Genetic Control of Specificity to Steroid-Triggered Responses in *Drosophila*

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ABSTRACT Steroid hormones trigger a wide variety of biological responses through stage- and tissue-specific activation of target gene expression. The mechanisms that provide specificity to systemically released pulses of steroids, however, remain poorly understood. We previously completed a forward genetic screen for mutations that disrupt the destruction of larval salivary glands during metamorphosis in *Drosophila melanogaster*, a process triggered by the steroid hormone 20-hydroxyecdysone (ecdysone). Here, we characterize 10 complementation groups mapped to genes from this screen. Most of these mutations disrupt the ecdysone-induced expression of death activators, thereby failing to initiate tissue destruction. However, other responses to ecdysone, even within salivary glands, occur normally in mutant animals. Many of these newly identified regulators of ecdysone signaling, including *brwd3*, *med12*, *med24*, *pak*, and *psg2*, represent novel components of the ecdysone-triggered transcriptional hierarchy. These genes function combinatorially to provide specificity to ecdysone pulses, amplifying the hormonal cue in a stage-, tissue-, and target gene-specific manner. Most of the ecdysone response genes identified in this screen encode homologs of mammalian nuclear receptor coregulators, demonstrating an unexpected degree of functional conservation in the mechanisms that regulate steroid signaling between insects and mammals.

STEROID hormones regulate developmental and homeostatic processes through their respective nuclear receptors, directly regulating expression of critical biological effector genes. Nuclear receptor activity is, in turn, modulated by specific coregulators (Lonard and O'Malley 2007). These coregulators may interact directly or indirectly with nuclear receptors and can increase (coactivators) or decrease (corepressors) the rate of target gene transcription. Importantly, nuclear receptor coregulators have been proposed to act in a combinatorial manner to provide specificity to hormone-induced gene expression (Mckenna and O'Malley 2002; Rosenfeld 2006; Lonard and O'Malley 2007), helping refine global hormonal cues into distinct local responses. However, our understanding of nuclear receptor coregulator function has been primarily restricted to biochemical and tissue culture studies in mam-

malian systems, making it difficult to define their role in regulating specificity to hormonal cues within the context of a developing animal.

In *Drosophila melanogaster*, pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) regulate the major developmental transitions during its life cycle. Each pulse triggers a wide variety of biological responses that end one developmental stage and start the next. Like steroid hormones in mammals, ecdysone acts by binding to its nuclear receptor, the ecdysone receptor (*EcR*), thereby directly regulating target gene expression. Transcriptional targets of ecdysone have been historically divided into two groups: primary response genes, whose expression starts within minutes of hormone exposure and does not require protein synthesis, and secondary response genes, whose expression starts a couple of hours later and requires protein synthesis (Thummel 1996). This hierarchical model of steroid-triggered transcriptional responses was originally proposed by Michael Ashburner and colleagues to explain the behavior of polytene chromosome puffs in insect larval salivary glands (Ashburner *et al.* 1974). Thus, according to this model, ecdysone initiates transcription of a small number of primary response genes that, in turn, encode transcription factors that initiate

expression of a larger set of secondary response genes, many of which were postulated to include regulators of biological processes. However, the relationship, if any, between this hierarchical model and the combinatorial model used to describe mammalian steroid responses has been unclear. These two conceptual frameworks for steroid action have been further confounded by the lack of evidence that nuclear receptor coregulators play a critical role in refining hormonal cues in invertebrates like *Drosophila*.

Our studies focus on a series of coordinated biological responses to the first two pulses of ecdysone during *Drosophila* metamorphosis. The late larval pulse of ecdysone triggers puparium formation, ending larval development and initiating prepupal development; ~12 hr later, the prepupal pulse of ecdysone triggers head eversion, marking the end of prepupal development and the beginning of pupal development (Riddiford 1993). This latter pulse of ecdysone also triggers destruction of larval salivary glands by initiating the stage- and tissue-specific activation of autophagy and caspases (Jiang *et al.* 1997, 2000; Lee and Baehrecke 2001). This death response depends on the *EcR* and three ecdysone primary response genes: *E74A*, *br-Z1*, and *E93* (Jiang *et al.* 2000; Lee *et al.* 2000, 2002b). *E74A* and *br-Z1* are induced after both the larval and prepupal pulses of ecdysone (Thummel *et al.* 1990; Karim and Thummel 1992) and thus are unlikely by themselves to provide the needed specificity to the death response. *E93*, on the other hand, is induced only after the prepupal pulse and may provide stage specificity to ecdysone even though this expression is not limited to the salivary glands (Lee *et al.* 2000). Importantly, these events at the onset of metamorphosis provide an ideal context within which to study the mechanisms that provide specificity to ecdysone-triggered biological responses.

To dissect the genetic control of specificity to steroid-triggered responses, we previously conducted a large-scale EMS-mutagenesis screen for mutations that disrupt the destruction of larval salivary glands (Wang *et al.* 2008). We generated >8600 lethal mutations and identified among them 37 complementation groups that had a persistent salivary gland (PSG) phenotype in otherwise normal-looking animals (Wang *et al.* 2008). Here, we characterize 10 complementation groups mapped to genes from this screen. Our results demonstrate that these mutations disrupt the death response in salivary glands without disrupting many other ecdysone-triggered responses during metamorphosis. For most PSG mutations, this selectivity is achieved by interrupting ecdysone signaling during the death response, with the mutations mapping to known and novel components of the ecdysone-triggered transcriptional hierarchy. We demonstrate that *brwd3*, *med12*, *med24*, *pak*, and *psg2* encode novel ecdysone secondary response genes required for robust expression of biological effector genes that mediate the death response. Our results indicate that these secondary response genes define an additional regulatory layer within the ecdysone-triggered transcriptional cascade that resides between primary response genes and biological effector genes. Importantly, most of these newly identified ecdysone secondary response genes encode homologs of vertebrate nuclear receptor coregulators, demonstrating a functional relationship between the hierarchical and combinatorial models of steroid action.

Materials and Methods

Drosophila strains

The Bloomington *Drosophila* Stock Center (BDSC) at Indiana University provided the following stocks: *Df(3R)Exel6149*, *Df(3R)BSC638*, *Df(3R)Exel6199*, *BRWD3[05842]*, *Df(3R)BSC677*, *Df(3R)Exel6188*, *Df(3R)BSC523*, *Eip93F[vno]*, *Df(3R)BSC503*, *Df(3R)ED6332*, *hdc[BG00237]*, *hdc[MI00406]*, *hdc[EY02460]*, *Df(3R)Exel6178*, *Df(3L)ED229*, *Df(3L)ED228*, *Df(3L)BSC445*, *kto1*, *Df(3L)Exel6112*, *Df(3R)BSC681*, *Df(3R)BSC745*, *Df(3R)BSC744*, *pak6*, *pak14*, *pak16*, *Df(3L)Exel6105*, *Df(3L)fz2*, *Df(3L)ED4789*, *Df(3L)ED4789*, *rept[EY12756]*, *rept[f01801]*, *rept[06945]*, *E74A[neo24]*, *Df(3L)81k19*, *br[rbp5]*, *hs-GAL4*, *UAS-GAL4*, *Sgs3-GAL4*, *UAS-EcR[F645A]*, and *UAS-p35*. Also from the BDSC but not listed are the complementing and lethal mutations used for mapping purposes. K. Moses (Janelia Farm, Asburn, VA) kindly provided the *brwd3/ramshackle* stocks *ram1*, *ram[p1]*, *ram[p2]*, and *ram[px3]*. C. Thummel (University of Utah, Salt Lake City, UT) kindly provided the *hs-E74A* stock. E. Baehrecke (UMass Medical School, Worcester MA) kindly provided the *E93[1]* and *E93[6]* stocks. The *UAS-Atg1[K38Q]* stock was generated in the Nuefeld laboratory (Scott *et al.* 2007). All PSG mutations were generated in an EMS-mutagenesis screen on the third chromosome (Wang *et al.* 2008). The allelic combinations used here for phenotypic and molecular analysis of each PSG locus are underlined in Table 1.

Mapping PSG mutations

Recombination mapping was conducted using pairs of dominant markers (Sapiro *et al.* 2013). The resulting recombination position was used to cross each mutation to deficiencies within the DK3 kit in the BDSC and, once mapped to a large deficiency, to progressively smaller deficiencies until a molecularly defined region was identified for each complementation group. Then, all publicly available lethal mutations in the BDSC within these defined regions were tested for complementation. To verify the gene assignment, we tested a number of alleles of the gene in question for complementation and used Sanger sequencing to identify lesions within genomic DNA. The mapping data are summarized in Table 1.

Developmental staging and PSG phenotypes

Animals were reared on cornmeal molasses media with yeast at 25°. Mutant animals were collected 7–10 days after seeding of bottles containing fresh media. For staging during metamorphosis, animals were synchronized as white prepupae [0 hr after puparium formation (0 APF)] and aged appropriately on moist black filter paper on petri dishes at 25°. Average duration of prepupal development was used to

