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The Ecdysone-Induced DHR4 Orphan Nuclear Receptor Coordinates Growth and Maturation in *Drosophila*

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Summary

A critical determinant of insect body size is the time at which the larva stops feeding and initiates wandering in preparation for metamorphosis. No genes have been identified that regulate growth by contributing to this key developmental decision to terminate feeding. We show here that mutations in the DHR4 orphan nuclear receptor result in larvae that precociously leave the food to form premature prepupae, resulting in abbreviated larval development that translates directly into smaller and lighter animals. In addition, we show that *DHR4* plays a central role in the genetic cascades triggered by the steroid hormone ecdysone at the onset of metamorphosis, acting as both a repressor of the early ecdysone-induced regulatory genes and an inducer of the βFTZ-F1 midprepupal competence factor. We propose that *DHR4* coordinates growth and maturation in *Drosophila* by mediating endocrine responses to the attainment of critical weight during larval development.

Introduction

Endocrine signaling plays a central role in the growth and maturation of all higher organisms. Although growth and maturation are coupled in vertebrates, these events are temporally distinct in holometabolous insects, with growth restricted to the larval stages and maturation occurring during metamorphosis. Larvae progress through a series of molts, with the duration of each molt determined by a pulse of the steroid hormone 20-hydroxyecdysone (20E, also referred to as ecdysone) (Riddiford, 1993). Larval development continues until a checkpoint is reached—the attainment of critical weight—that indicates that a sufficient size has been achieved to support pupariation and maturation (Stern and Emlen, 1999; Nijhout, 2003). In laboratory cultures of *Drosophila melanogaster*, the attainment of critical weight correlates with the so-called "70 hr change" (70 hr after egg laying, near the second-to-third-instar larval molt), after which larvae are able to pupariate at a relatively normal time, even if exposed to complete starvation (Beadle et al., 1938). Studies in other insects have demonstrated that the attainment of critical weight initiates an endocrine cascade, consisting of a low-titer ecdysone pulse that causes larval wandering behavior and a subsequent high-titer ecdysone pulse that initiates metamorphosis, thereby coordinating growth with maturation (Stern and Emlen, 1999; Nijhout, 2003). In spite of the importance of the larval feeding phase for determining final body size, no genes have been identified that are required for appropriate developmental responses to the attainment of critical weight in insects.

In contrast, we have a relatively detailed understanding of the molecular mechanisms by which ecdysone controls metamorphosis, based on studies of the puffing patterns of the giant larval salivary gland polytene chromosomes (Clever, 1964; Ashburner et al., 1974). The late-larval ecdysone pulse initiates the prepupal stage of metamorphosis, followed ~10 hr later by the prepupal ecdysone pulse that signals adult head eversion and the prepupal-pupal transition (Riddiford, 1993). Ecdysone rapidly and directly induces a small set of early puffs. The protein products of these early puffs appear to exert two opposing regulatory functions—they repress their own expression, self-attenuating the regulatory response to the hormone, and they induce a large set of late secondary-response puffs. The late puff genes, in turn, are thought to function as effectors that control the biological responses to each pulse of ecdysone during development. Molecular and genetic studies of genes corresponding to ecdysone-regulated puffs have provided strong support for this hierarchical model of ecdysone action. Three early puff genes, *E74*, *E75*, and the *Broad-Complex*, are induced directly by ecdysone and encode families of transcription factors (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991) that are required for developmental responses to ecdysone during metamorphosis (Kiss et al., 1988; Restifo and White, 1992) and directly regulate late gene transcription (Urness and Thummel, 1995; Crossgrove et al., 1996). In spite of these advances, however, several aspects of the hierarchical model of ecdysone action remain unclear. In particular, no ecdysone-regulated genes have been identified that are required for early gene repression, leaving this key aspect of the model untested.

Like vertebrate steroids, ecdysone acts through members of the nuclear receptor superfamily that function as ligand-regulated transcription factors. The ecdysone receptor is comprised of a heterodimer of two nuclear receptors, EcR and Ultraspiracle (USP) (Riddiford et al., 2001). Interestingly, approximately half of the remaining 16 canonical nuclear receptors encoded by the *Drosophila* genome are transcriptionally regulated by ecdysone and/or function during metamorphosis, implicating a central role for these factors in transducing the ecdysone signal (King-Jones and Thummel, 2005). These receptors are referred to as orphan

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nuclear receptors because they have no known hormonal ligand, although at least some of these factors may act as ligand-independent transcriptional regulators. Among the orphan receptors induced by ecdysone at puparium formation is DHR3, which acts together with E75B to direct appropriate expression of the βFTZ-F1 midprepupal competence factor (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997). The βFTZ-F1 orphan nuclear receptor is both necessary and sufficient for appropriate responses to the prepupal pulse of ecdysone, distinguishing these responses from those induced by the hormone a few hours earlier in late third-instar larvae (Woodard et al., 1994; Broadus et al., 1999; Yamada et al., 2000). Thus, the interplay between these three nuclear receptors directs appropriate temporal progression through the early stages of metamorphosis.

Studies in several insect species have suggested that another orphan nuclear receptor, encoded in *Drosophila* by *DHR4* (CG16902), may contribute to this crossregulatory network during the early stages of metamorphosis (Charles et al., 1999; Hiruma and Riddiford, 2001; Weller et al., 2001; Chen et al., 2002; Sullivan and Thummel, 2003). Here we test this hypothesis by characterizing *DHR4* regulation and function in *Drosophila*. *DHR4* is induced directly by 20E at the onset of metamorphosis. *DHR4* mutants display significantly reduced levels of *βFTZ-F1* expression and die as prepupae with defects characteristic of *βFTZ-F1* mutants, defining *DHR4* as a critical regulator of this key competence factor. In addition, *DHR4* is necessary and sufficient for repressing early gene transcription at puparium formation, fulfilling a central prediction of the hierarchical model of ecdysone action. We also found that *DHR4* mutants are small and display precocious wandering followed by premature puparium formation, as much as a day earlier than wild-type animals. *DHR4* thus plays a critical role in linking growth to maturation, ensuring appropriate temporal progression through larval and prepupal stages of insect development.

Results

DHR4 Is Induced Directly by 20E as an Early-Late Response

Northern analysis of RNA samples isolated from staged wild-type animals reveals that *DHR4* is induced in late third-instar larvae (L3), peaks in expression ~2–4 hr after puparium formation, and shifts from a 10 kb to a 9.5 kb mRNA in 4–6 hr prepupae (Figure 1A, upper panel). Upon prolonged exposure, a low level of *DHR4* mRNA can also be detected in the L3 time points (data not shown). The dramatic upregulation of *DHR4* just prior to pupariation suggests that it is induced by the late-larval ecdysone pulse. To test this hypothesis, we performed Northern analysis using RNA samples isolated from L3 organs cultured in the presence of either 20E, the protein synthesis inhibitor cycloheximide, or both 20E and cycloheximide (Figure 1B). *DHR4* is induced 1.5 hr after 20E addition, with maximum levels of expression by 3–4 hr. This induction is delayed and reduced in the presence of cycloheximide, indicating that *DHR4* is induced directly by 20E and that its maximal expression requires 20E-induced protein synthesis. This requirement is consistent with the delayed expression of *DHR4* in response to the late-larval ecdysone pulse and defines its regulation as an early-late response to the hormone. Northern blot hybridization using RNA isolated from late L3 organs cultured for 90 min with different concentrations of 20E revealed that *DHR4* requires at least 5×10^{-8} M 20E for its expression and shows a half-maximal response at $\sim 2.5 \times 10^{-7}$ M (Figure 1C). Both values are virtually identical to those of the *DHR3* and *E78* early-late 20E-inducible genes (Stone and Thummel, 1993; Horner et al., 1995).

