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CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is essential for metamorphosis

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ABSTRACT

Ecdysteroids are steroid hormones, which coordinate major developmental transitions in insects. Both the rises and falls in circulating levels of active hormones are important for coordinating molting and metamorphosis, making both ecdysteroid biosynthesis and inactivation of physiological relevance. We demonstrate that *Drosophila melanogaster Cyp18a1* encodes a cytochrome P450 enzyme (CYP) with 26-hydroxylase activity, a prominent step in ecdysteroid catabolism. A clear ortholog of *Cyp18a1* exists in most insects and crustaceans. When *Cyp18a1* is transfected in *Drosophila* S2 cells, extensive conversion of 20-hydroxyecdysone (20E) into 20-hydroxyecdysonoic acid is observed. This is a multi-step process, which involves the formation of 20,26-dihydroxyecdysone as an intermediate. In *Drosophila* larvae, *Cyp18a1* is expressed in many target tissues of 20E. We examined the consequences of *Cyp18a1* inactivation on *Drosophila* development. Null alleles generated by excision of a *P* element and RNAi knockdown of *Cyp18a1* both result in pupal lethality, possibly as a consequence of impaired ecdysteroid degradation. Our data suggest that the inactivation of 20E is essential for proper development and that CYP18A1 is a key enzyme in this process.

Introduction

Regulatory processes must be tightly timed in multicellular organisms to ensure appropriate cell growth and differentiation at each defined life-stage. In insects, ecdysteroids are the steroid hormones that coordinate major developmental transitions, and successive ecdysteroid pulses are necessary to induce larval molts and metamorphosis (Riddiford et al., 2003; Thummel, 2001). Both increases and decreases in the circulating levels of active hormones are important for the timing of developmental events (Apple and Fristrom, 1991; Thummel, 2001), making both biosynthesis and inactivation of ecdysteroids of physiological relevance. Despite this importance, the biosynthetic pathway leading to ecdysteroids is still not completely understood (Lafont et al., 2005; Warren et al., 2009) (Fig. 1). Moreover, only one gene encoding an ecdysteroid inactivating enzyme, ecdysone oxidase, has been so far identified in *Drosophila melanogaster* and no mutants have been characterized (Takeuchi et al., 2005).

Numerous ways to inactivate steroids have been identified in animals, consisting mainly of hydroxylation and conjugation reactions (Lafont et al., 2005; Rees, 1995; You, 2004). A widespread and prominent route of ecdysteroids inactivation is their irreversible conversion to 26-hydroxylated metabolites, and ultimately to the corresponding ecdysonoic acids (Lafont et al., 2005) (Fig. 1). In several insect orders, 26-hydroxylation of the active hormone 20-hydroxyecdysone (20E) has been reported (Lafont et al., 1983) with the formation of transient aldehyde intermediates (Chen et al., 1994; Kayser et al., 2002) (Fig. 1). Biochemical evidence suggests that 26-hydroxylation is catalyzed by a cytochrome P450 enzyme (CYP) (Kayser et al., 1997; Williams et al., 2000), but the identity of the gene encoding this enzyme has never been demonstrated (Bassett et al., 1997; Davies et al., 2006; Hurban and Thummel, 1993).

In the present study, we demonstrate that *D. melanogaster* CYP18A1 catalyzes the 26-hydroxylation of ecdysteroids and their further oxidation to 26-carboxylic acids. We show that, despite the apparent redundancy of hormone inactivation processes in insects, *Cyp18a1* loss-of-function mutants and *Cyp18a1* RNAi inactivation strains both result in an extended final larval instar and lethality during metamorphosis, whilst ectopic over-expression of *Cyp18a1* is

Abbreviations: CYP, cytochrome P450 enzyme; cpr, cytochrome P450 reductase; dib, disembodied; E, ecdysone; 20E, 20-hydroxyecdysone; 20, 20,26E, 20,26-dihydroxyecdysone; 20Eoic, 20-hydroxyecdysonoic acid; EIA, enzyme immunoassay; HPH, hours post hatching; HPL, hours post laying; ISH, in situ hybridization; JH, juvenile hormone; phm, phantom; PTTH, prothoracicotropic hormone; RP-HPLC, reverse phase high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; shd, shade.

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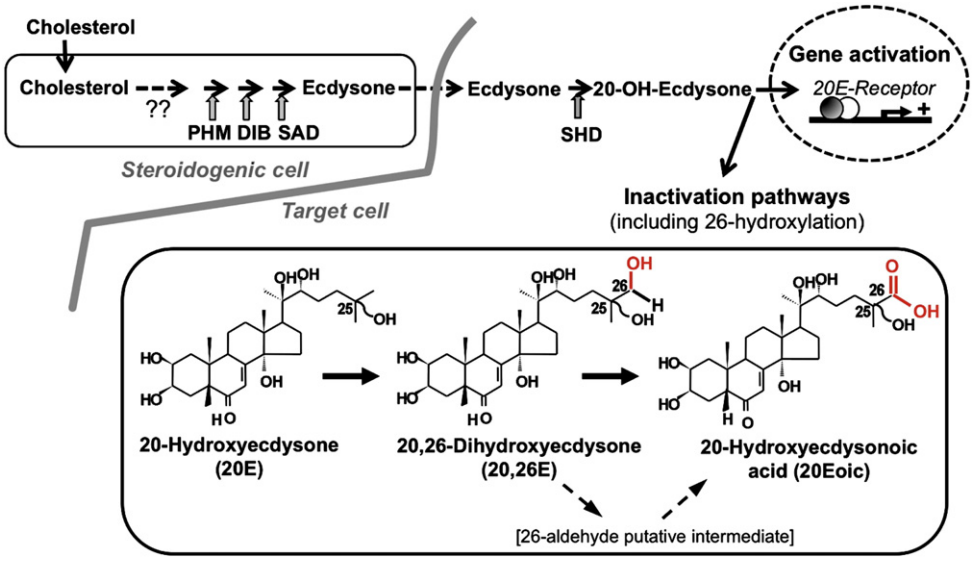

Fig. 1. Scheme of 20-hydroxyecdysone (20E) signaling pathway with emphasis on known steps of 20E biosynthesis and catabolism through 26-hydroxylation. The terminal steps of ecdysteroid biosynthesis have been identified and are catalyzed by the cytochrome P450 enzymes Phantom (PHM or CYP306A1), Disembodied (DIB or CYP302A1), Shadow (SAD or CYP315A1) and Shade (SHD or CYP314A1). After binding to the ecdysone receptor (EcR) and action at gene level, 20E is inactivated. 26-Hydroxylation and further oxidation to 20-hydroxyecdysonoic acid is a prominent inactivation pathway.

lethal. Our results therefore indicate that *Cyp18a1*, conserved in most arthropods, is essential for proper development of *D. melanogaster*.

Materials and methods

Drosophila feeding and staging

D. melanogaster strains were maintained at 25 °C on cornmeal agar medium. Control flies, unless specified, are w^{1118}. For experiments requiring staged larvae, animals were synchronized at the time of hatching by collecting the newly hatched larvae every hour and transferring to agarose plates coated with a paste of brewer's yeast. Larvae were synchronized again at the transition from the second to third larval instar at 30-minute intervals and transferred in groups of 30 into fresh tubes of cornmeal agar medium for further development.

