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Graphical Review

Lipofuscin: formation, effects and role of macroautophagy ☆

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ABSTRACT

Oxidative stress plays a crucial role in the development of the aging process and age dependent diseases. Both are closely connected to disturbances of proteostasis by protein oxidation and an impairment of the proteasomal system. The final consequence is the accumulation of highly cross-linked undegradable aggregates such as lipofuscin. These aggregates of damaged proteins are detrimental to normal cell functions. Here we provide an overview about effect of these aggregates on the proteasomal system, followed by transcription factor activation and loss of cell viability. Furthermore, findings on the mechanism of radical genesis, proteasomal inhibition and the required components of lipofuscin formation were resumed.

Contents

Introduction

In unstressed situations protein homeostasis is balanced by folding and stabilization of proteins by chaperones of the Hsp family [1] and the controlled degradation of proteins by the proteasomal system. The proteasome exists in different forms, and its activity is modulated by multiple regulators. The 20S core proteasome contains the proteolytic activity and selectively degrades a multitude of oxidized proteins [2–5], as well as other substrates [6], in an ubiquitin- and ATP-independent manner. When the core 20S proteasome combines with two 19S regulators, the 26S proteasome is formed [7] which selectively removes polyubiquitinated proteins [8–10]. Under stress conditions and, therefore, most notably during aging the balance between protein damage and clearance of damaged proteins is disturbed leading to a malfunctioning of proteostasis and an accumulating mass of oxidized proteins, aggregate and aggresome formation and finally to the accumulation of highly cross-linked materials such as lipofuscin, compromising cell viability. Accumulation of aggregates in postmitotic cells seems to be especially dramatic, since they are not able to dilute this material by cell division.

Previous work has shown that especially the 26S proteasome is highly susceptible to inactivation during oxidative stress [11,12] and although inhibition of 26S proteasomes could be caused by oxidation products such as protein aggregates, it is most likely a stress-triggered disassembly [13,14] (Fig. 1A). It could be suspected, that this disassembly of 26S proteasomes serves to increase 20S proteasome abundance, allowing cells to clear irreparably damaged proteins more effectively [9,15,16]. But inhibition of 26S is accompanied with the accumulation of undegraded, polyubiquitinated proteins, which are sensed by the ubiquitinbinding domain of HDAC6 (Histone deacetylase 6) [17].

Via HDAC6 this proteotoxic stress acts as a signal for a number of response mechanisms that cope with proteasomal inhibition, such as stabilization and prevention of aggregate formation by the induction of classical Hsps, elimination of polyubiquitinated proteins/aggregates by HDAC6 mediated aggresome formation and lysosomal uptake via autophagy, containment of inflammation by the induction of HO-1(heme oxygenase-1) and Nrf-2 (NF-E2-related factor 2) pathway and reduction of proteotoxic stress mediated apoptosis by the induction of classical Hsps and HO-1 [17–19] (Fig. 1B). Furthermore, nuclear translocation of NFκB (Nuclear factor κB) requires degradation of ubiquitinated phospho-IκB-a