Disruption of *DHR4* Function Affects Metamorphosis and Growth

A *DHR4* mutation was created by mobilizing a P element inserted ~6 kb downstream from the *DHR4* polyadenylation site in the viable line *P{EP}EP427* (hereafter referred to as *P427*). This lethal allele, *DHR4[1]*, carries the original *P427* element as well as a new P element insertion ~2 kb upstream of the *DHR4* start codon. Lethal-phase analysis revealed that hemizygous *DHR4[1]* mutant animals display highly penetrant prepupal lethality, with all animals failing to progress to head eversion (Table 1). Four lines of evidence indicate that this mutation is specific to *DHR4*. First, only low levels of *DHR4* mRNA can be detected upon prolonged exposure of a Northern blot of RNA samples isolated from *DHR4[1]* mutant animals (Figure 1A, lower panel). Second, the lethality associated with *DHR4[1]* maps to 2B3–2C3, spanning the *DHR4* gene at 2C1 (Figure S1A). Third, ectopic expression of *DHR4* from an *hsp70*-regulated transgene increases the percentage of normally shaped prepupae in a *DHR4[1]* mutant background more than 12-fold and allows ~15% of the population to progress through metamorphosis to the pharate adult stage (Figure S1B). A more complete rescue is likely complicated by the lethality associated with ectopic *DHR4* expression (data not shown). Finally, transgenic flies were established that carry the *hsp70* promoter upstream from a tandem inverted repeat of *DHR4* coding sequences, and one line, *hsDHR4i*, was selected for studies of *DHR4* function by heat-inducible RNAi (Lam and Thummel, 2000). Heat-induced expression of *DHR4* double-stranded RNA (dsRNA) during either embryonic, first instar (L1), or second instar (L2) larval stages had no significant effect on viability. In contrast, *hsDHR4i* pupae (n = 217) that were heat treated 12 hr prior to pupariation displayed significant prepupal (63%) and pupal (35%) lethality, with 2% of the animals eclosing. These observations are consistent with our lethal-phase analysis of *DHR4[1]* mutants and confirm that this gene has no essential functions prior to metamorphosis.

DHR4[1] mutants display a range of defects in ecdysone responses at pupariation, including the formation of misshapen prepupae, failure to evert the anterior spiracles, and a delay in tanning (Figure 2A). In addition, all mutant prepupae are smaller than wild-type. For example, in one collection of 116 *DHR4[1]* mutant prepupae, 67% were 80%–90% wild-type length, while the remaining animals were 60%–80% wild-type length. This length difference is reflected in a 40% weight defi-

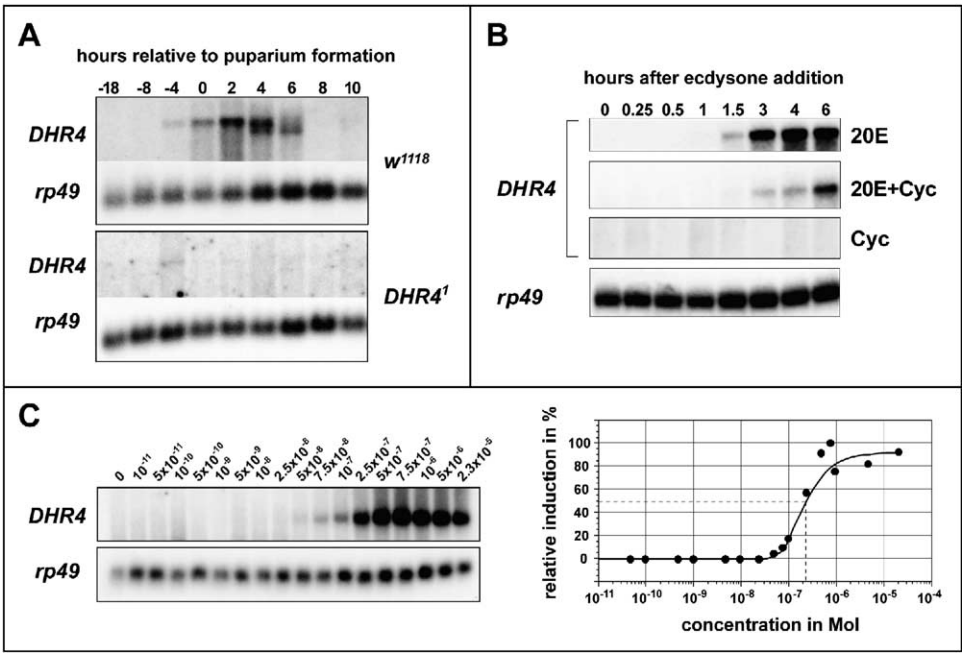

Figure 1. *DHR4* Is Induced Directly by Ecdysone as an Early-Late Response

(A) Temporal pattern of *DHR4* expression at the onset of metamorphosis. Total RNA isolated from staged w^{1118} control and $DHR4^{1}$ mutant animals was analyzed by Northern blot hybridization essentially as described (Karim and Thummel, 1991) to detect *DHR4* mRNA. The $DHR4^{1}$ mutant Northern blot was exposed ~10-fold longer than the wild-type blot to show low levels of *DHR4* expression.

(B) *DHR4* is induced directly by ecdysone. Mass-isolated late third-instar larval organs were maintained in culture and treated with either ecdysone (20E), cycloheximide (Cyc), or ecdysone and cycloheximide (20E + Cyc) for the periods of time shown. Total RNA was analyzed by Northern blot hybridization to detect *DHR4* mRNA.

(C) Ecdysone dose-response curve for *DHR4* transcription. Northern analysis of mass-isolated late third-instar larval organs maintained in culture and treated with different ecdysone concentrations, as shown, for 1.5 hr (Karim and Thummel, 1992). The autoradiograph was scanned, and intensities were adjusted relative to *rp49* and normalized to 100%. The dotted line indicates the half-maximal (50%) ecdysone dose response. 20E concentrations (M) are depicted on a $\log_{10}$ scale. *rp49* hybridization was used as a control for loading and transfer.

cit of *DHR4* mutant prepupae relative to controls (901 ± 133 μg compared to 1396 ± 125 μg in the controls, n = 80). Typically, the larger *DHR4* mutant prepupae have a relatively normal appearance (Figure 2A, second animal from left) but often fail to complete gut clearing, a process that normally precedes puparium formation (Figure 2D). Most of the smaller mutant prepupae display clear morphological defects, including a more larva-like shape, a failure to form the operculum, and defects in anterior spiracle eversion (Figure 2A, right two animals).

The defects caused by *DHR4* RNAi closely resemble those of $DHR4^{1}$ mutants (Figure 2B). By inducing RNAi at different stages, we were also able to observe additional later phenotypes. For instance, when L3 were heat treated at 18 hr and 10 hr before pupariation, ~5% of the pupae were cryptocephalic, failing to evert their head and arresting development several days later (Figure 2C, left animal). Of the animals that progressed to the pupal stage (n = 81), ~15% failed to progress beyond stage P10 (Figure 2C, right animal) (Bainbridge and Bownes, 1981). A small number of animals survived to late stages of metamorphosis, dying as either pharate adults or in the process of eclosion (data not shown). In addition to these developmental defects, expression of *DHR4* dsRNA by a single heat treatment in 0- to 3-hr-old L1 was sufficient to cause a significant growth defect, with 20% of the pupae developing to 60%–70% of wild-type length (n = 100), and a moderate reduction in size in 75% of the population (Figure 2B). In contrast to $DHR4^{1}$ mutants, many RNAi-induced small pupae continued development to form small, viable flies. This uncoupling of growth and developmental defects suggests a distinct earlier function for *DHR4* in growth during larval development.

