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Published in:
P L o S Genetics

DOI:
10.1371/journal.pgen.1004343

Publication date:
2014

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Transcriptional Control of Steroid Biosynthesis Genes in the Drosophila Prothoracic Gland by Ventral Veins Lacking and Knirps

E. Thomas Danielsen1,2, Morten E. Moeller1, Elad Dorry1, Tatsuya Komura-Kawa2, Yoshinori Fujimoto3, Jesper T. Troelsen4, Rachel Herder5, Michael B. O’Connor5, Ryusuke Niwa2,6, Kim F. Rewitz1*

1 Department of Biology, University of Copenhagen, Copenhagen, Denmark, 2 Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan, 3 Department of Chemistry and Materials Science, Tokyo Institute of Technology, Meguro, Tokyo, Japan, 4 Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark, 5 Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota, United States of America, 6 PRESTO, JST, Kawaguchi, Saitama, Japan

Abstract

Specialized endocrine cells produce and release steroid hormones that govern development, metabolism and reproduction. In order to synthesize steroids, all the genes in the biosynthetic pathway must be coordinately turned on in steroidogenic cells. In Drosophila, the steroid producing endocrine cells are located in the prothoracic gland (PG) that releases the steroid hormone ecdysone. The transcriptional regulatory network that specifies the unique PG specific expression pattern of the ecdysone biosynthetic genes remains unknown. Here, we show that two transcription factors, the POU-domain Ventral veins lacking (Vvl) and the nuclear receptor Knirps (Kni), have essential roles in the PG during larval development. Vvl is highly expressed in the PG during embryogenesis and is enriched in the gland during larval development, suggesting that Vvl might function as a master transcriptional regulator in this tissue. Vvl and Kni bind to PG specific cis-regulatory elements that are required for expression of the ecdysone biosynthetic genes. Knock down of either vvl or kni in the PG results in a larval developmental arrest due to failure in ecdysone production. Furthermore, Vvl and Kni are also required for maintenance of TOR/S6K and prothoracicotropic hormone (PTTH) signaling in the PG, two major pathways that control ecdysone biosynthesis and PG cell growth. We also show that the transcriptional regulator, Molting defective (Mld), controls early biosynthetic pathway steps. Our data show that Vvl and Kni directly regulate ecdysone biosynthesis by transcriptional control of biosynthetic gene expression and indirectly by affecting PTTH and TOR/S6K signaling. This provides new insight into the regulatory network of transcription factors involved in the coordinated regulation of steroidogenic cell specific transcription, and identifies a new function of Vvl and Knirps in endocrine cells during post-embryonic development.

Introduction

Steroid hormones have a conserved role in the regulation of developmental transitions, growth, metabolism and reproduction in animals [1-3]. Specialized endocrine tissues with cell-type specific complements of enzymes that form biochemical pathways mediate the biosynthesis of steroids. In Drosophila larvae, the steroid biosynthetic enzymes are expressed in the prothoracic gland (PG), the endocrine tissue of insects and the major source of the steroid hormone ecdysone. The production of ecdysone in the PG is regulated by a checkpoint control system in response to external and internal signals [2]. These checkpoints allow the endocrine system to assess growth and nutrient status before activating the biochemical pathway that increases the release of ecdysone, which triggers developmental progression.

Despite the importance of the coordinated expression in endocrine cells of the steroidogenic enzymes, the PG specific transcriptional regulatory networks that underlie steroidogenic cell function remain unknown. The steroidogenic function of the PG cells is defined by the restricted expression of the genes involved in ecdysone biosynthesis that mediate the conversion of cholesterol to ecdysone. The components of the ecdysone biosynthetic pathway include the Rieske-domain protein Neverland (Nvd) [4,5], the short-chain dehydrogenase/reductase Shroud (Sro) [6] and the P450 enzymes Spook (Spo), Spookier (Spok), Phantom (Phm), Disembodied (Dib) and Shadow (Sad) [7–12] collectively referred to as the Halloween genes. Ecdysone produced by the PG is released into circulation and converted into the more active hormone, 20-hydroxyecdysone (20E), in peripheral tissues by the P450 enzyme, Shade (Shd) [13,14].
Steroid hormones play important roles in physiology and disease. These hormones are molecules produced and secreted by endocrine cells in the body and control sexual maturation, metabolism and reproduction. We found transcriptional regulators that underlie the specialized function of endocrine steroid-producing cells. In the steroid-producing cells of the fruit fly Drosophila, Ventral veins lacking (Vvl) and Knirps (Kni) turn on all the genes required for steroid production. When Vvl or Kni were inactivated in the cells where the hormone is made, the genes involved in steroid production were not activated. Because of the reduced steroid production, the juvenile larvae failed to develop and undergo maturation to adulthood. Inactivation of Vvl and Kni also reduces endocrine cell growth by disturbing their response to growth promoting signals. Genetic variations in humans with the loss of a homolog of Vvl have been associated with disorders caused by insufficient steroid production. Together with the fact that Vvl is highly expressed in the steroid-producing cells of Drosophila, this suggests that Vvl may be a conserved master regulator of steroid production. Our findings provide insight into the network of factors that control endocrine cell function and steroid hormone levels that could have implication for human diseases.

The cell-type specific pattern and precise dynamics of the ecdysone titers suggest a tight transcriptional regulation of the biosynthetic enzymes in the PG. This is likely orchestrated by multiple transcription factors working in a network to achieve spatial and temporal control of steroid hormone production during development. The composition of this tissue-specific transcriptional regulation remains largely elusive, although some transcription factors are known to regulate ecdysone production in the PG [15-18]. The nuclear receptor DHR4 functions as a repressor of ecdysone biosynthesis in the PG and responds to prothoracotrophic hormone (PTTH) mediated activation of the mitogen-activated protein kinase (MAPK) pathway [17]. Loss of βFTZ-F1 in the PG has also been associated with reduced expression of phm and dib [18]. The zinc-finger protein Without children (Woc) is required for ecdysone biosynthesis [19], although the pathway component regulated by Woc has not been identified. However, it is unclear if Woc, βFTZ-F1 and DHR4 bind directly to the regulatory regions that control expression of the ecdysone biosynthetic genes. In contrast, we recently showed that the transcription factor Broad (Br) regulates expression of the genes involved in ecdysone biosynthesis by direct binding to their promoters/enhancers [20]. Although these factors may be important for steroidogenic gene expression, other factors are likely required for the transcriptional regulatory network that defines the PG cell-specific expression of the ecdysone biosynthetic pathway components.

We recently characterized cis-regulatory elements required for the expression of phm and dib in the Drosophila PG [20], including a 69 bp promoter element located in the upstream phm region and a 86 bp region in the third intron of dib. These elements are important for the temporal up-regulation of phm and dib by Br isoform 4 (Br-Z4) that increases the ecdysteroidogenic capacity of the PG and allows the production of the high-level ecdysone pulse that triggers puation. To further characterize the tissue-specific regulation of the ecdysone biosynthetic pathway, we analyzed PG specific regulatory elements for the presence of transcription factor binding sites.

Here, we report a novel role for Ventral veins lacking (Vvl) and Knirps (Kni) in regulating ecdysteroidogenesis in Drosophila. The cis-regulatory elements responsible for PG specific expression of spok, phm and dib contain conserved Vvl and Kni binding sites. Expression of vvl is high in the PG compared to the whole animal, while kni expression is less PG-specific. Knock down of vvl and kni in the PG results in larval developmental arrest due to impaired ecdysone production. We show that Vvl and Kni specifically regulate expression of all the ecdysone biosynthetic enzymes through functionally important regulatory sites. Furthermore, we find that Molting defective (Mld) specifically regulates enzymes that catalyze early steps in the ecdysone biosynthetic pathway. Our study identifies Vvl as a PG cell-specific transcription factor that underlies steroidogenic cell function. We conclude that Vvl and Kni are involved in the transcriptional regulatory network of the PG that coordinates expression of biosynthetic enzymes required for ecdysone production during Drosophila development.

