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Ecdysone-mediated programmed cell death in *Drosophila*

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ABSTRACT During *Drosophila* development, the steroid hormone ecdysone plays a key role in the transition from embryo into larva and then into pupa. It is during larval-pupal metamorphosis that extensive programmed cell death occurs to remove large obsolete larval tissues. During this transition, ecdysone pulses control the expression of specific transcription factors which drive the expression of key genes involved in cell death, thus spatially and temporally controlling programmed cell death. Ecdysone also controls cell death in specific larval and adult tissues. This review focuses on the current knowledge of ecdysone-mediated cell death in *Drosophila*.

KEY WORDS: *programmed cell death, ecdysone, transcription, apoptosis, autophagy*

Introduction

Programmed cell death (PCD) is a vital process that functions to remove damaged or potentially harmful cells, maintain homeostasis, and to shape and remove obsolete tissues during tissue morphogenesis (Fuchs and Steller, 2011). The latter function is particularly evident during development of the fruit fly *Drosophila melanogaster.* PCD occurs widely throughout different stages of the *Drosophila* life cycle including embryogenesis, larval development, and during larval-pupal metamorphosis where it functions to remove larval tissues and cells that are not required for the adult fly (Denton *et al.*, 2013a, Xu *et al.*, 2009). The genetic amenability and well-characterised involvement of PCD during its development has made *Drosophila* a powerful model organism for deciphering the cell death machinery and its regulation.

Steroid hormones play critical roles in regulating developmental PCD and morphogenesis across different species. For example, sculpting of the tadpole tail by PCD is mediated by thyroid hormone (Tata, 1966). Similarly, in mammals glucocorticoids mediate thymocyte apoptosis by controlling the transcription of key prosurvival transcription factors (Distelhorst, 2002). In *Drosophila,* developmentally timed pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) initiates processes such as molting and larval-pupal metamorphosis (Riddiford *et al.*, 2000, Thummel, 2001). Of these processes, ecdysone induces the histolysis of many obsolete larval tissues during larval-pupal metamorphosis such as the midgut, salivary gland, anterior and abdominal muscles, and distinct subsets of neurons in the nervous system and optic lobe (Choi *et al.*, 2006, Fahrbach *et al.*, 2005, Hara *et al.*, 2013, Jiang *et al.*, 1997, Kumar and Cakouros, 2004, Lee *et al.*, 2002a, Winbush and Weeks, 2011, Zirin *et al.*, 2013). Additionally, ecdysone is important for the remodelling of the fat body and nervous system (Boulanger and Dura, 2014, Kirilly *et al.*, 2009, Kuo *et al.*, 2005, T. Lee *et al.*, 2000, Loncle and Williams, 2012, Rusten *et al.*, 2004, Williams and Truman, 2005). In this review, we begin by providing a brief overview of the cell death machinery in *Drosophila* and then discuss the role of ecdysone in mediating and regulating PCD.

Cell death machinery in *Drosophila*

Apoptosis

The majority of cell death in *Drosophila* is mediated by apoptosis, a caspase-dependent cell death pathway that is highly conserved among metazoans (Fig. 1)(Kumar, 2007). Caspases are cysteine proteases consisting of initiator caspases activated in response to apoptotic stimuli, and effector caspases activated by initiator caspases that cleave the majority of substrates to cause cell death (Kumar, 2007). There are seven caspases in *Drosophila* (Dronc, Dredd, Strica, Drice, Dcp-1, Decay and Damm), of which Dronc, Dredd and Strica contain long amino-terminal prodomains that are a feature of initiator caspases (Chen *et al.*, 1998, Dorstyn *et al.*, 1999a,b, Doumanis *et al.*, 2001, Fraser and Evan, 1997, Harvey *et al.*, 2001, Song *et al.*, 1997). Of these initiator caspases, Dronc is the essential apical death caspase and has a function similar to CED-3 in *Caenorhabditis elegans* and caspase-9 in mammals (Chew *et al.*, 2004, Daish *et al.*, 2004, Dorstyn *et al.*, 1999a). Deletion or ablation of *dronc* causes a block in most developmental as well as stress-induced cell death (Chew *et al.*, 2004, Daish *et al.*, 2004,

Abbreviations used in this paper: Atg, Autophagy-related; BR-C, Broad Complex; EcR, Ecdysone Receptor; h APF, hours after puparium formation; PCD, Programmed cell death; RHG, Rpr, Hid and Grim; USP, ultraspiracle.

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Waldhuber *et al.*, 2005, Xu *et al.*, 2005). As is the case for activation of CED-3 and caspase-9, activation of Dronc requires its recruitment to the apoptosome complex consisting of Dark, an adaptor protein functionally similar to *C. elegans* CED-4 and mammalian Apaf-1 (Yang *et al.*, 1998, Yu *et al.*, 2006). Somewhat surprisingly however, cytochrome *c* is not required for the formation of the Dark apoptosome, Dronc activation or apoptosis in *Drosophila* despite the essential role of cytochrome *c* for apoptosome assembly in mammals (Dorstyn *et al.*, 2002, 2004, Dorstyn and Kumar, 2006, 2008, Kumar, 2007, Yuan *et al.*, 2011).

