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Ageing-associated changes of lysosomal compartment –  
implications on cellular functions

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**TO FATHER**



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## **ABSTRACT**

The lysosomal compartment is a major site for intracellular degradation. Lysosomal degradation of the cell's own constituents, so-called autophagy, not only provides a cell with nutrients, but also removes damaged and potentially dangerous endogenous structures, thus securing intracellular homeostasis. On the other hand, lysosomes have been shown to be involved in the initial stages of apoptosis, and the protective effect of autophagy has been suggested to switch to cell death when excessive.

Ageing-related changes of cellular structures result from damage caused by reactive oxygen species (ROS), which are an inevitable by-product of aerobic life. Intracellular turnover of compromised organelles and macromolecules, to which lysosomal degradation is a major contributor, does not function perfectly, even under favourable conditions. This inherent incompleteness of lysosomal degradation is responsible for the accumulation of a variety of non-degraded and functionally inefficient structures, which can be considered biological "garbage". Biological "garbage" includes damaged non-degraded macromolecules and organelles, as well as intralysosomal non-degradable polymer-like structure called lipofuscin, or age pigment. Although accumulation of biological "garbage" has been suggested harmful, little is known about the mechanisms of its deleterious effects.

To gain a better understanding of ageing-related changes of the lysosomal compartment and their influence on cell functions, we focused on studying: (1) the role of macroautophagy in the turnover of organelles and lipofuscin formation; (2) the role of biological "garbage" accumulation in the development of ageing-related changes and eventual death of growth-arrested, postmitotic-like cells; (3) the possible cell-protective effect of

mitosis; (4) the influence of lipofuscin on cell survival during complete starvation; and (5) the effects of lipofuscin on lysosomal stability.

As a model of induced biological “garbage” accumulation we used confluent human fibroblasts treated with the autophagy inhibitor 3-methyladenine (3MA). Alternatively, lysosomal degradation was suppressed by using the cysteine protease inhibitor leupeptin, or the cathepsin D inhibitor pepstatin A. As a cellular model of aged cells, we used lipofuscin-loaded human fibroblasts. Lipofuscin-loading was achieved by culturing confluent fibroblasts under hyperoxic conditions for 2-4 months. Using these *in vitro* models, the present study shows that: (1) inhibition of autophagy results in accumulation of lysosome-associated autofluorescent material and mitochondria with low membrane potential; (2) detrimental effect of biological “garbage” accumulation following inhibition of autophagy is prevented by continuous cell division; (3) lipofuscin-loaded cells are more resistant to starvation-induced cell death than control cells; (4) lysosomes of lipofuscin-loaded fibroblasts are more resistant to the organelle-targeted stress than lysosomes of control cells.

Based on the results of the present study we conclude that properly operating autophagic machinery plays a crucial role in preventing age-related changes associated with accumulation of biological “garbage”. We also suggest that continual proliferation is the natural mechanism by which cells cope with the accumulation of non-degradable material, employing mechanical dilution during the cell division. Finally, we introduce an idea of lipofuscin being a hormetic agent, and possibly possessing some lysosome-stabilising properties. Better understanding of the influence of the age-related accumulation of biological “garbage” on cellular functions may be helpful for future development of anti-ageing therapy and management of age-associated pathologies.

## **LIST OF PAPERS**

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Stroikin Y**, Dalen H, Löf S, Terman A, 2004. Inhibition of autophagy with 3-methyladenine results in impaired turnover of lysosomes and accumulation of lipofuscin-like material. *Eur J Cell Biol* 83, 583-90.
- II. Stroikin Y**, Dalen H, Brunk UT, Terman A, 2005. Testing the "garbage" accumulation theory of ageing: mitotic activity protects cells from death induced by inhibition of autophagy. *Biogerontology* 6, 39-47.
- III. Stroikin Y**, Johansson U, Asplund S, Öllinger K, 2006. Increased resistance of lipofuscin-loaded prematurely senescent fibroblasts to starvation-induced programmed cell death. *Biogerontology* (in press).
- IV. Stroikin Y**, Mild H, Johansson U, Roberg K, Öllinger K. Lipofuscin preserves lysosome integrity under conditions of organelle-targeted stress. *Manuscript*.



## ABBREVIATIONS

<b>3MA</b>	3-methyladenine
<b>AMC</b>	7-amino-4-methyl-coumarin
<b>ANOVA</b>	analysis of variance
<b>AO</b>	acridine orange
<b>BrDU</b>	bromodeoxyuridine
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase
<b>Hsp</b>	heat-shock protein
<b>LAMP</b>	lysosome-associated membrane protein
<b>LDH</b>	lactate dehydrogenase
<b>M6PR</b>	mannose 6-phosphate receptor
<b>MSDH</b>	<i>O</i> -methyl-serine dodecylamide hydrochloride
<b>NADH</b>	nicotinamide adenine dinucleotide (reduced form)
<b>NAG</b>	N-acetyl-D-glucosaminidase
<b>NzQ</b>	5,8-dihydroxy-1,4-naphthoquinone
<b>PBS</b>	phosphate buffered saline
<b>PCD</b>	programmed cell death
<b>PI3K</b>	phosphatidylinositol 3-kinase
<b>PI</b>	propidium iodide
<b>ROS</b>	reactive oxygen species
<b>Tor</b>	target of rapamycin



## INTRODUCTION

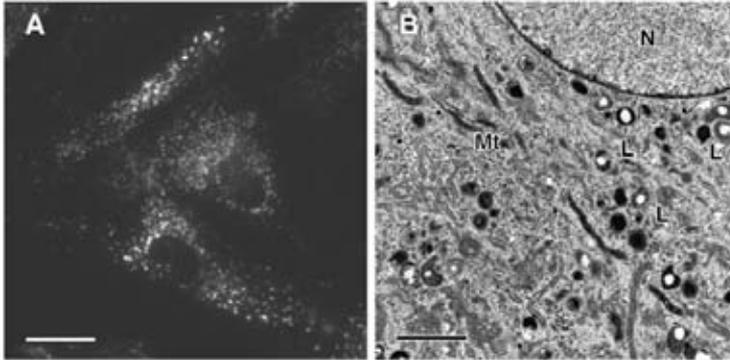
### **Lysosomes**

#### *General features*

The lysosome is commonly defined as a membrane-bound organelle containing many hydrolytic enzymes with optimum enzyme activity at acid pH. Characterisation of the lysosome as a discrete sub-cellular organelle was first outlined in 1951 (Berthet et al., 1951), and the term itself was coined in 1955 (de Duve et al., 1955). Lysosomes are present in all true eukaryotic cells, occupy 0.5-15% of cell volume, and are concentrated close to microtubule organizing centres (Matteoni and Kreis, 1987; Figure 1). A wide range of lytic enzymes, including proteases, nucleases, lipases, phosphatases, and glycosidases, is the main reason of naming these acidic organelles lysosomes, i.e. lytic particles (de Duve et al., 1955). Degradative capacity and low intra-organelle pH are distinctive features of lysosomes (de Duve and Wattiaux, 1966). Lysosomal pH is provided by the function of the vacuolar H<sup>+</sup>-ATPase, a pump that translocates protons from the cytosol into the lysosome (Ohkuma et al., 1982; Bowman et al., 1988), and is maintained at approximately pH 4.5 ± 0.5 (Riejngoud and Tager, 1973; Ohkuma and Poole, 1978). The acidic condition, indispensable for optimal activity of lysosomal enzymes, enables denaturation of proteins subjected to degradation helping the efficiency of proteolytic process.

The size of the lysosomes ranges between approximately 0.5 and 2 micrometers (Figure 1B), and depends on their content and the stage of lysosomal degradation process (Luzio et al., 2003). The lysosomal membrane is enriched in integral membrane glycoproteins, which are considered responsible for lysosomal resistance to self-degradation from the inside (Winchester, 2001). Although also acidic, endosomes are responsible for the delivery of endocytosed material to the lysosomal compartment, and

are deprived of specific lysosomal associated membrane proteins (LAMPs; Eskelinen, 2006; Figure 1A).



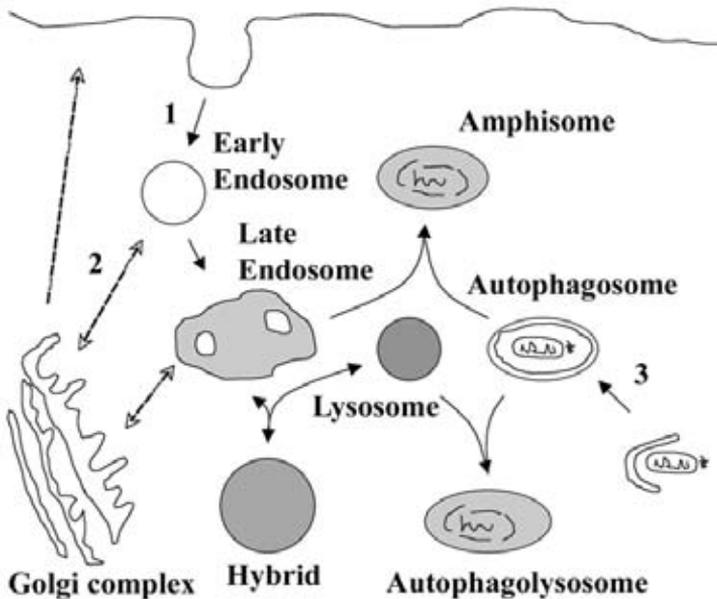
**Figure 1.** Fluorescent (A) and electron (B) microscopy of lysosomes in human fibroblasts. In (A) lysosomes are immunostained for the lysosomal associated membrane protein (LAMP-2). L, lysosome; Mt, mitochondria; N, nucleus. Scale bars, 20  $\mu\text{m}$  (A) and 2  $\mu\text{m}$  (B).

