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Steroid Hormone Inactivation Is Required during the Juvenile-Adult Transition in *Drosophila*

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SUMMARY

Steroid hormones are systemic signaling molecules that regulate juvenile-adult transitions in both insects and mammals. In insects, pulses of the steroid hormone 20-hydroxyecdysone (20E) are generated by increased biosynthesis followed by inactivation/clearance. Although mechanisms that control 20E synthesis have received considerable recent attention, the physiological significance of 20E inactivation remains largely unknown. We show that the cytochrome P450 Cyp18a1 lowers 20E titer during the *Drosophila* prepupal to pupal transition. Furthermore, this reduction of 20E levels is a prerequisite to induce *βFTZ-F1*, a key factor in the genetic hierarchy that controls early metamorphosis. Resupplying *βFTZ-F1* rescues *Cyp18a1*-deficient prepupae. Because Cyp18a1 is 20E-inducible, it appears that the increased production of steroid is responsible for its eventual decline, thereby generating the regulatory pulse required for proper temporal progression of metamorphosis. The coupling of hormone clearance to *βFTZ-F1* expression suggests a general mechanism by which transient signaling drives unidirectional progression through a multistep process.

INTRODUCTION

Animals develop from juveniles into sexually mature adults through successive life stages separated by temporally defined transitions. Steroid hormones are systemic signaling molecules that temporally coordinate the juvenile-adult transition in both mammals and insects through interactions with nuclear receptors (King-Jones and Thummel, 2005; McBrayer et al., 2007; Popa et al., 2008; Rewitz et al., 2009b). In insects, pulses of the steroid hormone, 20-hydroxyecdysone (20E), are responsible for this transition, a process known as metamorphosis (Riddiford, 1993).

Insight into how steroids control the genetic circuits during developmental transitions has mainly come from studies of *Drosophila melanogaster*, which led to a general model for gene regulation by steroid hormones in eukaryotes (Ashburner et al., 1974; Thummel, 1996, 2001, 2002). According to this model, only a pulse of 20E, i.e., a short period of high 20E titer, can trigger activation of some genes in the 20E-regulated cascade that initiates metamorphosis (Sun et al., 1994; Thummel, 1996; Woodard et al., 1994). Pulses of 20E are generated by two processes: synthesis that increases the titer and inactivation/removal that decreases the titer. Although the mechanisms that control the rise in 20E are well studied (Caldwell et al., 2005; Colombani et al., 2005; Gilbert et al., 2002; Layalle et al., 2008; McBrayer et al., 2007; Rewitz et al., 2009a, 2009b), the physiological significance of 20E inactivation is largely unexplored except for several in vitro studies that examined the importance of 20E decline during prepupal development (Fechtel et al., 1988; Richards, 1976).

One proposed route for 20E inactivation is through 26-hydroxylation catalyzed by the cytochrome P450 Cyp18a1 (Bassett et al., 1997; Guittard et al., 2010; Hurban and Thummel, 1993). Interestingly, Cyp18a1 was first identified based on its inducibility by 20E (Hurban and Thummel, 1993), consistent with the 20E-inducible 26-hydroxylase activity (Chen et al., 1994; Williams et al., 1997, 2000). If this is the case, inactivation is dependent on the concentration of the hormone itself, representing an elegant feedback regulation of steroid levels.

The aim of the present study was to examine the functional importance of steroid pulses during development by studying the role of Cyp18a1 in the decline of 20E levels. Here, we present evidence that Cyp18a1 is required for the decline of the 20E titer and that failure to reduce 20E levels after the late larval 20E peak disrupts metamorphic development and leads to animal death. Furthermore, we show that these animals die because elevated 20E levels repress the expression of the mid-prepupal gene *βFTZ-F1*, a factor necessary for providing competence to respond to 20E in a stage-specific manner during metamorphosis (Broadus et al., 1999).

RESULTS AND DISCUSSION

Cyp18a1 Overexpression Yields a Phenotype Similar to that of Ecdysone-Deficient Mutants

It has recently been demonstrated that Cyp18a1 hydroxylates 20E at position C26, a process believed to convert this hormone into inactive metabolites (Bassett et al., 1997; Guittard et al., 2010; Hurban and Thummel, 1993). To directly test the hypothesis that 26-hydroxylation inactivates 20E, we overexpressed this enzyme using the Gal4/UAS system during embryonic development. Mutants with reduced 20E titers during the embryonic stage show a characteristic "Halloween" phenotype that consists of a failure to secrete cuticle, a lack of head involution,

