INVITED REVIEW

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INTEGRATION OF AUTOPHAGY, PROTEASOMAL DEGRADATION, UNFOLDED PROTEIN RESPONSE AND APOPTOSIS

D.M. Benbrook[1,2,], A. Long[2]*

[1]*Department of Obstetrics and Gynecology and* [2]*Department of Biochemistry and Molecular Biology
University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA*

**A single cell has the potential to kill an entire human being. Efforts to cure cancer are limited by survival of individual cancer cells despite immune surveillance and toxic therapies. Understanding the intricate network of pathways that maintain cellular homeostasis and mediate stress response or default into cell death is critical to the development of strategies to eradicate cancer. Autophagy, proteasomal degradation and the unfolded protein response (UPR) are cellular pathways that degrade and recycle excess or damaged proteins to maintain cellular homeostasis and survival. This review will discuss autophagy and how it is integrated with proteasomal degradation and UPR to govern cell fate through restoration of cellular homeostasis or default into the apoptotic cell death pathway. The first response of autophagy is macroautophagy, which sequesters cytoplasm including organelles inside double-membraned autophagosome vesicles that fuse with lysosomes to degrade and recycle the contents. Ubiquitination patterns on proteins targeted for degradation determine whether adapter proteins will bring them to developing autophagosomes or to proteasomes. Macroautophagy is followed by chaperone-mediated autophagy (CMA), in which Hsc70 (Heat shock cognate 70) selectively binds proteins with exposed KFERQ motifs and pushes them inside lysosomes through the LAMP-2A (Lysosome-associated membrane protein type 2A) receptor. These two processes and the lesser understood microautophagy, which involves direct engulfment of proteins into lysosomes, occur at basal and induced levels. Insufficient proteasome function or ER stress induction of UPR can induce autophagy, which can mitigate damage and stress. If this network is incapable of repairing the damage or overcoming continued stress, the default pathway of apoptosis is engaged to destroy the cell. Induction of macroautophagy by cancer therapeutics has led to clinical trials investigating combinations of HCQ (hydroxychloriquine) suppression of autophagy with apoptosis-inducing agents. Further study of the complex integration of autophagy, proteasomal degradation, UPR and apoptosis is likely to provide additional targets for our fight against cancer. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".
Key Words: autophagy, proteasomal degradation, endoplasmic reticulum stress, unfolded protein response, apoptosis.**

INTRODUCTION

Understanding how cells die is critical knowledge needed for the development of health care strategies to prevent the death of cells in degenerative and acute diseases and to induce the death of diseased cells that

Abbreviations used: AMP — adenosine 5'-monophosphate; AMPK — AMP kinase; ATF — activating transcription factor; ATG — autophagy-related genes; BAG-1 — Bcl-2-associated athanogene 1; Bcl-2 — B-Cell CLL/Lymphoma 2; CHOP — CCAAT/-enhancer- binding protein homologous protein; CMA — chaperone-mediated autophagy; CQ — chloroquine; EF1α — elongation factor 1α; ER — endoplasmic reticulum; ERAD — ER-associated degradation; FIP200 — focal adhesion kinase interacting protein of 200 kD; GADD — growth arrest and DNA damage; Gal3 — galectin-3; GFAP — glial fibrillary acidic protein; GTPases — guanosine triphosphatases; HCQ — hydroxychloroquine; HDAC6 — histone deacetylase 6; HLA — human leukocyte antigen; HMGB1 — high mobility group box 1; IGF1 — insulin like growth factor 1; IRE1α — inositol-requiring enzyme 1α; LAMP-2A — lysosome-associated membrane protein type 2A; LC3 — light chain of the microtubule-associated protein 1; MDM2 — murine double minute 2; MEFs — murine embryonic fibroblasts; MLIV — mucolipidosis Type IV; mTOR — mammalian target of rapamycin; NBR1 — neighbor of BRC1; PE — phosphatidylethanoamine; PERK — double-stranded RNA-dependent protein kinase (PKR)-like ER kinase; PI3K — phosphoinositol 3-kinase; PIP3 — PtdIns(3,4,5)P3; PMN — piecemeal microautophagy of the nucleus; PS — phosphoatidylserine; Rheb — Ras homolog enriched in brain; TRPML1 — transient receptor potential mucolipin-1; TSC — tuberous sclerosis complex; ULK — uncoordinated 51-like kinase; UPR — unfolded protein response; V-ATPase — vacuolar H+ ATPase; Vps — vacuolar protein sorting.

can harm the body if not eliminated. Both morphological and molecular events that occur in dying cells have been characterized and categorized into 13 current modes [37, 68]. More recently, an iron-dependent form of non-apoptotic death termed "ferroptosis" has been described [30]. Autophagy, which was officially identified and named by Christian de Duve in 1963 [22, 59], is formally classified as a form of cell death, however the majority of evidence implicates autophagy as a mechanism to maintain cellular homeostasis and recover from stress, while unequivocal evidence that autophagy causes cell death is rare. The survival function is accomplished through digestion of long-lived or damaged proteins and organelles and release of the components for recycling. The death function is believed to be caused by excessive digestion of cellular components or selective digestion of survival factors over death factors. This review will discuss the integration of autophagy with other cellular processes that maintain homeostasis and mediate stress responses leading to cancer cell survival or death, sometimes through apoptosis, and how these mechanisms are being targeted to improve cancer therapy. For more details on the mechanism of autophagy, readers are directed to several recent reviews [7, 75, 76, 112, 123].

FORMS OF AUTOPHAGY

There are three natural processes of autophagy in the cell, MA (macroautophagy), CMA (chaperone-mediated autophagy) and microautophagy. Macroautophagy is a natural process in which portions

of the cytoplasm, including long-lived proteins and organelles, are sequestered inside double-membraned vesicles called autophagosomes. The autophagosomes eventually fuse with lysosomes to form autophagolysosomes where the contents are digested and their components released for recycling within the cells. CMA is driven by Hsc70 (heat shock cognate 70, also called HSPA8 [heat shock protein A8]), which binds specific proteins and transports them directly into the lysosome [76]. In microautophagy, defective molecules or organelles are directly engulfed into the lysosomes for degradation and recycling of their components [75]. All three forms of autophagy occur at basal levels to maintain the cell, while suprabasal levels are induced by nutrient or oxygen deprivation, endoplasmic reticulum stress, proteasome malfunction or damage caused by drugs or radiation. Most articles refer to macroautophagy as autophagy, however because this article addresses all three forms of autophagy in detail, the term macroautophagy is used herein to describe the specific process of autophagosome-mediated recycling. The term autophagy is used in situations where it is feasible that all three forms of autophagy could be actively involved in the process under discussion.

