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A steroid-controlled global switch in sensitivity to apoptosis during *Drosophila* development

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Abstract

Precise control over activation of the apoptotic machinery is critical for development, tissue homeostasis and disease. In *Drosophila*, the decision to trigger apoptosis--whether in response to developmental cues or to DNA damage--converges on transcription of inhibitor of apoptosis protein (IAP) antagonists *reaper*, *hid* and *grim*. Here we describe a parallel process that regulates the sensitivity to, rather than the execution of, apoptosis. This process establishes developmental windows that are permissive or restrictive for triggering apoptosis, where the status of cells determines their capacity to die. We characterize one switch in the sensitivity to apoptotic triggers, from restrictive to permissive, that occurs during third-instar larval (L3) development. Early L3 animals are highly resistant to induction of apoptosis by expression of IAP-antagonists, DNA-damaging agents and even knockdown of the IAP *diap1*. This resistance to apoptosis, however, is lost in wandering L3 animals after acquiring a heightened sensitivity to apoptotic triggers. This switch in sensitivity to death activators is mediated by a change in mechanisms available for activating endogenous caspases, from an apoptosome-independent to an apoptosome-dependent pathway. This switch in apoptotic pathways is regulated in a cell-autonomous manner by the steroid hormone ecdysone, through changes in expression of critical pro-, but not anti-, apoptotic genes. This steroid-controlled switch defines a novel, physiologically-regulated, mechanism for controlling sensitivity to apoptosis and provides new insights into the control of apoptosis during development.

Keywords

apoptosis; apoptosome-dependent; apoptosome-independent; ecdysone; mid-L3 transition; sensitivity to apoptosis

INTRODUCTION

The ability to commit suicide by apoptosis is a fundamental, and irreversible, cellular process in multicellular organisms. Accordingly, normal development and homeostasis depend on precise control over when and where apoptosis occurs. Apoptosis is regulated by

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a signaling cascade of cysteine proteases called caspases (Fuentes-Prior and Salvesen, 2004). Initiator caspases sit at the top of the signaling cascade; they exist as monomers and are activated by dimerization on specialized signaling platforms (Bratton and Salvesen, 2010). One such signaling platform, the apoptosome, is composed of oligomers of the caspase adaptor Apaf-1 and the initiator caspase Caspase-9 (Bratton and Salvesen, 2010). Unlike initiator caspases, effector caspases exist as inactive dimers and are activated by caspase-dependent proteolytic cleavage (Riedl and Shi, 2004). Although caspases are expressed in all cells, their inappropriate activation is prevented by Inhibitor of Apoptosis Proteins (IAPs) (Orme and Meier, 2009; Salvesen and Duckett, 2002).

Activation of apoptosis during development is regulated by relieving the IAP-dependent inhibition of caspases. This mechanism is best elucidated in *Drosophila* where the activation of caspases converges on transcription of the IAP-antagonists *reaper*, *hid* and/or *grim* (Steller, 2008). These IAP-antagonists bind to Diap1 (*Drosophila* IAP 1), disrupting its interaction with caspases and initiating caspase activation and apoptosis (Ryoo et al., 2002; Yoo et al., 2002). Elimination of all three IAP-antagonists blocks apoptosis, while ectopic expression triggers apoptosis (Chen et al., 1996; Grether et al., 1995; White et al., 1994; 1996). Similarly, loss of *diap1* has been shown to result in immediate activation of apoptosis in embryos (Goyal et al., 2000; Lisi et al., 2000; Wang et al., 1999). The mammalian death activator *Smac/Diablo* acts as an IAP-antagonist, inhibiting IAPs such as Survivin and XIAP, demonstrating that this pathway has been conserved through evolution (LaCasse et al., 2008).

Apoptosis is executed when the rapidly expanding cascade of caspase activation crosses a critical threshold; cells that do not cross this apoptotic threshold do not initiate apoptosis (Thompson, 1995). In turn, the ability to achieve this apoptotic threshold is predetermined by the endogenous expression levels of critical pro- and anti-apoptotic factors (Florentin and Arama, 2012; Lowe et al., 2004). This threshold model for activation of apoptosis was formulated primarily through characterization of oncogenic mutations and changes in gene expression observed in tumor cells, changes that disable the ability to trigger apoptosis in malignant cells (Adams and Cory, 2007; Lowe et al., 2004). It is now well established that the ability to evade apoptosis is a hallmark of cancer (Hanahan and Weinberg, 2011). However, physiological contexts for changes in the ability to trigger apoptosis and the mechanisms that regulate them remain poorly understood. Here, we characterize a dramatic and global switch in the sensitivity to apoptosis during *Drosophila* development at the onset of metamorphosis and demonstrate that this switch, mediated by changes in expression of critical pro-apoptotic genes, is regulated by the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone).

MATERIALS AND METHODS

Stocks

The following stocks were obtained from the Bloomington *Drosophila* Stock Center: *hs-Gal4*, *UAS-rpr*, *en-Gal4,UAS-RFP*, *Sgs3-Gal4*, *Sgs3-GFP*, *nub-Gal4*, *UAS-GFP*, *UAS-EcR^{F645A}* and *Nc^{51}*. The following stocks were kindly provided by colleagues in the fly community: *hs-rpr* (White et al., 1996), *hs-hid* (Grether et al., 1995), *hs-diap1 RNAi* (Yin and Thummel, 2004), *Ark^{82}* (Akdemir et al., 2006) and *drice$^{\Delta 1}$* (Muro et al., 2006).

Developmental staging

Early and wandering third instar larvae (eL3 and wL3, respectively) were identified by developmental age, wandering behavior and expression of a mid-L3-specific reporter (Sgs3-GFP)(Biyasheva et al., 2001). To collect eL3 animals, embryos were aged at 25°C about 76–

88 hours after egg lay (AEL) and Sgs3-GFP-expressing larvae, if any, were removed. The wL3 animals were collected as rapidly wandering larvae from the sides of un-crowded bottles with robust expression of Sgs3-GFP (these animals are within 10 hours from puparium formation). Sugar feeding experiments were performed at 25°C on agar plates with 20% sucrose and without yeast.

