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E93 Directs Steroid-Triggered Programmed Cell Death in *Drosophila*

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Summary

Steroid hormones coordinate multiple cellular changes, yet the mechanisms by which these systemic signals are refined into stage- and tissue-specific responses remain poorly understood. Here we show that the *Drosophila E93* gene determines the nature of a steroid-induced biological response. *E93* mutants possess larval salivary glands that fail to undergo steroid-triggered programmed cell death, and E93 is expressed in cells immediately before the onset of death. E93 protein is bound to the sites of steroid-regulated and cell death genes on polytene chromosomes, and the expression of these genes is defective in *E93* mutants. Furthermore, expression of E93 is sufficient to induce programmed cell death. We propose that the steroid induction of E93 determines a programmed cell death response during development.

Introduction

Programmed cell death, or apoptosis, is essential for the maintenance of homeostasis in all higher organisms, serving to eliminate unneeded cells and tissues, control cell number, and remove abnormal cells (Jacobson et al., 1997). Programmed cell death is distinguished from necrosis by a group of morphological and biochemical markers (Kerr et al., 1972; Wyllie, 1980) and is controlled by an evolutionarily conserved genetic regulatory pathway (Abrams, 1999).

A series of elegant genetic studies of programmed cell death in the nematode *C. elegans* led to the isolation of the *ced-3*, *ced-4*, and *ced-9* genes (Ellis et al., 1991). CED-3 is homologous to the mammalian family of caspases, which upon proteolytic activation are critical effectors of the programmed cell death signaling pathway (Cryns and Yuan, 1998). CED-4 is homologous to mammalian Apaf-1, which activates caspases in the presence of cytochrome c and dATP (Li et al., 1997; Zou et al., 1997). CED-9 is a member of the Bcl-2 family of cell death regulators (Vaux et al., 1992; Hengartner and Horvitz, 1994). While some Bcl-2 proteins impact Apaf-1 activity (Adams and Cory, 1998), the mechanism of this regulation remains controversial.

Genetic screens in *Drosophila* have revealed three cell death inducer genes: *reaper* (*rpr*), *head involution defective* (*hid*), and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996). *rpr*, *hid*, and *grim* reside next to one another in the genome, in a region defined by *Df(3L)H99*. The function of genes in this deficiency is required for virtually all programmed cell death during embryogenesis. Expression of *rpr* and *grim* immediately precedes and appears to be restricted to dying cells in *Drosophila*, whereas *hid* is also expressed in some cells that fail to die. Ectopic expression of each of these genes is also sufficient to induce caspase-dependent programmed cell death in a wide range of cell types (Grether et al., 1995; Chen et al., 1996; White et al., 1996). Although vertebrate homologs of *rpr*, *hid*, and *grim* have not yet been identified, expression of each of these genes is sufficient to induce apoptosis in mammalian cells (McCarthy and Dixit, 1998; Haining et al., 1999), suggesting that the downstream death pathway has been conserved between flies and mammals. In support of this proposal, *Drosophila* homologs of caspases including DCP-1, Dredd, DrICE, Dronc, and Decay have been identified (Fraser and Evan, 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999), along with the *Drosophila* homolog of Apaf-1/CED-4, named Dark, Hac-1, or Dapaf-1 (hereafter refered to as Ark, for Apaf-1-related killer) (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999), a *Drosophila* homolog of Bcl-2/CED-9 Drob-1 (Igaki et al., 2000), and *Drosophila* homologs of the inhibitors of apoptosis, DIAP1 and DIAP2 (Hay et al., 1995). The remarkable similarity between vertebrate and fly cell death pathways establishes *Drosophila* as a valuable model system for genetic studies of the regulation of programmed cell death.

An additional parallel between fly and vertebrate cell death is the conserved role of steroid hormones in triggering the death response. At the end of the third larval instar in *Drosophila*, a high titer pulse of the steroid hormone 20-hydroxyecdysone (ecdysone) triggers puparium formation and the onset of prepupal development. This transition is accompanied by morphogenesis of the adult legs and wings, as well as programmed cell death of the larval midgut (Robertson, 1936; Bodenstein, 1965). The subsequent ecdysone pulse, ~10 hr after puparium formation, signals the prepupal–pupal transition, and triggers salivary gland cell death, eversion of the adult head, and the onset of adult differentiation. The destruction of larval midguts and salivary glands are accompanied by DNA fragmentation and nuclear acridine orange staining, require caspase activation, and are dependent upon ecdysone, defining them as steroid-triggered programmed cell death responses (Jiang et al., 1997). In addition, larval midgut and salivary gland cell death is foreshadowed by the coordinate ecdysone induction of *rpr* and *hid*, implicating these genes as regulators of this cell death response (Jiang et al., 1997).

The mechanisms of steroid signaling have been extensively studied in *Drosophila* larval salivary glands by

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virtue of the giant polytene chromosomes that form ecdysone-induced puffs, reflecting a transcriptional regulatory hierarchy (Becker, 1959; Clever, 1964; Ashburner et al., 1974). The ecdysone receptor complex, a heterodimer of the EcR and USP nuclear receptors (Yao et al., 1992; Thomas et al., 1993), activates transcription of a small set of early regulatory genes (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). These genes encode transcription factors that, in turn, activate a larger set of late genes, which are thought to play a more direct role in controlling the appropriate biological responses to the hormone (Urness and Thummel, 1995; Crossgrove et al., 1996). Previous studies have implicated the *EcR*, *usp*, β*FTZ-F1*, *BR-C*, and *E74A* genes in steroid-activated larval cell death (Restifo and White, 1992; Bender et al., 1997; Hall and Thummel, 1998; Broadus et al., 1999; Jiang et al., 2000). The role of β*FTZ-F1* appears to be indirect, functioning as general competence factor for prepupal responses to ecdysone (Woodard et al., 1994; Broadus et al., 1999). In contrast, EcR, USP, BR-C, and E74A play a more direct role in triggering salivary gland cell death through the coordinate induction of *rpr* and *hid* transcription (Jiang et al., 2000). These factors, however, are not sufficient for the death response because they do not direct this pathway in response to the earlier pulse of ecdysone at puparium formation. Rather, one or more stage-specific regulators must be induced by ecdysone at the end of prepupal development that determine the stage specificity of salivary gland cell death. The *E93* early gene is an ideal candidate for fulfilling this function. *E93* is induced as a primary reponse to ecdysone in a stage- and tissue-specific manner (Baehrecke and Thummel, 1995). *E93* transcription increases immediately prior to larval midgut and salivary gland cell death and is coordinately induced with *rpr* and *hid* (Baehrecke and Thummel, 1995; Jiang et al., 1997). This correlation suggests that *E93* may contribute to the stage specificity of larval tissue cell death.

Here we define *E93* as a critical regulator of the appropriate spatial and temporal patterns of steroid-triggered programmed cell death during *Drosophila* metamorphosis. We show that *E93* mutant salivary glands fail to die and that expression of *E93* is sufficient to restore this cell death response. E93 encodes a novel nuclear protein that is expressed in doomed larval cells foreshadowing steroid-induced cell death and binds to specific sites in the larval salivary gland polytene chromosomes. Mutations in *E93* disrupt transcription of steroid-regulated genes and programmed cell death genes at chromosome loci where E93 protein is bound. Furthermore, ectopic expression of E93 is sufficient to kill cells that normally form adult structures. We conclude that *E93* specifies the appropriate patterns of steroid-activated programmed cell death during *Drosophila* development by regulating the transcription of key target genes involved in cell death.

