Supporting Information

Frömel et al. 10.1073/pnas.1206493109

SI Methods

Materials. The soluble epoxide hydrolase (sEH) inhibitors adamantyl-cyclohexyl-urea (ACU), *trans*- and *cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]-benzoic acid (*t*-AUCB, *c*-AUCB) were synthesized as previously described (1, 2). G-CSF was from Amgen. Fatty acid epoxides, diols, and deuterated analogs [e.g., 11,12-epoxyeicosatrienoic acid (11,12-EET) and 11,12-dihydroxyeicosatrienoic acid (11,12-DHET)] were from Cayman Europe. All other chemicals were purchased from either Sigma or Merck.

Animals. C57BL/6 mice (6–8 wk old) were purchased from Charles River. $sEH^{-/-}$ mice (3) were kindly provided by Frank Gonzalez (National Institutes of Health, Bethesda, MD) and crossbred for 10 generations onto the C57BL/6 background in the animal house facility at Frankfurt University. TOP-Gal/C57Bl6 transgenic mice (4), a reporter strain that expresses β-galactosidase in the presence of the lymphoid enhancer binding factor 1/transcription factor 3 (LEF/TCF)-mediated signaling pathway and activated β-catenin were from the Jackson Laboratory.

Mice were housed in conditions that conform to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication no. 85–23). All experiments were approved by the governmental authorities (Regierungspräsidium Giessen: F28/11, F28/10). Age-, sex-, and strain-matched animals were used throughout. In some experiments animals were given the sEH inhibitor, *t*-AUCB (4 mg/L) in the drinking water, as previously described (5).

The following transgenic zebrafish lines were used: *Tg(cmyb:EGFP)zf169* (6), *Tg(lmo2:dsRed)zf73* (7), and CD41-GFP *Tg(-6.0itga2b:EGFP)la2* (8), kindly provided by Leonard I. Zon (Children's Hospital, Boston, MA), and *Tg(kdrl-EGFP)s843* (9) and Tg(kdrl:HsHRAS-mCherry)s896 (10), kindly provided by Didier Stainier (University of California, San Fancisco, CA). Embryos were raised at 28 °C in embryo buffer containing 0.003% 1-phenyl-2-thiourea to prevent pigmentation. Age-matched embryos were exposed to sEH inhibitors (20 μmol/L in 0.1% DMSO) from the three-somite stage until 36 or 96 hpf. For microscopy, embryos were anesthetized in 0.02% tricaine and mounted in 1.5% (vol/vol) low melting-point agarose and stacks of optical sections were captured with a confocal microscope or an inverted fluorescence microscope. Projections of stacks of confocal images were generated with LSM image browser (Zeiss) and processed in Adobe Photoshop. In some studies 14,15-EET/DHET and 12,13-epoxyoctadecenoic acid (12,13-EpOME)/dihydroxyoctadecenoic acid (DiHOME) (all 10 μmol/L) were added to the embryo medium, as indicated in *Results*.

Morpholino Injection. Antisense morpholino modified oligonucleotides (GeneTools) were designed against the translational initiation site 5′-CTAAACAACACTGCCTTCTTCATTG-3′, and two different exon-intron boundaries 5′-CCACAGCTCT-GCGTATTAACAAAAT-3′ (Mo13), 5′ GACCTCTGAGA CA-CAAACACATGCA-3′ (Mo17) of the sEH. Standard control morpholinos (GeneTools) were also used. Oligonucleotides were diluted in water containing 1:10 phenol red and were injected (10 ng diluted 1:5 from a 1 mmol/L stock) with a volume of 4 nL into the yolk sac of one to four-cell stage embryos.

Immunoblotting. Samples were lysed in buffer containing Tris/HCl, 50 mmol/L (pH 7.5) NaCl, 150 mmol/L; EGTA, 2 mmol/L; EDTA 2 mmol/L; Triton X-100, 1% (vol/vol); NaF, 25 mmol/L; $Na_4P_2O_7$, 10 mmol/L; PMSF, 40 μg/mL and 2 μg/mL each of leupeptin, pepstatin A, antipain, aprotinin, chymostatin, and trypsin inhibitor. Detergent-soluble proteins were heated with SDS/PAGE sample buffer, separated by SDS/PAGE and specific proteins were detected as previously described (11). The sEH antibody for Western blotting was kindly provided by Christophe Morisseau (University of California, Davis, CA).

EET/DHET Measurements and sEH Activity Assay. Samples were extracted twice into ethyl acetate, evaporated under nitrogen, and resuspended in methanol/water (vol 1:1). The eicosanoid profiles generated were determined with a Sciex API4000 mass spectrometer operating in multiple reaction monitoring mode, as previously described (12).