**Results**

Regulatory regions of ecdysone biosynthesis genes contain conserved binding sites for Vvl and Kni

We analyzed the phm and dib PG specific regulatory elements for transcription factor binding sites. Our *in silico* search revealed conserved binding sites for the POU-domain transcription factor Vvl and the nuclear receptor Kni in the phm promoter and dib enhancer (Fig. 1A and S1). Analysis of the phm promoter identified one conserved Vvl site and four Kni sites of which three are highly conserved, indicating that they are important regulatory sites. In support of this, mutations disrupting the Vvl site and one of the conserved Kni sites eliminate PG specific GFP reporter expression [20]. In contrast, mutations in the non-conserved Kni binding sites do not reduce PG expression. The third intron dib enhancer also contains one Vvl site and two Kni sites, both in regions that have been conserved. We also identified a 300 bp PG specific promoter for spok, encoding an enzyme that acts at an early step in the ecdysone biosynthetic pathway [9]. This element located −331 to −32 bp upstream of translation start drives specific PG reporter GFP expression. This spok promoter contains three Vvl and three Kni binding sites, although these sites are less conserved compared to the Vvl and Kni sites identified in the phm and dib regulatory elements. Expression of spok has previously been reported to require Molting defective (Mld), a nuclear zinc finger protein [9]. Since the DNA binding sequence motif for Mld has not yet been characterized, we were unable to examine potential Mld binding sites in the spok promoter.

**Vvl and Kni are expressed in the prothoracic gland**

The observation of Vvl and Kni binding sites in the promoter/enhancer of the steroidogenic enzymes prompted us to verify if these transcription factors are expressed in the PG. We performed *in situ* hybridization on third instar larvae and observed an intense staining of vvl mRNA in the PG (Fig. 1B). Moreover, strong embryonic *vvl* expression is seen in the primordium of the PG from stage 13. Importantly, the appearance of *vvl* in the PG precedes that of the biosynthetic genes which are expressed by stage 15 in the PG primordium [11,12]. Although *in situ* expression of kni was undetectable in the PG of embryos, expression in the PG was observed at the L3 stage (Fig 1B). We also detected expression of *vvl* in nurse and follicle cells of adult female ovaries (Fig. S1).

Using specific antibodies, we also confirmed that Vvl and Kni are expressed in the PG and that these transcription factors localize in the nucleus (Fig. 1C). Although *kni* expression was not detected using *in situ* hybridization in the embryonic PG,
expression of Kni was found in the PG at the L2 stage (Fig. S1). Next, we quantified vvl and kni expression in the ring gland (an organ with by far the most of its volume constituted by PG cells) compared to the whole body in order to see if these transcription factors are enriched in the PG (Fig. 1D). As a control, we measured phm expression, which indeed is highly expressed in the PG compared to the whole animal and mld, encoding a factor with a specific role in the PG, but with a broader expression pattern.

Figure 1. Vvl and Kni have binding sites in the promoters and enhancers of the ecdysone biosynthetic genes and are expressed in the PG. (A) An illustration showing binding sites in the PG specific cis-regulatory elements of spok and phm and dib. Binding sites are indicated by squares (Vvl) and pentagons (Kni) with shades indicating the conservation of the site between Drosophila species. Conservation tracks were obtained from the UCSC genome browser. (B) In situ hybridization of embryos and third instar larval brains and ring glands with antisense probes for vvl (a’–d’) or kni (e’–g’). (a’) Stage 11, shows vvl expression in the primordial cells of the trachea, while (b’) stage 13, (c’) stage 16 and (d’) L3 show strong vvl expression in the PG cells of the ring gland. (e’) stage 4, (f’) stage 16 and (g’) L3 show kni expression in the PG of L3 larvae, but not clearly in embryos. (C) Immunostaining of the PG from L3 larvae with antibodies against Kni and Vvl (magenta) and Phm (green). Co-localization with nuclei staining (DAPI: gray) indicates that Vvl and Kni are expressed in the nucleus of the PG cells. (D) Expression of phm, vvl, kni and mld measured by qPCR in tissue from whole body L3 larvae or dissected ring glands containing the PG of L3 larvae (n = 4). vvl is highly expressed in the ring gland compared to whole body, like phm, while the expression of kni and mld show a minor enrichment in the gland. Error bars indicate s.e.m. **P<0.01, versus whole body. doi:10.1371/journal.pgen.1004343.g001
knock down of these factors does not compromise PG cell fate specification and survival. The developmental arrest indicates that loss of vvl and kni in the PG impair the cellular production of ecdysone. We therefore investigated the ecdysone levels in L1 larvae 36 hours AEL by measuring E75B mRNA expression in the whole animal, which has been used as a readout for ecdysone levels [20, 23]. Expression of E75B was significantly reduced in mid-first instar (36 hours AEL) by knock down of vvl, kni or mld in the PG. (C) Ecdysteroid levels measured by ELISA confirm that L1 larvae with reduced expression of vvl, kni or mld in the PG have lower levels of ecdysteroids 36 hours AEL compared to the control. Error bars indicate s.e.m. (n = 4). *P < 0.05, ***P < 0.001, versus the phm->+ control. doi:10.1371/journal.pgen.1004343.g002

**Figure 2. Knock down of vvl, kni and mld in the PG results in developmental arrest and reduces ecdysteroid levels.** (A) RNAi mediated knock down of vvl, kni or mld in the PG using a PG specific driver (phm->) results in developmental L1 arrest for phm->vvl-RNAi and phm->mld-RNAi and L1 and L2 arrest for phm->kni-RNAi larvae. The morphology of the cells in the PG (GFP; green in the top left corner) is normal in phm->GFP, vvl-RNAi, phm->GFP, kni-RNAi and phm->GFP, mld-RNAi animals 36 hours AEL (scale bars, 20 μm). Supplying phm->vvl-RNAi, phm->kni-RNAi and phm->mld-RNAi larvae with 20-hydroxyecdysone (20E) rescues the developmental arrest. (B) Ecdysone levels, as measured by the ecdysone inducible gene E75B, is reduced in the mid-first instar (36 hours AEL) by knock down of vvl, kni or mld in the PG. (C) Ecdysteroid levels measured by ELISA confirm that L1 larvae with reduced expression of vvl, kni or mld in the PG have low levels of ecdysteroids 36 hours AEL compared to the control. Error bars indicate s.e.m. (n = 4). *P < 0.05, ***P < 0.001, versus the phm->+ control.

(Fig. 1D and S1) [12, 21]. Expression of vvl was highly enriched in the ring gland, like phm, while kni expression was less specific to this tissue similar to mld.

**PG loss of vvl and kni result in developmental arrest due to failure in ecdysone production**

Based on the potential regulatory role of Vvl and Kni, we next sought to determine if these transcription factors are required for PG expression of the genes involved in ecdysone biosynthesis. We used the PG specific P9206-Gal4 (phm->) driver and observed that knock down of vvl in the PG using UAS-vvl-RNAi (vvl-RNAi) resulted in first instar (L1) arrest (Fig. 2A). Furthermore, RNAi mediated knock down of kni in the PG, by using phm-> with a UAS-kni-RNAi (kni-RNAi), led to an L1 and second instar (L2) arrest phenotype. To exclude the contribution of off-target effects, we tested PG specific knock down of vvl and kni using other transgenic RNAi lines that target different regions of the vvl and kni mRNA and found that they produce similar phenotypes (Table S1). To support this, we also used the P9206-Gal4 (P9206->) driver that promotes weak expression in the PG cells [22]. When expression of vvl and kni was reduced using P9206->, development was arrested during later stages compared to when crossed with phm->. Knock down of mld in the PG with phm-> driven UAS-mld-RNAi (mld-RNAi) also resulted in L1 arrest (Fig. 2A) consistent with mutant analysis [9, 21].