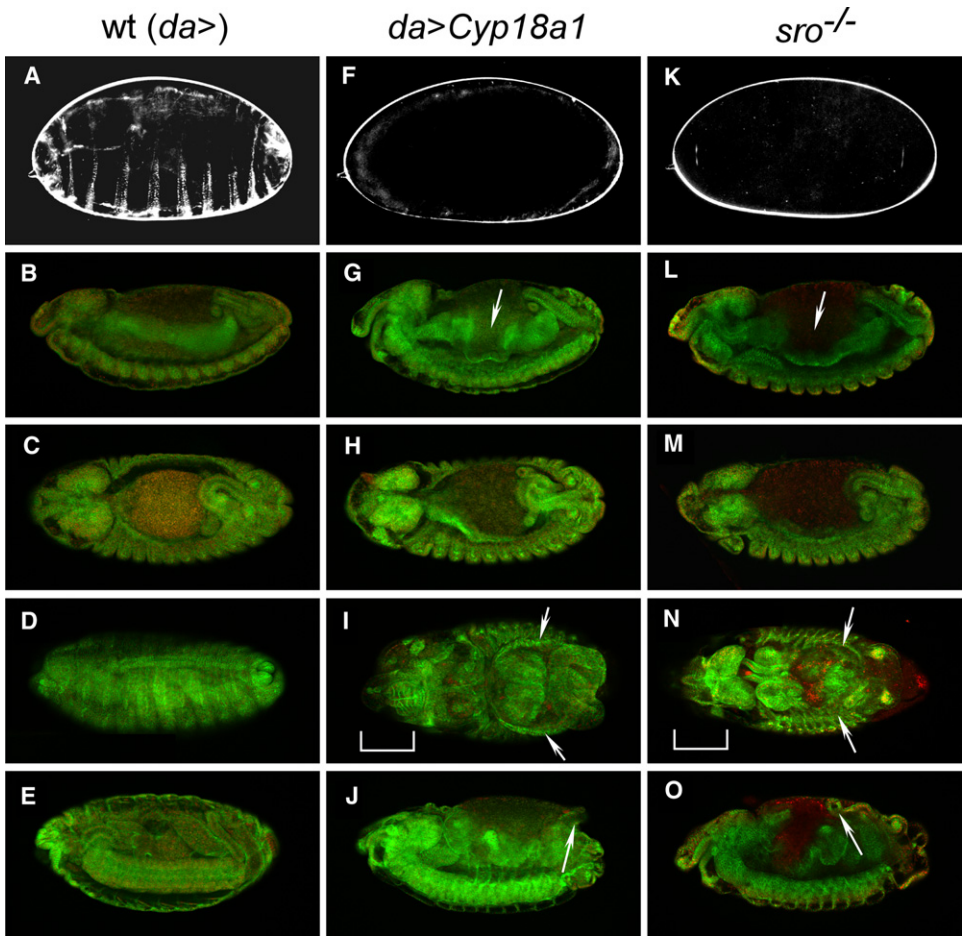


Figure 1. The Phenotype of *UAS-Cyp18a1* Overexpression Is Similar to that of Ecdysone-Deficient Mutants

(A, F, and K) Cuticle preparations of stage 17 embryos showing that embryos overexpressing *Cyp18a1* (*da > Cyp18a1*) fail to lay down an embryonic cuticle like the low ecdysone mutant *shroud*[1] (*sro*). Immunohistochemical staining with a spectrin antibody (green) and nuclear staining with DAPI (red) of embryos stage 14 lateral view (B, G, and L), stage 14 dorsal view (C, H, and M), stage 15–17 dorsal view (D, I, and N), and stage 15–17 lateral view (E, J, and O). The terminal phenotype in embryos overexpressing *Cyp18a1* (I and J) is similar to homozygous *sro* mutants (N and O). Note the defects in midgut morphogenesis (arrows in G and L), dorsal closure (arrows in I and N), head involution (brackets in I and N), and the protruding gut as a result of the morphogenesis defect (arrows in J and O) in these embryos. Embryos are viewed with anterior to the left.

and an inability of the midgut and dorsal epidermis to close (Figure 1). Ultimately, these embryos die during late embryonic development and fail to hatch as first instar larvae (Chavez et al., 2000; Petryk et al., 2003; Warren et al., 2002). To examine the effects of *Cyp18a1* overexpression, Gal4 drivers expressed in different tissues were used to overexpress *UAS-Cyp18a1* (see Table S1 available online). Ubiquitous strong (*da > Cyp18a1*) or weaker (*arm > Cyp18a1*) expression of *Cyp18a1* resulted in 100% embryonic lethality. Expression of *Cyp18a1* in the CNS alone (*elav-Gal4*) also caused embryonic death, whereas animals expressing *Cyp18a1* primarily in the fat body (*pumpless-Gal4* and *CG-Gal4*) died in the larval and pupal stages.

