Chapter 15
Autophagy in *Drosophila* and Zebrafish

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Abstract Autophagy is a highly conserved cellular process that delivers cellular contents to the lysosome for degradation. It not only serves as a bulk degradation system for various cytoplasmic components but also functions selectively to clear damaged organelles, aggregated proteins, and invading pathogens (Feng et al., Cell Res 24:24–41, 2014; Galluzzi et al., EMBO J 36:1811–36, 2017; Klionsky et al., Autophagy 12:1–222, 2016). The malfunction of autophagy leads to multiple developmental defects and diseases (Mizushima et al., Nature 451:1069–75, 2008). *Drosophila* and zebrafish are higher metazoan model systems with sophisticated genetic tools readily available, which make it possible to dissect the autophagic processes and to understand the physiological functions of autophagy (Lorincz et al., Cells 6:22, 2017a; Mathai et al., Cells 6:21, 2017; Zhang and Baehrecke, Trends Cell Biol 25:376–87, 2015). In this chapter, we will discuss recent progress that has been made in the autophagic field by using these animal models. We will focus on the protein machineries required for autophagosome formation and maturation as well as the physiological roles of autophagy in both *Drosophila* and zebrafish.

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15.1 Overview

There are three types of autophagy, known as macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Galluzzi et al. 2017; Mizushima et al. 2008, 2010). Macroautophagy is the most studied type of autophagy in which cytosolic materials are degraded through the formation of double-membraned autophagosomes, maturation, and fusion with lysosomes (Mizushima 2011; Mizushima and Komatsu 2011; Feng et al. 2014; Klionsky et al. 2016). Different from yeast, but similar to mammalian cells, the formation of autophagosomes in flies and zebrafish is initiated via the simultaneous formation of isolation membranes at multiple sites (Zhang and Baehrecke 2015). These membrane cisterns elongate and finally enclose cargoes to form double-membraned autophagosomes. These nascent autophagosomes fuse with endosomes and multivesicular bodies (MVBs). In this way, hybrid organelles are formed, which are named amphisomes (Lamb et al. 2013). The amphisomes fuse with lysosomes to form autolysosomes. The cargoes are digested in these autolysosomes, and nutrition is released to the cytosol. After that, the lysosomes are regenerated through a process called autophagic lysosome reformation (ALR) (Yu et al. 2010).

Microautophagy is a type of autophagy that is mainly found in yeasts and plants. During microautophagy, cytosolic materials are directly taken up by the vacuole through vacuole membrane invagination. In mammalian cells and flies, a similar process, known as "endosomal microautophagy", was found, in which endosomes engulf cytosolic material through the formation of MVBs (Huber and Teis 2016; Mijaljica et al. 2011).

CMA is an autophagic process to deliver specific cytosolic proteins to the lysosome for degradation (Kaushik and Cuervo 2018). Proteins destined for the lysosome contain a KFERQ-motif that binds to the chaperone proteins HSPA8/HSC70 to form a complex. The lysosomal membrane protein LAMP2A binds to the chaperone complex and translocates the protein into the lysosome for degradation (Kaushik and Cuervo 2012). LAMP2A is conserved in birds and mammals. Therefore, CMA was presumed to be restricted to the tetrapod clade. Recently, genes encoding proteins that are very similar to mammalian LAMP2A have been identified in several fish species, including zebrafish. This suggests that fish might also have CMA pathway (Lescat et al. 2018). Recent studies in flies and mammals indicated that proteins containing KFERQ-motif are degraded via endosomal microautophagy. This process also requires HSPA8/HSC70, suggesting that endosomal microautophagy as it exists in flies might be an ancient form of CMA known in birds and mammals (Issa et al. 2018; Mukherjee et al. 2016).

Since there have been very few studies about microautophagy and CMA in fly and zebrafish, we will mainly focus on macroautophagy in this chapter. Macroautophagy will be referred to as "autophagy" hereafter.

15.2 The Core Autophagic Machinery in *Drosophila* and Zebrafish

Pioneering work in yeast identified many autophagy genes, known as *ATG* genes, which are required for autophagic processes (Nakatogawa et al. 2009). The core autophagy-related proteins were grouped into complexes, and the hierarchy of these proteins was determined by the temporal sequences of their recruitment to the phagophore assembly sites (PAS) in yeast. In the past 10 years, novel autophagy genes specific for multicellular organisms were identified. Most of the autophagy-related genes are conserved in flies and zebrafish (Table 15.1).

Table 15.1 The genes encode proteins that participate autophagy in different species

	S.cerevisiae	*D.Melanogaster*	*D. rerio*	*H. sapiens*
Atg1 complex	*Atg1,* *Atg13,* *Atg17,* \	*Atg1,* *Atg13,* *Atg17,* *Atg101*	*ulk1a, ulk1b, ulk2,* *atg13,* *rb1cc1,* *atg101*	*ULK1, ULK2,* *Atg13,* *FIP200,* *ATG101*
VPS34 complex	*VPS34,* *VPS15,* *ATG6,* *ATG14* \	*Vps34,* *Vps15,* *Atg6,* *Atg14,* *Uvrag*	*pik3c3,* *pik3r4,* *beclin1,* *atg14L,* *uvrag*	*VPS34,* *VPS15,* *Beclin1,* *ATG14L,* *UVRAG*
PI3P effector	*ATG18*	*Atg18a, Atg18b,* *CG11975*	*wipi1, wipi2,* *wipi3, wipi4*	*WIPI1, WIPI2,* *WIPI3, WIPI4*
	ATG2	*Atg2*	*atg2a, atg2b*	*ATG2A, ATG2B*
	ATG9	*Atg9*	*atg9a, atg9b*	*ATG9A, ATG9B*
ATG8 conjugation system	*ATG8,* *ATG4,* *ATG7,* *ATG3*	*Atg8a,* *Atg8b,* *Atg4a,Atg4b,* *Atg7,* *Atg3*	*map1-lc3a,* *map1-lc3b,* *map1-lc3c,* *gabarap,* *gabarapl1,* *gabarapl2,* *atg4a,atg4b,* *atg4c,atg4da, atg4db* *atg7,* *atg3*	*MAP1-LC3A,* *MAP1-LC3B,* *MAP1-LC3C,* *GABARAP,* *GABARAPL1,* *GABARAPL2,* *ATG4A, ATG4B,* *ATG4C, ATG4D,* *ATG7,* *ATG3*
ATG12 conjugation system	*ATG5,* *ATG12,* *ATG10,* *ATG16*	*Atg5,* *Atg12,* *Atg10,* *Atg16*	*atg5,* *atg12,* *atg10,* *atg16l1,* *atg16l2*	*ATG5,* *ATG12,* *ATG10,* *ATG16L1, ATG16L2*
Receptor/ Adaptor	\ \ *YMR276W* \ \ \ \ \	*Ref(2)P* \ *CG31528, ubqn,* \ *Bchs,* \ \ *htt*	*sqstm1,* *optn,* \ *nbr1a, nbr1b,* *wdfy3,* *calcoco2,* *ncoa4,* *htt*	*P62/SQSTM1,* *OPTN,* *Ubqln2,* *NBR1,* *ALFY/WDFY3,* *NDP52,* *NCOA4* *HTT*

