Autophagy in Immunity and Infection

A Novel Immune Effector

Edited by
Vojo Deretic

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Contents

Preface

This book was inspired by a stream of nearly simultaneous reports in 2004 and 2005 demonstrating that the fundamental biological process of autophagy, primarily known for its role in cytoplasmic maintenance, represents a previously unrecognized innate and adaptive immunity mechanism that functions as a defense against intracellular pathogens and probably has other roles within the immune system. Although hints to the role of autophagy in immune defenses and other roles in immunity have existed in the literature, the most recent burst of publications made a compelling and definitive case for the importance of autophagy in immunity. A further motivation for this project came from the opportunity to merge these new findings with the superb recent progress on genetics, biochemistry and cell biology of autophagy. The product is a book covering the basic aspects of autophagy as a cytoplasmic maintenance process playing a role in cell survival and death, its role in health and disease in general, and the new cutting edge – the role of autophagy in immunity. Using this book, the reader can find a full range of information on autophagy in one place covering both its fundamental molecular mechanisms and its many physiological roles.

Autophagy is a homeostatic intracellular mechanism, whereby a cell digests parts of its own cytoplasm for removal or turn-over, as eloquently summarized in the Foreword by P. Seglen. The term autophagy represents a set of distinct yet related pathways. These range from the robust process of macroautophagy to a rather subtle process of chaperone mediated autophagy, as detailed in Chapter 1 by J. Legakis and D. Klionsky, which also provides the fundamentals of autophagy based on the powerful genetics in yeast and other organisms. Macroautophagy sequesters significant portions of the cytosol or whole organelles into a characteristic double membrane vacuole termed the autophagosome, for eventual degradation in autolysosomes, covered extensively in Chapter 2 by S. Tooze and colleagues and Chapter 3 by N. Mizushima. Chaperone mediated autophagy, covered in some detail in Chapter 4 by A. Cuervo and colleagues and touched upon in Chapter 12 by D. Schmid and C. Münz, is a degradative pathway whereby individual proteins are imported directly into the lysosomes. In macroautophagy, or its variant manifestation of microautophagy, the trapped cytosol or organelles are eventually delivered to degradative compartments (in mammalian cells – autolysosome) for digestion and removal. In its probably

most common presentation, autophagy recycles stable cytosolic macromolecules, such as proteins with long half-lives, to supply nutrients and maintain essential cellular anabolic needs and viability under starvation conditions. The organelle removal function of autophagy is a just as important housekeeping function, by controlling the pool of peroxisomes or removing compromised mitochondria, in the latter case potentially protecting cells from unscheduled apoptosis. Although autophagy is a cell maintenance mechanism, under certain conditions, excessive autophagy can cause non-apoptotic programmed cell death, covered in Chapter 5 by Y. Debnath and C. Fung. Autophagy has been implicated in cancer, degenerative disorders, such as Huntington, Parkinson, and Alzheimer diseases, normal development, and aging, covered in detail in Chapter 4 by A. Cuervo and colleagues.

A number of very precise studies on anti-viral action of autophagy have been the true forerunner of our present more general understanding of the role of autophagy in defense against intracellular pathogens, as covered in Chapter 13 by B. Levine. More recent studies demonstrate that autophagy is also an innate immunity effector against intracellular bacteria, a central theme of the second half of this book, encompassing: Chapter 6 on *Mycobacterium tuberculosis* elimination by autophagy (Harris et al.); Chapter 7 by T. Yoshimori and A. Amano on autophagic elimination of streptococci if they invade host cells and find themselves in the cytosol; Chapter 8 on the role of autophagy in capturing the intracellular *Shigella* and its ability to escape this process; and Chapter 9 by K. Rich and P. Webster on *Listeria*. Some highly evolved pathogens have mechanisms for harnessing autophagy to their own benefit, as suggested in Chapter 10 by M. Gutierrez and M. Colombo and discussed in Chapter 11 by M.-P. Stein and C. Roy. The duality of effects of autophagy is also reflected in the Addendum to B. Levine's Chapter 13 provided by J. Sparks and M. Denison. Significantly, autophagy has a strong impact on MHCII presentation (Chapter 12 by D. Schmid and C. Münz) and is controlled by cytokines (Chapters 6 and 13) clearly extending the role of autophagy to adaptive immunity.

