Chapter 22

Time Flies: Autophagy During Ageing in *Drosophila*

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Ageing

The process of ageing compromises the age-associated decrease in fertility, gradual loss of function, and increased vulnerability to disease, which progressively diminishes the capability of an organism to survive [1-3]. Unsurprisingly, in the past years it has been of great interest to understand which factors influence this inevitable and complex process. As a result a wide array of molecular and cellular damages has been identified and shown to accumulate during ageing. The lifelong accumulation of such damages will eventually result in frailty and disease [4]. The variety of identified age-dependent damages has given rise to different theories for molecular ageing mechanisms. These mechanisms include decreased cellular capacity to deal with DNA damage, and decline in cellular division capacity, which is linked to the progressive shortening of telomeres upon each cell cycle. Also an increased accumulation of damaged mitochondria and the involved increase in reactive oxygen species (ROS) production and decline in ATP synthesis has been shown to occur over time (reviewed in [5]). One of the phenotypic hallmarks of aged cells is the intracellular accumulation of damaged proteins and therefore protein turnover/protein degradation has attracted attention over the last years [2].

At the same time, forward genetics have allowed to investigate single gene alterations and their influence on lifespan of whole organisms. Even though the ageing process is without doubt influenced by stochastic and environmental factors, single gene mutations were shown to extend lifespan in worms, flies, and mice, suggesting the existence of a central process of ageing [6, 7]. Many of the genetic manipulations that alter longevity affect metabolism, nutrient sensing and stress response pathways. As all these pathways are connected to autophagy (an important player also in protein turnover), the question about the role of autophagy in ageing has come more and more to the fore. In this chapter we will focus on how research conducted

in the excellent genetic model system *Drosophila melanogaster* has contributed to understand more about the interplay of autophagy and ageing.

2. Autophagy

Autophagy, which literally means "self-eating" (coined by Nobel Laureate Christian de Duve in 1963), allows cells to digest cytosolic components via lysosomal degradation. Autophagy and the Ubiquitin Proteasome System (UPS) constitute together the main cellular pathways for protein and organelle turnover [8, 9]. Today, three different classes of autophagy are distinguished: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy.

During microautophagy, which is mainly studied in yeast (containing vacuoles instead of lysosomes), cytoplasmic material is delivered to the vacuolar lumen by direct invagination of the vacuolar boundary membrane and budding of autophagic bodies into the vacuolar lumen [10]. The molecular mechanisms underlying microautophagy in eukaryotic cells are largely unknown. However, Cuervo and colleagues described a microautophagy-like process (named endosomal microautophagy, e-MI) in mammalian cells, whereby soluble cytosolic proteins are selectively taken up by late endosomes/multivesicular bodies (MVBs). The cargo selection in e-MI depends on the chaperone Hsc70 and electrostatic interactions with the endosomal membrane [11]. Hsc70 is also involved in chaperone-mediated autophagy (CMA), in which cytosolic cargo is selectively recognized, bound by the lysosome-associated membrane type protein 2A (LAMP-2A) and finally taken up by the lysosome, thereby allowing for direct lysosomal degradation of cytosolic proteins. The requirement of protein unfolding and the binding of LAMP2-A is characteristic for CMA and thereby distinguishes CMA from e-MI [11, 12]. So far, CMA has not been investigated in *Drosophila melanogaster*. The third common type of autophagy, macroautophagy (henceforth referred to as autophagy), is highly conserved from yeast to mammalian cells [8]. Autophagy allows for cytosolic bulk degradation of long-lived macromolecules and organelles. Morphologically this process was already described in the 1960s but it was not before several decades later when genetic screens in *Saccharomyces cerevisiae* identified multiple genes involved in autophagy and thereby allowed to investigate the molecular mechanisms in further detail [13, 14]. Genetic screens in *S. cerevisiae* have since then led to the identification of numerous autophagy-related (ATG) genes and many homologs have been identified and characterized in higher eukaryotes [15]. In general, autophagy can be divided into three steps: 1) induction/nucleation; 2) expansion; and 3) maturation [16].

The formation of a cytosolic double membrane structure called the phagophore (also called isolation membrane) is an important step of autophagy initiation. It is subject of discussion about the origin of this initial autophagic membrane. Independent experiments identified ER, Golgi, or the outer membrane of mitochondria to contribute to the phagophore double membrane [17, 18]. Cytosolic components are enwrapped during the growth of the phagophore. Closure of the phagophore completes this engulfment and gives rise to a new structure called the autophagosome. These newly formed autophagosomes will further mature and

subsequently fuse with lysosomes where the captured cytosolic constituents will be degraded. Autophagy can achieve several purposes; it scavenges the cytosol from macromolecules and organelles but also provides a way to supply the cells with amino acids and if necessary with energy once the recycled amino acids are converted into intermediates of the tricarboxylic acid cycle (TCA) [15, 18-20]. It is therefore of little surprise that the autophagic machinery, which under normal conditions is running on low basal levels, can be set in motion by several intra- and extracellular stress factors, such as starvation, ER-stress, hypoxia and pathogen invasion [15]. Besides non-selective cytosolic bulk-degradation, autophagy is also implicated in selective turnover in yeast, a pathway known as the cytoplasm-to-vacuole targeting (CVT) pathway [21]. In analogy, cargo selective degradation of aggregated proteins (aggrephagy [22]), mitochondria (mitophagy [23]), ribosomes (ribophagy [24]), peroxisomes (pexophagy [25]), endoplasmic reticulum (reticulophagy [26]) and many more have been reported for mammalian systems [27]. The role of selective autophagy in ageing will be further addressed in a separate section of this chapter.

Several protein complexes are involved along the path from initiation to completion of autophagy. Induction of autophagy in *Drosophila* requires the Ser/Thr kinase Atg1 that forms a complex with Atg13. Phosphorylation of Atg13 by Atg1 directs phagophore initiation through a complex containing the class III PI(3)-kinase Vps34, the Ser/Thr kinase Vps15 and Atg6 (Beclin1 in mammals). The activation of this complex leads to localized generation of phosphatidylinositol-3-phosphate (PI3P), a critical step in autophagy. In mammalian systems, this core complex has several known interaction partners, e.g. Atg14, Ambra1, UVRAG, or Rubicon, that are all involved in autophagy. Several of these mammalian genes have orthologues in *Drosophila*, however their involvement in autophagy remains to be shown (reviewed in [28]). UVRAG has recently been found to be important in the regulation of Notch levels in the context of organ rotation during development. This role of UVRAG is coupled to endocytic degradation of Notch and, in this context, not to autophagy [29]. Autophagosome formation requires the ubiquitin-like proteins Atg12 and Atg8 and their respective ubiquitin-like conjugation systems [30]. Atg8 is processed by the cysteine protease Atg4 and covalently linked to phosphatidylethanolamine (PE) through the action of the E1 activating enzyme Atg7, the E2 activating enzyme Atg3 and the E3 like Atg12-Atg5-Atg16 complex, which is found at the phagophore membrane. The E3 like Atg12-Atg5-Atg16 complex itself requires also Atg7 and the E2 activating enzyme Atg10 for its assembly (reviewed in [31]). Once Atg8 is activated and lipid-conjugated it is localized to both sides of the phagophore and Atg4 later only removes the portion residing at the cytosolic side prior to autophagosome-lysosome/endosome fusion. It has also been reported that Atg8 can modulate the size of autophagosomes by influencing membrane curvature. For all these reasons, activation of Atg8/LC3 is widely used to monitor autophagy [15, 32]. The process from autophagy initiation until autolysosome formation is schematically illustrated in figure 1.

It is believed that stepwise fusion of autophagosomes with different endosomal populations account for maturation and culminates in the fusion with lysosomes, the organelle responsible for degradation [33]. Such stepwise fusion is supported by the findings that impairment of ESCRT machinery results in reduced autolysosome formation, measured as decrease in

lysotracker staining and accumulation of Atg8 positive punctate respectively [34, 35]. Similar accumulation of autophagosomes can be seen in flies with mutant *Drosophila* deep orange (dor) and dvps16A [36, 37]. Both proteins are known to play important roles in endocytic trafficking.