continued

Table 15.1 (continued)

	S.cerevisiae	*D.Melanogaster*	*D. rerio*	*H. sapiens*
HOPS complex	*VPS11,* *VPS16,* *VPS18,* *VPS33,* *VPS39,* *VPS41*	*Vps11/CG32350,* *Vps16A,* *Vps18/dor,* *Vps33A/car,* *Vps39/CG7146,* *Vps41/lt*	*vps11,* *vps16,* *vps18,* *vps33a,* *vps39,* *vps41*	*VPS11,* *VPS16,* *VPS18,* *VPS33A,* *AC048338.1,* *VPS39,* *VPS41*
Rab GTPase and effectors	\ \ \	*Rab7,* *Rab2,* *epg5*	*rab7a, rab7b,* *zgc:100918* *rab2a* *epg5*	*RAB7A,* *RAB2A, Rab2B* *EPG5*
SNARE proteins	*VAM3,* *VTI1,* *Vam7* *SEC9*[*] \ \ *YKL196C*	*Syx17,* *Vti1a, Vti1b* \ *Ubisnap* *Vamp7* \ *Ykt6*	*stx17,* *vti1a, vti1b,* *gosr2* \ *snap29* *vamp7* *vamp8* *ykt6*	*STX17,* *VTI1A, VTI1B,* *GOSR2,* *AC005670.2* \ *SNAP29* *VAMP7*[*] *VAMP8* *YKT6*

[*]These genes encode proteins have not been reported as a component of the indicated complex
\The ortholog does not exist in the indicated species

15.2.1 Proteins Required for Autophagosome Formation

15.2.1.1 Atg1 Complex

In metazoan, the target of rapamycin (TOR), a serine/threonine kinase, functions as a primary nutrient and energy sensor (Gonzalez and Hall 2017; Wullschleger et al. 2006). When nutrients are sufficient, TOR suppresses autophagy by phosphorylating and inhibiting Atg1, a serine/threonine kinase whose kinase activity is required for the initiation of autophagy. Upon starvation stimuli, TOR is inactivated, which facilitates the assembly and activation of the Atg1 complex, a multiprotein complex containing Atg1, Atg13, Atg17, and Atg101. Null mutant flies of *Atg1*, *Atg13*, or *Atg17* are able to reach adulthood, but the majority of animals fail to enclose from the pupal case (Chang and Neufeld 2009; Kim et al. 2013b; Nagy et al. 2014b). The lack of each of these proteins abolishes starvation and development induced autophagosome formation. The overexpression of *Atg1* induces autophagy in the absence of starvation stimuli. It inhibits cell growth and TOR signaling and finally leads to cell death (Lee et al. 2007; Scott et al. 2007). *Atg17* functions upstream of *Atg1*. The knockdown of *Atg17* prevents the formation of the characteristic starvation-induced punctate mCherry-Atg1 localization. *Atg17* overexpression-induced autophagy

depends on *Atg1* (Nagy et al. 2014b). Atg13 directly binds to Atg1, Atg17, and Atg101 (Hegedus et al. 2014). Upon starvation, Atg13 is hyperphosphorylated by Atg1 in *Drosophila*, which can be blocked in *Atg17* mutants. Atg13 stimulates the autophagy inducing activity of Atg1. However, overexpression of *Atg13* decreases the stability of Atg1 and facilitates Atg1's inhibitory phosphorylation by TOR. As a result, overexpression of *Atg13* inhibits autophagosome expansion (Chang and Neufeld 2009).

