Developmental Biology 481 (2022) 104–115

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Drosophila E93 promotes adult development and suppresses larval responses to ecdysone during metamorphosis

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ARTICLE INFO

Keywords:
Adult development
Cell death
Metamorphosis
Transcription
Ecdysone

ABSTRACT

Pulses of the steroid hormone ecdysone act through transcriptional cascades to direct the major developmental transitions during the *Drosophila* life cycle. These include the prepupal ecdysone pulse, which occurs 10 hours after pupariation and triggers the onset of adult morphogenesis and larval tissue destruction. *E93* encodes a transcription factor that is specifically induced by the prepupal pulse of ecdysone, supporting a model proposed by earlier work that it specifies the onset of adult development. Although a number of studies have addressed these functions for *E93*, little is known about its roles in the salivary gland where the *E93* locus was originally identified. Here we show that *E93* is required for development through late pupal stages, with mutants displaying defects in adult differentiation and no detectable effect on the destruction of larval salivary glands. RNA-seq analysis demonstrates that *E93* regulates genes involved in development and morphogenesis in the salivary glands, but has little effect on cell death gene expression. We also show that *E93* is required to direct the proper timing of ecdysone-regulated gene expression in salivary glands, and that it suppresses earlier transcriptional programs that occur during larval and prepupal stages. These studies support the model that the stage-specific induction of *E93* in late prepupae provides a critical signal that defines the end of larval development and the onset of adult differentiation.

1. Introduction

Temporal regulation is a critical part of normal development, with each stage occurring at the appropriate time, for the proper duration, and in the appropriate order. The regulation of developmental progression has been extensively studied in the fruit fly, *Drosophila melanogaster*, where pulses of the steroid hormone ecdysone trigger each stage in the life cycle (Ou and King-Jones, 2013; Truman, 2019; Yamanaka et al., 2013). These include embryonic maturation, the two larval molts, pupariation formation, and adult development during metamorphosis. Ecdysone exerts its effects through the EcR/USP ecdysone receptor, which directly induces primary-response genes, including a number of transcription factors. These primary-response transcriptional regulators, in turn, activate large batteries of secondary-response genes that play key roles in molting and metamorphosis. A central question that emerges from this work is how a single steroid hormone, acting through a common set of transcription factors, can direct the sequentially distinct developmental responses that are required for proper progression through the life cycle.

Classic studies by Richards and Ashburner used the giant *Drosophila* larval salivary gland chromosomes to address this question (Ashburner et al., 1974; Richards, 1976). They showed that a large transcriptional puff at 93F is induced directly by the prepupal pulse of ecdysone that occurs 10 hours after puparium formation. This puff, however, shows no response to the hormone at earlier stages of development and is dependent on the mid-prepupal expression of *FTZ-F1* for its competence to respond to ecdysone in a stage-specific manner (Broadus et al., 1999; Richards, 1976; Woodard et al., 1994). Subsequent molecular analysis revealed that *E93* (also referred to as *Eip93F*), which encodes a predicted HTH transcription factor, appears to be responsible for the 93F puff (Baehrecke and Thummel, 1995; Siegmund and Lehmann, 2002). Its induction in late prepupal salivary glands led to extensive studies to determine if it contributes to the stage-specific developmental responses to ecdysone that occur at this stage, including adult head eversion, leg

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https://doi.org/10.1016/j.ydbio.2021.10.001
Received 31 August 2021; Received in revised form 30 September 2021; Accepted 5 October 2021
Available online 11 October 2021

and wing development, and salivary gland cell death.

Consistent with an important role for *E93* in adult development, genetic analysis revealed that this locus is required for progression through metamorphosis, with mutants dying as pharate adults (Mou et al., 2012). These animals display widespread defects in multiple adult tissues, including the eyes, antennae, wings, legs, and abdomen. Detailed clonal analysis revealed that *E93* plays an important role in the responsiveness of *Dll* to EGFR signaling in the bract cells of the developing leg (Mou et al., 2012). These developmental functions for *E93* are consistent with the results of molecular studies, which showed that *E93* is required for the proper temporal induction of open chromatin domains in the developing wing (Uyehara et al., 2017). In particular, *E93* plays a key role in activating late-acting enhancers and repressing early-acting enhancers, contributing to the proper timing of gene expression during wing maturation (Nystrom et al., 2020; Uyehara et al., 2017). Interestingly, *E93* is also required for the proper arrest of neurogenesis and neuronal diversification in the developing mushroom body of the brain, supporting the model that it plays a widespread role in adult maturation (Pahl et al., 2019; Syed et al., 2017).

Studies in multiple insect species have confirmed and significantly expanded our understanding of how *E93* regulates adult development. This was first demonstrated in the hemimetabolous insect *B. germanica*, where it was shown that stage-specific induction of *E93* at the nymphal-adult transition is required for the repression of key transcriptional regulators encoded by *Kr-h1* and *BR-C* (Urena et al., 2014). These factors direct developmental programs that specify early developmental stages in the insect life cycle, prior to the onset of adulthood (Belles, 2020; Martin et al., 2020). In addition to suppressing juvenile developmental programs, *E93* is essential for adult development. Disruption of *E93* expression in *B. germanica* by RNAi results in nymphal arrest with a remarkable reiteration of supernumerary nymphal instars and a complete block in adult differentiation (Urena et al., 2014). Similar roles for *E93* are seen in the holometabolous insect *T. castaneum*, suggesting that these functions are conserved across insect species (Chafino et al., 2019; Urena et al., 2014). Taken together with studies in *Drosophila*, these observations support the model that *E93* plays a central role in specifying adult development in insects. Later papers extended these discoveries, demonstrating that *Kr-h1* represses *E93* expression, ensuring that a tight transition occurs between juvenile and adult transcriptional regulators (Belles and Santos, 2014). *E93* was also shown to be required for the larval-pupal metamorphosis during *Bombyx mori* development (Liu et al., 2015) as well as many other insects (Belles, 2020; Martin et al., 2020; Truman, 2019). This work has had a profound impact on our understanding of the molecular mechanisms by which hormonal signaling coordinates the larval-to-adult transition as well as provided important new insights into the evolution of insect metamorphosis (Belles, 2019; Martin et al., 2020; Truman, 2019).

Although the roles for *E93* in developing adult tissues are well established, its functions in dying larval tissues are more poorly understood. The original study of *E93* showed that it is induced by ecdysone in a stage-specific manner in *Drosophila* larval salivary glands immediately prior to the onset of tissue destruction (Baehrecke and Thummel, 1995). In addition, key death regulators including *rpr*, *hid*, and *dronc*, fail to be induced in *E93* mutant salivary glands, and the salivary glands fail to die (Lee et al., 2000, 2002). Subsequent work, however, revealed that the *E93* mutant allele used for these studies, $E93^{1}$, carries a second lesion in a neighboring locus in addition to the transition mutation in *E93* that changes a leucine at position 994 to a stop codon (Duncan et al., 2017). This second-site mutation maps to *Idh3*, which encodes an enzyme in the tricarboxylic acid (TCA) cycle. Moreover, the EMS-induced mutations that fail to complement $E93^{1}$, designated $E93^{2}$ and $E93^{3}$, carry missense mutations in *Idh3*, indicating that the essential functions defined by this complementation group correspond to the *Idh3* locus (Duncan et al., 2017). Further work showed that the larval salivary glands fail to exhibit markers of cell death in *Idh3* mutants, defining a critical role for this gene in larval salivary gland destruction at the onset of metamorphosis. These studies, however, leave us with the question of how *E93* functions in larval salivary glands and whether it is responsible for death gene regulation in this tissue. Moreover, the observation that ectopic *E93* expression is sufficient to drive cell death in the Malpighian tubules, wing imaginal discs, embryonic epithelia, and larval fat body suggest that it may normally play a role in this process during development (Lee and Baehrecke, 2001; Lee et al., 2000; Liu et al., 2014; Ojha and Tapadia, 2020). The cell death and clearance that is induced by *E93* expression in embryonic epithelial cells, however, occurs in the absence of key regulators of apoptosis, raising questions about the role of this gene in cell death (Lee and Baehrecke, 2001).

Here we use CRISPR/Cas9 technology to generate deletion mutations within the *E93* locus. We show that these mutations fail to complement each other as well as four alleles that were previously assigned to the *E93* complementation group (Duncan et al., 2017). Consistent with previous studies, the *E93* deletion mutants are pupal lethal and display delayed pupal development along with defects in cuticular tanning, eye pigmentation, and abdominal development. RNA-seq analysis of staged salivary glands revealed widespread mis-regulation of genes involved in development and morphogenesis. Only a few genes involved in cell death, however, are mis-regulated in *E93* mutants, with minor effects on their expression. Consistent with this, the destruction of larval salivary glands is unaffected in *E93* mutants. In contrast, we see widespread de-repression of ecdysone-inducible genes that are normally expressed at earlier stages of development, including the glue genes and *Eig71E* late genes. Taken together, our functional analysis of *E93* demonstrates that its ecdysone-induction in late prepupae determines the appropriate pattern of downstream gene expression that defines the larval-to-adult transition.