Delivery of apoptotic activators and survival assays

To trigger apoptosis in a temporally-controlled manner, we used transgenic lines with death activators directly fused to the *hsp70* heat-shock promoter (e.g., *hs-rpr* and *hs-diap1-RNAi*) or exposure to DNA-damaging UV-C light. Appropriately staged animals were heat-shocked for 30 minutes (unless otherwise noted) by submerging Parafilm-sealed grape agar plates in a water bath at the desired temperature; after heat-shock, animals were transferred to 25°C on grape agar plates with a thin layer of yeast. Exposure to UV-C light was done inside a Stratalinker 1800 (Stratagene) equipped with a UV-C bulb. Larvae were washed with distilled water, transferred as a single layer onto an empty petri dish and exposed to the appropriate dose of UV-C light. The larvae were then moved to grape agar plates with a thin layer of yeast and allowed to recover at 25°C. Death was assessed by touch response in larvae, heartbeat in prepupae, ability to head evert properly in pupae and by eclosion into adult flies.

Immunofluorescence

Tissues dissected from appropriately staged larvae were fixed and immunostained using standard methods (Yin et al., 2007). Staining for caspase activity was performed using an antibody raised against cleaved human Caspase-3 (rabbit α-cleaved caspase-3, Cell Signaling; used at 1:200 dilution), that detects activity of the *Drosophila* Caspase-9 homolog, *Nc* (Fan and Bergmann, 2010). Other primary antibodies used were mouse α-elav (1:50, Developmental Studies Hybridoma Bank), mouse α-Diap1 (1:200, gift from B. Hay), rabbit α-Nc and α-Drice (1:200, gift from P. Friesen). Secondary antibodies used were Cy3 α-rabbit (1:200, Jackson Immuno-Research Labs), AlexaFluor 488 α-mouse and α-rabbit (1:200, Invitrogen) and AlexaFluor 633 α-mouse and α-rabbit (1:200, Invitrogen). Images were taken on an Olympus FluoView FV1000 confocal microscope and optimized with the FV10-ASW software.

Quantitative RT-PCR

To measure mRNA expression levels of target genes, we used quantitative real time PCR (qPCR) with standard methods as previously described (Ihry et al., 2012). qPCR was performed using a Roche 480 LightCycler with the LightCycler 480 DNA SYBR Green I Master kit (Roche). Samples on the same graph were run simultaneously with three independent biological samples for each target gene (primer sequences shown in Table S2) and *rp49* was used as the reference gene. Relative Expression Software Tool (REST) (Pfaffl et al., 2002) was used to calculate changes in relative expression.

Western Blotting

To measure protein expression levels, we performed western blots using standard methods as previously described (Ihry et al., 2012). For whole animal lysates, eL3 or wL3 animals were homogenized in 75 μl of hi-salt lysis buffer. Primary antibodies used were rabbit α-DIAP1 (1:1,000, gift from B. Hay), rabbit α-Drice (1:5,000, gift from P. Friesen), guinea pig α-Nc (1:1,000, gift from H. Steller), α-β-tubulin (1:1,000, Millipore) and α-β-actin (1:1,000, Cell Signaling). Secondary antibodies used were alkaline phosphatase conjugated goat α-rabbit IgG (1:30,000, GE Healthcare), α-guinea pig and α-mouse IgG (1:30,000, Sigma). Membranes were developed for imaging with ECF substrate (GE Healthcare) and were

imaged using a Storm 840 Scanner (Amersham Bioscience) with ImageQuant TL software version 7.0 (GE Healthcare).

RESULTS AND DISCUSSION

Global changes in sensitivity to death activators define permissive and restrictive windows for apoptosis during development

To measure changes in the sensitivity to apoptotic triggers during development, we calibrated a heat-controlled promoter expressing the IAP-antagonist *reaper* (*hs-rpr*)(White et al., 1996). This *hs-rpr* transgene delivered ubiquitous and highly-reproducible bursts of *reaper* expression that lasted about two hours (Fig. 1A). The dose of *reaper* was controlled by adjusting the temperature of the heat treatment and the number of transgenes used (Fig. 1B). Our standard protocol, unless otherwise stated, is a 30 minute heat-shock at 37°C with one copy of *hs-rpr*; this treatment delivers ~120-fold induction of *reaper* and represents ten times the minimal lethal dose (Table S1). By comparison, an endogenous death response in larval tissues during metamorphosis generates a ~50-fold induction of *reaper* (Ihry et al., 2012).

The original description of the *hs-rpr* transgene reported that two developmental stages--the late embryo and the late pupae--were not sensitive to killing by *reaper* (White et al., 1996). We have extended that analysis to the entire fly life cycle and demonstrate that resistance to *reaper* is the norm, not the exception. Most stages during larval and pupal development appear to be highly resistant to *reaper* (Fig. 1C). In fact, sensitivity to killing by *reaper* appears to be primarily restricted to embryogenesis and to the larval-to-pupal transition when wandering larvae transform into immature pupae. These two developmental windows of sensitivity to *reaper* show a striking overlap with stages where most, if not all, programmed cell death occurs during the fly life cycle (Abrams et al., 1993; Wolff and Ready, 1991). Thus, the sensitivity to apoptosis appears to be regulated globally and may play an important role in restricting programmed cell death to discrete stages during development.

Animals within restrictive windows for apoptosis are 50 times more resistant to death activators and can resist knockdown of *diap1*

To understand the changes in sensitivity to apoptosis, we focused on the responses to *reaper* during L3 development. L3 development starts with the L2-L3 molt and ends about two days later with puparium formation and the onset of metamorphosis. The changes in sensitivity to apoptosis start half-way through L3 development and coincide with glue protein synthesis in salivary glands, a molecular marker of the mid-L3 transition (Andres and P. Cherbas, 1992; Andres et al., 1993). The mid-L3 transition, however, is strongly influenced by environmental conditions like crowding and nutrition, generating significant developmental asynchrony among individuals of the same age. To avoid this heterogeneity, we selected L3 animals well before and well after the mid-L3 transition: “early L3” (eL3) and “wandering L3” (wL3), respectively (Fig. 2A; see methods for details). These two populations of larvae have dramatically different sensitivities to *reaper*. Expression of *reaper* in wL3 kills animals within hours (Fig. S1A). In contrast, most *reaper*-treated eL3 animals, despite a comparable dose of *reaper* expression (Fig. S1B), eclose as adult flies (Fig. 2B). Heat-induced expression of *hid* gave similar results (Fig. 2B). The *hs-hid* transgene was not used extensively in this study because leaky expression of *hid* complicated phenotypic analysis (Fig. S1C). Consistent with the strong resistance to apoptosis in whole animals, expression of *reaper* does not initiate caspase activation in eL3 tissues. Staining wing imaginal discs with antibodies that detect caspase activity showed that *reaper* triggers caspase activation in wL3, but not in eL3, animals (Fig. 2C–F).