If kni and vvl are involved in specifying the gland during embryonic development, reducing their expression may cause a lack of PG cell differentiation. We used a phm->GFP to label and examine the morphology of the PG in L1 larvae 36 hours after egg lay (AEL). PG cell number and morphology of L1 larvae with reduced expression of vvl, kni or mld in the PG were indistinguishable from the phm->+ control (Fig. 2A). This demonstrates that vvl and Kni are required for ecdysone production by regulating steroidogenic gene expression

We next investigated if Vvl and Kni regulate the expression of the genes involved in ecdysone biosynthesis. phm->+ and...
phm, kni-RNAi larvae showed reduction in the expression of phm, dib and sad by the mid-first instar 36 hours AEL compared to the control (Fig. 3A). Knock down of kni also reduced expression of sro and spok, catalyzing early steps in the pathway, as well as a reduction of phm, dib and sad mediating the last three steps in the biosynthetic pathway. Knock down of kni results in down-regulation of phm, dib and sad, while knock down of mld causes a specific down-regulation of sro and a moderate reduction of sro. Expression was measured in mid-first instar larvae 36 hours AEL. Error bars indicate s.e.m. (n = 4). ±P<0.05, **P<0.01, versus the phm> control. (B, C) Direct binding of Vvl or Kni to the regulatory sites in phm promoter indicated by electrophoretic mobility shift assay (EMSA). Nuclear extract was incubated with [γ-32P]ATP-labeled oligonucleotide sequences of phm promoter containing the vvl (B) or the kni sites (C) and resulted in shifted DNA-protein bands (lane 1). Competition assays were performed with unlabeled non-specific random oligonucleotide sequences (lane 2), the phm promoter containing the vvl or kni sites (lane 3), the phm promoter with mutated vvl or kni sites (lane 4), an oligonucleotide sequence with vvl or kni consensus motif sequence (lane 5), or with the consensus motif mutated (lane 6).

doi:10.1371/journal.pgen.1004343.g003

Figure 3. vvl, kni and mld are required for the expression of genes in the ecdysone biosynthetic pathway. (A) Knock down of vvl, kni and mld in the PG reduces expression of genes in the steroidogenic pathway. vvl knock down results in a down-regulation of spok and sro, catalyzing early steps in the pathway, as well as a reduction of phm, dib and sad mediating the last three steps in the biosynthetic pathway. Knock down of kni results in down-regulation of phm, dib and sad, while knock down of mld causes a specific down-regulation of sro and a moderate reduction of sro. Expression was measured in mid-first instar larvae 36 hours AEL. Error bars indicate s.e.m. (n = 4). ±P<0.05, **P<0.01, versus the phm> control. (B, C) Direct binding of Vvl or Kni to the regulatory sites in phm promoter indicated by electrophoretic mobility shift assay (EMSA). Nuclear extract was incubated with [γ-32P]ATP-labeled oligonucleotide sequences of phm promoter containing the vvl (B) or the kni sites (C) and resulted in shifted DNA-protein bands (lane 1). Competition assays were performed with unlabeled non-specific random oligonucleotide sequences (lane 2), the phm promoter containing the vvl or kni sites (lane 3), the phm promoter with mutated vvl or kni sites (lane 4), an oligonucleotide sequence with vvl or kni consensus motif sequence (lane 5), or with the consensus motif mutated (lane 6).

doi:10.1371/journal.pgen.1004343.g003

phm>kni-RNAi larvae showed reduction in the expression of phm, dib and sad by the mid-first instar 36 hours AEL compared to the control [Fig. 3A]. Knock down of vvl also reduced expression of sro and spok, encoding enzymes believed to work in early steps in the pathway known as the black box [4,5,9]. However, expression of nvd, encoding a PG specific gene involved in the first step in the biosynthetic pathway, was not significantly reduced in the mid-first instar by knock down of vvl or kni in the PG. This further supports the notion that the PG is specified normally during embryogenesis. Previous studies have indicated that mld mutants have reduced ecdysone levels because of a specific lack of spok expression [9,21]. Our knock down results involving mld-RNAi in the PG support that Mld is required specifically for the expression of spok, but not for the later acting products of phm, dib and sad.

The binding sites of these factors in the PG specific regulatory elements indicate that Vvl and Kni are involved in a transcriptional network necessary for co-expression of the biosynthetic enzymes. We therefore sought to establish if Vvl and Kni can bind directly to the PG specific regulatory elements by performing a DNA/protein binding assay. For this purpose, we performed
electrophoretic mobility shift assays (EMSAs) with the conserved sites in the phm promoter since the functional importance of these sites has been confirmed [20]. Radiolabeled DNA oligonucleotide sequences that contained the conserved vvl or kni binding sites in the phm promoter required for PG expression (Fig. 1A) formed DNA/protein complexes with nuclear cell extract (Fig. 3B and C). These complexes were outcompeted by unlabeled oligonucleotide sequences containing consensus vvl or kni sites and by the unlabeled phm oligonucleotides containing the vvl or kni site, but not by the unlabeled phm oligonucleotides with mutated vvl or kni binding sites or by an unspecific oligonucleotide sequence. This finding demonstrates that the vvl and kni sites are required for

Figure 4. vvl, kni and mld have a specific role in regulating ecdysone biosynthesis in the L3 stage. (A) Expression of steroidogenic genes in ring glands from wild type (phm-GFP) larvae increases little from early (72 hours AEL) to mid (96 hours AEL) third instar, but rises dramatically in the late (120 hours AEL) third instar. vvl expression exhibits a minor increase in the late third instar, while mld, kni and especially Br-Z4 show a strong increase (n = 4). (B) Expression in the ring gland from larvae with knock down of vvl, kni or mld during the L3 stage two days after temperature induced activation of the RNAi with the Gal80ts;phm driver 96 hours AEL. Expression of all the steroidogenic genes were significantly reduced in animals with reduces vvl or kni expression. Knock down of mld results in a dramatic reduction in expression of nvd and spok that mediate two early steps in the biosynthesis of ecdysone (n = 4). (C) Quantified level of Phm protein in brain-ring gland complexes (BRGCs) from L3 larvae two days after temperature induced RNAi (96 hours AEL) normalized to Tubulin levels determined by immunoblotting (top panel) (n = 3). (D) Ecdysteroid levels determined by ELISA in L3 larvae with reduced PG expression of vvl, kni or mld two days after temperature induced activation of the RNAi effect (96 hours AEL) (n = 4). Error bars indicate s.e.m. *P<0.05, **P<0.01, ***P<0.001, versus the Gal80ts;phm control.

doi:10.1371/journal.pgen.1004343.g004
Figure 5. Ecdysone and 20E efficiently rescue loss of vvl and kni in the PG. (A) Ecdysone biosynthetic scheme showing steps in the conversion of cholesterol to 20-hydroxyecdysone (20E). Note that ecdysone produced and released from the PG is converted to its active form 20E in peripheral tissues. (B) Percentage of larvae developing to the indicated stage. L1; first instar larvae, L2; second instar larvae, L3; third instar larvae. Resupplying precursors later in the pathway is gradually more efficient in rescuing arrest of larvae with reduced expression of vvl and kni in the PG. In contrast, only precursors downstream of the black box efficiently rescue mld-RNAi larvae, indicating that Mld regulates a gene product(s) involved in the reactions upstream of the 5β-ketodiol. C; cholesterol, 7dC; 7-dehydrocholesterol, KD; 5β-ketodiol, KT; 5β-ketotriol, 2dE; 2-deoxyecdysone, E; ecdysone, 20E; 20-hydroxyecdysone. doi:10.1371/journal.pgen.1004343.g005

Vvl and kni are required to maintain expression of the biosynthetic genes during late larval development

