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Short communication

Drosophila 4EHP is essential for the larval–pupal transition and required in the prothoracic gland for ecdysone biosynthesis

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ABSTRACT

Maternal expression of the translational regulator 4EHP (eIF4E-Homologous Protein) has an established role in generating protein gradients essential for specifying the *Drosophila* embryonic pattern. We generated a null mutation of *4EHP*, which revealed for the first time that it is essential for viability and for completion of development. In fact, *4EHP* null larvae, and larvae ubiquitously expressing RNAi targeting *4EHP*, are developmentally delayed, fail to grow and eventually die. In addition, we found that expressing RNAi that targets *4EHP* specifically in the prothoracic gland disrupted ecdysone biosynthesis, causing a block of the transition from the larval to pupal stages. This phenotype can be rescued by dietary administration of ecdysone. Consistent with this, 4EHP is highly expressed in the prothoracic gland and it is required for wild type expression levels of steroidogenic enzymes. Taken together, these results uncover a novel essential function for 4EHP in regulating ecdysone biosynthesis.

1. Introduction

Translational control is an important component of the complex genetic regulation that is essential for the development of multicellular organisms. Translational regulation can occur *via* diverse molecular mechanisms (Kong and Lasko, 2012). Most often translation is regulated at initiation, a multiple step process involving the recruitment of the 40S ribosomal subunit to the 5′ end of an mRNA molecule and the positioning of the ribosome at the initiation codon (Hershey et al., 2012). Recognition of the mRNA 5′ cap m^7GpppN structure by the eukaryotic translation initiation factor eIF4F (composed of the subunits eIF4E, which binds the cap, eIF4A and eIF4G) is an integral part of this process.

Many translational control mechanisms target eIF4E. One such mechanism involves the eIF4E-homologous protein (4EHP), which acts by competitive inhibition; it binds to the 5′ cap structure, but cannot form a complex with eIF4G. 4EHP thus sequesters the cap and represses translation by blocking assembly of eIF4F (Rosettani et al., 2007). In *Drosophila*, the morphogen Bicoid (Bcd) binds directly to 4EHP and tethers it to the 3′ UTR of *caudal* mRNA to repress its translation in the anterior of the embryo where Bcd is present (Cho et al., 2005). *Drosophila* 4EHP also impairs translation of *hunchback* mRNA in the posterior of the embryo through interaction with several proteins, including Brain Tumor, Nanos and Pumilio (Cho et al., 2006). In mice, 4EHP interacts with the homeobox protein Prep1 and inhibits the translation of *Hoxb4* mRNA (Villaescusa et al., 2009). More recently, it was shown that GIGYF2 (Grb10-interacting GYF protein 2) directly interacts with 4EHP (Morita et al., 2012). The 4EHP-GIGYF2 complex functions as a translational repressor that is essential for normal development in mice.

Despite these insights, it is not yet known how widespread 4EHP-mediated regulation is and which other developmental processes it may control. Here, we report a novel role for 4EHP during *Drosophila* development and metamorphosis. Null mutation of *4EHP*, or knockdown of *4EHP* in the prothoracic gland, results in larval developmental arrest and in impaired ecdysone production. We further show that 4EHP is required to activate the expression of several genes encoding ecdysone biosynthetic enzymes. Taken together, our results show a specific requirement of 4EHP in regulating ecdysteroid biogenesis.

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2. Results and discussion

2.1. 4EHP is required for proper larval development that culminates in pupation

We produced a *4EHP* null allele (*4EHP*$^{\Delta}$) by deleting exon 2 and most of exon 3 through *trans*-recombination between two FRT-bearing transposon insertions (see Materials and Methods) (Kim and Rulifson, 2004). Since this deletion also removed the nested *Syntaxin 1A* (*Syx1A*) gene, we created a stock bearing the *4EHP*$^{\Delta}$ mutation and a second chromosome carrying a transgene encoding the wild type *Syx1A* gene (Schulze and Bellen, 1996) to compensate for the deletion of its endogenous copy. To enable easier detection of *4EHP*$^{\Delta}$ homozygotes we balanced the deletion with a GFP-expressing third chromosome balancer. The resulting stock was named *PSyx1A*; *4EHP*$^{\Delta}$. *4EHP*$^{\Delta}$ homozygotes exhibited a fully penetrant phenotype consisting of an extreme developmental delay. At 5 days after egg deposition (AED) the larvae were much smaller than their heterozygous siblings (Fig. 1A, compare II with I and Fig. 1B). These larvae arrested their development and showed mouth hooks morphology characteristic of larvae at L2 stage (Fig. 1A-II′), and without increasing their size, they died 2–3 days later. This lethality was not complemented by the chromosomal deletion *Df(3R)Exel6197*, which eliminates the region 95D8-95E1, covering the entire *4EHP* gene (Table S1), indicating further that the observed larval lethality resulted from loss of *4EHP*.