The goal of this volume was to provide the reader not only with the applications of autophagy in infectious diseases and immunity, but also to generate a definitive text for autophagy in general. In other words, a reader who is interested primarily in the fundamental principles and broad biological aspects of autophagy, should find this book an indispensable companion and a comprehensive source of information. For those who are primarily interested in the burgeoning field of autophagy in innate and adaptive immunity, the chapters covering the basic principles of autophagy are just as important to understand fully the underlying processes.

The book starts with a foreword by Professor Per Seglen, a doyen in the field of autophagy, who has defined many biochemical and cell biological features of autophagy and has also produced both classical and contemporary highly cited papers in this field. A careful reader of the foreword will extract many useful concepts on autophagosomes, amphisomes and autolysosomes, and precious cautionary notes on interpretations of cause and effect in diseases and in cell

survival vs. cell-death promoting faces of autophagy. The editor is indebted to Per for his willingness to write a foreword to this volume and give the reader both his sage advice on general aspects of autophagy and sum it all up including the latest developments in the context of defense against intracellular pathogens.

Furthermore, the reader is a true beneficiary of the combination of excitement and enthusiasm that pervades the field of autophagy research, and enormous expertise of the contributing authors in this area. The editor of this book is indebted immensely to all contributing authors. The chapters by Drs. Ana Maria Cuervo, Daniel Klionsky, Beth Levine, Sharon Tooze and Naboru Mizushima, taken together, can give a textbook on autophagy as a standalone product. Likewise, the chapters that link autophagy with innate and adaptive immunity by Drs. Christian Münz, Chichiro Sasakawa, Tamotsu Yoshimori, and others summarize the new breakthroughs in immunological applications of autophagy. They also define the nidus for the developing field of immunophagy, a term used by the Editor of this book in a recent review in Current Opinion in Immunology to describe collectively these processes.

I acknowledge the excellent coordination and open lines of communication with the publisher including the gentle prompts from Andreas Sendtko, importance of NIH funding (AI45148 and AI42999) for all my scientific activities including this one, great support and understanding at home beyond what a person can expect or deserves, and above all the collective responsiveness and enthusiasm for this book by the main protagonists in the field of autophagy. My great personal and professional respect for many of the contributors to this book has been reaffirmed in the process.

Placitas (between Albuquerque and Santa Fe), April 2006 Vojo Deretic

Foreword

Autophagy, the mechanism by which cells envelop and degrade their own cytoplasm, plays a dual role in cellular physiology. On the one hand, autophagy serves vital functions such as the supply of essential amino acids during nitrogen starvation, the mobilization of iron from intracellular stores, the sequestration of aggregated (and potentially harmful) abnormal proteins that cannot be digested by the proteasomes, and the containment and degradation of infectious organisms. On the other hand, autophagy is frequently turned on during programmed cell death, complementing the apoptotic caspases in the orderly liquidation of the cell. In certain cases, particularly if the major caspases are somehow incapacitated, autophagy can, by itself, complete the death process. Autophagy may thus either support or prevent cell survival, depending on the biological context.

In a pathological setting, this autophagic duality may cause problems of interpretation. Many diseases are accompanied by alterations in cellular membrane fluxes, often causing massive accumulations of intracellular vacuoles of varying morphologies. Do these changes represent an attempt to combat the disease or do they contribute to disease progression (or both – or neither)? What is the nature of the vacuoles that are the affected steps in the vacuolar dynamics and in what direction are they altered?

As a first step in the analysis, the observed vacuoles need to be identified; however, unfortunately, this is not a straightforward matter. In addition to the three major types of autophagic vacuoles, i.e. autophagosomes, amphisomes and (auto)lysosomes, endosomes may contain cellular material derived from disintegrated surrounding cells or, in late, multivesicular endosomes, internalized by invagination of the endosomal delimiting membrane. In certain diseases, such as Alzheimer's, both the endocytic and autophagic pathways are afflicted, causing the accumulation of an extremely heterogeneous array of vacuoles. It should be noted that a prolonged disturbance of vacuole fluxes may induce the formation of unusual vacuoles, which may be difficult to classify by morphological criteria.

