# Physiological Mini Reviews

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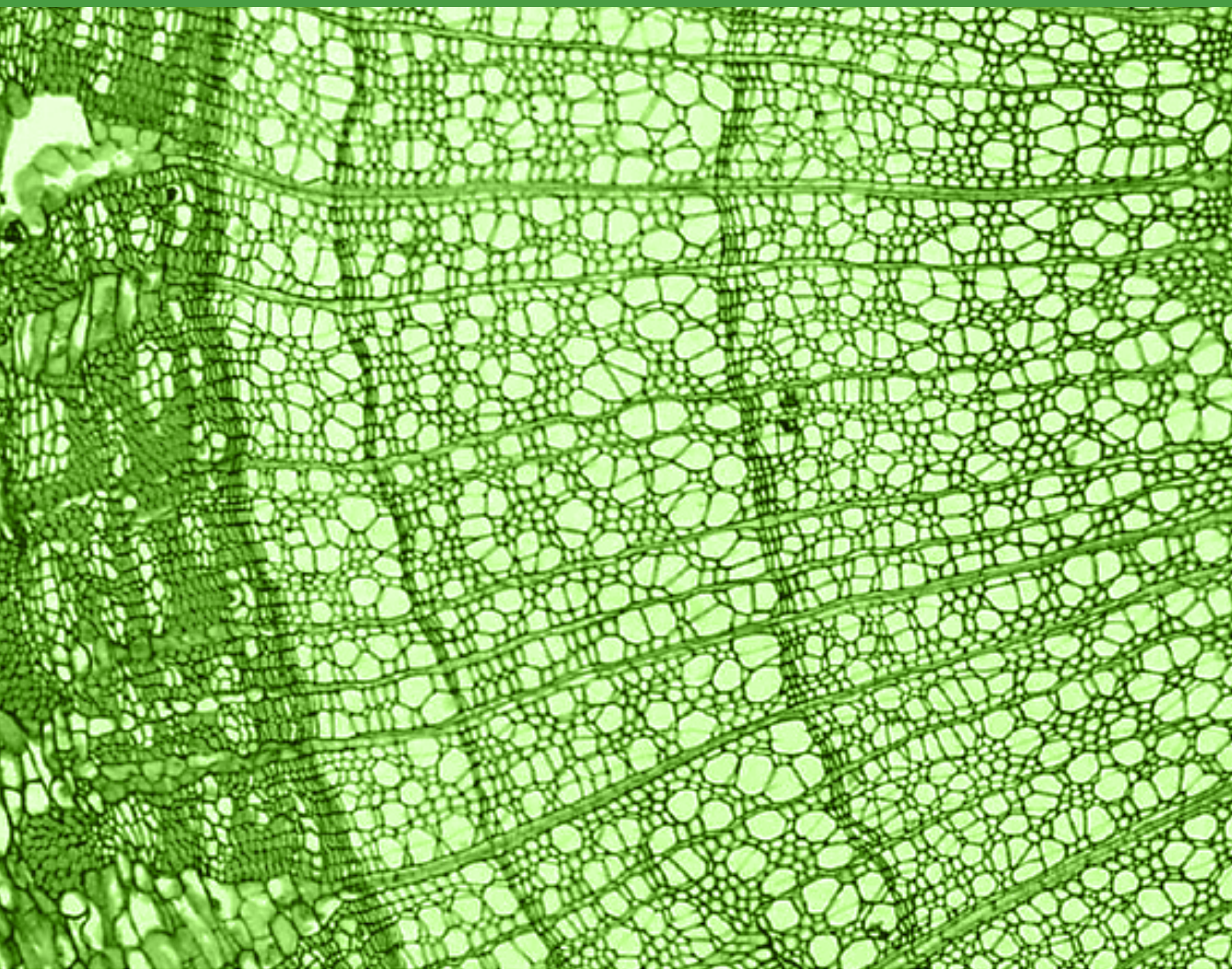

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# AUTOPHAGY, PHYSIOLOGY AND CELL PATHOLOGY

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## ABSTRACT

Autophagy plays an important role in human pathologies, such as miopathies and neurodegenerative diseases. Moreover, metabolic disease processes such as diabetes and pancreatitis, as well as tumor cells and cancer chemotherapy, activate autophagy. Furthermore, a selective type of autophagy of pancreatic zymogen granules has been discovered and characterized as a protective cellular process triggered by acute pancreatitis. In this review we describe the physiological bases and molecular mechanisms in the autophagic process and the possible role of autophagy in human disease. Understanding the events and molecular mechanisms of this complex cellular process will help in the design of new strategies for more effective diagnosis and treatment.

