PERSPECTIVES

OPINION

Autophagic cell death: the story of a misnomer

Guido Kroemer and Beth Levine

Abstract | Dying cells often display a large-scale accumulation of autophagosomes and hence adopt a morphology called autophagic cell death. In many cases, it is agreed that this autophagic cell death is cell death with autophagy rather than cell death by autophagy. Here, we evaluate the accumulating body of literature that argues that cell death occurs by autophagy. We also list the caveats that must be considered when deciding whether or not autophagy is an important effector mechanism of cell death.

Cell death is associated with at least three morphologically distinct processes that have been named apoptosis, autophagic cell death (ACD) and necrosis (BOX 1). ACD is characterized by the large-scale sequestration of portions of the cytoplasm in autophagosomes, giving the cell a characteristic vacuolated appearance[1]. Autophagosomes can be identified by transmission electron microscopy as double-membraned vesicles that contain cytosol or morphologically intact cytoplasmic organelles (such as mitochondria or the endoplasmic reticulum (ER)). Autolysosomes, which arise from the fusion of autophagosomes and lysosomes, are defined by a single membrane that contains degenerating (and often unrecognizable) organelles that undergo degradation through the process of macroautophagy (called autophagy throughout this article)[2].

Although there is no doubt that cells can manifest large-scale autophagy shortly before or during their death (referred to herein as cell death with autophagy), we share the opinion that autophagy is rarely, if ever, the mechanism by which cells actually die (referred to herein as cell death by autophagy) (FIG. 1). In this article, we will discuss whether the expression ACD, which is a linguistic invitation to think that cell death is executed by autophagy, is a misnomer.

A brief history of ACD

Richard Lockshin coined the expression programmed cell death (PCD) more than 40 years ago, when he described the developmental cell death process in the intersegmental muscles of silk moths[3]. Remarkably, this first example of PCD had the morphology of ACD. However, apoptosis was regarded as the principal cell death mechanism following its initial description by Wyllie and Kerr[4] and the discovery of the apoptotic machinery in *Caenorhabditis elegans*[5] and caspases in mammals[6] in the 1980s. After this eclipse by apoptosis, the notion of ACD regained momentum in the 1990s, prompted by the discovery of the autophagy-related (*ATG*) genes[7] and the increasing realization that cell death can occur in a caspase-independent manner, with a non-apoptotic morphology[8].

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Some of the renewed momentum of ACD came from observations that, on further consideration, had explanations unrelated to ACD. For example, some researchers in the cell death field interpreted as ACD the autophagic vacuolization that is observed in degenerative muscle and neuronal diseases, such as Danon disease (vacuolar skeletal myopathy and cardiomyopathy) and Parkinson's disease[9,10]. However, it is now well accepted that this increased autophagic vacuolization reflects defects in autophagosomal maturation and, hence, decreased, rather than increased, autophagy[2] (TABLE 1). Further, Kuchino and co-workers reported that spontaneously regressing neuroblastomas died from non-apoptotic cell death while exhibiting "ultrastructural features of autophagic generation" that could be mimicked by transfecting activated Ras into cancer cell lines[11]. However, later studies showed that activated Ras triggers death in glioblastoma cells by hyperstimulation of macropinocytosis rather than by autophagy[12] (TABLE 1). Moreover, several anticancer drugs, such as temozolomide[13] and suberoylanilide hydroxamic acid[14], a histone deacetylase inhibitor, were reported to induce ACD in glioblastoma cells, although later discoveries indicated that autophagy induced by these agents exerts cytoprotective rather than cytotoxic effects[15,16].

In 2004, Lenardo and colleagues[17] reported that, in U937 monocytoid cells and L929 fibrosarcoid cells, non-apoptotic cell death induced by caspase-8 inhibition is blocked by RNA interference (RNAi)-mediated knockdown of two essential *ATG* genes, *Becn1* (which encodes beclin-1 and is also called *Atg6*) or *Atg7*. During the same year, Tsujimoto and colleagues[18] reported that, in response to etoposide or staurosporine, *Bax*^{−/−}*Bak*^{−/−} mouse embryonic fibroblasts (MEFs) undergo non-apoptotic cell death accompanied by large-scale autophagic vacuolization, which can be reduced by knockdown of *Atg5* or *Becn1*. Subsequently, many additional studies have been published that show a reduction or alteration in cell death following *ATG* knockdown *in vitro*[8], and a few studies have reported similar findings *in vivo*[19–21] (TABLE 2).

In parallel with the growth in research on autophagy and cell death, there has been an explosion of research on autophagy and cell survival. The ability of autophagy to recycle nutrients, maintain cellular energy homeostasis and degrade toxic cytoplasmic constituents (and microbial intruders) helps to keep cells alive during nutrient and growth factor deprivation and other stressful conditions[7]. In cultured mammalian cells, *ATG* knockout or knockdown accelerates or enhances cell death that is induced by


Box 1 | Cell death modalities

The current nomenclature uses three terms to define morphologically distinct cell death modalities[1,65].