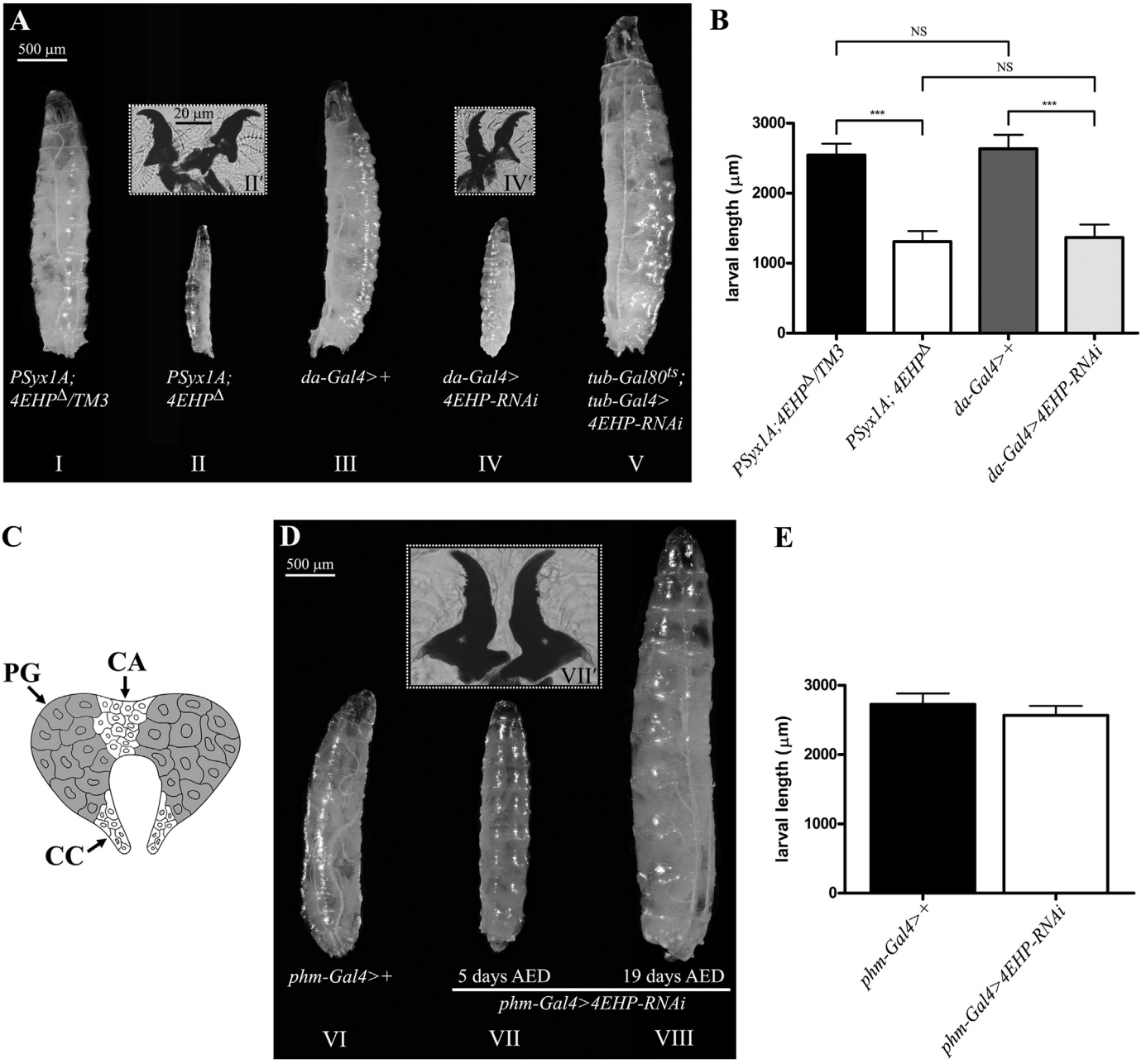

Fig. 1. 4EHP is necessary for larval development and metamorphosis. (A) Light micrographs of larvae of the indicated genotypes 5 days AED (I-IV) and 17 days AED (5 days at 21 °C plus 12 days at 29 °C) (V). (B) Length of larvae of the indicated genotypes, at 5 days AED. Ten larvae of each genotype were analyzed. Graph represents mean ± standard deviation. (C) Drawing of the ring gland, which is composed of the prothoracic gland (PG), the corpora allata (CA) and the corpora cardiaca (CC). (D) Light micrographs of 5 days AED larvae (VI and VII) and 19 days AED larva (VIII) of the indicated genotypes. In the insets (dotted boxes in A-II′, A-IV′ and D-VII′) are shown the maxillary hooks of larvae of the corresponding genotypes. The scale bar is the same for all of insets. (E) Length of larvae of the indicated genotypes, at 5 days AED. Ten larvae of each genotype were analyzed. Graph represents mean ± standard deviation.

To extend our analysis, we knocked down *4EHP* expression using RNAi at different times during larval development and in specific larval tissues. A lethal phenotype similar to that of the *4EHP* mutant was observed in larvae ubiquitously expressing a *UAS-4EHP-RNAi (4EHP-RNAi)* transgene using the *daughterless-Gal4* driver (*da-Gal4*). At 5 days AED these larvae were much smaller than control *da-Gal4 > + larvae* (Fig. 1A, compare IV with III, and Fig. 1B). They were arrested at L2 stage (see mouth hooks in Fig. 1A-IV′) and died after 3–4 more days. A similar lethal phenotype was also observed by expressing *4EHP-RNAi* with the ubiquitous *tubulin-Gal4* (*tub-Gal4*) driver (data not shown). These results recapitulate the $4EHP^{\Delta}$ larval lethal phenotype and confirm that 4EHP function is necessary for viability beyond the larval stage. We next analyzed the effect of knocking down *4EHP* expression specifically at the early L3 stage, using a temperature sensitive form of the Gal4 repressor Gal80, $Gal80^{ts}$ (McGuire et al., 2003), which allows modulation of *tub-Gal4* activity. *4EHP-RNAi (UAS-4EHP-RNAi/tub-*$Gal80^{ts}$*;tub-Gal4/+)* and control sibling (*UAS-4EHP-RNAi/tub-*$Gal80^{ts}$*; TM3, Ser, Act-GFP/+*) larvae were initially raised at 21 °C, and at 5 days AED they were shifted to the restrictive temperature (29 °C) to promote Gal4 activity. The temperature shift did not affect the development of control larvae, which pupated and produced normal adults. Conversely, the larvae expressing *4EHP-RNAi* did not develop to adulthood. Most did not pupate, instead forming abnormally large third-instar larvae that survived for 3–4 weeks before dying (Fig. 1A, V). The only 5 pupae we observed degenerated without forming pharates. In contrast, all the 63 sibling control pupae gave rise to normal adults.

2.2. Knockdown of 4EHP in the prothoracic gland blocks the larval–pupal molt

The developmental arrest induced by triggering ubiquitous expression of *4EHP-RNAi* in early L3 larvae suggested that 4EHP may have a role in controlling metamorphosis. The *Drosophila* ring gland is the major site of synthesis and release of developmental hormones. This gland (Fig. 1C) is composed of the prothoracic gland (PG), which synthesizes ecdysone (E); the corpora allata (CA) that produces juvenile hormone; and the corpora cardiaca (CC), which plays a key role in the regulation of metabolic homeostasis (Kim and Rulifson, 2004). If 4EHP regulates hormone production it may therefore be required in the prothoracic gland. To test this, we specifically induced the expression of *4EHP-RNAi* in PG cells using the *phantom-Gal4* driver (*phm-Gal4,* hereafter abbreviated as *phm > *). At 5 days AED the L3 *phm > 4EHP-RNAi* larvae (see mouth hooks for staging in Fig. 1D-VII′) were slightly smaller than the control *phm > +* larvae (Fig. 1D, compare VII with VI, and Fig. 1E). However, unlike the *phm > +* controls that underwent normal development into adult flies, none of the *phm > 4EHP-RNAi* larvae pupated, instead they continued to feed and to increase in size during their prolonged L3 larval life (Fig. 1D, compare VIII with VI) that extended up to 3–4 weeks AED. These results strongly indicated that 4EHP activity is required for PG function. Similar larval over-growth phenotypes and failure to