Table 1. Lethal-Phase Analysis of $DHR4^{1}$ Mutants

Genotype	1st Instar	2nd Instar	3rd Instar	Prepupa	Pupa	N
$DHR4^{1}/Y$	4%	3%	5%	88%	N/A	384
P427	3%	3%	6%	4%	3%	300

Surviving animals were scored from populations of first-instar larvae of *P427* control and hemizygous $DHR4^{1}$ mutant males throughout development. N = total number of animals.

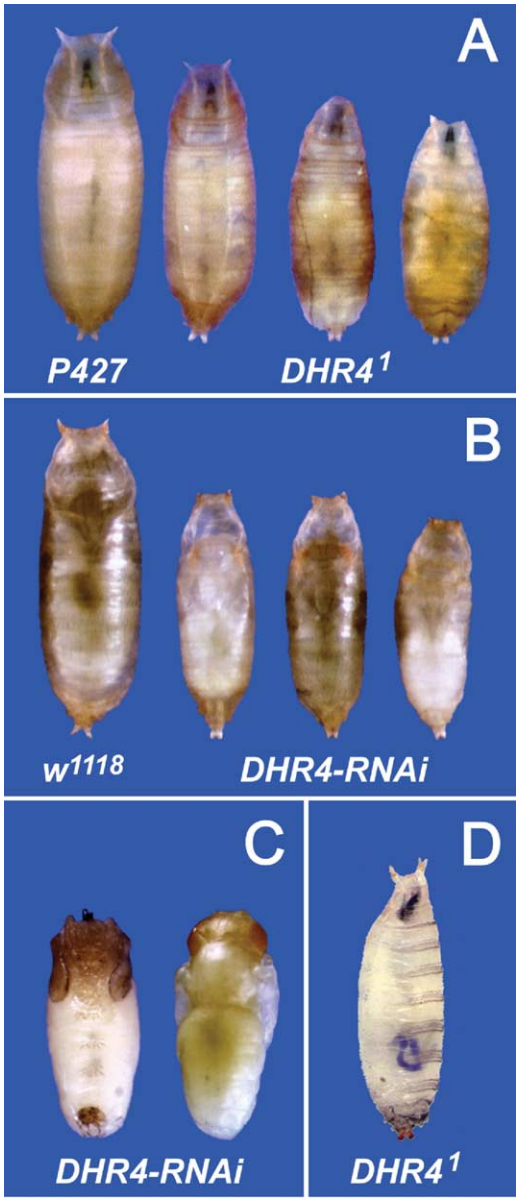

Figure 2. Disruption of *DHR4* Function Affects Metamorphosis and Growth

(A) *DHR4*[1] mutant phenotypes. From left to right: *P427* control prepupa; small, relatively normal *DHR4*[1] mutant prepupa; two smaller, abnormally shaped prepupae.

(B) *DHR4* RNAi growth and pupariation defects. Heat-treated *w*[1118] control pupa on left with three heat-treated *hsDHR4i* transformants that formed small, misshapen pupae.

(C) *DHR4* RNAi late lethal phenotypes. Left: cryptocephalic animal (head eversion defect, ventral view). Right: pupa arrested at stage P10 (dorsal view).

(D) Failed gut clearing in newly formed *DHR4*[1] prepupa.

DHR4 Protein Is Expressed in Larval and Neuroendocrine Tissues

In an effort to elucidate the roles of *DHR4* in growth and metamorphosis, antibodies were raised against the DHR4 protein and used to stain tissues dissected from staged larvae and prepupae (Figure 3). No DHR4 protein was detected during late L2 or L3 stages, except for strong constitutive expression in the ring gland (Figures 3A and 3B). It is likely that this expression accounts for the background level of *DHR4* mRNA detectable during larval stages by Northern blot hybridization (data not shown). Lower DHR4 expression could also be detected in specific neurons of the central nervous system (CNS) of mid and late L3 (Figure 3A). The ring gland stain was significantly reduced in larvae expressing *DHR4* dsRNA, confirming the specificity of this signal (Figure 3C). Results were more variable in *DHR4*[1] mutants, however, with some larvae showing highly reduced DHR4 protein in the ring gland and other animals showing little effect (data not shown). It is interesting to note that these effects on ring gland expression upon induction of RNAi or in *DHR4*[1] mutants correlate with the penetrance of growth defects in these genetic backgrounds, with highly penetrant effects upon RNAi and more variability in the *DHR4*[1] mutants, suggesting that the growth defects may arise from an endocrine function of DHR4. Close examination of the ring glands in wild-type animals reveals that DHR4 protein is most abundant in the cytoplasm of the prothoracic gland, with little or no protein in the nuclei of these cells or in the corpora allata or corpora cardiaca (Figures 3D and 3E). The high relative level of protein in the prothoracic cell cytoplasm, however, makes it impossible to determine whether DHR4 is absent from other regions of the ring gland.

The distribution of DHR4 protein expanded at pupariation formation to include the fat body and salivary glands (Figures 3F and 3H). DHR4 protein is located primarily in the nucleus of these tissues, consistent with its function as a transcription factor, although some DHR4 protein can also be detected in the cytoplasm of salivary gland and fat body cells (Figures 3F–3J). Tissues dissected from *DHR4*[1] mutant prepupae showed no nuclear signal, confirming the specificity of the antibody (Figure 3G). Little or no DHR4 protein was detected in the imaginal discs, muscle, and midgut of early prepupae (data not shown).

The spatial and temporal patterns of DHR4 expression provide a framework for interpreting our phenotypic studies, suggesting that the growth defects arise from neuroendocrine functions of *DHR4* during larval stages and the metamorphic requirement for *DHR4* arises from its ecdysone-induced expression at pupariation. Below, we examine these two regulatory functions of *DHR4*, focusing first on its roles in regulating gene expression at the onset of metamorphosis and then moving on to the mechanisms by which *DHR4* regulates growth during larval stages.

DHR4 Plays a Central Role in the Ecdysone Genetic Cascades

The temporal pattern of DHR4 expression and the prepupal lethality of *DHR4*[1] mutants suggest that this gene plays a critical role in ecdysone-regulated transcription at the onset of metamorphosis. To test this hypothesis, we analyzed staged mutant and control L3 and prepupae by Northern blot hybridization using probes to detect early (*EcR*, *E74*, *E75*), early-late (*DHR3*), or mid-prepupal (β*FTZ-F1*, *Imp-L1*) ecdysone-regulated gene