To measure the difference in sensitivity to apoptotic activators, we tested increasing doses of death activators for their ability to kill eL3 animals. The resulting dose response curves demonstrate that while wL3 animals are very sensitive to *reaper*, eL3 animals require 50 times higher doses to trigger lethality (eL3 LD_{50}=400 and wL3 LD_{50}=7; Fig. 2G). Surprisingly, similar dose response curves were observed with UV-C light (Fig. 2H), a known DNA-damaging agent (Zhou and Steller, 2003), demonstrating that the switch in sensitivity to apoptosis is not limited to expression of IAP-antagonists.

Given that *diap1* is thought to be essential for preventing activation of apoptosis, we tested if eL3 animals could resist knockdown of *diap1*. Expression of *diap1*-RNAi triggered caspase activation in wL3 tissues, shown by staining for activated caspases in ventral nerve cords dissected from RNAi-treated wL3 animals (Fig. 3C,D). However, caspase activation was not detected in ventral nerve cords dissected from RNAi-treated eL3 animals (Fig. 3A,B). In fact, knockdown of *diap1* in wL3 kills every animal within 24 hours while a similar treatment has little effect on eL3, with most animals eclosing as adults (Fig. 3E). Although the trace amounts of Diap1 protein remaining in RNAi-treated animals may be sufficient to block apoptosis in eL3 tissues (Fig. S1D–H), simultaneous expression of *reaper* does not dramatically alter the observed resistance (Fig. 3E). Thus, our data suggests that the resistance to apoptotic triggers in eL3 animals is mediated downstream of the *diap1*-dependent inhibition of apoptosis.

These results define a whole animal developmental context for understanding the mechanisms that control global changes in sensitivity to apoptosis. The unexpected resistance to a strong knockdown of Diap1 protein levels further demonstrate that expression of IAP-antagonists during development (i.e., “execution”) is not always sufficient to trigger apoptosis. Thus, our data suggests that apoptosis during development is regulated by two parallel processes whereby cells need to be “primed,” by increasing their sensitivity to apoptosis, before they can be “executed.”

The change in the sensitivity to death activators reflects a switch from apoptosome-independent to apoptosome-dependent execution of apoptosis

The striking difference in sensitivity to apoptosis between eL3 and wL3 animals prompted us to ask whether these two stages used different mechanisms to execute apoptosis. To our surprise, at the very high doses of *reaper* and UV-C light required to kill eL3 animals, the ensuing lethality occurs equally well in eL3 animals without functional *Ark* or *Nc* (orthologs of mammalian *Apaf-1* and *caspase-9*, respectively; Fig. 4A). Importantly, this *Ark*- and *Nc*-independent death response requires the effector caspase *drice* (ortholog of mammalian *caspase-3*; Fig. 4A), suggesting that *drice* is activated in an apoptosome-independent manner in eL3 animals. In wL3 animals, however, the death response requires *drice* and apoptosome components like *Nc* (Fig. 4B), highlighting a switch in the apoptotic pathways that execute apoptosis at these two stages.

To characterize the biochemical effect of this switch in apoptotic pathways, we examined the proteolytic activation of the Drice zymogen (p37). Cleavage of effector caspases like Drice define the point-of-no-return for apoptosis (Riedl and Salvesen, 2007). In wL3 animals, the large cleaved Drice product (p20) is observed within an hour after *reaper* treatment on western blots and this processing is *Nc*-dependent (lanes 6–8, Fig. 4C). In contrast, only negligible amounts of the activated Drice product is observed under the same conditions in eL3 animals (lanes 2–3; Fig. 4C). This difference in the processing of Drice results from a change in the kinetics of proteolytic activation. In response to the same extreme apoptotic stimuli, wL3 animals activate Drice within 15 minutes whereas it takes 120 minutes to process Drice in eL3 animals (Fig. 4D). In addition to approximately an order of magnitude increase in the rate of Drice cleavage, the fraction of pro-Drice that is

proteolytically activated is also considerably higher in wL3 animals (Fig. S2). Thus, the switch to an apoptosome-dependent pathway in wL3 animals confers a faster (increased rate) and higher capacity (increased efficacy) activation of Drice. In turn, these changes in kinetics of caspase activation translate into dramatic differences in the sensitivity to apoptotic triggers.

To understand how the switch in the ability to execute apoptosis through the apoptosome is regulated, we measured the expression levels of key pro- and anti-apoptotic genes at these stages. Our results indicate that endogenous mRNA levels, as measured by qPCR, of at least three critical regulators of apoptosis--the apoptosome components *Ark* and *Nc* and the effector caspase *drice*--are several fold higher in wL3 animals (Fig. 5A). On the other hand, endogenous mRNA levels of *diap1* and the caspases *dredd* and *dcp-1* do not change (Fig. 5A). Consistently, western blots of total protein extracted from eL3 and wL3 animals showed higher levels of Nc and Drice proteins in wL3, while Diap1 protein levels appear not to change (Fig. 5B). To demonstrate that changes in expression levels of three pro-apoptotic genes are sufficient to explain the dramatic switch in sensitivity to apoptosis, we examined the response to *reaper* in wL3 animals heterozygous for null mutations in *Ark, Nc* and *drice*. Animals carrying a single allele of any one of these mutations did not survive expression of *reaper*. Triple heterozygous wL3 animals, however, showed a striking resistance to *reaper*: 90% of *reaper*-treated animals survived 24 hours and nearly 65% eclosed as adults (Fig. 5C). Thus, the changes in endogenous levels of these three pro-apoptotic genes are sufficient to explain the switch in sensitivity to apoptosis.

Taken together, our results demonstrate that stage-specific, coordinately-regulated changes in gene expression of key pro-apoptotic genes, determine the ability to execute apoptosis through the apoptosome, thereby controlling the rate and efficacy of caspase activation and, as a result, establish windows during development that are either permissive or restrictive for apoptosis.

Single-cell quantitative studies of apoptosis have suggested that relatively minor changes in the endogenous levels of apoptotic regulators, even among cells within a clonal population, can change the outcome to uniform apoptotic stimuli (Spencer et al., 2009). Moreover, reduced expression of individual pro-apoptotic genes like *Apaf-1* has been described in many cancers (Dai et al., 2004; Fu et al., 2003; Soengas et al., 2001; Zlobec et al., 2007); although a causal relationship between changes in expression of pro-apoptotic genes and reduced sensitivity to apoptosis are difficult to establish from these studies because tumor cells often carry many mutations. Our results, however, demonstrate that a two-fold reduction in levels of three pro-apoptotic genes can effectively neutralize apoptotic outcomes in response to death activators. Our results also place more importance on the canonical apoptosome-dependent pathway for execution of cell death during development. Consistent with these results, most, but not all, developmentally-triggered apoptosis are disrupted in *Ark* and *Nc* mutant embryos (Mills et al., 2006; Xu et al., 2005). The mechanisms that regulate the apoptosome-independent activation of apoptosis, however, remain poorly characterized.