MECHANISM, MEASUREMENT AND MANIPULATION OF MACROAUTOPHAGY

The production and processing of autophagic vesicles is divided into 4 steps: 1) initiation, 2) nucleation, 3) maturation and 4) fusion with lysosomes (Fig. 1). These processes are mediated by a series of proteins encoded by autophagy-related genes (ATGs), which were originally characterized in yeast, and are highly conserved in higher eukaryotes [60].

During initiation, *de novo* synthesis of isolation membranes recruits lipids from several organelles depending on the cell type and stimulus. Electron micrograph documentation of ER located on both sides of isolation membranes, and what appears to be a single bridge connecting the ER to the isolation membrane, indicate that this organelle is a source of membrane lipids for the *de novo* formation of autophagosomes in the cytoplasm [42]. Formation of isolation membranes from the mitochondria appears to be dependent upon PS (phosphatidylserine) transfer from the ER to the mitochondria [41]. The mitochondrial enzyme, PS decarboxylase, converts PS to PE (phosphatidylethanolamine), which is needed

for autophagosome formation as described below [113]. Proteins associated with only the outer leaflet of the mitochondrial membrane appear to transfer to the autophagosome, while proteins that traverse the entire mitochondrial membrane are retained in the mitochondria [41]. The plasma membrane has also been shown to contribute lipids to the initiation membrane through a process dependent upon the interaction of clatherin on the plasma membrane with Atg16L on the forming autophagosome [100]. An alternative form of macroautophagy shown to occur during fetal development and erythroid maturation derives membrane lipids for *de novo* autophagosome formation from the trans-Golgi and late endosomes [87].

The complex of proteins that mediate initiation consists of ULK1 (uncoordinated 51-like kinase 1/Atg1), Atg13 and FIP200 (focal adhesion kinase interacting protein of 200 kD/Atg17). Basal levels of macroautophagy are kept in check by mTORC1 (mammalian target of rapamycin complex 1) phosphorylation of Atg13 and ULK1, which inhibits ULK1 phosphorylation of FIP200 [12, 38, 44, 49]. The mTORC1 complex is an important component of a network that senses the nutrient state of the cell and accordingly controls the levels of anabolism and catabolism to maintain homeostasis [46] (Fig. 2). High levels of amino acids maintain mTORC1 in an active state by enhancing binding of this complex to regulatory proteins Rag and Rheb (Ras homolog enriched in brain) GTPases (guanosine triphosphatases) [56, 105]. Insulin and IGF1 (insulin like growth factor 1) indirectly induce mTORC1 activity by stimulating class 1 PI3K (phosphoinositol 3-kinase) production of PIP3 (PtdIns(3,4,5)P3), which induces the Akt kinase at the plasma membrane, which in turn activates mTORC1 by inhibiting TSC (tuberous sclerosis complex) proteins 1 and 2, thereby relieving their repression of Rheb [4, 8]. Low glucose levels or high levels of AMP (adenosine 5'-monophosphate), which indicate low cellular energy status or stress, activate AMPK (AMP-activated protein kinase), which inhibits mTORC1 and stimulates macroautophagy [46, 95]. In summary, high levels of amino acids, insulin and IGF-1 inhibit macroautophagy by inducing the PI3K/Akt/mTORC1 pathway, while low glucose and high AMP levels induce autophagy by activating the AMPK and repressing mTORC1 activity (Fig. 2). Chemical inhibitors of mTORC1 currently in clinical use or in clinical trials, including rapamycin and analogs called
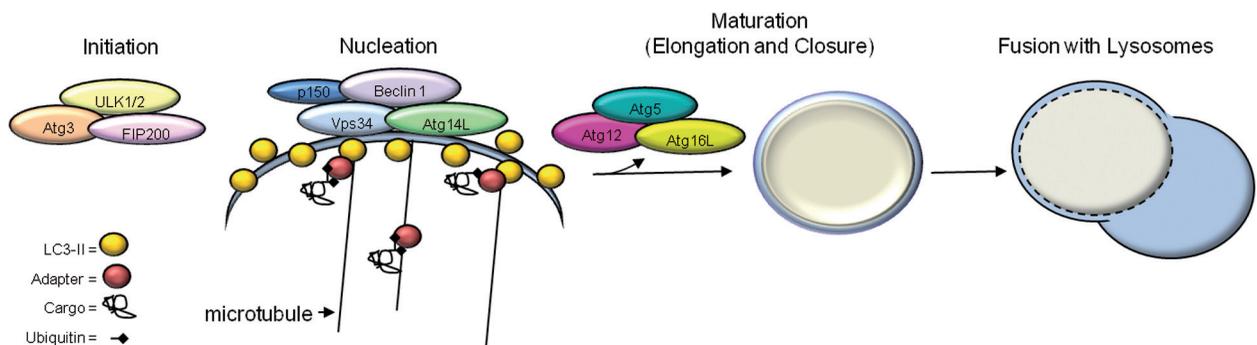

Fig. 1. Simplified Illustration of macroautophagy

rapalogs, such as Everolimus (RAD001), induce macroautophagy and are often used as tools to study autophagy [10, 57]. As discussed below, this induction of macroautophagy interferes with the clinical efficacy of these drugs as anti-cancer agents.

Nucleation is controlled by a class III PI3K called Vps34 (vacuolar protein sorting 34) that forms a complex with Beclin 1 (Atg6/Vp330), p150 (Vps15) and Atg14L [79]. Production of PIP3 by Vps34 recruits WIPI (WD40 repeat protein Interacting with phosphoInositides/Atg18) proteins to the isolation membrane allowing recruitment of LC3 (light chain of the microtubule-associated protein 1/Atg8) and further evolution of the autophagosome [97]. While specific inhibition of class I PI3K stimulates macroautophagy indirectly through downstream Akt/mTOR inhibition, specific inhibition of class III PI3K Vps34 inhibits macroautophagy through reduction of an autophagy-specific PIP3 pool [95]. As discussed below, nucleation can also be inhibited by binding of the anti-apoptotic protein Bcl-2 (B-Cell CLL/Lymphoma 2) to Beclin 1 [94].