The most important effector caspase activated by Dronc-mediated processing in *Drosophila* is Drice, a functional analogue of caspase-3 in mammals (Fraser and Evan, 1997, Kumar, 2007, Muro *et al.*, 2006). Dcp-1, another effector caspase which closely resembles Drice, plays a redundant role in apoptosis (Xu *et al.*, 2006). While *dcp-1* mutants do not show any significant phenotype, *drice; dcp-1* double mutants show more profound cell death defects than *drice* mutants alone. Once activated, Drice and Dcp-1, cleave many proteins to execute apoptosis (Kumar, 2007).

The function of the other caspases, Dredd, Strica, Damm and Decay in cell death is less well established. Strica has some level of redundancy with Dronc during PCD in the ovary and a subset of neurons that secrete the neuropeptide *Corazanin* (vCrz) (Baum *et al.*, 2007, Lee *et al.*, 2011). Along with Drice, Decay is responsible for high levels of caspase activity present in the larval midgut during PCD although its function here is unknown (Denton *et al.*, 2009). Dredd, a caspase-8-like caspase, is primarily involved in innate immunity, whereas the function of Damm is still unknown (Leulier *et al.*, 2000, Stöven *et al.*, 2000, 2003).

Interestingly, BH3-only proteins that provide an essential link between death signals and activation of the caspase cascade in mammals are absent in *Drosophila*. BH3-only proteins mediate their function through prosurvival and prodeath Bcl-2 family members (Happo *et al.*, 2012). While *Drosophila* has two Bcl-2-like proteins, Buffy and Debcl, neither is essential for cell death or survival except in specific contexts (Brachmann *et al.*, 2000, Colussi *et al.*, 2000, Quinn *et al.*, 2003, Doumanis *et al.*, 2007, Sevrioukov *et al.*, 2007, Wu *et al.*, 2010). In the absence of a BH3-only Bcl-2 controlled mechanism, the main proapoptotic factors in *Drosophila* are the RHG proteins consisting of Reaper (Rpr), Head involution defect (Hid) and Grim (Kornbluth and White, 2005). The levels of these genes are regulated by upstream signals such as stress, developmental cues or the steroid hormone ecdysone (Jiang *et al.*, 2000, Lohmann *et al.*, 2002, Zhang *et al.*, 2008). The key function of RHG proteins is to bind to the *Drosophila* inhibitor of apoptosis protein (Diap1), an essential protein for keeping caspases from being activated in the absence of apoptotic stimuli (Wang *et al.*, 1999). The binding of RHG proteins initiates the autoubiquitination and degradation of Diap1 alleviating the block on caspases to allow Dronc and downstream caspase activation (Yoo *et al.*, 2002).

Autophagy

Whilst most PCD in *Drosophila* is executed by caspase-dependent apoptosis, PCD involving macroautophagy (autophagy) also occurs in specific tissues (Denton *et al.*, 2012). Autophagy is an evolutionary conserved process through which cytoplasmic contents such as long-lived and damaged proteins, and organelles are degraded and recycled by the cell (He and Klionsky, 2009, Meléndez and Neufeld, 2008). Autophagy is primarily a cell survival mechanism induced in response to stress conditions to provide products for biosynthesis and energy (Lum *et al.*, 2005). However, autophagy also contributes to cell death in special circumstances as evident during PCD of the larval salivary glands and midgut in *Drosophila* (Berry and Baehrecke, 2007, Denton *et al.*, 2009, 2012). How autophagy causes the death of these tissues remains an unanswered question.

Autophagy is a multistep process that is regulated by distinct Autophagy-related (Atg) proteins (Chang and Neufeld, 2010, He and Klionsky, 2009). Autophagy is induced through activation of an Atg1 kinase complex triggering the formation of an isolation membrane which expands to enclose the cytoplasmic contents in a double-membrane vesicle called the autophagosome (Kabeya *et*

