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Comparative Modeling and Functional Characterization of Two Enzymes of the Cyclooxygenase Pathway in Drosophila melanogaster

Yan Qi
Graduate Center, City University of New York

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Yan Qi

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Date	**Chair of Examining Committee** **Dr. Shaneen Singh, Brooklyn College**
Date	**Executive Officer** **Dr. Laurel A. Eckhardt**
	Dr. Peter Lipke, Brooklyn College
	Dr. Shubha Govind, City College
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The City University of New York

Abstract

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Yan Qi

Mentor: Shaneen Singh

Eicosanoids are biologically active molecules oxygenated from twenty carbon polyunsaturated fatty acids. Natural eicosanoids exert potent biological effects in humans, and a great deal of pharmaceutical research has led to the discovery of compounds for selective inhibition of specific enzymes in eicosanoid biosynthesis. Coupled with different receptors, eicosanoids mediate various physiological and pathophysiological processes, including fever generation, pain response, vasoconstriction, vasodilation, platelet aggregation, platelet declumping, body temperature maintenance and sleep-wake cycle regulation. In mammals, the eicosanoid biosynthesis has three pathways: the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway and the epoxygenase pathway. The COX pathway synthesizes prostanoids, which are important signaling molecules in inflammation. Because of their central role in inflammatory disease and human health, COX enzymes continue to be a focus of intense research as new details emerge about their mechanism of action and their interactions with NSAIDs.

To date, the majority of studies dealing with the COX pathway are centered on mammalian systems. Although the literature is rich in speculations that prostaglandins are central signaling molecules for mediating and coordinating insect cellular immunity,

genes responsible for encoding COX or COX-like enzymes and other enzymes in the COX pathway have not been reported in insects. The value of *Drosophila melanogaster* as a model organism is well established, and the fundamental regulatory signaling mechanisms that regulate immunity at the cellular level in human and flies are conserved.

Given the importance of eicosanoids in mammalian and insect immunity, this study was designed to identify and characterize the enzymes that mediate eicosanoid biosynthesis in *D. melanogaster* computationally. After a preliminary extensive search for putative *D. melanogaster* homologues for all enzymes in the COX pathway, we conducted a systematic, comprehensive, and detailed computational investigation for two enzymes, COX and prostaglandin E synthase (PGES) in an endeavor to model and characterize the possible candidates and identify those that possess all the requisite sequence and structural motifs to qualify as valid COX(s)/PGE synthase proteins. In this study, we report the presence of qualified *D. melanogaster* COX(s)/PGE synthase proteins, characterize their biophysical properties, and compare them with their mammalian counterparts. This study lays the groundwork for further exploration of these proteins and establishing their role in *D. melanogaster* inflammation and immunity, opening up avenues for addressing the use of this model organism in COX signaling and its crosstalk with other signaling pathways.

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List of Abbreviations

AA	Arachidonic acid
alpha LeA	Alpha-linoleic acid
BLAST	Basic Local Alignment Search Tool
CBR	Corbonyl reductase
COX	Cyclooxygenase
cPGES	Cytosolic prostaglandin E synthase
$cPLA_2$	Cytosolic phospholipase A_2
CYP	Cytochrome
CYP450	Cytochrome P450
DHET	Diol dihydrooxygeicosatrienoic acid
Duox	Dual oxidase
EET	Epoxyeicosatrienoic acid
EFOXs	Exo-derivatives
ExPaSy	Expert Protein Analysis System
GSH	Glutathione
GST	Glutathione S-transferase
HETE	Hydroxyeicosatetraenoic acid
HMMs	Hidden Markov models
HPETE	Hydroperoxyeicosatetraenoic acid
Irc	Immune regulated catalase
LA	Linoleic acid
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LTCS	Leukotriene C_4 synthase
MAPEG	Membrane-associated proteins involved in eicosanoid and glutathione metabolism
MBD	Membrane-binding domain
Mgsl	Microsomal glutathione S-transferase
mPGES	Microsomal prostaglandin E synthase
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
PDB	Protein Data Bank
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFS	Prostaglandin F synthase

PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H synthase
PGI_2	Prostacyclin/prostaglandin I_2
PGIS	Prostacyclin synthase
POX	Peroxidase
PRS-BLAST	Reverse position-specific Basic Local Alignment Search Tool
PSI-BLAST	Position-Specific Iterated Basic Local Alignment Search Tool
PSSMs	Pre-calculated prosition-specific scoring matrices
PUFA	Polyunsaturated fatty acid
Pxn	Proxidasin
Pxt	Peroxinectin-like
RMSD	Root Mean Square Deviation
SIB	Swiss Institute of Bioinformatics
SMART	Simple modular architecture research tool
Su(2)P	Suppressor of ref(2)P sterility
TMPD	Tetramethyl-*p*-phenylenediamine
TX	Thromboxane
TxA_2	Thromboxane A_2

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Chapter 1 Introduction

The eicosanoid biosynthetic pathway

Eicosanoids are biologically active, oxygenated metabolites of twenty carbon polyunsaturated fatty acids that contain three to five *cis*, methylene-interrupted double bonds and play key roles in various physiological processes such as reproduction, immunity and inflammation, ion transport and various other signaling pathways (Buczynski, Dumlao, and Dennis 2009). Coupled with different receptors, eicosanoids mediate various biological processes, some examples of which are vasoconstriction, vasodilation, platelet aggregation, platelet declumping, bone resorption, fever generation and maturation of oocytes for ovulation (David W. Stanley-Samuelson 1987; Colin D. Funk 2001). In mammals, the C20 fatty acid substrates from which eicosanoids are derived include members from omega-3, omega-6 and omega-9 family of essential polyunsaturated fatty acids (William L Smith 1989; Lands and Samuelsson 1968; Needleman *et al.* 1979).

Mammalian eicosanoid biosynthetic pathway has three branches: (1) the cyclooxygenase (COX) pathway, which synthesizes prostanoids (prostaglandins (PGs) and thromboxanes (TXs)); (2) the lipoxygenase (LOX) pathway, which produces leukotrienes and mono-, di- and tri-hydroxy acids; and (3) cytochrome P-450 epoxygenase pathway, which generates epoxides (Buczynski, Dumlao, and Dennis 2009) (figure 1).

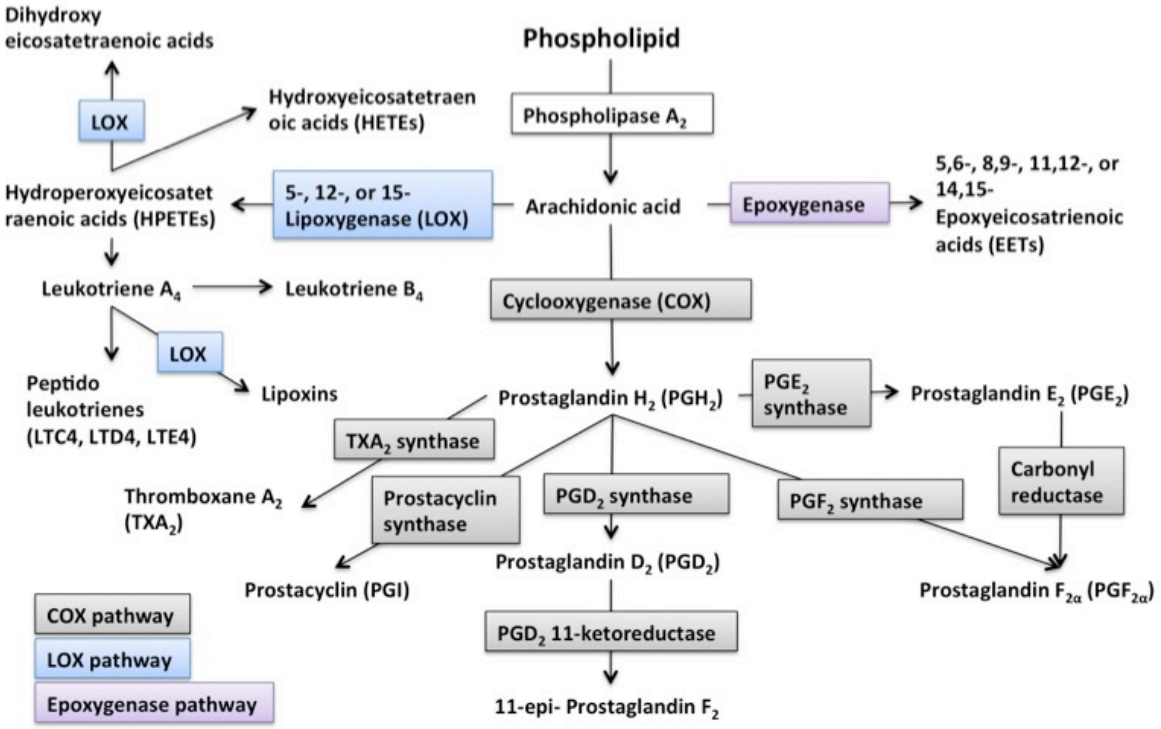


Figure 1. Eicosanoid biosynthetic pathway in mammals. Enzymes of COX pathway, LOX pathway and epoxygenase pathway are colored in grey, blue and violet, respectively.

COX Pathway

The products synthesized by the COX pathway are called prostanoids, which are important signaling molecules in inflammation. The COX pathway enzymes synthesize two types of prostanoids, PGs and TXs, both of which are short-lived signaling lipid molecules (Colin D. Funk 2001). The most common products from the COX pathway are the 2-series compounds, such as prostaglandin H_2 (PGH_2) and thromboxane-A_2 (TxA_2). The "2" indicates the number of carbon-carbon double bonds in the compounds (William L Smith, Urade, and Jakobsson 2011). PGH_2 is synthesized from arachidonic acid by the enzyme COX (also known as PGH_2 synthase; EC 1.14.99.1) in the first committed step of the pathway (W L Smith and Murphy 2002). Biologically active prostanoids, which include PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2, are biosynthesized from PGH_2 in a cell type-dependent manner through enzymatic reactions (W L Smith 1989). Even though formation of monohydroxy fatty acids has been observed during reactions catalyzed by prostaglandin H synthase, they do not seem to be of physiological significance (Serhan, Chiang, and Van Dyke 2008; Sharma *et al.* 2010; Groeger *et al.* 2010). The mechanism of prostanoid action is well established in various mammalian cell types and primarily involves signaling via G-protein-coupled receptors (Negishi, Sugimoto, and Ichikawa 1995; Gerlo *et al.* 2004).

Prostaglandin E_2 (PGE_2)

PGE_2, also called dinoprostone, is the best-studied prostanoid. Since PGE_2 was initially isolated from human seminal plasma in 1963 (Samuelsson 1963), more than 32,000 PGE_2 related articles have been indexed in PubMed till October 2013. PGE_2 is expressed in many different cells, including fibroblasts, macrophages and a series of malignant cells (Harris *et al.* 2002, 2). PGE_2 is involved in numerous physiological and pathophysiological processes, including sleep-wake cycle, maintenance of body temperature (Legler *et al.* 2010), cancer, arthritis, pain response (Harris *et al.* 2002; Colin D. Funk 2001), bronchodilation (Park, Pillinger, and Abramson 2006; Simmons, Botting, and Hla 2004), inhibition of apoptosis in tumor cells, and alteration of malignant tumor cells' morphology (Sumitani *et al.* 2001; Gately 2000; Jakobsson *et al.* 1999). The conversion of PGH_2 into PGE_2 is carried out by prostaglandin E synthase, which includes three structurally distinct prostaglandin E synthases (PGES; EC 5.3.99.3): microsomal prostaglandin E synthase-1, microsomal prostaglandin E synthase-2 and cytosolic prostaglandin E synthase.

Prostaglandin D_2 (PGD_2)

PGD_2 is a structural isomer of PGE_2. PGE_2 has a 9-keto and 11-hydroxy moiety, while PGD_2 has a 9-hydroxy and 11-keto moiety (Buczynski, Dumlao, and Dennis 2009). PGD_2 is the most abundant PG in the central nervous system in mammals (Narumiya *et al.* 1982), and is involved in sleep regulation, pain response, and hypothermia (Hayaishi *et al.* 2004; Hayaishi 1991). The biosynthesis of PGD_2 from PGH_2 is catalyzed by two structurally divergent prostaglandin D_2 synthases, hematopoietic prostaglandin D_2 synthase (HPGDS; EC 5.3.99.2) and prostaglandin D_2 synthase (PGDS; EC 5.3.99.2), that are differentially expressed in different organs (William L Smith, Urade, and Jakobsson 2011).

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

$PGF_{2\alpha}$ was also first isolated from human seminal fluid (Samuelsson 1963), and affects multiple biological processes, including embryo development, vasoconstriction and acute inflammation (Basu 2007). $PGF_{2\alpha}$ can be synthesized from different substrates: (a) from PGE_2 by two structurally diverse carbonyl reductases (CBR1 and CBR2; EC 1.1.1.184, EC 1.1.1.189), (b) from PGH_2 by PGF_2 synthase (EC 1.1.1.188), and (c) an isomer of $PGF_{2\alpha}$, 9α, 11β-$PGF_{2\alpha}$ can be synthesized from PGD_2, by PGD_2 11-ketoreductase (EC 1.1.1.188).

Prostaglandin I_2/prostacyclin (PGI_2)

Prostaglandin I_2, also known as prostacyclin, is a strong platelet aggregation inhibitor, and causes vasodilation and anti-platelet aggregation (Tateson, Moncada, and Vane 1977). PGI_2 is synthesized from PGH_2 by prostacyclin synthase (PGIS; EC5.3.99.4), which is a member of the cytochrome P450 superfamily.

Thromboxane A_2 (TxA_2)

TxA_2 was first identified in 1975 (Hamberg, Svensson, and Samuelsson 1975), and has an opposing function from that of PGI_2. TxA_2 causes vasoconstriction and platelet aggregation. TxA_2 is derived from PGH_2 by the enzyme thromboxane-A synthase (TBXAS1; EC 5.3.99.5), another member of the cytochrome P450 superfamily.

LOX pathway

Lipoxygenase (LOX; EC 1.13.11.12) belongs to a large family of non-heme iron containing fatty acid dioxygenases and is found to exist widely in various taxonomic divisions including fungi, plant and animals (Brash 1999; Grechkin 1998; Gerwick 1994; C D Funk 1996; S. Yamamoto, Suzuki, and Ueda 1997). LOXs have been linked with various biological functions and signaling pathways (Feussner and Wasternack 2002). LOXs are known to mediate peroxidation reactions and mobilization of lipids (Brash 1999), synthesize signaling molecules that provoke bronchoconstriction and inflammation, and catalyze oxygenation of polyunsaturated fatty acids (PUFAs) in plants during the process of germination in oil-seed plants (Andreou, Brodhun, and Feussner 2009).

LOX enzymes can use polyunsaturated fatty acids that contain multiple *cis* double bonds as substrates, for example, linoleic acid (LA), α-linolenic acid (α-LeA) or arachidonic acid (AA), and yield hydroperoxy derivatives of PUFAs. Lack of the necessary substrates in bacteria and yeast is mirrored by the absence of these enzymes in *Saccharomyces cerevisiae* and prokaryote genomes. Organisms lower down on the evolutionary scale, such as the unicellular organism *Chlorella* have only one gene that codes for a lipoxygenase (Zimmerman and Vick 1973), but higher plants and animals seem to possess more than one lipoxygenase. For example, multiple lipoxygenases have been identified in *Glycine max* (soybean) (Christopher and Axelrod 1971), seven lipoxygenase coding genes have been established in mice and five lipoxygenases genes in human (Krieg *et al.* 1998; Boeglin, Kim, and Brash 1998). Very little is known about lipoxygenases in insects, although hydroxyeicosatetraenoic acid (HETE), which is a

typical lipoxygenase product, has been identified in the primitive insect *Thermobia domestica* (Gadelhak, Pedibhotla, and Stanley-Samuelson 1995).

Epoxygenase pathway

In contrast to the extensively studied COX and LOX pathways, little is known about the epoxygenase pathway. In this pathway, cytochrome P450 (CYP450) epoxygenases use arachidonic acid as substrate and produce epoxyeicosatrienoic acids (EET), which function as autocrine and paracrine lipid mediators. Epoxygenase inserts an oxygen atom on a carbon that is attached to one of the double bonds of arachidonic acid. Arachidonic acid has four double bonds, and epoxidation can occur at any of the four bonds, resulting in the production of four regioisomers: 5,6-, 8,9-, 11,12- and 14,15-EETs, each of which can have either R, S or the S, R enantiomer (Zeldin 2001). Even though different EET regioisomers have quantitative or qualitative differences in various reactions, they have similar metabolic properties, so EETs are considered a single class of molecules (Spector 2008). EETs are involved in secretion of hormone peptides (Cashman, Hanks, and Weiner 1987; Falck *et al.* 1983; Snyder, Yadagiri, and Falck 1989), regulation of inflammation (Node *et al.* 1999) and homeostasis (Node *et al.* 2001). EETs are converted to less active diols dihydroxyeicosatetraenoic acids (DHETs) by soluble epoxide hydrolase (sEH) (Spector 2008; Elmarakby 2012). Till date, the functions of DHETs have not been elaborated (Elmarakby 2012). Although prevalent in mammals and other vertebrates including fish (Oleksiak *et al.* 2003), an enzyme with homology to cytochrome P450 epoxygenase has not been detected in insect genomes (D. Stanley 2011).

Eicosanoids in insects

Eicosanoids have primarily been linked with immunity and inflammation in insects (David Stanley, Haas, and Miller 2012). Insects possess three lines of defense to protect themselves from infections and invasive threats: (a) a physical barrier made up of cuticle and peritrophic membrane (David Stanley, Miller, and Tunaz 2009), (b) humoral response, which includes synthesis of antimicrobial peptides, the bacteriolytic enzyme lysozyme and activation of the prophenoloxidase (PPO) system (Kanost, Jiang, and Yu 2004), and (c) hemocyte-mediated cellular response, which includes three physiological processes: phagocytosis, nodulation and encapsulation (Strand 2008). Eicosanoids have been implicated as important mediators of insect immune response in multiple types of cellular defense responses, such as phagocytosis, microaggregation, nodulation, encapsulation, cell spreading and hemocyte migration toward bacterial peptides.

Eicosanoids are present and modulated during infection in insect immune tissues, and the presence of PGs has been established with full mass spectra of PGs (David Stanley 2006) in insect immune tissues, hemocytes and fat body (Gadelhak, Pedibhotla, and Stanley-Samuelson 1995). Multiple research studies showed that in insects, eicosanoids mediate immune reactions against different types of invaders, e.g., fungi, protozoan, virus, bacteria and parasitoids (Dean *et al.* 2002; Garcia, Machado, and Azambuja 2004; Carton *et al.* 2002). Stanley-Samuelson *et al.* and Miller *et al*.'s experiments show that eicosanoids mediate insect nodulation in response to bacterial invasions (D W Stanley-Samuelson *et al.* 1991; Miller, Nguyen, and Stanley-Samuelson 1994), and Downer *et al.* have suggested that eicosanoids play roles in three separate

cellular processes: phagocytosis, cell spreading and PPO activation in wax moths, *Galleria mellonella* (Downer *et al.* 1997). Morishima *et al.* suggest that eicosanoids mediate induction of cecropin and lysozyme in fat body of the silkworm, *Bombyx mori* (Morishima *et al.* 1997). Recent studies indicate that the *D. melanogast*er *pxt* (**P**ero**x**inec**t**in-like) may be a gene encoding an insect COX, and this has opened up new avenues for experimentation and investigation in immune signaling in *D. melanogast*er (Tootle and Spradling 2008).

In addition to immune tissues and cells, PGs or PG biosynthesis have been detected in cricket reproductive tissues (D. Stanley-Samuelson and Loher 1986), tobacco hornworm midgut (Büyükgüzel *et al.* 2002), and reproductive tissues of *D. melanogaster* (David Stanley 2006; Tootle and Spradling 2008; David Stanley, Miller, and Tunaz 2009; Toolson *et al.* 1994). Prostaglandins are thought to be involved in thermoregulation, control of hatching, egg-laying, and oogenesis in insects (David Stanley 2006; Tootle and Spradling 2008). All these studies strongly argue for significant conservation in eicosanoids biosynthesis and function in insects.

Although the literature is rich in speculations that prostaglandins are central signaling molecules for mediating and coordinating insect cellular immunity, genes responsible for encoding COX or COX-like enzymes have not been reported in insects. Most work on insect systems has focused on COX products, the prostaglandins and the eicosanoid hypothesis has been supported by experiments with a phylogenetically wide range of insect species, and a wide range of infecting agents, and several specific eicosanoid-mediated cellular actions have been identified in the process (David Stanley, Miller, and Tunaz 2009). In biochemical research on PG actions, the influence of PGs

on gene expression in insect cells has been recorded, linking PG actions to specific proteins (Morishima *et al.* 1997). Effects of aspirin and other NSAIDs on PG synthesis have also been reported for insects. Machado *et al.* found that cultured ovarioles treated with aspirin and other PG synthesis inhibitors have decreased choriogenesis, which can be reversed by $PGF_{2\alpha}$ (Machado *et al.* 2007). However, the mechanism of eicosanoid actions in insects is still not very well understood.

Specifically in *D. melanogaster,* Pxt, also known as Chorion peroxidase, a peroxidase with a clear role in reproduction has been suggested to function like a COX (Tootle and Spradling 2008; Vázquez, Rodríguez, and Zurita 2002): a *pxt* mutant causes infertility in *D. melanogast*er female, similar to COX-2 mutant mice and sterile *pxt* mutant *D. melanogast*er females can be rescued by mouse COX-1 (Tootle and Spradling 2008). Unpublished studies show a role of Pxt in immune function in *D. melanogaster* as well (personal communication, Dr. Shubha Govind).

Cyclooxygenase

COXs or prostaglandin H synthases (PGHS) are heme-containing peroxidase enzymes responsible for the biosynthesis of a family of eicosanoids and are key players in inflammation and immunity (W L Smith, Garavito, and DeWitt 1996). COX is the rate-limiting key enzyme of prostaglandin synthesis, and COX enzymes are known to be targets of various non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin (Vane 1971). Therefore, COX and the COX pathway have been the subjects of active research in academic and pharmaceutical studies.

COXs belong to the superfamily of animal heme peroxidases, which contains a large group of ubiquitous enzymes that catalyze the oxidation reaction of various substrates by hydrogen peroxide or organic hydroperoxide and usually contain a heme prosthetic group in their active site (Dunford 1999). COXs are unique amongst this group in possessing the capability to carry out two enzymatic activities: cyclooxygenase activity and peroxidase activity (Garavito and Mulichak 2003). COXs catalyze the committed step leading to the synthesis of prostaglandins, thromboxane, and prostacyclin (W L Smith, Garavito, and DeWitt 1996; Marnett *et al.* 1999).

Peroxidase (POX) and cyclooxygenase (COX) activities of COX enzymes are structurally distinct but functionally connected (W L Smith, DeWitt, and Garavito 2000). In the POX reaction, Compound I is formed by oxidation of the Fe^{3+} protoporphyrin IX (PPIX) into an oxyferryl heme porphyrin π-cation radical (Schulz *et al.* 1984; Patterson, Poulos, and Goodin 1995). Then Compound I receives an electron from Tyr385 through an intramolecular reduction, resulting in the formation of a tyrosyl

radical (Karthein *et al.* 1988; Dietz, Nastainczyk, and Ruf 1988), which is known as intermediate II. Intermediate II is the COX active form of this enzyme. In the COX reaction, the Tyr385 radical removes a hydrogen atom from C-13 of the substrate arachidonic acid to trigger the COX reaction (Benecky *et al.* 1993) and converts arachidonic acid into Prostaglandin G_2. The POX activity reduces PGG_2 into PGH_2. The POX activity is not dependent on the COX activity, so when the COX site is occupied by an inhibitor such as an NSAID, the POX activity can operate independently (Mizuno, Yamamoto, and Lands 1982). On the contrary, the COX activity is dependent on the POX activity because the generation of the tyrosyl radical of the COX activity requires the heme group, which is located at the POX site (W L Smith and Lands 1972; Landino *et al.* 1997).

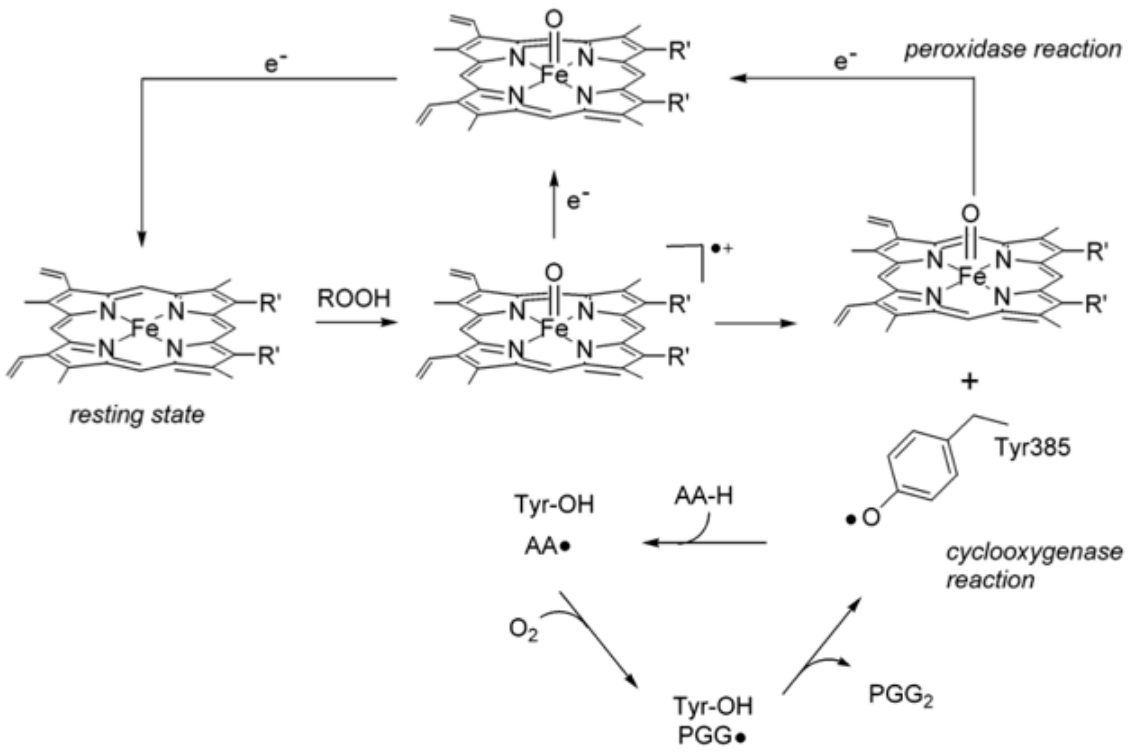


Figure 2. Mechanism of COX catalysis. Tyrosyl radical Tyr385, which is required for the COX reaction, is generated by the POX reaction. (Reprint of figure 3, (van der Donk, Tsai, and Kulmacz 2002))

There are two types of cyclooxygenases present in mammals: COX-1 and COX-2. COX-1 is a housekeeping enzyme, while COX-2 is an inducible enzyme (W L Smith, Garavito, and DeWitt 1996; Marnett *et al.* 1999). COX-1 is expressed constitutively in most tissues and systems, but COX-2 is induced by different factors, including growth factors and tumor promoters, to express rapidly and differentially in various cell types (W L Smith, Garavito, and DeWitt 1996). The COX-1 gene is known to regulate angiogenesis in endothelial cells and immune response (Tsujii *et al.* 1998; Rocca *et al.* 1999). Inducible expression of COX-2 is thought to be important for reproduction (Lim *et al.* 1999; Lim *et al.* 1997), immune response (Rocca *et al.* 1999), development

(Gilbert *et al.* 1994; Xie *et al.* 1991; T. Endo *et al.* 1995), kidney function (H. F. Cheng *et al.* 1999), liver function (Ledwith *et al.* 1997; Kraemer *et al.* 1996), neurotransmission (Breder, Dewitt, and Kraig 1995), bone formation regulation (Pilbeam *et al.* 1997; Pilbeam *et al.* 1993), muscle function (Pritchard *et al.* 1994; Rimarachin *et al.* 1994) and pancreatic regulation (Robertson 1998).

Separate genes encode COX-1 and COX-2, but the two genes share high sequence similarity (60% amino acid sequence similarity) and highly similar structural folds, as well as similar catalytic mechanisms (Järving *et al.* 2004). Mammalian COXs have been well studied, and several aspects of their structural folds and functional properties have been resolved based on experimental data (W L Smith, DeWitt, and Garavito 2000) and the solved structures of both COX-1 and -2 (Picot, Loll, and Garavito 1994; Kurumbail *et al.* 1996). Both COX-1 and COX-2 are thought to be membrane bound because both of them have a membrane-binding domain (MBD) (Otto and Smith 1996; A G Spencer *et al.* 1999). However, antibody staining experiments reveal that their sub-cellular localization varies from cytoplasmic to inner and outer membrane of the nuclear envelope, Golgi apparatus, lysosome, or the lumenal surface of the endoplasmic reticulum, depending on physiological conditions (Koumas and Phipps 2002; García-Bueno, Serrats, and Sawchenko 2009; Leclerc *et al.* 2008).

Both enzymes function as homodimers (Yuan *et al.* 2006), with each monomer composed of an EGF-like domain, a membrane-binding domain (MBD) and a catalytic animal heme peroxidase domain (W L Smith, DeWitt, and Garavito 2000). The core structures of the catalytic domain of COX-1 and COX-2 adopt nearly identical folds: the root mean square deviation (RMSD) of sheep COX-1 (PDB ID: 1CQE) and mouse

COX-2 (PDB ID: 1CVU) is only 0.4 Å (Tsai and Kulmacz 2010; W L Smith, DeWitt, and Garavito 2000). In both COX-1 and COX-2, functionally important residues include Arg120, Gln203, His207, Val349, His388, Tyr385, and Ser530 (W L Smith, DeWitt, and Garavito 2000; Loll, Picot, and Garavito 1995) (Figure 3). Arg120 contacts C-1 of the substrate arachidonic acid. Gln203, His 207, His 388 are crucial for the peroxidase activity, while Tyr385 is essential for the cyclooxygenase activity. The catalytic pocket of mammalian COX, with His207, Tyr385, and His388, is L-shaped and largely hydrophobic (W L Smith, DeWitt, and Garavito 2000). The acetylation of Ser530 (Loll, Picot, and Garavito 1995) is the structural basis of aspirin inhibition of COXs (Garscha and Oliw 2009).