Two interdependent ubiquitin-like conjugation systems mediate the maturation (elongation, curvature and closure) of the autophagosome. In one system, LC3 is first cleaved by the Atg4 serine protease and then conjugated to PE by the Atg7 and Atg3 enzymes [45]. The unmodified and lipidated forms of LC3 are termed LC3-I and LC3-II, respectively, and can be distinguished by Western blot analysis, a well accepted method to monitor macroautophagy [6]. The second system recruits LC3-II to the isolation membrane by the ubiquitin-like activity of Atg12, which is covalently bound to Atg5 and physically associated with Atg-16L to form a complex [86]. Once the isolation membrane is formed, the Atg-12, -5, -16L complex is released, which affords it's utilization as a marker of isolation membrane formation [86]. LC3-II on the other hand, remains associated with the autophagosome until fusion with the lysosome, and the transition of diffuse to punctuate pattern of a transfected LC3-green fluorescent protein fusion protein in the cytoplasm is a commonly used marker of autophagosome formation [6, 86].

Before fusion with the lysosome, ubiquitinated proteins can be brought to the inside of the developing autophagosome by adapter proteins. Proteins are targeted for degradation by E1, E2 and E3 ubiquitinases that attach the C-terminal glycine on ubiquitin to the ε-amino group of a lysine residue in the protein being targeted [96]. Multiple molecules of ubiquitin can be added to the same protein as individual components, and to Lys residues on existing ubiquitins that are already present on the target proteins to form branched chains [119]. Adapter proteins/macroautophagy receptors called p62, NBR1 (neighbor of BRC1), HDAC6 (histone deacetylase 6) and Alfy bind the ubiquitinated proteins and bring them inside developing autophagosomes [98, 121]. HDAC6 is a microtubule associated acetylase that binds to, and utilizes, dynein motors to transport ubiquitinated proteins along microtubles to a structure called the aggresome where they are recognized by developing autophagosomes [53]. As discussed below, the pattern of ubiquitination will determine whether the protein is targeted for autophagic versus proteasomal degradation [98, 121].

The fusion step is mediated by dynein transportation of the autophagosomes along microtubules to fuse with endosomes or lysomes [99]. Microtubule disrupting agents, such as taxanes, vinblastine or nocodazole, can prevent the fusion and cause accumulation of autophagic vacuoles [32]. Inhibition of lysosomal acidification by Bafilomycin A, a specific V-ATPase (vacuolar H+ ATPase) inhibitor, or by other lysosomal function inhibitors, CQ (Chloroquine) and HCQ (hydroxychloroquine), also cause accumulation of autophagosomes [122, 126]. Upon fusion with the lysosome, the contents are degraded and the components, including amino acids and lipids, are released for re-utilization in cellular metabolism. The TCA (tricarboxylic acid) cycle, which utilizes amino acids for generation of bioenergetic molecules and
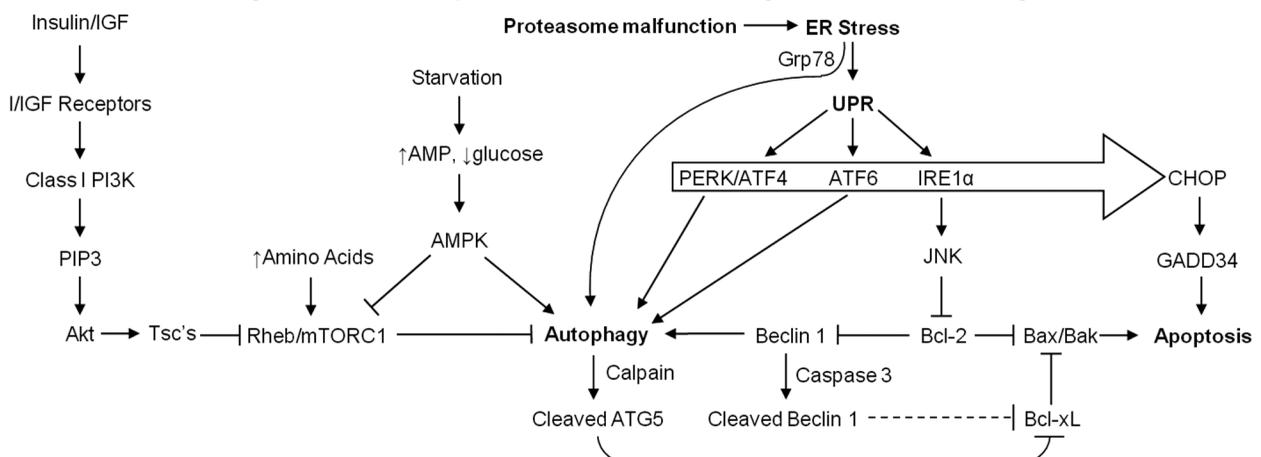

Fig. 2. Integration of macroautophagy with the regulatory network controlling the maintenance of cellular homeostasis versus apoptosis. The Grp78 under ER stress has two effects, increased Grp78 expression induces autophagic vesicle formation while release of Grp78 inhibition of PERK, EIF2α and ATF6 induce UPR. ATF4 is listed next to PERK because one study indicated that ATF4 stability is responsible for the induction of autophagy. Arrows indicate induction and crossed lines indicate repression. Dashed line indicate hypothesized, not proven, mechanism of Beclin 1 fragment induction of apoptosis. Large open arrow indicates that all three components contribute to CHOP induction

biosynthetic intermediates appears to co-ordinate with macroautophagy through negative feedback of the TCA substrate, pyruvate, on macroautophagy [81]. Although not specific for macroautophagy, staining with lysomotropic agents, such as acridine orange, is an acceptable marker for induction of macroautophagy [91]. Electron micrograph evidence of double-membraned vesicles containing cytoplasmic components is a gold standard for the presence of autophagosomes inside cells, while cleared vesicles provide evidence that the autophagic process is proceeding through to digestion of the contents and not blocked by lysomotropic agents or other situations. An example of electron micrographic images of autophagosomes and cleared autophagolysomes induced in human ovarian cancer cells by treatment with a novel anti-cancer drug called SHetA2 (NSC 726189) is shown in Fig. 3.

