Fork head controls the timing and tissue selectivity of steroid-induced developmental cell death

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Cell death during *Drosophila melanogaster* metamorphosis is controlled by the steroid hormone 20-hydroxyecdysone (20E). Elements of the signaling pathway that triggers death are known, but it is not known why some tissues, and not others, die in response to a particular hormone pulse. We found that loss of the tissue-specific transcription factor Fork head (Fkh) is both required and sufficient to specify a death response to 20E in the larval salivary glands. Loss of *fkh* itself is a steroid-controlled event that is mediated by the 20E-induced *BR-C* gene, and that renders the key death regulators *hid* and *reaper* hormone responsive. These results implicate the *D. melanogaster* FOXA orthologue Fkh with a novel function as a competence factor for steroid-controlled cell death. They explain how a specific tissue is singled out for death, and why this tissue survives earlier hormone pulses. More generally, they suggest that cell identity factors like Fkh play a pivotal role in the normal control of developmental cell death.

Introduction

Programmed cell death (PCD) is essential for the normal development of most, if not all, metazoans. The developmental time at which specific cells or tissues are removed is often specified by the release of systemic or locally acting signaling molecules. During amphibian metamorphosis, for instance, thyroid hormone signals cell death that leads to resorption of the tadpole tail and other larval tissues (Tata, 1994; Shi et al., 2001). During vertebrate limb development, separation of the limb digits requires death of the interdigital regions that is controlled by BMP signaling (Zuzarte-Luis and Hurle, 2005). Although much is known about the temporal aspect of regulation in these and other systems, it is less well understood why some cells and tissues, but not others, die in response to widespread signals (Vaux and Korsmeyer, 1999). A system that is particularly well suited to address this question is the removal of larval tissues by PCD during insect metamorphosis. In particular, the larval salivary glands of *Drosophila melanogaster* have been extensively used to unravel signaling pathways that control developmental cell death (Baehrecke, 2003; Yin and Thummel, 2005).

Death of the larval salivary glands takes place in the early pupa and is triggered by a pulse of the steroid hormone 20-hydroxyecdysone (20E). The salivary glands survive an earlier 20E pulse that leads to the destruction of the larval midgut (Jiang et al., 1997). The two consecutive hormone pulses that trigger these stage-specific responses are referred to in this study as the late-larval and the prepupal 20E pulse (Fig. 7). Salivary gland death is foreshadowed by transcriptional activation of the death genes *reaper* (*rpr*) and *head involution defective* (*hid*; Jiang et al., 2000). The protein products of both genes kill by interfering with caspase inhibition by the *D. melanogaster* inhibitor of apoptosis protein (IAP) 1 (DIAP1). A critical target of DIAP1 is the apical caspase Dronc, which is required for execution of salivary gland death (for review see Kornbluth and White, 2005). The mammalian cell death regulators Smac/Diablo and Omi/HtrA2, which are related to *hid* and *rpr*, act in a similar way by antagonizing IAP function (Du et al., 2000; Verhagen et al., 2000; Hegde et al., 2002). Loss of *hid*, but not *rpr*, leads to salivary gland persistence (Peterson et al., 2002; Yin and Thummel, 2004). However, *rpr* has been shown to synergize with *hid* in bringing about salivary gland death (Yin and Thummel, 2004). Induction of both *hid* and *rpr* requires the up-regulation by 20E of transcription factors encoded by *E93*, *Broad-Complex* (*BR-C*), and *E74A* (Jiang et al., 2000; Lee et al., 2000, 2002). In addition, full induction of *rpr* depends on direct binding of the 20E receptor EcR/Usp to a salivary gland enhancer of the gene (Jiang et al., 2000). Proper expression of the early hormone response genes and salivary gland death require the transient expression of the nuclear receptor βFtz-F1 in mid–prepupae (Broadus et al., 1999). Thus, βFtz-F1 has the properties

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Abbreviations used in this paper: 20E, 20-hydroxyecdysone; APF, after puparium formation; ds, double stranded; *hid*, head involution defective; DIAP, *D. melanogaster* IAP; IAP, inhibitor of apoptosis protein; PCD, programmed cell death.

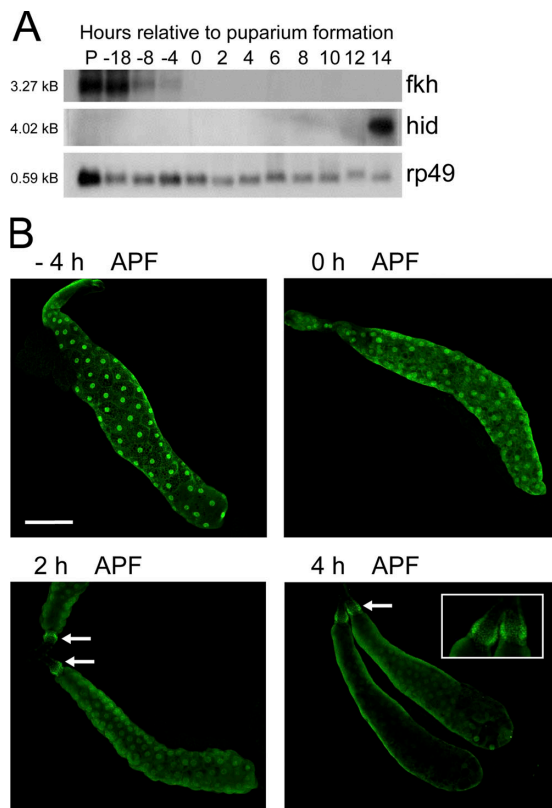The online version of this article contains supplemental material.

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Figure 1. Developmental profile of *fkh* mRNA and protein expression in the larval salivary glands. (A) Northern blot analysis of *fkh* expression during late-larval and prepupal/pupal development. Total RNA was extracted from salivary glands dissected at the indicated times. The 0-h time point corresponds to the time of puparium formation. P designates prewandering third instar larvae. Expression of *hid* at 14 h APF foreshadows histolysis of the salivary glands. Hybridization to detect *rp49* mRNA served as a control for loading and transfer. (B) Salivary glands were dissected at the indicated times and stained with an anti-Fkh antibody. Note that Fkh protein gradually disappears from the salivary gland nuclei, but remains strong in the imaginal rings that will later form the adult salivary glands (arrows and inset). Bar, 250 μm.

of a competence factor for stage-specific hormone signaling (Woodard et al., 1994; Broadus et al., 1999). However, βFtz-F1 expression is observed in almost all larval tissues (Yamada et al., 2000), leaving the question open of how the tissue specificity of salivary gland death is achieved.