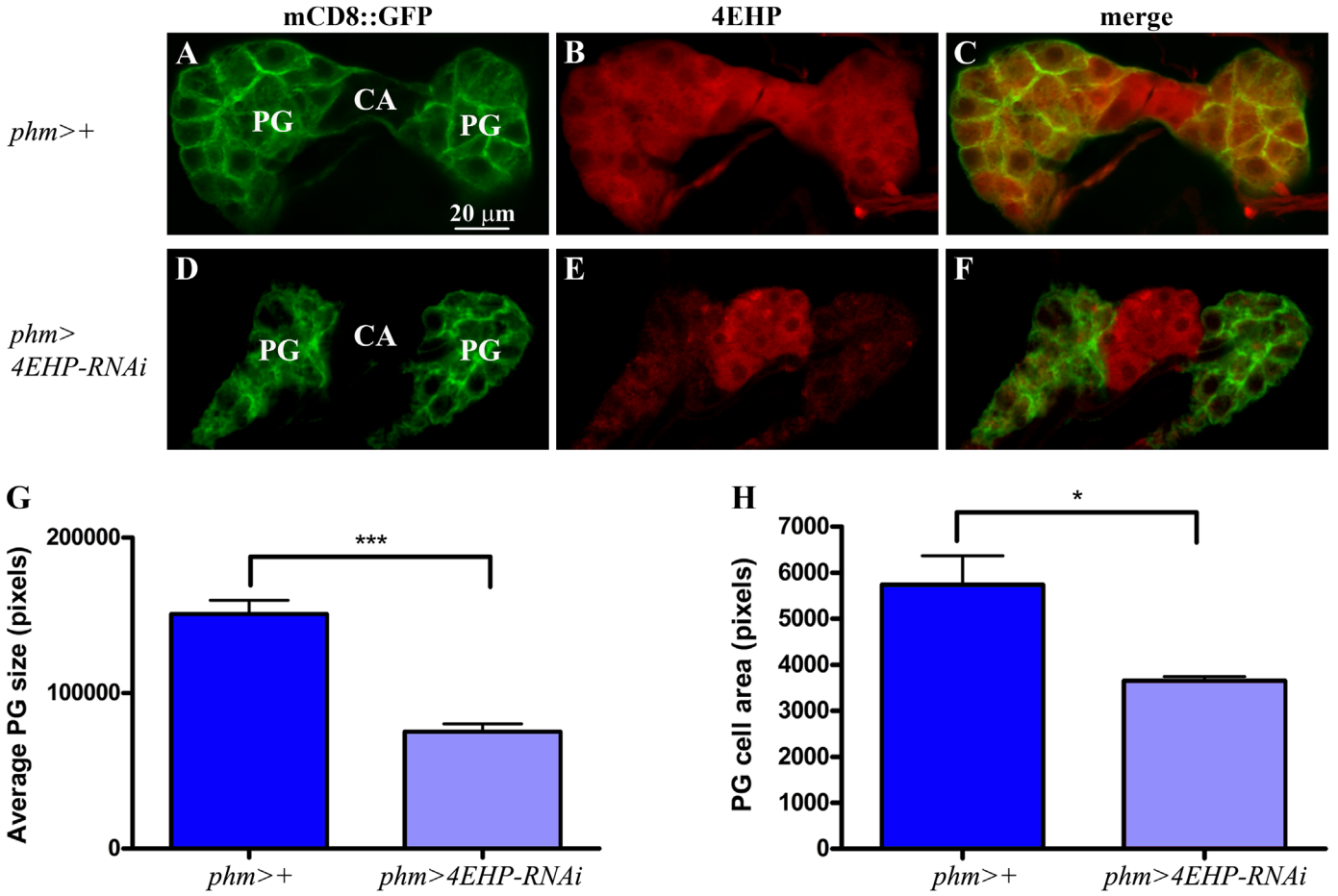

Fig. 2. 4EHP expression and its RNAi knockdown in the PG cells. (A–F) Immunolocalization with 4EHP antibody in 5 days AED larvae. In the control *phm > +* (A–C) 4EHP is highly expressed in cells of the prothoracic gland (PG, marked with mCD8::GFP) and corpora allata (CA) of the ring gland. Conversely, in the *phm > 4EHP-RNAi* (D–F) 4EHP is severely reduced in the PG cells. (G) The *phm > 4EHP-RNAi* PGs are significantly smaller (−50.2%, $n=10$) than PGs from control *phm > +* larvae. The graph represents the mean ± SD; $^{***}p<0.001$. (H) Measurement of PG cell areas showed a 36.4% ($n=17$) reduction in *phm > 4EHP-RNAi* samples compared to the *phm > +* control. The graph represents the mean ± SD; $^{*}p<0.05$.

puparíate have been reported by knocking down genes involved in PG function (Gibbens et al., 2011; Lin et al., 2011; Talamillo et al., 2008).

To investigate whether 4EHP is normally expressed in the PG, we conducted immunohistochemical experiments using an anti-4EHP antibody (Cho et al., 2005) on third instar control *phm > +*

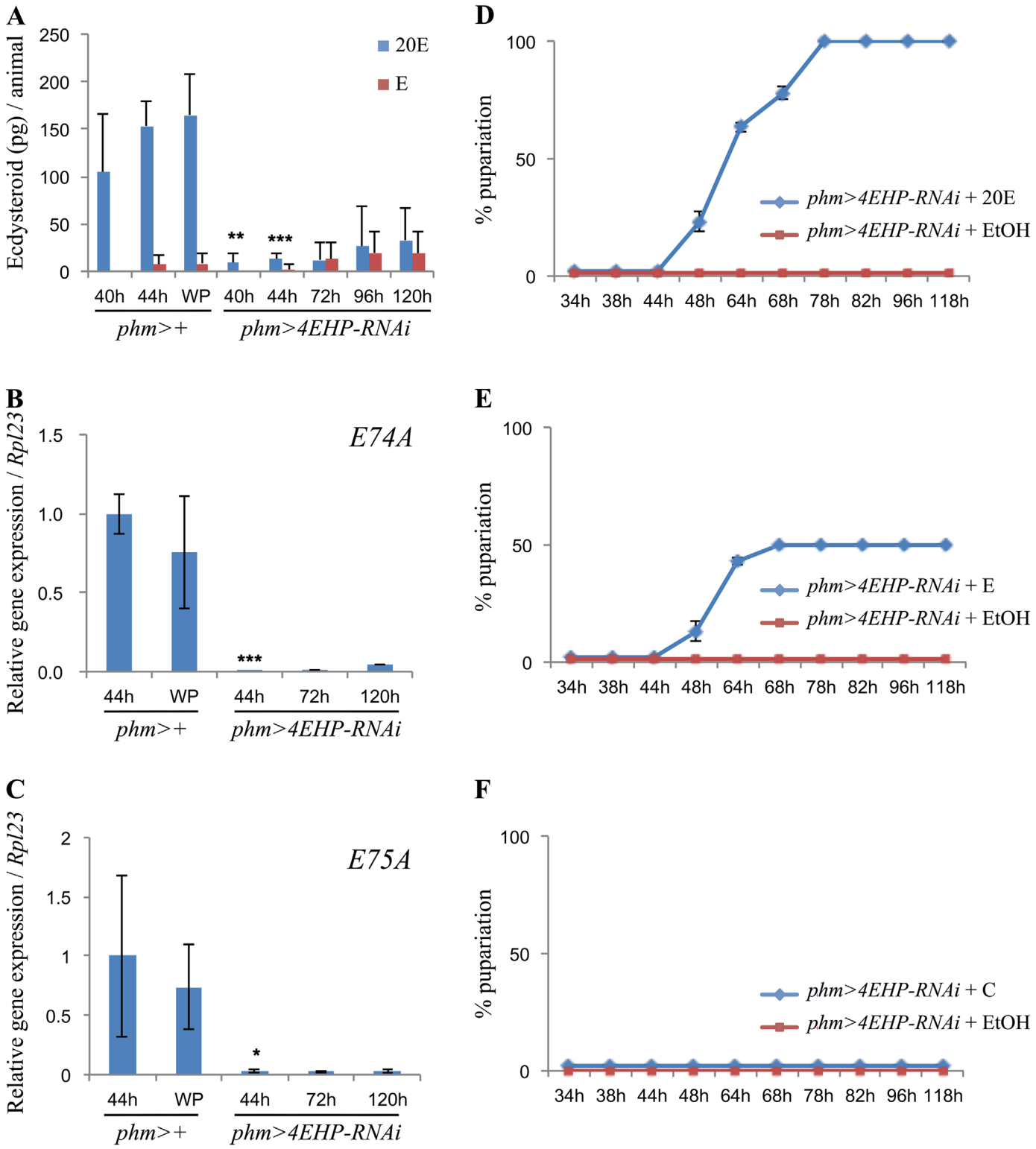

Fig. 3. *4EHP* knockdown in the PG impairs E biosynthesis. (A) Ecdysteroid titers in L3 larvae and white prepupae (WP). The indicated hours are after L3 ecdysis. Graph represents mean ± SD; n=5-7; **=$p < 0.01$; ***=$p < 0.001$. (B, C) Quantitative RT-PCR analyses of the mRNA levels of the 20E-inducible transcriptional factors (E74A, E75A) of individual L3 larvae and white prepupae (WP). The indicated hours are after L3 ecdysis. The gene expression level of control L3 larva *phm > +* at 44 h after L3 ecdysis is represented as 1 on the vertical axis. Graphs represent mean ± SD; n=4; *=$p < 0.05$; ***=$p < 0.001$. (D, E, F) Rescue experiments of *phm > 4EHP-RNAi* with 20-hydroxyecdysone (20E), ecdysone (E) and cholesterol (C). Values are the mean ± SD of two independent experiments. The indicated hours are after L3 ecdysis. Feeding *phm > 4EHP-RNAi* larvae with medium supplemented with 20E or E induces puparíation (blue; D, E), while *phm > 4EHP-RNAi* larvae fed with medium containing cholesterol do not reach the pupal stage (blue; F). *phm > 4EHP-RNAi* larvae in the medium containing ethanol (EtOH) served as controls (red).

and *phm > 4EHP-RNAi* larvae (Fig. 2). In our conditions, PGs could be easily visualized and dissected since the *phm-Gal4* stock expresses the mCD8::GFP cell membrane marker (*phm-Gal4,UAS-mCD8::GFP*) (Fig. 2A, C, D and F). In control larvae, 4EHP was intensely expressed in the PG ($n=10$) (Fig. 2B and C), while in *phm > 4EHP-RNAi* larvae its expression in the PG was strongly reduced ($n=10$) (Fig. 2E and F). Moreover, 4EHP was also detected in the CA, and, as expected, its expression was unaffected by silencing *4EHP* in the PG (Fig. 2E and F). This served as an internal control for the immunohistochemical experiments. Analysis of the gross morphology of the PG as visualized by the mCD8::GFP marker revealed that the PGs from *4EHP-RNAi* larvae were significantly smaller ($n=10$; $t=7.205$; $p<0.001$) than control PGs (~50%, Fig. 2G). Measurements of the PG cell area showed a significant reduction in *4EHP-RNAi* larvae ($n=17$; $t=3.283$; $p<0.05$) (Fig. 2H) indicating that *4EHP* knockdown influences cellular growth.