Apoptosis. Also known as type 1 cell death, apoptosis is accompanied by a rounding up of the cell, retraction of pseudopods, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), few or no ultrastructural modifications of cytoplasmic organelles, and plasma membrane blebbing, but the integrity of the cell is maintained until the final stages of the process (see figure, part **a**). Hence, the term apoptosis should be applied exclusively to cell death events that occur while manifesting these morphological features[66]. The term programmed cell death (PCD) refers to death that occurs during development, and is not synonymous with apoptosis, as PCD can occur with non-apoptotic features. There are several distinct subtypes of apoptosis that, although morphologically similar, can be triggered through different biochemical routes (for example, through the intrinsic or the extrinsic pathway and with or without caspase activation). Furthermore, the apparent uniformity of apoptotic cell death might conceal heterogeneous functional aspects, such as whether or not dying apoptotic cells are recognized by the immune system[1].

Autophagic cell death. Also known as type 2 cell death, autophagic cell death (ACD) is morphologically defined (especially by transmission electron microscopy) as a type of cell death that occurs in the absence of chromatin condensation but is accompanied by large-scale autophagic vacuolization of the cytoplasm (see figure, part **b**). Although the term ACD is a linguistic invitation to think that cell death is executed by autophagy, the term simply describes cell death with autophagy (FIG. 1).

Necrotic cell death or necrosis. Also known as type 3 cell death, necrotic cell death or necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles and plasma membrane rupture and subsequent loss of intracellular contents (see figure, part **c**). Necrosis has traditionally been considered merely as an accidental, uncontrolled form of cell death that only occurs in pathological circumstances. However, evidence is accumulating that the execution of necrotic cell death might be finely regulated by a set of signal transduction pathways and catabolic mechanisms[43,67]. The term 'necroptosis' has been created to define regulated (rather than accidental) necrosis that involves signalling through the kinase RIP1 (REF. 68). Scale bars, 1 μm. Images reproduced, with permission, from *Cell Death and Differentiation* REF. 65 © (2007) Macmillan Publishers Ltd. All rights reserved.
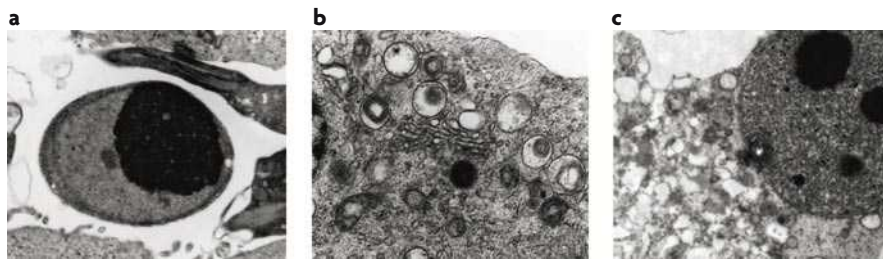
starvation, growth factor withdrawal, infectious agents and cytotoxic chemotherapeutic agents[8]. Moreover, *ATG* genes have been shown to be essential for organismal survival during starvation in diverse eukaryotic organisms[2,7].

Based mostly on the growing evidence that autophagy exerts cytoprotective effects, several investigators have proposed an operational definition of ACD that would clearly distinguish it from autophagy *per se* or from cell death with autophagy[22–26]. The underlying principle of this operational definition is that ACD is not only accompanied by the accumulation of autophagic vacuoles, but also involves an increase in autophagy that contributes to cell death. In other words, specific inhibition of autophagy should avoid irreversible loss of cellular functions and keep the cell alive.

