Supporting Information for

AIG1 and ADTRP are endogenous hydrolases of fatty acid esters of hydroxy fatty acids (FAHFAs) in mice

Meric Erikci Ertunc, Bernard P. Kok, William H. Parsons, Justin G. Wan, Dan Tan[1], Cynthia J. Donaldson, Antonio F. M. Pinto, Joan M. Vaughan, Nhi Ngo, Kenneth M. Lum, Cassandra L. Henry, Aundrea R. Coppola, Micah J. Niphakis, Benjamin F. Cravatt, Enrique Saez, Alan Saghatelian

Supporting experimental procedures

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Supporting Tables 1, 4

Supporting experimental procedures

Generation of antibodies against AIG1 and ADTRP

Animal Care

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Salk Institute and were conducted in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals (PHS Policy, 2015), the U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training, the NRC Guide for Care and Use of Laboratory Animals (8th edition) and the USDA Animal Welfare Act and Regulations. All animals were housed in an AAALAC accredited facility in a climate-controlled environment (65-72 degrees Fahrenheit, 30-70% humidity) under 12-hour light/12-hour dark cycles. Upon arrival, animals were physically examined by veterinary staff for good health and acclimated for at least two weeks prior to initiation of antiserum production. Each animal was monitored daily by the veterinary staff for signs of complications and weighed every two weeks. Routine physical exams were also performed by the veterinarian quarterly on all rabbits and guinea pigs.

For production of mouse AIG1 (mAIG1) antiserum using rabbits, three 10 to 12-week old, female New Zealand white rabbits, weighing 3.0 to 3.2 kg at beginning of the study, were procured from Irish Farms (I.F.P.S. Inc., Norco, California, USA). Rabbits were provided with ad libitum feed (5326 Lab Diet High Fiber), micro-filtered water and weekly fruits, vegetables and alfalfa hay for enrichment. For production of mouse ADTRP (mADTRP) antiserum in guinea pigs, four 10 to 12-week old, female Hartley guinea pigs, weighing 700-750 g at the beginning of the study, were procured from Charles River Laboratories. Guinea pigs were provided with ad libitum feed (5025 Lab Diet), micro-filtered water and weekly fruits and vegetables for enrichment.

Preparation of Antigens

Synthetic peptides were synthesized by RS Synthesis (Louisville, KY), HPLC purified to >95%, and amino acid sequenced verified by mass spectrometry. Cys^{59} mAIG1(59-87) and Cys^{22} mADTRP(22-45) were conjugated to maleimide activated Keyhole Limpet Hemocyanin (KLH) per manufacturer's instructions (ThermoFisher). Specific peptides used to generate antisera are as follows:
Cys^{59} mAIG1(59-87), CTDLSSLLTRGSGNQEQERQLRKLISLRD: and
Cys^{22}mADTRP, CHIPQIGRNEEKLREFHDGGRSKY.

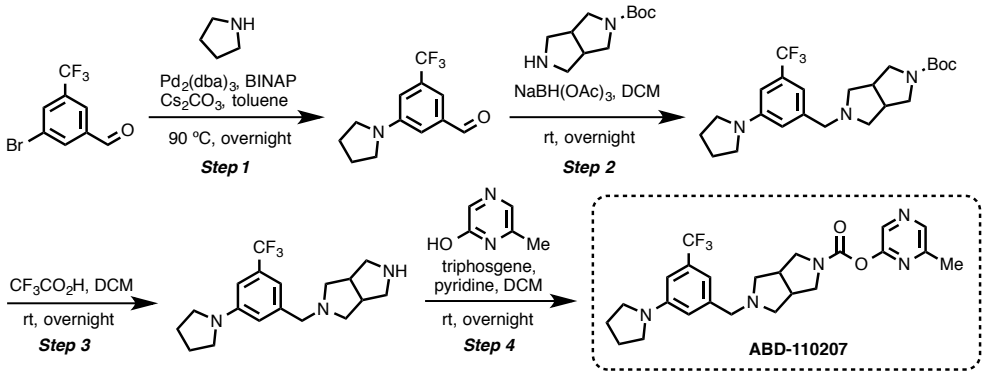
Injection and Bleeding of Animals

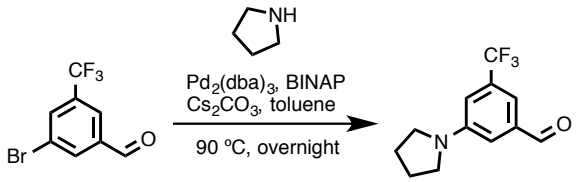
The antigen was delivered to host animals using multiple intradermal injections of peptide-KLH conjugate in Complete Freund's Adjuvant (initial inoculation) or incomplete Freund's adjuvant (booster inoculations) every three weeks for rabbits and once every four weeks for guinea pigs. Animals were bled, <10% total blood volume, one week (rabbits) or two weeks (guinea pigs) following booster injections and bleeds screened for titer and specificity. Rabbits were administered 1-2 mg/kg Acepromazine IM prior to injections of antigen or blood withdrawal. Guinea pigs were anesthetized using inhalation isoflurane maintained at 2 to 2.5% prior to injections and bleedings. At the termination of study, rabbits were exsanguinated under anesthesia (ketamine 50 mg/kg and aceprozamine 1 mg/kg, IM) and euthanized with an overdose of pentobarbital sodium and phenytoin sodium (1 ml/4.5 kg of body weight IC to effect). Guinea pigs were exsanguinated via cardiac puncture under inhalation anesthesia (isoflurane maintained at 2-2.5%). After blood was collected death of animals was confirmed. All animal procedures were conducted by experienced veterinary technicians, under the supervision of Salk Institute veterinarians.