2.3. 4EHP is required for ecdysteroid biosynthesis

Drosophila larval development occurs *via* a series of precisely timed developmental transitions. After egg hatching, larvae progress through three instar stages that are separated by molts, followed by pupariation and metamorphosis, which gives rise to the adults. The time of each developmental transition is influenced by nutritional and environmental cues and is dictated by pulses of the hormone ecdysone. Ecdysone is produced by the PG cells of the ring gland as an inactive molecule. Ecdysone is then secreted into the hemolymph, through which it reaches its target tissues where it is converted to its active form, 20-hydroxyecdysone (20E). 20E binds to a heterodimer composed of ecdysone receptor (EcR) and its partner Ultraspiracle (Usp) (King-Jones and Thummel, 2005), activating response genes and triggering genetic programs that drive developmental progression.

To assess whether 4EHP regulates ecdysone biosynthesis, we compared the ecdysteroid profiles between *phm > 4EHP-RNAi* and *phm > +* control larvae by LC/MS/MS analysis (Fig. 3A). Because approximately one half of L3 control larvae had completed white puparium formation at 44 h after L3 ecdysis, we measured ecdysteroid titers of L3 larvae and white prepupae at this stage. We also measured ecdysteroid titers of L3 larvae slightly earlier (40 h after L3 ecdysis). In control larvae, the 20E titer was already high at 40 h after L3 ecdysis and reached a maximum level of approximately 170 pg/animal in the white prepupal stage. Small amounts of ecdysone (~10 pg/animal) were also detected in L3 larvae at 44 h after L3 ecdysis and in white prepupae. In contrast, 20E levels never rose above ~20 pg/animal in *phm > 4EHP-RNAi* larvae at 40 or 44 h after L3 ecdysis. As *phm > 4EHP-RNAi* larvae failed to form pupae, we further measured their ecdysteroid titers at 72 h, 96 h and 120 h after L3 ecdysis to test the possibility of a delayed onset of the ecdysone peak. We detected both ecdysone and 20E in the prolonged L3 stages and observed slight increases in these ecdysteroids at 96 h and 120 h after L3 ecdysis. However, the level of 20E during the prolonged L3 stages remained much lower than that of control larvae at 44 h after L3 ecdysis. To confirm that such low ecdysteroid titers in *phm > 4EHP-RNAi* larvae were insufficient to activate the ecdysone receptor-mediated signaling pathway, we measured expression levels of two 20E-inducible transcription factors, *E74A* and *E75A*, which are required for entry into metamorphosis (Karim and Thummel, 1992). We found that expression of both *E74A* and *E75A* was severely reduced in *phm > 4EHP-RNAi* larvae as compared to control larvae (Fig. 3B and C). These results indicate that 4EHP is required for ecdysteroid biosynthesis.

2.4. The larval developmental arrest induced by 4EHP knockdown can be rescued by ecdysone

To investigate whether the block of the transition to pupal stage caused by knockdown of *4EHP* expression was due to a low level of 20E, we carried out sterol-feeding rescue experiments. Third instar *phm > 4EHP-RNAi* larvae at 34 h after L3 ecdysis were transferred to new vials containing yeast paste supplemented with either 20E, ecdysone, or cholesterol dissolved in ethanol. At 44 h the larvae fed with 20E started to pupate and by 78 h all had developed into pupae (100%, $n=20$) (Fig. 3D). We also obtained partial rescue when the larvae were fed with ecdysone, with 50% reaching the pupal stage ($n=20$) (Fig. 3E). Conversely, *phm > 4EHP-RNAi* larvae fed only with yeast and ethanol or with cholesterol, which is the most upstream substrate for ecdysteroid biosynthesis, did not form any puparia and persisted as third instar ($n=20$) (Fig. 3F). This result confirms that the developmental arrest of *phm > 4EHP-RNAi* larvae is due to a reduced level of 20E.

2.5. 4EHP knockdown affects the expression of steroidogenic genes

Although the ecdysone biosynthetic pathway is not completely characterized, a number of ecdysteroidogenic enzymes have been identified and characterized in *Drosophila* (Fig. 4A) (Gilbert and Warren, 2005; Niwa and Niwa, 2014). The Rieske-domain protein Neverland (Nvd) catalyzes the first enzymatic reaction of the pathway, the conversion of cholesterol to 7-dehydrocholesterol (7dC) (Yoshiyama et al., 2006; Yoshiyama-Yanagawa et al., 2011). 7dC is then converted to 5β-ketodiol through the 'Black Box', a biosynthetic step not yet characterized, in which the short-chain dehydrogenase/reductase Shroud (Sro) and the P450 enzymes Spook (Spo) and Spookier (Spok) seem to be involved (Namiki et al., 2005; Niwa et al., 2010; Ono et al., 2012; Ono et al., 2006). Finally, the P450 enzymes Phantom (Phm), Disembodied (Dib), Shadow (Sad) and Shade (Shd) catalyze the four sequential hydroxylation steps that convert 5β-ketodiol to active 20E (Chavez et al., 2000; Niwa et al., 2004; Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004).

We investigated by quantitative RT-PCR whether expression of steroidogenic genes was affected by knockdown of *4EHP*. We found that *spok* expression was severely reduced to nearly non-detectable levels in *phm > 4EHP-RNAi* larvae compared to the *phm > +* control (Fig. 4B). We also detected severe reduction of *sad* and endogenous *phm* mRNAs in *phm > 4EHP-RNAi* larvae. The expression levels of *nvd* and *dib* in *phm > 4EHP-RNAi* larvae at 44 h after L3 ecdysis were also significantly reduced as compared to control larvae of corresponding age. Thus RNAi-mediated knockdown of *4EHP* in the PG cells drastically reduced transcript levels of several genes involved in ecdysteroid biosynthesis, consistent with the low ecdysteroid titers found in *phm > 4EHP-RNAi* larvae.

2.6. The developmental arrest induced by 4EHP knockdown is not caused by reduced PG cell sizes

We next analyzed whether the effect of *4EHP* knockdown on the expression of steroidogenic enzymes could be due to the specific requirement of this gene for ecdysone biosynthesis or be caused by the reduced PG cell size in turn producing less steroidogenic enzymes. Because the membrane distribution of mCD8::GFP in the *4EHP-RNAi* PG cells is normal (Fig. 5, compare A and D), the reduced PG cell size does not seem to have broad effects on cell metabolism and physiology that could account for the low expression of steroidogenic genes. Moreover, we also analyzed the expression and localization of the transcription factor Without children (Woc), involved in the first step of ecdysone biosynthesis as it up-regulates the *cholesterol 7,8 dehydrogenase* gene (Warren

