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RESEARCH ARTICLE

Forward and feedback regulation of cyclic steroid production in *Drosophila melanogaster*

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ABSTRACT

In most animals, steroid hormones are crucial regulators of physiology and developmental life transitions. Steroid synthesis depends on extrinsic parameters and autoregulatory processes to fine-tune the dynamics of hormone production. In *Drosophila*, transient increases of the steroid prohormone ecdysone, produced at each larval stage, are necessary to trigger moulting and metamorphosis. Binding of the active ecdysone (20-hydroxyecdysone) to its receptor (EcR) is followed by the sequential expression of the nuclear receptors E75, DHR3 and βFtz-f1, representing a model for steroid hormone signalling. Here, we have combined genetic and imaging approaches to investigate the precise role of this signalling cascade within theprothoracic gland (PG), where ecdysone synthesis takes place. We show that these receptors operate through an apparent unconventional hierarchy in the PG to control ecdysone biosynthesis. At metamorphosis onset, DHR3 emerges as the downstream component that represses steroidogenic enzymes and requires an early effect of EcR for this repression. To avoid premature repression of steroidogenesis, E75 counteracts DHR3 activity, whereas EcR and βFtz-f1 act early in development through a forward process to moderate DHR3 levels. Our findings suggest that within the steroidogenic tissue, a given 20-hydroxyecdysone peak induces autoregulatory processes to sharpen ecdysone production and to confer competence for ecdysteroid biosynthesis at the next developmental phase, providing novel insights into steroid hormone kinetics.

KEY WORDS: Ecdysone, Prothoracic gland, Nuclear receptor, DHR3, *Drosophila*

INTRODUCTION

Animal development is controlled by a wide variety of messengers, including steroids that trigger developmental life transitions. In humans, the puberty phase is accompanied by an increase in steroid hormone production within the somatic cells of the gonads (Martos-Moreno et al., 2010). Although steroid production by the gonads has been shown to depend on positive- and negative-feedback loops (Navarro and Tena-Sempere, 2012), the molecular mechanisms that coordinate steroidogenesis with other intrinsic and extrinsic parameters remain poorly understood. *Drosophila* genetics has proved a powerful tool for deciphering how steroid production is controlled and subsequently signalled in target tissues (Ou and King-Jones, 2013; Rewitz et al., 2013; Yamanaka et al., 2013). In *Drosophila*, the steroid prohormone ecdysone is produced within the prothoracic gland (PG) cells, which are part of the ring gland (Bodenstein, 1994). Ecdysone is converted into the active hormone 20-hydroxyecdysone (20E) in the peripheral tissues (Petryk et al., 2003) to trigger moulting and metamorphosis (Riddiford et al., 2003). These processes require a precise kinetics of ecdysone production (Woodard et al., 1994).

Earlier studies on crustaceans and lepidopterans suggested that ecdysteroids may control their own biosynthesis (Beydon and Lafont, 1983; Dell et al., 1999; Sakurai and Williams, 1989) – a notion further supported by *Drosophila* studies. Binding of 20E to its receptor, EcR (Riddiford et al., 2000), induces a set of primary-response genes. These genes include *E75* (*Eip75B* – FlyBase), which encodes three distinct nuclear receptor (NR) isoforms: E75A, B and C (Segraves and Hogness, 1990). DHR3 (Hr46 – FlyBase) is another NR induced by ecdysone (Hiruma and Riddiford, 2004; Horner et al., 1995), which together with E75B regulates transcription of the downstream NR βFtz-f1, the β-isoform encoded by the *ftz-f1* gene (Palanker et al., 2006; Reinking et al., 2005; White et al., 1997). The idea of steroidogenesis autoregulation in *Drosophila* arises from phenotypic analyses showing that *E75A* mutants fail to produce ecdysteroids during the second larval stage (Bialecki et al., 2002). Furthermore, our past study has shown that the steroidogenic enzymes Phantom (Phm) and Disembodied (Dib) are not detected in *ftz-f1* mutant PG cells (Parvy et al., 2005), suggesting that the NRs induced by 20E signalling also act in PG cells to control steroidogenesis.

Here, we have investigated how the 20E-responsive NR cascade controls ecdysone biosynthesis within the steroidogenic tissue. Our findings indicate that 20E signalling does not directly activate ecdysone biosynthesis, but rather acts to potentiate and to narrow the cyclic production of the steroid hormone. Instead of transcriptional activation, we observed that DHR3 acts as a repressor of ecdysone production and that it downregulates expression of the steroidogenic enzymes at the onset of metamorphosis. In this process, βFtz-f1 and EcR moderate DHR3 levels. In addition, EcR is required earlier in development for the DHR3-mediated repression, whereas E75 counteracts the DHR3-inhibiting activity.

RESULTS

NRs mediating 20E signal are expressed in the PG cells

Based on an autoregulatory model, the PG cells should respond to 20E and thus should express the NRs induced by 20E stimulation. To investigate the expression of EcR, E75, DHR3 and βFtz-f1, we carried out ring gland immunostaining at stages corresponding to the major ecdysteroid peaks (Fig. 1A): mid/late L1 (12-20 h after hatching), mid/late L2 (12-20 h past the L1-L2 moult), wandering L3 (40-48 h past the L2-L3 moult), and early prepupae (0-6 h past pupariation) (Fig. 1A,B). EcR (Fig. 1D,H,L,P), DHR3 (Fig. 1F,J,N,R) and βFtz-f1 (Fig. 1G,K,O,S) were present in the nuclei of PG cells at all stages.

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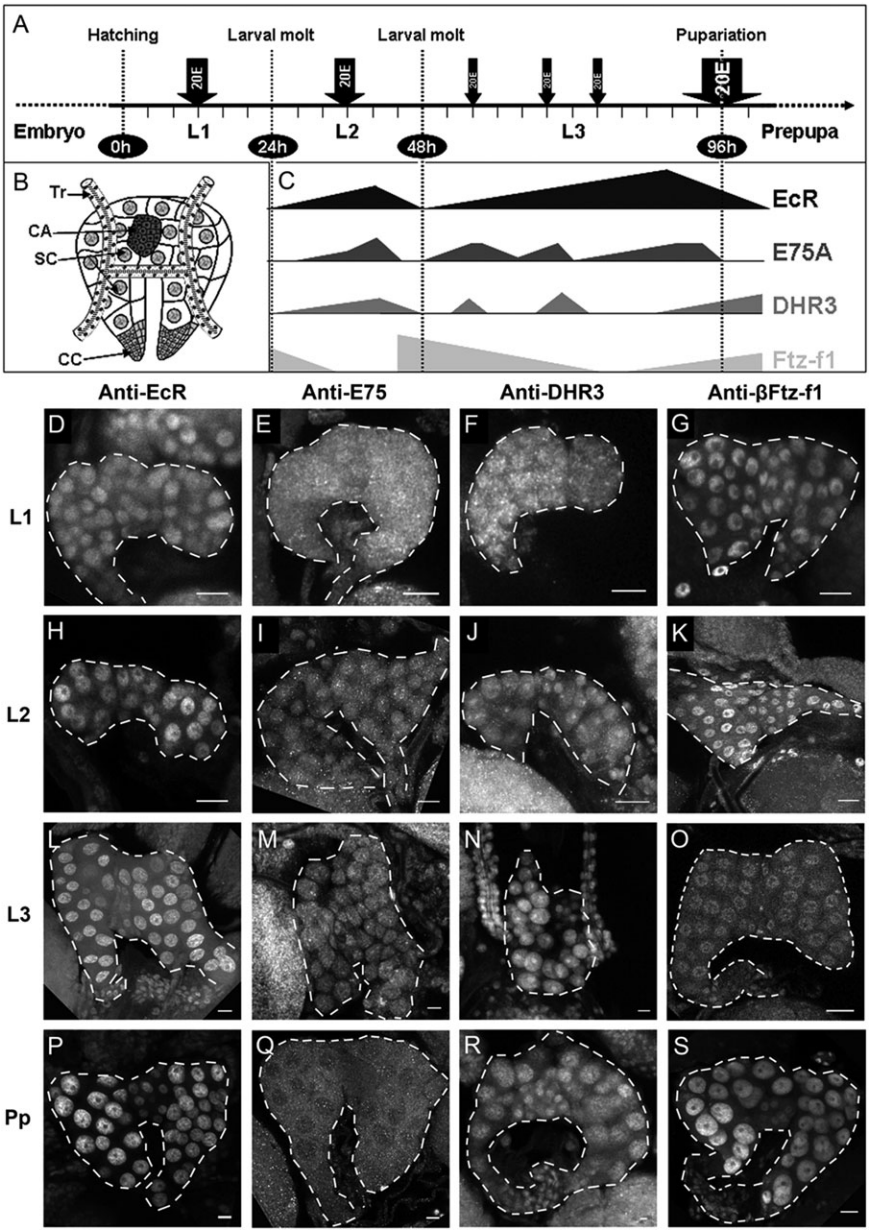

Fig. 1. Immunodetection of NRs mediating ecdysone signalling in the ring gland during larval development. (A) Representation of the cyclic ecdysone production during larval life. The time points of the ecdysone peaks are indicated by black arrows whose thicknesses are relative to steroid titres (Bialecki et al., 2002; Parvy et al., 2005; Richards, 1981; Warren et al., 2006). (B) Scheme of a ring gland: CA, corpora allata; CC, corpora cardiaca; SC, steroidogenic cells; Tr, trachea. In all the figures, the ring glands are oriented anterior to the top. (C) Scheme of the expression of EcR, E75A, DHR3 and βFtz-f1 in the steroidogenic cells during the L2 and L3 stages according to the supplementary material (supplementary material Figs S1, S2, S4 and S5). (D-S) Ring glands of mid L1 (D-G), mid L2 (H-K), late L3 (L-O) larvae and prepupae (P-S) were stained with antibody to EcR (D,H,L,P), E75 (E,I,M,Q), DHR3 (F,J,N,R) and βFtz-f1 (G,K,O,S). Scale bars: 10 µm.

Nuclear E75 was detected in the PG cells of L1, L2 and wandering L3 larvae but not in prepupae (Fig. 1E,I,M,Q).

