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Title: Caspase involvement in autophagy

Running title: Caspases and autophagy

Panagiotis Tsapras¹ and Ioannis P. Nezis^{1,*}

¹School of Life Sciences, University of Warwick, CV4 7AL, Coventry, United Kingdom

*corresponding author

Dr Ioannis P. Nezis

Associate Professor

School of Life Sciences

University of Warwick

Gibbet Hill Campus

Coventry CV4 7AL, UK

Phone: +44 (0) 24 76 150400

Fax: +44 (0) 24 76 522052

E-mail: I.Nezis@warwick.ac.uk

Abstract

Caspases are a family of cysteine proteases widely known as the principal mediators of the apoptotic cell death response, but considerably less so as the contributors to the regulation of pathways outside cellular demise. In regards to autophagy, the modulatory roles of caspases have only recently begun to be adequately described. In contrast to apoptosis, autophagy promotes cell survival by providing energy and nutrients through the lysosomal degradation of cytoplasmic constituents. Under basal conditions autophagy and apoptosis cross-regulate each other through an elaborate network of interconnections which also includes the interplay between autophagy-related proteins (ATGs) and caspases. In this review we focus on the effects of this crosstalk at the cellular level, as we aim to concentrate the main observations from research conducted so far on the fine-tuning of autophagy by caspases. Several members of this protease-family have been found to directly interact with key ATGs involved in different tiers across the autophagic cascade. Therefore, we firstly outline the core mechanism of macroautophagy in brief. In an effort to emphasize the importance of the intricate cross-regulation of ATGs and caspases, **we also present examples** drawn from *Drosophila* and plant models regarding the contribution of autophagy to apoptotic cell death during normal development.

Keywords and abbreviations

autophagy; macroautophagy; mammals; caspases; apoptosis; crosstalk; regulation

AMBRA-1 **Activating-molecule-in-Beclin1-regulated-autophagy-1**

AMPK **AMP-activated-protein-kinase**

ATG **Autophagy-related gene**

ATG14L **ATG14-like**

Bcl-2 **B-cell lymphoma 2**

DISC **Death-inducing signalling complex**

FADD **Fas-associated protein with death domain**

FIP200 **Focal-adhesion-kinase-family-interacting protein of 200 kD**

GABARAP **Gamma-aminobutyric acid receptor-associated protein**

GATE-16 **Golgi-associated adenosine triphosphatase enhancer of 16 kD**

GTPase **Guanosine triphosphatase**

LAMP	Lysosome-associated membrane glycoprotein
mTOR	mammalian target-of-rapamycin
MAP1LC3 (or simply LC3)	Microtubule-associated-proteins 1A/1B light-chain-3
MOMP	Mitochondrial outer membrane permeabilization
PCD	Programmed cell death
PI3K	Phosphatidylinositol-3 kinase
Raptor	Regulatory-associated-protein-of-mTOR
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
t-SNARE	target-Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
ULK	Uncoordinated (UNC) 51-like kinase
UVRAG	UV-radiation resistance-associated gene
VPS34	Vacuolar-protein-sorting-34

Facts

- Caspases have been shown to directly interact with core autophagy proteins
- Autophagy is implicated in many physiological and pathological processes, where apoptosis is also involved

Open Questions

- Does control of autophagy by caspases represent a central or a supplementary mode of regulation of this pathway?
- Is caspase activation required to control autophagy?
- To what extent do caspases affect autophagy and how exactly their effects on the pathway vary across different contexts?

Introduction

Apoptosis is the tightly controlled process for an orderly executed cell death, and its key mediators, the caspases (Cysteine-dependent aspartate-specific proteases), are known for their indispensable part in the cascade. They exist predominantly as inactive precursors within cells, activated frequently by oligomerization, cleavage of regulatory pro-domains, and/or dissociation from endogenous inhibitors [1]–[3]. In mammals, the ten major caspases (of the 14 characterized so far) can be roughly classified based on their primary apoptotic role, as initiators (caspase-2,-8,-9, and -10), or executioners (caspase-3, -6, and -7) [1]–[3]. The third group consists of inflammatory caspases (caspase-1, -4, and -5) which are largely involved in “pyroptosis”; a prominent mode of PCD during host-pathogen interaction, which also involves the immune system response [1]–[3]. While there is still a degree of dispute over the apoptotic roles of inflammatory caspases, the members comprising the other two groups have well-documented functions in the mediation of the apoptotic response [1]–[5]. Although caspase functions have been a commonly pursued research subject for decades, new properties are continuously attributed to these molecules [3], [6]. For instance, caspases owe half their name to their highly site-specific cleavage of their substrates on aspartate-containing recognition sites. Nevertheless, they have been recently reported to cleave their targets almost as efficiently on motifs bearing glutamate or phosphoserine in place of aspartate [6]. Despite their initial discovery as cellular assassins, the exponential increase of evidence that followed in support of their additional non-apoptotic functions has served to view caspases under a different scope; that of agents with many faces, contributing to the regulation of cellular processes that often function complimentary, or even oppositely to apoptosis [3], [7], [8]. One such example can be gleaned from their involvement in autophagy, a cellular process intricately connected with apoptosis; so much that often perturbations in one pathway considerably impact the other [9]–[13].

Autophagy (from the Greek for “*self-eating*”), was coined in 1963 by Nobel laureate C. de Duve [14]. 30 years later, the seminal work conducted by Y. Ohsumi in describing the core machinery of the process in yeast would in turn lead to his award with the 2016 Nobel Prize, in recognition of the pathways’ cardinal role in the well-being of cells [15], [16]. Since the first description of the process, autophagy is further subdivided nowadays into three types (macroautophagy, microautophagy, and chaperone-mediated autophagy) [17]–[19], of which macroautophagy is the most well-studied and in many texts including this review, the term is simply referred to as *autophagy*. The process is responsible for the recycling of intracellular constituents, in which a portion of the cytosol is sequestered by isolation membranes. This leads to the formation of a double-membrane-layered organelle called the autophagosome, which subsequently fuses with a lysosome to create an autolysosome, a structure with a single limiting membrane wherein its contents are degraded. The residual materials are then recycled back to the cytosol for effective use by the cell [20]. In this manner an assortment of intracellular residents including protein aggregates [21], [22], damaged organelles [23], [24], and invading pathogens [25]–[27], as well as caspases [28], [29] can be effectively cleared from the cytosolic environment. Therefore, autophagy is primarily regarded as a pro-survival mechanism and is triggered in response to a wide array of life-threatening stimuli. The nature of its function places the pathway at the kernel of many physiological

settings where apoptosis is also involved [30]. Their complex interplay is particularly highlighted during physiological processes such as developmental remodeling and cell differentiation [31], as well as aging [32]. Moreover, perturbations which impair the efficient removal of protein aggregates and cytotoxic oligomers by autophagy have been reported in a range of neurodegenerative diseases including Alzheimer's [33] and Parkinson's [34]. Additionally, it is posited that some cancer cells can utilize autophagy as a means to prolong their survival [35], [36].

Similarly to apoptosis, progression of autophagy follows a tightly supervised outline of events. Many steps in this cascade are controlled by the interactions of several ATGs with non-autophagy exclusive regulators including caspases [3], [9], [10], [13], [37]. In order to gain a clearer overview of the ways caspases can associate with ATGs and modulate autophagy, we firstly describe the stepwise assembly of the autophagic machinery in mammalian systems. Due to space limitations the process is presented in great summary.