Tissue-restricted expression of 20E-regulated genes in the larval salivary glands has been shown to require coregulation by the transcription factor Fork head (Fkh; Lehmann and Korge, 1996; Mach et al., 1996). *fkh* is already expressed in the salivary glands during embryogenesis, and is required for the proper development of this organ (Weigel et al., 1989; Myat and Andrew, 2000). Expression of *fkh* during larval development is restricted to the salivary glands and a small number of other tissues, including the lymph glands and Malpighian tubules (Weigel et al., 1989; Kuzin et al., 1994; Wang et al., 2004). The mammalian counterparts of Fkh are the FOXA1, 2, and 3 proteins (also known as HNF3α, β, and γ; Mazet et al., 2003; Lee and Frasch, 2004), which are members of the larger family of Fkh/HNF or Fox transcription factors (Weigel and Jäckle, 1990; Gajiwala and Burley, 2000; Kaestner et al., 2000). Similar to *fkh*, FOXAs

play a role in specifying tissue-specific responses to steroid signaling, suggesting that aspects of FOXA function are evolutionarily conserved (Friedman and Kaestner, 2006).

In *fkh* mutants, the embryonic salivary glands undergo extensive apoptosis, which is foreshadowed by *rpr* and *hid* expression. Whereas this indicates that the presence of Fkh is required for survival of the embryonic salivary glands, other data suggest that the protein has an independent developmental role in secretory cell invagination (Myat and Andrew, 2000). Thus, *fkh* is part of a long list of developmental genes that cause ectopic cell death when impaired in their function. It has been estimated that nearly 20% of all *D. melanogaster* genes can cause PCD when mutated (White et al., 1994). It is difficult to establish whether these genes normally participate in the control of apoptosis, or whether activation of the default death pathway is an indirect result of aberrant development (Abrams, 1996).

We show that *fkh* plays a key role in specifying a cell death response to steroid signaling during normal development. Fkh is lost from the larval salivary glands at the onset of metamorphosis, and this loss is required for the subsequent steroid-induced removal of the tissue. Ectopic expression of *fkh* rescues the salivary glands and premature knockdown of *fkh* leads to the premature 20E-induced activation of PCD and of the death genes *hid* and *rpr*. Transcription of *fkh* is down-regulated in a *BR-C*–mediated response to the late-larval 20E pulse, followed by a loss of the Fkh protein during prepupal development. These data indicate that Fkh protects the salivary glands from hormone-induced death until a stage-specific, hormone-induced loss of the protein earmarks the tissue for destruction in response to future hormone exposure.

Results

Salivary gland expression of *fkh* ceases at the onset of metamorphosis

We previously showed that *fkh* is transcriptionally down-regulated in the salivary glands in response to the late-larval 20E pulse, and that this response is mediated, at least in part, by the early 20E response gene *BR-C* (Renault et al., 2001). However, it was not clear whether this down-regulation is a transient event followed by a resumption of *fkh* expression, or whether *fkh* expression remains low or absent in prepupal and pupal salivary glands. To resolve this point, we dissected salivary glands from staged larvae, prepupae, and early pupae. Total RNA extracted from these glands was analyzed for *fkh* expression by Northern blot hybridization (Fig. 1 A). Consistent with our earlier results, we found that *fkh* is expressed in the salivary glands of early and mid-third instar larvae (Fig. 1 A, lanes P and -18), and that expression is turned off in an apparent response to the late-larval 20E pulse (Fig. 1 A, lanes -8 and -4). Our Northern analysis did not detect *fkh* mRNA in early prepupae (Fig. 1 A, lane 0), and it did not indicate that expression resumes at any time before the salivary glands die at ∼14 h after puparium formation (APF). These data suggested that Fkh protein is lost from the larval salivary glands before the tissue is removed in response to the prepupal 20E pulse. To test this prediction and to determine the temporal profile of Fkh protein expression at the larval–prepupal
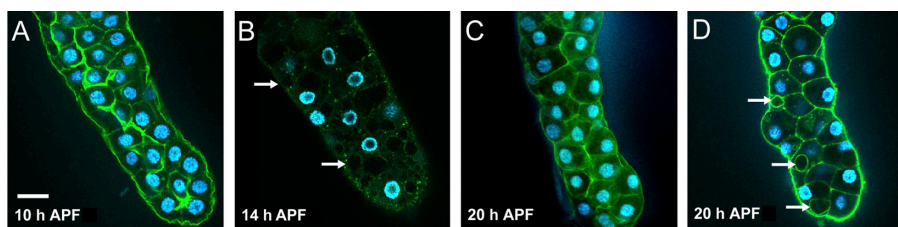
Figure 2. **Forced expression of *fkh* prevents destruction of the larval salivary glands during metamorphosis.** Prepupae of the line P[*hs-Fkh^111*] were heat shocked at 10 h APF to induce ectopic expression of *fkh*, or kept at 25°C as non–heat-shocked controls. The control animals' salivary glands were destroyed at the normal time of ~14 h APF, as they were in heat-shocked *w^1118* animals (not depicted). Salivary glands at the time of *fkh* induction (A), dying salivary glands at 14 hAPF (B), and persistent salivary glands at 20 h APF (C and D) were stained with FITC-conjugated phalloidin and Hoechst 33342 to visualize filamentous actin and the cell nucleus. (C) Example of a salivary gland rescued by *fkh* that did not undergo a major structural change. (D) Example of a salivary gland rescued by *fkh* whose cells have begun to round up and form vacuole-like structures (arrows). Similar structures are formed by dying glands at 14 h APF (arrows). Bar, 100 μm.

transition, we stained salivary glands dissected from staged animals with a Fkh antibody (Fig. 1 B). Strong immunostaining was observed in the cell nuclei at −4 h APF, a time at which *fkh* mRNA has almost disappeared from the salivary glands (Fig. 1 A). Fkh protein is still present in considerable amounts in the glands of freshly formed prepupae (0 h APF). 2 h later, the concentration has greatly diminished, and by 4 h APF the protein is reduced to very low levels. Collectively, these data show that *fkh* is transcriptionally down-regulated in response to the late-larval 20E pulse, and that the protein is still present in the cell nuclei in substantial amounts at the larval–prepupal transition. Subsequently, the protein is lost from the salivary glands during prepupal development.