To examine the phenotype of ubiquitous *Cyp18a1* overexpression, cuticles from *da > Cyp18a1* embryos were prepared and compared to the Halloween mutant *shroud* (*sro*) that encodes an enzyme involved in 20E synthesis (Niwa et al., 2010). Similar to *sro* mutants (and all other biosynthetic enzyme mutants), *da > Cyp18a1* embryos fail to produce cuticle structures such as denticle belts (compare Figures 1A, 1F, and 1K). Furthermore, after stage 14, these embryos exhibit morphological defects typically observed in Halloween family mutants, including abnormalities in gut morphogenesis (arrows in Figures 1G and 1L) and head involution and dorsal closure (brackets and arrows in Figures 1I and 1N). In these embryos the incomplete midgut closure leads to yolk granules that are located randomly through the dorsal opening. These animals never deposit a cuticle and die before hatching to the first larval instar. Thus, overexpression of *Cyp18a1* results in a phenotype identical to that of the 20E-deficient Halloween mutants, demonstrating that Cyp18a1 inactivates 20E.

Cyp18a1-Mediated Inactivation of 20E Is Required for Metamorphic Development

To determine if inactivation of 20E has an important developmental function, we characterized the *Cyp18a1* loss-of-function phenotype. An allele designated *Cyp18a1*[1] missing the region

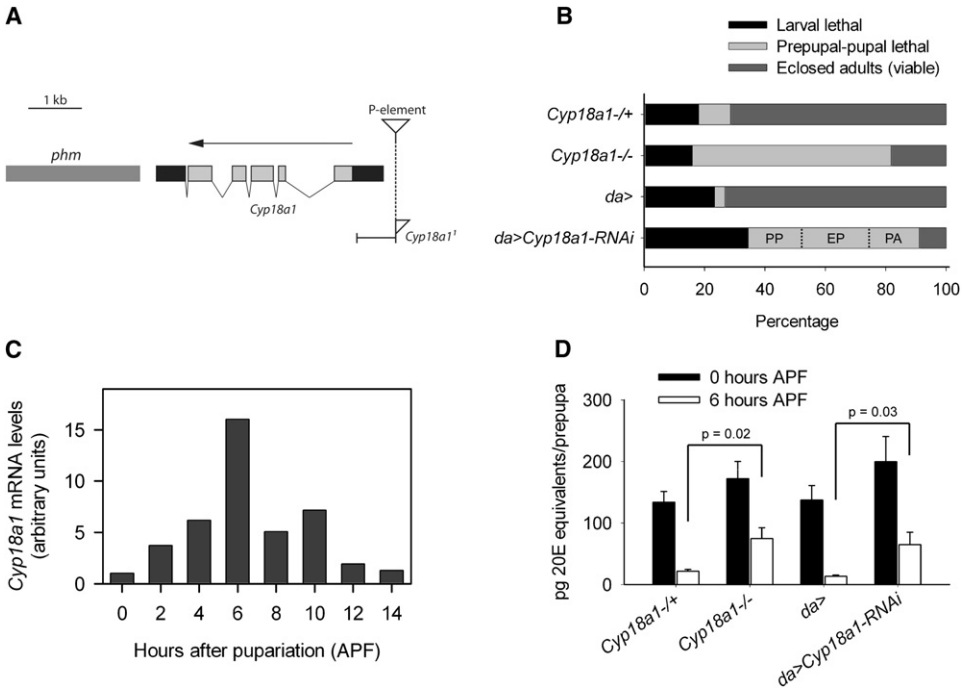


Figure 2. Cyp18a1 Is Required during Metamorphosis

(A) Illustration of the *Cyp18a1* locus and *Cyp18a1*^{1} mutant. Light-gray boxes indicate protein coding sequences, and black boxes show untranslated regions. An arrow indicates gene orientation, and the site of the excised *P* element is shown by an inverted triangle. The remaining part of the triangle in *Cyp18a1*^{1} indicates the part of the *P* element that is still present. *phm*, *phantom*.

(B) The lethal phase was analyzed in *Cyp18a1*^{1} and *da* > *Cyp18a1-RNAi* animals. Lethality during metamorphosis was scored for three phenotypic classes in *da* > *Cyp18a1-RNAi*: percentage of animals that died as prepupae (PP), early in the pupal stage (EP), or as pharate adults (PA) is shown.

(C) *Cyp18a1* expression during the prepupal-pupal transition detected using quantitative RT-PCR. High expression correlates with prepupal period where *Cyp18a1* is necessary for development.

