UC Riverside
UC Riverside Electronic Theses and Dissertations

Title

Functional Study of Uncharacterized Cytochrome P450 Enzymes in Drosophila Development

Permalink

https://escholarship.org/uc/item/9fd505fp

Author

Lu, Ruoying

Publication Date

2019

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Functional Study of Uncharacterized Cytochrome P450 Enzymes in *Drosophila*
Development

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

Ruoying Lu

September2019

Dissertation Committee:
      Dr. Naoki Yamanaka, Chairperson
      Dr. Anupama Dahanukar
      Dr. Michael Adams

The Dissertation of Ruoying Lu is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Prof. Yamanaka for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

Besides my advisor, I would like to thank the rest of my thesis committee: Prof. Dahanukar and Prof. Adams, for their insightful comments and encouragement to widen my research from various perspectives.

I thank my fellow labmates and super lab group members in for the supports they provided on my experiments and study. In particular, I am grateful to Dr. Okamoto for always being knowledgeable and helpful.

Last but not the least, I would like to thank my family and friends for supporting me spiritually throughout writing this thesis and my my life in general.

ABSTRACT OF THE DISSERTATION

Functional Study of Uncharacterized Cytochrome P450 Enzymes in *Drosophila*
Development

by

Ruoying Lu

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
University of California, Riverside, September 2019
Dr. Naoki Yamanaka, Chairperson

Cytochrome P450 (P450) enzymes encoded have generally been described as detoxifying

enzymes. However, study of the insect steroid hormone 20-hydroxyecdysone (20E)

revealed a group of CYP genes named Halloween Genes that participate in biosynthesis

of the 20E precursor ecdysone, as well as conversion of ecdysone to the active hormone

20E. 20E is the primary regulator of insect developmental transitions, triggering molting

and metamorphosis upon binding to the nuclear receptor heterodimer EcR-USP. In the

fruit fly *Drosophila melanogaster*, there are 18 identified nuclear receptor-encoding

genes presenting all 6 of the main nuclear receptor subfamilies in humans, while 20E is

the only known active steroid-derived ligand. In addition to 20E, some ecdysteroids are

present in insect hemolymph only at specific developmental stages, suggesting that there

might be some unknown ecdysteroids serving as ligands for nuclear receptors other than

EcR. Phylogenetic analysis showed a group of P450 genes evolutionarily close to

Halloween Genes with unidentified physiological functions. Therefore, we propose to

investigate the function of these uncharacterized P450 genes. The results of this thesis

work suggest that one of the uncharacterized P450 genes, *cyp303a1*, has important

functions in *Drosophila* development during pupal stage. Moreover, further study also

indicates that *cyp303a1* expression is required for activation of the dHNF4 nuclear

receptor signaling pathway.

Table of Contents

List of Figures

List of Tables

CHAPTER I

General Introduction

Cytochrome P450 Enzyme Superfamily

The cytochrome P450 (P450) is a large enzyme family that can be found in nearly all organisms. These thiolate hemoproteins were first identified in early 1960s as monooxygenases with CO-binding ability when in a reduced form. They were named after their specific characters: cytochrome represents hemoprotein, P stands for pigment and 450 comes from the absorbance peak of the reduced CO complex at 450 nm in the optical spectrum (Omura T and Sato R, 1962). Through around five decades of study, scientists revealed that these enzymes can catalyze many distinct oxidative reactions on a wide variety of molecules, from relatively simple molecules such as alkanes or fatty acids, to more complex compounds such as steroids and exogenous toxins (Bernhardt R, 2006; Coon MJ, 2005). Our knowledge about P450 enzymes is continuously growing, with ~3,000 new research papers appearing in PubMed database every year. So far more than 300,000 P450 sequences have been produced in all areas of the tree of life and more than 4,000 P450 genes were discovered in vertebrates through genome analysis (Nelson DR, 2018). Despite the large number and diversity of P450s, their functions can be classified broadly into two main roles. The first role P450s have been known for is detoxification, as they account for 70 to 80 percent of enzymes involved in drug and pollutant metabolism in humans (Rendic S and Guengerich FP, 2014). The cytochrome P450 superfamily is expressed primarily in the liver, small intestine and kidney in mammals (Thelen K and Dressman JB, 2009; Renaud HJ et al., 2011). Of the 57 P450 genes present in humans, five are involved in the metabolism of ~90% of small-molecule

drugs in use today, with varying selectivity and overlap. Several microbial and human P450s are drug targets, and the design of drugs is greatly facilitated by the structures of these P450s (Williams JA et al., 2004; Guengerich FP, 2016). The second subset of cytochrome P450 enzymes were reported as having a strong preference for single substrates and are important for steroidogenesis and eicosanoid biosynthetic pathways in animals (Miller WL, 2017; Jamieson KL et al., 2017). For example, synthesis of human steroid hormones is carried out by P450 enzymes of the adrenals, gonads, and other peripheral tissues (Martucci CP and Fishman J, 1993). Furthermore, a group of P450s also plays important roles in cancer, diabetes and atherosclerosis. Their activity or expression patterns are altered in patients, although most of the detailed molecular pathways remain unknown (Elfaki I et al., 2018).

Consequently, because of its diversity, this group of hemoproteins is often called "nature's most versatile biological catalyst". The versatility of P450s includes the ability to metabolize innumerable substrates of both physiological and xenobiotic importance, and to be markedly altered in activity by a wide variety of inducers and inhibitors. Ongoing studies of P450s include mechanisms of catalysis, oxygen activation and inhibition, gene regulation, and three-dimensional structure. Accordingly, knowledge about these enzymes is invaluable to scientists in fields as diverse as biochemistry, chemistry, biophysics, molecular biology, pharmacology, and toxicology. Better understanding of P450s functions is not only important for predicting drug metabolism but also essential for understanding endocrinology related to development and diseases.

Cytochrome P450 Enzymes in *Drosophila* Development

The fruit fly, *Drosophila melanogaster*, has been used as a model animal for over a hundred years, as there are many advantages of using flies for biological research. Compared to mammalian model animals, flies are easier and cheaper to maintain and manage and they have relatively shorter life cycle and are more fecund. Meanwhile, there are plenty of well-developed tools for gene functional studies, including genome sequence information, gene expression pattern information, cDNA clones, and Gal4-UAS system available for researchers. More than 70 percent of *Drosophila* genes have mammalian isoenzymes and they share many similar regulation mechanisms with other insects and even mammals. Compared to complicated mammalian systems, *Drosophila* has a relatively simpler system with fewer elements. Moreover, it only takes about 10 days to raise *Drosophila* from egg to adult in standard lab corn meal diet. Taken together, *Drosophila* is a perfect model organism for study of metabolism and development in insects and could inspire scientists in the field of mammalian study.

There are 87 P450 genes in *Drosophila melanogaster*. Like vertebrate P450s, they play critical roles in the metabolism of endogenous substrates and in the detoxification of xenobiotics. Interestingly, previous studies revealed that steroid hormone biosynthesis in flies is carried out by a group of P450 enzymes just like in humans. The most important hormone regulating maturation in *Drosophila* as well as other insects is a steroid

hormone, 20-Hydroxyecdysone (20E) (Riddiford LM, 1993). 20E is converted primarily from its precursor ecdysone by a P450 enzyme SHADE in peripheral tissues. Ecdysone is released into the hemolymph after being produced in the prothoracic glands by a group of P450s for conversion into the biologically active form 20E (Gilbert L et al., 2002). After completion of embryogenesis, *D. melanogaster* progresses through three larval stages, or instars, to enter metamorphosis and finally emerge as an adult fly. Each of the transitions between these stages is triggered by a pulse of 20E, defining the hormone as a crucial determinant of developmental timing (Kozlova T and Thummel CS, 2000). Without proper regulation of 20E, arthropods exhibit a series of developmental defects. Knockout of P450s for 20E synthesis in *Drosophila* results in embryonic lethality caused by cuticle defects, which makes the larvae look like ghost. Consequently, 20E synthesis genes were named Halloween genes (Niwa R and Niwa YS, 2014).

Other than Halloween genes and some P450s reported with roles in insecticide resistance, the rest of *Drosophila* P450s are uncharacterized. At the same time, in addition to ecdysone and 20E, there are many other ecdysteroids that were identified from the *Drosophila* hemolymph, while their functions remain unclear (Lavrynenko O et al., 2015). Study of uncharacterized P450 enzymes in *Drosophila* may reveal novel components in the steroid hormone signaling pathway and lead to better understanding of molecular mechanisms behind the regulation of development and maturation.

Nuclear Receptors and Ligands

20E controls molting and metamorphosis of arthropods through binding to a nuclear receptor heterodimer, the ecdysone receptor (EcR) and ultraspiracle (USP) (Yao TP et al. 1993). The nuclear receptor superfamily is the largest group of transcription factors in eukaryotes. Over 150 different genes encoding this group of structurally related proteins have been discovered in organisms ranging from early metazoans to humans. Members of the nuclear receptor superfamily are typically characterized by several conserved domains, including the DNA binding domain (DBD) in the N-terminal half, the ligand binding domain (LBD), and the dimerization domain in the C-terminal half of the protein (Evans RM and Mangelsdorf DJ, 2014; Mazaira GI et al., 2018). Generally, nuclear receptors tend to be activated by hydrophobic ligands such as steroids, retinoic acid, and thyroid hormones. Genes encoding putative receptors for unknown ligands, referred to as orphan receptors, have also been characterized and included in this superfamily based on the structural domain properties. They bind DNA upon activation in a dimerized form, either homodimer and/or heterodimer, and play major roles as transcription factors in development, metabolism, disease, and detoxification of foreign substances (Sever R and Glass CK, 2013).

Drosophila has only 18 nuclear receptor genes compared to a relatively large group of 48 nuclear receptor genes in humans, while they present all 6 subclasses of vertebrate receptors (King-Jones K and Thummel CS, 2005). Activities of nuclear receptors are

essential to the development and metabolism of fruit flies. Knockout of most of the nuclear receptors results in lethality at certain developmental stages (Table 1), which suggests that the activation of these receptors is required for survival of the flies. However, molecular mechanisms of the regulation or ligands of most nuclear receptors are still unknown. Many aspects of nuclear receptor functions have been conserved through evolution, making the fly an ideal system for study of the regulation and functions of nuclear receptors during development and metabolism. Knowledge about the ligands or regulating factors of the nuclear receptors would contribute to the study of the same field in mammals, identification of potential drug targets or the development of better insecticides.

In this thesis work, functional studies of uncharacterized P450 enzymes were performed to test the hypothesis that there are other members in P450 involved in steroid hormone signaling and important for *Drosophila* development/metabolism other than Halloween genes. Null mutants of five highly conserved P450s that are close to Halloween genes were generated for loss -of -function studies. Detailed phenotypic analyses following mutagenesis revealed that one of the uncharacterized P450 mutants phenocopies the mutant phenotype of the nuclear receptor, dHNF4. Results from further assays focusing on this gene and dHNF4 pathway suggests that this P450 may be involved in ligand production of the dHNF4 nuclear receptor.

Subfamily	Nuclear	Related Phenotype	References
1	E78	Partially pupal lethal - majority die	(Mummery-Widmer JL et al., 2009)
	E75	E75A mutants die throughout development and E75C mutants are pharate adult and adult lethal	(Bialecki, M et al., 2002)
	DHR3	Embryonic or Larval lethal	(Lam G et al., 1999)
	EcR	Embryonic lethal	(Kozlova T, 2003)
	DHR96	Viable	(Mummery-Widmer JL et al., 2009)
2	USP	Embryonic or larval lethal	(Gradilla AC et al., 2011)
	DHR78	Larval or Pupal lethal	(Astle J et al., 2003)
	HNF4	Pupal lethal Many die with their head protruding from the pupal case and remaining die within a day of eclosion.	(Palanker L et al., 2009)
	SVP	Embryonic or larval lethal	(Miller AC et al., 2008)
	TLL	Embryonic lethal	(Diaz RJ et al., 1996)
	DSF	Embryonic or larval lethal	(Pitman JL et al., 2002)

	DHR51	Pupal Lethal	(Neumüller RA et al., 2011)
	DHR83	Viable	(Mummery-Widmer JL et al., 2009)
3	ERR	Larval Lethal – before second instar	(Tennessen J et al., 2011)
4	DHR38	Pupal lethal	(Kahsai L and Cook ER, 2018)
5	FTZ-F1	Embryonic or larval lethal	(Yamada M et al., 2000)
	DHR39	Viable	(Mummery-Widmer et al., 2009)
6	DHR4	Larval lethal	(King-Jones K et al., 2005)
		Pupal Lethal – pharate adult stage	

Table 1.1 Nuclear receptor families and knockout phenotypes in *Drosophila melanogaster*. Subfamilies and the members in each of them are shown. Phenotypes are summarized and only 3 out of 18 nuclear receptor null mutants are viable.

CHAPTER II

Mutagenesis and Screening

Introduction

Molecular Evolution of P450 Enzymes in *Drosophila*

Since publication of the *Drosophila* genome sequence in March, 2000, more and more

genome information for predicting gene functions in flies has become available for

researchers over the world (Adams MD, 2000). There are 87 P450 genes in *Drosophila*

and their sequences are all available on flybase.org. Some of these P450s are reported to

detoxify exogenous compounds such as insecticides and plant toxins (Feyereisen R,

2011; Schuler MA, 2011; Feyereisen R, 2012). However, other than the Halloween genes

involved in the biosynthesis of the major insect hormone 20E, much less is known about

biosynthetic functions of insect P450 enzymes compared to plants and mammals (Gilbert

L et al., 2002). Studies of the molecular evolution of the *Drosophila* genome have

revealed that nonfunctional sequences are lost quickly, with the half-life of pseudogenes

estimated to be 18 Myr (Petrov DA and Hartl DL, 1998; Robin GC et al. 2000).

Interestingly, members of the P450 superfamily are conserved over time, as there were at

least 30 P450 genes showing no gain or loss in the *Drosophila* genome (Good RT et al,

2014). Phylogenetic analysis of P450s separated these enzymes into unstable and stable

groups. Most of the unstable P450s encode enzymes that function as detoxifiers, while

stable P450s usually take endogenous substrates (Thomas JH, 2007). Evolutionary

analysis of P450s found in 12 sequenced genomes of *Drosophila* species has also

identified stable and unstable P450s across the species (Clark AG, et al, 2007; Good RT

et al, 2014). Except Halloween genes, some uncharacterized P450s are also reported as

stable genes, which potentially have biosynthetic functions similar to those of the

Halloween genes.

Genome Editing Tools in *Drosophila*

Since the first mutant of *Drosophila* was discovered by Thomas Hunt Morgan and thus

began *Drosophila* experimental genetics, loss-of-function assays in flies have provided

deep insights into prediction of gene functions (Morgan TH, 1910). Techniques have

been rapidly developed from that time for generation of mutants or alteration of gene

functions in *Drosophila*. Traditionally, gene disruption was achieved by imprecise

excision of transposons or replacement by homologous gene targeting (Rong YS and

Golic KG, 2000; Ryder E and Russell S, 2003). Over the past decade, identification of

several specific DNA binding factors that can be used to target defined gene regions

contributed to development of new technologies for genome editing. By combining these

DNA binding modules and nucleases and introducing fusion proteins into the organism,

double- strand breaks in the DNA will be generated at target sites. When a double-strand

break is repaired by non-homologous end joining or homologous recombination leading

to a deletion or insertion, respectively, a mutation will occur at the defined gene region.

Zinc-finger nucleases (ZFN) and TALE nucleases (TALEN) are the best-known

examples of the application of programmable nucleases that have been successfully used

in various organisms (Carroll, D et al., 2008; Beumer KJ et al., 2013; Li T et al., 2011).

Both ZFN and TALEN have a DNA binding module that consists of units that can be

engineered so that the fused non-specific endonuclease domain can target desired

sequences within the genome (Choo Y and Klug A, 1994; Boch J et al., 2009). Despite

their specificity in DNA binding and high efficiency in gene targeting, ZFN and TALEN

were not as popular in *Drosophila* research as they are in other organisms (Beumer KJ et

al., 2008; Liu J et al. 2012). The main reason is that these technologies require assembly

of the DNA binding multimers and nuclease domain, and the resulting polypeptides are

usually too large to manipulate in *Drosophila* (Rong YS and Golic KG, 2000; Reyon D et

al., 2012).

The clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) system is

the latest addition to genome editing technologies. This system was discovered in

bacteria, which employ it as a defense against invading viruses and plasmids (Barrangou

R et al., 2007; Garneau JE et al., 2010). Cas9 is an endonuclease with a guide-RNA

(gRNA) component targeted to sequences from the invading pathogen. By base pairing

with the complementary sequence within the DNA, gRNA provides target specificity to

the Cas9 endonuclease. The 20-bp target-determining gRNA can be easily reprogrammed

and replaced so that the Cas9 associated can be recruited to the desired region within the

genome and induce a double-strand break in DNA (Jinek M et al. 2012; Cong L et al.