Autophagosomes, the autophagic vacuoles formed when the sequestering membrane cisternae (the phagophores) have completed the enclosure process, can be recognized in the electron microscope as areas of absolutely normal cytoplasm, circumscribed by delimiting membranes, but not deviating morpologi-

cally from their surroundings. The delimiting membrane can sometimes be seen as a double-membrane (cisternal) structure, sometimes as a thick, osmiophilic layer and sometimes (artificially) as an open, electron-lucent cleft. However, since later autophagic vacuoles may contain sequestered membraneous elements closely apposed to their (single) delimiting membrane, an apparent "double membrane" is a less reliable diagnostic criterion for an autophagosome than its contents of unaltered cytoplasm.

Amphisomes, the products of fusion between endosomes and autophagosomes, quickly get their contents denatured due to acidification by the proton pump brought in by the endosomal fusion partner. The denaturation is visible in the electron microscope as a darkening and a somewhat altered morphology relative to the cytoplasmic surroundings. Multiple inputs from both autophagy and endocytosis often make the amphisomes large and complex. The contents will usually serve to distinguish amphisomes from autophagosomes, but they cannot be reliably distinguished from early autolysosomes by morphological criteria alone, particularly because the endosomal fusion partner contributes small amounts of lysosomal enzymes that may initiate degradation of the amphisomal contents. With more advanced degradation, autolysosomes usually become distinctive.

Organelle markers can make the identification of autophagic and endocytic vacuoles considerably easier. Few markers are entirely specific, but by using them in combination, information can be obtained both from the presence and the absence of a marker. In immunogold labeling studies, a relatively degradation-resistant cytosolic enzyme such as superoxide dismutase (SOD) can be used to mark autophagic vacuoles (autophagosomes, amphisomes and autolysosomes), an endocytosed, gold-conjugated protein like bovine serum albumin (BSA) can be used to mark endosomes, amphisomes and lysosomes, and a lysosomal membrane protein, e.g. LGP120, can be used to mark lysosomes. The combination of positive and negative markers will then identify endosomes ($SOD^-/BSA^+/LGP^-$), autophagosomes ($SOD^+/BSA^-/LGP^-$), amphisomes ($SOD^+/BSA^+/LGP^-$) and autolysosomes ($SOD^+/BSA^\pm/LGP^+$). A similar approach can be used in light microscopic studies, using, for example, the lipidated mammalian Atg8 analogue, LC3-II, as a marker for all types of autophagic vacuoles, in combination with a marker of acidic vacuoles, e.g. monodansylcadaverine and suitable endosomal and lysosomal markers.

Markers may also give information about flux perturbations. The accumulation of a specific vacuolar organelle is not necessarily the result of an increased rate of its formation, but may equally well reflect a reduced rate of its disappearance due to a defective fusion step. For example, a microtubule poison like vinblastine will block all vacuole transport and fusion, and cause autophagosomes and endosomes to accumulate. An inhibitor of intralysosomal protein degradation, like leupeptin, will not only increase the size and visibility of autolysosomes, but the impaired fusion capacity of the congested lysosomes will also cause amphisomes to pile up. Similar changes in cellular vacuole populations may occur as a result of pathological alterations in vacuolar fusion rates. Even

moderate fusion defects may have large morphological consequences if they persist over long periods of time, as may be the case in many of the slowly progressing autophagy-related diseases.

Experimental interruption of the autophagic-lysosomal flux offers useful ways of measuring flux rates. Since inhibition of intralysosomal protein degradation has been shown not to affect autophagic sequestration on a short-term basis, the intravacuolar accumulation of an autophagocytosed cytosolic enzyme after leupeptin treatment provides a precise measure of the autophagic sequestration rate (an autophagic membrane marker like LC3-II is less suitable for this purpose, because its vacuolar dynamics are influenced by factors other than the rates of sequestration and intralysosomal degradation). However, by blocking the autophagic flux altogether with a sequestration inhibitor such as 3-methyladenine (3-MA), the flux rate can be calculated, e.g. as the 3MA-sensitive part of the degradation of long-lived cellular protein. The effectiveness of this inhibitor also makes it useful in assessing the secondary effects of autophagy: if a cellular response is *in*sensitive to 3-MA, an autophagic causation can be excluded. In contrast, 3-MA *sensitivity* is compatible with an involvement of autophagy, although it does not prove it (as is the case with inhibitors in general).