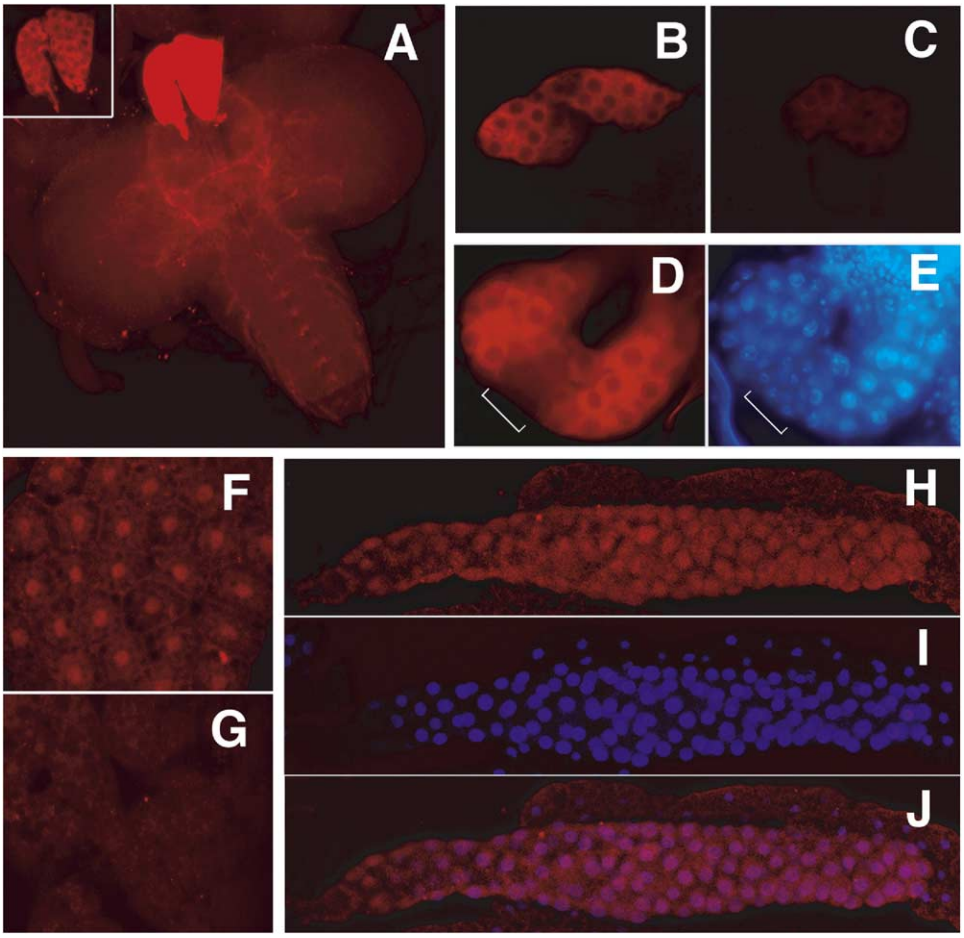

Figure 3. Spatial Patterns of DHR4 Expression

The spatial distribution of DHR4 protein expression was determined by antibody staining. DHR4 is expressed in the ring gland of a mid-third-instar larva ~18 hr prior to pupariation (A). Inset shows ring gland using 1/3 laser power used to detect low-level CNS expression in the main panel. Abundant DHR4 ring gland expression (B) is significantly reduced by RNAi (C). DHR4 protein is located primarily in the cytoplasm of prothoracic gland cells (D), as demonstrated by a DAPI stain of the nuclei (E). Corpora allata cells display little or no DHR4 protein ([D and E], brackets). DHR4 is expressed in the larval fat body (F) and salivary gland (H) of early prepupae and significantly reduced in a *DHR4[1]* mutant fat body from a 4 hr prepupa (G). DHR4 protein within and immediately surrounding larval salivary gland nuclei is revealed by nuclear staining with an anti-histone antibody (I) and an overlay with the antibody stain (J).

expression (Figure 4A). The *EcR*, *E74A*, *E75A*, and *E75B* early mRNAs are submaximally induced at puparium formation in *DHR4[1]* mutants, with *EcR*, *E74*, *E75A*, and *E75B* also failing to be repressed at the appropriate time. *DHR3* induction appears normal in *DHR4[1]* mutants; however, the repression of this gene is significantly impaired. β*FTZ-F1* expression is highly reduced in *DHR4[1]* mutant prepupae, with consequent defects in *E74A* and *E75A* induction in 10 hr prepupae, phenocopying a β*FTZ-F1* mutant (Broadus et al., 1999). *Imp-L1* expression, in contrast, accumulates to wild-type levels in *DHR4[1]* mutant prepupae, with an ~2 hr delay, demonstrating that the *DHR4[1]* mutation does not have a general effect on midprepupal gene expression. Similar effects on ecdysone-regulated gene expression are seen when *DHR4* function is disrupted by RNAi (data not shown).

As a further test of a role for *DHR4* in repressing early gene expression, we ectopically expressed *DHR4* in late L3 at the time when *EcR* and the classic early puff transcripts, *E74A* and *E75A*, are initially being induced by ecdysone (Ashburner et al., 1974) (Figure 4B). These transcripts are significantly downregulated under these conditions, resulting in almost complete suppression of the early transcriptional response to the hormone. Consistent with this effect on gene expression, most *hsDHR4* transformants subjected to an identical heat-treatment regime failed to initiate metamorphosis, dying as L3 (data not shown).

DHR4 Plays a Global Role in Repressing Ecdysone-Regulated Gene Expression

Microarray analysis of *DHR4[1]* mutant prepupae and heat-treated *hsDHR4* L3 was used to expand our understanding of *DHR4* function. Total RNA was isolated from *P427* control and *DHR4[1]* mutant prepupae staged at 0, 4, and 8 hr after pupariation, spanning the peak of *DHR4* expression (Figure 1A). Only *DHR4[1]* mutant pre-

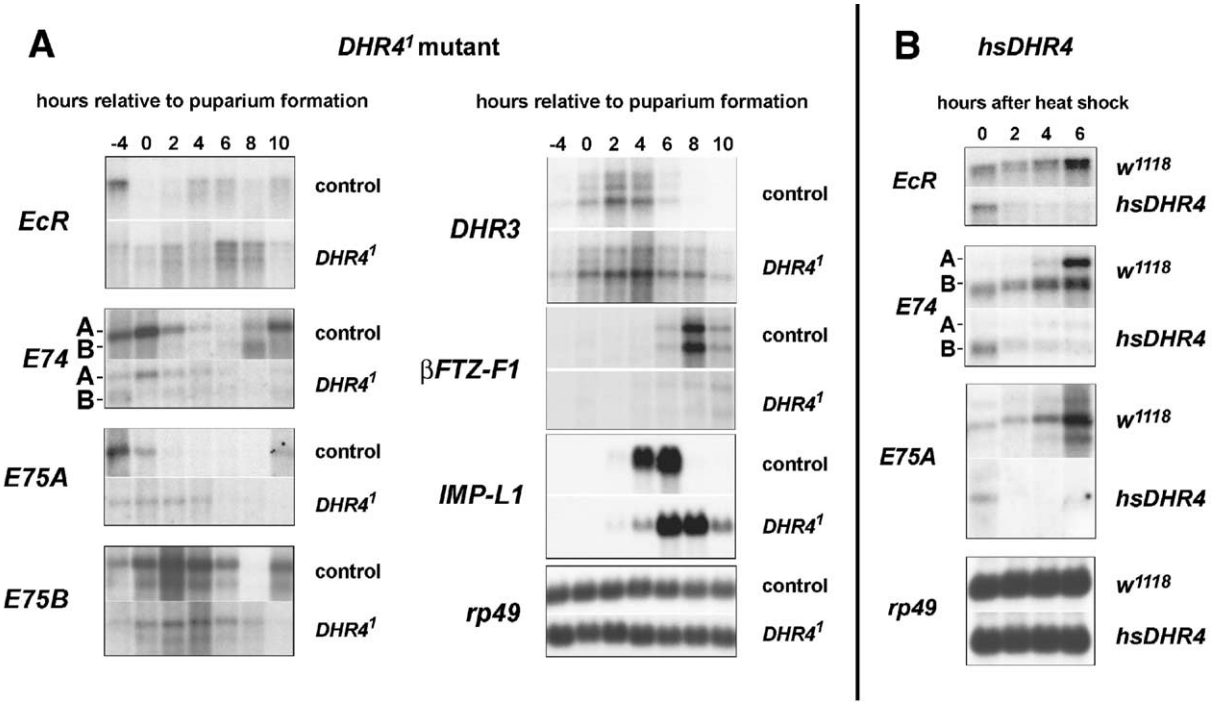

Figure 4. *DHR4* Plays a Central Role in the Ecdysone-Triggered Gene Cascades at the Onset of Metamorphosis

(A) *DHR4*[1] mutant Northern blot analysis. Total RNA from staged control and *DHR4*[1] mutant L3 or prepupae was analyzed by Northern blot hybridization to detect early (*EcR*, *E74A*, *E74B*, *E75A*, *E75B*), early-late (*DHR3*), or midprepupal (β*FTZ-F1*, *Imp-L1*) ecdysone-regulated gene expression and *rp49* as a control. Numbers at the top indicate hours relative to puparium formation. A and B indicate *E74* isoforms. Mutant and control blots were treated together to allow direct comparison. Controls are represented by RNA isolated from either w^{1118} (*EcR*, *DHR3*, β*FTZ-F1*) or *P427* (*E74*, *E75*, *Imp-L1*) animals.