2. Materials and methods

2.1. Drosophila strains and media

Drosophila were maintained on a standard cornmeal-molasses diet at 25°C. Mutations in *Drosophila E93* were generated using CRISPR-Cas9 as described (Gratz et al., 2014). Our strategy used two different sets of guide RNAs to generate deletions 11 and 12, and the forward and the most distal combination of guide RNAs to generate the large deletion 13, which thus extends from the 5′ endpoint of deletion 11 to the 3′ endpoint of deletion 12 (Fig. 1A and B). In each case, deleted *E93* genomic sequences were replaced with an eye-specific *DsRed* reporter to facilitate the identification of targeted mutants (Gratz et al., 2014). The *3XP3-DsRed* marker was inserted in an opposite orientation from *E93*. Guide RNAs for $E93^{\Delta 11}$: 5′ guide GTGCAGCCACTTGAATATCGAGG; 3′ guide GTCAGGTGAGTGCCGAATTGGGG. Guide RNAs for $E93^{\Delta 12}$: 5′ guide GGACAGCAATGCGTCGACACCGG; 3′ guide GTATGAAAACTACAATTATATGG. Guide RNAs for $E93^{\Delta 13}$: 5′ guide GTGCAGCCACTTGAATATCGAGG; 3’ guide GTATGAAAACTACAATTATATGG. The sequences of the deletion endpoints are depicted in Fig. 1B. The $E93^{\Delta 11}$, $E93^{\Delta 12}$, and $E93^{\Delta 13}$ mutant stocks are available from the Bloomington Stock Center (respective stock numbers: 93128, 93129, 93130). Mutations were maintained in a w^{1118} background with a *TM6C Sb Tb* balancer and studied in transheterozygous combinations with each other or with a wild-type third chromosome from w^{1118} as a control. Thus, unless otherwise stated, controls are w^{1118}; +/+; $E93^{\Delta 13}$/+ and mutants were established by crossing w^{1118}; +/+; $E93^{\Delta 11,\ 12,\ or\ 13}$/*TM6C* stocks with each other and selecting for pupae that lack the *Tb*-marked *TM6C* balancer. In some crosses, a *fkh-GAL4, UAS-GFP* (*fkh>GFP*) salivary gland reporter was included on the X chromosome to follow larval salivary gland destruction (Wang et al., 2008). The $E93^{\Delta 1}$, $E93^{4}$, $E93^{5}$, and $E93^{6}$ mutants were kindly provided by I. Duncan (Duncan et al., 2017; Mou et al., 2012).

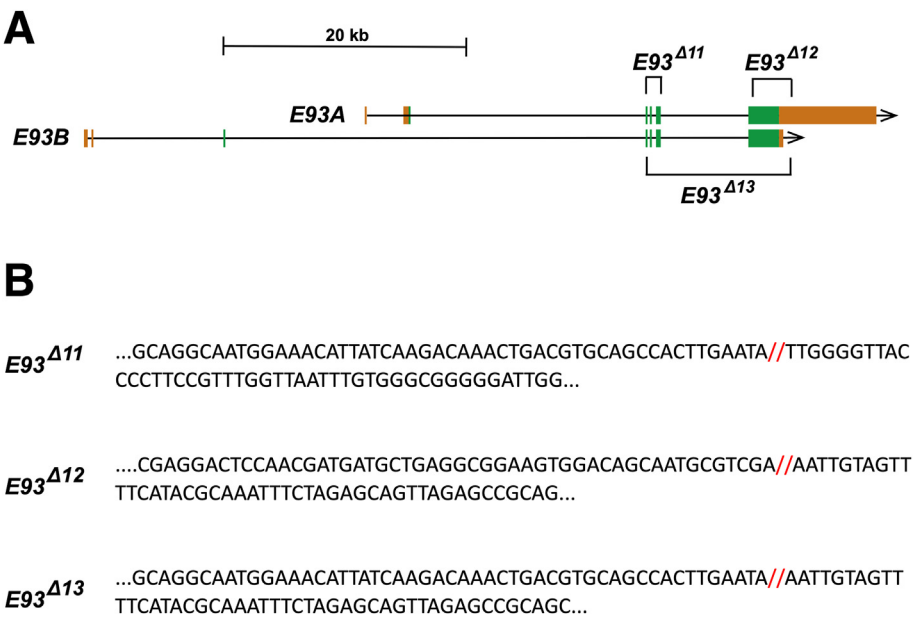

Fig. 1. A schematic representation of the *E93* genomic locus is depicted in panel A, including the protein-coding regions (green) and untranslated regions (brown), along with the *E93* deletions $E93^{\Delta 11}$, $E93^{\Delta 12}$, *and* $E93^{\Delta 13}$. (B) DNA sequences were determined from genomic DNA isolated from homozygous $E93^{\Delta 11}$, $E93^{\Delta 12}$, *and* $E93^{\Delta 13}$ mutants spanning the deleted region. For $E93^{\Delta 11}$, the DNA sequence is depicted from the third exon of the wild-type *E93A* mRNA isoform, corresponding to the region prior to the 1196 bp deletion, which is marked by two red slashes. This is followed by genomic DNA from exon 5 that lies downstream from the deletion. Similarly, genomic DNA sequences are depicted from the $E93^{\Delta 12}$ and $E93^{\Delta 13}$ mutants showing the deletion marked by two red slashes. $E93^{\Delta 12}$ carries a 3022 bp deletion within exon 6 of the *E93A* isoform, while the 11,629 bp $E93^{\Delta 13}$ deletion spans sequences from exon 3 and exon 6.

2.2. Mutant analysis

Controls and *E93* mutants carrying a *fkh>GFP* reporter (with a mini-*white* eye marker) were examined during metamorphosis to follow larval salivary gland cell death at head eversion. Mutants were also morphologically examined throughout metamorphosis to follow their developmental progression. Images were captured at 5–7 days after puparium formation. Pharate adult images of controls were taken at approximately 5 days after pupariation, while mutant images were captured on later days at similar stages of adult maturation, reflecting their delayed development. All images represent terminal phenotypes for each mutant combination. Images were captured on a Leica MZ125 dissecting microscope using a Sony ILCA-77M2 digital camera.

2.3. RNA-seq

RNA was isolated from collections of 13–15 pairs of salivary glands dissected from controls (w^{1118} *fkh>GFP*; +/+; $+/E93^{\Delta 13}$) and *E93* mutants (w^{1118} *fkh>GFP*; +/+; $E93^{\Delta 12}/E93^{\Delta 13}$) staged at either 12 or 14 h after puparium formation using the NucleoSpin RNA Kit (Macherey-Nagel 740955-50). Three independent control and mutant samples for each of the two time points were submitted for sequencing. Library generation of poly(A)-selected RNAs (Illumina RNA TruSeq Stranded mRNA Library prep Kit with oligo-dT selection) and sequencing (Illumina NovaSeq Reagent Kit v1.5_150X150 bp) were performed by the High-Throughput Genomics Core Facility at the University of Utah. The raw data from Illumina sequencing was analyzed using the BDGP6.28 genome and gene feature files and DESeq2 version 1.30.0 to identify differentially expressed genes with a 5% false discovery rate (Table S1). RNA quality control, library preparation, sequencing, and data analysis were performed at the University of Utah Huntsman Cancer Institute High Throughput Genomics and Bioinformatic Analysis Shared Resource. RNA-seq data from this study can be accessed at NCBI GEO (accession number: GSE181948).

2.4. RT-qPCR

RNA was isolated from salivary glands dissected from controls (w^{1118} *fkh>GFP*; +/+; $+/E93^{\Delta 13}$) and *E93* mutants (w^{1118} *fkh>GFP*; +/+; $E93^{\Delta 12}/E93^{\Delta 13}$) staged at 8, 10, 12 or 14 h after puparium formation using a NucleoSpin RNA Kit (Macherey-Nagel 740955-50). First-strand cDNA was synthesized using 0.5 μg RNA, Superscript Reverse Transcriptase II (ThermoFisher Scientific, 18064022), and oligo(dT) primers (Invitrogen, 18418012). qPCR experiments were performed on cDNA as described using an Applied Biosystem QuantStudio 3 device and the SYBR GreenER qPCR SuperMix Universal kit (ThermoFisher Scientific 11762100) (Storelli et al., 2019). ROX Reference Dye was diluted ten times before use and 0.1 μl was added for a final reaction volume of 20 μl. Fold inductions in transcript level were determined using the ΔΔCt method. Transcript levels were normalized to *rp49*. Each experiment was performed using 4–5 independent samples. The forward and reverse primers used in the qPCR experiments are listed in Table S3.