Although most disease-related alterations in autophagic-lysosomal traffic are likely to be secondary, they can be the primary causes of some pathological conditions, most notably the lysosomal storage diseases. In these diseases, a deficiency in a single lysosomal enzyme will cause a massive intralysosomal accumulation of undegradable material that eventually disrupts all lysosomal functions, resulting in complex cellular and pathological alterations. Autophagic and endocytic influxes to the lysosome will gradually slow down, and prelysosomal autophagic and endocytic vacuoles will accumulate, their contents of undegraded material representing a spreading of the storage syndrome beyond the lysosomes. In the closely related Danon disease, lysosomes have apparently become fusion-incompetent due to a mutation in the lysosomal membrane protein, LAMP-2, resulting in reduced influxes to the lysosome, and an accumulation of amphisomes and autophagosomes. During aging, the gradual intralysosomal accumulation of undegradable lipofuscin inclusions will similarly disturb lysosomal function and has been shown to cause a reduced chaperone-mediated lysosomal protein uptake as well as a reduced flux through the autophagic pathway.

In many neurodegenerative diseases, mutant proteins that somehow escape proteasomal degradation may instead become autophagocytosed and gradually form undegradable aggregates inside lysosomes. The resulting lysosomal storage syndrome, including the accumulation of prelysosomal autophagic vacuoles, may in the long run impair the autophagic sequestration of toxic protein aggregates and thus contribute to progression of the disease. Events that take place in the piled-up amphisomes may exacerbate the situation: in Alzheimer's disease, toxic peptides seem to be generated by intra-amphisomal proteolysis. If exocytic recycling from amphisomes takes place, it could possibly be involved in the formation of the extracellular aggregates (plaques) characteristic of several

neurodegenerative diseases. Preciously little is known about amphisome physiology; hopefully, a better understanding of this pivotal organelle, strategically located at the junction between the autophagic and endocytic pathways, may shed some light on the complex pathology of degenerative diseases.

In relation to infectious pathogens, autophagy has been shown to play a dual role. On the one hand, autophagy is a part of the innate and adaptive immune defense, participating in the generation of antigenic peptides for MHC class II presentation as well as in the sequestration, containment and degradation of bacteria like *Streptococcus*, *Shigella* and *Mycobacterium*. The bacteria fight back by attempting to suppress autophagic activity. On the other hand, bacteria like *Coxiella*, *Legionella* and several RNA viruses enter cells by a phagocytic route, but eventually become autophagocytosed intracellularly and take up residence inside autophagic vacuoles. In these cases, autophagy may promote infectivity.

Can better knowledge about autophagy help to combat autophagy-related diseases? Clearly, the slow progression of many of the degenerative diseases should leave a lot of room for therapeutic intervention. A stimulation of autophagic activity by intermittent amino acid starvation is one obvious strategy that seems to work well in mice (which can prolong their lifespan by fasting), but adequate data for humans are lacking. Autophagy-stimulatory drugs, such as rapamycin, represent another possibility that has shown considerable promise in several degenerative disease models. Conversely, in the case of infections or programmed cell death promoted by autophagy, autophagy suppressants like 3-MA have been demonstrated to be protective under experimental conditions. The development of autophagy modifiers that are pharmacologically acceptable and effective *in vivo* would seem like a promising therapeutic avenue.

Consideration should also be given to the possibility of overcoming or circumventing lysosomal dysfunctions. Some improvement has been reported with lysosomal enzyme replacement therapy to lysosomal storage disease patients, but the endocytic delivery of missing enzymes to lysosomes may be hampered by poor lysosomal uptake or fusion capacity. The amphisomes should, at least at an early stage, be more accessible. By supplying amphisomes, through the endocytic pathway, with lytic enzymes and other factors required for efficient degradation of problematic substrates, these organelles could possibly be turned into artificial lysosomes, tailored for a specific purpose. Hopefully, additional therapeutic strategies will be suggested by future research into the inner workings of the autophagic–endocytic-lysosomal network.