INTEGRATION OF THE PROTEASOMAL SYSTEM AND AUTOPHAGY

The proteasome partners with autophagy to recycle cellular proteins by digesting single soluble proteins and releasing peptides into the cytoplasm and nucleus to be digested by peptidases, or to be transferred to the ER where they are bound by HLA (human leukocyte antigen) proteins and eventually presented on the surface of the cell [25, 64]. Proteasomes are multi-protein complexes made up of an inner 20S cylindrical shaped core of subunits that have the proteolytic activity and a 19S regulatory cap of subunits on each end of the core which recognize ubiquitin-conjugated proteins and provide ATPase activity [36]. The proteasome degrades type I (short-lived) and type II (malfolded/dysfunctional) proteins, whereas autophagy degrades type II and type III (long-lived) proteins [14].

The cellular ubiquitination system specifies whether a protein will be transferred to the proteasome or an autophagosome for recycling. Proteins with attached polyubiquitin chains that are branched on Lys48 have a more closed conformation and are targeted for proteasomal degradation, while proteins with single ubiquitin moieties or polyubiquitin chains that are branched on other Lys residues are targeted to the autophagosomes as described above [98, 121]. Some proteins can be digested by both the proteasome and autophagic vesicles [64]. Degradation of the proteins via the proteasome or autophagy is ultimately determined through competition for binding by adapter proteins, p62 and NBR, which shuttle the proteins to autophagic vesicles, or to p97, which shuttles the proteins to the proteasome [98, 125]. The p62 protein has a higher affinity for the monoubiquitinated and Lys63 polyubiquitinated proteins targeted for autophagic degradation, but can also recognize Lys48 polyubiquitin chains targeted for proteasomal degradation, suggesting that p62 can compensate for loss of proteasomal function by bringing Lys48 ubiquitylated proteins to the autophagosome when these proteins accumulate during proteasome overload or disfunction [40, 121].

There are several additional levels at which the proteasome and autophagy are integrated. Inhibition of the proteasome leads to induction of autophagy [25–27], and induction of autophagy can protect cells from death induced by proteasomal inhibitors [52]. On the other hand, the proteasomal degradation pathway does not appear to compensate for loss of autophagy. Inhibition of early stages of autophagy results in buildup of the p62 adapter, which brings proteins, which would normally be degraded by the proteasome, instead to be accumulated in aggresomes where they cannot be accessed by the proteasomal machinery [63]. Inhibition of autophagy at that last stage of lysosomal degradation by CQ, which does not cause aggresome accumulation, but instead allows the buildup of the ubiquitinated proteins inside lysosomal compartments, also is not compensated by proteasome activity [11]. This unequal relationship between autophagy and proteasomal degradation is highlighted by the observations of proteasomes inside closed autophagic vesicles [21]. The induction of autophagy in response to proteasome inhibition is mediated through the unfolded protein response (UPR), which is induced when proteasomal inhibitors cause accumulation of polyubiquitinated proteins leading to ER stress (Fig. 2) [52].

INTEGRATION OF AUTOPHAGY WITH ER STRESS AND UPR

ER stress is caused by buildup of unfolded, misfolded or damaged proteins that exceeds the capacity of chaperones available in the ER to fold them. The UPR sets off a series of events that mitigate this stress and can also lead to induction of autophagy. The observation of ER stress, indicated by swollen ER, occurring in the same cell as autophagic vesicles upon treatment with the SHetA2 anticancer drug, but not upon treatment with solvent only, supports the interconnection of these two processes (Fig. 3). An excess of unfolded/misfolded proteins interferes with the repressive effect of the ER-resident chaperone, Grp78 (glucose regulated protein 78, also called HSPA5 [heat shock protein A5] or BiP [Binding immunoglobulin protein]) on three UPR-regulatory proteins called PERK (double-stranded RNA-dependent protein kinase (PKR)-like ER kinase), IRE1α (inositol-requiring enzyme 1α) and ATF6 (Activating transcription factor 6) [93] (Fig. 2). Once released, PERK, IRE1α and ATF6 work in concert to mediate the UPR survival response involving arrest of general protein synthesis, while selected synthesis of chaperone proteins is allowed to continue in order to restore the balance of unfolded proteins/chaperones needed for ER homeostasis [43]. To further reduce the ER load, UPR can cause retrograde translocation of ER proteins to the cytoplasm where they are degraded in the proteasome through ERAD (ER associated degradation) [84]. In situations of excess, irreparable or prolonged stress, UPR can transition into apoptosis by upregulation of CHOP (CCAAT/-enhancer- binding protein homologous protein) and downstream GADD34 (Growth arrest and DNA damage 34), but in some situations

autophagy can intervene and prevent cell death by removing the accumulated polyubiquitinated proteins and aggregates [29, 90].

ER stress induces autophagy directly through upregulation of Grp78 and through mechanisms downstream of Grp78 release of the three UPR signal transducers (Table 1, Fig. 2). A critical role for Grp78 in the induction of autophagy was demonstrated with knockdown of Grp78 in normal and cancer cells, which prevented autophagosome formation in response to starvation or in response to inhibition of protein processing with tunicamycin, an inhibitor of N-linked glycosylation [74]. This study also provided evidence for an integral co-dependency of intact ER and autophagy. The massively dilated and disrupted ER and the deficient autophagosome formation induced by Grp78 knockdown were both alleviated by simultaneous knockdown of the XBP-1 transcription factor, a downstream UPR mediator of IRE1α action required for ER expansion [71], suggesting that intact ER is maintained by and/or required for autophagy. The link between Grp78 and autophagy induction occurs downstream of nucleation, as the Grp78 knockdown had no effect on Beclin 1/Vps34 association.