The switch in sensitivity to apoptosis is regulated by ecdysone in a cell-autonomous manner

To identify regulators of the switch in sensitivity to apoptosis, we examined the role of ecdysone and the mid-L3 transition. The mid-L3 transition can be disrupted by transferring eL3 animals to sugar-only food (Britton and Edgar, 1998), arresting the progression of L3 development until a complete food source is re-supplied. These sugar-fed eL3-arrested animals remained resistant to apoptosis while their normal-fed counterparts switched their sensitivity to apoptosis (Fig. 6A). Accordingly, expression levels of core death genes

increased in normal-fed but not in sugar-fed eL3 animals (Fig. 6C). These results demonstrate that the switch in sensitivity to apoptosis during L3 development occurs during the mid-L3 transition. In turn, the mid-L3 transition coincides with a low titer systemic pulse of ecdysone (Warren et al., 2006). Although the role of ecdysone in the mid-L3 transition is not well understood, ecdysone is known to regulate expression of *Nc* and *drice* at other stages during development (Dorstyn et al., 1999; Kilpatrick et al., 2005). To examine the role of ecdysone in the switch in sensitivity to apoptosis during the mid-L3 transition, we used a dominant negative form of the ecdysone receptor *EcR* (EcR^{F645A})(L. Cherbas et al., 2003). eL3 animals treated with ubiquitous expression of EcR^{F645A} and aged for 24 hours in normal food remained highly resistant to apoptotic triggers (Fig. 6B). Moreover, this continued resistance to apoptosis was accompanied by a failure to increase expression of core death genes *Ark*, *Nc* and *drice* (Fig. 6C).

Strikingly, the effects of ecdysone on the switch in sensitivity to apoptosis were cell-autonomous and therefore independent of the global mid-L3 transition. Expression of EcR^{F645A} in the pouch region of the wing imaginal disc with the *nub-Gal4* driver, allowed animals to proceed through the mid-L3 transition. Importantly, however, EcR^{F645A}-expressing cells in the wing pouch did not activate caspases in response to ubiquitous *reaper*, while the region around the wing pouch did (Fig. 6D–F). Moreover, the resistance to caspase activation was accompanied by a reduction in levels of Nc and Drice but not of Diap1 proteins, as observed by staining with antibodies directed to these core death regulators (Fig. 6G–I). The cell-autonomous changes in expression of pro-apoptotic genes, parallel the changes observed in whole animals and provide a unique molecular signature to the ecdysone-regulated switch in sensitivity to apoptosis during L3 development.

In *Drosophila*, genetic manipulations of the *hippo* and *Jak/Stat* signaling pathways can alter the sensitivity to apoptosis through changes in the expression levels of critical apoptotic regulators. In fact, both of these pathways regulate transcription of *diap1* (Betz et al., 2008; Halder and Johnson, 2011; Staley and Irvine, 2012). The IKK-related kinase also regulates the sensitivity to apoptosis through its effects on *diap1*, controlling stability of Diap1 protein (Kuranaga et al., 2006). In contrast to these pathways, the ecdysone-controlled switch in sensitivity to apoptosis described here does not involve changes in levels of *diap1* transcripts or proteins. Thus, this ecdysone-regulated process represents a novel mechanism for regulating sensitivity to apoptosis during normal development. The effects of ecdysone are likely mediated through binding of the ecdysone nuclear receptor complex and recruitment of a unique subset of coregulators and chromatin-modifying complexes to the promoters of critical apoptotic genes. Although nuclear receptor coregulators remain poorly characterized in *Drosophila*, inappropriate function or expression of coregulators are implicated in a large number of human pathologies including endocrine-based diseases like breast, prostate and ovarian cancers (Lonard and O'Malley, 2012). Thus, our results may provide new insights into the role of steroid hormones in human disease.

Conclusions

Programmed cell death was originally described in the context of the hormonal regulation of insect and frog metamorphosis (Lockshin and Williams, 1964; Tata, 1966). Since then, the role steroid hormones have been shown to control apoptosis in many tissues during mammalian development (Evans-Storms and Cidlowski, 1995; Kiess and Gallaher, 1998). In *Drosophila*, the steroid hormone ecdysone controls the execution of apoptosis during metamorphosis by inducing expression of IAP-antagonists (Jiang et al., 2000; Yin and Thummel, 2005). The results described here define a parallel ecdysone-dependent process that regulates the sensitivity to, rather than the execution of, apoptosis. This independently controlled sensitivity to apoptosis generates permissive and restrictive windows for

apoptosis during development. Control of sensitivity to apoptosis may be used to protect essential cells or to allow IAP-antagonist-dependent initiation of non-apoptotic functions of caspases. These latter non-apoptotic roles include a growing list of functions like proliferation, differentiation, dendritic pruning and migration (Kuranaga and Miura, 2007; Lamkanfi et al., 2007; Rudrapatna et al., 2013; Yi and Yuan, 2009). Many of these non-apoptotic roles of caspases may be normally restricted to developmental stages with reduced sensitivity to apoptosis. Finally, our results highlight the importance of considering global changes in sensitivity to apoptosis when studying the regulation of cell death during development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- we define a process that controls sensitivity to, rather than execution of, apoptosis
- this generates permissive and restrictive windows for apoptosis during development
- during restrictive windows, cells fail to execute apoptosis through the apoptosome
- available pathways for apoptosis determined by levels of key pro-apoptotic genes
- this process is controlled, cell-autonomously, by the steroid hormone ecdysone