Oslo, December 1st, 2005 Per O. Seglen

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Color Plates

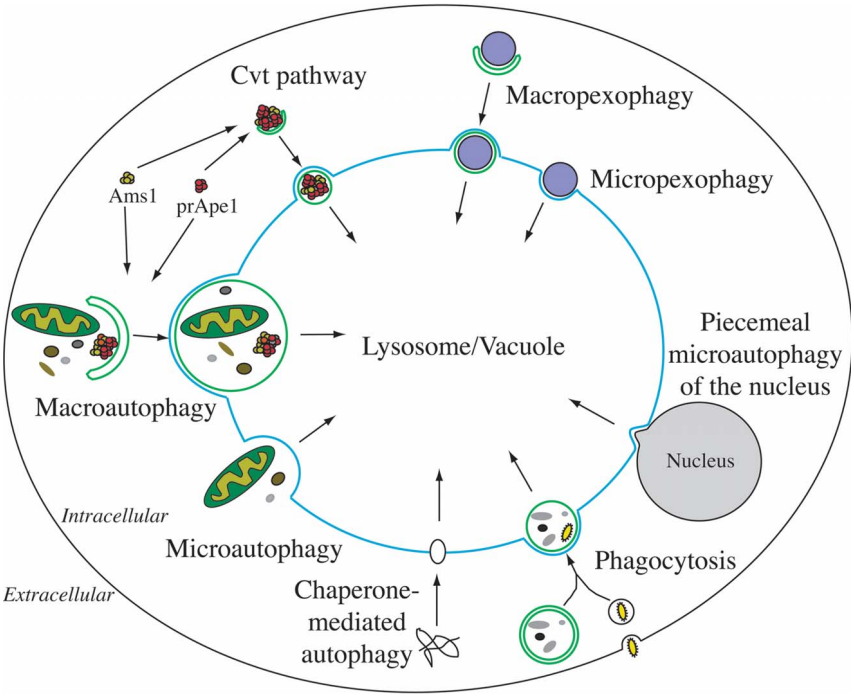

Fig. 1.1 Schematic representation of various transport routes to the lysosome/vacuole. There exist a number of pathways by which substrates are delivered to the lysosome/vacuole. Some of the sequestration events occur at the organelle membrane, these are denoted by the prefix "micro". In other cases, the enclosure of the substrate occurs spatially away from the lysosome/vacuole membrane. These pathways begin with the prefix "macro". Macro- and microautophagy are nonspecific degradation pathways, which include a variety of cargoes, depending on the organism and the particular stress conditions or stage of development. Selective degradation of peroxisomes, small parts of the nucleus or foreign pathogens occurs via macropexophagy, micropexophagy, piecemeal microautophagy of the nucleus or phagocytosis, respectively. Chaperone-mediated autophagy is a receptor-driven degradative pathway that is a secondary response to starvation conditions. The biosynthetic Cvt pathway is a method of delivery for at least two vacuolar hydrolases.