2013). Furthermore, gRNA design is flexible, the only limitation being that it must be

followed by a protospacer adjacent motif (PAM) of NGG in the DNA to induce efficient

cleavage (Mali P et al. 2013). The ease and flexibility of modifying Cas9 makes it an

ideal system that can be widely applied for all organisms with the possibility of engineering Cas proteins to bind to essentially any sequence.

In this project, we hypothesized that some of the uncharacterized P450 enzymes us ecdysteroids as their substrate and play roles in regulation of *Drosophila* development. Five candidate P450 genes were selected from phylogenetic study and null mutants were generated for loss-of-function assay. Based on phylogenetic analysis, selected uncharacterized P450s are structurally close to Halloween enzymes, which are responsible for the biosynthesis of 20E. Furthermore, they are conserved among major insect species, indicating importance of these enzymes across the Insecta. Mutants generated in this study were confirmed by PCR and sequencing. All five mutants exhibit no difference in developmental timing compared to control groups and most of them are fully viable. Only one of the null mutants shows a defect in eclosion. Double knockdown of closely related candidates was also examined and there was no obvious phenotype observed.

Materials and Methods

Phylogenetic Analyses

Protein sequence of all 87 P450 enzymes were obtained from Flybase (Table 2.1).

Homologs of selected proteins from other insect species were found through NCBI

protein BLAST (Table 2.2). Phylogenetic trees were generated by Claster W2 and

visualized by Tree-View.

Mutagenesis

Mutagenesis was carried out by CRISPR/Cas9 using methods developed by Kondo and

Ueda (Kondo and Ueda, 2013). A pair of gRNAs with PAM sequence was designed

targeting flanking regions of the coding sequence of target genes as indicated by the

white arrows (Figure 2.3). In the first step, one of the gRNAs was cloned into pBFv.U6.2

and the other into pBFv.U6.2B vector digested with *BbsI* restriction enzyme. Next, U6

promoter with gRNA was excised with *EcoRI* and *NotI* from pBFv.U6.2 and cloned into

pBFv.U6.2B with the other gRNA, resulting in a plasmid with a pair of gRNAs in pBFv.

U6.2B vector. gRNAs used for mutagenesis were listed in Table 2.3. The final constructs

were sent to the BestGene Inc. for injection into flies with nos-cas9 transgene, which

express Cas9 in the germline cells.

Mutants were screened by PCR with PCR Master Mix (2X) (Thermo Fisher Scientific).

PCR products were extracted with Gel DNA Recovery Kit (ZYMO Research) and sent

for sequencing by Retrogen. Primers used were listed in Table 2.4.

Plasmid Construction

cDNA clones of selected P450 genes were requested from *Drosophila* Genomics

Resource Center and cloned into pUAST vector to generate UAS transgenic flies.

Transformation was conducted with ELITE Electrocompetent Cells (Lucigen) and

plasmids were extracted with QIAprep Spin Miniprep Kit (QIAGEN).

General *Drosophila* Husbandry

Flies were reared in 25°C incubators with standard corn meal diet. Stocks were kept at

18°C and flipped every 4 weeks.

Developmental Timing Analysis

Eggs were collected from standard corn meal diet and transferred to 20% sucrose

solution, in which they hatch into the first instar (L1). L1 larvae of selected genotype

were transferred to the corn meal diet (15 animals/vial) and cultured in 25°C incubators

with approximately 25% humidity. Vials were checked at 12-hour intervals to record

number of prepupae formed for generation of developmental timing curves. Deficiency

lines used were listed in Table 2.6.

Gene Expression Pattern Analysis

L1 larvae were transferred to the corn meal diet and cultured in 25°C incubators with

approximately 25% humidity. Around 5 animals were collected and frozen at each

developmental stage for RNA extraction with Triton X and RNeasy kit (QIAGEN).

cDNA was generated by PrimeScript RT Master Mix (TAKARA) to use as a template for

qPCR with SYBR® Premix Ex Taq™ II (TAKARA) in CFX96 qPCR Instrument (Bio-

Rad). Primers for qPCR are listed in Table 2.5.

Results

<u>Determination of Candidate CYPs through Phylogenetic Analysis</u>

From the 87 sequences representing *Drosophila* P450 genes, a phylogenetic tree was generated. Halloween genes that carry out 20E biosynthesis fall in two clades – the CYP2 Clan and Mito Clan and they are phylogenetically close to each other. There are still some other P450 genes in the same clades with unknown functions. Among these genes, *cyp303a1*, *cyp304a1* and *cyp305a1* are the only genes other than Halloween genes in the CYP2 Clan. In the Mito Clan where the rest of the Halloween genes fall in, *cyp301a1* was reported to play an essential role in adult cuticle formation, a critical process during molting (Sztal T et al.,2012). Therefore, this gene was considered related to development and included in this study. Another gene, *cyp49a1*, was also included because it shares 40% similarity with *cyp301a1* and may share similar functions with *cyp301a1*.
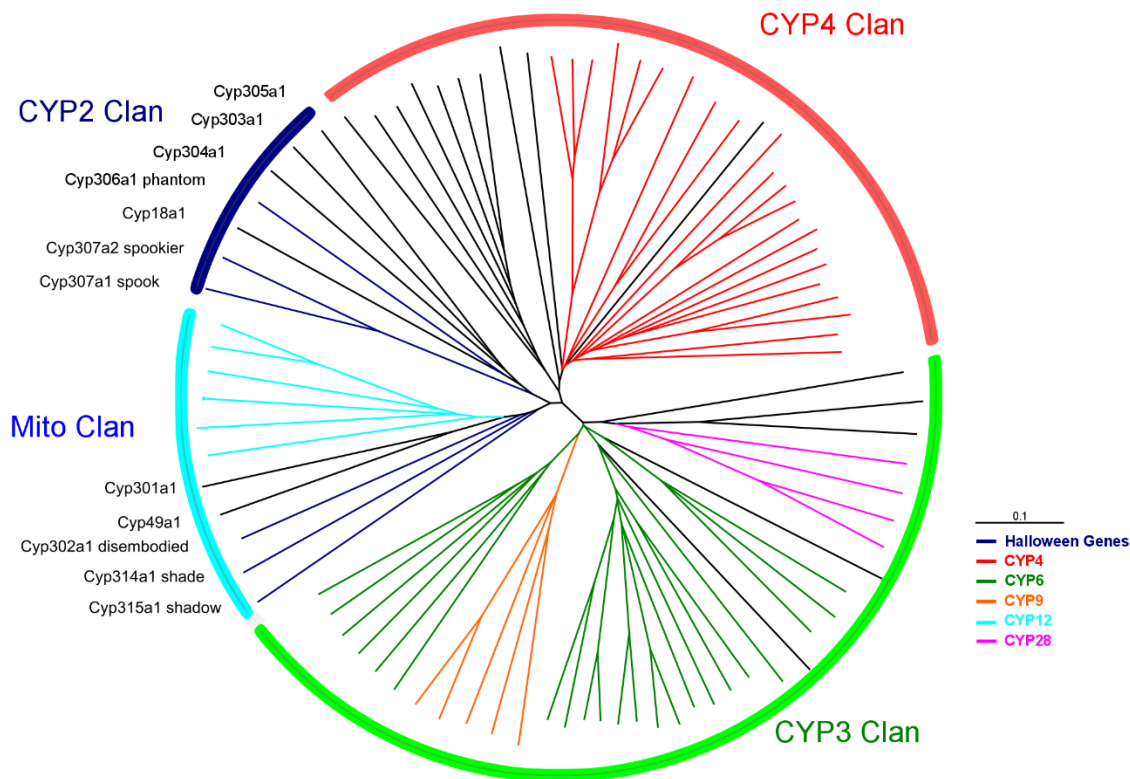
Figure 2.1 Phylogenetic tree of all reported P450s in *Drosophila melanogaster*.
Subfamilies are color-coded. Halloween genes that carry out biosynthetic reactions of
20E are found only in the CYP2 and Mito Clans.

Furthermore, stability and conservation of *cyp49a1*, *cyp301a1*, *cyp303a1*, *cyp304a1* and

cyp305a1 among insect species were also analyzed. Genes vary greatly in their long-term

phylogenetic stability, and there exists no general explanation for these differences.

Stable genes persist as a single copy over a wide range of distantly related species,

whereas unstable genes undergo frequent duplication and loss in a process called birth-

death evolution. It is known that P450 genes mainly related to xenobiotic metabolism are

more diverse than the other P450s, with less sequence conservation. Gain and loss events

are not evenly distributed among the P450 genes in *Drosophila*, indicating that the stable

genes are more likely responsible for processing of endogenous substrates that are potentially involved in regulation of metabolism and development. *Cyp49a1, cyp301a1, cyp303a1* and *cyp305a1* are reported as stable P450s in *Drosophila* with only one copy number among 12 *Drosophila* species. *Cyp304a1* is also stable with only 2 duplicates (Good Rt et al., 2014). In order to confirm the stability of these CYP450s, I generated another phylogenetic tree with protein sequences of these genes and their homologs in other insect species (Figure 2.2). The result clearly suggests that they are conserved among major species of different insect orders.
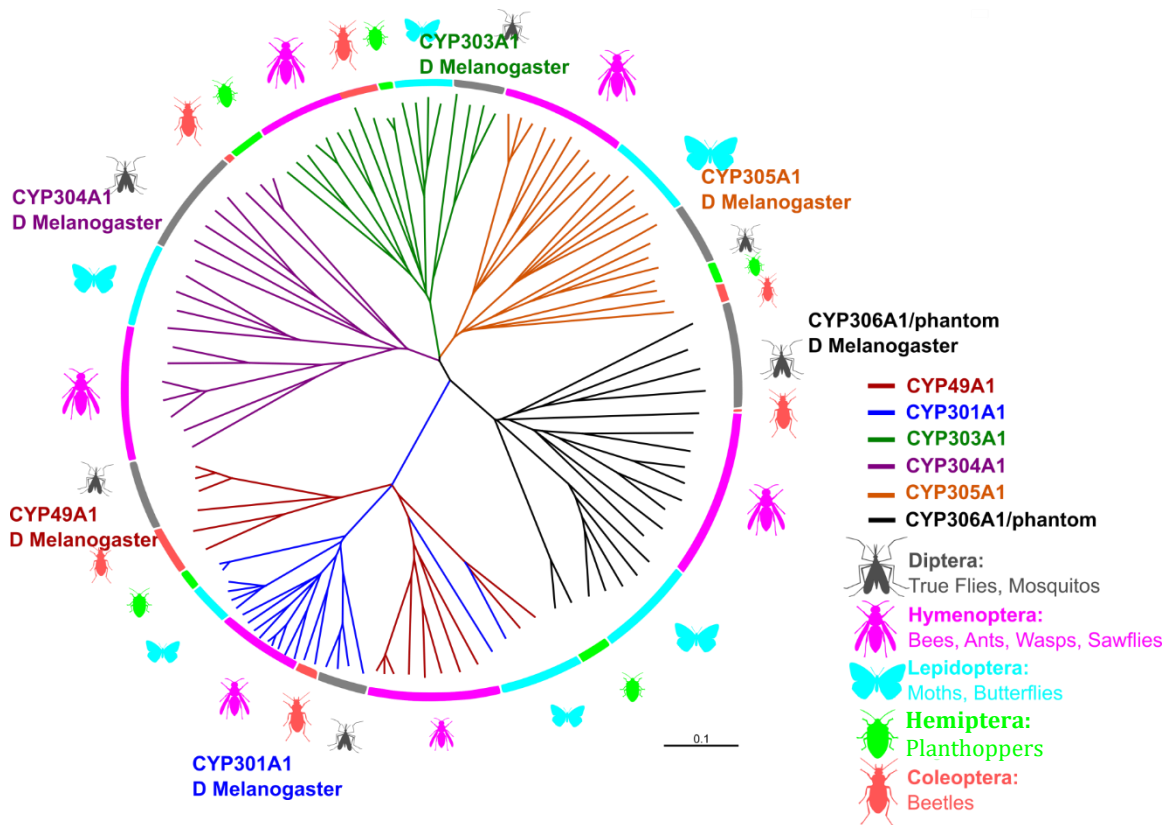

Figure 2.2 Phylogenetic tree of selected P450s in common insect model species. Different genes were color-coded. A Halloween gene *Phantom (cyp306a1)* was included as a reference to compare how these genes are conserved among insect species.

20

Being evolutionarily close to Halloween genes, as well as being stable and conserved during evolution, *cyp49a1*, *cyp301a1*, *cyp303a1*, *cyp304a1*, and *cyp305a1* were chosen as candidate genes for this project that are potentially involved in metabolism and development.

Mutagenesis and Viability of Null Mutants

Null mutants were generated by CRISPR/Cas9 technique and confirmed by genome

sequencing. The cleavage sites were close to PAM sequences as expected. In order to

eliminate potential off-target effects induced by CRISPR/Cas9, deficiency lines were

crossed to mutants and transheterozygote mutants over deficiency were used for further

study. Deficiency and mutant lines were crossed to *w1118* control strain, and these

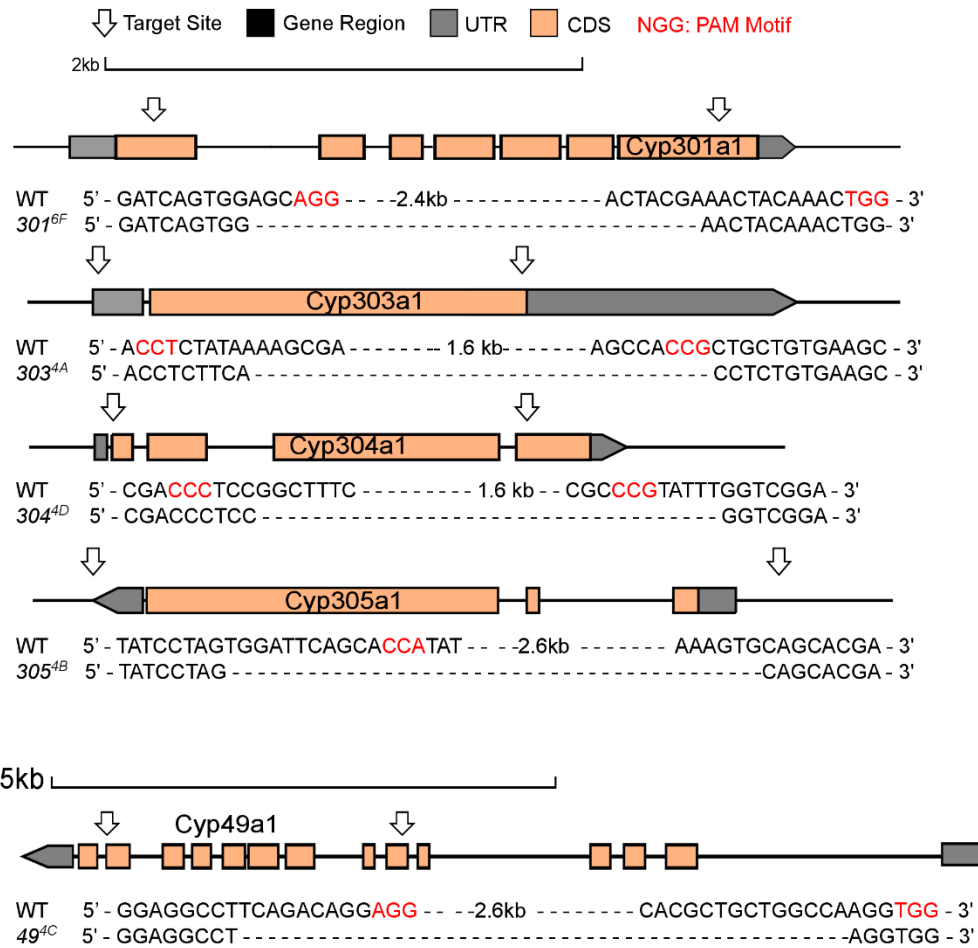heterozygotes were used as control groups of this work.

Figure 2.3 Genetic mapping and sequence information of mutants generated. gRNA targeted sites were designed on both sides of the coding regions and indicated by white arrows. Actual cleavage sites were confirmed by sequencing genomic PCR products. Deletion was detected as expected near PAM sequences marked in red.

For four of the target genes, *cyp49a1*, *cyp301a1*, *cyp304a1*, and *cyp305a1*, no obvious mutant phenotype was observed compared to control groups. The null mutants were homo-viable, fertile, and had no obvious defects on their morphology. However, deletion of *cyp303a1* caused a characteristic developmental defect: most null mutants died during

eclosion with their heads protruding from the pupal case (Figure 2.4). Around 80% of the

flies were stuck in the pupal case during eclosion, while the rest were able to complete

eclosion, but died within 24 hours.