Characterization and purification of antisera

Each bleed from each animal was tested at multiple doses for the ability to recognize the synthetic peptide antigen; bleeds with highest titers were further analyzed by western immunoblot for the ability to recognize the full-length endogenous protein and to check for cross-reactivity to other proteins. Antisera with the best characteristics of titer against the synthetic peptide antigen, ability to recognize the endogenous protein, and specificity were affinity purified and used for all studies. Rabbit PBL #7384 anti-mAIG1(59-87) and guinea pig PBL #096 anti-mADTRP(22-45) were purified using the homologous synthetic peptide covalently attached to Sulfolink agarose (ThermoFisher) per manufacturer's instructions. To ensure that the same batch of purified antibodies could be used for this and future studies, large volumes, 20 ml rabbit serum #7384, or 10 ml guinea pig #096 serum, from bleeds with similar profiles were purified.

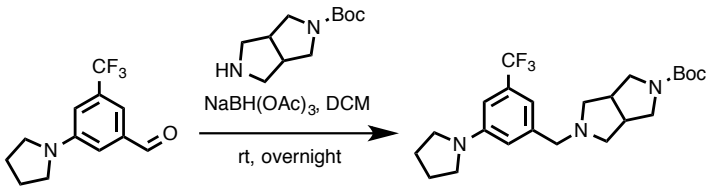
Synthesis of ABD-110207



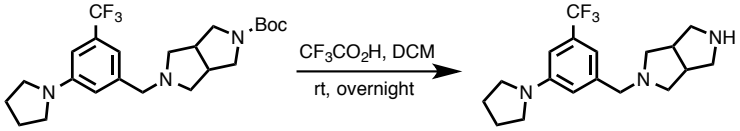


Step 1 - 3-(pyrrolidin-1-yl)-5-(trifluoromethyl)benzaldehyde: A 250-mL round-bottom flask was charged with 3-bromo-5-(trifluoromethyl)benzaldehyde (3.00 g, 11.9 mmol, 1.00 equiv), pyrrolidine (1.69 g, 23.8 mmol, 2.00 equiv), tris(dibenzylideneacetone)dipalladium (0.550 g, 0.600 mmol, 0.05 equiv), 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene (1.12 g, 1.80 mmol, 0.15 equiv), cesium carbonate (11.6 g, 35.6

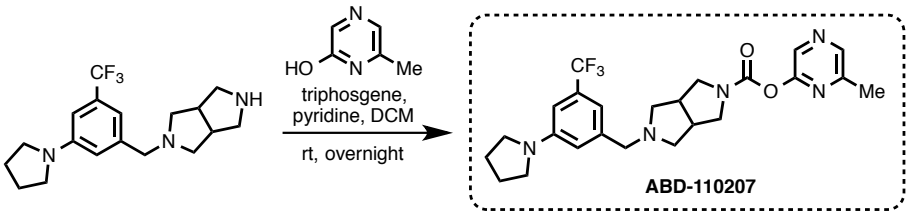
mmol, 3.00 equiv), toluene (40 mL) under nitrogen. The resulting solution was stirred overnight at 90 °C and then quenched with water (50 mL). The resulting mixture was extracted with ethyl acetate (3 x 50 mL) and the organic layers were combined, washed with brine (1 x 100 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with ethyl acetate/petroleum ether (1/5) to provide 1.89 g (66% yield) of 3-(pyrrolidin-1-yl)-5-(trifluoromethyl)benzaldehyde as a yellow solid. LCMS (ESI, *m/z*): 244 $[M+H]^+$.