Loss of Fkh is required for steroid induction of PCD in the salivary glands

We next asked whether the down-regulation of *fkh* might be required for the salivary glands to undergo PCD in response to the prepupal 20E pulse. When *fkh* is ectopically expressed from a heat-inducible transgene in the transformant P[*hs-Fkh^111*], the *Sgs4* gene, which is normally repressed by the late-larval 20E pulse, fails to be down-regulated (Renault et al., 2001). We used the same transgenic line to express *fkh* ectopically at 10 h APF, shortly before the prepupal 20E pulse signals salivary gland destruction. Pupae of the *fkh*-expressing line and heat-shocked control pupae (*w^1118*) were dissected 20 h APF, which is ~6 h after the salivary glands are normally destroyed. All *fkh*-expressing pupae still possessed larval salivary glands at this time (*n* > 37; penetrance = 100%), whereas no salivary glands could be found 20 h APF in any of the heat-shocked *w^1118* control pupae or in non–heat-shocked P[*hs-Fkh^111*] pupae. The structure of the cells and cell nuclei of most rescued salivary glands appeared well preserved, and the overall morphology of the glands was very similar to that of salivary glands before the onset of PCD (Fig. 2). In some of the rescued salivary glands, the cells appeared to be more round in shape and contained large vacuole-like structures, suggesting that the cell death program had been initiated in these glands. However, in contrast to dying cells at 14 h APF, which were largely depleted of filamentous actin, these structures were still supported by portions of a well-developed actin cytoskeleton. At 26 h APF, the number of persisting salivary glands was substantially reduced (11% persistence; *n* = 18), which is consistent with the interpretation that some of the glands at 20 h APF had entered the death pathway. We suspected that this was caused by a waning effect of

the ectopic Fkh. Therefore, we tested whether survival of the salivary glands could be further prolonged by sustained expression of *fkh*. After the first heat shock at 10 h APF, we applied a second heat shock at 16 h APF, which resulted in 67% salivary gland persistence at 26 h APF (*n* = 21). This suggests that activation of the cell death program can indeed be continuously suppressed by maintained *fkh* expression. Collectively, these data demonstrate that Fkh is sufficient to prevent PCD in the larval salivary glands. Importantly, they suggest that the down-regulation of Fkh before the prepupal 20E pulse provides competence to the salivary glands to respond to this pulse by activation of the death pathway.

We wondered whether rescue of the larval salivary glands by *fkh* was a tissue-specific effect or whether ectopic *fkh* would block PCD in other tissues as well. To address this question, we ectopically expressed *fkh* shortly before the larval midgut normally dies. This did not lead to a delay in the initiation or execution of PCD in this tissue (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200611155/DC1). Thus, our results support a model in which *fkh* specifically functions in the larval salivary glands in the developmental control of death.

Fkh represses expression of the death genes *hid* and *rpr*

In an attempt to identify genes that might mediate the survival function of Fkh, we analyzed the effect of *fkh* on the expression of genes that had previously been implicated in the control of salivary gland death. RNA was isolated from the salivary glands of P[*hs-Fkh^111*] and *w^1118* control animals at different times after heat treatment at 10 h APF. Northern blots of this RNA were first hybridized to detect expression of genes of the 20E-controlled signaling pathway (Fig. 3). The primary hormone response genes *E93*, *E74A*, and *BR-C*, which are required for proper salivary gland death (Jiang et al., 2000; Lee et al., 2000, 2002), were all expressed in the presence of ectopic Fkh. However, the amount of RNA and the timing of expression differed from the controls. Expression of all three genes started earlier when Fkh was present, and *E74A* and *BR-C* were also expressed more strongly. The *E93* mRNA level appeared to be somewhat diminished, but did not go down by 16 h APF, as it did in the control (Fig. 3). *BR-C* failed to be down-regulated in 16 h APF salivary glands as well. These data indicate that salivary gland survival in the presence of ectopic Fkh is not likely to be caused by a reduced expression of upstream regulators of steroid-induced death.
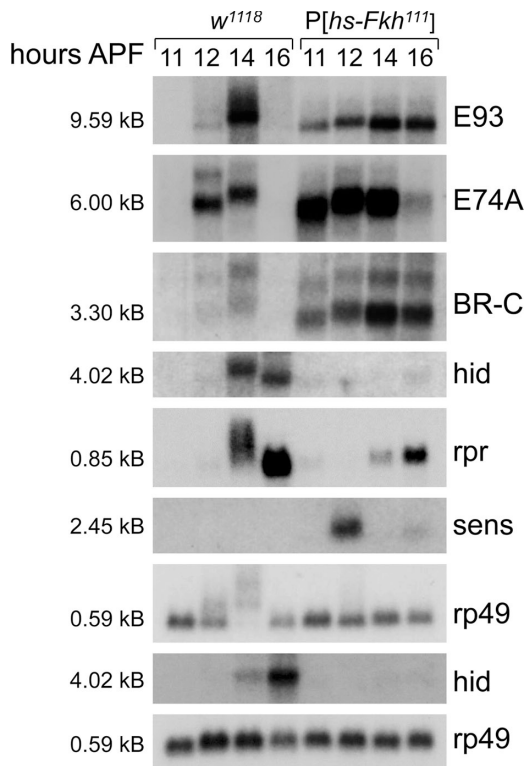
Figure 3. Gene expression in the absence and presence of ectopic Fkh. *w¹¹¹⁸* control prepupae and prepupae of the P[*hs-Fkh¹¹¹*] transformant were heat shocked at 10 h APF. Subsequently, RNA was extracted from the salivary glands at the indicated time points and analyzed by Northern blot hybridization. The bottom two autoradiographs show the results of a second, independent experiment for *hid* and *rp49*. Hybridization to detect *rp49* mRNA served as a control for loading and transfer.

Next, we tested whether expression of the downstream death activators *rpr* or *hid* was changed by Fkh. We found that *rpr* mRNA was diminished when compared with the control, but still detectable. More strikingly, expression of *hid*, which was strongly expressed in the control glands, appeared to be completely suppressed by the ectopic Fkh (Fig. 3). As *hid* is known to be required for proper salivary gland death, this

suggested that repression of *hid* was at least partially responsible for the suppression of salivary gland death by Fkh.

In *fkh* mutants, the transcription factor Senseless (Sens) is not properly expressed and, similar to *fkh* mutants, *sens* mutants exhibit embryonic salivary gland apoptosis (Chandrasekaran and Beckendorf, 2003). We were therefore interested to also determine the expression of *sens* in the presence and absence of Fkh. Hybridization with a *sens* probe showed that induction of *fkh* was followed by a brief burst of *sens* transcription at 12 h APF (Fig. 3), confirming that *sens* is a target gene of Fkh. However, although massive overexpression of *sens* from a *sens* transgene delayed salivary gland death, it did not affect the transcript levels of *hid* or *rpr* (unpublished data). Thus, *sens* uses a different pathway than *fkh* to protect cells from PCD (unpublished data). This conclusion is consistent with the finding that forced expression of *sens* in *fkh* mutants does not rescue the embryonic cell death phenotype of *fkh* (Chandrasekaran and Beckendorf, 2003).