Overview of the autophagy cascade

Initiation

The ULK-complex is regarded as the apical initiator of autophagy in mammals [38], [39]. Its key kinase ULK is the functional homologue of Atg1, with at least four known members in the family (ULK1-4) [40]. ULK1 and ULK2 are the most frequently studied in autophagy investigations and they are found to form distinct ULK-complexes within cells [39], mainly due to their different binding affinity for another prominent member of the ULK-complex; ATG13 [38]–[40]. Because of their characterized role in autophagy it is these two members that are being implied hereafter when mentioning either ULK or ULK-complex, unless explicitly specified. Together with ULK and ATG13, the core of the ULK-complex is further complemented by ATG101 [41] and the scaffold protein FIP200 [42], [43]. FIP200 is arguably the functional homolog of yeast Atg17 and binds ULK, indirectly promoting its kinase activity [38]. ATG13 facilitates the interaction between ULK and FIP200 and preferentially binds ULK1 over ULK2 [38]–[40].

Activity of the ULK-complex is in turn modulated further upstream by two Ser/Thr kinases, mTOR 1 and 2 [44], [45]. In an analogous fashion to ULK, these kinases associate with several different binding partners in distinct mTOR complexes (mTORC) with respectively distinct properties and signaling pathways [44], [45]. An example of the differential response of each complex related also to autophagy, is that activity of mTORC2 remains unaffected by treatment of cells with the autophagy inducer rapamycin, in contrast to mTORC1 [46]. mTORC2 interacts with the rapamycin-insensitive-companion-of-mTOR (Rictor) in place of Raptor [46]; the binding partner of mTORC1[44]–[46]. While they both regulate autophagy, the mTORC2-signalling cascade is still poorly defined therefore, the more well-characterized mTORC1-dependent pathway is often utilized to induce autophagy instead [44]–[46].

Under fed conditions, mTORC1 binds to and inhibits ULK [47]. In contrast, depletion of intracellular amino-acid levels following starvation results in the upregulation of energy level-dependent sensors such as AMPK, which phosphorylates and aids in the dissociation of mTORC

from ULK [47], [48]. In addition to other post-translational modifications, ULK further activates ATG13, FIP200 and ATG101 within the complex [38], [49]. The fully-engaged ULK-complex then translocates to the membrane compartment of phagophore assembly together with the class-III PI3K complex (PI3KC-III); the leading multi-protein structure of the next stage [38], [49]. A schematic representation of the step-wise progression of the autophagic cascade in mammalian systems after mTOR inhibition is given in Figure 1.

Phagophore nucleation and expansion

The lipid kinase VPS34, its regulatory subunit p150, and Beclin-1 (the orthologue of yeast *Atg6*) are the main components of PI3KC-III, which is essential through the steps of phagophore formation to its maturation to a complete *autophagosome* [43], [50]. Other important co-regulators of the complex include ATG14L, AMBRA-1 and UVRAAG [50]. ATG14L and UVRAAG compete for their binding of Beclin-1 and form separate PI3K-III complexes, which are respectively required in autophagosome biogenesis and in their maturation and fusion with lysosomes [20], [50], [51]. Beclin-1 is of particular interest as it sits at the crossroads between autophagy and apoptosis. As a Bcl-2-homology (BH)-3 domain only protein it interacts with BH3-domains of other Bcl-2 family proteins and is often bound inert to the anti-apoptotic Bcl-2 or Bcl-XL proteins at the outer mitochondrial membrane [50]. Upon initialization of autophagy, Beclin-1 undergoes phosphorylation and ubiquitination as well as associating with the ULK1-activated scaffold AMBRA-1 [50], [52]. These changes favour the uncoupling of Beclin-1 from Bcl-2; the freed protein in turn triggers the formation of the PI3K-III-complex by binding and switching-on VPS34 [50], [52]. The upregulated PI3KC-III generates a local pool of PI3-phosphate (PI3P) at the phagophore nucleation site, which serves as a beacon that attracts additional PI3P-sensing phospholipid effectors and ATG proteins on the scene [50]. Elongation of the nascent organelle depends on the constant incorporation of membrane material to its expanding body [43]. Among the many crucial modulators of this elaborate step, two transmembrane proteins, ATG9 and vacuolar-membrane-protein-1 (VMP1) often stand out due to their respective roles. VMP1 tethers PI3KC-III to the developing surface of the phagophore by binding to Beclin-1 in the complex. ATG9 is the only ATG member characterized so far, capable of shuttling between cytosolic compartments and transferring membrane cargo to the nascent autophagosome [20].

The mammalian Atg8 family consists of 3 subfamilies: LC3, GABARAP and GATE-16; all containing members with clarified or lesser known roles in autophagy [53], [54]. Of these, the variants of the LC3-subfamily (LC3A, LC3B, LC3C) and namely LC3B (hereafter LC3), are routinely used as markers in assays monitoring autophagosome formation [53], [54]. LC3 and the E3-ligase ATG12 are two ubiquitin-like proteins necessary for autophagosome expansion and closure and are recruited to the membrane site along with their associated ubiquitin-like conjugation systems [55], [56]. ATG7 activates ATG12 and ATG10 conjugates it to ATG5 [9], [50], [55]. The resulting ATG12-ATG5 heterodimer then binds ATG16L1, altogether completing the core of the ATG16L1 complex [9], [50], [55]. LC3 is cleaved by the cysteine protease ATG4B, yielding LC3-I,

which is subsequently transferred by ATG7 to the inner and outer membrane of the expanding phagophore [57]–[59]. There, LC3-I is passed over to ATG3 and by the localized operations of the PI3K-III and ATG16L1 complexes it is lipidated to LC3-II and anchored to the autophagosomal membrane [57]–[59].

Maturation and fusion with lysosomes

During fusion of the mature autophagosome with a lysosome, the ATG16L1-complex dissociates from the vesicle and ATG4B removes the cytosolic fraction of LC3-II [43], [57], [59].

A t-SNARE complex involving several syntaxins and ATG14L generally prime the cargo-bearing autophagosome for the fusion step with the lysosome [51]. Other reports suggest that an intermediate autophagosome-endosome fusion step might occur, thereby providing the factors necessary for the ensuing lysosomal fusion [60]. Acidification and further maturation to fully fledged autolysosomes is mediated with the aid of small GTPases of the Rab family, together with the lysosome-associated-membrane glycoproteins LAMP1 and LAMP2 [55], [61].

Table 1 summarizes the core ATG and co-regulator proteins discussed here, along with their position in the autophagic cascade and their function.

Caspases impact on autophagy

Autophagy and apoptosis are interlocked in an extensive crosstalk with each other, therefore it may not be surprising that many ATGs are recognized and cleaved by caspases [10], [37], [62], [63]. In most cases, interaction of the caspase with the ATG protein leads to abrogation of the autophagic function of the latter, and the homeostatic balance shifts in favor of an apoptotic profile (Figure 2) [11], [52], [64]. This is generally regarded as a commitment of dying cells to an apoptotic PCD by progressively shutting down other functions, including autophagy. Nevertheless, caspase-mediated cleavage of ATGs does not always result in idle waste products; instead several ATG-fragments have been shown to acquire new properties, which can differ greatly from those of their initial full-size isoforms and consequently affect apoptosis and autophagy in numerous ways [65], [66]. **On the other hand caspases have also been found to promote autophagy under certain contexts [66]–[68].**

The findings from the existing literature presented below are split between the *initiator* and the *effector* caspase groups, pertaining to their main role in apoptosis (as shown in Table 2).