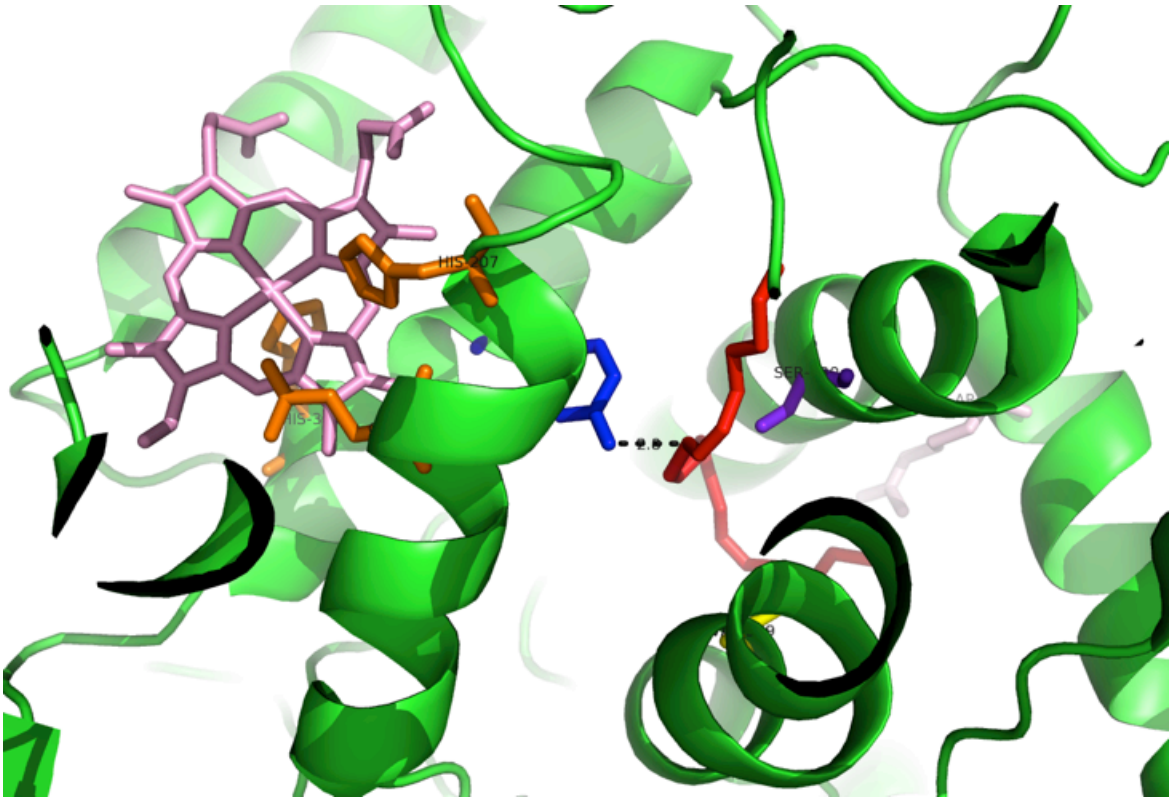


Figure 3. Structure of arachidonic acid bound to Sheep COX-1 (PDB ID: 1DIY) showing the location of the important residues. COX-1 structure is rendered in cartoon, residues 120, 203, 207, 349, 385, 388 and 530, cofactor protoporphyrin IX containing Co, and substrate arachidonic acid are rendered in sticks. Glu203, His207 and His388 are shown in orange. Tyr385 is shown in blue. Val347 is colored in yellow. Arg120 is colored in light pink. Ser530 is colored in purple. Rendering of the structure and distance between Tyr385 and C-13 of arachidonic was calculated using the program Pymol.

Despite extensive literature on mammalian COXs, several important questions remain regarding their *in vivo* biochemical functions. For example, recent studies have shown that even though COXs are pro-inflammatory, COX-2 can mediate the biosynthesis of anti-inflammatory electrophilic fatty acid exo-derivatives (EFOXs) (Groeger *et al.* 2010). Such studies underscore the need for a deeper understanding of structure-function relationships of these complex enzymes.

A phylogenetic analysis of peroxidases across the evolutionary scale shows that this superfamily can be divided into seven categories: chordate peroxidases, ecdysozoan

and deuterostomian peroxidasins, ecdysozoan and echinozoan peroxinectins, prokaryotic and eukaryotic cyclooxygenases, bacterial peroxicins, peroxidockerins, ecdysozoan and deuterostomian dual oxidases (Zamocky *et al.* 2008). Despite low conservation of primary structure, COX enzymes from fungi are able to catalyze the conversion of arachidonic acid to PGH_2 (Garscha and Oliw 2009). Mammalian COX-1 and COX-2 are highly conserved in primary structure with sequence identity of paralogues ranging from 60-65%, and orthologues from 85-90% (W L Smith, DeWitt, and Garavito 2000). However, more distant COX genes do exist in the genomes of lower vertebrates (Ishikawa *et al.* 2007), invertebrates (Koljak *et al.* 2001; Varvas *et al.* 1999), fungi (Lee *et al.* 2008), and plants (Lee *et al.* 2008) (table 1). Characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages (Järving *et al.* 2004). Insects are known to synthesize prostaglandins (D W Stanley-Samuelson and Ogg 1994; Bowman, Dillwith, and Sauer 1996), but the enzymes for their biosynthesis have not even been clearly identified. The presence of COX enzyme(s) in insects in general and *D. melanogaster* in particular remains debatable. In *D. melanogaster*, protein peroxinectin-like (Pxt) discovered in 2002 (Vázquez, Rodríguez, and Zurita 2002), is the only known putative COX-like enzyme and is important in oogenesis and eggshell production (Tootle and Spradling 2008; Tootle *et al.* 2011).

Table 1. Species with known cyclooxygenases

Species (common name/category)	**KEGG ID**
Homo sapiens (human)	HSA: 5742(PTGS1) 5743(PTGS2)
Pan troglodytes (chimpanzee)	PTR: 464713(PTGS1) 469616(PTGS2)
Macaca mulatta (rhesus monkey)	MCC: 698213(PTGS1) 716671(PTGS2)
Mus musculus (mouse)	MMU: 19224(Ptgs1) 19225(Ptgs2)
Rattus norvegicus (Norway rat)	RNO: 24693(Ptgs1) 29527(Ptgs2)
Canis lupus familiaris (dog)	CFA: 403544(PTGS1) 442942(PTGS2)
Bos taurus (cow)	BTA: 282022(PTGS1) 282023(PTGS2)
Sus scrofa (pig)	SSC: 397541(PTGS1) 397590(PGHS-2)
Equus caballus (horse)	ECB: 100034087(PTGS1) 791253(PTGS 2)
Monodelphis domestica (opossum)	MDO: 100016747 100024802
Ornithorhynchus anatinus (platypus)	OAA: 100081492 100086096
Gallus gallus (chicken)	GGA: 396451(PTGS2) 427752(PTGS1)
Taeniopygia guttata (zebra finch)	TGU: 100221909 100226968
Xenopus laevis (African clawed frog)	XLA: 100037245(ptgs1) 446781(ptgs2)
Xenopus (Silurana) tropicalis (western clawed frog)	XTR: 595089(ptgs2)
Danio rerio (zebrafish)	DRE: 246226(ptgs1) 246227(ptgs2a) 559 020(ptgs2b)
Branchiostoma floridae (Florida lancelet)	BFO: BRAFLDRAFT_129952
Ciona intestinalis (sea squirt)	CIN: 100183010 100183175
Podospora anserina (fungus)	PAN: PODANSg1229
Magnaporthe grisea (fungus)	MGR: MGG_10859
Aspergillus nidulans (fungus)	ANI: AN5028.2
Aspergillus fumigatus (fungus)	AFM: AFUA_3G12120
Aspergillus oryzae (fungus)	AOR: AO090003000772
Aspergillu niger (fungus)	ANG: An02g07930
Aspergillus flavus (fungus)	AFV: AFLA_030430
Aspergillus clavatus (fungus)	ACT: ACLA_039980
Penicillium chrysogenum (fungus)	PCS: Pc18g00240

Neosartorya fischeri (fungus)	NFI: NFIA_065200
Coccidioides immitis (fungus)	CIM: CIMG_00042
Uncinocarpus reesii (fungus)	URE: UREG_00168
Postia placenta (fungus)	PPL: POSPLDRAFT_98495
Laccaria bicolor (fungus)	LBC: LACBIDRAFT_315146
Ustilago maydis (fungus)	UMA: UM04571.1
Nitrosomonas europaea (bacteria)	NEU: NE1240
Methylobacterium sp. 4-46 (bacteria)	MET: M446_1624
Methylobacterium nodulans (bacteria)	MNO: Mnod_6498
Rhodobacter sphaeroides (bacteria)	RSH: Rsph17029_3626
Rhodobacter sphaeroides (bacteria)	RSK: RSKD131_4262
Roseobacter denitrificans (bacteria)	RDE: RD1_1072
Mycobacterium vanbaalenii (bacteria)	MVA: Mvan_3099
Streptosporangium roseum (bacteria)	SRO: Sros_8745
Gymnopilus obscurus (bacteria)	GOB: Gobs_1219
Nostoc punctiforme (bacteria)	NPU: Npun_R5469

Prostaglandin E synthase

Prostaglandin E synthases (PGES) are isomerases that catalyze the conversion of PGH_2 to PGE_2. These enzymes are unique because the same catalytic activity is manifested through three structurally distinct PGESs (M Murakami *et al.* 2000).

There are three PGESs: PGES-1, PGES-2 and PGES3. PGES-1 and PGES-2 are microsomal proteins that are also known as mPGES-1 and mPGES-2, while PGES-3 is a cytosolic protein, and also known as cPGES (T Tanioka *et al.* 2000). The two membrane-associated prostaglandin E synthases, PGES-1 and PGES-2, are inducible by inflammation, and can be down regulated by anti-inflammatory glucocorticoids; on the other hand, cytosolic PGES-3 is constitutively expressed (Toshihiro Tanioka *et al.* 2003; Weaver *et al.* 2000). Activity of both PGES-1 and PGES-3 is glutathione (GSH)-dependent, and PGES-2 requires the cofactor thiol for enzymatic activity. PGES-1 is co-expressed with and functionally linked to COX-2. PGES-3 is co-expressed with and functionally linked to with COX-1 (M Murakami *et al.* 2000). PGES-2 does not have a preference to couple with COX-1 or COX-2, and it can couple with either to produce PGE_2 (Makoto Murakami *et al.* 2003)(figure4).

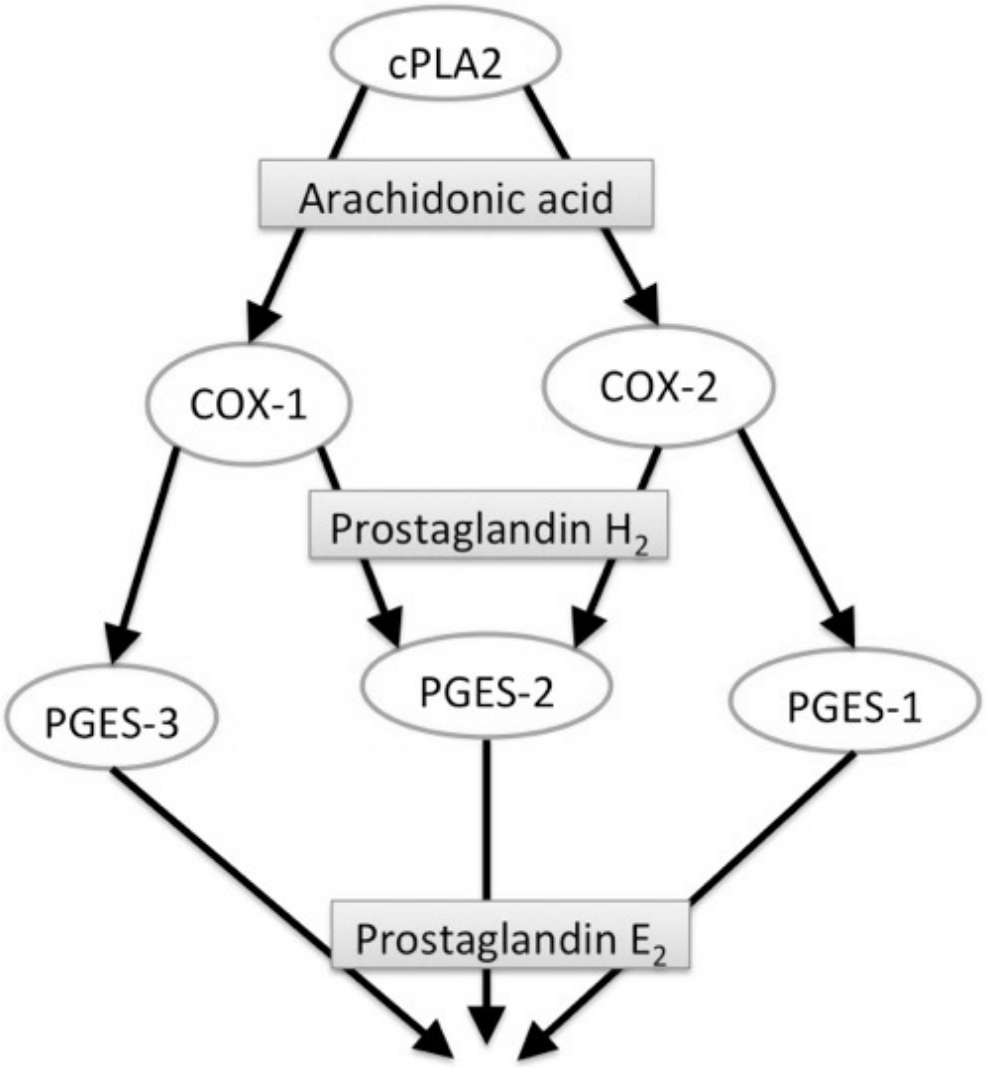


Figure 4. Coupling of COX-1/2 with PGES-1, PGES-2 and PGES-3

Prostaglandin E synthase-1 (PGES-1/mPGES-1)

Prostaglandin E Synthase-1 (PGES-1), also known as microsomal prostaglandin E synthase-1 (mPGES-1), is an inducible membrane-bound enzyme that catalyzes the isomerization of the pro-inflammatory molecule PGE_2 from PGH_2. Because of its importance in inflammation, PGES-1 is a potential and promising target for drugs in many diseases, including a variety of cancers (Nakanishi *et al.* 2010; Rådmark and Samuelsson 2010). Expression level of PGES-1 was found to be very low in normal rat tissues, and PGES-1 expression level is significantly induced by a stimuli of lipopolysaccharide or endotoxin in various tissues, including lung, brain, heart, testis, colon, spleen, and seminal vesicle tissues (Mancini *et al.* 1995; Yamagata *et al.* 2001).

The PGES-1 protein sequence contains approximately 150 amino acids and belongs to the MAPGE (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily. The MAPGE superfamily includes microsomal glutathione transferase-1 (MGST-1), MGST-2, MGST-3, FLAP and leukotriene C4 synthase (LTCS). Like most proteins in MAPGE family, PGES-1 functions using glutathione as a co-factor. Jegerschold *et al.* solved the first crystal structure of PGES-1 in 2008 using recombinant human PGES-1 expressed in *E. coli* with EM at a resolution of 3.5Å (Jegerschöld *et al.* 2008). The structure suggests that the enzyme functions as a homotrimer (figure 5), with each monomer composed of four transmembrane helices. Mutation of Arg67, Arg110 or Tyr117 to Ala lead to loss of enzyme activity, implying that these residues are crucial for enzyme activity (figure 6). Tyr117, Arg126, Tyr130 and Gln134 bind to glutathione. Other important residues include Glu66 and His72, the mutation of which results in reduction of enzyme activity by 50% and 70%,

respectively, compared to the wild type protein (Jegerschöld *et al.* 2008). The crystal structure of human mPGES-1 has elaborated three well-defined catalytic sites on the interface of the monomers and a small extra cytosolic domain inserted between helices I and II, which does not exist in members of the MAPGE family (Sjogren *et al.* 2013). Ser127 was suggested to play a critical role the catalytic mechanism of PGES-1 because the hydroxyl group of Ser127 assists the formation of glutathione thiolate and stabilizes it (Sjogren *et al.* 2013).

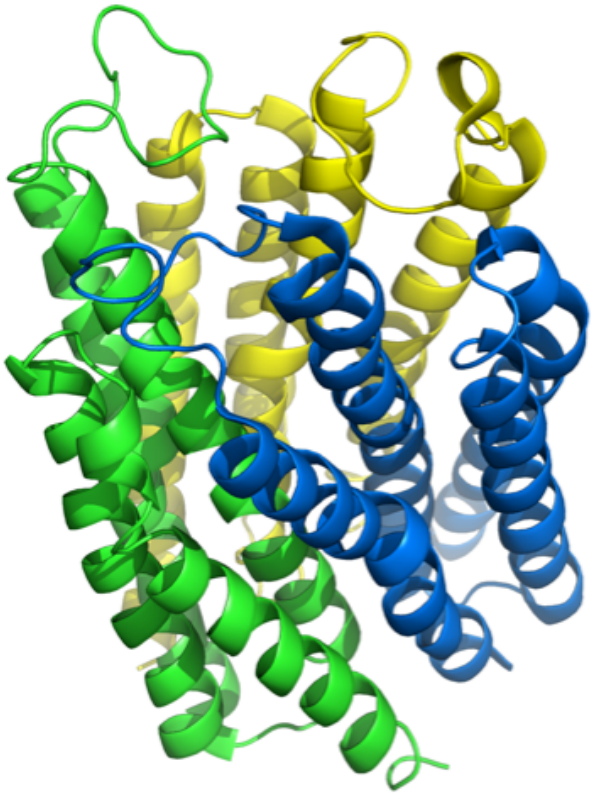


Figure 5. Human PGES-1 (PDB ID: 3DWW) forms a homotrimer. Each monomer is composed of four helices. Yellow, green and blue represent three monomers.

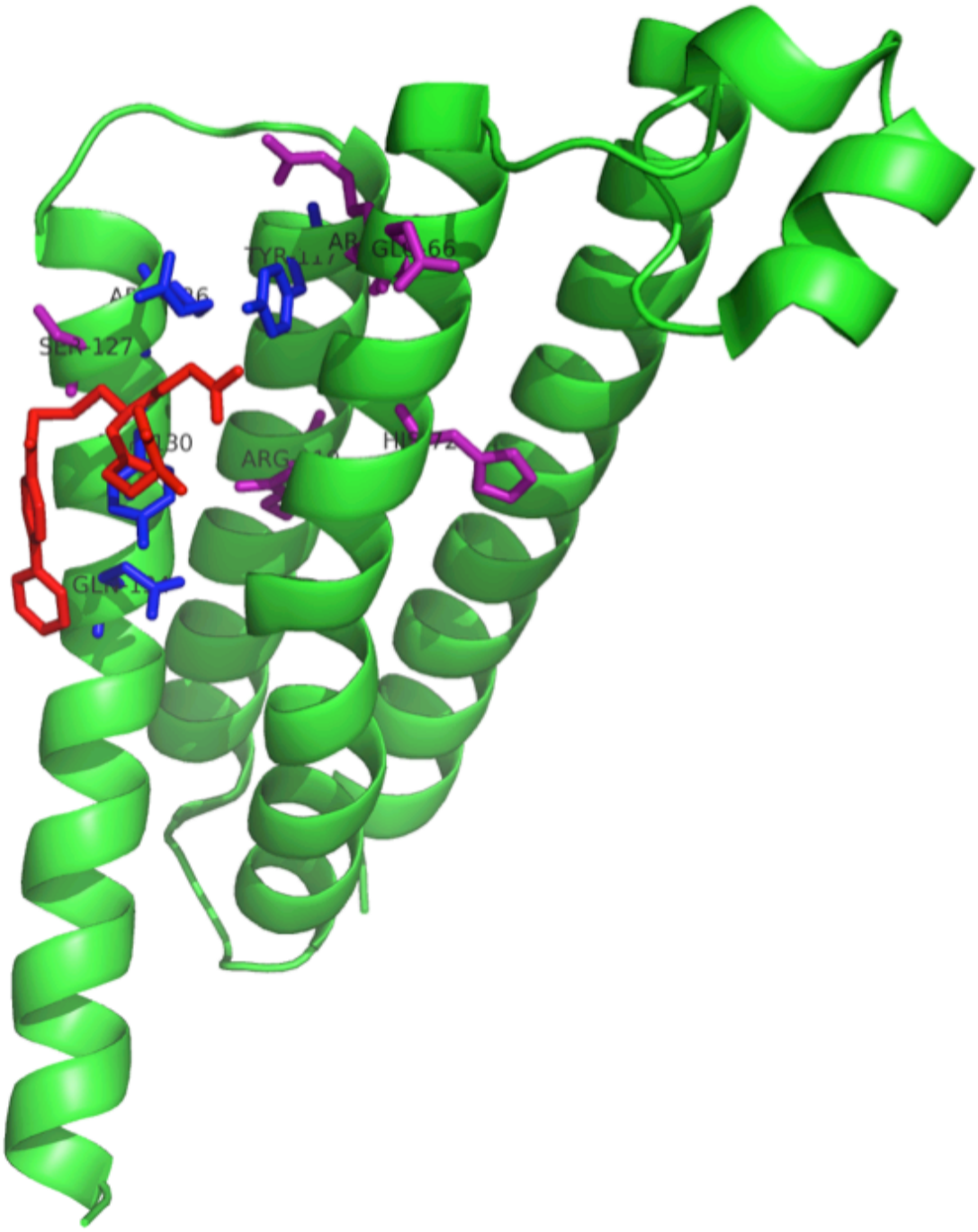


Figure 6. Catalytic and other important residues in human PGES-1 (PDB ID: 4AL1). Tyr117, Arg126, Tyr130 and Gln134 bind to glutathione analog 1-(4-phenylphenyl)-2-(S-glutathionyl)-ethanone (biphenyl-GSH). Tyr117, Arg126, Tyr130 and Gln134 are represented as blue sticks. Other functionally important residues including Glu66, Arg67, His72, Arg110 and Ser127 are shown as purple sticks. Glutathione analog 1-(4-phenylphenyl)-2-(S-glutathionyl)-ethanone (biphenyl-GSH) is shown as red sticks. PGES-1 monomer is rendered in green cartoon.

Yamamoto *et al.* identified, purified and crystalized a glutathione transferase that exhibits prostaglandin E synthase activity from silkworm *Bombyx mori* (K. Yamamoto *et al.* 2013). The isolated *B. mori* glutathione transferase (GSTS1) shares 44.5% sequence identity with *D. melanogaster* GSTS-1 (CG8938). The amino acid sequence alignment indicates *B. mori* GSTS1, *D. melanogaster* GSTS-1 and rat PGDS share high sequence identity and similarity.

Prostaglandin E Synthase-2 (PGES-2/mPGES-2)

PGES-2, also known as mPGES-2, is synthesized as a Golgi membrane-associated protein, but it functions as a cytosolic enzyme after its N-terminal hydrophobic domain is proteolytically removed (Makoto Murakami *et al.* 2003). PGES-2 consists of 378-385 amino acids, and is structurally distinct from PGES-1 (Jegerschöld *et al.* 2008; Yamada *et al.* 2005). It differs from PGES-1 because its activity is not glutathione dependent (Tanikawa *et al.* 2002).

Initial studies based on the crystal structure of PGES-2 suggested that it forms a dimer and is attached to the membrane by its N-terminal region (figure 7)(Yamada *et al.* 2005). However, Murakami *et al.* discovered that the formation of a mature PGES-2 required proteolytic removal of its hydrophobic N-terminus, which leads to the formation of a cytosolic protein (Makoto Murakami *et al.* 2003). Therefore, PGES-2 functions as a cytosolic protein, since its membrane-bound N-terminal region is truncated in the mature form. Adjacent to the hydrophobic domain, PGES-2 has a glutaredoxin/thioredoxin domain, which contains the thioredoxin consensus sequence Cys110-X-X-Cys113. Cys110 is the catalytic site of PGES-2, mutation of which results in loss of enzyme activity (Kikuko Watanabe *et al.* 2003). The substrate PGH_2 fits into the V-shaped catalytic pocket, and its endoperoxide moiety makes contacts with the SH functional group of Cys110 (figure 8). SH-reducing reagents, including dithiothreitol, GSH and β-mercaptoethanol are required for enzymatic activity *in vitro* (Kikuko Watanabe *et al.* 2003).

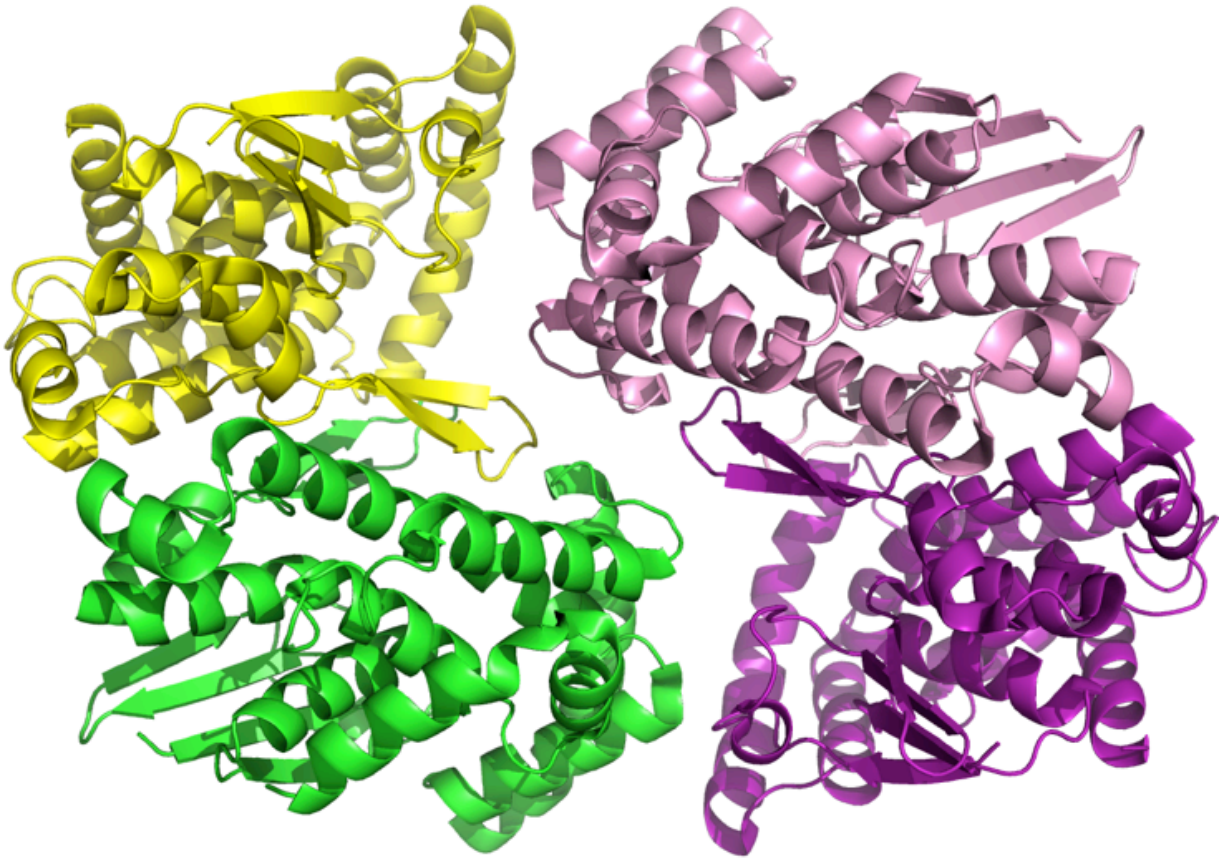


Figure 7. *Macaca fascicularis* PGES-2 (PDB ID: 1Z9H) functions as dimers. Two subunits (yellow and green, purple and pink) form a dimer. There is also weak dimer-dimer interaction between the two dimers (Yamada *et al.* 2005).

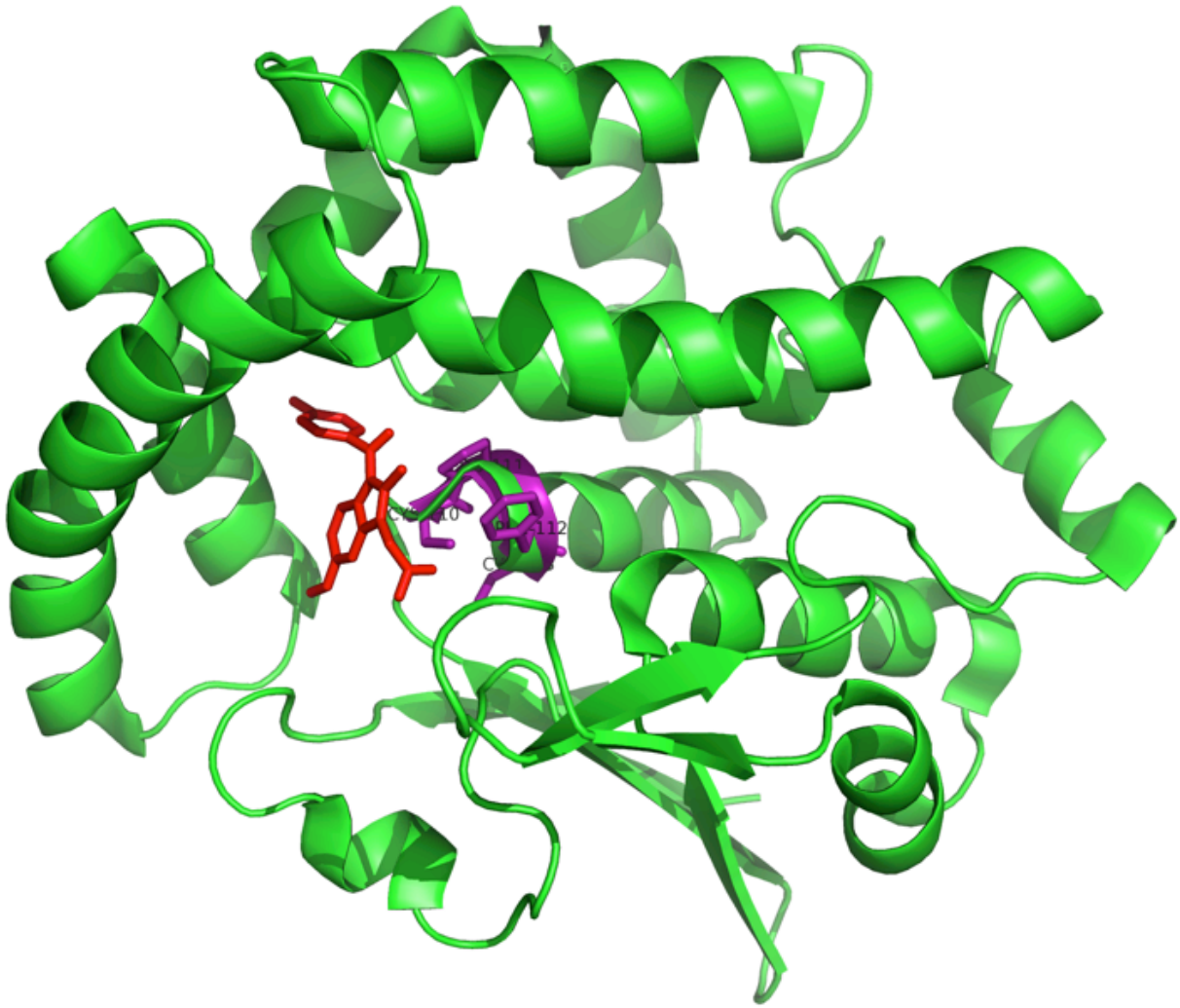


Figure 8. Important residues in *Macaca fascicularis* PGES-2 (PDB ID: 1Z9H). Cys110-X-X-Cys113 thioredoxin consensus sequence is shown as purple sticks. Indomethacin, the analog of substrate PGH_2, is shown as red sticks. Subunit of *Macaca fascicularis* PGES-2 is rendered in green cartoon.

Unlike PGES-1, which has very low basal expression level in normal tissues, basal transcriptional expression level of PGES-2 is much higher in a variety of tissues, such as brain, heart, skeletal muscle, kidney and liver, and the expression level of PGES-2 is tissue-dependent (Tanikawa *et al.* 2002). In most tissues, the expression level PGES-2 is not elevated dramatically by inflammation. However, transcriptional expression level of PGES-2 is elevated significantly in colorectal cancer and bone marrow stromal cells, where PGES-1 shows increased expression level as well (Makoto Murakami *et al.* 2003; Ueno *et al.* 2005).