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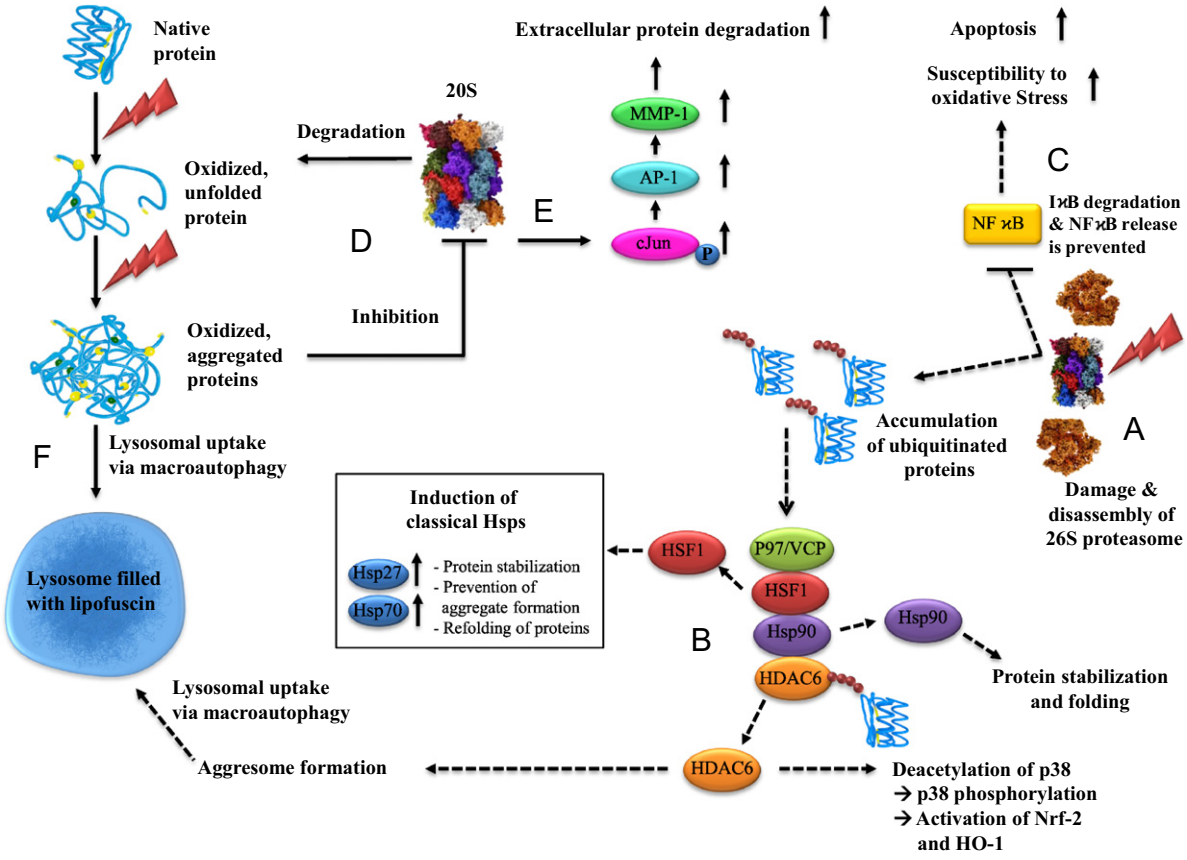

Fig. 1. Consequences of oxidative stress on proteins, the proteasomal system and transcription factors. (A) The 26S proteasome is highly susceptible to inactivation during oxidative stress (figured as flash symbol) leading to an excessive accumulation of polyubiquitinated proteins. (B) Histone deacetylase 6 (HDAC6) has the ability to sense and bind ubiquitinated proteins via the BUZ (bound to ubiquitin zinc finger) domain which induces the dissociation of a complex formed out of Hsp90, HSF-1, p97/VCP and HDAC6 [35]. Following the liberation of HDAC6 and p97/VCP [17,36], the latter uses either its ATP dependent segregase activity to dissolve the bond between Hsp90 and HSF-1 directly or p97/VCP stimulates the Hsp90 ATPase activity, which results in the release of HSF-1 and the consequence up-regulation of several heat shock proteins, such as Hsp70 and Hsp 27 [17]. Beyond up-regulation of classical Hsps upon proteasome inhibition, HDAC6 is also involved in an induction of HO-1 after proteasome inhibition [18]. Initiation of this pathway is also the detection of ubiquitinated proteins and the release of HDAC6 and mediated by a p38/MAPK-dependent activation of Nrf-2, which is the most important transcriptional activator of HO-1 gene translation [171]. Further HDAC6 has the ability to favor the accumulation of polyubiquitinated proteins in cellular aggresomes by interaction with ubiquitin and dynein motors. Aggresomes are inclusion bodies next to the nucleus at the proximity end of the microtubule organizing center, finally eliminated by an autophagy-mediated mechanism. (C) Furthermore, a pivotal regulatory protein function is disrupted by 26S proteasomal inhibition, the transcription factor NFκB. This factor binds to multiple DNA sequences, initiating the transcription of gene products including various cytokines, angiogenesis factors, cell adhesion molecules, enzymes and antiapoptotic factors [21]. NFκB is located in the cytoplasm in an inactive form, bound to an inhibitor molecule IκB. Stimulation of cells through a variety of mechanisms triggers a cascade of signaling events resulting in the degradation of IκB by the proteasome. This degradation releases active NFκB, which then translocates into the nucleus and binds to specific DNA sequences on its target genes [20]. Proteasomal inhibition blocks NFκB activation and leads among others to increased susceptibility to oxidative stress and apoptosis. (D) The activity of the 20S proteasome, which is mostly responsible for the degradation of oxidized proteins and less susceptible to direct oxidative stress, can be nevertheless diminished by aggregated oxidized proteins. These protein aggregates are formed under stress conditions as complexes of unfolded proteins which do not normally interact with each other. It might require several steps depending on the nature of the initial conditions, leading to unfolding and aggregate formation. Due to the complex process of intermolecular interactions, such as during physiological aging, the process of aggregation is slow [30,37,38]. The aggregate is independent from the original structure of the protein and introduces a new toxic element into cellular metabolism, partly by inhibiting 20S proteasome. (E) Another side effect of proteasome inhibition is leading to a higher amount of phosphorylated c-Jun leading to an activation of AP-1, known to control the expression of MMP-1 [22] and numerous other genes. Subsequently increased extracellular protein degradation can be assumed, as observed in skin aging. (F) When proteolytic capacity declines below a critical threshold of activity required to cope with oxidative stress, the final consequence is the accumulation of aggregated proteins which may, instead, cross-link with one another or form extensive hydrophobic bonds. This material might undergo further reactions and finally form the age pigment lipofuscin which has toxic properties and accumulates in the lysosomal system..

