UNIVERSITY OF CALIFORNIA, SAN DIEGO

Lipases of Branched Fatty Acid Esters of Hydroxy Fatty Acids in *Drosophila melanogaster*

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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The Thesis of Andrea Huang is approved and acceptable in quality and form for publication on microfilm and electronically:

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2017

DEDICATION

To my dearest family, whose loving care has shaped me to be the person I am today.

To my family at Redeemer's Grace Church, whose sacrificial love encourages me daily.

To God be all the glory.

EPIGRAPH

Though the fig tree should not blossom, nor fruit be on the vines, the produce of the olive fail and the fields yield no food, the flock be cut off from the fold and there be no herd in the stalls, yet I will rejoice in the Lord; I will take joy in the God of my salvation. God, the Lord, is my strength; he makes my feet like the deer's; he makes me tread on my high places.

Habakkuk 3:17-19

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LIST OF ABBREVIATIONS

FAHFAs	fatty acid esters of hydroxy fatty acids
GLUT 4	Glucose transporter type 4
AG4OX	adipose GLUT4 overexpressor
9-PAHSA	9-palmitic acid hydroxy stearic acid
LC-MS	liquid chromatography-mass spectrometry
AIG1	androgen induced gene-1
ADTRP	androgen-dependent TFPI regulating protein

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ABSTRACT OF THESIS

Lipases of Branched Fatty Acid Esters of Hydroxy Fatty Acids
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by

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A recently discovered class of endogenous lipids called fatty acid esters of hydroxy fatty acids (FAHFAs) display potent anti-diabetic and anti-inflammatory effects. This leads to the question of how these FAHFAs are regulated *in vivo*.

My thesis work focused on understanding how FAHFAs are biochemically degraded in an animal model. Prior to my work, androgen induced gene 1 (AIG1) and

androgen-dependent TFPI regulating protein (ADTRP) were discovered as FAHFA hydrolases and I endeavored to identify *Drosophila melanogaster* orthologs to provide an additional model to study FAHFA regulation. First, I confirmed that FAHFAs are present in flies, suggesting this is a well conserved lipid class. Conservation analysis revealed two putative FAHFA hydrolases, CG3625b and CG11601, in the *Drosophila melanogaster* genome. Like AIG1 and ADTRP, CG3625b and CG11601 have a conserved active site threonine, and testing the biochemical activity of these proteins demonstrated that CG3625b and CG11601 are FAHFA hydrolases. The results show that *D. melanogaster* can be used as a possible animal model to further elucidate how FAHFAs are physiologically regulated.

INTRODUCTION

1. Type 2 Diabetes

The prevalence of diabetes has been growing worldwide. In 2012, the United States alone had 22.3 million people diagnosed with diabetes. It is predicted that the number will double by 2050. Diabetes is a disease that is characterized by high blood glucose concentrations that can lead to a variety of complications such as cardiovascular disease, blindness, kidney failure, and neuropathy. The treatment for these complications are very costly with the United States spending about $245 billion in 2012 (American Diabetes Association 2013).

Obesity has long been recognized as the biggest risk factor in the development of type 2 diabetes (Allison 1930). Epidemiological studies have shown that the risk for diabetes was strongly related to weight gain (Colditz et al. 1995). The key pathogenic factor in this relationship is insulin resistance (Kahn, Hull, and Utzschneider 2006). In the body, the main tissues that are affected by insulin are adipose tissue and skeletal muscle. Insulin binds to its receptor, leading to the translocation of GLUT4 to the plasma membrane in these two tissues and glucose is able to be taken up (Epstein, Shepherd, and Kahn 1999). In insulin resistant individuals, downregulation of GLUT4 in adipose tissue serves as a marker for insulin insensitivity (Abel et al. 2001). Skeletal muscle, on the other hand, showed no difference in GLUT4 expression in insulin resistant individuals. GLUT 4-mediated glucose uptake in adipocyte biology was further examined.

2. FAHFAs

Transgenic mice overexpressing GLUT4 in adipose tissue (AG4OX) show improved glucose homeostasis and insulin sensitivity (Shepherd et al. 1993). A closer

look at altered adipose tissue metabolism have shown ChREBP activity to be induced by GLUT4-mediated glucose intake. In AG4OX mice, when ChREBP is knocked out, the improved insulin sensitivity is reversed. ChREBP is a transcriptional factor that leads to increased expression of lipogenic genes and de novo lipogenesis. Although insulin resistance is often characterized by higher levels of circulating fatty acids, expression of ChREBP shows improved metabolic effects (Herman et al. 2012). To determine what lipids could be causing these effects, an untargeted lipidomic profile of AG4OX mice led to the discovery of a 16-fold increase in the levels of a novel class of lipids in adipose tissue (Yore et al. 2014). Structural analysis of these lipids revealed that these lipids are fatty acid esters of hydroxy fatty acids (FAHFAs) (Figure 1A).

Within this class of lipids, the FAHFAs that were the most upregulated in AG4OX mice in comparison to wild type (WT) mice had palmitoleic acid (PO), palmitic (PA), or oleic acid (OA) as the fatty acid and hydroxy palmitic acid (HPA) or hydroxy stearic acid (HSA) as the hydroxy fatty acid. Palmitic acid esters of hydroxy stearic acids (PAHSAs) were the most upregulated in the adipose tissue of AG4OX mice so the physiological effects of PAHSAs were more closely examined. Targeted mass spectrometry for PAHSAs showed that there are different FAHFA isomers with the ester linkage appearing on the 5, 7, 8, 10, 11, 12, and 13 carbon of the hydroxy fatty acid (Figure 1B). 9-PAHSA is the most abundant in adipose tissue in WT mice and the most upregulated in AG4OX mice so that PAHSA was more closely examined for biological effects. When 9-PAHSA was fed to obese, diabetic mice, there was an improvement in glucose tolerance. When bone marrow derived cells were treated with 9-PAHSA, there was a decrease in inflammatory effects of LPS and the release of pro-inflammatory

cytokines. In human studies, PAHSAs were measured between insulin sensitive and insulin resistant individuals. Higher levels of 5-PAHSA were found in serum in insulin sensitive individuals when compared to the levels of 5-PAHSA in the serum of insulin resistant individuals. This further connects FAHFAs to insulin sensitivity and the blocking of diabetic affects. The beneficial effects of FAHFAs raises the questions of how they are being regulated and which enzymes are responsible for the synthesis and catabolism of these signaling lipids.

3. AIG1 and ADTRP

Different tissue lysates were profiled for FAHFA degrading potential. 9-PAHSA hydrolysis assays were performed and the brain was one tissue that was able to cleave this FAHFA. A database of serine hydrolases in the brain membrane was searched (Bachovchin et al. 2010) and inhibitor assays were performed to test which inhibitor could block 9-PAHSA hydrolysis. Any enzymes targeted by the inhibitor was a possible FAHFA hydrolase candidate. Several candidates were tested and androgen induced gene-1 (AIG1) was the only gene that hydrolyzed FAHFAs.

AIG1 is a multi-pass transmembrane protein that was originally discovered in human dermal papilla cells (Seo, Kim, and Kim 2001). It is a poorly characterized protein that is highly expressed in the brain and macrophages whose physiological function remains unknown. Androgen dependent TFPI regulating protein (ADTRP) is another poorly characterized protein (Lupu et al. 2011) and has 37% sequence homology to AIG1. It is highly expressed in many metabolic organs such as the kidney, liver, intestine, and brown adipose tissue. The physiological function of ADTRP is unknown but it has been linked to genetic studies of coronary heart disease (Huang et al. 2015).