Rescue from cell death: loopholes

The operational definition of ACD implies that suppression of autophagy reduces cell death. However, this definition fails to define what constitutes a biologically significant level of cell death suppression (in the setting of nearly complete inhibition of autophagy). It also fails to define whether abolition of autophagic features in dying cells without avoidance of cell death constitutes suppression of autophagic cell death, and whether the altered death kinetics in autophagy-inhibited dying cells signifies a role of autophagy as a lethal effector mechanism. We propose that these issues comprise quantitative and qualitative loopholes in the operational definition of ACD that allow most authors to 'correctly' use the term ACD, although, in many cases, the true role of autophagy in cell death remains uncertain.
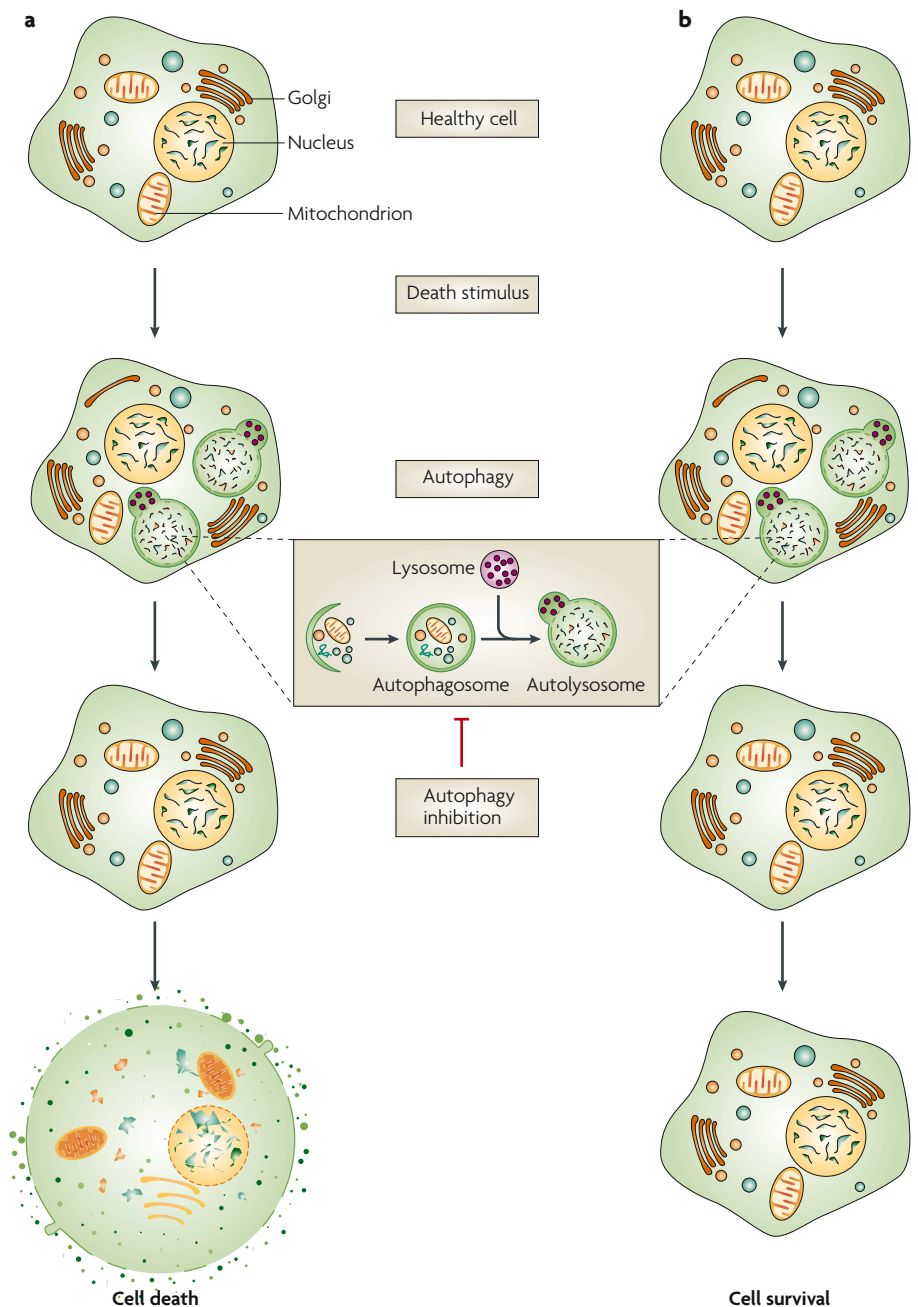
Quantitative loopholes. In several studies, inhibition of autophagy results in partial inhibition of cell death[27–34]. In many cases, some early signs of cell death (such as plasma membrane permeabilization) are inhibited, whereas others (such as DNA degradation) are not[35]. This is commonly interpreted as evidence that there is both autophagy-dependent cell death (for example, as measured by assays that detect plasma membrane permeabilization) and autophagy-independent cell death (for example, as measured by the quantification of cells in which DNA has been degraded). An important alternative interpretation, that autophagy inhibition might selectively affect some of the 'markers' of cell death, is rarely considered. For example, one marker of apoptotic cell death is phosphatidylserine exposure on the plasma membrane and, at least in a mouse embryonic developmental model system, this process requires *ATG*-dependent ATP production in dying cells[36]. It is theoretically possible that autophagy inhibition could alter other more general markers of cell death, such as changes in plasma membrane permeabilization.