Developmental Timing

According to our hypothesis, candidate genes may function similarly to Halloween genes, which carry out biosynthesis reactions of 20E and regulate *Drosophila* development. To test whether the selected genes also influence development, timing of puparium formation (pupariation) for null mutants was recorded. There was no significant difference observed between null mutants and respective controls (Fig. 2.4), indicating that the genes we selected for this study do not affect developmental timing during embryonic and larval stages.
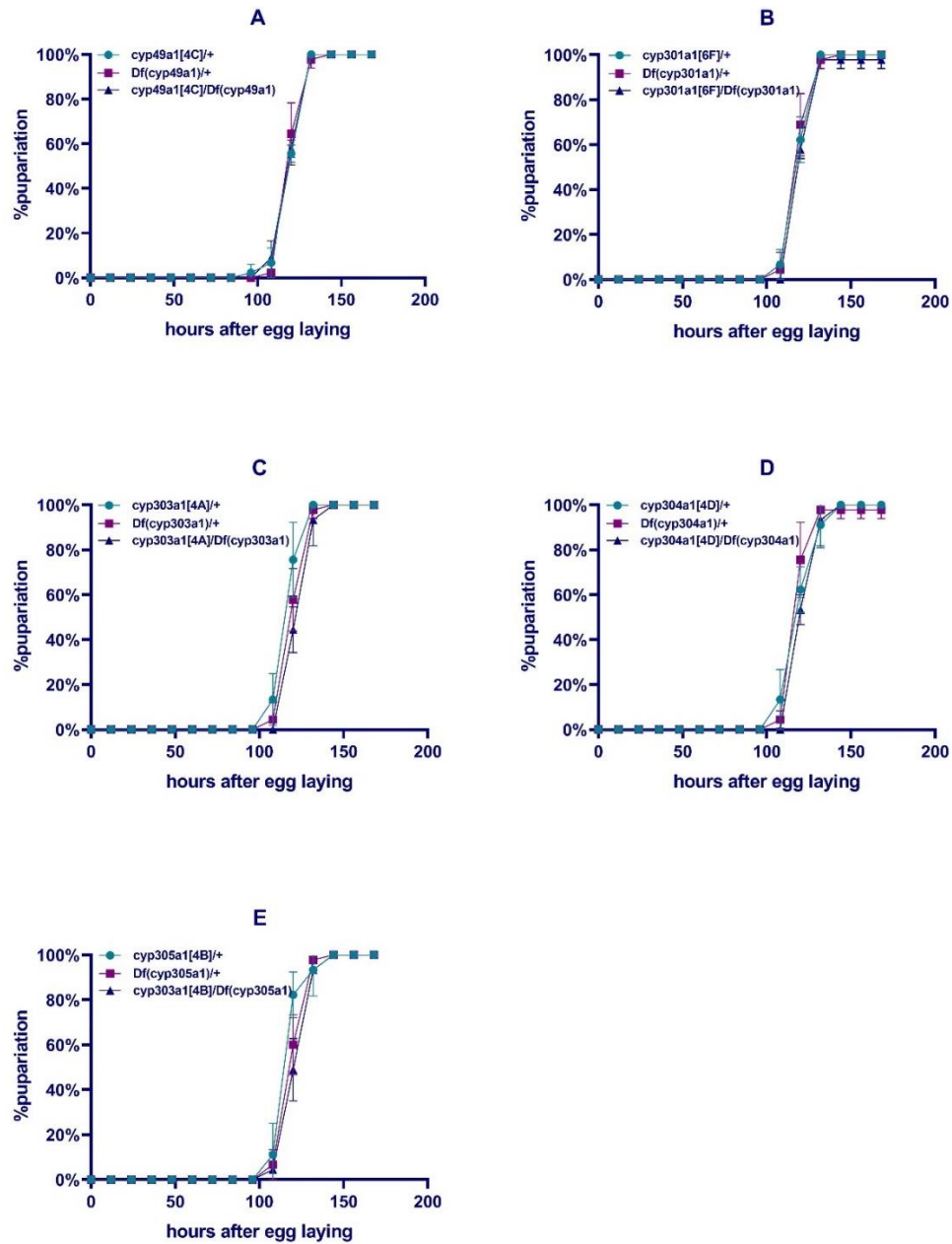
Figure 2.4 Developmental timing curve of selected P450 null mutants. (A-E) Pupariation rate was recorded at 12-hour interval after egg laying to generate a developmental curve for null mutants and corresponding controls of *cyp49a1*, *cyp301a1*, *cyp303a1*, *cyp304a1*, and *cyp305a1*, respectively. N= 3 vials, 15 flies per vial. Error bars = standard error.

Temporal Gene Expression Analysis

In order to gain a better understanding of functions of selected genes and the developmental stages involved, temporal expression patterns were analyzed by qPCR. RNA was extracted from flies of different developmental stages, and cDNA samples were prepared to use as templates for the analysis. All five genes are selectively highly expressed during pupal or adult stage, which is consistent with the observation that pre-pupal development is not affected in null mutants. While other genes have low to moderate expression in all stages, the expression pattern of *cyp303a1* was extremely specific. It was only highly expressed on the second day after puparium formation (Fig. 2.5?).
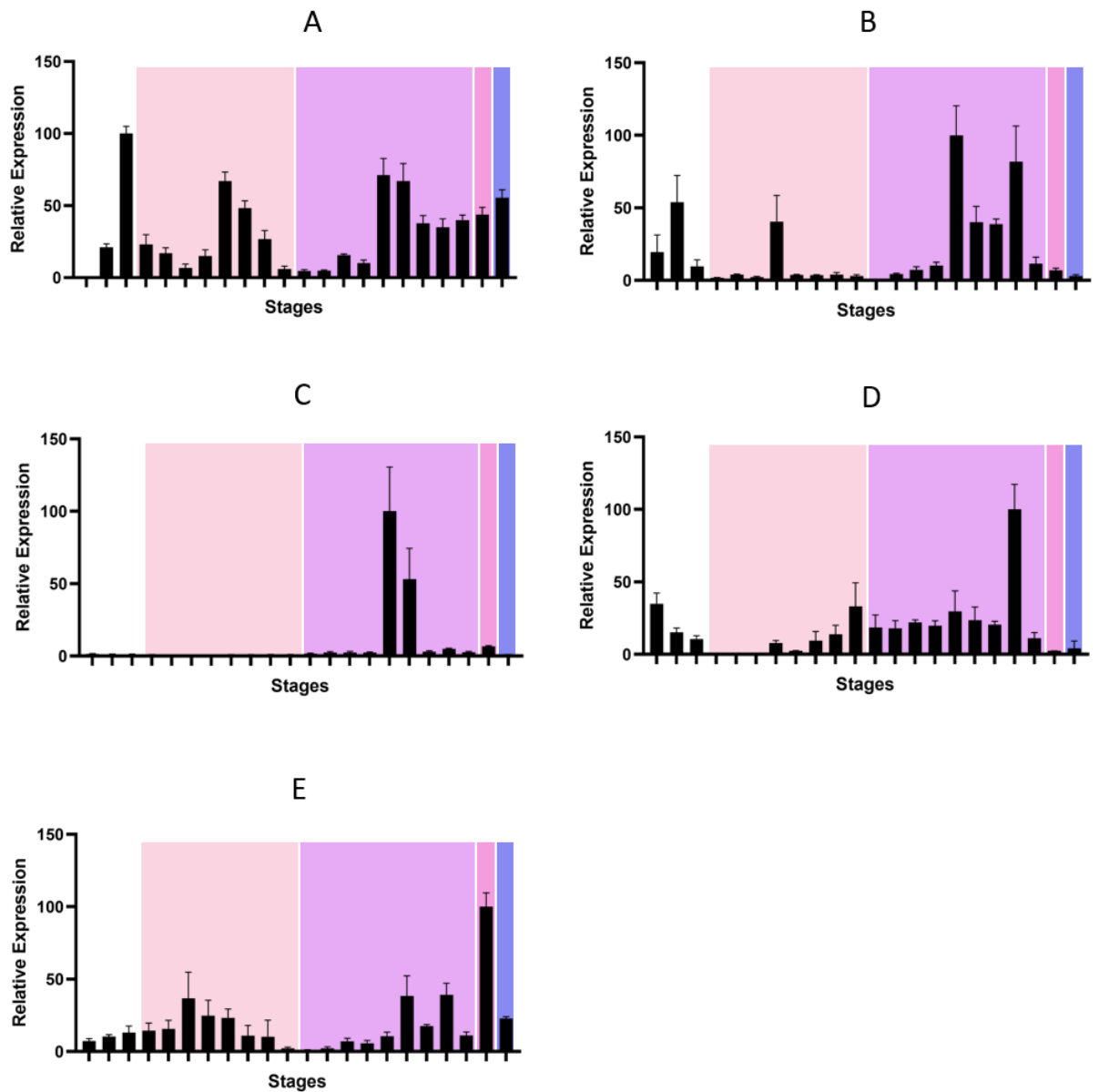
Figure 2.5 Temporal gene expression patterns of selected P450 null mutants. cDNA was reverse- transcribed from RNA extracted from *w1118* flies collected on different development stages. Expression levels of *cyp49a1* (A), *cyp301a1* (B), *cyp303a1* (C), *cyp304a1* (D), and *cyp305a1* (E) were tested by qPCR. Results were normalized with rp49. N= 3 samples, 3-5 flies per sample. Error bars = standard error. Each developmental stage is color-coded as following: Embryonic stage (white): 6, 14, 22 hours after egg laying; Larval stage (pink): 0, 12, 24, 36, 48, 60, 72, 84 hours after

hatching; Pupal stage (purple): 0, 12, 24, 36, 48, 60, 72, 84, 96 hour after pupal formation; Adult stage: female (hot pink) and male (blue) 24 hours after eclosion.

<u>Double mutants</u>

No obvious mutant phenotype was observed from knockout of *cyp49a1*, *cyp301a1*, *cyp304a1*, and *cyp305a1* individually. Meanwhile, *cyp49a1* shared 40% similarity in protein sequence with *cyp301a1*, and *cyp304a1* is also evolutionarily close to *cyp305a1*. This raises the possibility of possible redundancy in functions of these P450 enzymes. Therefore, *cyp49a1* and *cyp301a1* double knockout as well as *cyp304a1* and *cyp305a1* double knockout lines were generated by meiotic recombination. However, there was still no obvious phenotype observed when these lines were reared on the standard corn meal diet.

Discussion

In this chapter, five uncharacterized P450 genes *cyp49a1*, *cyp301a1*, *cyp303a1*, *cyp304a1*, and *cyp305a1* were selected for study through phylogenetic analysis. They are evolutionarily close to Halloween genes and conserved among insect species. Null mutants of these five genes were generated by CRISPR/Cas9 and brief phenotypical analysis was performed with the mutants.

Limitation of CRISPR/Cas9

A limitation of the CRISPR/Cas9 system is the relatively high occurrence of off-target effects (Ref?). Since the targeting specificity of the system is determined by a 20-bp gRNA only with the requirement for the neighboring PAM sequence (NGG), mismatch and cleavage at other regions within the genome were frequently observed (Wei C et al., 2013; Fu Y et al., 2013). Although it is less of a problem in *Drosophila* due to a relatively smaller genome size compared to other species, we have to consider the possibility that the mutant phenotype that we observed was not caused by disruption of other genes. To this end, mutant lines were crossed to corresponding deficiency lines that can provide independent null alleles. Other methods to prevent the influence of off-target effects are: 1) generate another mutant with a different pair of gRNAs for each gene and 2) use a transheterozygote mutant of those two alleles (mutant-1/mutant-2) for experiments, or perform backcrosses of the mutant to recover other parts of the genome while keeping the null mutation.

No Obvious Phenotype Was Observed in Most Uncharacterized P450 Null Mutants

Although they are conserved, stable, and have specific expression patterns, uncharacterized P450 candidates selected for this study did not show any phenotype as null mutants except for *cyp303a1*, the phenotype of which is examined in more detail in Chapter III. One explanation is that there might be redundancy in the functions of these genes, which is common in multigene superfamilies (Golby JA; Overton PM et al. 2002). For example*, Drosophila* the SNAP-25 paralogs, *Snap24*, *Snap25*, and *Snap29* encode proteins thought to be involved in secretion. Silencing each of these genes alone allows flies to survive until the late pupal stage. However, knock-out of all three *Snap* genes together leads to embryonic lethality (Poe AR, 2019).

In this study, double knockout was performed for two pairs of genes, but there was still no obvious mutant phenotype observed. It is possible that the genes are redundant with other P450s that were not silenced. More combinations of double knockout or triple knockout can be tested in the future to analyze potentially redundant functions of these genes.

On the other hand, observations of the null mutants generated are only performed with standard corn meal diet at 25℃, while some genes important for metabolism are only highly expressed and functional during adaptive responses to altered environment to enhance fitness and survival (Barghi N et al. 2018; Rane RV 2019). Further experiments can be done for investigating this possibility by observing mutant fitness and changes in gene expression levels under various treatments including cold-shock, starvation, and heat-shock.

Gene	Protein Sequence Flybase ID	Gene	Protein Sequence Flybase ID	Gene	Protein Sequence Flybase ID
Cyp4aa1	FBpp0113050	Cyp6a21	FBpp0086586	Cyp28c1	FBpp0305230
Cyp4ac1	FBpp0078701	Cyp6a22	FBpp0311630	Cyp28d1	FBpp0078698
Cyp4ac2	FBpp0289683	Cyp6a23	FBpp0086582	Cyp28d2	FBpp0078697
Cyp4ac3	FBpp0078703	Cyp6a8	FBpp0086585	Cyp49a1	FBpp0089308
Cyp4ad1	FBpp0304289	Cyp6a9	FBpp0289405	Cyp301a1	FBpp0086937
Cyp4ae1	FBpp0070372	Cyp6d2	FBpp0071839	Cyp302a1(disembodied)	FBpp0073061
Cyp4c3	FBpp0085074	Cyp6d4	FBpp0083697	Cyp303a1	FBpp0310367
Cyp4d1	FBpp0070412	Cyp6d5	FBpp0088707	Cyp304a1	FBpp0082159
Cyp4d14	FBpp0301778	Cyp6g1	FBpp0087100	Cyp305a1	FBpp0074694
Cyp4d2	FBpp0311678	Cyp6g2	FBpp0087117	Cyp306a1(phantom)	FBpp0309669
Cyp4d20	FBpp0072837	Cyp6t1	FBpp0076908	Cyp307a1(spook)	FBpp0312220
Cyp4d21	FBpp0079095	Cyp6t3	FBpp0087101	Cyp307a2(spookier)	FBpp0289444
Cyp4d8	FBpp0076543	Cyp6u1	FBpp0085516	Cyp308a1	FBpp0074375
Cyp4e1	FBpp0087825	Cyp6v1	FBpp0311867	Cyp309a1	FBpp0307161
Cyp4e2	FBpp0087824	Cyp6w1	FBpp0085332	Cyp309a2	FBpp0077428
Cyp4e3	FBpp0079515	Cyp9b1	FBpp0088127	Cyp310a1	FBpp0080627
Cyp4g1	FBpp0070094	Cyp9b2	FBpp0088126	Cyp311a1	FBpp0073473
Cyp4g15	FBpp0312025	Cyp9c1	FBpp0072228	Cyp312a1	FBpp0074901
Cyp4p1	FBpp0087673	Cyp9f2	FBpp0082070	Cyp313a1	FBpp0082413
Cyp4p2	FBpp0087672	Cyp9h1	FBpp0086933	Cyp313a2	FBpp0300785
Cyp4p3	FBpp0087674	Cyp12a4	FBpp0083145	Cyp313a3	FBpp0082086
Cyp4s3	FBpp0073845	Cyp12a5	FBpp0083144	Cyp313a4	FBpp0082136
Cyp6a13	FBpp0087770	Cyp12b2	FBpp0085792	Cyp313a5	FBpp0082088
Cyp6a14	FBpp0311645	Cyp12c1	FBpp0311798	Cyp313b1	FBpp0081379
Cyp6a17	FBpp0086581	Cyp12d1	FBpp0088437	Cyp314a1(shade)	FBpp0305245
Cyp6a18	FBpp0308423	Cyp12d2	FBpp0088436	Cyp315a1(shadow)	FBpp0081949
Cyp6a19	FBpp0086583	Cyp12e1	FBpp0290498	Cyp316a1	FBpp0304450
Cyp6a2	FBpp0085466	Cyp18a1	FBpp0074381	Cyp317a1	FBpp0311189
Cyp6a20	FBpp0289404	Cyp28a5	FBpp0080164	Cyp318a1	FBpp0073460

Table 2.1 FlyBase IDs of all P450 Enzymes in *Drosophila melanogaster*. Protein sequences of all 87 P450 enzymes in *Drosophila melanogaster* were obtained from flybase.org to perform phylogenetic analysis.

