

Review

# Autophagy: Many paths to the same end

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## Abstract

Different mechanisms lead to the degradation of intracellular proteins in the lysosomal compartment. Activation of one autophagic pathway or another, under specific cellular conditions, plays an important role in the ability of the cell to adapt to environmental changes. Each form of autophagy has its own individual characteristics, but it also shares common steps and components with the others. This interdependence of the autophagic pathways confers to the lysosomal system, both specificity and flexibility on substrate degradation. We describe in this review some of the recent findings on the molecular basis and regulation for each of the different autophagic pathways. We also discuss the cellular consequences of their interdependent function. Malfunctioning of the autophagic systems has dramatic consequences, especially in non-dividing differentiated cells. Using the heart as an example of such cells, we analyze the relevance of autophagy in aging and cell death, as well as in different pathological conditions. (*Mol Cell Biochem* **263**: 55–72, 2004)

*Key words:* lysosomes, cathepsins, protein targeting, aging, cellular death, cardiomyopathies

## Introduction

During the last 5 yrs, we have witnessed an astonishing advance in the understanding of the molecular mechanisms involved in the degradation of intracellular proteins in the lysosomal compartment (Autophagy). The identification of novel components of classically known lysosomal pathways and the discovery of alternative mechanisms for lysosomal degradation have definitely changed our perception of the lysosomal compartment. For many years lysosomes were considered to be responsible for non-selective degradation of proteins, but they have now revealed themselves capable of highly selective removal of intracellular components. In addition, the lysosome is now immersed in an intricate network of pathways that target cellular substrates for their degradation in this organelle.

Although some of the most recent findings on the molecular basis of each of the different lysosomal pathways are highlighted in the following sections, this review will focus on the selectivity of lysosomal degradation and the interdependence of the different lysosomal proteolytic pathways. Many of the mechanisms for lysosomal protein degradation

act interdependently. The same protein can be targeted to lysosomes by different pathways, and the switch between one pathway to another seems to rely on the existence of components common to all of them. We will analyze here the consequences of redundancy of the lysosomal system, and its possible implications in physiological and pathological conditions, with special emphasis in those pathologies affecting the cardiovascular system.

## Protein degradation: Why continuous turnover?

With different dynamics all intracellular proteins are continuously synthesized and degraded [1]. In part, the need for this continuous turnover is derived from the intrinsic nature of the intracellular environment that constantly generates compounds with putative harmful properties. Prompt degradation of irreversibly damaged proteins is essential to preserve normal cellular function [2]. Damaged proteins commonly develop abnormal intermolecular interactions resulting in the formation of aggregates. Those undigested complexes, which

are often trapped inside other cellular components, are toxic for the cells [2, 3].

However, despite this occasional removal of damaged proteins, the larger bulk of degraded proteins is still functional when eliminated. In a way, protein degradation is a 'preventive' mechanism that guarantees the replacement of cellular components by new ones before they lose functionality. This continuous degradation of normal proteins is not, as it could sound at a first hand, a waste of resources. The conservative nature of the degradative process allows the recycling of the constituent amino acids for future use inside the cell.

In addition to these housekeeping functions, intracellular degradation of proteins plays an important regulatory role, allowing rapid adaptation of cells to a changing environment. This regulatory role has been classically accepted for cytosolic proteolytic systems, such as the ubiquitin-dependent [4] or the calcium-dependent systems [5]. However, only recently the regulatory importance of the lysosomal system began to be understood.

## Lysosomal pathways for protein degradation

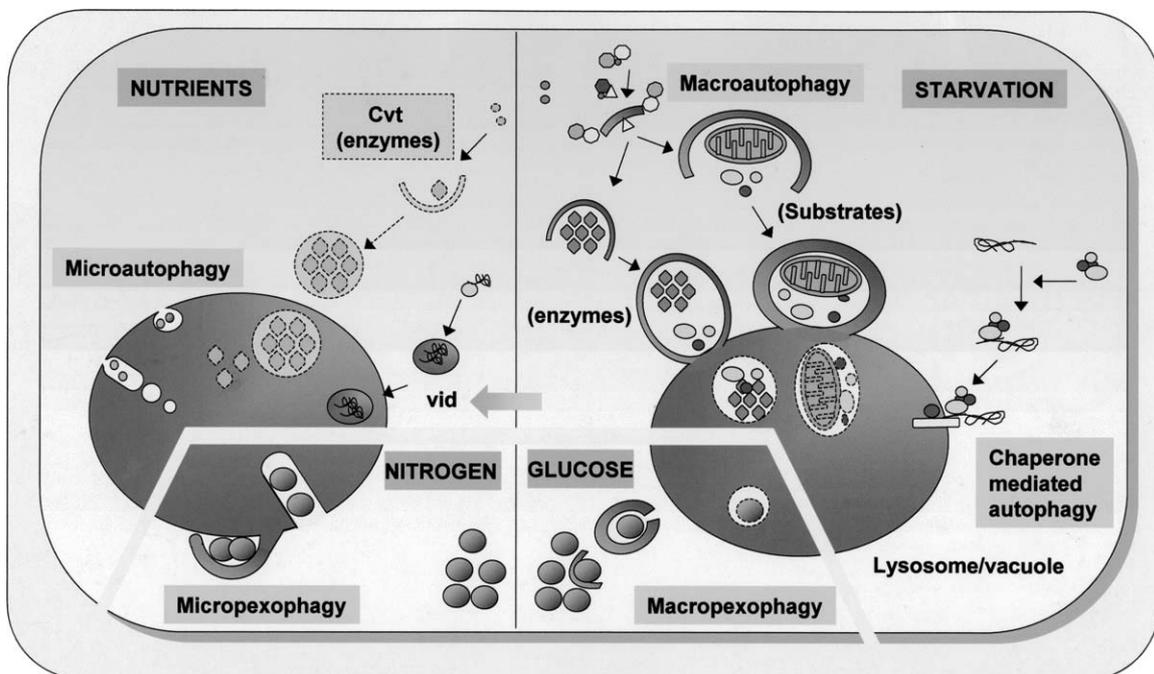
Different proteolytic pathways participate in both constitutive and induced turnover of proteins [1, 6, 7]. Although the same

proteolytic systems are present in almost all kinds of cells, the prevalence of one or the other varies from cell type to cell type, and with the cellular conditions. Intense 'cross-talking' among different intracellular proteolytic systems is obviously required to keep them in balance, and as described below, there is new evidence in support of it.

In the case of the major cytosolic proteases—20S proteasome, calpains and caspases—their activity and specificity relies in inhibitors and effectors, directly interacting with the proteolytic machinery [4, 5, 8]. In contrast, in the lysosomal system, the main regulatory step is the transport of the substrate to the lytic compartment. Different pathways for the transport of substrate proteins to lysosomes have been described for both mammals and yeast [6, 7, 9, 10]. A comparison of the main characteristics of each of those pathways and a schematic view of how transport of substrates takes place in each of them are shown in Table 1 and Fig. 1, respectively.

## Macroautophagy

Macroautophagy was first described in mammals as the sequestration of complete portions of cytosol, including not only soluble proteins but also complete organelles, into a double membrane vesicle known as autophagosome [11, 12]. After fusion with lysosomes, the autophagosome acquires



*Fig. 1.* Pathways for the degradation of intracellular proteins in lysosomes. A schematic model of the different mechanisms that lead to degradation of intracellular proteins in lysosomes and the stimuli that maximally activate them are depicted. The arrow indicates activation of vid after switching from poor to rich nutritional conditions. The cytosol to vacuole (cvt) pathway that transports hydrolases is also shown (dotted) because during the nutrient deprivation transport of the same enzymes is performed by macroautophagy.

Table 1. Comparison of the different pathways for the degradation of intracellular proteins in lysosomes

	MACRO	MICRO	CMA	VID	Pexophagy	
					MACRO	MICRO
Inducer	Starvation	No	Starvation	Glucose	Ethanol	Glucose
Organism described	Yeast Mammals	Yeast Mammals	Mammals	Yeast	Yeast Mammals	Yeast
Transport						
Vesicle mediated	Yes	Yes	No	Yes	Yes	Yes
Origin	<i>De novo</i>	Vacuole/lysosomes	–	?	<i>De novo</i>	Vacuole/lysosomes
Content	Soluble proteins Organelles	Soluble proteins Organelles		Single protein	Single peroxisome	Many peroxisome Soluble proteins
Receptor mediated	No	No	Yes	Likely	No	No
Requirements						
ATP	Yes	Yes	Yes	Yes	Yes	Yes
GTP/GTPases	Yes	Yes	No	?	?	?
Cytoskeleton	Yes	No	?	?	Yes	No
Chaperones	?	?	Yes	Yes	?	?
Acidic vacuolar pH	Yes	Yes	No	No*	Yes	Yes
Membrane potential	?	Yes	Yes	?	?	?
PI3 kinases	Yes	?	No**	No	No	Yes
Conjugation cascades	Yes	Yes	?	Yes	?	Likely
Substrates						
Organelles	All kinds	All kinds	No	No	Peroxisomes	Peroxisomes
Soluble cytosolic proteins	All kinds	All kinds	KFERQ-containing	Enzyme (1)	No	Yes?
Targeting signals	Ubiquitination Trimmed glucose	?	KFERQ-like motif	?	Peroxis inactivation	?
Unfolding	No	No	Yes	Likely	No	No
Study models						
Cell-free reconstituted	Yes	Yes	Yes	Yes	No	No
Yeast mutants	Yes	No	No	Yes	Yes	Yes

Data have been compiled from the literature cited along the text. Some of the findings have not been analyzed in both mammals and yeast, but they are expected to be valid for both systems based in the high homology of those already analyzed. \*Characteristics of vid transport refer mostly to the first step of translocation of proteins from the cytosol into the vid vesicles. \*\* (P. Finn and J.F. Dice, Personal communication). Abbreviations: MACRO (macroautophagy); MICRO (microautophagy); CMA (chaperone-mediated autophagy); VID (vacuole to degradation).

hydrolytic enzymes and its luminal pH acidifies, favoring the rapid degradation of the sequestered material. Macroautophagy is activated during nutrient deprivation and it becomes the major source of amino acids and other essential basic elements (lipids, sugars, nucleotides, etc.) during the first hours of starvation.

Recently, our understanding of how macroautophagy takes place and the major players involved in this catabolic process has grown considerably, thanks to genetic approaches carried out in yeast. With some exceptions, the yeast vacuolar system equals the lysosomal system in mammals. Studies in yeast are providing some initial answers to longstanding questions such as the following: How does the formation of autophagic vacuoles take place? What regulates the progres-

sion of autophagy? Or, how are selective organelles targeted for degradation? It is not the aim of the present review to describe in detail the function of more than 30 autophagy-related proteins identified, but instead to depict the general events leading to the formation, progression and final fate of the autophagic structures. The reader is directed to recent reviews containing thorough descriptions of autophagy-related proteins [10, 13, 14].

#### *The genetic dissection of macroautophagy*

In mammals, studies on macroautophagy have used either morphological observations or labeling and tracking of substrate proteins. These kinds of analysis have helped to

determine how macroautophagy is regulated, and the effect of inhibitors and effectors [12]. However, they have not been very informative in identifying the intracellular components involved in macroautophagy.