Another important feature that distinguishes lysosomes from endosomes is the absence of mannose 6-phosphate receptors (M6PRs) and recycling plasma membrane receptors (Kornfeld and Mellman, 1989; Luzio et al., 2001).

### ***Biogenesis of lysosomes***

Recent studies of biogenesis of lysosomes have revealed a dynamic complexity of lysosomal compartment, and argue against the dominating view of lysosomes as the static terminal degradative organelle of the endocytic pathway in animal cells (Luzio et al., 2003). The endocytosed material is first delivered to early endosomes, then late endosomes and, finally, lysosomes (Mellman, 1996; Mukherjee et al., 1997). To explain the mechanism of delivery of endocytosed material from late endosomes to lysosomes, different hypotheses have been suggested. Maturation, previously claimed to be a plausible mechanism of lysosomal biogenesis (Murphy, 1991), typically occurs in late endocytic pathway, and is not

regarded as the main means of delivery of material to lysosomes (Luzio et al., 2003).



**Figure 2.** A tentative model of lysosomal biogenesis. In this model the hypothesis of a direct fusion between late endosomes and lysosomes is employed. **1**, endocytosis; **2**, mannose 6-phosphate receptor associated vesicular trafficking; **3**, sequestration of cytosolic material. [Modified from Luzio et al., 2003, *Mol Membr Biol* 20: 141-54]

Another mechanism, so called vesicular transport, is mostly present in secretory and endocytic pathways (Mellman and Warren, 2000), with little evidence for such traffic between late endosomes and lysosomes. The “kiss and run” hypothesis (Storrie and Desjardins, 1996) employs an idea of repeated transient fusion and fission processes between the two organelles. Alternatively, complete fusion of late endosome and lysosome results in a formation of a hybrid organelle (Bright et al., 1997; Figure 2) with intermediate qualities between late endosomes and lysosomes (Mullock et al., 1998). Subsequent condensation of the content and removal of the excessive membrane by vesicular carriers lead to the re-formation of the

lysosome from the hybrid organelle (Pryor et al., 2000). In this manner, the hybrid organelle functions as a digestive organelle or “cell stomach” (Griffiths, 1996), and lysosomes are viewed as storage organelles for acid hydrolases (Luzio et al., 2003). Alternatively, lysosomes can fuse with autophagosomes with formation of autophagolysosomes, which are also a site for intracellular degradation.

### ***Functions of lysosomes***

Degradation of both extra- and endogenously derived material has been considered a primary function of lysosomes (de Duve and Wattiaux, 1966). Lysosomal engagement in degradation of endocytosed material serves mostly nutritional purposes, while degradation of cell’s own constituents, so-called autophagy, also secures intracellular homeostasis through the removal of damaged and potentially dangerous molecular structures and organelles (Klionsky, 2005). It has also been shown that the lysosomal compartment is involved in cell signalling (Miaczynska et al., 2004), especially initiation of cell death (Ferri and Kroemer, 2001).

The idea of lysosomal involvement in cell death, first suggested by de Duve and Wattiaux (1966), has received support from studies showing that lysosomal destabilisation is a triggering event in cell death induced by a variety of factors such as radiation, oxidative stress, staurosporine-treatment, exposure to lysosomotropic agents and oxidized low-density lipoprotein (Yuan et al., 1997; Brunk and Svensson, 1999; Roberg et al., 1999; Li et al., 2000; Johansson et al., 2003; Persson et al., 2005). The type of cell death, induced by lysosomal destabilisation, depends on the degree of lysosomal damage with necrosis being a result of extensive release of lysosomal content, and apoptosis progressing due to a moderate rupture of lysosomes (Brunk et al., 2001; Zhao et al., 2003).

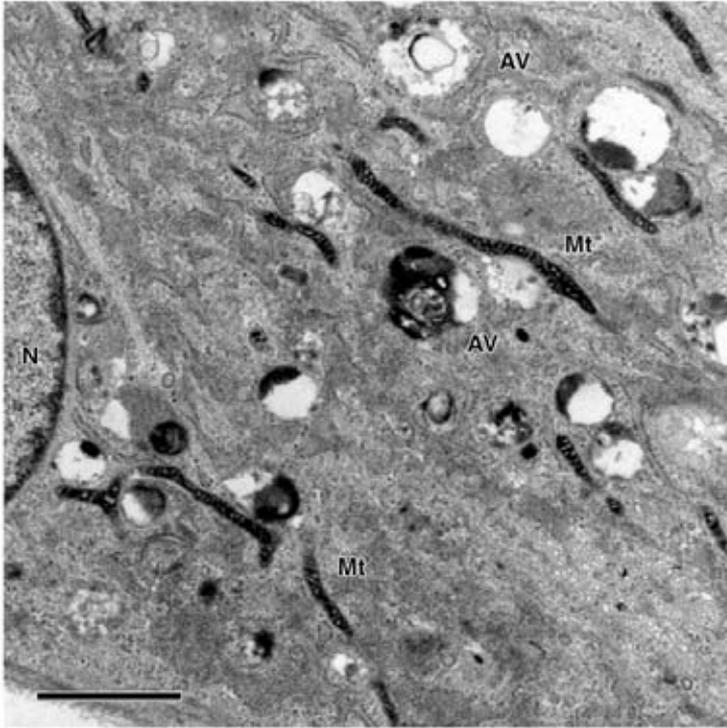
## **Autophagy**

### ***Definition and characteristics***

Autophagy (also called autophagocytosis) is an evolutionary conserved cellular pathway for the degradation of long-lived proteins and organelles (Klionsky, 2003). The word autophagy derives from Greek and means to eat oneself. The phenomenon of autophagy occurs in a wide range of eukaryotic organisms from yeast to mammals during starvation, cell and tissue development, and cell death (Levine and Klionsky, 2004; Figure 3). In mammalian cells, three types of autophagy are recognised depending on the way of delivery of material subjected to degradation to lysosomes, and include macroautophagy, microautophagy and chaperone-mediated autophagy (Klionsky and Emr, 2000; Cuervo, 2004).

Macroautophagy plays a major role in intracellular degradation and is often used as a synonym for autophagy (Yoshimori, 2004). During macroautophagy, portions of cytoplasm, and even entire organelles, are sequestered in a double-membrane organelle called autophagosome. Such organelles are subjected to a number of subsequent changes including membrane transformation, acidification, and, finally, fusion with lysosomes or late endosomes, and formation of autophagolysosomes or amphisomes, respectively (Fengsrud et al., 2004; Figure 2). Although macroautophagy is generally considered a non-selective process (Seglen et al., 1990), there have been reports of selective autophagy of peroxisomes and mitochondria (Yokota, 1993; Lemasters, 2005). The origin and nature of the autophagic sequestering membrane (phagophore) is still a matter of ongoing discussion with two considered alternatives for phagophore formation either by de novo synthesis, or by utilisation of pre-existing cytoplasmic membranes (Fengsrud et al., 2004).

The process when lysosomes sequester cytoplasmic components in invaginations of lysosomal membranes is defined as microautophagy (Dice, 2000). Digestion of the internalised material occurs after disappearance of the vesicle membrane. Microautophagy accounts for the degradation of peroxisomes and some cytosolic proteins (Subramani, 19989).



**Figure 3.** Electron micrograph of a human fibroblast exhibiting autophagic vacuoles (AV). Autophagy was induced by exposure to the lysosomotropic detergent MSDH at a concentration of 25  $\mu$ M for 60 minutes. Mt, mitochondria; N, nucleus. Scale bar, 2  $\mu$ m.

Chaperone-mediated autophagy is a selective pathway responsible for degradation of certain cytosolic proteins after their direct transport through the lysosomal membrane by means of molecular chaperones (Cuervo and Dice, 1998; Dice, 2000; Cuervo, 2004). Proteins selected for this type of autophagy, contain a specific amino acid sequence (KFERQ: lysine-phenylalanine-glutamate-arginine-glutamine), which is recognised by a heat-shock type chaperone protein, and is transported to the lysosomal-associated membrane protein type 2a (LAMP-2a) for translocation into the lysosomal lumen (Cuervo, 2004).

***Regulation***

Autophagy is a continuously ongoing process, present even under normal conditions, and is regulated by different agents. Mechanisms of regulation of macroautophagy are understood better than regulation of other types of autophagy. Therefore, the regulation mechanisms, in short described in this thesis, concern mostly macroautophagy, even though referred to as autophagy.

Nutrient deprivation is a potent inducer of autophagy, while amino acids, being the final product of autophagic degradation of proteins, act as negative feedback regulators (Blommaert et al., 1997). An important part of nutrient-dependent regulation pathway is the serine/threonine kinase Tor (target of rapamycin). Inhibition of Tor by rapamycin induces autophagy even in presence of amino acids (Blommaert et al., 1995). Although the exact mechanism of amino acid regulation of Tor activity is not yet understood, it has been shown that activation of Tor by nutrients induces phosphorylation of proteins involved in the initial step of autophagy, resulting in their disassembling and inhibition of autophagy (Levine and Klionsky, 2004; Meijer and Codogno, 2004). Tor has also been found sensitive to depletion of ATP (Dennis et al., 2001).

