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The *Drosophila* nuclear receptors DHR3 and βFTZ-F1 control overlapping developmental responses in late embryos

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SUMMARY

Studies of the onset of metamorphosis have identified an ecdysone-triggered transcriptional cascade that consists of the sequential expression of the transcription-factor-encoding genes *DHR3*, β*FTZ-F1*, *E74A* and *E75A*. Although the regulatory interactions between these genes have been well characterized by genetic and molecular studies over the past 20 years, their developmental functions have remained more poorly understood. In addition, a transcriptional sequence similar to that observed in prepupae is repeated before each developmental transition in the life cycle, including mid-embryogenesis and the larval molts. Whether the regulatory interactions between *DHR3*, β*FTZ-F1*, *E74A* and *E75A* at these earlier stages are similar to those defined at the onset of metamorphosis, however, is unknown. In this study, we turn to embryonic development to address these two issues. We show that mid-embryonic expression of *DHR3* and β*FTZ-F1* is part of a 20-hydroxyecdysone (20E)-triggered transcriptional cascade similar to that seen in mid-prepupae, directing maximal expression of *E74A* and *E75A* during late embryogenesis. In addition, *DHR3* and β*FTZ-F1* exert overlapping developmental functions at the end of embryogenesis. Both genes are required for tracheal air filling, whereas *DHR3* is required for ventral nerve cord condensation and β*FTZ-F1* is required for proper maturation of the cuticular denticles. Rescue experiments support these observations, indicating that *DHR3* has essential functions independent from those of β*FTZ-F1*. *DHR3* and β*FTZ-F1* also contribute to overlapping transcriptional responses during embryogenesis. Taken together, these studies define the lethal phenotypes of *DHR3* and β*FTZ-F1* mutants, and provide evidence for functional bifurcation in the 20E-responsive transcriptional cascade.

KEY WORDS: Nuclear receptor signaling, Ecdysone, Transcriptional cascade, Embryogenesis

INTRODUCTION

Animals develop through a series of distinct life stages separated by developmental transitions, including birth and sexual maturation. In *Drosophila*, pulses of the steroid hormone 20-hydroxyecdysone (20E) direct each of the major developmental transitions in the life cycle, including molting and metamorphosis. Each pulse of 20E is followed by a stereotypic sequence of 20E-responsive transcription factors. The regulatory interactions between these transcription factors have been elucidated through detailed studies of the onset of metamorphosis, when the late larval 20E pulse triggers pupariation (Fig. 1A, reviewed in Henrich et al., 1999; Riddiford et al., 2001; Thummel, 2001). The 20E signal, acting through its EcR/USP receptor, directly induces *E74A* and *E75A* transcription, and upregulates the early-late gene *DHR3* synergistically with 20E-induced protein synthesis. This additional level of regulation leads to *DHR3* accumulation at later times than transcription of direct primary-response genes such as *E74A* and *E75A* (Horner et al., 1995). *DHR3* then directly induces β*FTZ-F1* expression in mid-prepupae (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997). β*FTZ-F1*, in turn, functions as a competence factor for genetic responses to the prepupal 20E pulse, which occurs ~10 hours after puparium formation, directing adult head eversion and the prepupal-pupal transition (Woodard et al., 1994; Broadus et al., 1999; Yamada et al., 2000). These responses include reinduction of *E74A* and *E75A*, as well as stage-specific expression of the 20E primary-response gene *E93* (Thummel, 2001). These 20E-regulated genes are all widely expressed, suggesting that their primary function is to transduce temporal information provided by the sequential pulses of 20E. *E74A* encodes an ETS-domain transcription factor, whereas *EcR*, *usp*, *E75A*, *DHR3* and β*FTZ-F1* all encode members of the nuclear receptor superfamily. Nuclear receptors are defined by a zinc-finger DNA-binding domain and a C-terminal ligand-binding domain that can interact with small lipophilic compounds. Within this group of receptors, however, only EcR and E75A have known ligands (20E and heme/monoatomic gases, respectively; Koelle et al., 1991; Reinking et al., 2005).

Although the regulatory interactions between *DHR3*, β*FTZ-F1*, *E74A* and *E75A* at the onset of metamorphosis have been well defined, their individual developmental functions remain less clear. One model postulates that a central role for this transcriptional cascade is to provide an appropriate delay in β*FTZ-F1* expression, enabling it to regulate responses to the subsequent prepupal pulse of 20E (White et al., 1997; Lam et al., 1999). An alternative model is that the sequential expression of *DHR3* and β*FTZ-F1* directs successive temporal programs of gene expression that specify appropriate progression through prepupal development, similar to the genetic network that controls patterning of the *Drosophila* anteroposterior axis in early embryos (Arnone and Davidson, 1997). The similarity between *EcR*, *DHR3* and β*FTZ-F1* mutant phenotypes during metamorphosis, which include lethality at the prepupal-pupal transition with defects in head eversion and gas bubble translocation, supports the first model (Bender et al., 1997; Lam et al., 1997; Yamada et al., 2000). This interpretation, however, is complicated by the use of hypomorphic alleles or rescuing constructs that are required to overcome the early lethality associated with null mutations in *EcR*, *DHR3* and β*FTZ-F1*. Complete loss of function in any of these genes results in lethality

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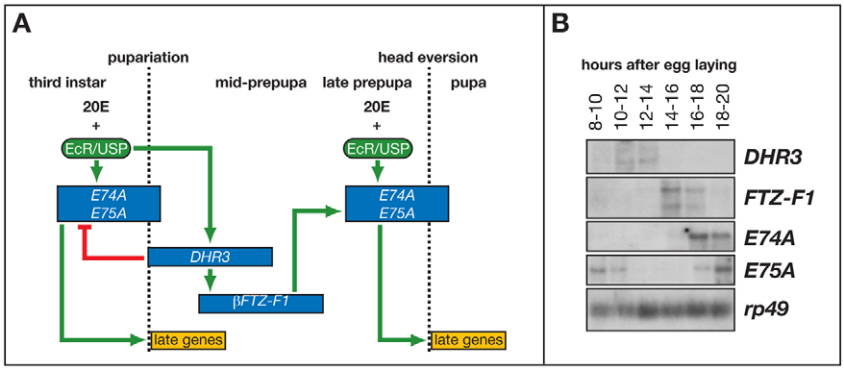

Fig. 1. A stereotypic transcriptional cascade occurs during metamorphosis and embryogenesis. (**A**) A schematic representation of the 20E-triggered regulatory interactions at the onset of *Drosophila* metamorphosis is depicted, adapted from Thummel (Thummel, 2001). The green ovals represent the EcR/USP 20E receptor heterodimer, blue boxes represent genes that encode 20E-regulated transcription factors and orange boxes represent secondary-response target genes. Green arrows represent inductive effects and red lines represent repressive effects. (**B**) Temporal profiles of transcription factor gene expression during embryogenesis. Total RNA from staged w^{1118} embryos was analyzed by northern blot hybridization to detect expression of the 20E-regulated genes *DHR3*, β*FTZ-F1*, *E75A* and *E74A*. *rp49* was used as a control for loading and transfer.

during embryogenesis (Bender et al., 1997; Carney et al., 1997; Yamada et al., 2000). Moreover, *DHR3*, β*FTZ-F1*, *E74A* and *E75A* are sequentially expressed during mid-embryogenesis in a pattern that is essentially identical to that seen in prepupae, starting a few hours after a high titer 20E pulse that occurs approximately 8 hours after egg laying (AEL) (Maroy et al., 1988; Sullivan and Thummel, 2003). These observations suggest that investigating the functions of *DHR3* and β*FTZ-F1* during embryonic development might help to discriminate between these two proposed models, and will provide further insights into the regulation and function of this transcriptional cascade during development.