Prostaglandin E Synthase-3 (PGES-3/cPGES)

In 2000, Tanioka *et al.* isolated, purified and identified cytosolic glutathione (GSH)-dependent PGES-3 from LPS-treated rat brain, and discovered that it is identical to the previously identified p23, which is a ubiquitous, highly conserved protein originally implicated to function as a co-chaperone for heat shock protein hsp90 (T Tanioka *et al.* 2000; Weaver *et al.* 2000). Constitutively expressed cytosolic PGES-3, also known as cPGES, has been observed in various types of cells and is functionally linked to the constitutively expressed COX-1 (T Tanioka *et al.* 2000).

PGES-3 and COX-1's functional coupling suggests that they have similar functions, which include gastrointestinal movement, reproduction and some neural functions. Research suggests that PGES-3 is essential for prenatal survival embryonic growth, and not as much for PGE_2 synthesis. PGES-3 deficient mice have various defective growth conditions including poor lung maturation and hindered skin development (Grad *et al.* 2006; Nakatani *et al.* 2007; Lovgren, Kovarova, and Koller 2007). Recent studies by Simpson *et al.* and Mattila *et al.* show that high level of cPGES results in elevated level of lymph node metastases and drug resistance, which in turn promotes tumor growth in breast cancer (Simpson *et al.* 2010; Mattila *et al.* 2009). Their study also shows overexpression of cPGES in human gliomas, suggesting that cPGES may play a role in inflammation. Similar to PGES-1, PGES-3 is GSH-dependent, and it requires GSH as its co-factor to reach optimal activity (T Tanioka *et al.* 2000).

PGES-3 belongs to the glutathione S-transferase (GSTs; EC 2.5.1.18) family, a large protein family composed of multifunction enzymes including structurally unrelated cytosolic enzymes and microsomal enzymes that are traditionally considered to be

involved in metabolic detoxication of electrophiles by glutathione conjugation (Johnson *et al.* 1993; Mannervik and Danielson 1988; Vos and Van Bladeren 1990; Hayes and Strange 2000). The structure of human PGES-3 was solved in 2000 by Weaver *et al.* (Weaver *et al.* 2000). Tyr9 in PGES is conserved in several other GSTs, and mutation of Tyr9 abolishes GST activity of cPGES (T Tanioka *et al.* 2000). Tanioka *et al.* found that Tyr9, which is known to be essential for enzymatic activity of PGES-3 as a glutathione S-transferase, is crucial for PGES synthesis activity as well (Johnson *et al.* 1993; T Tanioka *et al.* 2000). PGES-3 functions as a homodimer (figure 9).

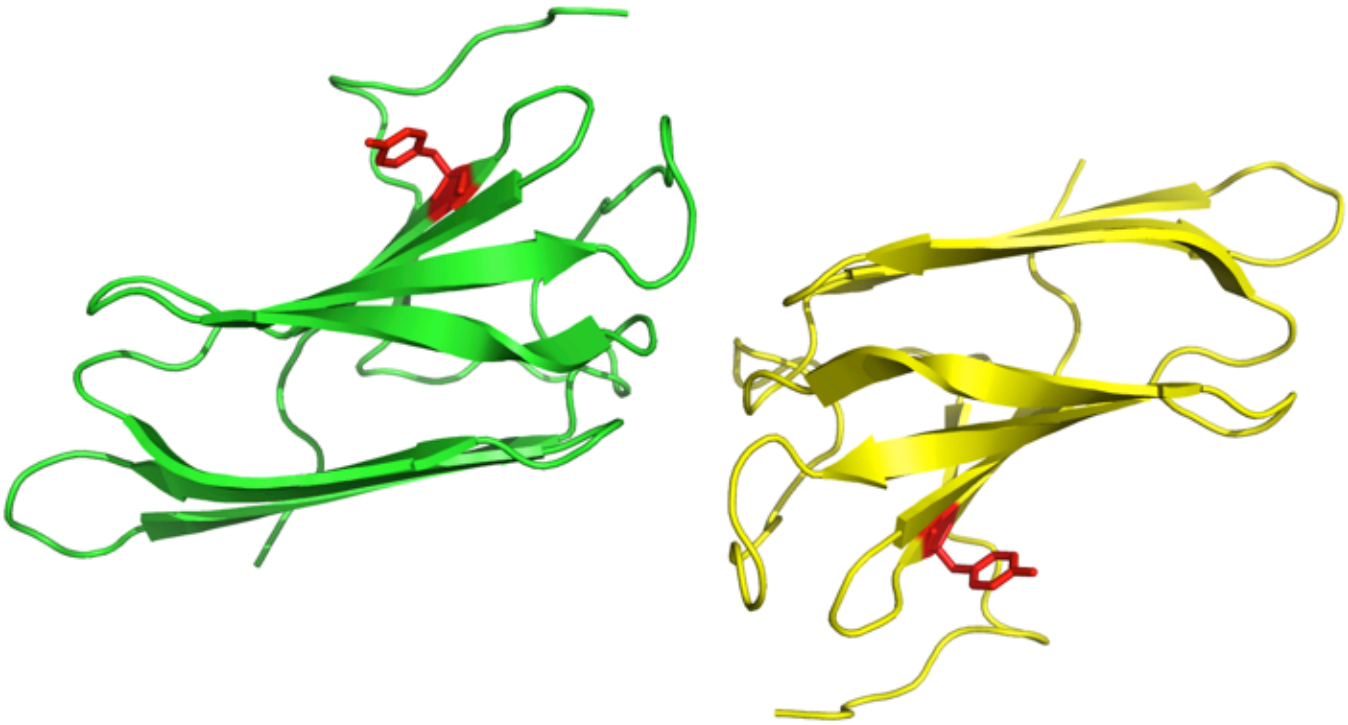


Figure 9. Human PGES-3 (PDB ID: 1EJF) forms a homodimer. Tyr9, which is required for the glutathione S-transferase activity of PGES-3, is essential for its PGES enzymatic activity. The two subunits were shown in green and yellow cartoons. Tyr9 is shown as red sticks.

Project Rationale

The value of the *Drosophila* as a model organism is well established, and multiple resources in terms of genetic tools, databases, and mutants are currently available to address important biological questions (Kalajdzic *et al.* 2012; Chintapalli, Wang, and Dow 2007). Overall, flies share extensive genetic similarities with vertebrates. Furthermore, it is well-documented that the fundamental regulatory signaling mechanisms that control cellular physiology in flies and humans are essentially conserved (Medzhitov and Janeway Jr 2000). Given the importance of eicosanoids in mammalian and insect immune systems, this study was designed to allow us to identify the enzymes that mediate eicosanoid biosynthesis in *Drosophila* computationally. After a preliminary scan for putative homologues for all enzymes in the COX pathway, a systematic, comprehensive, and detailed computational investigation was undertaken for two enzymes, COX and Prostaglandin E synthase in an endeavor to model and characterize the possible candidates and identify those that possess all the requisite sequence and structural motifs to qualify as valid COX(s) / PGE synthase proteins and make suitable candidates for further experimental investigations.

Chapter 2 Methods

Identification of homologues of COX, PGE Synthase and other enzymes of COX pathway in *D. melanogaster*

Putative COX homologous proteins, putative PGES homologous proteins, and other putative homologous proteins in COX pathway in *D. melanogaster* were identified using BLASTp, PSI-BLAST (position-specific iterated BLAST)(Altschul *et al.* 1990) and HMMER (Finn, Clements, and Eddy 2011). Then the amino acid sequences of the proteins were retrieved from NCBI protein sequence database in fasta format. BLASTp and PSI-BLAST were run against the *D. melanogaster* genome database (version FB2013_02, released March 8th, 2013) using human enzymes in COX pathway as input sequences. For BLASTp, E-value cutoff was set to 1E-5, and queries with coverage more than 45% were taken as candidates. Five iterations of PSI-BLAST were used, initial expect threshold was set to 0.01. HMMER implements profile hidden Markov models (profile HMMs) to searching sequence database for homologous proteins. Comprehensive results identified by BLASTp, HMMER and PSI-BLAST were kept as candidates. The individual accession numbers, chromosomal location, and residue lengths of the candidates were verified by scanning various databases manually to eliminate any ambiguities and annotation errors.

Domain architecture analysis

The retrieved candidate sequences were verified for existence of their characteristic catalytic domains: COX protein sequences for the animal peroxidase domain, PGES-1 protein sequences for the MAPGE domain, PGES-2 protein sequences

for glutathione S-transferase N-terminal domain (GST_N) and glutathione S-transferase C-terminal domain (GST_C), and PGES-3 protein sequence for p23/CS (CHORD-containing proteins and SGT1) domain respectively. Details of their domain architecture were verified using four different domain architecture analysis software: CDD (Marchler-Bauer *et al.* 2009), Prosite (de Castro *et al.* 2006; Sigrist *et al.* 2010; Sigrist *et al.* 2005), SMART (Letunic, Doerks, and Bork 2009; Schultz *et al.* 1998) and Pfam (Finn *et al.* 2010). CD-search is NCBI's online searching tool against CDD (Conserved Domains and Protein Classification), a protein annotation resource based on PSSMs (pre-calculated position-specific scoring metrices) and PRS-BLAST (reverse position-specific BLAST), a variant of PSI-BLAST (Marchler-Bauer *et al.* 2009). Pfam is a protein family database, and each family is represented by multiple sequence alignments and hidden Markov models (HMMS) (Finn *et al.* 2010). PROSITE is the database of protein domains, families and function site of SIB (Swiss Institute of Bioinformatics) as part of ExPaSy (Expert Protein Analysis System, the portal of SIB). PROSITE includes documentations describing protein domains, families and functional residues and associated patterns and motifs (de Castro *et al.* 2006). The domain boundaries were verified using secondary structure prediction programs as described below.

Secondary structure prediction

The boundaries of the domains in the protein sequences analyzed were ascertained based on the consensus output from a number of secondary structure prediction programs: Jpred (Cole, Barber, and Barton 2008), porter (Pollastri and McLysaght 2005), PredictProtein (Rost and Liu 2003), psipred (D. T. Jones 1999), sopma (Geourjon and Deléage 1995) and sspro (J. Cheng *et al.* 2005). The consensus secondary structure prediction for each sequence was also used to verify the accuracy of template–target alignments used in modeling their three dimensional structure.

Multiple sequence alignment and characterization of target sequence

In order to evaluate the key residues responsible for biochemical catalytic function of the protein, amino acid sequences of putative *D. melanogaster* were compared with mammalian sequences for the enzymes. Different programs such as T-Coffee (Notredame, Higgins, and Heringa 2000), CLUSTAL (Thompson, Higgins, and Gibson 1994), and Muscle (Edgar 2004) were used to generate optimal alignments, and ESPript was used to generate visualization of the alignments (Gouet *et al.* 1999).

Transmembrane helices in the target sequence were predicted using TMpred (Hofmann and Stoffel 1993), TMHMM (Möller, Croning, and Apweiler 2001) and SOSUI (Hirokawa, Boon-Chieng, and Mitaku 1998), and the consensus results were used. To predict the presence and location of a signal peptide in *D. melanogaster* putative COXs, four programs PrediSi (Hiller *et al.* 2003), SignalP4 (Petersen *et al.*

2011), SIGPred (Bradford 2001) and Signal-3L (H.-B. Shen and Chou 2007) were used. The consensus results were used.

Phylogenetic tree

To reconstruct the evolutionary relationship of COX proteins from different phyla, the PhyML (Guindon and Gascuel 2003) package from Phylogeny.fr (Dereeper *et al.* 2008) was used as follows: the multiple sequence alignment was built using Muscle (Edgar 2004) in default run mode and maximum 16 iterations; Gblock (Castresana 2000) was used for curation with the following parameters: smaller final blocks not allowed, gap position within the final blocks not allowed, less strict flanking positions not allowed, and many contiguous non-conserved positions allowed; Phylogentic tree was built based on maximum likelihood using the PhyML (Guindon and Gascuel 2003) package using the following parameters: 100 bootstraps, default substitution model with 4 substitution rate categories, automatic estimation of substitution parameter, automatic estimation of proportion of invariable sites, and removal of gaps in sequence alignment prior to building phylogenetic tree. The tree was visualized using FigTree v1.3 (Morris *et al.* 1998).

Structural modeling, model refinement and evaluation

Molecular modeling

A combination of comparative modeling techniques and *ab initio* approaches were used to generate high-quality three-dimensional models for the catalytic domains of *D. melanogaster* COX homologues Pxt, Pxd and CG4009, PGES-1 homologues Mgsl, CG33177 and CG33178, PGES-2 homologue Su(2)P and PGES-3 homologue CG16817 as outlined in the flowchart in figure 10.

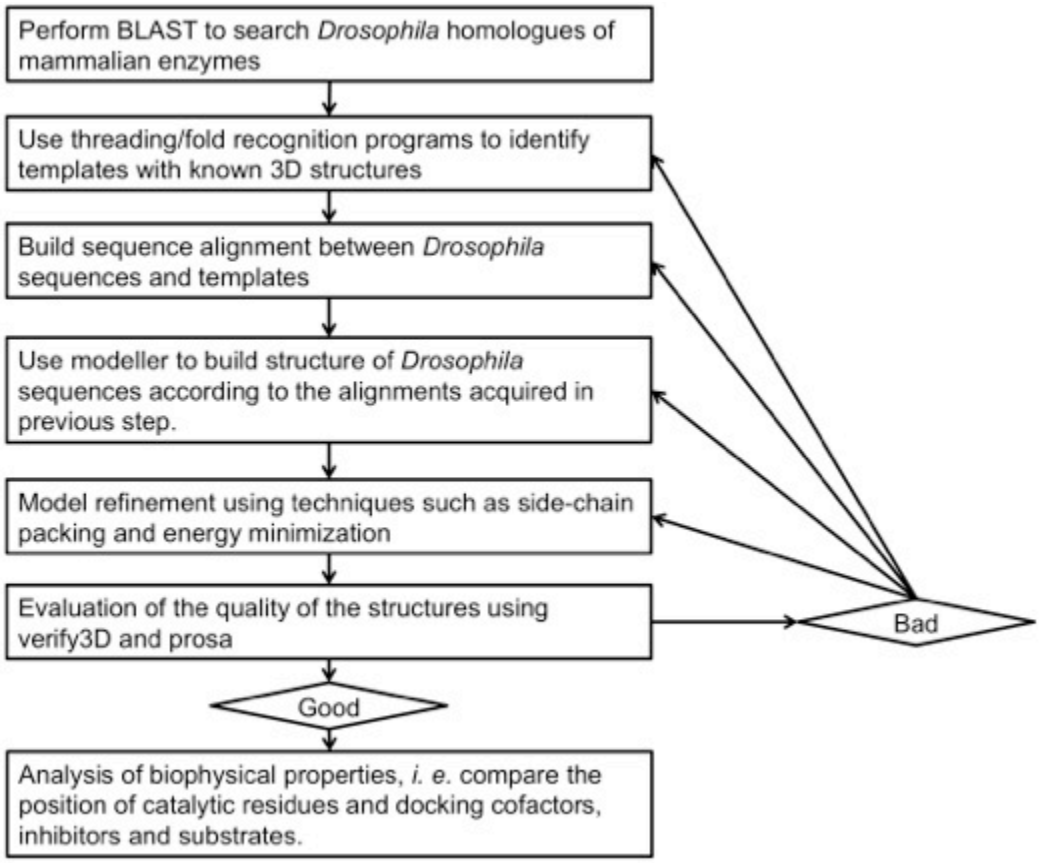


Figure 10. Flowchart of molecular modeling

Structural modeling of COX homologues Pxt, Pxd and CG4009

Multiple models for the catalytic domains of *D. melanogaster* Pxt, Pxd, and CG4009 proteins were built using various modeling programs. Human myeloperoxidase (PDB ID: 1MHL) was identified as the top ranked structural template by the fold-recognition software FUGUE (Shi, Blundell, and Mizuguchi 2001), while HHpred (Söding, Biegert, and Lupas 2005) identified *Bos taurus* lactoperoxidase (PDB ID: 3Q9K) as a high confidence structural template for the conserved catalytic domains of *D. melanogaster* COX candidates. The preliminary sequence alignments between the structural templates 1MHL and 3Q9K and the *D. melanogaster* proteins obtained from FUGUE (Shi, Blundell, and Mizuguchi 2001) and HHpred (Söding, Biegert, and Lupas 2005), respectively, were optimized and used to generate the three dimensional models using the program modeller 9v9 (Eswar *et al.* 2006).

Structural modeling of PGES-1 homologues Mgsl, CG33177 and CG33178

Using a similar approach as detailed above, the structure of the *Homo sapiens* microsomal prostaglandin E synthase-1 (PDB code 3DWW) identified by HHpred as a high confidence structural template was used for modeling for the full length sequence of *D. melanogaster* PGES-1 candidates, Mgsl, CG33177 and CG33178.

Structural modeling of PGES-2 homologue Su(2)P

HHpred (Söding, Biegert, and Lupas 2005) identified the structure of *Macaca fascicularis* microsomal prostaglandin E synthase-2 (PDB code 1Z9H) as the high confidence structural template for the conserved catalytic domain (residues 110-399) of *D. melanogaster* PGES-2 candidate Su(2)P. The sequence alignment between the structural template 1Z9H and *D. melanogaster* protein Su(2)P obtained from HHpred (Söding, Biegert, and Lupas 2005), was used to generate the three dimensional models using the program modeller 9v9.

Structural modeling of PGES-3 homologue CG16817

Homo sapiens co-chaperone P23 (PDB code 1EJF) was selected as the structural template for the conserved CS domain (residues 1-96) of *D. melanogaster* PGES-3 candidate CG16817, and used for creating its molecular models based on the HHPred generated alignment. The generated molecular models were refined in the same manner as the models for the other homologues.

Model refinement and evaluation

After models were constructed, loop refinement, side-chain packing and energy minimization were carried out by Scwrl4 (Canutescu, Shelenkov, and Dunbrack 2003). Besides the structural models of the catalytic domain, full-length models were also built for structural analysis of regions beyond the catalytic domains. Full-length models were constructed using I-TASSER (iterative threading assembly refinement) (Zhang 2008). I-TASSER is an automated integrated protein structure calculation server that utilizes the sequence-to-structure-to-function algorithm to calculate the three dimensional structure

of the query sequence. It first computes the secondary structure of the query sequence using multiple threading alignments and iterative structural assembly simulation, and then matches the 3D model with known protein structures in BioLip protein function database (Yang, Roy, and Zhang 2013). The best representative model was picked based on the z-score and knowledge-based energy score calculated using verify3D (Lüthy, Bowie, and Eisenberg 1992) and Prosa (Wiederstein and Sippl 2007). Structural alignment between catalytic domain of putative *Drosophila melanogaster* protein and sheep COX-1 (PDB ID 1CQE), human PGES-1 (PDB ID 3DWW), monkey PGES-2 (PDB ID 1Z9H), or human PGES-3 (PDB ID 1EJF) was calculated using CE protein server (Shindyalov and Bourne 1998). Finally, visualization of protein models was performed using Pymol (DeLano 2002).

Docking analysis

To understand the possible interactions between the ligands as co-factors and *D. melanogaster* COX candidates at the molecular level, flexible docking was performed using Autodock 4.0 (Morris *et al.* 1998). The structures of heme (PDB ligand ID HEM), linoleic acid (PDB ligand ID EIC) and arachidonic acid (PDB ligand ID ACD) ligands were obtain from Ligand Expo (Berman, Henrick, and Nakamura 2003) in PDB format. Polar hydrogen atoms were added to the protein, and then a total Kollman charge was assigned to the protein, and all non-polar hydrogen atoms were merged. For all the ligands, polar hydrogen atoms were added, and Gasteiger charge was calculated and assigned to the ligands, and then all the non-polar hydrogen atoms were merged, and torsion-tree was detected automatically using Autodock. After both receptor and ligand were processed and imported to AutoDock, grids were generated using AutoGrid. The

location and size of grids were manually adjusted, based on our prediction of the location of the ligand. Lamarckian Genetic Algorithm with the default parameter settings were performed for each docking, and the docked results were evaluated based on energy. The docked complex of the ligand and the protein with the lowest energy was further analyzed and visualized using Pymol (DeLano 2002).

COX activity assay

Fly stock

All fly stocks were maintained on standard cornmeal-agar-yeast medium at 25 °C. y w flies (wild-type) were used as the control in all the experiments. *pxt*^{f01000} strain was obtained from the Harvard Exelixis collection. *pxt*^{f01000} contains a Piggybac insertion within the 5' UTR of the gene, and the insertion is located 38 bp upstream of the start codon (Thibault *et al.* 2004). *pxt*^{f01000} strain has significantly lower transcriptional expression level of *pxt* in the whole fly, confirmed by Tootle *et al.* (Tootle and Spradling 2008).

Fly sample preparation

For each experimental group and control group, 40-80 adult flies were collected in a centrifuge tube, and the centrifuge tube was placed in a -20°C freezer for at least 45 minutes. Quiescent flies were weighed and transferred into a microtube, and mixed with 6.8 ml cold buffer (0.1 M Tris-HCl, pH 8.0 containing 1 mM EDTA) per mg of flies. Flies were then homogenized on ice. Homogenized flies were then centrifuged at 10,000 X g for 15 minutes at 4°C. After centrifuging, the supernatant was moved to new microtubes, stored on ice and used for the assay.

COX activity analysis

Cyclooxygenase is a bifunctional enzyme and has cyclooxygenase and peroxidase activities. The cyclooxygnase activity converts arachidonic acid into PGG_2, while the peroxidase activity converts PGG_2 to PGH_2, which is the precursor of prostaglandins, thromboxanes and prostacyclins.

Cayman's COX activity assay kit (Cayman Chemical Item Number 760151) measures the peroxidase activity of COX proteins. The peroxidase activity of COX catalyzes the reaction that converts tetramethyl-p-phenylenediamine (TMPD) to N, N, N', N'- tetramethyl-p-phenylenediamine (TMPD). The kit measures the peroxidase activity of COX by monitoring the appearance of N, N, N', N'- tetramethyl-p-phenylenediamine (TMPD) at 590 nm. (Van der Ouderaa *et al.* 1977; Kulmacz and Lands 1983)

The protocol in the Cayman's COX activity assay kit was used in this assay. Inactive samples were prepared as following: 50 µL of supernatant obtained after centrifuging the fly sample preparation was transferred to a 1.5 mL microtube, then placed in boiling water for five minute. Background wells contained 150 µL assay buffer, 10 µL heme solution and 10 µL inactive sample per well. Sample wells contained 150 µL assay buffer, 10 µL heme, 10 µL untreated supernatant sample per well. After adding reagents to background wells and samples wells, the 96-well plate was carefully shaken until the contents were thoroughly mixed, and then the plate was incubated for five minutes at 25°C. After the five-minute incubation, 20 µL of colorimetric substrate was added to every well. The reaction was initiated by adding 20 µL of arachidonic acid solution to each well. The plate was again carefully shaken and incubated for five

minutes at 25°C. Wells of the plate were read using a plate reader at the absorbance 590nm.

Total COX activity was calculated as following:

ΔA590 = A590 [sample] - A590 [background]

$$\textbf{Total COX Activity} = \frac{\Delta A_{590}/5 \text{ min.}}{0.00826\ \mu M^{-1}} \times \frac{0.21 \text{ ml}}{0.04 \text{ ml}} \div 2^{*} = \text{nmol/min/ml (U/ml)}$$

Three repetitions of COX activity assays were performed. The COX activity of each repetition was normalized by setting the total COX activity of y w female as 100%. A paired two-tail t-test was performed using Excel for each two of the three repetitions to determine whether the experimental results from two repetitions were significantly different.

Chapter 3 Mapping enzymes of the cyclooxygenase pathway in *Drosophila melanogaster*

The mammalian COX pathway is well documented and extensively investigated (Table 2). The mammalian COX pathway includes multiple proteins that catalyze nine well characterized enzymatic reactions (William L Smith, Urade, and Jakobsson 2011). Table 2 summarizes the nine-enzymatic reactions catalyzed by various enzymes with distinct structural folds and catalytic mechanisms.

Table 2. List of all the enzymes in COX pathway

Reaction	EC	Enzyme full names	NCBI locus (human)	Reference
AA → PGH_2	EC 1.14.99.1	Cyclooxygenase-1 (COX-1)	NP_000953.2	(Yokoyama and Tanabe 1989)
		Cyclooxygenase-2 (COX-2)	NP_000954.1	(D. A. Jones *et al.* 1993)
PGH_2 → TxA_2	EC 5.3.99.5	Cytochrome P450, family 5, subfamily A (CYP5A); thromboxane-A synthase (TBXAS1)	NP_001052	(Yokoyama *et al.* 1991)
PGH_2 → PGE_2	EC 5.3.99.3	Prostaglandin-E synthase 1 (PGES-1/mPGES-1)	NP_004869	(Jakobsson *et al.* 1999)
	EC 5.3.99.3	Prostaglandin-E synthase 2 (PGES-2/mPGES-2)	NP_079348	(Tanikawa *et al.* 2002)
	EC 5.3.99.3	Prostaglandin-E synthase 3 (PGES-3/cPGES)	NP_006592.3	(T Tanioka *et al.* 2000)
PGE_2 → $PGF_{2\alpha}$	EC 1.1.1.184; EC 1.1.1.189; EC 1.1.1.197	Prostaglandin-E_2 9-reductase; carbonyl reductase (NADPH) (CBR1)	NP_001748	(Wermuth *et al.* 1988)
	EC 1.1.1.184	Carbonyl reductase-2 (CBR2)	NA	
	EC 1.1.1.184; EC 1.1.1.189; EC 1.1.1.197	Carbonyl reductase-3 (CBR3, similar to CBR1)	NP_001227	(Koji Watanabe *et al.* 1998)
	EC 1.1.1.184	Carbonyl reductase-4 (CBR4)	NP_116172.2	(S. Endo *et al.* 2008)
PGH_2 → PGD_2	EC 2.5.1.18; EC 5.3.99.2	Hematopoietic prostaglandin D synthase (H-PGDS)	NP_055300	(Kanaoka *et al.* 2000)
	EC 5.3.99.2	Lipocalin-type prostaglandin D synthase (L-PGDS)	NP_000945	(White *et al.* 1992)
PGH_2 →$PGF_{2\alpha}$	EC 1.1.1.188	Prostaglandin F synthase (PGFS)	NP_001182665.1	(Moriuchi *et al.* 2008)
PGE_2 → $PGF_{2\alpha}$	EC 1.1.1.184; EC 1.1.1.189; EC 1.1.1.197	PGE 9-ketoreductase = CBR1	NP_001748	(Wermuth 1981)

$PGD_2 \rightarrow 9\alpha, 11\beta\text{-}PGF_{2\alpha}$	EC 1.1.1.213 EC 1.1.1.112 EC 1.1.1.188 EC 1.1.1.239 EC 1.1.1.64 EC 1.3.1.20	Aldo-keto reductase family 1 member C3; PGD 11-ketoreductase	NP_003730.4	(Suzuki-Yamamoto *et al.* 1999)
$PGH_2 \rightarrow PGI_2$	EC 5.3.99.4	Cytochrome P450, family 8, subfamily A (CYP8A); Prostacyclin synthase (PGIS)	NP_000952	(Miyata *et al.* 1994)

We have been able to identify a majority of the homologues in *D. melanogaster* based on sequence similarity with known enzymes from vertebrates and/or invertebrates (overall 14-39% sequence identity) in the COX pathway (figure 11), indicating that the enzyme structures show sufficiently high sequence conservation. In some instances (*e.g.*, COX), more than one homologue has been identified. To confirm that, in principle, a functional enzyme was identified, an initial investigation of each of the putative enzyme sequences was performed for (1) conserved catalytic domains, and (2) catalytic signatures (based on sequence alignments as well as structural superposition where the catalytic domain was successfully modeled). Our preliminary scan has identified putative homologues for COXs, PGES, carbonyl reductase (CBR), hematopoietic prostaglandin D synthase (H-PGDS), PGD 11-ketoreducatse, prostacyclin synthase and thromboxane-A synthase (figure 11).

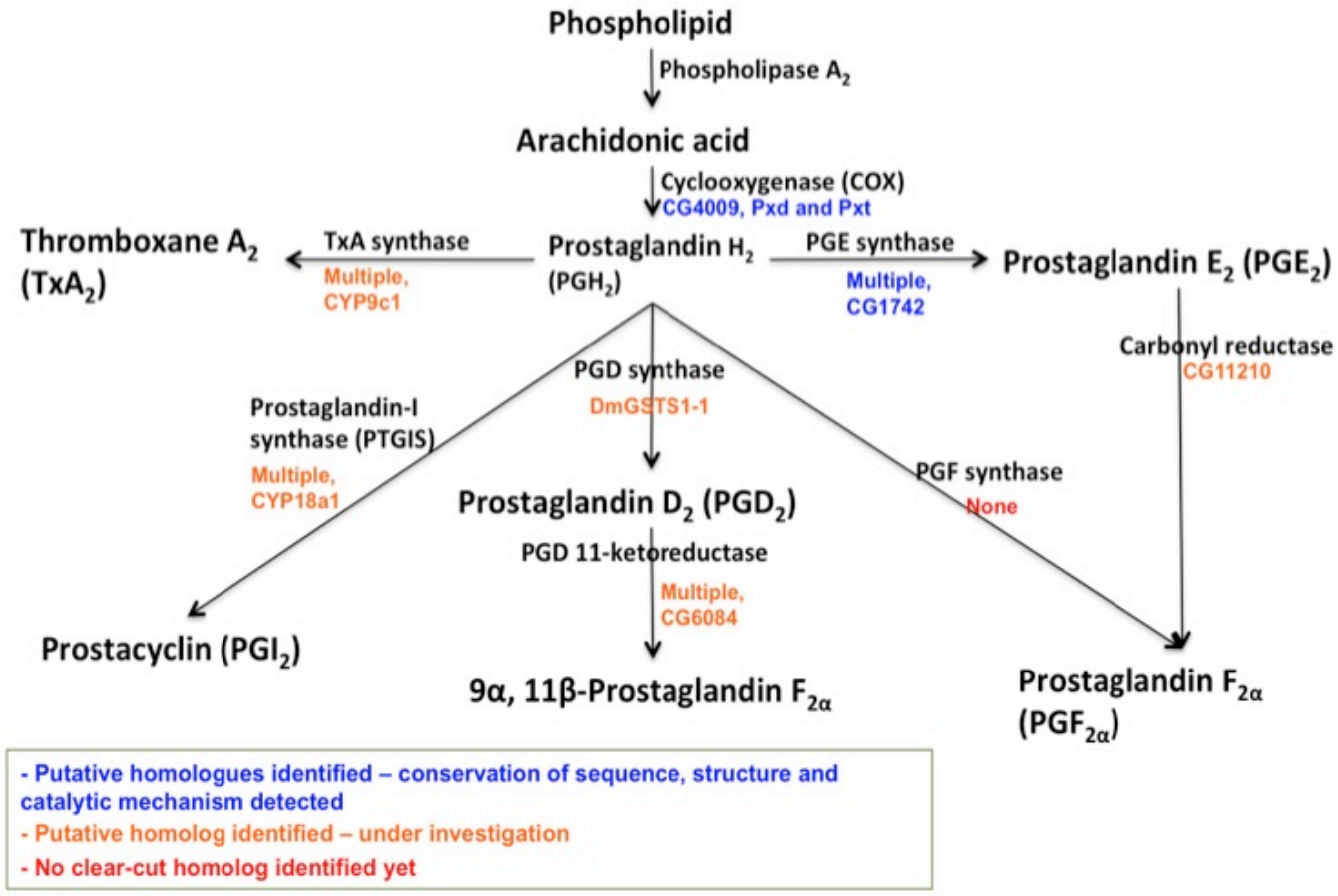


Figure 11. Putative *D. melanogaster* enzymes of the COX pathway

Two of these enzymes, COX and PGE synthase, were subsequently subjected to detailed investigation described in the following chapters (chapter 4 and chapter 5). The preliminary analysis upon which the assignment of putative homologs for the other enzymes is based on is described below.