Both of these proteins have been found to exclusively hydrolyze FAHFAs and cellular assays demonstrate that AIG1 knockdown dramatically reduces of FAHFAs hydrolysis in human cells (Parsons et al. 2016). However, whether these *in vitro* results are consistent with *in vivo* biochemistry of these enzymes still needs to be determined.

4. Project Goals

This project will look closely more at how FAHFAs are being degraded in the body. This study used AIG1/ADTRP orthologues in *Caenorhabditis elegans* and *Drosophila melanogaster* to look more closely at the physiological effects of AIG1 and ADTRP in the body. *C. elegans* and *D. melanogaster* were studied because many molecular pathways are conserved between these two organisms and mammals. They also have fully sequenced genomes. *C. elegans* have approximately 19,000 genes (The C. elegans Sequencing Consortium 1998) while D. melanogaster have approximately 13,600 genes (Adams 2000). The simpler genetics allows reverse genetic screening to be done. Reverse genetic screening starts at the genetic level and changes in the gene are made to see changes in phenotype. The process is often done through RNA interference (RNAi) which degrades the mRNA and changes are examined through loss of function phenotype (Fire et al. 1998).

From gene knockdowns of the AIG1/ADTRP orthologs in *C. elegans* and D. melanogaster, the role of AIG1 and ADTRP in FAHFA regulation will be further elucidated, but first candidate genes needed to be identified.

MATERIALS AND METHODS

Lipid Extraction

Lipids were extracted using a modified Bligh and Dyer's protocol. Organisms were Dounce homogenized on ice in a mixture of 1 ml: 1 ml: 2 ml PBS: methanol: chloroform. $13C_4$-9-PAHSA (5 pmol/sample) internal standard was added to chloroform prior to extraction. Mixture was vortexed then centrifuged at 2,200g, 6 minutes to separate top aqueous phase from bottom organic phase. Organic phase was collected and dried under a stream of nitrogen. Samples were reconstituted in 60ul methanol and 10ul was subjected to LC-MS analysis. Liquid chromatographer used Luna C18(2) (Phenomenex, 00G-4251-B0) column (3 mm, 100 A°, 2.0mm x 250 mm) with an in-line filter (Phenomenex, AF0-8497). Mobile phase was 93:7 methanol:water, 5 mM ammonium acetate, and 0.01% ammonium hydroxide LC run is 120 min with isocratic flow at 0.2 ml/min.

Cell Lines

HEK293T cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and maintained at 37°C and 5% CO_2.

Schneider 2 (S2) cells were maintained in Schneider's Drosophila Medium with 10% heat-inactivated fetal bovine serum and maintained at 28°C, no CO_2.

Cloning and Recombinant Expression of AIG1/ADTRP

Complete cDNA of *Caenorhabditis elegans* AIG1/ADTRP orthologs were cloned in-frame of the pcDNA3.1 V5/His B plasmid, which fused a V5 epitope to the C-terminus of the protein. C37E2.2 and C37E2.3 constructs were synthesized by IDT. cTel55X.1a construct was purchased from Dharmacon in pDONR201. cTel55X.1b

construct was made using the Q5 Site-Directed Mutagenesis protocol (NEB). Complete cDNA of *Drosophila melanogaster* AIG1/ADTRP orthologues were cloned in the pcDNA3.1 V5/His B plasmid. CG3625b (in pOT2) and CG11601 (in pOTB7) were purchased from Drosophila Genome Resource Center. HEK293T cells were grown in 10cm plates and transiently transfected with 12µg of construct with Lipofectamine 2000 (Thermo Fisher Scientific) at 90% confluency. "Mock" transfected cells were transfected with 12µg of the empty vector. 24 hours after transfection, cells were washed twice with PBS, harvested by scraping, and lysed by sonication in PBS. Membrane and soluble fractions were separated by spinning at 16,000g for 45 minutes at 4°C. Protein concentrations were measured using Quick Start™ Bradford 1X Dye Reagent (Bio Rad).

Western Blotting

Cell proteome was separated using SDS-PAGE gels and transferred to nitrocellulose membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). Immunoblot was blocked by Odyssey blocking buffer (LI-COR). Primary antibody anti-V5 (mouse, Thermo Fisher Scientific) was applied at dilution 1:2500 in blocking buffer. Secondary antibody anti-mouse IRDye 680RD (goat, LI-COR, 926-68170) was applied at dilution 1:10,000 in blocking buffer.

Gel-Based ABPP Analysis

The activity probe, FP-rhodamine, was synthesized and gifted by the Cravatt Lab at The Scripps Research Institute in La Jolla, CA. 50µg cell proteome was incubated with FP-rhodamine (1µM) at 37°C, 30 minutes. Reactions were quenched with 20µl 4X SDS-PAGE loading buffer, separated by SDS-PAGE gel, and visualized in-gel using a Typhoon FLA 9500 imaging system (GE Healthcare Life Sciences).

Site-Directed Mutagenesis

All mutations for CG3625b and CG11601 were done with Q5 Site-Directed Mutagenesis Kit (New England BioLabs) according to the manufacturer's instructions. Primers for each mutation are found in Table 1. Primers were purchased from Integrated DNA Technologies. All sequencing was done by Eton Biosciences Inc.

AIG1/ADTRP Ortholog Hydrolysis Assay

FAHFA substrates were purchased from Cayman Chemical Co. 20µg membrane proteome was incubated with FAHFA substrate (100µM final concentration) in a reaction volume of 100µl in PBS at 37°C for 20 minutes, shaking. Reaction was quenched with 400µl of 2:1 (v/v) chloroform:methanol doped with internal standard 20pmol 9-hydroxyheptadecanoic acid (9-HHDA). Mixture was vortexed then centrifuged at 2,200g for 6 minutes to separate the top aqueous phase from the bottom organic phase. Organic phase was collected and dried under a stream of nitrogen. The extract was re-solubilized in 60µl MeOH and a volume (10µl) was subjected to LC-MS analysis. Liquid chromatographer used Aquity UPLC BEH C18 column [1.7 µM, 2.1 mm × 100 mm]. Mobile phase A used H_2O, 0.01% NH_4OH, and 5 mM ammonium acetate. Mobile phase B used 95:5 ACN/H_2O mixture, 0.01% NH_4OH, and 5 mM ammonium acetate. LC run is 15 minutes long at a rate of 0.2 mL/min. The gradient for the mobile phases was 50 to 90% buffer B over 6 min, 90 to 100% B over 0.1 min, 100% buffer B for 3 min, 100 to 50% buffer B over 0.1 min, and 50% buffer B for 6 min.

AIG1/ADTRP Ortholog Inhibitor Assay

KC01 was synthesized and gifted from the Cravatt Lab in The Scripps Research Institute in La Jolla, CA. 20 µg of membrane proteome of HEK293T cells transiently

expressing AIG1/ADTRP orthologs were pre-treated with DMSO or KC01 (5μM) for 30 minutes. Proteome was then incubated with 100μM 9-PAHSA at 37°C, shaking for 30 minutes.