Results

E93 Mutants Die during Metamorphosis with Defects in Salivary Gland Cell Death

An F2 lethal screen was performed to isolate ethane methyl sulfonate (EMS)-induced mutations in *E93*. From a total of 11,134 F2 EMS-mutagenized lines, 29 lines were isolated that possess lethal mutations within the region defined by $Df(3R)93F^{X2}$, which removes *E93*. These mutations define 11 lethal complementation groups. Two of these complementation groups display pupal lethal phenotypes, while the other nine result in lethality at earlier stages in development. One of the pupal lethal complementation groups, represented by three alleles, dies late during metamorphosis with defects that are restricted to developing adult structures. The second pupal lethal complementation group, also represented by three alleles, dies earlier during pupal development. Because this lethal phase corresponds to the earliest expression of *E93*, we subjected these mutants to more detailed phenotypic and molecular characterization. The 3.6 kb *E93* open reading frame, as well as intron/exon boundaries, were sequenced from genomic DNA isolated from each of the three mutant alleles as well as the parental strain used for mutagenesis. The $E93^1$ allele has a T-to-A transition at nucleotide 3374 that changes a leucine at position 994 to a stop codon. While no mutations were detected in either $E93^2$ or $E93^3$, a significantly reduced amount of *E93* mRNA was detected in homozygous $E93^2$ mutants, and no *E93* mRNA was detected in homozygous $E93^3$ mutants (Figure 1). These results indicate that the $E93^2$ and $E93^3$ mutations affect either transcriptional regulatory elements or *E93* mRNA stability. Taken together, these results suggest that $E93^1$, $E93^2$, and $E93^3$ represent either strong hypomorphic or null *E93* mutations.

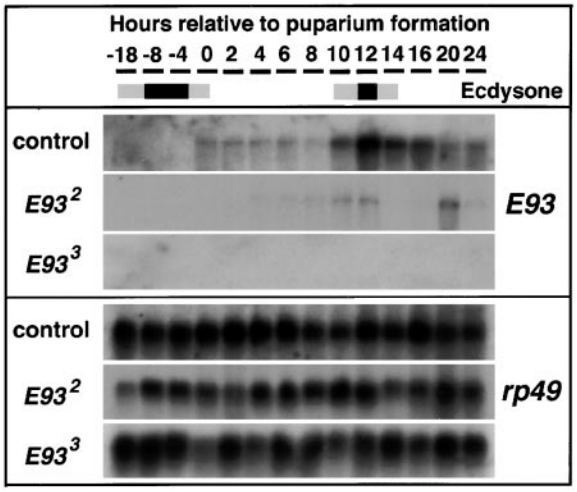

Figure 1. *E93* Transcription Is Reduced or Absent in $E93^2$ and $E93^3$ Mutants

Total RNA was isolated from $E93^1$/wild-type control, $E93^2/E93^2$, and $E93^3/E93^3$ staged larvae, prepupae, and pupae, and *E93* transcription was analyzed by Northern blot hybridization. Numbers at the top indicate hours relative to puparium formation, with boxes representing the two peaks in ecdyone titer. Control and mutant Northern blots were hybridized at the same time with probes to detect *E93* or *rp49* as a control for loading and transfer.

E93 mutants display little lethality during embryonic and larval development (<12%, n = 704, $E93^1$ homozygotes) and die during the early stages of pupal development (Figure 2). These mutants fail to shorten their body properly at puparium formation, often exhibit a defect in anterior spiracle eversion, and die following head eversion (Figure 2B). Although *E93* mutants possess a well-developed head, thorax, and abdomen, no pigmentation of adult structures occurs, even following prolonged aging. $E93^1$ mutants exhibit identical phenotypes

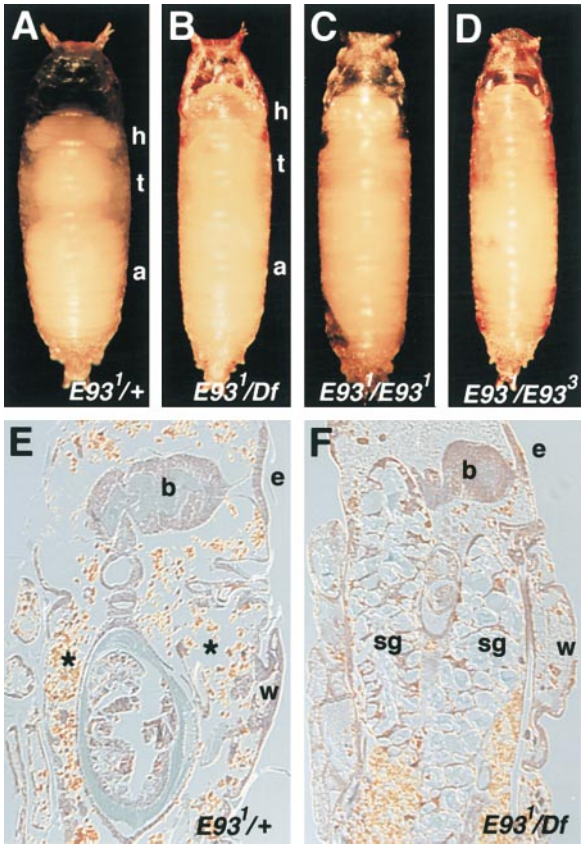

Figure 2. *E93* Mutants Die during Pupal Development with Persistent Larval Salivary Glands

(A–D) The four panels at the top are animals staged 24 hr after puparium formation; either (A) *E93*^{1}/+ control animals, (B) *E93*^{1}/*Df(3R)93F*^{X2} mutants, (C) *E93*^{1}/*E93*^{1} mutants, or (D) *E93*^{1}/*E93*^{3} mutants. While *E93* mutants always form pupae, as indicated by the development of a head (h), thorax (t), and abdomen (a), they never tan, always remain elongated, and have frequent defects in anterior spiracle eversion.

(E) Section of an *E93*^{1}/+ control animal 24 hr after puparium formation. Controls never possess salivary glands at this stage (asterisk), but have formed adult eyes (e) with connections to the brain (b), and adult wings (w).

(F) Section of an *E93*^{1}/*Df(3R)93F*^{X2} mutant 24 hr after puparium formation. All allelic combinations of *E93* mutants always exhibit persistent salivary glands (sg). Like controls, *E93* mutants have developed adult eyes (e) with connections to the brain (b), and adult wings (w).

when combined with a deletion of *E93* (Figure 2B) or as homozygotes (Figure 2C) and, therefore, fulfill the genetic definition of a null allele. Similarly, *E93*^{2} and *E93*^{3} were shown to behave as strong loss-of-function or null alleles (data not presented) and exhibit identical phenotypes when transheterozygous with the *E93*^{1} allele (Figure 2D).

In order to gain a better understanding of the developmental defects associated with *E93* mutations, animals were staged 24 hr following puparium formation, embedded in paraffin, and sectioned. At this developmental stage, control animals have formed adult structures, including eyes and wings, and the larval salivary glands have been completely destroyed (Figure 2E). In contrast, *E93* mutants possess persistent larval salivary glands, even though adult structures have formed, including

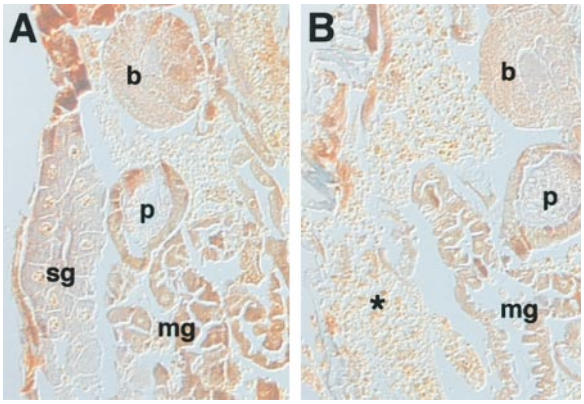

Figure 3. Expression of *E93* Rescues Salivary Gland Cell Death in *E93* Mutants

(A) Section of a *y w fkh-GAL4*; *E93*^{1}/*Df(3R)93F*^{X2} control animal, all of which possess larval salivary glands (sg).

(B) Section of a *y w UAS-E93(1)*/*y w fkh-GAL4*; *E93*^{1}/*Df(3R)93F*^{X2} animal. Expression of an *E93* transgene under the control of a salivary gland promoter (*fkh-GAL4*) rescues salivary gland cell death. Fat body is detected in the position previously occupied by the larval salivary gland (asterisk). The brain (b), proventriculus (p), and midgut (mg) are indicated.

eyes and wings (Figure 2F). This defect in salivary gland death is completely penetrant in all three *E93* mutant alleles ($n > 500$). Furthermore, mutant salivary glands can be detected for days after they would normally be destroyed. In addition to the salivary gland defect, *E93* mutants display defects in larval midgut destruction (C.-Y. L. and E. H. B., unpublished data). The observation that salivary gland cell death is blocked in *E93* mutants while adult head eversion occurs normally indicates that these animals have progressed through the ecdysone-regulated prepupal–pupal transition with specific defects in the destruction of larval cells.