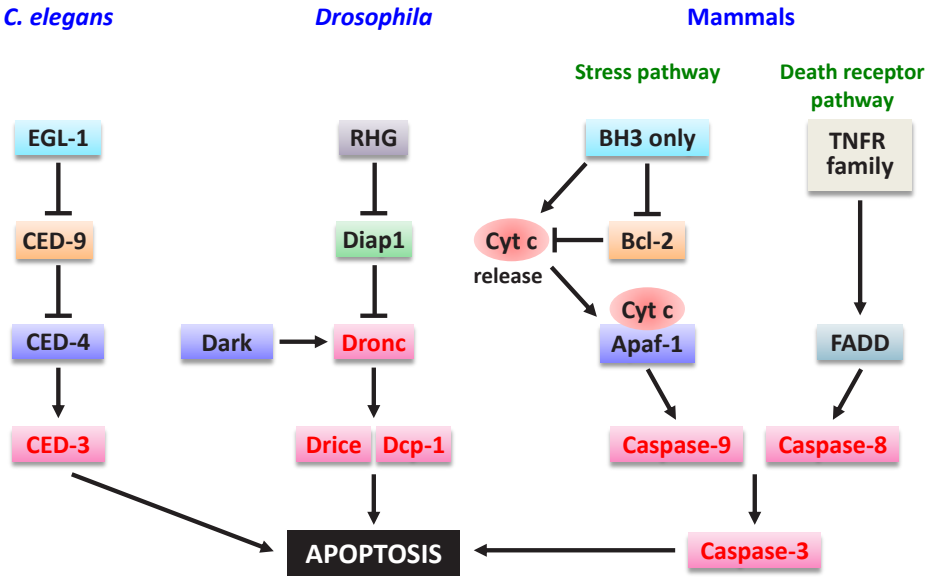

Fig. 1. The core cell death machinery in *C. elegans, Drosophila* and mammals. *In* C. elegans, *CED-9 (Bcl2-like) prevents the activation of CED-3 (caspase-9 like) by directly binding to CED-4 (Apaf-1 like). Upon cell death induction, EGL-1 sequesters CED-9 permitting the release of CED-4 to activate CED-3 and thus execution of apoptosis. In* Drosophila *in the absence of apoptotic stimuli, the* Drosophila *inhibitor of apoptosis protein, Diap1, prevents the activation of the caspases Dronc (caspase-9 like) and Drice (caspase-3 like). Upregulation of the inhibitor of apoptosis protein (IAP) antagonists RHG causes the autoubiquitination and degradation of Diap1 thereby facilitating activation of Dronc by Dark (Apaf-1 like) and downstream effector caspases Drice and Dcp-1. Upon apoptotic signalling in mammals, the BH3-only proteins are activated to cause cytochrome* c *release from the mitochondria, a process inhibited by Bcl-2 proteins in the absence of stimuli. Apoptosome formation is facilitated by binding of cytochrome* c *to Apaf-1 to activate caspase-9. Caspase-9 in turn activates caspase-3 to execute apoptosis. Alternatively, in the extrinsic pathway of apoptosis, effector caspases may be activated through activation of caspase-8 by death receptors of the tumour necrosis factor (TNF) family, which specifically requires its adaptor FADD for caspase-8 recruitment.*

al., 2005, Kamada *et al.*, 2000, Kawamata *et al.*, 2008). Formation of the isolation membrane requires the class III phosphatidylinositol 3-kinase (PI3K) complex to generate phosphatidylinositol (3)-phosphate (PtdIns(3)P) thereby recruiting other proteins involved in the autophagy pathway (Kametaka *et al.*, 1998, Obara and Ohsumi, 2008). During elongation and expansion of the autophagosomal membrane, two conserved ubiquitin-like conjugation systems are active, Atg12 and Atg8 (Geng and Klionsky, 2008). Atg12 is activated by Atg7, an E1-like enzyme, and is subsequently conjugated to Atg5 through the action of the E2-like enzyme Atg10. Atg12-Atg5 forms a complex with Atg16 through Atg5, and this Atg12-Atg5-Atg16 complex homodimerises through Atg16 and localises to the isolation membrane. Atg8 is also activated by Atg7 but requires initial cleavage by the cysteine protease Atg4 at its C terminus for this to occur. Atg8 is subsequently conjugated to phosphatidylethanolamine (PE) by the E2-like enzyme Atg3.

Ecdysone-regulated PCD

At the completion of embryogenesis, the developing embryos hatch as first instar larvae and undergo two molting events to become third instar larvae. During the middle of the third instar larval stage, low-titre pulses of ecdysone trigger a switch in the mechanism of caspase activation from apoptosome-independent to apoptosome-dependent through upregulation of the apoptosome components *dark* and *dronc* as well as *drice* (Kang and Bashirullah, 2014*).* Towards the end of the third instar larval stage a high-titre pulse of ecdysone initiates larval-pupal metamorphosis and destruction of the larval midgut, and abdominal and anterior muscles (Cakouros *et al.*, 2004b, Fahrbach *et al.*, 2005, Jiang *et al.*, 1997, Lee *et al.*, 2002a, Yin and Thummel, 2005, Zirin *et al.*, 2013). A second pulse of ecdysone approximately 12 hours after puparium formation (h APF) signals the transition from prepupal to pupal development and initiates PCD of the larval salivary glands (Jiang *et al.*, 1997, 2000, Lee *et al.*, 2002b). In addition, ecdysone regulates the PCD of the optic lobe and two distinct groups of neurons in the ventral central nervous system (Choi *et al.*, 2006, Hara *et al.*, 2013, Winbush and Weeks, 2011). Ecdysone-mediated PCD is not only restricted to larval-pupal metamorphosis but also occurs in the adult fly ovary (Pritchett *et al.*, 2009). Although not strictly cell death processes, ecdysone is also needed for larval fat body remodelling involving apoptosis and autophagy, and neuronal remodelling to remove obsolete axons and dendrites (Kirilly *et al.*, 2009, Kuo *et al.*, 2005, T. Lee *et al.*, 2000, Loncle and Williams, 2012, Rusten *et al.*, 2004, Williams and Truman, 2005).