Different kinds of screening tests have been used for genetic studies on yeast. The first mutants (APG) were identified by their inability to accumulate undigested vesicles in the vacuole when switched to a poor nutrient medium in the presence of hydrolase inhibitors [15]. A second strategy, based on the immunological tracking of a cytosolic marker protein normally degraded by macroautophagy, identified a new subset of mutants (AUT) [16], some of which overlapped with the APG mutants. To add more complexity, some mutants for a biogenic pathway for the targeting of hydrolases to the vacuole (CVT) are redundant with some of the APG and AUT mutants [17]. In the current literature most of those genes are named using double or triple nomenclature (i.e., *Aut7/Apg8/Cvt5*). Recently, thanks to the joint effort of the different groups that originally described these genes, a unified nomenclature has been adopted [18]. This review uses the new nomenclature in which autophagy-related genes are designated as ATG and their proteins as Atg. Although it is anticipated that some differences will arise between the autophagic process in yeast and mammals, most of the genes identified in yeast are conserved in mammals [19–23] and even in plants [24].

#### *The origin of the trapping membrane*

One of the major contributions of the studies in yeast has been determining the origin of the sequestering membrane that forms the autophagosome. Despite many reports describing the presence of endoplasmic reticulum, plasma membrane or Golgi markers in autophagosome membranes, there is now strong evidence supporting *de novo* formation of this membrane [25].

Fluorescence tagging of some of the new proteins identified revealed their arrangement in a perivacuolar structure, known now as the 'preautophagosome' [25, 26]. This is the starting point of the isolation membrane (nucleation) that elongates to form the autophagosome [26]. A similar structure was morphologically identified in mammals several years ago under the name of the 'phagophore' [27], but a complete molecular characterization was not performed until recently [28]. The preautophagosome includes both resident proteins and proteins that will be transferred to the forming autophagosome. It also contains lipids, but it is not clear if they organize as micelles or small vesicles.

#### *Assembling and progression of the autophagic machinery*

Different protein complexes assemble in the preautophagosome when macroautophagy is activated. Figure 2 shows a

scheme of some of these complexes and their interrelation. Formation of the autophagosome requires two different types of conjugation events: lipid to protein and protein to protein [14]. In the first conjugation event, a single lipid molecule (phosphatidylethanolamine) is conjugated to the c-terminus of a protein, Atg8 in yeast or LC3 in mammals, previously exposed by proteolytic cleavage [29]. This conjugate is one of the first ones to localize in the forming structure and can be used to monitor autophagosome formation [30]. In a second conjugation event, required for targeting of Atg8/LC3 to the limiting membrane and elongation [20, 31], the c-terminus of a protein, Atg12, covalently binds to a lysine in another protein, Atg5. Some of the enzymes that mediate this conjugation have homology to the ones involved in the covalent attachment of ubiquitin to proteins [22, 32, 33], but others are completely novel [34]. Atg5 associates to Atg16, which oligomerizes forming larger units required for the elongation also [35]. Atg7, and E1-like ligase, participates in both conjugation cascades [21].

In addition to conjugation, protein phosphorylation also plays an important role in nucleation and elongation of the autophagosome. The presence, in the preautophagosome, of a protein complex that includes an autophagy-specific phosphatidylinositol (PI) 3-kinase, Vps34p (homologue to human type III PI3-kinase) associated to Vps15p and Vps30p/Atg6 is necessary to localize the conjugation systems to the forming unit [36]. The mechanisms by which this kinase complex regulates targeting is not clear, but might be based on the properties of phosphorylated PI to recruit effector proteins to specific areas of the membrane and/or to modulate their activity [37].

A second kinase complex, Atg1, participates in the late stages (tubulation/elongation) of autophagosome formation [38]. This kinase forms different types of complexes inside the cell, which determine whether the preautophagosome evolves to autophagosome or to hydrolase carrier vesicle [9]. Interestingly, the kinase activity of Atg1 is only required for the formation of hydrolase carrier vesicles but not of autophagosomes [39]. When Atg1 interacts with Atg13, elongation of the preautophagosome structure and progression toward the complete autophagosome takes place [9].

The preautophagosome contains components of the two conjugation systems and the two kinase complexes and also other factors of still unknown function. Some of them, such as Atg2 or Atg9 are present only in the forming units but not in the mature autophagosome, and they might help to hold together these protein complexes [9, 40, 41].

Autophagosome nucleation does not require *de novo* synthesis of proteins, suggesting that changes in the composition of interacting protein complexes, as well as modifications (conjugation/phosphorylation) in their components, are the main initiators of this process.

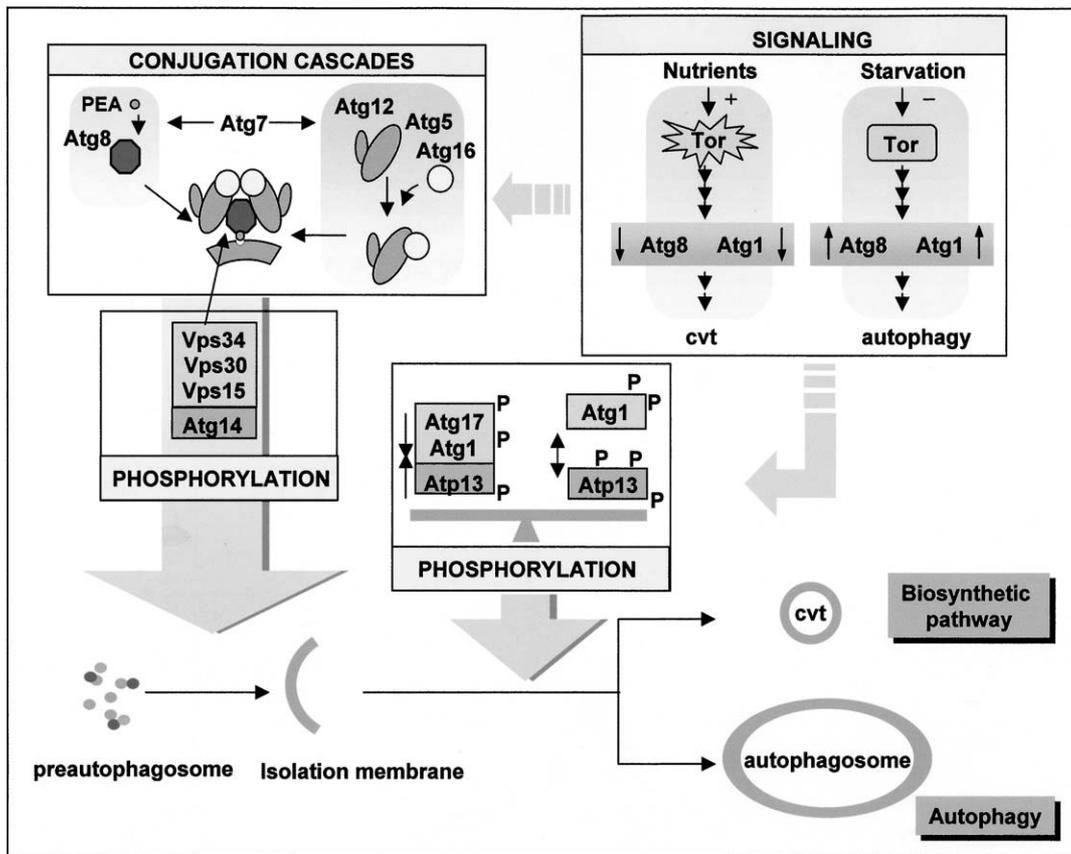


Fig. 2. Scheme of the events leading to the formation of autophagosomes. Assembly in the preautophagosome of two conjugation cascades and two phosphorylation systems leads to the nucleation/elongation of the isolation membrane. Progression of this isolation membrane toward the formation of a hydrolase carrying vesicle (cvt) or an autophagosome is regulated through the Tor-regulated signaling cascade. Abbreviations: PEA (phosphatidyl-ethanolamine); Tor (target of Rapamycin); cvt (cytosol-to-vacuole).

Once the two sides of the autophagosome fuse to each other, there is a second fusion event between the autophagosome and the lysosomes/vacuole that leads to the formation of the autolysosome. Little is known about the components required for this process, but participation of t- and v-SNARES is expected. Recent studies have shown defective autophagosome/vacuole fusion in mutants for components of the early secretory pathway such as Sec18p (a NSF) and Vti1p (a SNARE) [42]. Also Vam3, a member of the t-SNARE proteins, associates to vacuolar membranes, and its deletion results in impaired autophagy [43]. Dephosphorylation of some lysosomal component might take place since a type 2C protein phosphatase, *ptc4*, is required [44]. Finally, Rabz4 associates to autophagosome-like structures in a nutrient-dependent manner, but its role in the autophagic process is unknown [45]. When the external membrane of the autophagosome fuses with the lysosome, the phagocytosed content becomes accessible to the hydrolases [11]. In the case of the yeast, this content is transferred surrounded by a single limiting membrane that is first degraded.

#### Regulation of macroautophagy

Macroautophagy is a highly regulated process primarily at the sequestration/progression steps [11, 12, 46, 47]. Nutrient deprivation is the most generalized stimuli for macroautophagy, but decrease of specific regulator amino acids and increased levels of glucocorticosteroids and thyroid hormone also stimulate macroautophagy. Nutrient-independent activation of macroautophagy is also observed after some apoptotic stimuli. Recognized physiological inhibitors are insulin, various growth factors, AMPc and GMPc and conditions that lead to reduced intracellular levels of ATP, because sequestration, fusion and degradation are energy-dependent mechanisms [48]. The effect of some physiological regulators depends on the tissue analyzed. For example, glucagon and beta-adrenergic agonists inhibit rather than stimulate macroautophagy in cardiac and skeletal muscle, and the classical inhibitory effect of amino acids is not observed in pancreatic cells [49].

At the molecular level, the signaling cascades that connect the different regulators with the macroautophagic process are

still unclear. Intracellular signals for this pathway include cell swelling, mobilization of calcium pools, protein phosphorylation and GTP hydrolysis [47, 50–52]. Several small GTPases have been shown to participate in the autophagic process [53]. The role of kinase families, such as the PI3K has also been established [54]. Interestingly, different products of the PI3K-mediated reactions have opposite effects on macroautophagy [55].

Tor, a nutrient sensor kinase, has been extensively reported as a negative regulator of autophagy [47, 56]. When Tor is not stimulated by nutrients or when specific inhibitors such as Rapamycin inhibit it, the formation of autophagosomes is favored [47] (Fig. 2). Apparently, Tor inactivation increases levels of Atg8 and the kinase activity of Atg1 [57]. Stimulation of Tor leads to hyperphosphorylation of Atg13 decreasing its affinity for Atg1 [58]. Interestingly, Tor can act not only as an amino acid sensor but also as an ATP sensor [59]. However, in tissues such as muscle, Tor is not the only signaling pathway for macroautophagy, since activation of autophagy by amino acid removal, in this case, is not sensitive to Tor inhibitors [60]. Participation of other signaling pathways, such as the Notch pathway, in autophagy has been recently described in *Drosophila* [61] but further studies need to be carried on.