The link between the IRE1α arm and upregulation of autophagy is mediated by IRE1α activation of JNK (c-Jun N terminal kinase). Proteasomal inhibition with bortezomib or MG132 induced ER stress and autophagy in colon cancer cells, but not in the presence of siRNA reduction of IRE1α or chemical inhibition of JNK activity, or in IRE1α knockout MEFs (murine embryonic fibroblasts) [29]. In this study, the JNK inhibitor had no effect on XBP-1 activation and was equally effective in XBP-1-positive and -negative cells indicating that the UPR induction of autophagy occurs through JNK and not the IRE1α arm of UPR. As discussed above however, the XBP-1 function may be required to maintain the ER in a functional state to support autophagic vesicle formation. A similar study conducted in neuroblastoma cells using specific siRNA knockdown of IRE1α, PERK or ATF6, or using a JNK inhibitor,

demonstrated that ER stress up-regulated autophagy through a mechanism dependent on IRE1α activation of JNK, but independently of PERK and ATF6 [90]. The ER stress was induced with tunicamycin or thapsigargin, a chemical that blocks ER calcium uptake by inhibiting ER Ca^{2+}/ATPase. In this model, autophagy protected against cell death as demonstrated by the increase in cell death when autophagy was inhibited by chemical (3-MA [3-methyladenine]) or genetic manipulation (ATG7 siRNA), and inhibited cell death when autophagy was induced by chemical stimulation (rapamycin). The mechanism of autophagy induction downstream of JNK appears to be a result of JNK phosphorylation of Bcl-2, which releases Bcl-2 repression of Beclin mediation of autophagy [116].

Table 1. Mechanisms of ER stress and UPR induction of autophagy

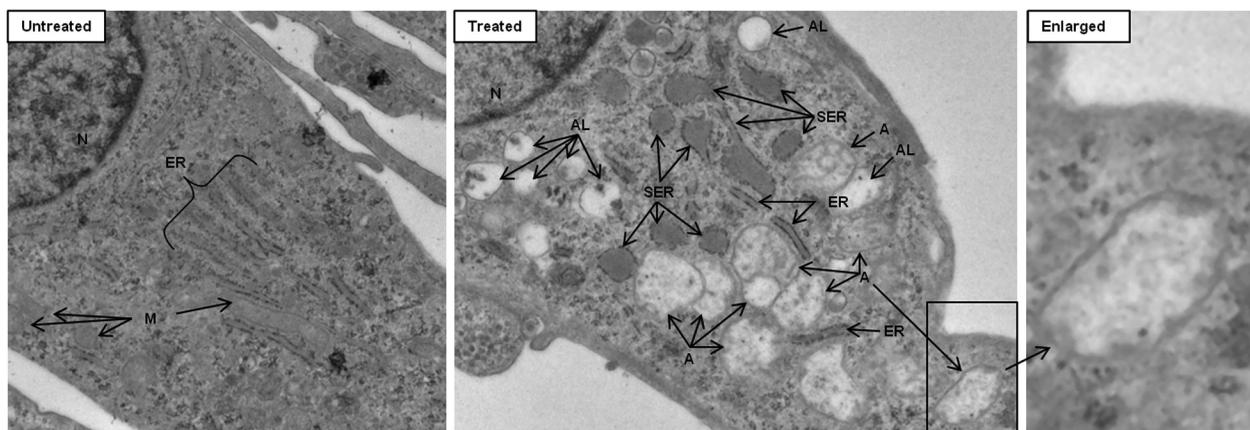| Stressor	Cell type	Mechanism of autophagy upregulation	R
Grp78:			
Tunicamycin, Starvation	Embryonal kidney, cervical cancer	Downstream of nucleation (Vps34/Beclin1)	[74]
IRE1α Arm:			
Bortezomib, MG132	Colon cancer, Prostate cancer	IRE1α → JNK → LC3B lipidation	[29]
Tunicamycin, Thapsigargin	Neuroblastoma	IRE1α → JNK → LC3B lipidation → autophagosomes	[90]
PERK Arm:			
Misfolded polyglutamine repeats	Embryonal carcinoma	PERK phosphorylation → eIF2α phosphorylation → LC3 lipidation (possibly through increased ATF12 transcription)	[65]
Bortezomib	Pancreatic cancer	PERK phosphorylation → eIF2α phosphorylation → ATF4 → Atg 5 and Atg 7 transcription	[129]
Bortezomib	Breast cancer	Reduction of proteasomal degradation of ATF4 → LC3B transcription	[85]
PERK, IRE1 α and ATF6 Arms:			
SPP1 depletion	Breast cancer	↑Grp78→ PERK+ IRE1α +ATF6 → LC3B lipidation	[72]
Indirect:			
BrefeldinA, Thapsigargin, Tunicamycin	Gingival fibroblast	p38MAPK → ↑Grp78 and ↑Beclin1 and autophagic vesicles (possibly through p38 inhibition of mTORC1)	[55]

R = reference

Fig. 3. Simultaneous induction of ER stress and autophagy. Electron micrographs (X3250 magnification) of the human SK-OV-3 ovarian cancer cell line treated with the SHetA2 anti-cancer drug (NSC 721689) for 17 hrs demonstrate normal nuclei (n) swollen ER (SER) and normal ER, double-membraned autophagic vessicles (A) and autophagolysosomes (AL) cleared of their contents, while control cells treated with the same volume of vehicle (dimethylsulfoxide) for 17 hrs do not exhibit SER, A or AL. The lack of clearly identified mitochondria (M) in the treated cells suggests that they may have been digested by autophagy. The inset is enlarged to show the double-membrane on an autophagosome

The link between the PERK arm and upregulation of autophagy is ultimately mediated by ATF4-driven transcriptional upregulation of the ATG genes. Treatment of embryonal carcinoma cells with misfolded polyglutamine repeats caused buildup of polyubiquitinated protein aggregates and induced LC3 lipidation through a mechanism dependent on PERK phosphorylation and activation of eIF2α, and resulting in autophagic elimination of the aggregates [65]. When the proteasomal inhibitor bortezomib was used in pancreatic cancer cells, the eIF2α led to activation of ATF4-driven transcription of the ATG5 and ATG7 genes [129]. Activation of eIF2α also was shown to mediate induction of autophagy by starvation and viral infection [110]. Bortezomib treatment of breast cancer cells led to reduction of proteasomal degradation of ATF4, thereby increasing ATF4-mediated LC3B gene transcription [85].