2.5. Histological examination of salivary gland cell death

Histology was performed as described (Velentzas and Baehrecke, 2021). Briefly, flies were maintained at 25°C and aged to 24 h after puparium formation. Whole pupae were fixed in FAAG (80% ethanol, 4% formaldehyde, 5% acetic acid, 1% glutaraldehyde) overnight at 4°C, embedded in paraffin, sectioned, and stained with Weigert's Hematoxylin and Pollack Trichrome. Stained sections were examined using a Zeiss AxioImager Z1 microscope.

2.6. Statistical analysis

Graphical representation and statistical analysis were performed using GraphPad Prism 8 software. In all graphs, the bars represent the mean and the error bars represent the standard deviation. For RT-qPCR, an unpaired *t*-test was used to compare mRNA levels between controls and *E93* mutants at each time point. Statistically significant differences ($p < 0.05$) between control and *E93* mutant samples are represented by asterisks: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; **** $p \leq 0.0001$.

3. Results

3.1. E93 mutants arrest development during late pupal stages

Although studies in hemimetabolous and holometabolous insects have demonstrated a key role for *E93* in adult development, little is known about its functions in salivary glands following its stage-specific induction in late prepupae. As a first step toward addressing these

Table 1
E93 mutants die during pupal development. *Inter se* complementation was performed between the w^{1118}; +/+; $E93^{\Delta 11,\ \Delta 12,\ or\ \Delta 13}$/*TM6C* stocks and the percent of *Tb*+ and *Tb*- pupae were scored for each cross. In all cases, the percentages are close to the 33% *Tb*+/66%*Tb*- that would be expected for a balanced lethal mutation.

		$E93^{\Delta 11}$		$E93^{\Delta 12}$		$E93^{\Delta 13}$	
		sum	percent	sum	percent	sum	percent
$E93^{\Delta 11}$	*Tb*	485	67%	435	67%	386	68%
	Tb+	237	33%	213	33%	182	32%
	sum	722		648		568	
$E93^{\Delta 12}$	*Tb*			269	66%	370	63%
	Tb+			138	34%	216	37%
	sum			407		586	
$E93^{\Delta 13}$	*Tb*					630	64%
	Tb+					357	36%
	sum					987	

activities, we generated three deletion mutations within the *E93* locus using CRISPR/Cas9 technology. $E93^{\Delta 11}$ is a 1196 bp deletion that removes part of exon 3 in the *E93A* isoform along with all of exons 4 and 5 (Fig. 1A and B). $E93^{\Delta 12}$ is a 3022 bp deletion that removes most of the protein coding region in the large exon 6, and $E93^{\Delta 13}$ is a 11,629 bp deletion that starts in exon 3 and ends after the stop codon in exon 6 (Fig. 1A and B). All three mutations result in major disruptions in the protein-coding capacity of *E93* and thus are likely to act as strong loss-of-function alleles (Fig. 1A). In particular, they remove the Psq domain that is predicted to form the helix-turn-helix DNA binding domain of the protein (Siegmund and Lehmann, 2002).

Inter se complementation was used to determine the lethal phenotypes of the *E93* deletion mutants. Consistent with previous studies, the three new mutants fail to complement each other and result in developmental delays and complete lethality during pupal stages (Table 1) (Mou et al., 2012; Urena et al., 2014). Morphological examination of the three mutants in all allelic combinations showed that the initial stages of metamorphosis appear to progress normally, with normal puparium formation, head eversion, and leg and wing elongation (Fig. 2). Abdominal development, however, is severely affected in mutants, with little or no cuticular differentiation. Eye pigmentation also fails to occur in *E93* mutants, the head, thorax and wings fail to tan, and widespread bristle defects can be seen in the head and thorax (Fig. 2). In addition, we see reduced wing size in *E93* mutant pupae, consistent with earlier functional studies of *E93* (Figs. 2 and 3) (Uyehara et al., 2017; Wang et al., 2019).

As expected, the four *E93* mutant alleles identified by Mou et al. (2012) fail to complement the large $E93^{\Delta 13}$ deletion (Table 2). Although all mutant combinations die during pupal development, the EMS-induced point mutants $E93^{4}$, $E93^{5}$, and $E93^{6}$ appear to be hypomorphic alleles, with less severe effects on development (Fig. 3). In particular, $E93^{4}/E93^{\Delta 13}$ and $E93^{5}/E93^{\Delta 13}$ mutants display eye pigmentation and cuticular tanning, unlike the strong loss-of-function alleles, as well as some abdominal development (Fig. 3). $E93^{6}/E93^{\Delta 13}$ mutants are more severely affected with some cuticular tanning and weak eye pigmentation but no abdominal development. In contrast, the deletion allele described by Duncan et al. (2017), $E93^{\Delta 1}$, displays pupal phenotypes that are similar to those of our deletion mutants, consistent with it being an amorphic allele (Fig. 3). We conclude that $E93^{4}$ and $E93^{5}$ are partial loss-of-function alleles, $E93^{6}$ represents a stronger loss-of-function mutant, and the remaining alleles, $E93^{\Delta 1}$, $E93^{\Delta 11}$, $E93^{\Delta 12}$, and $E93^{\Delta 13}$ represent strong hypomorphic or amorphic alleles.

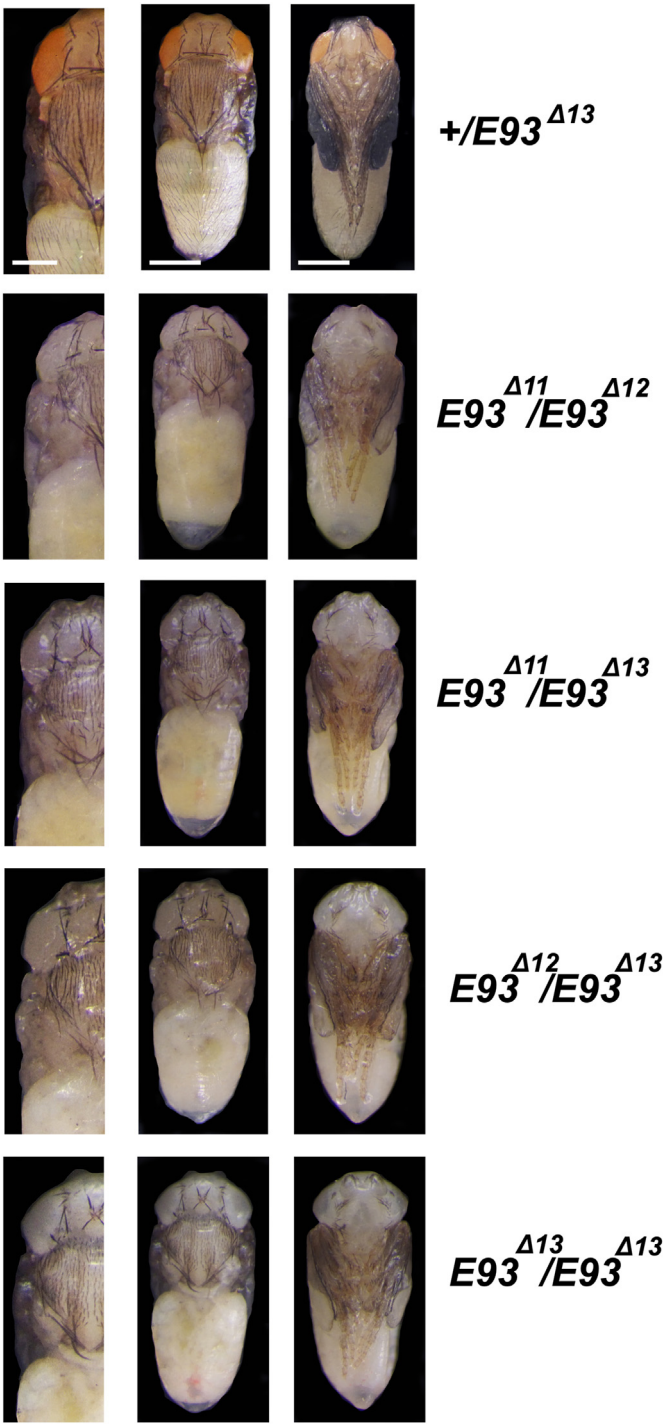

Fig. 2. *E93* mutants display defects in adult differentiation. Dorsal (left two panels) and ventral (right panel) images are depicted for control pharate adults (w^{1118} *fkh>GFP*; +/$E93^{\Delta 13}$) and animals carrying different combinations of *E93* mutations: w^{1118} *fkh>GFP*; $E93^{\Delta 11}/E93^{\Delta 12}$, w^{1118} *fkh>GFP*; $E93^{\Delta 11}/E93^{\Delta 13}$, w^{1118} *fkh>GFP*; $E93^{\Delta 12}/E93^{\Delta 13}$, and w^{1118} *fkh>GFP*; $E93^{\Delta 13}/E93^{\Delta 13}$. Dorsal views are presented at two different levels of magnification. *E93* mutants display normal head eversion and leg and wing elongation, but display defects in abdominal development, wing size, eye and cuticular pigmentation, and bristle morphology. Scale bars = 300 μm (left panel), 500 μm (right two panels).