Inactivation of Cyp18a1

Cyp18a1 knockdown flies were produced by generating flies with *Cyp18a1* RNAi transgenes. A 334-bp region specific to *Cyp18a1* was amplified from cDNA using *Cyp18a1*-RNAi-F and *Cyp18a1*-RNAi-R (Table S1) and cloned into pGEM-T Easy (Promega). This fragment was digested with XbaI, using restriction sites introduced in the primers, and sequentially cloned in both sense and antisense orientations downstream of the UAS sites in pWIZ (Lee and Carthew, 2003) using XbaI and SpeI. The resulting vector, marked with w^{+}, contained two copies of the *Cyp18a1* 334-bp fragment in an inverted repeat orientation separated by intron 2 of the *white* gene. Homozygous viable and fertile strains *Cyp18a1*RNAi-7a (transgene insert on chromosome 3) and *Cyp18a1*RNAi-14a (transgene insert on chromosome 2) were used in further analyses. Results obtained with strain *Cyp18a1*RNAi-7a are detailed in the results section. Similar results obtained with *Cyp18a1*RNAi-14a are shown in Supplementary Figs. S5A,B. The 6g1HR-GAL4 strain has been described previously (Chung et al., 2007). The 5′*phm*-GAL4 strain was established by generating transgenic flies carrying 674 bp upstream of the *phantom* gene fused to GAL4. The primers phm-F and phm-R (Table S1) were used to amplify the *phm* upstream region from w^{1118} gDNA. This was cloned into pGEM-T Easy (Promega) and, using NotI, subsequently cloned upstream of GAL4 in the pC3G4 vector. The construct was injected into the w^{1118} strain, and transgenic individuals recovered as w^{+} progeny. A single homozygous lethal insert on chromosome 2, maintained over a *CyO*, GFP balancer chromosome (5′*phm*-GAL4/*CyO*,GFP) was used. GAL4 expression is specific to the prothoracic cells of the ring gland in this strain (Fig. S5C). Other GAL4 drivers used were obtained from Bloomington Drosophila Stock Center, Indiana University. Two independent *Cyp18a1* null strains were generated by the imprecise excision of the modified *P* element (XP) located 601 bp upstream of *Cyp18a1* in the w^{1118} P(XP)*Cyp18a1*^{d07122} strain. The *P* element was mobilized using w^{*}; ry^{506} Sb^{1} *P*(*ry* + t7.2 = Delta2-3)99B/TM6B, Tb^{1}. Mobilization events creating deletions of *Cyp18a1* were detected by PCR using KO-F and KO-R primers (Table S1) spanning the *Cyp18a1* genomic region and confirmed by DNA sequencing. *Cyp18a1*^{null644} and *Cyp18a1*^{null748} are deletions of 644 bp and 748 bp respectively, including 43 bp and 147 bp of *Cyp18a1* coding sequence (from ATG). Both deletions remove the transcription and translation start sites of *Cyp18a1* (Fig. 6A and Fig. S6A). *Cyp18a1* null strains were maintained over a FM7i, *P*(w + mC = ActGFP)JMR3 (abbreviated FM7i-pAct-GFP) X-chromosome balancer. Results obtained with *Cyp18a1*^{null644} are detailed in the results section and similar results obtained with *Cyp18a1*^{null748} are shown as supplementary data (Figs. S6B,C). Survival of *Cyp18a1* null and knockdown individuals was determined by scoring for the presence/absence of GFP, or physical markers in adults. First instar larvae were reared in vials at 25 °C, with life-stage and mortality scored daily.

Cyp18a1 ectopic over-expression and rescue with 20-hydroxyecdysone

Ectopic over-expression of *Cyp18a1* was achieved using the GAL4/UAS system (Brand and Perrimon, 1993). The *Cyp18a1* ORF was PCR amplified using the primers 18a1-ORF-F and 18a1-ORF-R (Table S1) cloned into the pUAS vector (Brand and Perrimon, 1993) and transgenic flies generated in the w^{1118} strain. A single homozygous viable strain (UAS-*Cyp18a1*-6a) carrying an insert on chromosome III was used. All GAL4 driver strains were maintained as heterozygotes with GFP balancers (either *TM3*,GFP or *CyO*,GFP). GFP was used to distinguish control individuals from those driving *Cyp18a1* expression in crosses. Embryonic rescue experiments using 20E were conducted as described previously (Ono et al., 2006). 6–9 h old embryos were washed in (phosphate saline buffer with 0.1% Tween 20) and incubated with a 1 mL of 20E solution (100 μM) for 3 h before being placed on fly food media. UAS-*Cy18a1* survival to 1st instar of GFP and non-GFP was scored at 28 h post egg laying (HPL). Crossing experiments were conducted at 25 °C.

Molecular cloning into expression plasmid

Total RNA was extracted from total third instar larvae using SV Total RNA Isolation System (Promega) and full-length cDNA sequence of *Cyp18a1*was generated by reverse transcription using 1 μg of total RNA as template, with the M-MLV reverse transcriptase (Promega), primers 18a1-ORF-F and 18a1-ORF-R (Table S1) and using a high fidelity Taq polymerase (Invitrogen). After purification, the cDNA was cloned into the pIB/V5-His-TOPO expression vector (Invitrogen).

Reverse transcription (RT)-PCR

Total RNA was extracted from larvae using SV Total RNA Isolation System (Promega) and first strand cDNAs prepared from 200 ng total RNA, with the M-MLV reverse transcriptase (Promega). Analysis of *Cyp18a1* expression during the third larval instar used a RT-PCR protocol previously described (Parvy et al., 2005). After PCR was optimized for keeping within the exponential phase of amplification and optimal signal detection in Southern blots (20 cycles for *Cyp18a1* and 13 cycles for *rpL17*), PCR products were separated on agarose gel, transferred to positively charged nylon membranes and hybridized with DNA dig-labeled specific probes corresponding to the PCR amplification fragments. Chemiluminescent detection of the bound probes was performed using the Dig luminescent kit (Roche).

Quantitative RT-PCR was used to confirm *Cyp18a1* expression levels in RNAi and ectopic expression experiments. Total RNA was isolated from third instar larvae using TRI Reagent (Sigma), and then treated with RQ1 RNase-Free DNase (Promega). Reverse transcription was then performed on 1 μg of each RNA sample in a 10 μl reaction using Superscript III Reverse Transcriptase (Invitrogen Life Technologies) and oligo$(dT)_{20}$ primer, following the manufacturer's instructions. Real-time PCR was conducted on a RotorGene-3000 (Corbett Research) using a QuantiTect SYBR Green PCR kit (QIAGEN). PCR conditions were 95 °C for 10 min to activate the hot-start polymerase, followed by 50 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s, with fluorescence measured after each cycle. The housekeeping gene *RpL11* was used as a standard. PCR primers used are in Table S1. Relative quantification of *Cyp18a1* was determined using the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001) with *RPL11* as the reference gene control.

In situ hybridization

Early third instar larvae were dissected in PBT, and fixed with 4% paraformaldehyde. Sense and antisense ribo-probes were synthesized from *Cyp18a1* and labeled with digoxigenin according to protocols for SP6 and T7 RNA polymerases (Roche). In situ hybridization (ISH) was carried out according to standard protocols.

Transfection of S2 cells and incubation with ecdysteroid substrates

Schneider *Drosophila* S2 cell line was maintained at 22 °C in Schneider medium (Invitrogen) supplemented with 10% heat-inactivated FCS and antibiotics (100 μg/mL streptomycin and 60 μg/mL penicillin). Two days after subculture, cells were transfected using Effectene transfection reagent (Qiagen). Cells were transfected with *Cyp18a1* cloned in the pIB/V5-His vector and/or with GFP cloned in the same vector. GFP was used as a control for efficiency of transfection. Transfected cells were first incubated for 3 days at 22 °C to allow optimal protein expression. Enzymatic activity was lost when using cell homogenates, but optimal enzymatic activity was observed when intact cells were further incubated for 3 days with tritiated (1 nM) and unlabeled (up to 10^{-7} M) precursors resuspended in 5 μL ethanol and 400 μL fresh medium. After incubation, media plus cells were stored at −20 °C until further analysis. Alternatively, enzymatic activity was measured using membrane preparation of transfected cells (100 μL corresponding to approximately $5 \cdot 10^6$ cells) incubated with a mixture of tritiated and unlabeled 20E (up to10^{-5} M), 0.5 mM NADPH and an NADPH regenerating system, consisting of 2 mM glucose 6-phosphate and 0.2 U of glucose 6-phosphate dehydrogenase, in a phosphate buffer 50 mM, pH 7.5. Samples (final volume 250 μL) were incubated for 1 h at 35 °C. The reaction was stopped by adding 1 mL ethanol.

Extraction of ecdysteroids and HPLC analyses

Radiolabeled ecdysteroids (Text S1) were extracted from incubation media and cells by adsorption onto C_{18}-Sep Pak cartridges and eluted with 5 mL absolute methanol. Samples were routinely hydrolyzed prior to chromatographic analyses, as preliminary experiments showed that S2 cells constitutively express a kinase that actively converts 20,26E to 26-phosphate conjugate (Fig. S1 for details) and re-extracted using the same method. Ecdysteroids from membrane preparations were extracted by ethanol. The final extract was evaporated until dry and aliquots submitted to RP-HPLC. Non-radioactive references 20E and 20,26E were run simultaneously with the sample while 20Eoic, only available as a tritiated reference, was run as a separate injection using the same HPLC conditions. After re-suspension in 100 μL of HPLC initial mobile phase, samples were analyzed by RP-HPLC using a C18 column (150×4.6 mm i.d. Advanced Chromatography Technologies), a flow-rate of 1 mL min^{-1} and a linear gradient (5% to 35% in 60 min) of solvent B (acetonitrile: isopropanol, 5:2, vol/vol) in A (20 mM Tris buffer pH 7.5 or 0.1% trifluoroacetic acid TFA in water). Radiochromatograms were obtained by in-line analysis of the HPLC effluents with a radioactivity monitor (β-RAM; Lablogic) using EcoScint A (National Diagnostics) scintillation cocktail at a flow-rate of 3 mL min^{-1}.