Null mutations in the genes that encode components of the 20E biosynthetic pathway result in embryonic lethality, with defects in head involution, dorsal closure, gut development and cuticle deposition (Chavez et al., 2000; Ono et al., 2006). Inactivating *EcR* function leads to similar embryonic lethal phenotypes, although with earlier lethality and more severe defects, including aberrant germ band retraction (Bender et al., 1997; Kozlova and Thummel, 2003). Null mutations in either *DHR3* or β*FTZ-F1* also lead to fully penetrant embryonic lethality, although their developmental phenotypes are less well defined. *DHR3* null mutants die at the end of embryogenesis with relatively minor developmental defects (Carney et al., 1997). Approximately 50% of the embryos contain peripheral nervous system (PNS) defects, including loss of neurons, pathfinding and fasciculation defects, cluster organization defects, and displacement of neurons to inappropriate positions within the PNS. *DHR3* mutants also display a low frequency of defects in muscle patterning (5-15%). These defects, however, are unlikely to account for the embryonic lethality of *DHR3* mutants, as they are only partially penetrant and other mutants with similar phenotypes die later during development (Kolodkin et al., 1993). The *FTZ-F1* locus encodes two protein isoforms, α and β, which share a common C-terminal region with both a DNA-binding and ligand-binding domain (Lavorgna et al., 1991). α*FTZ-F1* is maternally deposited and functions as a cofactor for the *fushi tarazu* (*ftz*) segmentation gene (Guichet et al., 1997; Yu et al., 1997). Consistent with this, *FTZ-F1* maternal mutants die early during embryogenesis, with segmentation defects that resemble those of *ftz* mutants. In contrast, the β*FTZ-F1* isoform is zygotically expressed during late embryonic, larval and pupal stages. β*FTZ-F1* zygotic null mutants die during embryogenesis, with defects that remain to be characterized (Yamada et al., 2000).

We show that the regulatory interactions between *DHR3*, β*FTZ-F1*, *E74A* and *E75A* during embryogenesis are identical to those defined at the onset of metamorphosis. In addition, *DHR3* and β*FTZ-F1* null mutants not only exhibit common phenotypes, including a failure to fill their tracheal system with air, but also display gene-specific effects, with β*FTZ-F1* controlling the size and pigmentation of denticles, and *DHR3* directing proper ventral nerve cord (VNC) condensation. A microarray analysis of *DHR3* mutant embryos provides a molecular context for these phenotypic effects, revealing genes that are regulated by either *DHR3* and β*FTZ-F1*, or by both receptors together. This study defines the lethal phenotypes of *DHR3* and β*FTZ-F1* mutants, and provides evidence for functional bifurcation in the 20E-responsive transcriptional cascade.

MATERIALS AND METHODS

Fly strains

Fly stocks were maintained at 18-25°C on standard cornmeal-agar-yeast food. The w^{1118} strain was used as a control unless noted otherwise. dib^{F8} is a null allele described in Chavez et al. (Chavez et al., 2000). Two *DHR3* null alleles were used in this study: w^{-}*; DHR3*^{G60S} and w^{-}*; DHR3*$^{22\text{-}35}$ (Carney et al., 1997). *DHR3*^{G60S} encodes a protein with a single amino acid change at a highly conserved position within the DNA-binding domain, whereas *DHR3*$^{22\text{-}35}$ has a rearrangement that involves the exon that encodes the DNA-binding domain. Mutant phenotypes were examined in animals carrying a *DHR3* mutation in combination with *Df(2R)12*, a small X-ray-induced deficiency that removes the *DHR3* locus (Weber et al., 1995). *FTZ-F1*^{ex7} is an embryonic lethal allele that was generated by P-element excision from a maternal effect *FTZ-F1* mutation (Guichet et al., 1997; Yamada et al., 2000). Two heat-inducible rescue constructs were used: *P[*w^{+}*; hs-DHR3]* (Lam et al., 1999) and *P[*$y^{+}w^{+}$*; hs-*β*FTZ-F1]* (Yamada et al., 2000), neither of which express detectable amounts of protein in the absence of heat shock. A *breathless*-GAL4 transgene (a gift from M. Metzstein) and *elav*-GAL4 were used to drive *UAS-GFP* expression in the developing trachea and nervous system, respectively. Balancer chromosomes marked with *Dfd-GMR-YFP* and *twi-Gal4 UAS-GFP* were used to identify mutant embryos (Halfon et al., 2002; Le et al., 2006).

Heat-shock rescue of the embryonic lethal phenotype

For *hs-*β*FTZ-F1* rescue experiments, flies of the following genotype were crossed: *DHR3/CyO, P[Dfd-GMR-YFP]; P[*$y^{+}w^{+}$*, hs-*β*FTZ-F1]* with *DHR3/CyO twi-Gal4 UAS-GFP*, and β*FTZ-F1*^{ex7} *P[*$y^{+}w^{+}$*, hs-*β*FTZ-F1]/TM3, P[Dfd-GMR-YFP]* with β*FTZ-F1*^{ex7}*/TM3 twi-Gal4 UAS-GFP*. Four-hour egg lays were collected on molasses agar plates supplemented with yeast paste and allowed to develop at 25°C for 12 hours. The dishes

were floated in a water bath at 35°C for one hour, after which mutant embryos were transferred to fresh plates. Rescue was assessed by counting living first-instar larvae on the plate two days AEL. Similarly, rescue of *DHR3* lethality by *hs-DHR3* was tested in embryos derived from *DHR3/CyO, P[Dfd-GMR-YFP]; P[w$^+$, hs-DHR3]/TM3, Sb* parents. Embryos were collected, aged 6-10 hours AEL, and heat shocked at 35°C for 30 mins.