In addition to nutrients, autophagy is also regulated by some hormones especially insulin and glucagon. Activation of the insulin receptor induces activity of class I phosphatidylinositol-3-kinase (PI3K), with consequent activation of a cascade of intermediate enzymes, finally resulting in up-regulation of Tor and inhibition of autophagy (Levine and Klionsky, 2004). There has also been described a Tor-independent insulin receptor associated pathway, the activation of which results in inhibition of autophagy (Saeki et al., 2003). Glucagon, on the other hand, activates autophagy by inhibiting Tor via a protein kinase A-related mechanism (Kimball et al., 2004).

Among different regulators of autophagy, special attention is given to class III PI3K. Activation of this enzyme is required for sequestration, the initial step of autophagy (Petiot et al., 2000). Although a number of additional proteins, such as heterotrimeric G proteins, Ras, protein kinases A and B, and others, were shown to regulate autophagy, their role and mechanisms

of action are not yet well understood (Levine and Klionsky, 2004; Meijer and Codogno, 2004).

### ***Autophagy and cell death***

Programmed cell death (PCD) plays an important role in maintenance of organismal homeostasis by controlling the cell number and removing abnormal cells (Baehrecke, 2002). On morphological basis, three types of cell death have been described (Schweichel and Merker, 1973; Baehrecke, 2005). Type I PCD (or classical apoptosis), is characterised by cellular shrinkage, chromatin condensation and fragmentation of the nucleus, membrane blebbing, and subsequent engulfment of dying cell by neighbouring cells or specialised macrophages (Kerr et al., 1972). Distinctive feature of type II PCD (or autophagic cell death) is the presence of autophagosomes and autophagolysosomes (Ogier-Denis and Codogno, 2003; Bursch, 2004; Edinger and Thompson, 2004), which are the executors of self-degradation. Type III (also called non-lysosomal) cell death is similar to necrosis since it involves swelling of organelles and lysosome-independent formation of “empty spaces” in the cytoplasm (Leist and Jäättelä, 2001; Baehrecke, 2005). However, the distinction between different types of PCD is sometimes difficult to make. Thus, there are studies showing that autophagic vacuolisation precedes classical apoptosis (Uchiyama, 2001; Gonzalez-Polo et al., 2005), and that both types of PCD can be observed in the same cell (Bursch, 2004).

Implication of autophagy in apoptosis has been explained by different mechanisms. Thus, autophagy was found to sequester such pro-apoptotic factors as cytochrome c and AIF, which are released to the cytosol during mitochondrial permeability transition (Takano-Ohmuro et al., 2000; Elmore et al., 2001). Such findings led to the suggestion that autophagy primarily is a cell survival response to apoptotic stimuli, and PCD is activated when autophagy is overloaded (Lemasters et al., 1998). This idea is consistent with observations that moderately activated autophagy has beneficial effects on cell survival, but switches to cell suicide when excessive (Edinger and Thompson 2004; Gozuacik and Kimchi 2004; Lockshin and Zakeri, 2004).

Type II PCD is considered a phylogenetically old phenomenon (Bursch, 2001), and is found in both physiological and pathological states (Orier-Denis and Codogno, 2003). There are even suggestions that autophagic cell death evolutionary preceded apoptosis (Schwartz et al., 1993). Autophagic PCD is mainly activated when there is a necessity of massive cell elimination, for example during development and tissue remodelling. The important characteristic of type II PCD, distinguishing it from apoptosis, is that autophagy removes the bulk of cytoplasm before nuclear changes appear. This mechanism insures the possibility of autophagic removal of damaged constituents on the sub-cellular level, proceeding to elimination of the whole cell if the damage is not repairable (Bursch, 2004).

## **Ageing**

### ***Defining ageing***

Prolonged existence of the majority of complex systems, including biological ones, is inevitably accompanied by ageing. As viewed by biologists, life-associated process of ageing starts at conception and continues until death (Balcombe and Sinclair, 2001). According to the National Library of Medicine and the National Institutes of Health, ageing is regarded as “gradual irreversible changes in structure and function of an organism that occur as a result of the passage of time”. These changes are commonly viewed as harmful, decreasing normal functioning and adaptability, and simultaneously increasing probability of death (Comfort, 1979).

Regarding cellular ageing, or senescence, the emphasis is usually on decreased ability to proliferate as a result of either exceeded proliferative limit (replicative senescence; Hayflick, 1965; Blackburn, 2000; Weinert and Timiras, 2003) or cellular stress (stress-induced senescence; Toussaint et al., 2002; Campisi, 2003; Itahana et al., 2004). In this thesis, cellular ageing is regarded as changes in functions and structure occurring in an individual cell with the passage of time.

### ***Oxidative stress and cellular ageing***

From a wide range of theories introduced in order to explain the mechanisms of ageing (Medvedev, 1990), no single theory is generally accepted. Among theories that gained most attention is the free radical theory of ageing proposed by Harman (1956). According to this theory, ageing-related changes of cellular structures result from damage caused by highly reactive agents, which are called free radicals and are defined as atoms or molecules containing one or more unpaired electrons. Such molecules or ions, formed as a result of incomplete one-electron reduction of oxygen, are collectively called reactive oxygen species (ROS), and are an inevitable by-product of aerobic life (Beckman and Ames, 1998; Cadenas and Davies, 2000). Harman's hypothesis is supported by studies showing that ageing is accelerated under conditions of oxidative stress, which is a disturbance in the balance between pro- and antioxidants in favour of the former (Barja, 2002). Among endogenous sources of ROS, mitochondria play the most important role, because formation of free radicals is unavoidably coupled with mitochondria-associated oxidative phosphorylation and occurs even under favourable conditions (Harman, 1972).

Although any cellular structure can be a subject to ROS-induced damage, impairment of mitochondrial and lysosomal functions is the key factor in understanding why such damaged structures accumulate (Brunk and Terman, 2002). Thus, continuous damage of mitochondria results in even more pronounced decoupling of oxidative phosphorylation and leakage of ROS. In addition, compromised lysosomal system is unable to effectively remove either oxidatively damaged structures or the source of their damaging, i.e. defective mitochondria, promoting further oxidative stress (Terman et al., 2006).

### ***Functional characteristics of cellular ageing***

#### *Proliferative capacity of ageing cells*

Cellular ageing is associated with a variety of morphological and functional hallmarks, and is mostly attributed to so-called postmitotic cells, such as neurons and cardiac myocytes (Sachs et al., 1977; La Velle and Buschmann,

1983; Terman and Brunk, 2006). Among the changes of cellular functions, decrease of proliferative potential is a major feature of ageing cells. Age-dependant decrease of cellular proliferation has been explained by telomere shortening, acquired during previous cell divisions or exposure to stress-factors which induce DNA damage (Von Zglinicki, 2003; Itahana et al., 2004). Alternatively to a telomere shortening-dependent proliferative disability, it has been shown that p19/ARF-p53 and p16/pRb pathways, known as tumour suppressor mechanisms, also diminish cell proliferation during cellular senescence (Campisi, 2001; Itahana et al, 2004).

### *Autophagy and ageing*

Another functional characteristic of cellular ageing is a decreased autophagic degradation (Cuervo and Dice, 2000; Bergamini et al., 2004), and coupled with it a progressive accumulation of functionally inactive material, so-called “biological garbage” (Terman et al., 2006). This “garbage”, or intracellular “waste”-material including damaged non-degraded macromolecules and organelles, accumulates either intralysosomally, due to incomplete enzymatic degradation, or extralysosomally, as a result of defective autophagic sequestration. Since, mitochondria are the primary source of endogenously derived ROS, recycling of mitochondria has a particular importance in formation and accumulation of “biological garbage” (Brunk and Terman, 2002). Being originally a result of incomplete autophagic degradation, accumulation of “biological garbage” eventually contributes to the decrease of cellular degradative capacity, thus forming a vicious circle (Cuervo et al., 2005).

### *Cellular ageing and apoptosis*

Cell death is an inevitable outcome of finite life of normal cells. Ageing has been regarded as a precursor of cell death, but the mechanisms behind death of ageing cells are not yet entirely clear. It is known that cell death may occur by apoptosis or necrosis. While apoptosis is considered a regulated, programmed process, directed toward execution of “silent” death without activation of inflammatory response, necrosis, on the other hand, is a passive, destructive response to acute cellular damage, accompanied by a

marked inflammatory reaction (Kerr et al., 1972; Wyllie, 1997). Ratio between pro- and anti-apoptotic members of Bcl-2 family proteins has been considered a crucial factor in regulation of apoptosis (Adams and Cory, 1998; Antonsson and Martinou, 2000). Ageing cells were shown to be resistant to apoptosis (Pereira-Smith and Bertram, 2000). This resistance has been found to correlate with increased expression of Bcl-2 (Warner et al., 1997) and diminished activity of the major executor of apoptosis, caspase-3 (Spaulding et al., 1999; Marcotte et al., 2004). It has also been shown that ageing cells resist programmed cell death due to inability to suppress Bcl-2 (Wang, 1995). Inability of ageing cells to undergo p53-dependent apoptosis in response to DNA damage has been suggested to be responsible for the shift in cell death pathway from apoptosis to necrosis (Seluanov et al., 2001).