All three of the UPR arms appear to be involved in the induction of autophagy in breast cancer cells caused by accumulation of the sphingolipid metabolite S1P (sphingosine-1-phosphate) phosphatase [72]. In this study, S1P levels were increased by siRNA silencing of the SPP1 (S1P phosphatase) responsible for degradation of S1P in the ER. The resulting induction of autophagy was prevented by siRNA silencing of PERK, IRE1α, or ATF6 or a dominant negative PERK mutant. Other upstream inducers of Grp78 and autophagy that have not been characterized for specific UPR arm involvement in the mechanism include p38MAPK (p38 mitogen-activated protein kinase), which likely induces autophagy through inhibition of mTORC1 [55].

The ability of cellular UPR and autophagy responses to ER stress to sufficiently mitigate the stress and allow survival is dependent upon the transformation state of the cell and the degree of stress. Although autophagy was induced in both cancer and non-transformed cell lines by ER stress inducers tunicamycin, thapsigargin, A23187 (calcium ionophore) or brefeldin A (protein transport inhibitor) and could reduce the buildup of polyubiquitinated proteins, suppression of autophagy using chemical (3-MA) or genetic manipulation (siRNA to Beclin 1 or LC3B) reduced cell death in colon and prostate cancer cell lines, but increased cell death in normal non-transformed fibroblast and non-immortalized human colon cell line [28]. The authors of this study theorize that the macroautophagy in non-cancer cells may lead to cell death by digesting normal by-stander cellular constituents needed for survival. Cancer cells characteristically become growth-independent of these survival factors.

The switch between autophagy mediation of cell survival versus death has been shown to be related to the level of stress induced. In normal rat kidney cells, autophagy induced in response to a low dose of cisplatin was required for cell survival, while autophagy induced in response to a high dose of cisplatin was required for cell death [103]. ER stress and Grp78 appear to mediate the induction of autophagy in this model. In a study of ER stress inducers (thapsigargin and tunicamycin) in murine embryonal fibroblasts, low, sublethal doses induced Grp78 expression and activated PERK, IRE1α and ATF6α [104]. Although CHOP was also induced, the upregulation was lost within 24 hrs. Even when CHOP expression was persistently up-regulated, only expression of the down-stream GADD34 correlated with cell death. The authors of this study conclude that differential stability of the Grp78, CHOP and GADD34 mRNA's and proteins contribute to the ultimate fate of the cell.

Similar to the addiction of some cancer cells to certain oncogenes, cancer cells may become dependent upon certain aspects of macroautophagy for cell survival and tumor growth, while defects in macroautophagy that accumulate during tumor progression may allow adaptations that prevent apoptosis despite the presence of damaged proteins and organelles [118]. Consistent with this postulate, autophagy has been shown to be critical for K-ras transformation of breast cells [58]. In this model, K-ras induced transformation through a mechanism involving ROS (reactive oxygen species) induction of JNK. As discussed below, inhibiting autophagy is a current strategy in clinical trials to enhance chemotherapeutic response and overcome resistance of cancer cells.

AUTOPHAGY INTEGRATION WITH APOPTOSIS

Macroautophagy has been reported to play a role in cell death independently of apoptosis, but it remains unclear if this is a consequence of the severity and/or extended duration of autophagy, or as a deliberate mechanism of programmed cell death. Autophagic cell death is often described as cell death in the absence of apoptotic caspase activation and presence of autophagic vesicles, but there lacks mechanism that clearly defines autophagic cell death [37]. Whether autophagy contributes to, or reduces, apoptosis in cancer cells is dependent upon the type of cell and the type and duration of stimulus. For instance, autophagy can act as a either a survival or death mechanism within the human SK-OV-3 ovarian cancer cell line, depending on the molecule used to treat the cells [62, 70, 128]. Also, loss of autophagy promotes or prevents fibroblast apoptosis depending on the death stimulus [115]. In the majority of oncology studies however, autophagy appears to play a protective role. For example, in multiple myeloma cells, genetic and chemical inhibition of autophagy enhances induction of apoptosis *in vitro* and tumor growth inhibition by DNA-damaging drugs, doxorubicin and melphalan *in vivo* [92]. In rat C6 glioma cells, siRNA silencing of ATG5, ATG7 or ULK1 genes increased apoptosis caused by cyclosporine A [16]. A recent study screened over 1400 cytotoxic agents for their ability to induce autophagic cell death in the U2Os osteosarcoma cell line [107]. Of the 59 compounds that were validated to truly induce autophagic flux, none of them

were prevented from inducing cell death when the ATG7 gene critical for autophagy was knocked out.

Some fragments of the autophagic machinery are directly involved in the induction of apoptotic cell death through the intrinsic mitochondrial pathway. The transition to apoptosis appears to occur after autophagy has been working to save the cell, but activation of specific proteases cleave Atg5 or Beclin 1 releasing cleavage products that translocate to the mitochondria and induce the intrinsic apoptosis pathway (Fig. 2). This direct involvement of Atg5 in apoptosis was demonstrated by enhanced sensitivity of multiple cancer cell types to several cytotoxic chemotherapeutic agents upon increased expression of Atg5 protein *in vitro* and *in vivo*, while silencing of the ATG5 gene had the opposite effect [127]. In this study, the switch from macroautophagy to apoptosis was mediated by calpain cleavage of the Atg5 protein, releasing a truncated Atg5 that translocated to the mitochondria where it bound the anti-apoptotic Bcl-2 molecule, Bcl_{XL}, thereby relieving Bcl_{XL} inhibition of the BAX/BAK pore forming ability resulting in cytochrome c release from the mitochondria and caspase activation of apoptosis [127]. The Bcl-2 family of proteins also integrates macroautophagy and apoptosis through Beclin 1. Under non-stressed conditions, Bcl-2 binds and sequesters Beclin 1 resulting in suppression of autophagosome formation [94]. Under stressed conditions, JNK becomes activated and phosphorylates Bcl-2, leading to Bcl-2 degradation and release of Beclin 1 to allow macroautophagy to attempt to recover cellular homeostasis [116, 117]. Transition to apoptosis occurs when caspase 3 is activated resulting in a Beclin 1 C-terminal cleavage product that translocates to the mitochondria and enhances apoptosis by releasing pro-apoptotic factors [31, 120]. The known binding of Beclin 1 to the anti-apoptotic Bcl_{XL} protein is a possible explanation for the induction of apoptosis, similar to what has been reported for Atg5 [88].

