A balance between the *diap1* death inhibitor and *reaper* and *hid* death inducers controls steroid-triggered cell death in *Drosophila*

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The steroid hormone ecdysone directs the massive destruction of obsolete larval tissues during *Drosophila* metamorphosis, providing a model system for defining the molecular mechanisms of steroid-regulated programmed cell death. Although earlier studies have identified an ecdysone triggered genetic cascade that immediately precedes larval tissue cell death, no death regulatory genes have been functionally linked to this death response. We show here that ecdysone-induced expression of the death activator genes *reaper* (*rpr*) and *head involution defective* (*hid*) is required for destruction of the larval midgut and salivary glands during metamorphosis, with *hid* playing a primary role in the salivary glands and *rpr* and *hid* acting in a redundant manner in the midguts. We also identify the *Drosophila* inhibitor of apoptosis 1 as a survival factor in the larval cell death pathway, delaying death until its inhibitory effect is overcome by *rpr* and *hid*. This study reveals functional interactions between *rpr* and *hid* in *Drosophila* cell death responses and provides evidence that the precise timing of larval tissue cell death during metamorphosis is achieved through a steroid-triggered shift in the balance between the *Drosophila* inhibitor of apoptosis 1 and the *rpr* and *hid* death activators.

steroid hormone | autophagy

Small lipophilic hormones provide a critical signal for directing the appropriate patterns of programmed cell death during animal development, acting through members of the nuclear receptor family of ligand-regulated transcription factors. In frogs, thyroid hormone signals the destruction of the tadpole tail and remodeling of the intestine as the animal progresses from a juvenile to adult form (1–3). Similarly, steroid hormones regulate mammalian apoptotic pathways, including the glucocorticoid-induced apoptosis of immature thymocytes and mature T cells (4–6). In *Drosophila*, the steroid hormone ecdysone acts as a key signal to trigger the stage-specific destruction of obsolete larval tissues during metamorphosis. A high titer pulse of ecdysone at the end of larval development signals puparium formation and the destruction of the larval midgut as an adult gut forms around the dying cells (7, 8). A second ecdysone pulse, ≈10 h after puparium formation, triggers adult head eversion, marking the prepupal-to-pupal transition and signaling the rapid destruction of the larval salivary glands. Destruction of the larval midguts and salivary glands is accompanied by classic hallmarks of apoptosis, including acridine orange staining, DNA fragmentation, and caspase activation, although these larval tissues undergo a distinct form of programmed cell death referred to as autophagy, characterized by the formation of intracellular autophagic vesicles (9–11). *Drosophila* larval tissue cell death is foreshadowed by the coordinate transcriptional induction of two key death activator genes, *reaper* (*rpr*) and *head involution defective* (*hid*) (9). Ecdysone directly induces *rpr* transcription in doomed larval salivary glands (12). This effect is augmented by the ecdysone-induction of three transcription factor-encoding genes, *BR-C* (*Broad-Complex*), *E74A*, and *E93*, that, in turn, are required for appropriate *rpr* and *hid* expression and salivary gland cell death (12–14) (see Fig. 5). A similar steroid-triggered regulatory cascade is operative in doomed midgut cells, although *E74* plays no apparent role in this pathway (15).

Functions for *rpr* and *hid* are defined by *Df(3L)H99*, a chromosomal deficiency that removes these genes along with a third death activator gene, *grim*, resulting in a complete block in embryonic programmed cell death (16–18). The protein products of these genes bind to the *Drosophila* inhibitor of apoptosis 1 (DIAP1), releasing its complex with caspases and thereby initiating a proteolytic cascade that culminates in cell death (19–23). The death activators also down-regulate DIAP1 protein levels through at least two mechanisms involving a general suppression of protein synthesis (24, 25) and polyubiquitination and degradation (24–30). The mammalian death activators *Smac/Diablo* and *Omi/HtrA2* appear to act in the same manner as *rpr*, *hid*, and *grim*, inhibiting the action of death repressors, such as survivin and X-linked IAP (inhibitor of apoptosis), demonstrating that this pathway has been conserved through evolution (31–36).

Although the timing of ecdysone-induced *rpr* and *hid* expression correlates with the onset of larval midgut and salivary gland cell death, no functional studies have linked any death regulatory genes to the destruction of larval tissues during metamorphosis. *rpr* expression appears to accurately foreshadow cell death at other stages of development and is sufficient to drive programmed cell death (16, 37); however, *hid* can be expressed in cells that are fated to survive, consistent with posttranslational mechanisms for modifying Hid activity (38, 39). Similarly, no effects are seen on nurse cell apoptosis in *Df(3L)H99* germline clones despite the observation that *rpr*, *hid*, *grim*, and *diap1* are expressed in doomed nurse cells during *Drosophila* oogenesis (40). Recent analysis of a *rpr*-null mutant revealed no effects on larval midgut or salivary gland cell death, although significant defects were observed in the central nervous system, raising the possibility that *rpr* plays no role in larval tissue destruction (41). A possible independent or combinatorial role for *hid* in these pathways has been difficult to ascertain because the available genetic tools, *Df(3L)H99* and *hid*-null mutations, lead to early lethality (16, 18, 42). Moreover, the embryonic lethality of *diap1* mutants has prevented an understanding of its possible roles during metamorphosis (19–21).

In this study, we exploit heat-inducible RNA interference (RNAi) as a means of disrupting gene function at later stages of development (43). We show that *hid* is required for larval salivary gland cell death and demonstrate that *rpr* and *hid* act together in a redundant manner to direct efficient destruction of the larval midguts and salivary glands. *diap1* is required to block premature programmed cell death during larval development. In

Abbreviations: *rpr*, *reaper*; *hid*, *head involution defective*; dsRNA, double-stranded RNA; RNAi, RNA interference; APF, after puparium formation; PBST, PBS plus 0.1% Tween 20; *BR-C*, *Broad-Complex*; DIAP1, *Drosophila* inhibitor of apoptosis 1.