15.2.1.2 VPS34 Complex and PI3P Effectors

Upon starvation stimuli, the activation of the Atg1 complex facilitates the recruitment of the VPS34 complex to the isolation membranes (Ktistakis and Tooze 2016). The core VPS34 complex contains Atg6, the catalytic subunit of Class III PI3K Vps34, and its regulatory subunit Vps15. The VPS34 complex does not only play an important role in autophagy but is also essential for endocytosis. When Atg14 is bound to the core VPS34 complex components, it forms an autophagy-specific complex, but when Uvrag protein forms a complex with the core components, it regulates endocytosis (Itakura et al. 2008; Kim et al. 2013a). Null mutants of *Atg6*, *Vps34*, or *Vps15* die as early as *third instar* larvae, and only a few *Atg6* mutants are able to initiate pupariation (Juhasz et al. 2008). The loss of *Vps34* not only impairs autophagosome formation but also disrupts endocytosis. The kinase-dead form of Vps34 functions dominant negatively to reduce autophagosome formation upon stimuli. However, autophagosomes still form at a slow rate when the activity of Vps34 is lost, suggesting that there is a pathway to compensate for the loss of Class III PI3K activity. The overexpression of *Vps34* induces the formation of Atg8 puncta but not lysotracker positive structures in fed fat body tissue, which indicates that extra Vps34 is not sufficient to fully activate autophagy process (Juhasz et al. 2008). Vps15 is required for stress-induced or development programmed autophagosome formation and protein aggregate degradation. *Vps15* mutant animals are defective in the antibacterial immune-response and more susceptible to bacterial infection. Besides this, Vps15 is required for efficient salivary gland protein secretion (Anding and Baehrecke 2015b). Vps15 protein has a serine/threonine kinase domain, but whether its kinase activity is required for its function in autophagy is not known. As explained before, Atg14 and Uvrag bind mutually exclusive to the core VPS34 complex to form two functionally distinctive complexes. In fed fat body cells, the loss of *Atg14* does not significantly influence the patterns of PI3P positive vesicles, but the loss of *Uvrag* leads to a complete lack of PI3P in these cells. In contrast, the loss of *Atg14* abolishes starvation-induced PI3P formation in fat body cells, whereas *Uvrag* mutant cells form PI3P positive vesicles just as the controls upon starvation stimuli (Lorincz et al. 2014; Takats et al. 2014). The Uvrag containing class III PI3K complex is essential for the downregulation of *Patched* through the endolysosomal pathway to regulate axon pruning during neural development (Issman-Zecharya and Schuldiner 2014).

PI3P generated on the isolation membrane can recruit its effectors to regulate autophagosome formation. In yeast, Atg18 is an effector of PI3P. Atg18 forms a complex with Atg2 to regulate the recycling of Atg9, the only transmembrane protein encoded by *ATG* genes, from PAS (Reggiori et al. 2004). In worms and mammals, Atg2 appears to function downstream of the Atg8 family proteins (Polson et al. 2010). In flies, there is one gene encoding Atg2 and there are three genes encoding Atg18 like proteins: *Atg18a*, *Atg18b*, and *CG11975*. The functions of *Atg18b* and *CG11975* are not known. Both *Atg2* and *Atg18a* mutants are late pupal/pharate adult lethal. The lack of *Atg18a* abolishes the recruitment of Atg9 to the Ref(2)P concrete substrate upon starvation. In starved fat body tissues, Atg8 puncta are significantly reduced in *Atg18a* mutants. However, the patterns of both Atg9 and Atg8 are not influenced by the loss of *Atg2* (Nagy et al. 2014a). The Atg18 family proteins contain a WD40 domain with seven β-propellers, which enable their interaction with multiple proteins. In flies, both Atg2 and Atg9 show interaction with Atg18a (Nagy et al. 2014a). A lack of *Atg9* in flies is semilethal. Survivors are sterile and have locomotor defects, a reduced lifespan, and increased susceptibility to stress (Tang et al. 2013; Wen et al. 2017). The lack of *Atg9* abolished both starvations induced and developmental programmed autophagy. In addition to its autophagy functions, the loss of *Atg9* leads to an aberrant adult midgut morphology at the physiological condition. Atg9 also interacts with *Drosophila* tumor necrosis factor receptor-associated factor 2 (dTRAF2) to regulate ROS-induced c-Jun N-terminal kinase (JNK) signaling (Tang et al. 2013).

In mammals, a FYVE domain protein called DFCP1 binds to PI3P and labels omegasomes and autophagosome precursors (Matsunaga et al. 2010). There is no clear ortholog of DFCP1 in *Drosophila melanogaster*. However, other *Drosophila* species such as *Drosophila willistoni* and the *virilism* have DFCP1 orthologs. A tagged form of mammalian DFCP1 can be used as a marker to label early autophagosomal structures that are positive for PI3P in flies (Liu et al. 2018).

15.2.1.3 Ubiquitin-Like Protein and Its Conjugation System

Atg8 and Atg12 are two ubiquitin-like modifiers that need two ubiquitin-like conjugation systems. Phosphatidylethanolamine (PE) modified Atg8 labels autophagic vacuoles throughout different stages, from early isolation membranes to late autolysosomes (Ichimura et al. 2000; Matsushita et al. 2007). It is the most used autophagic marker to label autophagic vacuoles. Atg8 is cleaved by a cysteine protease Atg4 at the C-terminus to expose a glycine residue. Subsequently, the cleaved Atg8 is conjugated to an E1-like enzyme, Atg7, followed by its transfer to the E2-like Atg3. Similarly, with the help of Atg7 and an E2-like enzyme Atg10, Atg5 conjugates to Atg12 and finally forms a complex with Atg16. The Atg5-Atg12-Atg16 complex enhances the covalent conjugation of Atg8 to PE (Nakatogawa et al. 2007). There are two *Atg8* genes in flies: *Atg8a* and *Atg8b*. *Atg8a* is ubiquitously expressed in most tissues, whereas *Atg8b* is specifically expressed in testis. Mutants of *Atg7*, *Atg8a*, and *Atg16* are viable (Juhasz et al. 2007; Mulakkal et al. 2014; Simonsen

et al. 2008). They have a shorter lifespan and are more sensitive to different types of stress. There are two *Atg4* genes in *Drosophila*: *Atg4a* and *Atg4b*. It is not known whether both of them are required for Atg8 cleavage.

15.2.1.4 Autophagy Receptor Proteins

Many selective receptors or adaptors for autophagy cargo recognition have been identified in higher organisms (Deng et al. 2017). They all have orthologs in zebrafish, but only a few have *Drosophila* orthologs. In flies, Ref(2)P (the fly ortholog of *SQSTM1/p62*) acts as a selective receptor to recognize cargo during autophagy (Nezis et al. 2008). The Ref(2)P C-terminus has a ubiquitin-binding domain that binds to ubiquitin-modified cargoes. Ref(2)P multimerizes and triggers the formation of ubiquitin-positive protein aggregates both under physiological conditions and when healthy protein turnover is inhibited. Ref(2)P contains an Atg8-interacting motif that might help to enclose cargoes with Atg8 positive membranes. Homozygous *ref(2)P* null mutants are viable, but males are sterile with degenerated mitochondria in spermatids (Dezelee et al. 1989). In vertebrates, there are two oxidation-sensitive cysteine residues on SQSTM1 to sense stress and activate prosurvival autophagy. Although they are not conserved, introducing these oxidation-sensitive cysteine residues into the fly Ref(2)P protein increases protein turnover and stress resistance in flies (Carroll et al. 2018).