Theoretical assignment of CG11210 as carbonyl reductase (CBR)

Carbonyl reductases (CBRs, EC 1.1.1.184; EC 1.1.1.189; EC 1.1.1.197) are NADPH-dependent oxidoreductases that catalyze the production of $PGF_{2\alpha}$ using PGE_2 as the substrate. CBRs belong to short chain dehydrogenases/reductases (SDR) family (Forrest and Gonzalez 2000; Nelson *et al.* 1993). There are three CBRs in *Homo sapiens*, CBR-1, CBR-3 and CBR-4, and their sequences are highly similar (figure 12).

Our initial analysis has identified *D. melanogaster* protein CG11210 (accession NP_725952.1) as a CBR homologue. CG11210 shares 20% sequence identity and 33% sequence similarity with hCBR-1, and it contains the classic conserved sequences of all CBRs (figure 12), which include the cofactor binding region Rossmann fold GlyXXXGlyXGly at residues 12-18 and the conserved catalytic sequence TyrXXXLys at residues 194-198 (Forrest and Gonzalez 2000; Oppermann *et al.* 1998).

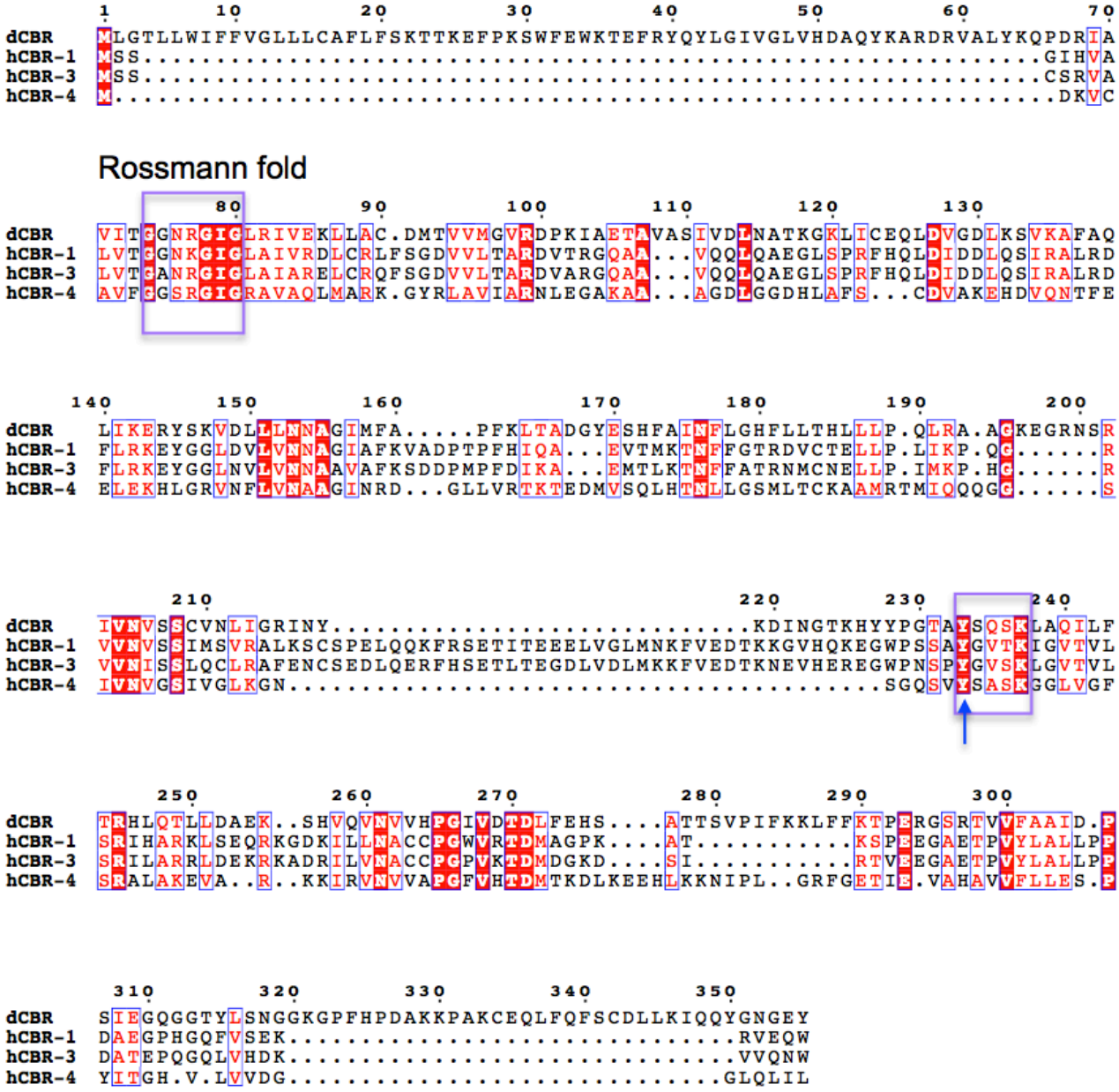


Figure 12. Alignment of human CBRs and *D. melanogaster* CBR. Identical/similar residues are boxed in red/white. Purple boxes show conserved sequences Rossmann fold GlyXXXGlyXGly (residues 12-18) and TyrXXXLys (residues 194-198). Conserved Tyr194 is marked with a blue arrow.

Theoretical assignment of DmGSTS1-1 as hematopoietic prostaglandin D synthase

PGDS catalyzes the synthesis of PGD_2. There are two different types of PGDSs in mammals: hematopoietic prostaglandin D synthase (H-PGDS) and lipocalin-type prostaglandin D synthase (L-PGDS), also called glutathione-independent PGD synthase (table 3). The two types of PGDS differ in tissue location, cofactor and activators, and they can be distinguished by their sequences through their differing catalytic signatures (Table 3; Urade *et al.* 1993; Gerena *et al.* 1998; Tokugawa *et al.* 1998; Eguchi *et al.* 1997; Lewis *et al.* 1982).

Table 3. Comparison of H-PGDS and L-PGDS

	Hematopoietic prostaglandin D synthase (H-PGDS)	Lipocalin-type prostaglandin D_2 synthase (L-PGDS)/Prostaglandin-H_2 D-isomerase
NCBI accession No./human	NP_055300.1	NP_000945.3
Amino acid	199aa	190aa
Molecular weight	23kDa	21kDa
Chromosomal location	4q22.3	9q34.2-q34.3
Subunit	Dimer	Monomer
Tissue locations	Mast cells, Th2 cells and microglia	Brain, male genital organs and heart
Activator	$Mg^{2+,}$ Ca^{2+}	
Co-factor	GSH	Sulfhydryl compounds
PDB	4EDY, 4EE0, 4EC0, 4EDZ, 3VI5, 3VI7, 2KXO, 3EE2,	4IMN, 4IMO, 3O19, 3O22, 3O2Y, 2WWP

	2VCQ, 2VCW, 2VCX, 2VCZ, 2VD0, 2VD1, 1V40, 1IYH, 1IYI	
EC	EC 2.5.1.18 EC 5.3.99.2	EC 5.3.99.2

Our initial analysis suggests that *D. melanogaster* possesses only one of the two types of enzymes. We identified DmGSTS1-1 (CG8938, glutathione S transferase S1), a well studied and characterized protein (Agianian *et al.* 2003; Singh *et al.* 2001; Agianian *et al.* 2001) as the putative H-PGDS. Structure-based sequence alignment of human H-PGDS and DmGSTS1-1 shows that they share 35% sequence identity and 58% sequence similarity. DmGSTS1-1 has the majority of key functional residues of human H-PGDS (figure 13). Key functional residues of hH-PGDS include (1) Tyr8 and Arg14, which are involved in stabilization of the thiol group of GSH; (2) Trp104, which is required to sustain the catalytic cleft in the active form of the enzyme (Pinzar *et al.* 2000); (3) Asp93, Asp96 and Asp97, which are the metal activation sites(Inoue *et al.* 2003). DmGSTS1-1 is missing Arg14, Trp104 and Asp96. In place of Arg14, Trp104 and Asp96, DmGSTS1-1 possesses Leu60, Ser152 and Asn142, respectively (figure 13). Despite the differences in the above residues, the residues of DmGSTS1-1 possess similar position as the human counterparts (figure 14). Structural alignment shows that the structural folds of DmGSTS1-1 and human H-PGDS are highly similar with RMSD 2.24Å (figure 14). Saisawang *et al.* discovered that DmGSTS1-1 is capable of utilizing 4-HNE (4-hydroxynonenal), adrenchrome and PEITC (phenethyl isothiocyanate) as substrates *in vitro* (Saisawang, Wongsantichon, and Ketterman 2012).

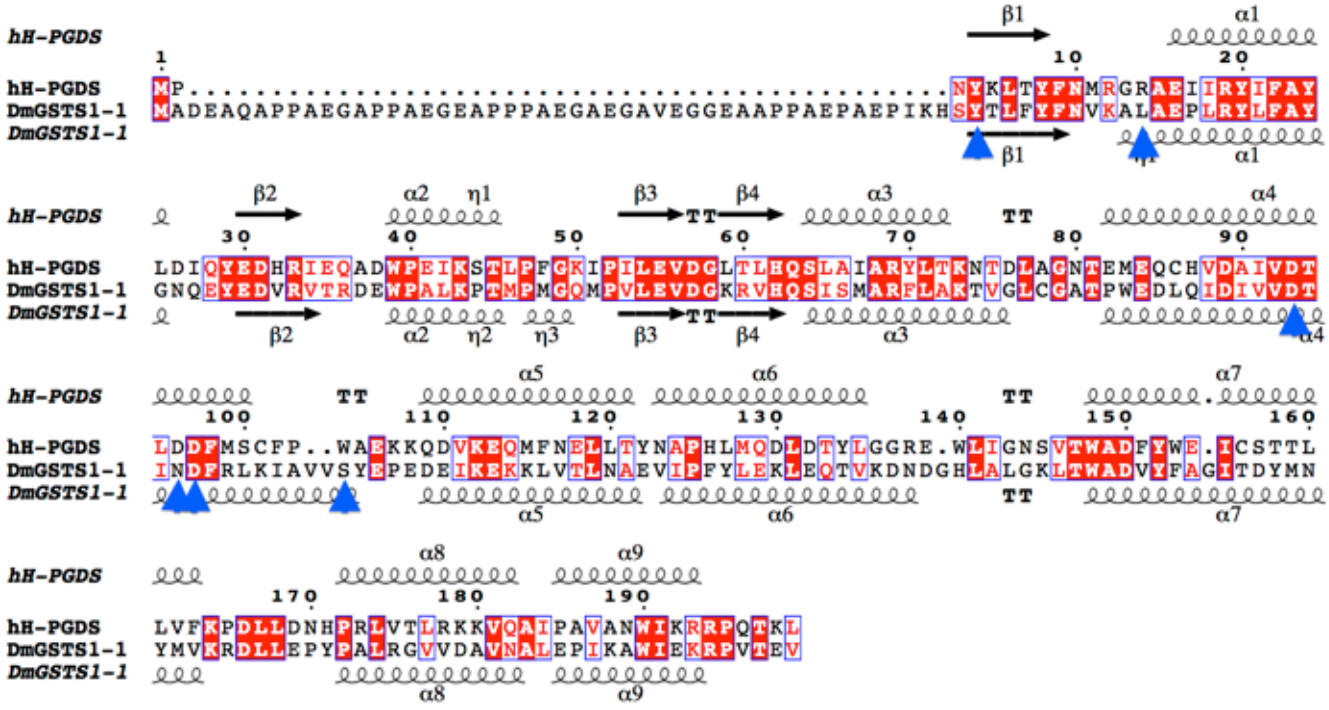


Figure 13. Sequence alignment of human H-PGDS (hH-PGDS) and DmGSTS-1. Top secondary structure was extracted from hH-PGDS (PDB ID 4EC0) and bottom secondary structure was extracted from DmGSTS-1 (PDB ID 1M0U). Key functional residues, Tyr8, Arg14, Trp104, Asp93, Asp96 and Asp97, are marked with blue arrows.

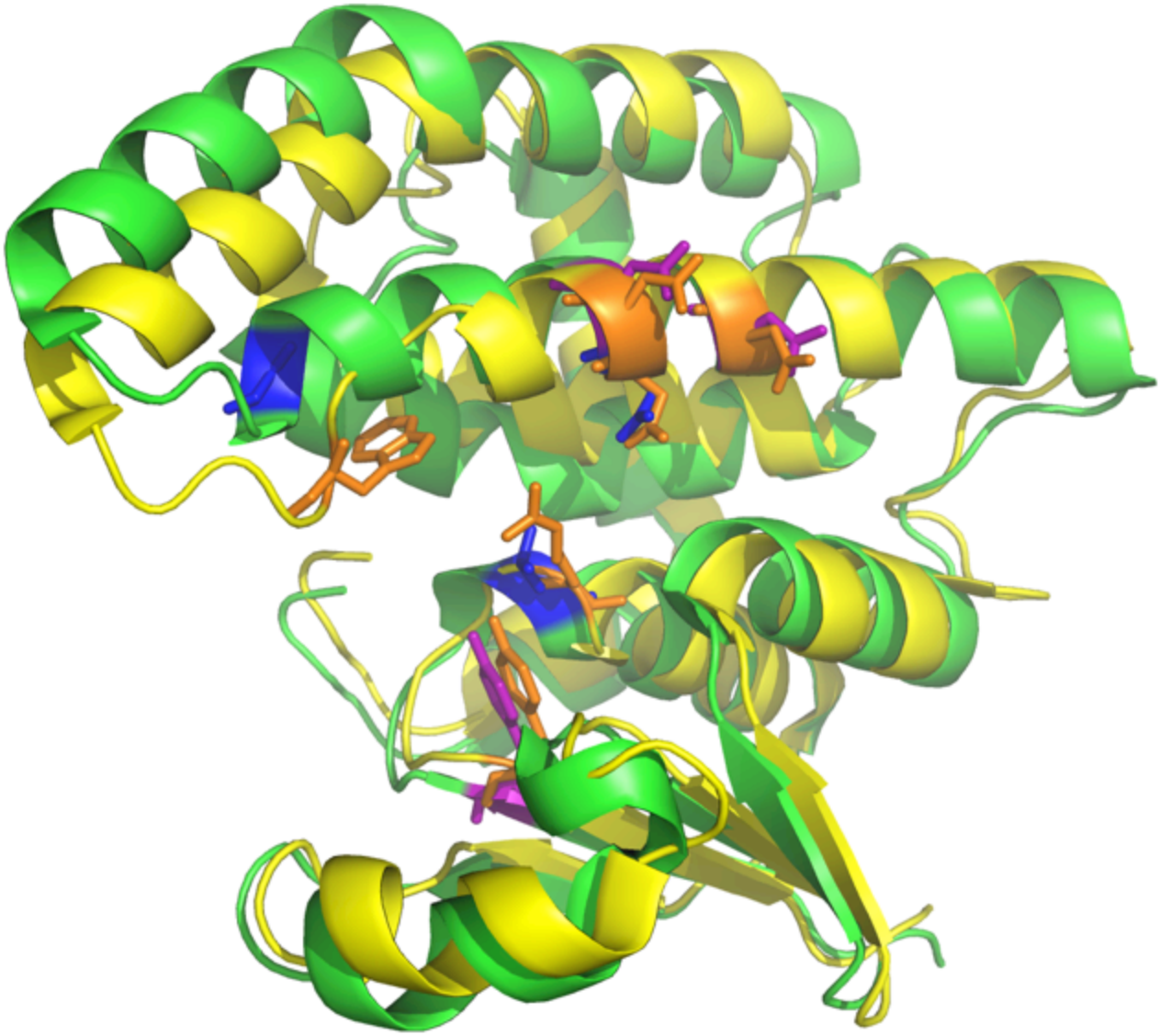


Figure 14. Structural alignment of hH-PGDS and DmGSTS1-1, showing the key functional residues. Protein structures are rendered in cartoon. hH-PGDS (PDB ID 4EC0) is shown in yellow, and DmGSTS1-1 (PDB ID 1M0U) is shown in green. Key residues - Tyr4, Arg13, Trp104, Asp93, Asp96 and Asp97 of hH-PGDS are shown as orange sticks. Key residues – Tyr54, Asp139 and Asp143 of DmGSTS1-1 are shown as purple sticks. Residues of DmGSTS-1, Leu60, Ser152 and Asn142 that are different from the human counterparts are shown as blue sticks.

Theoretical assignment of CG6084 *et al.* as PGD 11-ketoreducatse

PGD 11-ketoreductase, also known as aldo-keto reductase family 1 member C3 (AKR1C3) is one of the three enzymes that catalyze the synthesis of $PGF_{2\alpha}$ (table 4) (Kikuko Watanabe 2002). PGD 11-ketoreductase reduces the 11-keto group of PGD_2 and produces 9α, 11β-$PGF_{2\alpha}$, which is a $PGF_{2\alpha}$ stereoisomer (Chen, Watanabe, and Hayaishi 1992).

Table 4. Comparison of all the enzymes that catalyze the reactions that produce $PGF_{2\alpha}$

	Prostaglandin F synthase	PGE 9-ketoreductase = Carbonyl reductase 1	PGD 11-ketoreductase
EC	1.1.1.188	1.1.1.189	1.1.1.188
Substrate	PGH_2	PGE_s	PGD_2
Product	$PGF2_{2\alpha}$	$PGF_{2\alpha}$	9α, 11β-$PGF_{2\alpha}$

Our preliminary research identified multiple *D. melanogaster* proteins that could be PGD 11-ketoreductases. The proteins include CG4083, CG4084, CG10638, CG12766, CG10863, CG9436, CG2767, and CG40064. Sequence identity between *D. melanogaster* proteins and human PGD 11-ketoreductase ranges from 29% to 47%; Sequence similarity between *D. melanogaster proteins* and human PGD 11-ketoreductase ranges from 44% to 66%. All the *D. melanogaster* proteins contain the catalytic residues Tyr55, Lys84 and His117 (figure 15) (Liedtke *et al.* 2013).

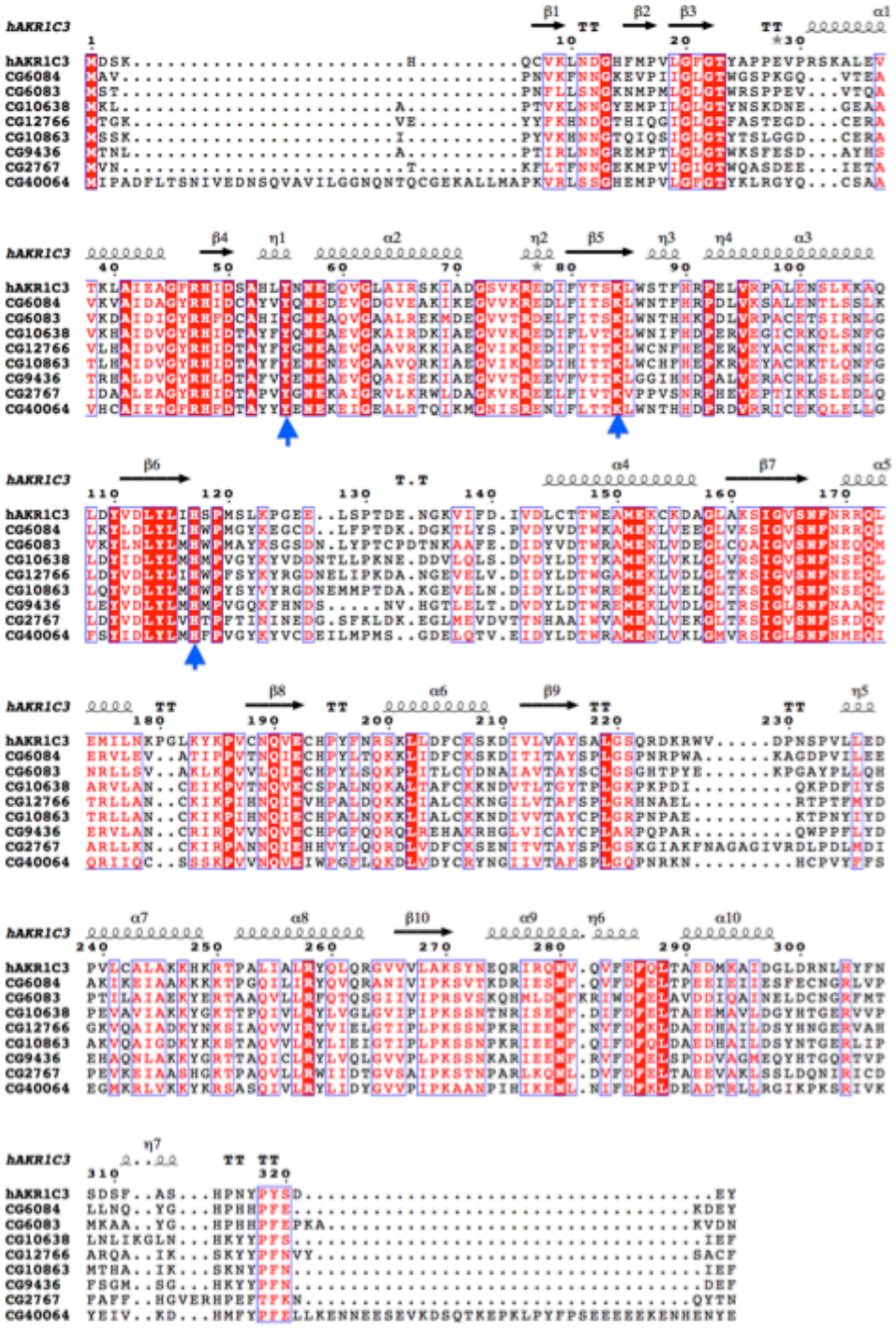
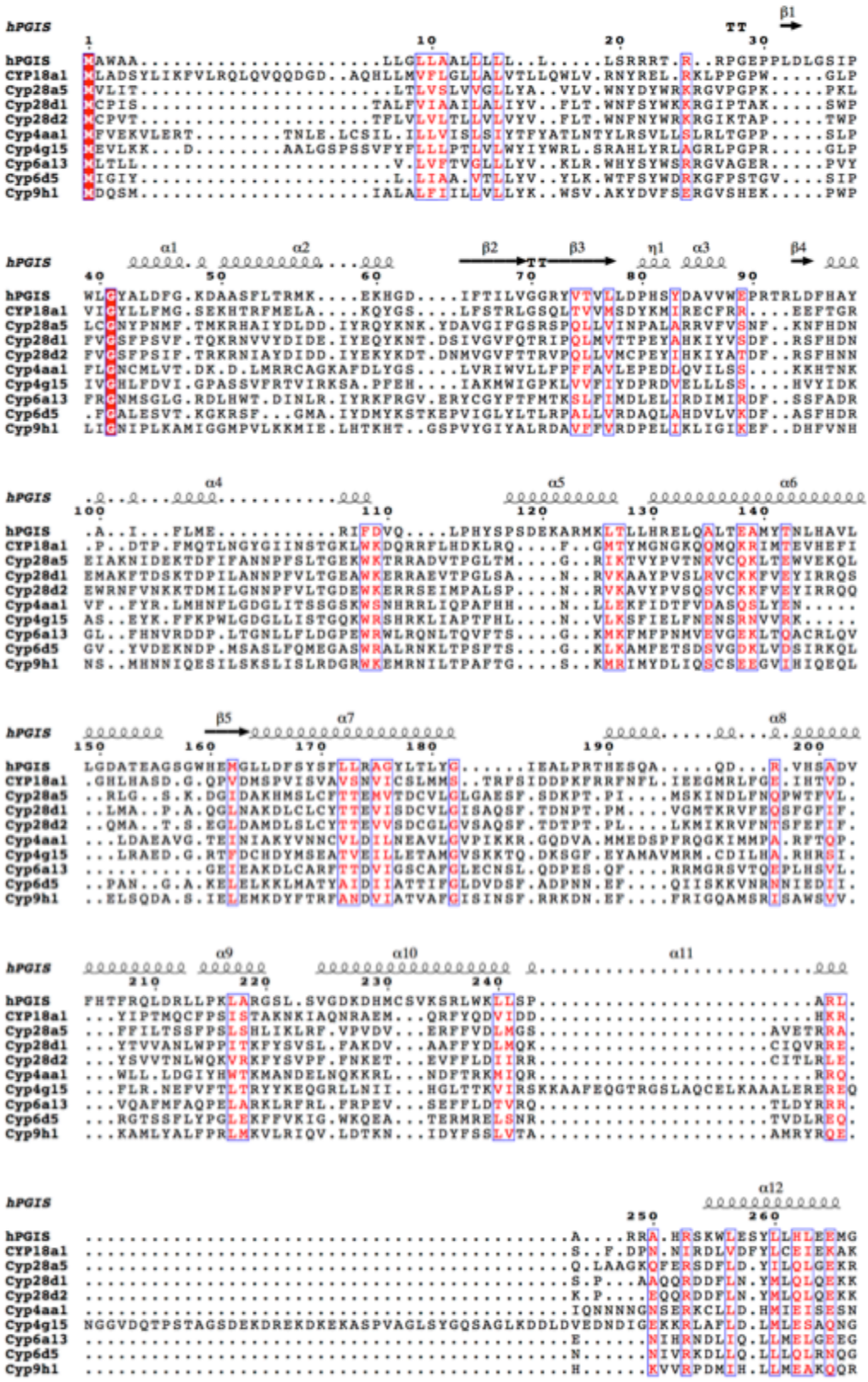


Figure 15. Alignment of human PGD 11-ketoreductase of *D. melanogaster* proteins CG4083, CG4084, CG10638, CG12766, CG10863, CG9436, CG2767, and CG40064. Secondary structure of human PGD 11-ketoreductase is obtained from human PGD 11-ketoreductase (PDB ID 4DBW). Identical/similar residues are boxed in red/white. Catalytic residues Tyr55, Lys84 and His117 are marked with blue arrows.

Theoretical assignment of CYP18a1 *et al.* as prostacyclin synthase (prostaglandin I synthase, PGIS)

PGIS catalyzes the reaction that converts PGH_2 into PGI_2. Our initial analysis identified nine *D. melanogaster* proteins, including CYP18a1, CYP28a5, CYP28d1, CYP28d2, CYP4aa1, CYP4g15, CYP6a13, CYP6d5 and CYP8h1, that share sequence similarity with human PGIS (hPGIS) and possess the key catalytic signature of a PGIS (figure 16). All the *D. melanogaster* CYP proteins have the key residues required for the catalytic reaction, which include Cys441 (heme iron binding), and Glu347 and Arg350, which are required for the catalytic activity (Hatae *et al.* 1996, 441). Sequence identity/similarity between hPGIS and *D. melanogaster* proteins is 12-15%/29-32%.


hPGIS
hPGIS MAWAA.....LLGLLAALLLL..L.....LSRRRT.R..RPGEPPLDLGSIP
CYP18a1 MLADSYLIKFVLRQLQVQQDGD..AQHLLMVFLGLLALVTLLQWLV.RNYREL.RKLPPGPW.....GLP
Cyp28a5 MVLIT.....LTLVSLVVGLLYA..VLV.WNYDYWRKRGVPGPK.....PKL
Cyp28d1 MCPIS.....TALFVIAAILALIYV..FLT.WNFSYWKKRGIPTAK.....SWP
Cyp28d2 MCPVT.....TFLVLVLTLLVLVYV..FLT.WNFNYWRKRGIKTAP.....TWP
Cyp4aa1 MFVEKVLERT.....TNLE.LCSIL.ILLVISLSIYTFYATLNTYLRSVLLSLRLTGPP.....SLP
Cyp4g15 MEVLKK...D.....AALGSPSSVFYFLLLPTLVLWYIYWRL.SRAHLYRLAGRLPGPR.....GLP
Cyp6a13 MLTLL.....V.LVFTVGLLLYV..KLR.WHYSYWSRRGVAGER.....PVY
Cyp6d5 MIGIY.....L.LIAA.VTLLYV..YLK.WTFSYWDRKGFPSTGV....SIP
Cyp9h1 MDQSM.....IALALFIILLVLLYK..WSV.AKYDVFSERGVSHEK.....PWP
hPGIS
hPGIS WLGYALDFG.KDAASFLTRMK....EKHGD....IFTILVGGRYVTVLLDPHSYDAVVWEPRTRLDFHAY
CYP18a1 VIGYLLFMG.SEKHTRFMELA....KQYGS....LFSTRLGSQLTVVMSDYKMIRECFRR....EEFTGR
Cyp28a5 LCGNYPNMF.TMKRHAIYDLDD.IYRQYKNK.YDAVGIFGSRSPQLLVINPALARRVFVSNF..KNFHDN
Cyp28d1 FVGSFPSVF.TQKRNVVYDIDE.IYEQYKNT.DSIVGVFQTRIPQLMVTTPEYAHKIYVSDF..RSFHDN
Cyp28d2 FVGSFPSIF.TRKRNIAYDIDD.IYEKYKDT.DNMVGVFTTRVPQLLVMCPEYIHKIYATDF..RSFHNN
Cyp4aa1 FLGNCMLVT.DK.D.LMRRCAGKAFDLYGS....LVRIWVLLFPFFAVLEPEDLQVILSS....KKHTNK
Cyp4g15 IVGHLFDVI.GPASSVFRTVIRKSA.PFEH....IAKMWIGPKLVVFIYDPRDVELLLSS....HVYIDK
Cyp6a13 FRGNMSGLG.RDLHWT.DINLR.IYRKFRGV.ERYCGYFTFMTKSLFIMDLELIRDIMIRDF..SSFADR
Cyp6d5 .FGALESVT.KGKRSF...GMA.IYDMYKSTKEPVIGLYLTLRPALLVRDAQLAHDVLVKDF..ASFHDR
Cyp9h1 LIGNIPLKAMIGGMPVLKKMIE.LHTKHT..GSPVYGIYALRDAVFFVRDPELIKLIGIKEF..DHFVNH
hPGIS
hPGIS .A..I...FLME.....RIFDVQ....LPHYSPSDEKARMKLTLLHRELQALTEAMYTNLHAVL
CYP18a1 .P..DTP.FMQTLNGYGIINSTGKLWKDQRRFLHDKLRQ....F..GMTYMGNGKQQMQKRIMTEVHEFI
Cyp28a5 EIAKNIDEKTDFIFANNPFSLTGEKWKTRRADVTPGLTM....G..RIKTVYPVTNKVCQKLTEWVEKQL
Cyp28d1 EMAKFTDSKTDPILANNPFVLTGEAWKERRAEVTPGLSA....N..RVKAAYPVSLRVCKKFVEYIRRQS
Cyp28d2 EWRNFVNKKTDMILGNNPFVLTGDEWKERRSEIMPALSP....N..RVKAVYPVSQSVCKKFVEYIRRQQ
Cyp4aa1 VF..FYR.LMHNFLGDGLITSSGSKWSNHRRLIQPAFHH....N..LLEKFIDTFVDASQSLYEN.....
Cyp4g15 AS..EYK.FFKPWLGDGLLISTGQKWRSHRKLIAPTFHL....N..VLKSFIELFNENSRNVVRK.....
Cyp6a13 GL..FHNVRDDP.LTGNLLFLDGPEWRWLRQNLTQVFTS....G..KMKFMFPNMVEVGEKLTQACRLQV
Cyp6d5 GV..YVDEKNDP.MSASLFQMEGASWRALRNKLTPSFTS....G..KLKAMFETSDSVGDKLVDSIRKQL
Cyp9h1 NS..MHNNIQESILSKSLISLRDGRWKEMRNILTPAFTG....S..KMRIMYDLIQSCSEEGVIHIQEQL
hPGIS
hPGIS LGDATEAGSGWHEMGLLDFSYSFLLRAGYLTLYG......IEALPRTHESQA.....QD...R.VHSADV
CYP18a1 .GHLHASD.G.QPVDMSPVISVAVSNVICSLMMS..TRFSIDDPKFRRFNFL.IEEGMRLFGE.IHTVD.
Cyp28a5 ..RLG..S.K.DGIDAKHMSLCFTTEMVTDCVLGLGAESF.SDKPT.PI....MSKINDLFNQPWTFVL.
Cyp28d1 ..LMA..P.A.QGLNAKDLCLCYTTEVISDCVLGISAQSF.TDNPT.PM....VGMTKRVFEQSFGFIF.
Cyp28d2 ..QMA..T.S.EGLDAMDLSLCYTTEVVSDCGLGVSAQSF.TDTPT.PL....LKMIKRVFNTSFEFIF.
Cyp4aa1 ...LDAEAVG.TEINIAKYVNNCVLDILNEAVLGVPIKKR.GQDVA.MMEDSPFRQGKIMMPA.RFTQP.
Cyp4g15 ...LRAED.G.RTFDCHDYMSEATVEILLETAMGVSKKTQ.DKSGF.EYAMAVMRM.CDILHA.RHRSI.
Cyp6a13 ..........GEIEAKDLCARFTTDVIGSCAFGLECNSL.QDPES.QF....RRMGRSVTQEPLHSVL.
Cyp6d5 ..PAN..G.A.KELELKKLMATYAIDIIATTIFGLDVDSF.ADPNN.EF....QIISKKVNRNNIEDII.
Cyp9h1 ..ELSQDA.S.IELEMKDYFTRFANDVIATVAFGISINSF.RRKDN.EF....FRIGQAMSRISAWSVV.
hPGIS
hPGIS FHTFRQLDRLLPKLARGSL.SVGDKDHMCSVKSRLWKLLSP.....ARL.
CYP18a1 ...YIPTMQCFPSISTAKNKIAQNRAEM...QRFYQDVIDD.....HKR.
Cyp28a5 ...FFILTSSFPSLSHLIKLRF.VPVDV...ERFFVDLMGS.....AVETRRA.
Cyp28d1 ...YTVVANLWPPITKFYSVSL.FAKDV...AAFFYDLMQK.....CIQVRRE.
Cyp28d2 ...YSVVTNLWQKVRKFYSVPF.FNKET...EVFFLDIIRR.....CITLRLE.
Cyp4aa1 ...WLL.LDGIYHWTKMANDELNQKKRL...NDFTRKMIQR.....RRQ.
Cyp4g15 ...FLR.NEFVFTLTRYYKEQGRLLNII...HGLTTKVIRSKKAAFEQGTRGSLAQCELKAAALERERE Q
Cyp6a13 ...VQAFMFAQPELARKLRFRL.FRPEV...SEFFLDTVRQ.....TLDYRRR.
Cyp6d5 ...RGTSSFLYPGLEKFFVKIG.WKQEA...TERMRELSNR.....TVDLREQ.
Cyp9h1 ...KAMLYALFPRLMKVLRIQV.LDTKN...IDYFSSLVTA.....AMRYRQE.
hPGIS
hPGIS .....A....RRA.HRSKWLESYLLHLEEMG
CYP18a1 .....S..F.DPN.NIRDLVDFYLCEIEKAK
Cyp28a5 .....Q.LAAGKQFERSDFLD.YILQLGEKR
Cyp28d1 .....S.P...AAQQRDDFLN.YMLQLQEKK
Cyp28d2 .....K.P....EQQRDDFLN.YMLQLQEKK
Cyp4aa1 .....IQNNNNGNSERKCLLD.HMIEISESN
Cyp4g15 NGGVDQTPSTAGSDEKDREKDKEKASPVAGLSYGQSAGLKDDLDVEDNDIGEKKRLAFLD.LMLESAQNG
Cyp6a13 .....E......NIHRNDLIQ.LLMELGEEG
Cyp6d5 .....N......NIVRKDLLQ.LLLQLRNQG
Cyp9h1 .....H......KVVRPDMIH.LLMEAKQQR