Ecdysteroid titer measurements

Total ecdysteroids were quantified by enzyme immunoassay (EIA). For ecdysteroid extraction, pools of five animals were crushed in 250 μL methanol using a close-fitting plastic pestle. The mixture was sonicated, centrifuged, and the residue was re-extracted with the same volume of methanol. Samples were dried and re-dissolved in EIA buffer. We used 2-succinyl-20-hydroxyecdysone coupled to peroxidase as enzymatic tracer (dilution 1:80,000) and the polyclonal L2 antiserum (gift from M. De Reggi [Marseille, France]; dilution, 1:40,000). This antibody recognizes both E and 20E, with a 3.8-fold higher affinity for E than for 20E, as calculated from the comparison of reference standard curves. Calibration curves were generated with 20E (16–2000 pg/tube) diluted in EIA buffer. All measurements were performed in duplicate and the results are expressed as 20E equivalents, mean values ± s.e.m. of several (n = 4–7) independent samples. Data were subjected to statistical analysis using Student's t test.

Results

CYP18A1 catalyzes the 26-hydroxylation and further oxidation of ecdysteroids to 26-carboxylic acids

The full-length cDNA of *Cyp18a1* was previously described (Bassett et al., 1997). We used *Drosophila* S2 cells transfected with *Cyp18a1* cloned into an expression vector to test the catalytic activity of CYP18A1. This system has proved a valuable tool for identifying several steps of ecdysteroid metabolism (Warren et al., 2002, 2004; Petryk et al., 2003; Niwa et al., 2004). We verified by RT-PCR that S2 cells express genes necessary for CYPs function such as the cytochrome P450 reductase, but show no constitutive expression of *Cyp18a1* (Fig. S1). Metabolites formed during incubation of tritiated 20E with S2 cells were first analyzed using RP-HPLC and a gradient of

organic solvent in a neutral Tris buffer. Samples were routinely hydrolyzed prior to chromatographic analyses, in order to cleave conjugates formed during incubation (see Materials and methods and Fig. S2 for details). No conversion of tritiated 20E by non-transfected cells incubated for up to 4 days was observed, while *Cyp18a1* transfected cells metabolized [^{3}H]20E into two more polar compounds (Fig. 2A). The major peak (P1) comigrated with reference tritiated 20Eoic run under the same chromatographic conditions (Fig. 2B), while the minor peak (P2) appeared as a double peak comigrating with the two epimers of 20,26E (see Text S1 for details about these epimers). To confirm the identity of P1 and P2, extracts were run under the same gradient of organic solvent using an acidic aqueous phase (Figs. 2D and E). Under these conditions, the retention time of P1 was increased, as expected for a weak acid, and it still comigrated with reference [^{3}H]20Eoic. P2 coeluted in both conditions with 20,26E and its retention time did not change with the pH. The formation of 20,26E, even as a minor peak, and of 20Eoic as the major conversion product of 20E only by transfected cells demonstrates that CYP18A1 is responsible for the 26-hydroxylation of 20E. It also suggests the involvement of CYP18A1 in the further oxidation of 20,26E into the corresponding 26-oic derivative. To confirm this, control or transfected cells were incubated with [^{3}H]20,26E and ecdysteroid extracts submitted to RP-HPLC analysis. Only *Cyp18a1* transfected S2 cells converted tritiated 20,26E to a single product comigrating with [^{3}H]20Eoic at both pH conditions (Figs. 2C and F). These data demonstrate that *Cyp18a1* codes for a 26-hydroxylase and that the same enzyme is responsible for the further oxidation of the primary C-26 alcohol to the corresponding carboxylic acid (Fig. 1).

Analysis using membrane preparations of transfected and non-transfected cells yielded additional information about the properties of the 26-hydroxylation (Fig. 3), even if a more extensive study would be necessary to calculate more precisely the kinetic parameters. Only membrane preparations of *Cyp18a1* transfected cells converted 20E to a mixture of 20,26E and 20Eoic. Similar percentages of 20E conversion were obtained for 10^{-9} to 10^{-7} M concentrations of 20E (Figs. 3A and B), while the percentage of conversion decreased by two-thirds when the concentration of 20E reached 10^{-6} M (Fig. 3C). According to the Michaelis–Menten equation, we expect that the percentage of conversion should be divided by 2 when [20E] = K_m; thus our results are consistent with an apparent K_m for 20E ranking between 10^{-6} and 10^{-7} M. The small peaks eluting after 20E are expected to correspond to 26-aldehyde intermediates (Kayser et al., 2002 and Fig. 1). Due to lack of complete identification, they have not been taken into account in the calculations, but this does not change the above conclusion.

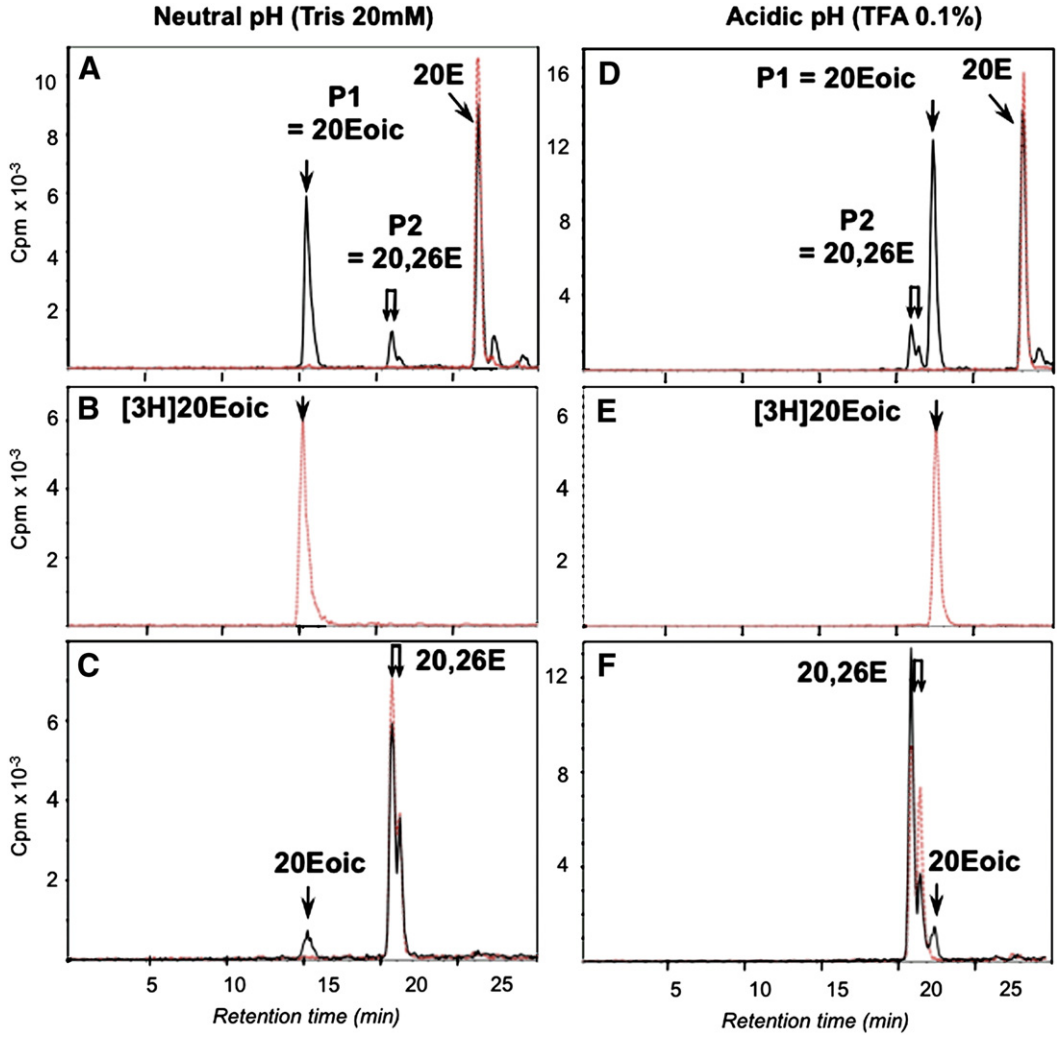

Fig. 2. Conversion of 20E by intact S2 cells transfected with *Cyp18a1*. RP-HPLC analyses were performed using a gradient of acetonitrile/isopropanol (5:2, v/v) in Tris buffer (A, B, C), or in 0.1% TFA (D, E, F). Biological extracts were hydrolyzed before HPLC. A, D: analysis of [^{3}H]20E conversion by S2 cells transfected with *Drosophila Cyp18a1* (black line) or control S2 cells (red dotted line). B, E: Migration of reference [^{3}H]20Eoic. C, F: Analysis of [^{3}H]20,26E conversion by transfected S2 cells (black line) or control S2 cells (red dotted line).