Alfy is an autophagy adaptor that is associated with the clearance of protein aggregates (Simonsen et al. 2004). Alfy is a very large protein. It binds to PI3P and interacts with LC3 through an LC3 binding motif. It can also associate with SQSTM1 and the ATG5-ATG12 complex (Isakson et al. 2013). In flies, the ortholog of *Alfy* is named *blue cheese* (*bchs*) after the brain morphology is observed in mutant flies (Finley et al. 2003). Bchs is required for autophagic degradation of Ref(2) P-associated ubiquitinated proteins. *bchs* mutants are viable but have a reduced adult life span. In the mutant animals, protein aggregates containing ubiquitinated proteins and amyloid precursor-like protein accumulate in the CNS in an age-dependent manner. The size of the CNS is reduced, and it shows extensive neuronal apoptosis (Finley et al. 2003; Lim and Kraut 2009).

Huntingtin (Htt), the protein encoded by the gene mutated in Huntington disease, was recently identified as a scaffold to regulate autophagy (Ochaba et al. 2014; Rui et al. 2015). It binds to SQSTM1 to facilitate the engulfment of ubiquitinated cargoes by LC3 positive membranes. Htt also binds to ULK1 to promote its activation. In flies, *htt* mutant animals are viable with no visible developmental defects. However, the life span and locomotor ability of *htt* mutants are reduced with aging. Lack of endogenous *htt* significantly enhances the neurodegenerative phenotypes associated with polyglutamine Htt toxicity (HD-Q93) (Zhang et al. 2009). Besides, *htt* and autophagy genes have genetic interaction in flies (Rui et al. 2015). Loss of *htt* in *Drosophila* disrupts starvation-induced autophagy (Ochaba et al. 2014).

For the autophagy receptor UBQLN2, there are several low homology orthologs in flies. However, their function has not been studied. There is no fly ortholog for autophagy receptors such as OPTN/Optineurin, NBR1, and NDP52 (Deng et al.

2017). For these genes, zebrafish might be an excellent model to study the *in vivo* functions.

15.2.2 Proteins Required for Autophagosome and Lysosome Fusion

Autophagosomes fuse with endosomes and lysosomes to deliver their cargo for degradation. The fusion requires Rab GTPases to label the membranes (Stenmark 2009), a tethering complex to facilitate docking and association between two organelles, and a complex of soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors (SNARE) proteins to mediate fusion (Hong 2005).

15.2.2.1 Rab GTPases

Rab7 and Rab2 are essential small GTPases mediating autophagosome-lysosome fusion. Rab7 is localized on endosomes, lysosomes, and autophagosomes. A lack of Rab7 leads to the abolishment of fusion between autophagosomes and lysosomes. The localization of Rab7 to the autophagosome does not depend on Rab5 and the autophagosome-lysosome tethering complex: the HOPS complex (see the below paragraph for a more detailed description of the function of this complex). The Rab7 guanine nucleotide exchange factor (GEF) Mon1–Ccz1 complex is required for the fusion process (Cabrera et al. 2014; Lawrence et al. 2014; Poteryaev et al. 2010). The Mon1–Ccz1 complex binds to the PI3P produced by the VPS34 complex to promote Rab7 recruitment to the autophagosomes (Cabrera et al. 2014; Hegedus et al. 2016; Lawrence et al. 2014). Rab2 is known to mediate the trafficking between the ER and the Golgi. Recently, it was shown to be required for autophagosome-lysosome fusion and the proper trafficking of lysosomal hydrolases (Csizmadia et al. 2018; Fujita et al. 2017; Lorincz et al. 2017b). The GTP-binding form of Rab2 (Rab2-GTP) localizes to the autolysosome. Overexpression of Rab2-GTP enhances the fusion between autophagosomes and lysosomes (Lorincz et al. 2017b). Both Rab7 and Rab2 bind to the tethering complex HOPS, to coordinate the fusion process (Lorincz et al. 2017b). PLEKHM1, the effector of Rab7, is also required for the fusion between autophagosomes and lysosomes (Csizmadia et al. 2018; McEwan et al. 2015). EPG5, initially identified in worms, is identified as an Rab7 effector that regulates autophagosome-lysosome fusion (Tian et al. 2010). In flies, the reduction of the *EPG5* ortholog *Epg5* leads to abnormal autophagy and progressive neurodegeneration (Byrne et al. 2016).

15.2.2.2 The HOPS Complex

The homotypic fusion and vacuole protein sorting (HOPS) complex is the tethering complex required for the fusion between the autophagosome and the lysosome (Solinger and Spang 2013; Spang 2016). In flies, *Vps11*, *Vps16A*, *Vps39*, *Vps18/dor*, *Vps33A/car*, and *Vps41/lt* encode proteins that form the HOPS complex (Lindmo et al. 2006). *Vps18/dor*, *Vps33A/car*, and *Vps41/lt* are classical eye color mutants (Lloyd et al. 1998). Hypomorphic mutations of these genes impair the biogenesis of eye pigment granules and lead to eye color defects. Null mutants in these genes lead to early instar lethality. Loss of *dor* or *car* or a hypomorphic mutation of *it* also leads to defects in endocytosis, and similar phenotypes have been reported based on RNA interference (RNAi) analysis of *Vps16A* (Akbar et al. 2009; Pulipparacharuvil et al. 2005; Sevrioukov et al. 1999; Swetha et al. 2011). The HOPS complex binds to Rab7 and Rab2 to tether autophagosomes and lysosomes. It also interacts with the autophagosomal Qa SNARE Syntaxin 17 (Syx17) to trigger SNARE complex assembly (Jiang et al. 2014; Takats et al. 2014).