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contrast, *diap2*, which is expressed immediately preceding salivary gland cell death, appears to play no independent role in these pathways (9, 44). This study provides a functional link between the genetic hierarchy that leads to *rpr* and *hid* induction and the destruction of larval tissues during *Drosophila* metamorphosis. This work also suggests that the integration and modulation of death activator and death repressor levels is vital for determining the proper timing of larval tissue cell death.

Materials and Methods

Supporting Information. Additional methods and details on the P element transformants for RNAi can be found as *Supporting Materials and Methods* and Fig. 6, which are published as supporting information on the PNAS web site.

Developmental Staging and Induction of Double-Stranded RNA (dsRNA) Expression. Third-instar larvae were staged by rearing on food containing 0.05% bromophenol blue (45). For larval midgut investigations, light-blue-gut, third-instar larvae were transferred to a 1.5-ml microcentrifuge tube plugged with cotton and subjected to a 30-min heat treatment in a 37.5°C water bath at 8 and 4 h before puparium formation; RNA was isolated from dissected larval midguts at 0 or 3 h after puparium formation (APF) for Northern blot hybridization. For salivary gland studies, control and *hs-hid-RNAi-30* prepupae were transferred to a 1.5-ml microcentrifuge tube and subjected to a sequential 30-min heat treatment in a 37.5°C water bath at 4 and 8 h APF. Salivary glands were dissected at the onset of head eversion, or 2 h later, for Northern blot hybridization. To study effects on salivary gland cell death, prepupae were subjected to the same heat-shock regime and salivary glands were dissected at 8 h after head eversion or analyzed by GFP fluorescence (with *34B-GAL4* and *UAS-GFP*) 12 h after head aversion. For induction of *diap1* or *diap2* dsRNA expression, dark-blue-gut, mid-third-instar larvae were transferred to a 1.5-ml microcentrifuge tube plugged with cotton, subjected to a 30-min heat treatment at 37.5°C in a water bath, and allowed to recover for 2 or 4 h at 25°C before total RNA was isolated for Northern blot hybridization. An identical heat treatment followed by 6 h of recovery at 25°C was used to study the phenotypes resulting from *diap1* or *diap2* dsRNA expression. *hs-diap2-RNAi-35* animals were also subjected to a 30-min 37.5°C heat treatment at either 3–5 h after egg lay or 36–48 h after egg lay to test for effects on viability.

Immunohistochemistry. Dark-blue-gut, w^{1118} control and *hs-diap1-RNAi-11*, third-instar larvae were subjected to a 30-min 37.5°C heat treatment. These animals were allowed to recover at 25°C for 6 h; they were then dissected and fixed with 4% formaldehyde, 1× PBST (PBS plus 0.1% Tween 20), and three volumes of heptane for 30 min at room temperature. Samples were washed four times in 1× PBST and blocked in 1× PBST/4% normal goat serum (NGS) for 2 h at room temperature. The samples were stained with antibodies directed against either DIAP1 (a generous gift from B. Hay, California Institute of Technology, Pasadena) at 1:400 dilution or against active caspase-3 (Cell Signaling Technology, Beverly, MA) at 1:1,000 in 1× PBST/4% NGS overnight at 4°C. Samples were washed four times in 1× PBST and stained with either Cy3 donkey anti-mouse secondary antibody (The Jackson Laboratory) for DIAP1 or Cy3 donkey anti-rabbit secondary antibody (The Jackson Laboratory) for active caspase-3, at 1:400 in 1× PBST/4% NGS for 2 h at room temperature. Samples were mounted in VECTAShield (Vector Laboratories) and images were captured as a Z-series by using a Bio-Rad 1024 confocal laser scanning microscope.

Results

***hid* Function Is Required for Efficient Larval Salivary Gland Cell Death.** Transgenic flies were generated that carry the heat-inducible *hsp70* (heat-shock protein 70) promoter upstream from a tandem inverted repeat of *hid* coding sequences. Several independent lines were established, one of which, *hs-hid-RNAi-30*, was used for studies of *hid* function. As an initial test of the efficiency of RNAi, we examined the levels of endogenous *hid* mRNA and cuticle preparations from embryos expressing *hid* dsRNA (Fig. 6). Whereas *hid* mRNA is readily detectable in heat-treated control embryos, this level is significantly reduced upon expression of *hid* dsRNA (Fig. 6). Consistent with this reduced level of *hid* expression, we see head involution defects in these embryos that phenocopy a *hid*-null mutant (42). The penetrance of head defects in the null mutant (70%, $n = 154$), however, is higher than that seen with RNAi (45%, $n = 96$). These observations indicate that the expression of *hid* dsRNA results in a strong loss-of-function *hid* mutant phenotype.