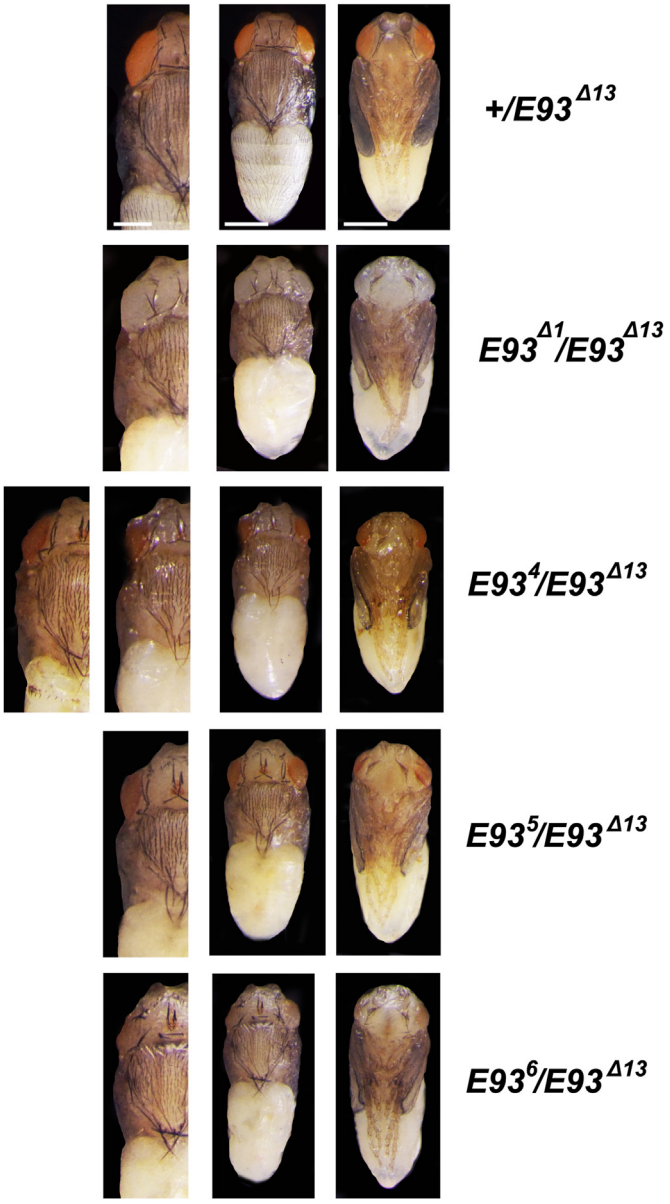

Fig. 3. Terminal phenotypes of *E93* missense mutations. Dorsal (left two panels) and ventral (right panel) images are depicted for control pharate adults (w^{1118} *fkh>GFP*; $+/E93^{\Delta 13}$) and animals carrying different combinations of *E93* mutations: w^{1118} *fkh>GFP*; $E93^{\Delta 1}/E93^{\Delta 13}$, w^{1118} *fkh>GFP*; $E93^{4}/E93^{\Delta 13}$, w^{1118} *fkh>GFP*; $E93^{5}/E93^{\Delta 13}$, and w^{1118} *fkh>GFP*; $E93^{6}/E93^{\Delta 13}$ (Mou et al., 2012). Dorsal views are presented at two different levels of magnification. $E93^{4}$ and $E93^{5}$ display more mild lethal phenotypes than the deletion alleles when carried in combination with $E93^{\Delta 13}$, although abdominal development is still severely affected. An additional dorsal perspective is included for $E93^{4}/E93^{\Delta 13}$ mutants (far left) to show less severe thoracic and abdominal defects, which are occasionally seen in this genotype. $E93^{6}$ mutants display more severe lethal phenotypes than $E93^{4}$ and $E93^{5}$, although some eye pigmentation and cuticular tanning can be seen in these animals. $E93^{\Delta 1}$ mutants display the most severe block in adult development, resembling the lethal phenotypes of $E93^{\Delta 11}$, $E93^{\Delta 12}$, *and* $E93^{\Delta 13}$ mutants (Fig. 2). Scale bars = 300 μm (left panel), 500 μm (right two panels).

3.2. *E93 coordinates developmental transcriptional programs in prepupal salivary glands*

In an effort to define the molecular functions of *E93*, we used RNA-seq to identify transcripts that are mis-regulated in *E93* mutant salivary glands. RNA was isolated from salivary glands staged at 12 and 14 hours after puparium formation, from both controls and *E93* mutants. This timing corresponds to the ecdysone-induced peak of *E93* expression and thus should cover the initial transcriptional response to this factor (Baehrecke and Thummel, 1995). RNA-seq revealed more than 500 genes that are either increased or decreased in their expression in *E93* mutant salivary glands relative to controls (Table S1). Gene Ontology (GO) analysis demonstrated that these genes correspond primarily to a wide range of developmental and morphogenetic pathways (Table 3). The p-values, however, are relatively low, indicating that no single pathway is over-represented in mutant glands. Interestingly, some GO categories, such as cellular morphogenesis, plasma membrane, and system development, are represented in both the down-regulated and up-regulated genes sets in 14-hour *E93* mutant glands (Table 3). These results suggest that *E93* normally plays a role in coordinating the proper regulation of developmental pathways in late prepupal salivary glands.

3.3. *E93 is not required for the proper expression of key cell death regulators*

The prepupal pulse of ecdysone at 10 hours after pupariation triggers the destruction of larval salivary glands as the animal enters metamorphosis (Jiang et al., 1997). The stage-specific induction of *E93* at this time raises the possibility that this transcription factor might play a central role in mediating this response to the hormone. Accordingly, we looked for significant effects on death gene expression in our RNA-seq datasets. The 340 genes that correspond to the *Drosophila* "programmed cell death" (PCD) GO category were compared to the genes that are expressed at either higher or lower levels in *E93* mutant salivary glands (Tables S1 and S2). Of the 724 genes expressed at reduced levels in 12-hour *E93* mutant salivary glands, 32 correspond to PCD genes (4%). Similarly, 25 PCD genes are included in the up-regulated gene set (685) at 12 hours (4%), 20 PCD genes are included in the down-regulated gene set (703) at 14 hours (3%), and 27 PCD genes are included in the up-regulated gene set (666) at 14 hours (4%) (Table S2). These relatively low numbers suggest that *E93* does not play a widespread role in regulating death gene expression in prepupal salivary glands. Moreover, only a few of the PCD genes mis-regulated in *E93* mutants correspond to direct regulators of cell death.

To confirm and extend these observations, we examined the expression of four death regulators in *E93* mutant salivary glands that are known to play an important role in directing its destruction: *rpr*, *hid*, *Dronc*, and *diap2* (Fig. 4) (Jiang et al., 1997, 2000; Yin and Thummel, 2004). Of these genes, *rpr* is significantly reduced in 14-hour mutant salivary glands, while *Dronc* and *diap2* are reduced in their expression in 8- or 10-hour glands (Fig. 4). These effects, however, are difficult to interpret since this is prior to the induction of *E93* that occurs between 10 and 12 hours (Fig. 6A). We conclude that the loss of *E93* does not appear to play a central role in PCD gene expression in salivary glands that are fated to die.

3.4. *E93 mutants display normal salivary gland destruction during metamorphosis*

The absence of a clear effect on cell death gene expression in *E93* mutants suggests that salivary gland destruction should occur normally in these animals. To test this hypothesis, control $+/E93^{\Delta 13}$ animals and $E93^{\Delta 11}/E93^{\Delta 13}$ mutants (Fig. 5A and B), $E93^{\Delta 12}/E93^{\Delta 13}$ mutants (Fig. 5C,D), $E93^{\Delta 13}/E93^{\Delta 13}$ mutants (Fig. 5E,F), and $E93^{\Delta 1}/E93^{\Delta 13}$ mutants (Fig. 5G and H) were staged at 24 hours after pupariation, embedded in paraffin, sectioned, stained, and examined for persistent salivary gland fragments. The timing of this collection corresponds to approximately 8 hours after salivary glands are fully cleared from wild-type animals (Velentzas and Baehrecke, 2021) (Fig. S1). The number of animals displaying residual salivary gland material was quantified for each genotype (Fig. 5B,D,F,H). In all cases, no effect was seen on salivary gland clearance in *E93* mutants. We conclude that *E93* is not required for the destruction of larval salivary glands during metamorphosis.