Initiator Caspases

Caspase-1 is primarily involved in the maturation of the pro-inflammatory cytokines interleukin-1B/interleukin-18 during oxidative stress in immune cells and endothelial cells [67], [69]. Additionally, a role of caspase-1 in facilitation of cytoprotective autophagy has been suggested during hypoxia-induced mitochondrial stress [67]. In hepatocytes of *casp-1* \sim mice levels of the

common autophagy markers LC3 and Beclin-1 were found to be decreased compared to wild-type (WT) [67]. When Beclin-1 was overexpressed in these cells the group reported that impaired autophagic clearance of damaged mitochondria was restored to WT rates [67]. It was therefore suggested that aside from its role in immune cells, caspase-1 in murine hepatocytes appears to favor autophagy and clearance of damaged mitochondria after redox stress by activating LC3 and Beclin-1 through an as-of-yet undefined mechanism [67].

Caspase-2 is the most evolutionary conserved member of the caspase family and possess features of both initiator and effector caspases [70]. It conveys the apoptotic signal chiefly after stimuli such as oxidative stress [71], DNA damage [70], heat shock [72] and cytoskeleton disruption [73]. In regards to its involvement in autophagy, under normal conditions caspase-2 is evidently a negative regulator of the process, as supported by results derived from mouse embryonic fibroblasts (MEFs) [74] and young adult mouse cortical neurons [71]. In both murine models, *casp2* knock-down at an early-stage lead to inhibition of apoptosis and mobilization of cytoprotective autophagy, particularly when the initial death-inducing stimulus was delivered either via heat shock or mitochondrial oxidative stress [71], [74].

While the exact mechanism of caspase-2-dependent modulation of autophagy remains to be fully delineated, it seemingly involves control of intracellular levels of reactive oxygen species by caspase-2 [71], [74]. Loss of caspase-2 favors the accumulation of oxygen radicals, which spark a cascade of events culminating in the concomitant downregulation of autophagy suppressors such as TORC and activation of pro-autophagy mediators including AMPK [74].

Caspase-8 is commonly upregulated during the extrinsic apoptotic cascade, shortly after activation of death receptors expressed at the cell-surface [2], [75]. DISC is the multi-protein signaling platform assembled on the scene, whereupon the inactive precursor pro-caspase-8 dimerizes and cleaves itself and subsequently released in its functional state to the cytoplasm, triggering upregulation of its effector caspases [75]–[77].

In addition to its conventional activation pathway, caspase-8 maturation has been reported even in the absence of DISC [78], [79]. When formation of this complex is inhibited, autophagy seemingly takes over, either as a death-escaping mechanism or as an alternative route for caspase-8 activation [78], [79]. Pro-caspase-8 can be localized to autophagosomes via the ATG8/LC3-interacting molecule p62 [80]. This is an adapter protein which binds poly-ubiquitinated (polyU) protein aggregates and targets them for sequestration at the site of autophagosome formation [81]. In this case, the autophagosomal surface may provide a platform where polyU pro-caspase-8 oligomers can be brought in close proximity with each other, thereby facilitating their interaction and maturation to caspase-8 dimers [80], [82], [83]. Conversely, it has also been proposed that sequestration of caspase-8 or its dormant precursor to the autophagosome may be a method utilized at least by Bax-/ Hct116 colon carcinoma cells to promote their survival instead [28]. It is important to note that these cells develop a resistance to apoptotic stimuli, despite having a fully functional DISC capable of switching on caspase-8 [28]. However, active caspase-8 and its inactive pro-form can still be

targeted and extensively retained at the autophagosome where they are eventually degraded along with the rest of the autophagosomal cargo within the ensuing autolysosome [28]. Since there is not enough caspase-8 to adequately propagate the cell death signal, cancer cells become increasingly unresponsive to apoptotic stimuli [28]. To rescue caspase-8 from autophagic degradation and re-sensitize cancer cells to apoptotic cell death, a viable option in this instance is to inhibit autophagosome formation [28]. As the above examples illustrate, the contradictory functions of the autophagosomal surface in regards to caspase-8 activity should be taken into account in investigative approaches to cancer-treatment by manipulating autophagy. As this effect seems to be context-dependent it is paramount to find the right balance between promoting targeting of the protease to the developing phagophore and inhibiting autophagy, **in order to re-establish an organized apoptotic dismantling of different type of tumor cells.** There are already reports where the ability of p62 to promote caspase-8 activation at the autophagosomal membrane has been successfully exploited to render cancerous cell-lines responsive to further pharmacological treatment [83], [84].

Moreover, functional caspase-8 has been shown to prevent autophagy from over activation [85]. In rapidly proliferating T-cells lacking either the DISC-adapter protein FADD or caspase-8, the autophagic response was initially mounted in order to provide the necessary energy to support their proliferation [85]. However, the process quickly became hyperactive and led to cell death with necroptotic features such as the upregulation of the Ser/Thr kinase RIPK1 [85]. This specific kinase as well as pro-caspase-8, associate with the ATG16L1 complex, which apart from its autophagosome-expanding role, has been quite interestingly shown to also serve as an auxiliary scaffold to DISC assembly [80], [85]. In WT T-cells, ATG5 is observed to interact with FADD, which also binds both pro-caspase-8 and RIPK1 [85]. The association of ATG5 with FADD can thereby promote DISC formation and maturation of pro-caspase-8 to active caspase-8 in a manner additionally involving the autophagosome-forming machinery [85]. The caspase can then cleave RIPK1, as part of a negative feedback loop to prevent excessive autophagy [85]. In the absence of either FADD or caspase-8, RIPK1 remains on and perpetuates the autophagic response, eventually resulting in necroptotic cell death [85]. RIPK1 also augmented autophagy in lung, bladder and prostate cancer cell-lines after stimulation of the external apoptotic pathway by the TNF-related apoptosis-inducing ligand (TRAIL) [86]. Based on these findings, caspase-8 and RIPK1 have emerged as valuable drugable targets to manipulate the balance between autophagy and apoptosis in under certain frameworks, in order to stall proliferation and induce recession of malignant tumors [85], [86].

The role of caspase-8 in modulating autophagy is further complemented by its interactions with ATG3, ATG5 and Beclin-1, which are all reportedly prone to cleavage by the protease *in vitro* [87], [88]. Following death-receptor activation in several cancer-derived cell lines, the subsequent downregulation of autophagic flux has been partly attributed to caspase-8-dependent cleavage of these autophagic proteins [50], [87], [88]. A peculiar yet noteworthy finding is that the processing of Beclin-1 by many initiator and effector caspases often returns a C-terminal fragment which in a

starvation-induced setting displays increased pro-apoptotic properties [50], [65]. It has been long accepted that both the extrinsic and intrinsic apoptotic pathway converge at the mitochondrial pro- and anti-apoptotic Bcl-2 proteins, which control the rate of cytochrome-c release [76]. The truncated Beclin-1 fragment has been observed to also localize to mitochondria and further promote MOMP and the release of pro-apoptotic factors to the cytosol, consequently amplifying the apoptotic response [50], [65]. The processing of ATG3 by caspase-8 was shown to effectively inhibit assembly of the autophagic machinery at an early stage and promote apoptosis [88]. The caspase-recognition site on ATG3 has been mapped in the region between amino-acids 166-169 which contains an evolutionary conserved LETD motif that is widely recognized by many caspases [88].