(inhibitor of κB) by the 26S proteasome [20]. NFκB regulates critical survival pathways such as apoptosis in a variety of different cell types [21]. During proteasomal inhibition apoptosis is enhanced through inhibition of IκB-a degradation (Fig. 1C). Although, the 20S proteasome is relatively resistant towards oxidants both *in vitro* and *in vivo* [11], it was shown that oxidized protein aggregates are able to inhibit the proteolytic activity of the 20S proteasome (Fig. 1D). So beside 26S disassembly as a consequence of oxidative stress the effectiveness of 20S is also diminished by accumulation of heavily damaged, oxidized, and aggregated proteins during postmitotic aging. It was further demonstrated that inhibition of 20S proteasome by UVA-irradiation followed aggregate formation subsequently leads to an accumulation of phosphorylated c-Jun and activation of activator protein-1 (AP-1), controlling MMP-1(matrix metalloproteinase-1) expression [22]. MMP-1 as a major protease of the extracellular matrix is thereby up-regulated causing increased extracellular protein degradation (Fig. 1E). Noteworthy, it can be expected that other AP-1 induced genes are also activated.

This accumulation of aggregates is the final fate of protein damage under stress conditions. Under such circumstances oxidized proteins may not undergo appropriate proteolytic digestion but instead, cross-link with one another or form extensive hydrophobic bonds. It is believed that the cross-linked proteins react further with other cellular components, forming a fluorescent material referred to as lipofuscin (Fig. 1F). Lipofuscin is accepted to consist of oxidized proteins (30–70%) as well as lipids (20–50%) [23] and from the fifth decade of life, bound sugar residues were also detected in human lipofuscin [24]. A