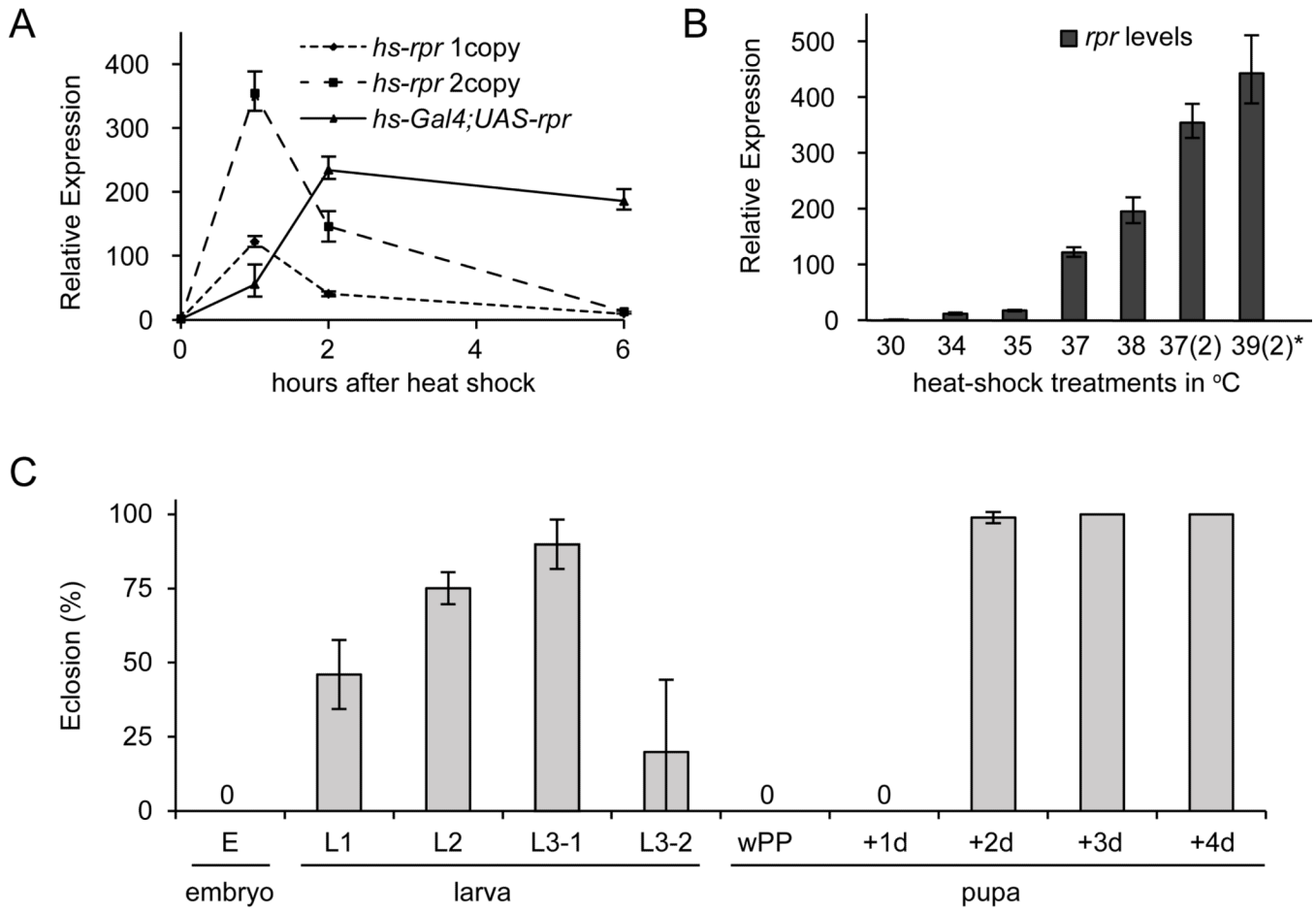

Fig. 1. Sensitivity to apoptosis is dynamically regulated during *Drosophila* development

(A) Expression of *reaper*, measured by qPCR in eL3 animals, peaks an hour after heat-shock and is back to basal levels within 6 hours. With the same heat-shock treatment (at 37°C for 30 minutes), animals carrying two copies of the *hs-rpr* transgene reach a higher peak of maximal *reaper* expression but maintain a similar temporally-restricted expression profile. On the other hand, the use of the *Gal4/UAS* binary expression system (*hs-Gal4;UAS-rpr*) results in a perduring and thus disproportionately higher cumulative dose of *reaper*. (B) The scalable nature of heat-induced expression was exploited by varying the temperature of heat-shock and the number of copies of the *hs-rpr* transgene (indicated in parenthesis). Shown are peak expressions, one hour after heat-shock. All heat-shock treatments were 30 minutes long, except one (marked by an asterisk) which was 60 minutes long. (C) Percent of animals eclosing as adults after temporally-restricted expression of *reaper* at representative stages during the life cycle. Embryos (6–18 hours after egg lay or AEL), first-instar (L1: 28–40 AEL), second-instar (L2: 52–64 AEL), third instars (L3-1: 76–88 AEL and L3-2: 100–112 AEL), newly pupariated animals (white prepupa or wPP), and various stages during metamorphosis (1–4 days after wPP). Partial lethality in L1 and L3-2 reflects additional developmental heterogeneity within these stages. Each experiment was done with three replicates of at least 20 animals each.