To evaluate the precise kinetics of 20E signalling in the PG, expression of EcR, E75, DHR3 and βFtz-f1 was analysed every 4 h from the early L2 to late L3 stages (Fig. 1C and supplementary material Figs S1-S5). EcR expression increased from early to mid-L2 (supplementary material Fig. S1A-E) and then decreased to reach a minimum in early L3 larvae (supplementary material Fig. S1F-H). During the L3 stage, EcR expression increased to a maximum in 40 h L3 larvae and then decreased at later stages (supplementary material Fig. S1I-T). The E75A (supplementary material Fig. S2) and E75B (supplementary material Fig. S3) isoforms were analysed with specific antibodies. E75A protein was weakly expressed in early L2 larvae (supplementary material Fig. S2A-C), reached a maximum at 12-16 h (supplementary material Fig. S2D,E) and decreased to a minimum at late L2 stage (supplementary material Fig. S2F,G). During L3 stage (supplementary material Fig. S2H-T), peaks of expression were observed at 8 h (supplementary material Fig. S2J), 24 h (supplementary material Fig. S2N) and 36-44 h (supplementary material Fig. S2Q-S), and then E75A levels dropped at late L3 stage (supplementary material Fig. S2T). E75B (supplementary material Fig. S3A-T) is weakly detected only in the cytoplasm. Low levels of nuclear DHR3 were detected at 4-8 h during L2 stage (supplementary material Fig. S4A-C). A peak of expression was observed at 12-16 h then DHR3 disappeared at late L2 stage (supplementary material Fig. S4D-G). During L3 stage, three peaks of nuclear DHR3 were detected at 8 h, 24-28 h and 44-48 h (supplementary material Fig. S4H-T). βFtz-f1 was highly expressed in early L2 (supplementary material Fig. S5A-C), became barely detectable at the mid-L2 stage (supplementary material Fig. S5D,E), and increased again after the L2 ecdysone peak (supplementary material Fig. S5F,G). βFtz-f1 expression was maintained in early L3 larvae (supplementary material Fig. S5H-J) and progressively decreased to become barely detectable past the mid-L3 stage (supplementary material Fig. S5K-P). At late L3 stage, βFtz-f1 expression slightly increased (supplementary material Fig. S5R-T). The observation that these four NRs are expressed in the PG at each larval stage suggests that this tissue responds to 20E.

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NR misexpression in the PG provokes developmental arrests rescued by 20E

To determine whether EcR, E75, DHR3 and βFtz-f1 are required for ecdysone production in the PG, we used the Gal4/UAS system to knock down each NR using specific *UAS-RNAi* transgenes (hereafter referred to as *NRx-RNAi*). To monitor gene silencing, we examined both transcript and protein levels in RNAi-expressing flies. Transcripts were quantified by real-time quantitative PCR using a ubiquitous *tub-gal4* driver combined with a ubiquitous thermosensitive form of the Gal4 inhibitor, Gal80^{ts} (*tub-gal80^{ts}*) that blocks Gal4 activity at 21°C but not at 29°C, thereby allowing RNAi expression after the temperature shift. Each *NR-RNAi* was ubiquitously induced during the late L3 stage and transcript levels were measured every hour during the prepupal stage. At each time point analysed, the RNAi dropped the total amount of the corresponding NR transcript (supplementary material Fig. S6A-D). Protein levels were analysed by immunostaining in PG containing clonal cells generated by random recombination (flip-out) to express each RNAi (supplementary material Fig. S6A′-D′). In this setting, each *RNAi* induced almost complete protein extinction (supplementary material Fig. S6A″-D″).

Next, we induced various *UAS* transgenes with the *phm-gal4* driver, which is active from late embryogenesis in the steroidogenic tissue. Considering that ecdysteroids induce moulting and metamorphosis, we anticipated that knocking down an NR required for steroid production would lead to developmental arrest. We carried out experiments at 25°C and 29°C, as higher temperatures increase Gal4/UAS efficacy. This silencing provoked differential developmental arrests depending on the targeted NR. Knockdown of *EcR* at 29°C provoked developmental arrest at the end of the L3 stage; however, at 25°C, several escapers underwent metamorphosis (Fig. 2A,B). The L3-arrested larvae kept foraging and continued to grow for several more days (supplementary material Fig. S7F-H). Consistently, directing an EcR-dominant

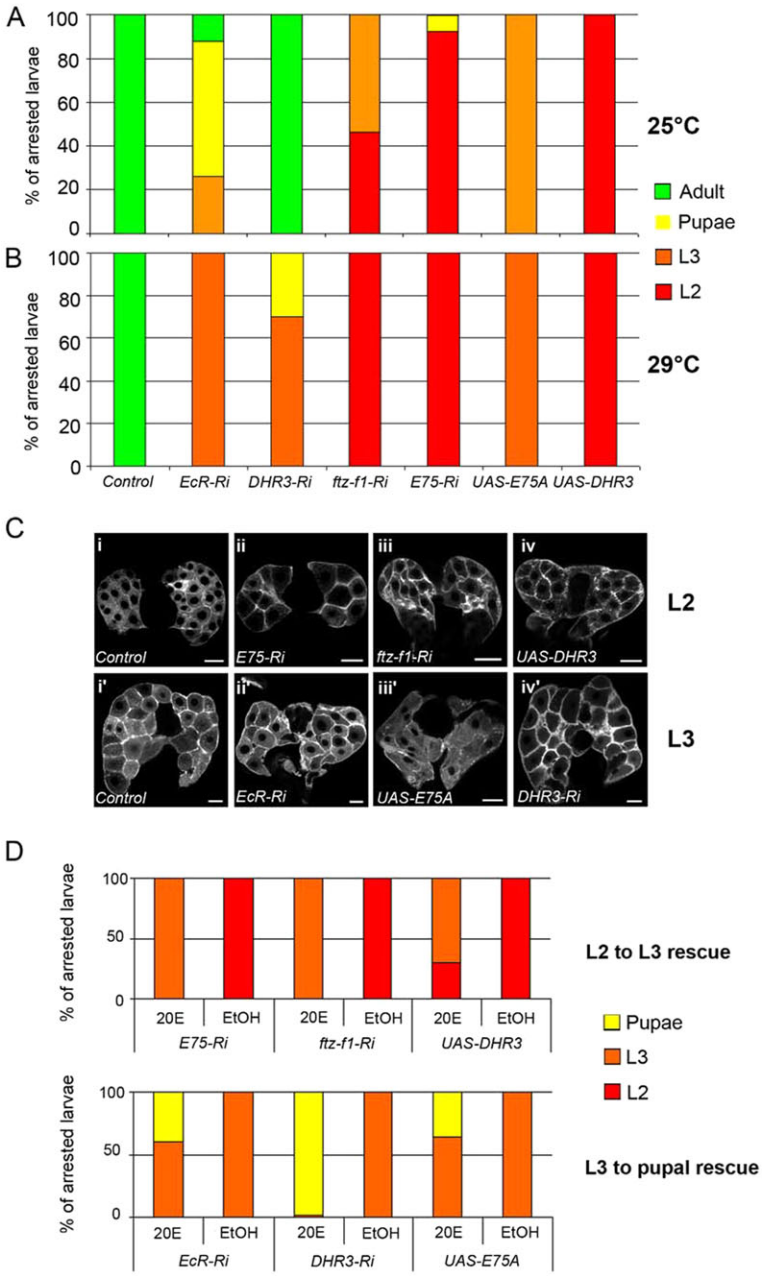

Fig. 2. NR misregulations provoke developmental arrests suppressed by ecdysone feeding. (A,B) Quantification of the phenotypes induced by misexpression of NRs using the *phm-gal4* driver. RNAi (*NRx-Ri*) and overexpressing (*UAS-NRx*) lines are indicated at the bottom. The percentages of the various phenotypes are represented as proportional bars corresponding to developmental arrest at the late L2 (red), late L3 (orange) and pupal (yellow) stages, or to adult survival (green). The experiments were performed at 25°C (A) or 29°C (B). (C) Ring glands of late L2 (top) or late L3 (bottom) larvae expressing various transgenes together with a *UAS-mCD8::GFP*, using the *phm-gal4* driver. Top panel from left to right: control (i), *E75-RNAi* (ii), *ftz-f1-RNAi* (iii), *UAS-DHR3* (iv). Bottom panel from left to right: control (i′), *EcR-RNAi* (ii′), *UAS-E75* (iii′), *DHR3-RNAi* (iv′). Scale bars: 20 µm. (D) The developmental arrests at late L2 (top) or L3 (bottom) stages induced by NR misregulation could be rescued on ecdysone-containing medium (20E) but not on control medium (EtOH). Rescue experiments were performed at 29°C. Phenotypes are indicated as in A,B. *DHR3-RNAi* shown (20157).

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negative form with the *phm-gal4* driver produced the same phenotype at 29°C (data not shown). At both temperatures, knockdown of E75 provoked a developmental arrest at the end of the L2 stage, although at 25°C, few larvae underwent pupariation (Fig. 2A,B and supplementary material Fig. S7A-C,K,L,N,O). Knockdown of DHR3 did not provoke developmental arrest at 25°C, whereas at 29°C it resulted in incomplete developmental arrest at the end of the L3 stage (Fig. 2A,B and supplementary material Fig. S7K,M). Expression of *ftz-f1-RNAi* in the PG led to developmental arrest at the late L2 stage either partial or total, when performed at 25°C or 29°C, respectively (Fig. 2A,B). The arrested L2 larvae continued to grow for up to 15 days and eventually died as giant L2 (supplementary material Fig. S7A,D,E). Using the same driver, overexpression of either DHR3 or E75A provoked developmental arrest at the late L2 and L3 stages, respectively (Fig. 2A,B and supplementary material Fig. S7F,I,J). Altogether, these findings indicate that these NRs differentially contributed to controlling the moulting transitions and suggest that in PG cells 20E signalling does not simply activate ecdysone biogenesis through the downstream NR βFtz-f1.