A related important concept is that autophagy inhibition might alter the kinetics of one marker compared with another marker of cell death, without altering the eventual percentage of cells that die. Unfortunately, most studies in the ACD literature fail to perform kinetic analyses of cell death at serial time points to confirm that cell death — not just one marker of cell death at one time point — is truly blocked, and few studies assess the effects of autophagy inhibition on clonogenic survival, which is often considered to be the gold standard for proving that an intervention has blocked cell death[37]. Results of kinetic studies often show delayed, rather than fully inhibited, cell death, and it is unclear how important the role of autophagy is in such cell death. Furthermore, results from clonogenic survival studies, although statistically significant, have been of unclear biological importance. For example, knockdown of *Atg5* or *Becn1* results in <10% clonogenic rescue in *Bax⁻/⁻Bak⁻/⁻* MEFs that have been treated with staurosporine or etoposide[18], or in human lung cancer cells that are irradiated in the presence of a caspase inhibitor[29]. This type of partial inhibition of cell death poses a dilemma. On the one hand, one might argue that the experimental abolition of autophagy (down to baseline levels) should lead to complete avoidance of cell death if autophagy is responsible for cell death. On the other hand, one might

argue, as is commonly done in the literature, that multiple cell death pathways operate concurrently, and that partial inhibition of cell death by autophagy inhibition reflects the coexistence of multiple death pathways. In this case, one would expect to see only a decrease in the total fraction of cell death that is truly autophagic following autophagy inhibition, except in scenarios in which autophagy is thought to lie upstream of apoptotic or necrotic cell death (see below).

How important is autophagy as a death effector mechanism if it is only responsible for an acceleration of cell death that would still occur in its absence, or for a fraction of the cell death that occurs in response to a certain stimulus in a given population of cells? In the case of apoptosis, there are many clear examples in which cell death is unequivocally blocked when components of the core apoptotic machinery are inactivated. For example, $Bax^{-/-}Bak^{-/-}$ mice have multiple developmental abnormalities that result from absent PCD, including the persistence of interdigital webs, an imperforate vaginal canal and excess cells in the nervous system and haematopoietic system[38]. Furthermore, cells derived from $Bax^{-/-}Bak^{-/-}$ mice are resistant to death that is induced by growth factor deprivation, B-cell lymphoma-2 (BCL2) homology-3 (BH3)-domain-only proteins, ultraviolet radiation, chemotherapeutic agents and ER stress[39,40]. To our knowledge, there is no similar evidence that autophagy-deficient cells show a complete resistance to cell death.

Qualitative loopholes. Beyond these quantitative loopholes in the operational definition of ACD, there are important qualitative loopholes that pose equally perplexing dilemmas. One of the clearest examples is with the model organism *Dictyostelium discoideum*, a soil amoeba that lacks Bcl2 and caspases, and hence is an ideal model for studying non-apoptotic death pathways[41]. In a *D. discoideum* monolayer system, starvation induces autophagy, whereas starvation together with differentiation-inducing factor (Dif) induces cell death that is characterized as autophagic or vacuolar cell death. This offers a unique opportunity to dissociate autophagy (which is Dif-independent) and cell death (which is Dif-dependent). In conditions of cell death induction (that is, starvation plus DIF), disruption of the autophagy gene *atg1* blocks signs of autophagy and vacuolization, but cell death still occurs and has a necrotic morphology[42]. These observations are interpreted as evidence that ACD is a bona fide mechanism of cell death. Parallel to the consequences of apoptosis inhibition

in animal cell death[21,43], disruption of ACD results in necrotic cell death in *D. discoideum*. It is not clear how to distinguish between this interpretation — that is, ACD is a bona fide

Figure 1 | **'Cell death with autophagy' versus 'cell death by autophagy'.** In both scenarios, a death stimulus results in morphological features of autophagy, and in both cases, pharmacological or genetic inhibitors of autophagy block these morphological features. **a** | In cell death with autophagy, the inhibition of autophagy does not alter the fate of the cell (that is, cell death), although it might change the kinetics of this fate and the morphological appearance of the dying cell, as well as the read-outs of some assays used to measure cell death, the type of cell death pathway used by the cell and/or the rate of degradation of the cell. If inhibition of autophagy accelerates cell death, autophagy is considered to be a pro-survival pathway in the dying cell. **b** | If cell death were to occur by autophagy, the inhibition of autophagy would alter the fate of the cell and result in long-term cellular survival. Note that the blockade of autophagy only leads to the disappearance of autophagosomes if it intervenes at the level of autophagosome formation.

mechanism of cell death — and the alternative interpretation that ACD is not a death mechanism because autophagy inhibition does not block cell death.