Treatment with inhibitors after autophagy stimulation leads to regression of elongating vacuoles and shrinking of the forming membranes [62]. On the basis of these observations, Kovac's group has proposed a very intriguing idea regarding the reversibility of autophagosome formation by disassembling of the different complexes that organize around the preautophagosome. This could be of special interest in diseases that present abnormally activated autophagy (i.e., myopathies) (see below).

## Microautophagy

Though worse understood than macroautophagy, a significant advance in the characterization of microautophagy has also been achieved during the last few years, especially regarding its role in selective degradation of organelles [7, 63, 64].

### *Morphological characterization of microautophagy*

Microautophagy also involves the trapping and degradation of complete regions of the cytosol by the lysosomal system, but the main difference when compared with macroautophagy is that the engulfing membrane in this case is the lysosomal membrane itself [65]. Formation of intermediate vesicles (i.e., autophagosomes) is not required through this pathway. As for macroautophagy, the cargo of microau-

tophagy can be both soluble proteins as well as complete organelles [66].

Microautophagy was morphologically described first in mammalian cells as the presence of lysosome-like organelles with multiple vesicles trapped in their lumen (multivesicular bodies) [66, 67]. These multivesicular bodies differ from the ones generated in endocytosis, in that they contain an acidic lumen and active mature proteases. Recent studies have shown that trapping of the cytosol by the lysosome/vacuole can adopt very different shapes going from invaginations, to tubulations, to other intermediate structures (some of them are depicted in Fig. 1) [68, 69].

Studies with isolated yeast vacuoles have made it possible to reconstitute *in vitro* the tubulation and pinching of microautophagic tubules. This system has revealed that the vacuole generates tubular structures with multiple ramifications that surround the internalized material. This process requires an intact membrane potential and participation of GTPases, but surprisingly, it is independent of the cytoskeleton and it does not require the presence of proteins that normally regulate homotypic membrane fusion [64]. The tubular invaginated structures are almost depleted of proteins, suggesting that a protein-free area, created probably by lateral mobilization of proteins in the lipid bilayer, becomes the initiating point of the invaginating complex [68]. This reorganization of proteins in the membrane and the progression of the tubule are probably mediated by protein complexes similar to the ones described for macroautophagy. In other types of yeast, direct engulfment of cytosolic components by the vacuole has been shown to include the formation of intravacuolar septums before the engulfed material gets in contact with the vacuolar lumen [69]. This tubulation or compartmentalization might maintain a relatively constant volume inside the lysosome/vacuole to accommodate the resident hydrolases during the engulfment.

### *Regulation of microautophagy*

Microautophagy is a constitutive mechanism for the continuous degradation of long-lived proteins inside many types of cells [70]. This form of lysosomal proteolysis is unresponsive to classical macroautophagic stimuli such as nutrient deprivation, glucagon or regulatory amino acids. Interestingly, some of these stimuli induce changes in the morphology of the multivesicular bodies, but without clear consequences in protein degradation [70].

Recently, a particular form of microautophagy, pexophagy, that leads to the preferential degradation of peroxisomes through direct engulfment by the vacuole, has been identified in yeast [69, 71]. The remarkable selectivity of pexophagy makes it difficult to directly extrapolate all the findings in this novel pathway to the classical microautophagy.

## Chaperone-mediated autophagy (CMA)

The selective degradation of particular cytosolic proteins in lysosomes during nutrient deprivation was described almost 20 yrs ago [72], but it was only recently that novel molecular components of this process, known as chaperone-mediated autophagy (CMA), and the basis for its regulation, began to be elucidated [6, 7].

The main characteristic of CMA, compared to macro- and microautophagy, is that the substrate proteins are directly translocated through the lysosomal membrane into the lumen without prior formation of intermediate vesicles. A unique feature of this pathway is that all substrate proteins contain in their amino acid sequence a motif, biochemically related to the pentapeptide KFERQ, required for targeting to the lysosomal compartment [73]. That motif is recognized in the cytosol by the heat shock protein of 73kDa (hsc73), a constitutively expressed member of the hsp70 family of chaperones (Fig. 3) [74]. Interaction between the substrate and hsc73 is regulated by nucleotides and by other chaperones and cochaperones [75]. The substrate/chaperone complex is then targeted to the lysosomal membrane where it binds to a receptor protein, the lysosome-associated membrane protein type 2a (lamp2a) [76]. The substrate interacts directly with lamp2a by a region, other than the KFERQ-related motif, occupied by the chaperone. Unfolding of the substrate is required for its transport into lysosomes [77]. Some of the chaperones associated to the targeted complex probably assist in the unfolding, but the presence of resident unfoldases in the lysosomal membrane could also be possible [75]. Multimerization of lamp2a is required for substrate translocation. It is thus possible that the association of multiple molecules of lamp2a could be enough to create discontinuity in the membrane required for transport of substrates (see inset, Fig. 3). Complete translocation is only achieved in the presence of a specific chaperone in the lysosomal lumen, the lysosomal hsc73 [78, 79]. By analogy with other intracellular transport system, this protein is believed to act as a 'pulling motor' for the substrates. It is however unclear how it obtains, in the lysosomal lumen, the ATP shown to be required for this function in other organelles.

CMA can be reconstituted *in vitro* with lysosomes isolated from culture cells or from different rodent tissues [79, 80]. This system allows for the separate analysis of the three main lysosomal steps of CMA: binding to the membrane, uptake into the lumen and degradation by lysosomal hydrolases.

### Substrates for CMA

Substrates for CMA include a very heterogeneous pool of cytosolic proteins with no functional or structural similarities, other than the presence of the lysosomal targeting motif.

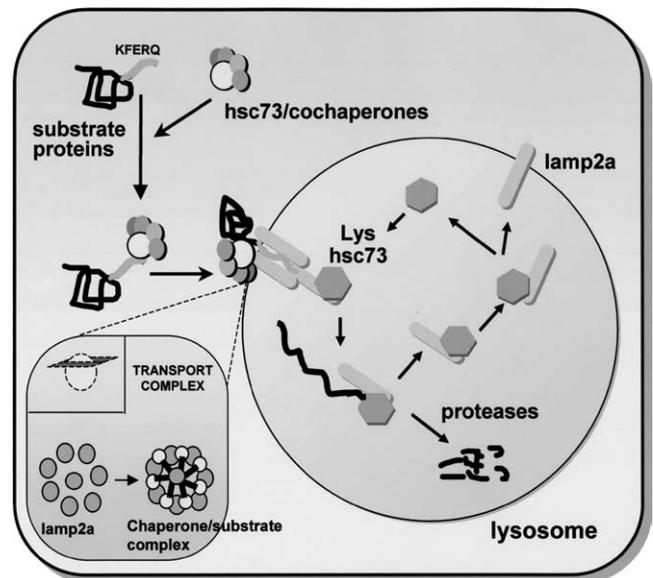


Fig. 3. Hypothetical model for the transport of cytosolic proteins to lysosomes by chaperone-mediated autophagy. Under stress conditions, a motif (KFERQ) in the amino acid sequence of specific cytosolic proteins is recognized by a cytosolic chaperone and its cochaperones. The substrate/chaperones complex binds to a lysosomal membrane protein and the unfolded substrate, along with the membrane receptor, are translocated into the lysosomal lumen assisted by a lysosomal chaperone. Unknown mechanisms dissociate chaperone and receptor from the substrate that is rapidly degraded by the lysosomal proteases. The internalized receptor, probably assisted by the luminal chaperone, is reinserted back into the lysosomal membrane. Inset shows in a transversal section (top view) a theoretical model of how molecules of the receptor organize in the lysosomal membrane to accommodate the multimeric substrate/chaperone complexes. It is hypothesized that the multi-spanning structure might act as a transporter. Abbreviations: hsc73 (heat shock cognate protein of 73 kDa); lamp2a (lysosome associated membrane protein type 2a); lys-hsc73 (lysosomal hsc73).

KFERQ is a relaxed targeting motif defined by the combination of a hydrophobic, a basic and an acid residue with a fifth hydrophobic or basic residue, and flanked in any of its sides by a glutamine [73]. The motif, present in about 30% of cytosolic proteins, works in both directions. Although it is a linear motif in all the substrate proteins identified so far, the existence of three-dimensional KFERQ-like motifs is, in theory, possible. It is not clear what determines the recognition of this motif by the chaperone. Some modifications (oxidation, denaturation) of the substrates increase their lysosomal uptake by CMA [81, 82]. It is possible that CMA stimulating factors induce small conformational changes on the substrate, making the KFERQ-like motif accessible to the chaperone. Those cytosolic changes might instead act by modifying the ability of hsc73 itself to recognize the motif.

Substrates identified for this pathway include, ribonuclease A, several glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, aldolase B, pyruvate kinase, aspartate

aminotransferase), some subunits of the 20S proteasome, the cytosolic form of a secretory protein (alpha-2-microglobulin), some of the members of the annexin family, a group of calcium-lipid binding proteins, glutathione transferase, the CMA cytosolic chaperone (hsc73) and some transcription factors (c-fos, c-jun and Pax2) and inhibitors of transcription factors (I $\kappa$ B) [6, 7]. In addition to functional proteins, a role for CMA in the degradation of damaged proteins is also well documented [81, 82].

### *Regulation of CMA*

Basal CMA activity can be detected in some tissues but maximal activation occurs in stress conditions such as prolonged nutrient deprivation or exposure to toxic compounds [6, 7]. As described above, the activation of macroautophagy by starvation initiates indiscriminate degradation of cytosolic components to provide the cell amino acids for protein synthesis. However, to prevent the cell from 'eating itself', macroautophagy activity declines after 6 h of starvation in some tissues and it is practically undetectable beyond 12 h [7, 83]. It is then that the activation of CMA becomes the new source of amino acids through the selective degradation of specific cytosolic proteins, the ones containing KFERQ motives [6, 7]. Activation of CMA by gasoline derivatives seems to be oriented to the removal of specific proteins damaged by those compounds [84].

CMA activation is tissue-dependent. Starvation preferentially activates CMA in liver, heart, kidney and spleen [84]. In the same tissue activation also varies from cell type to cell type. Thus, although nutrient-dependent activation of CMA in the brain is negligible, astrocytes in culture display high rates of CMA, when deprived of serum [85]. This pathway has only been described in mammals. Transport of specific proteins to the vacuole for degradation occurs in yeast but through different mechanisms (see below).

The signaling cascades leading to CMA activation during nutrient deprivation remain unknown. Recently, epidermal growth factor has been shown to reduce CMA activity in kidney epithelial cells, but secondary messengers for this inhibition have not been identified [86]. At the molecular level, a major regulator of CMA is lamp2a. Levels of this receptor at the lysosomal membrane determine rates of substrate uptake. We have described changes in the levels of lamp2a in all conditions known to change CMA activity [87]. Interestingly, the inhibitory effect of the epidermal growth factor was also associated with reduced levels of the receptor [86]. Lysosomal content of lamp2a is regulated by changes in its degradation rate as well as through lamp2a's dynamic distribution between the lysosomal membrane and lumen. Apparently, the cytosolic/transmembrane portion of lamp2a deinserts from the lysosomal membrane and is internalized in the lumen associated to the substrate protein

(Fig. 3). The substrate-binding region reappears again on the cytosolic side of the membrane, suggesting that lamp2a undergoes substrate-coupled cycles of insertion/deinsertion from the membrane [88]. This process is independent of the luminal pH but it requires an intact membrane potential. Prolonged starvation favors the mobilization of a portion of lamp2a present in the lysosomal lumen toward the membrane, thus increasing the number of receptors available for substrate binding [88]. Some of the proteases that participate in the degradation of lamp2a at the lysosomal membrane have been recently identified [89], but the components that mediate its dynamic lysosomal distribution remain unknown.