Caspase 9 is set in motion following cytochrome c-mediated assembly of the Apoptotic-protease-activating factor-1 (Apaf1)-apoptosome during the intrinsic apoptotic cascade [2], [89]. In regards to autophagic proteins being substrates for the protease, in several tumor cells caspase 9 cleaves and inactivates to a certain degree ATG5 and Beclin-1 [87], [90]. Another additional connection of the protease with autophagy has been proposed following consecutive observations on the MCF-7 breast cancer cell-line [91]. In this cell-line, the non-steroidal anti-inflammatory drug FR122047 (FR) is used to induce cell death via the extrinsic apoptotic signalling [91]. When MCF-7 cells deficient for caspase-9 were treated with FR, their susceptibility to cellular demise was increased, with a concomitant decrease in autophagic flux [91]. The authors postulated that caspase-9 possibly contributes to upregulation of cytoprotective autophagy by mediating proper acidification of lysosomes and the acid-dependent functions of cathepsins [91]. As a result, caspase-9-deficient MC7-cells appear to respond more positively to treatment with FR, due to their inability to mount a proper autophagic response [91].

Building on these findings, the mechanism by which caspase-9 exerts this peculiar effect on autophagy was later investigated in more detail. It was discovered in tumor cell-lines as well as MEFs with inducible knock-outs for an array of ATGs, that caspase-9 forms a stable complex with ATG7 [68]. This interaction does not result in cleavage of ATG7 but conversely the caspase-9-ATG7 complex is more efficient at binding and priming LC3, resulting in a corresponding increase of developing autophagosomes [68]. This is partly due to the fact that catalytic activity of caspase-9 while bound to ATG7 is abolished, leaving ATG7 affinity for LC3 unobstructed, thus leading the authors to posit that this mechanism may be utilized by several tumor cells in order to prolong their survival [68].

Caspase 10 is turned on in an analogous manner to caspase-8 during the extrinsic apoptotic pathway. In addition to its reported cleavage of ATG5 and Beclin-1 [50], [87], [90], caspase- 10 has also been discovered to modulate autophagic flux by processing Bcl-2-associated transcription factor 1 (BCLAF1) in a myeloma cell-line [92]. BCLAF1 is a known inducer of autophagy capable of indirectly activating Beclin-1 by antagonistically competing for their binding site at Bcl-2 [92]. This finding was the result of series of experiments utilizing RNA-interference to knock down an array of target proteins required for cell survival, identifying BCLAF-1 to be a substrate of caspase-

10 [92]. Proteolytic cleavage of BCLAF1 by caspase-10 abolished its function and therefore Beclin-1 remained tethered to Bcl-2 [92]. By effectively exploiting this mechanism, myeloma cells despite their requirement for a degree of background autophagy, seemingly ensure their survival by keeping the process in check and consequently escape demise [92].

Effector Caspases

Caspase-3, along with caspases -6 and -7, is the principal effector caspase whose role in autophagy regulation has been investigated the most. The first study to suggest a role of mammalian caspase-3 in autophagy utilized TRAIL-induced apoptosis in HeLa cells, during which Beclin-1 was cleaved in a caspase-dependent manner [90]. The research group disclosed that proteolytic processing of Beclin-1 by caspase-3 was found to be sufficient in significantly impairing autophagic flux when an apoptotic-inducing stimulus was applied [90]. In contrast either overexpression of Beclin-1 in this cell-line or introduction of a non-cleavable mutant effectively inhibited TRAIL-induced cell death in this cell line [90].

Two caspase-3-cleavage sites of Beclin-1 have been since identified on positions 124 and 149 [64]. Moreover, in murine bone marrow cells that underwent prolonged starvation, the initial autophagic response gradually shifted towards apoptosis [65]. During this transition stage Beclin-1 was shown to be cleaved by caspase-3, generating a C-terminal fragment that enhanced cell-death by the mechanism already mentioned [65]. ATG5 has also been proposed to be prone to cleavage by caspase-3, consequently contributing to autophagy downregulation [87].

Other studies argue that caspase-3 in nutrient-deprived human endothelial cells is able to aid towards the export of maturing LC3-containing autophagic vesicles through the plasma membrane, promoting cell-volume shrinkage, which is an indicative apoptotic marker [93], [94].

An isoform of the ATG4 protease, ATG4D, is another prominent substrate for caspase-3 processing [95]. Cleavage of ATG4D by caspase-3 has been shown to occur at the N-terminal DEVD⁶³K motif, which is a common caspase-cleavage site [66]. This action returns a truncated product (Δ N63K-ATG4D) with enhanced lipidation activity on the Atg8-related family member, GABARAP-L1 [66], [95]. The cleaved isoform was found to promote autophagy when it was the product of caspase-3 processing of endogenous ATG4D, but when overexpressed, Δ N63K-ATG4D was shown to enhance apoptosis instead [95]. This cytotoxic effect is possibly due to the combination of increased levels of Δ N63K-ATG4D and its enhanced affinity for damaged mitochondria where it augments with a currently undefined mechanism, MOMP and the subsequent release of pro-apoptotic factors in the cytosol [66].

Furthermore, AMBRA-1, which favors Beclin-1 binding to PI3KC-III, has being identified as an additional target for caspase-3-processing during apoptosis *in vitro* [52]. When AMBRA-1 was inactivated due to proteolytic cleavage by caspase-3, Beclin-1 consequently failed to couple effectively to PI3KC-III and autophagosome formation was inhibited as a net result [52].

Autophagy was thereby halted in an AMBRA-1-dependent fashion, favoring the progress of the apoptotic cascade [52].

Caspase 6 impact on autophagy progression has been suggested, along with other caspases, based on observations from cancer-derived cell cultures [87], [90]. In melanoma-derived cell-lines subjected to starvation and TRAIL-induced cell death, caspase-6 was shown to exacerbate degradation of Beclin-1 and ATG5, further inhibiting autophagy and shifting the balance in favor of an apoptotic response in these cells [87].

Moreover, an *in vitro* screening for identifying protease regulators of ATG proteins in mammalian cells showed that also ATG3 and p62 are potential substrates for caspase-6 processing [62]. When ATG3 is cleaved by the effector caspase, autophagy is subsequently downregulated, possibly due to inhibition of phagophore formation [62]. However, the exact reason why the pathway is inhibited as a result of p62 loss is yet unclear. A possible explanation as proposed by the authors might lie in the role of p62 as a promoter of autophagy by targeting LC3 and polyU-aggregates at the site of autophagosome formation [82], [96]. Its loss due to caspase-6 cleavage may therefore result in the less efficient recruitment of LC3 and polyU-cargo to the autophagosome, inevitably posing a setback in autophagy progression [62]. In a potential extension of this argument, in several settings p62 can modulate caspase-8 function by targeting its polyU-precursor to the autophagosome during apoptosis upregulation, as already mentioned [82], [83]. Caspase-8 then activates downstream executioners such as caspase-6, thereby enhancing the apoptotic response [1]. As such, it may be posited that in those same settings caspase-6 may potentially cleave p62 as part of a negative feedback loop that further prohibits the recruitment of LC3 and polyU-cargo to autophagosomes. No matter how tempting these explanations might be, due to its role p62 is involved in many cellular processes. As such its observed impact on autophagy because of its loss may just as likely be more indirect, through other p62-dependent cascades being affected, which additionally fine-tune autophagy. The precise function/s of p62 in autophagy is a subject still under intense scrutiny.