Expression of *E93* Rescues Salivary Gland Cell Death in *E93* Mutants

If the defects in salivary gland cell death are caused by mutations in *E93*, then we should be able to rescue this phenotype by ectopic expression of *E93*. For this purpose, we established a transgenic fly stock in which the *E93* gene is under control of the yeast GAL4 upstream activation sequence (Brand and Perrimon, 1993). This *UAS-E93* construct was combined with a *GAL4* transgene expressed in salivary glands in an *E93*^{1}/*Df(3R)93F*^{X2} genetic background. In all cases examined ($n = 23$/genotype), *E93* mutants that carry the *UAS-E93* transgene lacked salivary glands, while sibling controls that lack the transgene possess salivary glands (Figures 3A and 3B). This observation indicates that *E93* is required in the salivary gland for its appropriate programmed cell death response.

E93 Expression Foreshadows Steroid-Triggered Larval Cell Death

Previous studies indicated that *E93* encodes a novel protein with no matches in the sequence databases (Baehrecke and Thummel, 1995). In addition, *E93* transcription is induced in the larval midgut and salivary glands immediately before the onset of programmed cell death (Baehrecke and Thummel, 1995). To gain further

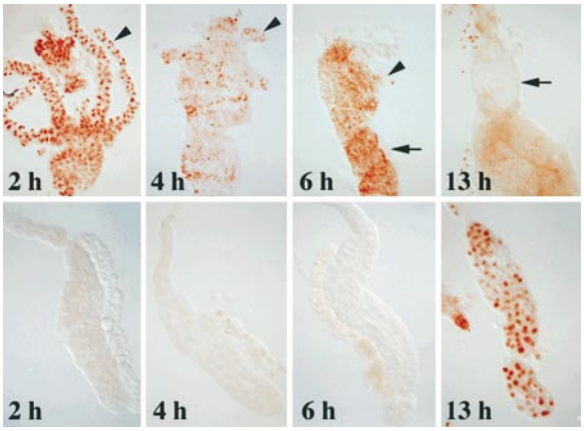

Figure 4. E93 Protein Is Expressed in Larval Midguts and Salivary Glands Prior to Programmed Cell Death

Larval midguts (top row) and salivary glands (bottom row) were dissected from wild-type Canton S prepupae and pupae at the indicated hours following puparium formation, fixed, and stained with antibodies to detect E93 protein. E93 is restricted to the nuclei of polytenized larval cells that are fated to die. Expression of E93 in midguts increases following the late larval pulse of ecdysone and foreshadows the shortening of the gastric caeca (arrowheads), persists in the dying larval cells 6 hr following puparium formation, and is not detected in the developing adult midgut epithelia (arrows). E93 protein is not detected in larval salivary glands of 2–6 hr prepupae but is highly expressed following the rise in ecdysone titer 10–12 hr after puparium formation.

insight into the cellular distribution and potential biochemical function of this protein, we raised antibodies against E93. Staged midguts and salivary glands were stained with affinity-purified E93 antibodies to determine the spatial and temporal patterns of E93 expression (Figure 4). E93 is not detected in the midguts of late third instar larvae (data not shown) but is expressed in the midguts of newly formed prepupae, paralleling the induction of *E93* mRNA at puparium formation (Baehrecke and Thummel, 1995). E93 is detected immediately prior to the destruction of midgut gastric caeca and midgut shortening, both of which coincide with the onset of programmed cell death (Jiang et al., 1997). Interestingly, E93 is not expressed in the diploid cells that form the adult midgut epithelium (Figure 4). Larval salivary glands do not express E93 in early and mid prepupae (Figure 4). Following the pulse of ecdysone in 10–12 hr prepupae, however, E93 expression is induced in salivary glands (Figure 4), reflecting the induction of *E93* mRNA at this time (Baehrecke and Thummel, 1995) and foreshadowing programmed cell death (Jiang et al., 1997). E93 is not expressed in leg and wing imaginal discs during prepupal development but is detected in a subset of cells in the developing eye and central nervous system (data not shown). These results are consistent with our hypothesis that E93 is expressed in dying cells, as the eye and central nervous system undergo programmed cell death at this developmental stage (Wolff and Ready, 1991; Truman et al., 1994). In addition, E93 is restricted to the nucleus, suggesting that it might regulate gene expression.

E93 Binds to Chromosome Sites Containing Ecdysone-Regulated Genes and Programmed Cell Death Genes

The nuclear localization of E93 in larval salivary glands provided an opportunity to determine if E93 binds to

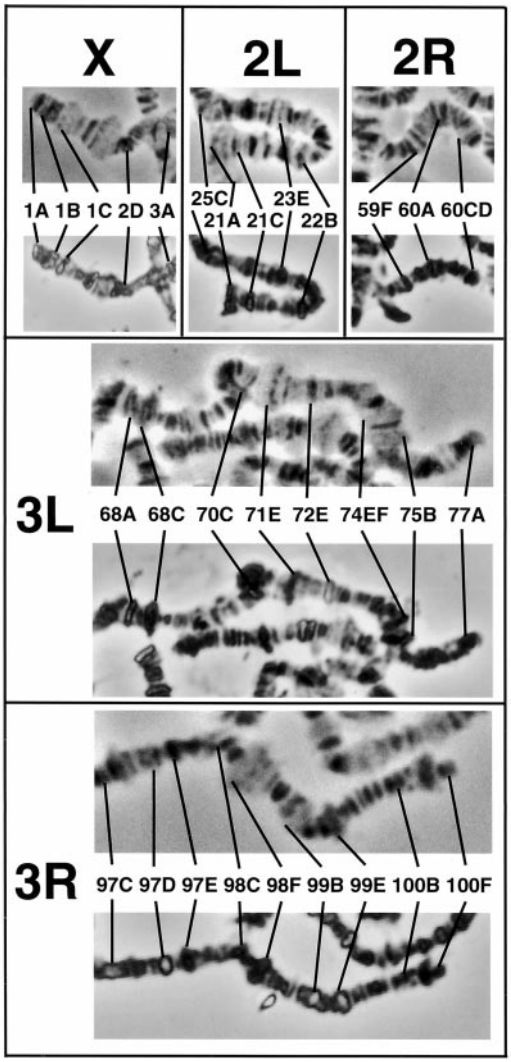

Figure 5. E93 Binds to Specific Sites on Salivary Gland Polytene Chromosomes

Larval salivary glands were dissected from wild-type Canton S prepupae 12–14 hr after puparium formation, fixed, squashed, and photographed to obtain accurate cytology for mapping. Chromosome spreads were then stained with affinity-purified E93 antibodies to determine the sites bound by E93 protein. Representative regions of each chromosome arm are presented, with the top panel depicting the region prior to antibody staining, and the lower panel depicting the region after antibody staining. The brown and gold refractile stains represent sites bound by E93 protein. A list of reproducibly bound stained sites is presented in Table 1.

the salivary gland polytene chromosomes and, if so, to identify the sites bound by the protein. Salivary glands were dissected 12–14 hr after puparium formation, fixed, squashed, and photographed to acquire accurate cytology of the banding and puffing patterns for mapping. The chromosomes were then stained with affinity-purified E93 antibodies, and these patterns were compared with the original set of photographs to allow accurate mapping of the bound sites. E93 clearly bound to the polytene chromosomes in a reproducible and site-specific manner and was consistently detected at 65 chromosome sites (Figure 5; Table 1), many of which contain ecdysone-regulated genes or programmed cell death