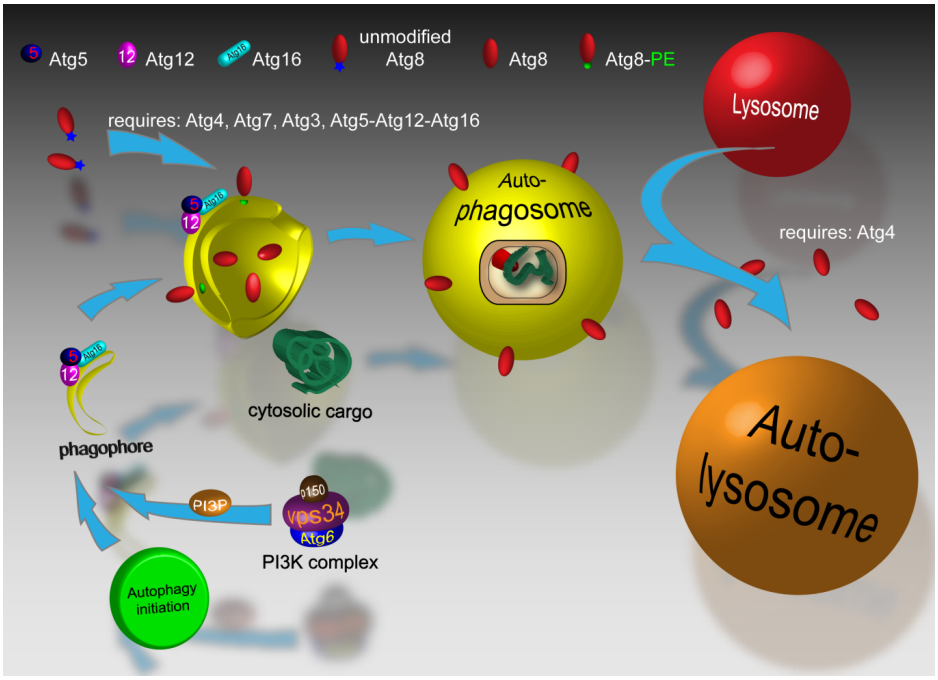

Figure 1. Schematic illustration of macroautophagy. Upon autophagy initiation the PI3K complex generates PI3P, which is then provided at high local concentrations at the initial step of phagophore membrane formation. The ubiquitin like proteins Atg12 and Atg8 with their respective conjugation system are recruited and activated once the phagophore is formed. Membrane expansion leads to phagophore maturation, which is finalized by vesicle closure and thereby autophagosome formation. This vesicle can fuse with different endocytic compartments or directly with lysosomes, forming autolysosomes. There, phagophore-sequestered cytosolic cargo is degraded and macromolecules can be recycled back to the cytosol. For further details see section 2 and references therein.

3. Role of autophagy in development, homeostasis and ageing

In general, the role of autophagy is predominantly described as cytoprotective. Intensive research over the last decade has increased our understanding of multiple cellular events that involve autophagy, e.g. dealing with low nutrient levels, development and morphogenesis, response to oxidative stress, turnover of protein aggregates and damaged organelles, immune response and lately also cell signaling (figure 2). Altogether the picture has emerged that the role and regulation of autophagy is extremely dependent on the cellular context [20, 38, 39].

With this review we therefore want to highlight what is known from research conducted in *Drosophila*, a model organism, which allows for elegant genetic manipulations of the cellular setup in a multi cellular organism.

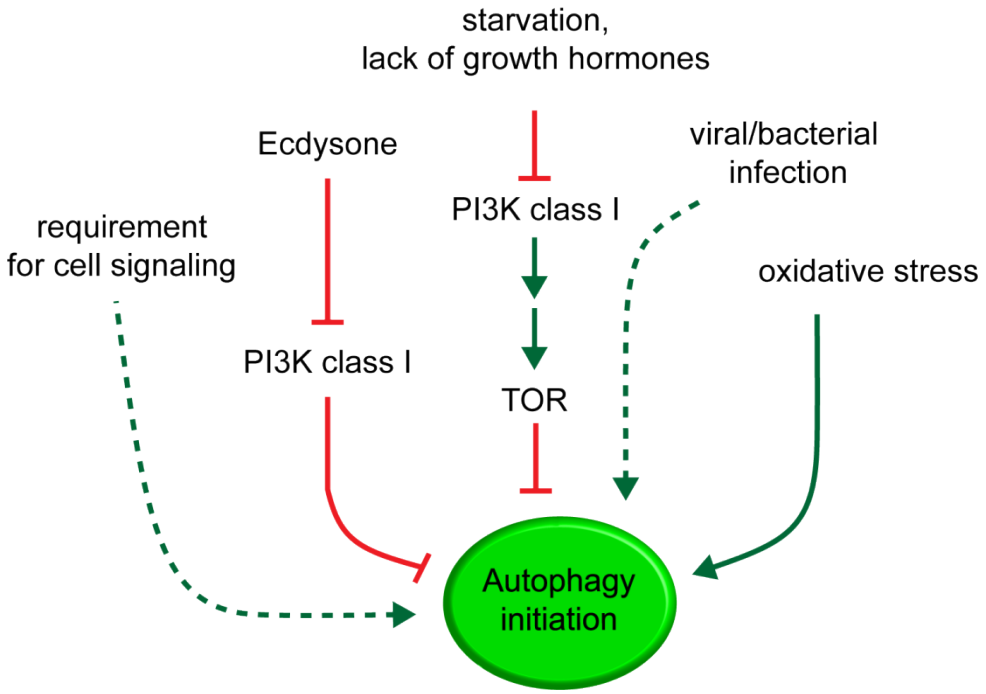

Figure 2. Autophagy can be initiated by multiple ways. Autophagy is involved in a variety of different cellular events (e.g. development, survival under conditions of low nutrient levels, oxidative stress response, immune response, and cell signaling), which requires several ways to initiate the core autophagy machinery (dashed lines: the exact pathway is still uncertain, however autophagy is shown to be upregulated as downstream effect). For further details see section 3 and references therein.

In *Drosophila*, autophagy plays a pivotal role during development and is crucial for a wide range of developmental processes. Cell growth depends on nutrients provided by autophagy as seen in the fat body. On the other hand autophagy has been reported to be necessary for targeted cell death and removal of tissue, e.g. during oogenesis and development of gut salivary glands.

More than ten years ago, both the class I phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase Target of Rapamycin (TOR) have been shown to control a signaling network that is important for development (reviewed in [40]). The growth of cells and tissues does require energy and building blocks. Hormones, such as insulin have been identified as important signals in order to meet these requirements by e.g. upregulation of protein synthesis. Already in 2003, Tom Neufeld speculated about the role of catabolic processes, such as

autophagy, to be important in development. This idea was supported by previous findings that established a connection between reduced basal autophagic protein turnover and cellular growth as well as that Apg6p, the yeast homologue of the tumor suppressor gene *Beclin 1*, is required for autophagy in yeast (reviewed in [40]). Furthermore, it was already shown that insulin, as well as class I PI3Ks can, besides their effect on protein synthesis, inhibit autophagic protein turnover, providing a plausible molecular link between autophagy and cell growth [41, 42]. Therewith the stage was set for two important findings published in 2004, revealing the regulation of programmed autophagy in the fat body and the importance for functional autophagy in cell growth [43, 44]. The levels of the hormone 20-hydroxyecdysone (ecdysone) rise during the development of *Drosophila*, leading to inactivation of the class I PI3K and subsequent autophagy activation [43]. This initiation of autophagy is necessary in order to supply the developing *Drosophila* larva/pupa with nutrients and to maintain survival and growth. The protective role of autophagy in this context is dominant over its otherwise known role in growth suppression [44, 45]. Noteworthy, dor (Deep orange), the *Drosophila* homolog to Vps18, can influence autophagy in the fat body in two separate ways. Dor is necessary for secretion of ecdysone from the salivary glands, thereby influencing the levels of this hormone. However, dor is also important in the fusion of autophagosomes with lysosomes, thereby directly controlling autophagy [36]. Autophagy in the fat body is dependent on the PI3K Vps34 [34]. Vps34 was initially identified to be involved in vacuolar protein sorting (Vps) in yeast [46]. Flies lacking Vps34 or its regulatory subunit, the protein kinase Vps15 (also referred to as p150), are hampered in their ability to initiate autophagy upon starvation in the fat body and die during development [34, 47]. Interestingly, the absence of Atg7 does not lead to lethality in the developing fly. Atg7 deficient flies have severe defects in autophagy but nevertheless are viable. However, such flies are short lived, show signs of accelerated ageing in the form of ubiquitin-positive aggregates in degenerating neurons and have very low resistance to nutrient deprivation and oxidative stress. This underscores the necessity of functional autophagy for cellular homeostasis and stress survival in the adult fly [48].