RESULTS

FAHFAs found in *Caenorhabditis elegans*

AIG1 and ADTRP have previously been characterized as FAHFA hydrolases. Not much is known about the physiological function of AIG1 and ADTRP. To further examine their physiological roles, we explored the potential use of *Caenorhabditis elegans* as a possible animal model of FAHFA regulation after discovering a presence of FAHFAs was found in *C. elegans* (Figure 2). Within the worm, the families LAHLAs, PAHSAs, and OAHSAs were found with LAHLAs being the most abundant. From the presence of FAHFAs, it was hypothesized that there were enzymes regulating these FAHFAs in the body of *C. elegans*. One way to examine how FAHFAs are processed in the body is to look at degradation. UniprotKB BLAST on human AIG1 and ADTRP was used to identify any *C. elegans* orthologs and C37E2.2, C37E2.3, cTel55X.1a, and cTel55X.1b were found in the search and further examined in this organism. However, the worm proteins were unable to express in HEK293T cells and could not be further characterized.

FAHFAs found in Drosophila melanogaster

D. melanogaster was looked at as a possible animal model due to its simpler genetics and the many conserved pathways that exist between *D. melanogaster* and mammals. To further examine whether the fly would be able to process FAHFAs similarly to mammals, we looked to see if the fly had endogenous FAHFAs. Lipids were extracted from whole adults flies (Bligh and Dyer 1959) and targeted LC-MS was used to profile the different FAHFAs found in the fly. Within the fly, we found PAHSAs, OAHSAs, LAHLAs, and POHSAs (Figure 3). LAHLAs were the most abundant

FAHFA in the fly. When looking at the extracted ion chromatograms of fly PAHSAs and mouse PAHSAs, the distribution of PAHSA isomers were similar between the fly and the mouse (Figure 4) which allowed us to further see the fly as an animal model that is comparable to a mouse model. And with the presence of FAHFAs in flies, we reasoned that there should be enzymes that biochemically regulate these signaling lipids. The number of hits found in a given database by chance (E-value), on UniProtKB BLAST was fairly small for the *C. elegans* orthologs but not as small as the orthologs for *D. melanogaster* when searched. Finding a way to express the worm proteins would be interesting, especially cTel55X.1a and b which have the conserved nucleophilic threonine and basic histidine, but the orthologs for *D. melanogaster* do seem more closely related to the human protein so were further examined.

Conserved Active Site in AIG1/ADTRP Orthologs

NCBI Protein BLAST searches were done on AIG1 and ADTRP to find orthologous proteins in Drosophila melanogaster. Within this data set, we were able to find three candidates to test: CG3625b, CG11601, and CG6149. When further searched on Uniprot, CG3625b and CG11601 had evidence at the transcript level while CG6149 was only a predicted protein. When overexpressing the candidates in HEK293T cells, CG3625b and CG11601 were able to be expressed and so are the candidates that were further tested for FAHFA degradation. AIG1 and ADTRP were identified to be of a novel class of threonine hydrolases. The Thr43 residue on hAIG1 and Thr47 residue on ADTRP were found to be conserved across many different species. The basic residue often found in active sites, His134 on AIG1 and His131 on ADTRP, is also conserved across species. In the two candidates (CG3625b and CG11601) found in *D.*

melanogaster, the two residues were found to be conserved as well (Figure 5) on Thr63 and His148 for CG3625b and Thr87 and His172 on CG11601. To confirm this, CG3625b and CG11601 were overexpressed in HEK293T cells. A western blot was run to see whether the overexpressed protein was in the membrane fraction or the cytosolic fraction (Figure 6A). Consistent with the fact that these proteins are transmembrane proteins, they were found in the membrane fraction and further assays used the membrane fraction. Activity based protein profiling (ABPP) was used to see if they were FP-reactive proteins and validate that these proteins were serine/threonine hydrolases as well. Membrane fraction of lysate was treated with a broad serine hydrolase probe tagged with a fluorophore (FP-rhodamine) and a gel was run. FP-rhodamine labeled both the proteins in the membrane fraction and CG3625b and CG11601 are shown to migrate to their expected molecular weights of 31.7 kDa and 28.5 kDa respectively (Figure 6B). Activity-based proteomics showed that the two proteins we were looking at were serine (or possibly threonine) hydrolases. To ascertain whether the active site was truly conserved in both candidates, we mutated the conserved threonine and histidine to alanine, a non-reactive amino acid. The conserved threonine was mutated into a serine as well to show that threonine is the nucleophilic residue for these orthologs while serine, although also a nucleophilic residue, is not. These mutants overexpressed the mutants in HEK293T cells and biochemical assays were run to compare them to their wild-type form. Activity-based proteomics was used to check whether the active site was affected by the mutations. FP-rhodamine was used to tag the active site of these mutant proteins. In CG3625b, the active site was unable to be tagged across all the mutants (Figure 7A). The same disappearance of bands was shown for CG11601 (Figure 7B) indicating that

the mutations of the supposedly conserved threonines and histidines affected the activity of the proteins. A western blot was run in conjunction to confirm that the disappearance of the bands on the ABPP gel was not due to decreased expression of protein but a change in the active site (Figure 7A and B).

Identification of AIG1/ADTRP Orthologs as FAHFA Hydrolases

Hydrolysis activity of membrane lysate overexpressing CG3625b and CG11601 was tested using 9-PAHSA as the substrate. Mock transfected cell lysate was used as a control. The hydrolysis was confirmed by the release of the product 9-hydroxy stearic acid (9-HSA) that can be quantified by LC-MS by integrating the peak for the m/z 299.3 on the extracted ion chromatogram. The mock control did have some background 9-PAHSA hydrolysis due to endogenous AIG1 in HEK293T cells. However, the cell lysate that overexpressed the fly candidates showed hydrolysis activity above that of the mock control (Figure 8). Hydrolysis of 12-PAHSA was also measured with the orthologs with mutated active sites. 9-PAHSA could be used as well, but this assay was showing more the comparison between the mutant proteins and the wild type protein so any FAHFA could be used. The hydrolysis assay revealed that the mutations in the predicted active site did quench the activity CG3625b when compared to the wild type protein (Figure 9A). When CG11601 had mutations in the corresponding threonine and histidine, hydrolysis activity was quenched as well (Figure 9B). The hydrolysis rate was decreased to the levels of the mock control when the conserved threonine and histidine were mutated which further confirms that the active site is conserved and that these orthologs are FAHFA hydrolases.

The difference between the activity of the mock control and the lysates overexpressing the CG3625b and CG11601 is not significantly different, but hydrolysis of FAHFAs is shown. The activity of the fly proteins was not expected to be as active as the human homolog since the fly is simpler organism. Also, the fly proteins were overexpressed in mammalian cells so there are potential discrepancies in protein trafficking. To further confirm hydrolysis activity, the enzymes were studied in their native fly cells.

FAHFA Hydrolysis in Schneider (S2) Cells

Schneider (S2) cells were tested for FAHFA degrading activity. 9-PAHSA was added to membrane and cytosolic fractions of cell lysate and the membrane lysate produced higher hydrolytic activity (Figure 10). Both enzymes are transmembrane proteins so we looked at the membrane fraction more closely. To confirm that the fly enzymes were producing the hydrolysis activity, inhibitors that could target the fly enzymes were used against the membrane fraction.

The inhibitor that was used against the fly enzymes was KC01, a covalent, irreversible inhibitor that selectively targets AIG1 and ADTRP. HEK293T lysate overexpressing CG11601 and CG3625b were treated with KC01 and compared to DMSO-treated lysate. A competitive ABPP gel was run to determine whether KC01 could target the fly proteins. The cell lysate was incubated with KC01 to first potentially block the active site and then FP-rhodamine was added to the lysate to tag serine hydrolases. Any disappearance of bands on the gel would indicate that KC01 targeted that particular serine hydrolase. In the ABPP gel run, the band for CG3625b disappeared when the cell lysate was treated with KC01 (Figure 11A). For CG11601, KC01 seemed

to block FP-rhodamine from tagging the protein as well (Figure 11B). This shows that KC01 is not only able to target human AIG1 and ADTRP, but is able to target the fly orthologs as well. KC01 abrogated 9-PAHSA hydrolysis of the overexpressed proteins showing that it could target the fly enzymes (Figure 11C and D). This confirmed that KC01 could be used as an inhibitor against the fly cells to determine whether or not our fly enzymes were contributing to FAHFA degrading activity.