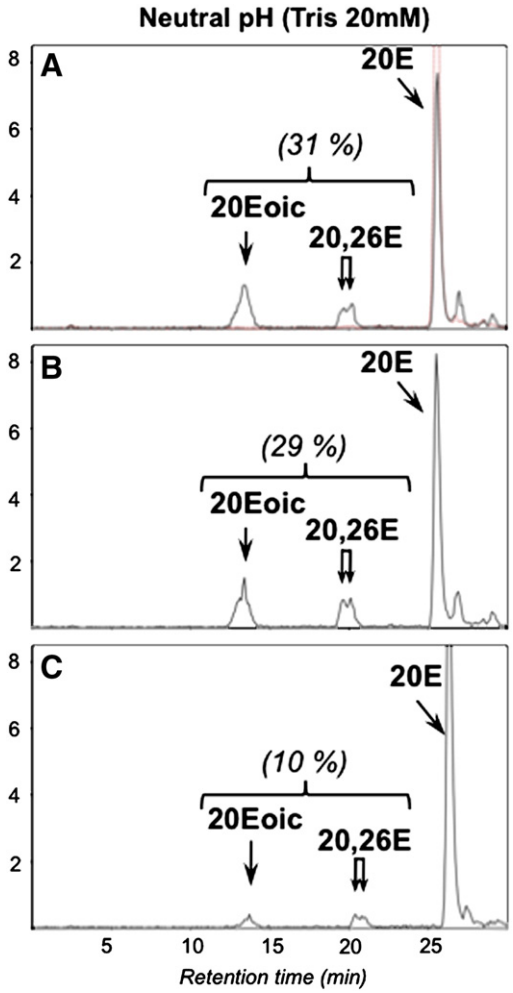

Fig. 3. Conversion of 20E by membrane preparations of S2 cells transfected with *Cyp18a1*. RP-HPLC analyses were performed using a gradient of acetonitrile/isopropanol (5:2, v/v) in Tris buffer. A: Analysis of [^{3}H]20E and 10^{-8} M 20E conversion by membranes from S2 cells transfected with *Drosophila Cyp18a1* (black line) or control S2 cells (red dotted line); B: analysis of [^{3}H]20E and 10^{-7} M 20E conversion by membranes from S2 cells transfected with *Drosophila* Cyp18a1; C: analysis of [^{3}H]20E and 10^{-6} M 20E conversion by membranes from S2 cells transfected with *Drosophila Cyp18a1*. Aliquots of the same membrane preparation (corresponding to approximately 5.10^6 cells) were used. (n%) indicates the percentage of conversion to 20,26E and 20Eoic. Similar results were obtained over three independent experiments.

CYP18A1 is expressed in many target tissues of ecdysteroids

In vivo, *Cyp18a1* expression was characterized using RT-PCR in precisely timed third instar larvae. *Cyp18a1* expression is higher in early third larval stage (48–60 h post hatching or HPH), a stage when ecdysteroids have returned to low levels after the peak preceding the second to third instar molt (Parvy et al., 2005), than at the end of the third larval instar (88–96 h post hatching or HPH) when ecdysteroid titers peak (Fig. S3). *Cyp18a1* transcript levels thus fluctuate in an opposite manner to genes encoding steroidogenic enzymes, such as *shd*, which encodes the 20-hydroxylase catalyzing the last step of ecdysteroid biosynthesis (Petryk et al., 2003) (Fig. 1).

At the time of high *Cyp18a1* expression (48–60 HPH), *in situ* hybridization (ISH) was performed to determine a precise tissue expression pattern (Fig. 4). Strong *Cyp18a1* expression was observed in the epidermis (Figs. 4A and A'), fat body (Fig. 4B), salivary glands (Fig. 4C) and eye-antenna imaginal discs (Figs. 4D and D'). *Cyp18a1* expression was also observed in gastric caecae and various sections of the midgut (Fig. 4E), but not in the hindgut or Malpighian tubules. *Cyp18a1* is thus expressed in many target tissues of ecdysteroids known to exhibit high 26-hydroxylase activity in different insect orders (Bassett et al., 1997; Davies et al., 2006). *Cyp18a1* expression was also detected in the prothoracic cells of the ring gland, the steroidogenic organ of *Drosophila* larvae (Figs. 4D and D'), only early in the third instar (48–60 HPH) and not later during the wandering stage (88–96 HPH). Specificity of this ISH labeling was assessed by comparison using a *Cyp18a1* sense probe and an antisense probe corresponding to *dib*, which encodes a steroidogenic enzyme (Fig. S4).

Cyp18a1 inactivation slows the end of larval development and causes pupal lethality

To understand its functional importance, *Cyp18a1* was first silenced by RNAi using the GAL4-UAS system (Fig. 5). Silencing *Cyp18a1* in all tissues, using a *tubulin*-GAL4 driver, resulted in limited mortality at third larval instar and high levels of lethality at the pupal stage (Figs. 5A, B and Ei–iii). Similar results were obtained with two different insertions of the transgene as detailed in Fig. S4. Development was delayed ca. 4 days at third larval instar (Fig. 5B). Melanotic masses were observed in some larvae (Fig. 5Ei). An estimated 90% reduction of *Cyp18a1* transcript levels was achieved by RNAi in early third instar larvae using the tub-GAL4 driver (Fig. 5G). Silencing *Cyp18a1* in the midgut, Malpighian tubules and fat body using the *6g1HR*-6c-GAL4 driver strain resulted in no obvious phenotype. Although midgut and fat body are important target tissues of ecdysteroids and do express *Cyp18a1*, expression in other tissues such as epidermis may provide enough 26-hydroxylase activity to adequately inactivate circulating 20E, or other degradation mechanisms may take place in these particular tissues. It is also possible that the level of *Cyp18a1* silencing was not sufficient to result in an observable phenotype. Silencing *Cyp18a1* specifically in the steroidogenic cells of the ring gland using the 5′*phm*-GAL4 driver was sufficient to induce lethality at third instar larval and pupal stages (Figs. 5C,D,Eiv–v and F), suggesting an important role for the transient expression of *Cyp18a1* in the steroidogenic cells during the third larval instar. Again, a similar delay in development of third instar larvae was documented (Fig. 5D), along with melanotic masses associated with the midgut and cuticle in many third instar larvae (Figs. 5Eiv–v and F).

Cyp18a1 was further inactivated by generating null alleles (*Cyp18a1^{null}*) using *P* element mobilization (Figs. 6A and B). Two independent *Cyp18a1* null alleles were generated. Both were homozygous lethal and resulted in similar phenotypes (see details in Fig. S6). As *Cyp18a1* is located on the X-chromosome, the null alleles were maintained using a *FM7i-pAct-GFP* X-chromosome balancer. GFP was used to distinguish between *Cyp18a1^{null}/Y* hemizygous males and *Cyp18a1^{null}/FM7i-pAct-GFP* heterozygous females at late embryo and larval stages. The phenotype of the *Cyp18a1^{null644}* mutant was characterized in detail. RT-PCR was used to confirm the absence of *Cyp18a1* transcript in *Cyp18a1^{null644}/Y* larvae (Fig. 6B). The phenotype of *Cyp18a1* null alleles is similar to ubiquitous inactivation of *Cyp18a1* by RNAi. Lethality in *Cyp18a1^{null}/Y* males occurred primarily during metamorphosis (Figs. 6C and E). Many animals died at the prepupal stage, failing to form distinguishable adult structures (Fig. 6Eii). Other individuals progressed to near the end of pupal development, but failed to emerge (Figs. 6Eiii and iv). A few *Cyp18a1^{null}/Y* males (approximately 5–10%) emerged as adults and died soon after. Limited mortality was observed at every developmental stage in control *Cyp18a1^{null}/FM7i-pAct-GFP* heterozygous females, which may be attributed to deleterious effects of the *FM7i-pAct-GFP* balancer chromosome. The average developmental rate of surviving *Cyp18a1^{null}/Y* males was slower than that of control *Cyp18a1^{null}/FM7i-pAct-GFP* females, with a ca. 1 day longer third larval stage and a ca. 4 days longer pupal development (Fig. 6D). Mutant pupae displayed an extended peak of ecdysteroids (Fig. 6F), consistent with impaired ecdysteroid degradation.