Cuticle preparation

Control embryos with air-filled tracheae and mutant embryos at least 27 hours AEL were dechorionated, hand picked and mounted in a drop of cuticle-mounting medium: 50 µl glacial acetic acid, 25 µl CMCP10 (Masters Co.), 25 µl 85% lactic acid. The slides were incubated overnight at 60°C with a weight on the coverslip, and observed under dark field and DIC optics on a Zeiss Axioskop 2 microscope.

Immunohistochemistry and microscopy

Developing tracheae were labeled using rhodamine-conjugated chitin-binding protein (CBP) at 1:500 dilution (New England Biolabs). GFP was detected using a rabbit anti-GFP primary antibody (1:500, MBL). A rabbit polyclonal anti-DHR3 antibody was used at 1:50 dilution (Lam et al., 1997). Secondary antibodies were Cy2- and Cy3-conjugated anti-rabbit and anti-mouse antibodies (1:200, Jackson ImmunoResearch). Staged embryos were stained as described by Reuter and Scott (Reuter and Scott, 1990), except that the primary antibody and CBP incubations were performed at room temperature. Embryos were visualized using confocal microscopy.

Microarray and northern blot analysis

RNA was isolated from 16-20 hour AEL w^{1118} and $DHR3^{G60S}/Df(2R)12$ embryos using TriPure isolation reagent (Roche) and purified on RNeasy columns (Qiagen). All samples were prepared in four replicates to facilitate subsequent statistical analysis. Probe labeling, hybridization to two-color Agilent *Drosophila* 44K arrays and scanning were performed by the University of Utah Microarray Core Facility. The data were quantile normalized using R, and the fold changes in gene expression and t-statistics were determined using GeneSifter (VizX Labs, Seattle, WA). p-values were calculated using the Benjamimi and Hochberg correction for false-discovery rate. Comparison between microarray datasets was performed using Microsoft Access. Microarray data from this study can be accessed at NCBI GEO (accession number: GSE18577). Northern blot analysis was conducted essentially as described by Karim and Thummel (Karim and Thummel, 1991).

RESULTS

The 20E-triggered *DHR3*, β*FTZ-F1*, *E74A*/*E75A* transcriptional cascade is present in embryos

Northern blot hybridization of staged embryos revealed that *E75A* is expressed in synchrony with the 20E pulse that occurs ~8 hours AEL (Fig. 1B) (Maroy et al., 1988). This is followed by transient bursts of *DHR3* and β*FTZ-F1* expression, and then co-expression of *E74A* and *E75A* in late embryos (Fig. 1B). A similar result was seen in an independent study (Sullivan and Thummel, 2003). These temporal profiles of expression raise the possibility that a functional transcriptional cascade triggered by 20E and involving *DHR3* and β*FTZ-F1* could play a role in embryonic development. As a first step toward testing this hypothesis, we examined *DHR3* expression in mutants deficient for 20E synthesis. Embryos carrying a null mutation for *disembodied* (*dib*), a cytochrome P450 required for 20E synthesis (Chavez et al., 2000), were immunostained with anti-DHR3 antibodies. DHR3 expression is absent in stage 14 dib^{F8} mutants when compared to a heterozygous control (Fig. 2A-B), indicating that embryonic DHR3 expression depends on 20E. We also analyzed the patterns of β*FTZ-F1*, *E74A* and *E75A* transcription in staged control embryos, *DHR3* mutants and β*FTZ-F1* mutants by northern blot hybridization (Fig. 2C). The $DHR3^{G60S}$ null allele was used in combination with a deficiency that removes the *DHR3* locus (*Df(2R)12*), together with animals homozygous for the $FTZ\text{-}F1^{ex7}$ mutation. β*FTZ-F1* expression is reduced in *DHR3* mutant embryos, similar to the effect seen in *DHR3* mutant prepupae, and undetectable in $FTZ\text{-}F1^{ex7}$ mutants, consistent with the nature of this null allele. In addition, *E74A* and *E75A* expression is significantly reduced in both *DHR3* and β*FTZ-F1* mutants during late embryogenesis (Fig. 2C). Similar results were observed with a second null allele for *DHR3*, $DHR3^{22\text{-}35}$, in combination with the *Df(2R)12* deficiency (data not shown). These results support the hypothesis that *DHR3* is required for proper β*FTZ-F1* induction in mid-embryos, and that the reduced expression of β*FTZ-F1* in *DHR3* mutants leads to reduced levels of *E74A* and *E75A* expression, paralleling the regulatory interactions seen in prepupae (Fig. 1A).

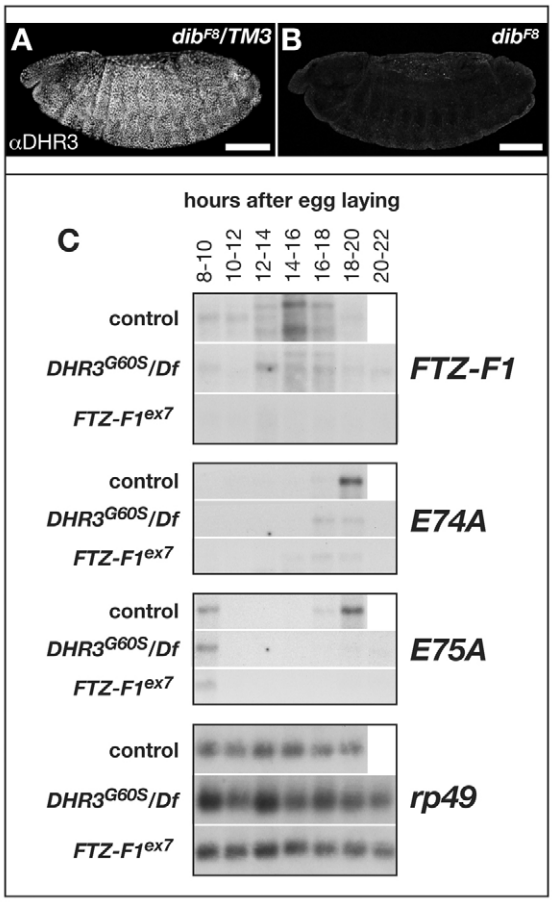

Fig. 2. Regulatory interactions in the 20E-triggered embryonic transcriptional cascade. (**A**,**B**) Stage 14 control ($dib^{F8}/TM3$) and dib^{F8} mutant embryos were immunostained with anti-DHR3 antibodies. Scale bars: 100 µm. (**C**) Total RNA isolated from staged control embryos, *DHR3* mutants and β*FTZ-F1* mutants was analyzed by northern blot hybridization to detect expression of the 20E-regulated genes β*FTZ-F1*, *E75A* and *E74A*. *rp49* was used as a control for loading and transfer. Most control animals have hatched by 20-22 hours AEL, whereas mutant embryos can still be collected.