	CYP49A1	CYP301A1
Drosophila	Drosophila Melanogaster NP_610588.2	Drosophila Melanogaster NP_610796.1
Mosquitos	Aedes aegypti XP_001659378.1 Anopheles darlingi ETN58108.1 Anopheles gambiae str. PEST XP_315789.4	Aedes aegypti XP_001649083.2 Anopheles darlingi ETN65528.1 Anopheles gambiae str. PEST XP_316138.4
Bees	Apis mellifera XP_006562546.1 Apis florea XP_012342168.1 Apis dorsata XP_006623577.1	Apis mellifera XP_397170.3 Apis florea XP_012342166.1 Apis dorsata XP_006623548.1
Moths	Bombyx mori NP_001274759.1	Bombyx mori BAM73846.1 Cnaphalocrocis medinalis AJN91157.1
Butterflies	Papilio xuthus XP_013171656.1 Danaus plexippus EHJ78479.1	Papilio xuthus XP_013171658.1 Danaus plexippus EHJ78478.1
Wasps	Fopius arisanus XP_011299698.1 Microplitis demolitor XP_008548459.1 Ceratosolen solmsi marchali XP_011500804.1 Nasonia vitripennis XP_001605687.2	Fopius arisanus XP_011299707.1 Microplitis demolitor XP_008548437.1 Ceratosolen solmsi marchali XP_011500803.1 Nasonia vitripennis XP_008206386.1
Ants	Solenopsis invicta XP_011175661.1	Acromyrmex echinatior XP_011056595.1 Solenopsis invicta XP_011175761.1
Beetles	Tribolium castaneum XP_970738.1 Dendroctonus ponderosae AFI45015.1	Tribolium castaneum XP_974014.1 Dendroctonus ponderosae ENN80608.1
True Bugs	Nilaparvata lugens AIW79956.1	Nilaparvata lugens AIW79955.1 Laodelphax striatella AGN52764.1
	CYP303A1	CYP304A1
Drosophila	Drosophila Melanogaster NP_652070.1	Drosophila Melanogaster NP_731751.1
Mosquitos	Aedes aegypti XP_001662278.1 Anopheles darlingi ETN63136.1 Anopheles gambiae str. PEST XP_319235.4	Aedes aegypti XP_001648725.1 XP_001648726.1 Anopheles darlingi ETN61335.1 Anopheles gambiae str. PEST XP_001237487.2
Bees	Apis florea XP_012342090.1 Apis dorsata XP_006624311.1	Apis mellifera XP_006617030.1 Apis florea XP_012339807.1 Apis dorsata XP_006623548.1
Moths	Bombyx mori XP_004932789.1 Helicoverpa armigera AKR53660.1	Helicoverpa armigera AID54853.1 Cnaphalocrocis medinalis AJN91164.1
Butterflies	Papilio xuthus XP_013179069.1 Danaus plexippus EHJ66608.1	Papilio xuthus XP_013165084.1 Danaus plexippus EHJ67475.1

Wasps	Fopius arisanus XP_011310802.1	Fopius arisanus XP_011301797.1
	Ceratosolen solmsi marchali XP_011495186.1	Microplitis demolitor XP_008561134.1
	Nasonia vitripennis NP_001161246.1	
Ants	Acromyrmex echinatior XP_011062020.1	Acromyrmex echinatior EGI67770.1
	Solenopsis invicta XP_011169921.1	Solenopsis invicta XP_011166286.1
Beetles	Tribolium castaneum EFA01261.1	Tribolium castaneum XP_973180.1
	Dendroctonus ponderosae ERL93649.1	
True Bugs	Nilaparvata lugens AIW79971.1	Nilaparvata lugens AIW79972.1
	Laodelphax striatella AFU86421.1	Laodelphax striatella AFU86426.1

	CYP305A1	CYP306A1 Phantom
Drosophila	Drosophila Melanogaster NP_649151.1	Drosophila Melanogaster NP_573319.1
Mosquitos	Aedes aegypti XP_001654598.1	Aedes aegypti XP_001649971.2
	Anopheles darlingi ETN65091.1	Anopheles darlingi ETN60005.1
	Anopheles gambiae str. PEST XP_315679.3	Anopheles gambiae str. PEST ABU42522.1
Bees	Apis mellifera XP_XP_623621.1	Apis mellifera XP_006557887.1
	Apis florea XP_003693153.1	Apis florea XP_003691685.2
	Apis dorsata XP_006611142.1	
Moths	Bombyx mori BAM73852.1	Bombyx mori BAD34476.1
	Cnaphalocrocis medinalis AJN91165.1	Cnaphalocrocis medinalis AJN91166.1
	Helicoverpa armigera AID54854.1	Helicoverpa armigera AID54855.1
Butterflies	Papilio xuthus XP_013179638.1	Papilio xuthus XP_013182088.1
	Danaus plexippus EHJ65529.1	Danaus plexippus EHJ76071.1
Wasps	Fopius arisanus XP_011299261.1	Fopius arisanus XP_011311381.1
	Microplitis demolitor XP_008551157.1	Microplitis demolitor XP_008546753.1
	Ceratosolen solmsi marchali XP_011495209.1	Ceratosolen solmsi marchali XP_011499554.1
	Nasonia vitripennis XP_001605600.2	Nasonia vitripennis XP_001600763.1
Ants	Acromyrmex echinatior XP_011060196.1	Acromyrmex echinatior EGI57771.1
	Solenopsis invicta XP_011167803.1	Solenopsis invicta XP_011161459.1
Beetles	Tribolium castaneum XP_970235.1	Tribolium castaneum XP_968477.1
	Dendroctonus ponderosae ERL90379.1	
True Bugs	Nilaparvata lugens AIW79974.1	Nilaparvata lugens AIW79976.1
	Laodelphax striatella AFU86441.1	Laodelphax striatella AGI92300.1

Table 2.2 NCBI Reference Sequences of Selected P450 Enzymes. Homologs of selected P450 enzymes from common insect species were obtained through NCBI protein BLAST to perform phylogenetic analysis. Name of species and reference sequence were listed for each enzyme. CYP306A1 was also included as a reference to show the conservation among insect species.

Target 1	gRNA forward	gRNA reverse
cyp49a1	CTTC GTGGAGGCCTTCAGACAGG	AAAC CCTGTCTGAAGGCCTCCCAC
cyp301a1	CTTC GTGCCGAGGATCAGTGGAGC	AAAC GCTCCACTGATCCTCGGCAC
cyp303a1	CTTC GTTGGGCGTCGCTTTTATAG	AAAC CTATAAAAGCGACGCCCAAC
cyp304a1	CTTC GAACCGGGTGGAAAGCCGGA	AAAC TCCGGCTTTCCACCCGGTTC
cyp305a1	CTTC GTATTATAGGTCAAGAGATA	AAAC TATCTCTTGACCTATAATAC
Target 2	gRNA forward	gRNA reverse
cyp49a1	CTTC GCACACGCTGCTGGCCAAGG	AAAC CCTTGGCCAGCAGCGTGTGC
cyp301a1	CTTC GGAGAATCCACATACCGGAC	AAAC GTCCGGTATGTGGATTCTCC
cyp303a1	CTTC GTCATATGGCTTCACAGCAG	AAAC CTGCTGTGAAGCCATATGAC
cyp304a1	CTTC GCTCCGGATCCGACCAAATA	AAAC TATTTGGTCGGATCCGGAGC
cyp305a1	CTTC GTCTGGGTATAGGTCTGGCA	AAAC TGCCAGACCTATACCCAGAC

Table 2.3 Guide RNA sequences for CRISPR/Cas9-mediated mutagenesis of uncharacterized P450s. gRNAs were designed as described by Kondo and Ueda (Kondo and Ueda, 2013) with online cas9 target finder provided on shigen.nig.ac.jp.

	checking primer forward	checking primer reverse
cyp49a1	GGTTTTAGCCACACCATACCCAGG	AACTCTCCGGAATTGTACGAGACC
cyp301a1	GCTAGAAGCCAGTTTCGTTTCCC	GGAGTACGATTGTCCCATTCTGG
cyp303a1	CCATACTACTTTTCGCAACCC	CGATTTAAGCGGAAGATGCAGG
cyp304a1	GTCCGGAATCCACGATGATCACGG	TTGAGGTTCTGGGAGAGATCGGGC
cyp305a1	GATCGCTGCTCGAATTTCTTCGGG	TTTCGAAGTGGTCCAGGACCAGGC

Table 2.4 Primers used for mutant screening of uncharacterized P450s. Primers were selected from upstream or downstream region of target 1 or target 2 gRNAs, respectively.

	qPCR primer forward	qPCR primer reverse
cyp49a1	CGATCCCGCATATTTCCCAGAACC	AACTCTCCGGAATTGTACGAGACC
cyp301a1	GCGTTTTGGAGGTCTTATAGGACGG	CCAGATCCCCAAAGAAATCCTTGC
cyp303a1	CAGTCACTTTCCCCTGCTCC	ATGCAGCTCGATCTCCTTGC
cyp304a1	CCTCAATAATCAGGTGTTCGATGG	TCTTAAGTAGCGCAGGATGAAGC
cyp305a1	CGTCCCAAGAACTTTCCACC	CGCTCGAACAGGATGTGC

Table 2.5 Primers for RT-PCR of uncharacterized P450s. Primers were designed with NCBI primer design tools. Product size of each pair of primers are 80-140 bp and primers are selected from two individual exons across introns.

Fly line	Resources	Fly line	Resources
cyp49a1[4D]/cyo-GFP	BestGene Inc	*Df(cyp301a1)*	BDSC#30585
cyp301a1[6F]/cyo-GFP	BestGene Inc	*Df(cyp49a1)/cyo*	BDSC#23666
cyp303a1[4A]/cyo-GFP	BestGene Inc	*Df(cyp303a1)/cyo-GFP*	BDSC#6915
cyp304a1[4C]/TM6C-GFP	BestGene Inc	*Df(cyp303a1)/cyo*	BDSC#1491
cyp305a1[4B]/TM6C-GFP	BestGene Inc	*Df(cyp304a1)/TM6C*	BDSC#8086
UAS-cyp303a1-3/cyo	BestGene Inc	*Df(cyp305a1)/TM6C*	BDSC#25691

Table 2.6 *Drosophila* lines used for mutant phenotype analysis. Mutants were generated by CRISPR, deficiency lines were obtained from Bloomington *Drosophila* Stock Center. BDSC# = Bloomington Drosophila Stock Center number

CHAPTER III

Cyp303a1 is required for successful transition to adulthood

Introduction

As mentioned in Chapter II, lethality associated with the *cyp303a1* knockout indicated an important role of this gene during *Drosophila* development. Furthermore, *cyp303a1* mutants did not show significant differences in developmental timing during early development. Specific expression of *cyp303a1* on the second day after puparium formation also suggested this enzyme is required during pupal development for successful transition to adults. To reveal molecular mechanisms underlying *cyp303a1* function, a series of assays was performed to determine the tissue and signaling pathways affected by this enzyme.

GAL4/UAS system is used in this study to perform tissue specific silencing and rescue experiments. The GAL4 protein, derived from yeast, serves as the transcriptional activator in this system. The upstream activation sequence (UAS) is an enhancer that is specifically activated by the GAL4 protein. Since there is no endogenous target of GAL4 within *Drosophila*, it only activates transcription of the gene that follows UAS (Duffy JB, 2002). Ease in incorporating the target gene combined with the specificity of GAL4 expression and efficiency of activation makes the system a favorable tool for functional analyses of genes in flies. In addition, this system has become more powerful as the heat-shock GAL4 became available to control timing of activation.

Since the *cyp303a1* null mutant has a unique eclosion defect phenotype, one report about dHNF4 captured our attention. dHNF4 is a close ortholog of human Hepatocyte nuclear factor 4α (HNF4 α), with 89% amino acid identity in the DNA-binding domain and 61% identity in the ligand-binding domain. Mammalian HNF4α was reported to be responsible for Maturity Onset Diabetes of the Young type 1 (MODY1) and regulates glucose transport and glycolysis (Iwasaki N et al., 1997; Navas MA et al., 1999; Gupta RK et al., 2005). One study from Carl Thummel's group suggested that dHNF4 directs lipid utilization and mobilization that are required for adaptation to starvation (Palanker L et al., 2009). In this study, the *dHnf4* knockout caused an eclosion defect very similar to that of *cyp303a1* null mutants. Therefore, a hypothesis was established that the product of CYP303a1 enzyme might be involved in the dHNF4 pathway as a ligand or intermediate in ligand biosynthesis. To test this hypothesis, comparison of *cyp303a1* and *dHnf4* null mutants was performed. Furthermore, *in vivo* receptor activities were analyzed to examine if dHNF4 activities are impaired in *cyp303a1* null mutants.

Materials and Methods

General Fly Genetics

Genotype of Df(*cyp303a1*)/*cyp303a1*[4A] was referred to as null mutants for this study.

Crosses made to produce null mutants were: $\frac{cyp303a1[4A]}{cyo-GFP} \times \frac{Df(cyp303a1)}{cyo-krGFP}$

Around 4 males and 15 females of the described genotype were mixed in vials with standard lab corn meal diet and placed in the dark for egg laying. After 4 hours, adult flies were removed and vials with eggs were incubated at 25°C. Numbers of GPF positive or negative pupa were counted and survival rate from embryonic stage through pupal stage was calculated as follows:

$$\text{Survival rate} = \frac{\text{Actual number of null pupa formed}}{\text{Expected number of null pupa}} = \frac{\text{Number of GFP nevative pupa}}{0.5 \times \text{Number of GFP positive pupa}}$$
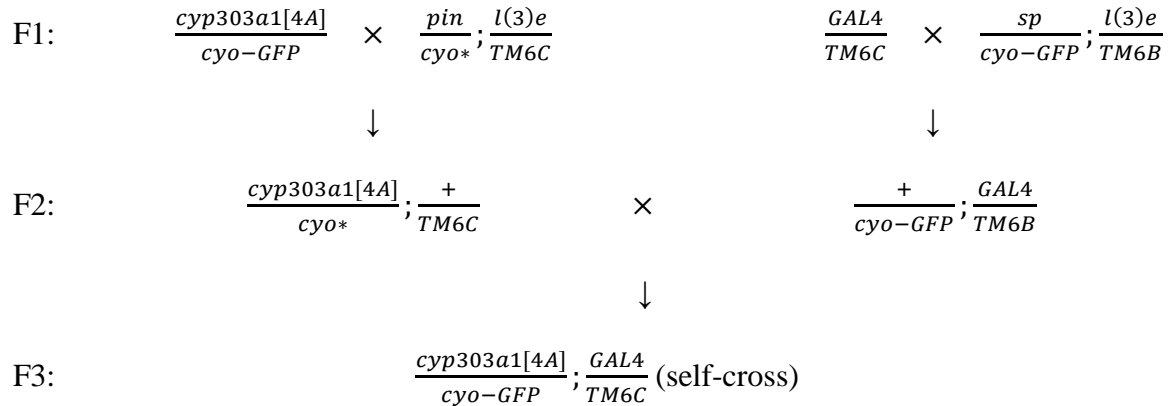
Tissue-Specific Rescue Experiment

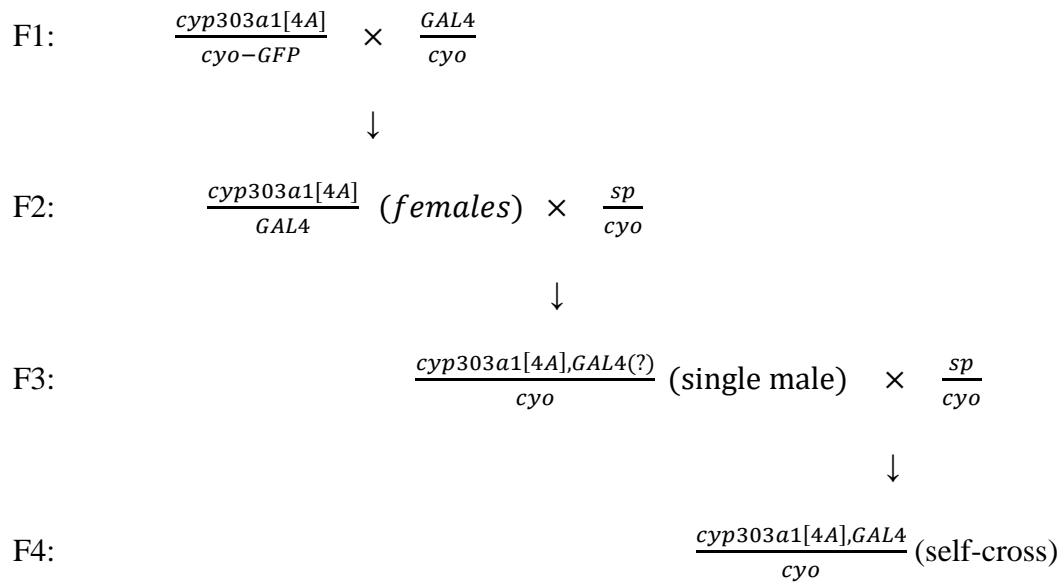Crossing schemes for the tissue-specific rescue experiments were as follows:

Recombination of *cyp303a1[4A]* and *UAS- cyp303a1*(on the third chromosome)

F1: $\frac{cyp303a1[4A]}{cyo-GFP} \times \frac{pin}{cyo*};\frac{l(3)e}{TM6C}$ $\quad$ $\frac{UAS-cyp303a1}{TM6C} \times \frac{sp}{cyo-GFP};\frac{l(3)e}{TM6B}$

$\downarrow$ $\qquad\qquad\qquad\qquad$ $\downarrow$

F2: $\frac{cyp303a1[4A]}{cyo*};\frac{+}{TM6C}$ $\qquad \times \qquad$ $\frac{+}{cyo-GFP};\frac{UAS-cyp303a1}{TM6B}$

$\downarrow$

F3: $\frac{cyp303a1[4A]}{cyo-GFP};\frac{UAS-cyp303a1}{TM6C}$ (self-cross)

Recombination of *cyp303a1[4A]* and *GAL4* drivers on the third chromosome

F1: $\quad \dfrac{cyp303a1[4A]}{cyo-GFP} \quad \times \quad \dfrac{pin}{cyo*};\dfrac{l(3)e}{TM6C}$ $\qquad\qquad \dfrac{GAL4}{TM6C} \quad \times \quad \dfrac{sp}{cyo-GFP};\dfrac{l(3)e}{TM6B}$

$\qquad\qquad\qquad\qquad\quad \downarrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow$

F2: $\quad \dfrac{cyp303a1[4A]}{cyo*};\dfrac{+}{TM6C} \qquad\qquad \times \qquad\qquad \dfrac{+}{cyo-GFP};\dfrac{GAL4}{TM6B}$

$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow$

F3: $\quad \dfrac{cyp303a1[4A]}{cyo-GFP};\dfrac{GAL4}{TM6C}$ (self-cross)

Recombination of *cyp303a1[4A]* and *GAL4* drivers on the second chromosome

F1: $\quad \dfrac{cyp303a1[4A]}{cyo-GFP} \quad \times \quad \dfrac{GAL4}{cyo}$

$\qquad\qquad\qquad \downarrow$

F2: $\quad \dfrac{cyp303a1[4A]}{GAL4} \; (females) \; \times \; \dfrac{sp}{cyo}$

$\qquad\qquad\qquad\qquad\qquad \downarrow$

F3: $\quad \dfrac{cyp303a1[4A],GAL4(?)}{cyo} \; (single\ male) \quad \times \quad \dfrac{sp}{cyo}$

$\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow$

F4: $\qquad\qquad\qquad \dfrac{cyp303a1[4A],GAL4}{cyo}$ (self-cross)

Recombination of *cyp303a1[4A]* and *GAL4* drivers on the second chromosome was confirmed by eye color marker and performing genomic PCR from the F3 single males using following primers:

forward primer: 5'- CCATACTACTTTTCGCAACCC -3'

reverse primer: 5'- CGATTTAAGCGGAAGATGCAGG -3'

Humidity Rescue Experiment

Eggs were transferred to 20% sucrose solution and L1 larva were collected upon hatching. 15 L1 larva were transferred to each vial containing mushy standard lab corn meal diet. Vials were kept at 25°C with 25% or 95% humidity.