Increased levels of ecdysone activate transcription through binding to the heterodimeric nuclear hormone receptor complex consisting of ecdysone receptor (EcR) and ultraspiracle (USP) (Hall and Thummel, 1998, Koelle *et al.*, 1991, Thomas *et al.*, 1993, Yao *et al.*, 1992). The EcR exists as three isoforms, A, B1 and B2 which share common DNA and ligand binding domains but have unique amino termini (Talbot *et al.*, 1993). These isoforms are expressed in different tissues and stages of the *Drosophila* lifecycle, and are required for different ecdysone-induced processes (Talbot *et al.*, 1993). *EcR-A* mutants arrest at pupal stages, and display persistent salivary glands and abnormal legs (Davis *et al.*, 2005, Talbot *et al.*, 1993). Alternatively, loss of *EcR-B1* results in developmental arrest at the onset of metamorphosis and defects in tanning of puparium, the midgut and abdomen, and neuronal pruning (Bender *et al.*, 1997, T. Lee *et al.*, 2000, Schubiger *et al.*, 1998). EcR-B2 has been difficult to study due to the lack of a specific antibody and mutant. In terms of regulating transcription, EcR-A has an inhibitory function whereas EcR-B1 and EcR-B2 have activation functions (Mouillet *et al.*, 2001).

Salivary glands

Among the various *Drosophila* tissues that undergo ecdysone-mediated PCD, the larval salivary gland is the best studied. Both autophagy and apoptosis are required for this process as inhibition of either pathway alone results in partial salivary gland removal whereas combined inhibition of both pathways completely delays removal (Berry and Baehrecke, 2007). Larval salivary gland PCD is triggered by a high-titre ecdysone pulse 12 h APF (Jiang *et al.*, 1997, 2000, Lee *et al.*, 2002b). Ecdysone released in this prepupal pulse initiates a transcriptional cascade firstly activating the expression of a set of primary response or 'early' genes encoding the transcription factors Broad-Complex (BR-C), E74A and E93 which are all required for larval salivary gland PCD (Fig. 2)(Baehrecke and Thummel, 1995, Burtis *et al.*, 1990, Cakouros *et al.*, 2002, DiBello *et al.*, 1991). BR-C, E74A and E93 in turn upregulate secondary response or 'late' genes including key prodeath genes *rpr, hid*, *dark*, *drice* and *dronc,* and downregulate the death inhibitor *diap1* (Fig. 2)(Cakouros *et al.*, 2002, Daish *et al.*, 2003, Jiang *et al.*, 2000, Kilpatrick *et al.*, 2005, C. Y. Lee *et al.*, 2000, Lee *et al.*, 2002b). EcR/USP also regulates the transcription of *rpr* and *dronc* by directly binding to regions in their promoters (Fig. 2)(Cakouros *et al.*, 2004a, Daish *et al.*, 2003, Jiang *et al.*, 2000). Although autophagy is also required for larval salivary gland PCD, of the autophagy genes transcriptionally upregulated during this process, *Atg1* is the only identified *Atg* gene to be directly regulated by EcR/USP (Fig. 2) (Denton *et al.*, 2013b, Gorski *et al.*, 2003, Lee *et al.*, 2003, Martin *et al.*, 2007). Other secondary response genes have been identified in the larval salivary gland such as *brwd3*, *pak*, *pgs2*, *med12* and

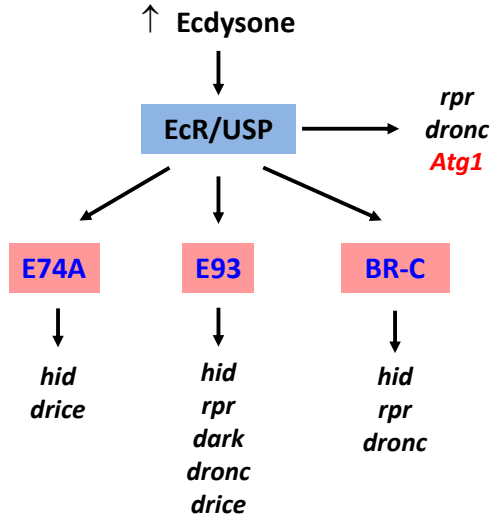

Fig. 2. Ecdysone induces a transcriptional cascade to regulate PCD of the larval salivary glands during larval-pupal metamorphosis. *A prepupal pulse of ecdysone induces larval salivary gland PCD by binding to the heterodimeric complex consisting of EcR/USP. This complex in turn directly regulates the transcription of genes in the case of* dronc, rpr *and* Atg1 *by binding to their respective promoters, or indirectly by upregulating the transcription factors E74A, E93 and BR-C that in turn regulate transcription of cell death genes.*

med24 as they require the function of all three primary response genes for their expression during larval salivary gland PCD and are needed for the downstream expression of *rpr* and *hid* (Ihry and Bashirullah, 2014). The function of these genes in ecdysone-mediated PCD however is yet to be thoroughly investigated. One such secondary response gene *med24* encodes a component of the RNA Pol II mediator complex that is required for salivary gland removal and the optimal but not temporal expression of *rpr* and *hid* (Ihry and Bashirullah, 2014, Wang *et al.*, 2008, Wang *et al.*, 2010).