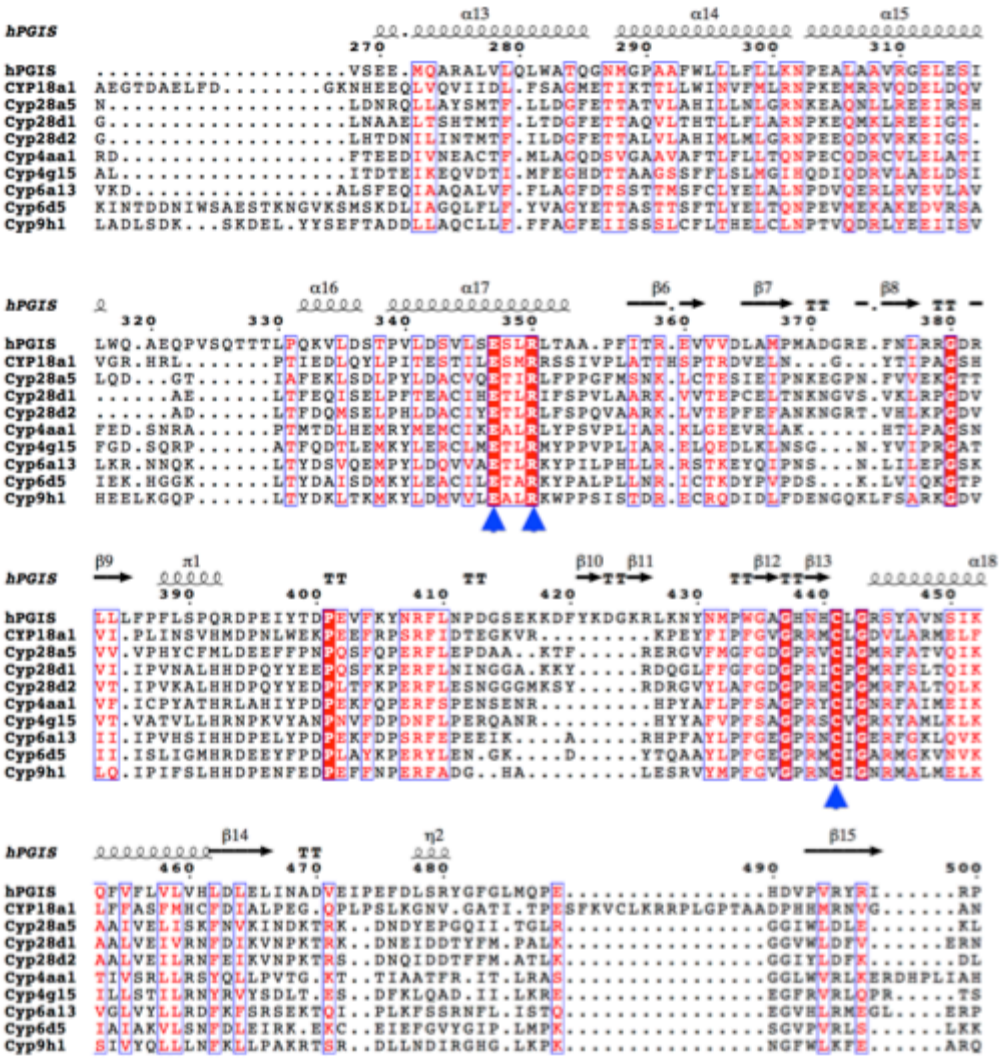


Figure 16. Alignment of hPGIS and *D. melanogaster* proteins CYP18a1, CYP28a5, CYP28d1, CYP28d2, CYP4aa1, CYP4g15, CYP6a13, CYP6d5 and CYP8h1. Secondary structure is obtained from hPGIS (PDB ID 2IAG). Identical/similar residues are boxed in red/white. Catalytically important residues Cys441, Glu347 and Arg350 are marked with blue arrows.

Theoretical assignment of CYP6a18 *et al.* as thromboxane-A synthase

Thromboxane-A synthase (TBXAS) catalyzes the reaction that converts PGH_2 into TxA_2. Our initial analysis identified many *D. melanogaster* CYP proteins that share sequence similarity with human thromboxane-A synthase (hTBXAS), which include CYP6a18, CYP6a19, CYP6a8, CYP6t1, CYP6w1 and CYP9c1 (figure 17). Sequence similarity and identity between hTBXAS and *D. melanogaster* proteins ranges from 24-26% and 43-48%, respectively. Very little is known about the catalytic mechanism of this enzyme (Ullrich 2003). The structure of thromboxane-A synthase has not been solved, and very little is known about the enzyme and its mechanism (William L Smith, Urade, and Jakobsson 2011).

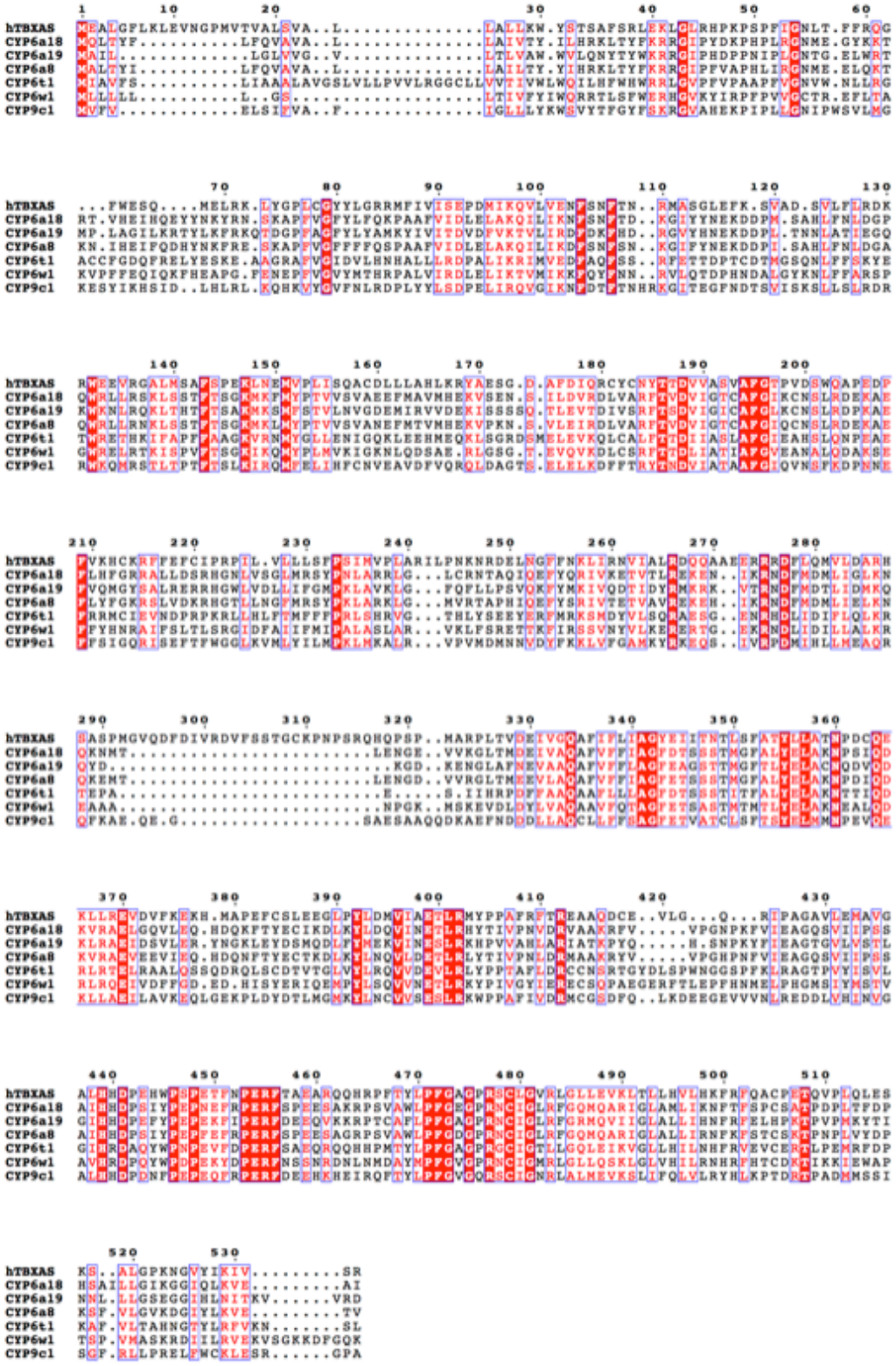


Figure 17. Sequence alignment of hTBXAS and *D. melanogaster* proteins CYP6a18, CYP6a19, CYP6a8, CYP6t1, CYP6w1 and CYP9c1. Identical/similar residues are boxed in red/white.

Chapter 4 Identification and Characterization of Putative Cyclooxygenase Enzymes in *Drosophila melanogaster*

Identification, chromosomal localization, and domain architectures of ten candidate COXs in *D. melanogaster*

We have identified ten candidate COX proteins from the *D. melanogaster* genome database (Release 5.30, May 16th, 2011) using a combination of iterative BLAST and HMMER searches based on sequence similarity with the hCOX-1 protein: CG3131 (dual oxidase (Duox)), CG3477 (peroxidase (Pxd)), CG4009, CG5873, CG6969 (Cardinal), CG7660 (peroxinectin-like (Pxt)), CG8913 (immune regulated catalase (Irc)), CG10211, CG12002 (peroxidasin (Pxn)) and CG42331 (Table 5). Among these genes, *pxd*, *pxn*, and CG6969 are predicted to encode two protein isoforms due to alternative splicing. Seven of the ten candidate COX encoding genes are located on the right arm on chromosome 3, CG3131 and CG10211 are located on left arm of chromosome 2, and CG12002 is located on the left arm of chromosome 3 (Figure 18).

Table 5. Length, CG number, chromosomal location and evidence level of the ten proteins in *D. melanogaster*

BLAST hits	GI	Length	CG number	Chromosomal location	Evidence level
CG4009	24647576	649aa	CG4009	3R	Transcript
CG5873	24647689	753aa	CG5873	3R	Transcript
CG6969, Cardinal	24649111	830aa	CG6969	3R	Transcript
CG10211	19921482	1394aa	CG10211	2L	Transcript
Irc	21356609	697aa	CG8913	3R	Protein (Ha *et al.* 2005)
Pxd	45553389	690aa	CG3477	3R	Transcript
Pxt	28571758	809aa	CG7660	3R	Protein (Sritunyalucksana *et al.* 2001)
CG3131	281364292	1537aa	CG3131	2L	Transcript
CG12002, Pxn	24656151	1527aa	CG12002	3L	Transcript
CG42331	221459132	1615aa	CG42331	3R	Transcripts

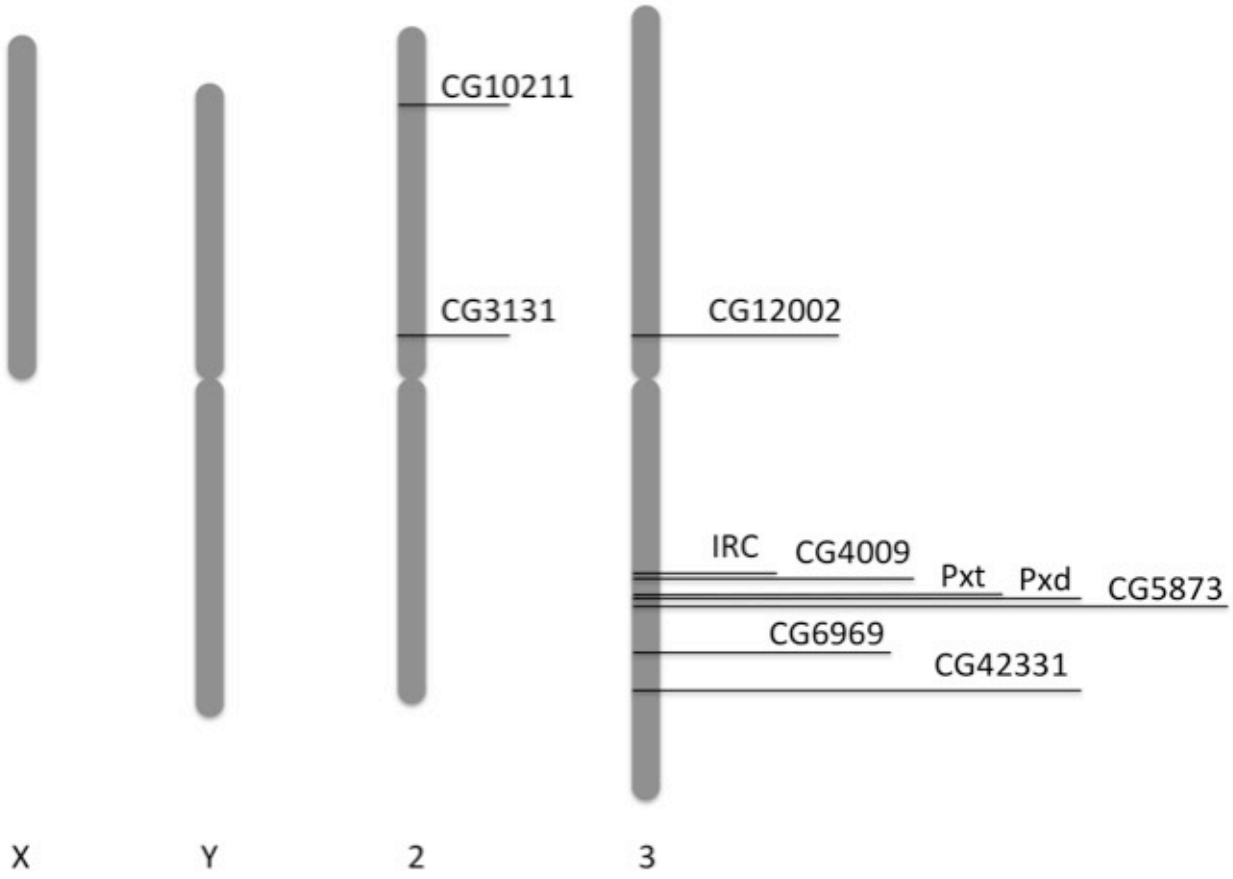


Figure 18. Chromosomal location of ten putative *Drosophila melanogaster* genes. CG10211 and CG3131 are located on the left arm of chromosome 2. CG12002 is located on the left arm of chromosome 3. *Irc*, CG4009, *pxt*, *pxd*, CG5873, CG6969 and CG42331 are located on the right arm of chromosome 3.

Mammalian COX proteins function as homodimers; each monomer contains (1) an N-terminal epidermal growth factor domain (EGF), (2) a membrane binding domain (MBD) with four amphipathic helices, and (3) a C-terminal catalytic domain of approximately 460 residues (Picot, Loll, and Garavito 1994; Luong *et al.* 1996; Kurumbail *et al.* 1996). Consensus from various domain architecture analysis programs

suggests that although all of the *D. melanogaster* proteins have at least one of the signature animal heme peroxidase domains located at the C-terminus (CG10211 is unique in housing two animal heme peroxidase domains), the rest of the domain architecture in the candidate *D. melanogaster* proteins bears little resemblance to the mammalian counterparts (Figure 19). In addition, none of the *D. melanogaster* proteins appears to have a detectable EGF-like domain at their N-termini. Although typical motifs associated with mammalian COX–like membrane domain are not detected in the *D. melanogaster* proteins, secondary structure analysis reveals helical content in their N-terminal regions raising the possibility that a helical structural domain similar to the mammalian COX MBDs may also be present in the fly proteins.

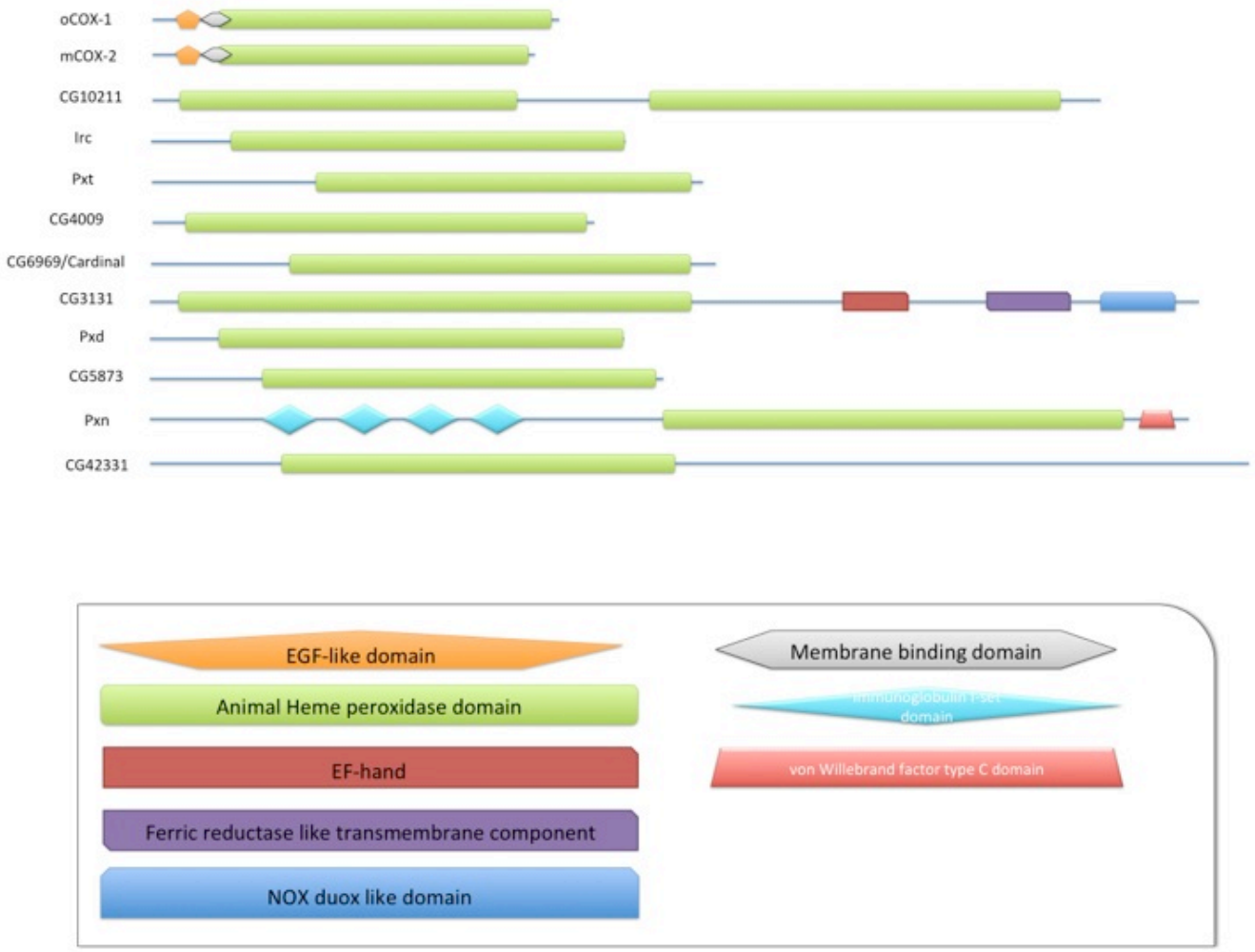


Figure 19. Schematic domain architecture of putative cyclooxygenase proteins in *Drosophila melanogaster*. Sequence length and domain position are proportional to actual amino acid sequences.

COX genes are conserved across different phylogenetic clades including insects

COXs genes have been found in the genomes of non-mammalian vertebrates, invertebrates (Koljak *et al.* 2001), plants (Sanz, Moreno, and Castresana 1998), bacteria, and fungi (Gao *et al.* 2003). A preliminary phylogenetic analysis of known and putative COX enzymes reveals that COX-1 enzymes and COX-2 are clustered into separate groups and fungal, bacterial, and insect COXs appear to be quite distant from vertebrate COXs, and closer to plant COXs. The existence of COX-2 like genes appears only in vertebrate genomes, whereas COX-1 gene is more widespread. The branches for bacteria, fungi, arthropods, and vertebrates are distinct, indicating that gene divergence of COX-1 and COX-2 occurred relatively early, before the speciation of arthropods (Koljak *et al.* 2001).

The putative *D. melanogaster* COXs are quite distinct in sequence from the known mammalian COXs, a pattern observed in most other insect genomes that have been sequenced except for two mammalian parasitic insects, *Pediculus humanus corporis* and *Acyrthosiphon pisum,* where the putative enzymes seem to be more closely related to mammalian COXs (figure 20). Our phylogenetic analysis suggests that although insects have more than one COX–like gene, they all cluster with COX-1, the housekeeping enzyme. Sequence and structural analysis detailed below also suggest COX-1-like features for the three putative *D. melanogaster* COXs, including residues His207, Tyr385, Tyr388 that are required for peroxidase and cyclooxygenase activities. The close proximity of the genes that encode CG4009 and Pxd, and high sequence

similarity of CG4009 and Pxd (Figure 18) suggest that they may have arisen from a gene duplication event. While all the insect COX homologues are significantly different from mammalian COXs from an evolutionary standpoint, all insects genomes analyzed (except for *Bombyx mori*), do possess putative homologous sequences of COX proteins, similar to the proteins identified in *D. melanogaster*.

Figure 20. Phylogenetic tree of COX proteins in different organisms. Bootstrap value is labeled at every node.

Sequence analysis of the candidate protein reveals only three of the candidate proteins possess key catalytic and other functional residues

To further scrutinize the candidate proteins for the conservation of key functional residues within the protein domains and to identify the hallmarks of COX proteins, we built a multiple sequence alignment of the animal heme peroxidase domain of ovine COX-1 (oCOX-1) and murine COX-2 (m-COX-2) (structural representatives for mammalian COX-1 and COX-2 respectively (Picot, Loll, and Garavito 1994; Kurumbail *et al.* 1996)) with the candidate proteins (table 6). The overall similarity in sequence between the seven candidate proteins and oCOX-1/mCOX-2 is low, but there seems to be some conservation of key functional residues as shown in table 5. (1) Functionally important residues for mammalian cyclooxygenase activity include the catalytic triad of His207, Tyr385, and His388, and Arg120, Gln203, Val349 and Ser530 (table 6; numbering based on oCOX-1). Although Arg120 is not housed in the catalytic domain, it is known to interact with the substrate arachidonate (Mancini *et al.* 1995). (2) Gln203 is conserved in all seven fly COX enzymes but the function of this residue in mammalian proteins remains unknown. (3) Val/Leu349 has been suggested to determine substrate specificity: All mammalian COXs have Val349 and utilize arachidonic acid as substrate, while in some invertebrate COXs, the valine is substituted by a leucine, and this changes their substrate specificity towards linoleic acid (Garscha and Oliw 2009). (4) Acetylation of Ser530 is the functional basis of inhibition of COX by aspirin (Loll, Picot, and Garavito 1995). (5) Alignment of the animal heme peroxidase domain of candidate fly COXs and oCOX-1/mCOX-2 reveals that only Pxt, Pxd and CG4009 have all three required residues for COX catalytic activity (Figure 21). A preliminary

structural modeling of the seven other candidates confirms the absence of the complete catalytic signature at the structural level as well (data not shown). (5) All three putative *D. melanogaster* COXs have residues corresponding to Gln203, but none of them have the residue corresponding to Arg120 in oCOX-1, located in the oCOX1 MBD. (6) While none of the seven fly candidates have a serine that aligns perfectly with mammalian Ser530, CG6969, the first of the two animal peroxidase domain of CG10211, CG5873 and Irc have a serine located in the vicinity of Ser530. (7) All seven *D. melanogaster* proteins have a leucine residue instead of valine at the position corresponding to Val349, indicating that *D. melanogaster* may have a different preferred substrate compared to the mammalian COXs. (8) Other striking features include a large insertion of around 60 residues in the N-terminus in *D. melanogaster* proteins outside the peroxidase domain.

Table 6. Comparison of ten COX candidate proteins from *Drosophila melanogaster*

	Arg120 (interact with arachidonate)	Gln203 (POX)	His207 (POX)	Tyr385 (COX)	His388 (POX)	Val349/Leu substrate determination	Ser530 (acetylation by aspirin)
CG3131	F	T	F	F	T	Leu	F
CG4009	F	T	**T**	**T**	**T**	Leu	F
CG5873	F	T	T	F	T	Leu	F
CG6969	F	T	T	F	T	Leu	F
CG10211	F	T	F	F	F	Leu	F
	F	T	T	F	T	Leu	F
CG42331	F	T	T	F	T	Leu	F
Irc	F	T	T	T	F	Leu	F
Pxd	F	T	**T**	**T**	**T**	Leu	F
Pxn	F	T	T	F	T	Leu	F
Pxt	F	T	**T**	**T**	**T**	Leu	F

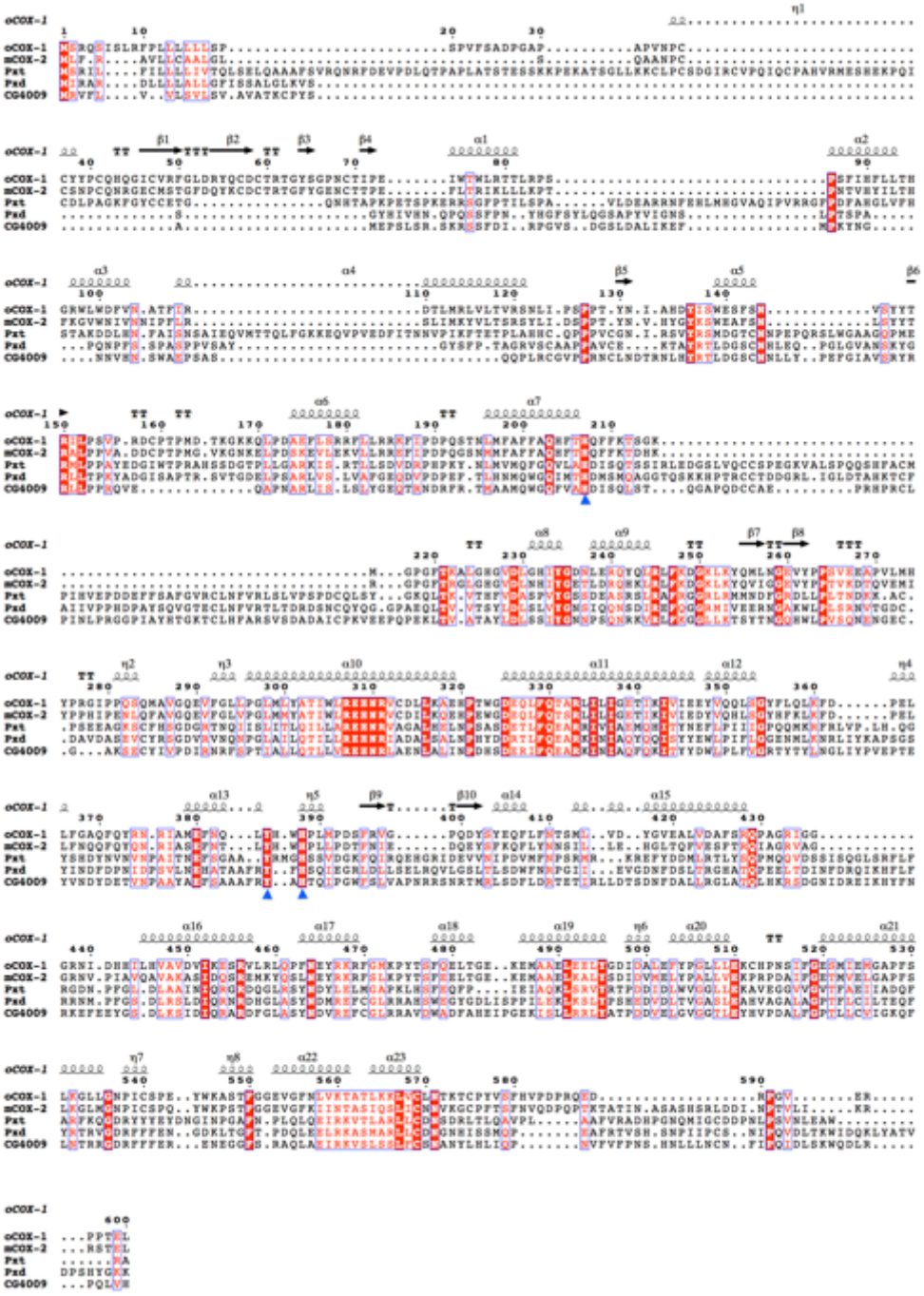


Figure 21. Sequence alignment showing conserved important functional residues in the three COX candidates. Pxt, Pxd and CG4009 have the three required residues – His207, Tyr385 and His 388. Structure profile of oCOX-1 is obtained from sheep COX-1 (PDB ID 1CQE). Identical/similar residues are boxed in red/white. Locations of His207, Tyr385 and His388 are marked with blue arrows.