A regulatory role has also been described for the lysosomal chaperone hsc73. Hsc73 is particularly enriched in the lumen of a specific group of lysosomes that show higher competence for CMA [78, 79]. Lysosomal content of hsc73 increases with the starvation time, but the mechanism followed by the protein to access the lysosomal lumen remains unknown.

## **The vacuole import and degradation pathway**

Another novel pathway for the transport of cytosolic proteins to the vacuole for their degradation (vacuole import and degradation; vid) has been described in yeast [7, 90]. Activity of vid is nutrient regulated, but in contrast to macroautophagy and CMA, both activated during nutrient removal, vid is activated when cells grown in poor carbon sources are switched to a glucose-enriched medium [90]. Apparently, activation of vid eliminates enzymes unnecessary under the new conditions, such as the gluconeogenic enzyme fructose-1, 6-biphosphatase, the only substrate identified for this pathway so far.

### *Targeting to a new kind of intracellular vesicle*

Vid requires the transport of the substrate from the cytosol to a newly identified type of small vesicles (30–40 nm diameter), before it is transferred to the vacuole for its degradation [91]. This transport is believed to be receptor-mediated because it is saturable and the substrate binds to the vesicles' surface before internalization. Degradation by vid requires ATP and is stimulated in the presence of cytosolic components [92]. Similar to CMA in mammals, a molecular chaperone, ssa2p, stimulates substrate transport into vid vesicles [93]. A plasma membrane protein, vid22, is required for vid activation [94]. It is likely that vid22 senses glucose replenishment initiating a signal cascade that leads, among other things, to up regulation of cyclophilin A, a protein also shown to stimulate vid. This is supported by the fact that vid activation requires *de novo* synthesis of proteins [92]. Another newly synthesized protein is vid24, a peripheral membrane protein of the small vesicles

required for their targeting/fusion with the vacuole [95]. Apparently, vid does not share common components with other lysosomal pathways (macroautophagy, pexophagy, biosynthetic pathway) that are normally functioning in mutants with complete vid blockage [90, 96]. As expected, import into vid vesicles does not depend on vacuole acidification or vacuole proteolysis, confirming that it takes place before vesicle fusion with the vacuole [97]. Interestingly, by analogy with the novel conjugation systems described for the other lysosomal pathways, vid requires ubiquitin chain formation and the ubiquitin-conjugating enzyme Ubc1p [97]. Furthermore, Vid vesicle–vacuole trafficking requires components of the SNARE membrane fusion machinery exhibiting characteristics similar to heterotypic membrane fusion events [98].

#### *Vid vs. CMA*

Vid has not been yet described in mammalian cells. The first step of this pathway (transport of the substrate into the small vesicles) shares certain similarities with CMA (i.e., dependence on ATP, chaperones and saturability). It is possible that the evolutionary change from a single vacuole in yeast to multiple lysosomes, ubiquitously distributed in the cellular cytosol in mammals, made it unnecessary to use carrier vesicles to transport cytosolic proteins. Also, the discontinuity in the membrane (channel?) required for the direct transport of substrates through CMA might be tolerable in lysosomes, but could lead to cytosolic leakage of vacuolar components in the case of the vacuole.

### **How does lysosomal degradation become selective?**

Substrate specificity in the lysosomal system depends on targeting the lysosome/vacuoles rather than in degradation once substrates reach the lysosomal lumen. Targeting signals in pathways such as CMA are well defined (KFERQ sequences, see above) [73]. Although a single substrate is preferentially degraded by vid, the targeting signal is unknown. In the case of macroautophagy, the forming vesicles can exclude specific cytosolic components. Thus during starvation, the cytoskeleton seems to be selectively excluded from autophagosome engulfment [99]. In the same way, the persistence of glucose in N-glycans groups of glycosylated proteins prevents them from sequestration by macroautophagy [100]. In addition to this trimming of glucose, ubiquitination is the only other modification described to promote degradation by macroautophagy of a single protein, aldolase B [101]. However, this modification is not required for the majority of macroautophagy substrates.

Selective macroautophagy is better understood for organelles. The two organelles that have recently received spe-

cial attention are the peroxisome and mitochondria. Other intracellular structures, such as specific regions of the endoplasmic reticulum [102], and even the nucleus [103] can also be selectively degraded by macroautophagy under specific conditions.

#### *Pexophagy*

The selective degradation of peroxisomes in the lysosome/vacuole (pexophagy) has been mostly analyzed in yeast [104], but also occurs in mammals after inducing peroxisome proliferation with different drugs [105, 106]. In methylotropic yeast, peroxisome degradation is dictated by changes in nutritional conditions. When the only source of carbon is methanol or oleic acid, peroxisome biogenesis is induced. Restoration of the normal carbon sources induces selective elimination of the no longer needed peroxisomes without changes in the degradation of any other intracellular organelle [107]. Depending on the type of yeast and the new source of carbon, peroxisome degradation takes place by processes that resemble either macroautophagy or microautophagy [108] (Fig. 1). Peroxisomes can be individually sequestered in double membrane vesicles and then targeted to the vacuole (macropexophagy). Alternatively, they can be directly engulfed by the vacuole along with other cytosolic components (micropexophagy). Many molecular components are common for both pathways but micropexophagy is slower, requires new synthesis of proteins, has intact functioning proteases in the vacuolar lumen and functional PI3kinases [63]. Studies of pexophagy mutants have revealed components unique for this process and others shared with other lysosomal pathways (macroautophagy, biosynthetic pathway, vacuolar protein sorting and endocytosis) [69, 108]. Surprisingly, despite the resemblance between macroautophagy and macropexophagy, and the fact that both share common components [109], kinetics of macropexophagy are faster than those of regular macroautophagy [110].

A very novel finding is that, at least in yeast, the vacuole has an inherent tendency for the engulfment of peroxisomes as soon as they proliferate (even before switching to the new nutrient medium). The protein that normally represses this engulfment, PAZ2, is homologous to Atg8 [69]. Whether or not Atg8 has a similar repressor function in macroautophagy is currently unknown.

What determines the selective degradation of peroxisomes? The best candidates are peroxisomal membrane proteins (peroxins), such as Pex14p [111, 112]. Pex14, also regulates peroxisome biogenesis and protein transport into the peroxisome matrix. In mature peroxisomes the inactive Pex14 is detected by a still unidentified, ‘terminator’ protein that activates macropexophagy [111]. Having the same protein controlling biogenesis and degradation allows, at the same

time, rapid turn-off of the synthesis of the organelle and the activation of its removal.

### *Removal of damaged mitochondria*

It is generally accepted that continuous turnover of mitochondria takes place by lysosomal sequestration along with other cytosolic components [99]. In addition, degradation of mitochondria, under specific conditions, is also selective. Inhibition of apoptosis progression in cells previously induced for apoptosis activates the selective elimination of a subset of mitochondria [113, 114]. A classical inhibitor of macroautophagy, 3-methyladenine, prevents this mitochondrial removal. Selective removal of mitochondria also takes place during development [115].

The marker that determines selective autophagic sequestration of mitochondria remains unclear, but there is strong evidence favoring mitochondria depolarization [116, 117]. In a series of elegant kinetic studies in which Lemasters's group simultaneously tracked both mitochondria membrane potential and changes in the lysosomal compartment, they showed that autophagy is induced about 20 min after isochoric depolarization [116]. Induction of autophagy by nutrient deprivation increases the number of spontaneously depolarizing mitochondria, which then move into the acidic vacuoles [116].

As described below, defect in the removal of damaged mitochondria is one of the most accepted theories in the biology of aging [118]. Eliminating mitochondria through autophagy might prevent exposure of the cell to cytochrome C and other compounds that, when released from the mitochondria, might activate apoptotic death [119]. Whether the selective removal of mitochondria occurs by classic macroautophagy or shares some of the components described only for pexophagy is currently unknown.

## **Degradation in the lysosomal lumen, the last step**

Independently of the targeting pathway, all substrates are rapidly degraded (5–10 min) once they reach the lysosome/vacuole lumen. This is in part due to the enormous enrichment in proteases (cathepsins) some of them with both endo- and exo-proteolytic activities [120, 121]. In addition, intralysosomal conditions and other lysosomal components are naturally designed to favor complete degradation of the internalized products. Thus substrate denaturation is achieved by the acid pH, and a thiol reductase reduces inter- and intrachain disulfide bonds [122]. Consequently, changes in the intralysosomal environment, primarily increase their luminal pH, impair degradation and, as described below, seem to be the basis for some pathological disorders. In contrast with the large number of agents known to block lysosomal protein

degradation, there is a lack of compounds with stimulatory properties, which might be desirable when intralysosomal degradation is impaired. Only recently, administration of vitamin C (ascorbic acid) to cells in culture has proven effective in stimulating degradation of proteins internalized into lysosomes by very different pathways [85]. The stimulatory effect of the vitamin is probably due to its ability to stabilize the intralysosomal pH at acidic values.

In contrast with other major intracellular proteases, esteric inhibitors of cathepsins do not play a significant regulatory role in intralysosomal degradation. Physiological cathepsin inhibitors (i.e., cystatins) are active on extracellularly released cathepsins but inactive inside lysosomes [123]. More important for regulation could be intralysosomal compartmentalization. Rather than an amorphous magma of enzymes and substrates, the lysosomal lumen is organized in dynamic supramolecular complexes that assemble and disassemble in a pH-dependant manner [124]. Specific release of some hydrolases but not others from those complexes might modulate the degradation rate inside the lysosomal compartment.

For macro- and microautophagy, but not for CMA, a limiting membrane blocks the direct access of proteases to the internalized substrates. Lipases and permeases are required to eliminate that membrane [119, 125, 126]. It is unknown, how the lysosomal membrane protects itself from degradation. Factors such as a different lipid composition or poor protein content might make the limiting membrane more susceptible. In fact, the tubular invaginations are often poor in proteins [68]. However, further biochemical characterization of these structures is needed.

In contrast with the classic idea of complete proteolysis of substrates in lysosomes, the transport of dipeptides from the lysosomal lumen into the cytosol, where they are completely degraded by dipeptidases, was recently described [127]. Whether those small peptides play some physiological function before their cytosolic degradation remains unclear.

## **Same substrate, different degradation pathways: Crosstalk is needed**

Numerous studies have reported degradation of a single protein by different proteolytic pathways. In many cases, degradation by one pathway or another depends on the cellular conditions. For example, I $\kappa$ B, a typical substrate for the 20S proteasome, can also be degraded by calpains, and during nutrient deprivation becomes a substrate for CMA [81]. Similarly, degradation of aldolase B via macroautophagy is induced during starvation, but if nutrient deprivation persists it becomes a substrate for CMA [101]. In addition, simultaneous degradation of the same protein by different pathways has also been reported [81]. Thus, it is not unusual to detect the presence of intracellular pools of the same protein with

different half-lives. Degradation of the short-lived pool probably facilitates rapid changes in the levels of the protein, while the long-lived pool, frequently not active yet, becomes an intracellular 'reservoir' for that specific protein. This reserve protein would allow large increases in intracellular levels of active protein without requiring protein synthesis. Though the molecular basis that would explain this multiple degradation is not well understood, it could be at least in part due to the coexistence of different targeting signals for degradation in the same protein. Conformational changes in the protein would lead to the exposure of one or the other signal.

