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Review Article

The complex interplay between autophagy and cell death pathways

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Autophagy is a universal cellular homeostatic process, required for the clearance of dysfunctional macromolecules or organelles. This self-digestion mechanism modulates cell survival, either directly by targeting cell death players, or indirectly by maintaining cellular balance and bioenergetics. Nevertheless, under acute or accumulated stress, autophagy can also contribute to promote different modes of cell death, either through highly regulated signalling events, or in a more uncontrolled inflammatory manner. Conversely, apoptotic or necroptotic factors have also been implicated in the regulation of autophagy, while specific factors regulate both processes. Here, we survey both earlier and recent findings, highlighting the intricate interaction of autophagic and cell death pathways. We, Furthermore, discuss paradigms, where this cross-talk is disrupted, in the context of disease.

Introduction

Cellular recycling is essential for both survival and organismal homeostasis. These clearance mechanisms bidirectionally interact with cell death pathways either to prevent them or to promote cell destruction due to extensive accumulation of damage. This complex interplay has been studied in both physiological and pathological context.

Autophagy from the Greek words 'auto', self, and 'phagy', eating, is an intracellular self-cannibalistic mechanism conserved in all eukaryotes. Although considered a bulk degradation pathway, it has emerged to be a highly selective catabolic mechanism regulated at multiple steps, which digests macromolecules (including proteins and lipids) and organelles via the lytic organelle, the lysosome. It can be subdivided into macroautophagy (hereafter referred to as autophagy), entailing autophagosome formation, chaperone-mediated autophagy and microautophagy [1]. Autophagy occurs both at basal levels, but can be up-regulated by nutrient, oxidative, hypoxic stress or DNA damage, all of which take place in a cancerous microenvironment.

Mechanistically, autophagy consists of multiple steps that are tightly controlled (Figure 1). The process is initiated upon activation and recruitment of the Unc-51-like kinase (ULK) complex, comprised of ULK1, focal adhesion kinase family interacting protein of 200 kDa (FIP200), ATG13 and ATG101, to the pre-autophagosomal structrures, mainly derived from Endoplasmic Reticulum (ER) membranes. Once formed, the ULK complex phosphorylates phosphatidylinositol 3-kinase (VPS34), part of the Beclin-1 complex, which is necessary for the initiation of the phagophore. The active VPS34 complex generates Phosphatidylinositol-3-phosphate (PI3P), which binds to WD-repeat protein Interacting with PhosphoInositides (WIPI) proteins. WIPIs in turn facilitate the recruitment of autophagy-related (ATG) proteins, which promote the elongation of the phagophore membrane through two ubiquitin-like conjugation systems. ATG7 and ATG10 conjugate ATG5–ATG12 which then bind to ATG16L1. The E1-like ATG7 and the E2-like ATG3 factors together with the E3-like ATG5–12–16 complex co-ordinate the conjugation of light chain 3 (LC3)/GABARAP (Gamma-aminobutyric acid receptor-associated protein) to phosphatidylethanolamine lipids of the expanding phagophore. The

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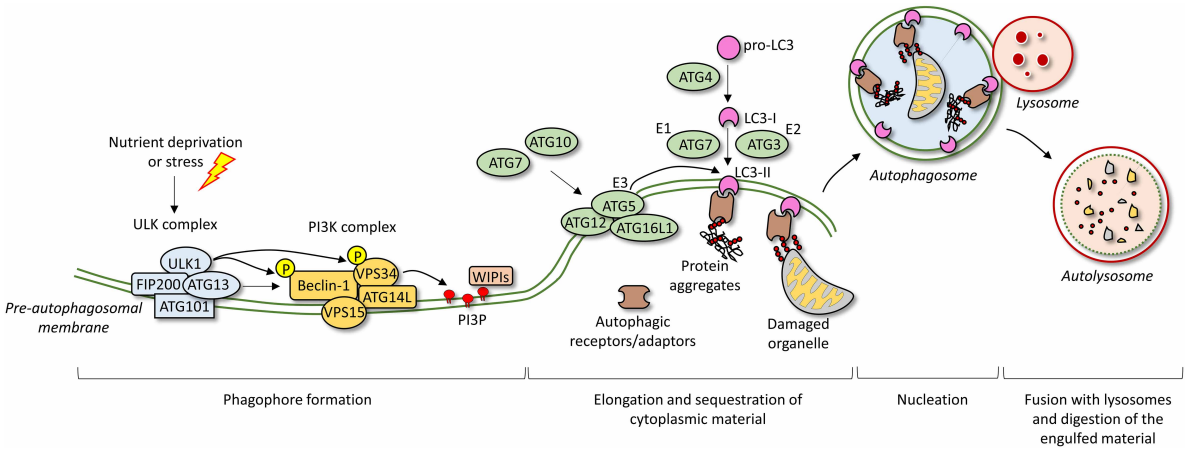

Figure 1. The main autophagic machinery.

Upon nutrient deprivation or other stress, the ULK complex is recruited to pre-autophagosomal membranes, where it enhances the recruitment of VPS34 and the formation of PI3K complex. Active PI3K complex generates PI3P which facilitates the recruitment of ATGs, by interacting with WIPI proteins. ATG7, ATG3 and the ATG5–12–16 complex conjugate LC3 to phosphatidylethanolamine lipids of the expanding phagophore, by acting as E1, E2 and E3 enzymes, respectively. During phagophore expansion, cytoplasmic components, including aggregated proteins and/or organelles, are sequestered, finally leading to phagophore enclosure and the formation of mature autophagosomes. Sequentially, the autophagosomes fuse with lysosomes, forming the autolysosomal structures, where the cytoplasmic cargo is finally degraded by resident hydrolases.

lipidated form of LC3 (LC3-II) in turn recognizes autophagic receptors, adaptors and substrates with an LC3-interacting region (LIR) [2]. Protein receptors include p62 (phosphotyrosine-independent ligand for the Lck SH2 domain of 62 KDa) or otherwise known as sequestosome (SQSTM), optineurin, NBR1 (neighbour of Brca1) and NDP52 (nuclear dot protein 52 kDa). For instance, NBR1 and p62 proteins act as cargo receptors for the selective autophagy of ubiquitinated targets [3]. Mutations in both core autophagic components and selective autophagy receptors cause a multitude of diseases, cell transformation and neurodegeneration.