15.2.2.3 The SNARE Complex

SNARE proteins mediate membrane fusion events. They are divided into four subfamilies: QA-SNARES, QB-SNARES, QC-SNARES, and R-SNARES. A tetrameric complex of one of each type of SNARES triggers membrane fusion (Hong and Lev 2014). In flies, mature autophagosomes have a Qa SNARE Syntaxin 17 (Syx17), which forms a complex with the Qbc SNARE Ubisnap (SNAP29 in mammals) and the R SNARE Vamp7 (VAMP8 in mammals) located on late endosomes and lysosomes (Itakura et al. 2012; Takats et al. 2013). Ykt6 is another R-SNARE localized on lysosomes. Ykt6 and Vamp7 compete to form a SNARE complex with Syx17 and Ubisnap. Both Vamp7 and Ykt6 bind to the HOPS complex and are required for the fusion between autophagosome and lysosome (Takats et al. 2018). It has been reported that ATG14 also binds to Syntaxin17 to promote the fusion events in mammals (Diao et al. 2015). It is not known whether Atg14 has a similar function in flies or not.

15.3 The Physiological Roles of Autophagy in *Drosophila* and Zebrafish

Autophagy plays important physiological roles in the development and tissue homeostasis in flies. Autophagy is required for tissue remodeling during metamorphosis, for ovary development, for neuronal homeostasis, and for the clearance of paternal mitochondria. During stress, such as starvation, autophagy also functions as a lifesaving strategy to remobilize nutrition to the proliferating tissues (Lorincz et al. 2017a).

15.3.1 *Autophagy in the Fat Body of* Drosophila

The *Drosophila* fat body is similar to the human liver and white adipose tissues and functions both as a metabolic organ and as a tissue for the storage of energy (Zheng et al. 2016). The larval fat body is the most used model system to study autophagy in *Drosophila* (Scott et al. 2004). It consists of a single layer of cells. From embryonic stage16 to the third *instar* larvae stage, the fat cell number remains constant at about 2200 cells per animal. At the embryonic stage, the size of the fat body is small. With feeding, the size of the fat body increases dramatically and the cells reach enormous sizes, but the cell number remains unchanged. The DNA in the fat body cells is endoreplicated to reach a ploidy level of 256-512n at the mid-third *instar* larval stage. Autophagy is induced at the initiation of the metamorphosis when the third *instar* larvae reach the wandering stage and crawl out of their food to start pupariation. Cytoplasmic components within the fat body are degraded by autophagy to provide nutrients to develop imaginal tissues (Butterworth et al. 1988; Juhasz et al. 2003; Rusten et al. 2004).

In addition to the programmed induction of autophagy during metamorphosis, autophagy can be induced in fat body tissues at the early third *instar* stage by starvation. When early third instar larvae are soaked in 20% sucrose for several hours (3–6 h), autophagy will be induced in the fat body tissue. The induction of autophagy can be observed by examining the patterns of lysotracker staining. Without autophagy induction, fat body cells only have a diffused background level of lysotracker staining. However, once autophagy is induced, a large amount of lysotracker positive puncta can be observed in the early third *instar* larval fat body tissues (Fang et al. 2016; Scott et al. 2004). Multiple autophagy markers, such as Atg5, Atg6, Atg8, Atg9, Atg16, Atg18, Syntaxin17, Rab7, and Ref(2)P, change patterns upon the induction of autophagy. Ectopic tagging of these proteins and examining their patterns or examining the endogenous proteins by immunostaining help to analyze whether there is a defect in autophagy. TEM analysis has been used to analyze autophagic vacuoles. Autophagosomes, amphisomes, and autolysosomes have distinct morphologies in this tissue and can be easily analyzed. The fusion between the autophagosome and the lysosome has been monitored by GFP- and RFP (mCherry)-double tagged Atg8a. Similar to mammalian cells, the GFP signals are observed to be quenched once the autophagosome fused with the lysosome, whereas the RFP signals remain. In the well-fed early third *instar* larval fat body, both the GFP and RFP signals were diffused. Occasionally, small RFP puncta could be observed. Upon starvation, GFP signals are still diffused, but a large amount of RFP puncta emerge. When autophagosomes fail to fuse with lysosomes or the acidification of the lysosome is defective, yellow puncta are observed upon induction of autophagy (Mauvezin et al. 2014; Nagy et al. 2015).

By using FRT-/FLP-mediated mitotic recombination, it is possible to generate mosaic tissues with both mutant cells and wild-type control cells in fat body tissues. This technique can also be used to overexpress a particular protein or RNAi knock down a gene's expression in a few cells surrounded by wild-type cells. Analyses on

this material make it possible to compare markers for autophagy in mutant cells and wild-type controls in the same piece of tissue. The internal control facilitates the analysis of mild defects caused by the loss or the gain of a gene's expression (Nagy et al. 2015).

15.3.2 Autophagy in the Salivary Gland of Drosophila

Autophagy is a self-protection mechanism promoting cell survival under stress conditions. However, it is also involved in cell death in animals (Anding and Baehrecke 2015a; Nelson and Baehrecke 2014). The *Drosophila* larval salivary glands die at the early pupal stage. A small ring of diploid cells located at the anterior end of the larval salivary glands divides and differentiates to form the adult salivary glands (Andrew et al. 2000). The elimination of larval salivary glands depends on a cell death mechanism that requires both autophagy and caspases (Berry and Baehrecke 2007; Martin and Baehrecke 2004).

The larval salivary gland is composed of two major cell types, secretory cells and duct cells. Secretory cells synthesize and secrete proteins and duct cells form tubes connecting the secretory cells to the mouth. The majority of salivary gland cells are polyploid cells with an enormous cell size. These cells have differentiated without further cell division, and their polyploidy level can reach 1024 *n*. At the end of the third *instar* stage, larvae crawl out of their food and search a suitable site to pupate. Glue is secreted from the salivary glands to the duct and out of the mouth and is used to adhere the pupa to their pupation place. About 10–12 h after puparium formation, a pulse of a steroid hormone 20-hydroxyecdysone (ecdysone) induces autophagy and prompts the destruction of the salivary glands (Berry and Baehrecke 2007; Lee and Baehrecke 2001; Martin and Baehrecke 2004).