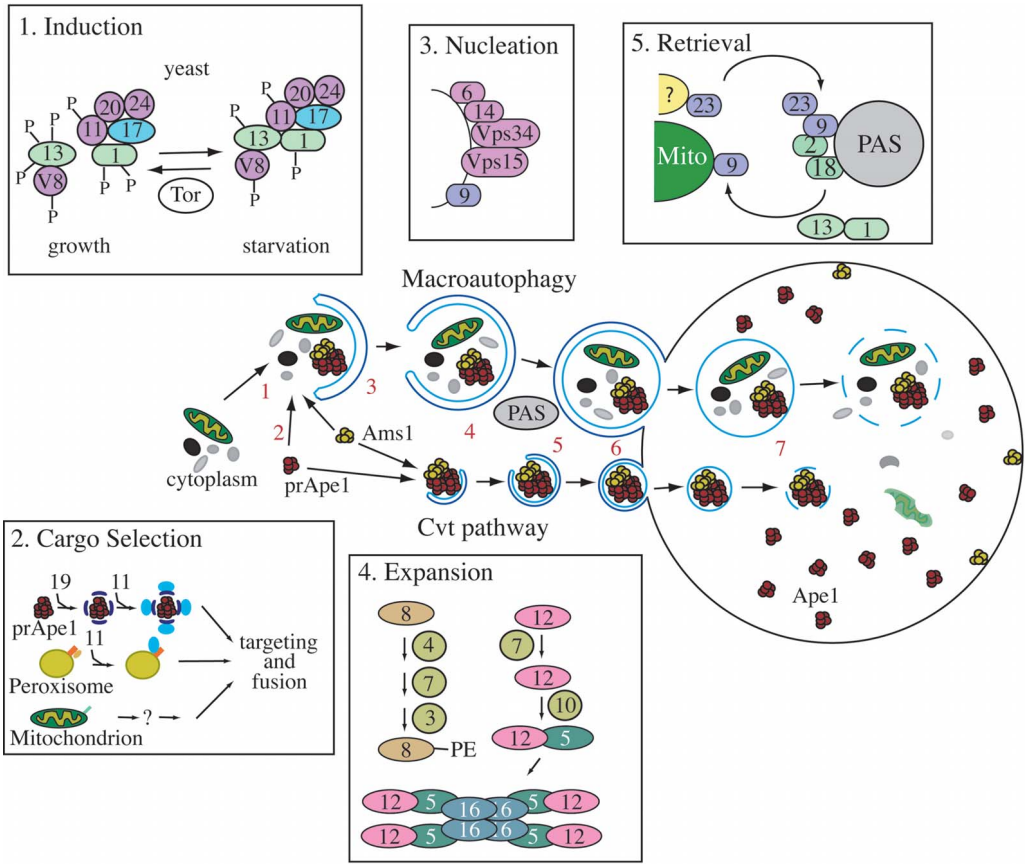

Fig. 1.2 Autophagy and the Cvt pathway. Autophagy and the Cvt pathway can be depicted as a series of separate steps. The roles of Atg and other proteins, shown to participate in different parts of the pathway, are depicted. The proteins classified by only a number are the corresponding Atg gene product. Otherwise, the protein name is specified, except for Vac8, which is indicated as "V8". "P" denotes phosphorylation of the indicated protein. (1) *Induction.* TOR kinase becomes inactivated upon nutrient limitation, eliciting a series of events, which result in the induction of autophagy. These include partial dephosphorylation of Atg13, which alters its association with Atg1. Atg1 is thought to play a key role in the switch between growth and starvation. Autophagy-specific proteins are shown in blue, whereas Cvt-specific proteins are depicted in purple. (2) *Cargo selection and packaging.* Examples of specific autophagy include the Cvt pathway, pexophagy and possibly mitophagy. During growth, the Cvt pathway is active. The cargo, prApe1, is synthesized as an inactive precursor and rapidly oligomerizes. Atg19, the cargo receptor, binds to the oligomer, followed by Atg11 binding to the complex. Upon induction of pexophagy, the peroxin, Pex3, is degraded, thus exposing the docking protein, Pex14. Although it is not proven, Atg11 is proposed to bind to the newly exposed Pex14. The mechanism of mitophagy is unknown. Once these binding events occur, the cargo are enwrapped by a double-membrane vesicle and delivered to the lysosome/vacuole. (3) *Vesicle nucleation.* Membrane is acquired from an unknown location and the cargo associates with the forming vesicle. Membrane formation re-