(D) Ecdysteroid titer at puparium formation (0 hr APF) and 6 hr APF in control, *Cyp18a1*^{1} and *da* > *Cyp18a1-RNAi* animals. Error bars represent SE; n = 3 batches. p values were calculated by Student's *t* test.

upstream of the translational start site, including most of the 5′-untranslated region, was obtained by the imprecise excision of a *P* element (Figure 2A). The excision removes from −34 to −649 bp upstream of the translational start and leaves a portion of the *P* element at the insertion position.

To investigate the developmental role of *Cyp18a1*, the lethal phase of *Cyp18a1*^{1} animals was analyzed. The majority of homozygous *Cyp18a1*^{1} embryos (93.2%; n = 73) developed normally to the first larval instar. Analysis of post-embryonic lethality showed that most homozygous *Cyp18a1*^{1} animals pupariate but fail to complete metamorphosis (Figure 2B). *Cyp18a1*^{1} is a strong loss-of-function allele because when placed in *trans* over *Df(1)BSC585*, a deficiency covering the *Cyp18a1* locus, the phenotype was indistinguishable from homozygous *Cyp18a1*^{1} mutants (data not shown). A similar metamorphosis defect was observed when *Cyp18a1* expression was reduced ubiquitously using *UAS-Cyp18a1-RNAi* (*da* > *Cyp18a1-RNAi*). During the prepupal to pupal transition, these animals die in three phenotypic classes (Figure 2B). Of those that pupariated, 72% of the animals died as prepupae or as early pupae after delayed head eversion. This shows that *Cyp18a1* is important for prepupal development. A smaller proportion of the animals died as pharate adults, and fewer escapers eclosed. All animals that pupate showed a malformed leg phenotype, and some also displayed head eversion defects (Figure 3). These results demonstrate that Cyp18a1 activity is critical for metamorphosis. Like *da-Gal4*, the strong ubiquitous drivers *αTub84B-Gal4* and *Act5C-Gal4* produced animals that died during metamorphosis (Table S2). Weaker ubiquitous expression of *Cyp18a1-RNAi* using *armadillo-Gal4* allowed most animals to develop to the adult stage.

If *Cyp18a1* is critical for prepupal development, it should be expressed at high levels during this stage. Therefore, *Cyp18a1* expression was analyzed using quantitative RT-PCR during the prepupal stage. After low expression at the time of pupariation when 20E peaks, expression of *Cyp18a1* rapidly increases in a manner that inversely correlates with the 20E level (Figure 2C). The expression of *Cyp18a1* reaches a peak in the mid-prepupal stage, 6 hr after puparium formation (APF), when 20E has declined to the basal level after the late larval 20E peak at pupariation. Consistent with this expression pattern, both *da* > *Cyp18a1-RNAi* and *Cyp18a1*^{1} prepupae had significantly higher 20E levels 6 hr APF (Figure 2D). These results demonstrate that Cyp18a1 has an important function in metabolizing 20E after pupariation.

It is interesting that loss of Cyp18a1 activity does not significantly arrest development at early embryonic or larval stages. Although this might indicate that the decline of the 20E level is

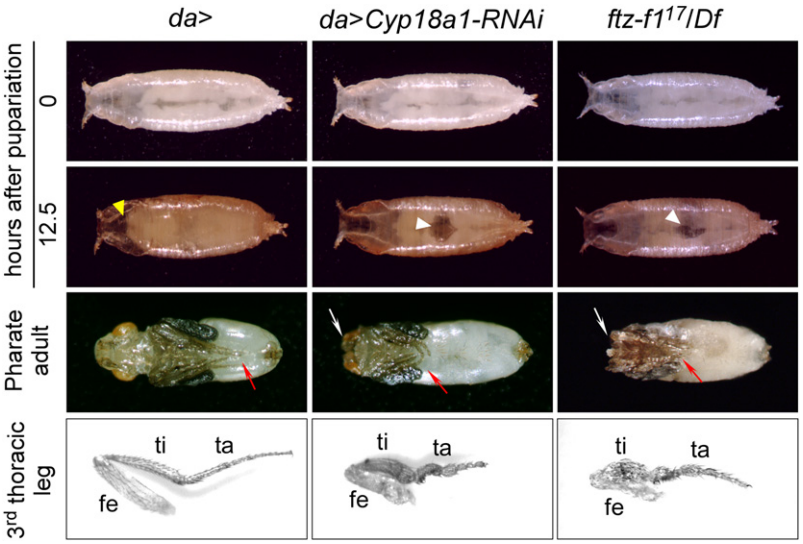


Figure 3. Reduction of Cyp18a1 Activity Results in a Phenotype Similar to the Loss of *βFTZ-F1* Function