Molecular models of the animal peroxidase domains of the putative Drosophila COXs predict similarity to mammalian COXs.

To understand the physiochemical properties of all three putative *D. melanogaster* cyclooxygenases, Pxt, Pxd and CG4009 and compare them with the mammalian COX enzymes, we built models of their catalytic domains (figure 22 and table 7). Structurally, all the three proteins are predicted to maintain the catalytic signature in a well–formed and largely hydrophobic catalytic pocket highly similar to the mammalian enzymes (figure 23). Pocket residues of CG4009 are Val161, Ile165, Thr348, Tyr349, Trp352, Leu353, Phe356, Val357, Glu375, Ser393, Ala396, Phe397, Ala400, Phe554, Leu559, Ile562, Gly563, Phe566, Leu567, Thr569 and Arg570. Pocket residues of Pxd include Met183, Met187, Ser384, Tyr385, Trp388, Leu389, Phe392, Leu393, Tyr412, His428, Ala432, Phe433, Phe436, Ala586, Leu591, Leu594, Thr595, Phe598, Tyr599, Thr601 and Arg602. Pocket residues of Pxt contain Leu318, Ile322, Thr517, Tyr518, Phe521, Leu522, Ile525, Ile526, Phe559, Ala563, Tyr564, Met566, Val713, Ala718, Ile721, Ala722, Phe725, Ala726, Phe728 and Lys729. The pocket volumes of CG4009, Pxd and Pxt are 521, 414 and 357 cubic angstroms, respectively, similar to the pocket volume of sheep COX-1, which is 344 cubic angstroms. In contrast, pocket volume of mouse COX-2, which is 1384 cubic angstroms, is much bigger. A structure based sequence alignment of oCOX-1 and the modeled structures of the *D. melanogaster* proteins marginally improves the sequence identity to the range of 12%-24%, and correspondingly, sequence similarity to 28% -39% over a purely sequence based alignment. Insertions in CG4009, Pxt, and Pxd relative to mammalian COXs manifest as three extra loops, one of which is located in the proximity of the

catalytic pocket, changing the shape of the pocket compared to the mammalian COX structures.

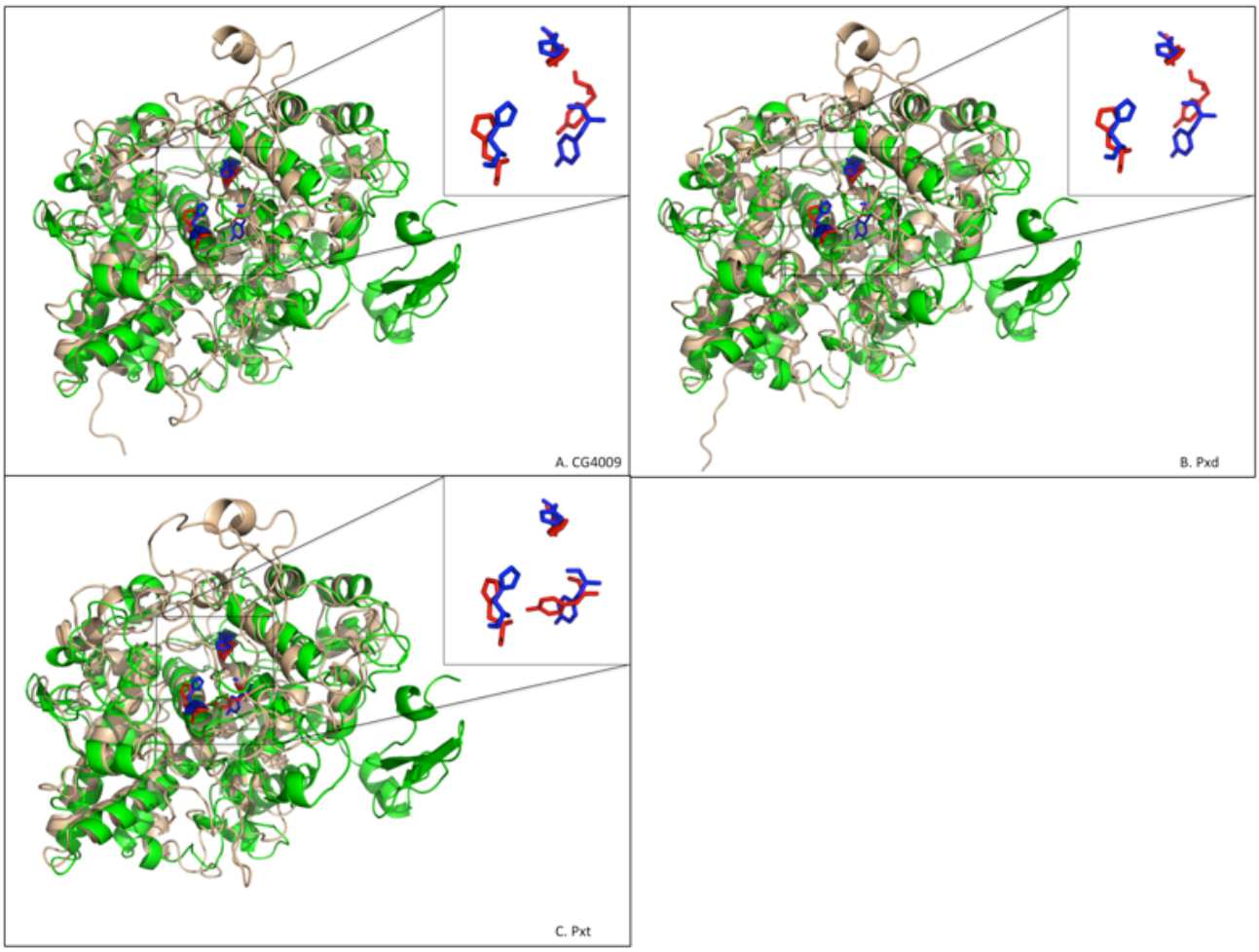
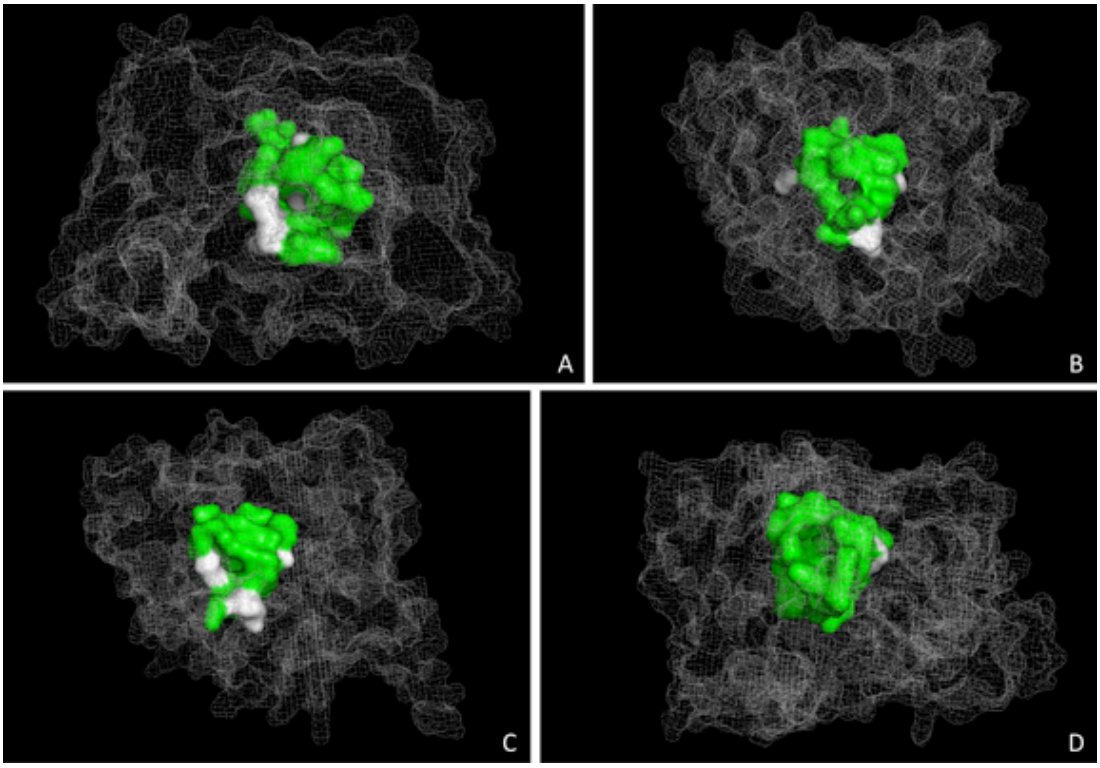


Figure 22. Structural alignment of molecular model of the catalytic animal peroxidase domain of *Drosophila melanogaster* putative COXs and sheep COX-1. Molecular models of the catalytic animal peroxidase domain of *D. melanogaster* putative COXs (silicon) aligned with sheep COX-1 (green, PDB code 1CQE). The catalytic residues (His, Tyr and His) are shown as sticks. His 207, Tyr 385 and His 388 from sheep COX-1 are shown in blue. The corresponding residues from *D. melanogaster* proteins are shown in red. A. Catalytic domain of CG4009 aligned with sheep COX-1. B. Catalytic domain of Pxd aligned with sheep COX-1. C. Catalytic domain of Pxt aligned with sheep COX-1

Table 7. Result of modeling of the catalytic domain of the three putative *D. melanogaster* COXs. RMSD, Z-score and sequence identity/similarity is based on structural alignment of the conserved domain from putative *D. melanogaster* protein and sheep COX-1 (PDB ID: 1CQE)

Protein	RMSD/Å	Sequence Identity	Sequence Similarity
CG4009	2.66	23%	39%
Pxd	2.85	20%	38%
Pxt	2.81	24%	37%

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                 *        20         *        40         *        60         *        80         *       100         *       120         *
oCOX-1 :  PVNPCCYYPCQHQGICVRFGLDRYQCDCTRTGYSGPNCTIPEIWTWLRTTLRPSPSFIHFLLTHGRWLWDFVNATFIRDTLMRLVITVRSNLIPSPPTYNIAHDYISWESFSNVSYYTRILPSVPRDCPTPMG : 133
CG4009 :  QPLRCGVPPRNCLNDTRNLHYRTLDGSCNNLL--YPEFGIAVSRYRRLLPPRQV--------EQ-------------------------------------------------------------------- :  54
Pxd    :  ------------------KTAYRTLDGSCNHLE--QPGLGVANSKYGRGLLTPKYADGISAPT-RSV-------------------------------------------------------------------- :  45
Pxt    :  --------------------YRSMDGTCNNPEPQRSLWGAAGQPMERMLPPAYEDGIWTPRAHSS-------------------------------------------------------------------- :  45

                140        *       160        *       180        *       200        *       220        *       240        *       260
oCOX-1 :  TKGKKQLPDAEFLSRRFLLRRKFIPDPQGTNLMFAFFAQHFTHQFFKTSGKM-------------------------------------------------------GP---------------- : 187
CG4009 :  ------APNARLISLSLYGEQTRNDR--FRTMAAMQWGQFVAHDLSQLSTQGAP------QDCCAEP-------R-------------HPRCLPINLPRGGPIAYHTGKTCLHFARSVSDADAICPKVEEPQP : 153
Pxd    :  --TGDELPSARLVSLVAFGEQDVPDP--EFTLHNMQWGQINTHDMSMQAGGTQSKKHPT-RCCTDDGRLIGLDTA-------------HKTCFAIIVPPHDPAYSQVGTECLNFVRTLTDRDS-NCQYQGGPA : 159
Pxt    :  --DGTPLLGARKISRTLLSDVDRPHP--KYNLMVMQFGQVLAHDLSQTSSIRLE--DGSLVQC-----------CSPEGKVALSPQQSHFACMPIHVEPDDEFFSAFGVRCLNFVRLSLVPSP-DCQL--SYG : 158

                *       280        *       300        *       320        *       340        *       360        *       380        *       40
oCOX-1 :  -GFTKALGHGVDLGHIYGDNLERQYQLRLFKDGKLKYQMLNGEVYPPSVEEAPVLMHYPRGIPP------------QSQMAVGQEVFGLLPGLMLYATIWLREHNRVCDLLKAEHPTWGDEQLFQTARLILIG : 307
CG4009 :  EKLTVA-TAYLDLSSIYGNNPSQNRKVRLFKGGLLKTSYTNGQHWLPVS--------------QNENGECGA---KSECYIVPDIRNRFSPTIALLQTLLVREHNRLAENLALINPDHSDERIFQEARKINIA : 268
Pxd    :  EQLTVV-TSYLDLSLVYGNSIQQNSDIREFQGGRMIVEERNGAKWLPLS--------------RNVTGDCDAVDASEVCYRSGDVRVNQNPGLAILQTILLREHNRIADALSALNPHYDDRTLFQEARKINIA : 277
Pxt    :  KQLTKV-THFVDASPVYGSSDEASRSLRAFRGGRLRMMNDFGRDLLPLT---------------NDKKACPSEEAGKSCFHSGDGRTNQIISLITLQILLARERHNRVAGALHELNPSASDETLFQEARRIVIA : 275

                0          *       420        *       440        *       460        *       480        *       500        *       520        *
oCOX-1 :  ETIKIVIEEVVQQLSG-------------------------------FLQLKFDPELLFGAQFQYRNRIAMEFNQLYHWHPLMPDSFRV------GPQDSYEQFLENTS-MLV-------DYGVEALVDAFS : 396
CG4009 :  QFQKITTYDMLPLFVGRTYTYLNGLIYPVEPTF----------Y----------VND-YDETVNPAAYAEFSAAAFRYAHTQIPGWFSLVAPNRRSNRTMRLSDFLDRTE----TIRLLDTSDNFDALLRGLA : 376
Pxd    :  QYQQISEYEMLPIFLGG-ENML----------KNRLIYKAPSGSF---------IND-FDPNIDPSVLNEMATAAFRYSHSQIEGRLDLLSELRQVLGSLTLSDWFNRPG-IIEV------GDNFDSLTRGHA : 382
Pxt    :  EMQHITTNEFLPILIGPQQMKRFRLVPLHQ--G----------Y---------SHD-YNVNVNPAITNEFSGAAYRWGHSSVDGKFQIRQEHGRIDEVVNIPDVMFNPSRMRKR------E-FYDDMLRTLY : 378

                540        *       560        *       580        *       600        *       620        *       640        *       660
oCOX-1 :  RQPAGRIGGGRNIDHHIL-------------HVAVDVIKESRVLRLQPFNEYRKRFGMKPYT---SFQELTGEKEMAAELELYGDIDALEFYPGLLLEKCHPNSIWGESMIEMGAPFSLKGLLGNPIC---- : 509
CG4009 :  TQLHKR--SDGNIDREIKHYFNRKEPEEYGSDLKSIDIQRARDFGLASYNDVREFCGLRRAVDWADFAHEIPGE-KISLLRRLYATPDDVELGVGGTLEYHVPDALFGPTLLCVIGKQFLNTRRGDRFFFERE : 506
Pxd    :  TQPEEL--TDINFDRQIKHFLFRR-NMPFGSDLRSLDIQRNRDHGLASYNDMREFCGLRRAHSWEGYGDLISPD-ILEKLKSLYPSHEDVDLTVGASLEAHVAGALAGPTFLCIITEQFIRTRVGDRFFFEN- : 510
Pxt    :  SQPMQQ--VDSSISQGLSRFLFRG-DNPFGLDLAAINIQRGRDQGLRSYNDYLELMGAPKLHSFEQFP----IE-IAQKLSRVYRTPDDIDLWVGGLLEKAVEGGVVGVTFAEILADQFARFVQGDRYYYEY- : 502

                *       680        *       700        *       720        *       740
oCOX-1 :  -SPE--YWKASTFGGEVGFNLVKTATLKKLVCLNTK---TCPVV--SFHVP---------------------------- : 552
CG4009 :  NE-----GGFS----RAQLAEIRKVSLSSLFCSNANY--LHLIQPNVFVFPNSH---------------------------- : 549
Pxd    :  -GDK--LTGFT----PDQLEELRKASMARLLCDNGNH--ISSMQPEAFRTVSHSNPIIPCSNIPQVDLTKWIDQKLYA : 579
Pxt    :  -DNGINPGAFN----PLQLQEIRKVTLARLLCDNSDRLTLQAVPLAAF---------------------------- : 545
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E

Figure 23. Comparison of catalytic pocket hydrophobicity in sheep COX-1, *Drosophila melanogaster* CG4009, Pxd and Pxt. Whole subunits were rendered in white mesh except residues forming the cavity. Hydrophobic residues are colored in green. Non-hydrophobic residues are colored in white. A. Catalytic pocket of sheep COX-1. Model shown is sheep COX-1 (PDB code: 1CQE). B. Catalytic pocket of CG4009. C. Catalytic pocket of Pxd. D. Catalytic pocket of Pxt. E. Sequence alignment based on multi-structural alignments of sheep COX-1 (oCOX-1) and *D. melanogaster* proteins showing hydrophobic/non-hydrophobic residues marked in green/yellow shadow.

Docking of substrate and heme in the catalytic pocket of the modeled animal peroxidase domain of the putative D. melanogaster COXs

Both CG4009 and Pxd possess a largely hydrophobic catalytic substrate pocket similar to those in mammalian COXs (W L Smith, DeWitt, and Garavito 2000) to accommodate the lipid substrate(s) (Figure 23). We docked the cofactor of COX, heme, as well as the lipid substrates in the molecular models of the *Drosophila* COXs to predict if they mimic the mammalian COXs in their binding orientation and satisfy the constraints to undergo catalysis. Based on the leucine substitution corresponding to the Val349 of oCOX-1 in the *D. melanogaster* COXs, we also docked linoleic acid in addition to the canonical COX substrate, arachidonic acid and compared their fit in the catalytic pocket, binding orientation and suitability as a substrate. We successfully docked both linoleic acid (data not shown) and arachidonic acid into the catalytic pocket in all three putative *D. melanogaster* peroxidase domains. The C-13 of the docked arachidonic acid and the phenolic oxygen of catalytic tyrosine in CG4009 are in close vicinity (figure 24B), suggesting the theoretical possibility for the reaction to occur. Docking of arachidonic acid in the catalytic pocket of Pxd and Pxt also suggest the same (figure 24C and D). The distance between the C-13 of arachidonic acid and the oxygen of Tyr 399 in CG4009 is 3.8 Å, and for Pxd, the C-13 of arachidonic acid is positioned 2.6 Å from the oxygen of Tyr 435, and for Pxt, the distance between the C-13 of arachidonic acid and the oxygen of Tyr 564 in CG4009 is 3.5 Å.

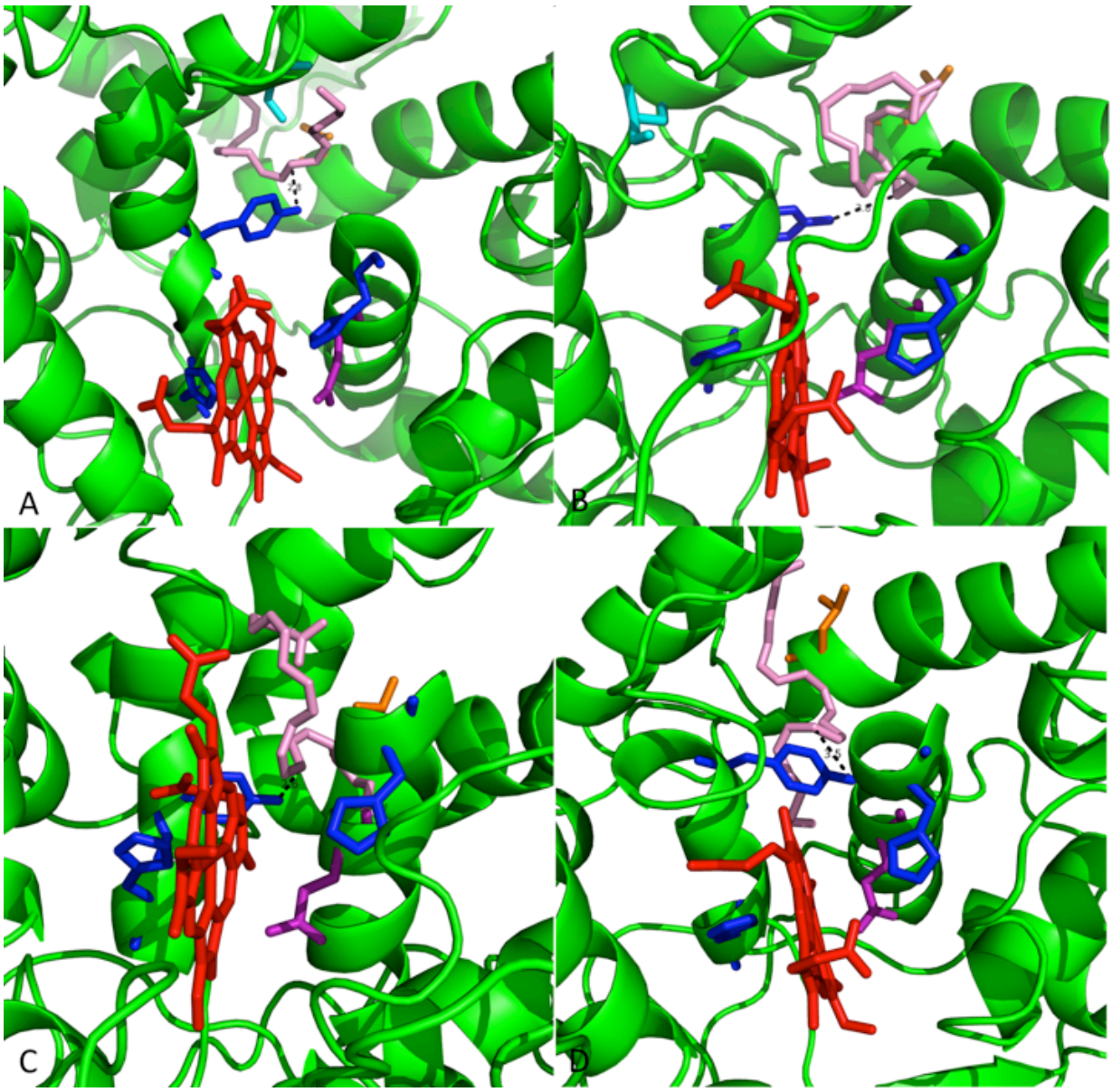

Figure 24. Docking of heme and arachidonic acid into the conserved domain of *D. melanogaster* COXs. Protein structures are rendered in cartoon in green color. Heme B is shown in red. Arachidonic acid is shown in pink. Catalytic residues are shown in blue. Gln203 and equivalents are shown in purple. Val349 and equivalents are shown in orange. Ser530 and equivalents are shown in cyan.
A. Structure of sheep COX-1 (Protein Data Bank code: 1DIY) shows the position of arachidonic acid and important residues. B-D. Computationally generated structures of the conserved catalytic domain from *D. melanogaster* proteins: CG4009, Pxd and Pxt.

Predicted subcellular localization of putative *D. melanogaster* COXs

COX proteins are primarily thought to be membrane-associated proteins. Homodimers of COX are thought to be located at the luminal side of the membrane of the endoplasmic reticulum and of the inner and outer membranes of the nuclear envelope (Otto and Smith 1994; Morita *et al.* 1995; Andrew G. Spencer *et al.* 1998), and anchor to the membrane via a MBD, which consists of four amphipathic helices of 50 amino acids. In comparison, both Pxd and CG4009 are predicted to have a single transmembrane domain each at their N-terminus, part of which encompasses a signal peptide motif (figure 25). It is uncertain if the signal sequence is cleaved and results in a partially cleaved transmembrane domain. On the other hand, the Pxt sequence does not show any characteristic features of a membrane–binding domain in its N-terminal region. Preliminary full length modeling based on combined template-based and *ab initio* approaches suggest that N-terminal region outside of catalytic domain in Pxd, Pxt, and CG4009 form structured helical regions that may manifest as independent domains similar to the MBD in oCOX-1 or integrate with the catalytic domain (figure 26). Mammalian COXs have a N-terminal 16 amino acids signal peptide for secretion, and it is translated and then cleaved by microsomal signal peptidase (Picot, Loll, and Garavito 1994). All three putative *D. melanogaster* COXs are predicted to have signal peptides for secretion similar to the mammalian COXs although divergent in sequence (figure 25).

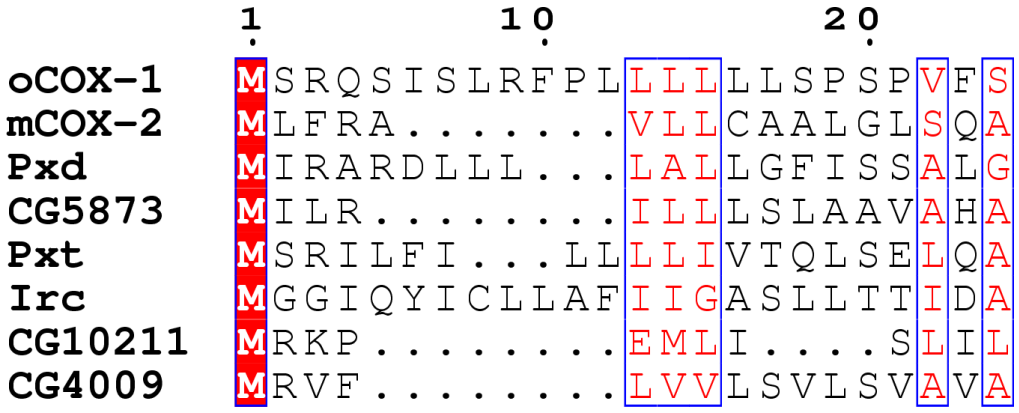

Figure 25. Sequence alignment of signal peptides from putative *D. melanogaster* COXs and human COX-1, COX-2, sheep COX-1 and mouse COX-2. Identical/similar residues are colored in red/white.

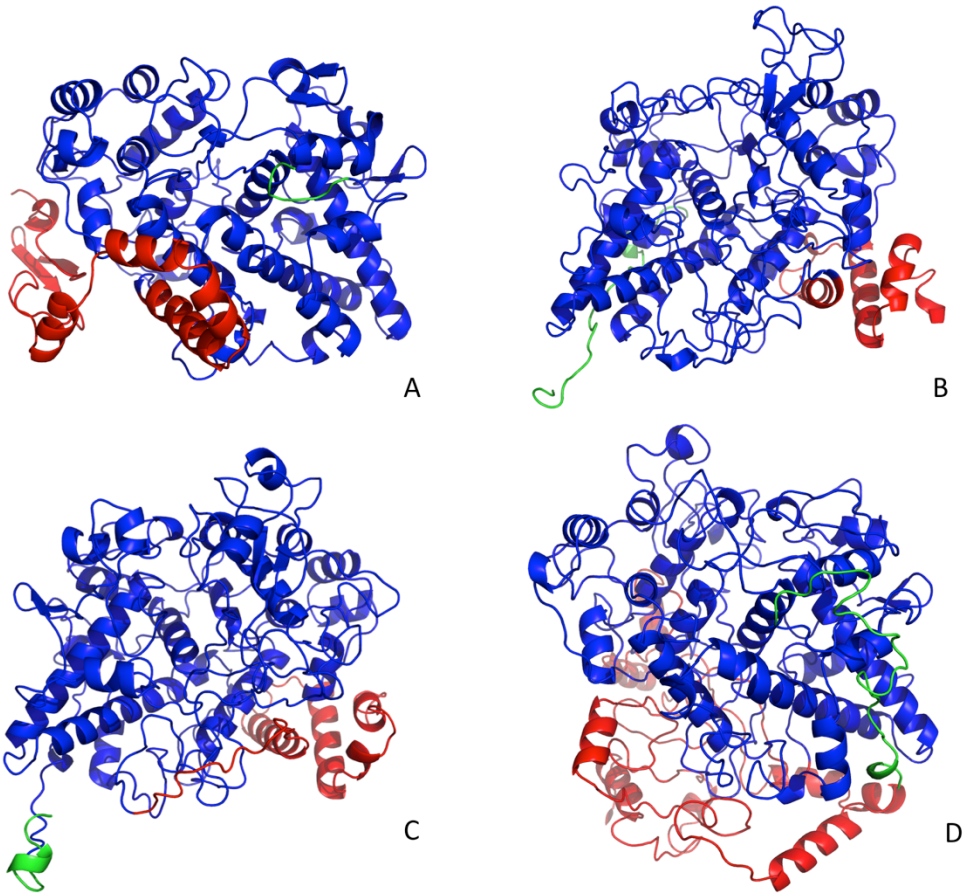

Figure 26. Full-length models of CG4009, Pxd and Pxt show that the N-termini of the proteins are similar to MBD in mammalian COXs. Full-length models built by I-tasser are rendered in cartoon. Blue regions are catalytic domains. Red regions are N-termini. Green regions are C-termini. A. sheep COX-1 (PDB ID: 1CQE). B.CG4009. C. Pxd. D. Pxt

COX activity assay

We used y w, *pxt*$^{f01000/+}$ and *pxt*$^{f01000/f01000}$ female and male flies to compare changes in COX activity in wild type and *pxt* mutant flies. y w files are wild-type flies that are used as the control group. *pxt*^{f01000} is a mutant allele of *pxt*, which contains a Piggybac insertion within the 5' UTR. The insertion is located 38 bp upstream of the start codon (Thibault *et al.* 2004). Tootle *et al.* confirmed that transcriptional expression level of *pxt* is significantly lower in the whole *pxt*$^{f01000/f01000}$ fly (Tootle and Spradling 2008).

We standardized COX activity in all the flies by setting the COX activity level in y w female flies as 100%. We performed paired two-tailed t-tests using Excel to verify whether the three repetitions of experiments are significantly different. The *p* (T<=t) values of three tests were 0.40, 012 and 0.11, which indicates that the data from three repetitions were not statistically different. Our preliminary results (figure 27) show that despite the noise (standard errors are marked in figure 27) in our activity assay there is a trend in COX activity in homozygous *pxt*$^{f01000/f01000}$ flies being lower than y w flies. In female *pxt*$^{f01000/f01000}$ flies, COX level is 50% of that in y w female flies; and in male *pxt*$^{f01000/f01000}$ flies, COX activity level is 40% lower than y w male flies. However, heterozygous male and female *pxt*$^{f01000/f01000}$ flies do not differ from y w male and female flies in COX activity level. In order to establish a clear link of COX activity associated with Pxt, further experiments would be required.