In part, redundant mechanisms of protein degradation are probably aimed to compensate for the failure of one proteolytic system. In addition, this redundancy could also serve a regulatory function. Keeping in mind that proteins frequently organize in functional complexes, changes in the kinetics of degradation for one of its components could completely switch on the function of that complex [128].

How is this cross talk among proteolytic pathways orchestrated? Many pieces of evidence support interdependent functioning of the different proteolytic systems inside cells. For example, the ubiquitin/proteasome pathway is involved in the endosomal sorting and degradation of selected membrane proteins in lysosomes [129]; vesicular structures of the endocytic pathway can interact and fuse with vesicles originated by macroautophagy [130]; mutations in a single protein (i.e., synuclein) result in altered proteasome and lysosomal function [131]. In addition, thorough studies in human fibroblasts have revealed that the contribution of the major different proteolytic pathways to the degradation of long-lived proteins changes under different cellular conditions [83]. Coordinate function is probably based in many of these examples on the existence of common components for several of these pathways. In the lysosomal system, some genes have been shown to be required for macroautophagy, microautophagy and pexophagy. The engagement of the shared component in one of these pathways could very well limit the amount of this common factor available for the other pathways, and consequently decrease their activity.

Sometimes, interdependent function involves not only proteolytic systems but also other intracellular pathways. For example, the preautophagosome is the origin of the autophagosome but also of carrier vesicles for a biosynthetic pathway, the cytosol-to-vacuole (cvt), that targets resident hydrolases to the vacuole [9, 10, 132] (Fig. 1). The cvt vesicles are smaller and do not contain any other cytosolic component. Maturation of the limiting membrane toward cvt vesicles or autophagosomes depends on the nature of the complexes formed by the Atg1 kinase, and it is regulated in a Tor-dependent manner [133]. Under conditions of nutrient deprivation, hydrolases are targeted to the vacuole through macroautophagy instead of the biosynthetic pathway. This alternative mechanism of transport is probably advantageous

for the vacuole that would receive along with substrate proteins, the enzymes required for their processing.

In other cases, the basis for the coordinate functioning of different proteolytic systems is not so well understood. For example, the sequential activation during nutrient deprivation first of macroautophagy and then of CMA could also obey to the existence of still unknown common elements for both pathways. However, it is also possible that targeting of hsc73 to lysosomes, along with other cytosolic components, during macroautophagy activation, progressively enriches hsc73 in the lysosomal lumen making them competent for CMA. Once CMA is activated, selective degradation by this pathway of a macroautophagy component could turn off macroautophagy. These and other possibilities would require future attention.

## Lysosomal degradation in the physiopathology of the cardiovascular system

As for any other tissue, proteins in the heart are continuously synthesized and degraded, at a rate that, under normal conditions, results in the replacement of most of the cardiac protein every 7 to 14 days [134]. All the lysosomal pathways described above, except for vid, have been shown to be active in the heart. In fact, lysosomal pathways with clear tissue-dependence, such as CMA, are greatly activated in cardiac muscle during nutrient deprivation [135]. Because the heart is mainly formed by terminally differentiated non-dividing cells (cardiomyocytes), proper functioning of the degradative systems is essential for the maintenance of cellular homeostasis and function. We will review three examples of alterations in the lysosomal system and how they directly affect heart function.

### *Lysosomes in the aged heart*

Total rates of protein degradation decrease with age in almost all animal and cellular models analyzed [136]. The consequences of reduced protein turnover, especially in post-mitotic cells, are easily inferred [137]. Added to the improper removal of abnormal proteins is the slowing down in the turnover of functional ones. Prolonging exposure of functional proteins to damaging agents increases their probability of becoming altered. In addition, loss of regulation of the proteolytic systems diminishes the ability of the cells to adapt to stress conditions.

Proteolytic systems are differently affected by age [137]. For the lysosomal system, impaired macroautophagy and CMA are well documented [138–140]. The defect in macroautophagy results from a decrease in both formation and elimination of autophagosomes [138]. The accumulated

autophagic vesicles often evolve toward multilamellar structures because the undigested material creates a microenvironment prone to the formation of membrane lamella [141]. The ability of senescent cells to respond to normal autophagic stimuli, such as glucagon or amino acid deprivation is impaired [140]. Whether the defect derives from alterations in the signaling cascades or in the molecular components of the preautophagosome complex needs to be determined. The defective removal of autophagosomes is probably a consequence of their impaired fusion with lysosomes. Age-related changes in the intralysosomal environment (i.e., increased of the intralysosomal pH, imbalance of their protease content, etc.) might contribute to the defective fusion. One of the most analyzed consequences of the altered autophagy is the effect on turnover of mitochondria in old cells. Persistence of damaged non-functional mitochondria inside old tissues increases intracellular levels of free radicals and consequently the risk of damage for other intracellular components [118].

A decrease in CMA activity has been shown in both senescent cells in culture and different tissues in old rodents [139, 142]. The cytosolic steps (interaction with the chaperone complex and targeting to the lysosomal surface) are well preserved until advanced ages. The primary defect locates in the binding of the substrates to the lysosomal membrane. We have found a progressive decrease with age in the levels of the lysosomal receptor that explains the reduced binding ability [139]. Our current studies are oriented to identify the reasons for the decrease in lamp2a levels with age.

In addition to the altered transport of substrates by those two pathways to lysosomes in old organisms, there is also a failure in the degradation of some of the internalized substrates that accumulate as a fluorescent pigment (lipofuscin). Lipofuscin forms within secondary lysosomes and it is not substantially eliminated from non-dividing cells by degradation or exocytosis [143]. Cells loaded with lipofuscin have an increased sensitivity to oxidative stress and decreased cellular survival. At the same time, accumulation of lipofuscin diminishes autophagocytic capacity [143]. This incomplete digestion of substrates results from both changes in the proteolytic susceptibility of the substrates and in the proteolytic machinery. Extensive covalent modifications of proteins such as oxidation or glycation make them insoluble and resistant to proteolytic cleavage [144]. In the heart, accumulation of undigested protein aggregates are common in the atrial regions [145]. Luckily, cells can maintain normal functioning for long time after these intracellular protein aggregates form. Recent evidence supports the participation of both the ubiquitin/proteasome and the lysosomes in the degradation of modified proteins (oxidized, glycated, nitrated, etc.) [146]. Levels and activity of several lysosomal hydrolases decrease with age in many tissues, but for others, an increase has been reported. This pattern of changes in the activity of cathepsins found in aged tissues (increase of cathepsin D, decrease

of cathepsin L and no changes in cathepsin B) can be reproduced, reducing lysosomal acidification. A shift in intralysosomal pH with age might thus be behind the described proteolytic changes [147].

A very interesting finding is the fact that dietary restriction, the only intervention known to slow down the aging process, reverses the effects of aging in protein degradation, mostly via autophagy [140]. Prolongation of life-span mediated by caloric restriction might be in part related with proper activation of the autophagic system. In fact, disruption of macroautophagy in long-lived mutants of *C. elegans* shortens their life-span [148].

### *Lysosomes and apoptosis*

Programmed cell death is especially relevant in the remodeling of the developing heart and in several pathological conditions such as hypoxia, ischemia, inflammation and exposure to toxic agents [149]. The participation of the lysosomal system in programmed cell death is now well accepted, though its specific role is still controversial. Two different aspects to consider are as follows: the role of the lysosomal components in the 'classic' caspase-mediated cellular death (apoptosis), and the cellular death type II also called autophagic death.

Apoptosis can be activated through intra or extracellular signaling, leading in both cases to activation of selective proteases (caspases). Although the best analyzed intracellular signal cascade originates in mitochondria, cytosolic leakage of lysosomal hydrolases also activates apoptosis through caspase-3-like proteases [150]. In some types of cells, lysosomal leakage/rupture precedes apoptosis regardless of the initiating agent (oxidative stress, growth factor, starvation) [151]. Interestingly, different cathepsins play different roles in apoptosis activation: Cathepsin D is a proapoptotic mediator whose action is blocked by cathepsin B. Probably a disturbance of the normal intracellular balance between cathepsin B and D determines apoptotic sensitivity [152]. In fact, levels of cathepsin B drastically decrease in apoptotic cells. The studies with microinjected cathepsin D have located its action upstream of cytochrome c release [153].

A second type of cellular death (type II) has a lysosomal origin. Vesicular structures with the characteristics of autophagosomes accumulate in this case in the cytosol of the dying cells [154]. The characteristics of this process are those of the macroautophagic pathway described above, but the initial stimuli and the signaling cascade involved are still poorly understood. Ceramide is so far the only proposed mediator of autophagic death because it induces enlargement of late endosomes and lysosomes [155].

Both types of cell death are not completely independent. Coexistence of both apoptosis and massive autophagy in the same cell is not unusual. In human failing hearts,

myocytes' disappearance involves both forms of cell death [156]. In those cases, blocking autophagy protects the cells from caspase-mediated apoptosis [157]. Overlapping between the control of apoptosis and autophagy has been demonstrated in a series of elegant studies on *Drosophila* [158]. Finally, serial analysis of gene expression in mammals has revealed that both processes also share similar components [159]. Typical apoptotic kinases are directly localized in the autophagic vacuoles [160] and Atg5, one of the yeast macroautophagy-related genes, is homologous to a human apoptosis specific protein [19].

### *Altered autophagy and cardiomyopathies*

The inherited deficiency of one or several lysosomal hydrolases, in what has been grouped as 'storage disorders', results in the accumulation of the undigested products inside lysosomes and the consequent loss of lysosomal function [161]. Interestingly, and supporting the relevance of the lysosomal system in cardiac muscle development and function, cardiomyopathy is common to most of the lysosomal storage diseases [161].

Although it is expected that alterations in most of the autophagic pathways have consequences in cardiac muscle function, a direct connection has only been established so far with macroautophagy. The consequences of impaired macroautophagy in heart have been demonstrated in genetic models such as the knock out mouse for lamp2 [162]. In this mouse cardiac, myocytes are ultrastructurally abnormal and heart contractility is severely reduced. This phenotype is similar to Danon disease patients (human lamp2 deficiency) who show severe vacuolation and accumulation of autophagic material in striated myocytes [163]. All patients with Danon disease have cardiomyopathy, and depending on the severity of the disease, also have other forms of muscle atrophy [164]. Metabolic studies in the lamp2 knockout mice revealed that the defect locates in the fusion autophagosome/lysosomes. It is not clear which of the different isoforms of lamp2, if not all, are responsible for that function. However, the fact that the whole phenotype is reproduced in a patient with mutation only in the lamp2b specific exon, and that lamp2b is expressed predominantly in heart and skeletal muscle [165], strongly supports lamp2b being essential for autophagosome/lysosome fusion.