A very different aspect of autophagy during development has been revealed in the context of programmed cell death. Autophagy is upregulated during the reorganisation of the salivary gland and gut [49, 50]. Inhibition of autophagy in salivary glands by activating the class I PI3K pathway reduces salivary gland cell degradation. In contrast, induction of autophagy in salivary gland cells results in premature cell death and it was shown that this cell death is dependent on both caspases and autophagy [49]. Similar events can be seen in the midgut. Even though caspases are highly expressed, the canonical apoptotis pathway is not required for midgut removal. Inhibition of autophagy on the other hand, impairs midgut degradation and simultaneously decreases caspase activity [50]. Additional ways how cell death and autophagy are connected are pointed out by the findings that autophagy can selectively degrade survival factors and thereby initiate cell death. During late oogenesis, autophagy is necessary to degrade the apoptosis inhibitor dBruce in nurse cells. Nurse cells lack the, under normal conditions typical, fragmentation of DNA and caspase-3 activity in the absence of autophagy [51]. A similar principle for cell death control is suggested by the finding that the valosin-containing protein (vcp), a ubiquitin-selective AAA chaperone, is required for degradation of the apoptosis inhibitor DIAP1 during regulated degeneration of dendrites of

class IV dendritic arborisation neurons [52]. It was already shown before that vcp is necessary for autophagy [53]. Altogether, this implies a role for autophagy in activating apoptosis by selective degradation of apoptosis inhibitors. It will be interesting to see if such a mechanism is limited to the programmed reorganization events during development or if this is a strategy employed even in other cellular contexts. If this is a general mechanism to initiate cell death, autophagic degradation of apoptosis inhibitors might become an interesting strategy for developing drugs aimed for cancer treatment.

The role of autophagy in *Drosophila* is not limited to development but instead autophagy is also important for various aspects during lifetime of eclosed flies. Any organism needs to be able to cope with oxidative stress, which itself is tightly linked to ageing [5]. In *Drosophila*, Jun N-terminal kinase (JNK) can protect the gut from oxidative toxicity due to feeding on paraquat, a well-established oxidative stress inducer. In addition, genetic upregulation of the JNK pathway extends lifespan of flies in a Foxo dependent manner [54, 55]. This cell protective effect of JNK is mediated by the transcriptional activation of autophagy. JNK cannot protect flies from oxidative toxicity when Atg1 or Atg6 activity is reduced [56].

A different putative way for autophagy to protect cells from oxidative stress is given by its involvement in the selective degradation of damaged mitochondria, termed mitophagy. So far, mitophagy has not been directly shown to occur in *Drosophila*, nevertheless several findings indicate that mitophagy also happens in flies. Studies in *Drosophila* have suggested that the E3 ubiquitin ligase Parkin normally facilitates mitochondrial fission and/or inhibits fusion [57]. In addition the PTEN-induced putative kinase protein 1 (PINK1) has been shown to genetically interact with Parkin in flies, and results from experiments in *Drosophila* S2 cells revealed that PINK1 is required for the recruitment of Parkin to damaged mitochondria leading to their degradation [58]. Interestingly, the finding that the level of the protrusion factor mitofusin (mfn) increased in the absence of PINK1 or Parkin, suggests that mfn might be ubiquitinated by Parkin, which can serve as putative label and targeting signal for degradation of damaged mitochondria [58]. In yeast and mammals it has been shown that ubiquitination of mitochondrial proteins by Parkin results in autophagic degradation of mitochondria (reviewed in [23]). This role of Parkin in the removal of damaged mitochondria might also explain the muscle degeneration, mitochondrial pathology and reduced lifespan in *parkin* mutant flies [59]. Lately, it has been reported that mitochondrial protein misfolding in *Drosophila* leads to degradation of mitochondria and that accumulation of an unfolded protein in the mitochondria phenocopies flies with mutations in PINK1 and Parkin. The requirement of Ref(2)P (refractory to Sigma P, the *Drosophila* homolog of p62) for this mitochondrial turnover resembles mitophagy as described in mammalian systems [60]. However, it remains to be proven that the turnover of damaged mitochondria in flies really is conducted by autophagy, hence that mitophagy also occurs in *Drosophila*.

Without doubt autophagy is crucial for cellular homeostasis and it is therefore of no surprise that autophagy is also induced upon viral or bacterial infections as both lead to changes in the intracellular environment. Flies with impaired autophagy are hampered in their immune defence. Even though this role of autophagy is much more studied in mammalian system, there are 4 different reports that highlight an involvement of autophagy in the *Drosophila*

immune response. When autophagy was impaired by the expression of RNAi against Atg5, Atg7, or Atg12, *Drosophila* displays a decreased resistance to injected *Escherichia coli*, which manifests in higher titers of *E. coli* and reduced survival rates. Interestingly, knockdown of any of these three Atg genes did not shorten lifespan of uninjected flies [61]. The latter finding is not in line with findings from Atg7 deficient flies, which show a significant shortening of life span [48]. Even though the conditional knockdown of Atg7 did lead to a decrease in lysotracker staining, a sign for reduced autophagy, it cannot be excluded that some remaining Atg7 activity is enough in order to allow for basal autophagy and thereby not altering lifespan. It can be expected that such basal autophagy is more severely affected in flies completely missing the gene for Atg7. On the other hand there is also the possibility that $Atg7^{-/-}$ flies already accumulate cell damage during development that might allow them to hatch normally but still will give them a severe survival disadvantage right from the start.

Autophagy does not only protect against bacterial but also against viral infection as shown in the case of the mammalian viral pathogen vesicular stomatitis virus (VSV) [62]. Autophagy protects flies against VSV by decreasing viral replication. Repression of autophagy has the contrary effect, increased viral replication and pathogenesis. The authors of this study were able to pinpoint the PI3K/Akt pathway to be responsible for autophagy regulation upon VSV infection [62]. Flies infected with *Mycobacterium marinum* are dependent on autophagy in order for mycobacteria drug treatment to be successful. *Drosophila* lacking the gene for Atg7 had a reduced survival rate upon *Mycobacterium marinum* infection and this phenotype could not be rescued with the help of antimycobacterial treatment [63].

An additional involvement of autophagy in immunity was found in the cortical remodelling of hemocytes (*Drosophila* blood cells). Integrin-mediated hemocyte spreading and Rho1-induced cell protrusions require continuous autophagy. As a consequence, flies with impaired autophagy in their hemocytes show severe defects in recruiting hemocytes to epidermal wounds. Furthermore, this study identified Ref(2)P to be crucial for functional autophagy, which suggests selective autophagy (see below) to be involved in this process [64]. The requirement for selective autophagic turnover of single proteins to maintain cellular homeostasis has been implicated in several different cellular contexts. E.g. activated rhodopsin is degraded via the endosomal pathway and mutations in rhodopsin leading to hampered endocytic turnover results in retinal degeneration [65]. Autophagy has also been connected to the turnover of activated rhodopsin and mutations in Atg7 or Atg8, or genes necessary for proper autophagosome formation, result in light-dependent retinal degeneration [66].

Another example for the necessity of functional selective autophagic degradation of proteins for proper homeostasis is given in muscle tissue maintenance. There, chaperone-assisted selective autophagy is necessary to remove contraction-induced damaged filamin from Z-discs in order to prevent Z disk disintegration and progressive muscle weakness in flies [67].