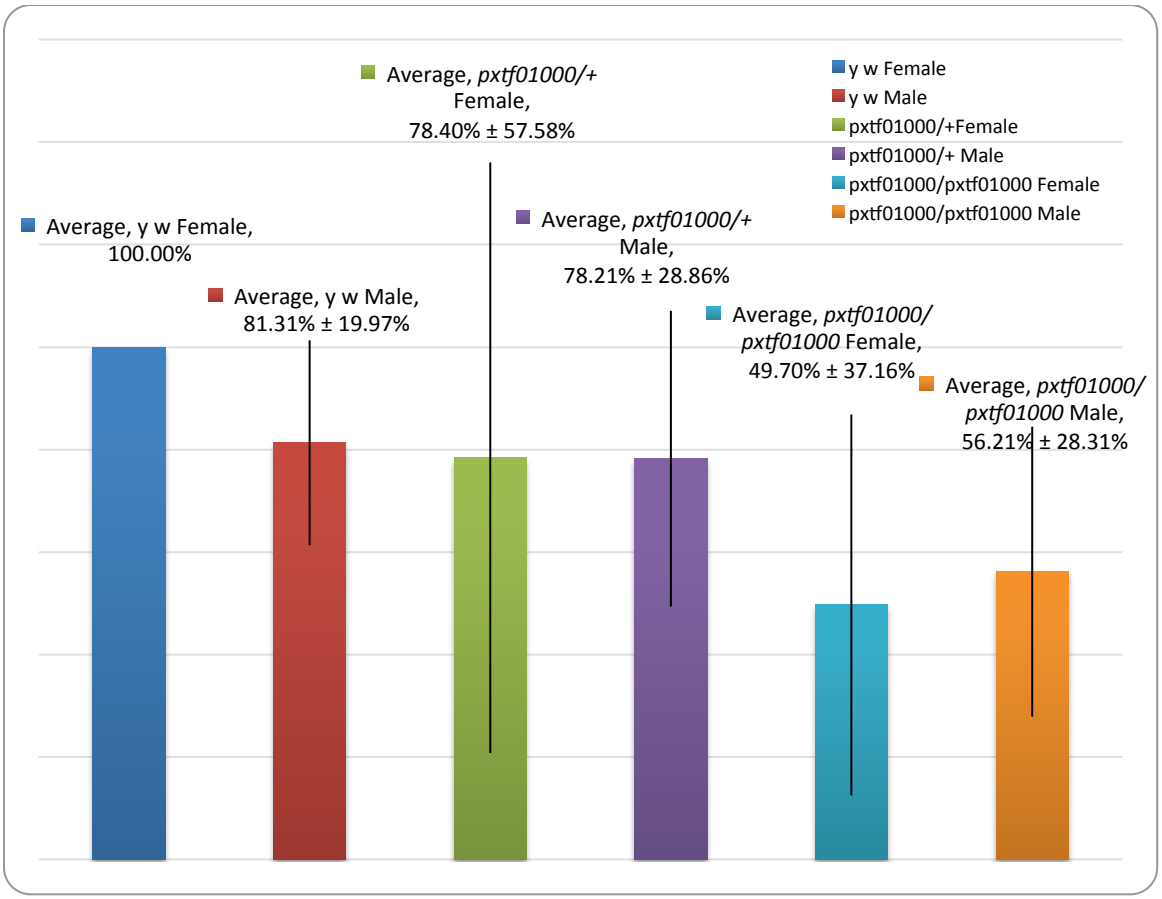

Figure 27. COX activity level in y w, *pxt*$^{f01000/+}$, and *pxt*$^{f01000/f01000}$ flies

Discussion

The major predictions of our studies are that Pxt, Pxd and CG4009 are highly likely to function as COX-like enzymes. All three proteins contain the conserved functionally crucial catalytic residues His207, Tyr385 and His388 found in mammalian COXs, suggesting their capability to catalyze the reaction that converts PUFAs to prostaglandins. The core structural fold of the conserved animal peroxidase domain is maintained in these proteins, and the biophysical features of the catalytic cavity mimic that of structurally solved mammalian COXs. The catalytic pocket of mammalian COX is L-shaped and largely hydrophobic; among the 24 residues that make up the catalytic pocket, only three are polar, and 21 are non-polar in human COX-1 and 2; the catalytic pockets of CG4009, Pxd, and Pxt follow a similar hydrophobic profile. All *Drosophila* COX proteins seem to be missing the mammalian MBD, which consists of four beta sheets and the last of which also forms a structural component of the catalytic cavity. A functionally important residue, Arg120, resides in this region and interacts with the carboxylate group of arachidonate through its arginine group (Bhattacharyya *et al.* 1996; Mancini *et al.* 1995). Pxd, Pxt and CG4009 do not have a direct counterpart for Arg120 in the primary structure, but Arg150 in CG4009 and Arg520 in Pxt could be envisioned to play a similar role. CG4009 also has Glu546 positioned on the opposing Arg150 and may be involved in forming a salt bridge when substrate in not present as proposed for Arg120 and Glu524 in oCOX-1 (Bhattacharyya *et al.* 1996; Mancini *et al.* 1995).

Other functionally important residues identified in the mammalian COXs include Glu203, Ser530 and Val349. Glu203 is important for the peroxidase activity of the

enzyme and mutating this residues reduces enzyme activity (Shimokawa *et al.* 1990; Shimokawa and Smith 1991; Landino *et al.* 1997). All three *Drosophila* proteins possess this conserved glutamine residue in the expected structural context. Ser530, the residue that is acetylated by aspirin and plays a critical role in aspirin mediated COX inhibition, is missing in all *Drosophila* COX candidates. However, CG4009 has a different serine residue, Ser393 in the catalytic pocket in the modeled catalytic domain. Although the serine does not align with Ser530 in mammals in the multiple sequence alignment, it presents in a similar structural context as the mammalian Ser530. This suggests that action of aspirin on *Drosophila* COXs may involve a different mechanism than the conventional acetylation of Ser530, or proceeds through the acetylation of Ser393 in CG4009. The role of Val349, which is present in the substrate binding channel, is more subtle; replacement of Val349 to isoleucine results in a change in stereochemistry of the product formed to 15R-configuration prostaglandins (Schneider *et al.* 2002), but substitutions with leucine affect substrate specificity changing preference from arachidonic acid to linoleic acid (Garscha and Oliw 2009). Previous research (Yoshioka *et al.* 1985; L. R. Shen *et al.* 2010) has shown *D. melanogaster* lacks C20 and C22 polyunsaturated fatty acids, including arachidonic acid in body tissues of larvae, pupae and adults, as well as the machinery to extend C18 fatty acids to C20 fatty acids (David W. Stanley-Samuelson *et al.* 1988). The leucine substitution corresponding to Val349 may be indicative of a preference of *Drosohila* Cyclooxygenase(s) to use linoleic acid as substrate. Our docking analysis, however, suggests that if arachidonic acid is available as a substrate, the substrate binding channel of CG4009, Pxd and Pxt can accommodate it and theoretically should be able to use it to generate prostaglandins. If indeed linoleic

acid is key substrate being utilized, then we expect to see hydroxyoctadecadienoic acids (HODEs) as the major products of CG4009, Pxd and Pxt. Further experimental studies can be designed to test our theoretical predictions to validate the substrate binding preferences as well as the capability of wild type flies/flies overexpressing CG4009/Pxd/Pxt to generate PGs if fed with a diet enriched with AA. A full profile of mass spectra of eicosanoid products from wild type, CG4009/Pxd/Pxt knockout/knockdown flies and flies overexpressing CG4009/Pxd/Pxt maybe be a useful indicator towards clues as to the preferred substrate of CG4009/Pxd/Pxt, which theoretically possess the capability of both cyclooxygenase and peroxidase catalytic activity.

Even though multiple COX candidates seem to exist in *Drosophila,* our preliminary analysis of the evolutionary origins of the sequences suggests that none of them clusters preferentially with COX-2. Mammalian COX-1 and COX-2 are very similar in sequence, sharing 60% sequence identity and virtually superimposable structures of their catalytic domains, but there are specific differences in some key residues lining the catalytic pocket and the volume occupied by the pockets that account for differences in substrate binding and affinity (Sharma *et al.* 2010; Yuan *et al.* 2009). A more select comparison of the key residues responsible for COX-1/COX-2 differences does not shed any further light, since the *Drosophila* sequences are quite different from the mammalian COXs. However, the sizes of the catalytic pocket for CG4009, Pxd and Pxt are much closer to that of the smaller pocket of COX-1. The existence of multiple COX-like sequences that do not correspond to a distinct COX-1/COX-2 type of lineage suggests that they may have arisen from gene-duplication events independent from those

that produced vertebrate COX-1 and COX-2; this seems highly plausible for CG4009, Pxd and Pxt since the locations of the genes on the genome are very close. It can be speculated that the multiple COXs code for either a functional redundancy or difference in function based on different expressional pattern. All three genes exist at transcript level based on EST analysis (Kalajdzic *et al.* 2012), and the anatomical and developmental expression level of these genes is quite distinct. Microarray studies suggest that whereas CG4009 is expressed only when flies reach adulthood, *pxt* expression peaks before the embryo reaches 6hr and is also high in adults (Chintapalli, Wang, and Dow 2007). Expression level of *pxd* has several peaks: during embryo 14-20hr, pupae 3 days and carcass (Chintapalli, Wang, and Dow 2007). *pxd* has high expression level in the fat body; CG4009 has moderately high expression in ovary in adult female flies, and *pxt* has extremely high expression in embryos and ovary in adult female flies (Chintapalli, Wang, and Dow 2007).

Structural analyses elucidate the mechanism for membrane binding for mammalian COX proteins (Picot, Loll, and Garavito 1994) and suggest their localization at membranes. Studies focusing on sub-cellular localization of mammalian COXs suggest that it predominantly located in ER/NE/Golgi system, but there are studies indicating that occasionally COX may be found both at the plasma membrane and cytosol (Yamashita *et al.* 2007; Koumas and Phipps 2002; Leclerc *et al.* 2008). Experimental studies following the cellular localization of COXs also show that COXs are both cytosolic and membrane-associated (Koumas and Phipps 2002; Yamashita *et al.* 2007; Perrone *et al.* 2007), and kinetics studies show that both microsomal and purified soluble proteins have the same Km using arachidonic acid as the substrate, indicating

that membrane-bound and soluble COXs behave in a similar way (Kulmacz, Pendleton, and Lands 1994). Additionally, it is known that the subcellular localization of COX is altered with changes in physical condition such as in cancer cells and treatment with various reagents such as heat-killed *Mycobacterium bovis* BCG. (Koumas and Phipps 2002; Roos and Simmons 2005; Perrone *et al.* 2007; Yamashita *et al.* 2007; Accioly *et al.* 2008; Leclerc *et al.* 2008; García-Bueno, Serrats, and Sawchenko 2009). Several sequence and structural features are suggested to contribute to the targeting mechanism of COXs to their correct subcellular compartment: signal peptides, membrane-binding domain, glycosylation sites, EGF-like domain and KDEL-like peptide (P/STEL in COX). The putative *Drosophila* COXs, CG4009, Pxd and Pxt all have signal peptide shared by proteins that are membrane resident or secreted. Even though the *Drosophila* sequences are distinctly missing the MBD, the N-terminus is not predicted to be unstructured. The preliminary full-length computational models suggest compact helical domains that associate with the catalytic domain with an overall hydrophobic and could serve as analogous membrane binding structures (data not shown). Some sequence features of mammalian cyclooxygenases such as N-glycosylation sites, and KDEL-like retention signal (P/STEL in COXs) are not apparent in the *Drosophila* sequences and would be worth investigating further experimentally if the *Drosophila* COXs parallel the mammalian COXs in glycosylation patterns and organelle retention possibly using divergent sequence signals.

We show that despite being quite divergent in sequence, the 3 putative COXs of *Drosophila* possess the same inherent enzymatic fold and components of domain architecture that would allow them to function in a very similar manner as mammalian

COXs. Out of the three candidates, CG4009 possess the most vertebrate COX-like features, but we suggest that all 3 proteins are capable of catalyzing enzymatic reactions characteristic of COX proteins. The preliminary COX activity assays for *pxt* mutants corroborate this prediction. Our findings raise some exciting and pertinent questions about the substrate requirements of all 3 proteins and the implications of downstream products being formed in *Drosophila* eicosanoid signaling. This study lays the groundwork for further exploration of these proteins and establishing their role in *Drosophila* inflammation and immunity, opening up avenues for addressing the use of this model organism in COX signaling and its crosstalk with other signaling pathways.

Chapter 5 Prostaglandin E Synthases in *Drosophila melanogaster*

Prostaglandin E Synthase-1 (PGES-1/mPGES-1)

Microsomal prostaglandin E synthase-1 (PGES-1/mPGES-1) is a downstream enzyme of COXs (COX-1/2).

Based on BLAST results, three genes CG33177, CG33178 and *mgsl* (microsomal glutathione S-tranferase-like, CG1742) in *D. melanogaster* genome encode four proteins that share sequence similarity with human PGES-1. Gene *mgsl* encodes two proteins: Mgsl isoform A and Mgsl isoform B due to alternative splicing. All the three genes are located on the *D. melanogaster* X chromosome. Residues that are crucial for enzyme activity include Glu66, Arg67, Arg70, Arg72, Arg100 and Tyr117, and all the residues are conserved in the three *D. melanogaster* proteins. In hPGES-1, residues required for GSH binding include Tyr117, Arg126, Tyr130 and Gln134. All the three proteins, Mgsl, CG33177 and CG33178 have the conserved Tyr117 and Arg126, but are missing Tyr130 and Gln134. In all the three *D. melanogaster* proteins, Tyr130 is replaced by a Phe. In CG33177, Gln134 is replaced by a Phe, but in CG33178 and Mgsl, the Gln is replaced by a Leu (Table 8; figure 28).

Table 8. Comparison of three PGES-1 homologues in *D. melanogaster*

Protein Name	Length	Accession No.	Chromosome	CG no.	Seq Identity with hPGES1	Seq Similarity with hPGES1	No of Transmembrane domain
Mgsl isoform A	152aa	NP_524696	X, 19E7-19E7	CG1742	33%	54%	4
CG33177	167aa	NP_788903.1	X, 13A9-13A9	CG33177	28%	49%	3
CG33178	165aa	NP_788904.1	X, 13A9-13A9	CG33178	30%	46%	3

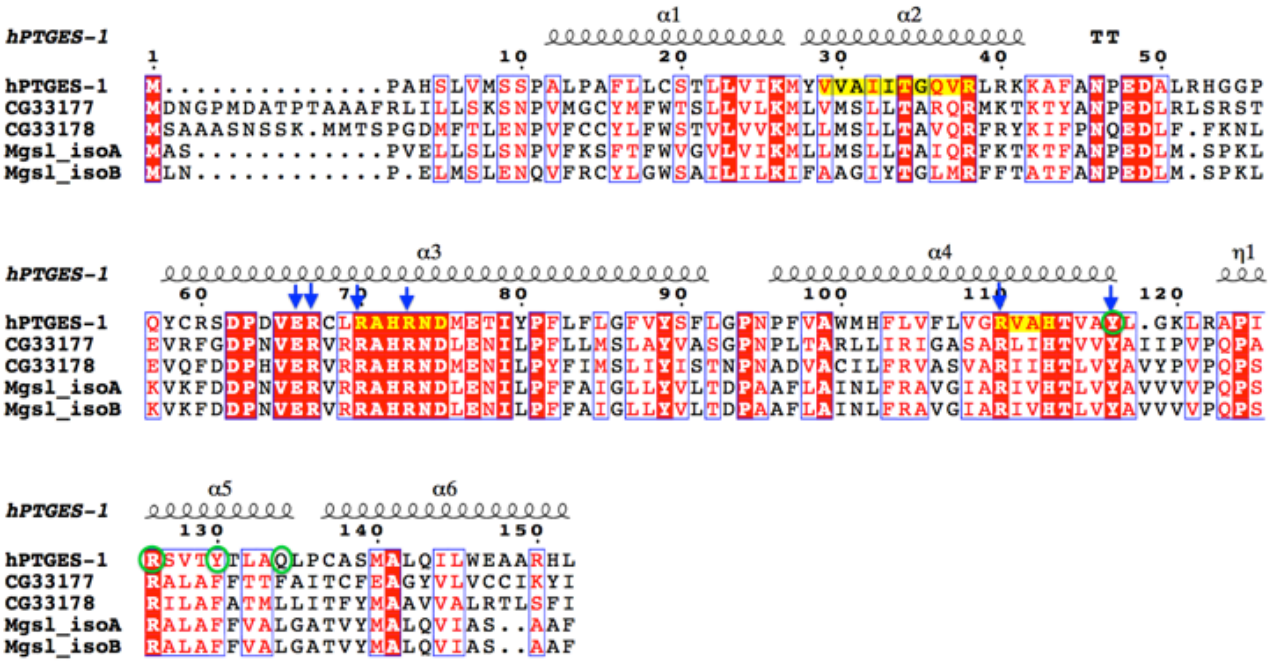

Figure 28. Sequence alignment of hPGES-1 and *D. melanogaster* proteins CG33177, CG33178, Mgsl isoform A and Mgsl isoform B. Secondary structure of hPGES-1 is based on the crystal structure of hPEGS-1 (PDB ID: 3DWW). Residues that share identity/similarity are boxed in red/white. Regions colored in yellow are glutathione-binding regions. Green-circled residues are glutathione-binding residues. Blue arrows indicate residues that are important for enzymatic reaction.

The secondary structure analysis shows that the four proteins are mostly helical, similar to human PGES-1. All the four proteins are predicted to have four transmembrane domains, similar to human PGES-1. Human PGES-1 has four transmembrane domains even though it has six helices, because the first and the second helices constitute the first transmembrane helix and the fifth and the six helices make up the fourth transmembrane helix (figure 29 and figure30).

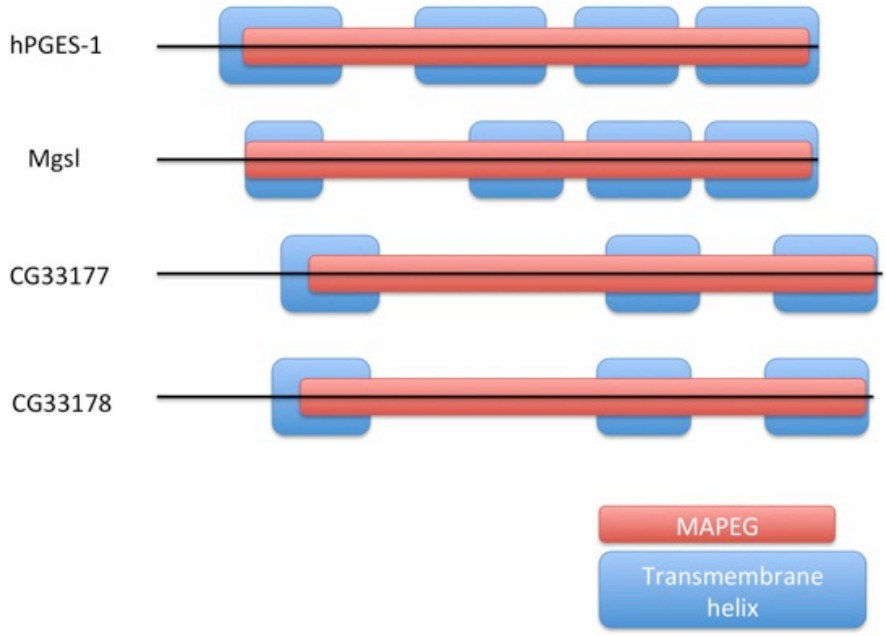

Figure 29. Conserved domain of human PGES-1, *D. melanogaster* proteins Mgsl, CG33177 and CG33178. Red rectangles represent MAPEG domains. Blue rectangles represent transmembrane helices.

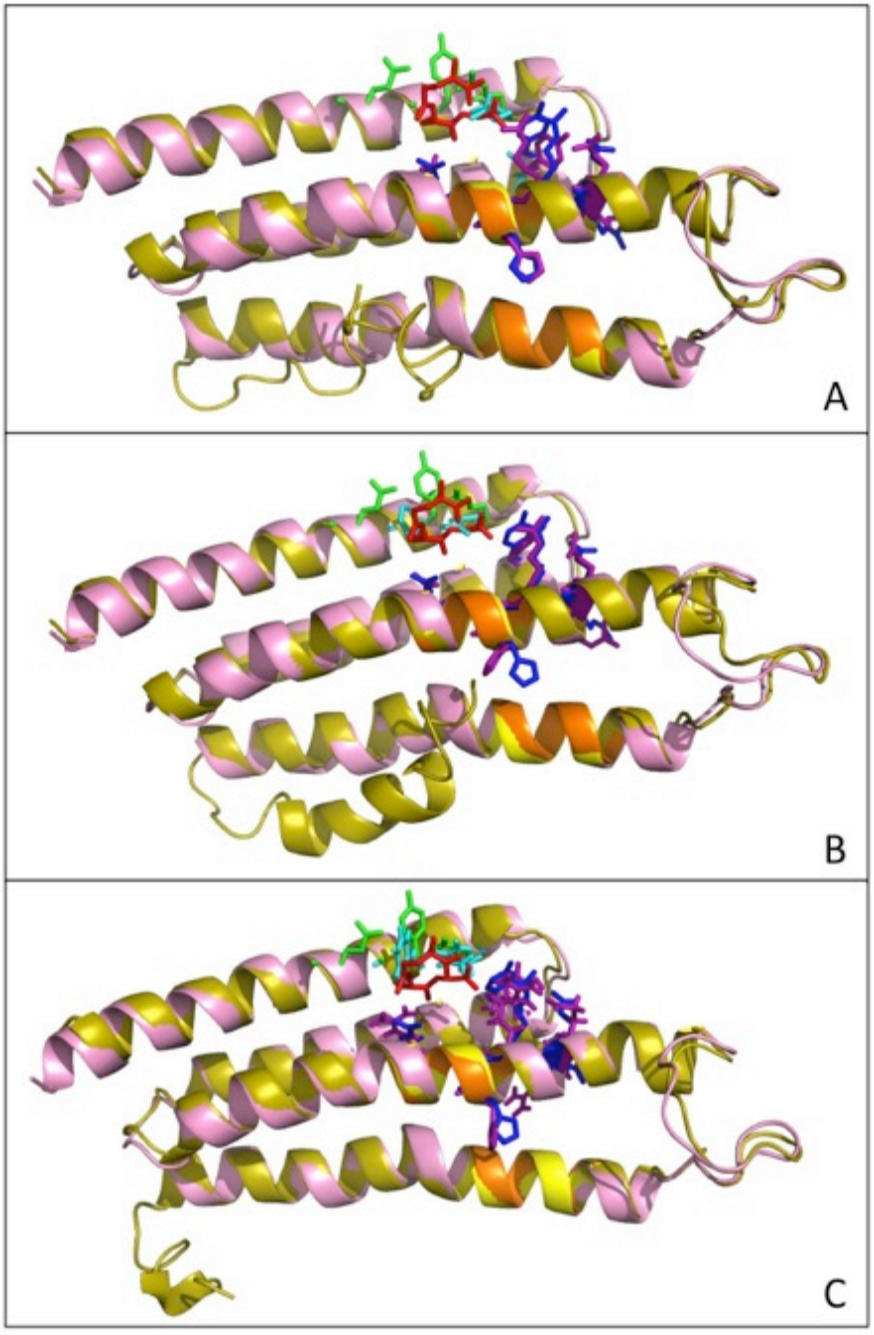

Figure 30. Structural alignment of hPGES-1 (PDB ID: 3DWW) and *D. melanogaster* proteins CG33177, CG33178 and Mgsl. Proteins are rendered in cartoon. hPGES-1 is shown in pink, and *D. melanogaster* proteins are shown in olive. Glutathione is shown as red sticks. In hPGES-1, glutathione-binding regions are colored in yellow. Glutathione-binding residues are shown in green. Other important residues are shown in blue. In *D. melanogaster* proteins, glutathione-binding regions are colored in orange. Glutathione-binding residues are shown in cyan. Other important residues are shown in purple.

A. Structural alignment of hPGES-1 and CG33177. B. Structural alignment of hPGES-1 and CG33178. C. Structural alignment of hPGES-1 and Mgsl.

Prostaglandin E Synthase-2 (PGES-2/mPGES-2)

We identified one protein, suppressor of ref(2)P sterility (CG4086, Su(2)P), in the *D. melanogaster* genome that shares sequence similarity and identity with monkey PGES-2. Monkey PGES-2, which has 377 amino acids, has two conserved domains, one glutathione S-transferase N-terminal region (GST_N) ranging from residue 90 to 193, and one glutathione S-transferase C-terminal region (GST_C), ranging from residue 263 to residue 377. Putative *D. melanogaster* PGES-2 Su(2)P has 417 amino acids, and similar to hPGES-2, it is predicted to have two conserved domains, GST_N and GST_C. GST_N domain in *D. melanogaster* Su(2)P ranges from residue 110 to 219, and GST_C domain in *D. melanogaster* Su(2)P is located from residue 287 to 399 (Figure 32).

Monkey and *D. melanogaster* PGES-2 share 34% sequence identity and 50% sequence similarity (figure 31). Important residues for monkey PGES-2 include residues that are involved in hydrogen bonding and glutathione binding. Residues that are involved in hydrogen bonding include Tyr104, Cys110, Phe112 and Cys113. *D. melanogaster* Su(2)P has all the equivalents except Tyr104. In *D. melanogaster* Su(2)P, Tyr104 is replaced by a Phe. The equivalent residues for monkey PGES-2 Cys110, Phe112 and Cys113 are Cys113, Phe135 and C136 in *D. melanogaster* Su(2)P. The residues that are involved in glutathione binding in monkey PGES-2 are Val148, Asp163 and Ser165, and *D. melanogaster* Su(2)P has equivalents for all the three residues: Val171, Asp164 and Ser165 (table 9).

We built a 3D structural model for the *D. melanogaster* Su(2)P conserved domain, which includes the two conserved domains, GST_N and GST_C, from residue 110 to residue 399, using monkey PGES-2 (PDB ID: 1Z9H) as structural template.

Monkey and *D. melanogaster* PGES-2 conserved domains have very similar structural folds with RMSD 0.6Å, Z-score 7.6 and sequence identity 42.8% (figure 33). The residues that are involved in glutathione-binding in the two proteins have similar locations and orientation.

Table 9. Comparison of important residues that are involved in hydrogen bond formation and GSH-binding in monkey PGES-2 and putative *D. melanogaster* PGES-2.

	H-bond chain				GSH-binding		
Monkey PGES-2	Y107	C110 Catalytic activity	F112	C113	V148	D164	S165
dPGES-2	F130	133	135	136	171	188	189

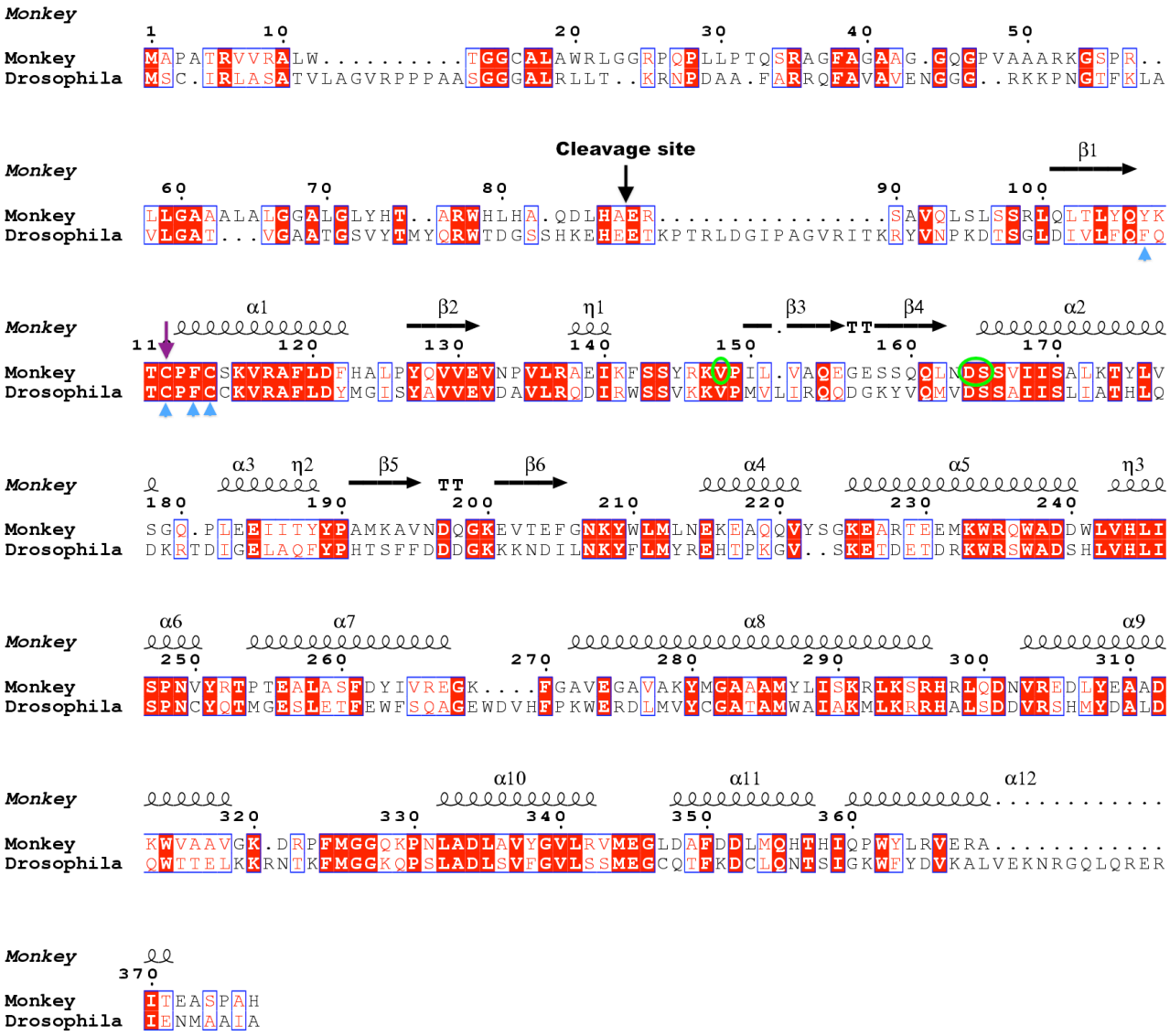

Figure 31. Sequence alignment of monkey PGES-2 and *D. melanogaster* suppressor of ref(2)P sterility.

Secondary structure of hPGES-1 is obtained from PDB 1Z9H.

Similar/identical residues are boxed in white/red boxes. Green circles show residues required for glutathione binding (V148, D164 and S165). Purple arrows indicate important residue required for catalytic activity (110C). Blue arrows indicate the characteristic H-bond chain formation (Y107-C113-C110-F112).