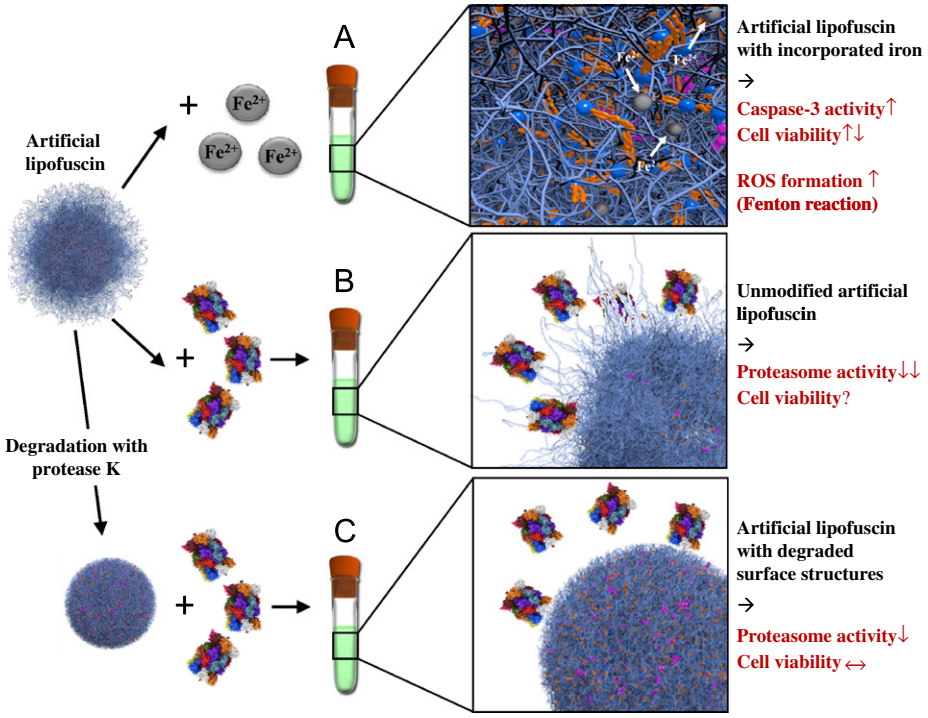

Fig. 2. Properties of lipofuscin. Studies have suggested that lipofuscin is not an inert waste product, but rather an active component influencing the cellular metabolism, which is especially relevant in senescent cells. It was proposed that lipofuscin is cytotoxic because of its ability to incorporate redox-active transition metals, resulting in a redox-active surface, able to catalyze the Fenton reaction: in the presence of Fe^{2+}, H_2O_2 is decomposed forming hydroxyl radicals ($OH^{\bullet}$) [39]. The resulting Fe^{3+} can be reduced by superoxide and the vicious cycle starts again. This ability to incorporate transition metals (iron) and form oxidants was tested by using artificial lipofuscin-like oxidized, cross-linked protein aggregates ("artificial lipofuscin"). Non-iron-loaded artificial lipofuscin was incubated with Fe^{2+} and was able to incorporate a maximum amount of 7 mass%, matching the characteristics of native lipofuscin very well [26]. *In vitro* this material is able to generate the formation of free radicals and initiates apoptotic cell death, resulting in a significant loss of cellular viability (A). Another major and already demonstrated characteristic of protein aggregates/lipofuscin is the ability to inhibit the degradation of oxidized proteins by competitively binding to the proteasome [40–42]. The proposed mechanism for proteasomal inhibition is binding to exposed hydrophobic amino acid structures on the surface of the highly oxidized and covalently cross-linked lipofuscin [5]. These structures seem to be main sites of proteasomal substrate recognition. After proteasomal binding to those structures the protease is unable to degrade the exposed structures completely because of sterical and/or mechanical inhibition by cross-links. So the proteasome is bound to the surface of lipofuscin in ineffective attempts of degradation detracting proteolytic capacity from other substrates and resulting in a measurable proteasomal inhibition. Furthermore, celluar viability is affected and significantly reduced (B). Degradation of these exposed structures by using the effective and nonspecific protease *K* demonstrated that such unfolded peptide binding sites are largely responsible for the inhibition of the proteasome. This degradation of these binding sites significantly reduces the proteasomal binding and the inhibition of the proteasome could be partially prevented. Consequently less proteasomal capacity is detracted in futile attempts of degradation and cellular viability was unaffected or increased referred to the undegraded material (C) [29].

hypothetical mechanism of lipofuscin formation was described in detail by Brunk and Terman in their widely accepted model known as "the mitochondrial–lysosomal axis theory of aging" [25]. According to this model, an intralysosomal accumulation of lipofuscin can be considered as the long-term result of a decreased degradation of oxidized proteins and an increase in intracellular free radical formation. Metals, including Fe, Cu, Zn, Al, Mn, and Ca, comprise up to 2% of lipofuscin [26] and especially catalytic iron seems to be an important factor in further oxidation reactions of the initial protein aggregate. In mammalian cells iron is the most abundant cellular transition metal and a fundamental player in the above mentioned mitochondrial–lysosomal axis theory of aging [25].

However, the detailed intracellular effects of lipofuscin in a cell are largely hypothetical and under discussion for a long time. By using an artificial lipofuscin it could be shown, that lipofuscin is a prominent source of oxidants and is able to incorporate iron in a redox-active manner (Fig. 2A). It could be shown that artificial lipofuscin and particularly iron-loaded artificial lipofuscin increase caspase-3 activity if taken up by cells and, therefore, apoptosis [27]. Furthermore, deferoxamine as chelating agent could reduce the effects of iron-loaded lipofuscin significantly. Thus, the amount of iron included in lipofuscin seems to play a crucial role in the intracellular effects of lipofuscin, particularly considering the ability to catalyze the formation of free radicals and the resulting cytotoxicity. These results match very well the hypothesis of Brunk and Terman [25], postulating that iron inclusions of lipofuscin result in a redox-active surface catalyzing the Fenton reaction.