Table 1 | **Difficulties in assessing the contribution of autophagy to cell death**

Category	Examples	Refs
Misdiagnosed 'autophagy'	Ras-induced vacuolization of glioblastoma cells, which was initially interpreted as a sign of autophagy, actually results from large-scale macropinocytosis	11,12
Discrepancy in results of different assays used to measure cell death	A partial inhibition of plasma membrane permeabilization by knockdown of *ATG* genes is not accompanied by an inhibition of DNA degradation	35
Increased numbers of autophagosomes might represent increased or decreased autophagy	Accumulation of autophagosomes in the muscle from patients with Danon disease, in $Lamp2^{-/-}$ mice or after chloroquine intoxication; this accumulation is due to the failure to remove autophagosomes (through fusion with lysosomes) and hence reflects an inhibition of autophagy	80,81
Quantitative discrepancy between the magnitude of autophagy inhibition and cell death inhibition	Complete inhibition of signs of autophagy (such as LC3 lipidation and GFP–LC3 aggregation in cytoplasmic dots) leads to only partial inhibition of cell death	18,32,33
Lack of specificity of autophagy inhibitors	Pharmacological inhibitors of autophagy are nonspecific, whereas specific knockout or knockdown of *ATG* genes can affect autophagy-unrelated functions	46,50,52
Autophagy for optimal corpse clearance by phagocytes	Failure to provide engulfment signals causes dying autophagy-deficient cells to persist in tissues, owing to inefficient clearance by phagocytic cells; lack of tissue regression can be misinterpreted as a block in cell death	36
Autophagy inhibition results in changes in morphology or rate of degradation of dead cells	Genetic inactivation of *ATG* genes in *Drosophila melanogaster* results in the persistence of vacuolated salivary gland cell fragments with active caspase-3 and DNA fragmentation	20

ATG, autophagy-related; GFP, green fluorescent protein.

Other technical considerations

A further limitation in assessing the role of autophagy in ACD arises from the use of pharmacological agents or genetic manipulations that are designed to suppress autophagy. Most studies of cell death by autophagy convincingly show efficient autophagy suppression. However, the specificity of agents that inhibit autophagy is an issue.

The pharmacological agent most commonly used to suppress autophagy is 3-methyladenine (3-MA), which interferes with the formation of autophagosomes by inhibiting the class III phosphoinositide 3-kinase (PI3K) VPS34. However, 3-MA is used at millimolar concentrations and might affect other targets, including class I PI3K (which inhibits autophagy), lethal stress kinases (such as p38 mitogen-activated protein kinase and c-JUN N-terminal kinase) and the mitochondrial permeability transition pore (which regulates mitochondrial outer membrane permeabilization (MOMP), one of the decisive events of both apoptosis and necrosis)[44,45]. A similar critique applies to lysosomotropic agents, such as chloroquine. Although such agents increase the lysosomal pH and inhibit the action of lysosomal hydrolases and the fusion of lysosomes with autophagosomes, they have multiple nonspecific effects on cellular function[46]. Thus, the observation that 3-MA or chloroquine modulate cell death cannot be used as proof that autophagy is involved in the lethal process.

Genetic manipulations that affect *ATG* genes might also be problematic. Small interfering (si)RNA-mediated knockdown of one gene usually affects thousands of transcripts, meaning that multiple non-overlapping siRNAs should be used independently from each other and that experiments should ideally be controlled by retransfection of the cells with a cDNA that is not affected in its expression by the siRNA[47]. These types of controls have been missing in all of the reports on ACD that we have observed. In addition, the inhibition of autophagy causes an increase in the proteasome-mediated turnover of proteins[48] and this might elicit the upregulation of cytoprotective antioxidant enzymes[49]. Finally, many, if not all, of the *ATG* gene products have functions outside of the autophagy pathway (BOX 2). In particular, this applies to beclin-1 (which might have a broad role in vesicular trafficking[50] and interacts with apoptosis-regulatory proteins, such as BCL2 and BCL-x$_L$ (REF. 51)) and to ATG5 (which can translocate to mitochondria and induce MOMP after calpain-mediated cleavage[52]). Hence, depletion of beclin-1 might augment cellular survival by affecting cellular signalling processes through its role in vesicular trafficking, and depletion of ATG5 might affect MOMP, which is viewed as one of the decisive events that mark the point of no return in lethal signalling.

Death effector or corpse remover?

It was once postulated that high levels of autophagy might be considered as a 'clean-up' or 'self-clearance' mechanism in cells that are committed to die by apoptosis or necrosis[53]. This theory was originally developed to explain the clearance of apoptotic corpses in developmental settings, such as embryogenesis and insect metamorphosis, in which it was presumed that insufficient phagocytes were available for dead cell clearance. In such cases, dying cells would activate autophagy to target the contents of the cell for degradation by its lysosomes.