Table 1 Summary of PSG complementation groups mapped to genes

| Gene | PSG loci | Allele(s) | Cytological position | Failed to complement | | Sequenced lesions | Source or reference |
				Deficiencies	Mutations		
belle	psg9	*psg9*	85A	*Df(3R)p712* *Df(3R)Exel6149* *Df(3R)BSC197*	*bel⁶* *belᴸ⁴⁷⁴⁰*	*psg9*: F469S	Ihry *et al.* (2012)
brwd3	psg13	*psg13*	95F	*Df(3R)BSC638* *Df(3R)Exel6199*	*BRWD3⁰⁵⁸⁴²* *ram¹, ramᵖ¹* *ramᵖ², ramᵖˣ³*	*psg13*: 1222(+4X)ᵃ	This study
Eip93F	psg11	*psg11*	93F	*Df(3R)BSC677* *Df(3R)Exel6188* *Df(3R)BSC523*	*Eip93F⁶* *Eip93Fⱽⁿᵒ*	*psg11*: Q485X	This study
hdc	psg12	*psg12*	99F	*Df(3R)BSC503* *Df(3R)ED6332*	*hdcᴮᴳ⁰⁰²³⁷* *hdcᴹᴵ⁰⁰⁴⁰⁶* *hdcᴱʸ⁰²⁴⁶⁰*	*psg12*: Q415X	This study
mdh2	psg7	*psg7aᵇ* *psg7bᵇ* *psg7c*	90F	*Df(3R)Exel6178* *Df(3R)LK19-1*	*mdh2ᴱʸ⁰¹⁹⁴⁰*	*psg7a*: D173G *psg7b*: Δ243-253 *psg7c*: N117I	Wang *et al.* (2008)
med12	psg14	*psg14*	76D	*Df(3L)ED229* *Df(3L)ED228* *Df(3L)BSC445*	*kto¹*	*psg14*: Q2175X	This study
med24	psg5	*psg5aᵇ* *psg5bᵇ*	66B	*Df(3L)ED4408* *Df(3L)Exel6112* *Df(3L)66C-G28*	*med24ᴮᴳ⁰¹⁶⁷⁰*	*psg5a*: M532I, Q893X *psg5b*: Q212X	Wang *et al.* (2008)
pak	psg4	*psg4a* *psg4b* *psg4c*	83E	*Df(3R)BSC681* *Df(3R)BSC745* *Df(3R)BSC744*	*pak⁶* *pak¹⁴* *pak¹⁶*	*psg4a*: Q325X *psg4b*: G439S *psg4c*: Δ512-604	This study
psg2	psg2	*psg2a* *psg2b*	64D	*Df(3L)Exel6105* *Df(3L)ZN47*	*psg2ᴱʸ⁰⁴⁴⁵¹*	*psg2a*: Q567X *psg2b*: Q650X	Wang *et al.* (2008)
reptin	psg10	*psg10*	76A	*Df(3L)fz2* *Df(3L)ED4789*	*reptᴱʸ¹²⁷⁵⁶* *reptᶠ⁰¹⁸⁰¹* *rept⁰⁶⁹⁴⁵*	*psg10*: I7V, P318L, V469M	This study

Underlined alleles and deficiencies indicate the allelic combinations used for phenotypic and molecular analysis.
ᵃ Donor splice site mutation in *brwd3ᵖˢᵍ¹³* that causes a premature stop codon after translating four ectopic residues within the unspliced intron.
ᵇ PSG alleles previously reported under different names: *med24¹, med24ᵖˢᵍ⁵ᵇ*; *mdh2², mdh2ᵖˢᵍ⁷ᵃ*; *mdh2¹, mdh2ᵖˢᵍ⁷ᵇ*.

determine the staging prior to head eversion. For staging during late larval stages, animals were reared in media containing bromophenol blue to select dark gut (-12 APF), light gut (-8 APF), and clear gut (-4 APF) stages (Andres and Thummel 1994). Block in the destruction of salivary glands was assayed at $+24$ APF in mutant animals carrying the *fkh-GAL4, UAS-GFP* transgenes as previously described (Ward *et al.* 2003). Only persistent salivary glands in animals that head everted relatively normally were counted as having a defect in the larval salivary gland death response. Lethal phases were determined using Bainbridge and Bownes stages (Bainbridge and Bownes 1981): P1–P3 for prepupae (PP), P4–P9 for pupae (P), P10–P15 for pharate adults (PA), and A1–A3 for eclosed adults (A). Images of dissected pupae and of persistent salivary glands were captured using an Olympus SZX16 stereomicroscope coupled to a digital camera. Staging and heat-shock treatments for ectopic expression of *E74A* were performed with control and mutant animals carrying one copy of the *hs-E74A* transgene as previously described (Ihry *et al.* 2012).

Quantitative RT-PCR

To quantify messenger RNA (mRNA) of target genes in whole animals and dissected salivary glands, quantitative real-time reverse transcriptase PCR (qPCR) was performed as previously described (Ihry *et al.* 2012). RNA was isolated using the RNeasy Plus Mini kit (QIAGEN, Valencia, CA). For each cDNA synthesis reaction, the SuperScript III First-Strand Synthesis System was used with 400 ng of total RNA template primed with Oligo(dT)$_{20}$ (Invitrogen, Carlsbad, CA). qPCR was conducted using the Roche 480 LightCycler and LightCycler 480 DNA SYBR Green I Master kit. In all cases, experimental samples and matched controls were analyzed simultaneously for expression of target genes and the reference gene *rp49*. Primer sequences for *reaper*, *hid*, *Nc*, *Ark*, *E74A*, *E75A*, *E93*, and *rp49* were previously described (Ihry *et al.* 2012); the remaining primers used in this study are listed in Supporting Information, Table S1. Relative expression was calculated using the Relative Expression Software Tool (REST) (Pfaffl *et al.* 2002) as previously described (Ihry *et al.* 2012). Each data point used represents the analysis of three independent biological samples (except as indicated in Figure 1B). For developmental expression profiles, the relative expression for each target gene was compared to samples from the stage with the lowest expression. The expression profiles and bar graphs in Figure 6 were compared to values for control salivary glands at 4 hr before head eversion [-4 hr after head eversion (-4 AHE)], a stage
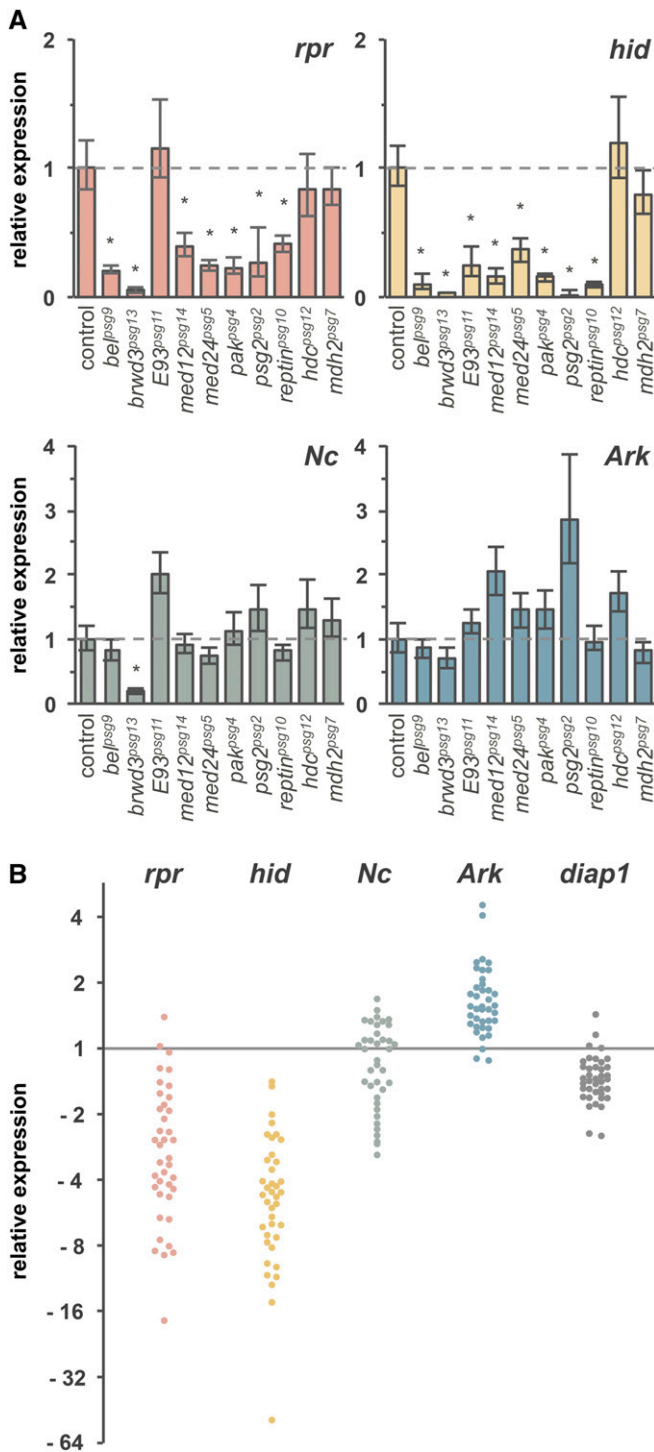
Figure 1 The ecdysone-induced expression of death activators is selectively disrupted in most PSG mutant salivary glands. (A) Eight of the 10 PSG mutations disrupt the ecdysone-induced expression of *reaper* (*rpr*) and/or *hid* in salivary glands dissected at 1.5 hr after head eversion (AHE). On the other hand, the ecdysone-induced expression of *Ark* and *Nc* is largely unaffected, indicating that the defects in hormone-induced transcription are target gene-specific. The *y*-axis plots relative expression of target genes measured by qPCR and compared to control. The *x*-axis shows control and mutant salivary glands. Each bar represents three independent biological samples normalized to *rp49*; asterisks indicate a significant reduction in expression compared to that in control glands (*P*-values <0.05). The allelic combinations used are

prior to the onset of the ecdysone-triggered expression of target genes *reaper* and *hid*.

Immunofluorescence

Salivary glands were dissected from control and mutant animals staged relative to head eversion and stained with antibodies as previously described (Ihry *et al.* 2012). For cleaved caspase-3 staining, salivary glands were permeabilized by heptane treatment prior to fixation. The following primary antibodies were used: 1:50 mouse α-BR-C Z1 (Developmental Studies Hybridoma Bank), 1:50 mouse α-lamin (Developmental Studies Hybridoma Bank), 1:200 rabbit α-cleaved caspase-3 (Cell Signaling), 1:300 mouse α-E74A primary antibodies (a generous gift from C. Thummel), and 1:300 mouse α-E93 (a generous gift from I. Duncan; Washington University, Saint Louis MO). The following secondary antibodies were used at 1:200: Cy3 α-mouse and α-rabbit (Jackson Immuno-Research Laboratories) and AlexaFluor 488 and 633 α-mouse (Invitrogen). Stained salivary glands were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and imaged on an Olympus FluoView FV1000 confocal microscope.

LysoTracker staining

Salivary glands dissected in PBS, from staged animals, were transferred to a 1.5-ml glass well plate containing a solution of LysoTracker Red at 50 nM and DAPI diluted 1:1000 (Invitrogen). Samples were covered and a rotator was used to agitate samples for 10 min at room temperature. Salivary glands were washed one time in PBS and mounted on glass slides in PBS to be imaged immediately by confocal microscopy.