Ectopic β*FTZ-F1* expression fails to rescue the lethality of *DHR3* mutants

If the major role of *DHR3* during embryogenesis is to ensure proper temporal expression of β*FTZ-F1*, then ectopic β*FTZ-F1* expression should rescue the embryonic lethality of *DHR3*

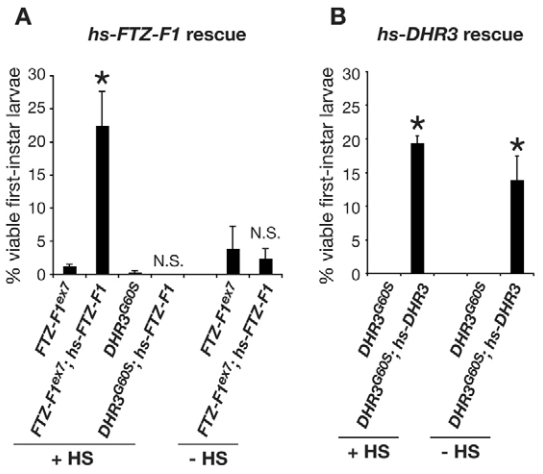

Fig. 3. Ectopic β*FTZ-F1* expression fails to rescue the lethality of *DHR3* mutants. (**A**) *FTZ-F1*^{ex7} and *DHR3*^{G60S} mutant embryos were heat treated (+HS) or not heat treated (–HS), in either the absence or presence of a *hs*-β*FTZ-F1* transgene, and scored for hatching into viable first-instar larvae. The transgene had no effect on *DHR3* mutants, but rescued β*FTZ-F1* mutants in a heat-dependent manner. (**B**) *DHR3*^{G60S} mutant embryos were heat treated (+HS) or not heat treated (–HS), in either the absence or presence of a *hs-DHR3* transgene, and scored for hatching into viable first-instar larvae. *DHR3* mutants are rescued by the transgene, independent of its heat-induced expression. Error bars represent s.e.m., $n \geq 3$ independent experiments, $N \geq 150$ embryos per condition. *$P<0.05$; N.S., not significant when compared with corresponding mutant embryos, Student's *t*-test.

mutants. To test this hypothesis, we used an *hs*-β*FTZ-F1* transgene to drive β*FTZ-F1* expression in *DHR3* mutant embryos during the short period of time when it is normally expressed. As expected, heat-induced β*FTZ-F1* expression 6-10 hours AEL in β*FTZ-F1* mutant embryos efficiently rescues their lethality (Fig. 3A), consistent with previous observations (Yamada et al., 2000). In contrast, no rescue was observed when the same treatment was applied to *DHR3* mutant embryos (Fig. 3A). It is possible that the inability of β*FTZ-F1* to rescue *DHR3* mutants is due to lethality unrelated to the *DHR3* mutation. Heat-induced *DHR3* expression, however, is sufficient to rescue *DHR3* mutants, indicating that this is not the case (Fig. 3B). Interestingly, no heat treatment is needed for this construct to rescue *DHR3* lethality, demonstrating that basal *DHR3* expression from this *hs-DHR3* transgene is sufficient to direct proper embryonic development. Taken together, these data suggest that *DHR3* exerts both β*FTZ-F1*-dependent and β*FTZ-F1*-independent functions to direct proper progression through embryogenesis.

β*FTZ-F1* mutants have abnormally small and unpigmented denticles

As a first step toward identifying developmental functions for *DHR3* and β*FTZ-F1* during embryogenesis, we examined cuticle formation in embryos mutant for these two nuclear receptors. Genetic and biological studies have demonstrated that 20E signaling plays a central role in cuticle synthesis and deposition during each stage in the life cycle (Chavez et al., 2000; Riddiford et al., 2001; Kozlova and Thummel, 2003). Examination of cuticle preparations from *DHR3* and β*FTZ-F1* mutants at low magnification revealed normal patterning of the embryonic cuticle, with the correct number and position of the ventral denticle belts (Fig. 4A-C). The dorsal cuticle is also normal in *DHR3* and β*FTZ-F1* mutants, indicating that dorsal closure occurred properly. Mouth hooks and denticle belts are fully developed in control embryos by 19 hours AEL; however, this process is not complete in *DHR3* and β*FTZ-F1* mutants until a few hours later, indicating a developmental delay in cuticle differentiation (data not shown). In addition, the denticles in β*FTZ-F1* mutants, but not *DHR3* mutants, are abnormally small and unpigmented (Fig. 4D-F). The number of denticles per belt appears to be wild type in β*FTZ-F1* mutant embryos, indicating that patterning is normal, but the denticles do not appear to fully differentiate. The specificity of this β*FTZ-F1* phenotype was confirmed by observing the same defects in animals carrying the β*FTZ-F1*^{ex7} mutation in combination with *Df(3L)Cat*^{DH104}, a deficiency that removes the *FTZ-F1* locus (Broadus et al., 1999) (data not shown). The absence of a cuticular phenotype in *DHR3* mutants was confirmed using a second allelic combination (*DHR3*^{G60S}/*Df*) and is in agreement with previous reports (Carney et al., 1997). Taken together, these results define a β*FTZ-F1*-specific function in controlling the size and pigmentation of ventral denticles.

DHR3 and β*FTZ-F1* mutants fail to air fill their trachea

To further define functions for *DHR3* and β*FTZ-F1* during embryonic development, we examined the major developmental events that occur after the peak expression of *DHR3* and β*FTZ-F1*: tracheal system maturation, initiation of muscular movements and central nervous system (CNS) condensation, three processes that remain relatively poorly understood (Campos-Ortega and Hartenstein, 1985). Tracheal development begins during the second half of embryogenesis, when 20 metameric placodes invaginate from the epidermis and undergo stereotypic branching and fusion events to form the tracheal tubular network. These tubes form as liquid-filled structures, with the epithelial cells depositing an apical chitinous matrix into the lumen that coordinates uniform tube growth (reviewed by Swanson and Beitel, 2006). The tubular network fills with gas approximately one hour before hatching. This event is easy to follow using bright-field microscopy because of the different refractive index of the gas compared to the surrounding

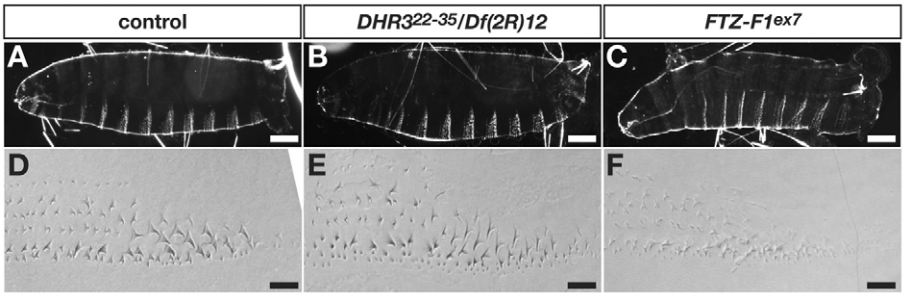

Fig. 4. β*FTZ-F1* mutants have abnormally small and unpigmented denticles. (**A-C**) Dark-field images of cuticle preparations of control, *DHR3* mutant and β*FTZ-F1* mutant embryos. The overall morphology of *DHR3* and β*FTZ-F1* mutant cuticles appears normal. Scale bars: 100 μm. (**D-F**) DIC images of the A3 denticle belt of embryos of the same genotype. β*FTZ-F1* mutants have small and unpigmented denticles (F) relative to the control (D), whereas *DHR3* mutant denticles appear normal (E). Scale bars: 10 μm.