Caspase-7 is the third member of the staple executioner caspase trio and the lesser known in regards to its impact on the autophagic pathway. Under nutrient scarcity, Beclin-1 is reportedly targeted directly by caspase-7 during apoptosis, which is preceded by autophagy upregulation [65]. A study demonstrated the ability of caspase-7 to bind and cleave a mutant of ATG16L1 (mutATG16L1) in cells from knock-in murine models bearing one amino-acid substitution on position 300 of ATG16L1, which turns threonine to alanine [97]. Binding affinity of caspase-7 was greater for mutATG16L1 compared to WT ATG16L1 [97]. In humans, this single coding polymorphism is arguably linked to increased risk for developing Crohn's disease [97], and caspase-7 binding to mutATG16L1 was correlated to an observed decrease of autophagy in response to pathogen-infection [97]. The group concluded that, even though the exact mechanism warrants further investigation, compromised cytoprotective autophagy in this context appears to be associated with increased bacterial load, further disrupting cellular integrity and potentially contributing to the disease manifesting in cells [97].

Functional conservation of the caspase-autophagy cross-regulation and its importance in developmental cell death

The role of autophagy as a cell death modality has sparked increased controversy among researchers. “Autophagic cell death” (ACD), also known as type-II PCD, refers to a particular phenotype of cellular demise whereupon an overabundance of autophagic vesicles is evident in dying cells, giving them a characteristic vacuolated appearance [12], [98]. However, researchers agree that, since it is often difficult to distinguish whether autophagy is a causal or an auxiliary phenomenon of cell death, the line between “cell death by autophagy” and “cell death with autophagy” must be drawn as clearly as possible in functional studies [99], [100]. There are excellent reviews available that deal in greater depth with this condition-specific duality of the pathway [12], [101].

The fruit fly *Drosophila* has provided substantial evidence favoring the contribution of autophagy to cell or tissue degradation, especially during developmental remodeling [102], [103]. Upon salivary gland removal in late larval stages, autophagy functions in parallel with caspase-dependent apoptosis to degrade the larval tissue [104]. Mutations of single key *Atg* genes or inhibition of the *Drosophila* initiator caspase *Dronc*, severely delay this degradation step [104]. Restoring expression of *Atg-1* in these cells re-vitalizes the autophagic response and leads to premature cell death in a caspase-independent fashion [104].

Furthermore, upon *larvae to pupae* transition stage, midgut cells of the intestine are disposed in a manner predominantly driven by autophagy and secondarily by apoptosis [105], [106]. In this setting, inhibition of core *Atgs* has been observed to negatively influence the rate of midgut cell degradation, which in contrast to salivary glands is not delayed further by inhibiting individual caspases or by using the pan-caspase inhibitor p35 [104], [106].

In addition to these examples, *Drosophila* and mammalian oogenesis also share the degradation of supporting nurse cells by PCD, which promotes the growth and maturation of oocytes [107]–[109]. In nurse cells the effector caspase Dcp-1 (the *Drosophila* homolog to *caspase-3*) and its upstream inhibitor of apoptosis dBruce were first observed to regulate autophagic flux, which contributed in turn to ovarian PCD [110]. Shortly after, it was shown that activation of PCD relied on the apical degradation of dBruce by autophagy, which enhanced Dcp-1 function and autophagy upregulation in a positive feedback loop, involving Dcp-1 translocation to mitochondria and its localized suppression of a recently identified autophagy-inhibitor [108], [111]. Nevertheless, since the effective removal of nurse cells has also been reported even when inhibiting both apoptosis and autophagy [112], the conditions under which autophagy functions as or contributes to death-mediating pathways require further elucidation.

Finally, in plants, fungi and protists, the caspase-like counterparts known as metacaspases are essential to plant PCD, particularly under several stress conditions [113]. However, despite their

functional homology as well as some sequence similarities with caspases, metacaspases also bear striking differences such as cleaving of their substrates after an arginine or lysine residue, instead of aspartate [113], [114]. Their involvement in controlling autophagic flux has been documented in vacuolar PCD (vPCD) during plant development [113], [115], [116] where activity of type II-metacaspase (*mcII-Pa*) was detected prior to vPCD of embryo-supporting tissue [116]. Metacaspase and autophagy upregulation was measured following the knock-down of either core *Atgs* or *mcII-Pa* respectively [115]. It was subsequently reported that *mcII-Pa* may operate as an upstream positive mediator of autophagic flux, by potentially cleaving or interacting in some other way with an as of yet uncharacterized suppressor of autophagy in plants [115], [116].

Conclusions

Although on many occasions caspases stay true to their role as death-propagators that cleave and inactivate their target substrates, under specific circumstances they can seemingly forego their primary directive and contribute instead to cell survival by polarizing cells towards autophagy. The precise mechanics by which a number of caspases manage to promote autophagy remain largely unknown. It is important to note that unless the initial stress-inducing stimulus is overwhelming, most cells will firstly aim to survive by mounting an autophagic response, before resorting to suicide [9], [10], [13]. In spite of its cytoprotective role, if autophagy progresses unimpeded over and above an especial threshold, it can lead to upregulation of cell-death markers, and activation of the apoptotic machinery or other cell death modalities if apoptosis is impaired [13]. In this regard, some caspases can evidently act as molecular brakes by constitutively degrading ATGs and other autophagy promoters as a means to keep the process from reaching a point of no return, past which the cellular suicide machineries are turned on. This is particularly exemplified with apical caspase-8, as it switches off the potent autophagy-enhancer RIPK1, therefore keeping the autophagic flux in check [85]. In contrast to this, the non-apoptotic role of caspase-9 appears to be mostly a matter of its limited availability due to its association with ATG7 in a complex, which nonetheless favors autophagy upregulation [68]. Furthermore, the differential proteolytic cleavage of autophagy-proteins by initiator and executioner caspases can result in fragments, which display either pro-apoptotic or pro-autophagic properties as discussed for Beclin-1 and ATG4D respectively.

The gradual unveiling of hidden functions for known regulatory proteins births new questions at an exponential rate. Is caspase-control of autophagy an essential mode for managing this pathway? Caspase involvement stands out particularly in apoptosis-inducing settings. As autophagy is a highly elaborate pathway, many signaling networks converge to regulate the process, therefore it is reasonable to expect that depending on setting, certain networks may impact the pathway more prominently than others. Do the caspase precursors have any type of control over the autophagic cascade? Perhaps. As the modulatory effects of pro-caspases in relation to autophagy is a largely unexplored avenue, it remains to be seen if and to what extent they can potentially affect the process. On the other hand, as already reported for pro-caspase-8, the autophagic machinery can be

indispensable as an alternative path to the maturation of the protease in conditions where the conventional mode of activation is blocked [78], [79]. Whether other cell-death proteases can be upregulated in a similar manner is a question in a long list of questions which warrant further investigation in order to decipher the underlying interconnections behind the mutual crosstalk of autophagy and apoptosis. Since caspases have been known to contribute to processes such as cell-proliferation and differentiation, which are starkly contradictory to apoptosis [3], [117], [118], it is entirely possible that these misunderstood hitmen may have additional and more extensive roles in autophagy awaiting discovery. Taking all the presented findings into account, the primary conclusions of the review are that caspases can directly interact with known ATGs and consequently affect autophagy in a number of ways. In the majority of cases autophagy is downregulated in favour of apoptosis. Interestingly enough, apical caspases -1, -8, and -9, as well as executioner caspase-3 have been found to promote autophagy on occasions. Overall, the net effect on autophagy due to caspase-ATG interactions appears to rely heavily on context, and further work is required to delineate the complex relationships between autophagic proteins and cell death proteases in health and disease.