KC01 was used against the membrane fraction of the fly cells. MAFP, a broad serine hydrolase inhibitor was used as a control. Addition of KC01 significantly decreased 9-PAHSA hydrolysis in comparison to DMSO-treated lysate (Figure 12). MAFP completely abolished hydrolytic activity. The difference between KC01 and MAFP shows that there are additional (i.e. not CG3625b and CG11601) serine hydrolases in the fly cell that can degrade FAHFAs. However, with the significant difference between DMSO-treated and KC01 treated lysate, it leads us to hypothesize that CG3625b or CG11601 enzymes are a major source of hydrolysis in the membrane fraction of these fly cells. This can be confirmed by specifically targeting the enzyme using RNAi.

DISCUSSION

Fatty acid esters of hydroxy fatty acids (FAHFAs) have anti-diabetic and anti-inflammatory effects. Using biochemical tools, I wanted to further elucidate how these beneficial lipids were being regulated. AIG1 and ADTRP make up a small class of FAHFA hydrolases in human cells. In this study, I wanted to further examine how these enzymes were degrading FAHFAs *in vivo*. I hypothesized that FAHFA pathways would be conserved between two model organisms, *C. elegans* and *D. melanogaster*, and mammals. A simple animal model would allow the Saghatelian lab to ascertain the physiological roles of AIG1 and ADTRP in processing FAHFAs more quickly since they have shorter life cycles and has a simpler genome in comparison to a mouse or any mammalian model. Genomic knockdown of AIG1 and ADTRP and examination of changes in FAHFA levels can be done to further elucidate the function of these two enzymes. Prior to genomic knockdown of the *D. melanogaster* orthologs, CG3625b and CG11601, the proteins were further studied.

To validate that the function of AIG1 and ADTRP is conserved in *D. melanogaster*, I tested whether the active site was conserved. The catalytic nucleophile and the basic residue, threonine and histidine respectively, were both conserved in CG3625b and CG11601, the two orthologs explored. The two proteins were overexpressed in HEK293T cells and a western blot confirmed that the two transmembrane proteins were in the membrane fraction of the cell lysate. To show that the active site was conserved, an ABPP gel was run to tag the proteins with FP-rhodamine. The ABPP gel did confirm that CG3525b and CG11601 were FP-reactive, thus were possible threonine hydrolases. To further show that the threonine and histidine

were conserved between the two species, the two residues were mutated into alanine to see if activity was abrogated. The threonine was also mutated into a serine to show that threonine is the nucleophilic residue for these orthologs. An ABPP gel was run to see if the active site was affected by the mutations and the FP-rhodamine was unable to react with the mutated active sites of both CG3625b and CG11601. These assays, in conjunction, show that the active site of the AIG1 and ADTRP orthologs is conserved, but they do not confirm whether they can hydrolyze FAHFAs.

Hydrolysis assays, analyzed by LC-MS, measured whether or not CG3625b and CG11601 could enzymatically cleave FAHFAs into a fatty acid and a hydroxy fatty acid. Using empty pcDNA3.1 vector as my negative control, I looked at the hydrolytic activity of the two enzymes. The hydrolysis activity confirmed that in comparison to the mock control, CG3625b and CG11601 could enzymatically hydrolyze FAHFAs, which suggested that they were enzymes that could regulate FAHFAs in flies. That FAHFA hydrolysis activity was further confirmed by hydrolysis assays with the overexpressed proteins that had mutated active sites. When the conserved threonine and histidine were mutated, the hydrolysis activity that was shown above the background mock control was quenched. The hydrolysis for the mutant proteins used 12-PAHSA which showed a higher level of hydrolysis. AIG1 and ADTRP have been shown to preferentially cleave esters farther from the carboxylate carbon (Parsons et al. 2016). A broad substrate assay with the different families and isomers of FAHFAs would need to be used to confirm that finding. Overall, the hydrolysis activity of both CG3625b and CG11601 were not significantly above the mock control. The mock control had activity due to endogenous AIG1 in HEK293T cells and the fly proteins did not appear to be as active as the human

protein when ABPP and hydrolysis assays were performed on the human protein (data not shown). As these studies were done in mammalian cells, they remain inconclusive as to whether CG3625b and CG11601 could endogenously process FAHFAs in flies.

If CG3625b and CG11601 are enzymes that were primarily degrading FAHFAs in flies, then blocking those orthologs would produce a decrease in hydrolytic activity. To test that, competitive ABPP was performed. Since the active site of AIG1 and ADTRP proved to be conserved in the orthologs, KC01, a covalent and irreversible inhibitor of AIG1 that selectively blocks the active site of AIG1, was hypothesized to also block the activity of the orthologs. KC01 was used against the membrane fraction of the HEK293T cell lysate overexpressing either CG3625b or CG11601 and then FP-rhodamine was added to see if the proteins could still react with the fluorophosphonate. In the ABPP gel run, the overexpressed proteins that were treated with KC01 could not be tagged by FP-rhodamine indicating that the active site was being blocked by KC01. To further validate that the activity was being blocked a hydrolysis assay was performed to see if hydrolysis would be inhibited by KC01. Hydrolysis was completely abrogated with the addition of KC01. These observations show that KC01 is able to target the fly proteins and inhibit FAHFA hydrolysis. This allowed me to use KC01 to further ascertain how the fly is able to process FAHFAs.

Schneider (S2) cells were fractionated into membrane and cytosol. I showed that FAHFA hydrolysis was more concentrated in the membrane fraction than the cytosolic fraction which is consistent with CG3625b and CG11601 being transmembrane proteins. Because I validated that the KC01 inhibitor was able to target the fly proteins of interest, I used KC01 against the membrane fraction to show that these enzymes were contributing

to the hydrolytic activity in the membrane. MAFP, a broad serine hydrolase inhibitor, was used as a positive control. There was a statistically significant decrease in hydrolysis between DMSO treated lysate and KC01 treated lysate. This change provides evidence that the two proteins of interest are FAHFA hydrolases in the fly. The fact that MAFP is able to further quench hydrolysis implies that there are other FAHFA hydrolases in the fly, but CG3625b and CG11601 may be major hydrolases. This would need to be further confirmed by a genetic knockdown of CG3625b and CG11601 to see if there is the same change in FAHFA hydrolysis in the fly cell.

If there is a significant decrease in FAHFA hydrolysis in the fly cells when CG3625b and CG11601 are knocked down, that would indicate that these two enzymes play a big role in FAHFA degradation in these fly cells. That would make us interested in further looking at their activity in the fly and checking for FAHFA levels in the fly when these proteins are knocked down. If the fly does show to be a good model for FAHFA regulation, that could open doors to many studies. The knockdown model could be used to measure other phenotypic changes connected to FAHFAs. Inhibitors for enzymes degrading FAHFAs could be fed to the flies to see if the inhibitors have an impact on FAHFA regulation *in vivo.* Because FAHFAs are beneficial, it would be interesting to see if targeting the enzymes that degrade FAHFAs will produce changes and be able to manipulate the levels of FAHFAs to produce more of the beneficial effects found previously.