Autophagy also serves several functions in neuron plasticity and homeostasis. An interesting finding was that synapse development is controlled by autophagy via the E3 ubiquitin ligase highwire. Highwire inhibits neuromuscular junction growth and is itself a substrate for selective autophagic turnover, indicating that autophagy activity might lead to synaptic overgrowth [68]. Tian et al. identified Rae1 to bind to highwire and thereby protecting highwire

from autophagic degradation [69]. The link between autophagy and synaptic growth at the neuromuscular junction is further strengthened by the observation that ROS can act as signaling molecules and mediate synaptic growth. At the same time, high ROS levels activate the JNK pathway, a previously reported activator of autophagy. As impairment of autophagy results in decreased synaptic size in a *Drosophila* model, whereas activation of autophagy has the opposite effect, one can speculate that ROS mediated synaptic growth is mediated by activation of JNK and subsequent autophagy (reviewed in [70]).

Off course, autophagy also plays a major role in simply keeping the cells "clean" by enabling the cells to turn over the cytosol. This recycling effect is especially pronounced in post-mitotic cells. Flies that are mutant for Atg8a are severely hampered in their efficiency to eliminate cellular material, which can be observed as an increase in ubiquitinated proteins and the increased presence of electron dense protein aggregates in young fly brains when investigated with transmission electron microscopy. Moreover, such Atg8a mutant flies display a drastic decrease of lifespan [71]. In addition, a very recent report by Fouillet et al., reveals an autophagy-mediated decrease of apoptosis in neurons upon mild ER-stress and further underscore a cytoprotective role of autophagy, that potentially can prolong survival of the whole organism [72]. Taken together, these data outline the versatile role of autophagy in homeostasis and normal survival of flies.

4. Autophagy and neurodegeneration

Ageing is a major risk factor for the development of neurodegenerative diseases and over the last decade autophagy has been implicated in many neurodegenerative diseases, such as Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), or Alzheimer's disease (AD). Many neurodegenerative diseases share the common phenotype of accumulations of protein aggregates [73]. Before reviewing the role of autophagy in *Drosophila* models for neurodegeneration we first want to give a short overview of key findings on the link between autophagy and neurodegeneration as known from mammalian systems and patient data.

Both, HD and PD are connected to elevated autophagy. In case of HD, autophagy can only be triggered by a mutant form of huntingtin that is prone to aggregate but not by wildtype huntingtin. Cytosolic aggregates of α-synuclein, the protein involved in PD, can be degraded by macroautophagy and CMA [74-77]. In ALS loss of motor neurons deprives patients of voluntary controlled muscle movements. The disease is associated with ubiquitinated, p62 positive protein inclusions of TDP-43 (TAR DNA binding protein 43) or SOD1 (superoxide dismutase 1) or rare mutations in a subunit of the ESCRT complex [78, 79]. A defective ESCRT complex in its turn has been shown to result in autophagosome accumulation [80], but also point mutations of the p150 subunit of dynactin resulting in defects in the transport machinery along microtubules have been implicated in ALS. Transport along microtubules is necessary for autophagosome-lysosome fusion and therefore crucial for functional autophagy [81, 82]. Extensive alterations in macroautophagy can also be found in patients with AD. An immuno-

electron microscopy study on neocortical biopsies from AD patients identified autophagosomes, multivesicular bodies, multilamellar bodies, and cathepsin-containing autophagolysosomes as the predominant organelles that occupied most of the cytosol of dystrophic neurites. Autophagy was detected in cell bodies with neurofibrillary pathology and associated with a relative depletion of mitochondria and other organelles. The authors of this study speculated that the accumulation of immature autophagic vacuoles results from impaired transport to and fusion with lysosomes thereby hampering the protective effects of autophagy [83]. Disruption of lysosomal proteolysis in primary mouse cortical neurons by inhibiting cathepsins, or by supressing lysosomal acidification, impairs transport of autolysosomes, endosomes and lysosomes, and leads to accumulation of these structures within dystrophic axonal swellings. Such a phenotype can also be seen in numerous mouse models of AD. The phenotype is not caused by general disruption of the axonal transport machinery, as mitochondria and cathepsin-lacking organelles were not influenced in their movements. Axonal dystrophy is reversed once lysosomal function is restored [84].

In the past, several independent groups have established *Drosophila* models for neurodegenerative diseases and/or investigated the role of aggregating proteins implied in neurodegenerative diseases in flies. Remarkably, already in 1982 Stark and Carlson characterized the degenerative phenotypes evoked by a mutant form of the rdgB (retinal-degeneration-B) protein in the fly compound eye and found amongst others lysosome-like bodies and vacuoles suggesting involvement of autophagy [85]. The compound eye of flies displays a highly structured order and degenerative properties of protein aggregates can easily be monitored as impairments of this structure. Expression of mutant huntingtin containing a polyQ-expansion of 120 glutamine leads to degeneration of the eye. However, treatment with rapamycin, an activator of autophagy, reduces this phenotype [86]. Treatment with new small-molecule enhancers (SMER) of the cytosolic effects of rapamycin, which were shown to induce autophagy in mammalian cells, also protected flies from polyQ huntingtin induced neurodegeneration [87]. Instead of treating flies with rapamycin in order to inhibit TOR by pharmacological means, Wang et al. highlighted the importance of TOR in neurodegeneration by genetical manipulations. Hyperactivation of TOR, achieved by expression of the TOR kinase activator Ras homologue enriched in brain protein (Rheb) or introduction of mutations in the TOR inhibitor dTsc1 increased age- and light-dependent photoreceptor loss [88]. The authors of this study were able to exclude TORs effects on growth to be responsible for this photoreceptor degeneration but instead pointed out autophagy as the downstream signaling of TOR mediating photoreceptor cell death. Activation of autophagy by overexpressing Atg1 protected not only cells from age- and light dependent photoreceptor degeneration, but also photoreceptor cells which either produced 120 polyQ-huntingtin or lacked a functional *Drosophila phospholipase C* gene *norpA* respectively. Both latter manipulations are commonly used to model neurodegeneration [88]

Macroautophagy in flies can also be upregulated by Rab5 over-expression and this approach also mitigates polyQ-huntingtin mediated degeneration in the eye [89]. However, there is a fine line between beneficial and detrimental consequences of autophagy activation in the context of neurodegeneration as shown in a dentatorubralpallidoluysian atrophy (DRPLA) fly

model [90]. This model is built upon the expression of atrophin with a polyQ expansion and is characterized by lysosomal dysfunction and blocked autophagosome-lysosome fusion, hence reduced autophagic flux [90]. Even though introduction of a mutant form of Atg1 intensified the neurodegenerative phenotype, upregulation of autophagy in this system had no rescuing effect but, in some case, even had the opposite outcome and increased neurodegeneration [90]. In other words, autophagy plays an important role in scavenging polyQ atrophin from the cytosol, but is only of beneficial nature as long as autophagy can proceed all the way to lysosomal degradation. Reaching a rate-limiting step in the autophagy cycle can have negative effects on the outcome of autophagy initiation. Work from the same lab also identified a mechanism how polyQ atrophin itself impairs autophagic flux. PolyQ-atrophin inhibits the tumor suppressor fat, which under normal conditions protects from neurodegeneration through the Hippo kinase cascade and subsequent increases autophagy [91]. How Hippo exactly activates autophagy is not completely understood yet [92]. Data obtained from studies in the salivary gland suggests that the phosphorylation of Warts (wts), a substrate of the Hippo kinase, acts upstream of TOR and thereby regulates autophagy [93]. It also has been reported that the Hippo pathway can directly interact with LC3 (the mammalian homolog of Atg8) and thereby initiate autophagy [92].

An additional, interesting link between polyQ sequence derived neurodegeneration and macroautophagy is given by puromycin-sensitive aminopeptidase (PSA). PSA is the only cytosolic enzyme capable of digesting polyQ sequences and it is therefore not surprising that there is inverse correlation between PSA expression and severity of neurodegeneration, e.g. over-expression of PSA has protective effects in cells expressing polyQ expanded ataxin-3, mutant α-synuclein and mutant superoxide dismutase (SOD) [94]. It comes as a surprise though that this beneficial role of PSA is mediated by its activation of macroautophagy rather than its role in degrading polyQ aggregates and thereby making them available for proteasomal degradation, although the putative involvement of the proteasome in cell protection in this process remains to be further understood [94].

