



Balancing crosstalk between 20-hydroxyecdysone-induced autophagy and caspase activity in the fat body during *Drosophila* larval-prepupal transition



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ABSTRACT

In the fruitfly, *Drosophila melanogaster*, autophagy and caspase activity function in parallel in the salivary gland during metamorphosis and in a common regulatory hierarchy during oogenesis. Both autophagy and caspase activity progressively increase in the remodeling fat body, and they are induced by a pulse of the molting hormone (20-hydroxyecdysone, 20E) during the larval-prepupal transition. Inhibition of autophagy and/or caspase activity in the remodeling fat body results in 25–40% pupal lethality, depending on the genotypes. Interestingly, a balancing crosstalk occurs between autophagy and caspase activity in this tissue: the inhibition of autophagy induces caspase activity and the inhibition of caspases induces autophagy. The *Drosophila* remodeling fat body provides an *in vivo* model for understanding the molecular mechanism of the balancing crosstalk between autophagy and caspase activity, which oppose with each other and are induced by the common stimulus 20E, and blockage of either path reinforces the other path.

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1. Introduction

Macroautophagy (herein referred to as autophagy) and apoptosis (a classical programmed cell death, PCD) play critical roles in development and diseases. Autophagy mediates a highly regulated self-degradation process that is initiated as an adaptive response in unfavorable conditions, such as nutrient deprivation, and is governed by a series of Atg genes. During autophagy, the cytoplasm and intracellular organelles are engulfed within autophagosomes and delivered to lysosomes for bulk degradation (He and Klionsky, 2009). Apoptosis results from a cascade of caspase activation, which leads to a rapid demolition of cellular structures and organelles. Apoptosis culminates in cellular shrinkage with nuclear chromatin condensation, nuclear fragmentation, and eventual phagocytosis (Green, 2005). Autophagy promotes cell survival against apoptosis, but extensive autophagy may also cause cell death in certain circumstances and is considered as the second type of PCD (Shintani and Klionsky, 2004). Common stimuli can induce combined autophagy and apoptosis; in many other

instances, autophagy and apoptosis develop in a mutually exclusively manner (Maiuri et al., 2007). Therefore, the functional relationship between autophagy and apoptosis is complex, due to the dual effects of autophagy. Although significant advances have recently been made regarding to the crosstalk between autophagy and apoptosis, the underlying mechanism is still largely unknown (Giansanti et al., 2010; Ryoo and Baehrecke, 2010). In particular, very little is understood about the transcriptional regulation of the crosstalk.

The fruitfly, *Drosophila melanogaster*, serves as a popular model for studies on apoptosis and autophagy, owing in part to the powerful genetic tools that have been developed in this model animal. Several significant advances about apoptosis and autophagy have been made in *Drosophila* over the past decade (Ryoo and Baehrecke, 2010). The core machinery of apoptosis in *Drosophila* includes the adaptor Ark, the initiator caspase Dronc, and the effector caspase Drice. The inhibitor of apoptosis proteins and death activators, such as Reaper, Hid and Grim, are critical regulators of apoptosis. In contrast, Cytochrome C and the Bcl-2 family, which are key regulators of apoptosis in mammals, play little to no role in *Drosophila* (Hay and Guo, 2006). The Atg genes, which tightly control the formation of autophagosomes in organisms ranging from yeast to human, are quite well conserved in

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Drosophila (Chang and Neufeld, 2010). A very important discovery in *Drosophila* is Draper, which was the first identified component that distinguishes autophagic cell death from autophagy-associated cell survival (McPhee et al., 2010).

Apoptosis plays critical roles during *Drosophila* development, for example, in larval imaginal discs, the nervous system and during embryogenesis (Ryoo and Baehrecke, 2010). The relationship between autophagy and caspases is context-specific in this organism. During the larval–pupal transition, many obsolete larval tissues undergo massive destruction mainly by autophagic cell death (Ryoo and Baehrecke, 2010; Yin and Thummel, 2005). Both autophagy and caspases, which function in parallel, contribute to autophagic cell death in the dying salivary gland, but autophagy plays a more important role than caspases (Berry and Baehrecke, 2007; Scott et al., 2007). Moreover, autophagy, but not caspases, governs cell death in the midgut (Denton et al., 2009). During oogenesis, caspases and autophagy function in a common regulatory hierarchy, where caspases are required for autophagy and *Atg* genes are required for caspase activation (Hou et al., 2008; Nezis et al., 2009, 2010).

In *Drosophila*, the molting hormone, 20-hydroxyecdysone (20E), is both necessary and sufficient to induce apoptosis and autophagy (Ryoo and Baehrecke, 2010; Yin and Thummel, 2005). The 20E-ecdysone receptor complex (20E-EcR-USP) and the 20E primary-response genes (including *Br-C*, *E74*, *E75* and *E93*) induce the expression of several 20E secondary-response genes that are responsible for apoptosis, including the caspases *Dronc* and *Drice* and the death activators *Reaper* and *Hid* (Hay and Guo, 2006; Yin and Thummel, 2005). TOR complex 1 (TORC1) is a central inhibitor of autophagy that integrates multiple upstream signals and, in nutrient-rich conditions, negatively regulates ATG1 by phosphorylation (Chang and Neufeld, 2010). 20E blocks the PI3K-TORC1 pathway to initiate autophagy (Colombani et al., 2005; Delanoue et al., 2010; Rusten et al., 2004). Meanwhile, 20E upregulates *Atg* genes for inducing autophagy (Tian et al., 2013; Yin and Thummel, 2005).