The data indicate that Vvl and Kni are critical for the steroidogenic activity of the PG during early post-embryonic development. Later during larval development the up-regulation of ecdysone biosynthetic genes and the growth of the gland are required to produce the high-level ecdysone pulse that triggers metamorphosis. To investigate the role of Vvl and Kni during later stages of postembryonic development, we analyzed their expression in third instar (L3) larval ring glands from early (72 hours AEL), mid (96 hours AEL) and late (120 hours AEL) L3 larvae. In wild type larvae, expression of the steroidogenic genes showed no or little increase from the early to mid L3, but a dramatic up-regulation in the late L3 (Fig. 4A), coinciding with the high-level ecdysone peak that triggers pupariation 120 hours AEL [20]. While the expression of vvl showed only a minor increase during the L3 stage, a stronger up-regulation of kni and mld was observed. Compared to both vvl, kni and mld, expression of Br-Z4 was highly up-regulated in the late L3 consistent with its role in the temporal up-regulation of the biosynthetic genes important for the high-level ecdysone pulse 120 hours AEL that triggers pupariation [24]. Considering the tissue-specificity and that vvl expression in the PG shows little relation with the ecdysone pulse, it seems likely that Vvl is important for the spatial control of ecdysone biosynthetic gene expression in the PG, but not for the temporal regulation during development. On the other hand, kni expression is less PG specific, but show more correlation with the ecdysone titer during L3.

To determine whether Vvl and Kni are only required to set the initial expression of the biosynthetic enzymes during embryonic and early larval development or also to maintain PG expression during late larval stages, we used tub-Gal80ts;phm-Gal4 (Gal80ts;phm>), to conditionally induce the UAS-RNAi effect. Gal80ts;phm>vvl-RNAi and Gal80ts;phm>kni-RNAi larvae develop normally at 18°C and were shifted to 29°C to induce the RNAi at different times during larval stages. Development of most Gal80ts;phm>vvl-RNAi, Gal80ts;phm>kni-RNAi and Gal80ts;phm>mld-RNAi larvae was arrested in L3 when larvae were shifted to 29°C 120 hours AEL or earlier, while larvae that were shifted 144 hours AEL or later pupariated normally (Fig. S2). Because development is slowed down at 18°C, 120 hours AEL is corresponding to the late L2 stage under these conditions [25]. Thus, inducing RNAi in late L2 or earlier causes developmental arrest in L3, suggesting that it prevents the production of the high-level ecdysone pulse in late L3 that triggers metamorphosis.

Since expression of the biosynthetic genes detected in L1 larvae with reduced expression of vvl and kni was measured on RNA extracted from whole animals (Fig. 3A), and normalized to RpL32 and Rpl32, ubiquitously expressed ribosomal housekeeping genes, it is possible that reduced PG cell growth could be responsible for the observed decrease in biosynthetic gene expression because of a reduced PG to whole animal size ratio. To exclude this possibility and to test whether Vvl and Kni are required to maintain PG specific expression of steroidogenic genes later during development, we analyzed expression in isolated ring glands from L3 larvae. We first confirmed the efficient reduction of vvl and kni mRNA levels compared to the control (Fig. S2). Additional analysis showed that expression of all of the steroidogenic enzymes was dramatically decreased in L3 ring glands in which vvl and kni were knocked down compared to the control (Fig. 4B). This demonstrates that the decreased expression of the ecdysone biosynthetic genes in vvl-RNAi and kni-RNAi animals is a consequence of a specific reduction in the transcription of steroidogenic genes, and not reduced glandular growth or a general reduction in transcription. Compared to vvl-RNAi and kni-RNAi, mld knock down had little or no influence on the

formation of the DNA/protein complex and supports that Vvl and Kni regulate transcription of the genes involved in ecdysone biosynthesis by direct binding to their promoters and enhancers.
transcription of the genes encoding enzymes acting in late steps in the biosynthetic pathway. However, spok and ned levels were strongly reduced in the ring glands of mld-RNAi larvae compared to the control, suggesting that these are direct targets of mld regulation. This indicates that Mld is involved in the transcriptional regulation of the enzymes mediating early biosynthetic conversions of cholesterol. The observation that mld-RNAi also regulates ned expression may explain why spok overexpression in the PG is insufficient to rescue mld mutants [9].

To examine the influence of vvl and kni knock down on the biosynthetic enzyme level, we measured Phm protein levels in brain-ring gland complexes (BRGCs) using immunoblotting analysis. Consistent with the reduced mRNA levels, these results show that Phm protein levels are dramatically reduced in vvl-RNAi and kni-RNAi larvae compared with the control (Fig. 4C). To reinforce that knock down of vvl, kni or mld in the PG impairs ecdysone biosynthesis, we also measured the ecdysteroid levels in L3 larvae. Ecdysteroid levels were reduced in L3 larvae where RNAi mediated knock down of vvl, kni or mld in the PG had been induced in the L2 stage (Fig. 4D).

The developmental arrest by PG inactivation of vvl and kni can be rescued by ecdysone and 20E

Taken together, the data suggest that the coordinated expression of steroidogenic enzymes in the PG requires Vvl and Kni function. To further corroborate our findings that Vvl and Kni are involved in co-regulating all components in the biosynthetic pathway, we examined whether supplementation of any 20E precursors to the larval growth medium was able to rescue the developmental arrest of vvl or kni RNAi larvae. When fed cholesterol, 7-dehydrocholesterol or 5β-ketodiol, most phm>vvl-RNAi and phm>kni-RNAi animals develop to small L2 larvae (Fig. 5A and B). Since phm>vvl-RNAi and phm>kni-RNAi arrest in L1 and L2 without supplementation, it appears that increasing the amount of substrate for ecdysone synthesis provides some compensation, but not complete rescue, when the pathway activity

Figure 6. vvl and kni knock down affects PG cell size and disturbs PTTH/Torso and insulin/TOR signaling. (A, B) PG (green) and nuclei (magenta; DAPI) show that the morphology of the gland is intact in late L3 larvae with reduced expression of vvl, kni, or mld in the PG. PG cell and nucleus size of L3 larvae (measured as the diameter) are reduced when vvl and kni are knocked down (n = 11). RNAi was induced using the Gal80ts; phm>96 hours AEL corresponding to the late L2 stage at 18°C. The PG was analyzed after two days at 29°C when control larvae are in the wandering stage. (C) Knock down of vvl and kni results in decreased expression of torso (tor), 4EBP and S6K in dissected ring glands containing the PG. Likewise, 4EBP and S6K are down regulated in ring glands of mld-RNAi animals that also exhibit reduced expression of the InR. Contrary, akt is increased in vvl-RNAi animals (n = 4). (D) Loss of vvl, kni or mld results in a strong increase of npc1a expression in the ring gland. In C and D, expression was measured in L3 larvae raised as described in A and B (n = 4). Error bars indicate s.e.m. *P<0.05, **P<0.01, ***P<0.001, versus the Gal80ts; phm> control.

doi:10.1371/journal.pgen.1004343.g006
is reduced. Supporting this notion, providing intermediates further downstream in the pathway gradually increased rescue of phm\( \rightarrow \)vel-RNAi and phm\( \rightarrow \)kni-RNAi larvae to the L3 stage. In particular, 20E and its precursor ecdysone efficiently rescue phm\( \rightarrow \)vel-RNAi and phm\( \rightarrow \)kni-RNAi larvae to the L3 stage (Fig. 5B).

We then tested whether increased availability of cholesterol substrate is sufficient to promote ecdysone biosynthesis. Indeed, supplementation with cholesterol increased E75E mRNA in wild type larvae and ecdysone levels in the control and in larvae grown on standard food (Fig. S3). Like rescue of the L1 arrest (Fig. 3B), cholesterol also provided minor rescue of the L3 developmental arrest observed when the RNAi effect was induced in the L2 stage (Table S2). Increasing cholesterol concentrations only provides minor rescue for loss of vvl and kni. In contrast, we confirmed that it provides complete compensation for loss of Niamann-Pick type C-1a (npc1a) (Fig. S3), which reduces substrate delivery for ecdysone biosynthesis [26,27]. These results suggest that the hormone deficiency observed in vel-RNAi and kni-RNAi larvae is a result of impaired ecdysone pathway activity and not compromised cholesterol substrate delivery, like in phm\( \rightarrow \)npc1a-RNAi larvae. These findings overall indicate that silencing vvl or kni in the PG specifically inhibits synthesis of ecdysone by reducing the activity of the biosynthetic pathway.