(B) Ectopic *DHR4* expression is sufficient to repress early gene transcription. w^{1118} and *hsDHR4* third-instar larvae were heat treated ~10 hr prior to pupariation and collected 0, 2, 4, or 6 hr later. Northern analysis was used to detect *EcR* and two classic early puff transcripts, *E74A* and *E75A* (Karim and Thummel, 1992), as well as the *rp49* control.

pupae with a normal appearance were selected for this study (Figure 2A, second from left). We also performed a gain-of-function study using w^{1118} or w^{1118}*; hsDHR4* L3 that were heat treated at ~10 hr prior to puparium formation and harvested 6 hr later. RNA was purified from each set of animals, labeled, and hybridized to Affymetrix *Drosophila* genome arrays. We compared the resultant gene lists with two data sets that are enriched for ecdysone-regulated genes: genes that significantly change their level of expression between 0 and 4 hr after pupariation in *P427* control animals, a time when ecdysone is known to exert global effects on gene activity (White et al., 1999), and the only published microarray study of *EcR* mutants, using larval midguts (Li and White, 2003). These comparisons revealed a robust correlation between genes that are normally downregulated in wild-type early prepupae, or *EcR*-dependent genes that are downregulated in the midgut, and genes that are upregulated in *DHR4*[1] mutants, suggesting a central role for *DHR4* in the repression of ecdysone-regulated genes (see Supplemental Data).

DHR4[1] Mutant Larvae Prematurely Terminate Feeding and Display Early Fat Body Autophagy

The microarray analysis of *DHR4*[1] mutants also revealed a significant correlation with data derived from *Drosophila* larvae subjected to either complete or sucrose-diet-induced starvation, indicating that these animals are undergoing a starvation response (see Supplemental Data). This result, combined with the small size of *DHR4* mutants, led us to ask whether mutant larvae display defects in feeding behavior that might contribute to these phenotypes. *P427* control and *DHR4*[1] mutant L1 (0–12 hr after hatching) were transferred to yeast paste supplemented with bromophenol blue dye, following standard protocols to assay larval feeding (Maroni and Stamey, 1983; Zinke et al., 1999). Initially, all mutant and control larvae remained inside the yeast and rapidly attained dark-blue stained guts. No differences were apparent in either the size or morphology of wild-type and mutant animals at this stage. During the next 24–48 hr, however, ~25% of the mutant larvae (n = 597) were found outside the yeast, while wild-type larvae continued to feed until they initiated wandering behavior ~60–72 hr after hatching. The nonfeeding mutant class was comprised of L2 (5%) and L3 (95%); were smaller than wild-type wandering larvae; and displayed a roughly even distribution of dark-blue, partial-blue, or clear gut staining, suggesting that they stop feeding at the blue-gut stage and then progress to a clear-gut stage, depending on the time spent outside the food (Maroni and Stamey, 1983). *DHR4*[1] mutant larvae also initiate premature mobilization of stored nutrients through autophagy, as visualized by

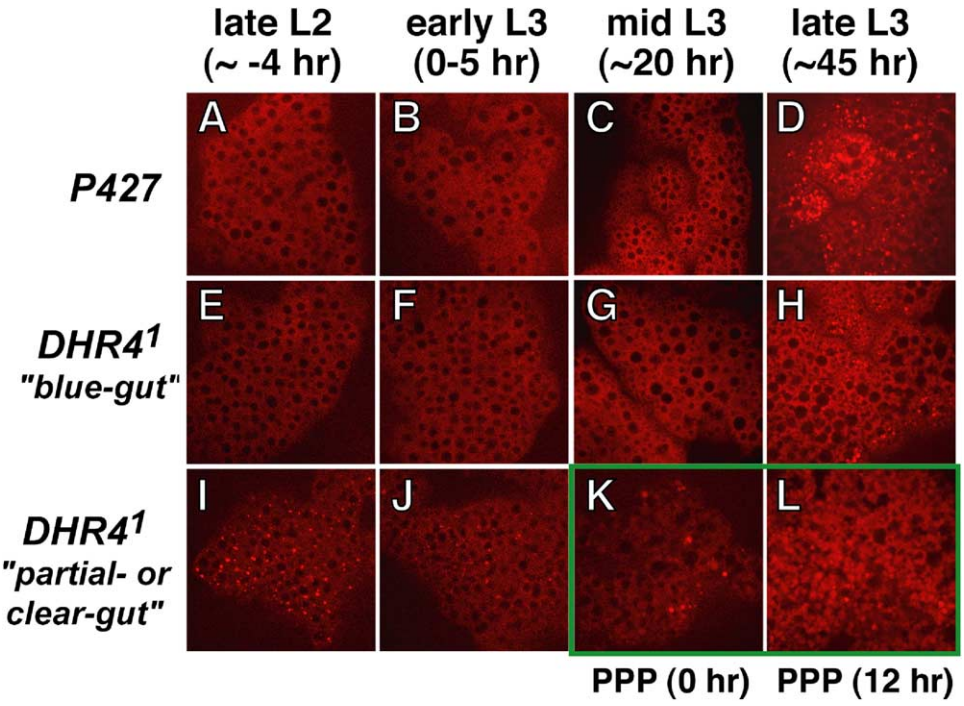

Figure 5. *DHR4¹* Mutants Display Premature Fat Body Autophagy

P427 control and *DHR4¹* mutant larvae were grown on yeast supplemented with 0.1% bromophenol blue for gut staging (Maroni and Stamey, 1983). Mutant nonfeeding larvae of submaximal size were separated into groups with similar gut color. Larvae were dissected and fat bodies were stained with Lysotracker Red essentially as described (Rusten et al., 2004), with larval stages indicated at top in approximate hours relative to the L2-to-L3 molt. Appearance of lysosomes in the fat body of *P427* control animals starts in late L3 (A–D). Most feeding and nonfeeding blue gut *DHR4¹* mutant animals show no difference to controls (E–H), whereas nonfeeding partial-blue or clear gut larvae consistently display positive Lysotracker staining in late L2 and early L3 stages (I and J). In (K), prematurely pupariating *DHR4¹* mutants (PPP) display fat body autophagy, while in (L), 12-hr-old PPP have less Lysotracker staining and an altered fat body morphology.

staining *P427* control and *DHR4¹* mutant larval fat bodies with Lysotracker Red (Rusten et al., 2004; Scott et al., 2004). As expected, no acidic vesicles were detected in *P427* larval fat bodies until shortly before puparium formation, ~45 hr after the L2-to-L3 molt (Figures 5A–5D), similar to wild-type wandering L3 (Rusten et al., 2004). A similar pattern was observed in most (6 of 8) normal-sized *DHR4¹* mutant feeding larvae (Figures 5E–5G) and all (7 of 7) normal-sized *DHR4¹* mutant wandering larvae (Figure 5H). In contrast, premature autophagy was observed in fat bodies from mutant late L2 and young L3 that displayed either partial-blue (4 of 6) or clear gut staining (7 of 7) (Figures 5I and 5J). These results demonstrate that *DHR4* function is required for the normal maintenance of feeding behavior and the normal timing of fat body autophagy.