Another level of integration between autophagy and apoptosis is controlled by HMGB1 (high mobility group box 1), a nonhistone DNA-binding protein that binds tightly to chromatin of apoptotic cells and is released from necrotic cells [2]. In response to autophagic stimuli, HMGB1 translocates from the nucleus to the cytosol where it binds directly to Beclin 1 causing the release of Bcl-2 and allowing Beclin 1 to increase autophagy [111]. Much less is known about the integration of autophagy and programmed necrosis. Although autophagic vesicles have been observed in necrotic cells [2], it is not clear if autophagy is contributing to the cell death in this situation. Based on the observation that combined inhibition of autophagy and apoptosis stimulates necrosis in cancer cells exposed to ischemic conditions, autophagy appears to prevent necrosis [24].

Inconsistent ordering of autophagy and UPR events reported to occur prior to apoptosis suggest that the transition from cell survival to apoptotic death is not linear, but instead driven by an integrated network

of events that can eventually tip the balance to survival or death. For example, JNK can be activated upstream of macroautophagy by ER stress, but can also be activated downstream of macroautophagy by etoposide or staurosporine treatment of apoptosis-deficient MEFs [108]. This inconsistency suggests that the pathways are not connected in a linear sequence, but instead integrated at multiple levels. A simplified interpretation may be that autophagic cell death is not a mechanism designed by the cell, but rather a rare consequence of too much autophagy in response to external stimuli, such as chemical reagents or prolonged stress. The unrestrained autophagy could lead to cell death in the absence of apoptosis by consuming the viable cell mass or by upsetting the balance of pro-survival versus pro-apoptotic proteins. This may explain why a definitive molecular mechanism for programmed autophagic cell death has not been defined.

TARGETING MACROAUTOPHAGY IN CANCER THERAPY

While there is evidence that macroautophagy can suppress the early development of cancer [13], upregulation of basal levels of autophagy in multiple cancers indicate that autophagy primarily drives survival once the tumor has formed [33]. A recently developed method to detect macroautophagy in clinical specimens using immunohistochemistry with an antibody to LC3B demonstrated that punctuate pattern staining of LC3B indicative of macroautophagy significantly correlated with heightened cell proliferation and nuclear grade, invasion and metastasis, and worse outcome [69]. This is of particular significance because multiple anti-cancer agents are known to induce macroautophagy, which could interfere with tumor response (Table 2).

Table 2. Anti-cancer agents that induce autophagy

Function	Name	R
Alkylating agents	Cyclophosphamide, Temozolomide	[3, 50]
Bcl2 inhibitor	GX15-070	[47]*
Farnesyltransferase inhibitor	Lonafarnib	[47]*
Glycolysis inhibitors	GX15-070, 2-deoxyglucose	[47]*
HDAC inhibitors	Vorinostat, Sodium butyrate, LAQ824, Panobinostat	[47]*
Hormone treatments	Tamoxifen,Toremifene	[47]*
Ionizing radiation	Cs-137	[91,124]
Microtubule inhibitor	Vinblastine	[101]
Monoclonal antibodies	Rituximab (to CD20), Panitumumab (to EGF-R)	[47]*
mTOR inhibitors	Sirolimus, Temsirolimus, Everolimus, NV-128	[47]*
Natural compounds	Arsenic, resveratrol	[47]*
PARP inhibitor	ABT-888	[47]*
Proteasome inhibitors	Bortezomib, NPI-0052114, Epoxomicin	[47]*
Topoisomerase poisons	Doxorubicin, Campothecin	[73, 78]
Tyrosine kinase inhibitors	Dasatinib, Sorafenib, Imatinib	[47]*
Vitamin D analog	EB1089	[47]*

R = references, * Multiple references are provided in this review.

Preclinical studies demonstrating a protective role for macroautophagy in response to these therapeutics have translated into multiple clinical trials evaluating CQ and the derivative HCQ (hydroxychloroquine) inhibition of autophagy in combination with a variety of current cancer treatment strategies. CQ and HCQ are "old drugs" that have been prescribed for malaria

[89], rheumatoid arthritis [66] and HIV [102]. The first reported clinical trial of combining autophagy inhibition with cancer therapy was a single-institution phase III trial in glioblastoma patients treated with conventional radiation and carmustine therapy with or without daily CQ [109]. Although this study was not adequately powered to detect a significant difference in survival, CQ increased overall survival from 11 months in the placebo arm to 24 months in the CQ arm. HCQ is a less toxic version of CQ and the best autophagy inhibitor currently commercially available for clinical trials [39]. Currently there are 84 clinical trials of HCQ listed on the United States Government website (ClinicalTrials.gov), of which 33 are cancer studies, including 15 Phase I, nine Phase I/II, and nine Phase II clinical trials of HCQ in combination with a range of chemotherapeutic agents.

In contrast to the sensitization of tumors to chemotherapeutic agents by autophagy inhibition, the sensitivity of tumors to radiation has been shown to be enhanced by induction of autophagy with the mTOR inhibitor everolimus (RAD001) and further enhanced by combined inhibition of apoptosis and induction of autophagy [9, 57]. There are several early phase clinical trials of everolimus or rapamycin in combination with radiation therapy for a variety of cancers listed on the ClinicalTrials.gov website. The translation of laboratory-based autophagy studies to clinical trials is based primarily on research of macroautophagy, however there is considerable integration of macroautophagy with the other forms of autophagy that could be taken advantage of in this cancer treatment strategy, and that needs to be taken into consideration to fully understand the impact of the interventions and the interpretation of results.