Collectively, our results show that at least part of the effect of Fkh on cell death is mediated by repression of the death genes *hid* and *rpr*. They further suggest a crosstalk between *fkh* and another survival pathway that acts through *sens*. We also note that *fkh* is epistatic to the cell death regulator *E93* (Lee et al., 2000), which is not sufficient to initiate activation of the death pathway as long as *fkh* is expressed.

fkh coordinately represses IAP inhibitors and affects other apoptosis-related genes

To provide a broader foundation for our conclusions, we performed a microarray analysis of gene expression at 14 h APF in the presence and absence of ectopic Fkh. Expression of *fkh* was induced by heat shock in P[*hs-Fkh¹¹¹*] animals, and RNA extracted from the salivary glands of these and heat-shocked *w¹¹¹⁸* control animals was hybridized to Affymetrix *Drosophila* Genome Arrays. The microarray analysis confirmed that *hid* and *rpr* were down-regulated in response to Fkh (16- and 2-fold, respectively). In addition, it revealed that another known IAP inhibitor, *Jafrac2* (Tenev et al., 2002), was down-regulated. Overall, the microarray analysis identified 55 genes annotated

Table I. Apoptosis-related genes that respond to Fkh

Gene	Fold change	Gene ontology	FlyBase ID
CG15097	−28.1	e. a.; actin binding	FBgn0034396
CG12789	−21.4	e. a.; scavenger receptor	FBgn0025697
Companion of reaper	−18.2	e. a.; DNA damage response	FBgn0030028
Wrinkled (hid)	−16.0	IAP antagonist	FBgn0003997
CG3571	−9.9	e. a.; actin binding	FBgn0037978
cactus	−4.6	survival signaling	FBgn0000250
Jafrac2	−4.4	IAP antagonist	FBgn0040308
CG7228	−4.4	e. a.; scavenger receptor	FBgn0031969
Rep1	+5.0	inhibitor of caspase-activated DNase	FBgn0024732
Protein kinase 61C (PDK1)	+8.7	Akt survival signaling	FBgn0020386
capricious	+9.2	e. a.; cell adhesion	FBgn0023095
Apc2	+9.8	e. a.; microtubule binding	FBgn0026598
decapentaplegic	+11.1	survival signaling	FBgn0000490

e. a. indicates that the association of the gene with apoptosis is inferred from electronic annotation. A complete list of all apoptosis-related genes that showed an at least 1.5-fold response to *fkh* is provided in Table S1.

as functioning in apoptosis whose expression was at least 1.5-fold changed by Fkh (Tables I and S1). *hid* was among the four most strongly down-regulated genes, as was *Companion of reaper*, a gene that has been reported to synergize with *hid* to promote apoptosis (Meier and Silke, 2003). Genes encoding the *D. melanogaster* Apaf-1 orthologue Ark and the apical caspase Dronc (*Nc*) were down-regulated by approximately twofold by Fkh. As these proapoptotic proteins are known to be required for the destruction of the larval salivary glands (Cakouros et al., 2004; Daish et al., 2004; Waldhuber et al., 2005; Mills et al., 2006), down-regulation of the corresponding genes is likely to contribute to the antiapoptotic effect of *fkh*. The two Bcl-2 family members of *D. melanogaster*, *Buffy* and *debcl*, were 2- and 2.5-fold up-regulated by *fkh*. We found that *fkh*-independent overexpression of either *Buffy* or *debcl* using the UAS/Gal4 system did not affect salivary gland death (unpublished data). These genes therefore have no, or at least no essential, role in mediating the effect of *fkh*. Interestingly, among the genes strongly up-regulated by *fkh* was the *D. melanogaster* PDK1 orthologue. PDK1 is an essential activator of the protein kinase Akt (Rintelen et al., 2001). As signaling through the PI3K–Akt pathway can protect salivary glands from PCD (Liu and Lehmann, 2006), PDK1 is likely to contribute to the survival function of *fkh*. In summary, the microarray data suggest that Fkh ensures survival by the coordinated repression of IAP antagonists and the regulation of other apoptosis-related genes. In particular, they confirm that the death gene *hid*, which is required for salivary gland death (Yin and Thummel, 2004), is a prime candidate for a target of the survival function of *fkh*.

Loss of *fkh* is sufficient to create competence for a death response to steroid signaling

Our results showed that the down-regulation of *fkh* in response to the late-larval 20E pulse is required for proper induction of *hid* and *rpr* in response to the prepupal 20E pulse and subsequent death of the salivary glands. This prompted us to ask whether down-regulation of *fkh* is also sufficient to specify these responses to steroid signaling. To address this question, we down-regulated *fkh* prematurely in early third instar larvae using RNAi. We generated transgenic fly stocks that use a heat-shock promoter to drive expression of a double-stranded (ds) *fkh* RNA (Lam and Thummel, 2000). Northern analysis confirmed that these lines strongly expressed *fkh* dsRNA upon heat treatment (unpublished data), followed by a dramatic decline in the amount of Fkh protein (determined by Western analysis; Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb .200611155/DC1). To further ascertain that expression of the dsRNA led to an effective knockdown of *fkh* activity in the salivary glands, we examined the expression of known target genes of *fkh* by Northern blot hybridization. RNA was isolated from the salivary glands of staged *fkh* dsRNA-expressing animals and *w1118* controls. A Northern blot of this RNA was hybridized to detect expression of the *Sgs4* and *sage* genes (Fig. 4). *Sgs4* is a well-characterized target gene of *fkh* that encodes a glue protein and is strongly expressed in third-larval instar salivary glands until puparium formation (Lehmann and Korge, 1996).
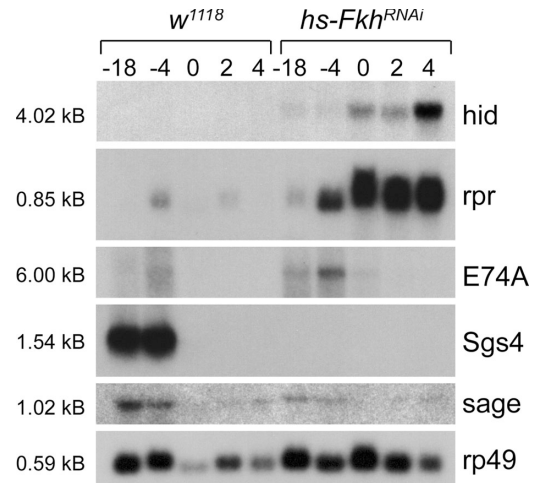

Figure 4. **The transcriptional response to the late-larval 20E pulse in the absence of Fkh.** *w1118* and P[*hs-fkhRNAi-4*] third instar larvae were heat treated, and RNA was extracted from salivary glands at the indicated times before and after pupariation (0 h is the time of puparium formation). A Northern blot of the RNA was hybridized with probes detecting mRNA of the indicated genes. The comparatively weak signal for *E74A* in the −4 h control lane is most likely caused by the very narrow window of *E74A* expression at this time (Jiang et al., 2000) and the difficulty in collecting synchronized larvae (see Materials and methods). Hybridization to detect *rp49* mRNA served as a control for loading and transfer.