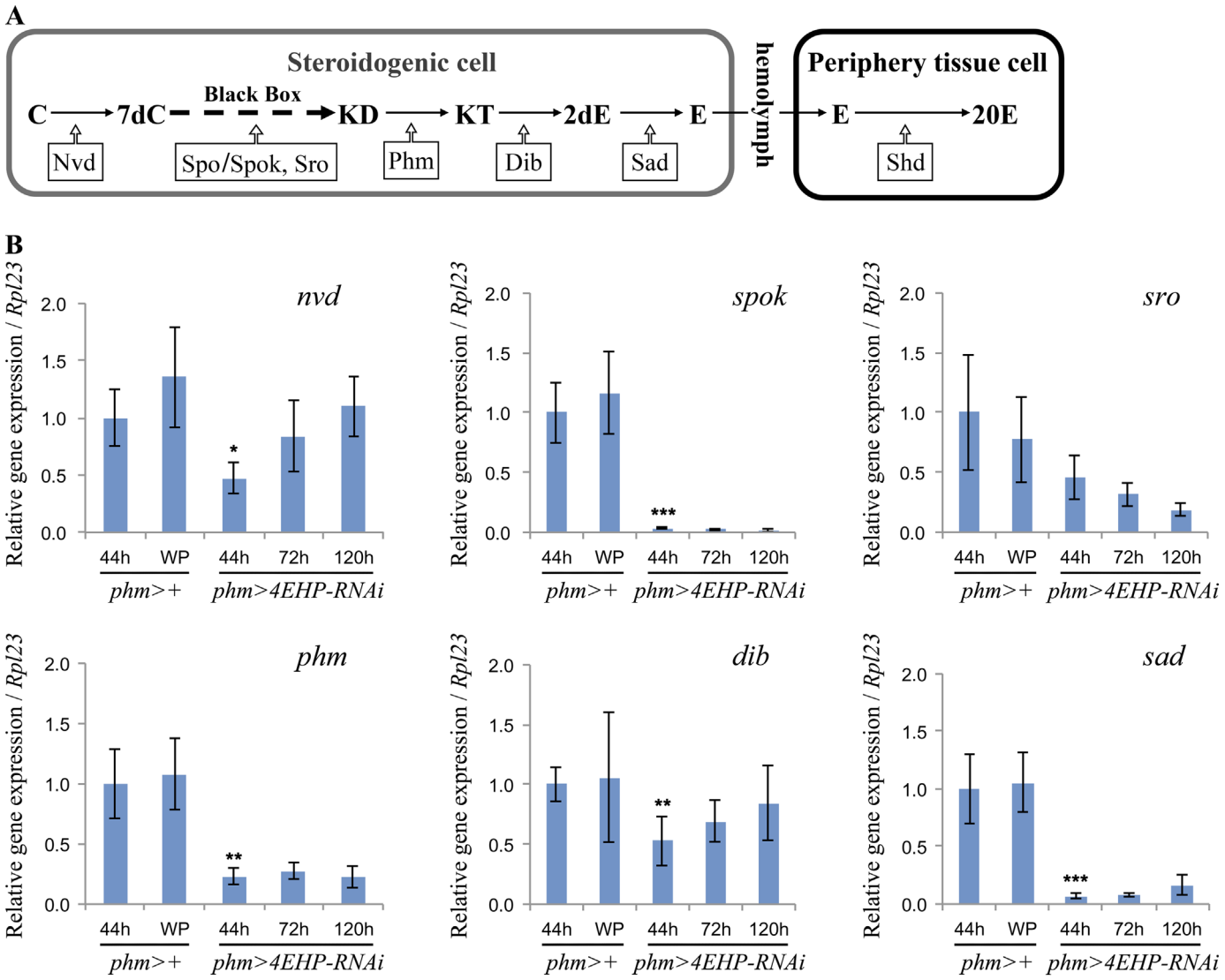

Fig. 4. Knockdown of *4EHP* reduces the expression of genes of the ecdysone biosynthetic pathway. (A) Scheme of the biosynthesis of 20E in *Drosophila*. Cholesterol (C) enters the steroidogenic cells and is converted to 7-dehydrocholesterol (7dC) by Neverland (Nvd). Spook (Spo) and Spookier (Spok) may operate in the Black Box along with Shroud (Sro) to convert 7dC in ketodiol (KD). Phantom (Phm) transforms KD in ketotriol (KT), Disembodied (Dib) converts KT in 2-deoxyecdysone (2dE) and Shadow (Sad) converts 2dE to ecdysone (E). Ecdysone is then transported by hemolymph to the periphery tissue cells where Shade (Shd) transforms E in 20E. (B) Quantitative RT-PCR analysis of the transcript levels of the ecdysone biosynthetic enzymes of individual L3 larvae and white prepupae (WP). The indicated hours are after L3 ecdysis. The gene expression level of control L3 larva *phm* > + at 44 h after L3 ecdysis is represented as 1 on the vertical axis. Graphs represent mean ± SD; $n=4$; $^{*}=p<0.05$; $^{**}=p<0.01$; $^{***}=p<0.001$.

et al., 2001). In wild type PG cells, Woc is localized to the nucleus (Fig. 5B and C) and we did not detect any differences in either the expression levels or cellular localization of this factor in *4EHP-RNAi* PG cells (Fig. 5E and F) suggesting that the *4EHP-RNAi* PG cells are functionally normal despite their small size.

We further analyzed whether the reduced PG size was correlated to the *4EHP-RNAi* larval developmental arrest. We used *tub-Gal80^{ts}; phm-Gal4* (*Gal80^{ts}; phm* >) to induce *4EHP* knockdown in the PG cells at different times during larval development. *4EHP-RNAi/tub-Gal80^{ts}; phm* > + larvae developed normally at 21 °C but when they were shifted to 29 °C at the early L2 (72 h AED) or earlier stages they arrested their development at L3 stage. Larvae shifted at the restrictive temperature at later stages pupariated normally. Thus, the *4EHP* knockdown induction in the PG cells at L2 stage is effective in blocking pupariation. We analyzed the PG size of these larvae when they were at L3 stage after 72 h at 29 °C, at the time when the control sibling (*4EHP-RNAi/tub-Gal80^{ts}; TM6B/+*) larvae pupariated. These glands not only did not show a reduction in their size but they were even larger than the PGs from *phm* >/+ raised at 25 °C (Fig. 5G and H). This indicates that the reduced PG cell size observed is a consequence of protracted 4EHP knockdown from early development and it is not correlated to the larval developmental arrest.

To corroborate these data, we also examined whether the induction of a PG extra growth by overexpressing *dMyc* (Colombani et al., 2005) could rescue the phenotype produced by the *4EHP* knockdown. While the *phm* > *dMyc* larvae pupariated, none of the *4EHP-RNAi* larvae overexpressing *dMyc* showed a rescued phenotype (data not shown). Collectively, these data indicate a specific requirement of 4EHP activity for the production of the 20E peak.

3. Conclusions

Our findings reveal that 4EHP is needed in the prothoracic gland to promote the expression of steroidogenic genes, providing new insights into the complex regulatory network controlling the ecdysone biosynthesis. The transient and temporally defined high-