FIGURES

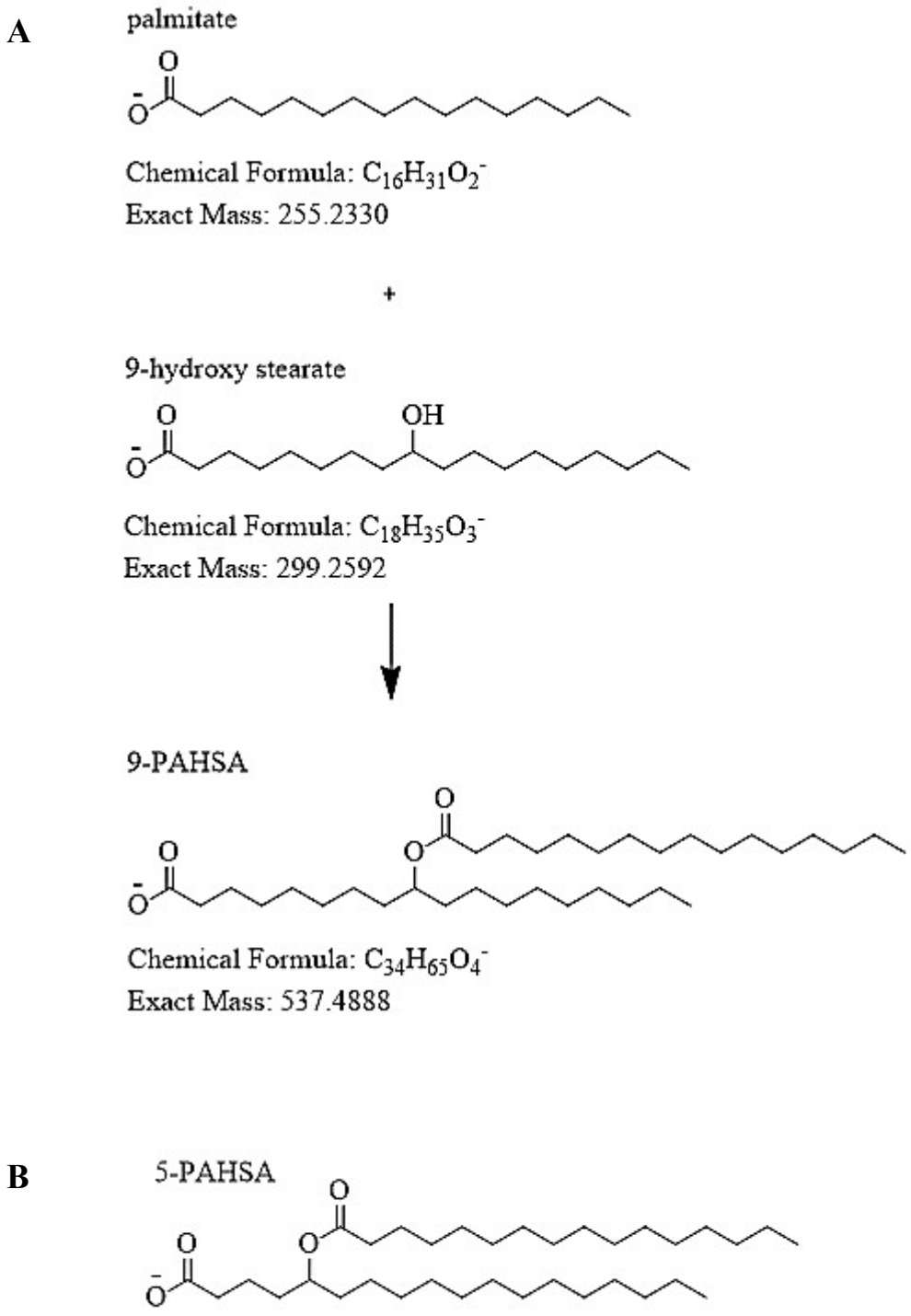

Figure 1. Structural Characterization of FAHFAs. (A) Molecular formula, mass, and name of components that go into 9-PAHSA. (B) Example of a different isomer (5-PAHSA) that is of the same family (PAHSA).

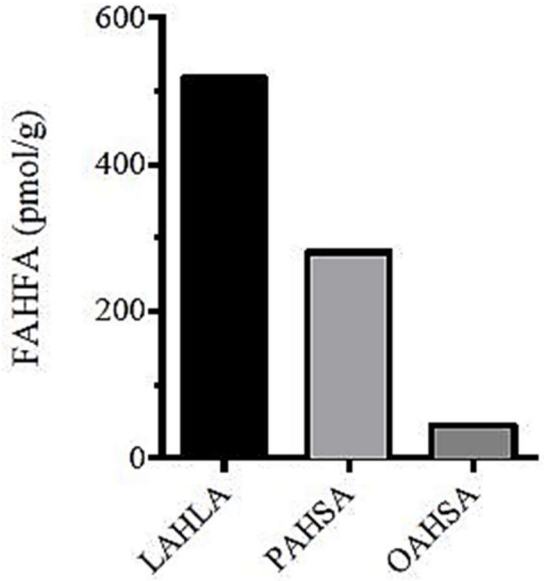

Figure 2. Identification of FAHFAs in *Caenorhabditis elegans*. Modified Bligh-Dyer lipid extraction followed by targeted LC-MS identified three FAHFA families present in *C. elegans*.

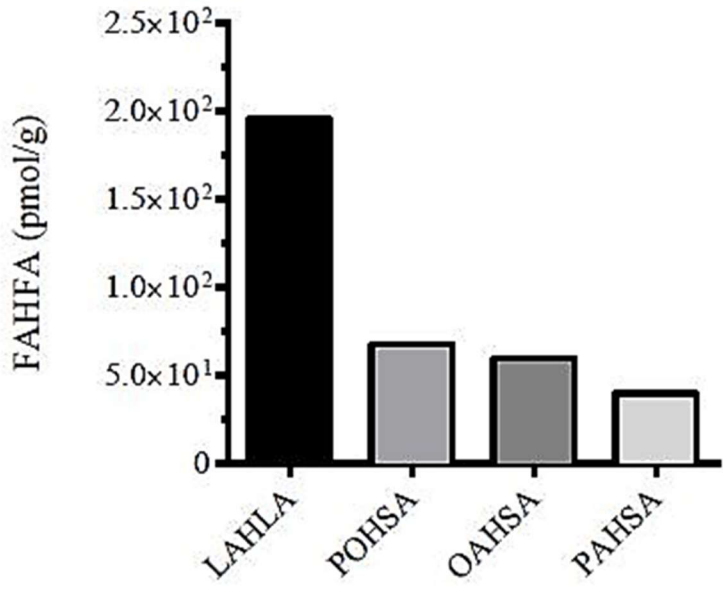

Figure 3. Identification of FAHFAs in *Drosophila melanogaster*. Modified Bligh-Dyer lipid extraction followed by targeted LC-MS identified four FAHFA families present in Drosophila