Cell death occurs physiologically to eliminate defective or superfluous cells or as a result of uncontrolled cell cycle progression in disease. There is increasing evidence of a variety of cell death pathways which are activated under specific conditions and cellular contexts. Apoptosis and necroptosis are well-characterized, while autophagic cell death has only recently gained attention as a distinct pathway [4]. Apoptosis is considered to be the classical programmed cell death pathway and is essential for development and tissue homeostasis. Specifically, cells undergo apoptosis to counterbalance cell proliferation and control tissue growth in order to maintain appropriate cell and tissue size. Recently, apoptosis has been proposed to promote tissue remodelling in the formation of hands, feet and aorta [5]. During this energy consuming process, DNA is fragmented, and the nucleus shrinks forming apoptotic bodies. Apoptosis can be further categorized into intrinsic and extrinsic. In the first case, B-Cell CLL/Lymphoma 2 (Bcl-2) family members are affected by several stimuli, including ageing, nutrient starvation or damage, and lead to cell death [6]. Anti-apoptotic Bcl-2 proteins antagonize the pro-apoptotic BH3-only and the death effectors Bcl-2 Associated X (BAX), Bcl-2 Homologous Antagonist/Killer (BAK), and Bcl-2 Related Ovarian Killer (BOK) act downstream to induce death. During extrinsic apoptosis, ligands of the tumour necrosis factor (TNF) such as TNF-α, Fas ligand (FASL), TNF-related apoptosis-inducing ligand (TRAIL) bind their cell surface receptors and activate a downstream death cascade of caspases. Necroptosis is a regulated inflammatory necrotic mode of cell death which is activated after induction of death receptors and ultimately converges to Receptor Interacting Serine/Threonine Kinase 3 (RIPK3) activation [7]. In autophagy-dependent cell death (ADCD), components of the autophagic machinery referred to above, such as ULK1, are responsible and essential for cell destruction, whilst in other forms of cell death autophagy can also be manipulated. The first subtype is dependent on autophagic flux while the second, autosis, does not require autophagosome-lysosome fusion [8].

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Autophagy as a negative regulator of cell death

Autophagy is primarily known for its cytoprotective role, since, in most cases, inhibition of essential Atg genes accelerates cell death [9–12]. It is generally accepted that under stress autophagy is activated as an adaptive response mechanism to alleviate stress-induced damage and preserve metabolic homeostasis [13–17]. These pro-survival effects are mainly indirect involving, among others, the restoration of cellular bioenergetics [18,19], as well as the elimination of toxic protein aggregates [20] or damaged organelles [21], which otherwise could trigger the activation of apoptotic or necroptotic mechanisms. However, autophagy can directly inhibit cellular demise through selective mechanisms that attenuate the function of pro-death proteins in different cellular contexts (summarized in Figure 2).

Autophagic degradation of caspases is one of the best-studied pro-survival responses [22]. In a BAX-deficient colon cancer cell line, autophagy selectively targets the large subunit of caspase-8, curtailing the activation of TRAIL-induced mediated apoptosis [23]. Supportively, liver-specific depletion of *ATG7* in mice enhances caspase-8 activity and promotes TNF-mediated cell death [24]. Another study conducted in human colon carcinoma cells has shown that up-regulation of the autophagic substrate p62/SQSTM1promotes apoptosis by enhancing caspase-8 self-aggregation and activation [25], highlighting the fact that autophagy-mediated sequestration of p62 attenuates caspase-8 activation and inhibits cell death.

A growing amount of evidence has recently revealed a pro-survival role of autophagy against necroptosis. *ATG16L1*-depleted macrophages display highly insoluble forms of the RIP homotypic interaction motif (RHIM)-domain proteins RIPK1, RIPK3, TIR (Toll/IL-1 receptor) domain-containing adaptor protein inducing interferon-β (TRIF) and Z-DNA binding protein 1 (ZBP1). Aggregation of these proteins activates their function and sequentially enhances cytokine production and necroptotic cell death, implying that autophagy inhibits necroptosis by actively sequestering the pro-necroptotic RHIM-domain containing proteins [26]. The RHIM-domain proteins RIPK1 and RIPK3 directly interact with LC3 through a functional LIR domain in the myocardium. Under hypoxia, autophagic flux is disrupted and the accumulated autophagosomes function as a platform for RIPK1/RIPK3 aggregation and subsequent formation of the necrosome complex [27]. Impaired autophagic flux in cell and mouse models of Alzheimer's disease (AD) promotes p62-mediated RIPK1 self-oligomerization and TNF-α induced necroptosis. In this context, overexpression of UV radiation resistance-associated gene (UVRAG), a key regulatory component for the initiation of autophagosome formation, hinders neuronal necroptosis [28]. Furthermore, it has been shown that RIPK1 is a substrate of the autophagy initiating kinase ULK1. The ULK1-dependent phosphorylation at Ser357 inhibits the assembly of the necrosome complex and reduces TNF-induced cell death [29].

Several studies highlight that selective autophagic degradation of dysfunctional mitochondria, a process known as mitophagy, delays cell death induction. Mitochondrial damage induces mitochondrial outer membrane permeabilization (MOMP), which in turn facilitates the release of mitochondria-localized apoptogenic factors including the caspase activators cytochrome C and Direct IAP-Binding Protein With Low PI (Diablo), as well as the caspase-independent death effectors apoptosis inducing factor (AIF) and endonuclease G (endoG) [30,31]. It has been shown that mitophagy protects against heat shock-induced apoptosis in HeLa cells by inhibiting cytochrome C release and caspase-3 activation [32]. Specifically, the PTEN Induced Kinase 1 (PINK1)/Parkin mitophagic pathway regulates the initiation of cell death mechanisms in different cellular contexts. Parkin overexpression in isolated rat cardiac myocytes is protective against hypoxia-induced apoptosis [33], while its deficiency in glial cells confers susceptibility to both apoptosis and necrosis [34]. Despite the limited number of studies with mechanistic insights regarding the cytoprotective role of Parkin, it has been shown that Parkin-dependent mono-ubiquitination of the outer mitochondrial membrane protein voltage dependent anion channel 1 (VDAC1) negatively regulates mitochondrial calcium uptake and protects against apoptosis in *Drosophila melanogaster* [35]. Interestingly, a study conducted in the TSM1 neuronal cell line has shown that Parkin, independently of its ubiquitin ligase activity, reduces the expression of the pro-apoptotic p53 gene, by acting as a transcriptional repressor [36]. PINK1, the upstream Parkin-activating kinase is required for long-term survival in differentiated human dopaminergic neurons [37], while it confers resistance in mice against oxidative stress [38]. Mechanistically, PINK1-dependent phosphorylation of the mitochondrial chaperone TNF-receptor associated protein 1 (TRAP1) is protective against hydrogen peroxide-induced cell death. Interestingly, fractionation analyses showed that endogenous PINK1 and TRAP1 are primarily localized in the mitochondrial intermembrane space and the inner mitochondrial membrane (IMM) fractions [39,40]. Another PINK1 substrate reported to negatively regulate cell death is Bcl-2 related extra-large (Bcl-xL). In depolarized