To determine whether expression of the above-mentioned RNAi-induced cellular defect in the PG cells may be responsible for the different phenotypes, the *NR-RNAi* were co-expressed with a *UAS-mCD8::GFP* using the *phm-gal4* driver. Observations of the ring glands did not reveal any visible cellular defect of the PG cells (Fig. 2C), indicating that the developmental arrests are not due to cell death but rather result from a dysfunction of steroidogenesis. To investigate whether ecdysteroid default was responsible for the developmental arrests, the feeding medium was supplemented with 20E. In the case of L2 arrest, feeding 20E rescued all the larvae expressing either *ftz-f1-RNAi* or *E75-RNAi* and 70% of the larvae overexpressing DHR3 (Fig. 2D). For late L3 arrest, feeding 20E rescued almost half of the larvae expressing either *EcR-RNAi* or *UAS-E75A* and the quasi-totality of the larvae expressing *DHR3-RNAi* (Fig. 2D). This 20E-dependent rescue demonstrates that the developmental arrests following NR misregulation in the PG cells are due to the lack of ecdysteroid at the L2 or L3 stages, showing that 20E controls its own biosynthesis.

NRs act through an unconventional hierarchy in the PG

To decipher the molecular mechanisms of 20E autoregulation, we investigated whether the canonical hierarchy of NRs is conserved in the PG. First, we analysed the expression of DHR3 and βFtz-f1 in flip-out clones expressing *EcR-RNAi*. In this setting, DHR3 levels were faintly decreased at L2 stage but strongly increased at late L3 stage (Fig. 3A-F). βFtz-f1 levels were decreased in *EcR-RNAi* flip-out cells of either late L2 or early L3 larvae (Fig. 3G-L). These findings indicate that EcR is required for the accurate dynamic expression of DHR3 and βFtz-f1.

These observations prompted us to investigate whether DHR3 controls βFtz-f1 expression in PG cells, as reported for other tissues (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997). Therefore, we analysed βFtz-f1 levels in ring glands containing homozygous *DHR3* mutant cells. We induced random mitotic recombination during embryogenesis and monitored βFtz-f1 expression at the mid-L2 (8-16 h past the L1/L2 moult), late L2 (16-24 h past the L1/L2 moult), early L3 (0-8 h past the L2/L3 moult), mid-L3 (20 to 28 h past L2/L3 moult) and late L3 (40 to 48 h past L2/L3 moult) stages. Surprisingly, the consequence of the *DHR3* mutation on βFtz-f1 protein expression varied with the developmental stage. At mid-L2 or late L3 stages, when DHR3 is normally expressed and βFtz-f1 barely detected, there was no effect on βFtz-f1 expression in *DHR3* mutant cells (Fig. 3M,N,Q and data not shown). At late L2 stage, when βFtz-f1 is strongly expressed, most of the *DHR3* homozygous mutant cells exhibited a severe drop in βFtz-f1 levels compared with control cells (Fig. 3O-Q). By contrast, at later stages, when βFtz-f1 expression decreased in control cells, far fewer *DHR3* mutant cells exhibited a visible drop in

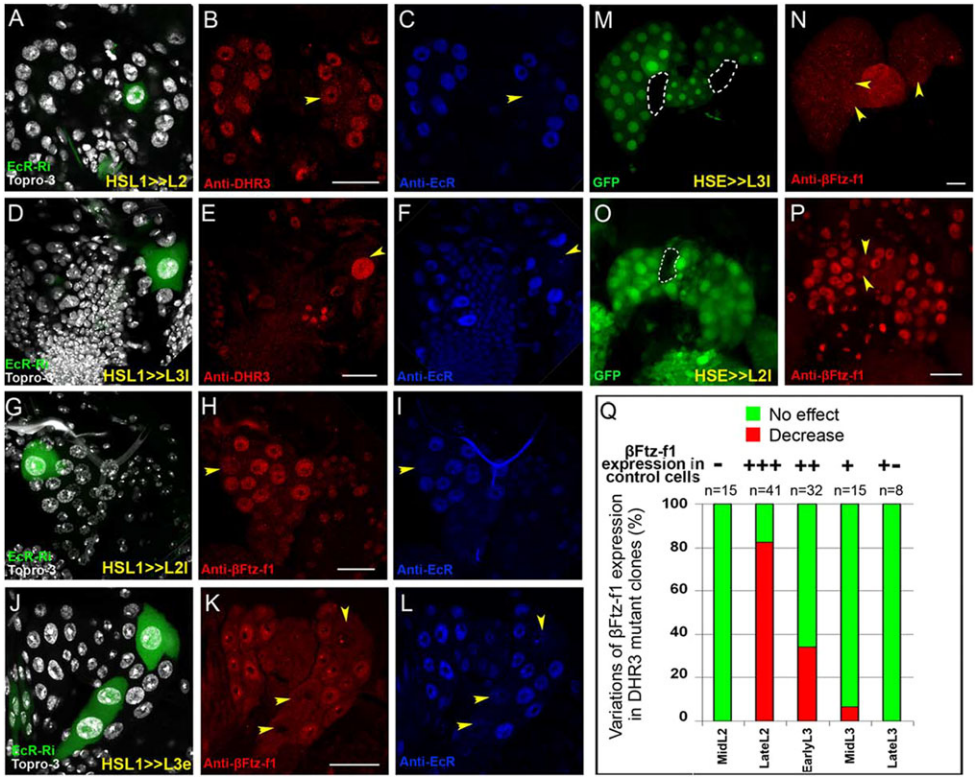

Fig. 3. EcR is not required for DHR3 and βFtz-f1 expression. (A-L) *EcR-RNAi* flip-out clones marked by GFP (A,D,G,J, arrowheads in the corresponding pictures) were stained for EcR (C,F,I,L) and DHR3 (B,E) or βFtz-f1 (H,K) in ring glands of either L2 (A-C,G-I) or L3 (D-F,J-L) larvae. Nuclear labelling (A,D,G,J). (M-P) βFtz-f1 expression (N,P) in homozygous $DHR3^{G60S}$ mutant clones in ring glands analysed at late L3 (M,N) or late L2 (O,P) stages. Clones are labelled by the lack of GFP (dashed lines in M,O and arrowheads in N,P). Scale bars: 20 µm. (Q) βFtz-f1 expression in homozygous $DHR3^{G60S}$ mutant clones. The percentages of the various phenotypes are represented as proportional bars corresponding to either a decrease in (red) or no effect (green) on βFtz-f1 expression. βFtz-f1 levels are indicated at the top. Number of ring glands analysed that exhibited $DHR3^{G60S}$ mutant clones are indicated for each developmental stage (*n*). In all pictures of clones, the timing of the heat shock (stageX) and of the dissection (stageY) is indicated as HSstageX≫stageY; developmental stages: embryogenesis (E), L1 stage (L1), L2 stage past the 20E peak (L2), late L2 stage (L2l), L2/L3 transition (L2/L3), early (L3e) or late (L3l) L3 stages.

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βFtz-f1 expression levels (Fig. 3Q and data not shown). In summary, although DHR3 is required for the highest βFtz-f1 expression levels before the L2/L3 moulting transition, it is dispensable at later stages, suggesting that βFtz-f1 can eventually be induced through a DHR3-independent mechanism.

Lowering DHR3 can rescue L2 developmental arrests

To investigate the hierarchy of E75, βFtz-f1 and DHR3 in PG cells, we performed genetic interactions using *UAS* transgene combinations. To normalize the number of UAS promoters in the combination settings, we buffered Gal4 activity with a *UAS-GFP* when driving only a single *UAS* transgene. In addition, to verify RNAi efficacy, knockdown has been checked in flip-out clones expressing RNAi combinations (supplementary material Fig. S8). Immunostaining confirmed that each RNAi line was efficient to drop protein levels of the targeted NR (supplementary material Fig. S8A,B,E-O), whereas co-expression of *ftz-f1-RNAi* and *UAS-E75A* did not impede E75A overexpression (supplementary material Fig. S8C,D). The genetic interactions were performed at 25°C using the *phm-gal4* driver. *E75-RNAi* led to developmental arrest at L2 (74%) or pupal (26%) stages, whereas *DHR3-RNAi* did not provoke a visible phenotype (Fig. 4A). Expressing *E75-RNAi* and *DHR3-RNAi* together provoked an intermediate phenotype: 40% of L2 larvae, 6% of pupae and 54% as adults (Fig. 4A). These observations indicate that DHR3 downregulation can partially rescue the phenotype induced by E75 knockdown.

The knockdown of βFtz-f1 provoked developmental arrest: at the late L2 (20%) or late L3 (80%) stages (Fig. 4A). This phenotype was partially rescued by co-expression of *ftz-f1-RNAi* with either *UAS-E75A* or *DHR3-RNAi* (Fig. 4A). In these genetic combinations, no arrested L2 larvae were observed, and some giant L3 (25%) and many pupae (75%) formed, although the pupae never emerged as adult. This phenotypic suppression indicates that lowering DHR3 or increasing E75 expressions in PG cells counteracts βFtz-f1 knockdown. Consistently, we observed that flip-out PG cells of L3 larvae expressing *ftz-f1-RNAi* exhibited a moderate increase of nuclear DHR3 levels (Fig. 4B,C), a phenotype that could also be observed in homozygous *ftz-f1*^{ex7} PG mutant cells at late L3 stage but barely at L2 stage (Fig. 4D-G). This increased nuclear DHR3 disappeared when co-expressing *ftz-f1-RNAi* and *DHR3-RNAi* (Fig. 4H,I). Importantly, the increase in DHR3 levels in *ftz-f1-RNAi* flip-out cells was observed in late L3 larvae while βFtz-f1 was weakly expressed in the PG (Fig. 4F,G). Considering that E75 has been described to counteract DHR3 activity (Palanker et al., 2006; Reinking et al., 2005) and that DHR3 overexpression provokes developmental arrest at late L2 stage, the developmental arrest due to *E75-RNAi* or *ftz-f1-RNAi* could be a consequence of higher DHR3 activity.