Both *da > CYP18-RNAi* and *βFTZ-F1^{17}/Df* animals show failure to displace the air bubble (white arrowheads) anteriorly during prepupal development and show defects in head eversion (yellow arrowhead in *da >*), resulting in microcephalic and cryptocephalic phenotypes, respectively (white arrows). The third leg pair (red arrows) is malformed in all animals that reach this stage. Dissected third legs from adults are shown. fe, femur; ti, tibia; ta, tarsus.

not important during these stages, there are a few things to consider when interpreting this result. First, neither *Cyp18a1^{1}* nor *da > Cyp18a1-RNAi* completely eliminates the transcript, so the residual Cyp18a1 activity might be high enough for the animals to make it through the embryonic and larval stages. Second, some reports indicate that other 20E inactivation routes also exist (Lafont et al., 2005; Rees, 1995), which may also explain why the 20E levels show some decline during prepupal stages, even in *Cyp18a1^{1}* and *da > Cyp18a1-RNAi* animals. Third, during larval stages, 20E can be eliminated not only by inactivation but also by excretion, whereas during metamorphosis where the animal is a closed system, a decline of 20E can only be achieved by the conversion into inactive metabolites. This might also contribute to the stage-specific sensitivity of the animal to the loss of Cyp18a1.

Reduction of Cyp18a1 Activity Results in a Phenotype Similar to the Loss of βFTZ-F1

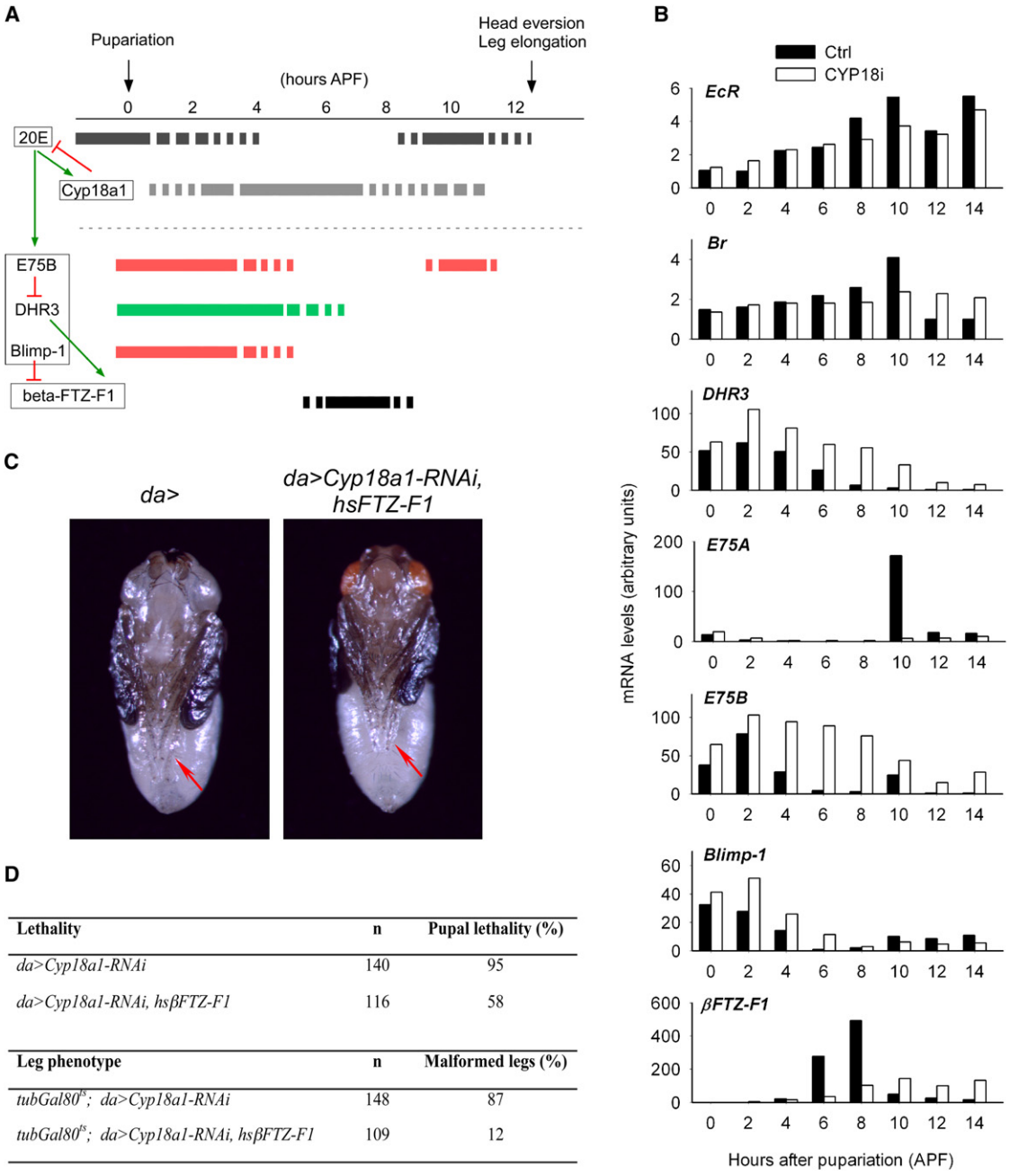
In considering the *Cyp18a1* loss-of-function phenotype, we realized that it closely matched that seen in hypomorphic *βFTZ-F1^{17}* mutants (Broadus et al., 1999; Sliter and Gilbert, 1992). Like animals heterozygous for *βFTZ-F1^{17}* and a deletion allele *Df(3L)Cat^{DH104}* that covers the *βFTZ-F1* locus, *da > Cyp18a1-RNAi* larvae pupariate normally but exhibit defects in pupation (Figure 3). *βFTZ-F1^{17}/Df(3L)Cat^{DH104}* and *da > Cyp18a1-RNAi* larvae contract their bodies and evert their anterior spiracles normally at pupariation. At this stage and shortly after pupariation when they sclerotize and tan their cuticle, these animals are morphologically normal. During subsequent prepupal development the abdominal air bubble normally translocates anteriorly in control animals, and pupation occurs approximately 12 hr APF with the eversion of the head. *da > Cyp18a1-RNAi* and *βFTZ-F1^{17}/Df(3L)Cat^{DH104}* prepupae show delayed or completely failed displacement of the abdominal air bubble and head eversion, generating microcephalic and cryptocephalic phenotypes, respectively. The third thoracic legs are also similarly short and malformed in *βFTZ-F1^{17}/Df(3L)Cat^{DH104}* and *da > Cyp18a1-RNAi* animals (Figure 3).