MECHANISM AND INTEGRATION OF CHAPERONE-MEDIATED AUTOPHAGY

CMA involves the selective degradation of individual molecules containing an amino acid sequence motif related to KFERQ (Lys-Phe-Glu-Arg-Gln) [27]. CMA activity can be measured in cells by the transition of diffuse to punctuate pattern of a photoconvertible KFERQ-PA-mCherryl reporter protein in transfected cells [61]. It is estimated that at least 30% of cytosolic proteins contain this sequence or a sequence that can be made mimic KFERQ through post-translational modifications [26]. The proteins may have KFERQ located on their surface for recognition by Hsc70 or this sequence may become exposed upon damage or denaturing of the protein, or upon separation of the protein with another protein subunit. A single molecular chaperone (Hsc70/HSPA8) appears to mediate the recognition of proteins with exposed KFERQ domains and transporting them to the lysosome [15]. At the lysosome, Hsc70 binds LAMP-2A (Lysosome-associated membrane protein type 2A), and interacts with a complex of other chaperones to unfold the transported protein and push it through LAMP-2A to another molecule of Hsc70 waiting inside the lysosome

[1]. Increased expression of LAMP2A and lysosomal Hsc70 correlate with CMA activity and are accepted markers of CMA [18, 19]. Hsc70 binds and hydrolyzes ATP in order to generate the energy required for this process, and the ADP-bound form of Hsc70 has the highest affinity for KFERQ proteins [48]. The proteins working with Hsc70 at the surface of the lysosome have unique functions. Hsp40 activates Hsc70 ATPase activity to increase affinity for protein substrates, Hip (heat shock protein 70 interacting protein) facilitates the assembly of the various proteins in the complex, cdc48 (cell division cycle 48) stimulates the activity of this protein complex, and Bag-1 (Bcl-2-associated athanogene 1) acts as a nucleotide exchange factor that stimulates substrate release [76]. The stability of Hsc70 inside the lysosome is tightly controlled by the pH of the lumen, which, if altered, could denature Hsc70 and make it susceptible to lysosomal proteases [19]. LAMP-2A also works as a complex with EF1α (elongation factor 1 α) to form a complex with GFAP (glial fibrillary acidic protein) that can be negatively regulated by GTP, which causes dissociation of the complex subunits [5].

CMA is a second line response to cell starvation [7]. Macroautophagy is the first line response to nutrient deprivation with maximal activity around 6 hours, followed by a gradual reduction [35, 83]. There appears to be mutual inhibition between macroautophagy and CMA, as CMA does not increase to suprabasal levels until the reduction of macroautophagy 6 to 8 hours after initiation of starvation [7]. Maximal CMA occurs 24 hours after initiation of starvation and continues for at least 3 days [20]. Although inhibition of macroautophagy or CMA can lead to upregulation of each other, the compensation is incomplete, as CMA cannot degrade organelles and macroautophagy cannot compensate for the selectivity of CMA. Inhibition of CMA by blocking expression of LAMP-2A in fibroblasts resulted in increased macroautophagy, however this compensatory action did not alleviate the increased sensitivity of CMA-deficient cells to stress [83]. Inhibition of macroautophagy with type III PI3K inhibitors (3-3-MA, wortmannin or LY294002) or activation of macroautophagy with rapamycin, had no effect on CMA [35], however macroautophagy-deficient cells caused by genetic deletion of ATG5 exhibit upregulation of both basal and induced CMA through different mechanisms [51]. Although there is little evidence to document direct integration of CMA with ER stress or UPR, integration of CMA with proteasomal degradation is indicated by the selective degradation of proteasomal catalytic core subunits by CMA [21].

Deregulated CMA has been shown to cause multiple diseases including MLIV (mucolipidosis Type IV), which is caused by defects in TRPML1 (transient receptor potential mucolipin-1) leading to ineffective docking with Hsc70 inside the lysosome [114], glaucoma and other diseases related to ineffective Hsc70 transport of proteins along neuronal axons [39]. In cancer, levels of the MDM2 (murine double minute

2) regulator of the p53 tumor suppressor protein and the Gal3 (Galectin-3) oncogenic protein, and likely multiple other oncongenes and tumor suppressor genes, are controlled by CMA [77, 80]. A recent study demonstrated that inhibition of constitutively upregulated CMA by silencing Lamp-2A expression caused increased p53 levels resulting in reduced proliferation and altered metabolism in human lung cancer cells *in vitro* and reduced tumor growth and metastases *in vivo*, indicating that CMA is a relevant target for anti-cancer therapy [61].

MICROAUTOPHAGY

Although microautophagy was originally described by de Duve and Wattiauz in 1963, the term was not coined until 1983 [23]. While much less well characterized than macroautophagy and CMA, studies of microautophagy have led to the delineation of 5 sequential steps that mediate microautophagy, namely: invagination, vesicle formation, vesicle expansion, vesicle scission and vesicle degradation and recycling [75]. The first step of invagination is an ATP-dependent process that occurs at areas of the lysosomal membrane with low concentrations of transmembrane proteins and that develops as tubes filled with cytoplasmic components, in contrast to the shapes of other types of lysosomal invaginations [82, 106]. The subsequent processes are mediated by mTOR regulated ubiquitin-like systems, LC3 lipidation and other Atg machinery similar to that discussed above for macroautophagy [75]. Selective microautophagy degradation of mitochondria (micromitophagy), the nucleus (PMN [piecemeal microautophagy of the nucleus]) and peroxisomes (micropexophagy) have been described [34, 54, 67]. Similar to macroautophagy and CMA, microautophagy occurs at basal levels and can be induced by nitrogen starvation or mitochondrial damage, however in contrast to the antagonism of macroautophagy with CMA, microautophagy appears to work synergistically with the other two forms of autophagy [17]. Although primarily characterized in yeast, defects in microautophagy have been linked to a number of human diseases, however a direct link with cancer has not been identified [75].

CONCLUSIONS

Are current definitions of pathways and modes of cell stress response, survival and death limiting our ability to comprehend the dynamic cell? Models are useful, but can introduce bias into our comprehension. This review has described multiple levels of integration between various pathways that can maintain cellular homeostasis or default into apoptosis. Upon stress to the system, the ultimate consequence of cell survival or death depends on balance of events linked at multiple network connections, similar to a hanging mobile. In this model, failure to balance the weights of proteasomal degradation, UPR and autophagy against apoptosis and other forms of cell death, such as programmed necrosis, can lead to excessive autophagy consuming the viable cell mass or to the relief of inhibition of the default apoptosis pathway that is always present and waiting for relief of inhibition to kill the cell. The detailed molecular interactions described in this review provide information on the nodes of this complex network that can be manipulated to control the network in development of strategies to induce apoptosis in cancer cells without harming normal cells.

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