DHR3 represses steroidogenic enzyme expression

Given that βFtz-f1 is required for expression of the steroidogenic enzymes Phm and Dib in L3 larvae (Parvy et al., 2005), we investigated the influence of DHR3 on steroidogenic enzyme expression in late L3 larvae. Consistently, Phm levels decreased in flip-out PG cells expressing *ftz-f1-RNAi* (Fig. 5A,B), a phenotype that we also observed in *E75-RNAi* flip-out cells (Fig. 5C,D). As lowering DHR3 counteracts the developmental arrests induced by *ftz-f1-* and *E75-RNAi*, we hypothesized that downregulation of Phm expression might directly depend on DHR3-mediated repression. Immunostaining revealed that PG cells overexpressing the *UAS-DHR3* transgene exhibited a dramatic drop in Phm, Dib and Sad expression in late L3 larvae (Fig. 5E,F; supplementary material Fig. S9A-D). Conversely, Phm and Dib expression significantly increased in homozygous *DHR3* mutant cells (Fig. 5G,H; supplementary material Fig. S9E,F) indicating that, at the late L3 stage, DHR3 represses steroidogenic enzymes. However, the expression of Phm did not appear to be increased in *DHR3-RNAi* flip-out clones (Fig. 5I,J) possibly due to incomplete DHR3 knockdown. Nevertheless, *DHR3-RNAi* was able to restore Phm

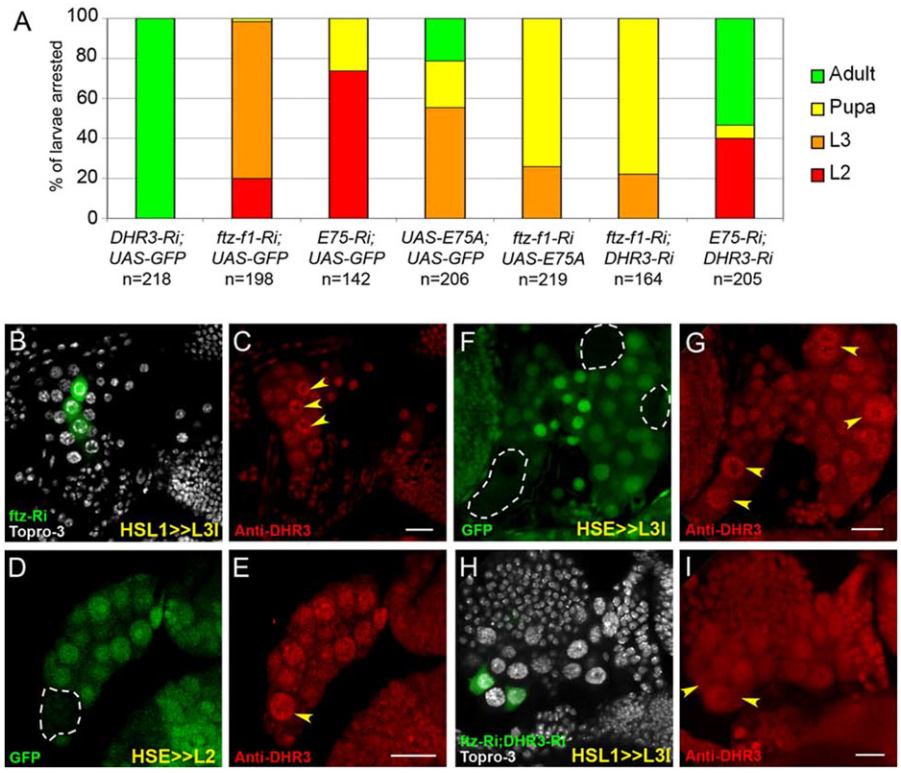

Fig. 4. Genetic hierarchy of NRs in the ring gland. (A) Quantification of the phenotypes obtained by inducing various transgene combinations in the ring gland. The *phm-gal4* driver has been used to induce combinations of two of these transgenes: *UAS-GFP*, *DHR3-RNAi* (20157 when used alone), *ftz-f1-RNAi*, *E75-RNAi* and *UAS-E75A*. Phenotype quantification is represented as in Fig. 2B (*n*: number of animals analysed). (B-I) DHR3 staining (C,E,G,I) of ring glands containing GFP-labelled flip-out clones (B,H, arrowheads in C,I) or homozygote *ftz-f1*^{ex7} mutant clones marked by the lack of GFP (dashed line in D,F, arrowheads in E,G). DHR3 levels in *ftz-f1-RNAi* flip-out clones of late L3 larvae (B,C), and in *ftz-f1*^{ex7} mutant clones of late L2 (D,E) or late L3 (F,G) larvae. The increased in nuclear DHR3 levels is lost in flip-out clones co-expressing *DHR3-RNAi* with *ftz-f1-RNAi* (H,I). Scale bars: 20 µm. For clonal analysis the stages of heat shock and dissection are indicated as in Fig. 3.

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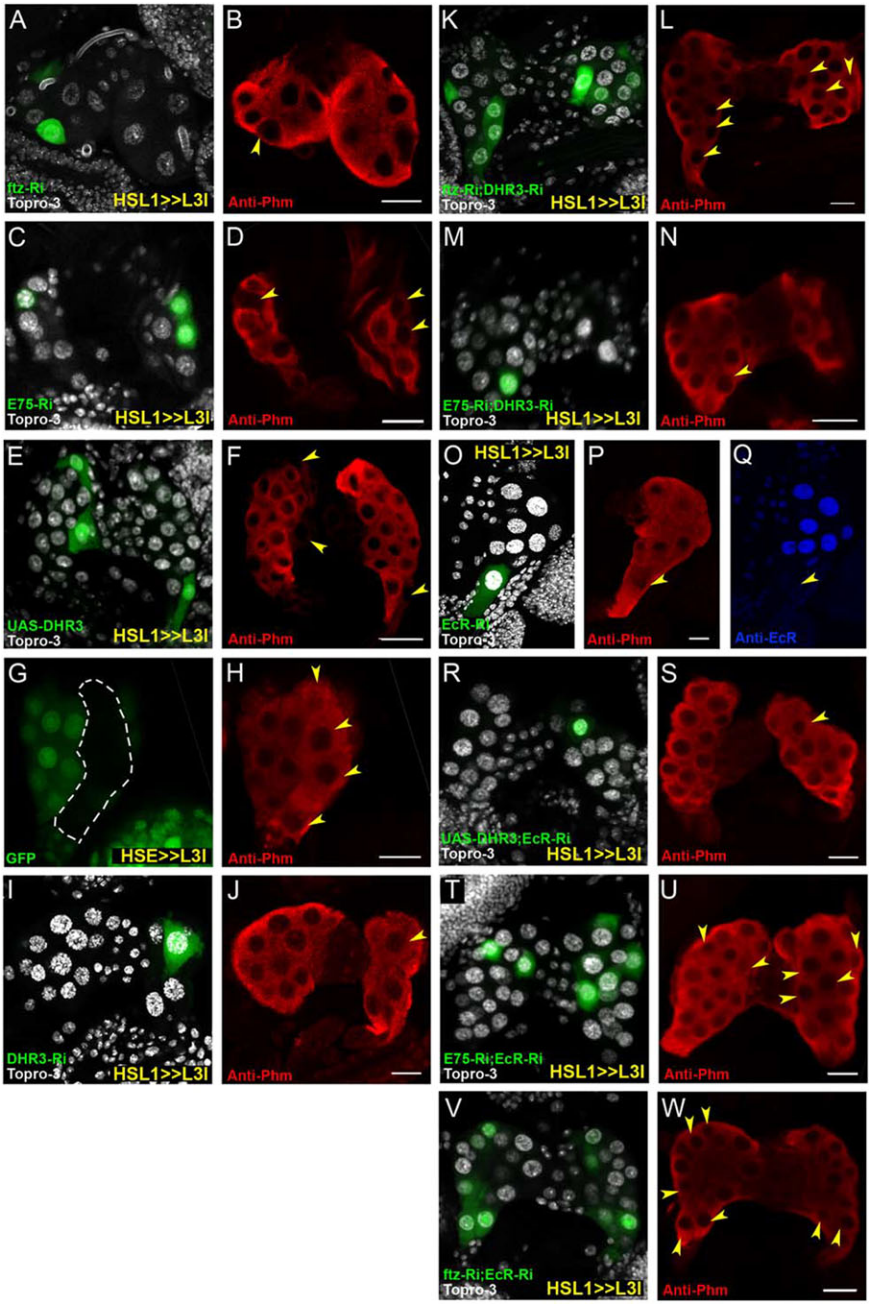

Fig. 5. DHR3 suppresses Phm protein levels. Nuclear (A,C,E,I,K,M,O,R,T, V), Phm (B,D,F,J,L,N,P,S,U,W) and EcR (Q) staining in ring gland containing flip-out clones labelled by GFP (A,C,E,I,K,M,O,R,T,V, arrowheads in B, D,F,J,L,N,P,Q,S,U,W) or *DHR3*^{G60S} mutant clones labelled by the absence of GFP (dashed line in G, arrowheads in H). Phm protein levels in flip-out clones expressing *ftz-f1-RNAi* (A,B), *E75-RNAi* (C,D), *UAS-DHR3* (E,F), in *DHR3*^{G60S} mutant clones (G,H), and in *DHR3-RNAi* (20157) flip-out clones (I,J). Phm levels in flip-out clones co-expressing *DHR3-RNAi* with *ftz-f1-RNAi* (K,L) or *E75-RNAi* (M,N). Phm and EcR expression in *EcR-RNAi* flip-out clone (O-Q). Phm levels in flip-out clones co-expressing *EcR-RNAi* with *UAS-DHR3* (R,S), *E75-RNAi* (T,U) or *ftz-f1-RNAi* (V,W). Scale bars: 20 µm. For clonal analysis the stages of heat shock and dissection are indicated as in Fig. 3.

levels when co-expressed with either *ftz-f1-RNAi* or *E75-RNAi* (Fig. 5K-N). The Phm levels were also restored when coexpressing *UAS-E75* with *ftz-f1-RNAi* (supplementary material Fig. S9G,H). Downregulation of Phm may be responsible for the developmental arrest induced by DHR3 overexpression or activity in late L3 larvae. By contrast, we could not detect repression of Phm in flip-out PG cells of L2 or early L3 larvae expressing either *E75-RNAi*, *ftz-f1-RNAi* or *UAS-DHR3* (supplementary material Fig. S9I-P) indicating that DHR3 can induce an ecdysone-dependent developmental arrest at the L2/L3 transition through a Phm-independent process. Taken together, these findings indicate that neither βFtz-f1 nor E75 directly activate the expression of steroidogenic enzymes, but rather maintain Phm levels by counteracting DHR3-mediated repression.