To determine a possible role for *hid* in larval salivary gland cell death, control w^{1118} and *hs-hid-RNAi-30* transformants were subjected to sequential 30-min heat treatments at 4 and 8 h APF. RNA was isolated from salivary glands of animals either at head eversion or 2 h later, ≈12 or 14 h APF, and analyzed by Northern blot hybridization. As expected, the levels of endogenous *hid* mRNA are greatly reduced upon expression of *hid* dsRNA (Fig. 1*A*). To determine the functional consequences of this reduction in *hid* expression, we scored for persistent larval salivary glands

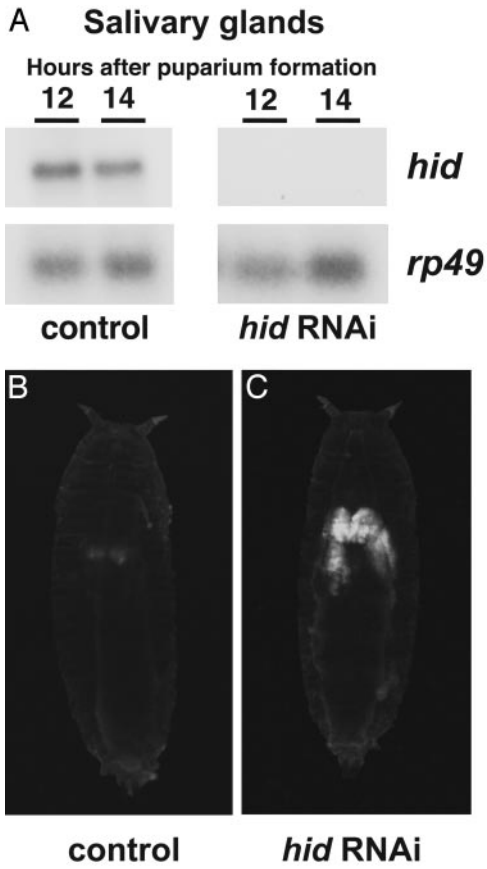

Fig. 1. Expression of *hid* dsRNA results in persistent larval salivary glands. (*A*) Control w^{1118} and *hs-hid-RNAi-30* prepupae were subjected to two sequential heat treatments at 4 and 8 h APF. Animals were allowed to recover until head eversion, ≈12 and 14 h APF, after which RNA was extracted from salivary glands for Northern blot hybridization. Hybridization to detect *rp49* was used as a control for loading and transfer. (*B* and *C*) Control *w*; *34B-GAL4*/+; *UAS-GFP*/+ (*B*) and *w*; *34B-GAL4*/+; *UAS-GFP*/*hs-hid-RNAi-30* (*C*) prepupae were subjected to the same heat-treatment regimen and scored at 24 h APF for GFP expression in the larval salivary glands. Although programmed cell death of control salivary glands is complete at this stage with only minimal residual fluorescence (*B*), salivary glands remain intact after expression of *hid* dsRNA (*C*).

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by either dissection or direct visualization of salivary glands by using the salivary gland-specific 34B-GAL4 driver in combination with a UAS-GFP reporter. Only residual GFP is detected in control *34B-GAL4*, *UAS-GFP* pupae at 24 h APF, consistent with the normal completion of salivary gland cell death (Fig. 1*B*), whereas persistent larval salivary glands are present in pupae that express *hid* dsRNA (Fig. 1*C*). Dissection of heat-treated *hs-hid-RNAi-30* pupae at 8 h after head eversion (≈20 h APF) revealed that 27% ($n = 37$) had persistent glands, compared with 0% ($n = 31$) in controls. A similar result was observed by scoring for GFP expression (25%, $n = 28$). We conclude that a severe reduction in *hid* expression results in a partial block in larval salivary gland cell death.

Consistent with an earlier study (41), *Df(3L)XR38/Df(3L)H99* mutant animals that lack *rpr* but have normal *hid* function display essentially normal salivary gland cell death, with only 4.2% ($n = 24$) displaying persistent larval salivary glands at 20 h APF (8 h after head eversion). Expression of *hid* dsRNA in this genetic background, however, increased this number to 65% ($n = 23$), significantly higher than that seen with *hid* RNAi alone. This result cannot be attributed to the reduced dose of *hid* in the *Df(3L)XR38/Df(3L)H99* mutant background, because heat-treated *Df(3L)H99*, *hs-hid-RNAi-30/+* pupae displayed a 24% frequency of persistent salivary glands at 20 h APF ($n = 17$), similar to the result obtained with *hid* RNAi alone. Taken together, these observations indicate that *rpr* and *hid* act together to drive efficient larval salivary gland cell death during the onset of metamorphosis.

***rpr* and *hid* Function in a Redundant Manner to Activate Cell Death in Larval Midguts.** To reduce the level of *hid* expression in larval midguts, control w^{1118} and *hs-hid-RNAi-30* transformants were subjected to two sequential 30-min heat treatments at 8 and 4 h before puparium formation. RNA was isolated from larval midguts at 0 and 3 h APF, the times when *hid* is normally induced in this tissue (9). Northern blot hybridization confirmed that the levels of endogenous *hid* mRNA are significantly reduced under these conditions (Fig. 2*A*). Dissection of these animals at 3 h APF revealed that neither reducing *hid* function with *hs-hid-RNAi-30* (4.4%, $n = 45$) nor the removal of *rpr* function with the *Df(3L)XR38/Df(3L)H99* chromosomal deficiencies (0%, $n = 20$) had a significant effect on larval midgut cell death (Fig. 2, compare *E* and *F* with *D*). In contrast, 80% ($n = 20$) of heat-treated *Df(3L)XR38/Df(3L)H99, hs-hid-RNAi-30* animals, which lack *rpr* activity and have reduced *hid* function, display persistent larval midguts (Fig. 2*G*). These larval midguts have failed to contract by 3 h APF and retain their gastric caeca (Fig. 2*G*, arrow), similar to the midguts of control mid-third-instar larvae (Fig. 2*B*). Midgut contraction normally begins at puparium formation (Fig. 2*C*), coinciding with the onset of cell death, and is complete by 3 h APF, when the gastric caeca are no longer present and the midgut is highly contracted (Fig. 2*D*) (9). These observations indicate that *rpr* and *hid* are functionally redundant in this tissue, acting in a cooperative manner to direct larval midgut cell death.