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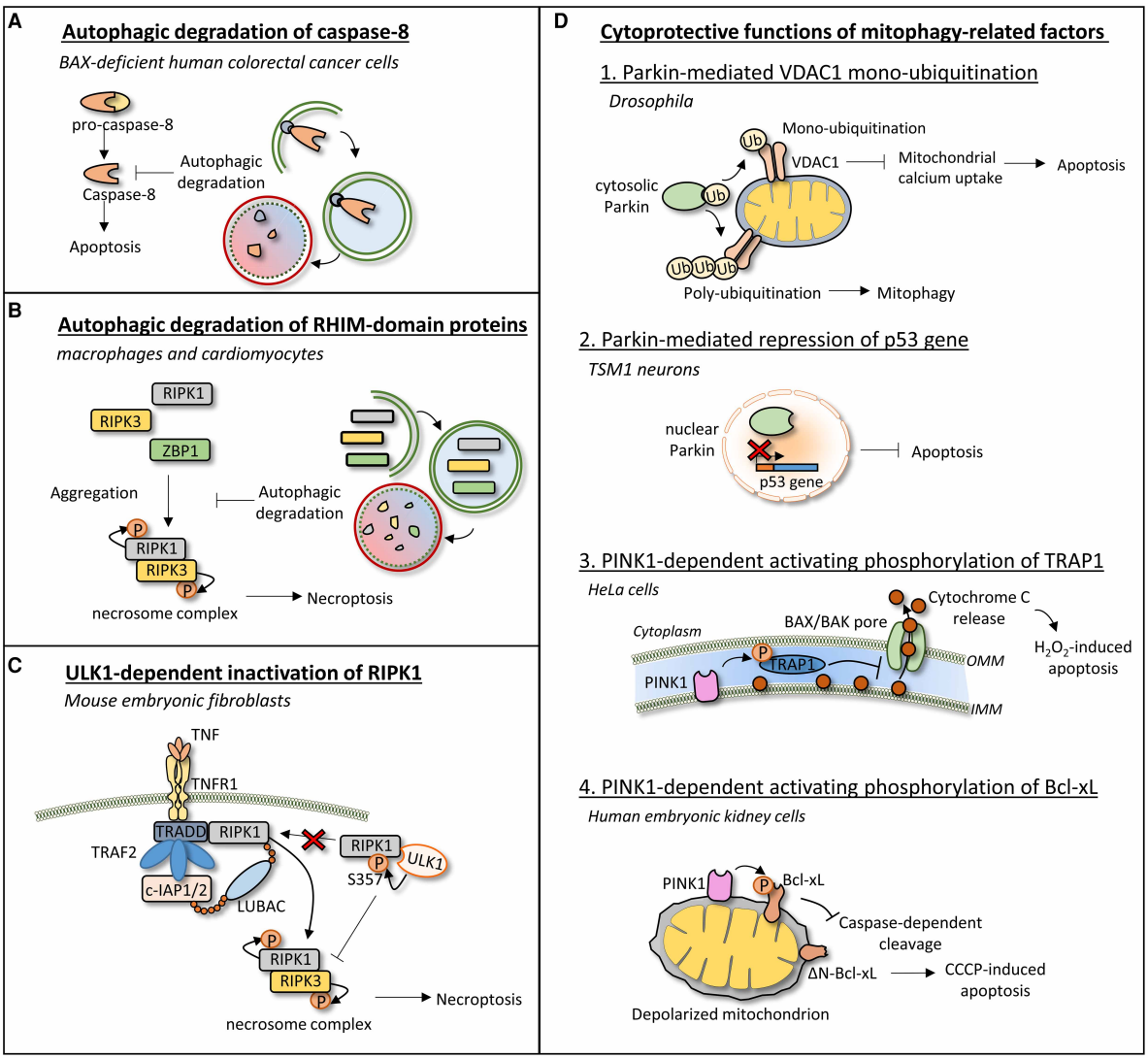

Figure 2. Autophagy as a negative regulator of cell death.

The figure summarizes the main mechanisms through which autophagy inhibits cell death machineries in different cellular contexts. (**A**) Selective autophagic degradation of caspase-8 blocks TRAIL-induced mediated apoptosis in BAX-deficient human colorectal cancer cells. (**B**) Studies conducted in macrophages and cardiomyocytes have shown that autophagic degradation of the RHIM-domain proteins RIPK1, RIPK3 and ZBP1 attenuates necrosome formation and necroptosis. (**C**) In TNF-α treated MEFs, ULK1-dependent phosphorylation of RIPK1 curtails necrosome formation and necroptosis. (**D**) The role of mitophagy factors Parkin and PINK1 in cell death inhibition. (1) In *Drosophila* Parkin-dependent mono-ubiquitination of VDAC1 blocks apoptosis by negatively regulating mitochondrial calcium uptake. (2) A study in TMS1 neurons showed that Parkin can act as a transcriptional repressor in the nucleus and block the expression of the pro-apoptotic p53 gene. (3) IMM-localized PINK1 phosphorylates TRAP1, which in turn inhibits cytochrome C release and blocks hydrogen-peroxide-induced apoptosis. (4) In response to CCCP, PINK1 phosphorylates Bcl-xL rendering it resistant to its pro-apoptotic cleavage.

mitochondria, PINK1 phosphorylates Bcl-xL and this phosphorylation impedes its pro-apoptotic cleavage, and thus enhances its anti-apoptotic activity [41]. Intriguingly, during mitophagy, the anti-apoptotic proteins Bcl-2 and FK506-Binding Protein 8–38kD (FKBP38) translocate from mitochondria to ER in a process which requires the ubiquitination activity of Parkin. This selective escape of anti-apoptotic proteins in response to mitochondrial damage is suggested to be a cytoprotective mechanism to prevent apoptosis commitment [42].

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Autophagy as an inducer and/or prerequisite of cell death

As previously mentioned, autophagy generally comes first as a response mechanism to salvage stressed cells from death. When stress exceeds a critical limit and the resulted damage cannot be resolved, 'suicide' programs are activated and the cell dies. A considerable amount of evidence during the last few decades supports a leading role of autophagy in cell death, since cells doomed to die often display features of enhanced autophagy [43]. However, in the respective studies it is not yet clear whether failure in autophagic flux triggers cell death or autophagic factors actively regulate cellular demise. In the literature, there have been reported cases of 'autophagy-mediated cell death', where autophagy acts upstream and promotes the activation of cell death modalities (Figure 3), and 'autophagy-dependent cell death' (ADCD), where death strictly depends on autophagy *per se*, without involving the activation of canonical cell death machineries [43,44]. In the current section, we summarize autophagy-mediated and autophagy-dependent types of cell death.