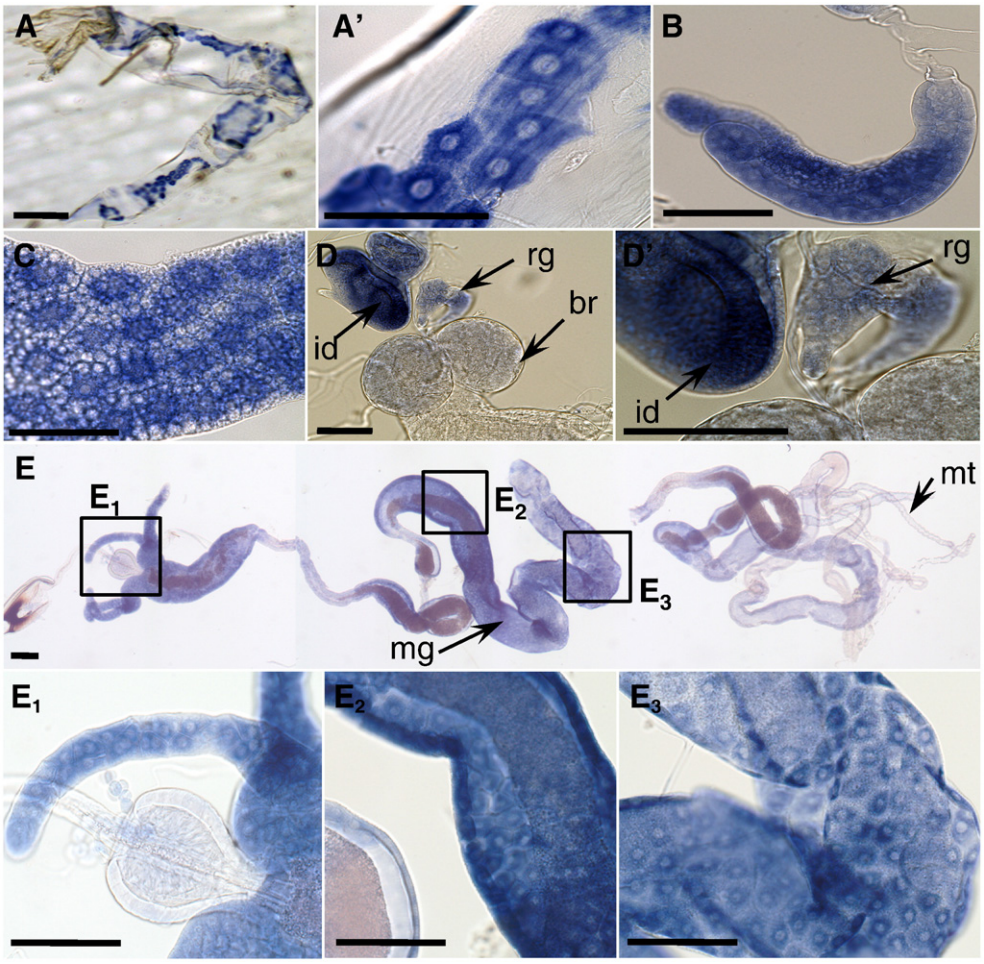

Fig. 4. Tissue expression of CYP18A1 in third instar larvae. A–E: *In situ* hybridization analysis using a *Cyp18a1* antisense probe; A and A': epidermis, B: salivary gland; C: fat body; D and D': brain, ring gland and eye-antenna imaginal disc; E and E1 to E3: gastric caecae and gut. A', D' and E1 to E3 are details at higher magnification. br brain, id eye-antenna imaginal disc, mg midgut, mt Malpighian tubules, rg ring gland. Scale bar is 100 μm.

Cyp18a1 ectopic over-expression causes lethality

Over-expression of *Cyp18a1* was achieved using the GAL4/UAS system and a UAS-*Cyp18a1* transgenic strain (Fig. 7C). *Cyp18a1* over-expression in the larval midgut, Malpighian tubules and fatbody, using the 5′HR-GAL4 driver, primarily resulted in third instar larval lethality, with a small number of individuals surviving until early pupal stages (Fig. 7A). As with ubiquitous *Cyp18a1* knockdown using RNAi, melanotic masses were observed in many larvae, often associated with the midgut and cuticle (Fig. 7B). Ubiquitous over-expression of *Cyp18a1* using *tub*-GAL4 resulted in late embryonic lethality. This is consistent with CYP18A1 having a role in 20E catabolism, as 20E is required to complete embryogenesis (Kozlova and Thummel, 2003). Over-expression of *Cyp18a1* in the prothoracic cells of the ring gland, using the *phm*-GAL4 driver, also resulted in late embryonic lethality. For both the *tub*-GAL4 and *phm*-GAL4 drivers, the embryonic lethality could be rescued to first instar larval stage by incubating embryos in 20E (Table 1), suggesting that lethality is 20E dependent.

Cyp18a1 gene is conserved among insects

Genes encoding cytochrome P450 enzymes metabolizing endogenous compounds are generally well conserved (Feyereisen, 2005; Rewitz et al., 2007; Rewitz and Gilbert, 2008) and 26-hydroxylation is a major inactivation pathway of ecdysteroids in arthropods, both in insects (Lafont et al., 2005) and crustaceans (McCarthy and Skinner, 1979; Lachaise and Lafont, 1984). CYP18A1 proteins contain conserved motifs characteristic of cytochrome P450 enzymes targeted to the endoplasmic reticulum (Fig. S7) and a BLAST search revealed that putative *Cyp18a1* orthologs are present in many insects and crustaceans (Fig. 8). A conserved tail to tail arrangement of *Cyp18a1* and *phm* (microsynteny) is present in *Drosophila* species, *Apis mellifera*, *Bombyx mori* and *Daphnia pulex* (Claudianos et al., 2006; Rewitz and Gilbert, 2008), also supporting orthology of *Cyp18a1* genes in insect species. In addition, we verified the functional conservation of the enzymatic activity in *D. melanogaster*, *Aedes aegypti* and *B. mori*, using transfection of *Cyp18a1* in S2 cells (C. Dauphin-Villemant and R. Lafont, unpublished data). A striking exception is *Anopheles gambiae*, where no clear ortholog of *Cyp18a1* could be detected (Feyereisen, 2006) but further studies are necessary to understand if another enzyme catalyses 26-hydroxylation in *A. gambiae* or if this reaction is absent. More generally, the appellation of 26-hydroxylase (Meaney, 2005) may be misleading as it is used in different organisms to refer to the modification of various cholesterol derivatives with different stereochemistry (Figs. 1 and 8). It is therefore not surprising that even the 26-hydroxylase of *C. elegans*, DAF-9 (CYP22A1), which belongs to the same microsomal CYP2 clade as CYP18A1 and CY306A1 (Feyereisen, 2006), is most likely non-homologous to CYP18A1 in a phylogenetic analysis including various members of CYP2s from Arthropods and *C. elegans* (Fig. 8). This independent origin applies also to the vertebrate CYP27s, which are mitochondrial 26-hydroxylases.