In addition to these secondary response genes, many other genes required for ecdysone-mediated PCD have been isolated from various screens. For example, components of the ribosome (RpS5, RpL13A, RpL37, RpLP1), the sorting nexin-like gene SH3PX1, a formin-like protein Fhos, a predicted malate dehydrogenase Mdh2 and the transcription factor Sox14 (Anhezini *et al.*, 2012, Chittaranjan *et al.*, 2009, Ritter and Beckstead, 2010, Wang *et al.*, 2010). Knockdown of *sox14* in salivary gland results in reduced transcripts of *rpr*, *dronc* and *E93* but not *Br-C*, however it is unknown whether Sox14 directly regulates their transcription and what position Sox14 sits in the ecdysone-mediated transcriptional cascade if it does at all (Chittaranjan *et al.*, 2009). Mutants for *mdh2* display larval salivary glands persisting past the normal developmental timing of removal (Ihry and Bashirullah, 2014, Wang *et al.*, 2008, Wang *et al.*, 2010). The expression of apoptosis genes upregulated by ecdysone during larval salivary gland PCD occurs as normal in *mdh2* mutant animals indicating that the function of *mdh2* is not related to that of the ecdysone transcriptional cascade (Wang *et al.*, 2010). However, *mdh2* mutants display inhibition of caspase activity and nuclear lamin breakdown, features of apoptosis, but not autophagy. *mdh2* encodes a predicted malate dehydrogenase in the mitochondria that functions in the citric acid cycle. *mdh2* mutants accumulate citric acid cycle products and have reduced ATP levels at the onset of larval salivary gland PCD perhaps indicating an energy requirement for ecdysone-mediated PCD of larval salivary glands (Wang *et al.*, 2010).

Ecdysone also plays an important role in the temporal and spatial regulation of PCD gene expression. For example, BR-C and E93 appear to regulate the temporal expression of *dronc* whereas direct binding of the *dronc* promoter by EcR/USP is important for the spatial expression of this caspase (Cakouros *et al.*, 2004a, Daish *et al.*, 2003). Animals which lack the EcR/USP binding element in the *dronc* promoter do not express *dronc* in the salivary gland. Additionally, caspase activation occurs in a distinct pattern in salivary glands from the anterior at 12h APF to the posterior (Takemoto *et al.*, 2007). However, caspase activation does not follow this pattern when salivary glands are exposed to ecdysone *ex vivo*, but occurs anteriorally to posteriorally when ecydsone is applied to media at the anterior region of the larval salivary glands.

The timing of larval salivary gland PCD is regulated by the transcriptional downregulation of *diap1* by CREB-binding protein (CBP), a transcriptional coregulator and histone acetyltransferase, as well as *rpr* and *hid* by the transcription factor Fork head (Fkh) (Cao *et al.*, 2007, Yin *et al.*, 2007). *diap1* is highly expressed during the early stages of larval development thereby preventing larval salivary gland PCD before the prepupal pulse of ecydsone (Yin *et al.*, 2007, Yin and Thummel, 2004). CBP is upregulated in response to a small pulse of ecdysone released during the mid-third instar transition and causes transcriptional downregulation of *diap1* (Yin *et al.*, 2007*).* The level of Diap1 is reduced to a level where it still enables the prevention of larval salivary gland PCD, however the balance between this prosurvival factor and prodeath genes is upset with the subsequent upregulation of *rpr* and *hid* with the prepupal ecdysone pulse, thus commencing larval salivary gland PCD. Fkh is also highly expressed prior to the initiation of metamorphosis and prevents the induction of *rpr* and *hid* expression before the prepupal pulse of ecdysone (Cao *et al.*, 2007, Renault *et al.*, 2001). Following this pulse, Fkh is downregulated by BR-C thereby allowing *hid* and *rpr* expression, and salivary PCD. In addition, the TATA box-binding protein (TBP) related factor 2 (TRF2) is also important for ensuring ecdysone-regulated genes are globally induced both to their required level and at the appropriate time for salivary gland PCD as well as other ecdysone-regulated processes (Bashirullah *et al.*, 2007).

Midgut

Larval midgut PCD is triggered by the release of ecdysone at the end of the third instar larval stage, and is affected in *EcR* or *usp* mutants along with many other ecdysone-mediated processes (Hall and Thummel, 1998, Jiang *et al.*, 1997, Lee *et al.*, 2002a). Cell death genes upregulated by ecdysone during this process include the IAP antagonists *rpr* and *hid*, and the effector caspase *dronc* activated by BR-C and E93 respectively (Daish *et al.*, 2003, Jiang *et al.*, 1997, Lee *et al.*, 2002a, Yin and Thummel, 2004).

Despite upregulation of apoptosis genes and other features of apoptosis that are observed during larval midgut PCD, removal proceeds normally despite mutations in the apoptotic machinery (Denton *et al.*, 2009). A high level of Decay and Drice caspase activity is observed during larval midgut PCD, but this seems dispensible for larval midgut PCD. However, deletion or ablation of key components of the autophagy pathway such as *Atg1*, *Atg2* and *Atg18* delays midgut removal (Denton *et al.*, 2009). This delay corresponds with reduced autophagy but no change in caspase activity indicating autophagy is the primary mechanism of larval midgut PCD. Many *Atg* genes are upregulated immediately prior to larval midgut PCD, however how these genes are activated by ecdysone signalling and their exact requirements for larval midgut PCD remain to be explored (Denton *et al.*, 2009; , Xu *et al.,* 2015).

