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Review

Aging: Central role for autophagy and the lysosomal degradative system

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ABSTRACT

The lysosomal network is the major intracellular proteolytic system accounting for more than 98% of long-lived bulk protein degradation and recycling particularly in tissues such as liver and muscles. Lysosomes are the final destination of intracellular damaged structures, identified and sequestered by the processes of macroautophagy and chaperone-mediated autophagy (CMA). In the process of macroautophagy, long-lived proteins and other macromolecular aggregates and damaged intracellular organelles are first engulfed by autophagosomes. Autophagosomes themselves have limited degrading capacity and rely on fusion with lysosomes. Unlike macroautophagy, CMA does not require intermediate vesicle formation and the cytosolic proteins recognized by this pathway are directly translocated to the lysosomal membrane. Aging is a universal phenomenon characterized by progressive deterioration of cells and organs due to accumulation of macromolecular and organelle damage. The continuous removal of worn-out components and replacement with newly synthesized ones ensures cellular homeostasis and delays the aging process. Growing evidence indicate that the rate of autophagosome formation and maturation and the efficiency of autophagosome/lysosome fusion decline with age. In addition, a progressive increase in intralysosomal concentration of free radicals and the age pigment lipofuscin further diminish the efficiency of lysosomal protein degradation. Therefore, integrity of the autophagosomal-lysosomal network appears to be critical in the progression of aging. Discovery of the genes involved in the process of autophagy has provided insight into the various molecular pathways that may be involved in aging and senescence. In this review, we discuss the cellular and molecular mechanisms involved in autophagy and the role of autophagosome/lysosome network in the aging process.

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1. Introduction

In the past two decades, there had been a constant accumulation of new knowledge in the field of research studying the aging process. The study of the biological basis of ageing, biogerontology, has so far unveiled mysteries of ageing by describing age-related changes in organisms, organs, tissues, cells and macromolecules (Troen, 2003). However, despite intense research, the molecular basis of the processes that cause loss of bodily functions and degeneration of cells and tissues is still unresolved. Aging is an essential, inevitable physiological phenomenon characterized by a progressive accumulation of deleterious molecular damages in cells and tissues during the post-maturational deterioration, which decreases the ability to survive and increases risk of death (Rajawat and Bossis, 2008). The aging process has many facets and multiple causes. The primary molecular phenotype of aging is the stochastic occurrence and accumulation of molecular damage leading to a progressive increase in molecular heterogeneity and functional impairment (Rattan, 2006). The failure of maintenance and repair pathways, effectively determines the course of aging, the origin of age-related diseases and eventual death (Holliday, 1995, 2000; Rattan, 2006; Rattan and Clark, 2005). Predetermined genetic factors, environmental influences, and certain diseases contribute to the process of aging. Accumulation of worn-out organelles and various cellular substructures over time reduces the cellular and molecular efficiency of various biological processes that are required to maintain homeostasis and survival.

Malfunctions in the biological processes required for the maintenance, repair and turnover pathways may be the main cause of the cumulative cellular damages during aging (Sohal et al., 1994). Aging, senescence and death are the final manifestations of unsuccessful homeostasis or failure of homeodynamics (Holliday, 2007; Rattan, 2006).

In various organisms, different types of cells have diverse kinds of machinery which accomplish their assigned functions. However, these heterogeneous cell populations have to operate in unison for proper functioning, adaptation and survival in harsh environments. During the lifespan of an organism, cells are subjected to

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various destructive forces, which may originate from either internal or external sources. The result of continuous exposure to these harmful forces is progressive accumulation of lesions. Over time these lesions become detrimental to cell and tissue survival. The precise molecular mechanism of aging is not yet completely understood. The elements that are responsible for oxidative damage and improper housekeeping are considered the main contributory causes and play pivotal roles in cell survival.

2. Characteristics of aging

In general, there are some common and universally accepted characteristics that manifest during the process of aging, such as, increased mortality after maturation (Gompertz, 1825), changes in the biochemical composition of tissues (Florini, 1981; Strehler, 1977), progressive decrease in physiological capacity (Lakatta, 1990; Shock, 1985; Lindeman et al., 1985), reduced ability to respond adaptively to environmental stimuli (Adelman et al., 1978) and increased susceptibility and vulnerability to diseases (Hitt et al., 1999). Among those, the increase in mortality with aging after maturation and the pattern of age-related survival is similar across species (Kaeberlein et al., 2001). To detect the changes in biochemical composition of tissues with age many markers of aging have been described (Florini, 1981). Two of the most notable biomarkers are increased accumulation of lipofuscin (age pigment) (Strehler, 1977) and increased cross-linking in extracellular matrix molecules such as collagen (Bjorksten, 1974; Kohn, 1978). Other notable age-related changes are alterations in the rate of transcription of specific genes, the rate of protein synthesis and turnover and numerous post-translational protein modifications, such as glycation and oxidation (Finch, 1990; Schneider and Rowe, 1996). These alterations with aging presumably lead to a diminished ability to maintain homeostasis and therefore reduced ability to respond adaptively to environmental stimuli (Adelman et al., 1978). As a result, there is increased susceptibility and vulnerability to various diseases and exponential increase in mortality with age (Brody and Brock, 1985). The basis for dramatic rise in mortality is incompletely understood, but presumably involves changes in the function of many types of cells, which lead to tissue/organ dysfunction and systemic illness.

3. Theories of aging

Aging is a multifactorial process and attempts at understanding the fundamental causes of aging are limited by the complexity of the problem (Kirkwood, 2005). Aging is manifested and easily observed at the organism level. However, studying the aging process at the cellular and molecular level is more complicated and subject to confounding experimental and environmental factors (Dice, 1993). The lack of precise, well defined and reliable cellular and biochemical markers of aging has hindered efforts to identify the primary mechanisms and separate those from secondary effects. Many theories about the causes of aging have been proposed (Weinert and Timiras, 2003), and could be divided into two broad categories (Troen, 2003): the stochastic theories and the genetic theories. The fundamental concept behind the stochastic theories is the build-up of "damage" that occurs throughout the entire lifespan of cells (Rattan, 2008). Such damage may accumulate from toxic byproducts of routine metabolism or inefficient repair/defensive systems. It is apparent that in long-lived cells such as, neurons or cardiomyocytes, accumulation of lesions can be more detrimental. In proliferating cells, lesions rarely accumulate with age while the process of cell division seems to efficiently remove damaged structures. The genetic theories consider aging as part of the genetically programmed and controlled lifespan that includes development, maturation, senescence, and death (Finch and Tanzi, 1997). These theories are heavily supported by two observations. The average life span is highly species specific, and the overall lifespan is much more similar in monozygotic twins compared to dizygotic twins. The recent discovery of longevity genes in lower eukaryotes (yeast and *Caenorhabditis elegans*) and the existence of accelerated aging syndromes (Turker and Martin, 1999) in humans have provided increased credibility in genetic theories of aging. The genetic and stochastic theories, however, are not mutually exclusive. As will be later described, the mitochondrial DNA theory of aging is related with accumulation of free radicals, by itself a stochastic event. In addition, it is generally accepted that as an organism moves from the early phases of development to the final phases of senescence, the influence of genes is reduced while that of stochastic events is increased. Since the focus of this review is the contribution of lysosomes and autophagy in the aging process, further discussion of the genetic theories is out of scope.

3.1. The stochastic theories of aging

The stochastic theories speculate that random environmental factors induce cumulative damages at various levels of an organism as the cause of aging (Fig. 1). These include damage to DNA or inability to repair, damage to tissues and cell organelles by oxygen free radicals, protein cross-linking and inability of the cellular turnover mechanisms to remove such damaged structures and molecules. The eventual accumulation of these damages to a level that inhibits physiological function leads to progressive aging and senescence.

3.1.1. The mutation accumulation theory

This theory, originally proposed by Medawar (1952), views aging as a side effect of natural selection. Somatic mutations that occur earlier in life are selected against, because they impair reproductive function. Mutations that occur after the reproductive age are not forcefully selected, because they are not passed on the offspring.

As a result of this natural selection, the rate of somatic mutations increases with age. An increase in DNA damage leads to declining fidelity of gene expression with age, and an increase fraction of abnormal proteins. Severely abnormal proteins are removed by cellular turnover mechanisms. However, functional but error-containing proteins may further compromise the DNA and protein-synthesizing mechanisms resulting in rapid accumulation of abnormal molecules and senescence (error catastrophe). Although there are several reports of changes in the rate of protein synthesis in aging, no direct evidence of age-dependent protein mis-synthesis has been documented. Instead, the abnormal proteins in aging cells and tissues are due to aberrant post-translational modifications, such as oxidation and glycation (Kristal and Yu, 1992; Levine and Stadtman, 1996; Gracy et al., 1985). Accumulation of these abnormal proteins with aging impairs the cellular turnover mechanisms leading to decreased clearance. Theoretically, this would further impair regulation of gene expression to a point that is incompatible with physiological function and life.