Table 1. Polytene Chromosome Loci Stained with E93 Antibodies

X	2L	2R	3L	3R
1A	21A	43A	61A	85D[a]
1B[b]	21C[ab]	47A[a]	61C[a]	92A[a]
1C[a]	22B[a]	49B[a]	61D	93F[a]
2D	23E[a]	49E[a]	61F	97A
3A[a]	25C[a]	50C	62A[a]	97C[a]
3C[a]	27C[a]	50CD[a]	62D	97D
4C	28A[a]	51A	63C	97E
5A	29E	59F[b]	64A[a]	98C
6D	30A[a]	60A	65C	98F[a]
7E		60CD	66D	99B[ab]
8E[a]			68A	99E[a]
9A			68C[a]	100B
9E			70C[a]	100F
13E[a]			71E[a]	
14B[a]			72E	
			74EF[a]	
			75B[a]	
			77A	

Only sites that could be mapped in at least two independent preparations are listed. Most listed sites stain strongly.
[a] Ecdysone-regulated puff loci (Ashburner, 1972).
[b] Loci containing programmed cell death genes (Chen et al., 1998; Franc et al., 1996; Fraser and Evan, 1997; Song et al., 1997).

genes. Among these sites are the 74EF and 75B early puffs, which contain the *E74* and *E75* ecdysone-inducible genes, as well as the 93F puff, which contains *E93*. In addition, 1B, 21C, 59F, and 99B are bound by E93 and contain the programmed cell death genes *dredd*, *crq*, *dcp-1*, and *drICE*, respectively. The 2B5 early puff, containing the *BR-C* ecdysone-inducible gene, and 75CD, containing β*FTZ-F1* and the programmed cell death genes *rpr*, *hid*, and *grim*, were not bound by E93. These data indicate that E93 may directly regulate the genes in bound chromosome loci and may either encode a site-specific DNA binding protein or a chromatin-associated protein that functions as a transcriptional regulator.

Transcription of Steroid-Regulated and Programmed Cell Death Genes Is Reduced or Absent in *E93* Mutants

The observations that *E93* is essential for salivary gland cell death and that E93 protein binds to specific sites in the salivary gland polytene chromosomes suggest that *E93* may regulate the transcription of target genes that function in steroid-triggered programmed cell death. If this hypothesis is true, then *E93* mutations should impact the transcription of genes that reside in salivary gland chromosome loci bound by E93. Salivary glands were dissected from staged late third instar larvae, prepupae, and pupae of control and mutant animals. Total RNA extracted from these tissues was analyzed by Northern blot hybridization. *E93* mutations had little or no effect on the timing and levels of *BR-C*, *E74*, and *E75A* transcription in the salivary glands of late third instar larvae and early prepupae (Figure 6). However, the level of expression of each of these regulatory genes was significantly reduced or absent in salivary glands 10–24 hr following puparium formation (Figure 6). Although the smaller *E74B* transcript is induced, the larger *E74A* RNA is not detected following the prepupal pulse of ecdysone. The levels of *EcR* expression in late third instar larval and prepupal salivary glands are not altered by *E93* mutations, although its timing is delayed by 4–6 hr at the prepupal to pupal transition (Figure 6). Like *EcR*, β*FTZ-F1* transcription was delayed but the level of this mRNA was not altered in *E93* mutant salivary glands (Figure 6). A similar delay was observed in the parental flies that were used for mutagenesis, indicating that this effect is due to the genetic background (data not presented). The induction of *EcR* and *E74B* in *E93* mutant prepupae, as well as the successful completion of adult head eversion, indicates that the prepupal pulse of ecdysone occurs in these mutant animals, signaling the prepupal–pupal transition.

E93 mutant salivary glands also exhibited little or no transcription of genes that play a key role in programmed cell death. As reported previously, *rpr* and *hid* are induced in control animals in a stage-specific manner, immediately preceding the onset of salivary gland cell death (Figure 6). Interestingly, the relative of the vertebrate *CD36* gene named *croquemort* (*crq*), *ark*, and the caspase *dronc* were also induced at this time, indicating that other components of the apoptotic signaling pathway are utilized during programmed cell death in salivary glands (Figure 6). Transcription of the caspases *dredd*, *dcp-1*, and *drICE* were not detected in salivary glands at these developmental stages (data not shown). The cell death genes *rpr*, *hid*, *crq*, *ark*, and *dronc* are transcribed at reduced levels in *E93* mutant salivary glands 12–24 hr following puparium formation (Figure 6). These observations indicate that *E93* functions as a key regulator by specifying the steroid activation of cell death genes.

Expression of E93 Is Sufficient to Induce Programmed Cell Death

The expression of E93 protein in dying cells, combined with the defects in *E93* mutant salivary gland cell death and transcription of apoptosis genes, indicates that *E93* is a key determinant of steroid-induced programmed cell death. Therefore, we tested if expression of E93 is sufficient to kill wing imaginal disc cells that have a well-defined response to ecdysone during metamorphosis. We initiated this experiment by crossing *UAS-E93* transformant flies with *Drosophila* strains that express GAL4 in wing imaginal discs (*vg-GAL4*). All progeny that possess both *UAS-E93* and *vg-GAL4* die at the start of pupal development (n = 1000). This lethal phase, combined with the wealth of information about ecdysone-triggered wing development (Fristrom and Fristrom, 1993) led us to detailed characterization of *E93*-induced cell death in *vg-GAL4 UAS-E93* animals. Control (*UAS-E93* alone or *vg-GAL4* alone) wing-thoracic imaginal discs dissected 2 hr following puparium formation exhibit little cell death (Figure 7A), consistent with previous reports (Milan et al., 1997). E93-expressing wing-thoracic imaginal discs, by contrast, exhibit extensive cell death in the wing blade and hinge regions at this developmental stage (Figure 7B). This pattern of cell death mimics the pattern of GAL4 expression in this *vg-GAL4* strain of *Drosophila*, as determined by crossing *vg-GAL4* flies with a *UAS-lacZ* reporter and detecting β-galactosidase activity (data not shown). Animals expressing E93 under the control of *vg-GAL4* die soon after head eversion during metamorphosis and exhibit defects in the presumptive adult notum and wing (data not shown). We examined these animals further by characterizing the

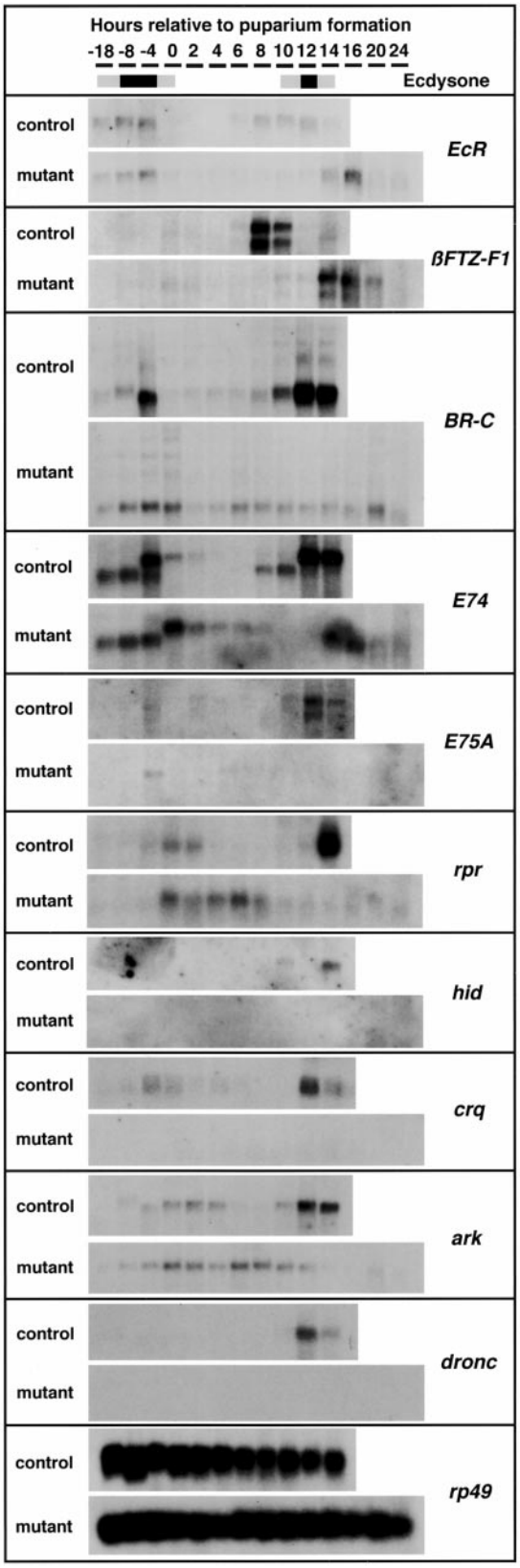

Figure 6. *E93* Mutant Salivary Glands Exhibit Defects in the Transcription of Ecdysone-Regulated Genes and Programmed Cell Death Genes