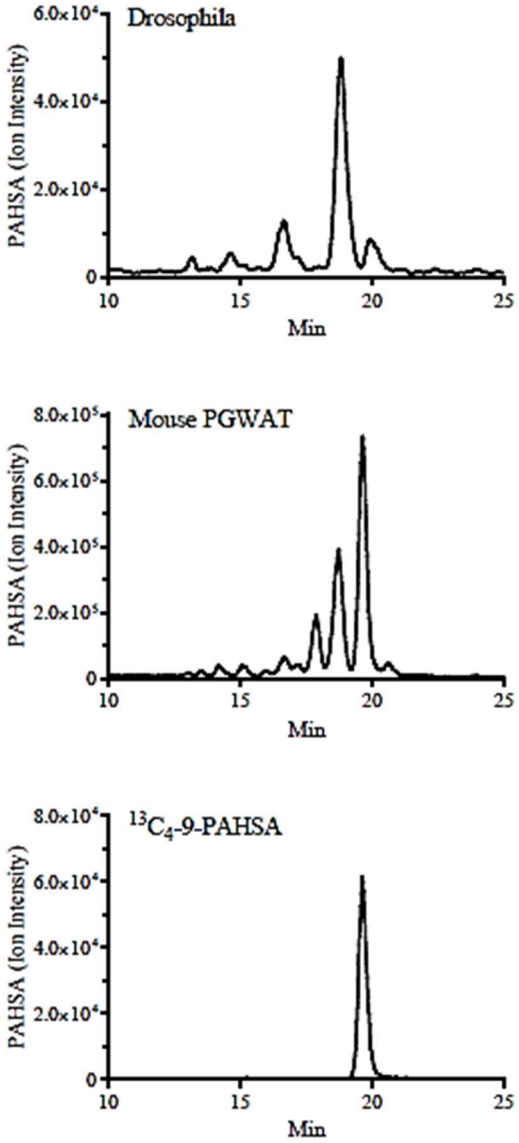

Figure 4. LC-MS Chromatograms for PAHSAs in Drosophila and Mice. Peaks for the different isomers of PAHSA in the full body of Drosophila and perigonadal adipose tissue in mice. $^{13}C_4$-9-PAHSA used as a synthetic standard.

Figure 5. Threonine and histidine in active site is conserved between species.
Sequence alignment of CG3625 and CG11601 against human AIG1 and ADTRP.

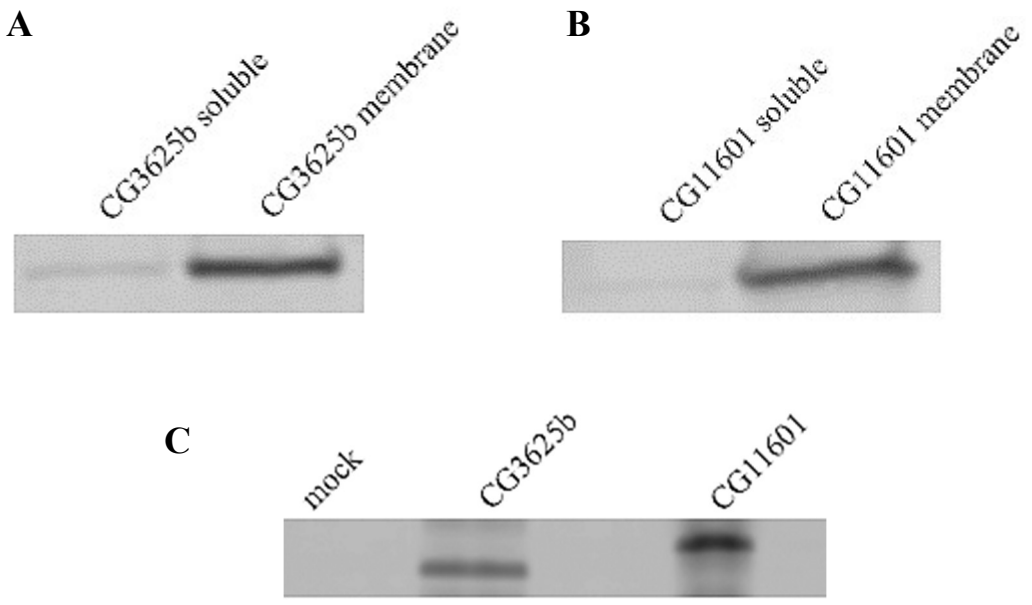

Figure 6. Western blot and ABPP of overexpressed Drosophila AIG1/ADTRP orthologs in HEK293T cells. (A and B) HEK293T cells were transfected with CG3625b and CG11601 recombinantly expressed in pcDNA3.1 V5/His B vector. Cytosolic and membrane fractions were separated. Mouse anti-V5 was used as primary antibody against expression vector. Overexpressed CG3625b and CG11601 were found in membrane fraction of cell lysate. (C) ABPP analysis of HEK293T cells transfected with CG3625b and CG11601 to identify serine hydrolases. 50µg membrane proteome was incubated with 1µM FP-rhodamine for 30 minutes at 37°C.

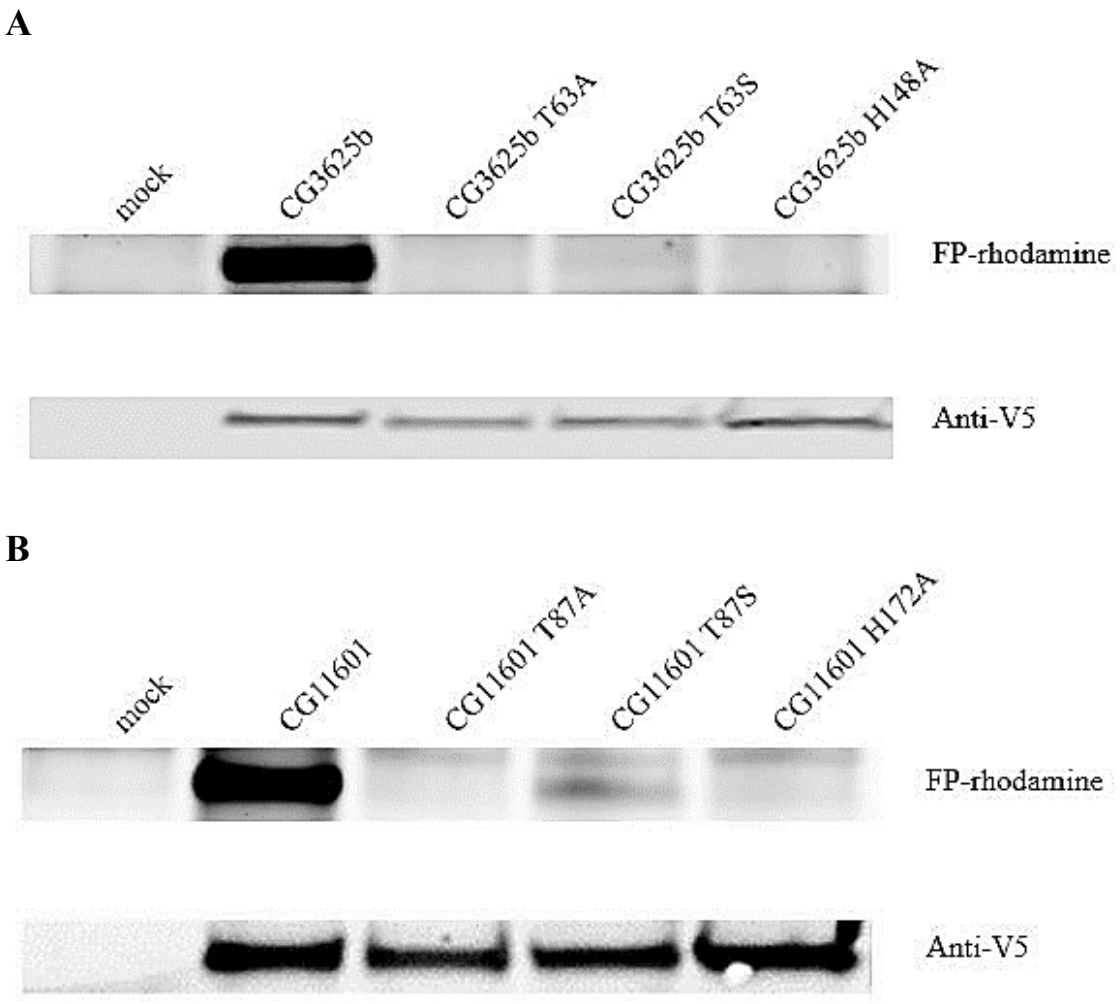

Figure 7. ABPP gels and western blots of Drosophila AIG1/ADTRP ortholog mutants. (A and B) FP-rhodamine tagged wild type CG3625b and CG11601 and not the mutant orthologs. 50µg membrane proteome was incubated with 1µM FP-rhodamine for 30 minutes at 37°C.

