necrosis due to other forms of cell 527 death, often observed in cultured cells, has been ruled out. Additional assays are discussed in 528 Box 2. Specific RN sub-forms can be revealed in some set ups by the use of inhibitory 529 compounds or mutations, although care should be applied to consider a possible switch to other 530 RCD forms. Necroptosis requires RIPK3 and/or MLKL, while the use of RIPK1 inhibitors (e,g, 531 532 Necrostatin-1) should consider the existence of RIPK1-dependent apoptosis. mPT-dependent 533 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 534 535 through mutations in required mitochondrial factors (e.g. Pinkl, EndoG). Ferrostatin is a reliable inhibitor of ferroptosis. 536

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 LaminB1⁺/LC3⁺ cytoplasmic puncta and, in cell cultures, of excreted LaminB1 and p62 in the microvescicular fraction in the medium.

548

549 Figure 3. Autophagy at a glance.

550 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 551 552 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 553 phosphatidylinositide 3-kinase and additional adaptors is activated and promotes the formation 554 of the phagophore. Thereafter the elongation is driven by the Ubiquitin-like cascades involving 555 Atg5/Atg12 conjugation and LC3 (Atg8) cleavage and lipidation with Phosphoethanolamine 556 (PE). The cargo recognition is determined through the interaction between autophagy receptors 557 like p62/SQSTM1 and lipidated proteins of the LC3 family present on the membrane of the 558 autophagosomes. Finally, upon autophagosome closure and maturation, the fusion with the 559 lysosome is determined through the HOPS machinery, Rab 7 and a set of SNARE proteins. 560

561

562 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 563 represented by different colours. Apoptosis, triggered by extrinsic death receptors on the 564 plasma membrane or by intrinsic BCL2-dependent release of Cytochrome C (CytC) from 565 566 damaged mitochondria, is antagonised by autophagy via the degradation of caspases in autophagic vacuoles and of mitochondria through mitophagy. In some contexts, however, 567 autophagosomes facilitate apoptosis by providing a scaffold for the activation of Caspase 8. 568 569 Apoptosis antagonizes autophagy through Caspase 8 cleavage of the p62 receptor. Effector caspases inhibit autophagy by cleaving Atg6/Beclin1 and other pro-autophagic factors. 570 Autophagy antagonizes necroptosis by degrading RIPK1 in some contexts, while in other 571 contexts both autophagosomes and the p62 autophagy receptors promote necroptosis by 572 helping through scaffolding the assembly of the RIPK1/RIPK3/MLKL necrosome. Autophagy 573 also promotes Ferroptosis through degradation of Ferritin. NDR1, MST1/2 kinases and p53 act 574

- as molecular switches that allow control of apoptosis, autophagy or RN depending on context.
- 576 MST1/2 kinases activate NDR1 kinase that either induces apoptosis or interacts with Beclin1
- 577 promoting autophagy. MST1/2 also regulates directly autophagy and apoptosis through
- 578 transcriptional regulation in the nucleus. p53 activates either autophagy, apoptosis or
- 579 mitochondria-dependent RN in different contexts either via transcription or via cytoplasmic
- 580 functions.
- 581
- 582

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Autophagic Cell Death

Karyoptosis

Napoletano et al. Figure 1

Step1: Identify Apoptosis

TUNEL / activated Caspase 3 co-labelling (antibodies or sensors)



Step2: Identify Necrosis

Incorporation of propidium iodide (in absence of secondary necrosis)

Step3: Measure Autophagy

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



Napoletano et al. Figure 2



mPT-dependent necrosis



Parthanatos

Germ cell necrosis

Ferroptosis



Napoletano et al. Figure 3



Napoletano et al. Figure 4