**Keywords:** Autophagy; VMP1; Zymophagy; mTOR

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## Autophagy, a self-eating cellular process

Autophagy is an evolutionarily conserved and highly regulated lysosomal pathway that degrades macromolecules (e.g. proteins, glycogen, lipids and nucleotides) and cytoplasmic organelles [1-3]. This catabolic process is involved in the turnover of long lived proteins and other cellular macromolecules, and it might play a protective role in development, aging, cell death, and defense against intracellular pathogens [4, 5]. Moreover autophagy has been linked to a variety of pathological processes such as neurodegenerative diseases and tumorigenesis, which highlights its biological and medical importance [6, 7].
Autophagy consists of several sequential steps, which are: induction, autophagosome formation, autophagosome-lysosome fusion and degradation. Although autophagy was first identified in mammalian liver upon glucagon treatment approximately 50 years ago, its molecular understanding started only in the past decade, largely based on the discovery of the autophagy related genes (ATG) in yeast.
Depending on the delivery route of the cytoplasmic material to the lysosome, there are three major types of autophagy in eukaryotes: 1) chaperone-mediated autophagy (CMA), 2) microautophagy and 3) macroautophagy, hereafter referred to as autophagy [8]. CMA allows the direct lysosomal import of unfolded, soluble proteins that contain a particular pentapeptide motif. In microautophagy, cytoplasmic material is directly engulfed into the lysosome at the surface of the lysosome by membrane rearrangement. Finally, autophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle, referred to as an autophagosome that subsequently fuses with a lysosome to form an autolysosome for the degradation by lysosomal hydrolases [9].

## The autophagy flux

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called autophagosomes, which eventually acquire lysosomal-like features [9, 10]. The autophagic process is described in the **Figure 1**.

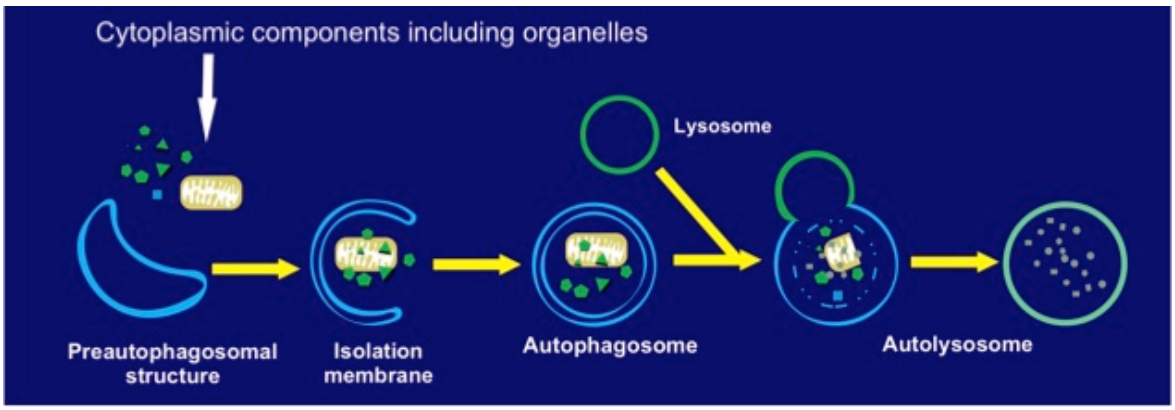


**Figure 1.** The autophagy process

Briefly, sequestration of cytoplasm into a double-membrane cytosolic vesicle is followed by the fusion of the vesicle with a late endosome or lysosome to form an autophagolysosome (or autolysosome). Then, inner membrane of the autophagosome and autophagosome-containing cytoplasm derived materials are degraded by lysosomal/vacuolar hydrolases inside the autophagosome. The molecular mechanisms underlying the transport and fusion of autophagosomes are just beginning to be

understood, and through active investigations, several major events involved in the process have recently been clarified including the recycling of lysosomes [11]. In mammalian cells, autophagosome maturation is a prior step for the fusion between autophagosomes and lysosomes. The degradation products, including macromolecules, are then exported to the cytosol for re-utilization by the cell.