Low Sugar Rescue Experiment

Sugar diets were made using 8% yeast, 1% agar, 0.05% $MgSO_4$, 0.05% $CaCl_2$, and either 3%, 9% or 15% dietary sugar (2:1 ratio of glucose to sucrose, percentages represent weight/final food volume. 10 ml/L tegosept and 6 ml/L propionic acid were added just prior to pouring). 7 mL of the sugar diets was aliquoted into regular fly vials for the experiment. L1 larvae were transferred to the vial and raised in 25°C incubators with 25% humidity.

Hnf4 Receptor Activity Assay

hs-HNF4-LBD-GAL4 was recombined with *cyp303a1[4A]* via meiotic recombination. *UAS-nls.GFP* or *UAS-lacZ* was recombined with *cyp303a1[4A]* using double balancer lines. Crosses were established to induce reporter expression specifically when ligand of dHNF4 is expected to present (i.e. the pupal stage) by using heat shock. White pupae were collected and placed in a petri dish with wet filter paper for 60 hours before heat

shock. GAL4 system was activated by heat shock in 37℃ incubator for an hour followed

by recovery in 25℃ incubator for 4 hours. Non heat-shocked controls were kept at 25℃

for the same time. After recovery, RNA was extracted from the whole body to generate

cDNA for qPCR analysis. GFP expression levels were quantified by qPCR to represent

dHNF4 activities by using following primers:

forward primer: 5'- GCTGACCCTGAAGTTCATCTGC -3'

reverse primer: 5'- CTTGTAGTTGCCGTCGTCCTTG -3'

Results

<u>Tissue-specific knockdown and mutant rescue experiments suggested a tissue non-autonomous role of CYP303A1</u>

The best way to identify tissues that express the CYP303A1 enzyme is to analyze the gene expression pattern in different tissues by qPCR. However, *cyp303a1* is only highly expressed at 48 hr and 60 hr after puparium formation, and it is hard to separate distinct tissues from this stage. Therefore, tissue-specific RNAi was carried out to determine if knockdown of *cyp303a1* in one specific tissue leads to the same phenotype as the null mutant. Surprisingly, none of the tissue-specific GAL4 lines tested in this study induced a discernible phenotype, while a ubiquitous GAL4-driven RNAi showed an effect similar to that of the complete knockout (Table 3.1).

Tissue	GAL4	With Dicer2	Without Dicer2
Ubiquitous	*Tubp*	Lethal	Lethal
PG	*phm22*	N	N
Muscle	*mef2*	N	N
Fat body	*CG*	N	N
SG	*FKH*	N	N
CA	*Aug21*	N	N
Oenocyte	*OK72*	N	N
Mid-gut	*Myo-1A*	N	N
Pan-neuron	*nSyb*	N	N
Pan-neuron	*elav*	N	N
Epidermis	*Epi71D*	N	N

Table 3.1. Tissue-specific knockdown phenotypes of *cyp303a1* using multiple GAL4 drivers. N, No phenotype.

On the other hand, tissue-specific mutant rescue experiments were conducted with the same set of GAL4 lines to identify tissues that require CYP303A1 enzyme function. Rescue experiments were conducted by expressing *cyp303a1* in the null mutant background using the GAL4-UAS system. By expressing *cyp303a1* with a ubiquitous GAL4 driver, lethality was fully rescued, as expected (Figure 3.1). Interestingly, expression of *cyp303a1* in all tissues tested led to partial or full rescue of the eclosion defects. Expression of *cyp303a1* in large tissues such as the salivary gland, epidermis, muscle, and fat body fully rescued most of the flies. Flies with *cyp303a1* expression in other tissues were able to eclose, but their wing expansion did not occur properly and some of them could not survive after eclosion.
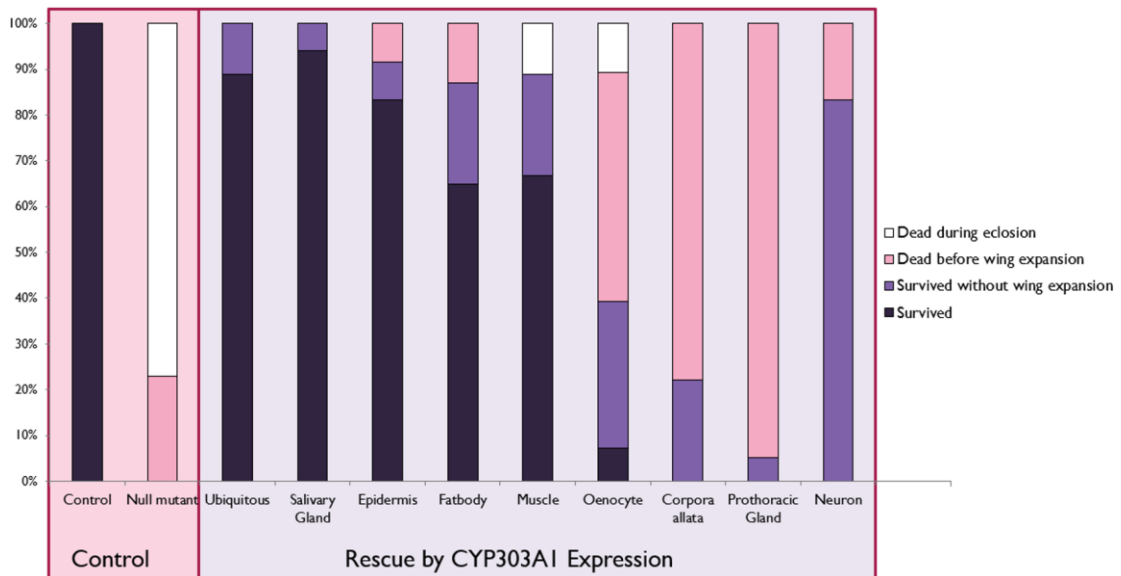
Figure 3.1 Rescue of the knockout phenotype by expression of *cyp303a1* in the homozygote mutant. Control: *cyp303a1[4A]/+; UAS-cyp303a1/+*. Null mutant: *cyp303a1[4A]/Df(cyp303a1)*. Rescue: *GAL4> UAS-cyp303a1* in *cyp303a1[4A]* homozygotes. GAL4 lines used for tissue-specific expression: *TubP-GAL4, FKH-GAL4, Epi71CD-GAL4, CG-GAL4, Mef2-GAL4; OK72D-GAL4; Aug21-GAL4; phm22-GAL4; Elav-GAL4*, respectively. N = 20 ± 2 animals collected from 2 independent vials.

Results from RNAi knockdown and mutant rescue experiments suggest that the function of CYP303A1 is not tissue-autonomous, which is consistent with the hypothesis that its direct or downstream products may be released into circulation to play hormonal roles in other tissues.

Similarities between *cyp303a1* and *dHnf4* null mutants

According to the results of tissue-specific functional assays, *cyp303a1* may have a regulatory role in a hormonal pathway. In our original hypothesis, products of uncharacterized P450 enzymes might function through nuclear receptor as ligands just like 20E produced by Halloween enzymes. This motivated us to look for previous literature describing nuclear receptor null phenotypes. Interestingly, several reports on one of the nuclear receptors in *Drosophila*, dHNF4, described a very similar eclosion defect phenotype (Palanker L et al., 2009). Considering that dHNF4 is an orphan nuclear receptor, I hypothesized that CYP303A1 and dHNF4 are in the same pathway. In order to test this hypothesis, the same set of assays was conducted with *cyp303a1* and *dHnf4* null mutants side by side, including larval starvation, humidity rescue, and low sugar rescue experiments.

As observed in *dHnf4* null mutants (Palanker L et al., 2009), *cyp303a1* null larvae were sensitive to starvation. Late L2s were collected 60 hours after egg laying and placed in water or 20% sucrose solution. The survival rate of both null mutants after 48 hour-starvation was significantly lower compared to controls (Figure 3.2).
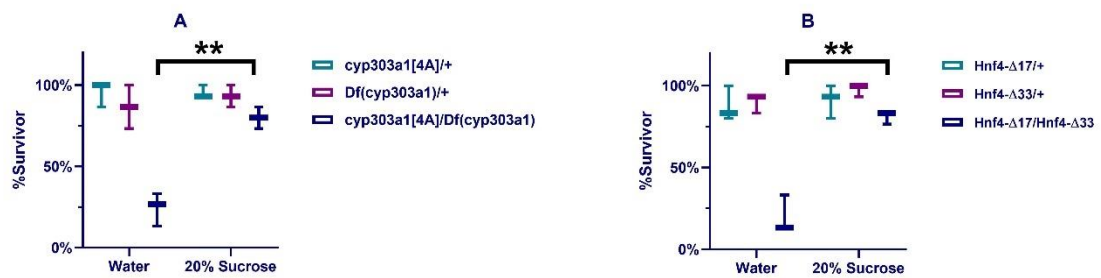
Figure 3.2 Survival rate of larvae in starved (water) or fed (20% sucrose) condition.
(A) *cyp303a1* null mutant and controls. (B) *dHnf4* null mutant and controls. n= 3 vials,
10-15 flies per vial. Error bars = min to max. ANOVA was used to test whether there is
significant difference between groups. ** $P<0.005$.

Another similarity between *dHnf4* and *cyp303a1* knockout flies is that both null mutants

can be rescued by increasing humidity. When humidity was increased from 25% to 95%,

about 40% of *cyp303a1* null mutants and 70% of *dHnf4* null mutants survived after

eclosion (Figure 3.3). However, *cyp303a1* null adults died within 48 hours, while *dHnf4*

null adults lived longer under 95% humidity. When the flies were transferred from 95%

humidity incubator to 25% humidity after eclosion, the majority of both mutants died
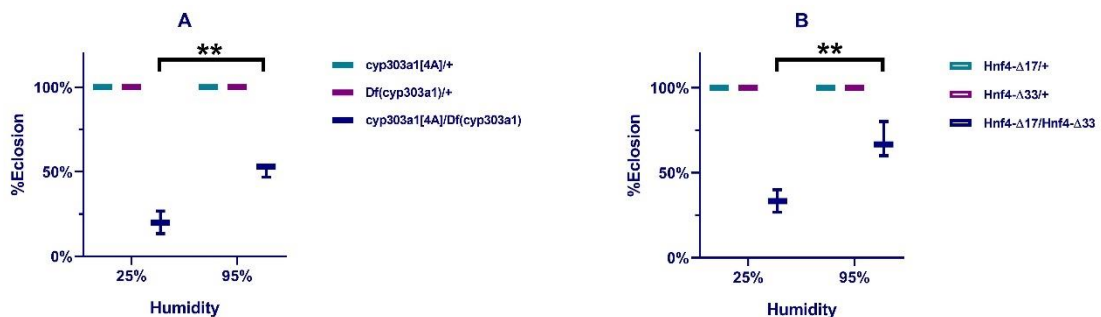
within 24 hours.

**Figure 3.3 Survival rate of flies in normal (25% humidity) or humid (95% humidity)
condition.** (A) *cyp303a1* null mutant and controls. (B) *dHnf4* null mutant and controls.

n= 3 vials, 10-15 flies per vial. Error bars = min to max. ANOVA was used to test whether there is significant difference between groups. ** P<0.005.

Next, based on a previous study (Barry WE and Thummel CS, 2016), rescue experiments with a low sugar diet were performed with both null mutants. Rearing larvae on a low sugar diet rescued the *dHnf4* null mutants, while the *cyp303a1* null mutants did not survive. With 3% dietary sugar, more than 80% *dHnf4* null mutants eclosed and about 40% survived after eclosion. Meanwhile, about 50% of *cyp303a1* null mutants eclosed with 3% dietary sugar and all emerged adults died within 24 hours. Compared to the 20% eclosion in standard lab corn meal diet and 15% sugar food, there was still a significant rescue of the eclosion defects with lower dietary sugar (Figure 3.4). Although none of the *cyp303a1* null mutants was able to survive as *dHnf4* mutants, they share a similar trend in this assay.

Figure 3.4 Rescue of flies with food of lower dietary sugar. (A) *cyp303a1* null mutant (B) *dHnf4* null mutant. Larvae were transferred into food with either 3%, 9% or 15% dietary sugar (glucose: sucrose = 2:1) as L1. Number of survivors in each status was

recorded to calculate percentage towards total larvae added to each vial. n= 3 vials, 10-15 flies per vial. Error bars = standard error.

Comparison of null mutants indicated that *cyp303a1* knockout generally causes a more severe phenotype than the *dHnf4* knockout. These results were consistent with the hypothesis that CYP303A1 is upstream of the dHNF4 nuclear receptor.

Combination of *cyp303a1* and *dHNF4* mutants did not cause additive effects.

Double knockout of *cyp303a1* and *dHnf4* was performed by recombining two mutants via meiotic recombination. There was no significant difference observed between recombined double mutant and *cyp303a1* knockout alone. This result suggested that CYP303A1 and dHNF4 likely belong to the same regulatory pathway.

In vivo analysis of dHNF4 activities in *cyp303a1* mutant background

In order to examine the possibility that CYP303A1 is directly involved in the dHNF4 regulatory pathway, a ligand-binding-GAL4-driven UAS reporter system (Palanker L et al., 2006) was used to monitor the change of dHNF4 activity in the *cyp303a1* mutant background. Without heat shock, GAL4 is not expressed and there is no GFP expression induced. Upon heat shock, a significant reduction of dHNF4 activity was observed in *cyp303a1* null flies in comparison to controls (Figure 3.5). This result is consistent with our hypothesis that CYP303A1 plays a role during the pupal stage through the dHNF4 pathway.
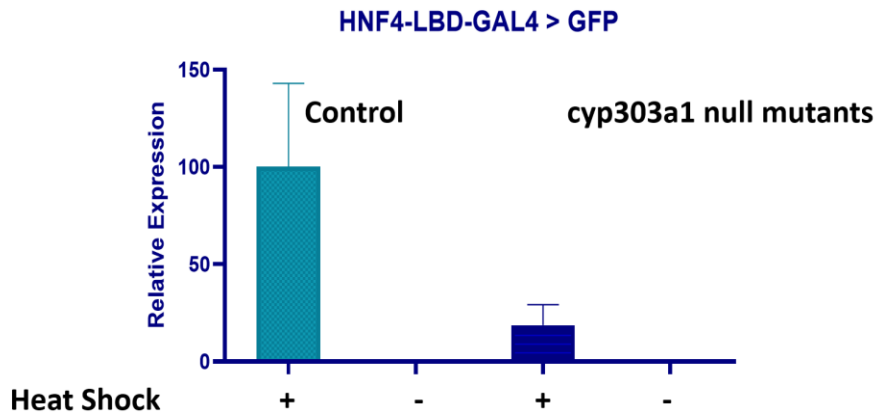
Figure 3.5 Altered dHNF4 activities in *cyp303a1* null mutants. Control (green): hs-HNF4-LBD-GAL4, *cyp303a1*[4A]/+; UAS-nls.GFP/+. Cyp303a1 null mutants (blue): hs-HNF4-LBD-GAL4*, cyp303a1*[4A]/ *cyp303a1*[4A]; UAS-nls.GFP/+. White pupae were collected and kept at 25°C for 60 hours before heat shock. Heat shock (+) group was transferred to 37°C for an hour and recovered at 25°C for 4 hours before RNA extraction. n= 3 samples, 3 pupae were extracted for each sample. Error bars = standard error.