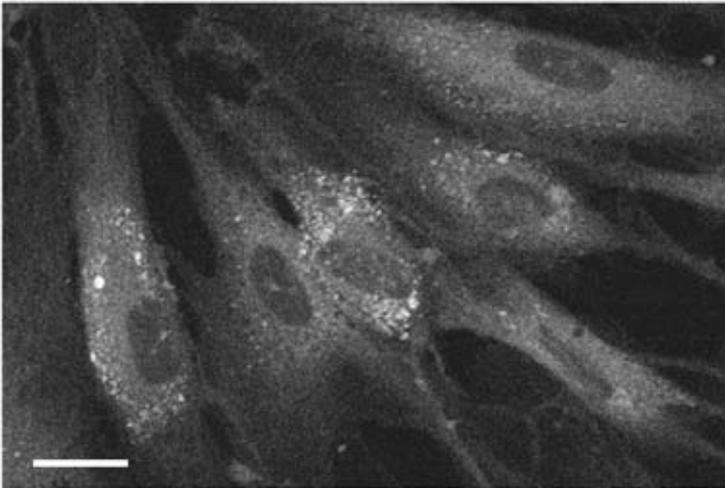
### ***Ageing and hormesis***

Ageing is generally characterised by a diminished adaptability due to accumulating insufficiency of homeodynamics (Rattan, 2004). It has been hypothesised that stimulation of pathways responsible for cellular maintenance and repair would result in improvement of adaptability and increased lifespan. Mild stress in the form of low doses of harmful agents has been proven such a stimulation that is able to activate homeodynamics without causing cellular demise. Such a phenomenon of adaptive response to low doses of otherwise harmful agents is termed hormesis (Rattan and Clark, 2005). Exposure to repeated periods of mild heat shock was reported to have beneficial anti-ageing effects, which were related to elevated levels of various heat shock proteins, diminished accumulation of oxidised proteins, and improved proteasomal activity resulting in stress resistance (Rattan, 1998; Fonager et al., 2002; Verbeke et al., 2002). Anti-ageing effect of calorie restriction can also be explained in terms of hormesis, if intermittent fasting is viewed as an example of mild stress (Anson et al., 2003; Masoro, 2005). Similarly, a wide variety of chemical and physical treatments were shown to have hormetic properties (Calabrese and Baldwin, 2000; Le Bourg et al., 2000; Le Bourg, 2001).

## Lipofuscin

### *Basic features*

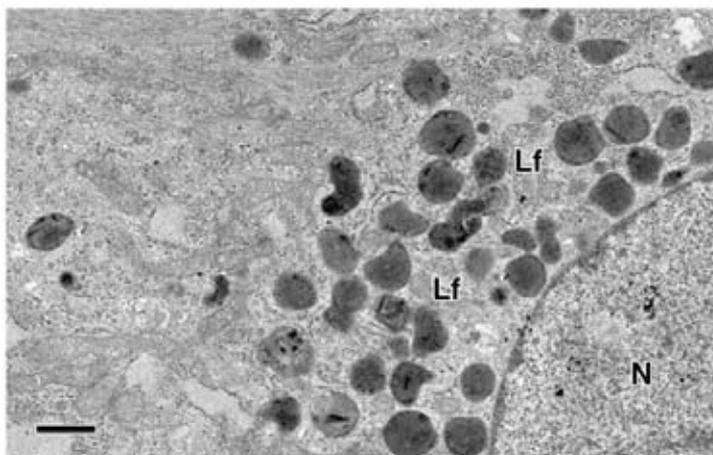
Morphological changes during cellular ageing include formation of yellow-brownish pigments, with typical broad-spectrum autofluorescence (Porta, 2002; Figure 4). Such pigments, found inside lysosomes, are called lipofuscin (Terman and Brunk, 2004; Figure 5) when age-related, or ceroid, when their accumulation is affected by pathological conditions (Porta, 2002; Seehafer and Pearce, 2006).



**Figure 4.** Lipofuscin-associated autofluorescence in human fibroblasts detected using confocal microscopy (488 nm laser). Accumulation of lipofuscin was accelerated by culturing cells in 40% oxygen for two months. Scale bar, 20  $\mu\text{m}$ .

Lipofuscin is commonly called “the age pigment”. It is an aggregated polymeric structure, composed of cross-linked oxidatively modified proteins and lipid degradation residues. Lipofuscin also contains carbohydrates and some metals, of which iron is the most important, due to its implication in Fenton-type chemistry and formation of free radicals (Terman and Brunk, 2004). The most distinctive feature of lipofuscin, despite differences of its

composition between different cell types, is that all lipofuscin pigments are undegradable. This is probably due to formation of complex polymer-like structures during cross-linking of peptides (Kikugawa et al., 1989).



**Figure 5.** Electron micrograph of a human fibroblast exhibiting a number of lipofuscin-containing lysosomes (Lf). Accumulation of lipofuscin was accelerated by culturing cells in 40% oxygen for two months. N, nucleus. Scale bar, 1  $\mu\text{m}$ .

### ***Mechanisms of formation and accumulation***

Lipofuscin can originate from both intra- (in most postmitotic cells) and extracellular material, e.g. in macrophages, glial cells and retinal pigment epithelium (Burke and Skumatz, 1998; Siddiqi and Peters, 1999). The auto-/phagocytosed material is delivered to lysosomes, but the process of degradation appears to be inherently imperfect, and results in intralysosomal accumulation of non-degraded products. Endogenously formed hydrogen peroxide freely diffuses through lysosomal membranes and causes radical damage of auto-/phagocytosed biomolecules. As previously mentioned, at low lysosomal pH redox active iron promotes formation of hydroxyl radicals via Fenton reactions, also causing radical damage and increasing resistance of intralysosomal material to lysosomal enzymes with final formation of

undegradable structure, i.e. lipofuscin (Terman and Brunk, 2004; Terman and Brunk, 2006).

### ***Physiological effects***

Having the working hypothesis that lipofuscin in itself might participate in free radical formation changed the long time dominating view of lipofuscin as an “innocent” marker of cellular ageing (Terman and Brunk, 2004). Thus, it has been shown that in retinal pigment epithelial cells lipofuscin accumulation reduces phagocytic capacity (Sundelin et al., 1998). In the same cellular model lipofuscin acts as a photosensitizer, especially when excited by blue light, causing lysosomal destabilisation and cell death (Wihlmark et al., 1997). It has also been shown that accumulation of lipofuscin increases susceptibility of fibroblasts to oxidative stress-induced lysosomal damage and subsequent cell death (Terman et al., 1999). Moreover, it has been suggested that newly produced lysosomal enzymes are misplaced to the lipofuscin-loaded lysosomes in an ineffective attempt to degrade lipofuscin, disengaging them from their useful functioning inside lipofuscin-free lysosomes (Terman and Brunk, 2004). Despite the presented evidence, the deleterious effects of lipofuscin are not yet generally accepted (Porta et al., 2002).



## **AIMS OF THE STUDY**

The general objectives of the present work were to study ageing-related changes of the lysosomal compartment and their influence on cell functions. The particular aims were to determine:

### ***Paper I***

- the source of lipofuscin in human fibroblasts
- the intracellular location of lipofuscin-like structures accumulating during inhibition of autophagy
- the role of macroautophagy in the turnover of organelles and lipofuscin formation

### ***Paper II***

- the role of biological “garbage” accumulation in the development of ageing-related changes and eventual death of postmitotic cells
- the feasibility of protective effect of cell division

### ***Paper III***

- the characteristics of cell death following complete starvation
- the influence of lipofuscin on cell survival during complete starvation

### ***Paper IV***

- the effects of lipofuscin on lysosomal stability



## MATERIALS AND METHODS

### Cells

Ageing mainly affects long-lived postmitotic cells such as neurons and cardiac myocytes. Confluent growth-arrested cells resemble true postmitotic cells and are widely used for the ageing studies. In the present work, human AG-1518 fibroblasts (obtained from Coriell Institute, Camden, NJ, USA; *Papers I-IV*) and U-1160CG astrocytes (a kind gift of Professor Bengt Westermark, Department of Pathology, University of Uppsala, Sweden; *Paper II*) were used.

Growing cells under hyperoxic conditions is known to accelerate age-related changes and accumulation of lipofuscin (Terman and Brunk, 1998; Grune et al., 2005). In order to induce lipofuscin accumulation confluent human fibroblasts (*Papers I-IV*) were exposed to hyperoxia (40% O<sub>2</sub>, 55% N<sub>2</sub> and 5% CO<sub>2</sub>) for two-five months and are referred to as lipofuscin-loaded.

### Experimental design<sup>\*</sup>

All cells were grown in Eagle's minimum essential medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin-G and 100 µg/ml streptomycin, in an atmosphere of 8% O<sub>2</sub>, 87% N<sub>2</sub> and 5% CO<sub>2</sub> at 37°C (normal conditions). The cells were sub-cultivated at a 1:2 ratio until they reached passage 22-24 (equivalent to ~50% of life span completed in terms of replicative limit [Hayflick, 1979]), and were then allowed to grow until confluency. All experiments were performed at 37°C, pH 7.4 under either normal or hyperoxic conditions.

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<sup>\*</sup> Detailed description of methodology is given in the papers the thesis is based on, and in the references.