**The autophagosome formation**

Since the discovery of yeast autophagy-related (ATG) proteins, autophagosome formation has been dissected at the molecular level but a lot of questions about the molecular mechanism underlying this process remain unanswered. Autophagosomes can be considered unique organelles because they do not contain marker proteins of other subcellular compartments [12]. In mammalian cells, the sequential association of at least a subset of the ATG proteins leads to the assembly of the pre-autophagosomal structures (PAS), which is believed to be the site where the precursor structure of the autophagosomes, the phagophores, are generated [13]. The PAS and phagophore formation also requires phosphatidylinositol 3-phosphate (PI3P) [14] and it is believed to be associated to specific subdomains of the endoplasmic reticulum (ER) termed omegasomes [15, 16]. Among the key mediators initiating autophagosome formation, there is a set of evolutionarily conserved ATG gene products; the kinase-containing Ulk1/2 complex (ATG1 in yeast), the Class III phosphatidylinositol 3-kinase (PI3K) complex (composed by BECN1/ATG6-hVps34, hVps15 and ATG14L), the ubiquitin-like conjugation systems leading to the formation of the ATG5–ATG12–ATG16L1 complex and the LC3/ ATG8 phosphatidylethanolamine- conjugate (e.g. LC3-II) [17]. A second group of ATG proteins, which does not have orthologous in yeast, has also recently emerged and appears to play a key role in regulating autophagy in high eukaryotes. One of these proteins is the transmembrane Vacuole-Membrane-Protein-1 (VMP1), whose expression triggers autophagy in mammalian cells even under nutrient-rich conditions [18, 19]. Conversely, autophagy is completely blocked in absence of VMP1 [18].

Autophagosome formation process is composed of isolation membrane nucleation, elongation and completion steps. In mammals, the Class III PI3K plays an essential role in isolation membrane nucleation during autophagy [20]. The Class III PI3K is associated with BECN1/ATG6 and p150, the homolog of Vps15 (phosphoinositide-3- kinase, regulatory subunit 4), to form the PI3K complex. This kinase catalyzes the generation of PI3P on the autophagosomal membrane favoring the localization of other ATG proteins to the PAS during autophagosomal formation. The autophagosome nucleation system is ATG12-ATG5-ATG16L, which is an ubiquitin-like protein conjugation system essential for the formation of the PAS. ATG12 is conjugated to ATG5 [21]. E1-like ATG7 activates the carboxy-terminal glycine residue of ATG12 through a high-energy thioester bond in an ATP-dependent manner. The ATG12-ATG5 conjugate further interacts with ATG16L1 to form a ~350 kDa multimeric ATG12-ATG5-ATG16L protein complex through the homo-oligomerization of ATG16L [22]. Another ubiquitin-like protein conjugation system is the modification of LC3 (a mammalian homolog of ATG8) by the phospholipid phosphatidylethanolamine (PE) [22], an essential process for the formation of autophagosomes. LC3 (LC3-I) is cleaved by cysteine protease ATG4 and then conjugated with PE by ATG7 and ATG3. This lipidated LC3 (LC3-II) then associates with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until its fusion with lysosomes [23, 24]. The conversion of LC3-I to LC3-II is thus well known as a marker of autophagy (**Figure 2**). However, the increase of LC3-II alone is not enough to

show autophagy activation because the inhibition of LC3-II degradation in the lysosome by the impaired autophagy flux can also cause its accumulation.