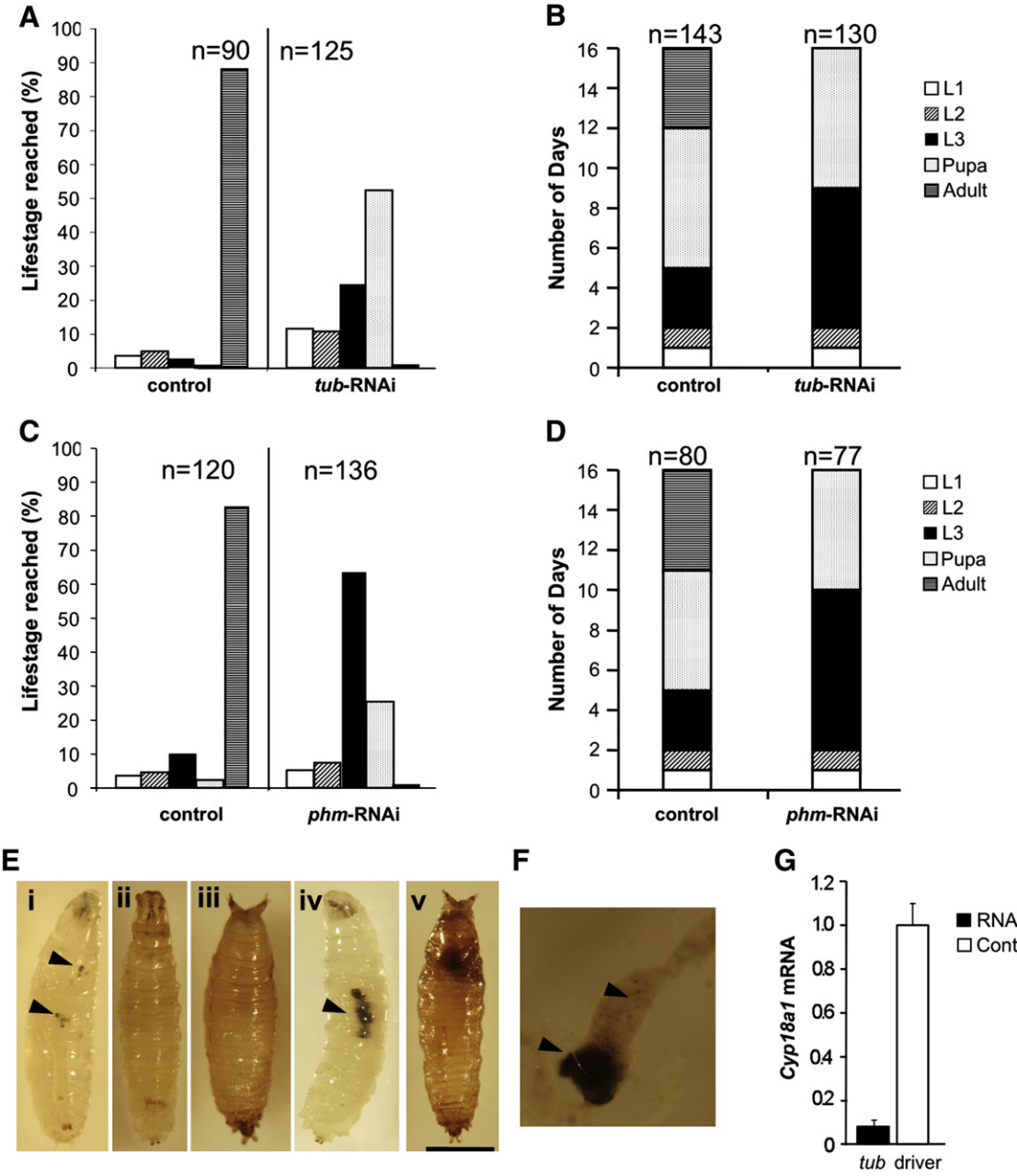

Fig. 5. RNAi knockdown of *Cyp18a1* slows larval development and results in pupal lethality. A: Percentage of individuals to reach different life-stages for *Cyp18a1* RNAi transgenic strain (UAS-*Cyp18a1*-RNAi-7a) crossed to the tub-GAL4/TM3,GFP strain. Key is the same for panel B. L1 = 1st instar larvae, L2 = 2nd instar larvae, L3 = 3rd instar larvae. n refers to the number of individuals of each genotype scored. B: Developmental rate for individuals of the same cross and compared to controls not driving the RNAi construct. C: Percentage of individuals to reach different life-stages for *Cyp18a1* RNAi transgenic strain (UAS-*Cyp18a1*-RNAi-7a) crossed to the *phm*-GAL4/CyO,GFP strain. L1 = 1st instar larvae, L2 = 2nd instar larvae, L3 = 3rd instar larvae. n refers to the number of individuals of each genotype scored. D: Developmental rate for individuals of the same cross and compared to controls not driving the RNAi construct. Ei–iii: UAS-Cyp18a1-RNAi-7a/tub-GAL4 individuals. *Cyp18a1* is knocked down ubiquitously in these individuals. Development is arrested at early prepupal and pupal stages (Eii–iii), with the appearance of melanized regions under the cuticle of some larvae (arrow head in Ei). Eiv–v: +/5′*phm*GAL4;+/UAS-*Cyp18a1*-RNAi-7a individuals. Knocking down *Cyp18a1* specifically in the steroidogenic cells of the ring gland results in lethality, at either prepupal (iv) or pupal (v) stages. Melanized regions form under cuticle and midgut of some larvae (arrow head in Eiv). Scale bar is 1 mm. F: Melanization in posterior section of midgut in third instar larva of +/5′*phm*GAL4;+/UAS-*Cyp18a1*-RNAi-7a. In many individuals, the melanization of individual cells can be seen, and in more extreme examples, large patches of melanization, most prominent at the border between the midgut and hindgut, are present (arrow heads in F). G: Quantitative PCR showing reduction of *Cyp18a1* transcript levels by RNAi in early third instar larvae using the *tub*-GAL4 driver. All values are relative to *RPL11* and normalized to UAS-*Cyp18a1*/CyO,GFP control. Values are the mean of five replicates ± SEM.

Discussion

In this paper, we demonstrate that *Cyp18a1* is an essential gene encoding an ecdysteroid 26-hydroxylase, a key enzyme of insect steroid hormone catabolism. CYP18A1 catalyses both the conversion of the active hormone 20E to its 26-CH_2OH derivative 20,26E and of the latter to the 26-COOH carboxylic acid 20Eoic. Both ecdysone (E) and 20E are substrates of CYP18A1 (data not shown), as expected from various metabolic studies (Chen et al., 1994; Kayser et al., 1997; Williams et al., 2000). We focused on 20E metabolism because S2 cells endogenously exhibited some 20-hydroxylase activity, which resulted in a complex conversion pattern. Efficient conversion of exogenous 20E to 20Eoic by *Cyp18a1* transfected S2 cells was observed. When membrane preparations were used, an apparent K_m between 10^{-7} and 10^{-6} M was found, which is in agreement with biochemical studies performed on various insects (Kayser et al., 1997;

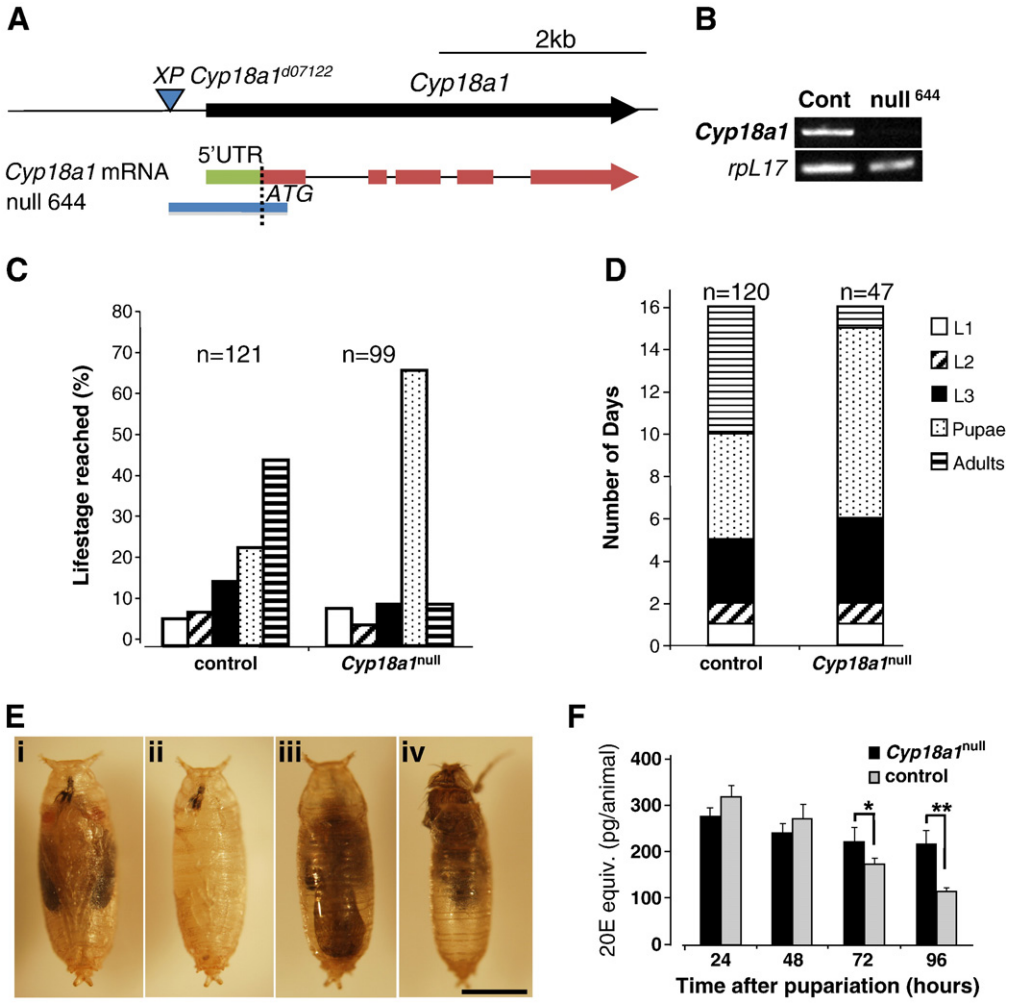

Fig. 6. *Cyp18a1* null mutants are pupal lethal. A: Generation of *Cyp18a1*^{null644} null strain. Blue triangle represents insertion site of *P* element in the XP*Cyp18a1*^{d07122} strain. *Cyp18a1*^{null644} is a *P* element mobilization deletion of 644 bp (blue box), including 43 bp of *Cyp18a1* coding sequence (from ATG). See also Materials and methods. B: Expression of *Cyp18a1* is not detectable in *Cyp18a1*^{null644} third instar larvae, as determined using RT-PCR analysis. *RpL17A* was used as a control. C: Percentage of individuals to reach different life-stages for *Cyp18a1*^{null644} hemizygous mutant males (*Cyp18a1*^{null}) and control animals (*Cyp18a1*^{null644}/*FM7i-pAct-GFP* heterozygous females). n refers to number of individuals of each genotype scored. D: Developmental rate of *Cyp18a1*^{null} mutants and control animals. Time taken for 50% of individuals to reach next development stage when reared individually. Stages are represented as in Fig. 5. E: Pupal phenotypes. i: pupae of *Cyp18a1*^{null644}/*FM7i-pAct-GFP* heterozygous females (control) develop normally and fertile and viable adults emerge. ii–iv: *Cyp18a1*^{null644}/*Y* males arrest their development at various times of prepupal (ii), pupal (iii) stage or fail to emerge as adults (iv). F: Variations of ecdysteroid levels in control and *Cyp18a1*^{null644} pupae. Total ecdysteroids were quantified by enzyme immunoassay and the results are expressed as 20E equivalents, mean values ± s.e.m. of several (n = 4–7) independent samples. Data were subjected to statistical analysis using Student's t test. *$p<0.05$; **$p<0.01$.