### ***Paper I***

Confluent cultures of AG-1518 fibroblasts were distributed in five groups: (i) early confluent cultures; (ii) cells cultured under normal conditions for two weeks; (iii) cells cultured under hyperoxic conditions for two weeks; (iv) cells under normal conditions exposed to 5 mM of autophagy inhibitor 3-methyladenine (3MA) for two weeks; (v) cells under hyperoxic conditions exposed to 5 mM 3MA for two weeks. Comparison was done between non-treated cells under normal conditions and other groups as well as between different groups.

Autophagy-inhibiting effect of 3MA is due to the inhibition of the class III phosphatidylinositol 3-kinase (PI3K). Active PI3K class III is required for sequestration, the initial stage of autophagy (Petiot et al., 2000).

### ***Paper II***

U-1160CG astrocytes and lipofuscin-loaded fibroblasts were distributed in the following groups: untreated confluent cells; confluent cells treated with 5 mM 3MA for two weeks; untreated sub-confluent cells; 3MA-treated sub-confluent cells. AG-1518 fibroblasts were distributed in the same groups plus additional: confluent cells treated with 5 mM 3MA for two weeks, then sub-cultivated under conditions of 3MA-exposure (3MA-treated cells released from confluency); confluent cells treated with 50  $\mu$ M of the lysosomal cysteine protease inhibitor leupeptin for 10 days; leupeptin-treated sub-confluent cells. Comparison was done both among different groups within the same cellular model and between respective groups of different cellular models.

In contrast to 3MA treatment-associated prevention of autophagic sequestration, leupeptin disturbs intralysosomal degradation of autophagocytosed material. We speculated that treatment with 3MA would primarily accelerate accumulation of extralysosomal “garbage”, while leupeptin-treatment would mostly result in accumulation of intralysosomal “waste”-material. In this manner, different approaches to acceleration of biological “garbage” formation were employed.

### ***Paper III***

Cells were distributed in following groups: (i) normal fibroblasts (referred to as controls); (ii) lipofuscin-loaded fibroblasts; (iii) normal fibroblasts pre-exposed to 5 mM 3MA for 24 hours; (iv) normal fibroblasts pre-exposed to 100  $\mu$ M of the cathepsin D inhibitor pepstatin A for 24 hours. In all groups starvation was induced by exposure of cells to phosphate-buffered saline (PBS) for different time intervals (24, 48, 72 and 96 hours). During starvation, cells in groups (iii) and (iv) were exposed to 5 mM 3MA and 100  $\mu$ M pepstatin A, respectively. Comparison was done between lipofuscin-loaded and non-loaded cells in relation to the time point at which significant changes had occurred.

Pepstatin A treatment was introduced in order to inhibit the lysosomal enzyme cathepsin D, known to be involved in initial stages of apoptosis (Roberg et al., 1999). Pepstatin A was also supposed to mimic the hypothetical lipofuscin-associated inhibition of cathepsin D.

### ***Paper IV***

Two groups of cells were compared: normal fibroblasts (referred to as controls) and lipofuscin-loaded. Both groups were exposed to three different treatments: (i) the lysosomotropic detergent MSDH at a concentration of 25  $\mu$ M for 15, 30 or 60 minutes; (ii) the redox-cycling quinone naphthazarin (inducer of acute oxidative stress) at a concentration of 0.75  $\mu$ M for 15, 30 or 60 minutes and; (iii) the vacuolar ATPase inhibitor bafilomycin A<sub>1</sub> at a concentration of 20 nM for 15 or 30 minutes.

The chosen treatments employ different mechanisms of action, and allow a multifaceted approach to study lysosome-targeted stress. Thus, MSDH- and NzQ-treatment cause structural changes in lysosomal membranes by inducing detergent-like and oxidative modifications, respectively, finally resulting in lysosomal leakage or rupture. Alternatively, bafilomycin A<sub>1</sub>-treatment affects acidification of lysosomes without apparent changes of lysosomal membrane integrity (Bowman et al., 1988).

### **Assessment of cellular autofluorescence**

The amount of lipofuscin-like material was estimated by measurements of cellular autofluorescence (*Paper I*). For quantification of autofluorescence, the non-confocal laser scanning images of live cultures were obtained. Unlike confocal images, which represent optical sections, non-confocal images represent whole cells. The measurements were performed using the National Institute of Health Image programme (<http://rsb.info.nih.gov/nih-image/>). Fluorescence intensity was expressed in arbitrary units (a. u.) being a product of average pixel value per cell (excluding background) and the cell area (Terman et al., 1999). From each specimen 90 to 140 randomly selected cells were analysed.

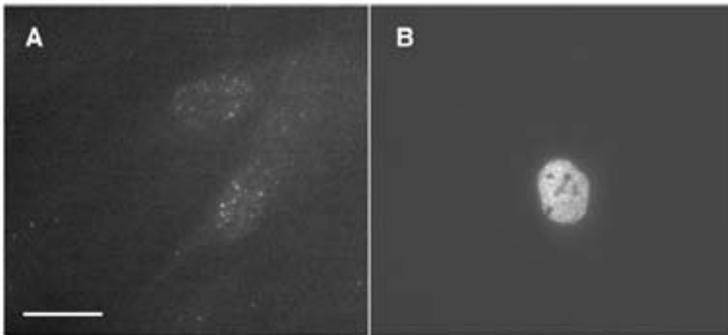
### **Co-localisation of autofluorescent material with lysosomes**

The co-localisation of autofluorescent material with lysosomes was estimated by comparison of autofluorescent images with images of lysosomes either immunostained for cathepsins (D or L; FITC-conjugated secondary antibody) or vitally stained with the lysosomotropic fluorochrome acridine orange (AO). Both types of imaging were performed on cells plated on chambered cover glasses with marked bottom areas (circles approximately 1 mm in diameter). During repeated imaging (detection of autofluorescence, followed by lysosomal staining), the same cells were identified and subjected to investigation. Since FITC and AO fluorescence were much brighter than lipofuscin-like autofluorescence, it allowed the attenuation of laser light to a level when lipofuscin was undetectable, not contributing to the fluorescence detected during lysosomal staining. Minimum 90 cells per sample were analysed (*Paper I*).

### **Assessment of proliferative potential**

Proliferative potential was estimated by immunocytochemical detection of DNA synthesis (*Paper II*). Bromodeoxyuridine (BrDU) incorporates into the nuclear- and mitochondrial-DNA instead of thymidine during replication and repair (Davis and Clayton, 1996). While the detection of mtDNA replication normally requires exposure to BrDU for 12 hours, exposure for 60 minutes is sufficient for the detection of nuclear DNA replication. According to this, cells were exposed to 15  $\mu$ M BrDU for 60 minutes, fixed

in 4% formaldehyde in PBS for 20 minutes in 4°C. After permeabilisation and denaturation of DNA, cells were exposed to monoclonal mouse anti-BrDU antibodies, followed by treatment with rabbit anti-mouse FITC-conjugated antibody. Cells were then rinsed in PBS and distilled water, mounted in Vectashield, and subjected to fluorescence microscopy (blue light excitation) and imaging. Cells with bright BrDU staining of nuclei were considered BrDU-positive (Figure 6). Minimum 100 cells from each specimen were analysed.



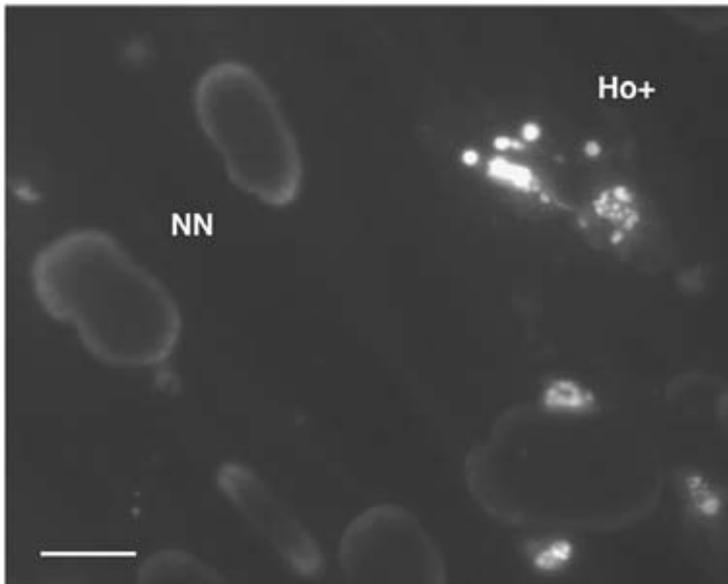
**Figure 6.** Nuclei of fibroblasts immunostained for BrDU. BrDU-negative (A) versus BrDU-positive (B) nuclei. Scale bar, 20  $\mu$ m.

### Detection of cell death

Both morphological and biochemical methods were used for the detection of cell death. For estimation of apoptotic cell morphology, formaldehyde-fixed cells were subjected to phase contrast (*Papers II and III*) and fluorescence microscopy (*Paper II*). Cellular shrinkage, pyknosis and nuclear fragmentation were considered typical for apoptotic cells. Cell death-associated morphological changes, revealed by combined Hoechst/propidium iodide vital staining, were analysed either by *in situ* fluorescence microscopy (*Paper II*) or by flow cytometry (*Paper III*). Biochemical detection of cell death included assessment of caspase-3-like activity (*Paper III*).