***diap1* Is Required for Third-Instar Larval Viability.** *diap1* is highly expressed in late third-instar larvae and significantly reduced at 6 h APF, with low constant levels of expression in both the larval midgut and salivary glands throughout the onset of metamorphosis (data not shown). To address possible roles for this death repressor in preventing premature larval tissue cell death, we reduced *diap1* expression during larval development by inducible RNAi. Transgenic flies were established that carry the *hsp70* promoter upstream from a tandem inverted repeat of *diap1* coding sequences; one line, *hs-diap1-RNAi-11*, was selected for studies of *diap1* function. Heat-induced *diap1* dsRNA expression in mid-third-instar larvae (≈18 h before puparium formation)

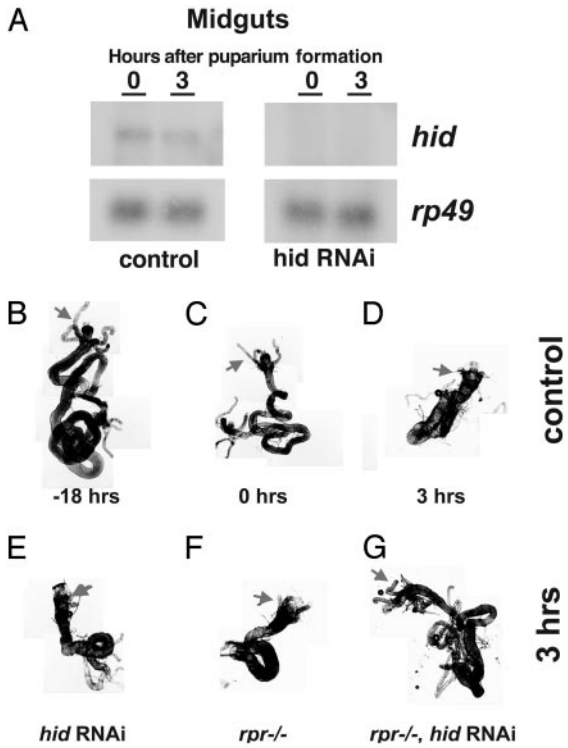

Fig. 2. Expression of *hid* dsRNA in a *rpr*-null mutant background leads to persistent larval midguts. (*A*) Control w^{1118} and *hs-hid-RNAi-30* third-instar larvae were subjected to two sequential heat treatments at 8 and 4 h before puparium formation, and RNA was isolated from larval midguts dissected at either 0 or 3 h APF for Northern blot hybridization. Hybridization to detect *rp49* was used as a control for loading and transfer (*B–G*). Larval midguts were dissected from a control w^{1118} mid-third-instar larva (*B*), newly formed prepupa (*C*), or 3 h APF (*D*) to depict the normal time course of cell death. Shortened gastric caeca (arrows) are evident at puparium formation, along with an overall contraction in the length of the midgut (*C*). This contraction is complete by 3 h APF, and gastric caeca are no longer present (*D*). Neither expression of *hid* dsRNA (*E*) nor a *rpr*-null mutation (*F*) has an effect on larval midgut cell death when examined at 3 h APF, whereas expression of *hid* dsRNA in a *rpr*-null mutant background leads to an efficient block in the destruction of this tissue (*G*).

led to a significant reduction in the levels of endogenous *diap1* transcripts (Fig. 3*A*). Some larvae stopped moving as early as 2 h after heat treatment, with necrotic patches often appearing in their larval tissues. By 6 h, most larvae exhibited visible signs of death (76%, $n = 100$), from relatively restricted death of larval tissues (Fig. 3 *C* and *D*) to widespread necrosis (Fig. 3*E*), compared with heat-treated, control third-instar larvae (Fig. 3*B*). Although the remaining animals formed morphologically normal prepupae, they all underwent massive death before head eversion, leaving only an empty pupal case.

***diap1* Is Required to Suppress Premature Destruction of Larval Tissues.** To determine the consequences of reducing *diap1* function on larval tissue cell death, *hs-diap1-RNAi-11* third-instar larvae were heat-treated at ≈18 h before puparium formation and dissected 6 h later. As expected, the levels of DIAP1 protein are significantly reduced under these conditions (Fig. 4, compare *D* and *J* with *A* and *G*). This observation is consistent with the reduced levels of *diap1* mRNA under these conditions (Fig. 3*A*) and the short half-life of DIAP1 protein (24). The larval midguts and salivary glands of heat-treated *hs-diap1-RNAi-11* larvae resemble those undergoing cell death. The larval midgut is