Discussion

CYP303A1 and dHNF4 Pathway

Ubiquitous knockdown of *cyp303a1*, one of the five selected uncharacterized P450

genes, resulted in a specific eclosion defect. This gene is only highly expressed on the

second day after puparium formation, suggesting that it has important functions during

the pupal stage (Figure 2.5C). Meanwhile, knockdown of *cyp303a1* in single tissues did

not result in the same phenotype observed following ubiquitous knockdown or the null

mutant. Interestingly, expression of *cyp303a1* in one of the tissues in the null mutant

background can significantly rescue the eclosion defects (Table 3.1 and Figure 3.1). This

result is consistent with our hypothesis that CYP303A1 has a hormonal role and its

products could be released into circulation and function in other tissues. Furthermore, we

noticed that knockout of the nuclear receptor dHNF4 leads to the same phenotype as that

resulting from the *cyp303a1* null mutant (Palanker L et al., 2009). To examine if

cyp303a1 is involved in the dHNF4 pathway, three assays were performed to compare

the phenotype of these two mutants. In all comparisons, the same trend was observed in

both *cyp303a1* and *dHNF4* knockout flies despite *cyp303a1* null mutants having more

severe defects overall (Figures 3.2-3.4). The higher severity of the *cyp303a1* knockout

phenotype could be explained by incomplete knockout of *dHNF4*. It was reported that

there is still a trace amount of dHNF4 expression in the mutants used for this study

(Storelli G et al., 2019). Alternatively, it is possible that products of CYP303A1 could be

substrates for other P450 enzymes and regulate other processes distinct from those

involved in dHNF4 signaling. Double knockout of *cyp303a1* and *dhnf4* did not lead to higher lethality or a more severe phenotype. No occurrence of additive effects in double knockouts also suggests that these two genes belong to the same signaling pathway. A stronger piece of evidence indicating that *cyp303a1* is required for dHNF4 activities in the pupal stage was obtained by investigation of dHNF4 activities in *cyp303a1* null flies. The activity of dHNF4 was significantly reduced in *cyp303a1* mutant pupae compared to controls (Figure 3.5). Combining all these results together, the uncharacterized P450 enzyme CYP303A1 is essential for adult emergence, and it is likely that the product of this enzyme is involved in activation of the dHNF4 nuclear receptor.

The HNF4α nuclear receptor has been known as an orphan receptor for decades. In more recent studies, acyl-CoAs were reported to be HNF4α ligands that regulate HNF4α activity (Hertz R et al., 1998; Rajas F et al., 2002). However, this reported role of acyl-CoA as a regulatory ligand does not fit well with either the crystal structure of HNF4α or the range of target genes, which are very broad and not limited to genes involved in fatty acid metabolism. Although crystallization of HNF4α revealed that it only forms a homodimer and that its ligand binding domain forms a complex with an endogenous fatty acid ligand *in vivo* (Bogan AA et al., 2000; Wisely GB et al., 2002; Dhe-Paganon S et al., 2002), HNF4α is still considered as a ligand-independent receptor for several reasons. First, the bound fatty acid is not removable, suggesting that it might be an integral structural component (Wisely GB et al., 2002). Moreover, fatty acid binding was observed in both active and inactive form of HNF4α receptor homodimer (Dhe-Paganon

S et al., 2002). A subsequent study showed that the HNF4α homodimer adopts an active conformation upon binding to a coactivator, suggesting that this event is key to its activity rather than fatty acid ligand binding (Duda K et al., 2004). HNF4α binds promoters of more than 1,000 genes in humans, regulating not only lipid metabolism but also carbohydrate metabolism, hormone biosynthesis, urea cycle, and alcohol metabolism (Odom DT et al., 2004; Adamson AW et al., 2006). In *Drosophila*, dHNF4 is expressed moderately throughout all stages of development, while *cyp303a1* is only highly expressed on the second day of the pupal stage. Based on current understanding about dHNF4 functioning mechanisms, the explanation of difference in temporal expression pattern of *cyp303a1* and *dHnf4* could be that at specific stage during development, dHNF4 binds to different coactivators and regulates multiple biological processes. The product of CYP303A1 may be required for coactivator binding and activation of dHNF4 in pupal stage for specific functions. This product might be a ligand that was not identified through crystallization, or a component required for the conformational change of the dHNF4 receptor to allow binding with co-activator peptides.

Biological Processes Potentially Regulated by CYP303A1 Through dHNF4

Previous reports regarding HNF4 in vertebrates and *Drosophila* indicate functional roles in glucose and lipid homeostasis (Stoffel M and Duncan SA, 1997; Gupta RK et al., 2007; Baker KD and Thummel CS, 2007). In this study, CYP303A1 and dHNF4 mutant phenotypes suggest that the product of CYP303A1 expression is required for dHNF4 activation. Based on the fact that eclosion defects could be rescued with increased

humidity, we hypothesize that waterproofing components are missing in the cuticle during pupal development of the mutants. In insects, hydrocarbons synthesized from fatty acids serve as cuticular waterproofing agents to provide desiccation resistance (Gibbs AG,1998). From studies of dHNF4 in newly emerged adults, expression levels of genes encoding enzymes responsible for very long chain fatty acid biosynthesis, including *kar*, *cg30008*, *cg18609*, and *cpr*, were significantly reduced in dHNF4 mutants (Storelli G et al., 2019), consistent with reports that silencing of dHNF4 in oenocytes impairs lipid mobilization (Palanker L et al., 2009). However, these studies were mainly performed in adults or larvae, and functions of dHNF4 during the pupal stage are still unclear. To test the hypothesis, levels and profiles of cuticular hydrocarbons of pharate adults and adults of different ages can be measured and compared to gain better understanding of the mutant phenotype.

Furthermore, although both mutants are sensitive to starvation, *cyp303a1* was not highly expressed during larval stages under normal conditions. This can be explained if *cyp303a1* is starvation inducible and altered dHNF4 activities are required to promote a switch from glucose to lipid metabolism for adaptation to starvation conditions. Expression levels of cyp303a1 need to be tested upon starvation to give insights into upstream regulating factors of the CYP303A1 pathway and pupal development.

Fly line	Resources	Fly line	Resources
cyp303a1[4A]/cyo-GFP	BestGene Inc	Df(cyp303a1)/cyo-GFP	BDSC#6915
cyp304a1[4C]/TM6C-GFP	BestGene Inc	Sp/cyo-GFP; l(3)/TM6B	Lab Stock
cyp305a1[4B]/TM6C-GFP	BestGene Inc	Pin/cyo*; l(3)2/TM6C	Lab Stock
UAS-cyp303a1-3/cyo	BestGene Inc	Sp/cyo-GFP	Lab Stock
UAS-cyp303a1-6/cyo	BestGene Inc	cyp303a1-RNAi	BDSC#51716
UAS-cyp303a1-1/TM6C	BestGene Inc	hs-GAL4-HNF4-LBD	BDSC#28856
UAS-cyp303a1-4/TM6C	BestGene Inc	Hnf4-33/cyo	BDSC#43634
cyp303a1[4A]/cyo-GFP; UAS-cyp303a1-1/TM6C	Recombination	Hnf4-17/cyo	BDSC#44218
cyp303a1[4A]/cyo-GFP; UAS-cyp303a1-4/TM6C	Recombination	UAS-GFP-nls.8	BDSC#4776
cyp303a1[4A]/cyo-GFP; TubP-GAL4/TM6C	Recombination	desat1-GAL4(III)	BDSC#65405
cyp303a1[4A]/cyo-GFP; mef2-GAL4/TM6C	Recombination	desat1-GAL4(II)/cyo	BDSC#65404
cyp303a1[4A]/cyo-GFP; fkh-GAL4/TM6C	Recombination	OK72-GAL4	BDSC#6486
cyp303a1[4A]/cyo-GFP; elav-GAL4/TM6C	Recombination	Aug21-GAL4	Lab Stock
Eip71CD-GAL4, cyp303a1[4A]/cyo-GFP	Recombination	Myo-1A-GAL4	Lab Stock
phm22-GAL4, cyp303a1[4A]/cyo-GFP	Recombination	fkh-GAL4	Lab Stock
CG-GAL4, cyp303a1[4A]/cyo-GFP	Recombination	Lsp2-GAL4	Lab Stock
OK72-GAL4, cyp303a1[4A]/cyo-GFP	Recombination	Eip71CD-GAL4	BDSC#6871
Myo-1A-GAL4, cyp303a1[4A]/cyo-GFP	Recombination	Elav(3A3)>dicer2/TM6C-GFP	Lab Stock
Aug21-GAL4, cyp303a1[4A]/cyo-GFP	Recombination	CG-GAL4(II)	Lab Stock
hs-GAL4-HNF4-LBD, cyp303a1[4A]/cyo-GFP	Recombination		
cyp303a1[4A]/cyo-GFP; UAS-GFP-nls.8/TM6C	Recombination		
cyp303a1[4A]/cyo-GFP; UAS-LacZ/TM6C	Recombination		

Table 3.2 *Drosophila* lines used for tissue specific RNAi, rescue and HNF4-LBD activity assessment. BDSC# = Bloomington Drosophila Stock Center number

CHAPTER IV

Development of an *in Vitro* Substrate Conversion Assay

Introduction

Results of genetic analyses in previous chapters suggest that CYP303A1 may mediate

substrate conversion whose product is involved in dHNF4 activation. In order to further

investigate this possibility, it is critical to identify the substrate and product of

CYP303A1-mediated enzymatic actions. While one possibility is to take a metabolomics

approach to identify metabolites with altered amounts in the *cyp303a1* null mutant, such

an unbiased approach can often be challenging. We therefore decided to take a candidate-

based approach, where potential substrates such as ecdysteroids are applied in

CYP303A1-mediated conversion assays *in vitro*. Reversed-phase high-performance

liquid chromatography (HPLC) with solvent gradient elution is particularly suitable for

analysis of ecdysteroids of widely differing polarities with relatively short retention time.

In this chapter, an *in vitro* conversion system was developed, whereby products of P450

enzymes can be separated from substrates incubated with P450-transfected *Drosophila*

S2 cells. This conversion system facilitates substrate screening for CYP303A1 as well as

the other evolutionarily conserved P450s in the future studies.

Materials and Methods

Transfection of *Drosophila* S2 Cells

Drosophila S2 cells were cultured in Shields and Sang M3 insect medium (SIGMA) supplemented with insect medium supplement (SIGMA) in a 10 cm diameter dish at 25°C. S2 cells were transfected with cDNA encoding *shade* packaged in the S2 expression vector pBRAcPA under control of the actin-5C promoter or with an empty control construct by using DDAB-mediated transfection (Warren JT et al., 2002). Cells were incubated at 25°C for 3 days before the substrate conversion assay.

HPLC Assay

Ecdysteroids were extracted with 30% methanol from collected S2 cells. After centrifugation at 12000 rpm for 30 min at 4°C, the supernatant was diluted in 10% methanol for sample injection and reversed- phase HPLC analysis with a C18 column (HPLC Column Vydac 218TP C18 100x4.6mm 5µm, GRACE). Ecdysteroids were separated using a 20 min 10–90% methanol gradient and detected an an absorbance setting of 242 nm with 1220 Infinity LC system (Aligent).

Results

<u>*In vitro* conversion of ecdysteroids</u>

An *in vitro* conversion system using *Drosophila* S2 cells was designed to test potential

substrates of candidate P450 enzymes. This system was established and optimized for the

reaction mediated by the P450 enzyme SHADE, which is responsible for conversion of

ecdysone to 20E (Petryk A et al., 2003). S2 cells were transfected with *shade* cDNA and

cultured at 25°C. On day 3, ecdysone dissolved in ethanol was added to the culture

medium to serve as a substrate at a final concentration of 600 ug/L, and the cells were

cultured for 4 days before collection. Ecdysteroids were extracted with methanol from

cell culture and fractionated by HPLC. When the sample extracted from control cells

transfected with empty expression vector was analyzed, a peak was observed around 31

min, which corresponds to the retention time of synthetic ecdysone (Figure 4.1). On the

other hand, when the sample extracted from cells transfected with *shade* was analyzed,

two peaks around 28 min and 31 min were detected (Figure 4.1). These peaks correspond

to retention times of synthetic 20E and ecdysone, respectively, confirming that substrate
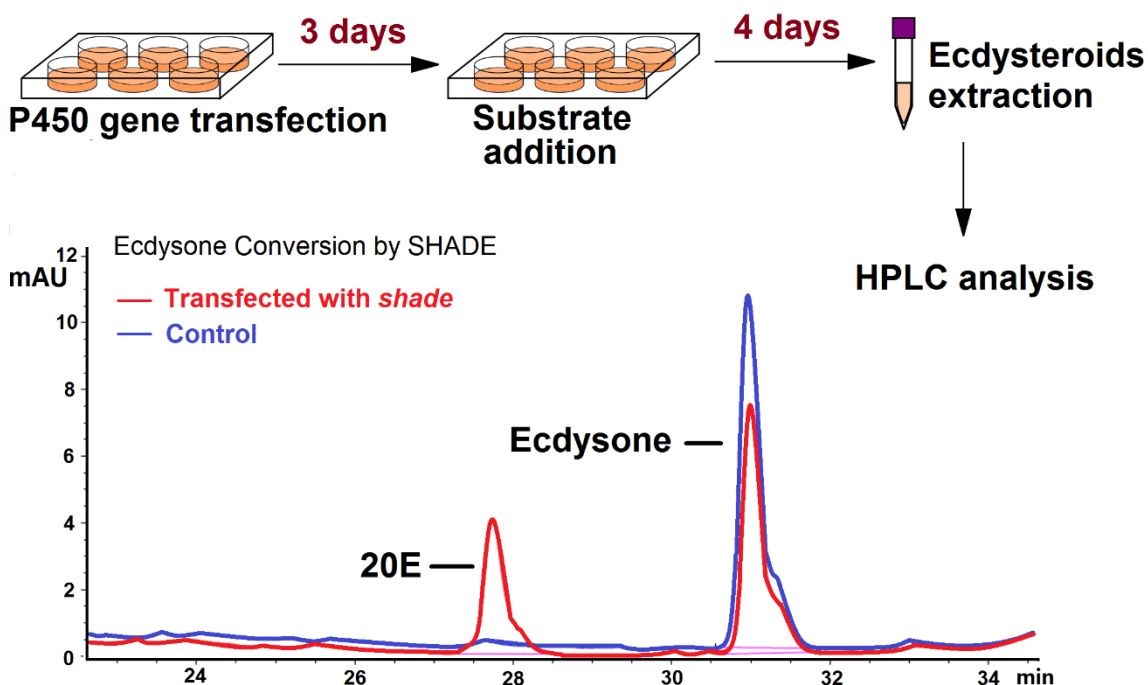
conversion occurred in S2 cells.

Figure 4.1 Experimental procedure and the result of HPLC analysis. Substrate peak was detected at around 31 min in both control (blue) and *shade*-tranfected (red) cell extracts. This peak corresponds to the retention time of synthetic ecdysone. As for the *shade*-transfected cell extract, another peak at around 28 min was detected, which corresponds to the retention time of synthetic 20E.

The incubation time with the substrate was optimized to maximize the rate of conversion for future studies. The conversion rate was calculated as a percentage of the reduced amount of the substrate (ecdysone) compared to control. After incubating with ecdysone for 2 days, the conversion rate reached about 20% and there was no further increase with extended incubation times (Figure 4.2). Since ecdysone requires a membrane transporter named EcI to enter cells (Okamoto N et al., 2018), co-transfection of *EcI* was performed to enhance the conversion. Interestingly, when *EcI* was co-transfected, the conversion rate reached the peak after the first day of incubation. There was no significant difference

between cells transfected with *shade* alone and those transfected with both *EcI* and *shade* after more than one day of incubation (Figure 4.2).
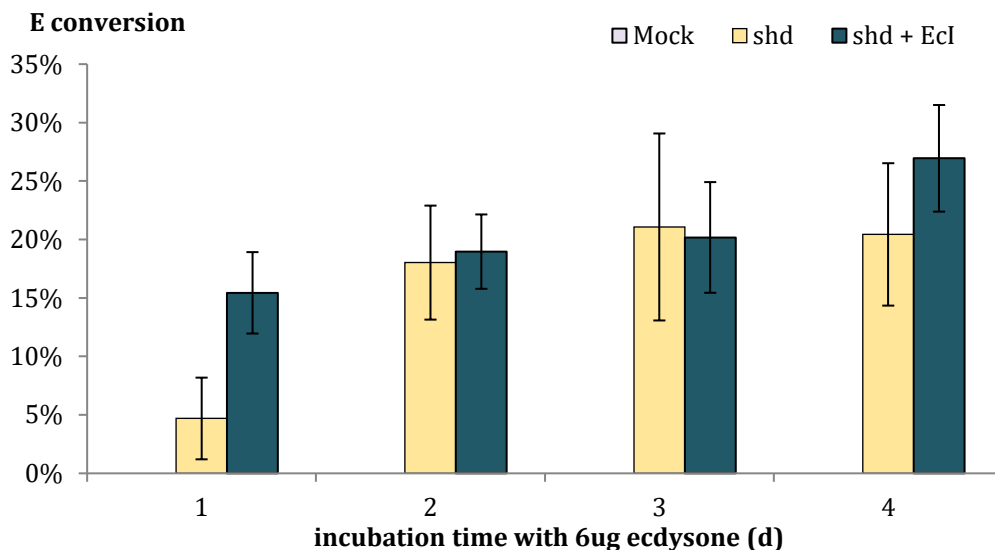

Figure 4.2 Optimization of the conversion rate of the system. S2 cells were transfected with empty pBRAcPA vector, *shade* (*shd*), or *shd* with *EcI*. Substrates were added to the cells 72 hours after transfection, and samples were collected 24, 48, 72, or 96 hours after substrates addition for HPLC analysis. N = 3 samples. Error bars = standard error.