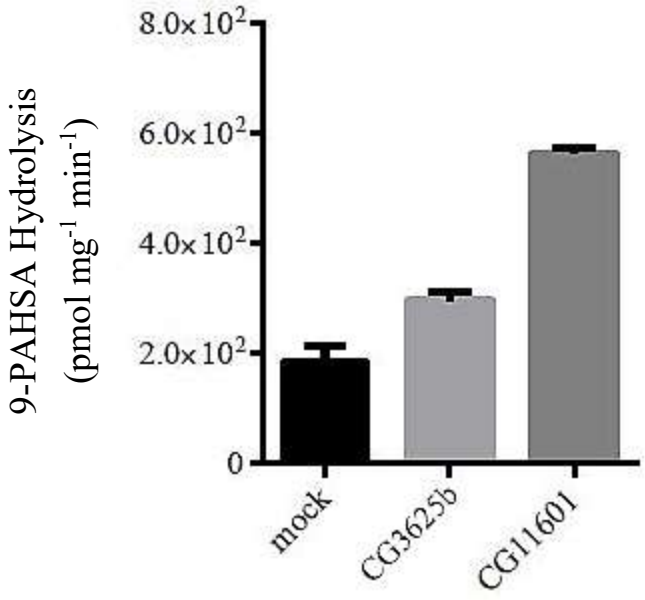

Figure 8. CG3625b and CG11601 hydrolysis of 9-PAHSA. 9-PAHSA hydrolysis assay for membrane proteome of HEK293T cells transfected with AIG1 and ADTRP orthologs. Mock is HEK293T cells transfected with empty pcDNA3.1 vector. 20µg membrane proteome was incubated with 100µM 9-PAHSA substrate for 20 minutes at 37°C. Data represents mean ± s.e.m. for two biological replicates.

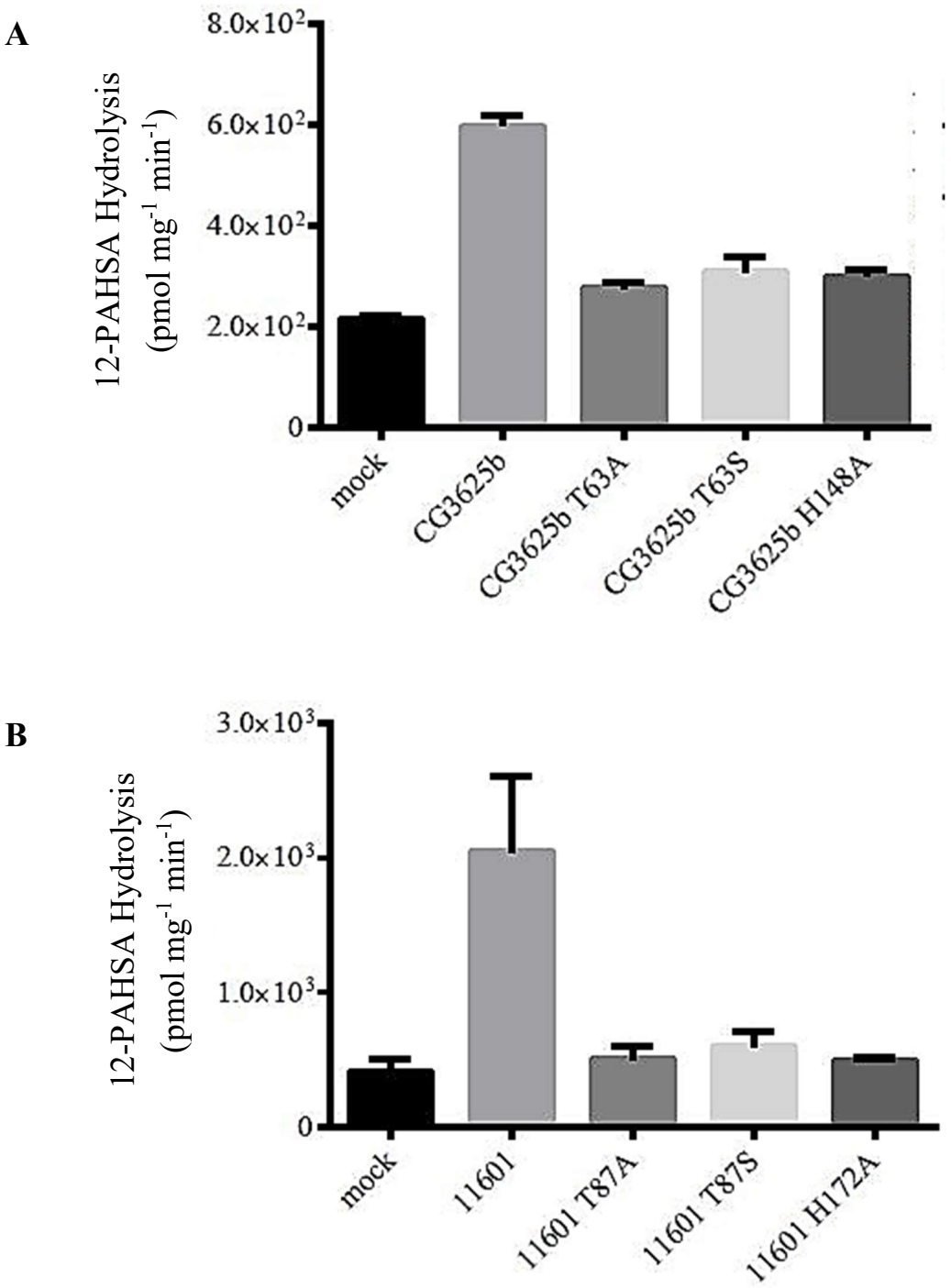

Figure 9. Mutant CG3625b and CG11601 hydrolysis of 12-PAHSA. (A and B) 12-PAHSA hydrolysis assay for membrane proteome of HEK293T cells transfected with mutant CG3625b and CG11601. Mock is HEK293T cells transfected with empty pcDNA3.1 vector. 20µg membrane proteome was incubated with 100µM 9-PAHSA substrate for 20 minutes at 37°C. Data represents mean ± s.e.m. for two biological replicates.