Another major characteristic of lipofuscin is its ability to inhibit the degradation of oxidized proteins by competitively binding to proteolytic enzymes including the 20S proteasome as mentioned above as well as lysosomal proteases. How exactly the proteasomal substrate recognition functions is still under investigation, but one of the recognition motifs might be exposed hydrophobic patches from oxidatively damaged and partially unfolded proteins [5,28]. It seems likely that the proteasome also binds to such exposed hydrophobic oligopeptides on the lipofuscin surface but is then unable to completely degrade or to release these peptides, resulting in proteasomal inhibition (Fig. 2B). Evidence for this hypothetical mechanism could be recently shown by partial degradation of these hydrophobic surface patches in artificial lipofuscin with protease K, resulting in decreased proteasomal inhibition and reduced cytotoxicity (Fig. 2C) [29].

Furthermore there has been some speculation about dysfunction of the lysosomal proteases due to the accumulation of lipofuscin in lysosomes, but it is still unclear where the initial cross-linking reactions are taking place: in the cytosol and afterwards uptake by macroautophagy, or whether lysosomes are required components of lipofuscin formation [30,31]. Macroautophagy is a process, which is beside organelle degradation

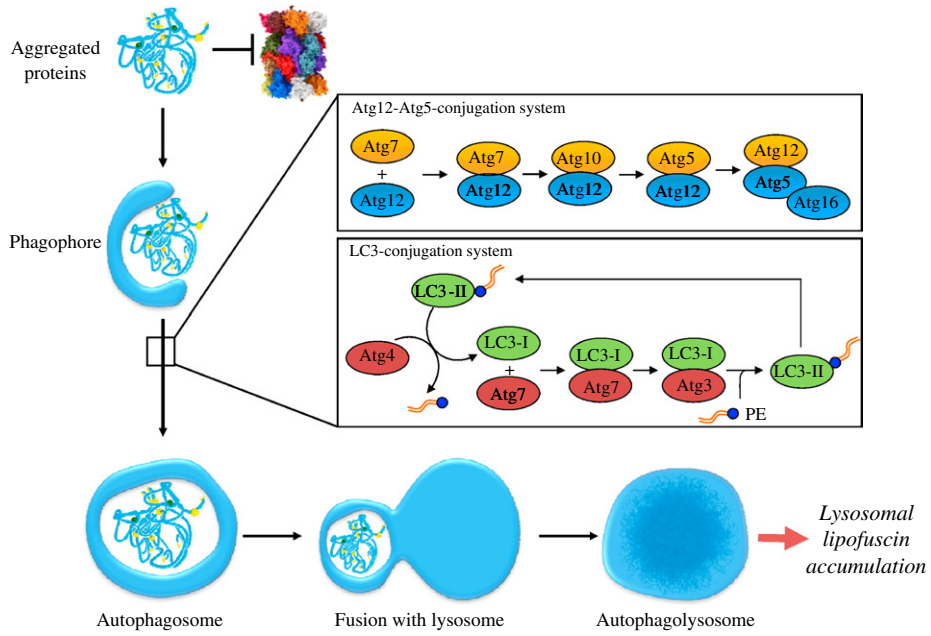

Fig. 3. The fate of aggregated proteins during functional macroautophagy. Macroautophagy is a process, which is responsible for the uptake of larger aggregates. Aggregates which are no longer degradable by the proteasome, but rather inhibit its proteolytic function are enclosed by a phagophore and transported towards the lysosomal system. Macroautophagy comprise the formation of an autophagosome, a double-membrane vesicle that engulfs substrates trough the expansion of an isolation membrane, called phagophore. This isolation membrane is build, expanded and closed to an autophagosome and delivered to the endo/lysosomal system. Afterwards both structures fuse and the final structure is called autophagolysosome. The elongation of the phagophore requires two processes similar to ubiquitination: Atg12-Atg5-conjugation and LC3 (microtubule-associated-protein-light-chain-3)-modification (illustration of Atg12-Atg5-system and LC3 conjugation system according to Levine and Deretic [43]). In the first conjugation system (Atg12-Atg5-system) Atg12 is conjugated with Atg5, in a process assisted by an E1-ubiquitin-like activating enzyme: Atg7 and the E2-ubiquitin-like conjugation enzyme: Atg10. Afterwards the Atg12-Atg5 conjugate forms a protein complex with Atg16 on the outside of the nascent autophagosome [44,45]. This complex is functionally necessary for the end product of the secondary conjugation system, lipidated LC3. First the cysteine protease Atg4 eliminates the C-terminal tail of LC3I to expose a glycine residue. A G116 is activated by Atg7, and LC3I is conjugated via a thioester bond with Atg7. Afterwards LC3I is transferred on the E2-ubiquitin-like conjugation enzyme Atg3, and again connected via a thioester bond. Finally LC3I is bound to the amino group of phosphatidylethanolamine (PE). This complex of LC3 with PE is called LC3II and localized via its lipid part to the autophagosomal membrane enabling membrane-elongation. Finally LC3II is deconjugated from the phospholipid-anchor via Atg4 and the luminal associated LC3II is degraded. This cycle of conjugation and deconjugation is important for the normal progression of autophagy. Especially under conditions of oxidative stress a huge amount of aggregated proteins accumulate. These aggregates are taken up by macroautophagy and react further with other cellular components, forming a fluorescent material referred to as lipofuscin, which accumulates in the lysosomal system over time.