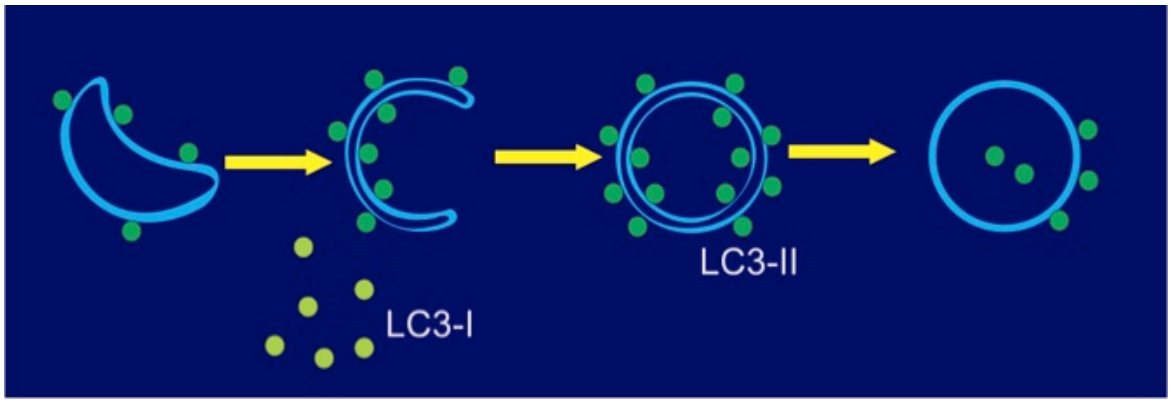


**Figure 2.** The conversion of LC3-I to LC3-II is thus well known as a marker of autophagy

While the origin of autophagic vacuoles remains disputable, several hypotheses have been proposed for the source of autophagosomal membrane during autophagosome formation. The first hypothesis is "de novo" formation of autophagosome by ATG9 reservoirs. In the second hypothesis, various organelles such as ER, mitochondria and plasma membrane are used as an origin for the formation of the phagophore. Recently, cup-shaped structures called omegasome, a discrete region of the ER, were identified as a platform for autophagosome formation [25]. The ATG5 complex, LC3 and ULK1 have been shown to recruit into the omegasome after starvation, and ATG5- and LC3-positive membranes seem to emerge from the omegasome. It was also observed that omegasomes form in close proximity to the PI3K- containing vesicles, which may synthesize the PI3P. This hypothesis is also supported by a notion of a physical association between the ER and early autophagic membranes [26].

## Regulation of autophagy induction

Basal autophagy in unstressed cells is kept down by the action of the mammalian target of rapamycin complex 1 (mTORC1). Key upstream regulators of mTORC1 include the class I phosphoinositide 3-kinase-Akt pathway, which keeps mTORC1 active in cells with sufficient growth factors, and the AMP-activated protein kinase (AMPK) pathway that inhibits mTORC1 upon starvation and calcium signals [27, 28].
Under stress conditions such as amino acid starvation, autophagy is strongly induced in many types of cultured cells. The effects of individual amino acids differ in their abilities to regulate autophagy. Amino acids including Leu, Tyr, Phe, Gln, Pro, His, Trp, Met and Ala suppress autophagy in ex vivo-perfused liver [29]. However, such profiles depend on cell types showing their different amino acid metabolisms in tissues. The questions on how cells sense amino acid concentration and physiological significance of autophagy regulation by amino acid starvation are not fully understood yet. It has been demonstrated that amino acid signaling pathways exist, which involve activation of mTORC1 and the subsequent regulation of the class III PI3K. The mTORC1 is involved in the control of multiple cell processes in response to changes in nutrient conditions [30]. Especially, mTORC1 requires Rag GTPase, Rheb and Vps34 for its activation and subsequent inhibition of autophagy in response to amino acids [31, 32]. Energy levels are primarily

sensed by AMPK, a key factor for cellular energy homeostasis. In low energy states, AMPK is activated and the activated AMPK then inactivates mTORC1 through TSC1/TSC2 and Rheb protein [33].
Thus, inactivation of mTORC1 is essential for the induction of autophagy and plays a central role in autophagy. In addition to amino acid signaling, hormones, growth factors and many other factors, including bcl-2 [34], have also been reported to regulate autophagy. But, not all autophagy signals are transduced through mTOR signaling. A recent study showed that small-molecule enhancers of the cytostatic effects of rapamycin (called SMERs) induce autophagy independently of mTOR [35]. Activities of the ULK1 are regulated by mTOR, depending on nutrient conditions. Under growing and high-nutrient conditions, the active mTORC1 interacts with the ULK1 and phosphorylates ULK1 and mATG13, and thus inhibits the membrane targeting of the ULK1. During starvation condition, on the other hand, the inactivated mTORC1 dissociates from ULK1 and results in the ULK1 complex formation (ULK1-mATG13-FIP200-ATG101) leading to autophagy induction [36].