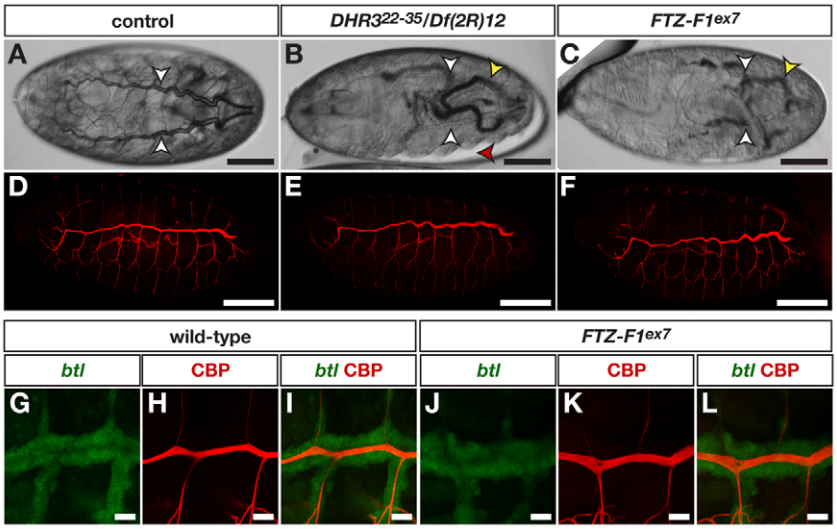

Fig. 5. *DHR3* and β*FTZ-F1* mutants fail to air fill their trachea. (**A-C**) Bright-field images of late-stage control, *DHR3* mutant and β*FTZ-F1* mutant embryos. Control embryos have completed liquid clearance and gas filling of their tracheal branches at 19 hours AEL (A), whereas *DHR3* and β*FTZ-F1* mutant embryos 24-28 hours AEL are defective for this process (B,C). White arrowheads indicate dorsal tracheal trunks and yellow arrowheads indicate Malpighian tubules. In B, the red arrowhead indicates a gap between the cuticle and epidermis as a result of muscle movement. Scale bars: 100 μm. (**D-F**) Staining of stage 16 embryos with rhodamine-conjugated CBP shows normal patterning, expansion and chitin accumulation in the tracheal systems of *DHR3* and β*FTZ-F1* mutant embryos. Scale bars: 100 μm. (**G-L**) At higher magnification, CBP staining (red) coincides with the lumen of the dorsal tracheal trunk and lateral branches marked with *breathless>GFP* (green) in both control and β*FTZ-F1* mutant embryos. Scale bars: 10 μm.

liquid. Examination of the tracheae in *DHR3* and β*FTZ-F1* late-stage mutant embryos revealed that air filling fails to occur in both genetic backgrounds, compared to the clearly visible air-filled tracheae present in controls (Fig. 5A-C). This phenotype is fully penetrant in animals of both genotypes (Table 1). The specificity of the *DHR3* mutant phenotype was confirmed by using a second allele, $DHR3^{G60S}$, in combination with a deficiency that removes the *DHR3* locus (Table 1). Interestingly, a gas bubble reminiscent of the first step of the air-filling process was observed in a few animals of this genotype (Table 1). Ectopic expression of β*FTZ-F1* in $DHR3^{G60S}$ mutants had no effect on the tracheal air-filling defect, suggesting that this represents a function of *DHR3* that is not solely dependent on its downstream target, β*FTZ-F1* (data not shown).

Failure to air fill the tracheal network could result from defects in earlier tracheal morphogenesis or from specific disruption of this late developmental event. To examine the tracheal morphology in *DHR3* and β*FTZ-F1* mutant embryos, we used rhodamine-conjugated CBP to specifically label the tracheal network. This showed that the branching pattern and size of the lumen in the developing tubes is indistinguishable from that seen in control animals (Fig. 5D-F). Closer examination of the tracheal cells, using *btl-GAL4* to mark these cells with GFP in combination with staining for CBP, confirmed that the development of the tracheal network, including differentiation, lumen formation and extension, chitin deposition and branch patterning, is unaffected in both *DHR3* and β*FTZ-F1* mutants (Fig. 5G-L and data not shown). The air-filling defect observed in *DHR3* and β*FTZ-F1* mutant embryos therefore arises from a specific inability to perform the last steps of tracheal development required for air filling of the tracheal network.

Observation of late *DHR3* and β*FTZ-F1* mutant embryos also indicated that organogenesis is apparently normal. Specifically, the midgut displayed its normal looping pattern (data not shown) and the four differentiated Malpighian tubules were clearly visible (Fig. 5B-C, yellow arrowheads). In addition, most *DHR3* and β*FTZ-F1* mutant embryos exhibited normal muscular movements, as shown in Fig. 5B (red arrowhead) by the gap observed between the cuticle and epidermis (quantified in Table 1). These results indicate that development is not simply arrested in *DHR3* and β*FTZ-F1* mutant embryos, but rather that specific developmental processes are selectively disrupted.

DHR3 mutants are defective for ventral nerve cord condensation

Similar to the tracheal system, the CNS undergoes terminal differentiation during the final stages of embryonic development (Campos-Ortega and Hartenstein, 1985). The CNS acquires its overall final morphology during germ-band retraction, when the germ-band neuroblasts follow the movement of the epidermis to generate the VNC, a neuronal structure that underlies the entire ventral side of the embryo. The VNC later increases in cellular density and decreases in size in a process called condensation. This process starts at late stage 15, with the majority of condensation occurring during stage 16 and early stage 17 (Campos-Ortega and Hartenstein, 1985), after the peaks of *DHR3* and β*FTZ-F1* expression. To visualize VNC condensation in living embryos, we drove GFP expression specifically in the nervous system using an *elav*-GAL4 driver, and assessed anteroposterior condensation by measuring the percentage of the embryo length occupied by the VNC in control and mutant animals (Fig. 6A). Condensation results

Table 1. Quantification of the tracheal gas-filling and muscle movement phenotypes

	Trachea filling (%)				Muscular movement	
Genotype	No	Partial	Full	*n*	%	*n*
$DHR3^{G60S}/Df(2R)12$	97	3	0	96	97	96
$DHR3^{22-35}/Df(2R)12$	100	0	0	124	95	124
$FTZ\text{-}F1^{ex7}$	100	0	0	126	96	126

Mutant embryos 24-28 hours AEL were scored for tracheal gas filling and muscle movement under a compound microscope equipped with bright-field optics. Results are presented as the fraction of animals in each category. No, no gas filling; partial, gas bubble in the posterior dorsal trunk; full, gas filling completed. *n* indicates the total number of embryos examined.