Table 2
The *E93*$^{\Delta 13}$ mutant fails to complement the *E93* mutants isolated by Mou et al. (2012). *Inter se* complementation was performed between the *E93*$^{\Delta 1, 4, 5, or 6}$*/TM6B* stocks (Mou et al. (2012)) and *E93*$^{\Delta 13}$*/TM6C*, and the percent of *Tb*+ and *Tb* pupae were scored for each cross. In all cases, the percentages are close to the 33% *Tb*+/66%*Tb*- that would be expected for a balanced lethal mutation.

		E93$^{\Delta 1}$		E93^{4}		E93^{5}		E93^{6}	
		sum	percent	sum	percent	sum	percent	sum	percent
***E93*$^{\Delta 13}$**	***Tb***	429	66%	351	66%	277	67%	332	66%
	***Tb*+**	225	34%	180	34%	136	33%	170	34%
	sum	654		531		413		502	

Table 3
Top Gene Ontology categories for *E93*-regulated genes in 14hour salivary glands.

Down-regulated genes (top 500)			
GO category	Number of genes (total)	P value	# additional similar categories
Signal transduction	35 (662)	3.0e-2	7
Peptidyl dipeptidase A activity	3 (4)	3.0e-2	1
Cellular morphogenesis during differentiation	21(340)	3.0e-2	3
Plasma membrane	29(537)	3.0e-2	3
N-methyl D-aspartate glutamate receptor activity	3(5)	3.1e-2	
System development	59 (1393)	3.1e-2	7
Chloride channel activity	4 (12)	3.1e-2	
Membrane lipid metabolic process	6 (40)	5.4e-2	
Procollagen-lysine 5-dioxygenase activity	2(2)	5.4e-2	
Up-regulated genes (top 500)			
GO category	Number of genes (total)	P value	# additional similar categories
Actin binding	13 (108)	5.8e-3	1
Cell morphogenesis	30 (554)	1.2e-2	6
Response to stimulus	45 (982)	1.6e-2	
Defense response	14 (164)	2.6e-2	
Plasma membrane	28(537)	2.6e-2	6
System development	56 (1393)	5.9e-2	
Regulation of nucleic acid metabolic process	8 (71)	5.9e-2	1
Glutamine-fructose-6-phosphate transaminase activity	2(2)	6.3e-2	
Serine-type endopeptidase inhibitor activity	6(48)	8.7e-2	

Determined using GOstat: http://gostat.wehi.edu.au/cgi-bin/goStat.pl

3.5. *E93 regulates ecdysone-inducible gene expression*

Visual inspection of our RNA-seq dataset revealed that many ecdysone-inducible genes are mis-regulated in *E93* mutant salivary glands (Table S1). Interestingly, all of these genes are increased in expression in *E93* mutants. They include ecdysone-inducible genes that correspond to canonical early puffs in the polytene chromosomes, encode transcription factors, and play a key role in directing ecdysone-induced developmental responses such as molting and metamorphosis: *BR-C* (*br*), *E74*, *E75*, *E78*, *HR38*, and *FTZ-F1* (Burtis et al., 1990; DiBello et al., 1991; Huet et al., 1995; Karim and Thummel, 1991; Kozlova et al., 1998; Lavorgna et al., 1993; Russell et al., 1996; Segraves and Hogness, 1990; Stone and Thummel, 1993). Other early ecdysone-inducible genes that are increased in expression in *E93* mutants include *ImpE2*, *Eip28/29*, and the *E63* puff gene (Andres and Cherbas, 1992; Andres and Thummel, 1995; Paine-Saunders et al., 1990). In addition, many stage-specific ecdysone-inducible genes are expressed at elevated levels in *E93* mutants. These include the *ng1*, *ng4*, *Sgs1*, *Sgs7*, and *Sgs8* glue genes, which are specifically induced by ecdysone in mid-third instar larval salivary glands (Crowley et al., 1983; Furia et al., 1993; Meyerowitz and Hogness, 1982). Similarly, nine *Eig71E* late genes, which are specifically expressed in the salivary glands of late prepupae, are increased in their expression in *E93* mutants (Restifo and Guild, 1986a). In addition, *CG43082*, which is in this gene cluster and displays the same temporal and spatial regulation as the *Eig71E* genes, responds in a similar manner, suggesting that this is an effect on the entire locus.

3.6. *E93 represses its own transcription*

In an effort to confirm and extend these observations, we used RT-qPCR to examine the temporal profiles of expression of each of these groups of ecdysone-regulated genes. RNA was isolated from control and *E93* mutant salivary glands dissected from prepupae staged at 8, 10, 12, or 14 hours after puparium formation, spanning the ecdysone pulse at 10 hours (Handler, 1982) (Figs. 6 and 7). As expected, *E93* expression is not detected in prepupal salivary glands when a primer that lies within the *E93* deletion is used for qPCR (Fig. 6A). In contrast, *E93* mRNA levels were found to be elevated when we used primer pairs that lie outside of the region deleted in mutants (Fig. S2). Consistent with this, *E93* is listed among the genes that increase in expression in *E93* mutants, as detected by RNA-seq (Table S1). This can be seen by aligning the RNA-seq coverage tracks for the *E93* locus in both control and *E93* mutant salivary gland RNA samples (Fig. S3). Transcription from the locus is elevated ~2-3-fold relative to controls in the sequences upstream from the deleted region. These observations suggest that *E93* negatively regulates its own transcription.

3.7. *E93 supports the proper temporal regulation of early gene expression in prepupae*

We also examined the temporal profiles of ecdysone-regulated puff gene expression in *E93* mutants (Fig. 6). As expected, *FTZ-F1* is expressed in control mid-prepupae and then repressed by the ecdysone pulse at 10 hours (Fig. 6B). Similarly, the switch in *E74* isoforms, from *E74B* to *E74A*, is seen as the ecdysone titer increases (Karim and Thummel, 1991) (Fig. 6C and D). *E74A*, *BR-C*, and *E75A* all peak at 12 hours after pupariation in controls, in synchrony with *E93*, reflecting their direct induction by the hormone (Fig. 6D–F). The levels of *FTZ-F1* mRNA trend downwards in 8-hour mutant prepupae, and upwards at 12 and 14 hours, but these changes are not significant (Fig. 6B). In contrast, levels of *E74B* mRNA are reduced in 8-hour mutant prepupae (Fig. 6C). Similarly, *E74A* and *E75A* are both expressed at reduced levels in 8- and 10-hour mutant prepupae, although they are also significantly elevated at 14 hours, following the peak of *E93* expression (Fig. 6D,F). *BR-C* transcripts are also elevated in mutant animals at the 14 hour timepoint. Taken together, these results indicate that *E93* maintains the proper temporal patterns of ecdysone-regulated puff gene expression in prepupal salivary glands.

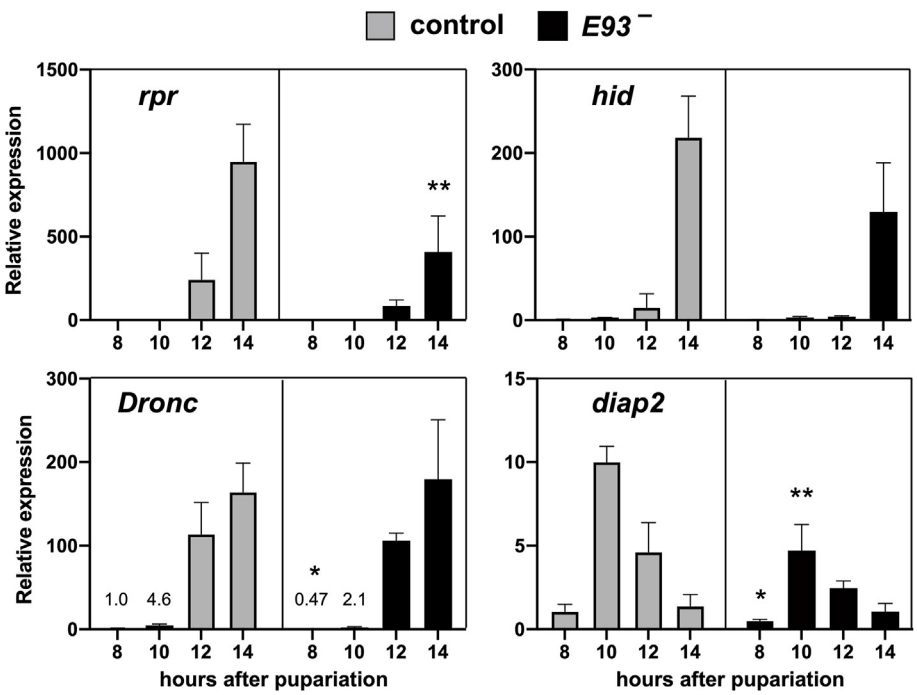

Fig. 4. *Reaper* and *diap2* expression is reduced in *E93* mutant salivary glands. RT-qPCR was performed using RNA isolated from staged control (grey) or *E93* mutant (black) salivary glands in order to detect the temporal patterns of *rpr, hid, Dronc,* or *diap2* death gene expression. The peaks of *rpr* and *diap2* expression are both reduced in *E93* mutant glands. Transcript levels are normalized to *rp49* and presented relative to controls at 8 h after pupariation. n = 4 independent samples with 13–15 flies per sample. P-values represent comparisons between control and *E93* mutant mRNA levels at the indicated time point: *p ≤ 0.05, **p ≤ 0.01. Data without an asterisk are not significant, *p*-value >0.05. Numbers have been added above some bars to show the corresponding y-axis value.