**BECN1**

BECN1 (former Beclin 1), the mammalian ATG6, is a haploinsufficient tumor suppressor and an important effector of autophagy. BECN1 is a subunit of the PI3K complex, the action of which is antagonized by Bcl-2 [36, 37]. BECN1 contains a BH3 domain that mediates its interaction with Bcl-2 [38, 39]. The interaction between Bcl-2 and BECN1 leads to inhibition of autophagy by interfering with the formation and activity of the autophagy promoter complex, BECN1- PI3K [40]. (**Figure 3**)

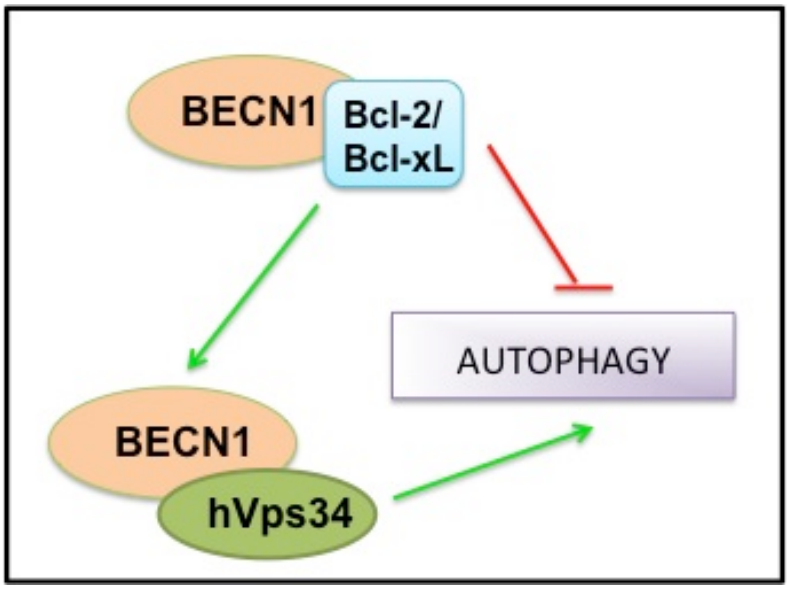


**Figure 3**. BECN1-PI3K promotes autophagy

**VMP1**

The pancreatitis-associated protein named vacuole membrane protein 1 (VMP1) is a transmembrane protein with not known homologues in yeast. VMP1 was found searching for new molecules that were differentially expressed during acute pancreatitis [41]. VMP1 expression is induced by mutated K-Ras in pancreatic cancer cells [42] and by hyperstimulation of Gq-coupled cholecystokinin receptor (CCK-R) in pancreatic acinar

cells during acute pancreatitis [43]. In the adult normal pancreas, VMP1 expression is not detectable, but it is highly induced early during experimental acute pancreatitis and its expression levels correlate with morphological features resembling autophagy [44]. Moreover, VMP1 expression can be found in pancreatic acinar cells from rats developing spontaneous chronic pancreatitis (WBN/Kob rats) [45] and it is rapidly and highly expressed in experimental diabetes [45-47]. Finally, Gemcitabine (2,2-difluorodeoxycytidine), the standard chemotherapy for the treatment of advanced pancreatic cancer, induces VMP1 expression in human pancreatic cancer cells [48-50]. We demonstrated that VMP1 expression triggers autophagosome formation in mammalian cells, even under nutrient-replete conditions [18, 19]. Remarkable, VMP1 pancreas-specific transgenic expression in mice promotes autophagosome formation in acinar cells. Therefore, VMP1 expression may be involved in autophagy induction during acute pancreatitis, a disease defined as pancreas self-digestion. Furthermore, VMP1 is the only human disease-inducible ATG-protein described so far.