Autophagy-mediated cell death

Several *in vivo* studies using model organisms support the existence of autophagy-mediated cell death modalities. Induction of autophagosome formation, as well as the downstream autophagic machinery, including autophagosome nucleation, expansion and completion, are all required for the necrotic cell death of mechanosensory neurons in a *C. elegans* established model of excitotoxicity [hyperactivation of the ion channel mechanosensory abnormality protein 4 (MEC-4)] [45]. Additionally, nematodes deficient in core autophagic factors display significantly reduced germline apoptosis in response to γ-ray induced genotoxic stress, indicating a pro-apoptotic role of autophagy. In the same study, they showed that autophagy is also required for the physiological apoptosis of germ cells and the post-embryonic death of ventral cord neurons upon partial inhibition of the executor caspase Cell death protein 3 (CED-3) [46]. In *Drosophila*, ablation of the core autophagic genes *Atg1* and *Atg7* compromises programmed cell death of the oocyte supporting nurse cells during late oogenesis [47]. Mechanistically, it was shown that autophagy-dependent sequestration of dBruce, the *Drosophila* inhibitor of apoptosis (IAP) mediates DNA fragmentation and development-induced cell death in nurse cells [48]. Another study in *Drosophila* revealed that overexpression of *Atg1* is sufficient to induce autophagy, which in turn triggers caspase-dependent apoptotic cell death in wing imaginal disc cells [49].

Mammalian studies have also reported cases of autophagy-inducing cell death pathways. In HeLa cells, Interferon-γ (IFN-γ) has been reported to elicit an autophagic type of cell death, necessitating the direct interaction of ATG5 with the death domain of Fas Associated via Death Domain (FADD). This does not represent an authentic case of ADCD, since the caspase inhibitor zVAD blocks cellular demise, without affecting vacuole formation, suggesting that the autophagic factor ATG5 acts upstream to positively regulate FADD-dependent extrinsic apoptotic pathway [50]. A pro-apoptotic function of ATG5 was also reported in cancer cell lines upon treatment with CD95, ceramide or anticancer drugs. Irrespectively of the apoptotic stimuli, ATG5 is processed in a calpain-dependent manner. Its truncated form translocates from cytosol to mitochondria, where it binds and inhibits the anti-apoptotic factor Bcl-xL, finally triggering the intrinsic apoptosis pathway [51]. Treatment of mouse embryonic fibroblasts (MEFs) with SKI-I (pan-sphingosine kinase inhibitor) or bortezomib (proteasome inhibitor) promotes autophagy-dependent activation of the extrinsic apoptotic pathway. Particularly, autophagosomes function as scaffolds for self-aggregation and activation of caspase-8. Caspase-8 is recruited to autophagosomes either by interacting with the autophagic adaptor p62/SQSTM1, or by forming a complex with ATG5 and FADD [52]. The autophagic regulator Tumour Protein P53 Inducible Nuclear Protein 2 (TP53INP2) was recently implicated in the regulation of extrinsic apoptosis. In death-stimulating mammalian cell lines, TP53INP2 promotes self-aggregation and activation of caspase-8 by enhancing its TNF Receptor Associated Factor 6 (TRAF-6)-dependent K63 ubiquitination [53]. Perturbation in intracellular iron and ROS homeostasis is another mechanism of autophagy-mediated cell death, since selective autophagic degradation of ferritin induces ferroptosis in erastin-treated fibroblasts and cancer cell lines [54,55]. Although the particular type of cell death is considered to be an example of ADCD, treatment of certain cancer cell lines with potent autophagy inhibitors, including bafilomycin A1, 3-methyladenine, and chloroquine could not block erastin-induced apoptosis [56]. Additionally, in response to mitochondria depolarization, the PINK1/Parkin mitophagic pathway can stimulate intrinsic apoptosis by promoting ubiquitination and subsequent degradation of the anti-apoptotic protein Myeloid Cell Leukemia Sequence 1(Mcl-1) [57]. Interestingly, it is suggested that the grade of mitochondrial damage mainly determines whether PINK1/Parkin pathway will mediate a cytoprotective or cytotoxic response, by enhancing mitophagy or cell death pathways respectively [57,58].

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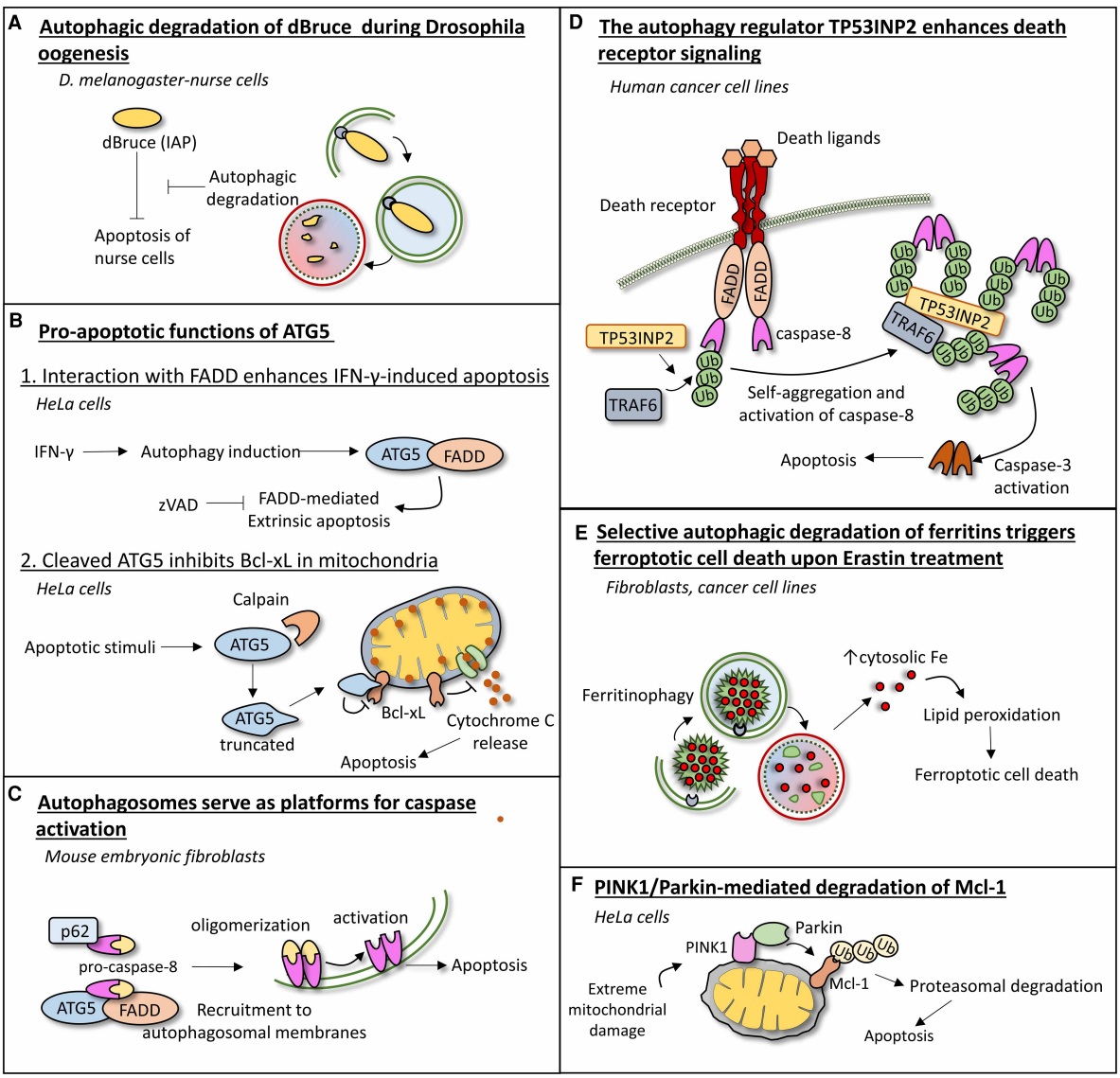

Figure 3. Mechanisms of autophagy-mediated cell death.