In 2007, the first evidence was published that indicated that autophagy is essential for the clearance of dying cells during developmental cell death, including in a mouse embryoid body cavitation model (which mimics the formation of the proamniotic cavity in early mammalian development)[36] and in *Drosophila melanogaster* salivary gland regression[20]. In addition to the previously postulated cell-autonomous function of autophagy in the clearance of dead cells (which is the presumed mechanism in *D. melanogaster* salivary gland regression[54]), autophagy was found to have an unexpected function in signalling corpse removal by phagocytes.

Qu *et al.*[36] showed that autophagy-dependent ATP production is essential for the generation of engulfment signals in mouse embryoid bodies, including the 'come-get-me' signal (lysophosphatidylcholine secretion) and the 'eat-me' signal (phosphatidylserine exposure). Embryoid bodies derived from $Atg5^{-/-}$ or $Becn1^{-/-}$ embryonic stem cells displayed impaired phagocytic clearance of corpses that contain degraded (TUNEL-positive) DNA and impaired cavitation, and these defects were reversed by providing an external substrate for ATP generation. Moreover, lung and retinal tissues from $Atg5^{-/-}$ embryonic mice contained an

increased number of apoptotic cells and showed ultrastructural evidence of impaired phagocytic removal of cell corpses. As the rate of cell death did not differ between wild-type and $Atg5^{-/-}$ or $Becn1^{-/-}$ embryoid bodies[36], these results were interpreted to signify that the activation of autophagy in dying cells — at least in this developmental model system — is neither a pro-survival nor a pro-death mechanism; rather, it might be a means, in dying cells, of supplying energy for the emission of proper signals to ensure cell removal by neighbouring phagocytes.

Interestingly, Berry and Baehrecke reported similar findings with respect to the effects of autophagy inhibition on salivary gland degradation during *D. melanogaster* metamorphosis[20]. However, in this context, the role of autophagy in cellular degradation is thought to be cell autonomous[54], and the authors interpreted these findings as "the first *in vivo* evidence that autophagy and *atg* genes are required for autophagic cell death"[54]. Transgenic expression of autophagy-inhibitory signalling molecules (such as DP110 (the active subunit of PI3K), AKT (also known as protein kinase B), activated Ras^V12 or the product of a dominant-negative, kinase-dead *atg1* mutant) or removal of *ATG* genes (that is, loss-of-function mutation of either *atg2, atg3, atg8* or *atg18* or knockdown of either *atg3, atg6, atg7* or *atg12* by siRNA) caused the persistence of TUNEL-positive salivary gland cell fragments[20]. However, this persistence only gave rise to a delay of histolysis by ~24 hours. This delay could be prolonged by transgenic expression of the caspase inhibitor p35 (an inhibitor of apoptosis (IAP) protein from baculovirus) or removal of either caspase activators (such as ARC, a *D. melanogaster* orthologue of apoptotic protease-activating factor-1 (APAF1) and CED4) or effector caspases (such as DRONC), indicating that both autophagy and apoptosis participate in the degradation of salivary gland cells[20]. However, it seems that salivary gland cells eventually disappear, even in conditions in which both autophagy and caspase activation are disabled, which suggests that both catabolic processes are dispensable for cell death to occur in this model. Furthermore, in a study by another group, the *D. melanogaster* APAF1 orthologue, DARK, was shown to be essential for salivary gland histolysis but dispensable for autophagy, which suggests that autophagy *per se* is not a 'killing event'[55].

These studies raise the question of whether autophagy inhibition-dependent blockade (or delay) of the degradation of salivary gland cell fragments (which display nuclear DNA fragmentation) meets the operational definition of ACD. The answer might depend more on how cell death, rather than how ACD, is defined. If one considers cell death as the failure to retain vital cellular functions, of which the best examples are perhaps clonogenic survival in dividing cells or persistent metabolic activity in terminally differentiated cells, these studies would suggest that autophagy is a crucial mechanism for the efficient clearance of dying cells, but not for death *per se*. However, if one defines cell death as the entire linear process that begins with the signal to irreversibly commit a cell to die and ends with the disappearance of the dead cell, these findings indeed constitute an important *in vivo* demonstration of an essential role for *ATG* genes in ACD.

Accomplice or sole perpetrator?

Another interesting question relating to the definitions of death and ACD is whether autophagy constitutes a death effector mechanism if it is genetically upstream of other death pathways, such as apoptosis or necrosis. In 2006, Espert *et al.*[56] published the first genetic evidence that autophagy lies upstream of apoptosis: they showed that siRNA against two *ATG* genes, *BECN1* and *ATG7*, completely suppressed apoptosis in CD4+ T lymphocytes that is triggered by C-X-C chemokine receptor-4 engagement by the HIV envelope glycoprotein. Subsequently, knockdown or knockout of *ATG* genes has been shown to block apoptotic death in other settings, including in photoreceptors that are exposed to oxidative stress[57], in murine embryonic fibroblasts that are exposed to ER stress[58] and in p53-overexpressing osteosarcoma cells[59].