VMP1 interacts with BECN1 through its hydrophilic C-terminal region (VMP1-ATGD), which is necessary for early steps of autophagosome formation. Hierarchical analyses in mammalian cells shows that VMP1 along with ULK1 localize in the autophagosome formation site [13]. VMP1-BECN1 interaction is required for the formation of the PI3K complex acting in mammalian autophagy. This complex, which is composed by BECN1-hVps34-ATG14 promotes PI3P generation on autophagosomal membrane favoring the localization of ATG16L1 and LC3 to the autophagosomal membrane during autophagosome formation [51, 52]. The interaction between VMP1 and BECN1 requires the BECN1 domain that binds to Bcl2 (BECN1-BH3 domain) [51]. During normal growth conditions Bcl-2 binding to BECN1 is maximal, and when autophagy is induced this interaction is strongly reduced [53]. VMP1 expression leads to the dissolution of the BECN1-Bcl-2 complex, indicating that VMP1 is involved in driving BECN1 into the autophagic process [38]. Thus, VMP1-BECN1 interaction through the VMP1-ATGD is required for the proper localization of PI3K activity on the autophagosomal membrane during mammalian autophagy, positioning VMP1 as a key regulator of the early steps of autophagosome formation possibly acting as a platform in the autophagosomal membrane.

## Selective types of autophagy

Early studies suggested that autophagy was a nonselective process in which cytoplasmic structures were randomly sequestered into autophagosomes before being delivered to the mammalian lysosome or the plant and yeast vacuole for degradation. Now there is growing evidence that unwanted cellular structures can be selectively recognized and exclusively eliminated within cells. This is achieved through the action of specific autophagy receptors, such as Nbr1 and p62, which is an ubiquitin-binding protein that interacts with LC3 [54-55]. Thus excess or damaged organelles including mitochondria, peroxisomes, lipid droplets, endoplasmic reticulum and ribosomes can be specifically sequestered by autophagosomes and targeted to the lysosome for degradation. Importantly, there is growing evidence that selective autophagy subtypes also have a wide range of physiological functions. Selective autophagic pathways target distinct cargoes to autophagosomes, including mechanisms for the clearance of aggregated protein, and for the removal of dysfunctional mitochondria (mitophagy). In pancreas cells, autophagy has recently been shown to specifically turn over secretory granules damaged by acute pancreatitis as a protective cellular response [43].

## Zymophagy

The pancreatic acinar cell activates VMP1-mediated autophagy early during acute pancreatitis [44]. Relevant data about the role of autophagy in pancreas was obtained using ElaI-VMP1 mice, in which the pancreas acinar-cell-specific elastase promoter drives VMP1 expression in pancreas. Pancreases of these transgenic mice show numerous vesicles that stain for endogenous LC3, indicating that VMP1 induces the autophagosome formation and, therefore, autophagy [18]. Interestingly, ElaI-VMP1 mice do not develop pancreatitis in normal conditions, confirming that autophagosome formation does not induce acute pancreatitis [18]. The immunomagnetic isolation of VMP1-autophagosomes containing zymogen granules from the ElaI-VMP1 transgenic mouse pancreas with acute pancreatitis, allowed the discovery of a new type of selective autophagy named zymophagy, which functions as an inducible cellular process that recognizes and degrades activated zymogen granules [43].

Zymophagy is characterized by the formation of autophagosomes containing zymogen granules. These organelles mediate the sequestration and degradation of pancreatitis activated zymogen granules. Cholecistokinin receptor (CCK-R) hyperstimulation with cerulein in wild-type animals, a classical model of acute pancreatitis, induced a markedly altered distribution pattern of the secretory granules such as fusion among zymogen granules as well as their fusion with condensing vacuoles. In addition, acinar cells lose their polarity, which results in the relocation of zymogen granules to the basolateral membrane. Surprisingly, ElaI-VMP1 mice subjected to CCK-R hyperstimulation reveal that acinar cells preserve their structure and polarity with negligible or no alteration in vesicular transport. Instead, pancreases from cerulein-treated ElaIVMP1 mice present autophagosomes containing zymogen granules displaying a distinct localization to the apical area of the acinar cell. This observation is confirmed using isolated mouse pancreas acini, revealing that 15 min after cerulein treatment, zymophagy is detected [43]. The finding of different maturation levels of selective autophagic vesicles as well as the degradation of p62 provide evidence that autophagic flow remains primarily unchanged under CCK-R hyperstimulation [43]