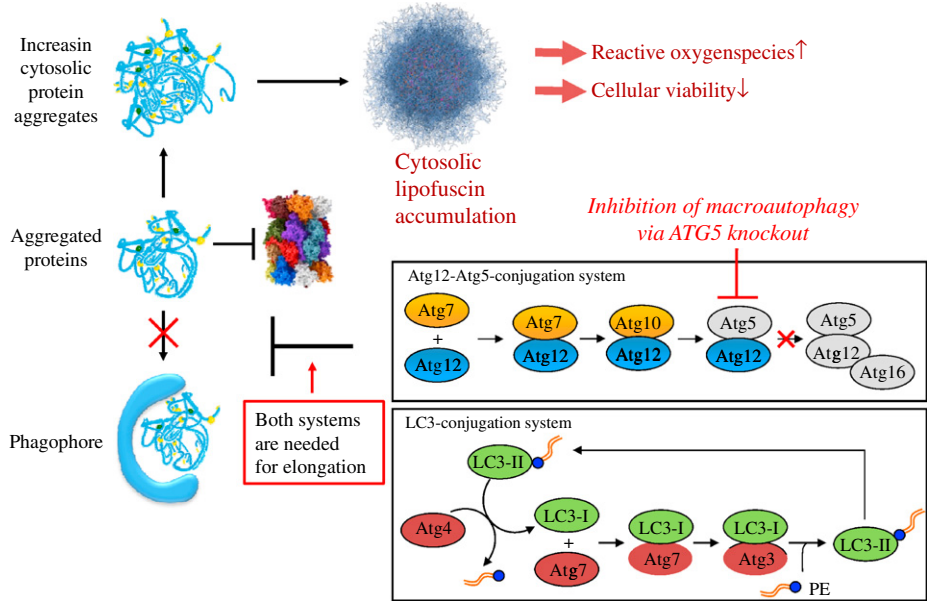

Fig. 4. The fate of aggregated proteins during impaired or non-sufficient macroautophagy. Macroautophagy is maximally activated under stress conditions such as starvation, oxidative stress and conditions leading to enhanced protein misfolding [46–50]. This essential role of macroautophagy is underpinned by the fact that Atg5 or Atg7 null mice die a few hours after birth [51]. Nevertheless it is possible to investigate the role of macroautophagy in knockout models such as fibroblasts from mouse embryos deficient in Atg5 (Atg5$^{-/-}$ MEFs) under stress conditions. This ATG5$^{-/-}$ MEFs naturally show no Atg5 expression and thus no formation of the Atg12-Atg5-complex. As the Atg12-Atg5-conjugation system and LC3 modification (illustration according to Levine and Deretic [43]) are both mandatory for the formation of a phagophore, the uptake of aggregated proteins (and other substrates) is prevented in this knockdown system. The consequence is an increasing amount of cytosolic protein aggregates and finally a dramatically enhanced cytosolic lipofuscin accumulation. These elevated levels of extralysosomal lipofuscin are combined with a decline in cellular viability and an increased amount of ROS production.

responsible for the uptake of larger aggregates. Normally aggregated proteins beyond proteasomal degradation are enclosed by a phagophore and mediated to the lysosomal system (Fig. 3). By application of oxidative stress to an ATG5 knockout model it was possible to manipulate the lipofuscin amount within lysosomes and the uptake of aggregates into lysosomes could be reduced. However this inhibition of macroautophagy actually did not prevent the formation of lipofuscin [32]. These findings indicate that in contrast to an earlier hypothesis [33,34] lipofuscin can also be formed in the cytosol (Fig. 4) and the uptake into an autophagosome seems to take place in a secondary step. Therefore, autophagosomes/lysosomes are not mandatory for the formation of lipofuscin but constitute a storage for aggregates reducing aggregate toxicity [32].

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