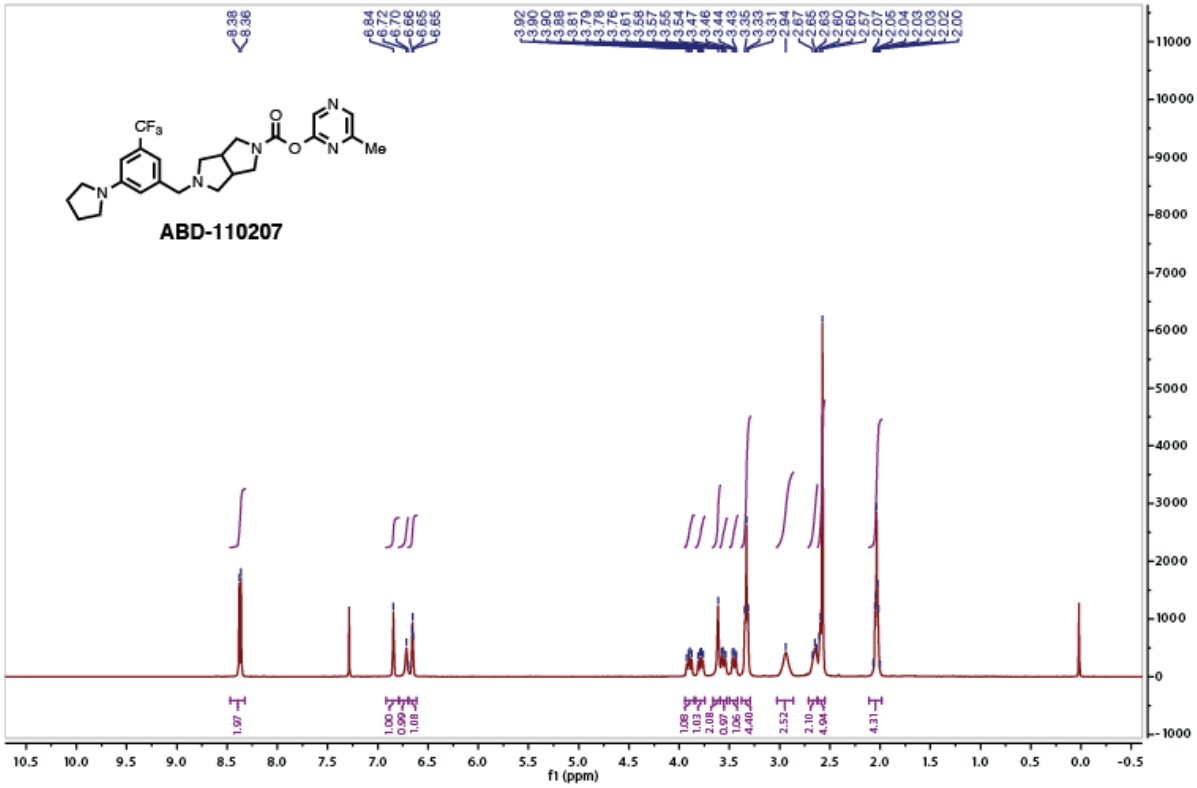
Step 2 - tert-butyl 5-[[3-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl]methyl]-octahydropyrrolo[3,4-c]pyrrole-2-carboxylate: A 100-mL round-bottom flask was charged with 3-(pyrrolidin-1-yl)-5-(trifluoromethyl)benzaldehyde (0.600 g, 2.47 mmol, 1.00 equiv), tert-butyl octahydropyrrolo[3,4-c]pyrrole-2-carboxylate (0.628 g, 2.96 mmol, 1.20 equiv), dichloromethane (10 mL). The mixture was stirred for 1 h at room temperature. Sodium triacetoxyborohydride (1.57 g, 7.41 mmol, 3.00 equiv) was added. The resulting solution was stirred overnight at room temperature and then quenched with water (10 mL). The resulting mixture was extracted with dichloromethane (3 x 15 mL) and the organic layers were combined, washed with brine (1 x 50 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with methanol/dichloromethane (1/5) to provide 1.02 g (94% yield) of tert-butyl 5-[[3-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl]methyl]-octahydropyrrolo[3,4-c]pyrrole-2-carboxylate as yellow oil. LCMS (ESI, *m/z*): 440 $[M+H]^+$.



Step 3 - 1-(3-[octahydropyrrolo[3,4-c]pyrrol-2-ylmethyl]-5-(trifluoromethyl)phenyl)pyrrolidine: A 250-mL round-bottom flask was charged with tert-butyl 5-[[3-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl]methyl]-octahydropyrrolo[3,4-c]pyrrole-2-carboxylate (1.07 g, 2.43 mmol, 1.00 equiv), trifluoroacetic acid (12 mL), dichloromethane (20 mL). The resulting solution was stirred overnight at room temperature and concentrated under reduced pressure. The crude product was dissolved in 1M NaOH solution (10 mL) and extracted with dichloromethane (3 x 20 mL). The organic layers were combined, washed with brine (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to provide 0.810 g (98% yield) of 1-(3-[octahydropyrrolo[3,4-c]pyrrol-2-ylmethyl]-5-(trifluoromethyl)phenyl)pyrrolidine as yellow oil. LCMS (ESI, *m/z*): 340 $[M+H]^+$.