As expected, large amounts of *Sgs4* mRNA were detected in the salivary glands of the heat-shocked control larvae. In striking contrast, *Sgs4* mRNA was absent from the salivary glands of the *fkh* dsRNA-expressing larvae. *sage* encodes a bHLH transcription factor that is enriched in larval salivary glands and down-regulated at the larval–prepupal transition (Li and White, 2003). Recently, it has been shown that *sage* is directly activated by *fkh*, although some *sage* expression is still observed in a *fkh* mutant (Abrams et al., 2006). Consistent with these findings, *sage* RNA was severely, but not completely, reduced after *fkh* dsRNA expression (Fig. 4). These data indicate that *fkh* function in the larval salivary glands is severely compromised by RNAi. Importantly, the RNAi knockdown had no effect on overall development and growth of the larvae, as heat-shocked P[*hs-fkhRNAi-4*] larvae pupariated at the same time and formed prepupae of similar size as heat-shocked *w1118* control larvae (unpublished data).

Next, we inspected salivary glands that had been treated with *fkh* dsRNA for signs of PCD. Staining with acridine orange revealed no difference between *fkh* RNAi and control glands before puparium formation (Fig. 5, A and B). This indicated that the premature down-regulation of *fkh* by itself was not sufficient to cause cell death. However, salivary glands after puparium formation exhibited a progressive loss of tissue integrity and showed strong nuclear acridine orange staining (Fig. 5, D and E). The salivary glands of heat-shocked control animals showed no nuclear acridine orange staining, or any signs of tissue disintegration, and died at the normal time in response to the prepupal 20E pulse (Fig. 5 C). The nuclei of the salivary glands of *fkh* dsRNA-treated animals were stainable with acridine orange as early as 4 h APF (Fig. 5 D). At 9 h APF, salivary glands could still be dissected from some, but not all, prepupae.
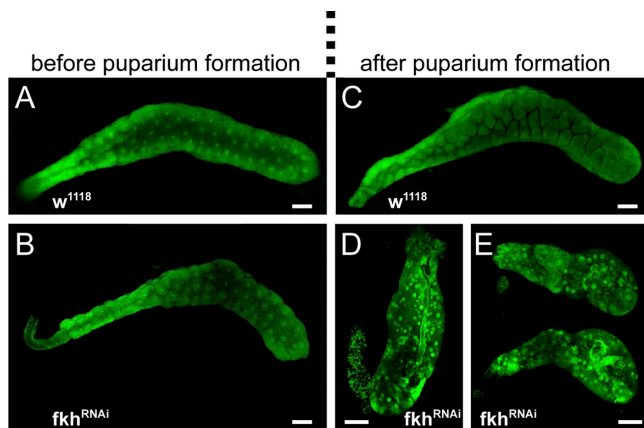
Figure 5. **RNAi-mediated knockdown of *fkh* leads to premature salivary gland death in response to the late-larval 20E pulse.** Third instar larvae of P[*hs-fkh*^RNAi-4^] and *w*^1118^ were collected after the second–third larval molt and subjected to three consecutive heat shocks. Salivary glands were dissected from animals before and after the late-larval 20E pulse and stained with acridine orange. (A and B) Salivary glands of early wandering larvae of neither *w*^1118^ nor P[*hs-fkh*^RNAi-4^] show nuclear acridine orange staining. However, the cytoplasm and nucleoli are stained (see Fig. S3), leading to a speckled appearance of the glands. (C and E) At 9 h APF, the salivary glands of P[*hs-fkh*^RNAi-4^] prepupae have lost their cellular structure and show strong nuclear acridine orange staining, whereas *w*^1118^ control glands show no nuclear acridine orange staining and have an intact cellular structure. Note that the staining of the nucleoli observed at earlier stages has disappeared. (D) P[*hs-fkh*^RNAi-4^] salivary gland already showing strong signs of cell death at 4 h APF. Bars, 100 μm.

These glands contained acridine orange–positive cell nuclei, but the cell boundaries, which were clearly discernable in the control glands, had disappeared (Fig. 5 E). Salivary glands could occasionally also be found at later time points. However, the cellular structure and shape of these glands were severely compromised. This suggests that, although the death program had been prematurely activated, the final steps of tissue disintegration and removal could not be performed properly. We overexpressed *hid* in early prepupae from a heat-inducible transgene and found that the salivary glands responded in a similar way, showing nuclear acridine orange staining, but persisting for several more hours (unpublished data). Collectively, these results strongly suggested that loss of *fkh* was sufficient to specify a cell death response to 20E signaling.

To further test this conclusion, we asked whether death gene expression was changed after the knockdown of *fkh*, and, if yes, whether this change occurred in response to the late-larval 20E pulse or earlier. Northern blot hybridization revealed that *hid* and *rpr* were strongly activated at or shortly before puparium formation in salivary glands that had been treated with *fkh* dsRNA. *hid* reached a peak in expression at 4 h APF, whereas *rpr* was induced earlier, already reaching a very high transcript level at 0 h APF (Fig. 4). This profile is strikingly similar to the temporal profile of *hid* and *rpr* expression observed in response to the late-prepupal 20E pulse. Also at this time, *rpr* shows maximal expression earlier than *hid*, which peaks in expression several hours after the early hormone response genes *E74A* and *BR-C* are first detected (Jiang et al., 2000). This suggests that, similar to the response in late-prepupal glands, *hid* induction after premature loss of *fkh* is a secondary hormone response
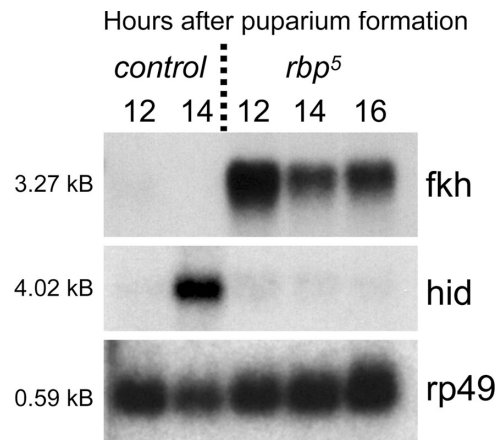