The figure summarizes the main mechanisms through which autophagy acts upstream to promote cell death machineries in different cellular contexts. (**A**) Autophagic degradation of the anti-apoptotic dBruce promotes the apoptotic death of nurse cells during late oogenesis in *D. melanogaster*. (**B**) Pro-apoptotic functions of ATG5 in cancer cell lines. (1) ATG5 promotes IFN-γ-induced extrinsic apoptotic pathway by interacting with FADD. (2) Calpain-dependent cleavage of ATG5 transforms ATG5 into a pro-apoptotic factor that is translocated to mitochondria, where it binds and inhibits the anti-apoptotic protein Bcl-xL, finally leading to cytochrome C release and apoptosis. (**C**) In response to various apoptotic stimuli, autophagosomes function as platforms for the oligomerization and activation of caspase-8, as shown in MEFs. (**D**) In human cancer cell lines, the autophagy regulator TP53INP2 facilitates TRAF-6-dependent K63 ubiquitination of caspase-8, leading to its aggregation, activation and finally to cell death. (**E**) Selective autophagic degradation of the iron-storage protein complexes of ferritin augments cytoplasmic iron levels, which in turn mediate lipid peroxidation and promote ferroptotic cell death in erastin-treated fibroblasts and cancer cell lines. (**F**) Extreme mitochondrial damage leads to Parkin-mediated ubiquitination and subsequent proteasomal degradation of the anti-apoptotic protein Mcl-1, leading to activation of the intrinsic apoptotic pathway.

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Autophagy-dependent cell death (ADCD)

The first *in vivo* case of autophagy-dependent cell death was described in *D. melanogaster*. During the developmental stage of metamorphosis, *Drosophila* midgut is eliminated through an autophagy-dependent process (requirement of *Atg1*, *Atg2* and *Atg18*). This death program is independent of the caspase-mediated canonical cell death pathway, since caspase inhibition does not affect midgut regression [59]. Another characteristic paradigm of autophagy-dependent cell death is presented in *Dictyostelium discoideum*, an amoeba parasite, the genome of which does not encode for caspases and other canonical apoptosis factors [60]. In response to starvation and the presence of differentiation-inducing factor (DIF-1), *Dictyostelium* cells aggregate, and through multiple developmental steps, form a multicellular structure known as the fruiting body. Fruiting body formation involves the programmed cell death of stalk cells, a caspase-independent process which requires *Atg1*, and thus functional autophagy [61].

ADCD has been also documented in multiple mammalian studies. Treatment of MEFs and human monocytoid cells with the pan-caspase inhibitor zVAD induces death, accompanied by the presence of numerous cytoplasmic vacuoles, reminiscent of autophagosomes. This autophagic type of cell death requires *ATG7* and the Beclin-encoding gene *BECN1* [62]. The autophagic factor ATG7 is also required for the caspase-independent ADCD observed in adult hypoxia/ischemia-injured mouse brains [63]. A subsequent study provided mechanistic insights into how autophagy promotes death in zVAD-treated MEFs. Particularly, they found that enhanced autophagy leads to the selective degradation of the ROS-scavenger catalase, supporting that elevated ROS contribute to ADCD [64]. In accordance to zVAD-mediated ADCD, apoptosis-deficient MEFs derived from BAX/ BAK double knockout mice are still able to die in response to apoptotic stimuli, through a non-apoptotic mechanism, dependent on the autophagic factors ATG5 and Beclin-1 [65,66]. In the same context (BAX/ BAK double knockout MEFs), lysosomal membrane permeability is also required for ADCD [67]. Additionally, it was recently reported that resveratrol treatment of the lung cancer cell line A549 induces a lethal program characterized by induced autophagic flux and the absence of apoptotic and necroptotic markers. In search of positive regulators of the particular autophagy-dependent death program, they performed a signalome-wide screen and identified glucocerebrosidase (GBA1) as a potent ADCD inducer [68]. A recent study revealed a regulatory role of Parkin in mediating mitophagic cell death in hippocampal neural stem cells upon low-insulin conditions [69]. Another study in breast cancer cells, identified a distinct type of ADCD, triggered by irradiation-dependent increase in intracellular iron abundance. Death in this case does not involve the activation of apoptotic, necroptotic or ferroptotic pathways, since treatment with the respective inhibitors cannot suppress irradiation-dependent mortality [70].

Modulation of autophagic activity by members of cell death pathways

Accumulating studies support that members of cell death pathways often inhibit autophagic factors as a means to attenuate possible cytoprotective activities during the death process. The best characterized mechanism of this anti-autophagic activity is the caspase- and calpain- dependent cleavage of autophagic factors. Upon stimulation of intrinsic or extrinsic apoptosis, Beclin-1 and VPS34, core factors of the autophagy inducing complex are cleaved by caspase-3, -7 and -8. Beclin-1 cleavage inhibits its autophagy-inducing activity and remarkably, the C-terminal fragment is recruited to mitochondria and sensitizes cells to apoptosis by inducing cytochrome C release [71,72]. In support of that, the pro-apoptotic protein BAX has been reported to enhance the caspase-mediated processing of Beclin-1 [73]. As previously mentioned in 'autophagy-mediated cell death' section, in response to apoptotic stimuli, ATG5 is selectively processed by calpains. This calpain-dependent cleavage transforms ATG5 into a death-promoting factor which blocks Bcl-xL anti-apoptotic function in mitochondria [51]. A similar mechanism has been described for ATG4, a protease required for both LC3 conjugation and delipidation during autophagosome formation [74,75]. ATG4D, one of the four ATG4 mammalian paralogs, is a direct target of caspase-3 in staurosporine-treated A431 and HeLa cancer cell lines. Caspase-mediated cleavage of ATG4D exposes a C-terminal BH3 (Bcl-2 homology-3 domain), which is suggested to facilitate ATG4D translocation to mitochondria, thus triggering apoptosis upon oxidative stress-induced mitochondrial damage [75,76]. In response to death receptor stimulation through TNF-α or TRAIL, the E2-like enzyme ATG3 is selectively targeted by caspase-8. This cleavage leads to ATG3 degradation and suppresses autophagosome formation during apoptosis [77]. Another study showed that early during apoptosis, the caspase-dependent cleavage and the

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calpain-dependent degradation of the autophagic factor Activating Molecule in Beclin-1-Regulated Autophagy 1 (AMBRA1) suppresses its autophagy-promoting capacity and stimulates cellular demise [78].