Supplementing the 5\( \beta \)-ketodiol and 5\( \beta \)-ketotriol, but not cholesterol or 7-dehydrocholesterol, rescued mld-RNAi larvae (Fig. 5B), consistent with Mld being required for expression of Nvd and Spok which mediate early steps in the pathway upstream of the 5\( \beta \)-ketodiol. We conclude, that Vvl and Kni are necessary for coordinating the tissue-specific expression of all steroidogenic genes in the endocrine cells of the PG, while Mld specifically regulates genes involved in early steps in the pathway responsible for the conversion of cholesterol to the 5\( \beta \)-ketodiol, an intermediate downstream of the black box reaction(s).

PTTH and insulin/TOR signaling in the PG is disturbed by loss of vvl and kni

Our data demonstrate that Vvl and Kni are specifically involved in transcriptional regulation of ecdysone biosynthetic components. However, when we analyzed the morphology of PG cells with reduced expression of vvl and kni, we found a mild decrease in PG cell size (Fig. 6A and B), indicating that knock down of these transcription factors also influence cellular growth. The major pathways that are thought to control PG cell growth are the PTTH and the insulin/TOR pathways [22,28–32]. Therefore, we investigated the possibility that Vvl and Kni affect PG cell growth and ecdysone synthesis indirectly by interfering with PTTH and/or insulin/TOR signaling. The neuropeptide, PTTH promotes PG growth and ecdysone synthesis through activation of its receptor Torso, a receptor tyrosine kinase (RTK) expressed specifically in the PG [33]. Activation of the insulin receptor (InR), another RTK, in the PG also regulates cell growth and stimulates ecdysone synthesis in response to circulating insulin levels. Although crosstalk between systemic insulin mediated growth regulation and TOR signaling might occur, the TOR pathway cell-autonomously regulates growth in response to cellular nutrient levels [34]. We therefore investigated whether PTTH and insulin/TOR signaling in the gland is affected by knock down of vvl and kni. Analysis of torso transcript levels revealed that, while mld-RNAi larvae have normal torso mRNA levels, expression of the PTTH receptor is reduced in ring glands from L3 vel-RNAi and kni-RNAi larvae (Fig. 6C). Consistent with down-regulation of the PTTH receptor, we found reduced levels of phosphorylated ERK, an indicator of MAPK activity and PTTH signaling [33], in BRGCs from vel-RNAi and kni-RNAi larvae (Fig. S4). However, unlike the biosynthetic enzymes (Fig. 3), expression of torso was not reduced in L1 phm\( \rightarrow \)vel-RNAi larvae 56 hours AEL (Fig. S4), indicating that torso expression is initiated normally despite the loss of vvl in the PG. When examining the expression of the InR and components mediating insulin signaling, we found reduced expression in vel-RNAi and kni-RNAi animals of 4EBP that encodes a negative growth regulator depressed by activation of the insulin pathway. Further, levels of aki, which encodes a serine/threonine kinase of the insulin signaling pathway [35], were increased, while levels of InR were decreased in vel-RNAi larvae. Increased insulin signaling is generally associated with decreased expression of both 4EBP and InR [36,37]. These results imply that loss of vvl and kni increases insulin signaling. The most likely explanation for increased insulin signaling in PG of animals with reduced vvl and kni expression is the low ecdysone levels, which cause a general increase of insulin release from the brain [29]. Thus, the disturbance of insulin signaling in the PG of vel-RNAi and kni-RNAi animals seems unlikely to account for the PG cell growth reduction. However, we observed a strong transcriptional reduction of the S6 kinase (S6K), an important positive growth regulator downstream of TOR. This suggests that the combined reduction of both PTTH/Torso and TOR/S6K signaling in the PG contributes to the negative influence of vel-RNAi and kni-RNAi on PG cell growth and ecdysone synthesis. Why does mld knock down not affect PG cell size negatively (Fig. 6A and B)? Since loss of mld does not affect torso expression (Fig. 6C), it is possible that disturbance of the TOR/S6K pathway alone is insufficient to impair growth, especially if this is combined with increased insulin signaling as indicated by the decreased InR and 4EBP mRNA levels in the ring glands of mild-RNAi larvae.

Knock down of vvl and kni affects Npc1a involved in cholesterol trafficking

Finally, we investigated whether loss of vvl and kni in the PG affects cholesterol substrate delivery for ecdysone synthesis. Surprisingly, we found that, whereas the biosynthetic genes show a strong decrease, npc1a exhibits a dramatic increase in the gland of vel-RNAi, kni-RNAi and mild-RNAi larvae (Fig. 6D). This finding indicates that up-regulation of npc1a in the PG of vel-RNAi, kni-RNAi and mild-RNAi larvae reflect a compensatory feedback regulation to maintain cholesterol homeostasis and/or increase substrate delivery to promote steroidogenesis. Down-regulation of biosynthetic activity in vel-RNAi, kni-RNAi and mild-RNAi larvae reduces cholesterol flux through the ecdysone pathway and may lead to intracellular redistribution of cholesterol to maintain homeostasis through feedback regulation. We therefore explored the possibility that npc1a, which is required for normal cholesterol distribution and availability for steroid synthesis, is controlled by feedback regulation of cholesterol. Expression of npc1a is repressed by cholesterol in wild type larvae (Fig. S4), indicating that npc1a is feedback regulated. Recently, we showed that ecdysone biosynthesis is controlled by feedback circuits in the PG [20]. We therefore also examined whether ecdysone signaling in the gland is affected by knock down vel, kni or mld. To test this, we measured mRNA levels of the ecdysone receptor (Ecr) ring glands isolated from L3 larvae where vel-RNAi, kni-RNAi or mld-RNAi had been induced in the PG during L2. Transcript levels of Ecr were not affected in ring glands from vel-RNAi and kni-RNAi larvae (Fig. S4), indicating that the responsiveness of the PG to ecdysone is not reduced. Taken together, these results suggest alterations of cholesterol uptake and trafficking in the PG when flow through the biosynthetic pathway is impaired.
Discussion

*Drosophila* developmental progression is dictated by tightly regulated ecdysone pulses released from the PG. Like any cell specialized for steroid biosynthesis, the PG expresses a set of enzymes that mediate steps in the conversion of cholesterol into steroids. The tissue-specific expression of these enzymes is key to the specialization of the cells that endows the PG with the competence to produce ecdysone. The transcriptional control mechanism underlying such regulation is likely orchestrated by a regulatory network of transcription factors. Here, we identify two transcription factors Vvl and Kni that are required for the expression of the biosynthetic enzymes in the ec dysone producing PG cells. Vvl is a POU domain transcription factor which has multiple important functions during *Drosophila* development. Mutations in *vvl* cause embryonic lethality with defects in the development of the trachea and the nervous system [38–41]. Moreover, Vvl is required for wing vein development and is involved in innate immunity by regulation of the expression of antimicrobial peptides [42,43]. We show that Vvl is expressed in the PG during late embryogenesis and in the larval stages. One important characteristic of Vvl is that it maintains its own expression by autoregulation [44]. Once activated, Vvl maintains its expression and likely also the expression of the ec dysone biosynthetic genes in the PG. Knock down of *vvl* in the PG reduces the expression of all genes in the biosynthetic pathway, showing that Vvl is required for maintaining expression of all pathway components. Together with the high expression of Vvl in the gland, this suggests that Vvl is a master transcriptional regulator involved in specifying the genetic program that dictates PG cell identity including its tissue-specific expression of steroidogenic enzymes. It is interesting to note that human chromosome 6 deletions that affect POU3F2, a homolog of Vvl, have been associated with hypogonadotropic hypogonadism and adrenal insufficiency [45,46], making it possible that Vvl is a conserved regulator of steroid biosynthesis.