Results

Mapping mutations with a PSG phenotype

As a first step toward functional characterization of the defects in mutant salivary glands, we mapped a subset of PSG complementation groups to their affected genes. We used recombination analysis with pairs of dominant markers and complementation tests with deficiencies to map 28 of the 37 PSG loci to discrete chromosomal regions (Sapiro *et al.* 2013). These 28 mutations were then tested for

shown in Table 1. (B) Molecular survey of 39 different PSG mutant salivary glands indicates that most PSG mutations disrupt initiation of the ecdysone-triggered death response. Expression of *reaper*, *hid*, *Nc*, *Ark*, and *diap1* was measured in salivary glands dissected at +15 APF (equivalent to ∼ +3 AHE). The majority of PSG mutant glands display selective defects in the ecdysone-induced expression of *rpr* and *hid*, while *Nc*, *Ark*, and *diap1* are expressed at near normal levels. Each dot represents a single sample of 30 salivary glands dissected from each PSG mutant analyzed. All samples were analyzed simultaneously and compared to three independent control samples dissected at +1.5 AHE (gray line at "1"). The *y*-axis shows relative expression (plotted on a log₂ scale) normalized to both *rpl18a* and *ubcd6*.

complementation with all publicly available lethal mutations within these mapped regions. The intent was to use an unbiased approach to rapidly identify a subset of PSG loci for further analysis. The PSG mutations identified in this manner map to the following 10 genes: *belle* (*bel*), *brwd3/ram*, *E93* (*Eip93F*), *headcase* (*hdc*), *mdh2*, *med12*, *med24*, *pak*, *psg2*, and *reptin* (*rept*) (Table 1). Of these, the mapping of PSG mutations in *belle* (Ihry *et al.* 2012) and in *med24*, *mdh2*, and *psg2* (Wang *et al.* 2008) have been reported. The remaining 19 PSG loci complemented all publicly available lethal mutations in their respective regions and were not pursued further.

All PSG mutations are lethal, with most animals arresting later during metamorphosis as pupae or pharate adults (Figure S1). Sanger sequencing shows that most of the newly mapped PSG alleles carry nonsense mutations that result in truncation of a significant portion of the encoded proteins (Table 1). Consistently, most mutations have similar lethal phases in homozygous and hemizygous (over a deficiency) combinations, suggesting that these mutations likely represent strong hypomorphic or loss-of-function alleles of the affected genes. On the other hand, hemizygous combinations of $med12^{psg14}$ die as prepupae while those of $brwd3^{psg13}$ and $reptin^{psg10}$ die earlier in development, suggesting that these PSG mutations are likely hypomorphic alleles. Our data also suggest that PSG mutations may represent unique alleles of their respective genes. For example, $E93^{psg11}$ complements the $E93^1$ allele but fails to complement the $E93^{vno}$ allele, even though both lethal mutations map to the same gene (Mou *et al.* 2012). A similar allele-specific phenotype was recently described for bel^{psg9}, which disrupts a subset of molecular functions regulated by *belle* (Ihry *et al.* 2012).

The PSG genes identified to date from this screen represent a functionally diverse group of proteins. *belle* is a DEAD box RNA-helicase required for translation of the ecdysone-induced transcription factor *E74A* (Ihry *et al.* 2012); in turn, *E74A* is required for the ecdysone-triggered destruction of salivary glands (Jiang *et al.* 2000; Wang *et al.* 2008). *brwd3/ram* contains a bromodomain and several WD40 repeats and likely regulates chromatin function (D'Costa *et al.* 2006). *E93* encodes an ecdysone-induced transcription factor required for the destruction of salivary glands (Lee *et al.* 2000). *hdc* encodes an evolutionarily conserved protein containing domains with currently unknown molecular functions (Weaver and White 1995; Chien *et al.* 2006). *mdh2* is a nuclear-encoded mitochondrial malate dehydrogenase (Wang *et al.* 2008, 2010). *med12* and *med24* are two subunits of the multiprotein mediator complex required for activator-dependent transcription (Malik and Roeder 2010). *pak* encodes a member of the highly conserved family of p21-activated kinases (Molli *et al.* 2009). *psg2* encodes a protein product without defined molecular functions (Wang *et al.* 2008). And finally, *reptin* is an AAA+ family member with biochemical roles in both transcription and chromatin remodeling (Jha and Dutta 2009). Impor-

tantly, two of the mapped PSG genes, *E93* and *belle*, encode regulators of ecdysone signaling required for the death response in salivary glands (Lee *et al.* 2000; Ihry *et al.* 2012). The roles of the remaining PSG genes in ecdysone signaling, if any, have not been previously characterized.

PSG mutations primarily disrupt the ability to initiate, rather than execute, the ecdysone-triggered death response

To begin to understand how PSG genes disrupt the death response in salivary glands, we measured the expression of ecdysone-induced target genes that initiate tissue destruction. The destruction of salivary glands requires activation of both caspases and autophagy (Berry and Baehrecke 2007). Although expression of several autophagy genes increases during the death response (Gorski *et al.* 2003; Lee *et al.* 2003), their role in activation of autophagic death and their relationship to ecdysone signaling have yet to be determined. In contrast, ecdysone activates caspases in salivary glands through transcriptional control of *reaper* and *hid* (Jiang *et al.* 1997, 2000). Because expression of *reaper* and *hid* is sufficient to initiate caspase activation (Grether *et al.* 1995; White *et al.* 1996), their induction defines a molecular readout, a point of no return, for the ecdysone-triggered death response. Failure to induce these genes, as occurs in *E74A*, *br-Z1*, or *E93* mutant salivary glands (Jiang *et al.* 2000; Lee *et al.* 2002b), indicates defects upstream of the death response, likely within the ecdysone signaling cascade. Conversely, normal induction of *reaper* and *hid* may indicate defects downstream of the death response, possibly within the machinery that executes tissue destruction (*e.g.*, caspases and/or autophagy). To measure whether *reaper* and *hid* were induced in response to ecdysone, we dissected salivary glands from control and mutant animals at +1.5 AHE, extracted total mRNA, and measured target gene expression using qPCR. This analysis was done at +1.5 AHE because *reaper* and *hid* are maximally induced in control glands at this stage. To our surprise, 8 of the 10 PSG mutations disrupt the ecdysone-dependent expression of these death activators (Figure 1A). Of these, only $E93^{psg11}$ differentially affects death activator expression, disrupting *hid* but not *reaper* expression (the other 7 mutations disrupt expression of both *reaper* and *hid*; Figure 1A). The $E93^{psg11}$ allele reveals a functional complexity within the *E93* locus since the $E93^1$ allele disrupts expression of both *reaper* and *hid* (Lee *et al.* 2000, 2002b); this also suggests that induction of *reaper* and *hid* are genetically separable. Mutations in *med24* have been previously reported to not affect *reaper* or *hid* expression (Wang *et al.* 2010), but these results were based on Northern blot analysis, which provides more qualitative than quantitative expression data. Importantly, despite the block in the ecdysone-triggered expression of *reaper* and *hid*, expression of other genes induced by the same pulse of ecdysone was not affected in these mutant glands. Ecdysone-induced expression of the caspase adaptor protein *Ark* (Lee *et al.* 2000; Ihry *et al.* 2012) is not disrupted in any mutant

glands, while the ecdysone-induced expression of the initiator caspase *Nc* (Dorstyn *et al.* 1999; Lee *et al.* 2000) is disrupted only in *brwd3^{psg13}* mutant glands (Figure 1A). Although these results do not represent a comprehensive list of ecdysone-dependent targets in salivary glands, they do demonstrate that PSG mutations do not disable ecdysone signaling. Instead, most PSG mutations disrupt the expression of a subset of ecdysone-induced target genes, target genes like *reaper* and *hid*, which then activate pathways that execute tissue destruction.

To further demonstrate that defects upstream of the death response occur in most PSG mutations, we measured expression of death activators in salivary glands dissected from 39 different PSG mutant animals. These mutations represent the top 30 complementation groups identified in the original mutagenesis screen. For practical reasons, this molecular survey was performed using one biological replicate of 30 salivary glands dissected from each of the mutant animals. These results show that the defects described above are representative of the entire collection of PSG mutations. Expression of *reaper* and *hid*, but not *Nc* and *Ark*, was strongly disrupted in most PSG mutant glands (Figure 1B). In addition, the expression of the critical anti-apoptotic gene, *diap1*, does not vary significantly among mutant glands, suggesting that the failure to initiate the death response is not caused by increased expression of apoptotic inhibitors. Despite the shortcomings of using one biological replicate, this molecular survey demonstrates that PSG mutations fall into two broad groups: those that disrupt the ability to initiate the death response (failing to induce *reaper* and *hid*) and those that disrupt the ability to execute the death response (inducing normal levels of *reaper* and *hid*). Most PSG mutations fall into the first group, those that disrupt the ability to initiate the death response.

PSG mutations disrupt a small subset of ecdysone-triggered responses during metamorphosis

Given that most PSG mutations appear to disrupt ecdysone signaling during the death response in salivary glands, we examined whether these mutations were also required for other ecdysone-triggered responses at the onset of metamorphosis (Figure 2A). The mid-L3 pulse of ecdysone initiates larval wandering behavior (Riddiford 1993). About 24 hr later, the late larval pulse of ecdysone triggers eversion of the anterior spiracles and contraction of the larval body into the barrel shape of the future pupa (Ashburner 1989). This late larval pulse of ecdysone also initiates the destruction of larval midguts (Jiang *et al.* 1997; Lee *et al.* 2002a). Approximately 12 hr later, the prepupal pulse of ecdysone triggers eversion of the head capsule, as well as inflation of the wing and leg imaginal discs (Fristrom and Fristrom 1993). Subsequent extension of pupal legs is also thought to be dependent on ecdysone signaling (D'Avino and Thummel 1998). To our surprise, most of these stage-specific responses to ecdysone occur normally in PSG mutant animals (Figure 2B, Figure S2A). In fact, of all the ecdysone-triggered responses

examined, PSG mutations disrupted, in addition to the destruction of larval salivary glands, only the destruction of larval midguts and the extension of leg imaginal discs. Importantly, however, the disruption of these two additional tissue-specific responses does not depend on the same set of PSG mutations, nor are they regulated by the same pulse of ecdysone, suggesting that each PSG gene regulates a small, but distinct, subset of ecdysone-triggered responses during metamorphosis.