3.1.2. The protein modification theory

In addition to age-related changes in the rate of protein synthesis, functional changes in the physiological and biochemical activity of proteins occur. Notably, the specific activity and heat stability of several enzymes is altered and the carbonyl content of proteins is increased (Levine and Stadtman, 1996). These changes can be caused by direct oxidation of amino acid residues, metal-catalyzed oxidation and modification by lipid oxidation products and glycation. Kohn (1978) and Bjorksten (1974) hypothesized

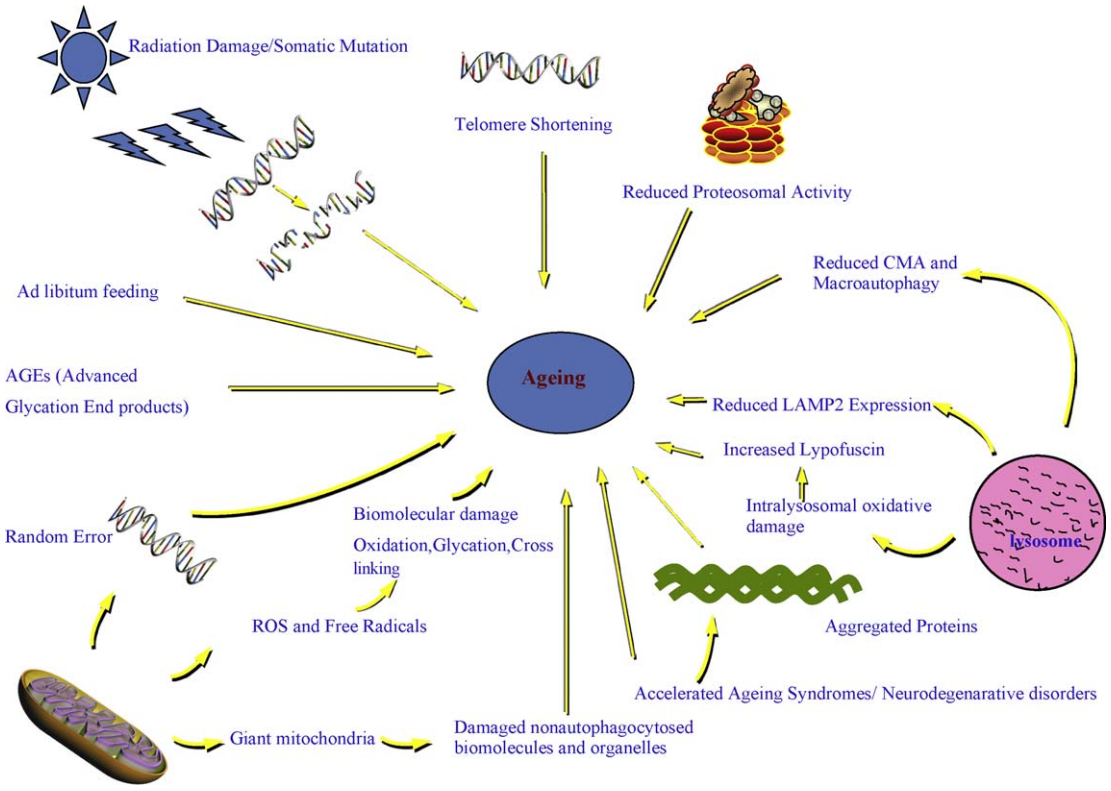

Fig. 1. Stochastic causes of aging: various factors induce cumulative damages at different levels of an organism as the cause of aging. These include damage to DNA or inability to repair, damage to tissues and cell organelles by reactive oxygen species, protein cross-linking, and inability of the cellular turnover mechanisms to remove such damaged structures and molecules.

that the accumulation of post-translationally altered proteins could impair cellular and ultimately, organ function.

The glycation/cross-linking hypothesis of aging is based on observations that our proteins, DNA and other structural molecules develop cross-links to one another with age (Cerami, 1985) The main way cross-linking occurs is through a process called glycation. Post-translationally processed proteins contain sugar moieties. These glycated molecules can be reduced through a complex series of reactions (Maillard reaction) to yield a multitude of end-products known as advanced glycation end-products (AGEs). When both of the sticky ends of AGEs adhere to neighboring proteins, they form permanent, disabling cross-links. Damaged proteins are normally broken down by proteases; however, in the presence of cross-linkages, proteases are inhibited and as a result, damaged proteins accumulate (Bonnefont-Rousselot et al., 2000).Several studies have strongly suggested involvement of glycation/cross-linking in aging. These AGEs increase with aging and are implicated in skin deterioration, diabetes, eye disorders and amyloid accumulation. Many extracellular matrix proteins exhibit increased cross-linking with age. Proper organ function depends upon a normal extracellular matrix for processes such as diffusion of essential molecules. In addition, the extracellular matrix plays an important role in the regulation of gene expression. Cross-linking of collagen has been shown to be at least partially responsible for some age-related changes in skin (Robins, 2007). Although, collagen undergoes increased cross-linking with age (Reiser et al., 1987), such alterations can lead to improved function at some sites and to impaired function at others (Hall, 1976). Cross-linking of proteins in the lenses of the eyes (crystalline) plays a significant role in age-related cataract formation (Kumar et al., 2007). Cross-linking of proteins in arterial walls (elastins) probably play a role in the increased stiffness of vascular walls with aging and may account for at least some forms of atherosclerosis, like diabetic angiopathy (Peppa and Vlassara, 2005), as well as, age-related decline in kidney function. Recent evidence has also indicated that glycation contributes to the formation of beta-amyloid, the protein that clumps together in the brains of Alzheimer's patients.

3.1.3. Free radical theory (oxidative stress)/mitochondrial DNA

In 1956 D. Harman developed the "oxidative damage" theory of aging after noticing similarities between ionizing radiation effects and aging (Harman, 1956). According to this theory, free radicals accumulate over time causing progressive deterioration in the function of cells, tissues and organ systems, which may cause system's death. Further it states that free radicals are the major determinants of molecular damage that cause aging. Twenty years later, Harman realized that mitochondria are a major producer of reactive oxygen species and heavily contributing to oxidative damage (Harman, 1981). Free radicals or reactive oxygen species (ROS) are ions or very small molecules in an unstable configuration because they contain an unpaired electron in the outermost shell of atom. Because of the their unstable nature free radicals are highly reactive with neighboring molecules picking up an electron to balance themselves and thereby turning the attacked molecule into a free radical. In this manner, the initial free radical attack creates a vicious dynamic cycle. The two major free radicals are superoxide radical ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) (Fridovich, 1989; Sohal and Weindruch, 1996).

Antioxidants including carotenoids, flavonoids, glutathione, uric acid, vitamins A, C and E, and enzymes like superoxide dismutase, catalase and glutathione peroxidase are free radical scavengers that protect the cells from oxidative damage. The level of oxidative damage is a function of pro-oxidant and antioxidant forces present in the cells. The balance between the two

determines the outcome of oxidative damage during aging. The oxidative damage theory suggests that ROS formation is inherent in biological systems. Indeed, ROS are a byproduct of normal aerobic metabolism of cells. The pool of free radicals that cannot be efficiently eliminated by antioxidants may cause deleterious effects, such as cross linking of various biomolecules, DNA-strand breaks, and lipid oxidation and accumulation of alkyl radicals and aldehydes. Especially susceptible to chronic ROS exposure is the mitochondrial DNA that accumulates somatic mutations with age, which result in defective mitochondria respiration, reduced energy output and further increase in ROS production. The mitochondria paradox is that they are both the main producers of energy and ROS in the cell, ROS appear to play a role in regulating cell signaling, differential gene expression, cell replication, differentiation, and apoptotic cell death (Sen and Packer, 1996; Suzuki et al., 1997). Production of free radicals in the heart, kidney, and liver of a group of mammals was found to be inversely proportional to the maximum lifespan (Sohal et al., 1989). The development of new experimental assays in the detection of ROS production has made such investigations more popular. As a result, substantial information has been brought to light and the theory of oxidative damage in aging has now both supporting (Balaban et al., 2005; Muller et al., 2007; Finkel and Holbrook, 2000) and opposing (Raha and Robinson, 2000; Gurber et al., 2008; Vijg and Campisi, 2008) evidence.

mtDNA has some properties that make it susceptible to deleterious mutations. mtDNA is maternally transmitted and replicate throughout the lifespan of an organism in both proliferating and nonproliferative post-mitotic cells. In addition, mtDNA lacks introns and has no efficient DNA repair mechanisms. These characteristics increase the possibility of deleterious random mutations which cannot be repaired. mtDNA has much higher mutation rate than nuclear DNA (Ozawa, 1997). In addition, in aging cells accumulation of mitochondria with mutated DNA is frequently seen and complete replacement of normal mitochondria with mutated mitochondria is also observed (homoplasmy) (Khrapko et al., 1999). Hypotheses given behind this observation is that mutated mtDNA may have a replicative advantage over normal mtDNA; consequently, there will be clonal expansion of defective mitochondria (Ozawa, 1997).