Apart from caspases, other members of cell death pathways have also been involved in the modulation of autophagic activity. In proliferating T cells, the DISC-adapter protein FADD and caspase-8 synergistically act to inhibit autophagy hyper-activation by binding to ATG5–ATG12 complexes. This mechanism protects against RIPK1-dependent cell death, through caspase-8-dependent cleavage of RIPK1 which is also recruited to the FADD–ATG5–ATG12–caspase-8 complex [79,80]. Additionally, during morphogenesis of human mammary cells, TRAIL, an inducer ligand of both necroptosis and extrinsic apoptosis, stimulates autophagy. Concurrent activation of autophagy and cell death in this particular context is suggested to be required for lumen formation during acinar morphogenesis [81].

Upstream modulators of autophagy and cell death pathways

Several upstream factors and signalling pathways, which can modulate both autophagy and cell death modalities, have been reported in the literature. The most important regulators are listed below.

Bcl-2 protein family

Bcl-2 family members are well-known regulators of the mitochondria-derived intrinsic apoptosis pathway and are subdivided into anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic Bcl-2 proteins contain four BH domains, while the pro-apoptotic ones are subdivided into multi-domain proteins (containing BH 1–3 domains) and BH3-only proteins (containing only the BH3 domain) [82]. The anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-xL, Bcl-2-like protein 2 (Bcl-2-L-2) and Mcl-1, can attenuate autophagy by directly binding to Beclin-1 and disrupting its interaction with VPS34 and class III phosphatidyl inositol 3-kinase (PI3K) [83–86]. Beclin-1 harbours a functional BH3 motif which is required for Bcl-2 interaction. Importantly, the subcellular localization of Bcl-2 determines its anti-autophagic or anti-apoptotic activity. Studies in yeast and mammalian cells have shown that ER-localized Bcl-2 exerts an inhibitory effect on both autophagy and apoptosis, by interacting with Beclin-1 and the ER-localized pro-apoptotic protein Bcl-2 interacting killer (BIK) respectively. On the contrary, mitochondrial localization of Bcl-2 predominantly enhances its anti-apoptotic function, by preventing BAX/BAK-mediated MOMP [82,87]. However, it has been reported that mitochondria-localized Bcl-2 can also inhibit autophagy by blocking the autophagy-inducing factor AMBRA1 [88]. Of note, nutrient deprivation autophagy factor-1 (NAF-1), an iron-sulfur cluster binding protein with anti-apoptotic function, has been found to enhance Bcl-2-Beclin-1 interaction by binding and inhibiting the BH3-only protein BIK [19]. Endogenous BH3-containing proteins or treatment with BH3-mimetics generally stimulate the autophagic process by blocking anti-apoptotic or activating the pro-apoptotic members of the Bcl-2 family. This is achieved by the antagonism of BH3 domains between BH3 containing proteins [84,89–93].

c-FLIPs

Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory proteins (c-FLIPs) are characterized by the presence of Death Effector Domains (DED) and have been implicated in the regulation of both death-receptor dependent extrinsic apoptotic and necroptotic pathways. Despite contradicting studies, the short c-FLIP isoform (c-FLIP$_{(S)}$) generally exerts anti-apoptotic effects by forming heterodimers with FADD and pro-caspase-8, and inhibiting pro-caspase-8 self-activation during DISC formation [94]. Instead, compelling evidence suggests that the long c-FLIP isoform (c-FLIP$_L$) has rather pro-apoptotic function by promoting the activation of pro-caspase-8 [95]. It is noteworthy that c-FLIP isoforms differentially modulate necroptosis. c-FLIP$_{(S)}$ stimulates necrosome formation by inhibiting pro-caspase-8 which acts as a negative regulator of RIPK1. Conversely, c-FLIP$_L$-pro-caspase-8 heterodimer cleaves RIPK1, without activating pro-caspase-8, thus enhancing cell survival versus apoptosis or necroptosis [94,96]. Apart from their well-studied role in regulating cell death, c-FLIP proteins can also modulate autophagic activity. Particularly, c-FLIPs interact with the E2-like enzyme ATG3, and prevent its association with LC3, thereby interfering with autophagosome elongation and ceasing autophagic activity [97].

P53

In response to diverse stressors, including oxidative stress and DNA damage, p53, the well-known tumour suppressor is activated and either promotes cell adaptation, or accelerates cell elimination upon failure of

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cell-defence mechanisms to mitigate stress. To achieve this, p53 primarily functions as a transcription factor, regulating the expression of numerous genes. P53 stimulates the intrinsic apoptotic pathway by transcriptionally activating the pro-apoptotic BH3-only protein-encoding genes *BAX*, p53-up-regulated modulator of apoptosis (*PUMA*), *NOXA* (latin for damage), BH3 Interacting Domain Death Agonist (*BID*), as well as the caspase-9 activator apoptotic peptidase activating factor 1(*APAF1*). Interestingly, p53 displays non-transcriptional activities, since portion of the cytoplasm-localized p53 is translocated to mitochondria and forms a complex with anti-apoptotic Bcl-2 and Bcl-xL proteins, thus enabling the activation of pro-apoptotic BAX and BAK proteins. Regarding the extrinsic apoptotic pathway, p53 transcriptionally activates the expression of *TRAIL* and *APO-1*/*Fas* receptors [98–100]. Apart from its role in apoptosis, p53 has also been implicated in the regulation of the autophagic machinery [101]. As shown in HCT116 and MCF7 cancer cell lines, cytoplasm-localized p53 inhibits autophagy by inactivating AMP-activated protein kinase (AMPK) and subsequently activating mechanistic target of rapamycin (mTOR) signalling [102]. However, nuclear p53 has opposing roles in autophagy regulation. Crighton et al. demonstrated that damage-regulated autophagy modulator (*DRAM*) gene, an autophagy-inducing lysosomal protein, is transcriptionally up-regulated by p53. *DRAM* is required for p53-dependent induction in both apoptosis and autophagy [103]. Interestingly, radiation of MCF-7 breast cancer cells triggers autophagic cell death [104]. The p53 transcriptional activity towards pro-apoptotic genes or the autophagic modulator *DRAM*, determines cell fate [105]. Another mechanism of p53-mediated regulation of autophagy was described in the human colorectal carcinoma cell line HCT116 [106]. P53 interacts with high mobility group box 1 (HMGB1) and this interaction regulates the cytoplasmic localization of either factors. P53 knockout HCT116 cells display increased cytoplasmic HMGB1 which in turn induces autophagy, possibly by interacting with Beclin-1 complex [107], while in HMGB1 knockout MEFs cytoplasmic p53 is increased and thus the apoptotic pathway is induced [106].