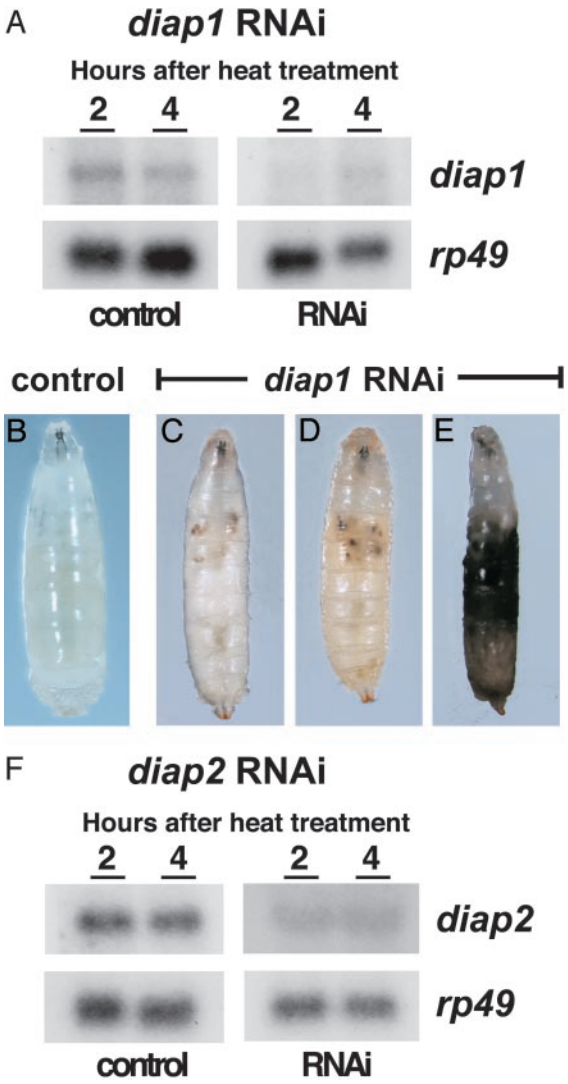

Fig. 3. Expression of *diap1* dsRNA leads to larval lethality. (*A*) Control w^{1118} and *hs-diap1-RNAi-11* mid-third-instar larvae were heat treated, and total RNA was isolated 2 or 4 h later for Northern blot analysis. Six hours after heat treatment, control larvae (*B*) exhibited no signs of premature cell death, whereas larvae expressing *diap1* dsRNA display patches of death in larval tissues (*C* and *D*) or overall necrosis (*E*). Control and *hs-diap2-RNAi-35* mid-third-instar larvae were subjected to the same heat-treatment regimen as *hs-diap1-RNAi-11* animals, and total RNA was isolated 2 or 4 h later for Northern blot analysis. Expression of *diap2* dsRNA leads to reduced levels of endogenous *diap2* mRNA (*F*).

contracted with a significantly reduced proventriculus and gastric caeca and an overall degraded morphology (Fig. 4*E*), similar to that normally seen 2 h APF (9) and contrasting dramatically with the healthy morphology of heat-treated control larval midguts (Fig. 4*B*). Similarly, the larval salivary glands of heat treated *hs-diap1-RNAi-11* larvae are severely degraded (Fig. 4*K*), resembling the morphology of wild-type glands dissected from 14.5-h pupae (9). In contrast, salivary glands dissected from heat-treated control larvae display a tight cellular structure and apparent lumen in the center of the gland (Fig. 4*H*). Consistent with these morphological indications of premature cell death, those tissues that lack *diap1* function are selectively stained with an antibody directed against active caspase-3, which detects the *Drosophila* DrICE effector caspase, indicating they are undergoing cell death (Fig. 4, compare *F* and *L* with *C* and *I*) (46).

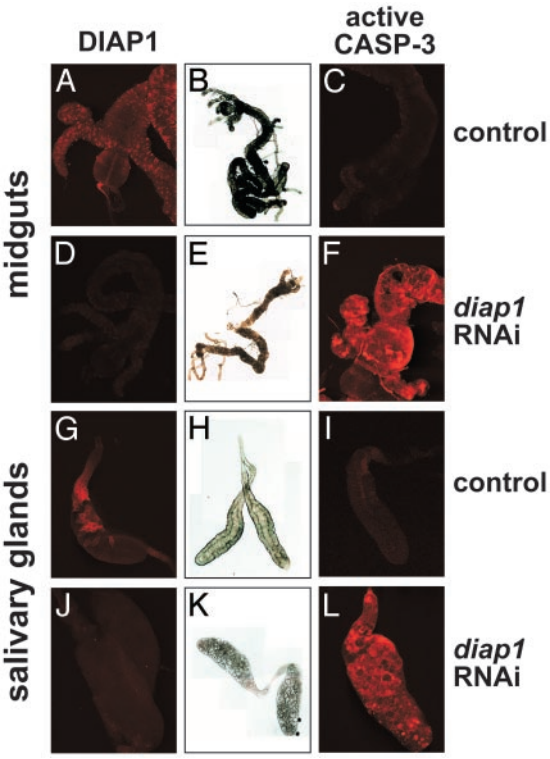

Fig. 4. Expression of *diap1* dsRNA leads to premature larval midgut and salivary gland cell death. Control w^{1118} and *hs-diap1-RNAi-11* mid-third-instar larvae were subjected to heat treatment and allowed to recover for 6 h. Larval midguts and salivary glands were then dissected from these animals and examined by light microscopy (*B*, *E*, *H*, and *K*), immunostaining to detect DIAP1 (*A*, *D*, *G*, and *J*), or immunostaining to detect active caspase-3 (*C*, *F*, *I*, and *L*). Physical signs of cell death (*E* and *K*) and caspase activation (*F* and *L*) are only seen when DIAP1 levels are reduced by RNAi (*D* and *J*).

***diap2* Has No Apparent Independent Role in the Regulation of Cell Death.** Previous studies have shown that a brief burst of *diap2* transcription precedes *rpr* and *hid* induction in doomed larval salivary glands (9), with expression that parallels that of *diap1* throughout late larval and prepupal stages (data not shown). To determine whether *diap2* contributes to the regulation of larval tissue cell death, we used inducible RNAi to inhibit *diap2* function during the onset of metamorphosis. Transgenic flies were established that carry the *hsp70* promoter upstream from a tandem inverted repeat of *diap2* coding sequences, and one line, *hs-diap2-RNAi-35*, was selected for studies of *diap2* function. Heat-induced *diap2* dsRNA expression in mid-third-instar larvae led to a significant reduction in the levels of endogenous *diap2* transcripts (Fig. 3*F*). To test for a role for *diap2* in preventing premature larval salivary gland cell death, we heat-treated *hs-diap2-RNAi-35* animals at 4 and 8 h APF and dissected salivary glands at 10 and 12 h APF. All salivary glands examined died on time (n = 15 animals), and all *hs-diap2-RNAi-35* animals exposed to this sequential heat treatment survived to adulthood (n = 50). No effects on viability were seen in heat-treated *hs-diap2-RNAi-35* embryos or first- and second-instar larvae (data not shown). In addition, a heat-shock regime identical to that used to inactivate *diap1* during third-instar larval development (Fig. 3*C–E*) resulted in no effects on *hs-diap2-RNAi-35* animals. These observations suggest that *diap2* has no significant independent role in the regulation of *Drosophila* programmed cell death.