As DHR3 expression increased in *EcR-RNAi* PG cells in late L3 larvae (Fig. 3D-F), the expression of Phm might also be downregulated in these cells. Surprisingly, Phm levels remained unaffected in *EcR-RNAi* flip-out clones of late L3 larvae (Fig. 5O-Q), albeit with higher DHR3 levels. Consistently, Phm expression levels were not affected in flip-out clones expressing the dominant negative form of EcR (supplementary material Fig. S9T,U). As EcR has been shown to physically interact with DHR3 (White et al., 1997), we hypothesized that EcR could be required for DHR3-mediated repression of Phm. Accordingly, the drop in Phm levels was not observed in *UAS-DHR3* PG cells that co-express *EcR-RNAi* (Fig. 5R,S), indicating that DHR3 requires EcR to repress Phm expression. In addition, repression of Phm was no longer observed in either *E75-RNAi* or *ftz-f1-RNAi* flip-out clones of late L3 larvae that co-express *EcR-RNAi* (Fig. 5T-W). Hence, DHR3 emerges as a crucial negative regulator of steroidogenic enzyme expression and requires EcR in this process.

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βFtz-f1 and EcR exert a forward effect to modulate DHR3 activity

To understand how EcR and βFtz-f1 restrain DHR3 expression in late L3 larvae, we first determined the window of time during which this regulatory process can be induced. Given that RNAi silencing is fully efficient 20 h after clonal recombination (supplementary material Fig. S10A-D), flip-out clones were induced at the L2/L3 transition and analysed 48 h later, before the L3/pupal transition. In this setting, DHR3 expression remained unaffected in either *EcR-RNAi* or *ftz-f1-RNAi* flip-out clones (Fig. 6A-D), whereas we previously showed an increase in DHR3 levels in early-induced clones (Fig. 3D-F and Fig. 4B,C). These findings indicate that both EcR and βFtz-f1 act early in development, to restrain DHR3 expression at the late L3 stage. Considering that EcR is required to maintain βftz-f1 expression in late L2 larvae (Fig. 3G-I), EcR might act through βftz-f1 to restrain DHR3 expression in late L3 larvae. Nevertheless, we wonder whether βftz-f1 could regulate EcR expression through a potential feedback loop. To address this issue, EcR levels were monitored in *ftz-f1-RNAi* flip-out clones. No effect were observed at mid-L2 stage (data not shown), while at late L2 stage EcR levels increased in the *ftz-f1-RNAi* clonal cells (Fig. 6E,F), indicating that depending on the development stage, βFtz-f1 may impede EcR expression. Consistently, EcR levels dropped dramatically in *UAS-βftz-f1* flip-out clones analysed at L2 or L3 stages (Fig. 6G-J). By contrast, most of the *ftz-f1-RNAi* clonal cells (70%) exhibited a mild decrease in EcR levels in early L3 larvae (Fig. 6K,L and data not shown); this phenotype was fully penetrant at the end of the L3 stage (Fig. 6M,N), indicating that βFtz-f1 is required to maintain EcR expression at this stage. Taken together, our findings unravel

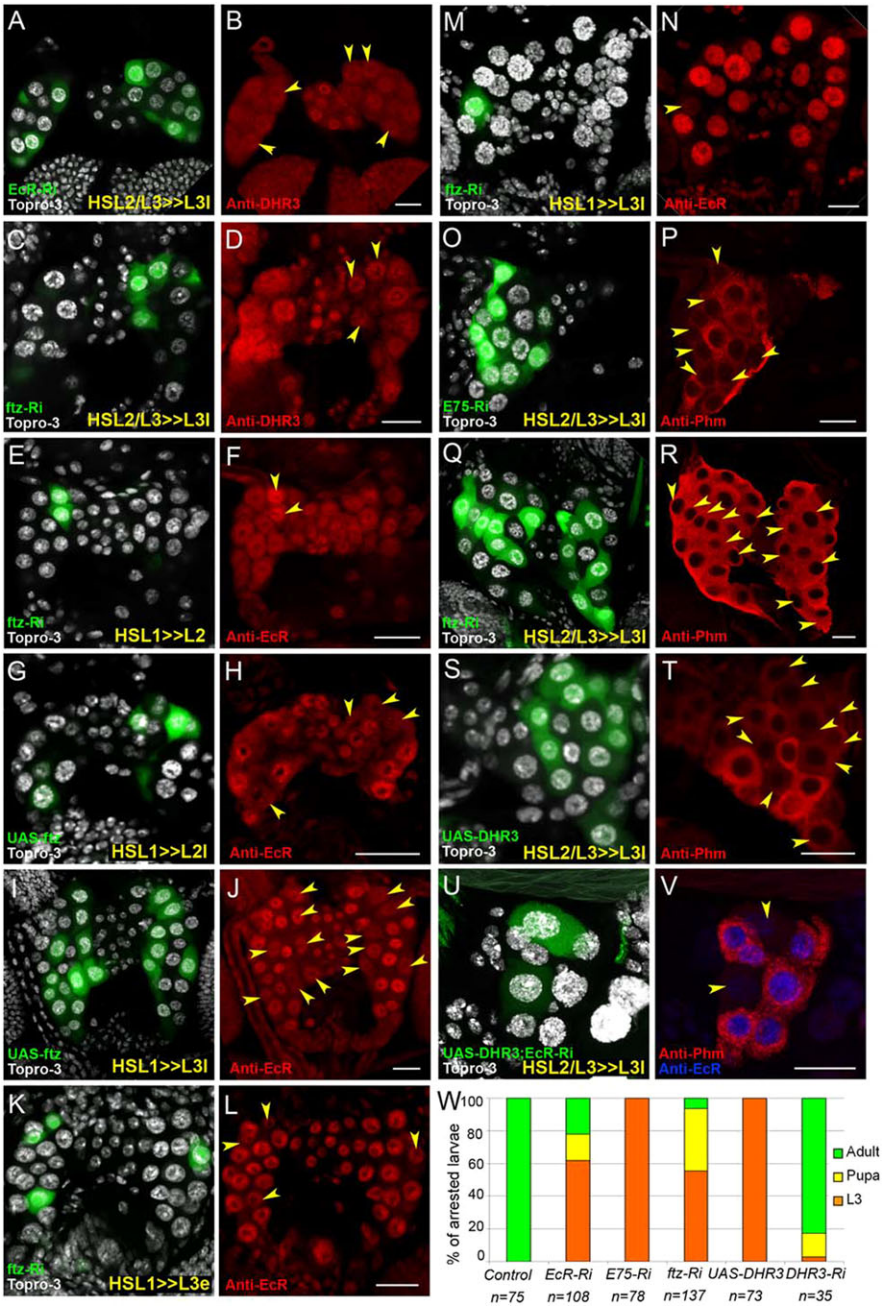

Fig. 6. Conditional misexpression of the NRs in the ring gland during development. (A-V) Flip-out clones labelled by GFP (A,C,E,G,I,K,M,O,Q,S,U, arrowheads in B,D,F,H,J,L,N,P,R,T,V) were stained for DHR3 (B,D), EcR (F,H,J,L,N,V) or Phm (P,R,T,V) protein. DHR3 protein levels in *EcR-RNAi* (A,B) or *ftz-f1-RNAi* (C,D) flip-out clones. (E-N) EcR protein levels in flip-out clones expressing *ftz-f1-RNAi* (E,F,K-N) or *UAS-βftz-f1* (G-J) analysed at L2 or L3 stages. Phm protein levels in flip-out clones expressing *E75-RNAi* (O,P), *ftz-f1-RNAi* (Q,R), *UAS-DHR3* (S,T). Phm and EcR protein levels in flip-out clones co-expressing *EcR-RNAi* and *UAS-DHR3* (U,V). For clonal analysis the stages of heat shock and dissection are indicated as in Fig. 3. (W) Quantification of the phenotypes produced by various transgenes induced in the PG following the temperature shift to 29°C at the L2/L3 transition. The induced transgenes are indicated from left to right: control; *EcR-RNAi*, *E75-RNAi*, *ftz-f1-RNAi*, *DHR3-RNAi*, *UAS-DHR3*. Phenotype quantification is represented as in Fig. 2B (*n*: number of animals analysed).

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an unexpected epistatic hierarchy between βFtz-f1 and EcR: βFtz-f1 exerts a negative input on EcR expression past the L2 ecdysone peak and a positive input in L3 larvae. Potentially, as the repression of EcR expression occurs when βFtz-f1 is highly expressed (Fig. 1A), it is conceivable that high βFtz-f1 levels repress EcR expression. In summary, although it is likely that EcR and βFtz-f1 act early through a common mechanism to restrain DHR3 expression at late L3 stage, it is not possible to decipher the hierarchy of this regulation.

EcR plays a dual role in the PG, as it is necessary for the DHR3-mediated repression of Phm and for limiting DHR3 expression in late L3 larvae. Our findings reveal an apparent paradox: we observed a decrease in both Phm and EcR levels in *ftz-f1-RNAi* flip-out cells of late L3 larvae (Fig. 5A,B and Fig. 6M,N), while we provide evidence that EcR is required for the repression of Phm (Fig. 5S,T). We therefore hypothesized that, when it comes to restricting DHR3 expression, EcR may be required early in development to potentiate Phm downregulation at the late L3 stage. To address this issue, we induced flip-out clones at L2/L3 transition and monitored Phm expression 48 h later. We observed a severe drop in Phm levels in *E75-RNAi* but not in *ftz-f1-RNAi* flip-out clones (Fig. 6O-R). Together with previous studies (Reinking et al., 2005; White et al., 1997), these findings suggest that E75 counteracts DHR3 activity, whereas βFtz-f1 acts early to restrict DHR3 expression and subsequently to sustain Phm expression. In addition, we observed that *UAS-DHR3* downregulated Phm expression in PG cells of late L3 larvae, whether or not *EcR-RNAi* was co-expressed (Fig. 6S-V; supplementary material Fig. S9Q-S), suggesting that EcR is not required during the second half of the L3 stage for the DHR3-mediated repression of Phm.

Finally, we wondered whether the developmental arrests induced by NR misregulation depend on an early effect. To this end, we used the *phm-gal4*;*tub-gal80^{ts}* driver to delay the knockdown in the PG. Animals were reared at 18°C, then *EcR-RNAi*, *E75-RNAi*, *ftz-f1-RNAi*, *DHR3-RNAi* and *UAS-DHR3* were induced following a temperature shift to 29°C at L2/L3 transition. In this setting, all the transgenes were highly effective 15 h past the temperature shift, although small amounts of DHR3 and βFtz-f1 can still be detected (supplementary material Fig. S10E-W). Developmental analysis revealed that roughly 40% of the larvae expressing either *EcR-RNAi* or *ftz-f1-RNAi* enter metamorphosis (Fig. 6W), further supporting that βFtz-f1 and EcR act, at least in part, through an early input to control the next ecdysone peak. By contrast, all of the larvae expressing either *E75-RNAi* or *UAS-DHR3* were blocked at the late L3 stage (Fig. 6W), suggesting that these two NRs acts during the L3 stage to control the concurrent moulting transition. Nevertheless, DHR3 also appears to act through an early input, as the quasi-totality of *DHR3-RNAi* expressing larvae underwent pupariation (Fig. 6W).