Recent studies suggest involvement of peroxisomes in the regulation of free radical and ROS production (Schrader and Fahimi, 2006). Peroxisomes are known as the organelles dedicated to fatty acid oxidation and generation, as well as, elimination of peroxides. They also harbor many enzymes involved in ROS catabolism. During several pathologic conditions, such as UV irradiation (Schrader and Fahimi, 2004), inflammation and aging (Terlecky et al., 2006), significant changes have been observed in peroxisomes which led to the hypothesis that peroxisomes may be involved in the process of aging (Perichon et al., 1998). Two major observations that implicate peroxisomes in the aging process are increased generation of peroxisomal ROS and alterations in fatty acid oxidation activity. Changes in fatty acid oxidation may lead to altered membrane lipid composition. Several investigators have suggested the essentiality of various membranes in maintaining intracellular homeostasis and that deterioration of membrane integrity is the underlying cause of the aging process (Zs-Nagy, 1994). Many age-related membrane alterations have been reported, including lipid peroxidation (Matsuo et al., 1993), as well as accumulation of the lipid dolichol, which can greatly impair transmembrane signaling (Dolfi et al., 2003). Interestingly, the degree of dolichol accumulation in the liver of growing rats is highly correlated with levels of autophagic activity (both macroautophagy and pexophagy) in the same organ (Marino et al., 1998).

3.1.4. Proteasome/ubiquitin degradation pathway

Eukaryotic cells have two major proteolytic systems for the regulated degradation of proteins, vesicle-dependent lysosomal pathway and the ubiquitin/proteasome pathway (Ciechanover, 2005b). The proteasome, a multicatalytic proteolytic complex, is an intracellular, nonlysosomal threonine type protease (EC 3.4.99.46). The 20S proteasome, a 700 kDa multisubunit enzyme complex, is a barrel-shape stack of four heptameric rings (Coux et al., 1996; Bochtler et al., 1999). The two outer α-subunits rings (α1–7) embrace two central head-to-head oriented rings containing β-subunits (β1–7). The internal chamber, which is composed by β-subunits, contains the proteolytic active sites. Three of the β-subunits, β1, β2, and β5, are responsible for the proteasome hydrolyzing activities that cleave peptide bonds on the carboxyl site of acidic (peptidylglutamylpeptide hydrolyzing activity, PGPH), basic (tryp sin-like activity, T-L), and hydrophobic (chymotryp sin-like activity, CT-L) amino acids, respectively (Goldberg, 2007; Bochtler et al., 1999). Binding of each side of 20S particle by 19S regulatory complex or "Dragons Head" or Cap gives rise to 26S proteasome that is responsible for the ATP/ubiquitin-dependent protein degradation (Voges et al., 1999). Proteolysis by the ubiquitin/proteasome involves two major steps: ubiquitination and degradation. Ubiquitin (Ub) is a small protein of 76 amino acids crucial to the degradation of many cytosolic, nuclear and endoplasmic reticulum proteins (Hochstrasser, 1996). Ubiquitination is a complex process involving the following sequence of events: (i) formation of a high energy thioester bond between Ub and a Ub-activating enzyme (E1) in a reaction that requires adenosine triphosphate (ATP) hydrolysis; (ii) formation of a thioester bond between the activated Ub and Ub-conjugating enzymes (E2); (iii) covalent attachment of the carboxyl terminal of Ub, most often to the ε-amino group of a lysine residue on protein substrates via an isopeptide bond; this reaction is mediated by Ub ligases (E3),which confer substrate specificity to the ubiquitin/proteasome pathway; and (iv) assembly of multi-Ub chains carried out by ubiquitination factors (E4),which promote the production of longer Ub chains (Hershko and Ciechanover, 1998). At least four molecules of Ub forming a tetra-Ub chain need to be attached to the substrate to ensure efficient recognition and degradation by the 26S proteasome machinery (Cook et al., 1994). These chains are formed by successive attachment of monomers by an isopeptide bond, most frequently formed between the side chain of Lys 48 in one Ub and the carboxyl group of the C-terminal Gly76 of a neighboring Ub. Ub is removed from ubiquitinated proteins by deubiquitinating enzymes, which also disassemble poly-Ub chains. Deubiquitinating enzymes are cysteine proteases that hydrolyze the amide bond immediately after the COOH terminal Gly76 (Wilkinson, 1997).

The eukaryotic proteasome is present in cytosol, nucleus and microsomes, and comprises 1% of cytosolic proteins (Tanaka et al., 1986). The proteasome recognizes and selectively degrades oxidatively damaged and ubiquitinated proteins. One of the hypothesis put forward to explain the accumulation of oxidized and ubiquinated proteins is the decrease of proteasome activity with age (Carrard et al., 2002). Indeed, accumulation of altered protein can be explained by increased protein alteration, decreased protein degradation or the combination of both. The peptidase activity of proteasome changes with age. Chymotrypsin- and trypsin-like activities have been reported to either increase (Shibatani et al., 1996), not change (Conconi et al., 1996; Anselmi et al., 1998) or decrease (Hayashi and Goto, 1998 and Keller et al., 2000) with increasing age. However, the PGPH activity exhibits a 50–60% decline with age (Conconi et al., 1996) and (Anselmi et al., 1998). Neither the level of free Ub, and Ub mRNA nor the overall rate of the Ub-mediated proteolysis changes significantly with age in mouse liver (Mura et al., 1996) or in cultured human fibroblasts

(Pan et al., 1993). Additionally, there were no consistent changes observed in the activities of the ubiquitination machinery enzymes E1, E2 and E3 (Ciechanover et al., 2000). However, a common age-related feature observed in many tissues is accumulation of the Ub-conjugated proteins (Jahngen et al., 1992; Ohtsuka et al., 1995; Scrofano et al., 1998; Pan et al., 1993). Such proteins, tagged for degradation but not efficiently removed, may be harmful to the cells. Excessive accumulation of the Ub-conjugates is observed in many pathological conditions such as Alzheimer's disease, Parkinson's disease and other neurodegenerative disorders (Ciechanover et al., 2000). Several reasons for this age-related accumulation of modified ub-conjugated proteins have been suggested. Due to chronic exposure to free radicals or accumulation of genetic errors aged cells accumulate more damaged and altered protein as compare to young cells. Even if the ubiquitination machinery is able to tag all the proteins, the proteasome may not be able to degrade them in a timely manner. Aging cells may also be unable to adequately upregulate expression of proteasome subunits; and/or the proteasomal subunits may lose their processivity due to nonoptimal kinetic conditions. It is interesting to note that an age-related twofold decrease in the expression of one of the ATPases residing in the "cap" and two of the Ub-specific proteases has been reported in mouse gastrocnemius muscle (Lee et al., 1999). These ATPases in particular are believed to be responsible for the recognition of the ubiquitinated substrates. Alternatively, the ubiquitination machinery does not loose activity however, decrease in fidelity may occur instead. Reduced fidelity may lead to an inability of the "cap" of the 26 S proteasome to recognize the proteins destined for degradation. In conclusion, the accumulation of ubiquitinated proteins in senescent cells can be a fault of the proteasome or of the ubiquitinating/deubiquitinating machinery or both.

4. Lysosomes

The concept of cellular protein turnover is about half a century old (Ciechanover, 2005a). The quest to identify a mechanism or enzymes responsible for maintaining the cellular protein pool started the era of cellular organelles discoveries. More than 50 years ago, this search led Belgian cytologist Christian de Duve to discover lysosomes, as a result of studying the intracellular distribution of enzymes using centrifugal fractionation (de Duve, 2005). The term lysosome derives from the Greek words lysis (dissolution or destruction) and soma (body). Within few years from their discovery, lysosomes were recognized as the terminal degradative compartment of the endocytic pathway (De Reuck and Cameron, 1963) and since then, lysosomes have been described as the cellular demolition crew or garbage disposal system. They are frequently nicknamed "suicide-bags" or "suicide-sacs" by cell biologists due to their role in autolysis. Lysosomes are heterogeneous in morphology and often contain electron-dense material and membranous aggregates (Holtzman, 1989). They can be distinguished from endosomes by the lack of mannose-6-phosphate receptors (MPRs). Lysosomes are single or double membrane organelles containing various proteolytic enzymes capable of digesting an array of biological polymers, including proteins, nucleic acids, carbohydrates and lipids. Lysosomes function as the cellular digestive organ, degrading material taken up from outside the cell and obsolete components of the cell itself (De Duve and Wattiaux, 1966). Lysosomes degrade both functional and damaged proteins and organelles. This ensures continuous renewal and recycling of cellular constituents and avoids accumulation of warn out components.

By electron microscopy, lysosomes can be visualized as dense spherical vacuoles, but they can display considerable variation in size and shape as a result of their content that has been taken up for digestion. The lysosomal lumen contains about 50 different digestive enzymes, all of which are acid hydrolases (active at the acidic pH of the lysosomal lumen). The lysosomal membrane contains primarily permeases. A proton pump, residing in the lysosomal membrane, is responsible for maintaining their acidic internal pH. This is accomplished by active transport of protons (H^+ ions) from the cytosol into the lysosome. The concentration of H^+ in the lysosome is about 100-fold higher than that of cytosol. The lysosomal acid hydrolases are responsible for degradation of the delivered cargo, whereas permeases are responsible for recycling essential nutrients (amino acids, fatty acids, sugars, etc.) from the degraded products back to the cytosol (De Duve and Wattiaux, 1966).