Larval salivary glands were dissected from *E93*^{1}/wild-type and *E93*^{3}/*E93*^{3} mutants staged as mid third instar larvae (−18), late third instar larvae (−8, −4), or at different times following puparium formation, as indicated at the top. Total RNA was extracted from these glands and analyzed by Northern blot hybridization. The late larval and prepupal peaks in ecdysone titer are depicted schematically at the top. Control salivary glands undergo programmed cell death 15–16 hr after puparium formation, preventing the collection of later time points. The blots were hybridized with probes to detect the ecdysone-regulated genes *EcR*, β*FTZ-F1*, *BR-C*, *E74*, and *E75A*, and the cell death regulators *rpr*, *hid*, *crq*, *ark*, and *dronc*. Hybridization with *rp49* was used as a control for loading and transfer.

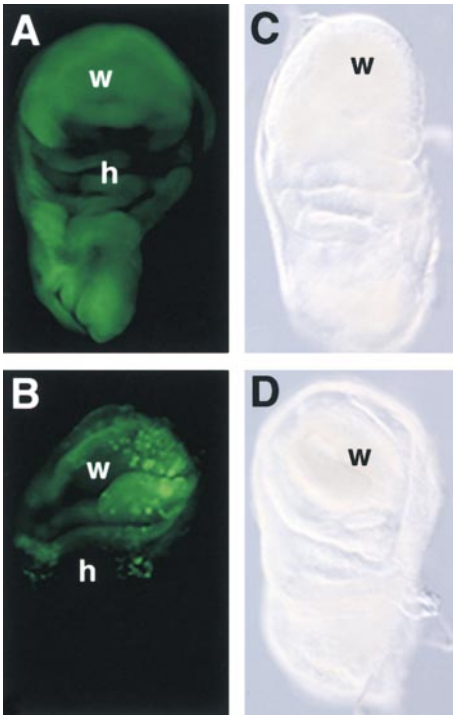

Figure 7. Ectopic Expression of E93 Is Sufficient to Induce Programmed Cell Death

(A) Control 2 hr prepupal wing imaginal discs (*vg-GAL4* or *UAS-E93*, *UAS-E93* is shown) exhibit no cell death as indicated by a lack of nuclear acridine orange staining at this stage.

(B) Two hour prepupal wing discs of *Drosophila* that contain both *vg-GAL4* and *UAS-E93* always possess large amounts of ectopic cell death in the wing (w) and hinge (h) regions of this tissue as indicated by nuclear acridine orange staining (green spots).

(C) Control 4 hr prepupal wing discs undergo normal changes associated with morphogenesis, including wing (w) elongation.

(D) Wing discs of 4 hr prepupae that contain both *vg-GAL4* and *UAS-E93* exhibit defects in wing morphogenesis, such that the wing (w) does not elongate and appears to fold back upon itself.

morphology of wing imaginal discs prior to the lethal phase but after the induction of ectopic cell death. While control wing-thoracic imaginal discs dissected from animals 4 hr following puparium formation have clearly progressed in elongation of the wing (Figure 7C), E93-expressing animals of the same age possess defective wing-thoracic imaginal discs that do not properly elongate (Figure 7D). These data demonstrate that expression of E93 is sufficient to induce programmed cell death.

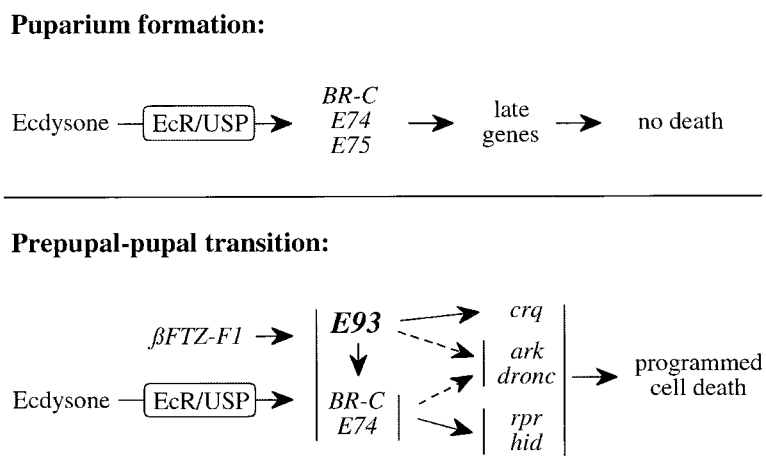

Figure 8. A Model for Ecdysone Triggered Salivary Gland Programmed Cell Death

Ecdysone induces early gene transcription through its interaction with the EcR/USP heterodimer. At early stages, the ecdysone receptor complex induces the *BR-C*, *E74*, and *E75* early genes that, in turn, regulate late genes that do not trigger cell death. At the prepupal–pupal transition, the competence factor β*FTZ-F1* facilitates ecdysone induction of the *BR-C*, *E74*, *E75*, and *E93* early genes. *E93* is essential for the proper transcription of the *BR-C*, *E74*, and *E75* genes, and the cell death regulators *rpr*, *hid*, *crq*, *ark*, and *dronc*. The dashed arrows indicate possible regulatory links that have not yet been tested. See text for more details.

Discussion

Steroid hormones coordinate a wide range of biological responses, including metabolic activity, growth, differentiation, and programmed cell death. The hormone and its receptor, however, are present throughout the animal and at many stages of development, raising the question of how this systemic signal is refined into distinct stage- and tissue-specific responses. Here we show that the precise temporal and spatial patterns of E93 induction by the steroid hormone ecdysone determines the biological fate of those target tissues, directing the massive programmed cell death of the larval salivary glands during metamorphosis. We also provide evidence that E93 acts both directly and indirectly to regulate the transcription of key effector genes that drive the cell death response.

E93 Is a Stage-Specific Regulator of Steroid-Triggered Cell Death

Initial studies of the ecdysone-triggered gene cascades speculated that early ecdysone-induced regulatory genes might be expressed in a tissue-specific manner, directing the different fates of larval and adult cells during metamorphosis (Thummel et al., 1990). In contrast, molecular characterization of the *BR-C*, *E74*, and *E75* early genes demonstrated that these genes are widely expressed throughout the animal (Boyd et al., 1991; Emery et al., 1994; T. Watanabe, personal communication). Localization of EcR and BR-C protein isoforms revealed that they are expressed in subsets of ecdysone target tissues; however, these expression patterns do not correlate with sets of tissues that undergo one fate in response to ecdysone (Talbot et al., 1993; Emery et al., 1994). Similarly, studies of *EcR*, *usp*, *BR-C*, and *E74* mutants have revealed multiple functions for these genes, affecting the development of both larval and adult cells during metamorphosis (Restifo and White, 1992; Fletcher et al., 1995; Bender et al., 1997; Hall and Thummel, 1998; Jiang et al., 2000). These observations led to the "tissue coordination model," which proposed that overlapping combinations of early ecdysone-induced transcription factors dictate the proper tissue-specific responses to ecdysone pulses during development (Thummel et al., 1990).

E93 stands in sharp contrast to these widely expressed early genes. *E93* expression is restricted to metamorphosis, with induction in the midguts of newly formed prepupae preceding induction in the salivary glands of late prepupae. The temporal correlation of this expression pattern with the onset of midgut and salivary gland cell death raised the possibility that *E93* might play a role in regulating the death response (Baehrecke and Thummel, 1995). Here we provide strong evidence in support of this model. Antibody stains show that E93 is induced in a cell type–specific pattern in the larval midguts, restricted to the polytene larval cells that are fated to die, and excluded from the diploid imaginal cells that form the adult gut (Figure 4). Similarly, E93 protein expression in the salivary gland parallels that of its mRNA, immediately preceding cell death. *E93* mutants die as pupae with persistent salivary glands, and this salivary gland cell death defect can be rescued by E93 expression from a transgene (Figures 2 and 3). Moreover, ectopic E93 expression is sufficient to direct a death response (Figure 7). Thus, the ecdysone induction of *E93* defines the fate of that tissue, directing its immediate and massive destruction through programmed cell death. E93 regulation therefore provides a molecular mechanism for refining the systemic ecdysone signal into a specific biological response during development.