Williams et al., 2000). Even if a mixture of 20,26E and 20Eoic was formed by membrane preparations of transfected cells, 20,26E never accumulated to a large extent when intact transfected cells were used. By contrast, exogenous 20,26E was poorly converted, suggesting that, in *D. melanogaster*, the multi-step conversion proceeds up to the formation of 20Eoic with a limited release of intermediates. This is consistent with the accumulation of 26-carboxylic acids previously detected *in vivo* in *D. melanogaster* (Sommé-Martin et al., 1988), *Pieris brassicae* (Lafont et al., 1983) and *Locusta migratoria* (Modde et al., 1984). This may not apply to all species, however, as a significant accumulation of 26-hydroxylated metabolites was reported *in vitro* with *Chironomus tentans* cells (Kayser et al., 1997) and *in vivo* in *Carausius morosus* (Lafont and Koolman, 1984). Precise characterization of CYP18A1 substrate specificities and kinetic properties will be necessary to understand the physiological importance of these differences among insects. An additional feature of 26-hydroxylation in *D. melanogaster* is that both 25*R*- and 25*S*-epimers of 20,26E are detected after 20E incubation with *Cyp18a1* transfected S2 cells. Both isomers were also detected after *in vitro* incubation with tritiated 20E and *P. brassicae* imaginal wing discs (Lafont et al., 1983) and *Trichoplusia ni* cells (Text S1) and *in vivo* in *Manduca sexta* eggs (Warren et al., 1986). Whether the situation observed here applies for all arthropods warrants additional experiments.

Cyp18a1 is expressed in many target tissues of ecdysteroids. As it is involved in hormonal catabolism, it is expected to be induced by the active hormone 20E. However, apparent discrepancies between *in vivo* and *in vitro* results were noticed. Hurban and Thummel (1993) observed that *Eig17-1*, which corresponds to *Cyp18a1*, was rapidly induced after *in vitro* incubation of *D. melanogaster* larval organs with E. On the other hand, in precisely staged larvae, we have observed that *Cyp18a1* expression peaks at the beginning of the third larval instar several hours after the peak of 20E of the second larval instar, i.e. when 20E concentration has already returned to basal levels. Such a delayed induction was also mentioned after injection of 20E agonists in Lepidopterans (Chen et al., 1994; Williams et al., 1997, 2000). These discrepancies may be due to the fact that another factor is involved in the *in vivo* regulation of CYP18A1 and would be responsible for the several hours delay between ecdysteroid peak and maximal CYP18A1 induction, as already proposed by Bassett et al. (1997).

The formation of ecdysonoic acids is not the only way to inactivate molting hormones in *Drosophila*. Ecdysteroid catabolism can occur via other reactions, such as epimerization at C3 and/or conjugation (Rees,

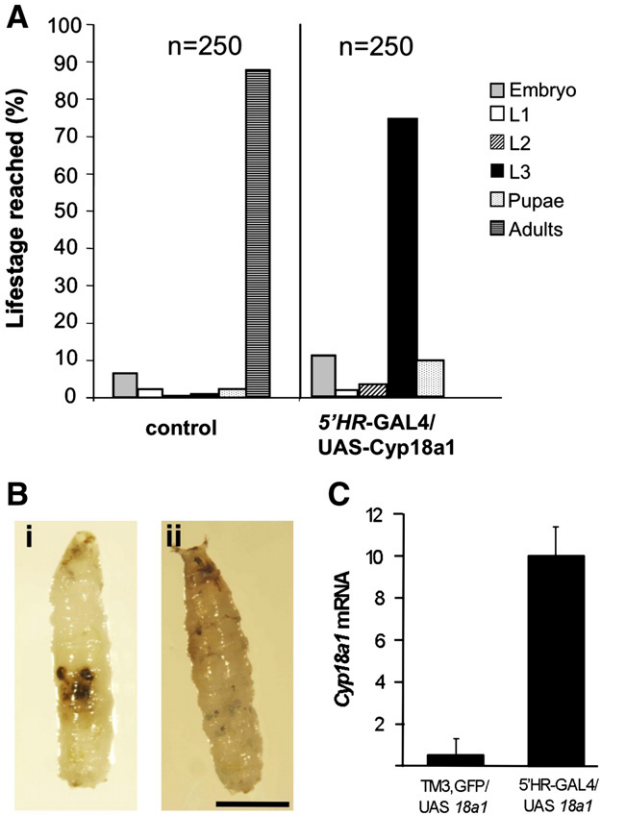

Fig. 7. Ectopic over-expression of *Cyp18a1* in the larval midgut, Malpighian tubules and fat body is larval lethal. A. Percentage of individuals to reach different life-stages for UAS-*Cyp18a1* transgenic strain crossed to the *5′HR-GAL4* strain. 5′HR-GAL4 drives expression in the larval midgut, Malpighian tubules and the fat body. Controls are UAS-*Cyp18a1/TM3*, GFP. L1 = 1st instar larvae, L2 = 2nd instar larvae, L3 = 3rd instar larvae. n refers to number of individuals of each genotype scored. B. Characteristic phenotype of dead 3rd instar larvae from 5′HR-GAL4/UAS-*Cyp18a1*. (i). Melanized patches on midgut and epidermis occur prior to death on many individuals. (ii). Development rarely proceeds past early pupal stages. Scale bar is 1 mm. C. Quantitative PCR showing *Cyp18a1* mRNA levels in 2nd instar larvae. All values are relative to *RPL11* and normalized to UAS-*Cyp18a1/CyO*,GFP control. Values are the mean of five replicates ± SEM.

1995; Lafont et al., 2005). Moreover, direct excretion of unmetabolized 20E can also occur (Lafont et al., 2005). Despite this apparent redundancy of steroid inactivation systems, *Cyp18a1* is necessary for metamorphosis in *D. melanogaster*. In *Cyp18a1* null mutants, or after ubiquitous inactivation of *Cyp18a1* by RNAi, massive pupal lethality occurs. This coincides with an extended pupal ecdysteroid peak, indicating that CYP18A1 is required for 20E catabolism, and that the phenotypes observed are the direct consequence of changes in 20E levels. Our data suggest that CYP18A1 is not essential for early development and primarily contributes to the regulation of ecdysteroid levels during third larval instar and at the onset of metamorphosis. The lack of an early observable phenotype, even though *Cyp18a1* is expressed in late embryos (Tomancak et al., 2002), and first or second instar larvae (Bassett et al., 1997), might be explained by the presence of alternative catabolic reactions or direct excretion of 20E, as stated above. In the absence of 26-hydroxylase, 20E could be excreted as such by open larval systems, but it might become toxic when retained in the closed pupal system. Extended presence of ecdysteroids would thus evoke some kind of "hyperecdysonism". The formation of melanotic tumors observed in some third instar larvae could be induced by a local excess of 20E, stimulating the innate immune system (Flatt et al., 2008). Our data resemble those obtained in several insect species including *Drosophila* when 20E was injected after the endogenous hormone peak, resulting in a delayed or even suppressed ecdysis (Truman, 2005; Zitnan et al., 2007). The onset of metamorphosis involves a complex cascade of 20E-dependent gene regulations (Thummel, 2001; Beckstead et al., 2007) that require exposure to hormone pulses, the decline of hormone concentration being as important as its rise. For instance, the pupal decline of ecdysteroids is necessary for the formation of pupal cuticle (Doctor et al., 1985; Apple and Fristrom, 1991) and for adult neuronal maturation (Weeks, 2003). By contrast, the prolonged L3 stage and delayed pupariation observed in *Cyp18a1* null or knockdown individuals appears to proceed from a paradoxal effect. In such animals, higher 20E levels were measured at mid-third instar, but thereafter the late-third instar peak was lower and delayed (data not shown). The phenotypes observed in third instar larvae when ecdysteroid degradation is impaired therefore differ from those observed after artificial stimulation of 20E production. For instance, when 20E production was stimulated by over-expression of the stimulatory factor PTTH (McBrayer et al., 2007) or the insulin signaling pathway (Colombani et al., 2005), no noticeable changes in the developmental timing of *Drosophila* were noted: metamorphosis proceeded normally, but smaller adults emerged. In both cases, positive/negative feedback mechanisms, that turn-on/off ecdysteroid production by the ring glands, are probably unaffected. This may help to understand the significance of the unexpected transient expression of *Cyp18a1* in the ring gland, as likewise in the prothoracic glands of two lepidopterans *Spodoptera littoralis* and *M. sexta* (Davies et al., 2006). The lethality caused when *Cyp18a1* expression is specifically knocked down in this organ suggests that regulation of ecdysteroid balance by CYP18A1 occurs directly in the ring gland, the site of E synthesis. A similar regulatory mechanism has been observed, with respect to the biosynthesis and degradation of juvenile hormones by the corpora allata of the cockroach *Diploptera punctata* (Helvig et al., 2004; Sutherland et al., 1998).