Degradation of material taken up from outside the cell by endocytosis relates not only to lysosomal function but also to lysosomal formation. Lysosomal formation represents an intersection between the endocytic and the secretory pathways. Extracellular material is taken up by clathrin-coated endocytic vesicles (originate from the plasma membrane), which then fuse with early endosomes that gradually mature into late endosomes (Luzio et al., 2007). These late endosomes fuse with transport vesicles budded from the *trans*-Golgi network to form the lysosome. The *trans*-Golgi vesicles contain mannose-6-phosphate receptors which function to attract acid hydrolases (contain mannose-6-phosphate residues). A hallmark during endosome maturation is the lowering of the internal pH to about 5 (Mellman et al., 1986). This is critical for the release of acid hydrolases from the mannose-6-phosphate receptors into the lysosomal lumen and recycling of the receptors back to the Golgi network.

There are various pathways by which lysosomes receive the extracellular and intracellular material that is to be degraded (Fig. 2) (Dice, 2000). Endocytosis can internalize extracellular proteins, as well as integral membrane proteins and sequester them in endosomes. Subsequently, these endosomes fuse with lysosomes and deliver the cargo (Luzio et al., 2007). Crinophagy is the process by which secretory proteins are delivered to lysosomes by fusion with secretory vesicles (Glaumann, 1989). Autophagy is the bulk degradation of cytoplasm and organelles by promoting the transfer of material from one, topologically distinct compartment, to another from the cytosol to the vacuole in yeast or to lysosomes in eukaryotic cells (Mizushima et al., 2008). Chaperone-mediated autophagy (CMA) is a more selective degradation process of autophagy in which a specific sequence in the target substrate protein is recognized by cytoplasmic molecular chaperones. Subsequently, the substrate-chaperone complex is delivered to lysosome for degradation (Dice, 2007).

Lysosomes are the major organelles responsible for proteolytic degradation and recycling. The main function of these microscopic organelles is the digestion of macromolecules derived from phagocytosis, endocytosis and autophagy (Fig. 2). Autophagy is a catabolic process that eliminates aggregates of aberrant proteins, superfluous or damaged organelles and sometimes even entire organisms such as, invading bacteria. Although, autophagosomes possess some hydrolytic activity, this activity is insufficient to complete proteolytic degradation. Thus, fusion of autophagosomes with lysosomes can be viewed as the Achilles heel in the process of autophagy.

5. Lysosomes and aging

Age-related decline in overall proteolytic activity has been observed in almost all organisms and progressive intracellular accumulation of damaged proteins with age has been extensively documented (Ward, 2002). Thus, the activity of lysosomes becomes pivotal in adequately removing damaged products in aged organisms and explains the reason why lysosomes are at

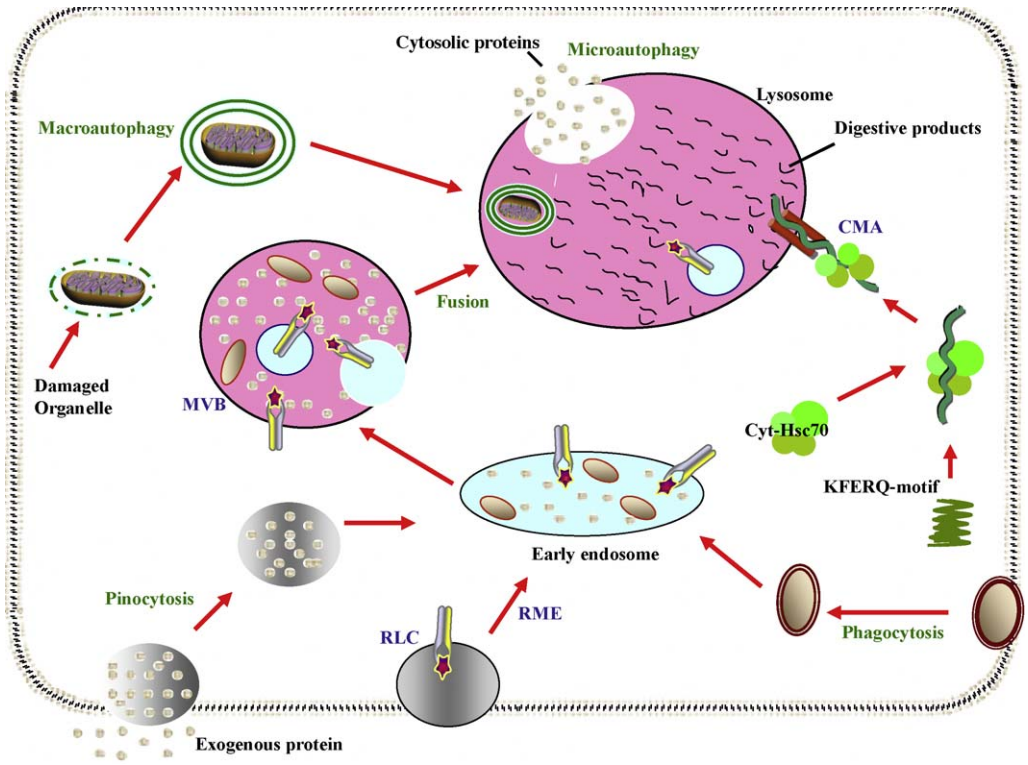

Fig. 2. The lysosome-mediated cellular degradative machine. Lysosomes are responsible for digestion of both exogenous material and endogenous proteins and cellular organelles. Exogenous proteins are targeted to the lysosome through phagocytosis, pinocytosis, and receptor-mediated endocytosis. Endogenous proteins and cellular organelles are targeted by microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). The various intermediate structures and organelles (endosomes, autophagosomes, multivesicular bodies) are mostly devoid of hydrolases. CMA: chaperone-mediated autophagy. RLC: receptor ligand complex. RME: receptor-mediated endocytosis. MVB: multivesicular bodies.

center stage of aging research looking for possible explanation for decreased proteolysis with age (Martinez-Vicente et al., 2005).

Most evident and convincing age-related alteration in lysosomes is the accumulation of lipofuscin. Lipofuscin is a pigmented product that accumulates in lysosomes and other lysosome-related vesicles due to incomplete digestion of engulfed components and subsequent intra-lysosomal oxidation (Brunk and Terman, 2002). Although lysosomes receive material for degradation from several intracellular mechanisms, autophagy is quantitatively the major process that delivers substrates to the lysosomal compartment. Since many autophagocytosed macromolecules contain iron (e.g., ferritin and mitochondrial electron-transport complexes), the lysosomal compartment becomes rich in this essential but potentially hazardous transition metal (Terman et al., 2007). Diffusion of cytoplasmically produced peroxides into the lysosome could result in Fenton-type reactions and hydroxyl radical production, which in turn can lead to peroxidation of the lysosomal protein content and formation of lipofuscin. Lipofuscin is a yellowish-brown, autofluorescent, nondegradable polymeric substance. Accumulation of lipofuscin reduces the degradative capacity of lysosomes, which in turn results in further accumulation of waste material inside the lysosome (Terman et al., 1999). Interestingly, leupeptin, a protease inhibitor of lysosomal proteases causes lipofuscin and ceroid accumulation in lysosomes (Ivy et al., 1984), while leupeptin-infusion in a transgenic mouse model of Alzheimer's disease (AD) results in the onset of neurodegenerative defects (Nixon et al., 2001) providing evidence that dysfunction of lysosomes is involved in age-related diseases. The rate of lipofuscin formation is inversely related to age and lysosomes filled with lipofuscin have a reduced ability to fuse with autophagic structures (Terman et al., 2007) The molecular basis of the age-related decline in different autophagic pathways is the subject of intense investigation (Levine and Klionsky, 2004).

As mentioned earlier, lipofuscin is nondegradable and cells cannot get rid of it (Terman and Brunk, 1998). However, actively dividing and growing cells can partially eliminate lipofuscin by diluting the pigment in each mitotic cycle. Post-mitotic cells do not have this capability. It has previously been observed that while accumulation of lipofuscin in postmitotic cells of short-lived animals is very rapid, in long-lived animals it is very slow (Nakano et al., 1995). This finding suggests a relationship between lipofuscin accumulation, function of the lysosomal compartment and cell survival. It is also worth mentioning that mitochondria of long-lived animals tend to produce significantly less superoxide and hydrogen peroxide than short-lived ones (Ku et al., 1993).