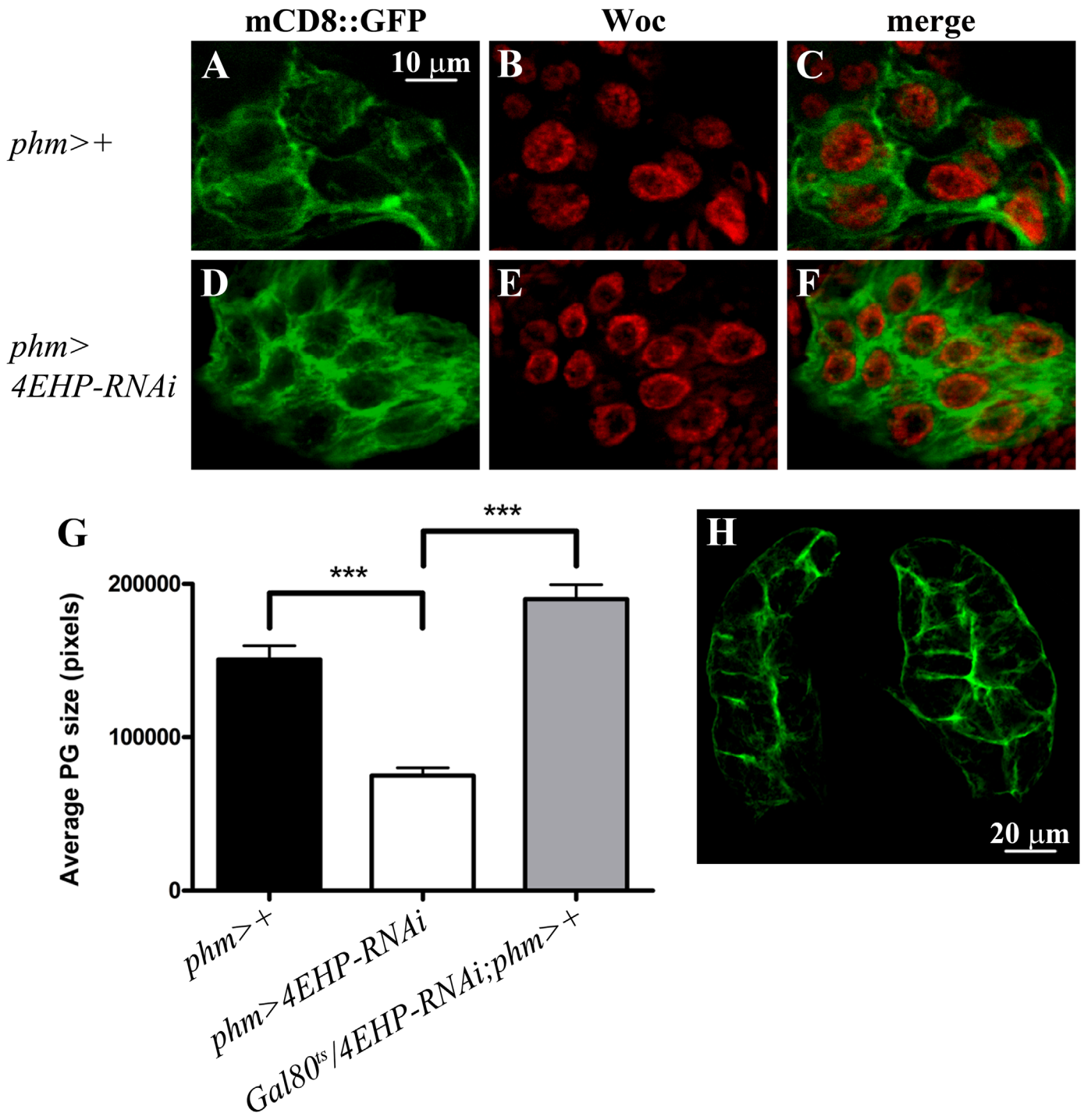

Fig. 5. Reduced PG size cannot account for the developmental arrest of the *phm > 4EHP-RNAi* larvae. (A–F) Confocal microscopy analysis of Woc immunostaining (red) of PGs dissected from *phm > +* (A–C) and *phm > 4EHP-RNAi* (D–F) larvae. In both genotypes, Woc is localized to the nucleus of the PG cells, as expected for this transcriptional factor. Note the reduced dimensions of PG cells in D–F with respect to A–C. (G) Similar to Fig. 2G, the *phm > 4EHP-RNAi* PGs are significantly smaller than PGs from control *phm > +* larvae. However, the knockdown of *4EHP* from L2 stage is able to induce the developmental arrest but the PG size is not reduced. The graph represents the mean ± SD; $^{***} = p < 0.001$. (H) Confocal image of a ring gland dissected from *tubGal80^{ts}/4EHP-RNAi; phm > +* larvae at L3 stage.

level pulse of ecdysone, which activates the larval to pupal transition is triggered by the action of the brain-delivered prothoracicotropic hormone (PTTH), which stimulates the production of ecdysone through the activation of its receptor Torso in the PG cells (McBrayer et al., 2007; Rewitz et al., 2009). Several other signaling pathways such as the Insulin and TOR (target of rapamycin) pathways can also regulate ecdysone synthesis (Yamanaka et al., 2013). Positive feedback is required for transcriptional upregulation of enzymes that act at late steps in the ecdysone biosynthetic pathway (Moeller et al., 2013). Two transcription factors, the POU-domain Ventral veins lacking (Vvl) and the nuclear receptor Knirps (Kni), regulate steroidogenic gene expression (Danielsen et al., 2014). It has also been previously reported that translational control plays a role in refining transcriptional responses to ecdysone (Ihry et al., 2012). Indeed, the DEAD box RNA helicase Belle directly regulates translation of E74A, a transcription factor that is a critical component of the ecdysone-induced transcriptional cascade.

According to its established role as translational repressor in embryonic development (Cho et al., 2006; Cho et al., 2005), 4EHP in the PG cells could play an indirect role in regulating the expression of steroidogenic genes. 4EHP could control the translation of factor(s) whose levels in the prothoracic gland regulate the expression of the steroidogenic genes. Moreover, the fact that *4EHP* knockdown induction in the PG cells at L2 stage is effective in blocking pupariation could also suggest a possible role of 4EHP on regulating the nuclear receptor cascade involved in the expression of the steroidogenic enzymes (Parvy et al., 2014).

4. Materials and methods

4.1. Fly strains

Flies were raised on standard cornmeal/yeast/agar medium at 18°C and crosses were made at 25 °C unless otherwise specified. *y w^{67c23}* was used as the wild type stock in this study. We used the following Bloomington stocks: #5460 (*w^{*}; P[Gal4-da.G32]UH1*); #4534 (*w^{*}; Sb^{1}/TM3, P[ActGFP]JMR2, Ser^{1}*); #7 (*P[hsFLP]1, y^{1} w^{1118}; $Dr^{Mi_{o}}$/TM3, ry** Sb^{1}); #2537 (*w^{*}; TM3, Sb^{1} Ser^{1}/TM6B, Tb^{1}*); #7676 (*w^{1118}; Df(3R)Exel6197, P[XP-U]Exel6197/TM6B, Tb^{1}*); #7019 (*w^{*}; P[w^{+mC}=tubP-Gal80^{ts}]20; TM2/TM6B, Tb^{1}*); #5138 (*y^{1} w^{*}; P[tubP-Gal4]LL7/TM3, Sb^{1} Ser^{1}*). *phm-Gal4* was a gift from C. Mirth (*phm-Gal4,UAS-mCD8::GFP/TM6B*). *UAS-HA::dMyc* stock was a gift from D. Grifoni.

The e03164 (*PBac[RB]4EHPe03164*) and e01165 (*PBac[RB]4EHPe01165*) stocks were obtained from Exelixis at Harvard Medical School.

The *UAS-4EHP-RNAi* line *v38399* (*w^{1118}; P[GD7071]v38399*) was obtained from Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). This RNAi line does not have any predicted off-target effect (see Dietzl et al. (2007) for VDRC's methods of detecting off-target genes).

4.2. Generation of the 4EHP null mutant (4EHP$^{\Delta}$)

Exelixis (Thibault et al., 2004) lines e03164 and e01165, both homozygous viable, were crossed to *P[hsFLP]1, y^{1} w^{1118}; Dr^{Mio}/TM3, ry** Sb^{1}. Progeny flies were crossed to obtain individuals containing both the *hsFLP* transgene and the two PBac (RB) element insertions. Flies were heat shocked three times (Parks et al., 2004; Thibault et al., 2004) and individual males were singly crossed to *w^{*}; TM3, Sb^{1} Ser^{1}/TM6B, Tb^{1}*.

76 different lines were established. The *4EHP$^{\Delta}$* line used here was verified by PCR using the GP1 and GP2 genomic primers (Fig. S1). This line carries the complete deletion of exon 2 and most of exon 3 of the *4EHP* gene, as well as the complete deletion of the nested *Syx1A* gene. Because this mutation removes the *Syx1A* gene we generated a stock carrying both the *4EHP$^{\Delta}$* allele and a functional transgenic *Syx1A* gene (*Syx1A$^{[wtC1\text{-}10]}$* line, a gift of Hugo Bellen) (Schulze and Bellen, 1996). We used this stock for all our analyses.