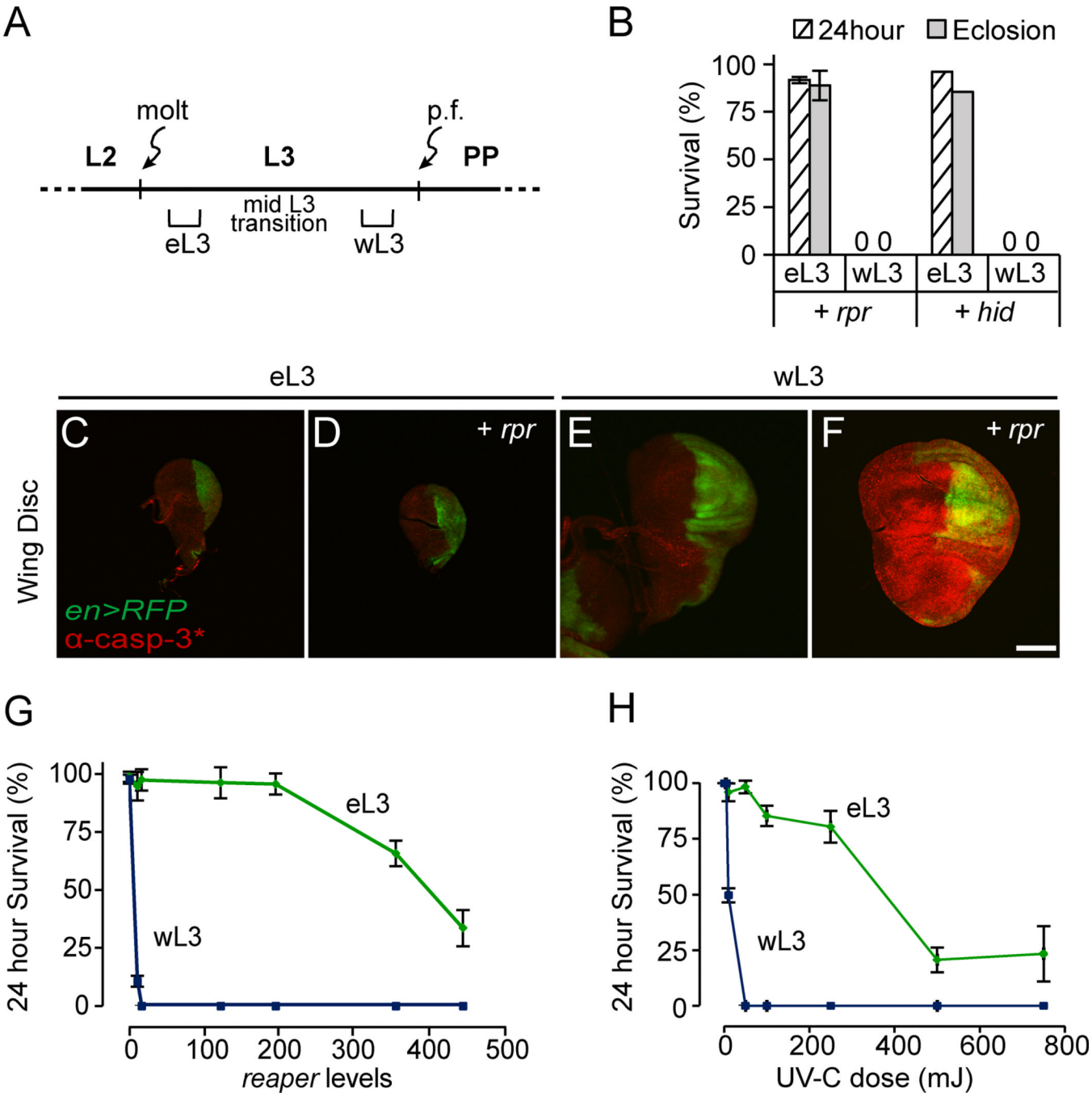

Fig. 2. Early third-instar larvae are highly resistant to expression of IAP-antagonists

(A) Developmental staging of our "early" and "wandering" third-instar larvae ("eL3" and "wL3," respectively). p.f.: puparium formation. PP: prepupae. (B) Most eL3 animals complete development after expression of *reaper* (*hs-rpr/+*) or *hid* (*hs-hid/+*). Each condition was tested in triplicate with at least 25 animals each. (C–F) Wing imaginal discs from control and *hs-rpr*-treated eL3 and wL3 animals dissected 30 minutes after heat-shock, stained with antibodies that detect caspase activity (in red; α-casp-3*: α-cleaved caspase-3); en-Gal4, UAS-RFP used as a morphological marker (RFP pseudo-colored green). Scale bars indicate 100 μM. (G–H) Dose response curves for 24 hour survival after expression of

reaper (G) or exposure to DNA-damaging UV-C light (H) in eL3 (green) or wL3 (blue) animals.

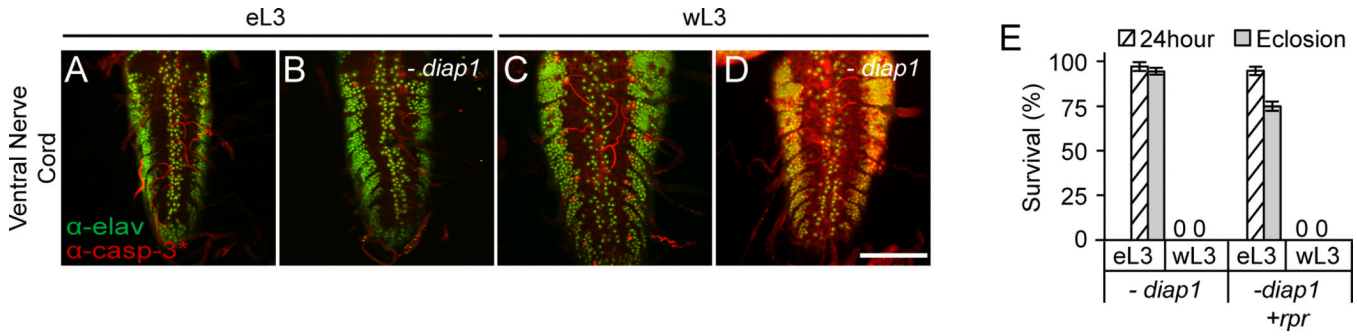

Fig. 3. Early third instar larvae are highly resistant to knockdown of *diap1*

(A–D) Staining with antibodies that detect caspase activity (in red; α-casp-3*: α-cleaved caspase-3) showing resistance to apoptotic triggers in eL3 tissues. Ventral nerve cords from control and *hs-diap1-RNAi*-treated eL3 and wL3 animals dissected two hours after heat-shock (staining with antibodies directed against elav used as a morphological marker). Scale bars indicate 100 μM. (E) Most eL3 animals complete development after knockdown of *diap1* (*hs-diap1-RNAi*), even with co-expresssion of *reaper*. Each condition was tested in triplicate with at least 25 animals each.