The gap gene *kni* is known for its role in embryonic segmentation patterning and development of the trachea and wing vein [47–51] similar to *vvl*. Kni is a nuclear receptor with a zinc-finger motif that is unlikely to be ligand activated since it lacks a ligand-binding domain. Our data show that Kni is required for expression of the genes involved in ec dysone biosynthesis in the PG, suggesting that Kni functions as an activator in this situation. Although Kni is generally considered a short-range repressor [52], it is required to activate hairy expression in stripe 6 during embryogenesis [53]. Thus, Kni may act either as a repressor or as an activator in a context-dependent manner. In mammals, nuclear receptors are also key regulators of steroidogenic target genes encoding P450 enzymes [54–56].

Although Vvl and Kni specifically control genes in the steroidogenic pathway, other targets of these factors could also be important for ec dysone synthesis in the PG. During development the continuous growth of the PG cells and endoreplication of DNA is important to scale its hormone production to the capacity required for developmental progression. We found that both *vvl-RNai* and *kni-RNai* larvae have mildly reduced PG nuclei and cell size, which is likely to contribute to the reduced ec dysone levels in these animals. Kni has been shown to suppress endoreplication activity in the gut by regulating cell cycle genes [48]. This is in contrast to our observation indicating that loss of *kni* results in a reduction in the nuclei size, and hence, reduced polyplody of the PG cells. Instead our results indicate that loss of *vvl* and *kni* decreases activity of PTTH/Torso and TOR/S6K signaling, two major pathways that promote growth and stimulate ec dysone biosynthesis [30,31,33,37]. However, loss of *vvl* and *kni* had no effect on *tusso* expression in the mid-first instar. This indicates that these factors are not required for the initial setting of *tusso* expression, but for the maintenance of high *tusso* expression during development. In tracheal cells, Vvl is required to maintain expression of the RTK *breathless*, but not for activating its initial expression [42,50]. It is unclear how the transcription of the biosynthetic enzymes fluctuates during the low level ec dysone peaks in L1 and L2, before the induction of the steroidogenic pathway by PTTH stimulation [17]. Unlike PTTH/Torso, Vvl and Kni are required in the PG during L1 and L2 for the transition to the L3 stage, which suggests that Vvl and Kni are important for the proper transcription of the biosynthetic enzymes throughout larval development. Altogether, these data suggest that in addition to being required to initiate and maintain expression of the biosynthetic enzymes, Vvl and Kni play an indirect role important for ec dysone production by enabling PG cells to be competent to respond to PTTH and by regulating the TOR/S6K pathway. In contrast, Vvl and Kni are not required for normal expression of *EcR* in the gland, indicating that feedback regulation of ec dysone biosynthesis is not influenced by knock down of these factors [20]. In contrast to the transcription factor *Br-Z4* involved in positive feedback regulation, which is strongly induced in the PG during late L3 to up-regulate expression of the biosynthetic pathway components, PG expression of *vvl* shows little relation with the high-level ec dysone peak that triggers pupariation. Taken together these data suggest that Vvl is required for maintaining PG specific expression (i.e. spatial control), while temporal regulation during development is controlled by other factors such as *Br-Z4*. Furthermore, our results confirm that Mld is required for PG expression of *spok* [9], but we also found that it controls Nvd, an enzyme that acts upstream of Spok in the biosynthetic pathway [4]. Thus, our data suggest that Mld is a specific regulator of the two early enzymes Nvd and Spok, while its function is not important for biosynthetic reactions that are downstream of Spok and the black box reaction(s) and the responsiveness of the PG to PTTH.

Our data show that Vvl and Kni are required in the PG during post-embryonic development to maintain PG specific expression of the ec dysone biosynthetic genes. During embryogenesis, *vvl* expression appears in the PG primordium by stage 13, after the embryonic ec dysone pulse (stage 8–12 [12]) that is required for morphogenesis and differentiation of the embryo. During early embryonic development where the PG primordium is not yet formed, the spatial expression patterns of *kni* and *vvl* (Fig. 1B) are different from the biosynthetic genes essential for the embryonic ec dysone pulse [8,9,11,12,59]. This suggests that Vvl and Kni regulate the biosynthetic genes in the PG, but not during early embryonic development. Consistent with this notion, *vvl* and *kni* mutants differentiate the embryonic cuticle [42,60], unlike the ec dysone deficient mutants that are unable to produce the embryonic ec dysone peak [11,59]. In adult females, the ovaries are believed to be the source of ec dysone consistent with expression of the ec dysone biosynthetic genes in the nurse and/or follicle cells [11–13]. In adult females, we find that *vvl* is expressed in both nurse and follicle cells, suggesting that Vvl may be involved in regulating expression of the ec dysone biosynthetic genes in the adult stage.

Interestingly, we observed that loss of *vvl*, *kni* or *mld* results in dramatic increase of *npcl* expression in the PG. Npcl is highly expressed in the PG where it is required for uptake and intracellular trafficking of cholesterol for steroidogenesis [26]. Larvae with loss of *npcl* exhibit a punctuate pattern of sterol accumulation in the PG cells, indicating defects in cholesterol
transplant within the cells. Normally cholesterol is taken up as low density lipoproteins (LDLs) and trafficked within endosomes to the lysosomes where hydrolysis releases free cholesterol that is delivered to the plasma membrane and endoplasmic reticulum (ER) [61] where the first step in the conversion of cholesterol to ecdysone likely takes place. Why is npc1a up-regulated in the PG when ecdysone synthesis and pathway activity is impaired? It seems unlikely that Vvl, Kni and Mid are all involved in repression of npc1a. The block of flux through the biosynthetic pathway in the PG of vvl-RNAi, kni-RNAi and midl-RNAi animals may change intracellular cholesterol pools in the gland and affect feedback regulation to maintain cholesterol homeostasis. Our results indicate that npc1a is regulated by cholesterol suggesting that the up-regulation of npc1a may be part of a feedback regulatory response to changes in cellular cholesterol levels. This may indicate a compensatory mechanism to redistribute cholesterol by increasing storage of cholesterol esters and/or efflux to reduce free cholesterol levels when ecdysone biosynthesis is blocked. Moreover, npc1a is regulated by Br [27], a factor induced by EcR in the PG [20], implying that npc1a may also be regulated by ecdysone feedback. Our study shows that cholesterol availability is an important parameter for ecdysone biosynthesis. Interactions between cholesterol and ecdysone feedback mechanisms may therefore be important for coordinating the supply cholesterol with the rate of steroidogenesis.

A key aspect of steroidogenesis is regulating the tissue-specific expression of the biosynthetic enzymes. We have shown here that the transcription factors, Vvl and Kni, are required for the coordinated expression of ecdysone biosynthetic genes in the PG. The transcriptional activation by Vvl and Kni is likely mediated by direct binding to cis-regulatory elements responsible for PG specific expression. This identifies an important new role for Vvl and Kni during post-embryonic development in the gene regulatory network of the steroid hormone producing cells in Drosophila.