Step 4 - 6-methylpyrazin-2-yl 5-[[3-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl]methyl]-octahydropyrrolo[3,4-c]pyrrole-2-carboxylate (ABD-110207): A 50-mL round-bottom flask was charged with triphosgene (92.0 mg, 0.310 mmol, 0.70 equiv), dichloromethane (5 mL). 6-methylpyrazin-2-ol (98.0 mg, 0.890 mmol, 2.00 equiv) was added at 0 °C. Pyridine (140 mg, 1.77 mmol, 4.00 equiv) was added at 0 °C. The mixture was stirred for 2 h at room temperature. 1-(3-[octahydropyrrolo[3,4-c]pyrrol-2-ylmethyl]-5-(trifluoromethyl)phenyl)pyrrolidine (150 mg, 0.440 mmol, 1.00 equiv) was added. The resulting solution was stirred overnight at room temperature and then quenched with water (10 mL). The resulting mixture was extracted with dichloromethane (3 x 15 mL) and the organic layers were combined, washed with brine (1 x 30 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product (400 mg) was purified by preparative HPLC using the following gradient conditions: 20% CH_3CN/80% Phase A increasing to 80% CH_3CN over 10 min, then to 100% CH_3CN over 0.1 min, holding at 100% CH_3CN for 1.9 min, then reducing to 20% CH_3CN over 0.1 min, and holding at 20% for 1.9 min, on a Waters 2767-5 Chromatograph. Column: Xbridge Prep C18, 19*150mm 5um; Mobile phase: Phase A: aqueous NH_4HCO_3 (0.05%); Phase B: CH_3CN; Detector, UV220 & 254nm. Purification resulted in 88.9 mg (42% yield) of 6-methylpyrazin-2-yl 5-[[3-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl]methyl]-octahydropyrrolo[3,4-c]pyrrole-2-carboxylate as a yellow solid. ^{1}H NMR (400MHz, Chloroform-*d*) □□8.35 (d, *J* = 6.0 Hz, 2H), 6.82 (s, 1H), 6.70 (s, 1H), 6.64 (s, 1H), 3.90 - 3.85 (m, 1H), 3.79 - 3.74 (m, 1H), 3.60 (s, 2H), 3.56 - 3.52 (m, 1H), 3.46 - 3.42 (m, 1H), 3.33 - 3.30 (m, 4H), 2.93 (br, 2H), 2.71 - 2.62 (m, 2H), 2.58 - 2.55 (m, 5H), 2.06 - 1.98 (m, 4H). LCMS (ESI, *m/z*): 476 [M+H]$^+$.