DHR4¹ Mutant Larvae that Terminate Feeding Early Display Premature Pupariation

The early cessation of feeding in *DHR4¹* mutant larvae could arise from several behavioral patterns, including food aversion, abnormal foraging (Sokolowski, 2003), or premature wandering behavior that would culminate in puparium formation (Riddiford, 1993). To distinguish between these possibilities, we set out to determine if the early onset of wandering we observed in *DHR4* mutants was associated with premature entry into metamorphosis. *P427* and *DHR4¹* mutant larvae were staged relative to the L2-to-L3 molt, and newly formed prepupae were scored every hour. As expected, *P427* animals displayed a tight window of pupariation between 48 and 51 hr after the molt, similar to wild-type animals (Figure 6A). In contrast, a virtually linear correlation was observed between time and pupariation of *DHR4¹* larvae, with the first emergence of *DHR4¹* prepupae as early as 28 hr after the L2-to-L3 molt, ~1 day earlier than wild-type (Figure 6A and data not shown). The proportion of larvae that form premature prepupae can vary from one experiment to another, within a range of ~20%–70% of the population. All animals that form premature prepupae are identical to the class of *DHR4¹* mutants that are small, exhibit a more larva-like shape, and only rarely evert their anterior spiracles (Figure 2A, right two animals). They contain acidic autophagic vesicles in their fat body (Figures 5K and 5L), similar to normal wild-type prepupae (Rusten et al., 2004). In addition, premature prepupae display changes in ecdysone-regulated gene expression that are diagnostic of entry into metamorphosis (Figure S4). To test whether prematurely pupariating *DHR4¹* mutants originate from larvae that precociously leave the food, we separated feeding from nonfeeding larvae and followed their respective developmental fates. In the class of prepupae that originated from nonfeeding larvae (n = 73), 34% formed small, abnormal prepupae characteristic of premature pupariation, while only 3.8% of the prepupae

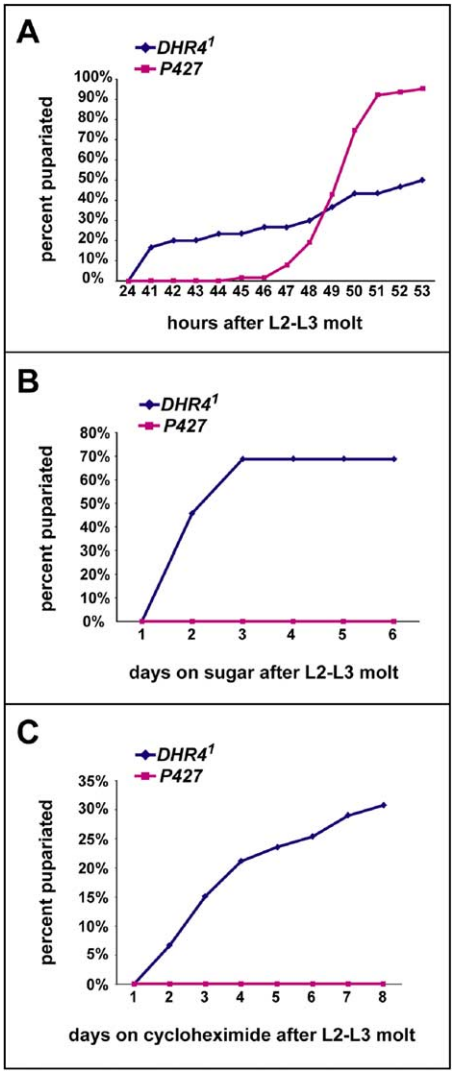

Figure 6. *DHR4¹* Mutants Pupariate Prematurely and under Conditions that Inhibit Growth

(A) *DHR4* mutants pupariate prematurely. *P427* controls (n = 60) and *DHR4¹* mutants (n = 30) were staged within 0–4 hr after the L2-to-L3 molt, and the number of prepupae was scored every hour. The results of one such experiment are shown.

(B) *DHR4¹* mutant early L3, but not *P427* controls, pupariate under starvation conditions. *P427* control (n = 54) and *DHR4¹* mutant L3 (n = 78) were staged within 0–5 hr after the molt and maintained for 12 hr at 25°C. Larvae were then transferred to petri dishes containing Whatman paper soaked in 20% sucrose/PBS, and prepupae/pupae were scored every 24 hr.

(C) *DHR4¹* mutant L3, but not *P427* controls, pupariate when provided with food supplemented with cycloheximide. Cycloheximide treatment was performed as described above, except that *P427* control (n = 151) and *DHR4¹* mutant L3 (n = 166) larvae were transferred to instant fly food (Carolina Biological) containing 500 μg/ml cycloheximide (Sigma).

that originated from feeding larvae (n = 342) displayed this phenotype. It is likely that the few premature prepupae in the latter group were derived from undetected nonfeeding larvae. We conclude that at least some of the early *DHR4* mutant L3 that leave the food are wandering in preparation for premature pupariation.

DHR4¹ Larvae Pupariate under Growth-Restricting Conditions

The timing of pupariation is linked to growth through the attainment of critical weight, which must be achieved before larvae can undergo adult differentiation in response to ecdysone (Nijhout and Williams, 1974b; Davidowitz et al., 2003). *Drosophila* larvae that have achieved critical weight will pupariate when completely starved; however, when transferred to a sugar-only diet that blocks further growth but allows for energy metabolism and thus survival, animals retain their larval identity and fail to pupariate (Beadle et al., 1938; Britton and Edgar, 1998; Zinke et al., 1999). We thus maintained *DHR4¹* mutant L3 on sucrose as a means of assessing whether these animals have achieved critical weight and can respond properly to a sugar-only diet. *DHR4¹* mutant and *P427* control L3 were selected at 12–17 hr after the molt and transferred to 20% sucrose medium. As expected, *P427* larvae remained arrested as L3 throughout the 6 day time course (Figure 6B). Remarkably, however, *DHR4¹* mutant larvae started to form small and misshapen prepupae within 48 hr after the molt, with 70% of the mutants pupariating within three days (Figure 6B). These L3 thus behaved as though they were completely starved rather than maintained on a sugar-only diet. To test if the *DHR4¹* mutant larvae were simply not feeding in spite of the presence of sucrose, we carried out a similar experiment using the protein synthesis inhibitor cycloheximide. Growth of wild-type *Drosophila* larvae on food supplemented with cycloheximide effectively blocks mitosis and prevents growth and pupariation (Britton and Edgar, 1998). These effects can be reversed after as long as 12 days by transferring animals back to normal food, demonstrating that the drug itself is not toxic (Britton and Edgar, 1998). Once again, control *P427* L3 arrested development as expected on cycloheximide-containing medium, while ~30% of *DHR4¹* mutant larvae formed small but normal-shaped prepupae in an almost linear manner during the 8 day time course (Figure 6C). These experiments indicate that some *DHR4¹* mutant L3 stop feeding prematurely. In addition, a significant number of mutant larvae appear to have achieved critical weight since they are capable of pupariating under starvation conditions.

Discussion

DHR4 Is a Central Regulator of Genetic Responses to Ecdysone at the Onset of Metamorphosis

Since the original proposal by Ashburner and colleagues (1974) of the hierarchical model of ecdysone action, efforts have focused on identifying the postulated ecdysone-induced repressor of the early regulatory genes. We propose that *DHR4* is at least one of these unknown factors (Figure 7). Not only do we observe efficient early gene repression upon ectopic *DHR4* expression in late L3 (Figure 4B), but we also see prolonged expression of *EcR*, *E74*, *E75*, and *DHR3* in *DHR4* mutant prepupae (Figure 4A). Like DHR4, DHR3 is sufficient for early gene repression upon ectopic expression in late L3 (Lam et al., 1997; White et al., 1997); however, DHR3 is not necessary for this response (Lam