### **Hoechst/Propidium iodide staining**

Using a combination of Hoechst and propidium iodide (PI) staining different kinds of cell death could be identified. Hoechst stains condensed chromatin of apoptotic cells more intensely than chromatin of normal nuclei. PI permeates only cells with impaired plasma membrane, and thus stains nuclei of necrotic cells. Vital staining, with a mixture of 5  $\mu\text{g/ml}$  Hoechst 33342 and 1  $\mu\text{g/ml}$  PI for 20 minutes on ice, was performed either *in situ* (*Paper II*) or on cells that had been trypsinised, centrifuged at  $\sim 300 \times g$  for 5 minutes, re-suspended in the culture medium and filtered through a 70  $\mu\text{m}$  cell strainer (*Paper III*). In the former case, the analysis of Hoechst/PI staining was done using fluorescence microscopy (330-380/420 nm and 510-560/590 nm excitation/barrier filters for Hoechst and PI staining, respectively), while in the latter case stained cells were analysed by flow cytometry (UV/blue dual excitation, and a 380 nm longpass and  $575 \pm 26$  nm barrier filters for measurements of fluorescence emission of Hoechst and PI, respectively).



**Figure 7.** Fluorescence microscopy of Hoechst-stained fibroblasts. NN, normal nuclei; Ho+, Hoechst-positive (also fragmented) nucleus of a cell undergoing apoptosis. Scale bar, 20  $\mu\text{m}$ .

During analysis of fluorescent images, normal viable cells showed low intensity of Hoechst-staining. In contrast, dying cells with condensed, and in some cases fragmented, nuclei, exhibited intense Hoechst-staining (Figure 7). Such kind of staining, obviously brighter than that of intact nuclei, was considered Hoechst-positive. According to the pattern of Hoechst/PI positive staining, cells were distributed in three groups: (i) Hoechst-positive only cells, considered as undergoing early stages of apoptosis; (ii) PI-positive only cells, regarded as necrotic; (iii) cells with combined Hoechst and PI positivity, judged as cells in the stage of post-apoptotic necrosis (Weber et al., 1997). Eighty to 120 randomly selected cells from each specimen were analysed.

During flow cytometric analysis, two populations of cells were gated according to the intensity of Hoechst/PI staining. Cells showing low intensity of staining were regarded viable, while those with higher intensity were considered as cells undergoing apoptosis or post-apoptotic necrosis. 10,000 cells from each sample were analysed.

### ***Caspase-3-like activity assay***

Caspases are known as major executors of classical apoptosis. In order to assess caspase-3-like activity (*Paper III*), cell lysates were incubated with a fluorophore conjugated substrate sequence (Ac-DEVD-AMC). Fluorescence intensity of liberated AMC (7-amino-4-methyl-coumarin) upon caspase cleavage of the substrate between D and AMC was measured by spectrophotometer and expressed as pmoles of AMC released per milligram of protein per hour. Protein content was assayed using the method described by Lowry (Lowry et al., 1951).

### **Lysosomal integrity assays**

#### ***Decrease of lysosome-associated red fluorescence of acridine orange***

Acridine orange (AO) is a lysosomotropic weak base with metachromatic features (Koenig, 1963; Robbins, 1963). Oligomeric form of highly concentrated and protonated AO ( $\text{AOH}^+$ ) is found in intact lysosomes and exhibits red fluorescence. Lysosomal alkalinisation and translocation of

lysosomal content to the cytosol results in the formation of the monomeric deprotonated form of AO with green fluorescence (Olsson et al., 1989). The degree of lysosomal leakage was estimated as a decrease of lysosome-associated red fluorescence of AO (*Paper IV*). Cells on cover-slips were briefly stained with 5 µg/ml AO for 15 minutes under normal culture conditions, rinsed in complete medium and exposed to appropriate treatments. After having obtained fluorescent images of the cells (blue light excitation), separate lysosomes were traced and measurements of lysosomal red AO-fluorescence were performed using the National Institute of Health Image programme (<http://rsb.info.nih.gov/nih-image/>). Fluorescence intensity was expressed in arbitrary units (a. u.) being a product of average pixel value per lysosome and the lysosome area. From 100 to 140 lysosomes from each specimen were analysed.

#### ***Immunocytochemical detection of location of cathepsin D***

Cathepsin D is normally present inside the lysosomal compartment. We used cathepsin D as a “marker enzyme” although other cathepsins are also released from lysosomes with disturbed membrane integrity. Cells with intact lysosomes exhibit grainy (lysosome-like) pattern of cathepsin D immunostaining. Destabilisation of lysosomal membrane is accompanied by translocation of cathepsin D to the cytosol, resulting in a diffuse (cytosolic) pattern of staining. Fibroblasts plated on cover-slips were processed for immunocytochemistry after having been fixed in 4% formaldehyde in PBS for 20 minutes in 4°C. Incubation with polyclonal rabbit anti-human antibodies to cathepsin D was followed by exposure to goat anti-rabbit IgG-Alexa 594 conjugate. Thereafter, the cells were rinsed in PBS and distilled water, mounted in Vectashield, and subjected to fluorescence microscopy and imaging (*Papers III and IV*).

#### ***Detection of cytosolic fraction of cathepsin D***

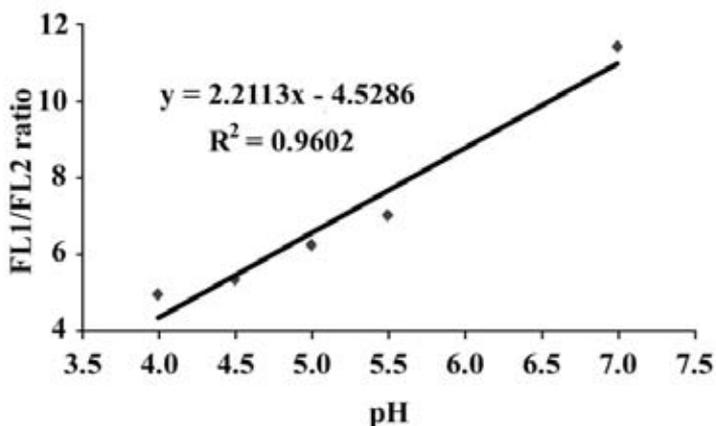
Extraction of cytosol was performed using the cholesterol-solubilising agent digitonin as described elsewhere (Johansson et al., 2003; *Paper IV*). Briefly, low concentrations of digitonin permeabilise the cholesterol-rich plasma membrane, while cholesterol-poor membranes of e.g. lysosomes remain more or less intact. Concentrations of digitonin were optimised to result in maximum release of cytosolic lactate dehydrogenase (LDH). LDH-activity

was also measured in order to verify an equal amount of extracted cytosol in all samples, and was spectrophotometrically estimated as a decrease of absorbance at 340 nm of nicotinamide adenine dinucleotide (NADH) during the LDH-catalysed reduction of pyruvate to lactate. Integrity of lysosomes during digitonin-treatment was verified by assessment of the lysosomal enzyme, N-acetyl-D-glucosaminidase (NAG), activity, which corresponds to the fluorescence of the cleaved NAG-substrate (4-methylumbelliferyl-2-acetamido-2-deoxy- $\beta$ -D glucopyranosid) at an excitation wavelength of 356 nm and an emission wavelength of 444 nm.

Exposure of cells to extraction buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA and digitonin at concentration 25  $\mu$ g/ml) for 12 minutes on ice was followed by collection of the supernatant and precipitation of proteins by means of treatment with 5% trichloric acid on ice for 10 minutes and subsequent centrifugation at  $\sim$ 20,800 x g for 15 minutes. The protein pellet was then subjected to immunoblotting. A monoclonal mouse anti-human cathepsin D primary antibody, and horseradish peroxidase-conjugated goat anti-mouse secondary antibody were used for western blot analysis. Bands were visualised using Western blotting Luminol Reagent. Equal loading was verified by re-probing the membrane with a mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH).

### ***Lysosomal pH measurement***

Low pH is a distinctive feature of lysosomes (De Duve and Wattiaux, 1966) and is representative of lysosomal functional integrity (Chen, 2002; Nilsson et al., 2003). The lysosomal pH was measured by flow cytometry as described elsewhere (Nilsson et al., 2003; *Papers III and IV*). Briefly, cells pre-loaded with FITC-conjugated dextran were subjected to appropriate treatments. Thereafter, cells were trypsinised, centrifuged at  $\sim$ 300 x g for 5 minutes, re-suspended in the culture medium, filtered through a 70  $\mu$ m cell strainer and analysed by flow cytometry. A 488 nm argon laser was used for the FITC excitation, and emission was detected in the FL1 and FL2 channels using a  $530 \pm 28$  nm and a  $610 \pm 20$  nm barrier filters, respectively. Data from 10,000 cells was analysed. Modified Britton-Robinson buffers (pH 4.0-7.0) were used for the preparation of a standard curve. The FL1/FL2 ratios were used to calculate the pH employing the standard curve (Figure 8).



**Figure 8.** Example of a standard curve obtained for lysosomal pH.

### Statistical analysis

All experiments were repeated at least three times. Values are given as means  $\pm$  S.D. Statistical comparisons of the lysosomal pH data were made using Kruskal-Wallis and post hoc Mann Whitney test. Data obtained from other experiments were analysed using the ANOVA and post hoc LSD test. P-values  $< 0.05$  were considered significant.

## RESULTS AND DISCUSSION

### Paper I

In this study, confluent cultures of AG-1518 human fibroblasts were exposed to hyperoxia and/or the autophagy inhibitor 3-MA for two weeks. The prolonged treatment with 3-MA is possible due to the relatively low toxicity of the drug, and enables the accumulation of non-autophagocytosed cellular structures to be studied. We speculated that identification of the accumulating non-degraded material would provide information regarding the source of lipofuscin in human fibroblasts.