A different way to induce neurodegeneration is to inhibit proteasomal function. Interestingly, proteasome impairment can be compensated for by autophagy, a rescue that depends on the histone deacetylase 6 (HDAC6) [95]. A protective role of autophagy in context of neurodegeneration was also demonstrated in a genetic screen conducted in *Drosophila* with pathogenic Ataxin-3-induced neurodegeneration. Knockdown of Atg5 in these flies reverts the polyQ containing Ataxin-3 mediated toxicity. Testing the effects of identified neurodegeneration-suppressors on autophagy revealed that these factors had different impact on autophagy. The authors of this study proposed a model in which some neurodegeneration-suppressors induce autophagy, thereby contributing to protein clearance whereas others mitigate autophagy in order to counteract autophagic cell death [96]. The role of autophagy in removal of protein aggregates in neurodegenerative diseases was further confirmed by the finding that depletion of subunits of the ESCRT complex in flies intensifies the toxic effects exerted by polyQ-expanded huntingtin [97]. Depletion of ESCRT subunits has autophagy inhibition as consequence, which manifests in accumulation of protein aggregates containing ubiquitinated proteins, p62 and Alfy [98].

The Alzheimer's disease related peptide $A\beta_{1-42}$ also induces neurodegeneration, mediated by age-dependent autophagy-lysosomal injury in a *Drosophila* model of AD [99]. The age dependence was shown to be of high importance as brain ageing is accompanied by an increasingly defective autophagy-lysosomal system and accumulation of dysfunctional autophagosomes and autolysosomes. As a consequence intracellular membranes and organelles are damaged. The expression of $A\beta_{1-42}$ resulted in similar changes already in young *Drosophila* and this raised the question if chronic deterioration of the autophagy-lysosomal system by $A\beta_{1-42}$ simply accelerates brain ageing [100]. This concept is supported the finding that expression of autophagy genes decreases with age, and disruption of the autophagy pathway reduces lifespan of flies [71].

5. Autophagy and its role in lifetime extension

The rate of ageing is reciprocally linked to lifespan and therefore are interventions that extend longevity of an organism the most direct indication that ageing is slowed down [101]. One well established, and long known intervention that extends lifespan is dietary restriction (DR), the limitation of food intake below the *ad libidum* level without malnutrition. DR has successfully been proven to extend lifespan in every organism tested, including yeast, worms, flies and rodents. In addition, DR not only extends lifespan, even the occurrence of age-associated pathologies, e.g. cardiovascular disease, multiple kinds of cancer, neurodegeneration, are drastically reduced or at least postponed in animal models [102]. The possibility to perform forward genetics in different model organisms has boosted the general understanding of underlying molecular mechanisms how DR, and other life extending interventions, can execute their effects. Studies in *Caenorhabditis elegans* by Cynthia Kenyon and co-workers have already almost two decades ago showed how mutations in the single gene daf-2 (the insulin receptor homologue in *C. elegans*) can increase survival by more than two-fold and that such extended survival is dependent on a second gene, namely daf-16 (a forkhead transcription factor) [103, 104]. Since then the role of nutrient-sensing pathways in ageing has been addressed by many independent groups, which has helped to identify numerous proteins that are crucial in lifespan determination. Amongst other pathways, both the insulin/insulin-like growth factor (IGF) and the Target of Rapamycin (TOR) network have been shown to be important modulators of longevity (reviewed in [101, 105, 106]). The fact that both these networks also are involved in the regulation of autophagy emphasizes a putative role of autophagy in lifetime extension and has been addressed in *Drosophila* by several groups.

Simonsen and co-workers showed that downregulation of autophagy genes in *Drosophila* neural tissue is part of the normal ageing process. This is accompanied by accumulation of insoluble ubiquitinated proteins (IUPs). Impairment of autophagy due to mutations in Atg8a aggravates the occurrence of IUPs at earlier time points and lowers survival rates [71]. As lipid-conjugation of Atg8 is essential for nucleation and phagophore elongation it can be speculated that Atg8 is a limiting factor in autophagic turnover. The over-expression of Atg8 in the central nervous system of *Drosophila* indeed extends average and maximum life span by approx. 50% [71]. Flies not only live longer upon Atg8a over-expression, but also showed a higher tolerance

to oxidative stress and lower occurrence of IUPs [71]. Interestingly, the longevity promoting effect of Atg8a over-expression cannot be seen when over-expression is initiated during development but decreases over time as seen in flies where Atg8a expression was driven by the early pan-neural driver line *Elav-Gal4* [71]

The question if IUPs are cause or a consequence of the ageing process remains to be answered though. Albeit, the age-dependent accumulation of ubiquitinated proteins that are positive for Ref(2)P, a protein necessary for cargo recognition in selective autophagy, can be employed as conserved marker of neuronal ageing and progressive autophagic defects [107].

Also Atg7 was recently reported to extend life span when over-expressed in neuronal tissues of flies [108]. The life-extending effect of Atg7 is not as pronounced when compared to Atg8. This might be due to different capabilities in inducing autophagic turnover, or non-autophagy related side effects of either Atg7 or Atg8a.

Proteostasis is not only important in neuronal tissues but also in muscles of flies. With increasing age polyubiquitinated proteins accumulate that co-localise with Ref(2)P in muscles and the cumulative appearance of such aggregates has been demonstrated to impair muscle fitness [109]. The build-up of such aggregates can be reverted in muscles by the constitutive activation of the transcription factor FOXO and its target 4E-BP (eukaryotic translation initiation factor 4E binding protein). Interestingly, the activation of FOXO/4E-BP signaling in muscles is sufficient to extend lifespan of the whole organism [109]. Furthermore it has been shown that the FOXO/4E-BP dependent delay in protein aggregate accumulation in muscles depends on functional autophagy, suggesting promotion of basal autophagy upon FOXO/4E-BP signaling [109]. The autophagy dependent beneficial effect of FOXO is well in line with earlier findings that revealed FOXO to be capable to upregulate autophagy [110]. In addition, the translational repressor 4E-BP is known to be upregulated upon DR and to mediate enhanced mitochondrial function and life span extension in *Drosophila* [111]. As already mentioned earlier, autophagy has a known role in the selective turnover of damaged mitochondria in yeast and mammals, and it is therefore tempting to speculate that autophagy can promote longevity by improving mitochondrial function in a FOXO/4E-BP dependent manner, however this remains to be proven.

Ageing in *Drosophila* can also be manipulated by pharmacological means. Feeding the TOR inhibitory drug rapamycin, a well-described drug for human use, significantly increases lifespan and resistance to starvation as well as the oxidative stress inducer paraquat [112]. Rapamycin fails to extend the lifespan of flies with downregulated Atg5 suggesting that autophagy has to be active in order for rapamycin to slow down ageing [112]. The finding that inhibition of TOR increases lifespan in *Drosophila* is well in line with earlier studies demonstrating that mutant, inactive TOR or over-expression of the TOR inhibitors dTsc1 or dTsc2 extend longevity [113, 114]. However, the specific role of autophagy was not addressed in those two studies.

Keeping *Drosophila* on food supplemented with the polyamine spermidine promotes increased longevity and this effect has been shown to be autophagy dependent, since depletion of Atg7 abrogates this anti-ageing effect [115].

Taken together, all these data indicate an anti-ageing effect of autophagy, however caution is advised in trying to merely upregulate autophagy pharmacologically in order to counter-act ageing. Autophagy is essential for the recycling of cellular content, which can serve two general purposes: autophagy can unburden cells from hazards by removal of those and autophagy can provide cells with new building blocks for cellular survival. During the lifetime of an organism, autophagy will most certainly switch forth and back between those roles. In order to completely understand the complex role of autophagy in ageing it is therefore important to understand the regulation and cellular outcome of autophagy in a tissue and time dependent manner.

6. Selective autophagy and ageing

In the following section we want to shed some light on the current knowledge about the selective removal of cellular contents by autophagy in *Drosophila melanogaster*. Above, we have already discussed some examples of selective autophagy in normal ageing and homeostasis. We therefore will focus more on the mechanistic insights of selective autophagy and what is known so far about the role of selective autophagy explicitly in ageing of *Drosophila*.