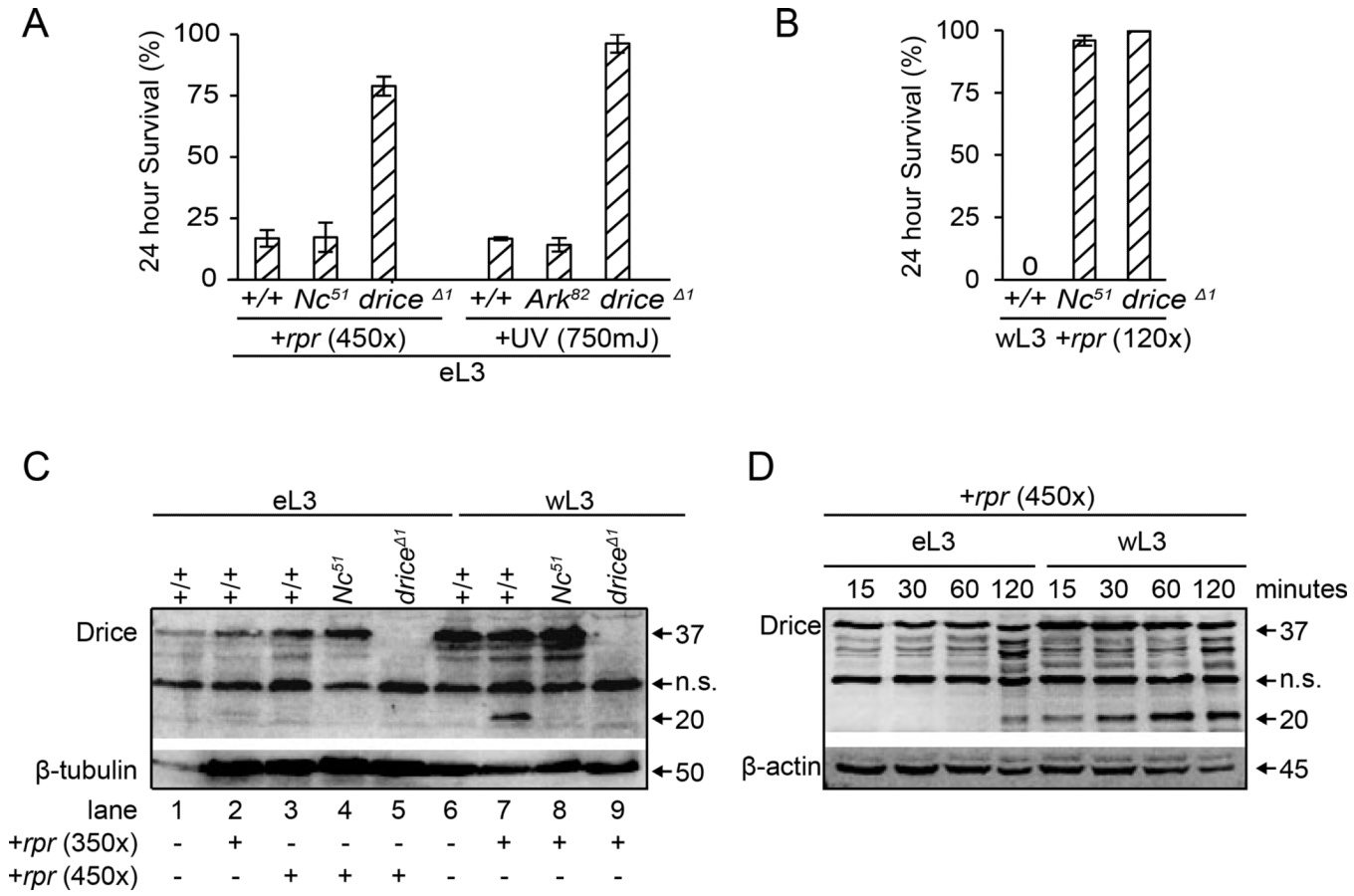

Fig. 4. eL3 and wL3 animals use different mechanisms for executing apoptosis

(A) Lethality after extreme doses of apoptotic triggers in eL3 animals is independent of the apoptosome. Control, *Ark*^{82} and *Nc*^{51} mutant eL3 animals show similar rates of lethality; however, most *drice*$^{\Delta 1}$ mutant animals survive the same treatment. (B) Lethality after expression of *reaper* in wL3 animals depends on the apoptosome. Most *Nc*^{51} and *drice*$^{\Delta 1}$ mutant wL3 animals are still alive 24 hours after our standard dose of *reaper*. Each condition was tested in triplicate with at least 20 animals each. (C) Western blot with antibodies directed to the large subunit of Drice was used to detect full-length (p37) and cleaved (p20) protein products in whole animal extracts. Extracts from eL3 or wL3 animals one hour after 350- or 450-fold expression of *reaper* (summarized below the blot) show that Drice is cleaved after reaper expression in wL3 (*cf*. lanes 6 and 7) but not in eL3 animals (*cf*. lane 1 with 2 and 3). This activation of Drice requires *Nc* activity (lane 8). (D) A time course of Drice products after 450-fold expression of *reaper* in eL3 and wL3 animals. Drice is activated within 15 minutes of *reaper* expression in wL3 animals but requires 120 minutes in eL3 animals. Antibodies to β-tubulin and β-actin used as controls. n.s.= non-specific band, present in all lanes including those from *drice* mutant animals (lanes 5 and 9 in C).

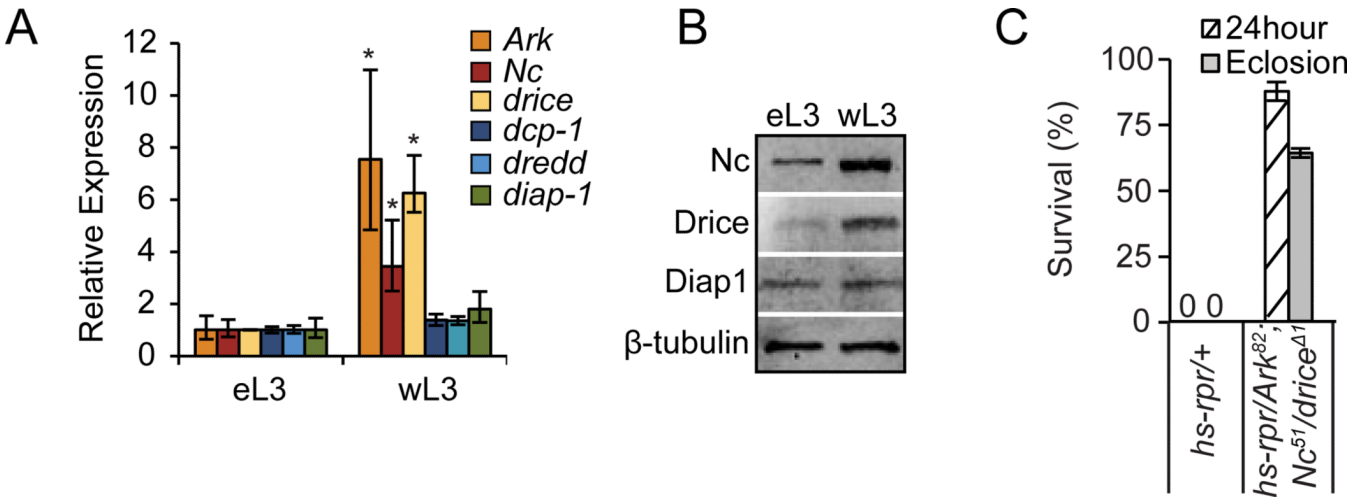

Fig. 5. Changes in expression levels of pro-apoptotic genes is sufficient to predetermine apoptotic outcome

(A) qPCR expression analysis shows that mRNA levels of *Ark*, *Nc*, and *drice* (but not *dcp-1*, *dredd* and *diap1*) significantly increase in wL3 animals. qPCR results reflect triplicate biological samples; asterisk indicates p-values <0.01. (B) Western blots show changes in protein expression of Nc and Drice; levels of Diap1 protein, however, are unchanged. β-tubulin was used as loading control. Total mRNA or protein was extracted from appropriately staged eL3 and wL3 whole animals. (C) Effects of *reaper* expression on animals heterozygous for mutations in Ark^{82}, Nc^{51} and $drice^{\Delta 1}$. All control animals die within hours after *hs-rpr* (120×) treatment while most triple heterozygous animals survive. Each condition tested in triplicate with at least 20 animals each.