Muscle atrophy similar to the one observed in Danon disease can be induced in rodents when macroautophagy is disrupted by chronic administration of inhibitors. This treatment results in the presence in muscle of multi-membrane components surrounded by structures positive for the autophagosome marker LC3 [166] and progressive mitochondria damage [167]. Finally, macroautophagy is also necessary for remodeling of the adult heart. During regenerative and plastic

myocardial insufficiency, autophagy is activated in cardiomyocytes leading to the formation of myelin-like structures, autophagosomes and lysosomes that focally degrade cytosolic organelles [168]. This process is probably directed to the adjustment of the cytoplasm volume to the functional state of the nucleus.

## **Concluding remarks**

The recent genetic studies in yeast have allowed the identification of novel components that participate in the different autophagic pathways. In addition, studies in both yeast and mammals have provided valuable information on new regulatory mechanisms, such as conjugation cascades or dynamic protein complexes. The identification of targeting signals in proteins and also in complete organelles is setting the basis for a better understanding of the selective degradation of intracellular components in the lysosomal system. Finally, the components behind the interdependent functioning of some of these pathways are starting to be identified.

There are, however, many aspects requiring further clarification. Some signaling cascades have been implicated in the regulation of autophagy, but a complete picture of how they modulate different pathways is missing. The process of fusion between the lysosomes and the carrier vesicle (autophagosome, vid vesicles, etc.) is still poorly understood. No clear explanation is available on how the lysosomal membrane is normally protected from degradation, while the microautophagic invaginations are not. Only the complete dissection of macro- and micropexophagy will help to determine what directs the same organelle through one or the other pathway. We do not know how the substrates for CMA are translocated into the lumen. Despite the similarity of this process to the transport of proteins into other organelles, a CMA transporter, channel or pore have not been identified yet. Also puzzling is the mechanism of insertion/deinsertion of the CMA receptor into the lysosomal membrane. How is it regulated? Does it require luminal chaperones? What is the role of the lipid components of the membrane in the reinsertion? Finally, little is known about the role of the chaperones in transport of substrate into vid vesicles, the requirement of substrate unfolding for that transport and the steps followed by the vid vesicles before the substrate reaches the vacuole.

Participation of the lysosomal system in aging and cellular death is now well established. The identification of a primary defect with age in the CMA receptor permits us now to carry on restorative attempts. If we are able to return values of lamp2a to normal, we could then analyze any favorable effect on CMA activity. Future studies are required to determine the reasons for the failure of other lysosomal pathways with age. The signaling cascades determining the type of cellular death

and the relation between these two forms remain unknown. The autophagic defect behind specific myopathies, such as the one observed in Danon disease, has been recently identified. Further studies need to be carried on to determine if the blockage of autophagy at a different step leads to similar pathologies.

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## References

- Kirschner M: Intracellular proteolysis. *Trends Cell Biol* 9: M42–M45, 1999
- Squier TC: Oxidative stress and protein aggregation during biological aging. *Exp Gerontol* 36: 1539–1550, 2001
- Kourie JI, Henry CL: Protein aggregation and deposition: implications for ion channel formation and membrane damage. *Croat Med J* 42: 359–374, 2001
- Imai J, Yashiroda H, Maruya M, Yahara I, Tanaka K: Proteasomes and molecular chaperones: Cellular machinery responsible for folding and destruction of unfolded proteins. *Cell Cycle* 2: 585–590, 2003
- Perrin BJ, Huttenlocher A: Calpain. *Int J Biochem Cell Biol* 34: 722–725, 2002
- Cuervo AM, Dice JF: Lysosomes, a meeting point of proteins, chaperones, and proteases. *J Mol Med* 76: 6–12, 1998
- Dice JF: *Lysosomal Pathways of Protein Degradation*. Landes Bioscience, Austin, TX, 2000, pp 107
- Stennicke HR, Ryan CA, Salvesen GS: Retrieval from execution: The molecular basis of caspase inhibition. *Trends Biochem Sci* 27: 94–101, 2002
- Kim J, Huang WP, Stromhaug PE, Klionsky DJ: Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. *J Biol Chem* 277: 763–773, 2002
- Wang CW, Klionsky DJ: The molecular mechanism of autophagy. *Mol Med* 9: 65–76, 2003
- Seglen PO, Berg TO, Blankson H, Fengsrud M, Holen I, Stromhaug PE: Structural aspects of autophagy. *Adv Exp Med Biol* 389: 103–111, 1996
- Mortimore GE, Miotto G, Venerando R, Kadowaki M: Autophagy. *Subcell Biochem* 27: 93–135, 1996
- Thumm M: Structure and function of the yeast vacuole and its role in autophagy. *Microsc Res Tech* 51: 563–572, 2000
- Ohsumi Y: Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol* 2: 211–216, 2001
- Tsukada M, Ohsumi M: Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett* 333: 169–174, 1993
- Thumm M: Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. *FEBS Lett* 349: 275–280, 1994
- Harding TM: Isolation and characterization of yeast mutants in the cytoplasm to vacuoles protein targeting pathway. *J Cell Biol* 131: 591–602, 1995
- Klionsky D, Cregg J, Dunn WJ, Emr S, Sakai Y, Sandoval I, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y: A unified nomenclature for yeast autophagy-related genes. *Dev Cell*: 539–545, 2003
- Hammond EM, Brunet CL, Johnson GD, Parkhill J, Milner AE, Brady G, Gregory CD, Grand RJ: Homology between a human apoptosis specific protein and the product of APG5, a gene involved in autophagy in yeast. *FEBS Lett* 425: 391–395, 1998
- Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, Yoshimori T: Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 152: 657–668, 2001
- Tanida I, Tanida-Miyake E, Ueno T, Kominami E: The human homolog of *Saccharomyces cerevisiae* Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3. *J Biol Chem* 276: 1701–1706, 2001
- Tanida I, Tanida-Miyake E, Komatsu M, Ueno T, Kominami E: Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. *J Biol Chem* 277: 13739–13744, 2002
- Marino G, Uria J, Puente X, Quesada V, Bordallo J, Lopez-Ortin C: Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy. *J Biol Chem* 278: 3671–3678, 2003
- Doelling J, Walker J, Friedman E, Thompson A, Vierstra R: The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in *Arabidopsis thaliana*. *J Biol Chem* 277: 33105–33114, 2002
- Noda T, Suzuki K, Ohsumi Y: Yeast autophagosomes: *de novo* formation of a membrane structure. *Trends Cell Biol* 12: 231–235, 2002
- Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y: The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J* 20: 5971–5981, 2001
- Seglen PO, Gordon PB, Holen I: Non-selective autophagy. *Sem Cell Biol* 1: 441–448, 1990
- Mizushima N, Ohsumi Y, Yoshimori T: Autophagosome formation in mammalian cells. *Cell Struct Funct* 3: 815–824, 2002
- Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y: A ubiquitin-like system mediates protein lipidation. *Nature* 408: 488–492, 2000
- Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, Noda T, Ohsumi Y: Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol* 147: 435–446, 1999
- Mizushima N, Yoshimori T, Ohsumi Y: Role of the Apg12 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 35: 553–561, 2003
- Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, Ohsumi Y: A protein conjugation system essential for autophagy. *Nature* 395: 395–398, 1998
- Komatsu M, Tanida I, Ueno T, Ohsumi M, Ohsumi Y, Kominami E: The C-terminal region of an Apg7p/Cvt2p is required for homodimerization

- and is essential for its E1 activity and E1-E2 complex formation. *J Biol Chem* 276: 9846–9854, 2001
34. Shintani T, Mizushima N, Ogawa Y, Matsuura A, Noda T, Ohsumi Y: Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J* 18: 5234–5241, 1999
  35. Kuma A, Mizushima N, Ishihara N, Ohsumi Y: Formation of the approximately 350-kDa Apg12-Apg5-Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. *J Biol Chem* 277: 18619–18625, 2002
  36. Kihara A, Noda T, Ishihara N, Ohsumi Y: Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol* 152: 519–530, 2001
  37. Simonsen A, Wurmser AE, Emr SD, Stenmark H: The role of phosphoinositides in membrane transport. *Curr Opin Cell Biol* 13: 485–492, 2001
  38. Matsuura A, Tsukada M, Wada Y, Ohsumi Y: Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. *Gene* 192: 245–250, 1997
  39. Abeliovich H, Zhang C, Dunn WJ, Shokat K, Klionsky D: Chemical genetic analysis of Apg1 reveals a non-kinase role in the induction of autophagy. *Mol Biol Cell* 14: 477–490, 2003
  40. Noda T, Kim J, Huang WP, Baba M, Tokunaga C, Ohsumi Y, Klionsky DJ: Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. *J Cell Biol* 148: 465–480, 2000
  41. Shintani T, Suzuki K, Kamada Y, Noda T, Ohsumi Y: Apg2p functions in autophagosome formation on the perivacuolar structure. *J Biol Chem* 276: 30452–30460, 2001
  42. Ishihara N, Hamasaki M, Yokota S, Suzuki K, Kamada Y, Kihara A, Yoshimori T, Noda T, Ohsumi Y: Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion. *Mol Biol Cell* 12: 3690–3702, 2001
  43. Darsow T, Rieder SE, Emr SD: A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *J Cell Biol* 138: 517–529, 1997
  44. Gaits F, Russell P: Vacuole fusion regulated by protein phosphatase 2C in fission yeast. *Mol Biol Cell* 10: 2647–2654, 1999
  45. Munafo DB, Colombo MI: Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24. *Traffic* 3: 472–482, 2002
  46. Codogno P, Ogier-Denis E, Houri J: Signal transduction pathways in macroautophagy. *Cell Signal* 9: 125–130, 1997
  47. Meijer A: Amino acids as regulators and components of nonproteinogenic pathways. *J Nutr* 133: 2057S–2062S, 2003
  48. Ohshita T: Suppression of autophagy by ethionine administration in male rat liver in vivo. *Toxicology* 147: 51–57, 2000
  49. Telbisz A, Kovacs AL: Intracellular protein degradation and autophagy in isolated pancreatic acini of the rat. *Cell Biochem Func* 18: 29–40, 2000
  50. Holen I, Gordon PB, Seglen PO: Inhibition of hepatocytic autophagy by okadaic acid and other protein phosphatase inhibitors. *Eur J Biochem* 215: 113–122, 1993
  51. Gordon PB, Holen I, Fosse M, Rotnes JS, Seglen PO: Dependence of hepatocytic autophagy on intracellularly sequestered calcium. *J Biol Chem* 268: 26107–26112, 1993
  52. Kadowaki M, Venerando R, Miotto G, Mortimore GE: Mechanism of autophagy in permeabilized hepatocytes: Evidence for regulation by GTP binding proteins. *Adv Exp Med Biol* 389: 113–119, 1996
  53. Petiot A, Ogier-Denis E, Bauvy C, Cluzeaud F, Vandewalle A, Codogno P: Subcellular localization of the Galphai3 protein and G alpha interacting protein, two proteins involved in the control of macroautophagy in human colon cancer HT-29 cells. *Biochem J* 337: 289–295, 1999
  54. Blommaert EFC, Krause U, Schellens JPM, Vreeling-Sindelárová H, Meijer AJ: The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur J Biochem* 243: 240–246, 1997
  55. Petiot A, Ogier-Denis E, Blommaert E, Meijer A, Codogno P: Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem* 275: 992–998, 2000
  56. Noda T, Ohsumi Y: Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* 273: 3963–3966, 1998
  57. Huang WP, Scott SV, Kim J, Klionsky DJ: The itinerary of a vesicle component, Aut7p/Cvt5p, terminates in the yeast vacuole via the autophagy/Cvt pathways. *J Biol Chem* 275: 5845–5851, 2000
  58. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y: Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* 150: 1507–1513, 2000
  59. Dennis PB, Jaeschke A, Saitoh M, Fowler B, S.C. K, Thomas G: Mammalian TOR: A Homeostatic ATP Sensor. *Science* 294: 1102–1105, 2001
  60. Mordier S, Deval C, Bechet D, Tassa A, Ferrara M: Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. *J Biol Chem* 275: 29900–29906, 2000
  61. Thumm M, Kadowaki T: The loss of *Drosophila* APG4/AUT2 function modifies the phenotypes of cut and Notch signaling pathway mutants. *Mol Genet Genomics* 266: 657–663, 2001
  62. Kovacs AL, Rez G, Palfia Z, Kovacs J: Autophagy in the epithelial cells of murine seminal vesicle in vitro. Formation of large sheets of nascent isolation membranes, sequestration of the nucleus and inhibition by wortmannin and 3-ethyladenine. *Cell Tissue Res* 302: 253–261, 2000
  63. Sakai Y, Koller A, Rangell LK, Keller GA, Subramani S: Peroxisome degradation by microautophagy in *Pichia pastoris*: identification of specific steps and morphological intermediates. *J Cell Biol* 141: 625–636, 1998
  64. Sattler T, Mayer A: Cell-free reconstitution of microautophagic vacuole invagination and vesicle formation. *J Cell Biol* 151: 529–538, 2000
  65. Marzella L, Ahlberg J, Glaumann H: Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation. *Virchows Arch B Cell Pathol Incl Mol Pathol* 36: 219–234, 1981
  66. Ahlberg J, Marzella L, Glaumann H: Uptake and degradation of proteins by isolated rat liver lysosomes. Suggestion of a microautophagic pathway of proteolysis. *Lab Invest* 47: 523–532, 1982
  67. Ahlberg J, Glaumann H: Uptake—microautophagy—and degradation of exogenous proteins by isolated rat liver lysosomes. Effects of pH, ATP, and inhibitors of proteolysis. *Exp Mol Pathol* 42: 78–88, 1985
  68. Muller O, Sattler T, Flotenmeyer M, Schwarz H, Plattner H, Mayer A: Autophagic tubes: vacuolar invaginations involved in lateral membrane sorting and inverse vesicle budding. *J Cell Biol* 151: 519–528, 2000
  69. Mukaiyama H, Oku M, Baba M, Samizo T, Hammond AT, Glick BS, Kato N, Sakai Y: Paz2 and 13 other PAZ gene products regulate vacuolar engulfment of peroxisomes during micropexophagy. *Genes Cells* 7: 75–90, 2002
  70. Mortimore GE, Lardeux BR, Adams CE: Regulation of microautophagy and basal protein turnover in rat liver. Effects of short-term starvation. *J Biol Chem* 263: 2506–2512, 1988
  71. Veenhuis M, Salomons FA, Van Der Klei IJ: Peroxisome biogenesis and degradation in yeast: a structure/function analysis. *Microsc Res Tech* 51: 584–600, 2000