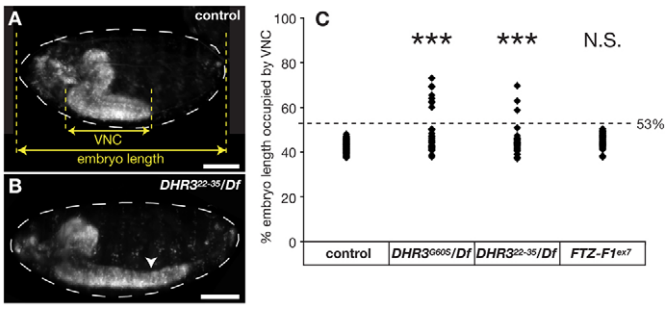

Fig. 6. *DHR3* mutants display defective VNC condensation. (**A**) Late stage 17 control embryo carrying an *elav*-GAL4 transgene driving GFP expression, showing normal VNC condensation. The arrows indicate the measurements that were used to calculate VNC condensation in the anteroposterior axis (Olofsson and Page, 2005). (**B**) *DHR3* mutant embryo showing an uncondensed VNC. The arrowhead marks where the VNC would terminate in a wild-type embryo (43% embryo length). Scale bars: 100 μm. (**C**) Quantification of VNC condensation phenotypes. *DHR3* mutants show a bimodal distribution, with 25% (*DHR3*^{G60S}*/Df*) and 11% (*DHR3*$^{22\text{-}35}$*/Df*) of the embryos exhibiting an uncondensed VNC. In contrast, VNC condensation is essentially normal in β*FTZ-F1* mutant embryos. Penetrance of mutant phenotypes was assayed by determining the percentage of embryos in which the VNC occupies ≥53% of the embryo length (which corresponds to the control average plus10%) (*N*≥28 individuals per genotype). ***P<0.001; N.S., not significant when compared to control, chi-square test.

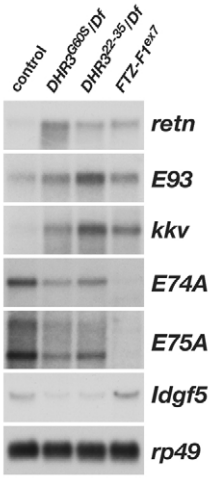

Fig. 7. Overlapping patterns of gene regulation in *DHR3* and β*FTZ-F1* mutants. Total RNA isolated from control, *DHR3*^{G60S}*/Df*, *DHR3*$^{22\text{-}35}$*/Df* and *FTZ-F1*^{ex7} mutant embryos 16-20 hours AEL was analyzed by northern blot hybridization to detect *retn*, *E93*, *kkv*, *E74A*, *E75A* and *Idgf5* expression. Blots were hybridized with *rp49* as a control for loading and transfer.

in a shortening of the VNC to an average value of 43% of embryo length at the end of stage 17 (air-filled tracheae) in control animals (Fig. 6C). Two *DHR3* mutant combinations displayed low penetrance VNC condensation defects, with 25% of *DHR3*^{G60S}*/Df* embryos and 11% of *DHR3*$^{22\text{-}35}$*/Df* animals exhibiting incomplete condensation (Fig. 6B-C). In contrast, VNC condensation is essentially normal in β*FTZ-F1* mutant embryos (Fig. 6C).

Altered developmental gene expression in *DHR3* and β*FTZ-F1* mutant embryos

Microarray studies were performed to investigate the molecular mechanisms by which *DHR3* contributes to late embryonic development. RNA was extracted from control and *DHR3* mutant embryos staged 16-20 hours AEL, a time period following *DHR3* and β*FTZ-F1* expression, and during which the affected developmental processes occur. RNA was labeled and hybridized to two-color Agilent *Drosophila* 44K arrays. All experiments were conducted in four replicates to facilitate statistical analysis. The raw data were quantile normalized using R, and the fold changes in gene expression and t-statistics were determined using GeneSifter. We chose *E74* as a positive control to determine the fold change cut-off and p-value for this analysis (no isoform-specific probe was available on the array for the other known *DHR3*-regulated gene, *E75A*). Our study revealed that 1352 transcripts are significantly affected in *DHR3* mutant embryos (≥2.1-fold, adjusted *P*-value<0.03), with 746 genes upregulated and 606 genes downregulated (see Table S1 in the supplementary material).

We used the web-based software DAVID to identify gene functions (gene ontologies) corresponding to transcripts that are misregulated in *DHR3* mutants (Dennis et al., 2003; Huang da et al., 2009). This analysis revealed that many genes involved in the innate immune and defense responses are expressed at increased levels in *DHR3* mutants, together with reduced expression of genes encoding cytochrome P450 enzymes and proteolytic enzymes, and genes that act in metabolism (data not shown). This is in agreement with the functions of the *DHR3* vertebrate homologs *RORα* and *RORβ* in controlling energy homeostasis and lipid metabolism in mice (Jetten, 2004). To determine whether metabolic dysfunction might contribute to the lethality of *DHR3* mutant embryos, we measured the levels of the major forms of stored energy and ATP. No significant difference, however, was detected between mutant and control animals (see Fig. S1 in the supplementary material).

The gene ontology analysis also identified 128 genes associated with development that are misregulated in *DHR3* mutants (see Table S2 in the supplementary material). These include several genes that have been well characterized as being directly induced by 20E, including *E74A*, *E75* and *IMPE3*, which are expressed at reduced levels in *DHR3* mutant embryos. An embryo-specific isoform of *E93*, another known 20E target gene, is normally repressed at the end of embryogenesis and is expressed at higher levels in *DHR3* mutants (Baehrecke and Thummel, 1995). Additional targets of 20E signaling are found among the list of *DHR3*-regulated genes and display increased levels of expression in *DHR3* mutants, including *Fbp1*, *Sgs4*, *Sgs5* and *Lsp1γ*. We also see reduced expression (2.6-fold) of the key ecdysone biosynthetic gene *phantom*. Although the time point we examined is significantly later than the known 20E pulse in embryos, this observation nonetheless raises the possibility that *DHR3* mutants may suffer from a hormone deficiency. To test this hypothesis, we determined whether exogenous 20E can rescue the embryonic lethality of *DHR3* mutants. Although exogenous 20E application is sufficient to rescue embryos that are mutant for the ecdysone-synthesis gene *spook* (11 viable larvae out of 126 embryos, 9% rescue), consistent with previous studies (Ono et al., 2006), this approach had no effect on *DHR3* mutants (0 viable larvae out of 319 embryos). This result suggests that hormone deficiency is not the sole cause of *DHR3* embryonic lethality.