The 20E-Regulated Genetic Hierarchy that Controls Metamorphic Development Is Disrupted in *Cyp18a1-RNAi* Animals

To investigate the molecular consequences of Cyp18a1 loss during prepupal development, we examined the temporal expression profile of 20E-regulated genes, including *βFTZ-F1*, that coordinate this particular developmental transition. Normally, *βFTZ-F1* is induced in the mid-prepupal stage only after a pulse exposure to 20E, which means a period with a high level of 20E followed by a low level (Broadus et al., 1999; Woodard et al., 1994; Yamada et al., 2000). The data presented above show that *Cyp18a1* loss-of-function animals experience an extended period of high 20E levels in the mid-prepupal stage that might repress *βFTZ-F1* expression.

When we examined the expression of EcR, which is required for the initial triggering of metamorphosis, we found that its expression is relatively normal in *da > Cyp18a1-RNAi* animals compared to the control (Figure 4B). In control animals, *βFTZ-F1* is induced in the mid-prepupal stage, 6–8 hr APF. At this point the 20E titer drops to the basal level in these animals, whereas it is still elevated in *da > Cyp18a1-RNAi* animals (Figure 2D). In agreement with the repressive role of 20E, reduced *βFTZ-F1* expression was observed in *da > Cyp18a1-RNAi* animals at this stage (Figure 4B). This supports the idea that misexpression of *βFTZ-F1* contributes to the phenotype of *da > Cyp18a1-RNAi* animals. Furthermore, increased expression of the 20E-inducible genes *DHR3*, *E75B*, and *Blimp-1* was observed during the early prepupal stage (Figure 4B). E75B is believed to prevent DHR3 from inducing *βFTZ-F1* in the first 4 hr APF, whereas *Blimp-1* is a direct repressor of *βFTZ-F1* (Figure 4A) (Agawa et al., 2007; Andres et al., 1993; Broadus et al., 1999; Yamada et al., 2000). In control animals, expression of *E75B* and *Blimp-1* has declined 6 hr APF, allowing DHR3 to induce *βFTZ-F1* (Figure 4B). In contrast, both repressors are expressed in *da > Cyp18a1-RNAi* animals 6 hr APF because these genes are induced by the higher 20E level. The prolonged expression of the *βFTZ-F1* repressors E75B and Blimp-1 in *da > Cyp18a1-RNAi* animals provides a molecular explanation for the lack of *βFTZ-F1* transcription in these animals.