Discussion

***rpr* and *hid* Cooperate to Destroy Larval Tissues During Metamorphosis.** Our understanding of the functional interactions between *rpr*, *hid*, and *grim* has been hampered by a paucity of genetic tools in

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this region. Extensive screens for lethal mutations within the *Df(3L)H99* interval resulted in the recovery of only *hid* alleles, suggesting that the three death activators might functionally compensate for one another and indicating that more directed efforts would be required to recover *rpr* and *grim* mutations (16, 18, 42). Reversion of a P element 225 kb proximal to *rpr* resulted in the *Df(3L)XR38* deficiency. This deletion, in combination with *Df(3L)H99*, leads to the selective elimination of *rpr*, although it remains a possibility that other unidentified modifiers of the death response could be affected by these deletions (41). Interestingly, these *rpr*-null mutants develop into morphologically normal adults with a highly enlarged central nervous system, indicating a relatively restricted requirement for *rpr* during development (41). Characterization of *hid* mutants and *Df(3L)X25* mutants, which lack *grim* and *hid*, have implied functional interactions between the death activators for embryonic head morphogenesis and programmed cell death of neurons that express crustacean cardioactive peptide (47, 48). This model is further supported by a gain-of-function study showing that targeted expression of both *rpr* and *hid* is required for the death of midline cells in the embryonic nervous system, suggesting that these genes act in a cooperative and dose-dependent manner in this cell type (49). A loss-of-function analysis to support this model, however, has not been possible because of the difficulty of recombining *hid* alleles onto the *Df(3L)XR38 rpr* mutant chromosome. Here, we used inducible RNAi to selectively inactivate *hid* function, both in the presence and absence of *rpr*, and showed that these genes act together in a combinatorial manner to drive the death of larval tissues during metamorphosis. This study provides the first direct genetic evidence that *rpr* and *hid* are functionally redundant for programmed cell death; it also supports the results of Peterson *et al.* (41) regarding the absence of larval tissue cell death in *rpr* mutants and indicates that this response requires the simultaneous induction of *hid*. The cooperativity and dose-dependency of death activator function is consistent with the ability of either *rpr* or *hid* to drive programmed cell death in the adult eye (16–18, 44) and their interchangeable role in inactivating the *diap1* death inhibitor both *in vivo* and *in vitro* (19–21).

We find that 27% of *hid* RNAi animals display persistent larval salivary glands, in contrast to the almost complete absence of this phenotype in *rpr*-null mutants. This observation supports the earlier proposal that, at least in some cells, *hid* is a more potent activator of programmed cell death than *rpr* (18, 41). Our results are also consistent with the observation that *E74A* mutant salivary glands, which display an almost complete absence of *hid* transcription but normal levels of *rpr*, fail to die in ≈20% of the pupae examined 24 h APF (12). Similarly, *BR-C* mutant salivary glands fail to express both *rpr* and *hid* and display highly penetrant persistent larval salivary glands (12, 50, 51).

In contrast to our results in larval salivary glands, larval midgut cell death occurs essentially normally in the presence of *hid* dsRNA. Both *hid* RNAi and the *rpr*-null mutation are required to see penetrant death defects in this tissue, indicative of complete functional redundancy. This observation supports the proposal that the ecdysone-induced death program in the larval midgut has differences from that activated by the hormone in doomed salivary glands (15).

***diap1* Acts as a Survival Factor to Prevent Premature Cell Death During Postembryonic Development.** With the exception of *Drosophila* adult eye development, functions for IAP death inhibitors at later stages of development have been undefined. Our studies indicate an essential role for *diap1* in holding back the death response during postembryonic development, restricting it to the appropriate space and time. We propose that the reduced levels of *diap1* expression seen in larval tissues during the early stages of metamorphosis prime the system for their destruction. The appropriate spatial and temporal patterns of ecdysone-induced *rpr* and *hid* expression then tip the balance in these cells, signaling their elimination.

It is interesting to note that visible necrotic patches appear rapidly in third-instar larvae that express *diap1* dsRNA (Fig. 3*C–E*). The distinction between apoptosis and necrosis was established in early studies of dying cells, based on morphology, arguing that the two pathways are distinct and involve different regulatory factors (52). More recent studies, however, have uncovered common features shared by these death responses (53, 54). For example, studies of neutrophil cell death showed that X-linked IAP is a target of calpain, a key component of the necrotic pathway (55), and that calpain inhibitors not only protect against necrosis in some neuronal cells but also inhibit apoptosis (56). Future studies should provide a better understanding of how IAPs may function at the intersection between necrotic and programmed cell death responses.

Earlier work showed that *diap2* is expressed in a brief burst, immediately before the induction of *rpr* and *hid* in doomed larval salivary glands, suggesting that it may contribute to the regulation of cell death in this tissue (9). The studies of *diap2* RNAi presented here, however, do not support this model. This conclusion is consistent with a complementary study, in which premature ectopic overexpression of *diap2* was shown to have no effect on larval salivary gland cell death (10). Thus, although *diap2* was originally identified by its ability to block *rpr*- or *hid*-induced programmed cell death in the adult eye, its ability to exert this function must be cell-type specific (44). Rather, we propose that *diap1* acts as the primary inhibitor of cell death in larval salivary glands. It is possible that *diap2* could contribute to biological pathways other than programmed cell death. For example, RNAi studies of survivin function have uncovered a role in cell cycle regulation (57). The characterization of specific *diap2* mutations should provide a better understanding of its functions during *Drosophila* development.