4.3. Molecular analyses

Genomic DNA from *4EHP$^{\Delta}$/TM3, Act-GFP, Ser^{1}* larvae was extracted as previously described (Cavaliere et al., 1998). A 7.3 kb fragment in the *4EHP* locus was amplified with Phusion high fidelity DNA polymerase (New England BioLabs) with 5′ GCTGCTTTCACTACCGACTTAT3′ (GP1, forward) and 5′GGCATGGCATCCTCCATAAT3′ (GP2, reverse) primers (Sigma) following the supplier's recommendations. The amplification was performed as follows: denaturation: 98 °C 10 s; annealing: 58 °C 40 s; elongation: 72 °C 5 min in 35 cycles. 1/50 of the PCR product reaction was resolved on a 0.8% agarose/TBE gel.

4.4. Crosses

UAS-4EHP-RNAi or control *y w^{67c23}* females were crossed to males of the different Gal4 lines.

For the *tub-Gal80^{ts}/tub-Gal4* experiment, *UAS-4EHP-RNAi* females were crossed to *tub-Gal80^{ts}; tub-Gal4/TM3, Act-GFP, Ser^{1}* males, and as control *yw^{67c23}* females were crossed to *tub-Gal80^{ts}; tub-Gal4/TM3, Act-GFP, Ser^{1}* males. Larvae were raised at 21 °C and transferred at 29 °C after 5 days AED.

For the *tub-Gal80^{ts}/phm-Gal4* experiment, *UAS-4EHP-RNAi* females were crossed to *tub-Gal80^{ts}; phm-Gal4/TM6B* males, and *yw^{67c23}* females were crossed to *tub-Gal80^{ts}; phm-Gal4/TM6B* males as control. Larvae were raised at 21 °C and at different days AED were transferred at 29 °C.

4.5. Immunofluorescence microscopy

Immunostaining on ring glands was performed as described previously (Valzania et al., 2014). Polyclonal rabbit anti-4EHP 1:100 (Cho et al., 2005) and anti-Woc 1:500 (Raffa et al., 2005) were detected using anti-rabbit Cy3-coniugated 1:2000 (Invitrogen) and mounted in Prolong Gold (Molecular Probes) or Fluoromount G (Electron Microscopy Sciences). Samples were analyzed by conventional epifluorescence with a Nikon Eclipse 90i microscope or with a Zeiss LSM510 or TCS SL Leica confocal system. Images were processed using Adobe Photoshop CS6.

4.6. Sterol rescue experiments

Two groups of ten *phm > 4EHP-RNAi* larvae were collected at 34 h after L3 ecdysis and placed at 25 °C in new tubes with yeast paste supplemented with ecdysteroid precursors. The following sterols were dissolved in 100% ethanol and their final concentrations were: cholesterol (Sigma) 0.14 mg/ml, 20-hydroxyecdysone (Sigma) 1 mg/ml and ecdysone (Sigma) 0.5 mg/ml. As a control the same experiments were carried out on *phm > 4EHP-RNAi* larvae fed with yeast paste containing only equal amounts of ethanol.

4.7. Larval ecdysteroids quantification

Sample collection, extraction of ecdysteroids and LC/MS/MS analyses were performed as previously described (Ono, 2014), with minor modification of the numbers of collected animals, LC/MS/MS system and HPLC conditions, as described below. Whole bodies of 10 larvae were extracted and the prepared samples were dissolved in 50 µl of ethanol. For quantification of ecdysteroids, 5-7 independent biological samples were collected. Ecdysteroids were analyzed in an LC/MS/MS system consisting of an Shimadzu HPLC system coupled to an API4000 triple quadrupole mass spectrometer (AB SCIEX, CA, USA) equipped with an electrospray ionization source as described previously (Miyashita et al., 2011). HPLC separation was performed on Poroshell 120 EC-C18 column (2.1×50 mm^2, Agilent, CA, USA) with a 0.3 ml/min flow rate at 40 °C by using 0.1% aqueous acetic acid (A) and acetonitrile containing 0.1% acetic acid (B). The LC mobile phase was a constant 10% of (B) in (A) for 1 min, and then a gradient of 10–50% (B) in (A) for 8 min.

4.8. Quantitative RT-PCR

Total RNA was extracted from individual larvae. For each experiment four independent biological samples were collected for each genotype. Purification of total RNA, reverse transcription and quantitative RT-PCR were performed as described previously (Ono,

2014). Transcript levels were normalized to *Rpl23* in the same samples. To assess this normalization we checked if there were any differences in the expression level of *Rpl23* with respect to *actin5C*, but there were not any (Fig. S2). The primers used for quantitative RT-PCR are listed in Table S2.

4.9. Prothoracic gland and cellular size measurements

For measurements of the PG area and cellular size, confocal images of PGs taken at 40× magnification were quantified with ImageJ.

4.10. Statistical analysis

GraphPad Prism 4 software was used for statistical analysis. Statistical significance was determined on the basis of unpaired *t*-test performed on the means and p values were calculated (NS=not-significant; $^{*}=p<0.05$; $^{**}=p<0.01$ and $^{***}=p<0.001$). $p<0.05$ was considered statistically significant. All results are expressed as the mean ± standard deviation (SD).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.12.021.