βFTZ-F1 is a nuclear receptor necessary for the stage-specific response to the prepupal 20E pulse at 10 hr APF that coordinates the prepupal-pupal transition (Broadus et al., 1999; Sliter and Gilbert, 1992). In control animals this prepupal 20E pulse induces



Lethality	n	Pupal lethality (%)
da>Cyp18a1-RNAi	140	95
da>Cyp18a1-RNAi, hsβFTZ-F1	116	58

Leg phenotype	n	Malformed legs (%)
tubGal80^{ts}; da>Cyp18a1-RNAi	148	87
tubGal80^{ts}; da>Cyp18a1-RNAi, hsβFTZ-F1	109	12

Figure 4. The Ecdysone-Induced Genetic Hierarchy Necessary for Metamorphic Development Is Disrupted in *Cyp18a1-RNAi* Prepupae

(A) A model for the regulation of *βFTZ-F1* and the role of *Cyp18a1* and 20E. The 20E peak at pupariation induces a set of genes including *E75B*, *DHR3*, *Blimp-1*, and *Cyp18a1*. Under these conditions, E75B represses *DHR3*, a transcriptional activator of *βFTZ-F1*, whereas Blimp-1 directly represses *βFTZ-F1* expression. As *Cyp18a1* lowers the 20E level, the expression of 20E-inducible genes *E75B*, *DHR3*, and *Blimp-1* declines. Low expression of *E75B* and *Blimp-1* at 6 hr APF allows residual DHR3 to induce *βFTZ-F1*.

(B) The expression of 20E-regulated genes analyzed using quantitative RT-PCR during the prepupal-pupal transition. Data are expressed as fold changes relative to the lowest expression level for each gene, arbitrarily set to 1.

(C) Expression of *hsβFTZ-F1* rescues the leg phenotype (red arrows) of *Cyp18a1-RNAi* animals. A *da* > *Cyp18a1-RNAi, hsβFTZ-F1* pharate adult with normally developed legs like the control (*da* >) is shown. (D) Ectopic expression of *hsβFTZ-F1* rescues lethality and leg abnormalities of *Cyp18a1-RNAi* animals.

transient high-level expression of the 20E-inducible gene *E75A* (Figure 4B). Because the mid-prepupal expression of *βFTZ-F1* is necessary for this induction, it is not observed in *βFTZ-F1^{17}/Df(3L)Cat^{DH104}* animals (Broadus et al., 1999). Consistent with the reduced mid-prepupal *βFTZ-F1* expression, the *E75A* peak at 10 hr APF was not observed in *da* > *Cyp18a1-RNAi* animals (Figure 4B). Together, the results suggest that after the late larval 20E pulse that triggers larval-prepupal transition,

da > *Cyp18a1-RNAi* animals do not acquire the *βFTZ-F1*-mediated competence to respond to the prepupal 20E pulse necessary for the prepupal-pupal transition.

Cyp18a1 Confers the βFTZ-F1-Mediated Competence to Respond to the Prepupal Ecdysone Pulse

The above data suggest that the reduced *βFTZ-F1* expression causes the metamorphic defect in Cyp18a1 loss-of-function animals. If this is the case, expression of *βFTZ-F1* in *da* > *Cyp18a1-RNAi* animals should rescue the phenotype. Indeed, we found that ectopic *βFTZ-F1* expression under control of a heat shock promoter rescued the lethality of *da* > *Cyp18a1-RNAi* animals. Forty-two percent of the *da* > *Cyp18a1-RNAi* animals containing *hsβFTZ-F1* eclosed as viable adults, compared to only 5% of the control *da* > *Cyp18a1-RNAi* animals (Figure 4D). This is comparable to the percentage of *βFTZ-F1* mutants rescued by expression of *hsβFTZ-F1*, which ranged from approximately 2% to 40% (Yamada et al., 2000).

Although the leg phenotype was also rescued in some of the *da* > *Cyp18a1-RNAi, hsβFTZ-F1* flies (Figure 4C), for a better comparison we chose to quantify rescue of this phenotype in the presence of Gal80^{ts}, a temperature-sensitive Gal4 inhibitor that partially inhibits Gal4 activity at 25°C. At this temperature, expression of Gal80^{ts} in *da* > *Cyp18a1-RNAi* animals (*tubGal80^{ts}; da* > *Cyp18a1-RNAi*) reduces the activity of the Gal4 and allows almost 100% of the pupae to eclose as adults, 87% of which exhibit the leg phenotype observed in *da* > *Cyp18a1-RNAi* animals (Figure 4D). In contrast, only 12% of the eclosed *tubGal80^{ts}; da* > *Cyp18a1-RNAi, hsβFTZ-F1* flies had abnormal legs. This clearly demonstrates that it is the lack of βFTZ-F1 activity that is causing the defects in leg morphogenesis in *Cyp18a1-RNAi* animals. Taken together, our results show that the defect in the prepupal-pupal transition in *Cyp18a1* loss-of-function animals can be attributed to the repression of *βFTZ-F1*, a factor necessary for the stage-specific response to 20E.