Discussion

In this chapter, an *in vitro* assay system was developed for conversion of ecdysone to 20E using *Drosophila* S2 cells expressing the P450 enzyme SHADE. The substrate conversion rate was around 20-30%. With the current solvent and gradient, the product of SHADE was clearly separated from the substrate. There was no significant increase in conversion rate when the incubation time of substrate was extended after 2 days, indicating that the transfection and conversion were efficient in S2 cells and that the system reaches its highest output within 48 hours.

Using the system established here, we can screen for substrates of uncharacterized P450s in the future. Furthermore, if any product peak is observed, we can optimize the HPLC conditions to obtain better separation of the substrate and product peaks, and collect the eluate to identify the structure of the product. Once the products of the candidate P450s are identified, they can be used for various functional studies; for example, CYP303A1 product can be tested for its potential interaction with dHNF4. It can also be applied to the *cyp303a1* null mutant and tested for its potential ability to rescue the mutant phenotype.

Chapter V

Summary and Discussion

With both traditional and innovated tools available, gene functions can be analyzed at both molecular and organismal levels in *Drosophila*. This thesis work has presented a study of uncharacterized P450 genes in *Drosophila* that may have similar roles as Halloween genes.

CYP303A1 is Required for Successful Transition from Pupa to Adult.

Among the mutants generated in this study, only *cyp303a1* knockout led to lethality. The phenotype of *cyp303a1* null mutants is very similar to *dHNF4* null mutants (Palanker L et al., 2009; Barry WE and Thummel CS, 2016; Storelli G et al., 2019). dHNF4 is a close ortholog of human HNF4α (Hepatocyte nuclear factor 4α), with 89% amino acid identity in the DNA-binding domain and 61% identity in the ligand-binding domain. Mammalian HNF4α was reported to be responsible for MODY1 (Maturity Onset Diabetes of the Young type 1) (Yamagata K et al., 1996; Navas MA et al., 1999). HNF4α is a nuclear receptor with high expression in liver, kidney, intestine, and pancreatic beta-cells in mammals (Duncan SA et al., 1994), where P450 genes are also enriched (Thelen K and Dressman JB, 2009; Renaud HJ et al., 2011). The spatial expression pattern is conserved in flies; dHNF4 is mostly expressed in 4 major tissues: the midgut and attached gastric caeca, fat body, Malpighian tubules, and oenocytes (Palanker et al., 2009). Unlike other orphan nuclear receptors, HNF4α does not heterodimerize with RXR; instead, HNF4α forms only a homodimer. Recent study suggested the ligand binding pocket of HNF4 can bind long chain fatty acids (Bogan AA et al., 2000; Wisely GB et al., 2002; Dhe-Paganon

68

S et al., 2002). Study from Carl Thummel's group suggested that dHNF4 directs fatty acid conversion to very long chain fatty acid and hydrophobic hydrocarbons, which are required for adaptation to dry conditions and dietary sugars (Storelli G et al., 2019)

Lipid stores are consumed rapidly after pupariation during metamorphosis to adulthood in *Drosophila*. A switch in lipid metabolism that supports this transition directed by dHNF4 is required for successful development (Storelli G et al., 2019). Results from our *cyp303a1* study indicate that CYP303A1 is a putative upstream player in the dHNF4 pathway. Knockout of *cyp303a1* caused a similar but more severe phenotype compared to *dHNF4* deletion, suggesting that the product of this enzyme might serve as a ligand or co-factor required for dHNF4 activity. Furthermore, this product may play roles in multiple pathways that are parallel to dHNF4, as the deletion of *cyp303a1* caused almost 100% lethality even under conditions that could rescue the dHNF4 knockout.

After decades of study, the complex of HNF4 bound to endogenous fatty acid was elucidated from the x-ray crystal structure of the HNF4α ligand binding domain (Dhe-Paganon S et al., 2002). However, most of the putative ligands reportedly bound to HNF4α in a non-specific manner or with low affinity (Wisely GB et al., 2002; Dhe-Paganon S et al., 2002; Duda K et al., 2004). Current evidence indicates that HNF4α is not regulated by a ligand but may bind to different fatty acid co-factors to regulate transcription in different tissues or developmental stages (Duda K et al., 2004). These previous studies of the HNF4 ligand binding domain provide valuable information for

substrate candidates of *cyp303a1* for our future study. Fatty acids can also be substrates of cytochrome P450 enzymes. More importantly, a human homolog of *cyp303a1*, *cyp2j2*, shares about 30% similarity to *cyp303a1* and was reported to take an acid as its substrate (Wu S et al., 1996). Loss of CYP2J2 leads to cardiac disease and diabetes (Aliwarga T et al., 2018). *Cyp2j2* is expressed in multiple tissues including heart, liver, kidney, skeletal muscle, lung, brain, pancreas, and gastrointestinal tract (Wu S et al., 1996; King LM et al., 2002; Dutheil F et al., 2009). It localizes to the endoplasmic reticulum and is thought to be a prominent enzyme responsible for metabolizing endogenous polyunsaturated fatty acids to signaling molecules (Wang X et al., 2014). More importantly, CYP2J2 metabolizes arachidonic acid to eicosatrienoic acid epoxides (EETs) (Wu S et al., 1996). Although EETs are considered to play regulatory roles through proliferator-activated receptors or G protein-coupled receptors, there may still be potential networks between these pathways and HNF4 activities yet to be described (Aliwarga T et al., 2018).

To further investigate CYP303A1 functions, we can potentially analyze fatty acid and hydrocarbon profiles of the *cyp303a1* mutant for screening of substrate candidates. Using the S2 cell conversion system developed in Chapter 4, we can test putative substrates and characterize products for any conversion. Using the isolated or synthetic products, HNF4 binding assay and *cyp303a1* mutant rescue experiments can be performed to further analyze the function of CYP303A1 enzyme at the molecular level.

Potential Functions of P450s in Conversion of Various Sterols into Active Hormones

Knockdown of other P450 enzymes examined in this work did not lead to any obvious phenotype. However, it remains possible that they are involved in *Drosophila* development, particularly under extreme conditions. It has been reported that Makisterone A and dh-Makisterone A serve as 20E substitutes in the absence of dietary cholesterol (Lavrynenko O et al., 2015). Makisterone A is produced from stigmasterol, sitosterol, campesterol, while the dh-Makisterone A is synthesized if there is only ergosterol available in the food (Maurer, P et al., 1993; Feldlaufer, MF et al.,1995). The last steps of the production of these 20E substitutes are most likely the same as 20E and the reactions are also carried out by Halloween enzymes (Blais C et al., 2010). However, the enzymes responsible for the conversion of early substrates that modify the backbone structure of the sterols remain unclear. Other uncharacterized genes, *cyp49a1*, *cyp301a1*, *cyp304a1* and *cyp305a1* are putative candidates for these conversions. To test this hypothesis, single sterol feeding experiment can be performed. If the null mutants of these genes are developmentally arrested in the food that only contains stigmasterol, sitosterol, campesterol, or ergosterol, these enzymes may be required to synthesize 20E alternatives when cholesterol is absent in the environment.

Uncharacterized P450s May Function in Altered Environmental Conditions

In most of the experiments we have done so far for study of the uncharacterized P450 enzymes, flies were reared in standard corn meal diet, which is a nutritionally rich condition. In fact, the actual environment is not always ideal and flies should be able to adapt to changing environments in nature. Their metabolism is altered in response to poor conditions and some genes may only have functions during such periods. It is possible that P450 genes that did not show any discernible null mutant phenotype are only required under poor or extreme environments. P450s responsible for detoxification are usually upregulated in response to chemicals causing oxidative stress, but little is known about the alteration in the P450s that are important for metabolism. Transcriptome analysis of *Drosophila* in response to various stress factors have shown that expression patterns of *cyp301a1, cyp303a1, cyp49a1*, and *cyp305a1* are altered under cold-shock or starvation in adults (Moskalev A et al., 2015; Sonn JY et al., 2018). Further studies need to be done with the mutants under altered nutritional conditions or environmental temperatures in order to obtain a better understanding of their functions.

In summary, in the present study, I generated null mutants of five evolutionarily conserved P450 genes in *Drosophila* and identified one that has a clear function in fly development. Functional analyses revealed that it is likely involved in the activation of HNF4, a highly conserved nuclear receptor that has critical roles in development and metabolism. I also developed an *in vitro* assay for analyzing substrates and products of

72

P450 enzymes. The results that uncharacterized P450 gene identified from this study is related to the regulation of nuclear receptor is inspiring to the study of *Drosophila* development. It supported the hypothesis that enzymes sharing highly structural similarities are likely to have similar functions. Revealing P450 functions and the mechanisms of the regulation in development and metabolism will promote the discovery of important molecules in the pathway, which could provide valuable information in insecticide development. Furthermore, since *Drosophila* shares many conserved pathways with human, studies in flies could also inspire the research of similar pathways in humans. For example, better understanding of the HNF4 pathway could yield further insights into diabetic conditions and provide information for development of novel therapies.

REFERENCES

Adams, M. D. (2000). The Genome Sequence of Drosophila melanogaster. *Science*, *287*(5461), 2185–2195. doi: 10.1126/science.287.5461.2185

Adamson, A. W., Suchankova, G., Rufo, C., Nakamura, M. T., Teran-Garcia, M., Clarke, S. D., & Gettys, T. W. (2006). Hepatocyte nuclear factor-4α contributes to carbohydrate-induced transcriptional activation of hepatic fatty acid synthase. *Biochemical Journal*, *399*(2), 285–295. doi: 10.1042/bj20060659

Aliwarga, T., Evangelista, E., Sotoodehnia, N., Lemaitre, R., & Totah, R. (2018). Regulation of CYP2J2 and EET Levels in Cardiac Disease and Diabetes. *International Journal of Molecular Sciences*, *19*(7), 1916. doi: 10.3390/ijms19071916

Astle, J., Kozlova, T., & Thummel, C. S. (2003). Essential roles for the Dhr78 orphan nuclear receptor during molting of the Drosophila tracheal system. *Insect Biochemistry and Molecular Biology*, *33*(12), 1201–1209. doi: 10.1016/j.ibmb.2003.06.011

Baker, K. D., & Thummel, C. S. (2007). Diabetic Larvae and Obese Flies—Emerging Studies of Metabolism in Drosophila. *Cell Metabolism*, *6*(4), 257–266. doi: 10.1016/j.cmet.2007.09.002

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., … Horvath, P. (2007). CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science*, *315*(5819), 1709–1712. doi: 10.1126/science.1138140

Barry, W. E., & Thummel, C. S. (2016). The Drosophila HNF4 nuclear receptor promotes glucose-stimulated insulin secretion and mitochondrial function in adults. *ELife*, *5*. doi: 10.7554/elife.11183

Bernhardt, R. (2006). Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology*, *124*(1), 128–145. doi: 10.1016/j.jbiotec.2006.01.026

Beumer, K. J., Trautman, J. K., Bozas, A., Liu, J.-L., Rutter, J., Gall, J. G., & Carroll, D. (2008). Efficient gene targeting in Drosophila by direct embryo injection with zinc-finger nucleases. *Proceedings of the National Academy of Sciences*, *105*(50), 19821–19826. doi: 10.1073/pnas.0810475105

Beumer, K. J., Trautman, J. K., Mukherjee, K., & Carroll, D. (2013). Donor DNA Utilization During Gene Targeting with Zinc-Finger Nucleases. *G3 &Amp;#58; Genes|Genomes|Genetics*, *3*(4), 657–664. doi: 10.1534/g3.112.005439

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W. A., & Thummel, C. S. (2002). Loss of the Ecdysteroid-Inducible E75A Orphan Nuclear Receptor Uncouples Molting from Metamorphosis in Drosophila. *Developmental Cell*, *3*(2), 209–220. doi: 10.1016/s1534-5807(02)00204-6

Blais, C., Blasco, T., Maria, A., Dauphin-Villemant, C., & Lafont, R. (2010). Characterization of ecdysteroids in Drosophila melanogaster by enzyme immunoassay and nano-liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, *878*(13-14), 925–932. doi: 10.1016/j.jchromb.2010.02.018

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., … Bonas, U. (2009). Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors. *Science*, *326*(5959), 1509–1512. doi: 10.1126/science.1178811

Bogan, A. A., Dallas-Yang, Q., Ruse, M. D., Maeda, Y., Jiang, G., Nepomuceno, L., … Sladek, F. M. (2000). Analysis of protein dimerization and ligand binding of orphan receptor HNF4α. *Journal of Molecular Biology*, *302*(4), 831–851. doi: 10.1006/jmbi.2000.4099

Carroll, D., Beumer, K. J., Morton, J. J., Bozas, A., & Trautman, J. K. (2008). Gene Targeting in Drosophila and Caenorhabditis elegans With Zinc-Finger Nucleases. *Chromosomal Mutagenesis Methods in Molecular Biology*, 63–77. doi: 10.1007/978-1-59745-232-8_5

Chen, W., Zheng, G., Yang, S., Ping, W., Fu, X., Zhang, N., … Wang, J. (2014). CYP2J2 and EETs Protect against Oxidative Stress and Apoptosisin Vivoandin VitroFollowing Lung Ischemia/Reperfusion. *Cellular Physiology and Biochemistry*, *33*(6), 1663–1680. doi: 10.1159/000362950

Choo, Y., & Klug, A. (1994). Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions. *Proceedings of the National Academy of Sciences*, *91*(23), 11168–11172. doi: 10.1073/pnas.91.23.11168

Clark, G. A., & G, D. 12 G. C. A. (2007). Evolution of genes and genomes on the Drosophila phylogeny. *Nature*, *450*(7167), 203–218. doi: 10.1038/nature06341

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., … Zhang, F. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science*, *339*(6121), 819–823. doi: 10.1126/science.1231143

Coon, M. J. (2005). CYTOCHROME P450: Natures Most Versatile Biological Catalyst. *Annual Review of Pharmacology and Toxicology*, *45*(1), 1–25. doi: 10.1146/annurev.pharmtox.45.120403.100030

Dhe-Paganon, S., Duda, K., Iwamoto, M., Chi, Y.-I., & Shoelson, S. E. (2002). Crystal Structure of the HNF4α Ligand Binding Domain in Complex with Endogenous Fatty Acid Ligand. *Journal of Biological Chemistry*, *277*(41), 37973–37976. doi: 10.1074/jbc.c200420200

Diaz, R. J., Harbecke, R., Singer, J. B., Pignoni, F., Janning, W., & Lengyel, J. A. (1996). Graded effect of tailless on posterior gut development: molecular basis of an allelic series of a nuclear receptor gene. *Mechanisms of Development*, *54*(1), 119–130. doi: 10.1016/0925-4773(95)00467-x

Duda, K., Chi, Y.-I., & Shoelson, S. E. (2004). Structural Basis for HNF-4α Activation by Ligand and Coactivator Binding. *Journal of Biological Chemistry*, *279*(22), 23311–23316. doi: 10.1074/jbc.m400864200

Duffy, J. B. (2002). GAL4 system indrosophila: A fly geneticists swiss army knife. *Genesis*, *34*(1-2), 1–15. doi: 10.1002/gene.10150

Dutheil, F., Dauchy, S., Diry, M., Sazdovitch, V., Cloarec, O., Mellottée, L., … Loriot, M.-A. (2009). Xenobiotic-Metabolizing Enzymes and Transporters in the Normal Human Brain: Regional and Cellular Mapping as a Basis for Putative Roles in Cerebral Function. *Drug Metabolism and Disposition*, *37*(7), 1528–1538. doi: 10.1124/dmd.109.027011

Elfaki, I., Mir, R., Almutairi, F. M., & Duhier, F. M. A. (2018). Cytochrome P450: Polymorphisms and Roles in Cancer, Diabetes and Atherosclerosis. *Asian Pacific Journal of Cancer Prevention*, *19*(8), 2057–2070. doi: 10.22034/APJCP.2018.19.8.2057