Supporting Figures

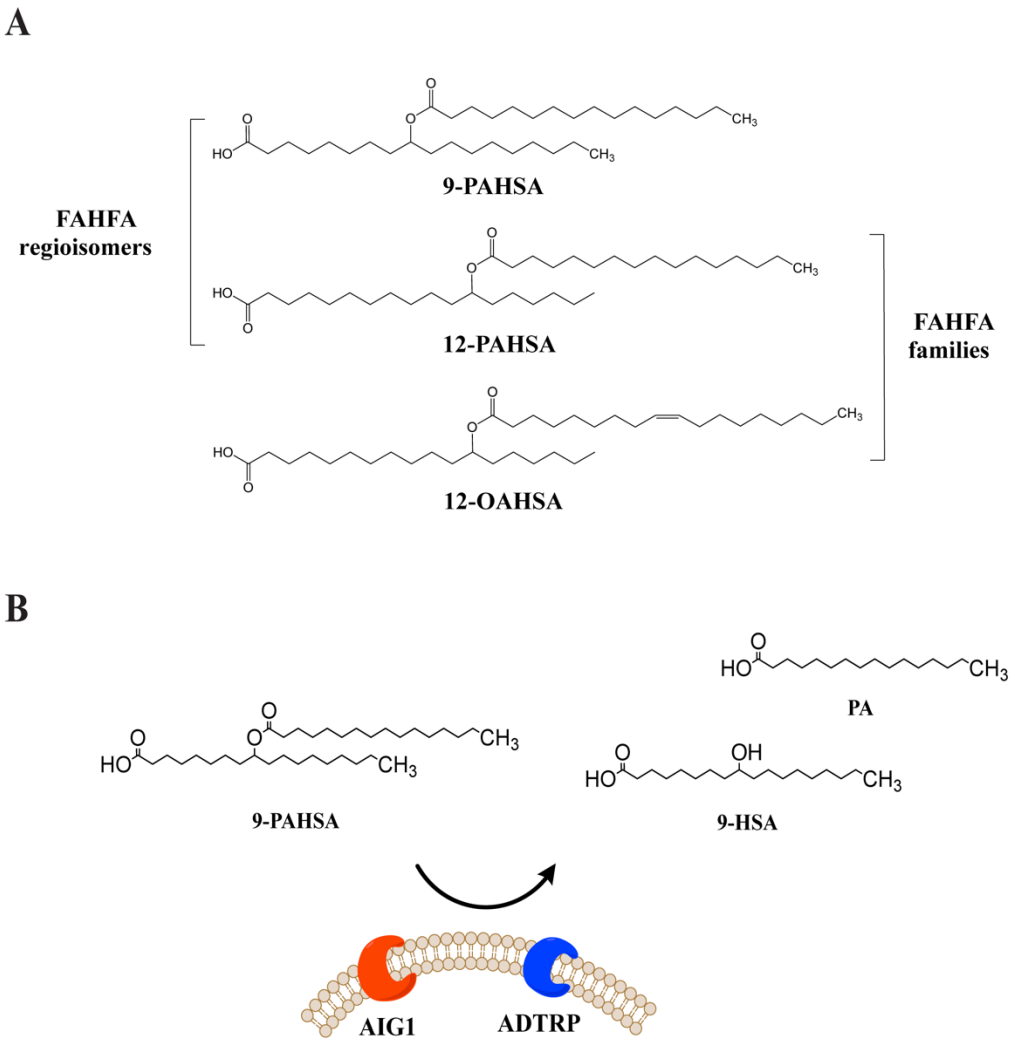


Figure S1. FAHFA structures. (A) FAHFA families have different acyl chains such as palmitic acid ester of hydroxy stearic acid (PAHSA) and oleic acid ester of HSA (OAHSA) and each family contains multiple FAHFA regioisomers (e.g., 9-PAHSA and 12-PAHSA). **(B)** Transmembrane AIG1 and ADTRP hydrolyze FAHFAs at the ester bond leading to generation of end products, free fatty acids (e.g. PA: palmitic acid) and hydroxy fatty acids (e.g. 9-HSA: 9-hydroxy stearic acid).