The use of sterol oxidation to generate signaling molecules is a general occurrence as a result of several independent evolutionary processes (Markov et al., 2009). However, is there any common origin between P450 genes encoding enzymes involved in ecdysteroid biosynthesis and degradation? The closest gene to *Cyp18a1*, both in sequence similarity and physical location, is *Phm* (*Cyp306a1*) (Rewitz and Gilbert, 2008). *Phm* encodes a 25-hydroxylase essential for ecdysteroid biosynthesis (Lafont et al., 2005). CYP18A1 and PHM share both a high level of conservation (36% amino acid similarity, microsomal localization) and only present a slight difference in enzymatic reaction (26-hydroxylation versus 25-hydroxylation). It is not uncommon that minor sequence variations in steroidogenic CYPs induce changes in hydroxylation positions (Bulow and Bernhardt, 2002; Swart et al., 2010). It is thus conceivable that this represents an example of neofunctionalization after gene duplication, although further study would be necessary to assess this hypothesis.

Taken together, our results demonstrate that *Cyp18a1* is a key and conserved gene in the regulation of ecdysteroid levels during insect metamorphosis. This is the first time that a mutant, which interferes directly with the degradation of ecdysteroids is described in *Drosophila*. In addition, ectopic over-expression of *Cyp18a1* appears to provide an effective means of reducing the 20E titer at any stage of development. Manipulating *Cyp18a1* expression therefore represents a useful tool in order to better understand the systemic and local effects of ecdysteroids and their decline during metamorphosis.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.09.023.

Table 1
Cyp18a1 induced embryonic lethality rescued by 20E[a].

Genotype	Untreated	20E
UAS-*Cyp18a1*/5′*tub*-GAL4	0 (115)	38.9 (90)
UAS-*Cyp18a1*/*TM3*,GFP	51.7 (120)	42.5 (106)
+/5′*phm*-GAL4; +/UAS-*Cyp18a1*	4.7 (128)	30.0 (140)
+/*CyO*,GFP; +/UAS-*Cyp18a1*	53.6 (140)	44.8 (172)

[a] % survival to 1st instar, total number scored is in parentheses.

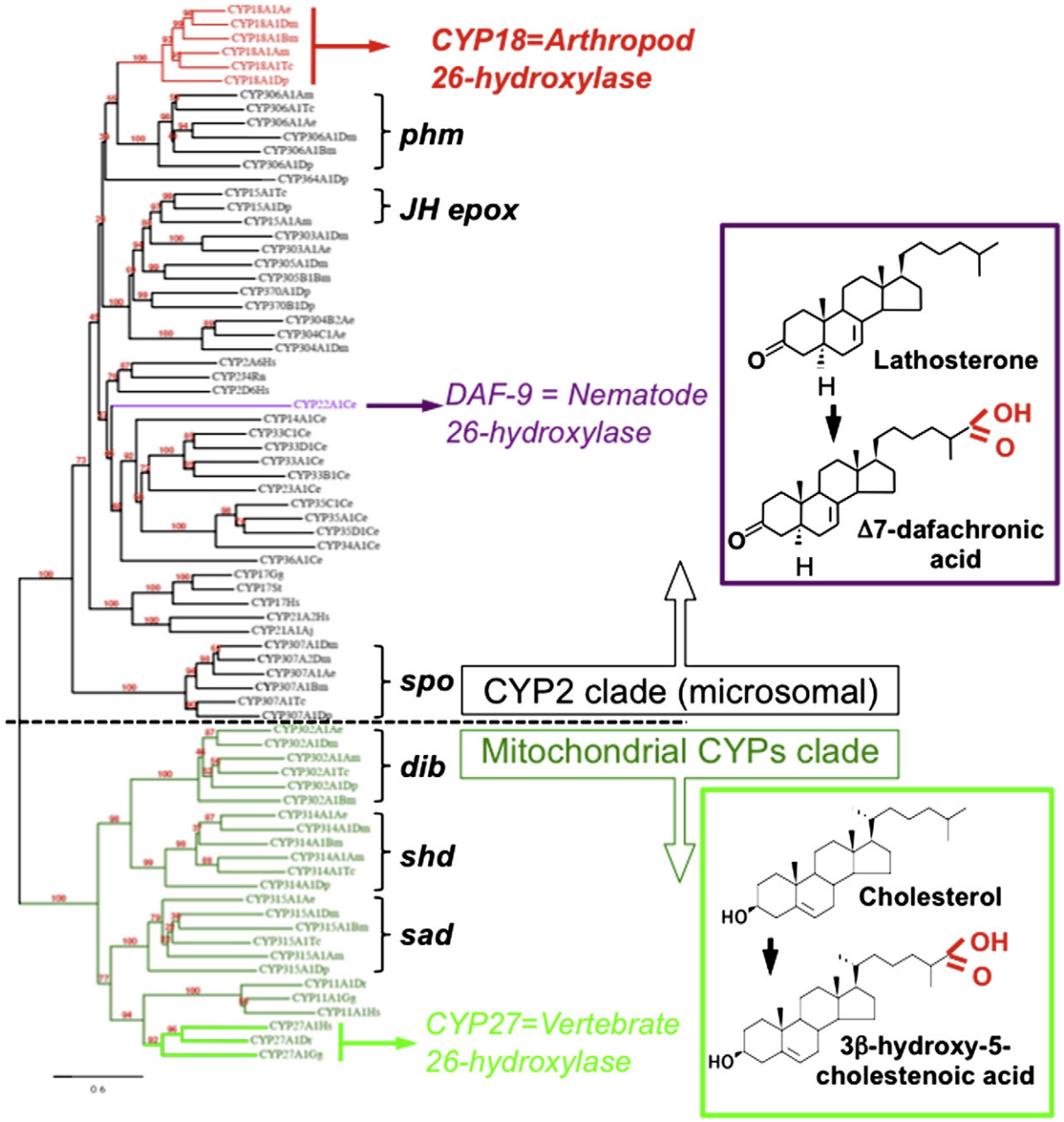

Fig. 8. A simplified phylogeny of CYPs focusing on CYP18A1. A maximum likelihood tree based on ClustalX multiple alignments. Numbers indicate bootstrap values as percentage of 1000 replicates. CYPs are included to infer relationship to major classes of CYPs related to steroidogenesis, to members of CYP2 clade or to known 26-hydroxylases in animals. Abbreviations of enzyme names: 26-hydroxylases: DAF-9 or CYP22A1, CYP18A1, CYP27A1; other steroidogenic enzymes: 25-hydroxylase is CYP306A1 or PHM (Phantom), 22-hydroxylase is CYP302A1 or Dib (Disembodied), 2-hydroxylase is CYP315A1 or SAD (Shadow), 20-hydroxylase is CYP314A1 or SHD (Shade), CYP307A1/A2 (Spook/Spookier), P450scc or cholesterol side chain cleavage enzyme is CYP11A1. CYP15 is the juvenile hormone epoxidase (JH epox). Abbreviations of species names: Ae *Aedes aegypti*, Ag *Anopheles gambiae*, Am *Apis mellifera*, Bm *Bombyx mori*, Ce *Caenorhabditis elegans*, Cp *Culex pipiens*, Dm *Drosophila melanogaster*, Dp *Daphnia pulex*, Ms *Manduca sexta*, Sl *Spodoptera littoralis*, Tc *Tribolium castaneum*, Bt *Bos taurus*, Dr *Danio rerio*, Gg *Gallus gallus*, Hs *Homo sapiens*, Rn *Rattus norvegicus*. Detailed accession numbers are given in Table S2.

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