Other ecdysone-regulated genes involved in larval midgut PCD include *sox14* and the phosphatase *Ptp52F* (Chittaranjan *et al.*, 2009, Santhanam *et al.*, 2013, 2014). RNAi-mediated knockdown of *sox14* delays larval midgut PCD (Chittaranjan *et al.*, 2009). Sox14 has been implicated in larval salivary gland PCD (as described above) and neuronal pruning (as discussed below), however its role in larval midgut PCD is currently undetermined (Chittaranjan *et al.*, 2009, Kirilly *et al.*, 2009, Kirilly *et al.*, 2011). PTP52F regulates larval midgut PCD through dephosphorylation of the transitional endoplasmic reticulum ATPase, TER94, and deletion or ablation of *Ptp52F* delays midgut PCD (Santhanam *et al.*, 2014). This event causes ubiquitin-dependent degradation of many proteins including Diap1 to enhance both autophagy and apoptosis. The significance of Diap1 downregulation given that midgut PCD occurs in the absence of caspase activity is yet to be determined.

Other tissues

Muscle

The abdominal muscles known as dorsal external oblique muscles (DEOMs) undergo PCD during *Drosophila* metamorphosis

with a small subset, the dorsal internal oblique muscles (DIOMs), evading this process (Kimura and Truman, 1990, Zirin *et al.*, 2013). This subset aids in the formation of the adult musculature but dies shortly after fly eclosure (Bate *et al.*, 1991, Broadie and Bate, 1991). Ecdysone-mediated apoptosis is required for DEOM PCD as knockdown of *EcR-B1* that is highly expressed in this tissue delays degradation and caspase activation (Zirin *et al.*, 2013). Autophagy is observed but is not essential for caspase activation or DEOM degradation as this occurs normally despite knockdown of key components of the autophagy pathway. In contrast, DIOMs which also highly express *EcR-B1* do not undergo ecdysone-mediated apoptosis. How apoptosis is mediated by ecdysone downstream of the receptor, and why DEOMs but not DIOMs undergo ecdysone-mediated apoptosis at this time requires further investigation.

DEOMs begin degrading at 8h APF with the majority of DEOMs degraded by 12h APF (Zirin *et al.*, 2013). The timing of DEOM cell death is regulated by the nuclear receptors βFTZ-F1 and HR39 which have opposite expression profiles in both muscle types. Knockdown of *ftz-f1* or overexpression of *Hr39* in muscles results in delayed caspase activation and persistence of DEOMs past the normal timing of removal. *ftz-f1* overexpression causes premature degradation but not caspase activity, whereas *Hr39* mutants have premature caspase activation and DEOM removal.

Nervous system

Ecdysone-mediated PCD is required to remove specific subsets of neurons during *Drosophila* metamorphosis in the ventral nervous system, peptidergic neurons that secrete the neuropeptide *Corazonin* (*Crz*)(vCrz) and RP2 motorneurons (Choi *et al.*, 2006, Winbush and Weeks, 2011). PCD of vCrz neurons requires both EcR-B isoforms but not EcR-A (Choi *et al.*, 2006). *EcR-B* mutants have persisting vCrz neurons past the normal timing of removal (6h APF), and expression of either *EcR-B1* or *EcR-B2* in an *EcR-B* mutant background rescues PCD in the majority of vCrz neurons. On the other hand, *EcR-A* mutants do not display any PCD defects consistent with lack of *EcR-A* expression in these neurons. The RP2 neurons are removed between 14-20h APF in response to the prepupal ecdysone pulse (Winbush and Weeks, 2011). Isolating abdominal portions of the ventral glia where these neurons are found before the ecdysone prepupal pulse, and culturing these in ecdysone-containing media, results in PCD. In addition, expression of a dominant negative *EcR-B1* mutant but not an *EcR-A* mutant results in persisting neurons past the normal developmental time of removal.

The optic lobe in the adult fly is responsible for sending visual information received by the eye to the brain. The developing optic lobe undergoes two stages of PCD with the first occurring during metamorphosis where the majority of cells are removed in distinct clusters by 24h APF using apoptosis (Hara *et al.*, 2013, Togane *et al.*, 2012). The time between 48 h APF and eclosion is when the second round of cell death occurs removing a small number of cells. EcR isoforms -A and -B1 are expressed in a cell-specific and temporal manner throughout the developing optic lobe (Hara *et al.*, 2013). EcR-B1 but not EcR-A is required for PCD of the optic lobe after 24h APF however, PCD of the optic lobe occurs normally in *EcR-B1* and *EcR-A* mutants at an earlier time of PCD. This indicates that PCD at this stage may be independent of ecdysone.