Death-associated protein kinase (DAPK) family

The Ca^{2+}/Calmodulin (CaM)-dependent serine/ threonine kinases of the Death-associated protein kinase (DAPK) family are involved IFN-γ-mediated cell death but also in autophagy regulation. In HeLa and breast cancer cells, constitutive activation of DAPK and its closest homolog DAPK-related protein kinase (DRP-1), promotes a caspase-independent autophagic type of cell death, characterized by membrane blebbing and increased autophagic vesicles [108]. Similarly, *C. elegans* nematodes deficient in the single DAPK ortholog DAPK-1, display significantly reduced autophagy in response to starvation [109]. Mechanistically, DAPK mediates its autophagy-inducing effects by phosphorylating Beclin-1 at Thr-119. This DAPK-dependent phosphorylation promotes Beclin-1 dissociation from its negative regulators Bcl-xL and Bcl-2 [110,111]. A subsequent study revealed that upon oxidative stress, DAPK phosphorylates protein kinase D (PKD), which in turn phosphorylates and activates directly VPS34, an indispensable factor for autophagy induction [112].

Cross-talk between autophagy and cell death pathways in the context of disease

Cancer

Autophagy connects nutrient sensing pathways with cell death cascades. In the context of cancer, this interlink is crucial for the tumours to sense nutrient availability and adapt accordingly. During tumour progression, autophagy can have either a tumour-activating or suppressing role, by promoting cancer cell survival or death, respectively. Moreover, autophagy can contribute to cell death type conversions. Experimental setting is an important parameter when analysing the role of autophagy in tumourigenicity, as relevance of using mammalian cancer cell lines or *in vivo* mouse models is still under dispute. Additionally, tissue specificity, variability and genetic background of different cancer cell types are factors which differentiate the role of autophagy.

Autophagy as a tumour suppressor has been demonstrated in various settings, either directly or indirectly, inducing various cell death mechanisms. Genetic ablation of *BECN1* and interacting proteins can cause spontaneous tumour formation [113]. Similarly, *ATG5* and *ATG7* deletion after *Ras* activation can cause pre-neoplastic lesions and tumour appearance [114]. Moreover, selective autophagic degradation of anti-apoptotic Fas-Associated Protein-Tyrosine Phosphatase 1, FAP-1 induces cancer cells to FAS-induced apoptosis [115].

ADCD has been greatly debated as a separate cell death mechanism. Quite recently though, ADCD induction was observed during replicative crisis [116]. That is, before cell transformation, when there is cell cycle

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progression dysfunction, this mechanism is activated to eliminate any potential cancerous cells via extensive cell death. Experiments performed in epithelial cells and fibroblasts reveal that autophagy is activated after telomere dysfunction and specifically intra-chromosomal breaks, which cause nuclear fragility, cytoplasmic DNA species and subsequent activation of the cyclic GMP–AMP synthase (cGAS)- stimulator of interferon genes (STING) pathway. This hyperactivation of autophagy leads to excessive cell death and could have unwanted side effects, including the production of neoplastic lesions. Etoposide, a DNA damaging agent, can cause autophagic cell death under hypoxic conditions, in contrast with normoxia where it triggers apoptosis [117]. Similarly, oxidative stress induces autophagic cell death in transformed cells [118]. Nutrient stress induced by resveratrol can also selectively trigger autophagic cell death [68]. Cell death mode switch is also modulated by autophagy. Genetic ablation of Mitogen-Activated Protein Kinase Kinase Kinase 7 (*MAP3K7)* activates RIPK1 and the necrosome which are recruited by p62 autophagic receptor to the autophagosomal membrane to facilitate TRAIL-induced necroptosis. When necroptosis complex recruitment is hindered, TRAIL induces apoptosis instead [119]. In another type of cell death, when Cdc25 is up-regulated in cervical cancer cells, autophagy-dependent ferroptosis is hindered [120].

However, after the tumour is established, autophagy acts as a survival mechanism to withstand nutrient stress and other harsh situations. Thus, timing of autophagy activation is critical, as autophagy initially prevents tumour formation but subsequently promotes tumour progression. Less aggressive types of cancer are detected upon autophagic inhibition. Specifically, genetic ablation of *ATG5* and *ATG7* in various tissues can cause tumour shrinkage primarily due to cell death. Cell death is triggered either directly or due to accumulation of damage. In the former case, lack of catabolic products leads to decreased fatty acid oxidation and glycolytic activity; while in the latter, accumulated damage could be a result of defective mitophagy due to gradual aggregation of mitochondria or increased DNA damage [121–123]. Thus, autophagy can act as a cell death evasion mechanism. Autophagy inhibition in HRAS transformed fibroblasts, triggers attachment-induced apoptosis, known as 'anoikis' and induces caspase-3 cleavage. Pharmacological inhibition of autophagy has also been shown to be therapeutic in tumour cells which are dependent on autophagy [124]. Evasion of chemotherapy-induced apoptosis can be mediated by autophagy, namely chemoresistance, while combinatorial chemotherapy and autophagic inhibition can be used as a drug regimen for more successful patient outcomes. Cancerous glial cells are resistant to treatment with cytotoxic chemotherapeutic agents due to autophagy-dependent elevation in ATP production. Genetic or pharmaceutical inhibition of autophagy triggers a non-apoptotic cell death mechanism associated with micro-nucleation [125]. Interestingly enough, cancer cells are extremely adaptable and can evade autophagic inhibition-triggered death. In particular, autophagy-deficient clones of cancer cell lines where autophagy was initially essential for their survival and proliferation, gradually adapted and had similar replicative rates as their wild type counterparts [126]. Thus, from a clinical perspective, the decision as to whether to inhibit or stimulate autophagy to augment an anticancer treatment depends on the type of cancer and its stage of progression. One should not overlook post-drug treatment effects on autophagy levels. However, more research is required on all fronts and contradicting studies make drawing conclusions even more difficult.