DHR3 represses steroid synthesis

To ascertain that DHR3 may act as a repressor of 20E synthesis, we measured ecdysteroid titres in larvae overexpressing DHR3 in their PG using the *phm-gal4*. Compared with control larvae, overexpression of DHR3 totally abolished steroid production at mid L2 stage (Fig. 7A). To analyse ecdysteroid titres in L3 larvae the *tub-gal80ts;phm-gal4* driver was used to trigger transgene expression 24 h past the L2/L3 transition by switching animals from 18°C to 29°C. In this way, DHR3 overexpression induced a total arrest at late L3 stage (data not shown) and a severe drop in steroid titres at the timing of prepupal transition (Fig. 7B). Importantly, the steroid levels in these arrested larvae did not increase 24 h and 48 h later (Fig. 7B), indicating that DHR3 overexpression abolishes the

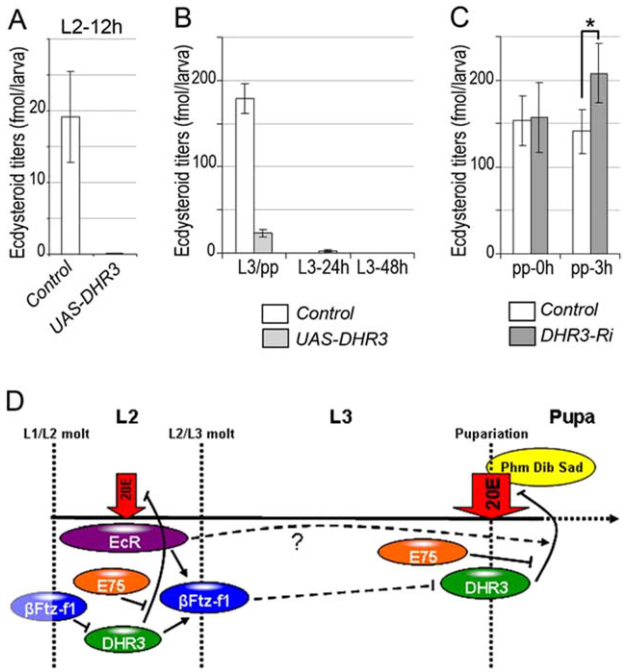

Fig. 7. Model for steroidogenesis autoregulation. (A) Ecdysteroid measurement (fmol per larva) in 12 h L2 larvae of the corresponding genotypes: *phm-gal4* (control) and *phm-gal4>UAS-DHR3* (*UAS-DHR3*). (B) Ecdysteroid measurement in *phm-gal4;tub-gal80^{ts}* (control) and *phm-gal4;tub-gal80^{ts}>UAS-DHR3* (*UAS-DHR3*) animals at the time of L3/prepupal transition (L3/pp) and 24 h or 48 h later in *phm-gal4;tub-gal80^{ts}>UAS-DHR3* larvae. (C) Ecdysteroid measurement in *phm-gal4;tub-gal80^{ts}* (control) and *phm-gal4;tub-gal80^{ts}>DHR3-RNAi* (*DHR3-Ri*, 20157) prepupae at the stage of puparium formation (pp-0 h) and 3 h later (pp-3 h). Animals were shifted to 29°C, 24 h past the L2/L3 transition (B,C). Statistical analysis: *t*-test *P<0.05. (D) DHR3 acts as a key repressor of steroidogenesis at the L2/L3 and L3/pupal transitions. At the late L3 stage, DHR3 activity contributes to shorten ecdysone biosynthesis through the inhibition of steroidogenic enzyme expression. To avoid premature DHR3-mediated repression, E75 counteracts DHR3 activity This DHR3-mediated repression requires early activity of EcR. In addition, βftz-f1 and EcR restrains DHR3 protein levels through an effect induced early in development. The ecdysone peaks are represented by red arrows. Dashed lines correspond to time-delayed effects, whereas bold lines correspond to concurrent effects.

ecdysteroid peak responsible for the prepupal transition. By contrast, triggering *DHR3-RNAi* 24 h past the L2/L3 transition did not impede metamorphosis onset (data not shown). Consistently, ecdysteroid titres in *DHR3-RNAi* animals remained unaffected at the prepupal transition, but significantly increased 3 h later (Fig. 7C). Taken together, these findings demonstrate that DHR3 is a bona fide repressor of steroid production.

DISCUSSION

Unconventional 20E signalling regulates steroidogenic activity of the PG

During the past few years, the *Drosophila* steroidogenic tissue has been extensively used to investigate how extrinsic and intrinsic parameters integrate to coordinate growth with developmental progression. Here, we show that EcR, E75, DHR3 and βFtz-f1, which mediate ecdysone signalling (King-Jones and Thummel, 2005), are expressed in the steroidogenic cells and are required for ecdysone synthesis during *Drosophila* larval development. These findings are consistent with previous studies showing that *E75A* mutation and βFtz-f1 disruption in the PG induce developmental arrest as a consequence of steroid deficiency (Bialecki et al., 2002;

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Talamillo et al., 2013), and that DHR3 downregulation leads to late L3-arrested larvae (Caceres et al., 2011). In addition to E75, DHR3 and βFtz-f1, our study reveals that disrupting EcR in the PG also leads to developmental arrest that can be rescued by ecdysone feeding. To investigate the role of this NR cascade in the steroidogenic tissue, we generated somatic clones (Xu and Rubin, 1993) so that the steroid titres are maintained in the whole animal, but some cells are defective for a given NR. In this way, we observed that flip-out clones expressing a dominant negative form of EcR do not affect Phm expression, whereas a recent study reported that expression of the same EcR variant in the whole PG represses steroidogenic enzyme expression (Moeller et al., 2013). This discrepancy suggests that affecting 20E regulators in the entire PG induces a systemic response and does not allow studying cell-autonomous regulations.

Gene expression analysis following 20E stimulation supports a model in which the ternary complex EcR/USP/20E activates the transcription of E75 and DHR3, which together control the delayed expression of βFtz-f1 (King-Jones and Thummel, 2005). Our findings confirm that the dynamics of βFtz-f1 expression relies on DHR3 stimulation (Kageyama et al., 1997; Lam et al., 1999; White et al., 1997), although we observed that βFtz-f1 can be induced later in *DHR3* mutant cells. Moreover, that EcR is dispensable for DHR3 expression in the steroidogenic tissue of L3 larvae raises the question of whether an EcR-independent 20E signalling (Srivastava et al., 2005) is active in PG cells. Unexpectedly, disrupting E75 or βFtz-f1 provokes developmental arrest at the end of the L2 stage, whereas disrupting EcR or DHR3 provokes developmental arrest at the end of the L3 stage. Moreover, downregulating DHR3 can rescue L2 arrest due to disruption of either E75 or βFtz-f1, which further supports the idea that an unconventional NR cascade regulates steroidogenesis. Alternatively, as these NRs respond to 20E cyclic production and can act through a delayed effect in PG cells (see below), it is possible that disrupting the 20E response at a given peak may affect the 20E response at the next peak, thus leading to an apparent unconventional response. Nonetheless, our findings place DHR3 rather than βFtz-f1 (Ou and King-Jones, 2013; Parvy et al., 2005; Rewitz et al., 2013; Yamanaka et al., 2013) as the downstream effector of the NR cascade in the control of steroid production (Fig. 7D).

DHR3 mediates a feedback control on steroid biosynthesis

Our study reveals that DHR3 represses steroidogenesis both at the L2 and L3 stages. At the late L3 stage, DHR3 downregulates expression of the steroidogenic enzymes Phm, Dib and Sad. Several studies describe DHR3 as a transcriptional activator whose activity is inhibited by E75 (Caceres et al., 2011; Palanker et al., 2006; Reinking et al., 2005; White et al., 1997), suggesting that DHR3 may act indirectly to repress steroidogenesis. We previously reported that βFtz-f1 was necessary for Phm and Dib expression (Parvy et al., 2005). Here, we show that βFtz-f1 does not directly activate the transcription of these enzymes, but acts by restraining DHR3 expression. Furthermore, we provide evidence that E75 also acts to restrict DHR3-mediated repression of Phm. Hence, DHR3 emerges as a major repressor of steroidogenesis. Nonetheless, discriminating between direct transcriptional suppression and indirect repression through the activation of an unknown intermediate suppressor will require an in-depth analysis of the direct DHR3 DNA targets.

The increased expression of Phm and Dib in DHR3 mutant cells together with the increased ecdysteroid levels in DHR3 deficient animals past the L3/prepupal transition indicates that DHR3 acts in a feedback loop to sharpen the peak of ecdysone (Fig. 7D). Consistent with this, previous works revealed that progression through metamorphosis requires 20E inactivation, an enzymatic reaction catalysed by Cyp18A1 (Guittard et al., 2011; Rewitz et al., 2010). These observations underscore the notion that the transient increase of ecdysone titres is as critical as the inactivation/clearance of the hormone. However, the fact that the development of Cyp18a1 mutants is not arrested earlier than the prepupal stage (Guittard et al., 2011; Rewitz et al., 2010) and that DHR3 does not repress the steroidogenic enzymes at the late L2 stage suggests that the drop in 20E titre is more critical at the onset of metamorphosis than it is for the L2/L3 transition. This discrepancy may be a consequence of the different 20E titres at the L2 versus late L3 stage, or alternatively because a larva excretes organic compounds, whereas a prepupa is a closed system (Lafont et al., 2012).