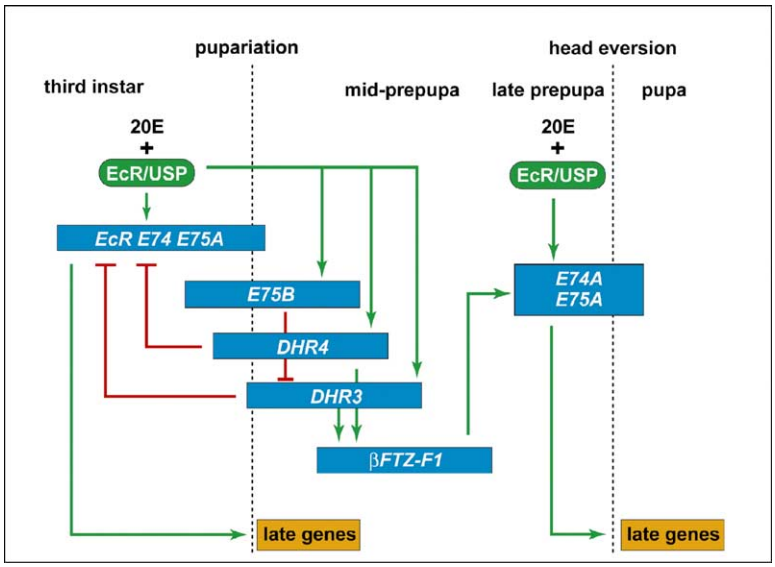

Figure 7. Model for *DHR4* Function at the Onset of Metamorphosis

This figure summarizes regulatory interactions described in the text. The green ovals represent the EcR/USP ecdysone receptor heterodimer, blue boxes represent genes that encode ecdysone-regulated transcription factors, and orange boxes represent secondary-response late genes. The length of the boxes represents the approximate duration of expression. Green arrows represent inductive effects, and red lines represent repressive effects based on genetic studies. The major developmental transitions and stages are listed on top.

et al., 1999), suggesting that DHR3 and DHR4 act together, in a redundant manner, to direct early gene repression (Figure 7).

A broad survey of *DHR4* regulatory targets using microarray analysis demonstrates a wider role for this factor in gene repression at puparium formation (see Supplemental Data). Among these *DHR4* targets are known ecdysone-regulated genes such as *Lsp1γ*, *Ddc*, and *Kr-h1* as well as 13 ecdysone-regulated muscle genes identified in an earlier microarray study as coordinately downregulated at pupariation (White et al., 1999) (Table S2). We conclude that *DHR4* function is not limited to early gene repression but, rather, acts more widely to downregulate gene expression at the onset of metamorphosis.

Metamorphic functions for *DHR4* are not restricted to puparium formation but also extend to prepupal stages through its essential role in β*FTZ-F1* regulation. Like *DHR3*, *DHR4* is required for maximal expression of this midprepupal competence factor (Figure 7). The effects seen on *EcR*, *E74*, and *E75* transcription in 10 hr *DHR4* mutant prepupae (Figure 4A) and the lethal phenotypes associated with *DHR4* RNAi (Figure 2C) are indistinguishable from those seen in β*FTZ-F1* mutants (Broadus et al., 1999), suggesting that most if not all of the effects of *DHR4* are channeled through β*FTZ-F1* at this stage in development. Thus, as originally proposed based on the timing of *DHR4* expression (Charles et al., 1999; Hiruma and Riddiford, 2001; Sullivan and Thummel, 2003), this factor contributes to the crossregulatory interactions among orphan nuclear receptors in prepupae. Together with *DHR3* and through β*FTZ-F1*, *DHR4* directs appropriate genetic and biological responses to the prepupal pulse of ecdysone, ensuring that this response will be distinct from that induced by the hormone several hours earlier at pupariation (Figure 7).

DHR4 Mutants Display Premature Cessation of Feeding and Larval Wandering

Despite an abundance of food, many *DHR4*^{1} mutants stop feeding prematurely as early L3. Three lines of evidence indicate that most of these animals do not resume feeding. First, once larvae have left the food and initiated gut clearing, they typically do not return to the food source. They will either pupariate or die as small larvae, likely reflecting whether or not they have achieved the critical weight necessary for pupariation. Second, unlike their control counterparts, *DHR4*^{1} mutant L3 can pupariate successfully despite being reared on cycloheximide-containing food (Figure 6C). Although it is possible that *DHR4*^{1} mutants have gained the ability to inactivate this drug, we favor the interpretation that they have stopped feeding altogether. Finally, in a similar experiment, rather than arresting development like wild-type L3, *DHR4*^{1} mutant L3 maintained on a sugar-only diet pupariate as if they were experiencing complete starvation in the absence of sugar (Figure 6B) (Beadle et al., 1938; Zinke et al., 1999). We conclude that *DHR4* is required for the proper duration of larval feeding. Moreover, defects in feeding behavior are likely to extend to all *DHR4* mutant animals because even those larvae that pupariate at the normal time display changes in gene expression indicative of starvation.

Drosophila larvae display two basic forms of behavior, either foraging for food or wandering in search of a substrate for pupariation (Sokolowski, 2003). Foraging for food is intermittent, with animals continuing to feed, while wandering larvae stop feeding, purge their gut contents, display fat body autophagy, and eventually pupariate (Riddiford, 1993; Sokolowski, 2003; Rusten et al., 2004). The late-larval behavioral patterns and increased autophagy associated with wandering are similar to the phenotypes observed in *DHR4*^{1} mutant L3, suggesting that these animals prematurely receive the cue to wander. In support of this proposal, we find that precociously nonfeeding *DHR4*^{1} mutant larvae also pupariate prematurely, while their feeding counterparts do not. We conclude that *DHR4* is required for the correct timing of the signal that triggers the cessation of feeding and the onset of wandering behavior.

DHR4 Appears to Be Required for Appropriate Responses to the Attainment of Critical Weight

As has been emphasized in several reviews (Stern and Emlen, 1999; Nijhout, 2003; Stern, 2003), the control of insect body size is not simply a matter of growth control via insulin signaling and nutrition but, rather, includes a more profound determinant of size, the decision of when to stop growing and initiate maturation. This decision depends on the attainment of critical weight, which is defined as the minimum weight needed to successfully initiate a normal time course to pupariation (Nijhout and Williams, 1974b; Davidowitz et al., 2003; Nijhout, 2003; Stern, 2003). To date, no genetic studies have addressed this level of growth control. Our characterization of *DHR4* mutants implicates a critical role for this orphan nuclear receptor in assessing critical weight and determining the duration of larval development. *DHR4* mutants begin to pupariate ~28 hr after the L2-to-L3 molt, almost a day earlier than wild-type animals. They pupariate at random times, independent of their state of gut clearing, with variable wandering phases. Premature *DHR4* mutant prepupae are small (Figure 2A), display fat body autophagy like normal prepupae (Figures 5K and 5L), and express ecdysone-regulated genes specific for the prepupal stage in development although they are the chronological age of third-instar larvae (Figure S4).

Studies in *Manduca* and other insects have shown that the attainment of critical weight triggers a drop in juvenile hormone (JH) titer, leading to release of the neuropeptide prothoracicotropic hormone (PTTH) and subsequent release of ecdysone from the ring gland. The resulting ecdysone pulse, in turn, initiates wandering behavior, with a subsequent high-titer ecdysone pulse triggering pupariation and maturation (Nijhout and Williams, 1974a; Dominick and Truman, 1985). Interestingly, removal of the *Manduca* corpora allata—the gland that produces JH—leads to premature activation of this endocrine cascade, resulting in precocious pupariation and the formation of small adults. Similarly, if JH levels are artificially increased by injecting hormone, PTTH secretion is delayed, and a prolonged larval feeding phase results in the formation of giant adults (Nijhout and Williams, 1974a; Rountree and Bollenbacher, 1986). These observations implicate a central role for JH in determining the duration of larval development and adult body size. Moreover, they provide a critical endocrinological link between growth and maturation and thus a point at which *DHR4* could potentially intervene.