A Steroid-Triggered Regulatory Cascade Directs the Stage-Specific Destruction of Larval Tissues During Metamorphosis. The steroid-triggered destruction of larval tissues during *Drosophila* meta-

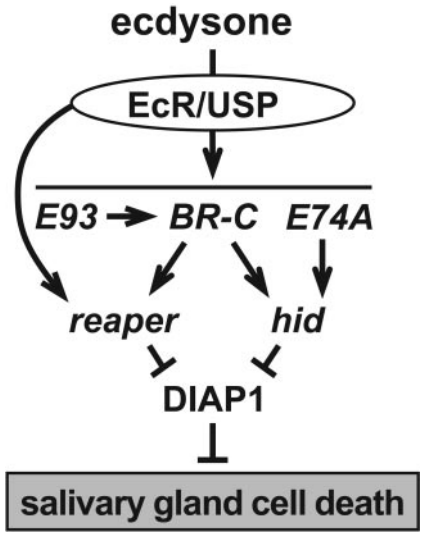

Fig. 5. A model for the steroid regulation of salivary gland cell death. Ecdysone functions through the heterodimeric ecdysone receptor (EcR)/Ultraspiracle (USP) receptor to directly induce primary response transcription factors, *E93*, *BR-C*, and *E74A*, which are required for proper *rpr* and *hid* induction. Ecdysone can also regulate *rpr* directly through an ecdysone response element in its promoter. *reaper* and *hid* act together in a combinatorial manner to overcome the inhibitory effect of DIAP1, triggering salivary gland cell death.

morphosis provides one of the best systems for understanding the temporal and spatial control of programmed cell death. Several transcription factors that are induced directly by ecdysone have been linked to the regulation of key death genes in these tissues, defining a genetic pathway for the control of programmed cell death (12, 14, 15). This cascade has been most extensively characterized in the larval salivary gland, in which numerous death regulators are induced just before the destruction of this tissue (58, 59). What has been missing from these studies, however, is a functional link between the death regulators and the destruction of larval tissues during metamorphosis. Indeed, the recent report that *rpr*-null mutants have no effect on larval midgut and salivary gland cell death raised the possibility that an alternate pathway might be operative, as appears to be the case in nurse cell apoptosis (40, 41). The results presented here complete the pathway, showing that ecdysone-induced *rpr* and *hid* expression, mediated by *EcR* (ecdysone receptor), *E93*, *BR-C*, and *E74A*, is required for the stage-specific destruction of the larval midgut and salivary glands (Fig. 5). Moreover, this study shows that *diap1* is required to hold back the death response, adding a new critical player to the pathway. Further studies of *Drosophila* metamorphosis should provide a better understanding of the regulation of programmed cell death during development as well as a model system for understanding how steroid hormones control apoptotic responses in vertebrate organisms.