Our findings suggest that EcR acts through a mechanism that occurs early in development to potentiate the DHR3-dependent Phm downregulation at late L3 stage. In contrast to a previous study (White et al., 1997), it is unlikely that EcR and DHR3 physically interact in this downregulation, as the DHR3-mediated repression of Phm is observed even when EcR is knockdown from early L3 stage. Interestingly, EcR has been shown to work as a modifying cofactor of the chromatin structure (Sedkov et al., 2003). Moreover, in mammalian steroidogenic tissues, specific histone modifications are associated with the rapid increase in expression of the steroidogenic acute regulatory protein (StAR), the rate-limiting factor of steroidogenesis (Hiroi et al., 2004a,b). Therefore, it is conceivable that once past the L2 ecdysone peak, EcR might induce chromatin modifications, allowing DHR3 to mediate Phm repression at the L3/pupal transition. At this stage, this repression will stop ecdysone biosynthesis so that metamorphosis can proceed.

A forward control restrains the DHR3-mediated repression of steroidogenesis

Concerning the L3/pupal transition, our study indicates that βFtz-f1 and EcR act early in development to regulate DHR3 expression. By contrast, E75-RNAi and DHR3 overexpression provokes a developmental arrest at the end of the stage concurrent to their induction. These findings revealed that early and concurrent regulatory processes control steroidogenesis. Interestingly, we provide evidence that DHR3 act through both processes, as its downregulation provokes an L3 developmental arrest when knockdown is induced early in development but not past the L2/L3 transition. In addition, E75A overexpression provokes a phenotype similar to the one induced by DHR3 knockdown. Therefore, as shown in previous studies (Palanker et al., 2006; Reinking et al., 2005; White et al., 1997), E75A might directly counteract DHR3 activity to regulate steroidogenesis. Furthermore, E75 has been proposed to sustain and amplify ecdysone production through a feed-forward effect (Bialecki et al., 2002) suggesting that the developmental arrest observed in *E75A* mutants is due to the defect in ecdysone auto-amplification. In light of our study, the developmental arrest of *E75A* mutants is likely to be a direct consequence of premature DHR3-repressing activity, whereas our observation of early induced events unravels a step-forward process. This step-forward effect roughly originates soon after the L2 20E peak to moderate later DHR3 expression. Earlier studies have reported that in the salivary glands, the response to 20E requires a gap to acquire competence for re-induction (Richards, 1976a,b) and that βFtz-f1 is a competent factor in this process (Broadus et al., 1999; Woodard et al., 1994). Competence is described as the acquired ability of one group of cells to respond to a developmental

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signal, while other cells do not respond to this signal (Waddington, 1940). In the case of 20E responsiveness, the gap for acquisition of competence is associated with low ecdysone titres and high βFtz-f1 levels. Our findings support the notion that βFtz-f1 also acts as a competent factor for ecdysone biogenesis through a step-forward moderation of DHR3 expression. In addition, as both EcR and DHR3 knockdown also act through an early induced event and affect βFtz-f1 expression, it is conceivable that they participate in the competence acquisition through βFtz-f1. Although the molecular mechanisms that link βFtz-f1 and EcR to DHR3 must still be elucidated, our study reveals that the response following the L2 ecdysone peak is necessary to confer competence for ecdysone biogenesis at the late L3 stage by delaying the DHR3-mediated repression of steroidogenic enzymes.

In summary, our study unravels an autoregulatory mechanism in cyclic ecdysone production. This autoregulation is likely to be coordinated with the processes that adjust ecdysone biogenesis at the L3 stage in response to environmental cues. These include nutrition, insulin signalling and the circadian rhythm that integrates through the prothoracicotropic hormone (PTTH) (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; McBrayer et al., 2007; Mirth, 2005). Interestingly, a downstream effector of PTTH is the NR DHR4, also described to modulate the ecdysone response at the onset of metamorphosis (King-Jones et al., 2005; Ou et al., 2011). However, the fact that *DHR4* mutants are not arrested earlier than the prepupal stage, while each *RNAi* tested here provokes a significant arrest at larval stages, suggests that DHR4 acts through an independent mechanism. Moreover, as intermediates of the nutrient, insulin and PTTH signalling interact with NRs that respond to 20E (Colombani et al., 2005; Montagne et al., 2010; Ou et al., 2011), our study provides a framework to further investigate how environmental parameters integrate with the autoregulation of cyclic ecdysone production.

MATERIALS AND METHODS

Drosophila handling and genetics

Fly strains: w^{1118}; *UAS-DHR3,* (Montagne et al., 2010), $DHR3^{G60S}$ (Carney et al., 1997), $ftz\text{-}f1^{ex7}$ (Yamada et al., 2000), *UAS-βftz-f1*, *UAS-E75A*, $UAS\text{-}EcR^{F645A}$ (Cherbas et al., 2003), *phm-gal4*, $tub\text{-}gal80^{ts}$;*tub-gal4, hs-flp;ubi-nls-GFP,FRT2A, hs-flp;ubi-nls-GFP,FRT42D, hs-flp,actin5C-FRT-CD2-FRT-gal4,UAS-nls-GFP*; *UAS-RNAi* lines from VDRC (Dietzl et al., 2007) targeting *EcR* (37058), *E75* (44851), *ftz-f1* (2959), *DHR3* (20157, 106837, 12044). The various DHR3 RNAi lines provided similar results when used alone. For transgene combinations, the 20157 or 12044 were used depending on the chromosome location of the associated transgene.

Standard feeding medium contained 7.5% corn flour, 7.5% yeast, 1% agar and 0.35% moldex. For synchronization, larvae were grown in agar plates supplemented with standard medium and staged each hour, at the L1/L2 and L2/L3 transitions. For immunostaining, ten to 20 ring glands were examined at each time point. FRT-mediated mitotic recombination and flip-out clones were generated as previously described (Parvy et al., 2005, 2012). Larvae raised at 25°C were heat shocked for 1 h at 37°C at early L1 stage or L2/L3 transition.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature, rinsed in PBT (phosphate-buffered saline plus 0.5% Triton X-100) and blocked for 30 min in PBT containing 2% bovine serum albumin (PBT-BSA). Samples were incubated overnight in PBT-BSA containing the primary antibody at 4°C, washed in PBT and incubated for 2 h with goat anti-rabbit A568 or goat anti-mouse A488 secondary antibodies at a 1:1000 dilution (Molecular Probes). Nuclear labelling was performed by incubating the tissues with a 0.01 mM solution of TO-PRO-3-iodide (Invitrogen) in DABCO (Sigma) for 2 days at 4°C. To study PG cells, phenotypes of arrested larvae, ten brain-ring gland complexes were dissected for each genotype of either late L2 or late L3 larvae, fixed as described above and rinsed three times in PBT. Samples were mounted in DABCO and examined using Nikon (TE-2000-U) and Leica (SP8) confocal microscopes.

Primary antibodies: anti-DHR3 at 1:50 (Montagne et al., 2010); anti-Ftz-f1 at 1:20,000 (Ohno et al., 1994); anti-Phm and anti-Sad at 1:1000; anti-EcR and anti-Dib at 1:200 (Ag102 at Developmental Studies Hybridoma Bank). E75 core antiserum was produced by Eurogentec with the peptides ^{925}RKLDSPTDSGIESGN^{939} and ^{1125}AEPRMTPEQMKRSDI^{1139} to immunize rabbits and affinity purified as previously described (Montagne et al., 2010) and used at 1:200 dilution. The E75A or E75B antiserums were previously described (Hill et al., 1993; Schubiger and Truman, 2000).

Ecdysteroid feeding and measurement

Ecdysteroid feeding rescue was performed using 1.5 ml of warm-melted medium mixed with either 50 µl or 150 µl of a 20E stock solution (10 mg/ml 20E in 95% ethanol) for L2 to L3 rescue or L3 to pupal rescue, respectively. Controls contained either 50 µl or 150 µl of a 95% ethanol solution. For 20E rescue mid L2 or late L3 larvae were transferred on appropriate media, and the larvae that underwent moulting transition were counted the following day.

Ecdysteroids were extracted and quantified as previously described (Parvy et al., 2005) using the L2 antiserum. Each determination was made on two (UAS-DHR3 in Fig. 7A) or at least five replicates and the results were expressed as mean values±s.e.m. For each replicate, 20 (Fig. 7A) or 10 (Fig. 7B,C) animals were used.

RT-QPCR experiments

Reverse transcription and quantitative PCR were performed as previously described (Parvy et al., 2012) using two independent groups of 20 larvae for each time point.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.-P.P. and J.M. conceived the study, designed and analysed experiments, wrote the manuscript. All authors performed experiments.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102020/-/DC1

References

Beydon, P. and Lafont, R. (1983). Feedback inhibition of ecdysone production by 20-hydroxyecdysone in Pieris brassicae pupae. *J. Insect Physiol.* **29**, 529-533.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W. A. and Thummel, C. S. (2002). Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in Drosophila. *Dev. Cell* **3**, 209-220.

Bodenstein, D. (1994). The postembryonic development of Drosophila. In *Biology of Drosophila* (ed. M. Demerec), pp. 275-367. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Broadus, J., McCabe, J. R., Endrizzi, B., Thummel, C. S. and Woodard, C. T. (1999). The Drosophila beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell* **3**, 143-149.

Caceres, L., Necakov, A. S., Schwartz, C., Kimber, S., Roberts, I. J. H. and Krause, H. M. (2011). Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. *Genes Dev.* **25**, 1476-1485.

Caldwell, P. E., Walkiewicz, M. and Stern, M. (2005). Ras activity in the Drosophila prothoracic gland regulates body size and developmental rate via ecdysone release. *Curr. Biol.* **15**, 1785-1795.

Carney, G. E., Wade, A. A., Sapra, R., Goldstein, E. S. and Bender, M. (1997). DHR3, an ecdysone-inducible early-late gene encoding a Drosophila nuclear receptor, is required for embryogenesis. *Proc. Natl. Acad. Sci. USA* **94**, 12024-12029.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E. and Cherbas, P. (2003). EcR isoforms in Drosophila: testing tissue-specific requirements by targeted blockade and rescue. *Development* **130**, 271-284.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carré, C., Noselli, S. and Léopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in Drosophila. *Science* **310**, 667-670.

Dell, S., Sedlmeier, D., Böcking, D. and Dauphin-Villemant, C. (1999). Ecdysteroid biosynthesis in crayfish Y-organs: feedback regulation by circulating ecdysteroids. *Arch. Insect Biochem. Physiol.* **41**, 148-155.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448**, 151-156.