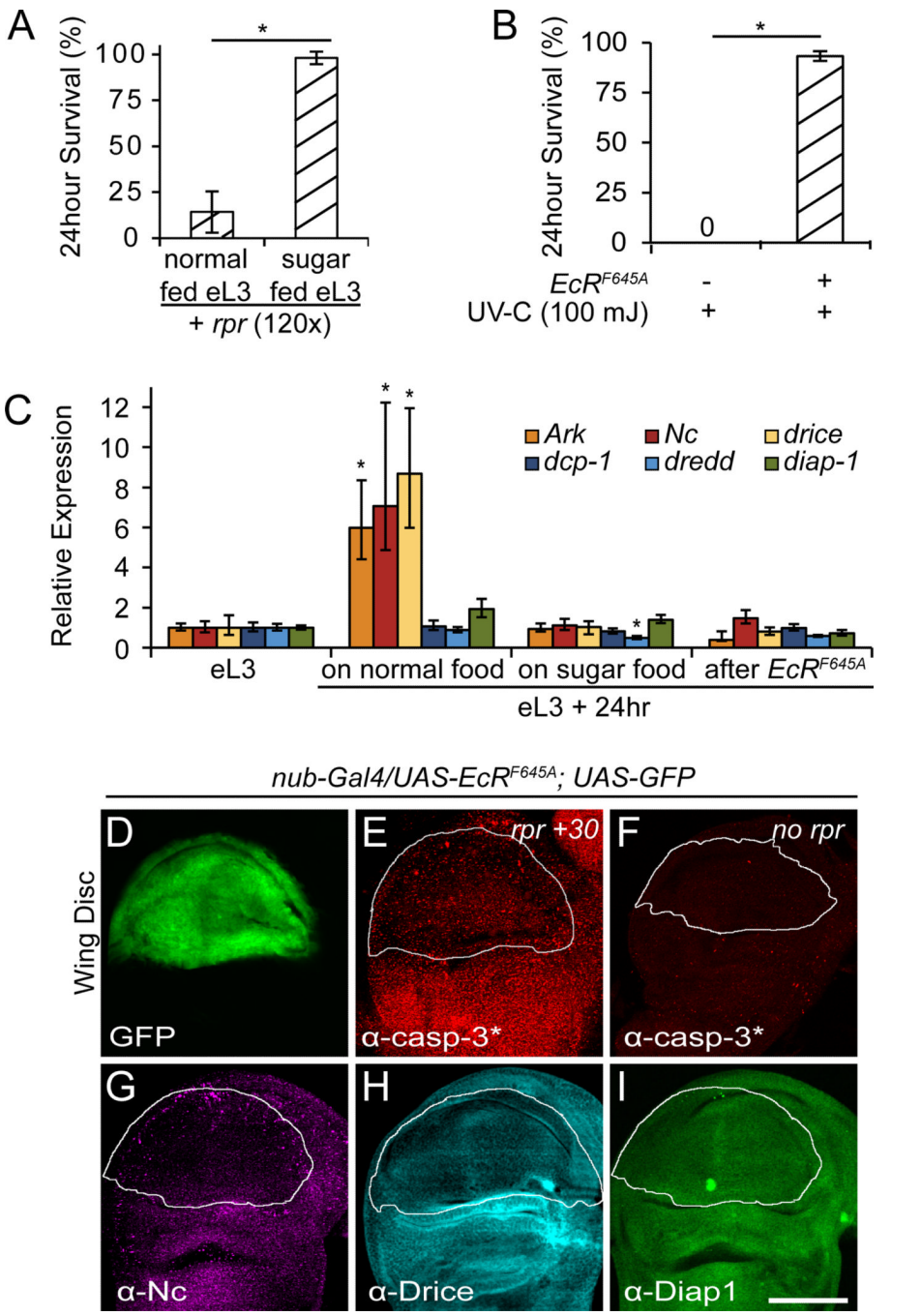

Fig. 6. The switch in sensitivity to apoptosis during L3 development is regulated by ecdysone in a cell-autonomous manner during the mid-L3 transition

(A) Preventing the mid-L3 transition by transferring eL3 animals to sugar food for 24 hours, blocks the switch in sensitivity to apoptosis. Sensitivity to apoptosis measured by 24 hour survival after exposure to ubiquitous *reaper*. (B) Ubiquitous expression of a dominant negative ecdysone receptor (EcR^{F645A}) in eL3 animals also blocks the switch in sensitivity to apoptosis. Tested by measuring 24 hour survival after exposure to UV-C light. Survival examined with three replicates of at least 20 animals for each condition. (C) Sugar-fed and EcR^{F645A}-treated eL3 animals also block the increase in expression of *Ark*, *Nc* and *drice* observed in similarly aged control siblings. All qPCR results are in triplicate; asterisks

indicate p-values <0.01. (D–I) Wing imaginal discs, dissected from wL3 animals, expressing the dominant negative ecdysone receptor in the pouch region using *nub-Gal4* (*nub-Gal4, UAS-EcR^{F645A}, UAS-GFP*). (D) Expression of GFP used to outline the pouch region. (E–F) Wing discs before and 30 minutes after ubiquitous expression of *reaper* (from *hs-rpr*), stained with antibodies that detect caspase activity (in red; α-casp-3*: α-cleaved caspase-3). Expression of *reaper* does not induce caspase activation in *EcR^{F645A}*-expressing cells. Staining with antibodies directed to Nc (E), Drice (F) and Diap1 (G) proteins showing that *EcR^{F645A}*-expressing cells have lower levels of Nc and Drice but not Diap1.