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Figure legends

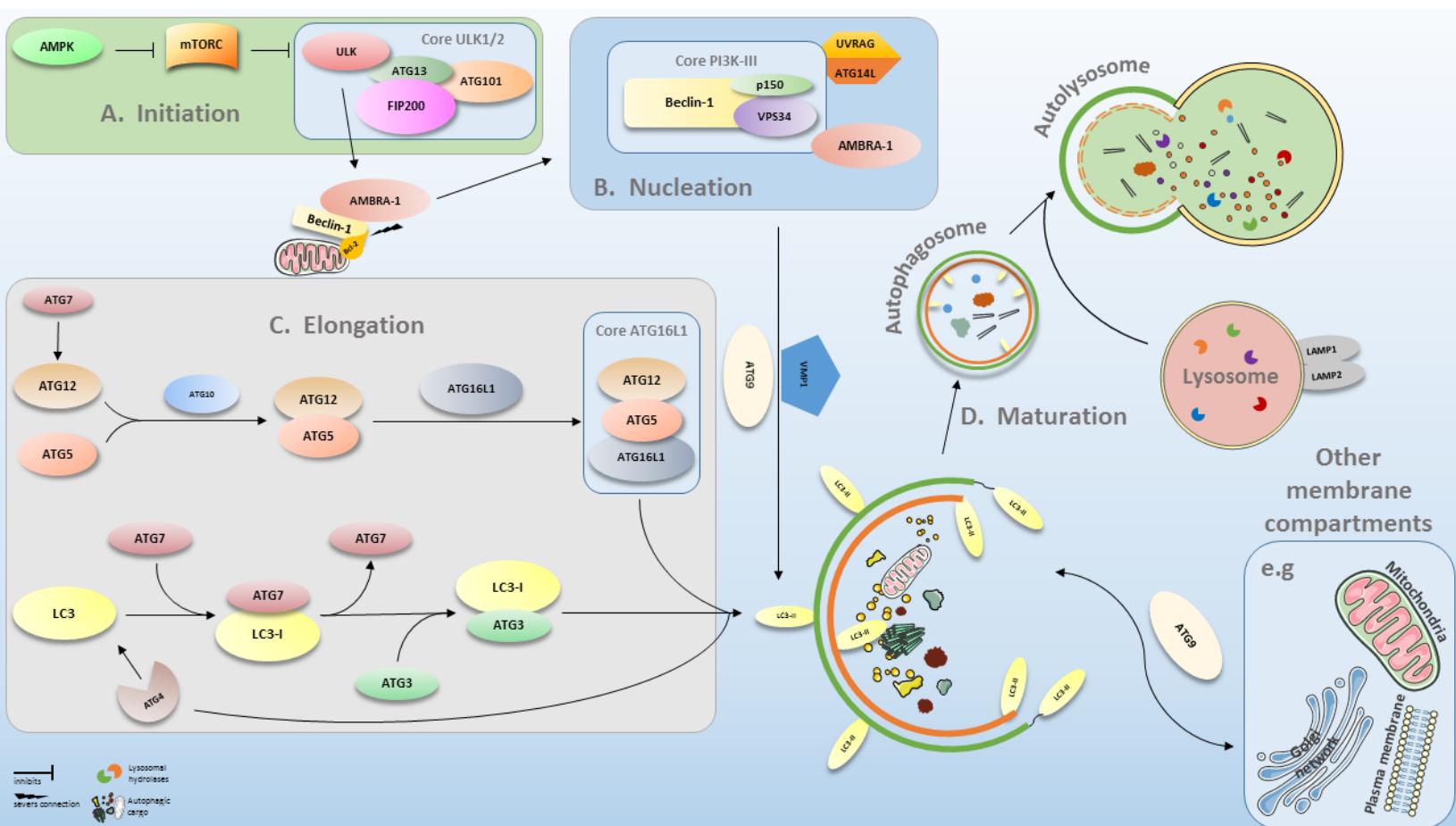
Figure 1

Overview of the autophagy mechanism in mammalian cells.

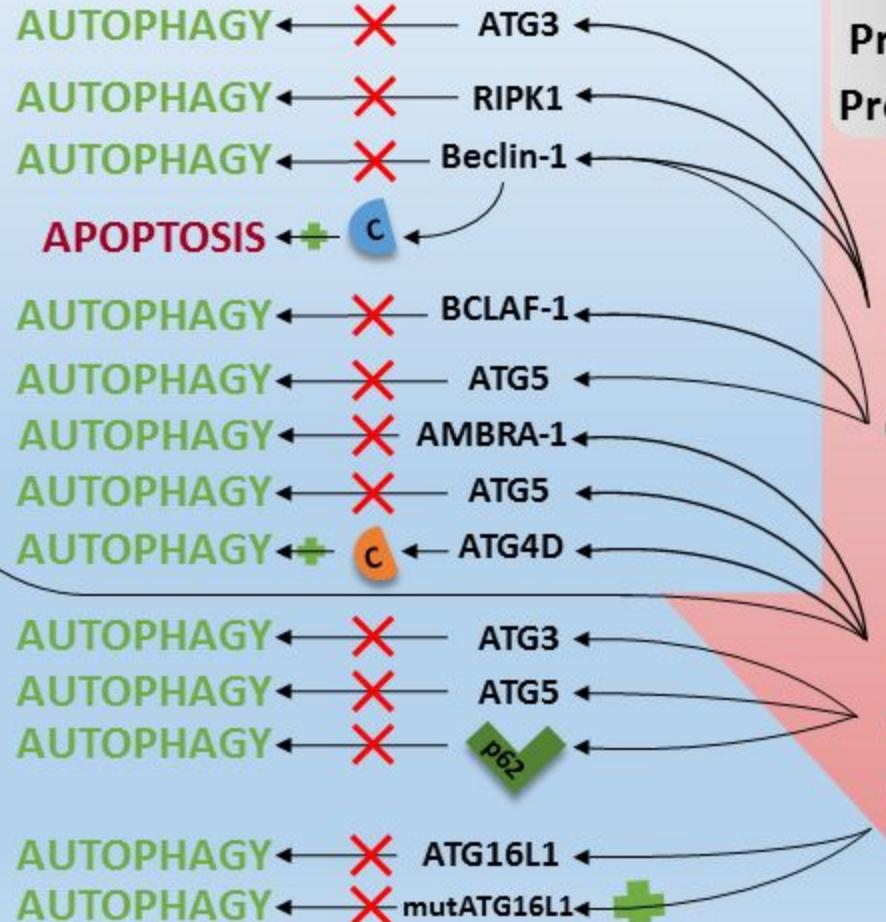
The proteins are grouped according to their position in the cascade. “Core” contains within its boundaries the key proteins that make up each complex. **A. Initiation.** Upon nutrient withdrawal AMPK phosphorylates mTORC1, thereby lifting its inhibitory effect on ULK, and allowing the initiation complex to be activated. ULK phosphorylates AMBRA-1, which subsequently severs the binding between Beclin-1 and Bcl-2 proteins, and facilitates Beclin-1 interaction with p150 and VPS34 in the PI3K-III-complex. UVRAG and ATG14L differentially bind to form distinct PI3K-III-complex populations within cells, while AMBRA-1 also interacts with the PI3K-III-complex. **B. Nucleation.** The ULK- and PI3K-III- complexes then translocate to the nascent phagophore site with the aid of ATG9 and VMP1, where the localized generation of PI3P leads to the recruitment of additional effectors, and the formation of the initial isolation membrane (not shown). **C. Elongation.** Shortly after autophagy upregulation, ATG7 and ATG10 are activated and instigate the formation of the ATG5-ATG12 dimer which then binds ATG16L1 to create the ATG16L1 elongation complex. ATG7 also oversees together with ATG4B and ATG3 the cleavage and lipidation of LC3 to LC3-I and then LC3-II, which is bound to the inner and outer membrane of the forming phagophore. LC3-II acts as a recognition marker by LC3-II sensors, which bring cargo targeted for degradation to be sequestered to the autophagosome during its formation, as well as by ATG9 (shown) and other transporter proteins (not shown) which shuttle between various intracellular membrane compartments (e.g. mitochondria, Golgi, plasma membrane, shown above) and transfer phospholipid fragments to be incorporated to the expanding structure. **D. Maturation.** Upon closure of the loaded phagophore, and the removal of the outer fraction of LC3-II, the now-complete autophagosome can then fuse with a lysosome, through a docking process involving Syntaxin members and the lysosomal LAMP1 and 2 proteins, among others, to create an autolysosome, wherein its contents are degraded by lysosomal hydrolases, and recycled back to the intracellular space to be taken up by numerous effectors (not shown).