72. Auteri J, Okada A, Bochaki V, Dice J: Regulation of intracellular protein degradation in IMR-90 human diploid fibroblasts. *J Cell Physiol* 115: 159–166, 1983
73. Dice JF: Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends Biochem Sci* 15: 305–309, 1990
74. Chiang H, Terlecky S, Plant C, Dice J: A role for a 70 kDa heat shock protein in lysosomal degradation of intracellular protein. *Science* 246: 382–385, 1989
75. Agarraberes FA, Dice JF: A molecular chaperone complex at the lysosomal membrane is required for protein translocation. *J Cell Sci* 114: 2491–2499, 2001
76. Cuervo A, Dice J: A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* 273: 501–503, 1996
77. Salvador N, Aguado C, Horst M, Knecht E: Import of a cytosolic protein into lysosomes by chaperone-mediated autophagy depends on its folding state. *J Biol Chem* 275: 27447–27456, 2000
78. Agarraberes F, Terlecky S, Dice J: An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. *J Cell Biol* 137: 825–834, 1997
79. Cuervo A, Dice J, Knecht E: A lysosomal population responsible for the hsc73-mediated degradation of cytosolic proteins in lysosomes. *J Biol Chem* 272: 5606–5615, 1997
80. Terlecky S, Dice J: Polypeptide import and degradation by isolated lysosomes. *J Biol Chem* 268: 23490–23495, 1993
81. Cuervo A, Hu W, Lim B, Dice J: I $\kappa$ B is a substrate for a selective pathway of lysosomal proteolysis. *Mol Biol Cell* 9: 1995–2010, 1998
82. Cuervo A, Hildebrand H, Bomhard E, Dice J: Direct lysosomal uptake of alpha2-microglobulin contributes to chemically induced nephropathy. *Kidney Int* 55: 529–545, 1999
83. Fuertes G, Martin De Llano J, Villarroya A, Rivett A, Knecht E: Changes in the proteolytic activities of proteasomes and lysosomes in human fibroblasts produced by serum withdrawal, amino-acid deprivation and confluent conditions. *Biochem J* 375: 75–86, 2003
84. Wing S, Chiang HL, Goldberg AL, Dice JF: Proteins containing peptide sequences related to KFERQ are selectively depleted in liver and heart, but not skeletal muscle, of fasted rats. *Biochem J* 275: 165–169, 1991
85. Martin A, Joseph J, Cuervo A: Stimulatory effect of vitamin C on autophagy in glial cells. *J Neurochem* 82: 538–549, 2002
86. Franch HA, Sooparb S, Du J, Brown NS: A mechanism regulating proteolysis of specific proteins during renal tubular cell growth. *J Biol Chem* 276: 19126–19131, 2001
87. Cuervo AM, Dice JF: Unique properties of lamp2a compared to other lamp2 isoforms. *J Cell Sci* 113: 4441–4450, 2000
88. Cuervo AM, Dice JF: Regulation of lamp2a levels in the lysosomal membrane. *Traffic* 1: 570–583, 2000
89. Cuervo AM, Mann L, Bonten E, d'Azzo A, Dice J: Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor. *EMBO J* 22: 12–19, 2003
90. Chiang HL, Schekman R, Hamamoto S: Selective uptake of cytosolic, peroxisomal, and plasma membrane proteins into the yeast lysosome for degradation. *J Biol Chem* 271: 9934–9941, 1996
91. Huang PH, Chiang HL: Identification of novel vesicles in the cytosol to vacuole protein degradation pathway. *J Cell Biol* 136: 803–810, 1997
92. Shieh HL, Chiang HL: In vitro reconstitution of glucose-induced targeting of fructose-1, 6-bisphosphatase into the vacuole in semi-intact yeast cells. *J Biol Chem* 273: 3381–3387, 1998
93. Brown CR, McCann JA, Chiang HL: The heat shock protein Ssa2p is required for import of fructose-1, 6-bisphosphatase into Vid vesicles. *J Cell Biol* 150: 65–76, 2000
94. Brown CR, Cui DY, Hung GG, Chiang HL: Cyclophilin A mediates Vid22p function in the import of fructose-1,6-bisphosphatase into Vid vesicles. *J Biol Chem* 276: 48017–48026, 2001
95. Chiang MC, Chiang HL: Vid24p, a novel protein localized to the fructose-1, 6-bisphosphatase-containing vesicles, regulates targeting of fructose-1,6-bisphosphatase from the vesicles to the vacuole for degradation. *J Cell Biol* 140: 1347–1356, 1998
96. Hoffman M, Chiang HL: Isolation of degradation-deficient mutants defective in the targeting of fructose-1,6-bisphosphatase into the vacuole for degradation in *Saccharomyces cerevisiae*. *Genetics* 143: 1555–1566, 1996
97. Shieh HL, Chen Y, Brown CR, Chiang HL: Biochemical analysis of fructose-1,6-bisphosphatase import into vacuole import and degradation vesicles reveals a role for UBC1 in vesicle biogenesis. *J Biol Chem* 276: 10398–10406, 2001
98. Brown CR, Liu J, Hung GC, Carter D, Cui D, Chiang HL: The Vid vesicle to vacuole trafficking event requires components of the SNARE membrane fusion machinery. *J Biol Chem* 278: 25688–25699, 2003
99. Stromhaug PE, Berg TO, Fengsrud M, Seglen PO: Purification and characterization of autophagosomes from rat hepatocytes. *Biochem J* 335: 217–224, 1998
100. Ogier-Denis E, Bauvy C, Cluzeaud F, Vandewalle A, Codogno P: Glucose persistence on high-mannose oligosaccharides selectively inhibits the macroautophagic sequestration of N-linked glycoproteins. *Biochem J* 345: 459–466, 2000
101. Lenk SE, Susan PP, Hickson I, Jasionowski T, Dunn WA, Jr.: Ubiquitinated aldolase B accumulates during starvation-induced lysosomal proteolysis. *J Cell Physiol* 178: 17–27, 1999
102. Masaki R, Yamamoto A, Tashiro Y: Cytochrome P-450 and NADPH-cytochrome P450 reductase are degraded in the autolysosomes in rat liver. *J Cell Biol* 104: 1207–1215, 1987
103. Roberts P, Moshitch-Moshkovitz S, Kvam E, O'Toole E, Winey M, Goldfarb D: Piecemeal microautophagy of nucleus in *Saccharomyces cerevisiae*. *Mol Biol Cell* 14: 129–141, 2003
104. Bellu A, Kiel J: Selective degradation of peroxisomes in yeasts. *Microsc Res Tech* 61: 161–170, 2003
105. Locci Cubeddu T, Masiello P, Pollera M, Bergamini E: Effects of antilipolytic agents on rat liver peroxisomes and peroxisomal oxidative activities. *Biochim Biophys Acta* 839: 96–104, 1985
106. Nardacci R, Sartori C, Stefanini S: Selective autophagy of clofibrate-induced rat liver peroxisomes. *Cytochemistry and immunocytochemistry on tissue specimens and on fractions obtained by Nycodenz density gradient centrifugation. Cell Mol Biol* 46: 1277–1290, 2000
107. Tuttle DL, Lewin AS, Dunn WA, Jr.: Selective autophagy of peroxisomes in methylotrophic yeasts. *Eur J Cell Biol* 60: 283–290, 1993
108. Yuan W, Stromhaug PE, Dunn WA, Jr.: Glucose-induced autophagy of peroxisomes in *Pichia pastoris* requires a unique E1-like protein. *Mol Biol Cell* 10: 1353–1366, 1999
109. Bellu AR, Kram A, Kiel JA, Veenhuis M, van der Klei IJ: Glucose-induced and nitrogen-starvation-induced peroxisome degradation are distinct processes in *Hansenula polymorpha* that involve both common and unique genes. *FEM Yeast Res* 1: 23–31, 2002
110. Hutchins MU, Veenhuis M, Klionsky DJ: Peroxisome degradation in *Saccharomyces cerevisiae* is dependent on machinery of macroautophagy and the Cvt pathway. *J Cell Sci* 112: 4079–4087, 1999
111. Bellu A, Komori M, van der Klei I, Kiel J, Veenhuis M: Peroxisome biogenesis and selective degradation converge at Pex14p. *J Biol Chem* 276: 44570–44574, 2001
112. Subramani S, Koller A, Snyder WB: Import of peroxisomal matrix and membrane proteins. *Ann Rev Biochem* 69: 399–418, 2000
113. Xue L, Fletcher GC, Tolkovsky AM: Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. *Curr Biol* 11: 361–365, 2001