The only apparent phenotype in common for all PSG mutations is the failure to destroy larval salivary glands (Figure 2B, Figure S2B). This tissue specificity may indicate a role for PSG genes during the development of salivary glands. To address this possibility, we examined salivary gland-specific responses to the same three pulses of ecdysone described above (Figure 2A). These pulses of ecdysone initiate distinct responses in developing salivary glands. The mid-L3 pulse of ecdysone initiates synthesis of glue proteins (Beckendorf and Kafatos 1976; Korge 1977); the late larval pulse initiates secretion of synthesized glue proteins (Biyasheva *et al.* 2001), allowing newly-formed pupae to stick to solid surfaces; and, finally, the prepupal pulse of ecdysone triggers the death response (Jiang *et al.* 1997). Synthesis of glue proteins was examined using qPCR to measure ecdysone-dependent induction of the glue gene *Sgs3* (Figure 2B, Figure S3) and secretion of glue proteins was examined by the ability of pupae to firmly adhere to the sides of vials (Figure 2B). As expected, all of these responses to ecdysone were blocked by salivary gland-specific expression of a dominant negative version of the ecdysone receptor (*EcR^{F645A}*; Figure 2B, Figure S3). PSG mutant animals, however, disrupted only the death response, not the responses to the prior two pulses of ecdysone (Figure 2B, Figure S3), suggesting that PSG mutations do not disrupt the overall development of salivary glands. Taken together, the PSG mutations encode genes that regulate a highly-selective subset of stage- and tissue-specific biological responses initiated by systemic pulses of ecdysone.

Most PSG genes are induced in a stage- and tissue-specific manner during the ecdysone-triggered death response

To understand how PSG mutations regulate the death response in such a specific manner, we examined when and where these PSG genes were expressed. Total mRNA was extracted from either dissected salivary glands or whole animals at various developmental stages from early wandering third instar larvae until after the death response (−12 APF through +13.5 APF). The two systemic pulses of ecdysone that occur during this period, the late larval pulse at −4 APF and the prepupal pulse at +10 APF, are highlighted by the induction of the ecdysone primary response genes *E74A* and *E75A* (Figure 3A). Consistent with published reports (Baehrecke and Thummel 1995), *E93* showed a strong stage-specific induction in response to the prepupal pulse of ecdysone in salivary glands (Figure 3A). Strikingly,
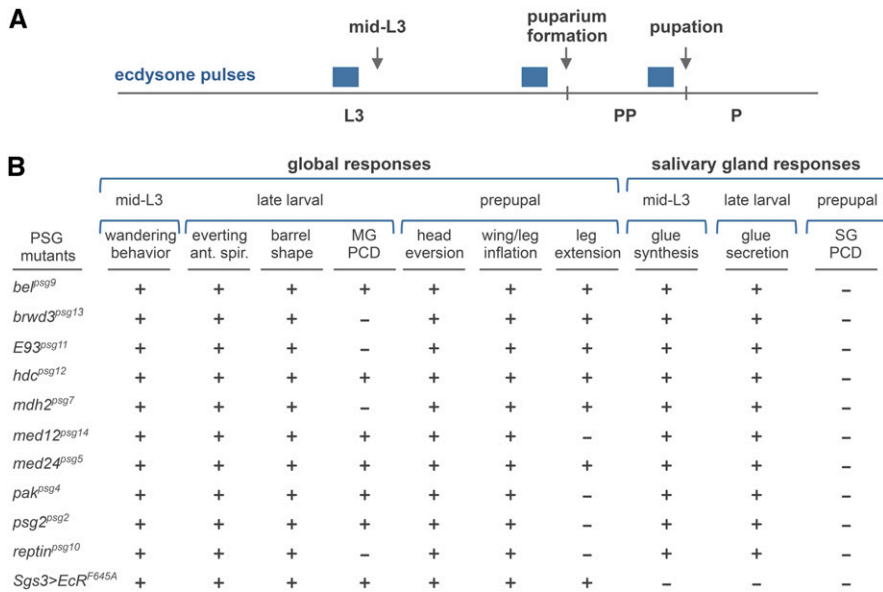
Figure 2 PSG genes are required for a subset of stage- and tissue-specific ecdysone-induced responses during the onset of metamorphosis. (A) Schematic showing the three systemic pulses of ecdysone (blue boxes) at the onset of metamorphosis. Arrows mark the major developmental transitions triggered by each of these pulses of ecdysone: the mid-third instar transition (mid-L3), puparium formation, and pupation. L3, third instar larvae; PP, prepupae; P, pupae. (B) Global and salivary gland-specific responses to each of the three pulses of ecdysone (referred to as the mid-L3, late larval, and prepupal pulses) were scored in PSG mutant animals. The "+" and "−" indicate whether or not appropriate responses were observed. Ant. spir, anterior spiracles; MG PCD, midgut programmed cell death; SG PCD, salivary gland programmed cell death. Genotype of $Sgs3 > EcR^{F645A}$ is $UAS\text{-}EcR^{F645A}/+$; $Sgs3\text{-}GAL4/+$. Allelic combinations used are shown in Table 1.

most PSG genes also showed a stage-specific expression profile in salivary glands similar to that of *E93*. *belle*, *brwd3*, *med12*, *pak*, *psg2*, and, to a lesser but statistically significant extent, *med24* and *rept*, show increased expression during the death response (Figure 3A). Moreover, these stage-specific expression profiles were also tissue-specific: whole animal expression profiles of these genes, other than that of *E93*, are relatively flat throughout the stages examined (Figure 3B). In contrast, the two remaining PSG genes, *hdc* and *mdh2*, have different expression profiles (Figure 3). *hdc* shows a gradual increase in expression after puparium formation in glands and in whole animals, with a spike in expression immediately after head eversion in salivary glands. *mdh2* remains relatively flat throughout this period in both expression profiles. Thus, most PSG genes are induced in a stage- and tissue-specific manner during the ecdysone-triggered death response in salivary glands.

PSG genes brwd3, med12, med24, pak, and psg2 encode novel ecdysone secondary response genes

The expression profiles of most PSG genes suggest that they may be induced by the prepupal pulse of ecdysone. In fact, one of the best-characterized stage-specific targets of ecdysone is *E93* (Baehrecke and Thummel 1995; Lee *et al.* 2000). *E93* is an ecdysone primary response gene, directly induced by the prepupal pulse of ecdysone, and thought to provide specificity to ecdysone responses (Lee *et al.* 2000; Mou *et al.* 2012). To determine whether the remaining PSG genes were also targets of ecdysone, we first tested whether ecdysone was required for expression of these genes. Indeed, blocking ecdysone signaling immediately prior to the prepupal pulse of ecdysone, with EcR^{F645A}, disrupts the salivary gland-specific induction of all PSG genes except *reptin* (Figure 4A). As expected, expression of *mdh2*, which is not upregulated during this stage, is also unaffected by EcR^{F645A} (Figure 4A). We then tested whether these newly-identified

genes were primary or secondary response genes within the ecdysone transcriptional hierarchy. To do this, we measured expression of the ecdysone-dependent PSG genes in salivary glands dissected at +1.5 AHE from animals carrying null mutations in the ecdysone primary response genes *E74A* ($E74A^{neo24}$), *E93* ($E93^1$), and *br-Z1* (br^{rbp5}). Ecdysone primary response genes are directly induced by ecdysone and their induction does not depend on activity of other primary response genes. In contrast, expression of the newly-identified ecdysone-dependent genes is regulated by primary response genes. Six of the PSG genes (*belle*, *brwd3*, *med12*, *med24*, *pak*, and *psg2*) require all three ecdysone primary response genes for their expression during the death response (Figure 4B). Together with expression profiles that coincide with ecdysone pulses (Figure 3A), these six genes fit the classical definition of ecdysone secondary response genes (Ashburner 1974). On the other hand, expression of *hdc*, although dependent on a subset of primary response genes (Figure 4B), does not display a classical ecdysone-dependent expression profile (Figure 3A).

We have recently shown that the PSG gene *belle* is critical for translation of *E74A* mRNA (Ihry *et al.* 2012). Here we show that induction of *belle* during the death response depends on the primary response gene it regulates, demonstrating cross-talk between layers of the ecdysone-triggered transcriptional hierarchy, whereby an ecdysone secondary response gene is required for translational control of an ecdysone primary response gene. Although the functional significance of this cross-talk has yet to be determined, it raises the possibility that other PSG genes may also disrupt translation of ecdysone primary response genes. To examine this possibility, we stained salivary glands, dissected at head eversion, with antibodies directed to E74A, E93, and BR-Z1 proteins. These three proteins are the only ecdysone primary response genes known to be required for the death response in salivary glands (Jiang *et al.* 2000; Lee *et al.* 2002b). As
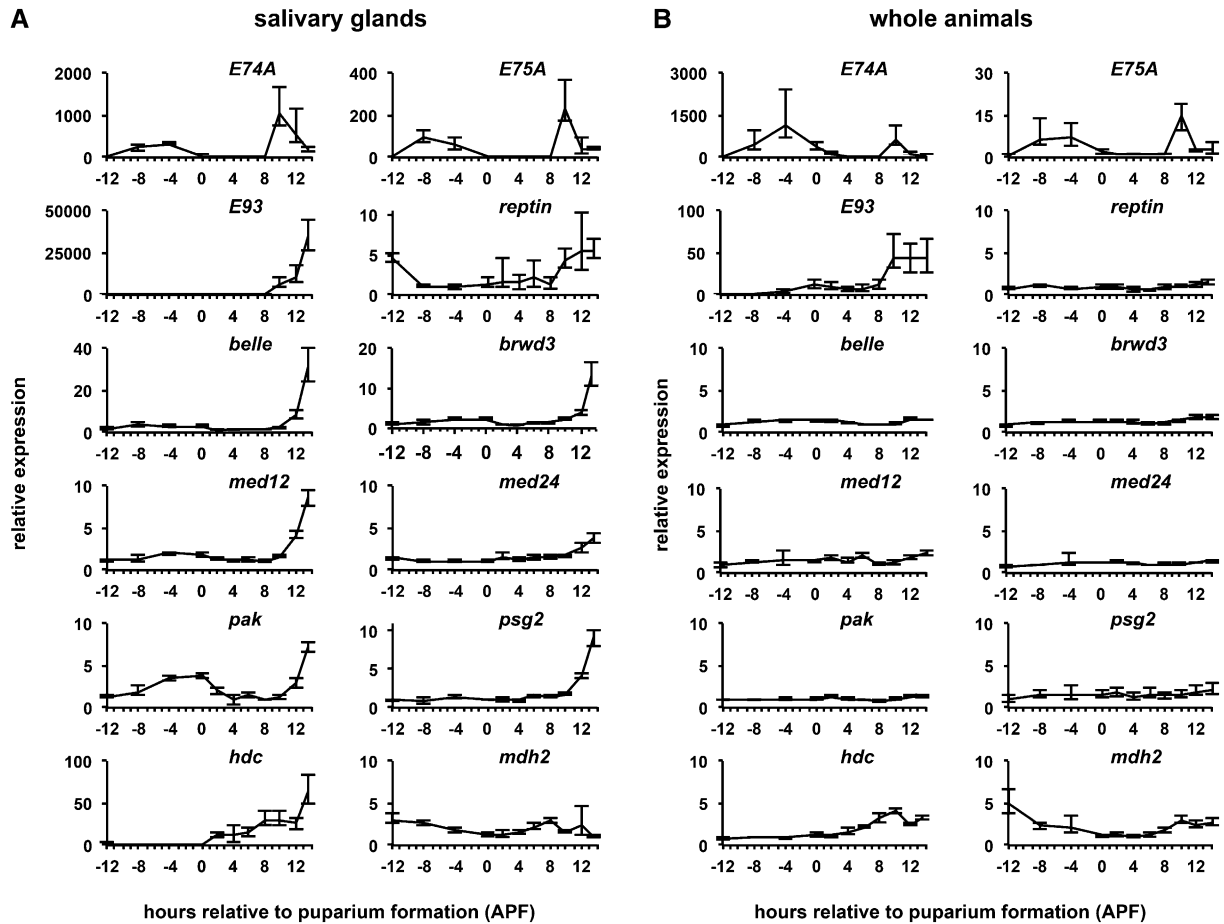
Figure 3 Most PSG genes show stage- and tissue-specific expression profiles. (A and B) Developmental expression profiles of target genes measured by qPCR in dissected salivary glands (A) or in whole animals (B). Previously characterized ecdysone target genes *E74A* and *E75A* show strong induction in response to the late larval (at −4 APF) and prepupal (at +10 APF) pulses of ecdysone, while *E93* responds only to the prepupal pulse. Most PSG genes, however, show strong induction after the prepupal pulse of ecdysone in salivary glands yet remain relatively flat in whole animals. The y-axis plots relative expression measured by qPCR for each target gene compared to the stage with the lowest expression. The x-axis plots developmental stage relative to puparium formation in control salivary glands and whole animals. Each time point represents three independent biological samples normalized to *rp49*.