Figure 2**Schematic depiction of reported effects of mammalian caspases in autophagy-related effectors.**

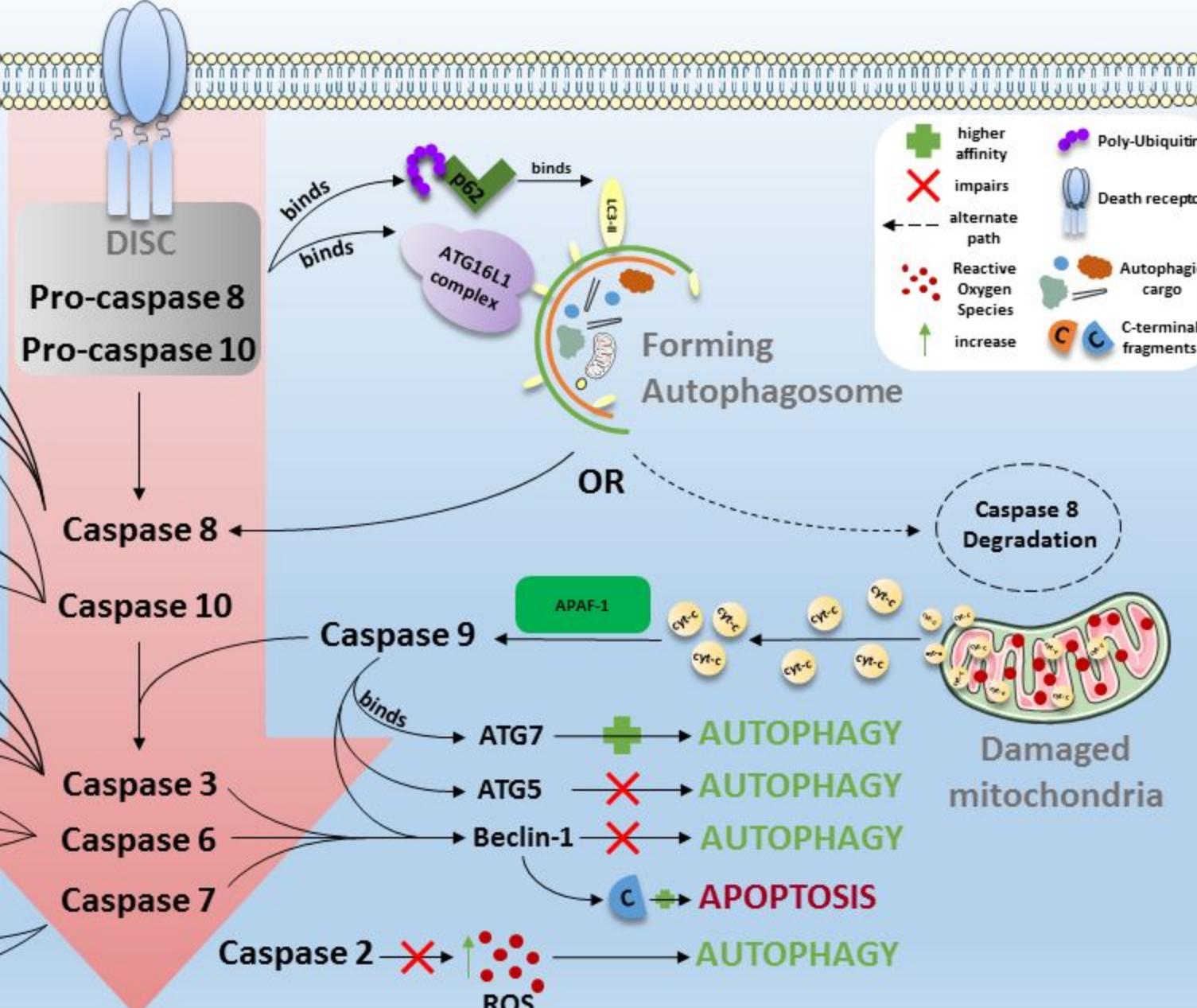
Central red arrow depicts the activation of caspases during the apoptotic cascade for the extrinsic and intrinsic pathway. Following activation of cell-surface expressed death receptors and DISC formation, pro-caspase 8 is converted to caspase-8 in ways which can also involve the ATG16L1-complex, and targeting of pro-caspase-8 oligomers to the autophagosomal surface by p62. Sequestration of pro-caspase-8 to the autophagosome can lead to caspase-8 activation, or downregulation, due to the extensive retention of the protease by the autophagosome, and its consequent degradation by lysosomal hydrolases upon autolysosome formation (not shown). It remains unclear whether pro-caspase-10 is regulated in a similar manner. Initiator caspase-9 is activated during the intrinsic pathway by cytochrome-c release from damaged mitochondria and the assembly of the apoptosome. The initiator caspases then cleave the pro-forms of their effectors, resulting in the upregulation of the executioner caspases -3, -6, and -7, altogether leading to the apoptotic dismantling cells. The side arrows on each caspase depict its known interactions with ATG targets and other autophagy-related proteins. The resulting outcome on autophagy is shown with an “X” over, or a “+” behind the arrows that indicate the otherwise physiological effect of the autophagic protein on the pathway. Alternatively the arrows may point towards other effectors and functions which directly impact autophagy. As shown above the same protein can be targeted by multiple caspases. (Not shown: Caspase-1 interactions, as it is a member of the inflammatory caspase group and associated mostly with the pyroptotic rather than the apoptotic response).