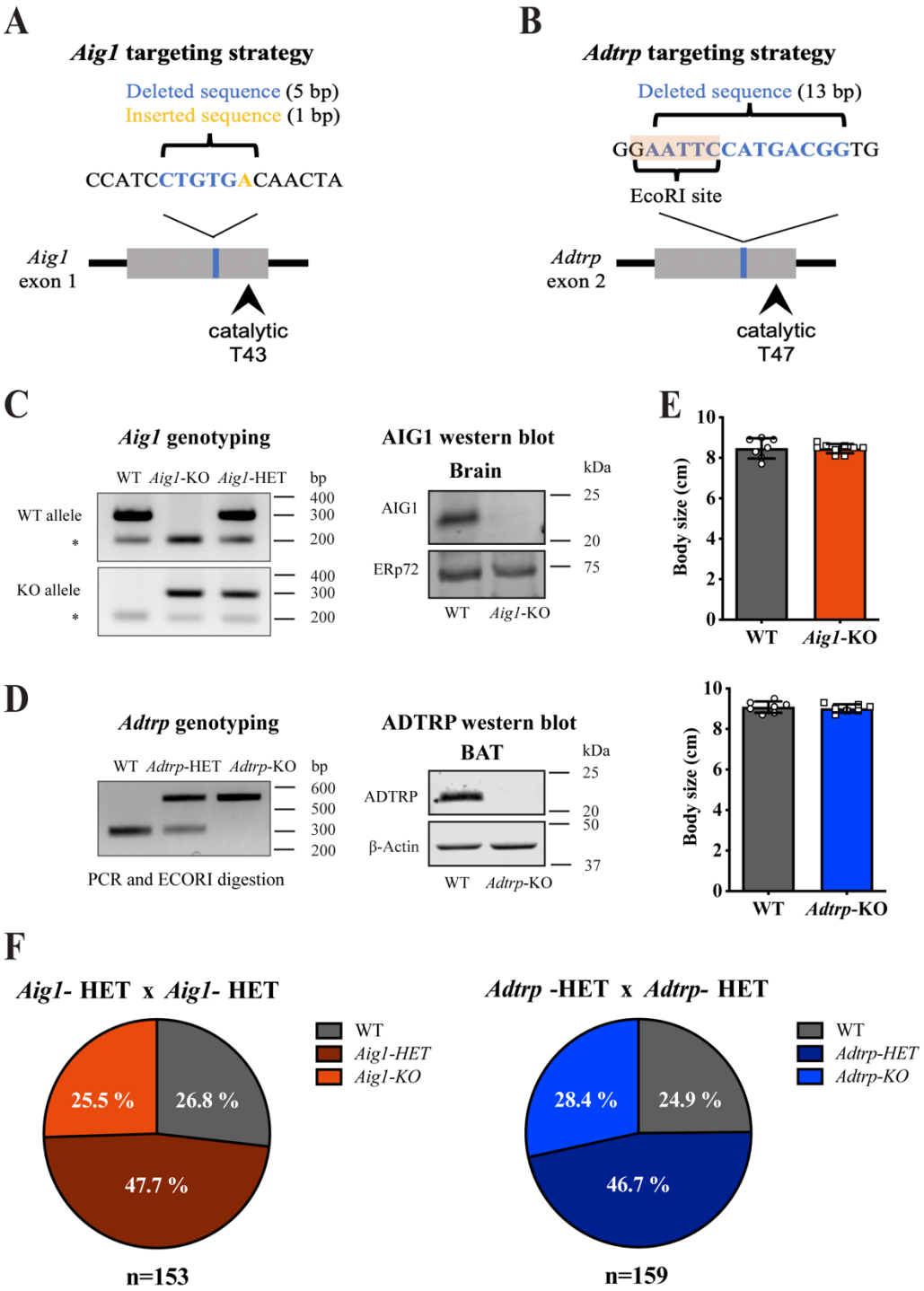


Figure S2. Generation of AIG1 and ADTRP deficient mouse models. (A) Exon 1 of mouse *Aig1* (left panel) and **(B)** exon 2 of mouse *Adtrp* gene were targeted using CRISPR/Cas9 resulting in frameshift indel mutations and immature stop codons prior to the catalytic threonine residues. **(C)** Genotyping strategy for *Aig1* via allele specific PCR amplification (left) and western blot for AIG1 in brain microsomal fractions (right). * indicates PCR band used as internal control. ERp72 was used as a loading control for microsomal preparations. **(D)** Genotyping strategy for *Adtrp* utilizing EcoRI sensitivity to assess presence of mutant allele, which lacks the EcoRI site leading to resistance to restriction enzyme digestion (left panel). Western

blot of BAT lysates for ADTRP (right). β-Actin was used as loading control. **(E)** Body size of approximately 3.5-month old female WT and *Aig1*-KO mice (top) and 7-month old male WT and *Adtrp*-KO mice (bottom) measured as the distance between anus and chin. Error bars represent SD (n=7-9 per group). **(F)** Percent of genotypes of pups born from *Aig1*- (left) or *Adtrp*- (right*)* HET crosses.

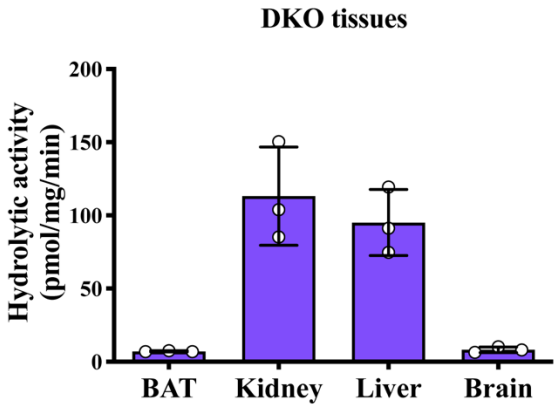


Figure S3. Absolute hydrolytic activity in DKO tissues. 9-PAHSA hydrolysis assay on membrane lysates from BAT, kidney, liver, and brain from DKO mice. Error bars represent SD (n=3 per group).

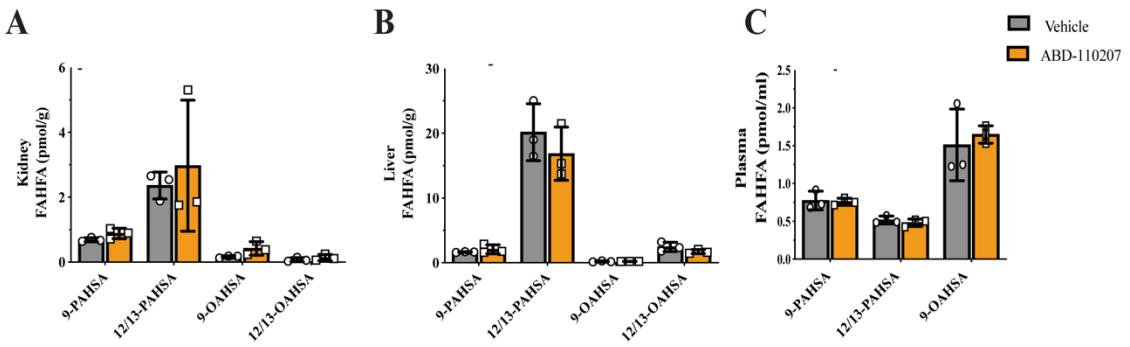


Figure S4. FAHFA levels in Vehicle vs ABD-110207 treated mice. Free FAHFA levels in **(A)** kidney, **(B)** liver, and **(C)** plasma of mice treated with vehicle or ABD-110207. Error bars represent SD (n=3 per group).