expected, E74A protein was absent in *bel^psg9* mutant glands, and E93 protein was absent in *E93^psg11* mutant glands (Figure 4C). However, all the remaining combinations were positive (Figure 4C), demonstrating that PSG genes, with the exception of *belle* and *E93*, do not disrupt translation of ecdysone primary response genes. Taken together, these results indicate that half of the PSG genes, *brwd3*, *med12*, *med24*, *pak*, and *psg2*, encode *bona fide* secondary response genes within the ecdysone transcriptional hierarchy.

The ecdysone secondary response gene *med24* is required to amplify target gene expression during the death response

Our results indicate that all of the newly-identified ecdysone secondary response genes are required for the ecdysone-triggered death response, disrupting the ecdysone-triggered expression of *reaper* and *hid*. However, the role of these secondary response genes during ecdysone signaling remains unclear. To address this problem, we focused on one of the

ecdysone secondary response genes, *med24*. Hemizygous *med24^psg5* mutant animals have one of the most highly penetrant PSG phenotypes (Figure S2B), making it an ideal candidate for further analysis. We first examined the developmental expression profiles of *reaper* and *hid* in control and mutant salivary glands. In control glands, induction of *reaper* and *hid* starts at head eversion in response to the prepupal pulse of ecdysone and continues to increase thereafter (Figure 5A). In *med24^psg5* glands, induction of *reaper* also starts at head eversion but at three times lower levels: 23-fold induction in *med24^psg5* compared to 71-fold induction in control glands (Figure 5A). Although the expression of *reaper* increases thereafter in mutant glands, it remains significantly lower than in control glands. The expression of *hid* shows a similarly submaximal expression profile in mutant glands (Figure 5A). In contrast to these results, expression of *EcR^F645A* results in a complete disruption of *reaper* and *hid* expression (Ihry *et al.* 2012), suggesting that within the ecdysone transcriptional hierarchy *med24^psg5* regulates
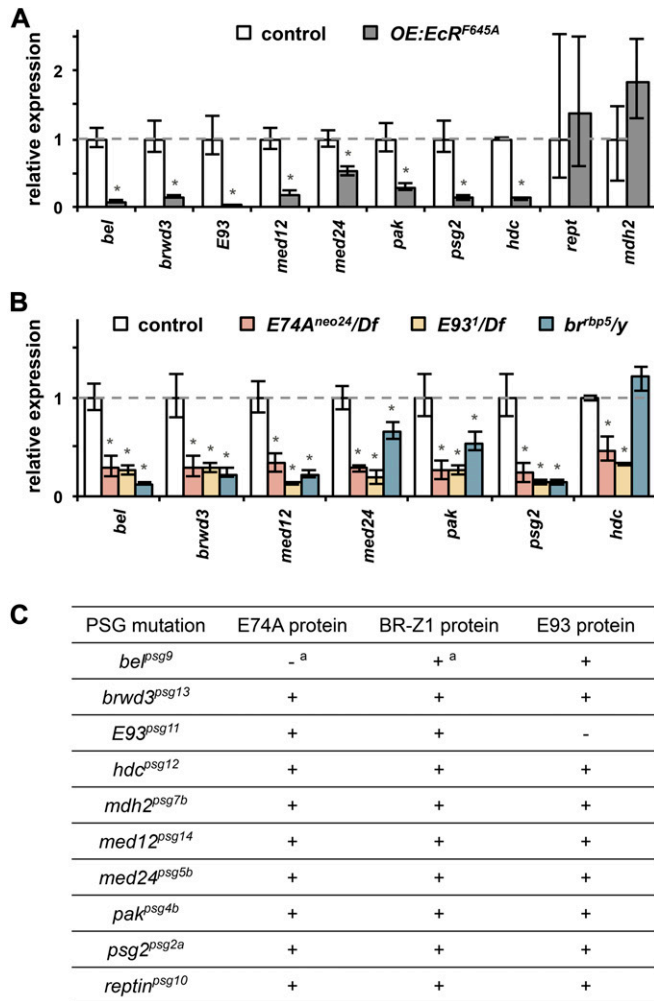
Figure 4 Many PSG genes encode components of the ecdysone-triggered transcriptional hierarchy. (A) Expression of a dominant negative version of the ecdysone receptor (EcR^{F645A}) prior to the prepupal pulse of ecdysone blocks expression of most PSG genes in salivary glands, showing that the stage-specific induction in salivary glands is hormone-dependent. Control salivary glands (white bars) were dissected 1.5 hr after head eversion (AHE). $OE:EcR^{F645A}$ (UAS-$GAL4/UAS$-EcR^{F645A}; hs-$GAL4/+$) glands (gray bars) were dissected at +1.5 AHE, after prepupae were heat-shocked 4 hr before head eversion (−4 AHE). The x-axis shows all 10 PSG genes and the y-axis plots relative expression compared to control and normalized to $rp49$. (B) The ecdysone primary response genes $E74A$, $E93$, and br-$Z1$ are required for the ecdysone-dependent induction of PSG genes in salivary glands. Shown is qPCR analysis of PSG gene expression in salivary glands dissected from control and mutant animals at +1.5 AHE. The x-axis shows all ecdysone-dependent PSG genes (except $E93$, which is a known ecdysone primary response gene). The colors of the bars indicate the genotype of the dissected salivary glands at +1.5 AHE: control (white), $E74A^{neo24/Df}$ (pink), $E93^{1/Df}$ (yellow), or $br^{rbp5/y}$ (blue). The y-axis plots relative expression of target genes compared to control and normalized to $rp49$. All qPCR data represent results from three independent biological samples with asterisks indicating significant change in expression compared to that in control glands (P-values <0.05). (C) Translation of ecdysone primary response proteins E74A, E93, and BR-Z1 in PSG mutant salivary glands. All mutant glands show robust expression of the three primary response genes (marked by "+"), except for absence of E74A protein in bel^{psg9} and E93 protein in $E93^{psg11}$. Salivary glands were dissected at head eversion and analyzed by immunofluorescence with antibodies directed to E74A, E93, and BR-Z1 proteins. [a], previously reported in Ihry et al. (2012).

the amplitude, not the onset, of the ecdysone-induced transcriptional response. This defect is consistent with the observed submaximal expression of reaper and hid in all PSG mutations that disrupt the death response (Figure 1A) and reflects the biological significance of inducing sufficiently high levels of these death activators (Yin and Thummel 2004).

Defects in amplitude of transcriptional responses reflect a slower rate of transcriptional initiation; however, defects in the rate of transcription are often difficult to distinguish from defects in the timing of transcription. This is important for hormonal signaling cascades because, for example, a relatively minor delay in the timing of the hormone pulse may result in significantly lower levels of accumulated transcripts. To separate defects in amplitude and timing, we first identified a critical regulator of the timing of the death response downstream of ecdysone. Ecdysone primary response genes $E74A$, $E93$, and br-$Z1$ are coordinately transcribed in response to the prepupal pulse of ecdysone (Thummel et al. 1990; Karim and Thummel 1992; Baehrecke and Thummel 1995). Their protein products, however, are translated at different times. Using antibodies directed to the E74A, E93, and BR-Z1 proteins, we show that translation of E74A protein coincides with head eversion, while translation of E93 and BR-Z1 proteins occurs at least 1 hr earlier (Figure 5B). This result raises the possibility that translation of E74A protein may determine the timing of the death response. Accordingly, precocious expression of E74A protein from a heterologous promoter (Ihry et al. 2012), ~1.5 hr before head eversion (−1.5 AHE), is sufficient to trigger an ~44-fold induction of reaper expression (Figure 5C). On the other hand, the same treatment in $med24^{psg5}$ mutant glands triggered an ~14-fold induction of reaper (Figure 5C), three times lower than the induction observed in control glands. Precocious E74A protein had a smaller, but statistically significant, effect on expression of hid in control salivary glands (Figure 5C). This $E74A$-dependent response of hid is absent in $med24^{psg5}$ mutant glands (Figure 5C). Taken together, these results suggest that $med24$, and most likely the other ecdysone secondary genes, regulate the maximal induction of reaper and hid, consistent with a role in amplification during hormone-dependent transcription.