Autophagosome
Exocytosis



APOPTOSIS



Protein	Phase	Main function in Autophagy
ULK-complex		
ULK	Initiation	<i>Atg1</i> homologues; Ser/Thr kinases that mediate mTOR signaling and upregulate downstream autophagy mediators [38]–[40]
ATG13	Initiation	ULK substrate that modulates the activity of the ULK complex [38]
FIP200	Initiation	<i>Atg17</i> homologue; ULK substrate that also modulates the activity of the ULK complex [37]
ATG101	Initiation	Interacts with ULK1 and ATG13 and protects it from degradation [41]
PI3K-complex		
Beclin 1	Initiation; Elongation; Maturation	<i>Atg6</i> orthologue; Part of the PI3K complex and functions on many levels during autophagosome assembly [43], [50]
VPS34	Elongation; Maturation	Catalytically active subunit of the PI3K complex [50]
p150	Elongation; Maturation	<i>Vps15</i> orthologue; Regulatory subunit that recruits the PI3K complex to membranes [50]
ATG14L	Elongation; Maturation	<i>Atg14</i> orthologue; Also known as Barkor; Directs the PI3K complex to the omegasome [51]
ATG16L1 complex		
ATG5	Elongation; Maturation	Conjugated to ATG12 during formation of the Atg16L1 complex in autophagosome assembly step [16], [56]
ATG12	Elongation; Maturation	Ubiquitin-like protein; member of the Atg16L1 complex that aids in the activation of ATG3 [16], [56]
ATG16L1	Elongation; Maturation	Atg16 homologue; Scaffold protein; Binds to the ATG5–ATG12 heterodimer and contributes to LC3 conjugation at the phagophore birth site [19], [56]
Other key ATGs		
ATG3	Elongation; LC3–PE conjugation	E2-like conjugating enzyme; Binds LC3 to phosphatidylethanolamine on forming phagophore and further promotes its lipidation [56], [88]

ATG4	Elongation; Maturation	Atg4B most active in mammals; Cysteine protease priming LC3 for lipidation by cleaving carboxy-terminal Gly residues; removes LC3 from the autophagosome outer membrane during maturation [56], [57], [59]
ATG7	Elongation	E1-like enzyme; activates ATG12 for conjugation to Atg16L1 complex; Primes LC3 for lipidation [16], [43]
LC3/ GABARAP/ GATE16,	Elongation; Maturation	The mammalian family of Atg8 homologues; ubiquitin-like proteins that are recognized in their lipidated form by adapter proteins bringing cargo to autophagosomes for engulfment, or elongation; contribute to membrane fusion; widely used markers for monitoring autophagy [53], [54], [56], [96]
ATG10	Elongation	E2-like enzyme; links ATG12 to ATG5 during formation of the Atg16L1 complex [43], [56]
ATG9	Elongation; Membrane trafficking	Atg9A and Atg9B are the mammalian Atg9 orthologues; Cargo-bearing and only transmembrane Atg capable of shuttling between intracellular compartments; Brings membrane cargo to elongating phagophore [20], [43], [50], [54]

Table 1. Essential proteins in mammalian autophagy and their function in the pathway. The proteins listed under each complex comprise its core while the ones under “Other key ATGs” are central in the progress of autophagy while not necessarily belonging to a complex. The complexes are presented by order of their activation in the autophagic cascade (to reduce complexity, here nucleation has been incorporated to elongation), but they may function, along with their constituent proteins in more than one steps during autophagy upregulation. A short information about the main function of each protein in autophagy is also supplied, with mammalian homology being compared to the identified genes in yeast.

Caspase	Role in Apoptosis (primary)	Interactions with Autophagy-effectors	Reported effect on autophagy
Inflammatory			
Caspase-1	Involved in inflammatory apoptotic response during host-pathogen interactions	LC3 (<i>putative</i>) Beclin-1 (<i>putative</i>)	↑ Autophagy possibly due to upregulation of LC3, and Beclin-1 by unidentified mechanism [67]
Caspase-4			Under investigation
Caspase-5			Under investigation
Initiators			
Caspase-2	Upregulates apoptotic response to redox stress, DNA damage, heat shock, and cytoskeleton disruption; Does not process executioners; Substrate for both caspase-8 and caspase-3	Controls mitochondrial ROS levels	↓ Autophagy by preventing accumulation of ROS, which when elevated enable dissociation of mTORC from autophagy initiation complex [71], [74]
Caspase-8	Apical caspase of extrinsic pathway; Cleaves target proteins and activates executioner caspases	RIPK1 p62 ATG3 ATG5 Beclin-1	↓ Hyperactive autophagy by cleaving RIPK1, which normally augments autophagy. The adapter FADD binds to pro-caspase-8, RIPK1 and ATG5, and facilitates caspase-8 activation [79], [80], [86] ↑ Autophagy, depending on retention time of pro-caspase-8 on the autophagosome [83], [84] ↓ Autophagy by cleaving ATG3, ATG5, and Beclin-1 during autophagosome assembly. May return C-terminal Beclin-1 fragment with pro-apoptotic properties [50], [65], [88]
Caspase-9	Apical caspase of intrinsic pathway; Activated by cyt-c and APAF1; Cleaves target proteins and activates executioner caspases	ATG7 ATG5 Beclin-1	↑ Autophagy because caspase-9 loses catalytic activity when binding to ATG7, resulting in complex with higher affinity for LC3 than ATG7 alone [91] ↓ Autophagy by cleaving ATG5, and Beclin-1. May sometimes return C-terminal Beclin-1 fragment with pro-apoptotic properties [50], [87]

Caspase-10	Apical caspase of extrinsic pathway; Cleaves target proteins and activates executioner caspases	ATG5 Beclin-1	↓ Autophagy by cleaving ATG5, and Beclin-1. May sometimes return C-terminal Beclin-1 fragment with pro-apoptotic properties [50], [87]
		BCLAF-1	↓ Autophagy by cleaving BCLAF-1 which impairs Bcl-2-Beclin-1 dissociation upon autophagy induction [92]
Executioners			
Caspase-3	Main effectors of initiator caspases, activated during both the extrinsic and intrinsic apoptotic pathway; Shut down cellular functions by cleaving many protein substrates during programmed cell death	Beclin-1 ATG5 ATG4D Autophagosome exocytosis AMBRA-1	↓ Autophagy by cleaving ATG5 and Beclin-1. May return C-terminal Beclin-1 fragment with pro-apoptotic properties [65], [87] ↑ Autophagy by cleaving ATG4D resulting in C-terminal fragment with higher lipidation activity [66] ↓ Autophagy by aiding in exocytosis of autophagic vesicles during apoptotic dismantling [93] ↓ Autophagy by cleaving AMBRA-1, which normally contributes to dissociation of Beclin-1 from Bcl-2 [52]
		ATG3 ATG5 Beclin-1 p62	↓ Autophagy by cleaving p62, ATG3, ATG5, and Beclin-1. May sometimes return C-terminal Beclin-1 fragment with pro-apoptotic properties [62], [87], [90]
		ATG16L1 Beclin-1	↓ Autophagy by cleaving Beclin-1, ATG16L1, and mutant of ATG16L1 associated with increased risk for Crohn's disease. May sometimes return C-terminal Beclin-1 fragment with pro-apoptotic properties [65], [97]

Table 2. Effects of mammalian caspases on autophagy through their autophagy-related interactors. Inflammatory caspases are grouped separately, while the remaining caspases are listed in order of their main apoptotic function as initiators or executioners, with a brief mention of their role in apoptosis. Up arrows (\uparrow) denote upregulation of autophagy as the net effect observed based on the described interaction, while down arrows (\downarrow) represent downregulation of the pathway.