#### *Lipofuscin-like material accumulates following exposure to hyperoxia and/or 3-MA*

Exposure of confluent fibroblast cultures to 40% ambient oxygen or 3-MA, but especially the combination of both, produced enhanced autofluorescence compared to control cells, maintained under normal conditions and displaying only age-related increase of autofluorescence. The accumulating material exhibited lipofuscin-like broad-spectrum autofluorescence following excitation with ultraviolet, blue, or green light. Although resembling common, age-related lipofuscin, the accumulating material had some particular morphological characteristics revealed by electron microscopic studies. Thus, the electron density of the vacuoles containing accumulating material was lower than that of lipofuscin inclusions. Some of these vacuoles also contained electron-lucent spaces and fibril-like structures.

***Inhibition of autophagy increases the number of lysosomes and mitochondria with low membrane potential***

Lysosomes were found more abundant in 3-MA exposed cells than in controls. Since exposure to 3-MA did not alter the late endosomal compartment (as deduced from the absence of appreciable changes in MPR immunoreactivity), which was much smaller than the area occupied by autofluorescent lysosomal markers-positive granules (see following section), it was suggested that it was mainly mature lysosomes that accumulated following block of autophagy. This was consistent with the electron microscopy findings, showing that most vacuoles accumulating during 3-MA exposure did not have multivesicular structure, typical for late endosomes (Luzio, et al., 2003).

Exposure to 3-MA also induced a general moderate increase in the number of mitochondria, especially those with low inner membrane potential. On electron microscopical level, mitochondria of 3-MA treated cells displayed changes similar to those found in ageing cells, namely partially condensed matrix and dilated cristae. These findings are in agreement with previous studies showing that prolonged 3-MA treatment induces accumulation of cellular structures excluded from recycling (Terman et al., 2003).

***Lipofuscin-like autofluorescence in 3-MA treated cells co-localises with lysosomes***

Autofluorescent material, accumulated during two-week-exposure to 3-MA, co-localised with lysosomes, as deduced from the similarity of the patterns of autofluorescence with immunostaining for cathepsins D and L, and vital staining with AO of the very same cells.

Since lipofuscin is regarded as an intralysosomal pigment, which is formed from the material delivered to the lysosomes mostly by means of autophagy (Brunk et al., 1992; Terman and Brunk, 2004), the observed accumulation of lysosomes containing lipofuscin-like material under conditions of inhibited autophagy can be explained if the possibility of autophagy of lysosomes is considered. The term “lysophagy” originally described electron microscopy

findings of lysosomal membrane invaginations (de Duve and Wattiaux, 1966; Glaumann et al., 1981) appearing after lysosomal fusion or microautophagy, and was considered an evidence of partial membrane recycling (Marzella et al., 1981; Lloyd, 1996). In the light of present findings, “lysophagy” could describe not only the excess fluid-phase autophagy, but also the bulk-phase macroautophagy of entire lysosomes. This idea of lysosomal autophagy is supported by previous immunoelectron microscopic findings of lysosomal membrane proteins inside lysosomes (Barriocanal et al., 1986), and of acid phosphatase activity-positive membrane-bounded structures inside autophagosomes after vinblastine-induced block of fusion (Miettinen and Reunanen, 1991).

The observed reduction of Golgi complex and decreased immunostaining for procollagen in 3-MA treated cells may be considered examples of harmful effects of the accumulating material on cellular functions. Undegraded material accumulating due to prolonged inhibition of autophagy, especially when combined with chronic oxidative stress, might be viewed as biological “garbage” typically found in ageing cells (Sheldrake, 1974; Hirsch, 1978; Terman, 2001). Similarity of the present findings to common ageing-related changes emphasises the suitability of the cell model used in this study for ageing research.

## **Paper II**

Cellular ageing is associated with a progressive accumulation of functionally inefficient material often called biological “garbage” or “waste” (Terman, 2001). Biological “garbage” includes extralysosomally localised damaged non-degradable molecular structures and organelles, e.g. “giant” mitochondria, and intralysosomal indigestible polymeric material called lipofuscin. In order to accelerate biological “garbage” accumulation, AG-1518 human fibroblasts (loaded with lipofuscin or not) and U-1160CG astrocytes were exposed to the autophagy inhibitor 3-MA for two weeks. Alternatively, intralysosomal degradation was inhibited in AG-1518 fibroblasts by means of exposure to the lysosomal cysteine protease inhibitor leupeptin for 10 days. We hypothesised that cell proliferation would prevent

“garbage” accumulation by its mechanical dilution, and improve cell survival by diminishing the concentration of potentially hazardous “waste”.

### ***Confluent cells frequently die following prolonged inhibition of autophagy***

In all cell models used in this study, spontaneous cell death even in untreated confluent cultures was detected. The number of dying cells increased significantly following inhibition of autophagy. Compared to non-loaded cells, lipofuscin-loaded fibroblasts displayed less pronounced decrease of viability following treatment with 3-MA. Cells dying following prolonged exposure to leupeptin contained large amounts of autofluorescent lipofuscin-like material and showed typical apoptotic nuclear fragmentation. In cells dying following 3-MA treatment, electron microscopy studies revealed apoptotic-like chromatin condensation and nuclear fragmentation, combined with large numbers of vacuoles loaded with undegraded material.

### ***Sub-cultivation increases proliferation of both untreated and 3-MA exposed fibroblasts***

Both lipofuscin-loading and 3-MA treatment diminished sub-cultivation induced proliferation, which, however, remained significantly higher than in confluent cultures. Exposure to 3-MA did not cause further decrease of already compromised proliferative potential in sub-confluent lipofuscin-loaded fibroblasts.

### ***Sub-cultivation improves viability of cells under conditions of inhibited autophagy***

In all cell models of “garbage” accumulating cells presented in this study, sub-cultivation resulted in a significant decrease in the number of dead cells, even when sub-cultivation was combined with continuous exposure to autophagy inhibitors. The protective effect of sub-cultivation, although still significant, was less pronounced in lipofuscin-loaded fibroblasts probably due to their decreased proliferative potential.

The presented decrease of cell viability due to prolonged inhibition of autophagy and associated accumulation of biological “garbage” is consistent with the idea that accumulation of “waste” material compromises cellular functions (Terman et al., 1999; Terman, 2001; Terman and Brunk, 2006). This idea is supported by previous findings of progressive death of cardiac myocytes following 3-MA induced inhibition of autophagy (Terman et al., 2003). Detrimental effects of “garbage” accumulation can be explained by the toxicity of protein oxidation products (Goldberg, 2003; Grune et al., 2004), and the increased amount of ROS generated by damaged mitochondria (Sohal et al., 1995). Alternatively, by occupying a certain amount of physical space inside a cell, biological “garbage” could interfere with intracellular signalling, transport and metabolism.

The beneficial effect of cell proliferation is probably due to the dilution of hazardous non-degradable “garbage” during mitosis, which provides a more or less equal distribution of material between the daughter cells. Continuous employment of such a mechanism could be the only possibility for cells to escape ageing-associated changes and eventual death. In agreement with this idea are the findings of continual replacement of all cells in a primitive organism *Hydra*, known to lack features of senescence (Martinez, 1998). Overall, the results of this study support the “garbage” accumulation theory of ageing and emphasise the role of cell division as a natural anti-ageing mechanism.

### **Paper III**

From the wide range of cell death inducers, complete starvation was chosen as the most “physiological” one. In this study, human AG-1518 fibroblasts, normal and those pre-cultured under hyperoxic conditions in order to induce accumulation of lipofuscin, were exposed to phosphate-buffered saline (PBS) for up to 96 hours. Since lysosomes have been shown to be involved in the initial stages of programmed cell death (Yuan et al., 1997; Brunk and Svensson, 1999; Roberg et al., 1999; Li et al., 2000; Ferri and Kroemer, 2001; Johansson et al., 2003; Persson et al., 2005), we speculated that lipofuscin-loading, 3-MA induced inhibition of autophagy, or inhibition of

lysosomal enzyme cathepsin D with pepstatin A would interfere with lysosomal function that might affect cellular sensitivity to starvation-induced cell death.

### ***Lipofuscin-loaded cells endure complete starvation***

In control cells, significant increase in the number of dead cells occurred after 72 hours of complete starvation. Compared to controls, cell death was delayed until 96 hours in pepstatin A treated cells, and accelerated in cells exposed to 3-MA (observed after 48 hours of exposure to PBS). Cell death was impeded in lipofuscin-loaded cells, and significant increase in the number of dead cell was not found even after 96 hours of starvation.

### ***Caspase-3 activity declines during starvation***

Caspase-3 activity, present at some basal level in non-starving cells, declined during starvation. Subsequent caspase-3 re-activation was found to coincide with the time of significant increase in the percentage of dead cells in corresponding experimental groups. No significant changes in caspase-3 activity during 96 hours of starvation were observed in lipofuscin-loaded cells. The present findings of a significantly lower level of the basal caspase-3 activity in lipofuscin-loaded cells compared to controls is in agreement with previous reports showing diminished caspase-3 activity in ageing cells (Spaulding et al., 1999; Marcotte et al., 2004).