med24 is required to initiate tissue destruction in salivary glands

To demonstrate that the defects in amplification of the death response result in the subsequent failure to execute tissue destruction, we stained $med24^{psg5}$ mutant glands for markers of caspase activation and autophagy. Phenotypic analysis of autophagy is difficult and labor-intensive, often requiring electron microscopy to evaluate the number and size of vesicles during autophagosome formation (Klionsky et al. 2012). We used LysoTracker as a potential surrogate for autophagy since this vital dye stains all acidic structures within cells. At head eversion, salivary gland cells are filled with small LysoTracker-positive acidic structures (Figure 6A). About 1.5 hr after head eversion (+1.5 AHE), after the death response has initiated, LysoTracker stains much
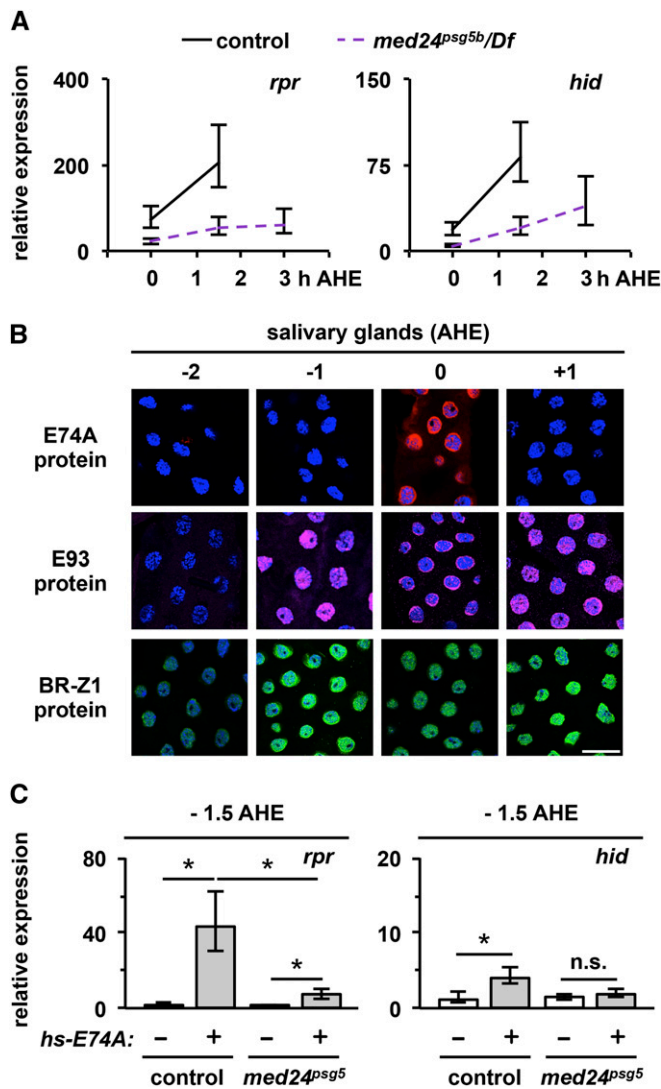
Figure 5 The PSG gene *med24* amplifies hormone-dependent expression of effector genes *reaper* and *hid*. (A) Developmental expression profiles of *rpr* or *hid* in control (black solid line) and *med24* mutant (purple dashed line) salivary glands. *rpr* and *hid* are induced, but at submaximal levels in mutant glands. The *y*-axis plots relative expression of *reaper* or *hid* as measured by qPCR. The expression ratios were calculated relative to those in −4 AHE control salivary glands, a stage prior to the prepupal pulse of ecdysone and the onset of *reaper* and *hid* expression. The *x*-axis plots developmental stage relative to hours after head eversion (AHE). (B) E74A protein expression coincides with the onset of *rpr* and *hid* transcription at head eversion, while E93 and Z1 proteins are robustly expressed earlier. Salivary glands were dissected and analyzed by immunofluorescence for expression of E74A (red), E93 (magenta), and BR-Z1 (green) proteins. Nuclei were costained with DAPI (blue). Bar, 50 μm. (C) Precocious expression of E74A protein is sufficient to induce expression of *reaper* and *hid*. Although ectopic E74A can induce *reaper* in *med24* mutant glands, this induction is significantly lower than the response observed in control glands. Ectopic E74A cannot induce *hid* in *med24* mutant glands. Control and mutant animals carrying one copy of the *hs-E74A* transgene (gray bars) were heat-shocked for 30 min at −4 AHE and salivary glands dissected 2 hr later (at −1.5 AHE). The same heat-shock treatment was given to control and mutant glands without the *hs-E74A* transgene (white bars). The *x*-axis shows the genotypes and the presence (+) or absence (−) of the *hs-E74A* transgene. The *y*-axis plots relative expression of *reaper* or *hid* as measured by qPCR. The expression ratios

larger structures (note white asterisks in Figure 6B). Expression of the inhibitor of autophagy *Atg1*[K38Q], however, blocks formation of these large acidic structures (Figure 6, E and F). In contrast, expression of the apoptotic inhibitor *p35* does not disrupt formation of large LysoTracker-positive structures (Figure 6, I and J). Conversely, expression of *p35*, but not *Atg1*[K38Q], disrupts the activation of caspases during the death response in salivary glands. This is shown by staining with antibodies that detect activity of the initiator caspase *Nc* (Fan and Bergmann 2009) and nuclear lamin, a direct target of caspases (Figure 6, C, G, and K).

Disrupting ecdysone signaling by expression of *EcR*[F645A] blocks activation of caspases and formation of any acidic structures (Figure 6, M–O), resulting in a strong PSG phenotype (Figure 6P). On the other hand, blocking autophagy or apoptosis alone does not result in a strong PSG phenotype (Figure 6, H and L). The persistent GFP fragments likely reflect slower tissue breakdown when either caspases or autophagy are blocked. Accordingly, the salivary glands in these animals are too fragile to dissect at +24 APF (Figure S4). In contrast, *EcR*[F645A]-expressing glands can be easily dissected at +24 APF (Figure S4), a faithful measure of the failure in tissue histolysis. Importantly, consistent with its role within ecdysone signaling, *med24*[psg25] mutant salivary glands disrupt caspase activation and the formation of large LysoTracker-positive structures (Figure 6, Q–S), resulting in a strong PSG phenotype with glands that can be dissected at +24 APF (Figure 6T, Figure S4). *med24* was previously reported not to affect autophagy (Wang *et al.* 2010); however, that conclusion was based solely on the presence of LysoTracker staining. Our analysis demonstrates that *med24* mutant glands, like *Atg1*[K38Q]-expressing glands, block formation of the larger acidic structures (Figure 6, F and R), suggesting a possible influence of *med24* on autophagy. Although the ecdysone-dependent target genes that initiate autophagy during the death response have yet to be identified, our results provide genetic evidence that the activation of autophagy and caspases is likely regulated by a common set of genes within the ecdysone transcriptional hierarchy.

Discussion

Systemically-released pulses of steroid hormones initiate a wide variety of biological responses during development and homeostasis. Our results demonstrate that components within the steroid-triggered transcriptional hierarchy itself play a critical role in refining these systemic signals into distinct local responses. These components include novel ecdysone secondary response genes; genes like *brwd3*, *med12*, *med24*, *pak*, and *psg2* that are themselves induced by ecdysone in a stage- and tissue-specific manner. These ecdysone secondary response

were calculated relative to those in −4 AHE control salivary glands. All qPCR data represent three independent salivary gland samples normalized to *rp49*; asterisks indicate significant differences in expression (*P*-values <0.05).
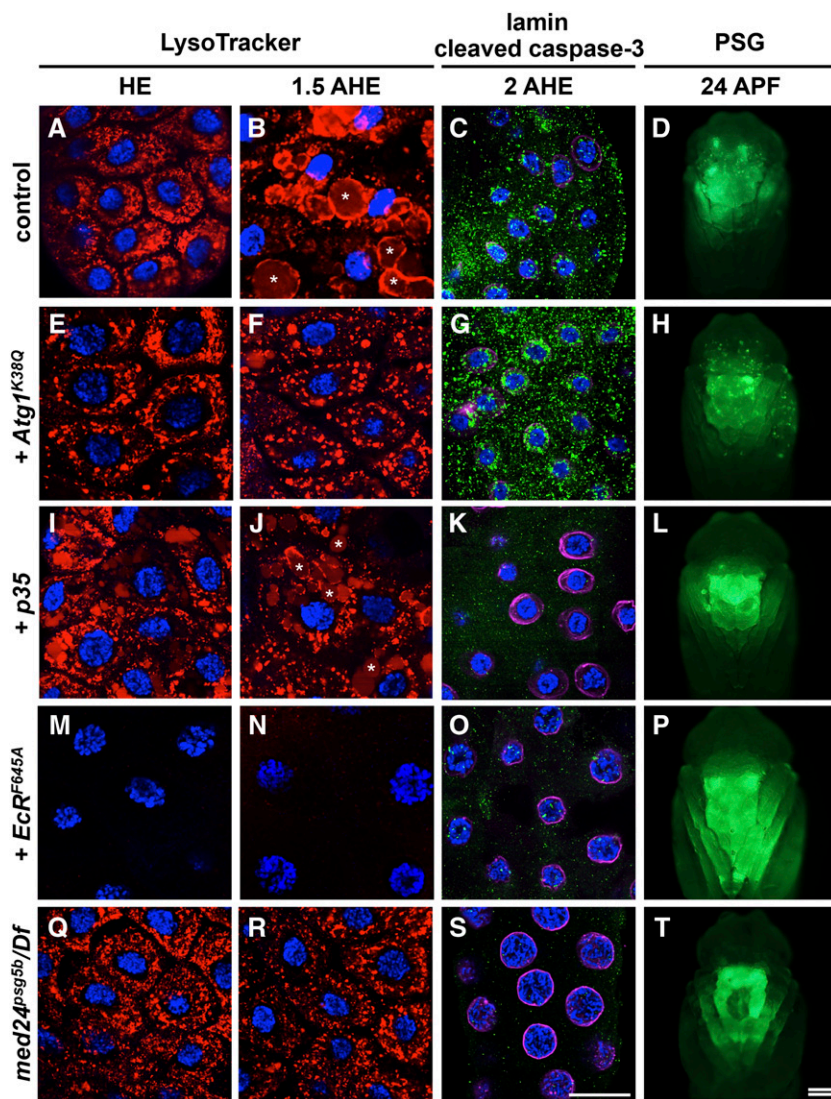
Figure 6 *med24* is required for the ecdysone-triggered death response in larval salivary glands. (A–D) The death response in control salivary glands activates autophagy and caspases, resulting in tissue histolysis. LysoTracker staining (in red), used as a surrogate marker for autophagy, shows formation of large acidic structures only after the death response has been triggered (examples marked by white asterisks in B). In parallel, the death response triggers caspase activation (C), shown by staining with antibodies against cleaved caspase-3 (in green) and loss of staining of a target of caspases, nuclear lamin (in magenta). (D) The resulting tissue destruction is assayed by loss of the salivary gland-specific GFP expression (*Sgs3-GAL4*, *UAS-GFP*) at 24 hr after puparium formation (APF). (E–H) Salivary gland-specific expression of an inhibitor of autophagy, *Atg1^{K38Q}* (*UAS-Atg1^{K38Q}/+; Sgs3-GAL4/+*), blocks formation of the large Lyso-Tracker-positive structures (E and F), but does not block caspase activation (G). Blocking only autophagy does not block tissue breakdown as shown by the absence of an intact PSG phenotype (H). (I–L) Conversely, expression of the caspase inhibitor *p35* (*UAS-p35/+; Sgs3-GAL4/+*) does not block formation of large Lyso-Tracker-positive structures (examples marked by white asterisks in J), but blocks activation of caspases (K). Blocking only caspase activation does not block tissue breakdown (L). (M–P) In contrast, blocking ecdysone signaling by expressing a dominant negative ecdysone receptor (*UAS-EcR^{F645A}/+; Sgs3-GAL4/+*) blocks formation of any LysoTracker-positive structures (M and N) and blocks activation of caspases (O), resulting in a persistent salivary gland phenotype (P). (Q–T) In *med24^{psg5}* mutant glands, LysoTracker staining is similar to that in *Atg1^{K38Q}*-expressing glands (Q and R); moreover, markers of caspase activity are similar to those of *p35*-expressing glands (S), suggesting that activation of autophagy and caspases does not occur. *med24^{psg5}* mutant animals have a PSG phenotype (T). Double bar in T, 200 μm for D, H, L, P, and T; bar in S, 50 μm for A–C, E–G, I–K, M–O, and Q–S. DAPI-costained nuclei are shown in blue. APF, hours after puparium formation; HE, head eversion; AHE, hours after head eversion; PSG, persistent salivary gland.