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1. Yoshizato, K. (1996) in *Cell Death and Histolysis in Amphibian Tail Metamorphosis*, eds. Gilbert, L. I., Tata, J. R. & Atkinson, B. G. (Academic, New York), pp. 647–671.
2. Tata, J. R. (1966) *Dev. Biol.* **13,** 77–94.
3. Shi, Y. B., Wong, J., Puzianowska-Kuznicka, M. & Stolow, M. A. (1996) *BioEssays* **18,** 391–399.
4. Evans-Storms, R. B. & Cidlowski, J. A. (1995) *J. Steroid Biochem. Mol. Biol.* **53,** 1–8.
5. Winoto, A. & Littman, D. R. (2002). *Cell* **109,** Suppl., S57–S66.
6. Smith, S. W., McLaughlin, K. A. & Osborne, B. A. (1995) *Curr. Top Microbiol. Immunol.* **200,** 147–162.
7. Robertson, C. W. (1936) *J. Morphol.* **59,** 351–399.
8. Bodenstein, D. (1965) in *The Postembryonic Development of Drosophila*, ed. Demerec, M. (Hafner, New York), pp. 275–367.
9. Jiang, C., Baehrecke, E. H. & Thummel, C. S. (1997) *Development (London)* **124,** 4673–4683.
10. Lee, C. Y. & Baehrecke, E. H. (2001) *Development (London)* **128,** 1443–1455.
11. Martin, D. N. & Baehrecke, E. H. (2004) *Development (London)* **131,** 275–284.
12. Jiang, C., Lamblin, A.-F., Steller, H. & Thummel, C. S. (2000) *Mol. Cell* **5,** 445–455.
13. Lee, C. Y., Wendel, D. P., Reid, P., Lam, G., Thummel, C. S. & Baehrecke, E. H. (2000) *Mol. Cell* **6,** 433–443.
14. Lee, C. Y., Simon, C. R., Woodard, C. T. & Baehrecke, E. H. (2002) *Dev. Biol.* **252,** 138–148.
15. Lee, C. Y., Cooksey, B. A. & Baehrecke, E. H. (2002) *Dev. Biol.* **250,** 101–111.
16. White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. & Steller, H. (1994) *Science* **264,** 677–683.
17. Chen, P., Nordstrom, W., Gish, B. & Abrams, J. M. (1996) *Genes Dev.* **10,** 1773–1782.
18. Grether, M. E., Abrams, J. M., Agapite, J., White, K. & Steller, H. (1995) *Genes Dev.* **9,** 1694–1708.
19. Lisi, S., Mazzon, I. & White, K. (2000) *Genetics* **154,** 669–678.
20. Goyal, L., McCall, K., Agapite, J., Hartwieg, E. & Steller, H. (2000) *EMBO J.* **19,** 589–597.
21. Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H. A. & Hay, B. A. (1999) *Cell* **98,** 453–463.
22. Vucic, D., Kaiser, W. J. & Miller, L. K. (1998) *Mol. Cell Biol.* **18,** 3300–3309.
23. Vucic, D., Kaiser, W. J., Harvey, A. J. & Miller, L. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10183–10188.
24. Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M., Clem, R. J., Muller, H. A. & Hay, B. A. (2002) *Nat. Cell Biol.* **4,** 416–424.
25. Holley, C. L., Olson, M. R., Colon-Ramos, D. A. & Kornbluth, S. (2002) *Nat. Cell Biol.* **4,** 439–444.
26. Hays, R., Wickline, L. & Cagan, R. (2002) *Nat. Cell Biol.* **4,** 425–431.
27. Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E. & Meier, P. (2003) *Nat. Cell Biol.* **5,** 467–473.
28. Ryoo, H. D., Bergmann, A., Gonen, H., Ciechanover, A. & Steller, H. (2002) *Nat. Cell Biol.* **4,** 432–438.
29. Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D. A., Agapite, J., Steller, H. & Meier, P. (2002) *Nat. Cell Biol.* **4,** 445–450.
30. Wing, J. P., Schreader, B. A., Yokokura, T., Wang, Y., Andrews, P. S., Huseinovic, N., Dong, C. K., Ogdahl, J. L., Schwartz, L. M., White, K. & Nambu, J. R. (2002) *Nat. Cell Biol.* **4,** 451–456.
31. Goyal, L. (2001) *Cell* **104,** 805–808.
32. Martin, S. J. (2002) *Cell* **109,** 793–796.
33. Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C. & Fesik, S. W. (2000) *Nature* **408,** 1004–1008.
34. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. & Takahashi, R. (2001) *Mol. Cell* **8,** 613–621.
35. Wu, G., Chai, J., Suber, T. L., Wu, J. W., Du, C., Wang, X. & Shi, Y. (2000) *Nature* **408,** 1008–1012.
36. Wu, J. W., Cocina, A. E., Chai, J., Hay, B. A. & Shi, Y. (2001) *Mol. Cell* **8,** 95–104.
37. White, K., Tahaoglu, E. & Steller, H. (1996) *Science* **271,** 805–807.
38. Bergmann, A., Agapite, J., McCall, K. & Steller, H. (1998) *Cell* **95,** 331–341.
39. Kurada, P. & White, K. (1998) *Cell* **95,** 319–329.
40. Foley, K. & Cooley, L. (1998) *Development (London)* **125,** 1075–1082.
41. Peterson, C., Carney, G. E., Taylor, B. J. & White, K. (2002) *Development (London)* **129,** 1467–1476.
42. Abbott, M. K. & Lengyel, J. A. (1991) *Genetics* **129,** 783–789.
43. Lam, G. & Thummel, C. S. (2000) *Curr. Biol.* **10,** 957–963.
44. Hay, B. A., Wassarman, D. A. & Rubin, G. M. (1995) *Cell* **83,** 1253–1262.
45. Andres, A. J. & Thummel, C. S. (1994) in *Methods for Quantitative Analysis of Transcription in Larvae and Prepupae*, eds. Goldstein, L. S. B. & Fyrberg, E. A. (Academic, New York), pp. 565–573.
46. Yu, S. Y., Yoo, S. J., Yang, L., Zapata, C., Srinivasan, A., Hay, B. A. & Baker, N. E. (2002) *Development (London)* **129,** 3269–3278.
47. Nassif, C., Daniel, A., Lengyel, J. A. & Hartenstein, V. (1998) *Dev. Biol.* **197,** 170–186.
48. Draizen, T. A., Ewer, J. & Robinow, S. (1999) *J. Neurobiol.* **38,** 455–465.
49. Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L. M., Steller, H. & Nambu, J. R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5131–5136.
50. Restifo, L. L. & White, K. (1992) *Roux's Arch. Dev. Biol.* **201,** 221–234.
51. Zhimulev, I. F., Belyaeva, E. S., Mazina, O. M. & Balasov, M. L. (1995) *Eur. J. Entomol.* **92,** 263–270.
52. Kerr, J. F., Wyllie, A. H. & Currie, A. R. (1972) *Brit. J. Cancer* **26,** 239–257.
53. Proskuryakov, S. Y., Konoplyannikov, A. G. & Gabai, V. L. (2003) *Exp. Cell Res.* **283,** 1–16.
54. Syntichaki, P. & Tavernarakis, N. (2002) *EMBO Rep.* **3,** 604–609.
55. Kobayashi, S., Yamashita, K., Takeoka, T., Ohtsuki, T., Suzuki, Y., Takahashi, R., Yamamoto, K., Kaufmann, S. H., Uchiyama, T., Sasada, M. & Takahashi, A. (2002) *J. Biol. Chem.* **277,** 33968–33977.
56. Wang, K. K. (2000) *Trends Neurosci.* **23,** 20–26.
57. Li, F., Ackermann, E. J., Bennett, C. F., Rothermel, A. L., Plescia, J., Tognin, S., Villa, A., Marchisio, P. C. & Altieri, D. C. (1999) *Nat. Cell Biol.* **1,** 461–466.
58. Gorski, S. M., Chittaranjan, S., Pleasance, E. D., Freeman, J. D., Anderson, C. L., Varhol, R. J., Coughlin, S. M., Zuyderduyn, S. D., Jones, S. J. & Marra, M. A. (2003) *Curr. Biol.* **13,** 358–363.
59. Lee, C. Y., Clough, E. A., Yellon, P., Teslovich, T. M., Stephan, D. A. & Baehrecke, E. H. (2003) *Curr. Biol.* **13,** 350–357.