Another interesting requirement of ecdysone is for the transdifferentiation of Rhodopsin 5-photoreceptors (Rh5-PRs) into Rh6-PRs in the larval eye during development of the adult *Drosophila* eyelet (Sprecher and Desplan, 2008). The larval eye is composed of four Rh5-PRs and eight Rh6-PRs, the former switching to Rh6 and the latter removed by apoptosis during the early stages of metamorphosis (Sprecher *et al.*, 2007). Deletion or ablation of *EcR* prevents apoptosis of Rh6-PRs, and the binary switch between expression of Rh5 to Rh6 is also prevented with expression of mutant *EcR* (Sprecher and Desplan, 2008).

Fat body and neuronal remodelling

Although not strictly a cell death process, ecdysone is required for remodelling during *Drosophila* development in the fat body and nervous system. The *Drosophila* larval fat body is the equivalent of the mammalian liver and adipose tissue, functioning to store nutrients and generate energy. During metamorphosis, the fat body is extensively remodelled dissociating from a single layer of cells to become individual cells which are then eliminated during early adulthood (Aguila *et al.*, 2007, Nelliot *et al.*, 2006). As well as undergoing autophagy in response to starvation, the *Drosophila* fat body undergoes ecdysone-mediated autophagy during the transition from larval to pupal development via downregulation of the PI3K pathway (Lindmo *et al.*, 2006, Liu *et al.*, 2013, Rusten *et al.*, 2004, Scott *et al.*, 2004). This is also accompanied by an ecdysone-dependent increase in caspase activity and apoptotic gene transcription but not other classical features of apoptosis such as nuclear membrane breakdown and chromatin condensation (Liu *et al.*, 2013). In fact, caspase activity is enhanced in the fat body when autophagy is inhibited and vice versa indicating the balance between these two activities is important for fat body remodelling. In the fat body knockdown of *Br-C* reduces caspase activity but has no effect on autophagy, *E74* knockdown inhibits autophagy and increases caspase activity, and both are reduced upon *E93* knockdown (Liu *et al.*, 2014). An important regulator of ecdysone-mediated autophagy in the fat body is the RING finger protein Deep Orange (Dor) (Lindmo *et al.*, 2006). Dor is required not only to indirectly induce the level of ecdysone to a threshold so that PI3K signalling is downregulated, but also for the fusion between autophagosomes and lysosomes.

Neuronal pruning is an important process for the development of the *Drosophila* adult nervous system that selectively rids a neuron of its processes without inducing cell death of the neuron itself (Truman, 1990). This occurs in certain classes of the dendritic arborisation (da) sensory neurons in the peripheral nervous system and mushroom body γ neurons in the central nervous system (Kirilly *et al.*, 2009, Kuo *et al.*, 2005, Lee *et al.*, 1999, Williams and Truman, 2005, Zhu *et al.*, 2003). Class IV (ddaC) neurons undergo extensive dendrite pruning and then develop new dendrites that give rise to the adult nervous system before eclosion (Kuo *et al.*, 2005, Satoh *et al.*, 2012, Williams and Truman, 2005). This initially involves severing of the proximal dendrite at 6h APF with the severed dendrites subsequently fragmented at 10-12h APF and the remaining debris removed by phagocytosis at 16-18h APF (Kirilly *et al.*, 2009). The pruning of mushroom body γ neurons, both dendrites and axons, begins around 4 and 8h APF respectively and are completely removed by 18h APF (Lee *et al.*, 1999, Zhu *et al.*, 2003). The axons are then regrown a little later in development.

These pruning events are initiated by the prepupal ecdysone pulse. In ddaC neurons, this activates both EcR and USP to transcriptionally upregulate *sox14* and *headcase* (*hdc*), both of

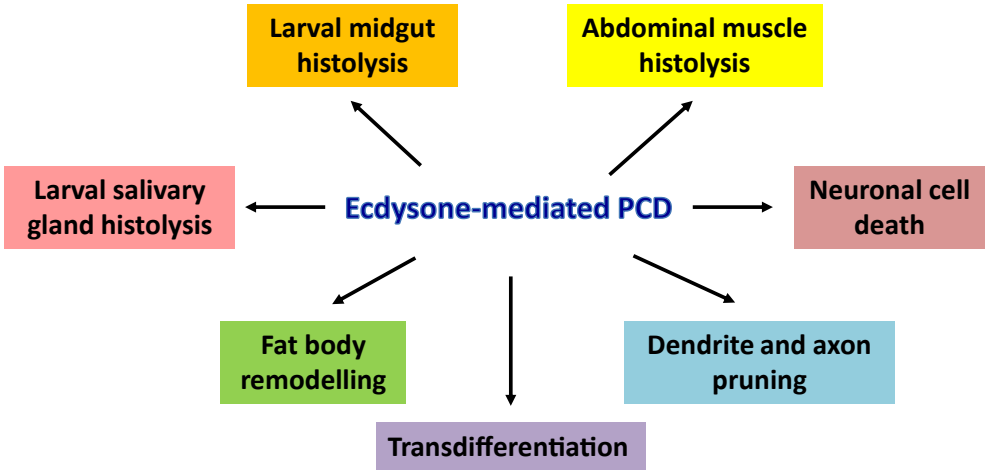

Fig. 3. Various functions of ecdysone-mediated PCD in *Drosophila*. *Ecdysone-mediated PCD is vital for histolysis of the larval salivary glands, midgut and abdominal muscles. It also functions to regulate the death of certain neurons, transdifferentiation of larval eye photoreceptors, and remodelling of the larval fat body and nervous system through dendrite and axon pruning.*