The presence of undigested materials in lysosomes could be responsible for their impaired ability to fuse and/or degrade the autophagosomal contents. Lysosome-associated membrane protein type 2a (LAMP-2a) is a receptor protein present on lysosome membrane and responsible for binding and uptake of chaperone–substrate complex in chaperone-mediated autophagy. A decrease in the lysosomal levels of the LAMP-2A is the primary defect responsible for the diminished CMA activity during aging (Terman et al., 2007). Normal CMA activity is initially maintained (during middle age) by increasing the amount of luminal chaperone. At advanced ages, the levels of the receptor are so low that compensation by the chaperone is no longer possible (Cuervo and Dice, 2000). The reasons behind the decline in receptor numbers are not known and are currently under investigation. LAMP-2A is not only critical for CMA but also for maturation of autophagosomes and efficient fusion with lysosomes. In recent studies (Zhang and Cuervo, 2008), induction of LAMP-2A over expression in the liver of aged mice lowered intracellular accumulation of damaged proteins and dramatically improved overall organ functionality. Restoring the levels of LAMP-2A, didn't only increase CMA but also upregulated macroautophagic and

proteasomal degradative pathways, increased the levels of intracellular ATP and reduced the levels of cytosolic waste (oxidized and polyubiquitinated aggregated proteins). Interestingly, these changes coincided with a dramatic increase in the number of autophagosomes indicating the importance of autophagy in the aging process.

6. Autophagy

Autophagy (derived from the Greek meaning "to eat oneself") is present in all eukaryotic cells and is evolutionarily conserved from yeast to humans (Levine and Klionsky, 2004). Autophagy is a ubiquitous catabolic process that involves the bulk degradation of cytoplasmic components through a lysosomal pathway. This process is characterized by the engulfment of part of the cytoplasm inside double-membrane vesicles called autophagosomes. Autophagosomes subsequently fuse with lysosomes to form an autophagolysosome in which the cytoplasmic cargo is degraded and the degradation products are recycled for the synthesis of new molecules (Lee and Marzella, 1994). Turnover of most long-lived proteins, macromolecules, biological membranes, and whole organelles, including mitochondria, ribosomes, endoplasmic reticulum and peroxisomes, is mediated by autophagy (Cuervo, 2004). The autophagic machinery actually mediates the majority of intracellular housekeeping tasks. Short-lived nuclear and cytoplasmic proteins are degraded by multicatalytic proteinase complexes (proteasomes) and by calcium-dependent neutral proteases (calpains). Mitochondria can autonomously turn over certain defective proteins, because they possess their own proteolytic systems (Bakala et al., 2003; Arnold and Langer, 2002)

The cellular process of autophagy was initially described over 40 years ago. Only recently though, we have started understanding its role in various pathophysiological conditions and unraveling the basic cell biology mechanism. Discovery of the *ATG* genes in yeast has greatly advanced our understanding of the molecular mechanisms involved in autophagosome biogenesis and the various pathways that regulate autophagic activity (Suzuki and Ohsumi, 2007). Most yeast *ATG* genes have orthologues, in multicellular organisms (nematodes, flies) and mammals, which suggests that the basic machinery for autophagy has been evolutionarily conserved in eukaryotic organisms. These genes, also called APG/AUT/CVT, were initially identified from studies that investigated defects in the formation of autophagic vacuoles and protein degradation in *Saccharomyces cerevisiae* (Takeshige et al., 1992; Thumm et al., 1994). In these studies, knockout strains of individual ATG genes showed defects of starvation-induced autophagy. It is now widely accepted that at least 30 yeast genes are actively involved in autophagy. More than 50 additional yeast genes may be required for autophagy, which also play key roles in other pathways (Klionsky et al., 2003). The precise cellular and biochemical function of these genes in the autophagic pathway remains to be elucidated. Although the role of autophagy in the aging process has yet to be fully defined, studies in model organisms (*C. elegans*) and mammals (mice) have begun to understand a growing number of aging processes that are influenced by autophagy. It is now largely accepted that autophagy affects several cellular activities crucial for longevity and healthy aging (eulongevity).

6.1. Forms of autophagy

Eukaryotic cells are equipped with several degradation systems, predominantly two proteolytic systems contribute to cellular clearance: the ubiquitin-proteasome and the lysosomal systems. Autophagy is responsible for the degradation of whole organisms and structures (bacteria, viruses), whole organelles, as well as long-lived soluble proteins (Reggiori and Klionsky, 2002; Wang and Klionsky, 2003). The differences among the various autophagic mechanisms reside in the type of cargo/waste, the route and mechanism for its delivery to lysosomes and the physiological conditions in which each process is activated. Despite the variety of autophagic processes that have been described, they all fall into one of three main types: macroautophagy, microautophagy and chaperone-mediated autophagy (Mizushima et al., 2008; Klionsky, 2005; Cuervo, 2004).

Classic autophagy or macroautophagy is an inducible form of autophagy that becomes activated under stress conditions (nutrient deprivation, infections, and toxins) (Dice, 2000). Macroautophagy is the most extensively studied and quantitatively the most important form of autophagy. In this process, entire regions of the cytosol are sequestered by a *de novo*-synthesized membrane that seals into an autophagosome (Fig. 4) (Yorimitsu and Klionsky, 2005). These double-membrane vesicles fuse with secondary lysosomes, and in the process, acquire the proteases required for degradation of the sequestered material. Two major intracellular kinase complexes, the beclin-VPS34 complex and the mTOR (mammalian target of rapamycin, as it is referred in mammalian cells) complex, act in coordination to modulate formation, circularization, and fusion of the autophagic compartments with lysosomes (Yorimitsu and Klionsky, 2005; Ohsumi, 2001). For a long time, macroautophagy was considered as an inducible form of autophagy. Growing evidence though, indicate the existence of basal macroautophagy, essential for maintenance of cellular homeostasis in different organs (Hara et al., 2006; Komatsu et al., 2006; Nakai et al., 2007).

Most eukaryotic cells are equipped with another form of autophagy, known as CMA (Dice, 2007). CMA is selective for a particular group of soluble cytosolic proteins that contain a specific sequence signature. Unlike autophagy, CMA does not require intermediate vesicle formation (Majeski and Dice, 2004). Once the specific signature sequence is identified, these soluble proteins are directly translocated to the lysosomal membrane. The specific signature sequence, which is present in all CMA substrates, is biochemically related to the pentapeptide KFERQ (Dice, 1990). All substrates with this sequence are then targeted to lysosomes. The cytosolic molecular chaperone heat shock protein 70 (hsp70) and its co-chaperones recognize the signature motif in substrate proteins (Hayes and Dice, 1996). 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 (LAMP-2a)(Cuervo and Dice, 1996). A second chaperone, lysosomal hsc73 (lys-hsc70), is required for complete translocation of the substrate protein/hsc70 complex into the lysosomal matrix, where it is completely degraded by lysosomal proteases (Terlecky et al., 1992).

The third form of autophagy is microautophagy (Klionsky, 2005). The characteristic feature of the microautophagy process is that the lysosomal membrane itself either invaginates or exvaginates to engulf an organelle. Unlike macroautophagy, there is no sequestering double-membrane formed in the cytoplasm (Mortimore et al., 1988). Pexophagy (Farre and Subramani, 2004) and Mitophagy (Kim et al., 2007) involve the selective sequestration or engulfment of peroxisomes and mitochondria, respectively, and their delivery to lysosomes. The process of microautophagy is poorly characterized, at least in mammalian cells. Variations of microautophagy have been described in yeast for the selective degradation of peroxisomes (micropexophagy) or even nuclear regions (piecemeal microautophagy) (Klionsky et al., 2007).

6.2. The autophagy process

The stages involved in the process of autophagy have not as yet been precisely delineated, especially for mammalian cells. Studies

in the yeast *S. cerevisiae* have provided insight into the mechanisms involved and based on these studies, the following steps of autophagy are largely accepted: induction and cargo selection, vesicle nucleation and expansion, lysosome targeting, lysosome docking and autophagosome-lysosome fusion, vesicle breakdown and recycling (Klionsky and Emr, 2000).

Autophagy may be either a selective or nonselective process (Reggiori and Klionsky, 2005). In *S. Cerevisiae,* Cvt is a transport mechanism involved in the recognition and packaging of cargo and is considered a selective process. However, this pathway is limited only to yeast; it is not present in any other organism (Shintani and Klionsky, 2004). In general, macroautophagy is considered to be nonspecific, however there are also specific type of macroautophagy such as in certain cellular conditions mitochondria are selectively trapped by autophagosomes. Further some types of microbes are selectively killed by autophagy. Growing line of evidence has revealed that there are reliable substrate for selective autophagy in mammals such as p62 and Nbr1 (Bjørkøy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007; Ichimura et al., 2008; Kirkin et al., 2009a,b). SQSTM1/p62 is the first proposed example of receptor proteins that mediate recognition and targeting of ubiquitinated substrates for their degradation via selective autophagy (Bjørkøy et al., 2005). Recently it has been shown that NBR1 (neighbor of BRCA1 gene 1) also act as receptors for selective autophagosomal degradation of ubiquitinated targets (Kirkin et al., 2009a). Further, it is proposed that NBR1 together with p62 promotes autophagic degradation of ubiquitinated targets and simultaneously regulates their aggregation when autophagy becomes limited (Kirkin et al., 2009b).