Northern blot hybridizations were conducted to test the response of selected *DHR3*-regulated genes in *DHR3* and β*FTZ-F1* mutant embryos (Fig. 7). This analysis identified three major classes of genes. First, some genes that are misregulated in *DHR3* mutants are similarly affected in β*FTZ-F1* mutant animals, such as *retn* (*retained*), *E93* (*Eip93F*) and *kkv* (*krotzkopf verkehrt*). Second, *E74A* and *E75A* are submaximally expressed in *DHR3* mutants, but are not detectably expressed in β*FTZ-F1* mutant embryos. Finally, we identified one gene, *Idgf5* (*imaginal disc growth factor 5*), that is downregulated in *DHR3* mutants, but not significantly affected by the β*FTZ-F1* mutation. Taken together, these results validate the *DHR3* target genes identified in the microarray analysis, and provide a molecular basis to support the existence of overlapping developmental functions for *DHR3* and β*FTZ-F1* during embryogenesis.

DISCUSSION

Studies of the onset of metamorphosis have identified a transcriptional cascade that leads to the sequential expression of the transcription-factor-encoding genes *DHR3*, β*FTZ-F1*, *E74A* and *E75A*. Although the regulatory interactions between these genes have been characterized, the functional consequences of their expression remain more poorly understood. In this study, we show that the *DHR3*-β*FTZ-F1* transcriptional cascade is functional in embryos and is required for progression through this stage of development. We describe the essential roles of *DHR3* and β*FTZ-F1*, and demonstrate their contributions to both common and distinct developmental and transcriptional responses during embryogenesis. This study also reveals an evolutionarily conserved role for DHR3 and its vertebrate homolog, RORα, in nervous system development.

A functional embryonic transcriptional cascade

The regulatory interactions between *DHR3*, β*FTZ-F1* and *E74A*/*E75A* that are described here in embryos are indistinguishable from those seen in prepupae. First, *DHR3* expression in embryos is dependent on 20E signaling. Second, *DHR3* mutants display reduced levels of β*FTZ-F1*, *E74A* and *E75A* expression at both stages in the life cycle, and β*FTZ-F1* mutants have reduced levels of *E74A* mRNA and no detectable *E75A* expression (Broadus et al., 1999; Lam et al., 1999) (Fig. 2). Taken together with studies that show that ectopic β*FTZ-F1* is sufficient to drive maximal expression of *E74A* and *E75A* (Woodard et al., 1994), our results indicate that *DHR3* exerts its effect on these genes through its induction of β*FTZ-F1* in embryos. Third, a loss of *DHR3* function during embryogenesis does not eliminate β*FTZ-F1* expression (Lam et al., 1999). This is probably due to other upstream factors that contribute to this response. One candidate for this function is the DHR4 nuclear receptor, which is coexpressed with *DHR3* in both embryos and prepupae (Sullivan and Thummel, 2003). *DHR4* mutants have no effect on *DHR3* expression, but display significantly reduced levels of β*FTZ-F1* mRNA in prepupae (King-Jones et al., 2005). These mutants, however, have no effect on embryonic development, suggesting that *DHR4* does not play a major role in β*FTZ-F1* induction at this early stage in the life cycle.

The late larval pulse of 20E both directly and indirectly induces *DHR3* and represses β*FTZ-F1* (Woodard et al., 1994; Horner et al., 1995). Taken together with the inductive effect of *DHR3* on β*FTZ-F1* expression, this regulation ensures that the peak of β*FTZ-F1* expression will be delayed until the proper time during development (White et al., 1997; Lam et al., 1999). The observation that the embryonic 20E pulse, at ~8 hours AEL, immediately precedes *DHR3* expression suggests that similar regulatory interactions are acting in embryos. However, unlike prepupae, there is no known hormone peak in late embryos that could account for the coordinated induction of *E74A* and *E75A* mRNA at this time, as is known to occur in late prepupae (Thummel, 2001). It is possible that these transcripts are fully dependent on trans-acting factors such as β*FTZ-F1* for their expression in embryos. Alternatively, these 20E primary-response genes might be induced by a novel temporal signal that remains to be identified.

It is interesting to note that a similar temporal profile of *DHR3*, β*FTZ-F1* and *E74A*/*E75A* expression is also seen in larvae. A burst of *DHR3* expression in mid-second instar larvae immediately follows the peak in the 20E titer and precedes the transient expression of β*FTZ-F1*, which is followed by co-expression of *E74A* and *E75A* at the end of the instar (Sullivan and Thummel, 2003). Curiously, *E75A*, but not *E74A*, is expressed at an earlier time as well, in apparent synchrony with the 20E pulse, recapitulating the timing seen in embryos (Sullivan and Thummel, 2003) (Fig. 1B). It is thus likely that a common set of regulatory interactions function in both embryos and larvae to dictate the precise timing of these expression patterns at each stage in the life cycle, prior to the third instar. Moreover, the observation that *EcR*, *E75A* and β*FTZ-F1* mutants display defects in larval molting indicates that their expression is essential for proper progression through these stages in development (Li and Bender, 2000; Yamada et al., 2000; Bialecki et al., 2002).

DHR3 and β*FTZ-F1* exert overlapping functions during embryogenesis

DHR3 and β*FTZ-F1* null mutations lead to fully penetrant embryonic lethality, with relatively minor and partially penetrant phenotypes reported in *DHR3* mutant embryos and no phenotypic description of β*FTZ-F1* mutant embryos (Carney et al., 1997; Yamada et al., 2000). The studies described here define both common and unique functions for these two nuclear receptors during embryogenesis. *DHR3* and β*FTZ-F1* null mutants both display a highly penetrant defect in air filling of the tracheal tree (Fig. 5). In addition to this common function, β*FTZ-F1* is required for the proper differentiation of the denticles in the ventral cuticle and *DHR3* is required for VNC condensation (Figs 4, 6). Both *DHR3* and β*FTZ-F1* mutants display apparently normal muscle movements at the end of embryogenesis, indicating that only some developmental responses are blocked at this stage (Table 1). These processes of cuticle differentiation, tracheal air filling, muscular movements and VNC condensation represent the major developmental events that can be described in late embryos. Defects in three of these four pathways thus define a central role for *DHR3* and β*FTZ-F1* in late embryonic development. In addition, unlike prepupae, in which *DHR3* and β*FTZ-F1* mutants have essentially identical phenotypes, these studies establish independent functions for these two nuclear receptors during development. Together with the previously identified early embryonic roles of the 20E receptor *EcR* in dorsal closure, head involution and midgut morphogenesis, these data indicate that each step of the 20E-induced transcriptional cascade controls sequential developmental programs during embryogenesis (Chavez et al., 2000; Kozlova and Thummel, 2003). Moreover, as mentioned above, the observation that this transcriptional cascade is also required for larval molting suggests that it represents a stereotypic 20E response that is required for progression through each major transition in the life cycle.