Guittard, E., Blais, C., Maria, A., Parvy, J.-P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P. J. and Dauphin-Villemant, C. (2011). CYP18A1, a key enzyme of Drosophila steroid hormone inactivation, is essential for metamorphosis. *Dev. Biol.* **349**, 35-45.

Hill, R. J., Segraves, W. A., Choi, D., Underwood, P. A. and Macavoy, E. (1993). The reaction with polytene chromosomes of antibodies raised against Drosophila E75A protein. *Insect Biochem. Mol. Biol.* **23**, 99-104.

Hiroi, H., Christenson, L. K., Chang, L., Sammel, M. D., Berger, S. L. and Strauss, J. F., III. (2004a). Temporal and spatial changes in transcription factor binding and histone modifications at the steroidogenic acute regulatory protein (stAR) locus associated with stAR transcription. *Mol. Endocrinol.* **18**, 791-806.

Hiroi, H., Christenson, L. K. and Strauss, J. F., III. (2004b). Regulation of transcription of the steroidogenic acute regulatory protein (StAR) gene: temporal and spatial changes in transcription factor binding and histone modification. *Mol. Cell. Endocrinol.* **215**, 119-126.

Hiruma, K. and Riddiford, L. M. (2004). Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3. *Dev. Biol.* **272**, 510-521.

Horner, M. A., Chen, T. and Thummel, C. S. (1995). Ecdysteroid regulation and DNA binding properties of Drosophila nuclear hormone receptor superfamily members. *Dev. Biol.* **168**, 490-502.

Kageyama, Y., Masuda, S., Hirose, S. and Ueda, H. (1997). Temporal regulation of the mid-prepupal gene FTZ-F1: DHR3 early late gene product is one of the plural positive regulators. *Genes Cells* **2**, 559-569.

King-Jones, K. and Thummel, C. S. (2005). Nuclear receptors–a perspective from Drosophila. *Nat. Rev. Genet.* **6**, 311-323.

King-Jones, K., Charles, K., Lam, G. and Thummel, C. S. (2005). The ecdysone-induced DHR4 orphan nuclear receptor coordinates growth and maturation in Drosophila. *Cell* **121**, 773-784.

Lafont, R., Dauphin-Villemant, C., Warren, J. and Rees, H. (2012). Ecdysteroid chemistry and biochemistry. In *Comprehensive Molecular Insect Science*, Vol. 3 (ed. L. I. Gilbert, K. Iatrou and S. S. Gill), pp. 106-176. Oxford: Elsevier.

Lam, G. T., Jiang, C. and Thummel, C. S. (1997). Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during Drosophila metamorphosis. *Development* **124**, 1757-1769.

Lam, G., Hall, B. L., Bender, M. and Thummel, C. S. (1999). DHR3 is required for the prepupal-pupal transition and differentiation of adult structures during Drosophila metamorphosis. *Dev. Biol.* **212**, 204-216.

Layalle, S., Arquier, N. and Léopold, P. (2008). The TOR pathway couples nutrition and developmental timing in Drosophila. *Dev. Cell* **15**, 568-577.

Martos-Moreno, G. A., Chowen, J. A. and Argente, J. (2010). Metabolic signals in human puberty: effects of over and undernutrition. *Mol. Cell. Endocrinol.* **324**, 70-81.

McBrayer, Z., Ono, H., Shimell, M., Parvy, J.-P., Beckstead, R. B., Warren, J. T., Thummel, C. S., Dauphin-Villemant, C., Gilbert, L. I. and O'Connor, M. B. (2007). Prothoracicotropic hormone regulates developmental timing and body size in Drosophila. *Dev. Cell* **13**, 857-871.

Mirth, C. (2005). Ecdysteroid control of metamorphosis in the differentiating adult leg structures of Drosophila melanogaster. *Dev. Biol.* **278**, 163-174.

Moeller, M. E., Danielsen, E. T., Herder, R., O'Connor, M. B. and Rewitz, K. F. (2013). Dynamic feedback circuits function as a switch for shaping a maturation-inducing steroid pulse in Drosophila. *Development* **140**, 4730-4739.

Montagne, J., Lecerf, C., Parvy, J.-P., Bennion, J. M., Radimerski, T., Ruhf, M.-L., Zilbermann, F., Vouilloz, N., Stocker, H., Hafen, E. et al. (2010). The nuclear receptor DHR3 modulates dS6 kinase-dependent growth in Drosophila. *PLoS Genet.* **6**, e1000937.

Navarro, V. M. and Tena-Sempere, M. (2012). Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. *Nat. Rev. Endocrinol.* **8**, 40-53.

Ohno, C. K., Ueda, H. and Petkovich, M. (1994). The Drosophila nuclear receptors FTZ-F1 alpha and FTZ-F1 beta compete as monomers for binding to a site in the fushi tarazu gene. *Mol. Cell. Biol.* **14**, 3166-3175.

Ou, Q. and King-Jones, K. (2013). What goes up must come down: transcription factors have their say in making ecdysone pulses. *Curr. Top. Dev. Biol.* **103**, 35-71.

Ou, Q., Magico, A. and King-Jones, K. (2011). Nuclear receptor DHR4 controls the timing of steroid hormone pulses during Drosophila development. *PLoS Biol.* **9**, e1001160.

Palanker, L., Necakov, A. S., Sampson, H. M., Ni, R., Hu, C., Thummel, C. S. and Krause, H. M. (2006). Dynamic regulation of Drosophila nuclear receptor activity in vivo. *Development* **133**, 3549-3562.

Parvy, J.-P., Blais, C., Bernard, F., Warren, J. T., Petryk, A., Gilbert, L. I., O'Connor, M. B. and Dauphin-Villemant, C. (2005). A role for betaFTZ-F1 in regulating ecdysteroid titers during post-embryonic development in Drosophila melanogaster. *Dev. Biol.* **282**, 84-94.

Parvy, J.-P., Napal, L., Rubin, T., Poidevin, M., Perrin, L., Wicker-Thomas, C. and Montagne, J. (2012). Drosophila melanogaster Acetyl-CoA-carboxylase sustains a fatty acid-dependent remote signal to waterproof the respiratory system. *PLoS Genet.* **8**, e1002925.

Petryk, A., Warren, J. T., Marques, G., Jarcho, M. P., Gilbert, L. I., Kahler, J., Parvy, J.-P., Li, Y., Dauphin-Villemant, C. and O'Connor, M. B. (2003). Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl. Acad. Sci. USA* **100**, 13773-13778.

Reinking, J., Lam, M. M. S., Pardee, K., Sampson, H. M., Liu, S., Yang, P., Williams, S., White, W., Lajoie, G., Edwards, A. et al. (2005). The Drosophila nuclear receptor e75 contains heme and is gas responsive. *Cell* **122**, 195-207.

Rewitz, K. F., Yamanaka, N. and O'Connor, M. B. (2010). Steroid hormone inactivation is required during the juvenile-adult transition in Drosophila. *Dev. Cell* **19**, 895-902.

Rewitz, K. F., Yamanaka, N. and O'Connor, M. B. (2013). Developmental checkpoints and feedback circuits time insect maturation. *Curr. Top. Dev. Biol.* **103**, 1-33.

Richards, G. (1976a). Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster: IV. The mid prepupal period. *Dev. Biol.* **54**, 256-263.

Richards, G. (1976b). Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster: V. The late prepupal puffs. *Dev. Biol.* **54**, 264-275.

Richards, G. (1981). The radioimmune assay of ecdysteroid titres in Drosophila melanogaster. *Mol. Cell. Endocrinol.* **21**, 181-197.

Riddiford, L. M., Cherbas, P. and Truman, J. W. (2000). Ecdysone receptors and their biological actions. *Vitam. Horm.* **60**, 1-73.

Riddiford, L. M., Hiruma, K., Zhou, X. and Nelson, C. A. (2003). Insights into the molecular basis of the hormonal control of molting and metamorphosis from Manduca sexta and Drosophila melanogaster. *Insect Biochem. Mol. Biol.* **33**, 1327-1338.

Sakurai, S. and Williams, C. M. (1989). Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, Manduca sexta. *Gen. Comp. Endocrinol.* **75**, 204-216.

Schubiger, M. and Truman, J. W. (2000). The RXR ortholog USP suppresses early metamorphic processes in Drosophila in the absence of ecdysteroids. *Development* **127**, 1151-1159.

Sedkov, Y., Cho, E., Petruk, S., Cherbas, L., Smith, S. T., Jones, R. S., Cherbas, P., Canaani, E., Jaynes, J. B. and Mazo, A. (2003). Methylation at lysine 4 of histone H3 in ecdysone-dependent development of Drosophila. *Nature* **426**, 78-83.

Segraves, W. A. and Hogness, D. S. (1990). The E75 ecdysone-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4**, 204-219.

Srivastava, D. P., Yu, E. J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T. and Evans, P. D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. *J. Neurosci.* **25**, 6145-6155.

Talamillo, A., Herboso, L., Pirone, L., Pérez, C., González, M., Sánchez, J., Mayor, U., Lopitz-Otsoa, F., Rodriguez, M. S., Sutherland, J. D. et al. (2013). Scavenger receptors mediate the role of SUMO and Ftz-f1 in Drosophila steroidogenesis. *PLoS Genet.* **9**, e1003473.

Waddington, C. H. (1940). *Organisers & Genes*. Cambridge: Cambridge University Press.

Warren, J. T., Yerushalmi, Y., Shimell, M. J., O'Connor, M. B., Restifo, L. L. and Gilbert, L. I. (2006). Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of Drosophila melanogaster: correlations with changes in gene activity. *Dev. Dyn.* **235**, 315-326.

White, K. P., Hurban, P., Watanabe, T. and Hogness, D. S. (1997). Coordination of Drosophila metamorphosis by two ecdysone-induced nuclear receptors. *Science* **276**, 114-117.

Woodard, C. T., Baehrecke, E. H. and Thummel, C. S. (1994). A molecular mechanism for the stage specificity of the Drosophila prepupal genetic response to ecdysone. *Cell* **79**, 607-615.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E. and Ueda, H. (2000). Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in Drosophila melanogaster. *Development* **127**, 5083-5092.

Yamanaka, N., Rewitz, K. F. and O'Connor, M. B. (2013). Ecdysone control of developmental transitions: lessons from Drosophila research. *Annu. Rev. Entomol.* **58**, 497-516.