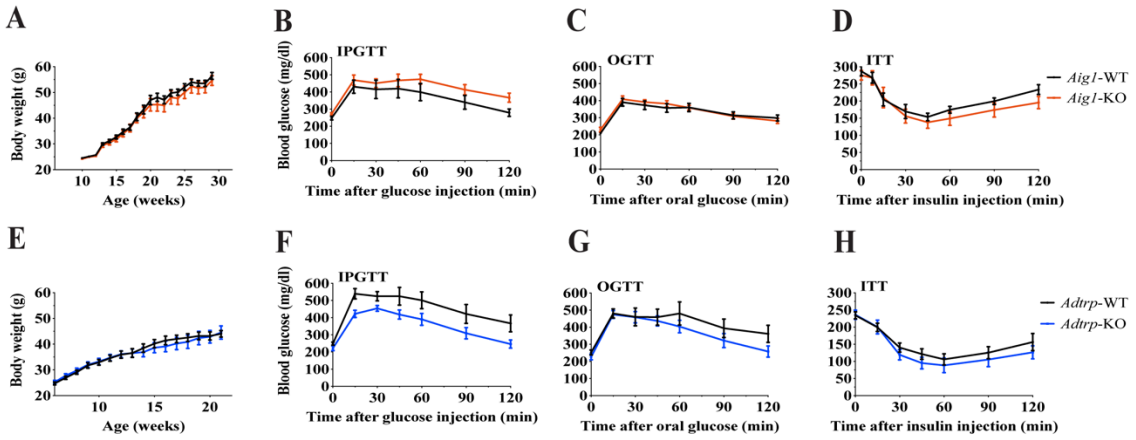


Figure S5. Metabolic studies in HFD-fed *Aig1*-KO or *Adtrp*-KO male mice with corresponding WT controls. WT and *Aig1*-KO mice: **(A)** Body weight. Blood glucose levels during **(B)** IPGTT after 8-hour food withdrawal and 1 g/kg glucose administration **(C)** OGTT after 16-hour fasting and 2 g/kg glucose administration **(D)** ITT after 4-hour food withdrawal and 0.5 u/kg insulin administration. Error bars represent SEM (n=6-8per group). WT and *Adtrp*-KO mice: **(E)** Body weight. Blood glucose levels during **(F)** IPGTT after 1 g/kg glucose administration **(G)** OGTT after 2 g/kg glucose administration **(H)** ITT after 1 u/kg insulin administration. Error bars represent SEM (n=6-7 per group).

Supporting Tables

Table S2. Tissue FAHFA levels from *Aig1*, *Adtrp*, and DKO mice

FAHFA	*Aig1*-WT	*Aig1*-KO	*Adtrp*-WT	*Adtrp*-KO	WT	DKO
BAT						
9-PAHSA	51.0±7.6	33.3±4.7	26.4±4.8	145.7±14.8***	19.2±1.5	265.7±39.5***#
12/13-PAHSA	15.6±3.8	9.0±1.4	8.2±1.0	14.0±1.6*	6.6±1.1	20.1±1.5***#
9-OAHSA	43.9±4.6	31.9±5.3	23.9±4.2	161.9±17.9***	18.5±2.4	293.1±34.9***#
12/13-OAHSA	6.9±1.1	4.1±0.4*	3.6±0.6	7.6±1.1*	2.4±0.4	10.2±1.8**
SQWAT						
9-PAHSA	27.2±7.0	30.1±6.3	43.0 ±17.1	227.9±19.2***	34.6±2.8	226±3 4.7***
12/13-PAHSA	6.5±1.8	7.7±2.8	12.3±3.4	12.5±3.7	5.5±1.4	9.0±0.8
9-OAHSA	23.4±6.8	26.0±6.5	32.3±14.1	158.3±19.8**	30.3±2.7	179.4±3.8***
12/13-OAHSA	3.2±1.1	3.9±1.9	7.1±3.1	6.8±2.5	3.0±0.5	5.3±0.9
PGWAT						
9-PAHSA	27.7±9.2	21.6±1.9	34.5±12.2	167.0±24.7**	19.1±2.3	138.4±28.3**
12/13-PAHSA	9.3±2.1	5.3±1.3	17.5±5.9	9.8±0.8	5.4±0.9	6.4±1.0
9-OAHSA	19.3±6.2	15.5±2.1	25.1±9.3	146.5±22.1**	13.2±1.6	85.8±14.6**
12/13-OAHSA	3.8±1.7	3.3±1.1	6.3±3.1	5.8±0.3	1.5±0.2	1.7±0.3
Kidney						
9-PAHSA	0.6±0.1	0.6±0.1	1.1±0.5	1.2±0.2	0.6±0.1	2.0±0.3**
12/13-PAHSA	1.1±0.3	0.8±0.3	1.1±0.1	0.7±0.1	1.2±0.4	1.0±0.4
9-OAHSA	0.4±0.1	0.3±0.1	0.5±0.3	0.8±0.1	0.3±0.1	1.8±0.5*
12/13-OAHSA	0.11±0.01	0.08±0.01	0.2±0.1	0.14±0.02	0.07±0.01	0.07±0.02
Liver						
9-PAHSA	0.9±0.1	0.8±0.1	1.1±0.1	1.4±0.3	0.8±0.1	1.1±0.2
12/13-PAHSA	13.4±1.5	12.9±0.4	14.5±0.3	16.2±1.2	16.5±1.2	14.9±1.6
9-OAHSA	0.10±0.02	0.21±0.03*	0.25±0.04	0.32±0.04	0.12±0.01	0.28±0.03**
12/13-OAHSA	1.6±0.3	1.6±0.3	1.7±0.2	1.9±0.2	1.8±0.1	1.9±0.3
Plasma						
9-PAHSA	0.9±0.1	1.0±0.1	0.9±0.01	1.4±0.1	0.9±0.1	1.3±0.2
12/13-PAHSA	0.5±0.04	0.6±0.1	0.6±0.03	0.7±0.04	0.6±0.1	0.5±0.1
9-OAHSA	1.4±0.02	2.0±0.3	1.8±0.1	2.2±0.3	1.8±0.1	1.8±0.2