At the molecular level, the serine/threonine protein kinase TOR (target of rapamycin, as it is referred in yeast) is involved in the induction of autophagy (Petiot et al., 2000). Phosphorylated TOR is part of the induction complex and acts as a negative regulator of autophagy (Schmelzle and Hall, 2000). Phosphorylated TOR negatively controls autophagy, primarily by acting on the signaling cascade that controls general translation and transcription. In addition, activated TOR induces hyperphosphorylation of Atg13, which lowers its binding affinity to other Atg interacting proteins, thereby inhibiting autophagy (Kamada et al., 2000).

The initial formation of an autophagosomal membrane takes place by enwrapping the degradative cargo within a double membrane, which eventually elongates to form a vesicle called an autophagosome. The exact origin of autophagosomal membranes is controversial. Studies in mammalian cells have suggested that autophagosomal membranes originate from the ribosome-free region of the rough endoplasmic reticulum (Dunn, 1990). Alternatively, a poorly characterized organelle called a phagophore, has also been suggested as the origin of autophagosomes and other vesicular structures (Seglan et al., 1996). In yeast, a unique perivacuolar structure, called PAS (preautophagosomal structure), has been proposed as the precursor of autophagosomes due to the transient association of several Atg proteins with it (Noda et al., 2002). A PAS-like structure has not yet been identified in any other endomembrane system (Kim et al., 2002).

Vesicles will elongate and extremities will fuse to complete the double-membraned structure. Phagophores elongate and completely encircle the cargo to form an "autophagosome" or "autophagic vacuole" (Fig. 4) (Reggiori and Klionsky, 2002). Most of the proteins involved in vesicle expansion and maturation steps are retrieved to the original pool because they do not associate with the complete and mature autophagosome (Levine and Klionsky, 2004; Reggiori and Klionsky, 2002; Klionsky, 2005). Therefore, it has been suggested that the proteins involved in autophagosome formation are retrieved for future use. Atg8 (LC3) is the exception; it is found on the mature autophagosome and can be used as a valuable marker to track these structures (Ohsumi, 2001).

The next critical step is transport and fusion of the autophagosome with the lysosomes. The outer membrane of the autophagosome completely fuses with the outer membrane of the lysosome to make a path for the inner membrane bound autophagic vacuole. The inner membrane and its enclosed cytoplasmic contents, which together are called an "autophagic body", are released into the lumen of the lysosome (Fig. 4). The machinery required for the process of vesicle fusion includes SNARE proteins and the class C Vps/HOPS complex (Wang and Klionsky, 2003). The ultimate step in the autophagic process is degradation of the autophagic body content by lysosomal enzymes, which ensures recycling of essential cytoplasmic contents.

6.3. Molecular machinery of autophagy

Regulation of autophagosome biogenesis, maturation, and fusion with lysosomes is a complicated process and a number of diverse signaling complexes and pathways are involved (Fig. 3). Induction of autophagosome biogenesis requires two complexes. The first one, that initiates vesicle formation, contains the Class III PI3K (Vps34), Beclin1/Atg6, Atg14 and Vps15/p150. The second one, responsible for the vesicle nucleation, contains Atg1, Atg11, Atg13 and Atg17, the association of which is controlled by TOR. TOR is a classic serine/threonine kinase. Nutrient starvation or treatment of yeast cells with rapamycin (a specific inhibitor of TOR) rapidly induces an increase in autophagosome formation (Beck and Hall, 1999). Inhibition of TOR results in dephosphorylation of Tap (Klionsky et al., 2003) causing its dissociation from PP2A. PP2A becomes activated, causing dephosphorylation of downstream targets and activation of Atg1. In mammalian cells, mTOR appears to modulate autophagy in a manner similar to that observed in yeast. Likewise, the activity of the mammalian PP2 A orthologue is strongly correlated to induction of autophagy (Holen et al., 1992).

The observation that 3-methyladenine (PI3K inhibitor) inhibits autophagy was the first evidence that implicated the Vps34/class III PI3K family of kinases in the process (Seglen and Gordon, 1982). In addition, other PI3K inhibitors, such as wortmannin and LY294002, have also been found to inhibit autophagy. In yeast, Vps34 is primarily involved in vacuolar protein targeting, through the endosomal/prevacuolar compartment, to form a complex with Vps15-Vps38-Atg6 (Kihara et al., 2001a). At the same time, Vps34 has been found to associate with Vps15-Atg6-Atg14 on pre-autophagosomal structure (PAS) or phagophore assembly site (Obara et al., 2006). PAS is a perivacuolar site where transient accumulation of most of the Atg proteins takes place and consider as autophagosomes origin site (Suzuki et al., 2001). Atg 14 and Atg 6 are important in mediating the localization of other Atg protein to the PAS (Suzuki et al., 2007). However, the mechanism by which Vps34 affects autophagosome formation is largely unknown. In mammalian cells, there are three classes of PI3Ks. So far, only Class I and Class III PI3K have been implicated in autophagy, primarily in the early steps of autophagosome formation (Petiot et al., 2000) Class III PI3K has been found to associate with beclin-1 and p150, which are the orthologues of yeast Atg6 and Vps15, respectively. Class III PI3K regulates diverse molecular pathways, including several involved in tumor formation.

The first autophagy-related tumor suppressor gene reported was *Beclin-1,* the mammalian orthologue of yeast Atg6, which showed a relationship between autophagy and cancer (Liang et al., 1999). Beclin 1 was identified as a new binding partner for Bcl-2 by Liang et al. (1999) using yeast two-hybrid system (Liang et al., 1998) and later on Beclin 1 was the first protein shown to indispensible for autophagy (Liang et al., 1999). Beclin1/Vps34 complex produces Phosphatidylinositol (3) phosphate which is an early step in the autophagosome formation (Kihara et al., 2001b).

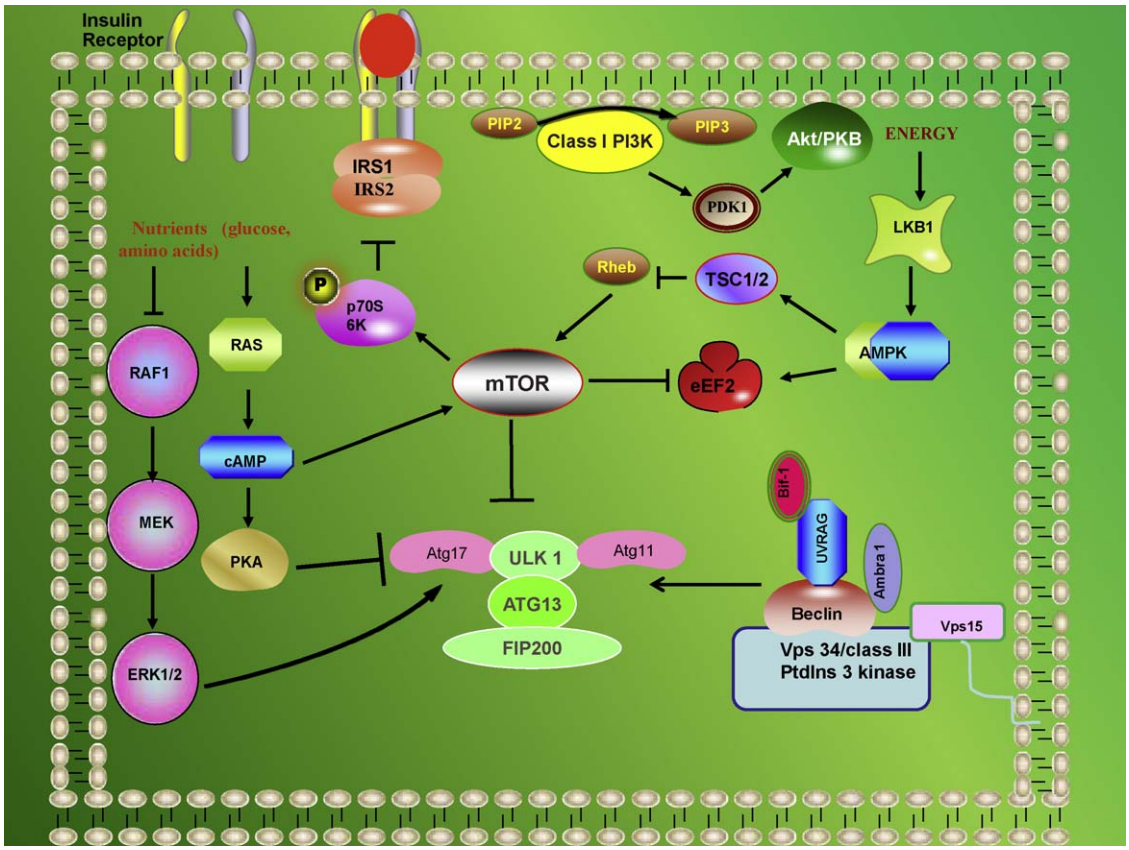

Fig. 3. Regulation of autophagy: important pathways regulating autophagy includes TOR (target of rapamycin), which is at the center of the diverse pathways linked to autophagy, Class I and Class III PI3 kinase pathways.