The death of the salivary gland cells requires both caspases and autophagy. The expression of the majority of *Drosophila Atg* and *caspase* genes are induced. Dying salivary gland cells contain a large number of autophagosomes (Lee and Baehrecke 2001; Martin and Baehrecke 2004). Sixteen hours after puparium formation, the larval salivary glands are degraded. In most *Atg* gene mutants, such as the mutants of *Atg1*, *Atg2*, *Atg3*, *Atg6*, *Atg7*, *Atg8a*, *Atg12*, or *Atg18a*, the salivary glands are not properly degraded. Similarly, the reduction of caspase activity leads to an incomplete degradation of the salivary glands. A combined inhibition of caspases and autophagy further blocks gland degradation. A provocation of autophagy by the induction *Atg1* expression leads to a premature destruction of the salivary gland. Interestingly, this Atg1-triggered autophagy is sufficient to induce salivary gland cell death without the requirement of caspase activity (Berry and Baehrecke 2007).

During salivary gland cell death, autophagy is regulated by calcium and inositol-1,4,5 trisphosphate (IP3) signaling pathway components. IP3 binds to the IP3 receptor to trigger calcium release from the ER (Nelson et al. 2014). The calcium ions bind to Calmodulin to activate autophagy. miR14 was found to target IP3 kinase 2 to regulate autophagy in salivary glands (Nelson et al. 2014). Recently, it has been

shown that Hermes, a proton-coupled monocarboxylate transporter, is required for autophagy during steroid-triggered salivary gland cell death. Hermes preferentially transports pyruvate over lactate. In *Hermes* mutant flies, mTOR signaling is elevated and the salivary glands cannot be adequately degraded. The cell death defect could be suppressed by decreasing the mTOR function (Velentzas et al. 2018).

The death and elimination of salivary glands is a cell-autonomous process, and no phagocytosis is involved. Surprisingly, autophagy-induced salivary gland cell death and phagocyte-mediated clearance of dying cell corpses use a similar machinery. Draper, an immunoreceptor, is required for autophagy in the salivary gland. Loss of draper prevents autophagy in dying salivary glands and leads to an incomplete larval salivary gland degradation (McPhee et al. 2010). Draper is also known as the critical engulfment receptor to recognize cell debris during phagocytosis. Draper-dependent phagocytic activity is mediated by Src and Syk family kinase signaling in glial cells (Ziegenfuss et al. 2008). Interestingly, these factors downstream of Draper are also required for autophagy during salivary gland cell death.

The elimination of salivary gland could be examined by histochemistry. The induction of autophagy could be monitored by TEM and the immunostaining of various autophagy markers.

15.3.3 Autophagy in the Intestine of Drosophila

Autophagy-induced cell death also occurs in the larval intestine. A pulse of ecdysone in the late third *instar* larvae induces a wave of autophagy in midgut cells. The elimination of these cells begins after puparium formation. Small islands of imaginal cells that are committed to forming the future adult gut surround the larval midgut cells. They proliferate and enclose the condensed degenerating larval midgut. The dead midgut cells form the yellow body, which is excreted as meconium right after adult flies enclose (Denton et al. 2009; Lee et al. 2002).

The death of the larval midgut cells occurs with many features similar to the cell death in salivary glands, including the formation of autophagosomes, DNA fragmentation, and caspase activation. Disrupting *Atg* genes such as *Atg1*, *Atg2*, *Atg18*a, as well as blocking the initiation of autophagy by modulating growth signaling, delays midgut cell death. However, different from autophagic cell death in salivary glands, caspase activity is not required for larval midgut cell death (Denton et al. 2009; Xu et al. 2015). The inhibition of caspases fails to enhance the *Atg* mutant phenotype in the midgut, suggesting that autophagy is required for larval midgut cell death, but caspases are not. Surprisingly, not all the *Atg* genes encoding the core machinery of autophagy are required. The E1-activating enzyme Atg7 and the E2-conjugating enzyme Atg3 are required for Atg8 lipidation. Although *Atg8* is essential for midgut cell death, *Atg3* and *Atg7* are dispensable for this process. Instead, the E1-activating enzyme encoded by *Uba1* is required for autophagy and the reduction of cell size during midgut cell death. These data indicate that there is

a specific mechanism regulating the process of autophagy in midgut cell death (Chang et al. 2013).

In addition to the larval cell death, several *Atg* genes are required for the homeostase of the adult midgut. The *Drosophila* adult midgut is a tubular structure with monolayered epithelium cells surrounded by visceral muscles (Micchelli and Perrimon 2006). Intestinal stem cells (ISCs) divide asymmetrically to generate renewal ISCs and enteroblasts (EBs). The EBs differentiate further to produce either absorptive enterocytes (ECs) or secretory enteroendocrine cells (EEs). The ablation of *Atg9* leads to a significantly shortened and thickened adult midgut in the posterior region. Atg9 acts on ECs to control their size and morphology (Wen et al. 2017). Similar midgut defects can be observed when *Atg1*, *Atg13*, and *Atg17* encode components of Atg1 kinase complex, but not when *Atg7*, *Atg12*, *Atg16*, *Atg18a*, or *Vps34* is reduced in ECs (Wen et al. 2017). Further study found that the defects are likely due to an increased TOR activity since inhibiting it could primarily rescue the midgut growth defects in *Atg9* mutants. Although Atg9 functions downstream of the Atg1 complex during autophagy, it seems to use different mechanisms to inhibit TOR activity in ECs (Wen et al. 2017).