Table S2. Tissue FAHFA levels from *Aig1*, *Adtrp*, and DKO mice. Absolute levels (pmol/g tissue) of non-esterified 9- and 12/13-PAHSA and OAHSA in BAT, SQWAT, PGWAT, kidney, and liver of *Aig1*-KO, *Adtrp*-KO, and DKO mice and the corresponding WT controls. Values shown ± SEM (n=4 per group. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, compared to WT. T-test; #, $p<0.05$, DKO compared to *Adtrp*-KO, T-test).

Table S3. Tissue FAHFA-TG levels from *Aig1*, *Adtrp*, and DKO mice.

FAHFA	*Aig1*-WT	*Aig1*-KO	*Adtrp*-WT	*Adtrp*-KO	WT	DKO
BAT						
9-PAHSA	12098±1149	12658±1063	9374±890	22494±2333***	7950±226	34969±2026***,#
12/13-PAHSA	3741±545	3326±387	2662±260	3716±390	1860±131	4078±660*
9-OAHSA	10869±922	11446±931	8872±785	26035±1619***	7921±275	39752±1245***,#
12/13-OAHSA	2681±321	2455±253	1964±184	2301±165	1382±106	2746±381*
SQWAT						
9-PAHSA	3532±436	2978±403	4183 ±264	4992±288	3304±205	4823±206**
12/13-PAHSA	3574±357	3147±309	4586±228	4792±364	3541±305	3902±133
9-OAHSA	3128±446	2765±346	3688±241	4163±419	2944±128	4135±154**
12/13-OAHSA	2582±326	2248±188	3378±271	3326±337	2514±211	2823±178
PGWAT						
9-PAHSA	3120±787	2233±125	3368±419	3563±371	1906±101	2490±382
12/13-PAHSA	4498±867	3434±411	4819±475	4430±537	2959±203	3291±435
9-OAHSA	2553±723	1908±133	2767±362	2942±260	1556±127	1927±252
12/13-OAHSA	3066±592	2453±239	3422±309	3099±260	2162±109	2337±273
Kidney						
9-PAHSA	39.8±7.2	34.9±4.8	67.0±23.2	39.6±12.1	40.4±15.6	49.0±9.1
12/13-PAHSA	15.5±2.4	23.0±9.8	23.2±6.5	13.8±4.0	13.7±4.3	13.8±2.6
9-OAHSA	30.6±6.2	25.1±3.2	53.5±20.5	28.9±7.8	31.8±12.2	36.1±7.7
12/13-OAHSA	9.6±1.8	9.0±1.6	16.0±5.5	8.5±2.1	9.1±3.1	9.1±1.9
Liver						
9-PAHSA	3.8±0.4	4.7±0.5	3.8±0.5	3.9±0.3	4.4±0.4	4.9±0.2
12/13-PAHSA	60.4±3.5	60.7±4.5	61.8±13.9	54.7±5.0	66.7±3.9	62.5±5.7
9-OAHSA	2.4±0.4	2.6±0.02	2.0±0.4	1.9±0.1	2.5±0.4	2.6±0.4
12/13-OAHSA	8.2±1.1	7.3±0.7	7.1±1.3	6.6±0.5	7.7±0.6	8.4±0.6

Table S3. Tissue FAHFA-TG levels from *Aig1*, *Adtrp*, and DKO mice. Absolute levels (pmol/g tissue) of TG-esterified 9- and 12/13-PAHSA and OAHSA in BAT, SQWAT, PGWAT, kidney, and liver of *Aig1*-KO, *Adtrp*-KO, and DKO mice and the corresponding WT controls. Values shown ± SEM (n=4 per group. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, compared to WT, T-test; #, $p<0.05$, DKO compared to *Adtrp*-KO, T-test).

Table S5. Oligos used for CRISPR/Cas9 targeting and primers used for mouse genotyping.

Aig1 CRISPR oligo 1	5’ CACCGCGATGGCCTTGTAGTTGCAC 3’
Aig1 CRISPR oligo 2	5' AAACGTGCAACTACAAGGCCATCGC 3'
Adtrp CRISPR oligo 1	5’ CACCGTAAGGGAATTCCATGACGGT 3’
Adtrp CRISPR oligo 2	5' AAACACCGTCATGGAATTCCCTTAC 3'
Aig1 (MUT) Primer F	5' TCCTATTGCTCCATCACAAC 3'
Aig1 (WT) Primer F	5’ CTATCCTATTGCTCCATCCTG 3'
Aig1 (Common) Primer R	5’ CGTTTGGCTGTACTCCCATG 3'
Adtrp Primer F	5’ GAGATGCAAAGTCCAGCCTG 3'
Adtrp Primer R	5’ CAAGTTGGGCTCGGAAAGTC 3'