The insect fat body is an organ analogous to the vertebrate adipose tissue and liver (Liu et al., 2009). During *Drosophila* metamorphosis, the fat body undergoes a significant remodeling process. Autophagy gradually occurs in the larval fat body during the larval–prepupal transition (Rusten et al., 2004). The single-cell layer of fat body tissues dissociates to individual cells during the prepupal–pupal transition (Nelliott et al., 2006). Apoptosis continuously occurs in the fat body cells during the pupal life, while a portion of them survive to the first three days in adults (Aguila et al., 2007; Butterworth et al., 1988). However, the function and regulation of autophagy and caspase activity in the remodeling fat body are largely unknown. In this study, we discovered that during the larval–prepupal transition in *Drosophila*, a balancing crosstalk occurs between the 20E-induced autophagy and caspase activity in the remodeling fat body, both of which play roles during metamorphosis.

2. Material and methods

2.1. Fly strains and genetics

All fly strains were grown on standard cornmeal/molasses/agar medium at 25 °C. The following fly strains were collected from the Bloomington *Drosophila* Stock Center: *w¹¹¹⁸*, *Act-GAL4*, *Adv/Cyo::arm-GFP* and *TM6B/TM3::arm-GFP*. *Lsp2-GAL4* is a fat-body-specific GAL4 line. In preliminary experiments, we observed no difference of 20E-induced autophagy and caspase activity between *w¹¹¹⁸* and *Lsp2-GAL4*, and in most cases only one control was shown if not specifically mentioned. The UAS lines used include the

following: *UAS-Atg1^{DN}* (Scott et al., 2007), *UAS-p35* (Mergliano and Minden, 2003), *UAS-Dronc^{DN}* (Meier et al., 2000), *UAS-Dronc* (Meier et al., 2000), *UAS-EcR^{DN}* (IIA, IIB2 and IIIB2) (Cherbas et al., 2003), *UAS-GFP-LC3* (Rusten et al., 2004) and *UAS-mCherry-Atg8a* (Chang and Neufeld, 2009). *UAS-Atg1^{DN}::UAS-Dronc^{DN}*, *Lsp2-GAL4::UAS-GFP-LC3*, *Act-GAL4::UAS-GFP-LC3* and *Act-GAL4::UAS-mCherry-Atg8a* were produced by genetic recombination. For the GAL4/UAS experiments, *Lsp2-GAL4* or the flies recombined with *Lsp2-GAL4* and *Act-GAL4* was crossed with the UAS lines. For the Flp-out experiments, *hsFlpase; Act > CD2 > GAL4; UAS-GFP* (Pignoni and Zipursky, 1997) was crossed with the UAS lines. The mutants used are *Atg8a^{KG07569}* (Scott et al., 2007), *Dronc^{I24}* (Xu et al., 2005). *Dronc^{I24}* were recombined with *Lsp2-GAL4* to generate *Dronc^{I24}::Lsp2-GAL4*, with *UAS-GFP-LC3* to obtain *Dronc^{I24}::UAS-GFP-LC3*.

2.2. Fluorescence microscopy and immunohistochemistry

GFP- and non-GFP-containing larvae were separated under an Olympus SZX16 fluorescence stereomicroscope. The fat body tissues of different genotypes were dissected at the indicated developmental stages. For detecting cleaved Caspase-3 (Fan and Bergmann, 2010) by immunohistochemistry, the dissected fat body was fixed in 4% paraformaldehyde for 45 min at room temperature, blocked in phosphate buffered saline containing 5% BSA and 0.1% Triton-X (PBSBT) for 1 h, and incubated with the cleaved Caspase-3 antibody at a dilution of 1:200 at 4 °C overnight. The fat body was washed for 1 h in PBSBT and incubated with a FITC- (green) or Alexa Fluor 647- (red) conjugated secondary antibody (diluted 1:200) for 2 h. DAPI (Sigma) was added to label nuclei and the stained fat bodies were imaged using a Zeiss LSM 510 META confocal microscope at ×20 magnifications. For detecting Lamin Dm0 (Berry and Baehrecke, 2007) by immunohistochemistry, the staining procedures were the same as above except using the Lamin Dm0 primary antibody and a Cy3-conjugated secondary antibody. According to the manufacturer's instructions (Beyotime, Shanghai, China), DNA fragmentation was monitored using TUNEL staining under the above Zeiss microscope at ×20 magnifications. GFP-LC3 was monitored under an Olympus FV10-ASW confocal microscope at ×40 magnifications, and LysoTracker Red DND-99 (Invitrogen) was monitored using the same microscope at ×40 or ×60 magnifications. We used the Photoshop program to calculate % values of GFP-LC3, LysoTracker Red and cleaved Caspase-3 staining with the Color Range function. It is necessary to note that the brightness and contrast of all the images of GFP-LC3 are adjusted using the same parameters in the Photoshop program.

2.3. Transmission electron microscopy (TEM)

The animals collected from different genotypes were fixed over 24 h at 4 °C in 2% paraformaldehyde, 4% glutaraldehyde, 2% sucrose in 100 mM phosphate buffer (Scott et al., 2004) or 4% glutaraldehyde (0.1 M Na-cacodylate buffer, pH 7.4) (Franzetti et al., 2012), thoroughly washed in 0.1 M PBS, pH 7.2, postfixed in 0.5% osmium tetroxide for 2 h, and embedded in resin according to the manufacturer's recommendations. From the fixed tissue, 70-nm sections were cut, stained in Reynolds lead citrate, and viewed on a transmission electron microscope (H7650, Hitachi) for observing autolysosomes in the fat body. An important criterion for autolysosomes is the visible remnants of organelles and the undigested materials (Klionsky et al., 2012). The autolysosomes are typically of 1–5 μm sizes, which gradually increase from the early wandering stage (EW), late wandering stage (LW) to white prepupal stage (WPP), while the autophagosomes are typically of 0.2–1 μm sizes showing double-membrane structures.

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