A lack of *Atg16* leads to a shorter and thicker posterior midgut in adult flies. The differentiation of EE cells is compromised. However, the lack of *Atg8a* and *Atg5* does not influence EE differentiation. The WD40 domain on Atg16 is not required for autophagy but is essential for EE differentiation. Together, it suggests that Atg16 plays an autophagy-independent role in adult midguts (Nagy et al. 2017). The functions of Atg9 and Atg16 in adult midguts are not directly related to the autophagy process, and it needs further efforts to elucidate whether autophagy per se plays a role in the adult midgut homeostasis.

The clearance of the larval midgut could be analyzed by histochemistry. The adult midgut morphology, cell proliferation, cell death, ISC self-renew, and differentiation could be monitored by immunostaining of specific markers. Autophagy markers such as mCherry-tagged Atg8 and Ref2(P) could also be analyzed via immunostaining and western blots in this tissue.

15.3.4 Autophagy in the Ovary of Drosophila

The adult fly ovary contains 15–20 ovarioles with developing egg chambers. Each egg chamber consists of one oocyte and 15 nurse cells surrounded by a layer of somatic follicle cells (King 1970; Spradling 1993). During the late stage of oogenesis, the nurse cells transfer their cytoplasmic contents to the oocyte to support its growth. Then, the nurse cells undergo programed cell death. This process depends on the autophagy-mediated degradation of Bruce, an inhibitor of apoptosis. The degradation of Bruce enables caspase activation and cell death (Nezis et al. 2009).

In addition to this late-stage developmental cell death, nutrient starvation or other stresses can induce the egg chambers to die at two earlier stages, during germarium formation (in region 2) and mid-oogenesis (Drummond-Barbosa and

Spradling 2001; McCall 2004). This stress-induced cell death requires caspases to activate high levels of autophagy. Therefore, autophagy seems to function either upstream or downstream of caspases in the cell death of fly ovary (Hou et al. 2008).

The cell death during mid-oogenesis is accompanied by the remodeling of the mitochondrial network in the dying nurse cells followed by the fragmentation of nurse cells and engulfment of their clustered mitochondria and the cytoplasm by the surrounding follicle cells. The lack of *Atg* genes such as *Atg1* or *Atg7* interferes with the engulfment of the fragments of nurse cells by follicle cells and cell death. The remodeling of mitochondria is a pivotal mechanism to regulate autophagy flux and cell death during mid-oogenesis (DeVorkin et al. 2014).

Autophagy can be modulated at the translational level during oogenesis. *Orb*, the fly ortholog of mammalian translation regulator *CPEB*, prevents cell death through the repression of autophagy. It does so by directly repressing the translation of *Atg12* mRNA (Rojas-Rios et al. 2015).

Autophagy not only plays a role in germ cell death but also triggers the loss of follicle stem cells (FSCs). Hedgehog signaling-induced autophagy drives FSC loss and premature sterility. During aging, Hh-dependent autophagy increases. Insulin-IGF signaling (IIS) suppresses Hh-induced autophagy and promotes a stable proliferative state. The balance between cell proliferation and autophagy determines the reproductive lifespan of flies (Singh et al. 2018).

In *Drosophila* ovaries, *bam* mutant stem cells function as tumor-like stem cells to promote tumor growth. Autophagy is low in wild-type stem cells but elevated in bam mutant stem cells. Loss of either *Atg6* or *Atg17* decreases the bam mutant stem cell niche occupancy, slows the cell cycle, and inhibits *bam* mutant-induced tumor growth in the ovary (Zhao et al. 2018).

15.3.5 Autophagy in the Nerve System of Drosophila

Developmentally programmed autophagy mostly occurs in polyploid cells, as we mentioned above. These polyploid cells undergo autophagic cell death in response to specific signals. Environmental stimuli such as starvation can prematurely induce autophagic responses in these polyploid cells. *Drosophila* neurons are diploid cells. Autophagy cannot be induced by starvation in these cells (Mulakkal et al. 2014). However, a basal level of autophagy is critical for neuronal homeostasis. Autophagy is particularly crucial for neurons since they are long-lived cells with a complex morphology and lengthy processes. The loss of most *Atg* genes or factors required for autophagy results in neurodegeneration in flies. For example, the loss of *Atg7*, *Atg8a*, *Atg5*, *Epg5*, *Ref(2)P*, *bchs*, or *htt* leads to neurodegeneration (Chang and Neufeld 2010; Mulakkal et al. 2014).

Because of the importance of autophagy for neuronal survival, neurons have developed some specific mechanisms to regulate the autophagy process locally at the synapses. Cacophony (Cac), a Drosophila voltage-gated calcium channel

(VGCC), is required for the fusion between autophagosomes and lysosomes in neurons. Photoreceptor terminals mutated in *Cac* accumulate a large number of autophagosomal structures. In cultured cerebellar neurons from mice, the protein encoded by the *cac* ortholog *CACNA1A* localizes on lysosomes and is required for lysosomal fusion (Tian et al. 2015). The presynaptic lipid phosphatase Synaptojanin is required for macroautophagy. In flies that contain a Parkinson's disease mutation *synaptojanin*^{R258Q} knock-in, Atg18a is accumulated on nascent synaptic autophagosomes. Autophagosome maturation is blocked in the synapses of these flies (Vanhauwaert et al. 2017). Endophilin A, a protein that is highly enriched at the synapse of flies, induces macroautophagy at the synapses. Kinase LRRK2 phosphorylates the BAR domain of Endophilin A to promote the formation of highly curved membranes, which serve as docking stations for autophagic factors (Soukup et al. 2016).

15.3.6 Autophagy in Zebrafish

Although most genes participating in autophagy are highly conserved between zebrafish and human, the study of autophagy in zebrafish is still at an early stage. It is relatively easy to study embryogenesis and organogenesis in zebrafish since the embryos and larvae are small and transparent and develop *ex utero*. These characteristics make zebrafish an excellent system to generate disease models and discover the underlying mechanisms (Mathai et al. 2017). For example, polyglutamine expansion diseases, such as Huntington's disease (Williams et al. 2008), tauopathy (Bai et al. 2007), and amyotrophic lateral sclerosis (ALS) (Ramesh et al. 2010), have been successfully modeled in zebrafish.