3.8. *E93 represses earlier developmental responses to ecdysone*

The *ng* and *Sgs* glue genes are tightly temporally and spatially regulated, being restricted to the salivary glands of late third instar larvae (Crowley et al., 1983; Furia et al., 1993; Meyerowitz and Hogness, 1982). The protein products of these genes are used by the animal to adhere to a substrate immediately prior to puparium formation (Beckendorf and Kafatos, 1976; Biyasheva et al., 2001). We were thus surprised to see these genes ectopically expressed in the salivary glands of *E93* mutant prepupae (Table S1). RT-qPCR confirmed this observation for *ng-1*, *Sgs-5*, and *Sgs-8*, showing that these genes are all expressed at elevated levels in *E93* mutant salivary glands (Fig. 7A–C). This effect can also be seen when the data are normalized to glue gene expression levels in third instar larval salivary glands (Fig. S4). These results indicate that *E93* normally suppresses ectopic salivary gland glue gene expression following puparium formation.

3.9. *E93 contributes to the timely repression of Eig71E late gene expression*

The *Eig71E* late ecdysone-inducible genes are tightly temporally regulated to a brief period of time in control mid-prepupae (Restifo and Guild, 1986a, b) (Fig. 7D–G). Interestingly, these genes appear to be reinduced at 12 hours after puparium formation in *E93* mutant salivary glands, in synchrony with the peak of *E93* expression (Fig. 7D–G). These results indicate that *E93* normally contributes to the efficient coordinate repression of the *Eig71E* genes in late prepupae.

4. Discussion

Pulses of the steroid hormone ecdysone trigger the major developmental transitions in *Drosophila*, driving the animal forward from larva to adult. This activity raises the central question of how a single hormone can direct different developmental responses at different times to ensure proper temporal progression through the life cycle. The stage-specific ecdysone induction of *E93* in late prepupae, as the animal initiates adult morphogenesis, provides a mechanistic framework to address this question. Studies performed initially in *Drosophila*, and later in a wide range of insects, demonstrated that the stage-specific ecdysone induction of *E93* specifies adult differentiation. It does so by both promoting adult developmental pathways and repressing earlier programs that specify larval and pupal identity. The induction of *E93* thus represents a critical switch that defines the end of larval life and the onset of adult maturation. Our work reported here supports this pivotal role for *E93* and extends it to the larval salivary glands of *Drosophila*. Below we discuss these regulatory functions and present possible directions for future study that could provide mechanistic insights into the role of *E93* in specifying adult fate.

4.1. *E93 is required for adult differentiation*

The *E93* deletion mutations reported here appear to act as null alleles, consistent with their loss of the predicted DNA binding domain of the protein (Fig. 1). When examined in different allelic combinations, these mutants die during late pupal stages with defects in eye pigmentation, tanning of the adult cuticle, reduced wing size, a block in abdominal development, and widespread bristle defects (Fig. 2). These phenotypes resemble those reported in earlier functional studies of *E93* using either mutants (Mou et al., 2012) or RNAi (Urena et al., 2014; Wang et al., 2019) (Fig. 3). In addition, they are consistent with studies in multiple insect species that have established a central role for *E93* as a specifier of adult development (Belles, 2020; Martin et al., 2020; Truman, 2019). Indeed much has been learned about the developmental functions of *E93* from studies in insects other than *Drosophila*. In part this is due to the simpler metamorphosis that occurs in hemimetabolous insects such as *B. germanica*. In addition, functional studies of *E93* could be more readily integrated with juvenile hormone signaling in other insect species, providing a clearer understanding of the molecular mechanisms that drive insect maturation (Belles, 2020; Martin et al., 2020; Truman, 2019). In contrast, only a few studies have investigated roles for *E93* in *Drosophila* adult developmental pathways, primarily focused on bract development in the leg and neurogenesis (Mou et al., 2012; Pahl et al., 2019; Syed et al., 2017; Wang et al., 2019). Future mosaic clonal studies using null *E93* mutations in *Drosophila* should expand our understanding of this key regulator of adult development and provide new insights into its roles in specifying adult fate. It will also be interesting to continue studies of how *E93* regulates target gene expression in developing adult structures. These studies should provide important insights into the molecular mechanisms that drive adult maturation.