Neurodegeneration

In the nervous system, the role of autophagy in mediating or inhibiting cell death is rather complex. Apoptosis has been implicated in reduced adult mouse hippocampal neural stem cell survival upon depletion of *ATG5*. Thus, autophagic deficiency leading to apoptosis could cause cognitive/memory impairment [127]. In accordance, chronic blockage in the canonical autophagic pathway has been correlated with neuronal death in mouse models of the polyglutamine disease dentatorubral-pallidoluysian atrophy (DRPLA). This specific type of cell death, termed karyoptosis, involves nucleophagy-based disintegration of the nuclear envelope and subsequent excretion of nuclear components in a Golgi-dependent mechanism, finally leading to cell shrinkage, nucleus degeneration and death [128]. The autophagic pathway acts as a mediator of stress-induced neuronal cell death. Specifically, inhibition, by either 3-methyladenine or knockdown of *ATG7* or *BECN1,* blocks stress-induced caspase-independent cell death. Beclin-1 seems to have a more crucial and complex role in controlling this type of neuronal cell death, as its absence blocked both staurosporine- and hydrogen peroxide- induced death, while *ATG7* ablation only prevented the latter [129]. More recently, an *in vivo* brain injury mouse model of hypoxia–ischemia, which usually occurs after perinatal asphyxia in the neonatal brain and can lead to hypoxic–ischemic encephalopathy, revealed that ATG7 is a mediator of cell death. In its absence, there is 42% reduction in tissue loss. Interestingly, in the neonatal brain both caspase-3-dependent and independent cell death are induced

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while in the adult brain cell death is caspase-independent [130]. Thus, a severe hypoxic insult causes excessive induction of autophagy, which is detrimental for cell survival.

Similarly, in neurodegenerative diseases, the level of autophagy activation might be significant in determining the balance between cell survival and death. In a Huntington's disease mouse model, berberine alleviates cellular stress; while, in hepatic cancer induces apoptotic and autophagic cell death. Whether berberine acts promiscuously on multiple cellular pathways, or differentially, depending on the level of administration, cellular context remains to be investigated. One cannot exclude the possibility that berberine-mediated hyperactivation of autophagy ultimately leads to cell death of diseased neurons [131,132]. In a Parkinson's disease model of 6-hydroxydopamine (6-OHDA) injured rat substantia nigra neurons, there is excessive production of autophagosomes which triggers cell death [133]. However, it must be noted that it might not be autophagy *per se* that causes cell death, but the failure to properly degrade the overproduction of autophagosomes. Namely, lysosomal dysfunction might be more relevant in directly causing cell death. This is the case for lysosomal storage disorders, where autophagy failure causes neurodegeneration. Lack of cathepsin D causes autophagosome accumulation in neurites leading to cell death [134]. When degradation itself is blocked, mutant oxidized proteins accumulate in the lysosomes, where they increase lysosomal membrane permeability, finally leading to the release of hydrolases into the cytoplasm. This lysosomal dependent cell death pathway can be triggered by various endogenous agents which induce lysosome permeabilisation such as ceramide, sphingosine, oxidized lipids, ROS, even amyloid beta and apo-lipoprotein E, which are involved in Alzheimer's disease [135]. In any case, *in vivo* disease models are required to discern the cross-talk between autophagy levels in neuronal cell death.

Concluding remarks

A tremendous amount of research during the last decade has revealed a complex reciprocal communication between autophagy and cell death pathways. Notwithstanding, it is difficult to construct a generalized and defined model of how mechanistically these self-destructive processes interact. Most of the studies which support a cytoprotective or cytocidal role of autophagy are conducted *in vitro*, in cancer cell lines upon genetic or pharmaceutical manipulations, and thus do not correspond to physiological cellular conditions. In these experimental settings, the crucial role of the tumour microenvironment and its signals is overlooked. Thus, the results obtained from cell lines are context-dependent and need further verification in *in vivo* systems. In addition, drugs that are used to block autophagy usually result in pleiotropic effects that could interfere with cell fate in an autophagy-independent manner. Another issue which adds to the complexity and often results in misleading interpretations, is the dynamic nature of both processes. In most cases, the conclusions obtained regarding autophagy and cell death interactions, are based on qualitative and rather descriptive studies which only represent a very specific time frame of the cellular status. Scientific advances in live imaging techniques, combined with large-scale systemic and single-cell analytic approaches will definitely shed light on the highly dynamic cross-talk between autophagic and cell death machineries. The expanding knowledge can support the development of effective therapeutic strategies for diseases, since manipulations in autophagic activity can differentially modulate cellular viability in distinct disease contexts.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

AIF, apoptosis inducing factor; AMBRA1, activating molecule in Beclin1-regulated autophagy 1; AMPK, AMP-activated protein kinase; APAF1, apoptotic peptidase activating factor 1; ATG, autophagy-related; BAK, Bcl-2 homologous antagonist/killer; BAX, Bcl-2 associated X; Bcl-2, B-cell CLL/lymphoma 2; Bcl-2-L-2, Bcl-2-like protein 2; Bcl-xL, Bcl-2 related extra-large; BID, BH3 interacting domain death agonist; BIK, Bcl-2 interacting killer; BOK, Bcl-2 related ovarian killer; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; CED-3, cell death protein 3; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; DAPK, death-associated protein kinase; DIABLO, direct IAP-binding protein with low PI; DIF-1, differentiation-inducing factor; DRAM,

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damage-regulated autophagy modulator; DRP-1, DAPK-related protein kinase; DRPLA, dentatorubral-pallidoluysian atrophy; EndoG, endonuclease G; ER, endoplasmic reticulum; FADD, Fas associated via death domain; FAP-1, Fas-associated protein-tyrosine phosphatase 1; FASL, Fas ligand; FIP200, focal adhesion kinase family interacting protein of 200 kDa; FKBP38, FK506-binding protein 8 (38 kD); GBA1, glucocerebrosidase; HMGB1, high mobility group box 1; IFN-γ, Interferon-γ; IMM, inner mitochondrial membrane; LC3, light chain 3; LIR, LC3-interacting region; MAP3K7, mitogen-activated protein kinase kinase kinase 7; MCL-1, myeloid cell leukemia sequence 1; MEC-4, Mechanosensory abnormality protein 4; MEFs, mouse embryonic fibroblasts; mTOR, Mechanistic target of rapamycin; NAF-1, nutrient-deprivation autophagy factor-1; NBR1, neighbor of Brca1; NDP52, nuclear dot protein 52 kDa; p62, phosphotyrosine-independent ligand for the Lck SH2 domain of 62 KDa; PI3K, phosphatidyl inositol 3-kinase; PI3P, phosphatidylinositol-3-phosphate; PINK1, PTEN Induced Kinase 1; PKD, protein kinase D; PUMA, P53-up-regulated modulator of apoptosis; RHIM, RIP homotypic interaction motif; SQSTM, sequestosome; TIR, Toll/IL-1 receptor; TNF, tumor necrosis factor; TP53INP2, tumor protein P53 inducible nuclear protein 2; TRAF-6, TNF receptor associated factor 6; TRAIL, TNF-related apoptosis-inducing ligand; TRAP1, TNF-receptor associated protein 1; TRIF, TIR domain-containing adaptor protein inducing interferon-β; ULK, Unc-51-like kinase; UVRAG, UV radiation resistance-associated gene; VDAC1, voltage dependent anion channel 1; VPS34, phosphatidylinositol 3-kinase; ZBP1, Z-DNA binding protein 1.

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