The functions of most zebrafish autophagy genes were revealed by morpholino-mediated knockdown experiments. The reduction of *atg5*, *atg7*, *beclin1*, *atg4da*, *ambra1a*, and *ambra1b* all leads to developmental defects during embryogenesis. One of the common phenotypes seen is a cardiac defect, suggesting that autophagy plays a specific role in cardiac development (Benato et al. 2013; Hu et al. 2011; Kyostila et al. 2015; Lee et al. 2014). Transient depletion of *sqstm1* in zebrafish embryos increases the susceptibility to bacterial infection (Mostowy et al. 2013; van der Vaart et al. 2014). The ablation of *sqstm1* causes a specific locomotor defect (Lattante et al. 2015). The knockdown of *optineurin* also leads to motor axonopathy, an increase of protein aggregates, defective vesicle trafficking, and an increased susceptibility to bacterial infection (Chew et al. 2015; Korac et al. 2013; Paulus and Link 2014). Morpholino-mediated depletion of *spns1*, a lysosomal transporter, increases embryonic cellular senescence, and the phenotype is reversed by the depletion of the lysosomal v-ATPase (Sasaki et al. 2014, 2017). *Sorting nexin 14* knockdown causes defective autophagic degradation and neurodegeneration in zebrafish (Akizu et al. 2015).

The development of genome editing techniques greatly facilitated mutant generation. Recently, an *epg5* knockout zebrafish was generated. The zebrafish

epg5$^{-/-}$ mutants were viable and without visible developmental defects. The *epg5*$^{-/-}$ mutants developed age-dependent locomotor defects and muscle thinning, together with the accumulation of nondegradative autophagic vacuoles. The human *EPG5* mutation leads to Vici syndrome. Zebrafish *epg5*$^{-/-}$ mutants could serve as a model to study this disease (Meneghetti et al. 2019).

15.4 Mitophagy in *Drosophila*

Mitophagy is the most studied type of selective autophagy in *Drosophila*. The Parkinson disease-related genes *PINK1* and *PARKIN* are well-known as mitophagy regulators in cultured mammalian cells (Durcan and Fon 2015). Upon damage of mitochondria, the kinase PINK1 phosphorylates the E3 ubiquitin (Ub) ligase PARKIN to stimulate PARKIN activation and translocation to damaged mitochondria. Mitochondrial proteins such as MFN are ubiquitinated. The mitochondrial surface polyUb chain is also phosphorylated by PINK1, and that phosphorylated polyUb chain facilitates mitophagy (Pickles et al. 2018). In flies, the functions of *Pink1* and *park* (the fly ortholog of *PARKIN*) are required to maintain proper mitochondrial morphology and activity. The loss of *Pink1* or *park* leads to male infertility and the degeneration of dopaminergic neurons and muscles (Guo 2012). However, whether *Pink1* and *park* participate in the mitophagic process in flies is still controversial. Using live imaging monitor mt-Keima signals and correlative light and electron microscopy (CLEM), one group showed that mitophagy occurred and increased with aging in muscle cells and dopaminergic neurons. However, the age-dependent increase of mitophagy was abrogated by the loss of *Pink1* or *park* (Cornelissen et al. 2018). Another group using a similar tool observed that basal mitophagy occurred in multiple tissues, but the loss of *Pink1* and *park* had little effects on mitophagy (Lee et al. 2018). These controversial results are likely due to the low basal mitophagy level under physiological conditions. In addition to *Pink1*/*park*, the mitophagy receptor FUNDC1 also has orthologs in flies (Liu et al. 2014). However, their functions have not been studied.

It has been reported that elevating autophagy by *Atg1* overexpression can significantly rescue mitochondrial defects in *Pink1* and *park* mutants in *Drosophila* (Ma et al. 2018). This suggests that defective mitochondria quality control is the leading cause of the mitochondrial defects in *Pink1/park* mutants. A knockdown of the mitochondrial deubiquitinating enzyme USP30 or the inhibition of USP14 improves the mitochondrial integrity in *park*- or *Pink1*-deficient flies (Bingol et al. 2014; Chakraborty et al. 2018). Mitochondrial ubiquitin ligase 1 (MUL1, also known as MAPL or MULAN), an E3 protein ligase, functions in parallel with Pink1/park to regulate the Marf level. Overexpression of *MUL1* rescues the mitochondrial defects of the *Pink1*/*park* mutants (Yun et al. 2014). A mitochondrial protein Clueless (clu) binds to VCP/p97 and promotes the degradation of Marf. An overexpression of *clu* complements the *Pink1* mutant defects (Wang et al. 2016).

In addition to the basal level of mitophagy that maintains the homeostasis of adult fly tissues such as neurons and muscles, there are also developmental processes that require mitophagy. The paternal mitochondria are eliminated after fertilization through a process displaying multiple features of the endocytic and autophagic pathways in flies. Park is not required for this process. However, the ubiquitin pathway and Ref(2)P are required (Politi et al. 2014).

At the onset of larval midgut cell death during intestine development, autophagy is required for the reduction of the cell size and for mitochondrial clearance in the dying cells. *Vps13D* mutant cells retain their mitochondria, indicating a defect in mitochondrial clearance. The autophagy function of Vps13D is context dependent since no defect is observed in starvation or rapamycin-induced autophagy in the fat body or intestine. Vps13D also regulates mitochondrial fission downstream of known mitochondrial fusion regulators such as Drp1 and Mff. It is still not clear how Vps13D regulates autophagy/mitophagy (Anding et al. 2018).

In cells, mutant mtDNA often coexists with the wild-type mtDNA, a phenomenon known as heteroplasmy. By studying a fly model that carries a heteroplasmic lethal mtDNA deletion ($mtDNA^{\Delta}$) in adult muscle, one group found that stimulation of autophagy, activation of the Pink1/park pathway, or decreased levels of Marf resulted in a selective decrease in $mtDNA^{\Delta}$. It suggests that mitophagy may help to eliminate damaged mtDNA selectively (Kandul et al. 2016).

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