114. Tolkovsky AM, Xue L, Fletcher G, Borutaite V: Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? *Biochimie* 84: 233–240, 2002
115. Takano-Ohmuro H, Mukaida M, Kominami E, Morioka K: Autophagy in embryonic erythroid cells: its role in maturation. *Eur J Cell Biol* 79: 759–764, 2000
116. Elmore SP, Qian T, Grissom SF, Lemasters JJ: The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J* 15: 2286–2287, 2001
117. Lemasters JJ, Quian T, He L, Kim J, Elmore S, Cascio W, Brenner D: Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. *Antioxid Redox Signal* 4: 769–781, 2002
118. Brunk U, Terman A: The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. *Eur J Biochem* 268: 1996–2002, 2002
119. Suriapranata I, Epple UD, Bernreuther D, Bredschneider M, Sovarasteanu K, Thumm M: The breakdown of autophagic vesicles inside the vacuole depends on Aut4p. *J Cell Sci* 113: 4025–4033, 2000
120. Katunuma N, Kominami E: Structures and functions of lysosomal thiol proteinases and their endogenous inhibitor. *Curr Top Cell Regul* 22: 71–101, 1983
121. Barrett AJ, Kirschke H: Cathepsin B, Cathepsin H, and cathepsin L. *Meth Enzymol* 80: 535–561, 1981
122. Arunachalam B, Phan UT, Geuze HJ, Cresswell P: Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl Acad Sci* 97: 745–750, 2000
123. Reed CH: Diagnostic applications of cystatin C. *Br J Biomed Sci* 57: 323–329, 2000
124. Jadot M, Dubois F, Wattiaux-De Coninck S, Wattiaux R: Supramolecular assemblies from lysosomal matrix proteins and complex lipids. *Eur J Biochem* 249: 862–869, 1997
125. Epple UD, Suriapranata I, Eskelinen EL, Thumm M: Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. *J Bacteriol* 183: 5942–5955, 2001
126. Teter SA, Eggerton KP, Scott SV, Kim J, Fischer AM, Klionsky DJ: Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. *J Biol Chem* 276: 2083–2087, 2001
127. Thamotharan M, Lombardo YB, SZ, Adibi S: An active mechanism for completion of the final stage of protein degradation in the liver, lysosomal transport of dipeptides. *J Biol Chem* 272: 11786–11790, 1997
128. Klionsky DJ, Emr SD: Autophagy as a regulated pathway of cellular degradation. *Science* 290: 1717–1721, 2000
129. van Kerkhof P, Alves dos Santos C, Sachse M, Klumperman J, Bu G, Strous G: Proteasome inhibitors block a late step in lysosomal transport of selected membrane but not soluble proteins. *Mol Biol Cell* 12: 2556–2566, 2001
130. Liou W, Geuze HJ, Geelen MJ, Slot JW: The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J Cell Biol* 136: 61–70, 1997
131. Stefanis L, Larsen K, Rideout H, Sulzer D, Greene L: Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J Neurosci* 21: 9549–9560, 2001
132. Hutchins MU, Klionsky DJ: Vacuolar localization of oligomeric alpha-mannosidase requires the cytoplasm to vacuole targeting and autophagy pathway components in *Saccharomyces cerevisiae*. *J Biol Chem* 276: 20491–20498, 2001
133. Scott SV, Nice DC, 3rd, Nau JJ, Weisman LS, Kamada Y, Keizer-Gunnink I, Funakoshi T, Veenhuis M, Ohsumi Y, Klionsky DJ: Apg13p and Vac8p are part of a complex of phosphoproteins that are required for cytoplasm to vacuole targeting. *J Biol Chem* 275: 25840–25849, 2000
134. Gevers W: Protein metabolism of the heart. *J Mol Cell Cardiol* 16: 3–32, 1984
135. Wing SS, Chiang HL, Goldberg AL, Dice JF: Proteins containing peptide sequences related to KFERQ are selectively depleted in liver and heart, but not skeletal muscle, of fasted rats. *Biochem J* 275: 165–169, 1991
136. Ward WF: The relentless effects of the aging process on protein turnover. *Biogerontology* 1: 195–199, 2000
137. Goto S, Takahashi R, Kumiyama AA, Radak Z, Hayashi T, Takenouchi M, Abe R: Implications of protein degradation in aging. *Ann N Y Acad Sci* 928: 54–64, 2001
138. Terman A: The effect of age on formation and elimination of autophagic vacuoles in mouse hepatocytes. *Gerontology* 41: 319–325, 1995
139. Cuervo AM, Dice JF: Age-related decline in chaperone-mediated autophagy. *J Biol Chem* 275: 31505–31513, 2000
140. Donati A, Cavallini G, Paradiso C, Vittorini S, Pollera M, Gori Z, Bergamini E: Age-related changes in the autophagic proteolysis of rat isolated liver cells: effects of antiaging dietary restrictions. *J Gerontol* 56: B375–383, 2001
141. Hariri M, Millane G, Guimond MP, Guay G, Dennis JW, Nabi IR: Biogenesis of multilamellar bodies via autophagy. *Mol Biol Cell* 11: 255–268, 2000
142. Dice J: Altered degradation of proteins microinjected into senescent human fibroblasts. *J Biol Chem* 257: 14624–14627, 1982
143. Terman A, Brunk U: Lipofuscin- Mechanisms of formation and increase with age. *APMIS* 106: 265–276, 1998
144. Lockwood TD: Redox control of protein degradation. *Antioxid Redox Signal* 2: 851–878, 2000
145. Takahashi M, Hoshii Y, Kawano H, Gondo T, Yokota T, Okabayashi H, Shimada I, Ishihara T: Ultrastructural evidence for the formation of amyloid fibrils within cardiomyocytes in isolated atrial amyloid. *Amyloid* 5: 35–42, 1998
146. Vittorini S, Paradiso C, Donati A, Cavallini G, Masini M, Gori Z, Pollera M, Bergamini E: The age-related accumulation of protein carbonyl in rat liver correlates with the age-related decline in liver proteolytic activities. *J Gerontol* 54: B318–323, 1999
147. Bednarski E, Lynch G: Selective suppression of cathepsin L results from elevations in lysosomal pH and is followed by proteolysis of tau protein. *Neuroreport* 9: 2089–2094, 1998
148. Melendez A, Tallozy Z, Scaman M, Eskelinen EL, Hall DH, Levine B: Essential role of autophagy genes in dauer development and lifespan extension in *C. elegans*. *Science* 301: 1387–1391, 2003
149. Bromme HJ, Holtz J: Apoptosis in the heart: when and why? *Mol Cell Biochem* 163–164: 261–275, 1996
150. Ollinger K: Inhibition of cathepsin D prevents free-radical-induced apoptosis in rat cardiomyocytes. *Arch Biochem Biophys* 373: 346–351, 2000
151. Brunk UT, Svensson I: Oxidative stress, growth factor starvation and Fas activation may all cause apoptosis through lysosomal leak. *Redox Rep* 4: 3–11, 1999
152. Shibata M, Kanamori S, Isahara K, Ohsawa Y, Konishi A, Kametaka S, Watanabe T, Ebisu S, Ishido K, Kominami E, Uchiyama Y: Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation. *Biochem Biophys Res Comm* 251: 199–203, 1998
153. Roberg K, Kagedal K, Ollinger K: Microinjection of cathepsin d induces caspase-dependent apoptosis in fibroblasts. *Am J Pathol* 161: 89–96, 2002
154. Bursch W, Ellinger A, Gerner C, Frohwein U, Schulte-Hermann R: Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? *Ann N Y Acad Sci* 926: 1–12, 2000

155. Monney L, Olivier R, Otter I, Jansen B, Poirier GG, Borner C: Role of an acidic compartment in tumor-necrosis-factor-alpha-induced production of ceramide, activation of caspase-3 and apoptosis. *Eur J Biochem* 251: 295–303, 1998
156. Kostin S, Pool L, Elsasser A, Hein S, Drexler H, Arnon E, Hayakawa Y, Zimmermann R, Bauer E, Klovekorn W, Schaper J: Myocytes die by multiple mechanisms in failing human hearts. *Circ Res* 92: 715–724, 2003
157. Uchiyama Y: Autophagic cell death and its execution by lysosomal cathepsins. *Arch Histol Cytol* 64: 233–246, 2001
158. Thummel CS: Steroid-triggered death by autophagy. *Bioessays* 23: 677–682, 2001
159. Gorski S, Chittaranjan S, Pleasance E, Freeman J, Anderson C, Varhol R, Coughlin S, Zuyderduyn S, Jones S, Marra M: A SAGE approach to discovery of genes involved in autophagic cell death. *Curr Biol* 13: 358–363, 2003
160. Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A: DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* 157: 455–468, 2002
161. Winchester B, Vellodi A, Young E: The molecular basis of lysosomal storage diseases and their treatment. *Biochem Soc Trans* 28: 150–154, 2000
162. Saftig P, Tanaka Y, Lullmann-Rauch R, von Figura K: Disease model: LAMP-2 enlightens Danon disease. *Trends Mol Med* 7: 37–39, 2001
163. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, Janssen PM, Blanz J, von Figura K, Saftig P: Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature* 406: 902–906, 2000
164. Sugie K, Yamamoto A, Murayama K, Oh S, Takahashi M, Mora M, Riggs J, Colomer J, Iturriaga C, Meloni A, Lamperti C, Saitoh S, Byrne E, DiMauro S, Nonaka I, Hirano M, Nishino I: Clinicopathological features of genetically confirmed Danon disease. *Neurology* 58: 1773–1778, 2002
165. Konecki D, Foetisch K, Zimmer K, Schlotter M, Lichter-Konecki U: An alternatively spliced form of the human lysosome-associated membrane protein-2 gene is expressed in a tissue-specific manner. *Biochem Biophys Res Comm* 215: 757–767, 1995
166. Suzuki T, Nakagawa M, Yoshikawa A, Sasagawa N, Yoshimori T, Ohsumi Y, Nishino I, Ishiura S, Nonaka I: The first molecular evidence that autophagy relates rimmed vacuole formation in chloroquine myopathy. *J Biochem* 131: 647–651, 2002
167. Terman A, Dalen H, Eaton JW, Neuzil J, Brunk UT: Mitochondrial recycling and aging of cardiac myocytes: the role of autophagocytosis. *Exp Gerontol* 38: 863–876, 2003
168. Nepomnyashchikh LM, Lushnikova EL, Semenov DE: Focal degradation of cytoplasmic organelles in cardiomyocytes during regenerative and plastic myocardial insufficiency. *Bull Exp Biol Med* 130: 1190–1195, 2000