quires the PI3K complex I; the components of this complex are shown in Step 3. The PI3-phosphate (PI3P) generated by this complex recruits a number of Atg proteins to the PAS, including Atg18, Atg20, Atg21, and Atg24 [24]. (4) *Vesicle expansion and completion*. There are two sets of Atg proteins, which participate in a series of ubiquitin-like (Ubl) conjugation reactions. These generate Atg12–Atg5–Atg16 and Atg8–PE (see text for details). The functions of these proteins are not known but they are needed for expansion and completion of the sequestering vesicle. (5) *Retrieval*. As most of the Atg proteins are not included in the completed vesicle, there must be a mechanism to release and return these components back to their original site. Atg9 and Atg23 have been shown to be cycling proteins, moving between the PAS and other punctate structures. Atg9 has been shown to cycle betweent he mitochondria and the PAS. The non-PAS localizations of Atg23 are as yet unidentified. These two proteins may aid in the recovery of Atg components, allowing them to be reused for another round of delivery. (6) *Targeting, docking and fusion of the vesicle with the lysosome/vacuole*. The docking and fusion of the completed vesicle requires a number of components (see text for details). The fusion event results in a single-membrane vesicle within the lumen of the lysosome/vacuole. (7) *Breakdown of the vesicle and its contents*. Once inside the lysosome/vacuole, the autophagic or Cvt body must be degraded in order for the cargo to be released. The lipase responsible for vesicle lysis is thought to be Atg15. Upon release into the lumen, the cargoes of pexophagy and bulk autophagy are broken down for re-use in the cell, while the cargoes of the Cvt pathway carry out their function as hydrolases.

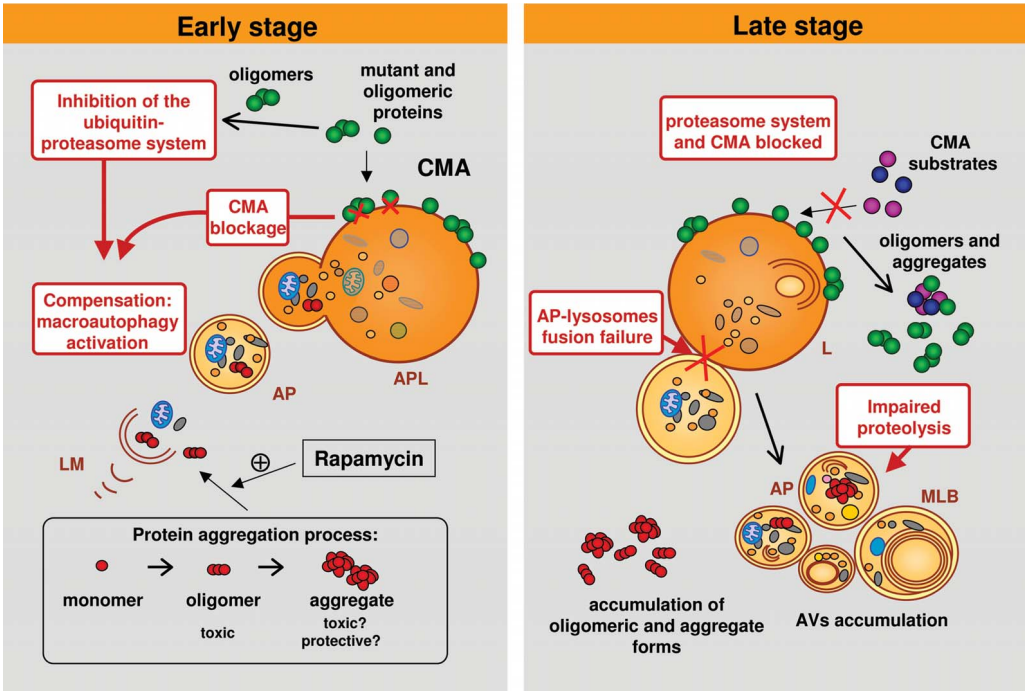

Fig. 4.1 Autophagy in protein conformational disorders. Protein conformational disorders result from abnormal conformational changes in particular proteins (due to mutations or post-translational modifications) that make them prone to aggregation. In the early stages of the disorder, the abnormal proteins often block the activity of proteolytic systems normally responsible for the degradation of soluble proteins (proteasome and CMA by the lysosome), resulting in compensatory activation of macroautophagy to eliminate the oligomeric toxic forms. As the diseases progress (late stage), a macroautophagic failure often occurs, probably due to problems in the clearance of the autophagocytosed materials, leading to the accumulation of AVs with partially degraded contents and eventually to cell death. Abbreviations: L = lysosome; LM = limiting membrane; AP = autophagosome; APL = autophagolysosome; MLB = multilamelar bodies.