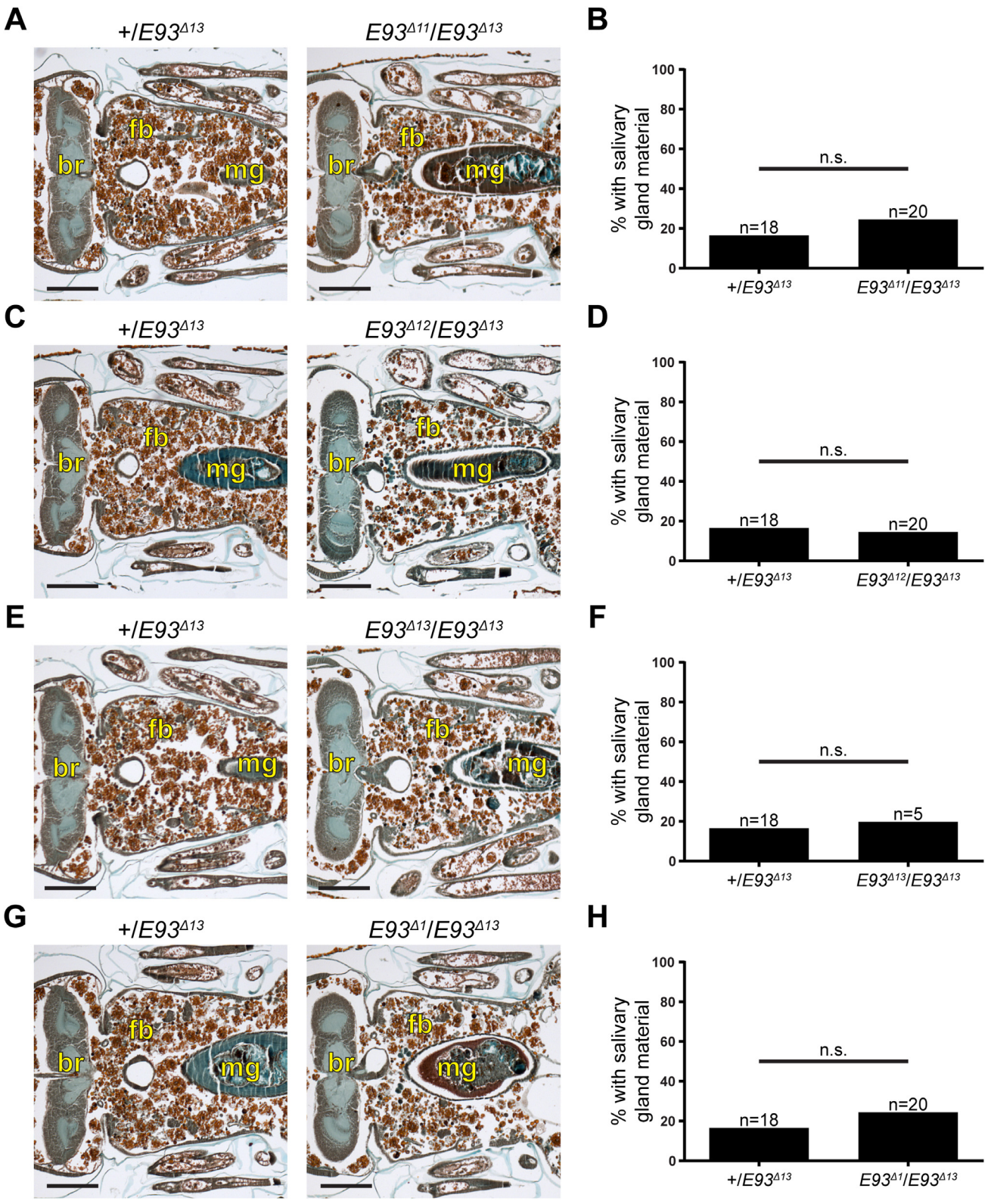

Fig. 5. Larval salivary gland cell death occurs normally in *E93* mutants. (A) Samples from control animals (w^{1118}; +; $+/E93^{\Delta 13}$), n = 18 (left) and *E93* transheterozygote mutants (w^{1118}; +; $E93^{\Delta 11}/E93^{\Delta 13}$), n = 20 (right), analyzed by histology for the presence of salivary gland material 24 h after puparium formation. (B) Quantification of data from (A). (C) Samples from control animals (w^{1118}; +; $+/E93^{\Delta 13}$), n = 18 (left) and *E93* transheterozygote mutants (w^{1118}; +; $E93^{\Delta 12}/E93^{\Delta 13}$), n = 20 (right), analyzed by histology for the presence of salivary gland material 24 h after puparium formation. (D) Quantification of data from (C). (E) Samples from control animals (w^{1118}; +; $+/E93^{\Delta 13}$), n = 18 (left) and *E93* mutants (w^{1118}; +; $E93^{\Delta 13}$), n = 5 (right), analyzed by histology for the presence of salivary gland material 24 h after puparium formation. (F) Quantification of data from (E). (G) Samples from control animals (w^{1118}; +; $+/E93^{\Delta 13}$), n = 18 (left) and *E93* transheterozygote mutants (w^{1118}; +; $E93^{\Delta 1}/E93^{\Delta 13}$), n = 20 (right), analyzed by histology for the presence of salivary gland material 24 h after puparium formation. (H) Quantification of data from (G). All sections are oriented with anterior on the left and posterior on the right. The major tissues are labeled: (br: brain, fb: fat body, mg: midgut). Scale bar = 200 μm.

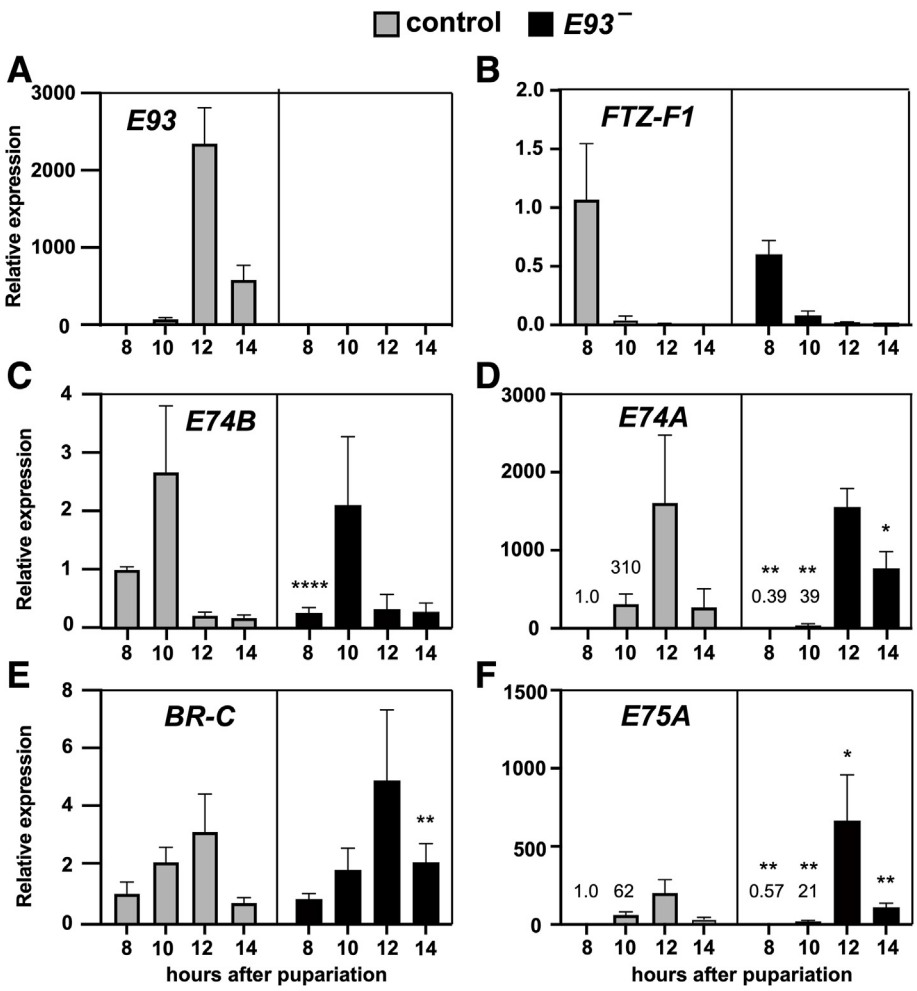

Fig. 6. *E93* is required for the proper timing of early ecdysone-inducible gene expression. RT-qPCR was performed using RNA isolated from staged control (grey) or *E93* mutant (black) salivary glands in order to detect the temporal patterns of *E93* (A), *FTZ-F1* (B), *E74B* (C), *E74A* (D), *BR-C* (E), or *E75A* (F) early ecdysone-induced transcription. Transcript levels are normalized to *rp49* and presented relative to controls at 8 h after pupariation. n = 4–5 independent samples with 13–15 flies per sample. P-values represent comparisons between control and *E93* mutant mRNA levels at the indicated time point: *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. Data without an asterisk are not significant, p-value >0.05. Numbers have been added above some bars to show the corresponding y-axis value.

4.2. *E93 is not required for the destruction of larval salivary glands during metamorphosis*

E93 was discovered based on its stage-specific induction in *Drosophila* salivary glands immediately prior to their ecdysone-triggered destruction at 12 hours after pupariation (Richards, 1976). Earlier genetic studies proposed that *E93* was directly responsible for this biological response through the stage-specific induction of key death inducer genes (Lee et al., 2000, 2002). Subsequent work, however, showed that the mutants used for this study carried lesions in a neighboring locus encoding *Idh3b*, and demonstrated that this TCA cycle enzyme is required for salivary gland cell death (Duncan et al., 2017). Our results here confirm this observation and show that a loss of *E93* has no major effect on either death gene expression or salivary gland destruction (Figs. 4 and 5). Ectopic expression of *E93*, however, is sufficient to trigger cell death in embryonic epithelial cells, imaginal discs, Malpighian tubules, and larval fat body, suggesting that it contributes to these pathways (Lee and Baehrecke, 2001; Lee et al., 2000; Liu et al., 2014; Ojha and Tapadia, 2020). Moreover, *E93* RNAi in *B. germanica* prevents the destruction of the prothoracic gland (Kamsoi and Belles, 2020). One possibility to explain these observations is that *E93* acts in a redundant manner with other regulators to direct salivary gland cell death. Earlier studies have shown that the ecdysone induced transcription factors encoded by *BR-C*, *E74A*, and *FTZ-F1* are each required for proper death gene expression and salivary gland destruction (Jiang et al., 2000; Lee and Baehrecke, 2001; Lee et al., 2002; Restifo and White, 1992). Both *BR-C* and *E74A* are induced by the prepupal ecdysone pulse in synchrony with *E93*, raising the possibility that functional interactions between these factors could contribute to the death response. Consistent with this, only 20% of *E74A* mutants display a defect in salivary gland clearance, reflecting a partially penetrant effect on cell death (Jiang et al., 2000). Genetic studies could address possible functional interactions between *BR-C*, *E74A*, and *E93* in the regulation of ecdysone-triggered salivary gland cell death at the onset of metamorphosis. In addition, it is important to note that our studies do not address the tissue-specific functions of *E93* in the salivary glands. It is possible that the systemic loss of *E93* in mutants could indirectly affect gene expression and cell death in the salivary glands. Further studies are needed to examine the tissue-specific roles of *E93* in regulating the divergent fates of larval and adult cells at the onset of metamorphosis.

4.3. *E93 represses early ecdysone-inducible gene expression*

Our molecular studies have shown that *E93* is required for the proper repression of *BR-C*, *E74A*, and *E75A* transcription in late prepupae, at the prepupal-pupal transition (Fig. 6). This observation is consistent with earlier work showing that *E93* is required for the proper down-regulation of *BR-C* expression in both hemimetabolous and holometabolous insects at the onset of metamorphosis (Ishimaru et al., 2019; Urena et al., 2014, 2016). This functional interaction is critical for arresting earlier developmental programs associated with *BR-C* function and directing the transition to adulthood (Belles, 2020; Martin et al., 2020). Detailed molecular studies in *Drosophila* have demonstrated that this regulation occurs at the level of chromatin accessibility, where key enhancers for *BR-C* expression are shut down by *E93* in early pupae (Uyehara et al., 2017).