References

Cavaliere, V., Taddei, C., Gargiulo, G., 1998. Apoptosis of nurse cells at the late stages of oogenesis of Drosophila melanogaster. Dev. Genes. Evol. 208, 106–112.
Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J.E., O'Connor, M.B., 2000. The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development 127, 4115–4126.
Cho, P.F., Gamberi, C., Cho-Park, Y.A., Cho-Park, I.B., Lasko, P., Sonenberg, N., 2006. Cap-dependent translational inhibition establishes two opposing morphogen gradients in Drosophila embryos. Curr. Biol. 16, 2035–2041.
Cho, P.F., Poulin, F., Cho-Park, Y.A., Cho-Park, I.B., Chicoine, J.D., Lasko, P., Sonenberg, N., 2005. A new paradigm for translational control: inhibition via 5′-3′ mRNA tethering by Bicoid and the eIF4E cognate 4EHP. Cell 121, 411–423.
Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., Leopold, P., 2005. Antagonistic actions of ecdysone and insulins determine final size in Drosophila. Science 310, 667–670.
Danielsen, E.T., Moeller, M.E., Dorry, E., Komura-Kawa, T., Fujimoto, Y., Troelsen, J.T., Herder, R., O'Connor, M.B., Niwa, R., Rewitz, K.F., 2014. Transcriptional control of steroid biosynthesis genes in the Drosophila prothoracic gland by ventral veins lacking and knirps. PLoS Genet. 10, e1004343.
Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., Dickson, B.J., 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448, 151–156.
Gibbens, Y.Y., Warren, J.T., Gilbert, L.I., O'Connor, M.B., 2011. Neuroendocrine regulation of Drosophila metamorphosis requires TGFbeta/Activin signaling. Development 138, 2693–2703.
Gilbert, L.I., Warren, J.T., 2005. A molecular genetic approach to the biosynthesis of the insect steroid molting hormone. Vitam. Horm. 73, 31–57.
Hershey, J.W., Sonenberg, N., Mathews, M.B., 2012. Principles of translational control: an overview. Cold Spring Harb. Perspect. Biol. 4, a011528.
Ihry, R.J., Sapiro, A.L., Bashirullah, A., 2012. Translational control by the DEAD Box RNA helicase belle regulates ecdysone-triggered transcriptional cascades. PLoS Genet. 8, e1003085.
Karim, F.D., Thummel, C.S., 1992. Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. EMBO J. 11, 4083–4093.
Kim, S.K., Rulifson, E.J., 2004. Conserved mechanisms of glucose sensing and regulation by Drosophila corpora cardiaca cells. Nature 431, 316–320.
King-Jones, K., Thummel, C.S., 2005. Nuclear receptors—a perspective from Drosophila. Nat. Rev. Genet. 6, 311–323.
Kong, J., Lasko, P., 2012. Translational control in cellular and developmental processes. Nat. Rev. Genet. 13, 383–394.
Lin, J.I., Mitchell, N.C., Kalcina, M., Tchoubrieva, E., Stewart, M.J., Marygold, S.J., Walker, C.D., Thomas, G., Leevers, S.J., Pearson, R.B., Quinn, L.M., Hannan, R.D., 2011. Drosophila ribosomal protein mutants control tissue growth non-autonomously via effects on the prothoracic gland and ecdysone. PLoS Genet. 7, e1002408.
McBrayer, Z., Ono, H., Shimell, M., Parvy, J.P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., O'Connor, M.B., 2007. Prothoracicotropic hormone regulates developmental timing and body size in Drosophila. Dev. Cell. 13, 857–871.
McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., Davis, R.L., 2003. Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302, 1765–1768.
Miyashita, M., Matsushita, K., Nakamura, S., Akahane, S., Nakagawa, Y., Miyagawa, H., 2011. LC/MS/MS identification of 20-hydroxyecdysone in a scorpion (Liocheles australasiae) and its binding affinity to in vitro-translated molting hormone receptors. Insect Biochem. Mol. Biol. 41, 932–937.
Moeller, M.E., Danielsen, E.T., Herder, R., O'Connor, M.B., Rewitz, K.F., 2013. Dynamic feedback circuits function as a switch for shaping a maturation-inducing steroid pulse in Drosophila. Development 140, 4730–4739.
Morita, M., Ler, L.W., Fabian, M.R., Siddiqui, N., Mullin, M., Henderson, V.C., Alain, T., Fonseca, B.D., Karashchuk, G., Bennett, C.F., Kabuta, T., Higashi, S., Larsson, O., Topisirovic, I., Smith, R.J., Gingras, A.C., Sonenberg, N., 2012. A novel 4EHP-GIGYF2 translational repressor complex is essential for mammalian development. Mol. Cell. Biol. 32, 3585–3593.
Namiki, T., Niwa, R., Sakudoh, T., Shirai, K., Takeuchi, H., Kataoka, H., 2005. Cytochrome P450 CYP307A1/Spook: a regulator for ecdysone synthesis in insects. Biochem. Biophys. Res. Commun. 337, 367–374.
Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., Kataoka, H., 2004. CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of Bombyx and Drosophila. J. Biol. Chem. 279, 35942–35949.
Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T., Banno, Y., Fujimoto, Y., Shigenobu, S., Kobayashi, S., Shimada, T., Katsuma, S., Shinoda, T., 2010. Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. Development 137, 1991–1999.
Niwa, R., Niwa, Y.S., 2014. Enzymes for ecdysteroid biosynthesis: their biological functions in insects and beyond. Biosci. Biotechnol. Biochem. 78, 1283–1292.
Ono, H., 2014. Ecdysone differentially regulates metamorphic timing relative to 20-hydroxyecdysone by antagonizing juvenile hormone in Drosophila melanogaster. Dev. Biol. 391, 32–42.
Ono, H., Morita, S., Asakura, I., Nishida, R., 2012. Conversion of 3-oxo steroids into ecdysteroids triggers molting and expression of 20E-inducible genes in Drosophila melanogaster. Biochem. Biophys. Res. Commun. 421, 561–566.
Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T., Marques, G., Shimell, M.J., Gilbert, L.I., O'Connor, M.B., 2006. Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. Dev. Biol. 298, 555–570.
Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., Deal-Herr, M.E., Grant, D., Marcinko, M., Miyazaki, W.Y., Robertson, S., Shaw, K.J., Tabios, M., Vysotskaia, V., Zhao, L., Andrade, R.S., Edgar, K.A., Howie, E., Killpack, K., Milash, B., Norton, A., Thao, D., Whittaker, K., Winner, M.A., Friedman, L., Margolis, J., Singer, M.A., Kopczynski, C., Curtis, D., Kaufman, T.C., Plowman, G.D., Duyk, G., Francis-Lang, H.L., 2004. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat. Genet. 36, 288–292.
Parvy, J.P., Wang, P., Garrido, D., Maria, A., Blais, C., Poidevin, M., Montagne, J., 2014. Forward and feedback regulation of cyclic steroid production in Drosophila melanogaster. Development 141, 3955–3965.
Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., 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.
Raffa, G.D., Cenci, G., Siriaco, G., Goldberg, M.L., Gatti, M., 2005. The putative Drosophila transcription factor woc is required to prevent telomeric fusions. Mol. Cell. 20, 821–831.
Rewitz, K.F., Yamanaka, N., Gilbert, L.I., O'Connor, M.B., 2009. The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. Science 326, 1403–1405.

Rosettani, P., Knapp, S., Vismara, M.G., Rusconi, L., Cameron, A.D., 2007. Structures of the human eIF4E homologous protein, h4EHP, in its m7GTP-bound and unliganded forms. J. Mol. Biol. 368, 691–705.
Schulze, K.L., Bellen, H.J., 1996. Drosophila syntaxin is required for cell viability and may function in membrane formation and stabilization. Genetics 144, 1713–1724.
Talamillo, A., Sanchez, J., Cantera, R., Perez, C., Martin, D., Caminero, E., Barrio, R., 2008. Smt3 is required for Drosophila melanogaster metamorphosis. Development 135, 1659–1668.
Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H.L., Ryner, L., Cheung, L.M., Chong, A., Erickson, C., Fisher, W.W., Greer, K., Hartouni, S.R., Howie, E., Jakkula, L., Joo, D., Killpack, K., Laufer, A., Mazzotta, J., Smith, R.D., Stevens, L.M., Stuber, C., Tan, L.R., Ventura, R., Woo, A., Zakrajsek, I., Zhao, L., Chen, F., Swimmer, C., Kopczynski, C., Duyk, G., Winberg, M.L., Margolis, J., 2004. A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat. Genet. 36, 283–287.
Valzania, L., Romani, P., Tian, L., Li, S., Cavaliere, V., Pennacchio, F., Gargiulo, G., 2014. A polydnavirus ANK protein acts as virulence factor by disrupting the function of prothoracic gland steroidogenic cells. PLoS One 9, e95104.
Villaescusa, J.C., Buratti, C., Penkov, D., Mathiasen, L., Planaguma, J., Ferretti, E., Blasi, F., 2009. Cytoplasmic Prep1 interacts with 4EHP inhibiting Hoxb4 translation. PLoS One 4, e5213.
Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., Dauphin-Villemant, C., O'Connor, M.B., Gilbert, L.I., 2002. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 99, 11043–11048.
Warren, J.T., Petryk, A., Marques, G., Parvy, J.P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M.B., Dauphin-Villemant, C., Gilbert, L.I., 2004. Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem. Mol. Biol. 34, 991–1010.
Warren, J.T., Wismar, J., Subrahmanyam, B., Gilbert, L.I., 2001. Woc (without children) gene control of ecdysone biosynthesis in Drosophila melanogaster. Mol. Cell. Endocrinol. 181, 1–14.
Yamanaka, N., Rewitz, K.F., O'Connor, M.B., 2013. Ecdysone control of developmental transitions: lessons from Drosophila research. Annu. Rev. Èntomol. 58, 497–516.
Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., Niwa, R., 2006. Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development 133, 2565–2574.
Yoshiyama-Yanagawa, T., Enya, S., Shimada-Niwa, Y., Yaguchi, S., Haramoto, Y., Matsuya, T., Shiomi, K., Sasakura, Y., Takahashi, S., Asashima, M., Kataoka, H., Niwa, R., 2011. The conserved Rieske oxygenase DAF-36/Neverland is a novel cholesterol-metabolizing enzyme. J. Biol. Chem. 286, 25756–25762.