genes help amplify the hormonal cue, but do so in a tissue-, stage-, and target-specific manner. The result is a dramatic, hormone-dependent, transcriptional activation of genes that initiate the appropriate biological responses.

The experimental context for this study is the ecdysone-triggered destruction of salivary glands during metamorphosis. The original motivation in screening for mutations that disrupt this process was to identify novel regulators of cell death (Wang *et al.* 2008). However, about half of the pupal lethal mutations examined during the mutagenesis screen had a persistent salivary gland phenotype (Wang *et al.* 2008). To winnow this common phenotype, we selected only those mutations with "intact" salivary glands inside pupae with relatively normal morphology. By adding this criterion, we had hoped to select against mutations in global regulators of ecdysone signaling. In hindsight, however, it appears we have also selected against mutations in regulators of caspase activation or autophagy because individually blocking these processes results in incomplete or partial de-

struction of salivary glands (Leulier *et al.* 2006; Berry and Baehrecke 2007). In fact, an independent screen for suppressors of ectopic *reaper* in the eye [Su(*GMR-rpr*)] identified a gain-of-function mutation in *diap1* as well as two loss-of-function mutations in the caspase *Nc*, all present among the original, but "non-PSG", pupal lethal mutations (A. Bashirullah, unpublished data). Nevertheless, a number of mutations within our collection of PSG mutations, like those in *hdc* and *mdh2*, may provide insights into the mechanisms that execute tissue destruction. *mdh2* is required to generate sufficient ATP, a key and rate-limiting ingredient during tissue destruction (Wang *et al.* 2010). The role of *hdc* during tissue destruction, however, remains to be determined. Importantly, our results demonstrate that the selection used to identify PSG phenotypes has primarily enriched for mutations that disrupt the initiation, rather than the execution, of the death response, highlighting the role of the ecdysone-triggered transcriptional hierarchy during this stage- and tissue-specific biological response.

Our data suggest that many, if not most, PSG mutations disrupting ecdysone signaling within salivary glands encode components of the ecdysone-triggered transcriptional hierarchy. All of these genes are induced in a stage- and tissue-specific manner by ecdysone and they are, in turn, required for induction of a subset of ecdysone-dependent target genes. These newly-identified components of the ecdysone transcriptional hierarchy greatly expand the number of genes known to provide specificity to ecdysone pulses during development. Strikingly, most of these components of the ecdysone signaling hierarchy encode *Drosophila* homologs of vertebrate nuclear receptor coregulators. Both Med12 and Med24 bind to and are required for function of a large number of nuclear receptors, including the thyroid receptor (Ito *et al.* 1999; Zhang and Fondell 1999; Fondell 2013), the androgen receptor (Wang *et al.* 2002), the estrogen receptor (Kang *et al.* 2002), and the vitamin D receptor (Ito *et al.* 1999; Zhang and Fondell 1999). One of the *pak* homologs in vertebrates, *pak6*, can directly regulate androgen and estrogen receptor function (Yang *et al.* 2001; Lee *et al.* 2002). In addition, the mammalian ortholog of *E93* is the ligand-dependent corepressor *LCoR* that is required for transcriptional activation by the estrogen, androgen, and progesterone receptors (White *et al.* 2004; Palijan *et al.* 2009; Asim *et al.* 2011). Moreover, although nuclear receptor coactivator functions have not been reported for human *brwd3*, recent proteomic studies have reported interaction of the human BRWD3 with nuclear receptors (Miyamoto-Sato *et al.* 2010).

Our results highlight important parallels between the role of these newly-identified ecdysone response genes in insects and those of nuclear receptor coregulators in vertebrates. Both appear to be required for amplification, but not initiation, of hormone-dependent transcription (Mckenna and O'Malley 2002). Moreover, there is evidence that vertebrate coregulators may also be targets of the hormonal responses they amplify. For example, expression of a subset of androgen receptor coregulators can be upregulated by androgens in human cell lines (Urbanucci *et al.* 2008). Given that increased expression levels of nuclear receptor coactivators generate increased hormone-dependent transcription (Chen *et al.* 2000), the relationship between coactivator function and their increased expression is likely to confer a faster and stronger hormonal response. Moreover, in addition to this hierarchical relationship between hormones and their coregulators, our results also highlight a combinatorial relationship between hormones and their coregulators. Like vertebrate coregulators, ecdysone secondary response genes appear to play a critical role in providing specificity to systemic hormonal pulses, whereby each coactivator regulates a small, but largely nonoverlapping, subset of steroid-triggered biological responses. These results suggest that the same coregulators may be used in different combinations in different tissues to regulate distinct biological processes.

Improper function or expression of nuclear receptor coregulators has been implicated in a rapidly growing list of human diseases (Lonard and O'Malley 2012). However, the number of human nuclear receptor coregulators estimated by proteomics may be in the hundreds and even thousands (Malovannaya *et al.* 2011; O'Malley *et al.* 2012), making it difficult to identify those that play more important roles in development and disease. The degree of functional conservation in the regulation of nuclear receptor signaling described here suggests that forward genetic approaches in *Drosophila* may help identify novel and clinically relevant coregulators of steroid hormone signaling. Accordingly, the human homologs of five of the six components of ecdysone signaling we identified in this genetic screen have been directly linked to endocrine-based disorders like breast, prostate, and ovarian cancers (Kim *et al.* 2005; Siu *et al.* 2010; Asim *et al.* 2011; Barbieri *et al.* 2012; Hasegawa *et al.* 2012).

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Communicating editor: M. Wolfner