Regarding the pathophysiological relevance of zymophagy during acute pancreatitis, it was demonstrated that zymophagy protects acinar cells from intracellular trypsinogen activation triggered in vivo by experimental pancreatitis induced by cerulein. Upon CCK-R hyperstimulation, wild type mice developed acute pancreatitis with high amylase and lipase serum levels. On the contrary, enzymatic levels in cerulein-treated ElaI-VMP1 mice were significantly lower compared with wild type mice. Consistently, ElaI-VMP1 mouse pancreas showed remarkably less macroscopic evidence of acute pancreatitis compared with wild type animals, which showed marked edema and hemorrhage. Histological analyses displayed a high degree of necrosis as well as infiltration in wild type pancreas with acute pancreatitis. In contrast, neither necrosis nor significant inflammation was seen in cerulein-treated ElaI-VMP1 mice [41, 43]. Thus, results obtained in the transgenic animal model showed that zymophagy functions as a protective pathophysiological mechanism against pancreatitis-associated injury.

Upon CCK-R hyperstimulation, acinar cells from wild-type mice show early cytoplasmic trypsinogen activation, which is a hallmark of pancreatitis pathophysiology. Surprisingly, in acinar cells from ElaI-VMP1 mice, CCK hyperstimulation causes almost no activation of trypsinogen. Microscopic examinations using BZiPAR (rhodamine 110 bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride), a cell permeable substrate that becomes fluorescent after the cleavage by the protease, reveal only few activated granules that highly colocalize with VMP1, showing that zymophagy selectively sequester the

activated zymogen granules. Zymogen activation is an enzymatic chain reaction where initial zymogen granule alterations trigger rapid spread of active trypsin within the acinar cell. We think that the degradation of early-activated zymogen granules by zymophagy prevents this deleterious event. Interestingly, the inhibition of autophagic flow markedly increases trypsin activity within acinar cells in ElaI-VMP1 mouse pancreases under CCK-R hyperstimulation, confirming that zymophagy specifically degrades those zymogen granules that are initially activated by acute pancreatitis [43]. This function is confirmed in the in vivo animal model of acute pancreatitis, where the ability of the ElaI-VMP1 mouse developing zymophagy clearly prevents the increment of enzymatic markers of pancreatic damage and pancreas morphological changes characteristic of acute pancreatitis.

Analysis of autophagosomes containing zymogen granules magnetically immunopurified from the pancreas of ElaI-VMP1 mice treated with cerulein revealed that apart from zymogen granules, isolated vesicles contains LC3-II and, notably, strong signals of p62. Moreover, GFP-Ubiquitin-transfected acinar cells subjected to CCK-R hyperstimulation show co-localization between activated granules and ubiquitin aggregates but do not show colocalization between unaffected or normal zymogen granules and ubiquitin, indicating that the ubiquitin system serves as a targeting signal for activated zymogen granules during zymophagy. Therefore, activated zymogen granules are directly or indirectly ubiquitinated for their recognition by autophagic membranes, in which ubiquitin acts as a label for selective engulfment. Nevertheless, activated zymogen granules are ubiquitinated upon acute pancreatitis and the VMP1-mediated selective autophagic pathway sequesters these ubiquitinated granules [43]. p62 may function as a cargo receptor during zymophagy. This data demonstrates for the first time that ubiquitin modifications may possess an additional function in acinar cells by promoting the degradation of highly harmful activated zymogen granules [56] (**Figure 4**).