The proposal that *DHR4* plays a central role in transducing responses to the attainment of critical weight fits with its temporal and spatial patterns of expression. DHR4 is expressed in the ring gland of late L2 and throughout L3, with a lower level and complex pattern of expression in the L3 CNS (Figure 3A). Curiously, DHR4 is most abundant in the cytoplasm of the prothoracic gland and is low or absent in the corpora allata, which produces JH (Figures 3D and 3E). Although we did not observe nuclear localization or clear corpora allata expression at any of the time points examined, we cannot exclude low levels of expression or expression within a tight temporal window. Moreover, the variable levels of cytoplasmic DHR4 that we observed in the larval fat body and salivary glands at later stages of development suggest that nuclear/cytoplasmic shuttling may contribute to DHR4 regulatory function (Figures 3F and 3H and data not shown). Future tissue-specific rescue experiments should help to clarify the critical neuroendocrine cell types that may confer *DHR4* function. Clearly, an intriguing possibility is that *DHR4* either directly or indirectly regulates JH levels or JH signal transduction, contributing to the critical decrease in JH signaling that sets the endocrine cascade in action. Alternatively, *DHR4* could regulate the neuroendocrine signaling that leads to ecdysone release. Identification of the *Drosophila PTTH* gene would provide a critical step toward testing this possibility. The fact that *DHR4* encodes an orphan nuclear receptor, and thus may be modulated by binding one or more small lipophilic compounds, provides the most direct means of integrating this transcription factor into an endocrine circuit that defines the end of larval growth and the timing of pupariation.

The studies described above provide a genetic and molecular framework for understanding critical weight and the coordination of larval feeding and pupariation—key aspects of insect growth control that, in the past, have only been approached through physiological studies in nongenetic organisms. Future studies of *DHR4* should provide new insights into the mechanisms by which growth is coupled to maturation, a key aspect of postembryonic development in all higher organisms.

Experimental Procedures

Generation of a *DHR4* Mutant

The *P{EP}EP427* insertion, hereafter referred to as *P427*, is located 7486 bp downstream of the *DHR4* stop codon (GenBank accession number AY971884). We confirmed its location by PCR using P element primer pP (5′-CGACGGGACCACCTTATGTTATTTCATCATG) in combination with primers p+240 (5′-GTTGCCACGCTCTTCATCG GTCTCCTC) or p–680 (5′-GAGTATCGACTGCTCGATAGCACGCAG). To mobilize *P427*, we crossed *P427/Y* males to *w*; ry^{506}*, *Sb^{1}*, *P{Δ2–3}99B/TM6*, *Tb^{1}* virgin females carrying the *Δ2–3* transposase. Virgin dysgenic F1 females of genotype *w*/P427; ry^{506}*, *Sb^{1}*, *P{Δ2–3}99B/+* were then crossed to *FM7h* males (a kind gift of P. Heitzler). Candidate lines were established from single virgin females from this cross and balanced over *FM7h*. Potential insertions in the 5′-flanking region of *DHR4* were identified by PCR using the pP primer (see above) with primer F1 (5′-CCTCTTCCCCTTCTCGATTGCAC TGCG) or R1 (5′-CTCCATATTAAGGGCGTGCGGTGTGCG), which are located at positions 229598 and 234671, respectively, in the AE003422 contig. Of ~1000 independent lines tested, we obtained a single insertion that lies 5′ of the *DHR4* ORF, creating the *DHR4^{1}* mutant allele. This new *P{EP}* insertion is located between positions 230227 and 230228 of contig AE003422, 1870 bp upstream of the putative start codon of *DHR4*. Further PCR and genomic Southern blot analyses revealed that the original *P427* element is still present in this line, although its flanking DNA remained unchanged (data not shown). Transposase-mediated precise excision of the *DHR4^{1} P{EP}* insertion restored viability in all cases examined, indicating that it is this P element insertion that is the cause of lethality (data not shown).

RNA Interference

Two fragments of 1 kb and 1.1 kb were amplified from a *DHR4* cDNA template using primer F4Xbai2 (5′-GTTCGTCTAGAGACC GACAGATCTCGTACGAGCACGCC) with either primer R4BSPe1 (5′-TTGCTGTCCGGAGCCGCTCCGGGATCGTATCAGGACCAGG) or R4BSPe2 (5′-TCTTCAAAATGGACGTGGATGCGGCTGAGG). Both PCR

fragments were cut with BspEI, ligated to each other and inserted into the XbaI site of *pCasper-hs-act*. Three independent lines of transgenic flies were generated by P element-mediated transformation, and one line, *P{hsDHR4i$^{60/3}$}*, was selected to use for inactivating *DHR4* function. Control and *P{hsDHR4i$^{60/3}$}* larvae were maintained on standard cornmeal-agar food supplemented with 0.05% bromophenol blue, and the vials were incubated in a water bath for 60 min at 37.5°C to induce RNAi when sufficient wandering larvae were present. After a 1 hr recovery, larvae that displayed partial-blue gut staining were transferred to yeast and scored the following days for phenotypes (Andres et al., 1993). Northern analysis revealed that the levels of *DHR4* mRNA are significantly reduced in heat-treated *hsDHR4i* animals (data not shown).

Antibody Stains

An 890 bp SacI-XhoI fragment encoding the DHR4 ligand binding domain was excised from the cDNA clone NB5 and inserted into pET24c (Novagen) in frame with the coding region for a 6X Histidine tag, resulting in a fusion protein carrying 299 amino acids of DHR4. Purified His-DHR4 protein was resolved by preparative SDS-PAGE, excised from the gel, and injected into three rabbits (Covance), and antisera was screened by Western blotting. Antiserum was affinity purified as described (Carroll and Laughon, 1987), using a 304 amino acid region of DHR4 (coding sequences were amplified using primers F4ABgl [5′-GCTGGTGGAAGATCTCAACTGCCGC CGC] and R4AXho [5′-GCTCTCGAGCAGCAGGCTAGCCGCAGC]) that corresponded to the original antigen but was flanked by maltose binding protein on the N terminus and β-galactosidase on the C terminus. Animals were dissected and stained with affinity-purified anti-DHR4 antibody at a dilution of 1:50, using 1:200 Cy3-labeled anti-rabbit secondary antibodies (Jackson). Nuclei were detected by either DAPI staining or staining with a 1:1000 dilution of mouse anti-histone antibody (MAB052, Chemicon) and 1:200 Cy2-labeled anti-mouse secondary antibodies (Jackson). The stains were imaged on a Bio-Rad MRC1024 confocal laser scanning microscope. To test the specificity of ring gland staining, *P{hsDHR4i$^{60/3}$}* larvae were heat treated at 37°C for 60 min 1 day prior to dissection. Confocal imaging of these samples was done under identical conditions to allow a direct comparison of the data.

Microarray Analysis

Total RNA was isolated from staged animals using Trizol (Gibco) and purified on RNAeasy columns (Qiagen). All samples were prepared in triplicate to facilitate subsequent statistical analysis. Probe labeling, hybridization to Affymetrix GeneChip *Drosophila* Genome Arrays, and scanning were performed by the University of Maryland Biotechnology Institute Microarray Core Facility. Raw data were normalized using dChip1.2 (Li and Wong, 2001a; Li and Wong, 2001b) to the array with the median background intensity of the data sets being analyzed. Replicate arrays were pooled and single outliers were detected. Expression analysis using dChip was based on the PM-only model, and expression-level differences between control and experimental were analyzed by assigning a fold-change value, a confidence interval, and a paired t test p value for each probe set. Data sets were filtered with Microsoft Access for a minimum fold change of $f \geq 1.5$, a minimum difference in absolute expression values of $\Delta E \geq 50$, and a minimum lower confidence threshold of $LB \geq 1$. Comparisons between different microarray data sets were performed using Microsoft Access.

Supplemental Data

Supplemental Data include supplemental text, Supplemental References, four figures, and five tables and are available with this article online at http://www.cell.com/cgi/content/full/121/5/773/DC1/.

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