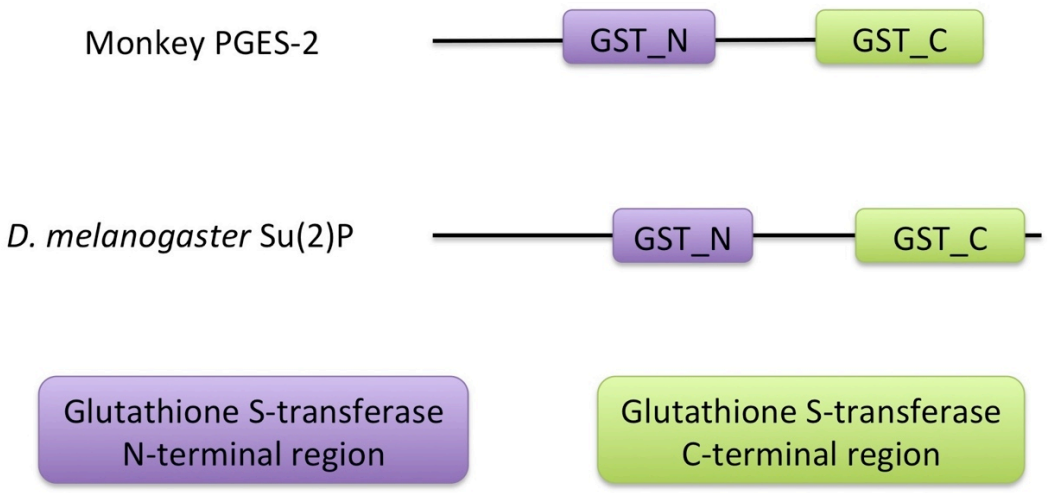

Figure 32. Conserved domain of monkey PGES-2 and *D. melanogaster* suppressor of ref(2)P sterility. Purple rectangles represent glutathione S-transferase N-terminal region (GST_N). Green rectangles represent glutathione S-transferase C-ternimal region (GST_C).

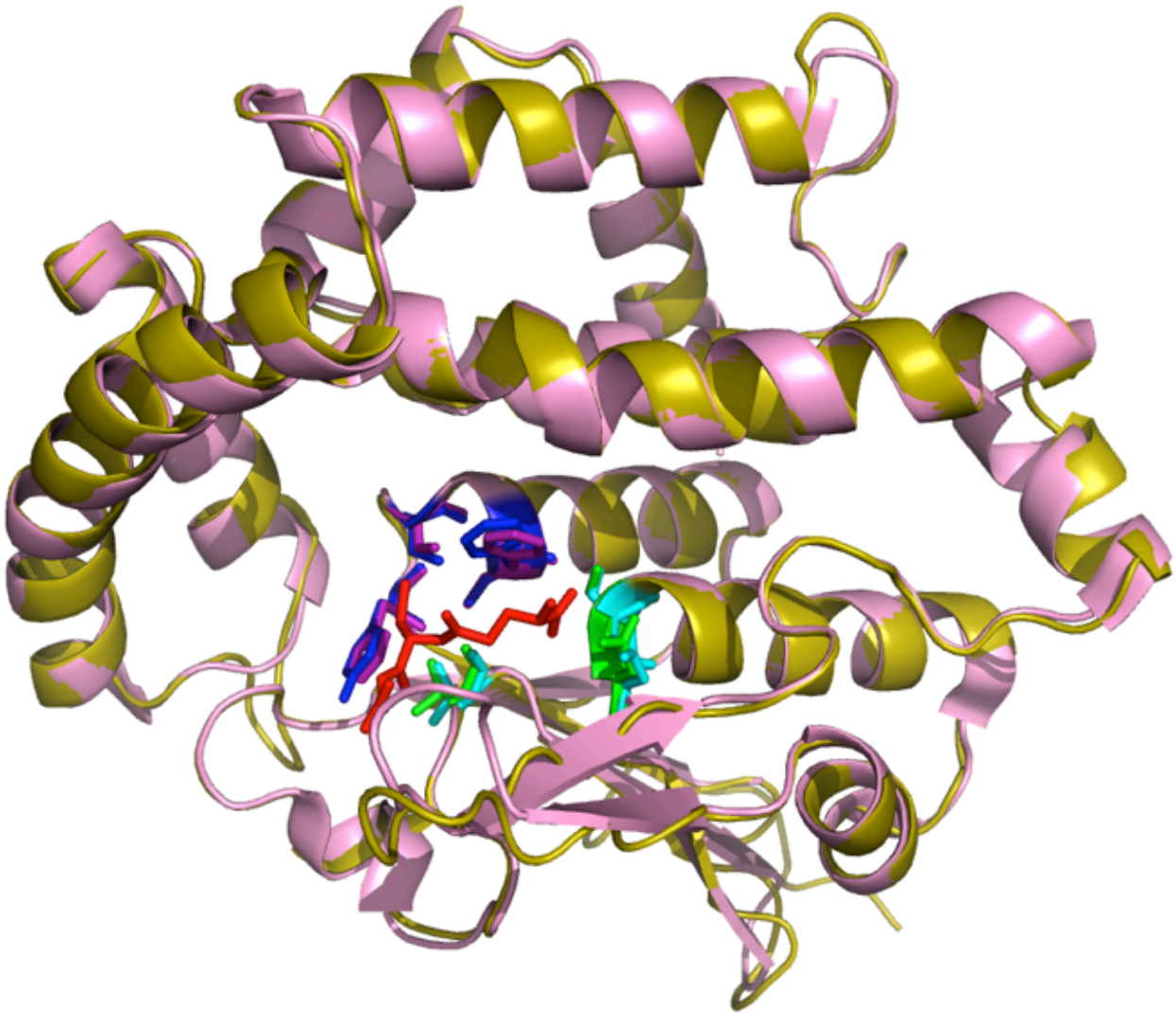

Figure 33. Structural alignment of conserved domain of monkey PGES-2 (PDB code: 1Z9H) and *D. melanogaster* suppressor of ref(2)P sterility.

Monkey PGES-2 is shown in pink. *D. melanogaster* suppressor of ref(2)P sterility is shown in olive.

For monkey PGES-2: four residues (Y107, C110, F112, C113) that form H-bond chain are shown as blue sticks, and three residues (V148, D164, S165) that are involved in glutathione-binding are shown as green sticks.

For *D. melanogaster* suppressor of ref(2)P sterility: four residues (F130, C133, F135, C136) that form H-bond chain are shown as purple sticks, and three residues (V171, D188, S189) that are involved in glutathione-binding are shown as cyan sticks.

Glutathione molecule is shown as red sticks.

Prostaglandin E Synthase-3 (PGES-3/cPGES)

We identified one putative PGES-3 in *D. melanogaster*, CG16817. Similar to mammalian PGES-3, the secondary structure of CG16817 consists of mostly beta-sheet. Sequence identity/similarity between the two proteins is 24%/41% (figure 34).

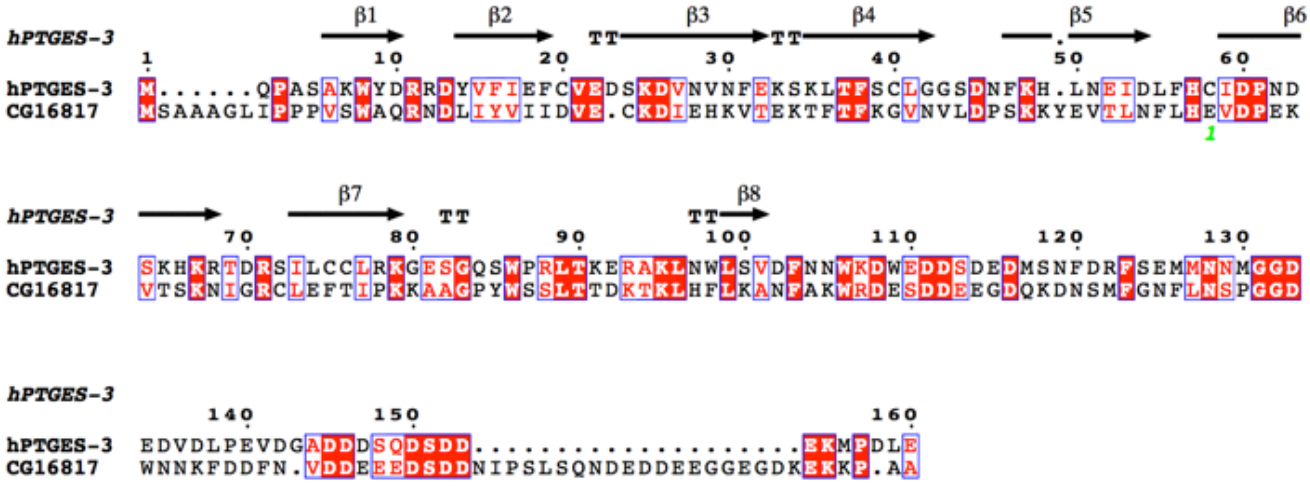

Figure 34. Sequence alignment of human PGES-3 and *D. melanogaster* protein CG16817.

Secondary structure shown above the sequences is obtained from Protein Data Bank from crystal structure of the human co-chaperon p23 (PDB code 1EJF). Eight beta sheets are shown and numbered. Similar/identical residues are boxed in white/red.

Human PGES-3 is 160 amino acids long. The crystallized structure of human PGES-3 (PDB ID: 1EJF) includes residues 1-110. Human PGES-3 has eight beta sheets. The functional domain in human PGES-3 is co-chaperone p23 domain, also called CS domain (CHORD-containing proteins and SGT1 domain), which ranges from residues 1-110, and this conserved domain includes all the eight beta sheets. According to prediction by multiple servers, CG16817 also is composed of eight beta sheets, in addition to an extra C-terminal alpha helix. Conserved p23 domain in CG16817 is made

up with the eight N-terminal beta sheets, ranging from residue 1 to 120 (figure 35 and figure 36).

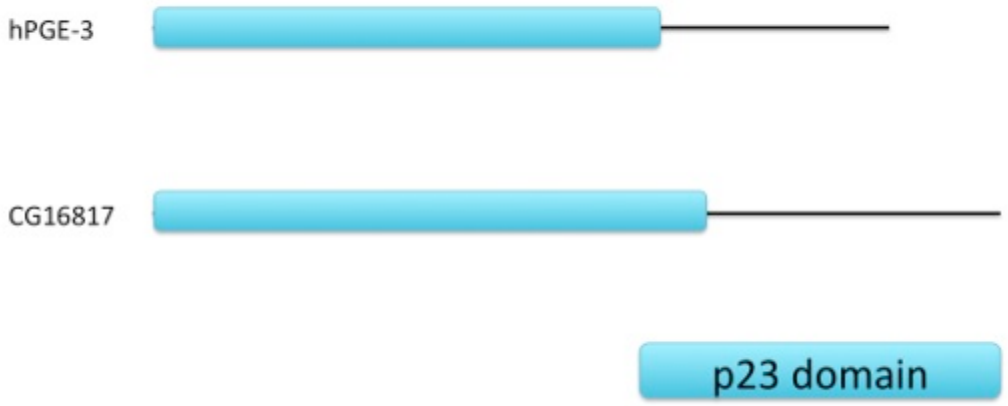

Figure 35. Conserved domain of hPGES-3 and *D. melanogaster* CG16817.

```
                  *        20         *        40         *        60         *        80
CG16817 : MSAAAGLIPPPVSWAQRNDLIYVIIDVECKDIEHKVTEKTFTFKGVNVLDPSKKYEVTLNFLHEVDPEKVTSKNIGRCLE :  80
psipred : CCCCCCCCCCCEEEEEECCEEEEEEEECCCCCEEEEEECEEEEEEECCCCCCEEEEECCCCCCCCCCCEEEECCCEEE :  80
Jpred   : ----------EEEEE-----EEEEEEEE-----EEEEE--EEEEEEEE------EEEEEEE----------EEEE---EEE :  40
prof    : CCCCCCCCCCCEEEECCCCEEEEEEEEECCCEEEEEECEEEEEEEECCCCCCCEEEEEEEEEEECCCCCCCCCCCCEEEE :  80
APSSP2  : CCCCCCCEEEEEEECCCEEEEEEEECCCCCEEEEEECEEEEEEEECCCCCCEEEEEEECCCCCCCCHHCECECCEEEEEE :  80

                  *       100         *       120         *       140         *       160
CG16817 : FTIPKKAAGPYWSSLTTDKTKLHFLKANFAKWRDESDDEEGDQKDNSMFGNFLNSPGGDWNNKFDDFNVDDEEEDSDDNI : 160
psipred : EEEECCCCCCCCCCCCCCCCCCCCEEECCCCCCCCCCCCCCCCCCCCCHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCC : 160
Jpred   : EEEEE------------------EEEE------------------HHHHHHHHH-------------------------- :  58
prof    : EEEECCCCCCCCHHHCCCCCCEEEEEECCCCCCCCCCCCCCCCCCCHHHHHHHHCCCCCCCCCCCHHHHCHHCCCCCCCCC : 160
APSSP2  : EEECCCCCCCCHHCCCCCCCCECCECCCCCCCCCCCCCCCCCCCHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCC : 160

                  *       180
CG16817 : PSLSQNDEDDEEGGEGDKEKKPAA : 184
psipred : CCCCCCCCCCCCCCCCCCCCCCCC : 184
Jpred   : ------------------------ :   -
prof    : CCCCCCCCCCCCCCCCCCCCCCCC : 184
APSSP2  : CCCCCCCCCCCCCCCCCCCCCC-- : 182
```

Figure 36. Secondary structure of putative *D. melanogaster* PGES-2 (CG16817).

Secondary structure of CG16817 is calculated by four servers: psipred, jpred, prof, and APSSP2. Beta sheets are shaded in yellow color, and alpha helices are shaded in red color.

We have built a three-dimensional structural model of the conserved p23/CS domain of CG16817 using human PGES-3 (PDB ID: 1EJF) as the structural template and alignment generated by HHpred. The structural alignment of the conserved CS domains from the two proteins has RMSD 2.94 Å, Z-score 6.35, and sequence identity 25% (figure 37).

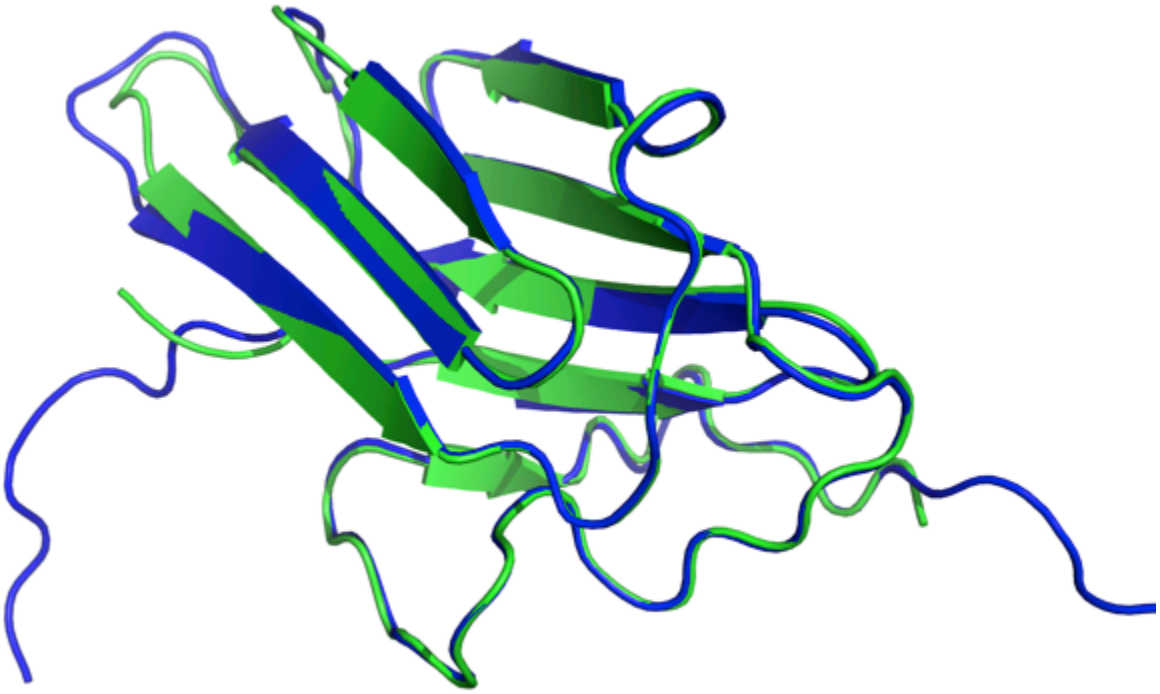

Figure 37. Structural alignment of hPGES-3 and *D. melanogaster* CG16817. Proteins are represented in cartoon. hPGES (PDB code 1EJF) is shown in green. p23/CS domain of CG16817 (residues 1-120) is shown in blue.

Currently, there has not been any solved PGES-3 structure with co-factor GSH bound. Therefore, to compare and analyze the GSH-binding structural motifs of hPGES-3 and *D. melanogaster* CG16817, we docked GSH onto the predicted model of *D.*

melanogaster CG16817 and hPGES-3 (PDB ID: 1EJF) (figure 38) and show that the GSH binding motifs are similar in the two proteins. Based on our docking results we identified that Trp8, Arg88, Thr90, Glu92, Arg93, Ala94, Lys95, Leu96, Asn97, Trp98, Leu99, Ser100 and Val101 of hPGES-3, and Trp14, Ser93, Ser94, Leu95, Thr96, Asp98, Lys99, Thr100, Lys101, Leu102, His103 and Leu105 of CG16817 are involved in GSH-binding (figure 38). GSH-binding motifs of the two proteins are closely related on both primary sequence and 3D structures (figure 39).

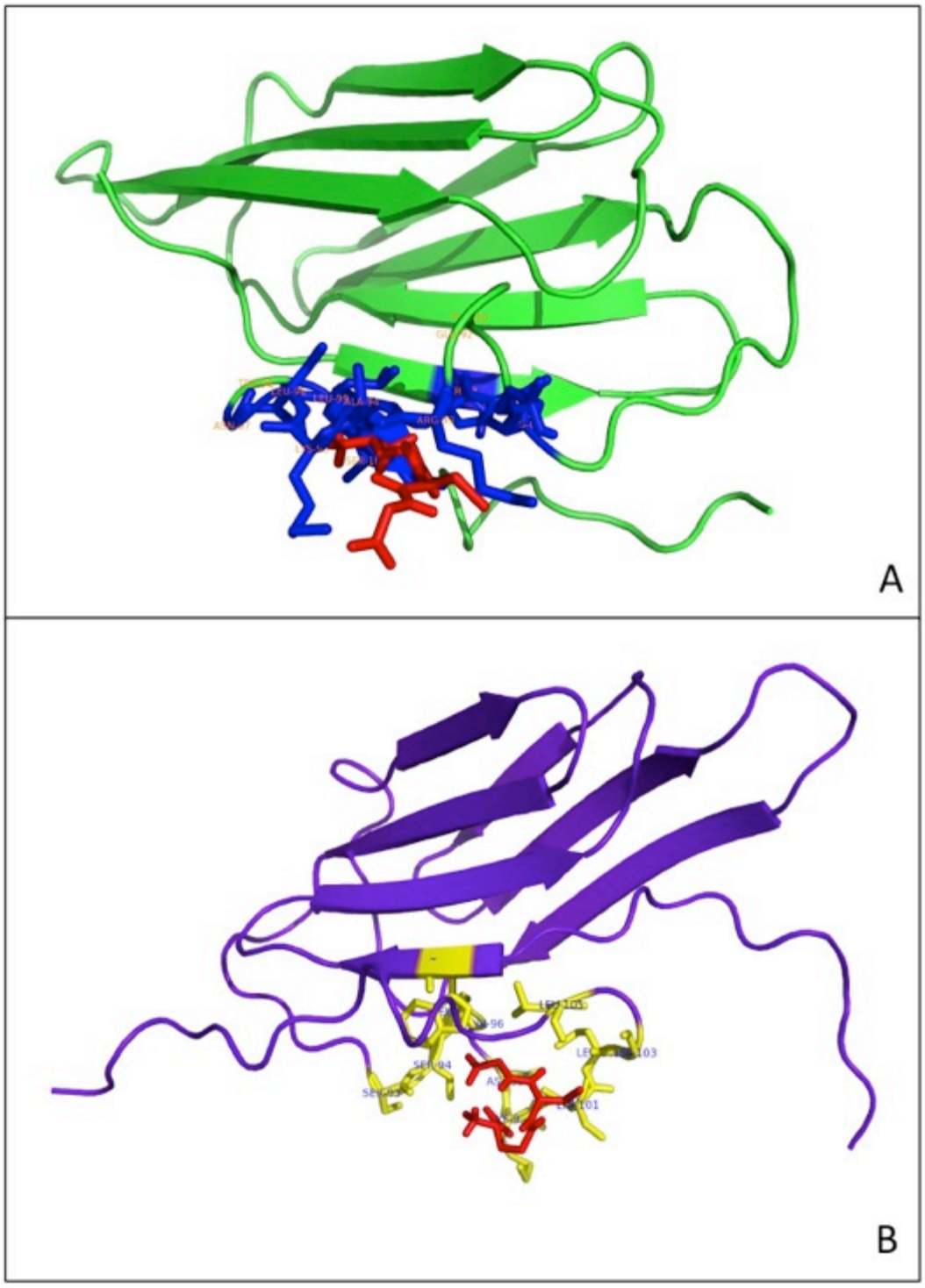

Figure 38. Docking of GSH on hPGES-3 and CG16817 showing that the GSH binding motifs are similar in the two proteins. GSH is shown as red sticks. hPGES is rendered in green cartoon, and CG16817 is rendered in purple cartoon. hPGES-3 residues involved in GSH-binding are shown as blue sticks. CG16817 residues that bind to GSH are shown as yellow sticks. A. hPGES-3 (PDB ID 1EJF). B. *D. melanogaster* CG16817.

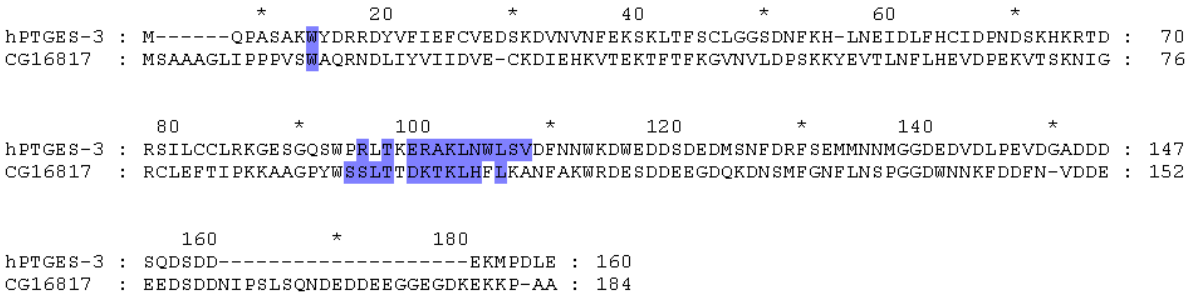

Figure 39. Sequence alignment of hPGES-3 and CG16817 showing the GSH-binding motifs.
Residues predicted to be involved in GSH-binding are marked in purple shadow.

Discussion

In our studies, we predict that the three PGES-1 homologues, CG33177, CG33178 and Mgsl are highly likely to function as PGES-1 enzyme. All three proteins have the signature multi-transmembrane domains and conserved GSH-binding regions (Tyr117, Arg126, Tyr130 and Gln134) that exist in mammalian PGES-1, suggesting the three proteins are capable of utilizing GSH as a cofactor, and each isozyme should be able to catalyze the reaction that converts PGH_2 into PGE_2. The computational 3D structures of the conserved MAPEG domain in the three proteins are highly similar to human PGES-1, and all three *D. melanogaster* proteins have the conserved catalytic motif (Glu66, Arg67, Arg70, Arg72, Arg100 and Tyr117) similar to that of human PGES-1.

Our computational studies of *D. melanogaster* protein Su(2)P show that Su(2)P and monkey PGES-2 are highly similar at both sequence level (34% sequence identity and 50% similarity) and structural level (RMSD 0.6Å). *D. melanogaster* protein Su(2)P contains that signature H-bond chain required for catalytic activity in human PGES-2, which consists of Tyr107, Cys110, Phe112 and Cys113. Also, GSH-binding residues, Val148, Asp164 and Ser165, are conserved in human PGES-2 and *D. melanogaster* Su(2)P, suggesting that *D. melanogaster* protein Su(2)P is likely to function as a PGES-2.

Our prediction shows that CG16817 has the capability to function as a PGES-3 enzyme. The sequence of the catalytic domain is highly conserved in human PGES-3 and CG16817. The docking results of cofactor GSH show that the two proteins have

similar GSH-binding residues, suggesting that CG16817 is highly likely to function as PGES-3.

In mammals, all three PGESs are involved in the biosynthesis of PGE_2, but they are expressed in different tissues and under different conditions. PGES-1 is coupled with COX-2 and various pathogens induce its expression, while PGES-3 is ubiquitously expressed and coupled with COX-1. PGES-2 couples with either COX-1 or COX-2.

Three PGES-1 homologues, CG33177, CG33178 and Mgsl are proteins that share high sequence identity, and all three of them are located on X chromosome within close proximity. It is highly likely that the emergence of the three genes is due to a gene duplication event.

Mammalian PGES-1 belongs to MAPGE superfamily, which includes six transmembrane proteins: PGES-1, leukotriene C_4 synthase (LTC_4S), 5-lipoxygenase activating protein (FLAP), microsomal glutathione S-transferase 1 (MGST-1), MGST-2 and MGST-3. All the six members of MAPGES superfamily use GSH as their cofactor (Martinez Molina, Eshaghi, and Nordlund 2008). To our knowledge, structures of four out of six MAPGE superfamily members were solved. The four crystallized structures are human PGES-1 (PDB code: 3DWW, Jegerschöld et al. 2008), rat MGST1 (PDB code: 2H8A, Holm et al. 2006), human LTC_4S (PDB code: 2UUH, 2UUI, 2PNO, Ago et al. 2007; Martinez Molina et al. 2007) and human FLAP (PDB code: 2Q7M, 2Q7R, Ferguson et al. 2007). To date, no crystallized structure of non-mammalian MAPGE proteins has been published. Our studies are the first to identify a MAPGE superfamily member in *D. melanogaster*.

High-throughput expression data in FlyAtlas anatomy microarray shows that Mgsl has low expression level in larvae, and it is highly expressed in head, eye, hindgut, fat body and heart in adult tissues. The expression levels of CG33177 in larval and adult tissues are similar, and CG33177 is moderately expressed in various types of tissues. CG33178 is highly expressed in adult crop, head, eye and hindgut and larval trachea. PGES-2 homologue Su(2)P is ubiquitously expressed in all adult and larval tissues at moderate level, indicating it is more involved in development instead of immune response, similar to human PGES-2. PGES-3 homologue CG16817 is ubiquitously expressed in all adult and larval tissue at high level, indicating that it is more involved in maintaining homeostasis, similar to the function of its counterpart PGES-3 in human.

Recently, Yamamoto *et al* discovered that a *Bombyx mori* sigma-class glutathione transferase exhibits prostaglandin E synthase activity (K. Yamamoto et al. 2013). This *Bombyx mori* sigma-class glutathione transferase (bmGSTu) shares much higher sequence identity with mammalian PGDS (32.4% identity with rat PGDS) than PGES-1 (12.7% identity with human PGES-1) or PGES-3 (8.3% identity with human PGES-3), but when incubated with PGH_2, it exhibits prostaglandin E synthase activity rather than prostaglandin D synthase activity (K. Yamamoto et al. 2013). This discovery suggests that perhaps enzymes that are involved in prostaglandin synthesis in insects may act differently from those in mammals.

Our studies identified homologous enzymes of all three types of PGESs in *D. melanogaster*. *D. melanogaster* PGESs and human PGESs are highly similar in primary sequences, and the comparative modeling in our studies suggests that they have very similar structural folds. We demonstrate that all putative *D. melanogaster* PGESs

possess the same essential enzymatic structures and key residues, and closely related domain architectures that will enable them to catalyze the same reaction that converts PGH_2 into PGE_2 in a similar manner as the mammalian PGESs. Our studies suggests that PGE_2 biosynthesis mechanism in *D. melanogaste*is similar to PGE_2 synthesis in mammals, indicating the possibility of using *D. melanogaster* in studying physiological and pathophysiological processes that involve PGE_2.

Chapter 6 Conclusions and future directions

D. melanogaster has served well as a model organism to dissect signaling mechanisms governing developmental processes that underlie human disease. A fly model with hallmarks of acute and chronic mammalian inflammatory responses will be highly useful in understanding how signaling networks and feedback regulatory mechanisms are involved in chronic inflammation. Despite the knowledge that lipid mediators can modulate immunity in insects, the biochemical nature of eicosanoids, the enzymes essential for their biosynthesis, and their role in innate immunity remains uncharacterized. The characterization of these pathways in *D. melanogaster* will enhance the mechanistic understanding of the underlying biology in mammalian models and underlies our attempt to chart out the components of the COX pathway with detailed functional characterization using computational tools.

Our findings from a preliminary sequence-based scan identified the majority of the components of COX pathway in *D. melanogaster*. This work is the first description of the pathway essential for prostaglandin biosynthesis in any invertebrate.

Detailed comparative modeling and functional characterization of two enzymes of this pathway, COX and PGES reveal that despite overall low sequence similarity, *D. melanogaster* enzymes possess similar structural folds and the catalytic motifs that characterize their mammalian counterparts, which is consistent with previous findings that prove the existence and important biological functions of prostaglandin. Our studies build a preliminary foundation for future studies of prostaglandin biosynthesis in *D. melanogaster*.

Our studies show that *D. melanogaster* proteins CG4009, Pxd and Pxt are all COX-like enzymes. All three of them (1) have the key catalytic residues, His207, Tyr385 and His388, which are required for the dual peroxidase and cyclooxygenase functions, and (2) have a L-shaped mostly hydrophobic catalytic cavity that is required to hold the unsaturated fatty acid substrate. A Leu349->Val replacement suggests that Pxd and CG4009 may prefer linoleic acid instead of arachidonic acid as a substrate. The small substrate-binding cavity of Pxt raises new and intriguing questions regarding its substrate(s).

We have identified *D. melanogaster* homologues of all the three PGES in mammals, PGES-1, PGES-2 and PGES-3. Functional characterization of the *D. melanogaster* PGESs show that the *D. melanogaster* and mammalian PGESs are structurally conserved and share similar enzymatic structures and key catalytic residues. *D. melanogaster* PGES-1 candidates Mgsl, CG33177 and CG33178 possess the signature GSH-binding residues and key catalytic residues. Su(2)P, the only putative *D. melanogaster* PGES-2, is highly similar to mammalian PGES-2 at both primary sequence and structural levels, and possesses the functional catalytic residue Cys110. The molecular mechanism of the catalytic reaction of hPGES-3 is not well studied, but our finding showed that CG16817, the sole putative PGES-3 in *D. melanogaster*, shares similar GSH-binding motifs as hPGES-3.

Our studies show that the prostaglandin synthesis pathway in *D. melanogaster* largely parallels mammalian COX pathway: (1) *D. melanogaster* has the majority of the enzymes involved in mammalian prostaglandin biosynthesis pathway; (2) *D. melanogaster* enzymes and mammalian counterparts are similar in amino acid sequence

level and structural level; (3) *D. melanogaster* enzymes have the same catalytic motifs of their mammalian equivalents; suggesting the possibility of using *D. melanogaster* as a model organism for studying eicosanoid biosynthesis pathway and physiological/pathophysiological processes that involve prostaglandins. Even though the putative *D. melanogaster* COX proteins do not share high sequence similarity and identity with the mammalian counterparts, the highly conserved downstream enzymes PGES in *D. melanogaster* suggest that prostaglandin synthesis in *D. melanogaster* parallels the mammalian pathway.

COX and PGES are targets of anti-inflammatory drugs in pharmaceutical research, and our studies unveil the possibility of using *D. melanogaster* for screening novel inhibitors of COX and PGES.

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