Selective autophagy in the form of CVT has been known in yeast for a long time and has gained major attention in mammalian systems over the last years. Selectivity requires crucial, additional steps to the above described autophagy process: cargo has to be recognized by specific receptors and must be delivered to the autophagic machinery.

Ubiquitin has emerged as a molecule to tag proteins that are determined for degradation [116]. Conjugation of ubiquitin depends on a complex reaction cascade that requires activation of ubiquitin (by E1 enzymes), conjugation (E2 ubiquitin conjugating enzyme), and ligation of ubiquitin with a target substrate (E3 ubiquitin ligase). As a result, ubiquitin is covalently bound via an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue on the substrate protein. Substrate specificity is given by the E3 ubiquitin ligase that specifically recognizes a protein substrate and brings it to the E2 ubiquitin conjugating enzyme. A wide spectrum of E1, E2, and E3 enzymes provide cells with selectivity for this signaling machinery [117]. Ubiquitin itself contains seven lysine residues enabling ubiquitin to self-attach, thereby forming a polyubiquitin tag. The best-characterised linkages occur via K48, targeting the substrate for proteasomal degradation, and via K63, which is preferred by ubiquitin-binding autophagy receptors. Furthermore, K63 ubiquitination has been reported to be a potent enhancer of inclusion formation and leads to substrate degradation via the autophagy/lysosome degradation pathway [116, 118-120]. Also more atypical sites for polyubiquitination, such as K6 or K29, have been reported but the exact role of these ubiquitin chains is still poorly understood [121].

Taken together, ubiquitin conjugation offers several possibilities to flag proteins and organelles in different ways by variation of chain length and various sites for ubiquitin self-attachment and thereby act as a signal for distinct subsequent cellular processing. Molecular links between ubiquitinated proteins and autophagy were identified in form of the cargo receptors seques-

tosome marker SQSTM1/p62 and NBR1 (neighbour of BRCA1 gene) [122]. The conserved functional homologue for p62/NBR1 in *Drosophila* is Ref(2)P. Ref(2)P is a 599 amino acid long protein with an N-terminal Phox Bem1p (PB1) domain, followed by a ZZ-type Zinc finger domain and a C-terminal UBA (ubiquitin-associated) domain [123]. The PB1 domain allows for self- and hetero-oligomerisation, while the UBA domain enables Ref(2)P to recognize and directly interact with ubiquitin. Both domains are necessary for formation of protein aggregates normally found in brains of adult *Drosophila* [124]. Flies mutant for Atg8 display an increased amount of deposited protein aggregates in the brain, however such aggregates are absent in double-mutant Atg8/Ref(2)P flies [124]. This suggests that Ref(2)P is a selective cargo receptor for selective autophagy in *Drosophila*, similar as its homologue p62 in mammals. This is supported by the presence of a putative LIR (LC3 interacting) domain in Ref(2)P as identified by bioinformatics analysis [122]. The LIR domain is known to be essential for p62 to interact with LC3, but it remains to be elucidated if Ref(2)P really interacts with Atg8 via its putative LIR domain. Independent of the absence of final proof of direct interaction between Atg8 and Ref(2)P, protein aggregations containing Ref(2)P serve as excellent markers for neuronal ageing and autophagic defects in *Drosophila* [107].

Filimonenko et al. were able to identify the mammalian phosphatidylinositol-3-phosphate (PI3P) binding protein Alfy (PI3P-binding Autophagy-linked FYVE domain protein) to be actively involved in autophagic degradation of polyglutamine (polyQ) expanded, aggregated proteins [125]. Albeit harbouring a FYVE domain Alfy is usually not found on endosomes but instead resides in the nucleus decorating the nuclear membrane. The presence of ubiquitinated, aggregated proteins in the cytosol leads to relocalization of Alfy to these aggregates [126]. Alfy can directly interact with p62 and Atg5 [125, 127]. *In vitro*, Alfy is necessary to recruit Atg5 to polyQ protein aggregates. In addition, Alfy scaffolds the Atg5-Atg12-Atg16L complex to p62- and ubiquitin-positive polyQ inclusions [125]. The Atg5-Atg12-Atg16L complex on the other hand is important for LC3 lipidation [128]. Taken together, all these interactions allow for LC3 lipidation in close spatial proximity to ubiquitinated, aggregated proteins and explain the absence of other cytosolic components in aggregate filled autophagosomes [125]. Primary neurons expressing polyQ Htt (Huntingtin) have fewer polyQ inclusions upon ectopic Alfy expression. These results were confirmed *in vivo* with a *Drosophila* model where polyQ production provokes a phenotype that is due to toxicity. The outcome of polyQ-mediated toxicity was much milder once bchs (blue cheese, the *Drosophila* homologue of Alfy) was co-expressed [125]. Reduced levels of bchs in mutant flies had opposite effects and led to shortened live span and extensive neurodegeneration [129]. It remains to be elucidated if Alfy/bchs directly recognizes ubiquitinated aggregates or if this interaction is mediated by p62/Ref(2)P [22].

Accumulation of damaged mitochondria and increased production of ROS are generally believed to account for age associated pathologies [5]. The efficiency of selective removal of damaged mitochondria, mitophagy, might therefore play a major role in the outcome of the ageing process. Although several lines of evidence suggest the existence of mitophagy in *Drosophila* (see section 3) the molecular details in flies still have to be further unravelled.

7. Summary and outlook

The cytoprotective role of autophagy has been shown in many different cellular contexts and induction of autophagy by either pharmacological or genetical means has life extending effects. However, research conducted in *Drosophila* has also identified situations during development when autophagy is necessary for controlled tissue removal and cell death initiation. These two rather contrary roles, cytoprotection versus cell death initiation, highlight the complexity of the autophagy pathway and also underscore the importance to understand the molecular mechanisms by which autophagy exerts its role. As autophagy most likely is regulated in a tissue and time dependent manner it is of great interest to pinpoint those time points and tissues in which autophagy has the biggest impact on the general ageing processes.

Ageing is not only influenced by one single pathway but in contrary is a multifaceted process. Age is a major risk factor for a variety of diseases, e.g. neurodegenerative diseases, metabolic syndrome, cancer and more. In the past, extensive research has been undertaken to model neurodegenerative diseases in the fruitfly and has helped to push our understanding, not the least concerning the involvement of autophagy, to new levels. Today, *Drosophila* is getting growing attention as cancer model and it will be exciting to follow future research in order to get new insights from *Drosophila melanogaster* about the complex role of autophagy in cancer. By putting several different pieces of puzzle together, *Drosophila* already has helped us to get a clearer picture about the role of autophagy in various aspects of ageing and for sure the fruitfly will continue to help the research community to reveal more of this complex picture in the future.

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References

[1] Kirkwood, T. B, & Austad, S. N. Why do we age? Nature, (2000). , 233-238.

[2] Vellai, T. Autophagy genes and ageing. Cell death and differentiation, (2009). , 94-102.

[3] Lionaki, E, Markaki, M, & Tavernarakis, N. Autophagy and ageing: Insights from invertebrate model organisms. Ageing research reviews, (2012).

[4] Kirkwood, T. B. A systematic look at an old problem. Nature, (2008). , 644-647.

[5] Kirkwood, T. B. Understanding the odd science of aging. Cell, (2005). , 437-447.

[6] Guarente, L, & Kenyon, C. Genetic pathways that regulate ageing in model organisms. Nature, (2000). , 255-262.

[7] Hekimi, S, & Guarente, L. Genetics and the specificity of the aging process. Science, (2003). , 1351-1354.

[8] Reggiori, F, & Klionsky, D. J. Autophagy in the eukaryotic cell. Eukaryotic cell, (2002). , 11-21.

[9] De Duve, C. The lysosome. Scientific American, (1963). , 64-72.

[10] Uttenweiler, A, et al. The vacuolar transporter chaperone (VTC) complex is required for microautophagy. Molecular biology of the cell, (2007). , 166-175.

[11] Sahu, R, et al. Microautophagy of cytosolic proteins by late endosomes. Developmental cell, (2011). , 131-139.