E93 Encodes a Novel Regulator of Gene Expression

Although *E93* encodes a novel protein with little similarity to other proteins in the sequence databases, it shares several characteristics of *Drosophila* transcription factors. These include homopolymeric tracts of acidic amino acids that can serve as transcriptional activation domains, a potential nuclear localization signal, and two basic domains that could serve as DNA binding motifs (Baehrecke and Thummel, 1995). E93 is localized to nuclei (Figure 4) and binds to specific sites on polytene chromosomes (Figure 5; Table 1), further suggesting that *E93* regulates gene activity. Significantly, *E93* mutants impact the transcription of genes from chromosome loci that are bound by E93 protein (Figure 6). While these data do not provide conclusive evidence that distinguish the biochemical characteristics of DNA and chromatin binding, these results are consistent with the hypothesis that E93 encodes a novel transcription regulator.

E93 Is Required for Induction of the Death Program in Doomed Larval Salivary Glands

E93 appears to exert its effects by both directly and indirectly regulating genes required for programmed cell

death (Figure 8). Prior to the prepupal stage, ecdysone triggers regulatory hierarchies that do not result in salivary gland cell death. In late third instar larvae, for example, the ecdysone receptor complex activates the primary response genes *BR-C*, *E74*, and *E75*. These early genes, in turn, direct a switch in salivary gland late gene expression, repressing the glue genes and inducing more than 100 late genes including the *L71* genes (Figure 8). The following pulse of ecdysone, at the end of prepupal development, triggers the *BR-C*, *E74*, *E75*, and *E93* early genes. This response is dependent on the prior expression of the β*FTZ-F1* competence factor, which is necessary and sufficient for early gene induction in late prepupae (Woodard et al., 1994; Broadus et al., 1999). As expected, β*FTZ-F1* is expressed at a normal level in *E93* mutants with a delay due to genetic background (Figure 6). In contrast, *E93* is required for ecdysone induction of the *BR-C*, *E74A*, and *E75A* genes in prepupal salivary glands (Figure 6). *E93* mutants do not impact *EcR* and *E74B* transcription (Figure 6) and head eversion (Figure 2), indicating that the prepupal pulse of ecdysone is normal in these animals. Thus, the effect of *E93* mutants on transcription of early genes is not caused by the absence of ecdysone. E93 protein binds to the 74EF and 75B puffs that contain the *E74* and *E75* genes (Figure 5; Table 1), suggesting that these are direct regulatory targets. E93 does not bind to the 2B5 puff containing the *BR-C* gene, suggesting that this regulation is indirect (Figure 8).

Several cell death genes are transcribed immediately prior to larval salivary gland programmed cell death (Figure 6). Components of the core apoptosis machinery, including *ark* and the caspase *dronc*, as well as the death genes *rpr*, *hid*, and *crq*, increase in transcription in late prepupal salivary glands. The synchronous induction of these cell death genes indicates that salivary glands die by a mechanism that is similar to that utilized in apoptosis during *Drosophila* embryogenesis, where *rpr* and *hid* are involved in caspase activation. The increase in *crq* transcription in dying salivary glands suggests that these cells are unique, however, since CRQ is expressed in phagocytes and functions in removal of dying cells during embryogenesis (Franc et al., 1999). This increase in *crq* transcription is not due to the adhesion of phagocytes to the dying salivary gland, as CRQ protein is expressed at a high level in the dying cells (C.-Y. L. and E. H. B., unpublished data). While salivary gland cell destruction involves genes that function in apoptosis, these cells also have characteristics of autophagy (C.-Y. L. and E. H. B., unpublished data), and this form of cell death may utilize *crq* in the terminal stages of cell removal.

E93 mutants impact transcription of cell death genes, consistent with the model that *E93* serves as a regulator that specifies the cell death response to ecdysone. *E93* mutants exhibit defects in transcription of *rpr*, *hid*, *crq*, *ark*, and *dronc* (Figure 6). The 75C locus that contains *rpr* and *hid* and the 53F locus that contains *ark* are not bound by E93 protein (Figure 5; Table 1), suggesting that the regulation of these genes is indirect (Figure 8). Recent studies of *rpr* and *hid* regulation support this conclusion, as mutations in *BR-C* and *E74A* alter *rpr* and *hid* RNA levels (Jiang et al., 2000), and *E93* is required for *BR-C* and *E74A* expression (Figure 6). The 21C locus, which contains *crq*, was bound by E93 protein (Figure 5; Table 1), suggesting that *E93* may directly regulate *crq* transcription. Thus, *E93* plays an essential role in regulating cell death genes, thereby directing steroid-triggered programmed cell death.

This study demonstrates that components of the central cell death pathway, including *ark* and *dronc*, exhibit dynamic changes in RNA transcription that immediately precede salivary gland cell death. While our model emphasizes regulation at the level of transcription, it is important to consider that many of these factors may also be regulated at the posttranscriptional level. For example, *rpr* and *hid* direct programmed cell death during *Drosophila* embryogenesis by repressing the inhibitory activity of DIAP1 on caspase activation (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). Thus, while *rpr*, *hid*, and *dronc* are regulated by the ecdysone-induced primary response genes at the transcriptional level, *dronc* may also be regulated by a secondary mechanism. Future studies of the genetic pathways that mediate steroid-regulated destruction of larval salivary glands will provide further insights into the conserved molecular mechanisms that underlie cell death during development.

Experimental Procedures

Isolation of *E93* Mutants

The smallest exisiting deficiency that removes *E93* is *Df(3R)e*^{BS2}, which deletes from 93B-93F14, encompassing the 93F9-10 locus that contains *E93*. We isolated a smaller deficiency in the 93F locus by irradiating males that contain a P element with a w^+ eye color marker that is inserted 30 kb from the 3′ end of *E93* with a ^{137}Cs source (Baehrecke, 1997). This resulted in the isolation of *Df(3R)93F*^{X2}, which spans from 93F5 to 94A8 as determined by polytene chromosome squashes. We isolated *E93* mutants by mutagenizing isogenized *ru h th st cu sr e ca* males with either 25 or 37 mM EMS following previously described methods (Lewis and Bacher, 1968). These flies were mated with *TM3 Sb e/TM6B Hu Tb e* virgin females, and the male progeny from this cross were pair mated to *Df(3R)93F*^{X2}*/TM6B Hu Tb e* virgin females. The progeny of this cross were screened for lethality and adult phenotypes. After screening 11,132 lines, 11 lethal complementation groups were isolated that reside within the interval between 93F5 and 93F14, as defined by the deficiencies *Df(3R)e*^{BS2} and *Df(3R)93F*^{X2}.

Characterization of *E93* Mutants

The 11 lethal complementation groups in the 93F region were analyzed for late larval, prepupal, and pupal lethality after crossing these putative *E93* mutants/*TM6B Hu Tb e* to *Df(3R)93F*^{X2}*/TM6B Hu Tb e*. This was accomplished by distinguishing putative *E93* mutant/*Df(3R)93F*^{X2} from siblings containing the balancer chromosome *TM6B Hu Tb e* using the dominant larval and pupal marker *Tb*. Markers adjacent to *E93* were removed from mutants by recombination with wild-type chromosomes. The *E93* open reading frame and intron boundaries were sequenced from genomic DNA of *E93* mutants and the parental strain that was used for mutagenesis as previously described (Baehrecke, 1997). The lethal phase and phenotype of *E93* homozygous mutants, heteroallelic combinations, and controls were determined by staging animals in hours relative to puparium formation at 25°C, inspecting whole animal morphology using a Zeiss SV11 stereomicroscope, and determining the stage of developmental arrest. Animals were fixed at the stage of developmental arrest, embedded in paraffin, sectioned, and stained as described previously (Restifo and White, 1992). Images of sections were collected using a Zeiss Axiophot 2 microscope.