As the mechanisms that link *ATG* genes to apoptotic cell death are still poorly defined, it is unclear whether these observations indicate essential roles of the autophagy pathway in triggering apoptosis or, rather, alternative functions of components of the autophagy machinery in apoptosis signalling or execution. In either case, the above studies suggest that, in many settings, even if autophagy somehow participates in cell death, it is an accomplice in apoptotic cell death rather than a sole perpetrator in autophagic cell death.

Autophagy might also serve a similar accomplice role in necrotic cell death. In *C. elegans*, a gain-of-function mutation in the mechanosensor MEC-4 (the *mec-4d* mutant) that causes uncontrolled Ca2+ influx into neurons leads to neurodegeneration with features of excitotoxin-induced necrosis. This cell death (as well as cell death induced by a gain-of-function mutation in the acetylcholine receptor channel subunit gene *deg-3*) is accompanied by increased autophagy and is inhibited for at least several days by mutations that affect the nematode orthologues of *ATG1* or by RNAi of the orthologues of yeast *ATG6, ATG8* or *ATG18* (REF. 19). How autophagy might have such an accomplice role is an open question. Autophagy might disrupt the pro-death–anti-death equilibrium, for instance by the selective removal of antioxidant enzymes, such as catalase[60].

Does autophagic cell death exist?

Based on the considerations discussed above, the question arises whether there are physiological situations in which autophagy truly kills cells that would otherwise manifest long-term survival. There are some examples in which experimental induction of excessive autophagy can kill cells in model organisms *in vivo* or in mammalian cells

Table 2 | Removal of *ATG* genes for cell death avoidance *in vivo*

Species	Cell type and death inducer	Experimental removal of *ATG* genes that affects cell death	Refs
Caenorhabditis elegans	Neurons that degenerate as a result of uncontrolled ion fluxes through mutated channel proteins (MEC-4 and DEG-2)	Mutation of *atg-1* and RNAi of *bec-1* (the orthologue of *ATG6* (yeast) and *BECN1* (mammals)), *atg-8* (the orthologue of mammalian *LC3*) and *atg-18*	19
Drosophila melanogaster	Transgenic expression of ATG1 in larval salivary glands	RNAi of *atg12*	20
Mus musculus	Neonatal hippocampal neurons dying after ligation of the carotid artery and transient hypoxia	Neuron-specific knockout of *Atg7* (*Atg7^{flox/flox}* and nestin–Cre) strongly reduces autophagy, caspase-3 activation, pyknosis, TUNEL-positivity and neuronal loss	21

ATG, autophagy-related; DEG-2, degenerin-2; MEC-4, mechanosensory protein-4; RNAi, RNA interference.

Box 2 | **Non-autophagic functions of *ATG* gene products**

Several autophagy-related (ATG) proteins are known or are thought to have alternative, autophagy-independent functions. The *Caenorhabditis elegans* orthologue (UNC-51) and mammalian orthologue (ULK1) of ATG1 function in axonal elongation and branching[69,70]. ATG5 can be proteolytically activated to become a pro-apoptotic molecule that translocates to mitochondria and triggers mitochondrial outer membrane permeabilization[52], and the ATG5–ATG12 conjugate functions as a suppressor of innate immune signalling through interactions with the caspase recruitment domain of retinoic acid-inducible gene-1 (RIG1) and interferon-β promoter stimulator (IPS)[71]. Yeast Atg6, and perhaps its mammalian orthologue beclin-1, function in autophagy-independent vesicular trafficking pathways[72]. The BAX-interacting factor-1 (BIF1, also known as endophilin B1), which binds to beclin-1 through ultraviolet (UV) irradiation resistance-associated gene (UVRAG), is required for autophagy[73] but was originally described as a pro-apoptotic factor[74]. UVRAG was originally discovered as a factor that affects UV sensitivity[75] and is deficient in malformations of the left–right axis[76]. Beclin-1 has a B-cell lymphoma-2 (BCL2) homology-3 (BH3) domain[77], which raises the theoretical possibility that it could function as a pro-apoptotic molecule. The knockout of AMBRA1 (activating molecule in beclin-1-regulated autophagy-1), another beclin-1 interactor, results in exencephaly[78]. Indeed, the knockout of *ATG* genes in mice results in rather distinct phenotypes that range from early embryonic to perinatal lethality or an increased incidence of tumours in adulthood[2], which suggests that these genes have distinct functions outside of the autophagy-regulatory system. ATG16-like-1 (ATG16L1) might also have alternative functions, as a polymorphism in its non-conserved WD40 domain is associated with Crohn's disease[79], but is predicted not to affect its autophagy function.