[12] Cuervo, A. M. Chaperone-mediated autophagy: selectivity pays off. Trends in endocrinology and metabolism: TEM, (2010). , 142-150.

[13] Tsukada, M, & Ohsumi, Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS letters, (1993). , 169-174.

[14] Thumm, M, et al. Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. FEBS letters, (1994). , 275-280.

[15] He, C, & Klionsky, D. J. Regulation mechanisms and signaling pathways of autophagy. Annual review of genetics, (2009). , 67-93.

[16] Yamamoto, A, & Simonsen, A. The elimination of accumulated and aggregated proteins: A role for aggrephagy in neurodegeneration. Neurobiol Dis, (2010).

[17] Mcewan, D. G, & Dikic, I. Not all autophagy membranes are created equal. Cell, (2010). , 564-566.

[18] Tooze, S. A, & Yoshimori, T. The origin of the autophagosomal membrane. Nature cell biology, (2010). , 831-835.

[19] Seglen, P. O, Gordon, P. B, & Holen, I. Non-selective autophagy. Seminars in cell biology, (1990). , 441-448.

[20] Mizushima, N, & Komatsu, M. Autophagy: renovation of cells and tissues. Cell, (2011). , 728-741.

[21] Yorimitsu, T, & Klionsky, D. J. Autophagy: molecular machinery for self-eating. Cell death and differentiation, (2005). Suppl 2: , 1542-1552.

[22] Knaevelsrud, H, & Simonsen, A. Fighting disease by selective autophagy of aggregate-prone proteins. FEBS letters, (2010). , 2635-2645.

[23] Youle, R. J, & Narendra, D. P. Mechanisms of mitophagy. Nature reviews. Molecular cell biology, (2011). , 9-14.

[24] Kraft, C, et al. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3ubiquitin protease. Nature cell biology, (2008). p. 602-10., Bre5p.

[25] Dunn, W. A, et al. Pexophagy: the selective autophagy of peroxisomes. Autophagy, (2005). , 75-83.

[26] Bernales, S, Mcdonald, K. L, & Walter, P. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS biology, (2006). , e423.

[27] Klionsky, D. J, et al. How shall I eat thee? Autophagy, (2007). , 413-416.

[28] Chang, Y. Y, & Neufeld, T. P. Autophagy takes flight in Drosophila. FEBS letters, (2010). , 1342-1349.

[29] Lee, G, et al. UVRAG is required for organ rotation by regulating Notch endocytosis in Drosophila. Developmental biology, (2011). , 588-597.

[30] Ohsumi, Y. Molecular dissection of autophagy: two ubiquitin-like systems. Nature reviews. Molecular cell biology, (2001). , 211-216.

[31] Mcphee, C. K, & Baehrecke, E. H. Autophagy in Drosophila melanogaster. Biochimica et biophysica acta, (2009). , 1452-1460.

[32] Simonsen, A, & Tooze, S. A. Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. The Journal of cell biology, (2009). , 773-782.

[33] Razi, M, Chan, E. Y, & Tooze, S. A. Early endosomes and endosomal coatomer are required for autophagy. The Journal of cell biology, (2009). , 305-321.

[34] Juhasz, G, et al. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. The Journal of cell biology, (2008). , 655-666.

[35] Rusten, T. E, et al. ESCRTs and Fab1 regulate distinct steps of autophagy. Current biology : CB, (2007). , 1817-1825.

[36] Lindmo, K, et al. A dual function for Deep orange in programmed autophagy in the Drosophila melanogaster fat body. Experimental cell research, (2006). , 2018-2027.

[37] Pulipparacharuvil, S, et al. Drosophila Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules. Journal of cell science, (2005). Pt 16): , 3663-3673.

[38] Backues, S. K, & Klionsky, D. J. Autophagy gets in on the regulatory act. Journal of molecular cell biology, (2011). , 76-77.

[39] Rubinsztein, D. C, Marino, G, & Kroemer, G. Autophagy and aging. Cell, (2011). , 682-695.

[40] Neufeld, T. P. Body building: regulation of shape and size by PI3K/TOR signaling during development. Mechanisms of development, (2003). , 1283-1296.

[41] Petiot, A, et al. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. The Journal of biological chemistry, (2000). , 992-998.

[42] Pfeifer, U. Inhibition by insulin of the formation of autophagic vacuoles in rat liver. A morphometric approach to the kinetics of intracellular degradation by autophagy. The Journal of cell biology, (1978). , 152-167.

[43] Rusten, T. E, et al. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. Developmental cell, (2004). , 179-192.

[44] Scott, R. C, Schuldiner, O, & Neufeld, T. P. Role and regulation of starvation-induced autophagy in the Drosophila fat body. Developmental cell, (2004). , 167-178.

[45] Lee, S. B, et al. ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. EMBO reports, (2007). , 360-365.

[46] Banta, L. M, et al. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. The Journal of cell biology, (1988). , 1369-1383.

[47] Lindmo, K, et al. The PI 3-kinase regulator Vps15 is required for autophagic clearance of protein aggregates. Autophagy, (2008). , 500-506.

[48] Juhasz, G, et al. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. Genes & development, (2007). , 3061-3066.

[49] Berry, D. L, & Baehrecke, E. H. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell, (2007). , 1137-1148.

[50] Denton, D, et al. Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Current biology : CB, (2009). , 1741-1746.

[51] Nezis, I. P, et al. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late Drosophila melanogaster oogenesis. The Journal of cell biology, (2010). , 523-531.

[52] Rumpf, S, et al. Neuronal remodeling and apoptosis require VCP-dependent degradation of the apoptosis inhibitor DIAP1. Development, (2011). , 1153-1160.

[53] Ju, J. S, et al. Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. The Journal of cell biology, (2009). , 875-888.

[54] Wang, M. C, Bohmann, D, & Jasper, H. JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila. Developmental cell, (2003). , 811-816.

[55] Wang, M. C, Bohmann, D, & Jasper, H. JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. Cell, (2005). , 115-125.

[56] Wu, H, Wang, M. C, & Bohmann, D. JNK protects Drosophila from oxidative stress by trancriptionally activating autophagy. Mechanisms of development, (2009). , 624-637.

[57] Deng, H, et al. The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proceedings of the National Academy of Sciences of the United States of America, (2008). , 14503-14508.

[58] Ziviani, E, Tao, R. N, & Whitworth, A. J. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. Proceedings of the National Academy of Sciences of the United States of America, (2010). , 5018-5023.

[59] Greene, J. C, et al. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proceedings of the National Academy of Sciences of the United States of America, (2003). , 4078-4083.

[60] Pimenta de Castro I., et al., Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster. Cell death and differentiation, (2012). , 1308-1316.

[61] Ren, C, Finkel, S. E, & Tower, J. Conditional inhibition of autophagy genes in adult Drosophila impairs immunity without compromising longevity. Experimental gerontology, (2009). , 228-235.

[62] Shelly, S, et al. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity, (2009). , 588-598.

[63] Kim, J. J, et al. Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. Cell host & microbe, (2012). , 457-468.

[64] Kadandale, P, et al. Conserved role for autophagy in Rho1-mediated cortical remodeling and blood cell recruitment. Proceedings of the National Academy of Sciences of the United States of America, (2010). , 10502-10507.

[65] Chinchore, Y, Mitra, A, & Dolph, P. J. Accumulation of rhodopsin in late endosomes triggers photoreceptor cell degeneration. PLoS genetics, (2009). , e1000377.

[66] Midorikawa, R, et al. Autophagy-dependent rhodopsin degradation prevents retinal degeneration in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience, (2010). , 10703-10719.

[67] Arndt, V, et al. Chaperone-assisted selective autophagy is essential for muscle maintenance. Current biology : CB, (2010). , 143-148.

[68] Shen, W, & Ganetzky, B. Autophagy promotes synapse development in Drosophila. The Journal of cell biology, (2009). , 71-79.

[69] Tian, X, et al. Drosophila Rae1 controls the abundance of the ubiquitin ligase Highwire in post-mitotic neurons. Nature neuroscience, (2011). , 1267-1275.