This step is important for the recruitment of other ATG proteins. Afterwards, the sequential recruitment of ATG12 and its covalent binding with the ATG5 pool on the isolation membrane triggers autophagosome formation. Now, ATG12–ATG5 conjugate binds with ATG16 and this trimeric complex oligomerizes and localize on the external surface forming the autophagosome (Fig. 4). ATG12–ATG5–ATG16 and other ATG complex signals the recruitment of LC3 (ATG8) and forms ATG12–ATG5 and LC3 conjugate. Interaction of Beclin 1 with Vps34 and Vps15 is conserved in mammals (Kihara et al., 2001b). Additionally, Beclin 1 interacts with UVRAG (the UV irradiation resistance-associated gene protein) (Liang et al., 2006), Ambra1 (activating molecule in Beclin 1-regulated autophagy) (Fimia et al., 2007) and Bif-1 (also called endophilin B1) (Takahasi et al., 2007). UVRAG, Ambra1 and Bif-1 are all positive regulator of autophagy and indispensable for the activation of autopagy, as well as for the optimal activation of Vps34.

In yeast, Atg1 is involved in both the Cvt and autophagic pathways, and it is found in association with Atg11 and Atg 13 (Kamada et al., 2000). Under nutrient-rich conditions, TOR activation causes hyperphosphorylation of Atg13 that prevents its association with Atg1 and it enables interaction of Atg1 with Atg11; this may determine the use of the autophagic machinery in the Cvt pathway. Under nutrient starvation or treatment with rapamycin, Atg13 becomes partially dephosphorylated, leading to an Atg1–Atg13 interaction, which subsequently triggers autophagy and generation of autophagosomes instead of Cvt vesicles. Atg17 is an Atg13 interacting protein that is part of the Atg1 complex and modulates the kinase activity of Atg1 (Kamada et al., 2000). Recently, hierarchy map analysis of Atg proteins involved in PAS organization has suggested that phosphorylated Atg 17 is the most basic protein in PAS organization: when it is specifically targeted to the plasma membrane, other Atg proteins are recruited to that location, suggesting that Atg17p acts as a scaffold protein to organize Atg proteins to the PAS (Suzuki et al., 2007).

In yeast, vesicle expansion and maturation is controlled by two ubiquitin like conjugation complexes; the Atg12–Atg5 conjugation complex and the Atg8 lipidation complex. In mammalian cells, the Atg8 orthologue MAP-LC3 (microtubule-associated protein light chain 3) is associated with GATE-16 (Golgi associated ATPase enhancer of 16 kDa) and GABARAP (γ-aminobutyric acid type A receptor-associated protein) (Okazaki et al., 2000). This complex was found to interact with ULK1, the mammalian orthologue of Atg1 (Yan et al., 1998). GATE-16 and GABARAP are the other two mammalian Atg8 orthologues. For vesicle expansion, the carboxy terminus of Atg8 (MAP-LC3) must first be removed by the action of the cysteine protease Atg4. This cleavage exposes a reactive glycine in the C-terminus of Atg8 (MAP-LC3-I) that is subsequently activated by the Atg3 and Atg7 enzymes, and covalently linked to phosphatidylethanolamine (MAP-LC3-II-PE) on the autophagosomal membrane. This action of Atg3 and Atg7 requires activation by the Atg12–Atg5–Atg16 conjugation system, which dissociates from the membrane once autophagosome maturation is completed. The subsequent fusion with the lysosome (vacuole in yeast) leads to engulfment of the autophagosome, breakdown of its internal membrane and degradation of its cytosolic content by the action of lysosomal hydrolases.

7. Autophagy and aging

Genetic studies in yeast were pivotal in identifying the role of autophagy genes in cellular aging. As a result of this discovery, yeast homologues were identified in higher eukaryotic organisms that fueled aging-related research in more evolutionary advanced species. *C. elegans* has been extensively used as a model organism

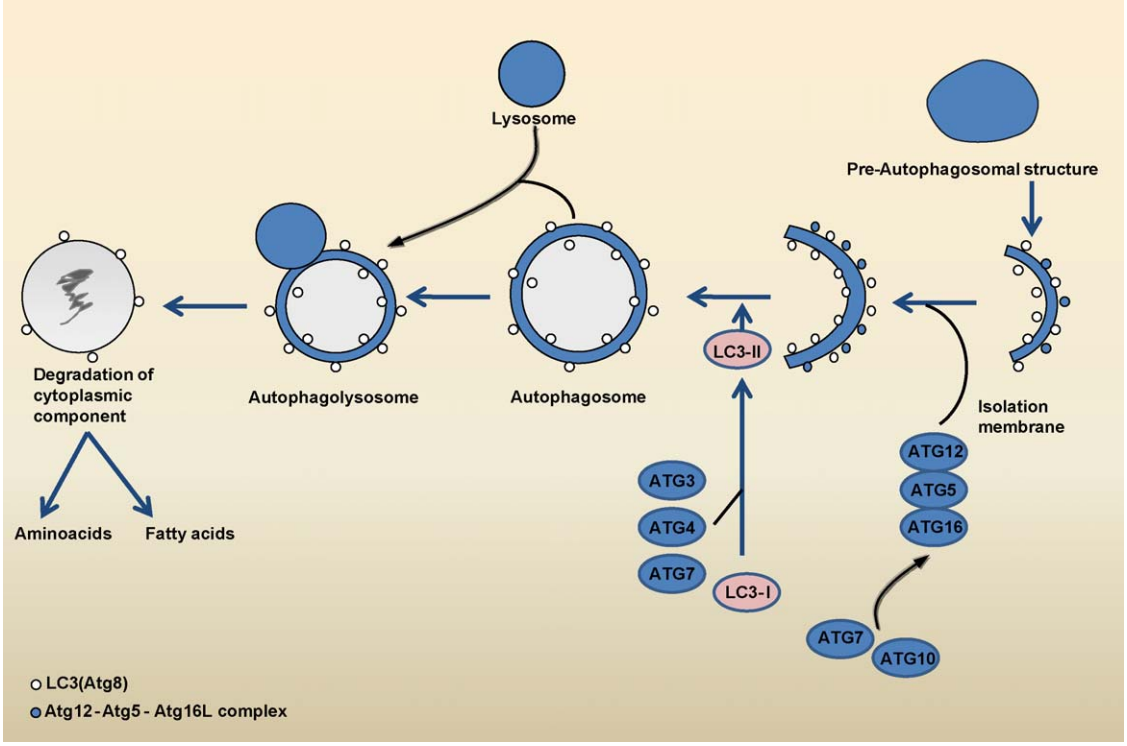

Fig. 4. Autophagosome formation and lysosomal fusion. LC3(ATG8) is well known autophagosome marker (LC3-II-PE).

to study the mechanisms controlling life span and has provided the first evidence linking autophagy to the aging process. In this organism, RNA-i mediated depletion of Beclin-1 leads to incomplete dauer morphogenesis, increased deposition of abnormal structures and pigment, and reduced life span (Meléndez et al., 2003). Moreover, down regulation of Beclin-1 reverses the increased life span of Daf-2 mutants, indicating the importance of autophagy genes in longevity. Similar evidence has also been obtained from studies in Drosophilla (Toth et al., 2008; Simonsen et al., 2008). In *Arabidopsis* plants, knockout mutants of ATGs (Atg7, Atg9, Atg4) have normal growth but accelerated leaf senescence and are hypersensitive to nitrogen and carbon starvation (Doelling et al., 2002; Hanaoka et al., 2002 and Yoshimoto et al., 2004). Timosaponin A-III, a saponin isolated from the rhizome of *Anemarrhena asphodeloides* induces apoptotic death in HeLa cells through autophagic response. The autophagic response was blocked by the autophagy inhibitor 3-methyladenine or small interfering RNA against the autophagic gene Beclin 1 (Sy et al., 2008). A transgenic mouse model of progeroid syndrome with premature aging phenotype has increased basal autophagic activity due to the activation of LKB1-AMPK and inhibition of mTOR, suggesting that autophagy may be a compensatory mechanism to restrain the progression of the disease (Mariño et al., 2008). Importantly, this mutant mouse has short lifespan indicating that the dynamic expression of the autophagic pathway is more important than a fixed level. Conditional knockout of *atg7* gene in liver (essential for ATG congugation and autophagosome formation) caused tissues with abnormal mitochondrial morphologies and ubiquitin-containing aggregates (Komatsu et al., 2005). Comprehensive proteomic studies in the livers of these mice revealed increased protein mass and pathological conditions resembling oxidative damage (Matsumoto et al., 2008). Dolichol, an isoprenoid compound similar to cholesterol, accumulates over time and precedes liver deterioration; therefore, it has been proposed as a biomarker of aging (Parentini et al., 2005). Similarly, targeted deletion of the Atg5 gene in mice neurons resulted in loss of motor and cognitive functions, and accumulation of cytoplasmic inclusion bodies (Hara et al., 2006). These findings are consistent with the role of autophagy in aging and promotion of survival in nutrient-deficient conditions as it was seen in yeast and other eukaryotes.