E93 Germline Transformants and Rescue

The *UAS-E93* transgene was constructed by polymerase chain reaction (PCR) amplification of the 5′ end of the *E93* open reading frame (ORF), utilizing cDNA as a template (Baehrecke and Thummel, 1995). A 5′ PCR primer containing an NdeI restriction site that encodes an AUG triplet followed by 13 bases of *E93* coding sequence was used in combination with a 3′ primer on the opposite side of the first XhoI

site in the *E93* ORF to amplify the 513 base pair 5′ end of the ORF. This fragment was combined with the remainder of the *E93* ORF in pBS-KS(−). The sequence of the ORF was confirmed by DNA sequencing. This plasmid was linearized with NdeI, the 3′ recessed termini of the NdeI site were filled with the Klenow fragment of DNA polymerase I, and a BglII linker was ligated to the 5′ end of the ORF. Following ligation and restriction digestion with BglII and KpnI, the entire ORF was inserted into the BglII and KpnI sites of pUAST (Brand and Perrimon, 1993). P element–mediated germline transformation of this construct into w^{1118} *Drosophila melanogaster* was performed following standard procedures (Rubin and Spradling, 1982). After obtaining a single homozygous viable *UAS-E93* transformant on the X chromosome, we mobilized this transgene to generate 12 transformant lines. Lines were tested for the ability to induce a specific pattern of E93 protein expression during embryogenesis (a stage when E93 is not expressed) using the GAL4 system (Brand and Perrimon, 1993)—all exhibited similar abilities to induce E93 protein expression. Thus, all studies reported were conducted using the *UAS-E93(1)* transformant that contains a homozygous viable insertion on the X chromosome.

To rescue homozygous *E93* mutant salivary gland destruction, *fkh-GAL4* transgenic flies that express GAL4 in salivary glands were used to drive expression of the *UAS-E93(1)* transgene. Virgin females of the genotype *y w fkh-GAL4*/*y w fkh-GAL4*; $Df(3R)93F^{X2}$/*TM6B Hu Tb e* were crossed to *y w UAS-E93(1)*; $E93^{1}$/*TM6B Hu Tb e* males. Male progeny of this cross all lack the *UAS-E93(1)* transgene, all progeny contain the *fkh-GAL4* activator transgene, and $E93^{1}/Df(3R)93F^{X2}$ mutants can be distinguished based on the absence of the dominant *Tb* larval/pupal marker on the *TM6B Hu Tb e* balancer chromosome. Thus, male *y w fkh-GAL4*; $E93^{1}/Df(3R)93F^{X2}$ served as controls, and female *y w UAS-E93(1)*/*y w fkh-GAL4*; $E93^{1}$/$Df(3R)93F^{X2}$ animals were analyzed for rescue of salivary gland destruction. Animals were genotyped and sexed as late third instar larvae. Control and rescue animals were either dissected and analyzed for the presence of salivary glands, or fixed, embedded in paraffin, and sectioned as described (Restifo and White, 1992).

E93 Antibody Preparation and Stains of Tissues and Polytene Chromosomes

A bacterial plasmid was constructed by inserting the PstI fragment that encodes amino acids 279–837 of E93 (Baehrecke and Thummel, 1995) into pUR292 (Ruther and Muller-Hill, 1983). The resultant E93/β-galactosidase fusion protein was expressed in *E. coli* and purified from inclusion bodies (Rio et al., 1986). Rats were immunized five times with 400 μg of protein at 3 week intervals, and antisera were screened using standard procedures. E93 antibodies were affinity purified as described (Carroll and Laughon, 1987).

Wild-type Canton S animals were raised at 25°C and were staged in hours relative to puparium formation. Staged tissues were dissected and immunostained as described previously (Boyd et al., 1991). For polytene chromosome antibody stains, salivary glands were dissected 12–14 hr following puparium formation, fixed, squashed, and photographed to obtain accurate cytology for mapping. These spreads were then stained with affinity-purified E93 antibodies, following previously described methods (Zink and Paro, 1989; Urness and Thummel, 1990).

Northern Blot Analysis

For analysis of *E93* transcription in *E93* mutants, total RNA was isolated from $E93^{1}$/Canton S control, $E93^{2}/E93^{2}$, and $E93^{3}/E93^{3}$ whole animals. Larvae, prepupae, and pupae were staged, RNA was extracted and analyzed by Northern blot hybridization as previously described (Andres and Thummel, 1994; Baehrecke and Thummel, 1995). Control and mutant Northern blots were cohybridized with probes to detect *E93* and *rp49* as a loading and transfer control. All probes were prepared by random labeling gel-purified DNA fragments (Stratagene Prime-It). Exposures of control and mutant Northern blots were normalized based on the time required for equal detection of *rp49*.

To determine the impact of *E93* mutations on target gene transcription in larval salivary glands, total RNA was isolated from $E93^{1}$/Canton S control and $E93^{3}/E93^{3}$ salivary glands. Total RNA was extracted from salivary glands dissected from staged larvae, prepupae, and pupae, and analyzed by Northern blot hybridization. Control and mutant Northern blots were hybridized at the same time with probes to detect the ecdysone-regulated genes *EcR*, β*FTZ-F1*, *BR-C*, *E74*, and *E75A*, the cell death genes *rpr*, *hid*, *crq*, *ark*, and *dronc*, and *rp49* as a loading and transfer control. Exposures of control and mutant Northern blots were normalized based on the time required for equal detection of *rp49*.

E93 Induction of Programmed Cell Death

E93 was ectopically expressed in wing imaginal discs by crossing *y w UAS-E93(1)*/*y w UAS-E93(1)*; *CyO*/*Sco*; *TM2 Ubx*/*Sb* virgin females and males of the genotype *y w*; *vg-GAL4*/*vg-GAL4* (transgenic flies were constructed by the laboratory of F. M. Hoffman). Embryos of this cross were collected overnight, allowed to develop on standard medium, and the number of eclosing adults were counted. In addition, prepupae expressing *E93* in wings (and parental controls) were dissected at 2 hr intervals following puparium formation for analyses of *E93*-induced cell death and phenotypes. Programmed cell death was detected by acridine orange staining following previously described methods (Abrams et al., 1993).

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References

Abrams, J.M. (1999). An emerging blueprint for apoptosis in *Drosophila*. Trends Cell Biol. *9*, 435–440.

Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322–1326.

Abrams, J.M., White, K., Fessler, L.I., and Steller, H. (1993). Programmed cell death during *Drosophila* embryogenesis. Development *117*, 29–43.

Andres, A.J., and Thummel, C.S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. In *Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology, L. Goldstein and E. Fyrberg, eds. (New York: Academic Press), pp. 565–573.

Ashburner, M. (1972). Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. VI. Induction by ecdysone in salivary glands of *D. melanogaster* cultured in vitro. Chromosoma *38*, 255–281.

Ashburner, M., Chihara, C., Meltzer, P., and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. Cold Spring Harb. Symp. Quant. Biol. *38*, 655–662.

Baehrecke, E.H. (1997). *who* encodes a KH RNA binding protein that functions in muscle development. Development *124*, 1323–1332.

Baehrecke, E.H., and Thummel, C.S. (1995). The Drosophila E93 gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. Dev. Biol. *171*, 85–97.

Becker, H.J. (1959). Die puffs der speicheldrüsenchromosomen von *Drosophila melanogaster*. I. Beobachtungen zur verhalten des puffmusters in normalstamm und bei zwei mutanten, *giant* und *lethal-giant-larvae*. Chromosoma *10*, 654–678.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., and Hogness, D.S. (1997). *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell *91*, 777–788.

Bodenstein, D. (1965). The postembryonic development of *Drosophila*. In Biology of *Drosophila*, M. Demerec, ed. (New York: Hafner Publishing Co.), pp. 275–367.

Boyd, L., O'Toole, E., and Thummel, C.S. (1991). Patterns of *E74A* RNA and protein expression at the onset of metamorphosis in *Drosophila*. Development *112*, 981–995.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., and Woodard, C.T. (1999). The *Drosophila* βFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol. Cell *3*, 143–149.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D.S. (1990). The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. Cell *61*, 85–99.