which are ecdysone-responsive genes and required for dendrite pruning (Kirilly *et al.*, 2009, Loncle and Williams, 2012). Sox14 in turn directly activates *mical*, a cytoskeletal regulator required for dendrite pruning through an unknown mechanism (Beuchle *et al.*, 2007, Kirilly *et al.*, 2009, Suzuki *et al.*, 2002, Terman *et al.*, 2002). Similarly, an unknown function of Hdc is needed for dendrite pruning and appears to be an ecdysone-responsive gene independent of the transcriptional role of Sox14 (Loncle and Williams, 2012). γ neurons in which EcR-B1 is absent do not undergo pruning however the mechanism by which EcR-B1 acts and its downstream targets for this process are undetermined (T. Lee *et al.*, 2000).

Adult ovary

During development of the adult ovary, PCD occurs periodically in the germarium and throughout the mid-stages (Pritchett *et al.*, 2009). This PCD is dependent on nutrient availability and involves ecdysone in mid-stage egg chambers (Pritchett *et al.*, 2009). Much of the cell death in *Drosophila* ovaries is mediated by non-canonical pathways that do not require RHG proteins (Peterson *et al.*, 2007). Interestingly, similar to the larval salivary glands, starvation-induced cell death in the ovary is dependent on both apoptosis and autophagy (Hou *et al.*, 2008, Nezis *et al.*, 2009). Increased levels of ecdysone accompany nutrient deprivation and control both cell death and cell survival, via an apparently complex interplay involving the ecdysone-induced transcription factors BR-C, E74 and E75 (Buszczak *et al.*, 1999). The exact mechanism of this type of cell death remains unknown.

Epigenetic regulation of ecdysone-mediated PCD

One of the main questions that remains is how ecdysone mediates the spatio-temporal control of PCD. As summarised below, emerging evidence suggests that, at least in part, this control may be mediated via epigenetic regulators.

The interaction of the arginine methyltransferase CARMER (Coactivator Arg Methyltransferase for EcR/USP) with EcR/USP is important for the regulation of apoptosis in response to ecdysone (Cakouros *et al.*, 2004b). Expression of CARMER in cells increases cell death in response to ecdysone treatment, and it is also important for the transcriptional activation of ecdysone-regulated apoptosis genes such as the caspases *drice*, *dcp-1* and *dronc*, the adaptor *dark* and the IAP antagonists *rpr* and *hid*. The role of CARMER in cell death is specific to ecdysone-induced apoptosis as knockdown of *carmer* in cells does not affect cycloheximide-induced apoptosis. The *Drosophila* lysine ketoglutarate reductase/saccharopine dehydrogenase (dLKR/SDH) is an important corepressor of EcR/USP that acts to repress the function of CARMER to ensure cell death genes are transcribed at the appropriate developmental time (Cakouros *et al.*, 2008).

Additional regulation of ecdysone-mediated salivary gland PCD, comes from a histone-modifying enzyme (Denton *et al.*, 2013b). During larval salivary gland PCD, the *Drosophila* H3K27me3 demethylase UTX (dUTX) interacts with EcR/USP to coordinately regulate the transcription of key apoptosis and autophagy genes by demethylation of H3K27me3, a histone modification associated with inactively transcribed chromatin (Kooistra and Helin, 2012). The salivary glands of *dUTX* mutant animals fail to degrade by the normal developmental time and display a reduction in both caspase activity and autophagy. This corresponds with reduced transcription of several apoptosis and autophagy genes, and an enrichment of H3K27me3. dUTX is also required for regulating the transcription of the primary response gene *E93* but not *Br-C* in response to ecdysone.

Transcriptional activation of *sox14* by EcR-B1 in ddaC neurons is regulated by the interaction of EcR-B1 and CBP facilitated by the chromatin remodelling factor Brahma (Brm) (Kirilly *et al.*, 2011). CBP forms a complex with EcR-B1 to transcriptionally activate *sox14* through the histone acetyltransferase activity of CBP, specifically by enrichment of H3K27Ac. EcR/CBP complex formation is facilitated by Brm as knockdown of *brm* results in a significant decrease in the formation of this complex.

Conclusions

In conclusion, we have summarised the role of ecdysone in mediating and regulating cell death in *Drosophila* (Fig. 3). From the discussion presented here it is apparent that the use of *Drosophila* as a model has provided remarkable insights into the mechanisms and functions of hormone-regulated PCD. It is interesting to note that, in the salivary glands and midgut, where autophagy plays a critical role in the removal of larval tissues, ecdysone controls the expression of both apoptosis and autophagy genes. It is possible that similar regulation exists in other tissues, such as the adult ovaries, where starvation-induced cell death is accompanied by

increased ecdysone levels and autophagy. From emerging data it appears that epigenetic modifiers, such as histone methyltransferases and demethylases, through interaction with EcR, regulate both the timing and tissue specificity of cell death. It is likely that similar control mechanisms also operate in mammals where coordinate regulation of hormone-induced genes may be necessary for specific cellular outcomes.

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