[70] Milton, V. J, & Sweeney, S. T. Oxidative stress in synapse development and function. Developmental neurobiology, (2012). , 100-110.

[71] Simonsen, A, et al. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. Autophagy, (2008). , 176-184.

[72] Fouillet, A, et al. ER stress inhibits neuronal death by promoting autophagy. Autophagy, (2012). , 915-926.

[73] Knaevelsrud, H, & Simonsen, A. Fighting disease by selective autophagy of aggregate-prone proteins. FEBS Lett, (2010). , 2635-2645.

[74] Nagata, E, et al. Autophagosome-like vacuole formation in Huntington's disease lymphoblasts. Neuroreport, (2004). , 1325-1328.

[75] Ravikumar, B, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet, (2004). , 585-595.

[76] Webb, J. L, et al. Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem, (2003). , 25009-25013.

[77] Cuervo, A. M, et al. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science, (2004). , 1292-1295.

[78] Parkinson, N, et al. ALS phenotypes with mutations in CHMP2B (charged multivesicular body protein 2B). Neurology, (2006). , 1074-1077.

[79] Talbot, K, & Ansorge, O. Recent advances in the genetics of amyotrophic lateral sclerosis and frontotemporal dementia: common pathways in neurodegenerative disease. Human molecular genetics, (2006). Spec (2), R182-R187.

[80] Rusten, T. E, & Stenmark, H. How do ESCRT proteins control autophagy? Journal of cell science, (2009). Pt 13): , 2179-2183.

[81] Munch, C, et al. Point mutations of the subunit of dynactin (DCTN1) gene in ALS. Neurology, (2004). p. 724-6., 150.

[82] Rubinsztein, D. C. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature, (2006). , 780-786.

[83] Nixon, R. A, et al. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. Journal of neuropathology and experimental neurology, (2005). , 113-122.

[84] Lee, S, Sato, Y, & Nixon, R. A. Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer's-like axonal dystrophy. The Journal of neuroscience : the official journal of the Society for Neuroscience, (2011). , 7817-7830.

[85] Stark, W. S, & Carlson, S. D. Ultrastructural pathology of the compound eye and optic neuropiles of the retinal degeneration mutant (w rdg BKS222) Drosophila melanogaster. Cell and tissue research, (1982). , 11-22.

[86] Ravikumar, B, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nature genetics, (2004). , 585-595.

[87] Sarkar, S, et al. Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nature chemical biology, (2007). , 331-338.

[88] Wang, T, Lao, U, & Edgar, B. A. TOR-mediated autophagy regulates cell death in Drosophila neurodegenerative disease. The Journal of cell biology, (2009). , 703-711.

[89] Ravikumar, B, et al. Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. Journal of cell science, (2008). Pt 10): , 1649-1660.

[90] Nisoli, I, et al. Neurodegeneration by polyglutamine Atrophin is not rescued by induction of autophagy. Cell death and differentiation, (2010). , 1577-1587.

[91] Napoletano, F, et al. Polyglutamine Atrophin provokes neurodegeneration in Drosophila by repressing fat. The EMBO journal, (2011). , 945-958.

[92] Calamita, P, & Fanto, M. Slimming down fat makes neuropathic hippo: the Fat/Hippo tumor suppressor pathway protects adult neurons through regulation of autophagy. Autophagy, (2011). , 907-909.

[93] Dutta, S, & Baehrecke, E. H. Warts is required for PI3K-regulated growth arrest, autophagy, and autophagic cell death in Drosophila. Current biology : CB, (2008). , 1466-1475.

[94] Menzies, F. M, et al. Puromycin-sensitive aminopeptidase protects against aggregation-prone proteins via autophagy. Human molecular genetics, (2010). , 4573-4586.

[95] Pandey, U. B, et al. HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature, (2007). , 859-863.

[96] Bilen, J, & Bonini, N. M. Genome-wide screen for modifiers of ataxin-3 neurodegeneration in Drosophila. PLoS genetics, (2007). , 1950-1964.

[97] Rusten, T. E, et al. ESCRTing autophagic clearance of aggregating proteins. Autophagy, (2007).

[98] Filimonenko, M, et al. Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. The Journal of cell biology, (2007). , 485-500.

[99] Ling, D, et al. Abeta42-induced neurodegeneration via an age-dependent autophagic-lysosomal injury in Drosophila. PloS one, (2009). , e4201.

[100] Ling, D, & Salvaterra, P. M. Brain aging and $Abeta_{1-42}$ neurotoxicity converge via deterioration in autophagy-lysosomal system: a conditional Drosophila model linking Alzheimer's neurodegeneration with aging. Acta neuropathologica, (2011). , 183-191.

[101] Partridge, L, et al. Ageing in Drosophila: the role of the insulin/Igf and TOR signaling network. Experimental gerontology, (2011). , 376-381.

[102] Bishop, N. A, & Guarente, L. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nature reviews. Genetics, (2007). , 835-844.

[103] Kenyon, C, et al. A C. elegans mutant that lives twice as long as wild type. Nature, (1993). , 461-464.

[104] Lin, K, et al. daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science, (1997). , 1319-1322.

[105] Kapahi, P, et al. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell metabolism, (2010). , 453-465.

[106] Kenyon, C. J. The genetics of ageing. Nature, (2010). , 504-512.

[107] Bartlett, B. J, et al. Ref(2)P and ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects. Autophagy, (2011). p. 572-83., 62.

[108] Chen, S. F, et al. Autophagy-related gene 7 is downstream of heat shock protein 27 in the regulation of eye morphology, polyglutamine toxicity, and lifespan in Drosophila. Journal of biomedical science, (2012). , 52.

[109] Demontis, F, & Perrimon, N. FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. Cell, (2010). , 813-825.

[110] Juhasz, G, et al. Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. Cell death and differentiation, (2007). , 1181-1190.

[111] Zid, B. M, et al. E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. Cell, (2009). , 149-160.

[112] Bjedov, I, et al. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell metabolism, (2010). , 35-46.

[113] Kapahi, P, et al. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Current biology : CB, (2004). , 885-890.

[114] Luong, N, et al. Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity. Cell metabolism, (2006). , 133-142.

[115] Eisenberg, T, et al. Induction of autophagy by spermidine promotes longevity. Nature cell biology, (2009). , 1305-1314.

[116] Kirkin, V, et al. A role for ubiquitin in selective autophagy. Molecular cell, (2009). , 259-269.

[117] Hershko, A, & Ciechanover, A. The ubiquitin system. Annual review of biochemistry, (1998). , 425-479.

[118] Tan, J. M, et al. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Human molecular genetics, (2008). , 431-439.

[119] Welchman, R. L, Gordon, C, & Mayer, R. J. Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nature reviews. Molecular cell biology, (2005). , 599-609.

[120] Wooten, M. W, et al. Essential role of sequestosome 1/in regulating accumulation of Lys63-ubiquitinated proteins. The Journal of biological chemistry, (2008). p. 6783-9., 62.

[121] Ikeda, F, & Dikic, I. Atypical ubiquitin chains: new molecular signals.'Protein Modifications: Beyond the Usual Suspects' review series. EMBO reports, (2008). , 536-542.

[122] Johansen, T, & Lamark, T. Selective autophagy mediated by autophagic adapter proteins. Autophagy, (2011). , 279-296.

[123] Nezis, I. P. Selective autophagy in Drosophila. International journal of cell biology, (2012). , 146767.

[124] Nezis, I. P, et al. Ref(2)P, the Drosophila melanogaster homologue of mammalian is required for the formation of protein aggregates in adult brain. The Journal of cell biology, (2008). p. 1065-71., 62.

[125] Filimonenko, M, et al. The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alfy. Molecular cell, (2010). , 265-279.

[126] Simonsen, A, et al. Alfy, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes. Journal of cell science, (2004). Pt 18): , 4239-4251.

[127] Clausen, T. H, et al. SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. Autophagy, (2010). p. 330-44., 62.

[128] Fujita, N, et al. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Molecular biology of the cell, (2008). , 2092-2100.

[129] Finley, K. D, et al. blue cheese mutations define a novel, conserved gene involved in progressive neural degeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience, (2003). , 1254-1264.