In both insects and mammals, initiation of sexual maturation is controlled by the neuroendocrine system that triggers the production of systemic steroid signals (McBrayer et al., 2007; Rewitz et al., 2009a, 2009b). To date, most insect studies have concentrated on how the rise in steroid titer induces new genetic cascades that program a developmental transition such as metamorphosis. However, as illustrated here, correct temporal progression through the metamorphic genetic program requires properly timed removal of steroid as well. Gene regulation by the consecutive rise and fall of steroids, produced by feedback regulation, may provide a general mechanism for control of temporal progression through development.

EXPERIMENTAL PROCEDURES

Drosophila Stocks

Flies were maintained on standard cornmeal media at 25°C under 12 hr/12 hr light/dark cycle. *Drosophila* stocks were obtained from the Bloomington Stock Center unless otherwise specified. The following stocks were obtained from the indicated sources: *hsβFTZ-F1* (Yamada et al., 2000); *βFTZ-F1^{17}* and *Df(3L)Cat^{DH104}* (Broadus et al., 1999); *UAS-Cyp18a1-RNAi* (Vienna *Drosophila* RNAi Center No. 5602); and *Cyp18a1^{1}* and *UAS-Cyp18a1* (Ethan Bier, University of California, San Diego).

Lethal Phase Determination and Phenotype Analysis

Flies were allowed to lay eggs for 2 hr on apple juice agar plates supplemented with yeast paste. Embryonic lethality was assayed in embryos allowed to develop for 24 hr. To assay post-embryonic lethality, newly hatched first instar larvae were transferred to standard cornmeal food, with 25 larvae per vial. To examine the exact lethal phase during the prepupal-pupal stages, white prepupae were isolated and allowed to develop on moist filter paper and scored for lethality and morphological phenotype.

Quantification of Ecdysteroids

Frozen prepupae (ten animals/tube) were homogenized and extracted as described previously (Warren et al., 2006). The extracts were evaporated, redissolved, and subjected to ecdysteroid ELISA. The ELISA was performed in a competitive assay format using anti-20E rabbit antiserum (Cayman Chemical), acetylcholinesterase-conjugated 20E (Cayman Chemical), and standard 20E (Sigma). The acetylcholinesterase activity was quantified by Ellman's Reagent (Cayman Chemical), and the absorbance at 415 nm was detected with a Benchmark microplate reader (Bio-Rad).

Analysis of Embryonic Phenotypes

Cuticle from developed embryos was prepared (Rewitz et al., 2009b), and embryonic structures were visualized by staining for spectrin (a cytoskeletal protein) using a mouse anti-spectrin antibody (Developmental Studies Hybridoma Bank, University of Iowa, IA) at 1/50 dilution as described (Chavez et al., 2000), except that staining was visualized using a fluorescent conjugated secondary antibody (Molecular Probes) at 1/500 dilution. Confocal images were obtained using a Zeiss LSM 710 confocal laser-scanning microscope.

Quantitative RT-PCR

For analysis of gene expression, RNA was extracted from prepupae and pupae precisely timed from the white puparium stage. Total RNA was prepared from six individuals using the RNeasy Mini Kit (QIAGEN), DNaseI treated on-column to remove genomic DNA, and reverse transcribed using the Superscript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was carried out with SYBR Green Master mix using a real-time PCR LightCycler 480 (Roche) as described (Rewitz et al., 2006). To ensure homogeneity of the amplified PCR product, melting curve analysis was performed on all reactions. The *Drosophila ribosomal protein L23* (*rpL23*) was used to normalize transcript levels (McBrayer et al., 2007). The primers used are shown in Table S3.

Rescue Experiment

A *hsβFTZ-F1* transgene was recombined with *UAS-Cyp18a1-RNAi* by standard genetic methods. In order to avoid the elevated lethality of *da* > *Cyp18a1-RNAi* animals raised at 29°C (100%), we performed the rescue experiment at 25°C, where the heat shock promoter shows high enough activity to rescue mutant phenotypes (Guo et al., 2000; Qian et al., 1988; Ruaud et al., 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2010.10.021.

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