Given the implication of *ATG* genes in cellular processes other than autophagy, it is an oversimplification to assume that the inhibition of cell death that results from the knockout or knockdown of one or a few *ATG* genes is due to suppression of the autophagic process. In this context, it is noteworthy that cytoprotective effects are mostly described for the knockout or knockdown of three genes — *BECN1* (which encodes beclin-1 and functions in vesicle nucleation), *ATG5* and *ATG7* (which function in autophagosome elongation and completion). As noted above, at least two of these, *ATG5* and *BECN1*, have been postulated to exert non-autophagic functions.

in vitro. For example, in *D. melanogaster* larvae, transgenic expression of a highly active *atg1* mutant induces large-scale autophagy and premature degradation of salivary glands, which, although accompanied by an increase in apoptosis[61], is prevented by *atg12* knockdown but not a *p35* (apoptosis-inhibitory) transgene[20]. In cultured mammalian cells, *BECN1* mutants that escape physiological regulation by BCL2 induce excessive levels of autophagy and cell death that can be inhibited by siRNA against *ATG5* (REF. 51). In many other studies, cell death associated with excessive autophagy activation in response to cytotoxic agents or other stress stimuli has been shown to be blocked by 3-MA[8].

Although experimentally induced excessive autophagy can cause cell death in non-mammalian organisms and in cultured mammalian cells, we are not aware of any model system that convincingly shows that physiological (or even pathological) cell loss in mammalian cells *in vivo* is executed by autophagy. One report shows that neuron-specific knockout of *Atg7* can block neuronal cell loss that is induced by neonatal ischaemia or reperfusion damage of the brain[21]. However, in this system, neuronal cell loss occurs through an apoptotic pathway that can be interrupted by pharmacological

inhibition of caspases and by a hypomorphic mutation of the gene that codes for apoptosis-inducing factor (AIF)[21,62]. The absence of *Atg7* abolishes all signs of apoptosis that affect hippocampal pyramidal neurons of wild-type mice: proteolytic maturation of caspase-3 and caspase-7, oligonucleosomal DNA fragmentation, TUNEL-positivity and pyknosis[21]. These results suggest that *Atg7* is involved in a signal transduction pathway that facilitates activation of an apoptotic programme of cellular self-destruction, perhaps (but not necessarily) by autophagy. Alternatively, sublethal cellular stress induced by deficient autophagy (which in *Atg7*−/− neurons causes severe neurodegeneration in early adulthood)[48] might elicit neuroprotective mechanisms that are similar to those triggered by ischaemic preconditioning[63]. Thus, whether autophagy is really involved in cellular demise remains elusive.

Conclusions and perspectives

As outlined above, the question of whether mammalian cell death can occur by autophagy cannot be answered definitively, although there are many examples of cell death with autophagy. In this sense, the term ACD should be considered to be a misnomer, and there are indeed overwhelming arguments (not discussed in this article) to

suggest that autophagy has, above all, a role in protecting mammalian cells both *in vitro* and *in vivo* from nutrient stress, aggregates of misfolded proteins, organelle damage and microbes[2,64]. Importantly, at least some of the proteins that are essential for autophagy might also participate in signalling pathways that lead to autophagy-unrelated lethal processes (such as MOMP and caspase activation), and it will be important to distinguish between lethal signalling that depends on the expression of particular *ATG* gene products and true ACD in the future.

We propose two non-mutually exclusive approaches to tackle this problem: to develop specific genetic or pharmacological inhibitors of autophagy, or to inhibit autophagy by removing or depleting multiple *ATG* gene products and to provide solid evidence that autophagy inhibition (rather than off-target effects) has a major cytoprotective effect that leads to clonogenic survival or the permanent persistence of functional post-mitotic cells. Hence, investigators working in the field should either redouble their efforts to provide unequivocal evidence for the existence of death by autophagy or avoid the term ACD if it only describes death with autophagy.

Guido Kroemer is at the Institut Gustave Roussy, F-94805 Villejuif, France, Université Paris Sud, Paris 11, F-94805 Villejuif, France and INSERM, U848, F-94805 Villejuif, France.

Beth Levine is at the Howard Hughes Medical Institute and the Department of Internal Medicine and the Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390, USA.

*Correspondence to G.K.
e-mail: kroemer@igr.fr*

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Atg5 | *Atg7* | *Bak* | *Bax* | *Becn1*
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
Crohn's disease | Danon disease | Parkinson's disease
UniProtKB: http://www.uniprot.org
AKT | APAF1 | VPS34

FURTHER INFORMATION
Guido Kroemer's homepage:
http://www.igr.fr/index.php?p_id=1072
Beth Levine's homepage: http://www4.utsouthwestern.edu/idlabs/Levine/Levine_intro.htm

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