GENETICS

Genetic Control of Specificity to Steroid-Triggered Responses in *Drosophila*

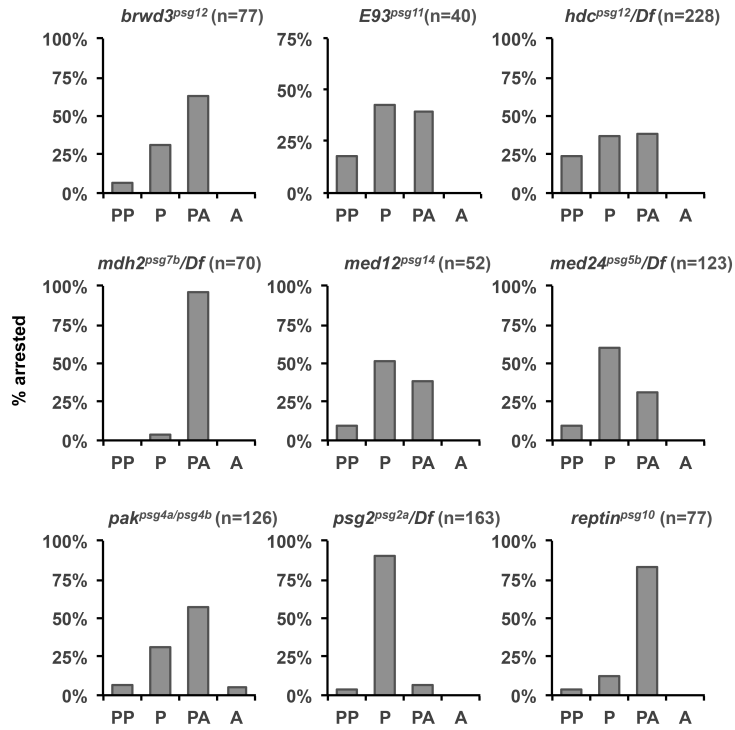**Robert J. Ihry and Arash Bashirullah**

Figure S1 Lethal phase analysis of PSG mutations. The lethal phase analysis shows that the majority of PSG mutant animals arrest after head eversion and die as pupae or pharate adults. The genotype and number of animals used for lethal phase analysis is shown above each graph. PP: prepupae, P: pupae, PA: pharate adults, A: adults eclosed.
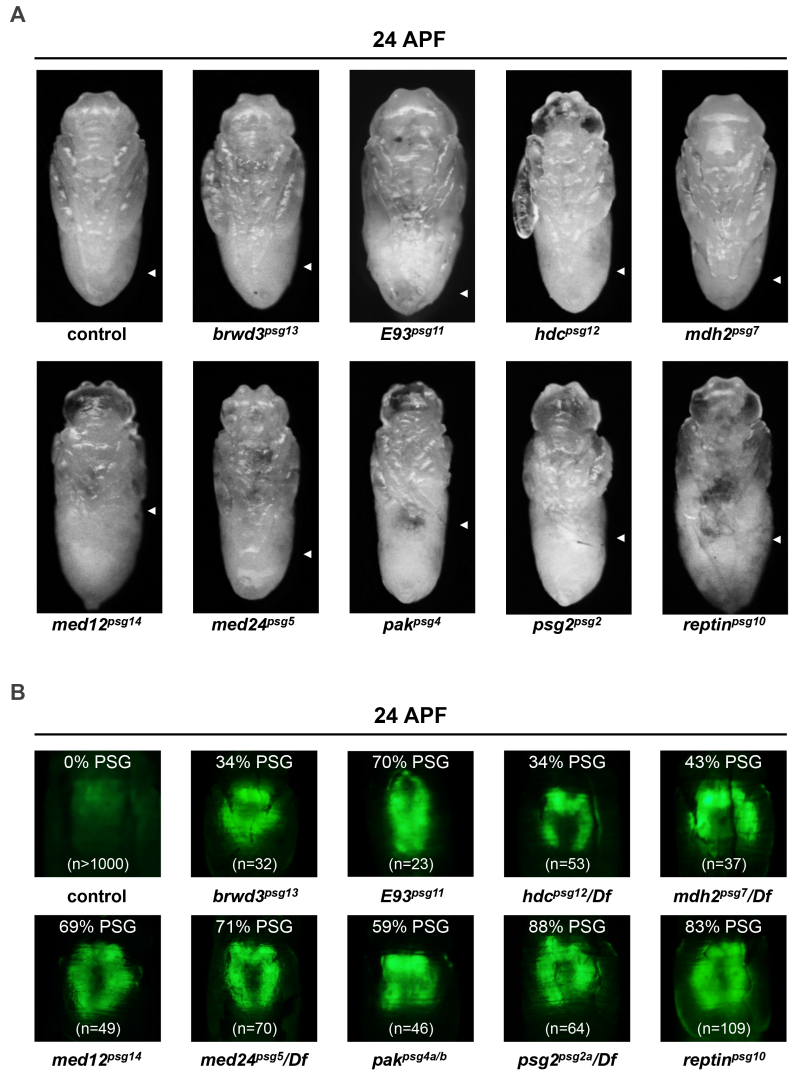
R. J. Ihry and A. Bashirullah

A

24 APF

control   brwd3^{psg13}   E93^{psg11}   hdc^{psg12}   mdh2^{psg7}

med12^{psg14}   med24^{psg5}   pak^{psg4}   psg2^{psg2}   reptin^{psg10}

B

24 APF

0% PSG	34% PSG	70% PSG	34% PSG	43% PSG
(n>1000)	(n=32)	(n=23)	(n=53)	(n=37)
control	brwd3^{psg13}	E93^{psg11}	hdc^{psg12}/Df	mdh2^{psg7}/Df
69% PSG	71% PSG	59% PSG	88% PSG	83% PSG
(n=49)	(n=70)	(n=46)	(n=64)	(n=109)
med12^{psg14}	med24^{psg5}/Df	pak^{psg4a/b}	psg2^{psg2a}/Df	reptin^{psg10}

Figure S2 Pupal morphology and PSG penetrance for PSG mutations. (A) Pupal morphology was examined by dissecting animals out of their pupal casing at 24 hours after puparium formation (APF). All PSG mutants display head capsule eversion, wing and leg inflation. White triangles mark the progression of leg extension. *med12, pak, psg2*, and *reptin* display defects in leg extension. (B) PSG mutants display a highly penetrant block in salivary gland cell death. Each image shows salivary gland-specific expression of GFP (*fkh-Gal4*, *UAS-GFP*) at 24 APF. Within each image, the percent PSG and the number of animals examined are shown in white text.
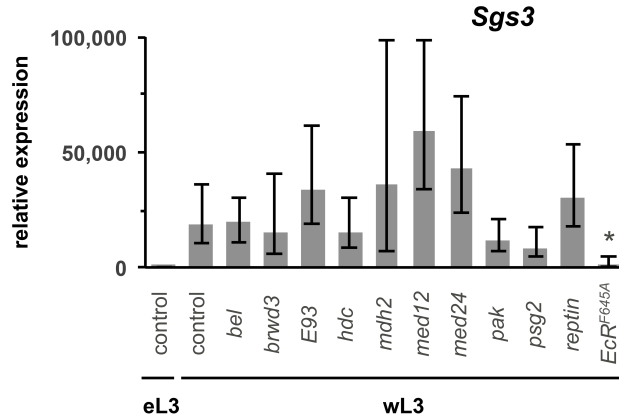
Figure S3 Induction of the glue gene *Sgs3* in response to the mid-L3 pulse of ecdysone. *Sgs3* is massively induced in salivary glands after the mid-L3 pulse of ecdysone; the amount of *Sgs3* mRNA made is easily detectable by qPCR in extracts of total mRNA from whole animals at wandering third instar stages (wL3). wL3 controls display an ~18,500-fold increase of *Sgs3* mRNA when compared to third instar animals prior to the mid-L3 pulse (early L3 or eL3). All PSG mutant wL3 animals show robust induction of *Sgs3* mRNA. In contrast, salivary gland-specific expression of EcR^{F645A} using the Sgs3 promoter (*uas-EcR^{F645A}/+; Sgs3-GAL4/+*) shows a nine-fold decrease in the induction of *Sgs3* mRNA, highlighting the requirement of ecdysone signaling for continued expression of *Sgs3*. y-axis plots relative expression of target genes compared to eL3 controls and normalized to *rp49*. x-axis shows control animals at eL3 and wL3 stages, and mutant animals at wL3 stages. Each bar represents 3 independent biological samples; asterisks indicate a significant changes in expression compared to control animals at wL3 (*p*-values < 0.05).

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dissected salivary glands

A

control

12 APF

B

med24^psg5b^/Df

24 APF

C

salivary gland recovery by dissection at 24 APF

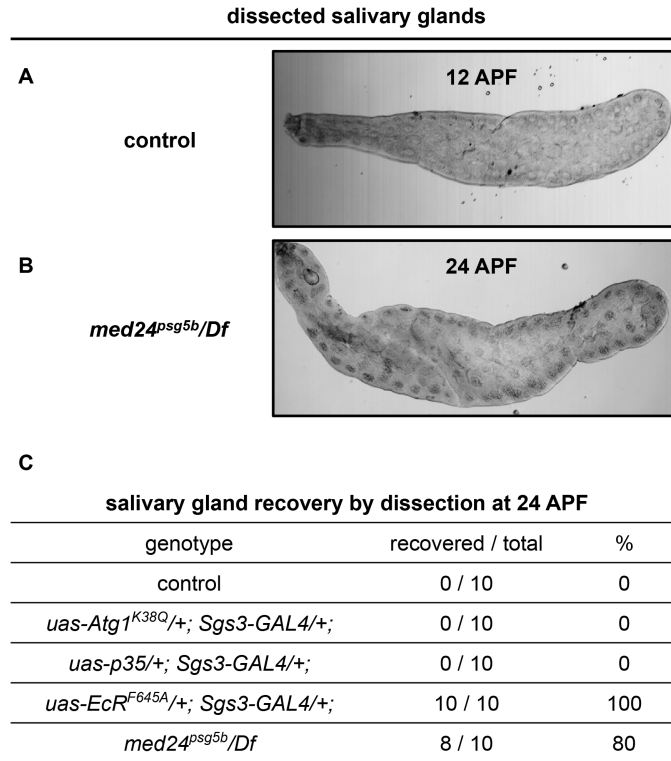| genotype	recovered / total	%
control	0 / 10	0
uas-Atg1^K38Q^/+; Sgs3-GAL4/+;	0 / 10	0
uas-p35/+; Sgs3-GAL4/+;	0 / 10	0
uas-EcR^F645A^/+; Sgs3-GAL4/+;	10 / 10	100
med24^psg5b^/Df	8 / 10	80

Figure S4 Ability to recover larval salivary glands 24 hours after puparium formation (APF). (A) Although control salivary glands are easily recovered at 12 APF, immediately before tissue destruction begins, they cannot be recovered a few hours later. (B) *med24* mutant glands, however, can be recovered by dissection 12 hours later at 24 APF. Note that mutant glands appear morphologically "intact," similar to control glands at 12 APF. (C) Table summarizing the ability to recover larval salivary gland by dissection at 24 APF. Column one indicates the genotype, column two the number recovered over the total number of glands dissected and column three the percentage recovered. Only an upstream block in salivary gland destruction caused by overexpressing *EcR^F645A^* or a mutation in *med24* leads to a complete block in gland destruction, which allows for recovery by dissection at 24 APF. Blocking activation of caspases or autophagy, by expression of either the caspase inhibitor *p35* or the autophagy inhibitor *Atg1^K38Q^*, does not allow gland recovery at 24 APF.

TABLE S1   Primer sequences for qPCR

Gene	Primer Sequence	Source
belle F	ACCGCAGCAATGGAAACTC	this study
belle R	GGTCCTTTGCCTGAAGCTG	
brwd3 F	AGCAGTACAATCGCCGACAC	this study
brwd3 R	GGTACACATCCGCAAGCTG	
hdc F	CTCTCATCGCTGGCCCAATC	this study
hdc R	GTGCGTCCCTCGTATTTAACCT	
mdh2 F	TCACCGACTTGGCGCTCTAC	this study
mdh2 R	GAGATGTCCTTGATGATGCCGG	
med12 F	GCAGCAACAGTGGCACAATG	this study
med12 R	GAGTTGGCATTCAACGGCGG	
med24 F	CGTTGCTCCAGTACGTGGTG	this study
med24 R	CATTTCCTTTGCGGCTCTCCAG	
psg2 F	GCTACCAACGCAAGGACCTC	this study
psg2 R	GTAGTTTCCGCTTTAGTCCTGCA	
pak F	GGTCCATGTGGGATTCGATGC	this study
pak R	CTTCTTCTGCTCCTGCTTGCT	
reptin F	CCAACTGATTACCACAGCCAAC	this study
reptin R	GTCCCTTTCCACCTCCTCGG	
Sgs3 F	CTACCGCCCTAGCGAGCAT	Cʜɪᴀɴɢ *et al.* 2003
Sgs3 R	GCATCCACAATCGCAACAGT	
Rpl18a F	ACGTCCAACATGAGAGCCAAG	this study
Rpl18a R	CTGCTTGATGGACACGATCTC	
UbcD6 F1	ACATATTGCAGAACCGCTGG	this study
UbcD6 R1	GCTTTCACACGCTTCTCGT	