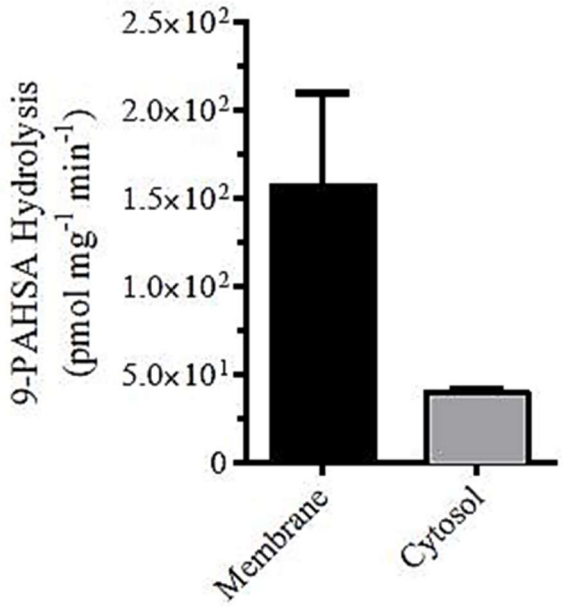

Figure 10. Schneider Cell FAHFA Hydrolysis. Membrane and cytosol fractionated at 16,000g for 45 minutes at 4°C. 20µg membrane proteome treated with 5µM inhibitor for 30 minutes at 37°C followed by 100µM 9-PAHSA substrate for 20 minutes at 37°C. Data represents mean values ± s.e.m. for two biological replicates.

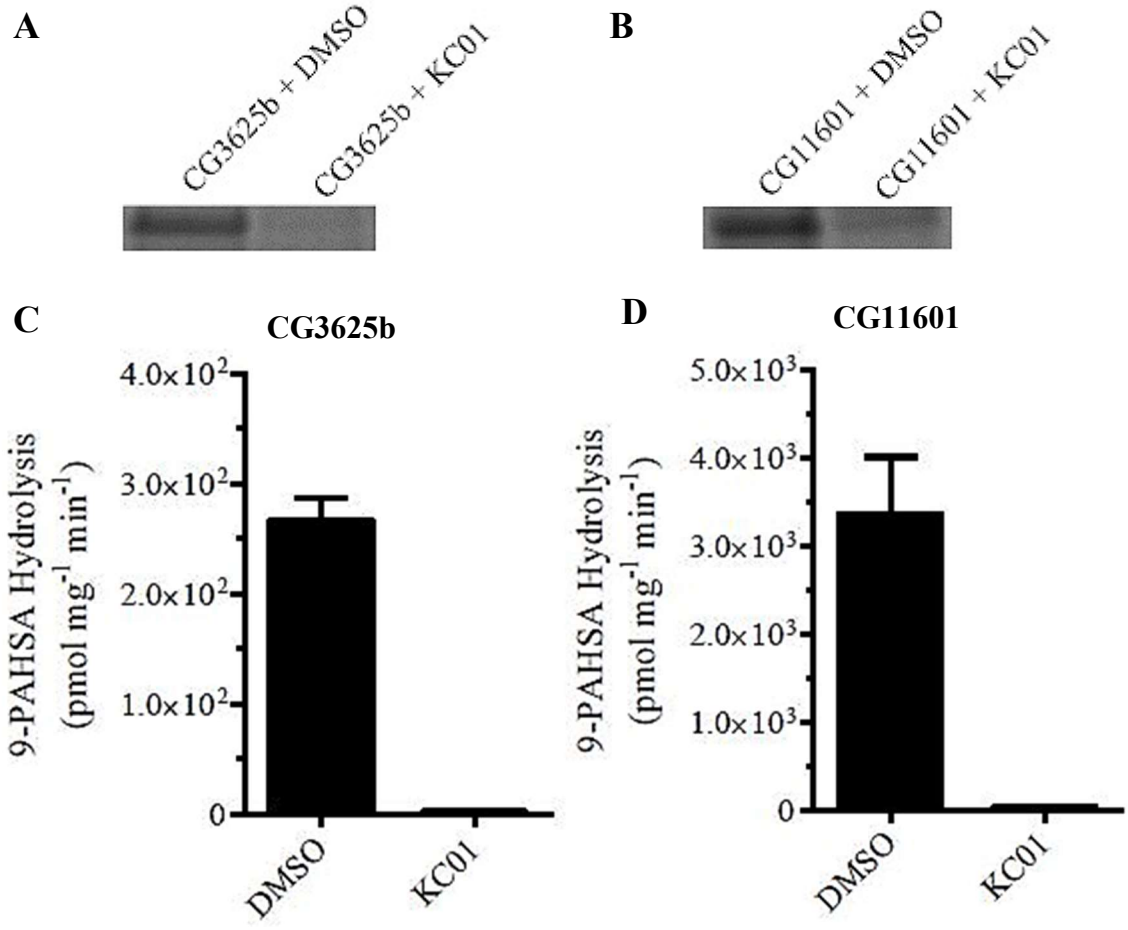

Figure 11. KC01 able to block activity of CG3625b and CG11601. (A and B) Competitive ABPP with KC01 inhibitor. Proteome was treated with 5µM KC01 for 30 minutes at 37°C followed by 1µM FP-rhodamine for 30 minutes at 37°C. (C and D) Inhibition of 9-PAHSA hydrolysis in HEK293T cells transfected with CG3625b and CG11601. 20µg membrane proteome treated with 5µM inhibitor for 30 minutes at 37°C followed by 100µM 9-PAHSA substrate for 20 minutes at 37°C. Data represents mean values ± s.e.m. for three biological replicates.

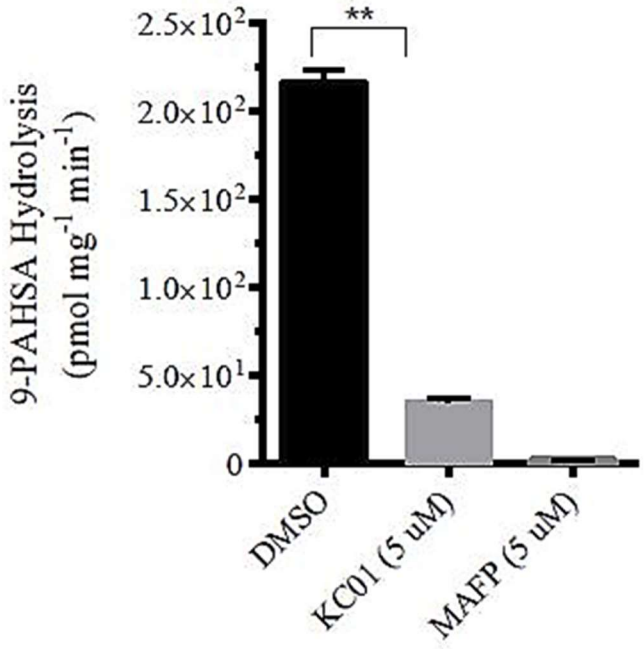

Figure 12. KC01 blocks FAHFA hydrolysis in Schneider cells. Inhibition of 9-PAHSA hydrolysis in membrane fraction of Schneider cells. 20μg membrane proteome treated with 5μM inhibitor for 30 minutes at 37°C followed by 100μM 9-PAHSA substrate for 20 minutes at 37°C. Data represents mean values ± s.e.m. for three biological replicates. $^{**}P < 0.01$ by two-sided Student's *t*-test for inhibitor-treated versus control cell lines.

TABLES

Table 1. Primers used in site-directed mutagenesis of CG3625b and CG11601.

Mutation	Forward Primer
CG3625b	
T63A	CAAATACCTAGCGTTCCTGGATGCG
T63S	CAAATACCTATCGTTCCTGGATGCGATAATTC
H148A	CCACGTCCTGGCCACCAACATAGTGG
CG11601	
T87A	CAAGTACCTTGCCTTCTTGGACGTCATTCTACAG
T87S	CAAGTACCTTTCCTTCTTGGACGTCATTCTACAGG
H172A	TCACGTGGTAGCCACGAATGTGGC

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