Materials and Methods

Drosophila strains and husbandry

The following Drosophila strains were used in this study: w1118, UAS-vvl-RNAi (#110723), UAS-kni-RNAi (#2980), UAS-midl-RNAi (#101867) and UAS-npc1a-RNAi (#105405) from the Vienna Drosophila RNAi Center (VDRC); UAS-vvl-RNAi (#26228), UAS-kni-RNAi (#34705), tub-Gal40 and UAS-CD8-GFP (UAS-GFP) from the Bloomington Drosophila Stock Center (BDSC); phm2-Gal4 (phm-Gal4) [9] and P9206-Gal4 [29]. A transgenic line phm-291-4B (phm-GFP) with a 69 bp phm promoter in a pH-stinger GFP reporter vector generated in [20] was used to collect ring glands by dissection for analyzing the development expression profile in the gland. Flies were raised on standard cornmeal food under a 12:12 hour light:dark cycle. For experiments involving staged or timed larvae, flies were allowed to lay eggs at 25°C for 2-4 hours on apple juice agar plates supplemented with yeast paste in a humidified chamber. After 24 hours, 25 L1 larvae were collected and transferred to vials containing standard food. For experiments using tub-Gal40, eggs deposited at 25°C were immediately transferred to 18°C and 25 larvae were transferred to vials containing food 48 hours later. Images of phenotypes were captured with an Olympus SZX7 camera and analyzed using AxioVision software (Zeiss). Characterization of the PG specific spok element was done as described [20] by generating transgenic animals with constructions of 5′-UTR spok elements in a pH-stinger GFP reporter vector.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as previously described [20]. DNA oligonucleotide sequences (Table S3) were designed to cover Vvl and Kni binding sites in the phm promoter based on in silico analysis using Transfac and Jaspur databases. Oligos containing Vvl (Vvl-wt) or Kni (Kni-wt) consensus binding sites and oligos with mutations that disrupt the Vvl (Vvl-mut) or Kni (Kni-mut) binding sites were adapted from [62,63]. The complementary oligonucleotides were annealed and labeled at the 5′-end labeling by [γ32P]ATP (Perkin Elmer) using T4 polynucleotide kinase (Fermentas) and purified using Microspin G-25 columns (GE Healthcare). The EMSA reaction was performed on ice by mixing Drosophila S2 cell nuclear extracts (Active Motif), dialysis buffer (25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol), gelshift buffer (25 mM Tris-HCl pH 7.5, 5 mM MgCl2, 60 mM KCl, 0.5 mM EDTA, 5% Ficoll 400, 2.5% glycerol, 1 mM DTT and protease inhibitors) and poly(dI-dC) (Invitrogen). The reaction mixture was supplemented and incubated with 25-50-fold molar excess of unlabeled competitor nucleotides before adding the radiolabeled probe. After incubation the mixture was supplemented with gelshift loading buffer and run on a 5% non-denaturing polyacrylamide gel and dried on a slab gel dryer (Savant) followed by exposure onto a phosphorimager screen. The image was acquired using a Storm 840 scanner (Molecular Dynamics) and processed with ImageQuant software version 5.2.

Immunostaining

Tissue dissections were performed in PBS followed by fixation in 4% formaldehyde for 20 minutes at room temperature. For this study, the following primary antibodies were: mouse anti-GFP 1:200 (Clontech, #632830); rabbit anti-Phm 1:200 [18]; rat anti-Kni, 1:1000 [64] and rat anti-Vvl 1:1000 [65]. Tissues were incubated over night with primary antibodies at 4°C. Fluorescent conjugated secondary antibodies used were goat anti-mouse Alexa Fluor 488 (A11001, Invitrogen), goat anti-rabbit Alexa Fluor 555 (A21429, Invitrogen) and goat anti-rat Alexa Fluor 555 (A21434, Invitrogen). Secondary antibodies were diluted 1:200 and incubated for two hours at room temperature. DAPI was used in 1:500 for nuclei staining. Confocal images were captured using Zeiss LSM 710 laser scanning microscope and processed using ImageJ (NIH). Images of mid-first instar PG morphology were obtained by confocal imaging of live L1 larvae (36 hours AEL) mounted in 80% glycerol.

Feeding experiment with steroids and precursors

Preparation and synthesis of 3β,14α-Dihydroxy-5β-cholest-7-en-6-one (5β-ketodiol) and 3β,14α,25-Trihydroxy-5β-cholest-7-en-6-one (5β-ketotriol) were previously described [8]. For the steroid feeding rescue experiment, 30 mg of dry yeast was mixed with 57 μl H2O and 3 μl ethanol or supplemented with 3 μl of the following sterols dissolved in ethanol: 20E (Sigma; 450 μg), ecdysone (Sigma; 100 μg), cholesterol (Sigma; 45 μg), 7-dehydrocholesterol (Sigma; 200 μg), 5β-ketodiol (450 μg), or 5β-ketotriol (280 μg). Thirty larvae were transferred to the yeast paste on an apple juice agar plate and allowed to develop in a humid chamber at 25°C. The phenotype of the larvae was scored at day 5 prior to pupariation of w1118 control for rescue to the L3 stage. For other experiments with cholesterol supplementation of the food, standard cornmeal was supplied with cholesterol (Sigma) dissolved in ethanol to a final concentration of 40 μg/ml.
In situ hybridization

DIGoxigenin (DIG)-labeled antisense RNA probes were synthesized using DIG RNA labeling mix (Roche) and T3 (Fermentas), T7 (Fermentas) or SP6 (Roche) RNA polymerase according to the manufacturer’s instructions. For the bin probe, an EST clone GH19318 [66] was used as a templates. For the vvl probe, a portion of vvl gene was amplified by PCR with cDNA derived from \( \omega_1 \) larvae and the following primers: vvl_PA_CDS_F (5′-ATGGCCCGGACCTGTACATGAC-3′) and vvl_PA_CDS_R (5′-CTAGTGGCAGGCACCACTGTATGC-3′). For the mld probe, a portion of mld gene was amplified by PCR with the plasmid mld-\( \mu \)UAST [21]; a gift from S. M. Cohen and the following primers: mld_CDS_1_F (5′-ATCAGTGCCAAACCAGGAGACGC-3′) and mld_CDS_1_R (5′-GCTCATGAGATTGGTCATGA-GATTGTACTTTAGG-3′). PCR products containing the vvl and mld fragments were subcloned into Small-digested pBluescript II SK(-) and pCRII-Blunt-TOPO (Invitrogen), respectively, and then used as the templates for synthesizing RNA probes. Fixation, hybridization and detection were performed as previously described [8,67].

Quantitative RT-PCR

For gene expression experiments using the animals, 30 L1 larvae or 4 L3 larvae were used for each replicate. For analysis of ring gland expression, 10–15 ring glands were dissected in PBS and directly transferred to RNA lysis buffer. RNA was extracted using the RNeasy mini kit (Qiagen) and DNase treated to avoid genomic DNA contamination according to the manufacturer’s instructions. RNA was quantified using a NanoDrop (Thermo Scientific) and the integrity was assessed using agarose gel electrophoresis. Total RNA was used for cDNA synthesis with the SuperScript III First-Strand Synthesis kit (Invitrogen). Primers were designed using the Primer3 software [68] (Table S4). Relative gene expression was analyzed using a Mx3000P qPCR System (Agilent Technologies) with the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer’s instructions as described [10,33,69]. All reactions were subjected to 95°C (10 min), followed by 45 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 15 sec. Dissociation curve analysis was applied to all reactions to ensure the presence of single specific PCR product. Non-reverse transcribed template controls and non-template controls were included to check for background and potential genomic contamination. No product was observed in these reactions. Efficiencies were calculated for each primer pair from standard curves generated from serial dilutions of a mix of cDNA from all control samples. PCR efficiencies were always close to 100%, which was therefore used as the standard in all calculations. Expression of target genes was normalized to reference gene, Rpl23 and Rpl32. We confirmed that these reference [32,33,70–74] are stably expressed across tissues and experimental conditions, by comparing Rpl23 and Rpl32 mRNA levels in cDNA synthesized from equal amounts of RNA extracted from different tissues and developmental stages (Fig. S2). Reference gene stability determined using qBASE Plus (Biogazelle NV, Zwijnaarde, Belgium) was within the recommended limits (M = 0.274 and CV = 0.095). For definition of these stability factors see [75].