Carroll, S.B., and Laughon, A. (1987). Production and purification of polyclonal antibodies to the foreign segment of β-galactosidase fusion proteins. In DNA Cloning, D.M. Glover, ed. (Oxford: IRL Press), pp. 89–111.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J.M. (1996). *grim*, a novel cell death gene in *Drosophila*. Genes Dev. *10*, 1773–1782.

Chen, P., Rodriguez, A., Erskine, R., Thach, T., and Abrams, J.M. (1998). *Dredd*, a novel effector of the apoptosis activators *Reaper*, *Grim*, and *Hid* in *Drosophila*. Dev. Biol. *201*, 202–216.

Clever, U. (1964). Actinomycin and puromycin: effects on sequential gene activation by ecdysone. Science *146*, 794–795.

Crossgrove, K., Bayer, C.A., Fristrom, J.W., and Guild, G.M. (1996). The *Drosophila Broad-Complex* early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade. Dev. Biol. *180*, 745–758.

Cryns, V., and Yuan, J. (1998). Proteases to die for. Genes Dev. *12*, 1551–1570.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., and Guild, G.M. (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics *129*, 385–397.

Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H., and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. Proc. Natl. Acad. Sci. USA *96*, 4307–4312.

Dorstyn, L., Read, S.H., Quinn, L.M., Richardson, H., and Kumar, S. (1999). DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. J. Biol. Chem. *274*, 30778–30783.

Ellis, R.E., Yuan, J., and Horvitz, R.H. (1991). Mechanisms and functions of cell death. Annu. Rev. Cell Biol. *7*, 663–698.

Emery, I.F., Bedian, V., and Guild, G.M. (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. Development *120*, 3275–3287.

Fletcher, J.C., Burtis, K.C., Hogness, D.S., and Thummel, C.S. (1995). The *Drosophila E74* gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. Development *121*, 1455–1465.

Franc, N.C., Dimarcq, J.-L., Lagueux, M., Hoffmann, J., and Ezekowitz, A.B. (1996). Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity *4*, 431–443.

Franc, N.C., Heitzler, P., Ezekowitz, A.B., and White, K. (1999). Requirement for Croquemort in phagocytosis of apoptotic cells in *Drosophila*. Science *284*, 1991–1994.

Fraser, A.G., and Evan, G.I. (1997). Identification of a Drosophila melanogaster ICE/CED-3-relatedprotease, drICE. EMBO J. *16*, 2805–2813.

Fristrom, D., and Fristrom, J. (1993). The metamorphic development of the adult epidermis. In The Development of *Drosophila melanogaster*, vol. 2, M. Bate and A. Martinez-Arias, eds. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), pp. 843–897.

Goyal, L., McCall, K., Agapite, J., Hartwieg, E., and Steller, H. (2000). Induction of apoptosis by *Drosophila* reaper, hid and grim through inhibition of IAP function. EMBO J. *19*, 589–597.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., and Steller, H. (1995). The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev. *9*, 1694–1708.

Haining, W.N., Carboy-Newcomb, C., Wei, C.L., and Steller, H. (1999). The proapoptotic function of *Drosophila* Hid is conserved in mammalian cells. Proc. Natl. Acad. Sci. USA *96*, 4936–4941.

Hall, B.L., and Thummel, C.S. (1998). The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. Development *125*, 4709–4717.

Hay, B.A., Wassarman, D.A., and Rubin, G.M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell *83*, 1253–1262.

Hengartner, M.O., and Horvitz, H.R. (1994). C. elegans cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. Cell *76*, 665–676.

Igaki, T., Kanuka, H., Inohara, N., Sawamoto, K., Nunez, G., Okano, H., and Miura, M. (2000). Drob-1, a *Drosophila* member of the Bcl-2/CED-9 family that promotes cell death. Proc. Natl. Acad. Sci. USA *97*, 662–667.

Jacobson, M.D., Weil, M., and Raff, M.C. (1997). Programmed cell death in animal development. Cell *88*, 347–354.

Jiang, C., Baehrecke, E.H., and Thummel, C.T. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development *124*, 4673–4683.

Jiang, C., Lamblin, A.-F.J., Steller, H., and Thummel, C.T. (2000). A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. Mol. Cell *5*, 445–455.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H., and Miura, M. (1999). Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. Mol. Cell *4*, 757–769.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239–257.

Lewis, E.B., and Bacher, F. (1968). Methods of feeding ethyl methane sulfonate (EMS) to *Drosophila* males. Dros. Info. Service *43*, 193.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479–489.

Lisi, S., Mazzon, I., and White, K. (2000). Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. Genetics *154*, 669–678.

McCarthy, J.C., and Dixit, V.M. (1998). Apoptosis induced by *Drosophila* Reaper and Grim in a human system. J. Biol. Chem. *273*, 24009–24015.

Milan, M., Campuzano, S., and Garcia-Bellido, A. (1997). Developmental parameters of cell death in the wing disc of *Drosophila*. Proc. Natl. Acad. Sci. USA *94*, 5691–5696.

Restifo, L.L., and White, K. (1992). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle, and gut. Wilhelm Roux's Arch. Dev. Biol. *201*, 221–234.

Rio, D.C., Laski, F.A., and Rubin, G.M. (1986). Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. Cell *44*, 21–32.

Robertson, C.W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. J. Morphol. *59*, 351–399.

Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., and Abrams, J.M. (1999). Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nat. Cell Biol. *1*, 272–279.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science *218*, 348–353.

Ruther, U., and Muller-Hill, B. (1983). Easy identification of cDNA clones. EMBO J. *2*, 1791–1794.

Segraves, W.A., and Hogness, D.S. (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev. *4*, 204–219.

Song, Z., McCall, K., and Steller, H. (1997). DCP-1, a *Drosophila* cell death protease essential for development. Science *275*, 536–540.

Talbot, W.S., Swyryd, E.A., and Hogness, D.S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell *73*, 1323–1337.

Thomas, H.E., Stunnenberg, H.G., and Stewart, A.F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. Nature *362*, 471–475.

Thummel, C.S., Burtis, K.C., and Hogness, D.S. (1990). Spatial and temporal patterns of *E74* transcription during *Drosophila* development. Cell *61*, 101–111.

Truman, J.W., Talbot, W.S., Fahrbach, S.E., and Hogness, D.S. (1994). Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. Development *120*, 219–234.

Urness, L.D., and Thummel, C.S. (1990). Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the *Drosophila* ecdysone-inducible *E74A* protein. Cell *63*, 47–61.

Urness, L.D., and Thummel, C.S. (1995). Molecular analysis of a steroid-induced regulatory hierarchy: the *Drosophila* E74A protein directly regulates *L71–6* transcription. EMBO J. *14*, 6239–6246.

Vaux, D.L., Weissman, I.L., and Kim, S.K. (1992). Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. Science *258*, 1955–1957.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Müller, H.-A.J., and Hay, B.A. (1999). The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. Cell *98*, 453–463.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. Science *264*, 677–683.

White, K., Tahaoglu, E., and Steller, H. (1996). Cell killing by the *Drosophila* gene *reaper*. Science *271*, 805–807.

Wolff, T., and Ready, D.F. (1991). Cell death in normal and rough eye mutants of *Drosophila*. Development *113*, 825–839.

Woodard, C.T., Baehrecke, E.H., and Thummel, C.S. (1994). A molecular mechanism for the stage-specificity of the Drosophila prepupal genetic response to ecdysone. Cell *79*, 607–615.

Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*, 555–556.

Yao, T.-P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). *Drosophila ultraspiracle* modulates ecdysone receptor function via heterodimer formation. Cell *71*, 63–72.

Zhou, L., Song, Z., Tittel, J., and Steller, H. (1999). HAC-1, a *Drosophila* homolog of APAF-1 and CED-4, functions in developmental and radiation-induced apoptosis. Mol. Cell *4*, 745–755.

Zink, B., and Paro, R. (1989). In vivo binding pattern of a transregulator of homeotic genes in *Drosophila melanogaster*. Nature *337*, 468–471.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell *90*, 405–413.