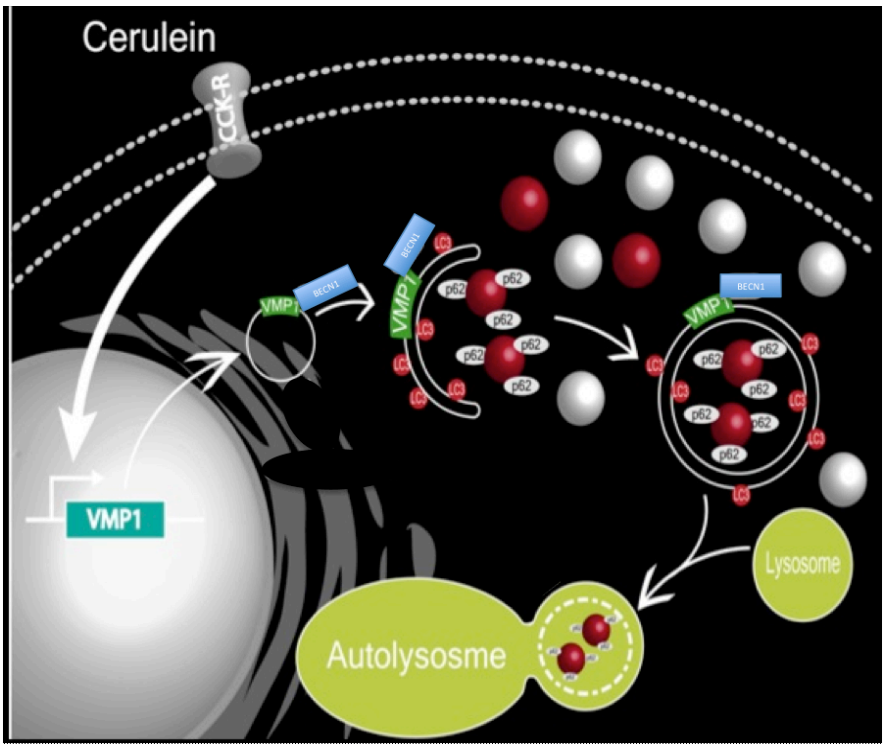


**Figure 4**. Zymophagy: The selective type of autophagy that sequesters and degrades activated zymogen granules (red circles) mediated by VMP1 expression, early during acute pancreatitis

Zymophagy prevents pancreatic acinar cell death induced by CCK-R hyperstimulation [43]. Autophagosome formation inhibition with 3-methyl adenine as well as autophagy flux interruption with vinblastine significantly reduces acinar cell survival in a cell model of acute pancreatitis. Moreover, VMP1 downregulation (shVMP1) also significantly decreases acinar cell survival under CCK hyperstimulation showing that VMP1 expression and autophagy is required to prevent acinar cell death in acute pancreatitis. These results indicate that zymophagy prevents pancreatic cell death induced by the activation of zymogen granules [56] and confirm that endogenous VMP1 expression is activated in acinar cells to mediate zymophagy as a protective cellular response against cell death.
Furthermore, VMP1 expression and zymophagy are present in human pancreas affected by acute pancreatitis [43, 56]. VMP1 is not detectable in human normal pancreas tissue, but its expression is activated in human pancreatitis pancreas specimens and highly colocalizes with LC3 in autophagosomes. Moreover, autophagosomes markedly colocalize with zymogen granules. Remarkable, the finding of large autolysosomes without trypsin signal in pancreas of human pancreatitis suggests that affected zymogen granules are eventually degraded by zymophagy during human pancreatitis.

## Perspectives

There is ample evidence supporting an active role for autophagy in cell physiology and disease. During the last decade, autophagy has turned from a morphological finding to a cellular process involving a membrane transport system and complex molecular machinery. Moreover, since the discovery of ATG genes, there have been many studies on the physiological and pathological roles of autophagy in a variety of autophagy knockout models. However, direct evidence of the connections between ATG gene dysfunction and human diseases has emerged only recently [57]. Here we have overviewed the physiological bases and molecular mechanisms of the autophagic process. We have introduced the reader to a novel autophagy-related transmembrane protein -VMP1- whose expression triggers autophagy and to the role of this protein in the cell response to disease. Elucidation of the specific extracellular and intracellular conditions that stimulate autophagy and the linkage of these conditions to either cell survival or cell injury and death in different cell types and during different pathological processes, is a rapidly evolving and fruitful field of research. The development of therapies to take advantage of the potential cytoprotective effect of autophagy in several pathologies such as cancer or neurodegenerative diseases is a potentially promising avenue of investigation.

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