Ecdysteroid measurements

For ecdysteroid measurements, ecdysteroids were extracted from whole animals as described [24]. Briefly, whole larvae were rinsed in water and stored at \(-80°C\). Samples were homogenized in 0.5 ml methanol and the supernatant was collected following centrifugation at 14,000 g. The remaining tissue was re-extracted first in 0.5 ml methanol then in 0.5 ml ethanol. The pooled supernatants were evaporated using a SpeedVac and redissolved in ELISA buffer (1 M phosphate solution, 1% BSA, 4 M sodium chloride and 10 mM EDTA). ELISA was performed according to the manufacturer’s instructions using a commercial ELISA kit (ACE Enzyme Immunoassay, Cayman Chemical) that detects ecdysone and 20-hydroxyecdysone with the same affinity [76]. Standard curves were generated using 20E (Sigma). Absorbance was measured at 405 nm on a plate reader, ELx80 (BioTek) using the Gen5 software (BioTek).

Western blotting

Four brain-ring gland complexes were dissected in cold PBS and transferred to 20 µl Laemmli Sample Buffer (Bio-Rad) supplemented with 2-mercaptoethanol. Samples were boiled for 5 minutes, centrifuged at 14,000 g and 10 µl supernatant were loaded on a 4–20% polyacrylamide gradient gel (Bio-Rad) followed by transfer onto a PVDF membrane (Millipore). Primary antibodies used were: mouse anti-tubulin, 1:5,000 (T9026, Sigma Aldrich), rabbit anti-Phm, 1:1,000 [18] and rabbit anti-phospho-ERK, 1:1,000 (9101, Cell Signaling Technology). Secondary antibodies were goat anti-mouse IRDye 680RD, 1:10,000 (926-68070, LI-COR) and goat anti-rabbit IRDye 800CW, 1:10,000 (926–32211, LI-COR). The blot was scanned on an Odyssey Fc (LI-COR) and the software, Image Studio for Odyssey Fc, was used for image processing and protein quantification.

Statistics

The statistical differences between data sets were calculated using two-tailed Student’s t-test and error bars represent standard error of the mean (s.e.m.).

Supporting Information

Figure S1 PG-specific cis-regulatory elements of spok, phm and dbb, immunostaining and in situ hybridization. (A) Vvl and Kni binding sites are indicated on the promoter and enhancer sequences. (B) Immunostaining of the PG from an L2 larva with antibodies against Kni (magenta) and Phm (green). Scale bars, 25 µm. (C) Staining with an antisense mld probe indicates expression of mld in the ring gland PG cells of L3 larvae (b’), but no staining was observed in the embryonic PG (a’). (D) In situ hybridization of adult female ovaries with antisense probes for vvl indicate strong staining in the nurse cells and weaker staining in the follicle cells. (TIF)

Figure S2 Effect of inducing the RNAi at different times during development, RNAi knock down efficiency and reference gene stability. RNAi mediated knock down of vvl, kni or mld was induced at different times using (A) tubGal80\^\#phm-Gal4 (Gal80\^\#phm>) or (B) tubGal80\^\#phm-Gal4,UAS-GFP (Gal80\^\#phm>GFP) by shifting larvae from 18°C to 29°C at the indicated times. (A) Inducing the RNAi effect until 120 hours AEL at 18°C blocks pupariation, while shifting larvae 144 hours and later has little influence on pupariation. This indicates that inducing knock down by Gal80\^\#phm> of vvl, kni and mld as late as 120 hours AEL reduces ecdysone biosynthesis and prevents formation of the high level pulse that triggers pupariation. (B) The effect is strongest when inducing the RNAi 96 hours AEL with the Gal80\^\#phm>GFP driver including GFP. To facilitate analysis of the ring gland, we chose to use Gal80\^\#phm>GFP that labels the PG by expression of GFP (for simplicity hereafter referred to as Gal80\^\#phm>) for all further experiments. (C) Knock down efficiency of vvl, kni and mld in the PG. When the RNAi was induced in the PG 96 hours AEL, expression of vvl and kni was reduced to 20 or 10 percent,
respectively, in dissected ring glands two days later, at the time when the control larvae were in the wandering stage. Expression of mid was reduced to 50 percent at this time. Black bars are the control (Gal80ts;phm) and gray bars show the indicated RNAi animals (n = 5). *P<0.05, **P<0.001, versus the Gal80ts;phm control. (D) Stability of reference gene expression in different stages and tissues. Expression of the reference genes RpL32 and RpL32 in first instar (L1) and third instar (L3) whole larvae shows that these reference genes are stably expressed in the different developmental stages analyzed. Comparison of RpL32 and RpL32 relative quantities in the ring gland (prothoracic gland) of L3 larvae and whole L3 larvae shows stable expression of these genes. Error bars indicate s.e.m.

Figure S3  Effect of a high-cholesterol diet on ecdysteroid levels. (A) Effect of substrate concentrations on ecdysone biosynthesis was determined by measuring the transcription of E75B, as a proxy for ecdysone levels. Expression of E75B was determined in wild type (w1118) late L3 larvae 120 hours AEL, grown either on standard food (−) or a high-cholesterol diet (+). Elevated E75B expression in L3 larvae grown in the presence of cholesterol indicates that the amount of ecdysone produced depends on the supply of substrates (n = 4), **P<0.01, versus the control grown on standard food. (B) RNAi was induced in larvae 96 hours AEL by switching larvae from 18°C to 29°C and ecdysteroid levels were analyzed 36 hours later when control (Gal80ts;phm) larvae raised on high cholesterol exhibited wandering behavior, while animals raised on a standard diet were still in the pre-wandering stage. Ecdysteroid levels are increased in larvae raised on a high-cholesterol diet compared to standard food conditions, consistent with the accelerated development, indicated by the wandering behavior normally associated with the high-level ecdysone peak [24] (n = 4). *P<0.05, **P<0.001, versus the Gal80ts;phm control. (C) The PG must take up cholesterol from circulation to support ecdysone synthesis, a process that requires the function of Npc1a [26], phm>npc1a-RNAi animals, with impaired delivery of cholesterol to the ecdysteroid biosynthesis, arrest development in L1 when grown on a standard diet, but develop normally when cholesterol is increased by dietary supplementation. Error bars indicate s.e.m.

Figure S4  Effect of loss of vvl and kni on PITH and ecdysone signaling and cholesterol feedback regulation of npc1a. (A) Quantification of phosphorylated ERK (p-ERK) levels in brain-gland complexes (BRGCs) from L3 animals determined by immunoblotting with an antibody specific for p-ERK. RNAi was induced 96 hours AEL by switching larvae from 18°C to 29°C and p-ERK levels were measured in BRGCs two days later, p-ERK levels were normalized to Tubulin. *P<0.01, versus the Gal80ts;phm control. (B) Expression of torso was analyzed 36 hours AEL in mid-first instar larvae. (C) mRNA levels of npc1a were measured in wild type (w1118) L3 larvae 120 hours AEL, grown either on standard food (−) or on a high-cholesterol diet (+). Expression of npc1a was repressed by cholesterol (n = 4), **P<0.01, versus the control grown on standard food. (D) Expression of EcR in ring glands from L3 larvae two days after temperature induced activation of vvl-RNAi, kni-RNAi or mid-RNAi in the PG by switching larvae 96 hours AEL from 18°C to 29°C (n = 5). *P<0.01, versus the Gal80ts;phm control. Error bars indicate s.e.m.

Acknowledgments
We thank Thomas Gregor and Makoto Sato for Vvl and Kni antibodies, Stephen M. Cohen and Hideki Yoshida for providing us with plasmids and Yota Hirano for a technical assistance. For use of confocal microscopy, we acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

Author Contributions
Conceived and designed the experiments: ETD MEM YF RH MBO RN KFR. Performed the experiments: ETD MEM ED TKK YF RH MBO RN KFR. Analyzed the data: ETD MEM ED TKK YF JTT RH MBO RN KFR. Contributed reagents/materials/analysis tools: YF JTT MBO RN KFR. Wrote the paper: ETD MEM TKK YF RH MBO RN KFR.

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