Ectopic expression of wild-type β*FTZ-F1* is sufficient to rescue the lethality of β*FTZ-F1* mutants, but has no effect on the viability of *DHR3* mutants, indicating that *DHR3* exerts essential functions

Table 2. Altered expression of genes involved in nervous system development in *DHR3* mutant embryos

Probe ID	FlyBase ID	CG	Gene name	Fold change
A_09_P011601	FBgn0005630	CG12052	*longitudinals lacking*	–3.1
A_09_P043346	FBgn0002543	CG5481	*leak*	2.1
A_09_P103140	FBgn0011592	CG8581	*frazzled*	2.3
A_09_P221615	FBgn0053208	CG33208	*Molecule interacting with CasL*	2.5
A_09_P010386	FBgn0004369	CG11516	*Protein tyrosine phosphatase 99A*	3.3
A_09_P012971	FBgn0011259	CG18405	*Sema-1a*	3.3
A_09_P045606	FBgn0013733	CG18076	*short stop*	3.7
A_09_P052206	FBgn0015774	CG10521	*Netrin-B*	3.9
A_09_P054701	FBgn0036101	CG6449	*Ninjurin A*	6.3

List of genes with nervous system development functions that are misregulated in *DHR3^{G60S}/Df(2R)12* mutants compared to *w^{1118}* controls 16-20 hours AEL, as determined by microarray analysis. The following data columns are included: Agilent probe designation, FlyBase identification number, CG number, gene name and fold change in expression level.

independently of its downstream partner (Fig. 3). The causes of lethality in *DHR3* and β*FTZ-F1* mutant embryos, however, remain unclear. Strong loss-of-function mutations in the *signal peptide peptidase* (*Spp*) gene result in tracheal air-filling defects; however, *Spp* mutant embryos hatch normally and die as first or second instar larvae (Casso et al., 2005). Similarly, embryos with severe defects in VNC condensation can hatch into first instar larvae and survive to later stages of development (Page and Olofsson, 2008). These results indicate that the lethality of *DHR3* and β*FTZ-F1* mutant embryos cannot be directly attributed to defects in these pathways. Rather, *DHR3* and β*FTZ-F1* may participate in a developmental checkpoint necessary to trigger the last steps of embryogenesis required for hatching and survival.

Our microarray study revealed that a number of 20E-responsive genes are misregulated in *DHR3* mutants, consistent with studies in prepupae that indicate a crucial role for *DHR3* in 20E signaling (Lam et al., 1999). The microarray analysis also identified several genes that are involved in chitin metabolism and protein secretion, which could account for the defects in tracheal gas filling seen in *DHR3* mutants. These included the chitinase genes *Idgf5* (–8.6-fold) and *kkv* (+2.4-fold) (Devine et al., 2005), the CBP *Cht12* (+2.6-fold) and the COPII coat subunit *sec13* (+2.5-fold) (Tsarouhas et al., 2007). This study also identified a number of genes that play a role in axon guidance (Table 2). Interestingly, most of these genes have dose-dependent effects, whereby either reduced or increased expression can disrupt nervous system development (see, for example, Winberg et al., 1998). Failure of *DHR3* mutant embryos to express these genes at normal levels could thus contribute to the PNS defects described previously (Carney et al., 1997).

Northern blot hybridization studies to examine the effects of *DHR3* and β*FTZ-F1* mutants on selected *DHR3*-regulated genes confirm and extend our phenotypic studies of these mutants. Some genes, such as *retn*, *E93* and *kkv*, display similar transcriptional responses in *DHR3* and β*FTZ-F1* mutants, whereas *E74A* and *E75A* are more significantly affected in β*FTZ-F1* mutants and *Idgf5* is selectively reduced in *DHR3* mutants (Fig. 7). These transcriptional effects support our phenotypic studies and provide further evidence that *DHR3* and β*FTZ-F1* exert common and independent regulatory roles during embryogenesis. This conclusion is consistent with experimental and theoretical studies of gene regulatory networks, which indicate that transcriptional cascades provide an effective means of amplifying signals and integrating multiple cues to provide specificity in biological responses. Transcriptional cascades can also direct temporal programs of successive gene expression, as observed in the formation of flagella in *Escherichia coli* (Kalir et al., 2001) and the specification of anteroposterior patterning in the *Drosophila* embryo (Arnone and Davidson, 1997). In addition, the *DHR3*-β*FTZ-F1* transcriptional cascade involves nuclear receptors that could potentially act as ligand-regulated transcription factors, introducing an additional level of control by small lipophilic compounds. These observations support the proposal that the sequential expression of *DHR3* and β*FTZ-F1* at multiple stages of development can specify successive biological programs that promote appropriate progression through the life cycle. By combining insect endocrinology with the predictive power of genetics, the 20E-triggered transcriptional cascades in *Drosophila* provide an ideal context to define how a repeated systemic signal can be refined into precise stage-specific temporal responses during development.

Conserved functions for the ROR family of nuclear receptors

DHR3 is required for VNC condensation, a terminal step in embryonic nervous system morphogenesis that is dependent on nervous system activity, glial cell function and apoptosis (Page and Olofsson, 2008). In addition, previous studies have identified roles for *DHR3* in PNS development (Carney et al., 1997). Interestingly, these functions, which are specific for *DHR3* and are not shared with its direct target, β*FTZ-F1*, parallel the role of the mammalian DHR3 homolog RORα in brain development. *RORα* was initially identified as the gene associated with the spontaneous *staggerer* mutation in mice, which display ataxia associated with cerebellum developmental defects and degeneration (Hamilton et al., 1996; Gold et al., 2007). The cerebellum in *staggerer* mutants is dramatically smaller than in controls, containing fewer of the two major cell types: granule cells and Purkinje cells (Sidman et al., 1962). Further investigation showed that this phenotype arises primarily from reduced expression in Purkinje cells of Sonic hedgehog (Shh), a mitogenic signal for granule cells (Gold et al., 2003). These data support the hypothesis that there is an evolutionarily conserved role for the ROR/DHR3 family of nuclear receptors in nervous system development and suggest that further functional studies of *DHR3* may provide new insights into its ancestral functions in this pathway.

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Supplementary material

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