Figure 6. **The *BR-C* mutant *rbp*^5^ exhibits strong derepression of *fkh*.** Salivary glands were dissected at the indicated time points from males of the genotype *y rbp*^5^/*Y* and from *Binsn/Y* control males. Total RNA extracted from the glands was analyzed by Northern blot hybridization for the presence of *fkh* and *hid* RNA. Hybridization with an *rp49* probe served as a control for loading and transfer. RNA from 16 h APF control animals could not be obtained because of the advanced histolysis of the salivary glands at this time.

mediated by *E74A* and *BR-C*. This hypothesis is supported by the expression profiles of *E74A* and *BR-C*, which are very similar in late-larval and -prepupal salivary glands (Jiang et al., 2000). Hybridization with an *E74A* probe confirmed that this early response gene is induced at the normal time after knockdown of *fkh* (Fig. 4). Collectively, the morphological and gene expression data demonstrate that loss of *fkh* leads to a premature activation of the death program in response to the late-larval 20E pulse. They identify *hid* and *rpr* as two key death regulators whose hormone responsiveness is controlled by Fkh.

fkh is derepressed in *BR-C* mutants that are defective in salivary gland death

The response of *fkh* to 20E signaling at the end of larval development is mediated, at least in part, by the early 20E-inducible gene *BR-C* (Renault et al., 2001). The down-regulation of *fkh* that is normally observed at this time does not occur in *2Bc* mutants of *BR-C*. The continued expression of *fkh* in these mutants is sufficient to maintain expression of the *Sgs4* gene in prepupal salivary glands. *2Bc* mutants also show defects in salivary gland death, which are even more pronounced in mutants of the *rbp* subfunction of *BR-C* (Restifo and White, 1992; Jiang et al., 2000). Collectively, these observations raised the possibility that a derepression of *fkh* might be responsible for the persistence of the larval salivary glands in *BR-C* mutants. To test this possibility, we performed a Northern analysis of *fkh* expression in the salivary glands of late prepupae and early pupae of the *rbp*^5^ mutant (Fig. 6). As expected, the salivary glands of control animals did not show expression of *fkh* shortly before their destruction, at 12 or 14 h APF. In striking contrast, salivary glands of hemizygous *rbp*^5^ mutant animals exhibited strong expression of *fkh* mRNA both at these times and also in 16-h pupae. In 16-h control pupae, the disintegration of the salivary glands was too far advanced to obtain RNA for a Northern analysis. These data indicate that *fkh* is indeed derepressed in the persisting salivary
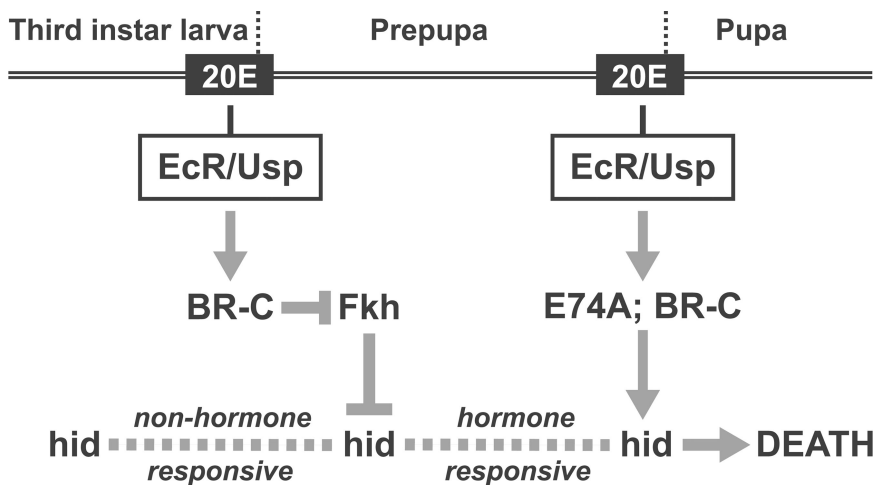
Figure 7. **Model for the acquisition of competence by the salivary glands to respond to steroid signaling with PCD.** Salivary gland death is triggered by the prepupal 20E pulse. This pulse is preceded by the late-larval 20E pulse that triggers the destruction of the larval midgut. The model illustrates, using *hid* as an example, how a *fkh*-dependent switch in the hormone responsiveness of critical death genes makes the salivary glands susceptible to steroid-triggered death. Our data show that *rpr* and possibly other death genes are subject to a similar control by *fkh*. The concerted action of these genes ensures the demise of the salivary glands in response to the prepupal 20E pulse. See the text for further discussion of the model.

glands of *BR-C* mutants. Hybridization of the Northern blot with a *hid* probe confirmed that this death gene was not properly induced in the *rbp⁵* mutant (Jiang et al., 2000). The expression of *fkh* in *rbp⁵* provides an explanation for the persistence of larval salivary glands in *BR-C* mutants and identifies *fkh* as at least one critical target of the function of *BR-C* in programmed cell death. These results lend further support to a model in which *fkh* is tied into the hormonal signaling pathway through the 20E-controlled *BR-C* gene (Fig. 7).

Discussion

Developmental cell death in invertebrates and vertebrates is often controlled by systemic signals, which provide the trigger for cell and tissue destruction (Jacobson et al., 1997; Baehrecke, 2002). However, it is not well understood why these signals induce death only at a particular time and only in some cells and tissues, but not in others. The experimental data presented in this study support a model that explains how a specific tissue of the fruit fly *D. melanogaster* is singled out for destruction in response to the steroid hormone 20E (Fig. 7). It explains why the larval salivary glands are destroyed in response to a particular 20E pulse, the prepupal pulse, and why they survive earlier pulses of the same hormone. It thus provides a framework for our understanding of how tissue-specific developmental cell death is precisely timed.