Evans, R. M., & Mangelsdorf, D. J. (2014). Nuclear Receptors, RXR, and the Big Bang. *Cell*, *157*(1), 255–266. doi: 10.1016/j.cell.2014.03.012

Feldlaufer, M., Weirich, G., Imberski, R., & Svoboda, J. (1995). Ecdysteroid production in Drosophila melanogaster reared on defined diets. *Insect Biochemistry and Molecular Biology*, *25*(6), 709–712. doi: 10.1016/0965-1748(95)00009-k

Feyereisen, R. (2011). Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics*, *1814*(1), 19–28. doi: 10.1016/j.bbapap.2010.06.012

Feyereisen, R. (2012). Insect CYP Genes and P450 Enzymes. *Insect Molecular Biology and Biochemistry*, 236–316. doi: 10.1016/b978-0-12-384747-8.10008-x

Garneau, J. E., Dupuis, M.-È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., … Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves

bacteriophage and plasmid DNA. *Nature*, *468*(7320), 67–71. doi: 10.1038/nature09523

Gibbs, A. G. (1998). Water-Proofing Properties of Cuticular Lipids. *American Zoologist*, *38*(3), 471–482. doi: 10.1093/icb/38.3.471

Gilbert, L. I., Rybczynski, R., & Warren, J. T. (2002). Control and Biochemical Nature of the Ecdysteroidogenic Pathway. *Annual Review of Entomology*, *47*(1), 883–916. doi: 10.1146/annurev.ento.47.091201.145302

Good, R. T., Gramzow, L., Battlay, P., Sztal, T., Batterham, P., & Robin, C. (2014). The Molecular Evolution of Cytochrome P450 Genes within and between Drosophila Species. *Genome Biology and Evolution*, *6*(5), 1118–1134. doi: 10.1093/gbe/evu083

Gradilla, A.-C., Mansilla, A., & Ferrús, A. (2011). Isoform-Specific Regulation of a Steroid Hormone Nuclear Receptor by an E3 Ubiquitin Ligase in Drosophila melanogaster. *Genetics*, *189*(3), 871–883. doi: 10.1534/genetics.111.132191

Guengerich, F. P. (2016). Intersection of the Roles of Cytochrome P450 Enzymes with Xenobiotic and Endogenous Substrates: Relevance to Toxicity and Drug Interactions. *Chemical Research in Toxicology*, *30*(1), 2–12. doi: 10.1021/acs.chemrestox.6b00226

Gupta, R. K., Vatamaniuk, M. Z., Lee, C. S., Flaschen, R. C., Fulmer, J. T., Matschinsky, F. M., … Kaestner, K. H. (2005). The MODY1 gene HNF-4α regulates selected genes involved in insulin secretion. *Journal of Clinical Investigation*, *115*(4), 1006–1015. doi: 10.1172/jci22365

Iwasaki, N., Oda, N., Ogata, M., Hara, M., Hinokio, Y., Oda, Y., … Bell, G. I. (1997). Mutations in the hepatocyte nuclear factor-1alpha/MODY3 gene in Japanese subjects with early- and late-onset NIDDM. *Diabetes*, *46*(9), 1504–1508. doi: 10.2337/diabetes.46.9.1504

Jamieson, K. L., Endo, T., Darwesh, A. M., Samokhvalov, V., & Seubert, J. M. (2017). Cytochrome P450-derived eicosanoids and heart function. *Pharmacology & Therapeutics*, *179*, 47–83. doi: 10.1016/j.pharmthera.2017.05.005

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, *337*(6096), 816–821. doi: 10.1126/science.1225829

Kahsai, L., & Cook, K. R. (2017). Mapping Second Chromosome Mutations to Defined Genomic Regions in Drosophila melanogaster. *G3(Bethesda) Genes|Genomes|Genetics*, *8*(1), 9–16. doi: 10.1534/g3.117.300289

King, L. M., Ma, J., Srettabunjong, S., Graves, J., Bradbury, J. A., Li, L., … Zeldin, D. C. (2002). Cloning of CYP2J2 Gene and Identification of Functional Polymorphisms. *Molecular Pharmacology*, *61*(4), 840–852. doi: 10.1124/mol.61.4.840

King-Jones, K., & Thummel, C. S. (2005). Nuclear receptors — a perspective from Drosophila. *Nature Reviews Genetics*, *6*(4), 311–323. doi: 10.1038/nrg1581

King-Jones, K., Charles, J.-P., Lam, G., & Thummel, C. S. (2005). The Ecdysone-Induced DHR4 Orphan Nuclear Receptor Coordinates Growth and Maturation in Drosophila. *Cell*, *121*(5), 773–784. doi: 10.1016/j.cell.2005.03.030

Kondo, S., & Ueda, R. (2013). Highly Improved Gene Targeting by Germline-Specific Cas9 Expression in Drosophila. *Genetics*, *195*(3), 715–721. doi: 10.1534/genetics.113.156737

Kozlova, T. (2003). Essential Roles for Ecdysone Signaling During Drosophila Mid-Embryonic Development. *Science*, *301*(5641), 1911–1914. doi: 10.1126/science.1087419

Kozlova, T., & Thummel, C. S. (2000). Steroid Regulation of Postembryonic Development and Reproduction in Drosophila. *Trends in Endocrinology & Metabolism*, *11*(7), 276–280. doi: 10.1016/s1043-2760(00)00282-4

Lam, G., Hall, B. L., Bender, M., & Thummel, C. S. (1999). DHR3 Is Required for the Prepupal–Pupal Transition and Differentiation of Adult Structures during Drosophila Metamorphosis. *Developmental Biology*, *212*(1), 204–216. doi: 10.1006/dbio.1999.9343

Lavrynenko, O., Rodenfels, J., Carvalho, M., Dye, N. A., Lafont, R., Eaton, S., & Shevchenko, A. (2015). The ecdysteroidome of Drosophila: influence of diet and development. *Development*, *142*(21), 3758–3768. doi: 10.1242/dev.124982

Li, T., Huang, S., Jiang, W. Z., Wright, D., Spalding, M. H., Weeks, D. P., & Yang, B. (2010). TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. *Nucleic Acids Research*, *39*(1), 359–372. doi: 10.1093/nar/gkq704

Liu, J., Li, C., Yu, Z., Huang, P., Wu, H., Wei, C., … Jiao, R. (2012). Efficient and Specific Modifications of the Drosophila Genome by Means of an Easy TALEN Strategy. *Journal of Genetics and Genomics*, *39*(5), 209–215. doi: 10.1016/j.jgg.2012.04.003

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., Dicarlo, J. E., … Church, G. M. (2013). RNA-Guided Human Genome Engineering via Cas9. *Science*, *339*(6121), 823–826. doi: 10.1126/science.1232033

Martucci, C. P., & Fishman, J. (1993). P450 enzymes of estrogen metabolism. *Pharmacology & Therapeutics*, *57*(2-3), 237–257. doi: 10.1016/0163-7258(93)90057-k

Maurer, P., Girault, J.-P., Larchevêque, M., & Lafont, R. (1993). 24-Epi-makisterone a (not makisterone A) is the major ecdysteroid in the leaf-cutting antAcromyrmex octospinosus(reich) (hymenoptera, formicidae: Attini). *Archives of Insect Biochemistry and Physiology*, *23*(1), 29–35. doi: 10.1002/arch.940230105

Mazaira, G. I., Zgajnar, N. R., Lotufo, C. M., Daneri-Becerra, C., Sivils, J. C., Soto, O. B., … Galigniana, M. D. (2018). The Nuclear Receptor Field: A Historical Overview and Future Challenges. *Nuclear Receptor Research*, *5*. doi: 10.11131/2018/101320

Miller, A. C., Seymour, H., King, C., & Herman, T. G. (2008). Loss of seven-up from Drosophila R1/R6 photoreceptors reveals a stochastic fate choice that is normally biased by Notch. *Development*, *135*(4), 707–715. doi: 10.1242/dev.016386

Miller, W. L. (2017). Steroidogenesis: Unanswered Questions. *Trends in Endocrinology & Metabolism*, *28*(11), 771–793. doi: 10.1016/j.tem.2017.09.002

Morgan, T. H. (1910). Sex Limited Inheritance In Drosophila. *Science*, *32*(812), 120–122. doi: 10.1126/science.32.812.120

Moskalev, A., Zhikrivetskaya, S., Krasnov, G., Shaposhnikov, M., Proshkina, E., Borisoglebsky, D., … Kudryavtseva, A. (2015). A comparison of the transcriptome of Drosophila melanogaster in response to entomopathogenic fungus, ionizing radiation, starvation and cold shock. *BMC Genomics*, *16*(S13). doi: 10.1186/1471-2164-16-s13-s8

Mummery-Widmer, J. L., Yamazaki, M., Stoeger, T., Novatchkova, M., Bhalerao, S., Chen, D., … Knoblich, J. A. (2009). Genome-wide analysis of Notch signalling in Drosophila by transgenic RNAi. *Nature*, *458*(7241), 987–992. doi: 10.1038/nature07936

Navas, M. A., Munoz-Elias, E. J., Kim, J., Shih, D., & Stoffel, M. (1999). Functional characterization of the MODY1 gene mutations HNF4(R127W), HNF4(V255M), and HNF4(E276Q). *Diabetes*, *48*(7), 1459–1465. doi: 10.2337/diabetes.48.7.1459

Nelson, D. R. (2018). Cytochrome P450 diversity in the tree of life. *Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics*, *1866*(1), 141–154. doi: 10.1016/j.bbapap.2017.05.003

Neumüller, R. A., Richter, C., Fischer, A., Novatchkova, M., Neumüller, K. G., & Knoblich, J. A. (2011). Genome-Wide Analysis of Self-Renewal in Drosophila Neural Stem Cells by Transgenic RNAi. *Cell Stem Cell*, *8*(5), 580–593. doi: 10.1016/j.stem.2011.02.022

Niwa, R., & Niwa, Y. S. (2014). Enzymes for ecdysteroid biosynthesis: their biological functions in insects and beyond. *Bioscience, Biotechnology, and Biochemistry*, *78*(8), 1283–1292. doi: 10.1080/09168451.2014.942250

Odom, D. T. (2004). Control of Pancreas and Liver Gene Expression by HNF Transcription Factors. *Science*, *303*(5662), 1378–1381. doi: 10.1126/science.1089769

Omura, T., & Sato, R. (1962). A New Cytochrome in Liver Microsomes. *Journal of Biological Chemistry*, *237*, 1375–1376.

Palanker, L., Tennessen, J. M., Lam, G., & Thummel, C. S. (2009). Drosophila HNF4 Regulates Lipid Mobilization and β-Oxidation. *Cell Metabolism*, *9*(3), 228–239. doi: 10.1016/j.cmet.2009.01.009

Petrov, D. A., & Hartl, D. L. (1998). High rate of DNA loss in the Drosophila melanogaster and Drosophila virilis species groups. *Molecular Biology and Evolution*, *15*(3), 293–302. doi: 10.1093/oxfordjournals.molbev.a025926

Petryk, A., Warren, J. T., Marques, G., Jarcho, M. P., Gilbert, L. I., Kahler, J., … Oconnor, M. B. (2003). Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proceedings of the National Academy of Sciences*, *100*(24), 13773–13778. doi: 10.1073/pnas.2336088100

Pitman, J. L., Tsai, C.-C., Edeen, P. T., Finley, K. D., Evans, R. M., & Mckeown, M. (2002). DSF Nuclear Receptor Acts As a Repressor in Culture and in Vivo. *Developmental Biology*, *245*(2), 315–328. doi: 10.1006/dbio.2002.0648

Renaud, H. J., Cui, J. Y., Khan, M., & Klaassen, C. D. (2011). Tissue Distribution and Gender-Divergent Expression of 78 Cytochrome P450 mRNAs in Mice. *Toxicological Sciences*, *124*(2), 261–277. doi: 10.1093/toxsci/kfr240

Rendic, S., & Guengerich, F. P. (2014). Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Xenobiotic and Natural Chemicals. *Chemical Research in Toxicology*, *28*(1), 38–42. doi: 10.1021/tx500444e

Reyon, D., Tsai, S. Q., Khayter, C., Foden, J. A., Sander, J. D., & Joung, J. K. (2012). FLASH assembly of TALENs for high-throughput genome editing. *Nature Biotechnology*, *30*(5), 460–465. doi: 10.1038/nbt.2170

Riddiford, L. M., & Truman, J. W. (1993). Hormone Receptors and the Regulation of Insect Metamorphosis. *American Zoologist*, *33*(3), 340–347. doi: 10.1093/icb/33.3.340

Robin, G. C. D. Q., Russell, R. J., Cutler, D. J., & Oakeshott, J. G. (2000). The Evolution of an α-Esterase Pseudogene Inactivated in the Drosophila melanogaster Lineage. *Molecular Biology and Evolution*, *17*(4), 563–575. doi: 10.1093/oxfordjournals.molbev.a026336

Rong, Y. S., & Golic, K. G. (2000). Gene Targeting by Homologous Recombination in Drosophila. *Science*, *288*(5473), 2013–2018. doi: 10.1126/science.288.5473.2013

Ryder, E. (2003). Transposable elements as tools for genomics and genetics in Drosophila. *Briefings in Functional Genomics and Proteomics*, *2*(1), 57–71. doi: 10.1093/bfgp/2.1.57

Ryder, E., & Russell, S. (2003). Transposable elements as tools for genomics and genetics in Drosophila. *Briefings in Functional Genomics and Proteomics*, *2*(1), 57–71. doi: 10.1093/bfgp/2.1.57

Schuler, M. A. (2011). P450s in plant–insect interactions. *Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics*, *1814*(1), 36–45. doi: 10.1016/j.bbapap.2010.09.012

Sever, R. K., & Glass, C. K. (2013). Signaling by Nuclear Receptors. *Cold Spring Harbor Perspectives in Biology*, *5*(3), a016709. doi: 10.1101/cshperspect.a016709

Sonn, J. Y., Lee, J., Sung, M. K., Ri, H., Choi, J. K., Lim, C., & Choe, J. (2018). Serine metabolism in the brain regulates starvation-induced sleep suppression in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *115*(27), 7129–7134. doi: 10.1073/pnas.1719033115

Stoffel, M., & Duncan, S. A. (1997). The maturity-onset diabetes of the young (MODY1) transcription factor HNF4 regulates expression of genes required for glucose transport and metabolism. *Proceedings of the National Academy of Sciences*, *94*(24), 13209–13214. doi: 10.1073/pnas.94.24.13209

Storelli, G., Nam, H.-J., Simcox, J., Villanueva, C. J., & Thummel, C. S. (2019). Drosophila HNF4 Directs a Switch in Lipid Metabolism that Supports the Transition to Adulthood. *Developmental Cell*, *48*(2). doi: 10.1016/j.devcel.2018.11.030

Tennessen, J. M., Baker, K. D., Lam, G., Evans, J., & Thummel, C. S. (2011). The Drosophila Estrogen-Related Receptor Directs a Metabolic Switch that Supports Developmental Growth. *Cell Metabolism*, *13*(2), 139–148. doi: 10.1016/j.cmet.2011.01.005

Thelen, K., & Dressman, J. B. (2009). Cytochrome P450-mediated metabolism in the human gut wall. *Journal of Pharmacy and Pharmacology*, *61*(5), 541–558. doi: 10.1211/jpp.61.05.0002

Thomas, J. H. (2005). Rapid Birth-Death Evolution Specific to Xenobiotic Cytochrome P450 Genes in Vertebrates. *PLoS Genetics*, *preprint*(2007). doi: 10.1371/journal.pgen.0030067.eor

Williams, J. A. (2004). Drug-Drug Interactions For Udp-Glucuronosyltransferase Substrates: A Pharmacokinetic Explanation For Typically Observed Low Exposure (Auci/auc) Ratios. *Drug Metabolism and Disposition*, *32*(11), 1201–1208. doi: 10.1124/dmd.104.000794

Wisely, B., Miller, A., Davis, R., Spitzer, T., Shearer, B., Moore, J., … Williams, S. (2002). Hepatocyte Nuclear Factor 4 is a Transcription Factor that Constitutively Binds Fatty Acids. doi: 10.2210/pdb1lv2/pdb

Wu, S., Moomaw, C. R., Tomer, K. B., Falck, J. R., & Zeldin, D. C. (1996). Molecular Cloning and Expression of CYP2J2, a Human Cytochrome P450 Arachidonic Acid Epoxygenase Highly Expressed in Heart. *Journal of Biological Chemistry*, *271*(7), 3460–3468. doi: 10.1074/jbc.271.7.3460

Yamada, M.-aki, Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., & Ueda, H. (2000). Temporally restricted expression of transcription factor βFTZ-F1: significance for embryogenesis, molting and metamorphosis in Drosophila melanogaster. *Development*, *127*(23), 5083–5092.

Yao, T.-P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J.-D., Mckeown, M., … Evans, R. M. (1993). Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature*, *366*(6454), 476–479. doi: 10.1038/366476a0