### ***Starvation is associated with lysosomal alkalinisation***

Lysosomal alkalinisation occurred in both control and lipofuscin-loaded cells, but was more pronounced in the former. Significant increase of lysosomal pH in control cells was found to coincide with the time of significant increase in the percentage of dead cells in that group (after 72 hours of starvation). This is consistent with previously reported involvement of lysosomal alkalinisation in apoptosis (Nilsson et al., 2003). Importantly,

control and lipofuscin-loaded cells displayed similar lysosomal pH before initiation of starvation.

***Starvation-induced cell death is associated with relocation of cathepsin D to the cytosol***

Immunostaining for cathepsin D revealed relocation of the enzyme to the cytosol after 72 hours of starvation, which coincided with the time point at which significant increase of the percentage of dead cells in control group was found. In dying cells, light microscopy studies revealed a dramatic decrease of cytoplasmic volume without apparent changes of nuclear morphology. In contrast, lipofuscin-loaded cells preserved normal morphology and lysosomal pattern of cathepsin D staining.

***Hyperoxia does not induce overexpression of Hsp70***

Mild stress, including mild oxidative stress, is regarded as an activator of the systems responsible for cellular maintenance and repair, and is a basis of hormesis (Rattan and Clark, 2005). Protective effect of repeated exposures to mild stress has been found related to an increased expression of heat-shock proteins, namely Hsp70 (Fonager et al., 2002; Hercus et al., 2003). In this study no apparent changes in the expression of Hsp70 were found following exposure to 40% ambient oxygen for up to 4 months. This is in agreement with previously reported findings showing decreased expression and inducibility of heat-shock proteins with advancing age (Njemini et al., 2002; Jin et al., 2004; Starnes et al., 2005).

The distinction between PCD type I, or classical apoptosis characterised by caspase activation, and PCD type II, or autophagic cell death, is not always clear (Bursch, 2001; Lockshin and Zakeri, 2004). According to the present findings, starvation-induced cell death is associated with notable shrinkage of cytoplasm before apparent nuclear changes, which is typical for autophagic cell death (Bursch, 2001). On the other hand, the finding that inhibition of autophagy with 3-MA accelerated death of starving cells argues against an autophagic nature of starvation-induced cell death. In addition,

nuclear chromatin condensation, observed in dying cells, is more characteristic for apoptosis and post-apoptotic necrosis (Weber et al., 1997). Finally, although the role of caspases in starvation-induced programmed cell death is not entirely clear, their activation is also more typical for apoptosis (Bursch, 2004; Edinger and Thompson, 2004). Owing to the possibility of a simultaneous co-existence of both types of PCD in the same cell (Bursch, 2004; Mills et al., 2004), we do not specify the type of cell death detected in this study.

The present findings of a decreased cell death response of lipofuscin-loaded cells are in agreement with the view that ageing cells develop resistance to apoptosis. Alternatively to the previously described mechanisms behind this resistance (Wang, 1995; Spaulding et al., 1999; Pereira-Smith and Bertram, 2000; Marcotte et al., 2004), which are shortly covered in *Introduction*, the hormetic effect (Rattan and Clark, 2005) of moderate levels of lipofuscin is also possible. We further speculate that lipofuscin might stabilise lysosomal compartment through the attraction of lysosomal enzymes, disabling their translocation to the cytosol during PCD. This idea is supported by the present findings of delayed cell death in fibroblasts with inhibited cathepsin D activity. Overall, the results of this study warrant a novel view of lipofuscin accumulation as a mechanism for preservation of cellular integrity at the expense of functionality and general reactivity.

## **Paper IV**

Based on our previous findings of increased resistance of lipofuscin-loaded cells to starvation-induced cell death, we speculated that the anti-apoptotic effect of lipofuscin is due to stabilisation of lysosomes and, in this manner, prevention of PCD-triggering under conditions of lysosomal stress. In order to test if lipofuscin could influence lysosomal stability, human AG-1518 growth-arrested fibroblasts (lipofuscin-loaded versus non-loaded cells) were exposed to lysosome-targeted stress induced either by a lysosomotropic detergent (MSDH; Li et al., 2000) or acute oxidative stress by exposure to a redox-cycling quinone-treatment (NzQ; Roberg et al., 1999). Additionally,

functional integrity of lysosomes was affected by exposure to the vacuolar ATPase inhibitor bafilomycin A<sub>1</sub> (Bowman et al., 1988).

### ***MSDH-induced lysosomal destabilisation activates autophagy***

Exposure to MSDH resulted in pronounced autophagic vacuolisation observed on both electron- and light-microscopical level. We speculated that the increased autophagy could be viewed as an adaptive cellular response in order to limit the damage caused by the leakage of protons and proteolytic enzymes into the cytosol because of impairment of lysosomal membrane. This idea is in agreement with the previously suggested role of autophagic vacuolisation in protection of intracellular homeostasis (Henics and Wheatley, 1999). In this manner, autophagic vacuolisation, albeit present in both control and lipofuscin-loaded fibroblasts, but less pronounced in the latter, could be a sign of a lower degree of lysosomal damage in lipofuscin-loaded cells.

### ***Lysosomal destabilisation following the organelle-targeted stress is more pronounced in control than in lipofuscin-loaded fibroblasts***

Lysosomal destabilisation was significantly more pronounced in controls than in lipofuscin-loaded fibroblasts, especially during NzQ-treatment when lipofuscin-loaded cells did not show any signs of lysosomal impairment. Decrease in the number of lysosomes, following the organelle-targeted stress, was also found characteristic for control but not for lipofuscin-loaded cells.

According to the present findings of decreased sensitivity of lipofuscin-loaded lysosomes to organelle-targeted stress, we hypothesise that the observed phenomenon is due to intralysosomal lipofuscin. We further speculate that the lipofuscin-dependent lysosomal stabilisation could be explained by the following tentative mechanisms:

- Permanent occupation of the active sites of lysosomal enzymes by non-degradable (but targeted for degradation) lipofuscin, which

prevents translocation of the enzymes to the cytosol during lysosomal stress. This idea, based on the present results of decreased cytosolic translocation of cathepsin D in lipofuscin-loaded fibroblasts, is in agreement with our previous findings of increased resistance of lipofuscin-loaded cells to starvation-induced death (*Paper III*).

- Proton-trapping due to the polymeric structure of lipofuscin containing high number of OH<sup>-</sup> groups, which are potentially capable of reacting with H<sup>+</sup>. The idea is based on the present results of decreased cytosolic alkalinisation in lipofuscin-loaded cells following exposure to MSDH, NzQ, and, especially, the specific vacuole ATPase inhibitor bafilomycin A<sub>1</sub> (Bowman et al., 1988). The idea is also supported by our previous findings of decreased lysosomal alkalinisation in lipofuscin-loaded fibroblasts during starvation (*Paper III*).
- Lipofuscin is a site of free radical attraction. This might explain the observed high resistance of lipofuscin-loaded cells to acute oxidative stress.

Overall, the results of the present study support our previously suggested idea of lysosome-stabilising properties of lipofuscin.

## **CONCLUSIONS AND FUTURE PERSPECTIVES**

The introduced idea of lysosomal autophagy (*Paper I*) considers the possibility of the degradation of lysosomes as entire organelles. The presented tentative mechanism provides both a more efficient turnover of lysosomal membrane, when compared with partial recycling following lysosomal fusion or microautophagy, and an alternative way of delivery of lysosomal enzymes to the sites of ongoing degradation.

Properly operating autophagic machinery has been shown to play an important role in preventing age-related changes associated with (and dependent on) accumulation of so called biological “garbage” (*Papers I and II*). Biological “garbage” is constituted of two groups of non-degradable intracellular structures: polymer-like material known as lipofuscin, and non-autophagocytosed damaged bio-molecules and organelles. The most important difference between the two, in my view, is that the former is compartmentalised to the lysosomes, while the latter is located in the cytosol. According to the present study, biological “garbage” accumulating as a result of inhibited autophagy is deleterious for the cells (*Papers I and II*), probably due to the toxic effects of oxidised proteins and, especially, damaged mitochondria, which had not been sequestered from the cytosol (*Paper I*). Continual proliferation could be the natural mechanism by which cells cope with the accumulation of non-degradable material, employing mechanical dilution during the cell division (*Paper II*).

On the other hand, intralysosomal lipofuscin, albeit also a non-degradable material but removed from the site where the apoptotic machinery is unleashed, could be viewed as a marker of the damage that the cell had successfully withstood. In this manner, cellular ageing, characterised by lipofuscin accumulation, would be viewed as a continual process of coping

with damage to cellular constituents by either degradation of potentially hazardous structures, or their removal to a topologically different compartment. In addition, lipofuscin has been found to express lysosome-stabilising properties (*Papers III and IV*) and to improve cellular viability under stress conditions (*Paper III*). Thus, a novel view on lipofuscin is introduced, although the mechanisms of cell-protective effects of lipofuscin, if any, are still a matter of speculations and aims for the future investigations.

In order to better understand the influence of age-related changes of lysosomal compartment on cellular functions, further research is required. Thus, the presented idea of possible lysosomal turnover through macroautophagy (*Paper I*) requires more evidence to be fully confirmed. The possibility to consider lipofuscin as a hormetic agent urges further research focused on finding relationships between amount of lipofuscin and its effects on cellular functions, especially lysosomal stability. Considering heterogeneity of so-called biological “garbage”, further investigation of physiological/pathological effects of different components of “waste”-material is needed. Understanding the mechanisms and consequences of age-related accumulation of biological “garbage” may be helpful for future development of anti-ageing therapy and management of age-associated diseases.

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