In more general terms, our data suggest that a key event in acquiring competence for a cell death response to a systemic signal is the loss of a tissue-specific survival factor. This loss occurs in response to a temporal signal that precedes the death-inducing signal. In the salivary glands, the tissue-specific survival factor is Fkh, and the signal that leads to the loss of Fkh is provided by the late-larval 20E pulse. Our data show that loss of Fkh is required for the death response to the prepupal 20E pulse (Fig. 2). They further suggest that the salivary glands survive all previous hormone pulses because they are protected by the presence of Fkh (Fig. 1). Thus, Fkh not only plays a key role in determining the tissue selectivity of salivary gland death, but also in the proper timing of this event. Elimination of Fkh is mediated by the 20E-induced transcription factor *BR-C*

(Renault et al., 2001). We find that *fkh* is strongly expressed at the normal time of salivary gland death in the *rbp⁵* mutant of *BR-C*, which demonstrates that *BR-C* is required for the continued repression of *fkh* beyond the larval–prepupal transition (Fig. 6). The derepression of *fkh* in *rbp⁵* is sufficient to explain why the salivary glands do not die in the mutant.

Loss of the survival factor renders critical death regulators responsive to the death-inducing signal. In the salivary glands, these death regulators are the IAP antagonists *hid* and *rpr*, which together are required for salivary gland death (Yin and Thummel, 2004). In the absence of Fkh, the two genes are inducible by hormone, as shown by the premature induction of *hid* and *rpr* after RNAi knockdown of *fkh* (Fig. 4). Importantly, loss of *fkh* by itself is not sufficient to activate *hid* and *rpr*, or to kill the salivary glands within the ∼36 h between *fkh* knockdown and the late-larval 20E pulse. Strong activation of *hid* and *rpr* and death only occur in response to the hormonal signal. After elimination of Fkh, the hormone induces expression of *hid* and *rpr* at a level that is sufficient to kill (Fig. 5). This observation supports the conclusion that there are no other repressors present in prepupal salivary glands that are sufficient to prevent hormonal induction of cell death. All that seems to be needed to induce death is the hormone 20E and one or more 20E-induced transcriptional activators.

Previous work has shown that *E74A* and *BR-C* play the role of hormone-induced activators of *hid* and *rpr* in late-prepupal salivary glands (Jiang et al., 2000). Both *E74A* and *BR-C* are required for the induction of *hid*, which has the characteristics of a secondary-response gene. Intriguingly, the activation of *hid* after premature loss of *fkh* shows the same secondary-response characteristics, suggesting that the same 20E-induced transcription factors are responsible for the activation of *hid* by the late-larval 20E pulse (Fig. 4). Full induction of *rpr* depends not only on *BR-C* but also on direct binding of the hormone receptor EcR/Usp to the gene (Jiang et al., 2000). Thus, *rpr* has characteristics of both a primary- and secondary-response gene, leading to an earlier induction of the gene in response to the prepupal 20E pulse. Again, premature activation in response to the late-larval pulse shows the same temporal characteristics (Fig. 4). This suggests that *E74A* and *BR-C* are responsible for the

premature activation of *hid* and *rpr* after knockdown of *fkh*, and that such an activation is normally prevented by the presence of Fkh. Our immunostaining data support this conclusion by showing that Fkh protein is still present in the larval salivary glands at the time when the two genes are active. It only disappears from the tissue 2–4 h APF (Fig. 1 B). These data explain why *E74A* and *BR-C* mediate a death response exclusively to the prepupal 20E pulse, despite a very similar induction pattern of the two genes in response to the preceding late-larval pulse.

Our results exclude that repression of *hid* and *rpr* is mediated by the *fkh* target *sens*. Repression may thus be mediated by another downstream target of *fkh* or by direct binding of Fkh to transcriptional control regions of *hid* and *rpr*. In support of the latter possibility, we found that the first intron of *hid* contains a cluster of 13 Fkh binding sites. One of these sites exhibits strong binding of Fkh in in vitro DNA-binding assays, whereas the other sites have weak to moderate binding affinity (unpublished data; de Banzie, J., personal communication). Although this region may function as a silencer of *hid* expression in vivo, *lacZ* reporter gene assays in transgenic flies did not reveal that it has an enhancer function. We were not able to identify a similar binding site cluster in *rpr*.

Our microarray data identify other apoptosis-related genes that are down- or up-regulated by Fkh. Therefore, it is likely that Fkh protects cells from death by interfering with the cell death program at multiple levels. Regulation of genes such as *Ark*, *Dronc*, or *PDK1*, is likely to mediate a general function of *fkh* as a survival factor. This function appears to be required for the survival of the developing salivary glands during embryogenesis (Myat and Andrew, 2000). However, it is not essential for the survival of postembryonic salivary glands, as demonstrated by the failure of the glands to die in the absence of Fkh during prepupal development. Our data confirm this conclusion by showing that the salivary glands fail to undergo PCD within the ∼36 h between the premature knockdown of *fkh* and the steroid induction of death. They separate a general protective function of Fkh from a specific function that Fkh has in the control of steroid-induced developmental PCD.

Tissue-specific developmental cell death controlled by steroid hormone plays an important role not only in insects but also in humans and other vertebrates. Glucocorticoids, for instance, control the development of the immune system by killing specific types of thymocytes (Ashwell et al., 2000). Many genes regulated by glucocorticoids are coregulated by the vertebrate FOXA counterparts of Fkh (Friedman and Kaestner, 2006). It will be interesting to see whether FOXAs have evolutionarily conserved functions in glucocorticoid-induced death and in other types of developmental cell death.

Materials and methods

Plasmid construction and *P* element transformation
For construction of the P[*fkh^RNAi*] transformation plasmid, a segment of the coding region of *fkh* (corresponding to amino acid positions 189–435) was amplified by PCR from the plasmid fkh-pET3b. Two copies of the product were then sequentially cloned in a head–head orientation into the transformation vector pCaSpeR-hs-act (Thummel et al., 1988), leaving a 130-bp

spacer between the copies. Five independent transformant lines were obtained, which all expressed *fkh* dsRNA upon heat shock; a detailed description of the construction steps can be obtained upon request). *P* element injections were performed by BestGene, Inc.

Developmental timing and Northern blot hybridizations
Third instar larvae were staged using the blue gut method as previously described (Andres and Thummel, 1994). For the −4-h time point (clear gut) in Fig. 1 A, only salivary glands that had completed glue protein secretion into the gland lumen were used. For the −4-h time point in Fig. 4, this additional criterion could not be used because glue production was severely affected in heat-shocked P[*hs-fkh^RNAi-4*] animals. Prepupae and pupae were staged by collecting freshly formed prepupae within 30 min of puparium formation and keeping them on damp filter paper at 25°C for the indicated lengths of time.