In contrast, we found that *E74A* and *E75A* are expressed at reduced levels in *E93* mutant salivary glands at 8 and 10 hours after pupariation,

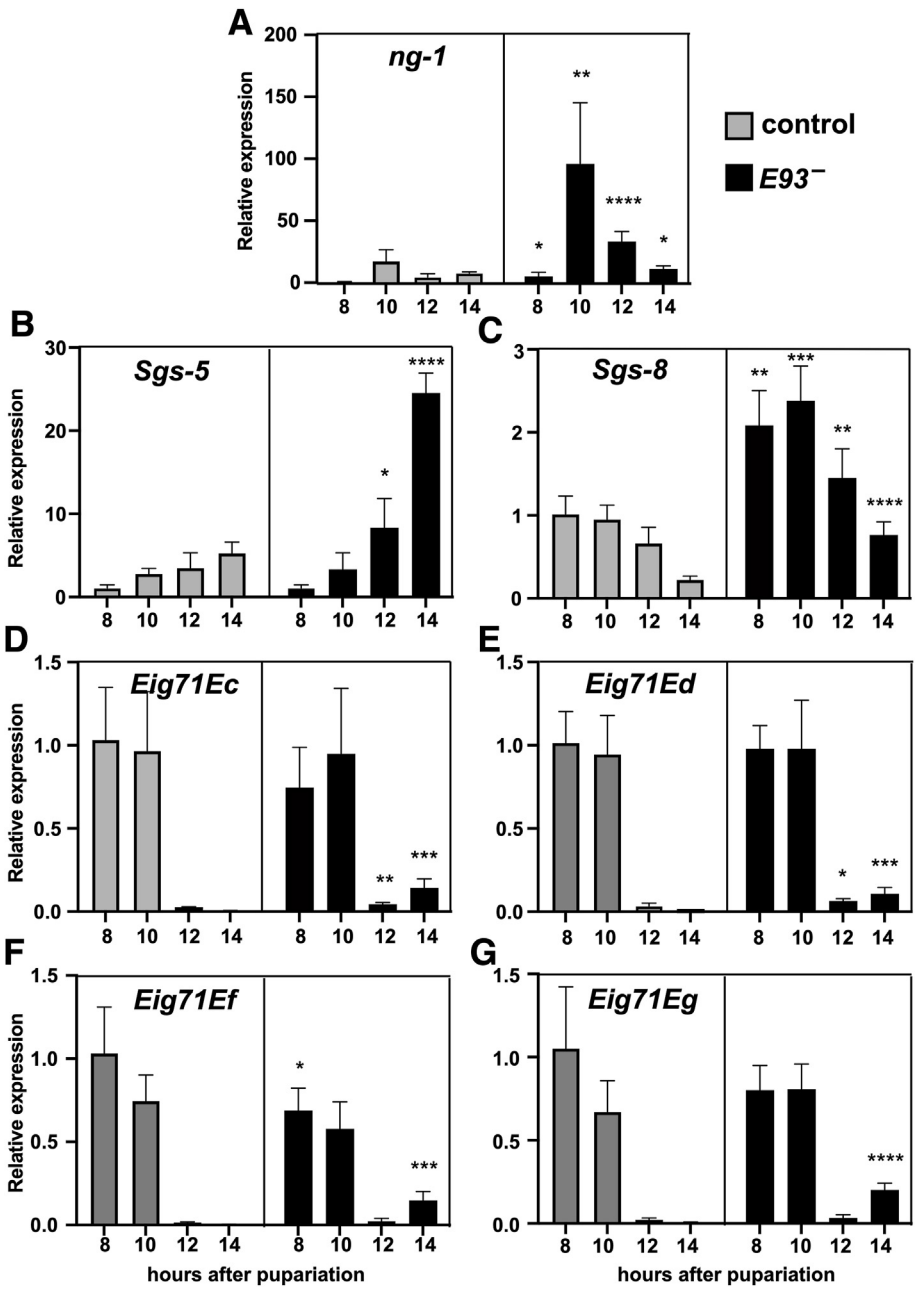

Fig. 7. Larval salivary gland genes and *Eig71E* late genes are mis-regulated in *E93* mutants. RT-qPCR was performed using RNA isolated from staged control (grey) or *E93* mutant (black) salivary glands in order to detect the temporal patterns of *ng-1* (A), *Sgs-5* (B), *Sgs-8* (C), *Eig71Ec* (D), *Eig7*1Ed (E), *Eig71Ef* (F), and *Eig7*1Eg (G) ecdysone-induced transcription. Transcript levels are normalized to *rp49* and presented relative to controls at 8 h after pupariation. n = 5 independent samples with 13–15 flies per sample. P-values represent comparisons between control and *E93* mutant mRNA levels at the indicated time point: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Data without an asterisk are not significant, p-value >0.05.

when *E93* is at basal levels of expression (Fig. 6A,D,F). This effect could represent an earlier requirement for this factor in establishing an appropriate chromatin state at specific loci (Uyehara et al., 2017). Further studies are required to determine how *E93* exerts this early repressive role on ecdysone-inducible gene transcription.

4.4. *E93 represses earlier transcriptional responses to ecdysone*

Another unexpected aspect of our work was the discovery that glue genes are ectopically expressed in *E93* mutant salivary glands (Fig. 7A–C). These genes have been extensively characterized based on their tightly restricted expression in late third instar larval salivary glands (Beckendorf and Kafatos, 1976; Biyasheva et al., 2001). Interestingly, the glue genes continue to be expressed in mid- and late prepupal *E93* mutant salivary glands (Fig. 7A–C). Once again, *E93* appears to be acting as a repressor at 8 and 10 hours after pupariation, when it is expressed at only basal levels. Given that *E93* controls chromatin accessibility, it is possible that this regulator contributes to the silencing of specific glue gene enhancers in late prepupae to ensure their restricted expression in third instar larvae (Uyehara et al., 2017). Further work is needed to understand the mechanisms that underlie this repressive effect on glue gene expression. Regardless, these studies support the model that the ecdysone-induced expression of *E93* defines the end of larval developmental programs and the onset of adulthood, and demonstrates that these regulatory functions extend to the salivary glands.

In addition, we discovered that a loss of *E93* function results in persistent low-level expression of the *Eig71E* late genes in 12- and 14-hour prepupae (Fig. 7D–G). These genes are normally briefly expressed in mid-prepupae as an indirect transcriptional response to the late larval pulse of ecdysone that triggers puparium formation (Guay and Guild, 1991; Restifo and Guild, 1986a). Similar to the glue genes, the rapid repression of these genes in *E93* mutant mid-prepupae is followed by an abnormal second wave of expression (Fig. 7D–G). In part, this might be an indirect effect due to the inefficient repression of *BR-C* and *E74A*

expression in late prepupal salivary glands (Fig. 6D and E). Earlier work has shown that *BR-C* and *E74A* are each required for proper *Eig71E* late gene expression in early prepupae (Fletcher and Thummel, 1995a; Guay and Guild, 1991; Urness and Thummel, 1995). In addition, functional interactions between *BR-C* and *E74A* amplify this effect, resulting in the tight coordinate regulation of *Eig71E* induction (Fletcher et al., 1997; Fletcher and Thummel, 1995b). Further studies could address whether *E93* directly or indirectly represses *Eig71E* gene expression in late prepupae.

Taken together, our results indicate that *E93* plays a pivotal role in determining the stage-specificity of the transcriptional response to the prepupal pulse of ecdysone, and contributes to the proper coordination of gene expression during the onset of metamorphosis. Future studies using the deletion mutants described here should further our understanding of this critical regulator and provide directions for defining the transcriptional switches that establish the end of larval developmental programs and the onset of adult differentiation.

Acknowledgements

We thank I. Duncan for providing the $E93^{\Delta 1}$, $E93^{4}$, $E93^{5}$, and $E93^{6}$ mutants and for informative discussions about the genetics of the *E93* locus, A. Bashrullah for providing the *fkh>GFP* salivary gland reporter, C. Stubben for help with the RNA-seq analysis, FlyBase for informatic support, and the Bloomington Stock Center (NIH P40OD018537) for providing fly stocks. RNA-seq was performed by the HCI High-Throughput Genomics and Bioinformatic Analysis Shared Resource at the University of Utah, which is supported by the NCI (P30CA042014). This research was supported by the NIH: R01 DK075607 (CST) and R01 GM079431 (EHB).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2021.10.001.

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