Like in aging, there is great sensitivity of the autophagic response to nutritional state implying that certain nutrients regulate this response. Nutrient deprivation and especially reduction in certain intracellular glucogenic amino acids, mainly glutamine, triggers the autophagic protein degradation in liver (Schworer and Mortimore, 1979). Similarly, older organisms benefit from undernutrition (caloric restriction diet, CR) as it improves lysosomal degradation of liver cells and slows down aging (Donati et al., 2001). Studies on different CR regimens are needed to address whether prolonged CR or interrupted CR increases life span.

Studies in *C. elegans*, Drosophila and mice show an evolutionary conserved regulation of longevity by insulin (Klass, 1983; Kenyon et al., 1993; Holzenberger et al., 2003; Blüher et al., 2003). Binding of insulin to its cell surface receptor induces autophosphorylation of the receptor and activation of its kinase activity (Fig. 3). Subsequently, the receptor phosphorylates intracellular substrates such as insulin receptor substrate IRS-1 and IRS-2 (Sun et al., 1991; Frattali et al., 1992). In mice, disruption of IRS-2 causes type-2 diabetes, a disease prevalent in elderly (Withers et al., 1998). Lifespan in genetically engineered mice with brain-specific IRS-2 knockout increased significantly although they became overweight and hyperinsulinemic (Taguchi et al., 2007). Likewise, in fruit fly loss of insulin-like receptor (Tatar et al., 2001) or its substrate *chico* (IRS) (Clancy et al., 2001) extended lifespan. In *C. elegans* mutations in positive regulators of insulin signaling, such as Daf-2 (insulin receptor), Daf-16 (HOXO transcription factor) and Age-1 (PI3K), increase lifespan by up to 200%, whereas mutations in negative regulators of this pathway such as Daf-18 (PTEN) shorten lifespan (Finch and Ruvkun, 2001; Libina et al., 2003). These observations indicate that insulin signaling negatively

regulates aging, autophagy and longevity in model organisms. Studies in humans are more complicated due to the presence of insulin receptors in many organs and the pleiotropic actions of insulin.

Accumulating evidence has suggested that lifespan extension is dependent on efficient maintenance of autophagy (Hansen et al., 2008; Bergamini et al., 2007; Zhang and Cuervo, 2008; Salminen and Kaarniranta, 2009). The yeast histone deacetylase Sir2 (silent information regulator-2) is known to regulate cellular senescence and the lifespan of budding yeast (Sinclair et al., 1998). It has also been shown that increased expression of Sir2 orthologues is sufficient to extend life span in lower organisms Guarente and Picard (2005). Mammalian sirtuins (SIRTs) are homologues of yeast Sir2 (with SIRT1 being the closest) and are known to regulate a variety of physiological processes, such as metabolism, stress responses, cellular survival, and senescence (Michan and Sinclair, 2007; Guarente, 2006; Longo and Kennedy, 2006). The sirtuin family consists of seven members (SIRT1-7), each containing a conserved catalytic core domain. SIRTs have diverse cellular localization and a large number of target substrates destined for deacetylation or ribosylation, thus affecting numerous cellular functions (Haigis and Guarente, 2006). Most sirtuins (SIRT1, SIRT2, SIRT3, and SIRT5) catalyze the NAD± dependent deacetylation of target proteins, whereas SIRT4 and SIRT6 mediate the ADP-ribosylation of protein substrates. Recent in vitro and in vivo studies have suggested a direct role of SIRT1 in autophagosome formation and regulation of the autophagic process (Lee et al., 2008). Several autophagy proteins, such as Atg5, Atg7, and Atg8 that are known to be major regulators of autophagy, are deacetylated by SIRT1 in an NAD± dependent manner (Lee et al., 2008). Embryonic fibroblasts of SIRTH- mouse contain high levels of acetylated autophagy proteins and do not display starvation-induced autophagy. This defect can be largely corrected by transient overexpression of SIRT1 (Lee et al., 2008). It is interesting that SIRT−/− and Atg5−/− mice have extensive phenotypic similarities, such as accumulation of damaged organelles, and particularly mitochondria. Moreover, caloric restriction can extend the lifespan of several lower eukaryotes and rodents, and this effect is believed to be mediated by increased expression of SIRT1 (Boily et al., 2008). These pioneering studies have largely suggested acetylation as an important post-translational modification in the mechanism of autophagosome formation. The exact mechanism however still remains unknown.

An important observation with yeast strains deficient in autophagy was the accumulation of damaged mitochondria. Mitochondria, the power house of the cells, plays a central role in the biology of the cell. Physiologic cellular function requires that damaged mitochondria must be engulfed in autophagosomes and delivered to lysosomes or vacuole for degradation and possible recycling. Aged cells tend to accumulate mitochondria with abnormal phenotypes including point mutations and deletions of mtDNA. To test whether there is a causative relation between mtDNA defects and aging, Trifunovic et al. (2004) created a mice that carries a mutant PolgA (catalytic subunit of mtDNA polymerase) (Trifunovic et al., 2004). The genetically engineered mice exhibited phenotypes of premature ageing characterized by premature weight loss, hair loss, spine defects (kyphosis), reduced fertility and lifespan. Another study, using similar genetic approach (Kujoth et al., 2005), confirmed these results and additionally found that aging acceleration was not due to increased ROS production but rather increased apoptosis. Other aging-related phenotypes found in the mitochondria-defective mice were loss of muscle mass (sarcopenia) and hearing loss (loss of spiral ganglion neurons). These findings demonstrate beyond correlation that defective mitochondria accelerate aging. Therefore, elimination of damaged mitochondria would prolong life (Tolkovsky et al., 2002).

Neuronal degeneration is evident during aging due to environmental toxins, ischemic or diabetic-related damage and may lead to loss of cognitive (memory, attention) and physical functions. An upregulation of Beclin 1 has been detected in neurons undergoing degeneration but is role in neuronal loss is not well understood (Erlich et al., 2006). Several studies have suggested a protective role for autophagy against the development of a subset of neurodegenerative diseases associated with misfolded and aggregated proteins such as Alzheimer's disease, Huntington's disease (HD), Parkinson's disease and oculopharyngeal muscular dystrophy (Ravikumar et al., 2004, 2005). At early stages of Alzheimer's disease neuronal autophagy is significantly upregulated while lysosomal and mitochondrial overload is evident as the disease progresses. Autophagy may also be involved in β-amyloidogenesis because β-amyloid production decreases when autophagic vacuole formation is blocked (Rubinsztein et al., 2005).

Damaged cells are found scattered both in healthy and tumor tissues and are being eliminated through apoptosis, so tissue inflammation is prevented. If apoptosis however fail to occur prolonged inflammation may result in autoimmune diseases (Bratton and Henson, 2005) and even cancer. Studies investigating the relationship of autophagy and apoptosis have yielded conflicting results. Evidence in *Droshophila* has suggested that the two processes occur at the same time (Riddle and Gorski, 2003; Lee et al., 2003). However, studies in primary neurons and cell lines found autophagy preceding apoptosis (Xue et al., 1999). These findings are very interesting and more studies are required to address this issue.

8. Perspective

Aging is an intrinsic property of biological systems that results from the accumulation of defects after exposure to harsh environmental conditions, such as, nutrient limitation, temperature extremes, osmotic changes, hormone stimulation, radiation exposure and pollutants. Aging phenotypes were described long before the molecular basis of the phenomenon was understood. Studying the molecular basis of aging poses many challenges because it involves interactions at many levels of organization occurring at different rates and for prolonged periods. Whole body phenotypes of aging are crucial in studying the role autophagy in the process. They will enable experimental approaches on organs and cellular entities that have been most affected by it. The efforts of the many research teams involved in this field brought exciting new evidence on the different roles of autophagy in cell biology that created an explosion of interest. However, many unanswered questions still remain. Autophagy is a dynamic process; to better understand how autophagy is regulated, we need more comprehensive datasets of the co-expression of several genes involved in this process under different conditions (i.e. stimulus levels, genetic backgrounds). It is already evident that extensive cross-talk between different signaling pathways and the cell cycle exists and results in different autophagic responses. The evolutionary conservation of the pathway and its molecular components makes it likely that cells are using the same mechanisms to regulate this process. Understanding how autophagy is regulated in simple organisms like yeast has provided important clues on the inner workings of the pathway that are applicable to cells from higher organisms. Future studies should take advantage of noninvasive techniques such as in vivo time-lapse imaging of fluorescently tagged proteins to gain useful information on the spatio/temporal expression of the signaling molecules. Further genetic and biochemical characterization of pathways and components involved may reveal new cellular markers of autophagy that are very much needed. More than 40 years after the initial identification of the autophagosomal structures, the origin of the

membranes involved is still unknown, and the subject of intense investigations. As more cellular markers of autophagy are revealed, we will be able to better understand the process and its qualitative and quantitative changes that determine life or death of the cell.

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