For the Northern analysis of *fkh* expression in a *BR-C* mutant background, we used the *y rbp^5/Binsn* stock (Belyaeva et al., 1980). Hemizygous mutant male larvae of this stock (*y rbp^5/Y*) can be distinguished from males carrying the *Binsn* X balancer (*Binsn/Y*) by the yellow color of their mouth hooks and denticles (*y* phenotype; Binsn carries *y⁺* allele). The males were separated based on this phenotype, and salivary glands were dissected for RNA extraction.

RNA extraction, fractionation by gel electrophoresis, transfer to nylon membranes, and hybridization with radioactive DNA probes were performed as previously described (Lehmann et al., 2002). Probes were derived by restriction digest from the following plasmids: *fkh*, 848-bp AflII–BglII fragment from RE03865; *Sgs4*, 1-kb EcoRI–HindIII fragment from pOW3Sal; *sens*, 1.4-kb EcoRI fragment from pBS-sens (provided by H. Bellen; Baylor College of Medicine, Houston, TX); *BR-C*, 480-bp StuI–PvuII fragment from paaDM527; and *E93*, 1.6-kb AccI fragment from *E93* cDNA (provided by C. Thummel; University of Utah, Salt Lake City, UT). *hid*, *rpr*, *E74A*, and *rp49* probes were prepared as previously described (Lehmann et al., 2002).

Ectopic expression and RNAi experiments
For ectopic expression of *fkh*, prepupae of the transformant line P[*hs-Fkh^111*] and *w^1118* control prepupae were collected at 9.5 h APF and incubated for 30 min in a 37°C water bath. The animals were transferred to damp filter paper in a Petri dish and kept at 25°C until the salivary glands were dissected for RNA extraction or microscopic analysis. For RNAi knockdown of *fkh*, third instar larvae of P[*hs-fkh^RNAi-4*] and *w^1118* were collected within 3 h of the second–third larval instar molt and transferred to fresh yeast paste. Larvae were kept at 25°C and subjected to heat shocks, as described in the previous section, at 12, 26, and 40 h after collection. The salivary glands of larvae expressing *fkh* dsRNA were defective in glue protein production and showed a reduced size compared with *w^1118* control glands (Fig. 5). Glue proteins make up >30% of the total gland protein in wandering third instar larvae (Korge, 1977), suggesting that the reduced organ size was caused by smaller cell size and not by reduced cell number. Staining of the salivary glands with the nuclear dye Hoechst 33342 confirmed this prediction. Impaired glue production was an expected result of the knockdown of *fkh*, as expression of all glue protein genes, including *Sgs4* (Fig. 4), depends on *fkh* (Mach et al., 1996; Roth et al., 1999; our microarray data).

Histochemical staining and Western analysis
Acridine orange staining of salivary glands and midguts was performed as previously described (Jiang et al., 1997). Independent of the genotype of the animals, the salivary glands of both heat-shocked and non–heat-shocked wandering larvae had a speckled appearance after acridine orange staining (Fig. 5). Microscopic analysis of single nuclei at high magnification revealed that this was caused by staining of the nucleoli, which stain brightly with acridine orange (Nash and Plaut, 1964). The polytene chromosomes did not show staining above background (Fig. S3).

Staining of salivary glands with Fkh antibodies, FITC-labeled phalloidin (Alexis Biochemicals), and Hoechst 33342 (AnaSpec, Inc.), was performed using standard procedures. The anti-Fkh antibody was affinity purified and used at a dilution of 1:500 (Lehmann and Korge, 1996). Bound Fkh antibodies were detected with a Cy2-conjugated goat anti–guinea pig secondary antibody (1:400; Jackson ImmunoResearch).

For Western blot analysis (Fig. S2), Fkh antiserum was used at a dilution of 1:2,000, and bound antibody was detected using a peroxidase-conjugated goat anti–guinea pig secondary antibody (1:5,000; Jackson ImmunoResearch Laboratories).

Images of the fluorescently labeled tissues shown in Figs. 1, 2, and 5 were taken with an inverted confocal microscope (TCS SP2; Leica), over-laying a z series of 30–50 sections. Tissues were mounted in PBS, and images were acquired with a 10×/0.40 NA (Figs. 1 and 5) or 20×/0.70 NA (Fig. 2) HC PL APO objective (eyepiece lens 10×/22 HC PLAN; Leica). Bright-field images shown in Fig. S1 were taken with a 3–charge-coupled device digital imaging camera (KY-F75U; JVC) using a microscope (Axioskop 2 Plus; Carl Zeiss MicroImaging, Inc.) and Auto-Montage imaging software (Syncroscopy). The differential interference contrast and fluorescent images shown in Fig. S3 were taken with a camera (AxioCam MRm; Carl Zeiss MicroImaging, Inc.) using an Axioskop 2 Plus microscope and AxioVision 4.1 software. 2.5×/0.075 NA (Fig. S1) and 40×/0.75 NA (Fig. S3) Plan-Neofluar objectives (Carl Zeiss MicroImaging, Inc.) were used for image acquisition (10×/23 eyepiece lens). Images were processed using Photoshop 7.0 (Adobe), with uniform adjustments made to brightness and contrast, and were assembled using Illustrator CS2 (Adobe). All images were taken at room temperature.

Microarray analysis

P[*hs-Fkh*^{111}] prepupae and w^{1118} control prepupae were heat shocked at 9.5 h APF for 30 min at 37°C and the salivary glands dissected 4 h later. Samples were prepared in three replicates from the P[*hs-Fkh*^{111}] glands and in two replicates from the w^{1118} control glands. Total RNA was isolated using Trizol (Invitrogen) and purified on RNAeasy columns (QIAGEN). Hybridization to Affymetrix *Drosophila* Genome Arrays was performed by the microarray facility of the University of Maryland Biotechnology Center. Raw data provided by the Center were normalized, pooled, and compared using dChip (Li and Wong, 2001). Analysis was performed using the PM-only model with outlier detection. The datasets were filtered for genes that showed an at least 1.5-fold relative change in their mean expression and an absolute expression change of at least 400. Query for apoptosis-related genes was performed based on annotation using dChip and Microsoft Access.

Online supplemental material

Fig. S1 shows that misexpression of *fkh* does not affect PCD in the larval midgut. Fig. S2 shows a Western blot analysis of Fkh protein expression after knockdown of the gene by RNAi and after overexpression from P[*hs-Fkh*^{111}]. Fig. S3 shows that acridine orange strongly stains the nucleoli of salivary gland nuclei of normal w^{1118} third instar larvae. Table S1 lists all apoptosis-related genes identified by microarray analysis that showed an at least 1.5-fold response to *fkh*. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200611155/DC1.

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