Soluble epoxide hydrolase activity was determined using cytosolic cell lysates generated as previously described (13). Reactions were performed at 37 °C for 10–20 min in 10–100 μL of 100 mmol/L potassium phosphate buffer (pH 7.2) containing 0.1–5 μg protein, started by the addition of a fatty acid epoxide (10 μmol/L), stopped on ice, and immediately extracted twice with ethyl acetate (0.5 mL). For LC/MS-MS analysis one-tenth of the sample was spiked with a deuterated internal standard. sEH and microsomal EH inhibitors were used at a concentration of 10 μmol/L.

Ethyl acetate extracts from homogenized embryos (200 embryos), murine plasma (100 μL), or bone marrow extracellular fluid, obtained from flushed-out femurs were spiked with the corresponding deuterated internal standard and subjected to LC/MS-MS.

Expression in *Spodoptera frugiperda* Insect Cells. Recombinant baculoviruses were produced with the Bac-To-Bac-System (Invitrogen) and expression of the sEH was performed as described previously (14). *Spodoptera frugiperda* (Sf9) cells were grown in ExCell 405 medium supplemented with 10% (vol/vol) heat-inactivated FCS. After reaching a density of 2×10^6 cells/mL, the cultures were infected with the recombinant sEH-baculoviruses with a multiplicity of infection of 5. Cells were harvested after 48 h, resuspended in 0.1 mol/L potassium phosphate buffer (pH 7.4, containing 20% (vol/vol) glycerol and 1 mmol/L EDTA), and lysed by sonication. The cytosolic fraction was prepared by differential centrifugation (10 min at $1,000 \times g$ and 20 min at $10,000 \times g$), resuspended in the same buffer, and stored at −80 °C.

In Vitro Progenitor Cell Assays. For the colony-forming unit (CFU-C) assays, mice were injected with G-CSF (250 μg·kg·d s.c.) for 5 consecutive days. Peripheral blood was obtained by retro-orbital bleeding under isoflurane (Forene, Abbott) anesthesia and full blood profiles (EDTA buffered, 10 μg/ mL) were determined using a hemocytometer (Hemavet).

Peripheral blood derived mononuclear cells (PBMCs) were prepared via density centrifugation using Lympholiter-M (Cedarlane; Sanbio), at $800 \times g$ for 20 min at 10 °C without brake. PBMC (10^5 cells) were plated after density centrifugation as described above in 35-mm dishes in methylcellulose medium supplemented with growth factors (MethoCult; Stem Cell Technologies). The colonies formed were counted with an inverted microscope (three independent and blinded scorers) and characterized as CFU-granulocytes (CFU-G), CFU-monocytes/macrophages (CFU-M), mixed CFU-granulocytes/macrophages (CFU-GM), and the most

immature granulocytes, erythrocytes, macrophages, and megakaryocytes (CFU-GEMM).

Proangiogenic circulating cells (previously referred to as "early endothelial progenitor cells") were obtained from the spleen of mice pretreated with G-CSF (250 μg·kg·d s.c.) for 3 consecutive days, as previously described (15). The spleen was disrupted with a syringe and passed through a 70-μm tissue strainer (BD Biosciences). After density centrifugation, as described above, 4×10^6 cells were seeded in a 24-well dish in EGM medium [20% (vol/vol) FCS, PAA] and incubated for at least 7 d. Cells were stained with Dil-Ac-LDL (Harbor Bio Products) and quantified.

Lineage Depletion and FACS Analysis of Progenitor Cells. Lineage depletion and $cKit^+$ enrichment of the PBMC population was achieved using magnetic beads according to the manufacturer's instructions (Miltenyi). Lineage-negative (Lin^-) cells were labeled with R-phycoerythrin (PE)-Sca-1 (E13; BD Biosciences) and allophycocyanin (APC)-c-kit (clone 2B8; BD Biosciences); the control staining included the appropriate isotype control antibodies. Nonspecific binding was prevented by addition of rat anti-mouse CD16/CD32 antibody (Fc receptors; BD Biosciences). Flow cytometric analysis was performed using a FACSCalibur flow cytometer equipped with CellQuest software (version 3.3; BD Biosciences).

Murine CFU-Spleen Assay. Mobilized PBMCs (1.5×10^5) from WT and $sEH^{-/-}$ mice or unfractionated bone marrow-derived cells (2×10^5 cells) from WT C57BL/6 mice were transplanted via tail vein injection into total-body irradiated (8 Gy) syngeneic WT recipients. After 12 d, the spleens were removed, fixed in Bouin's solution, and the hematopoietic colonies formed were documented with a stereomicroscope (Lumar; Zeiss). In some experiments, unfractionated bone marrow-derived cells (2×10^5 cells) from WT C57BL/6 mice were pretreated ex vivo with solvent (0.1% DMSO) 11,12-EET, 11,12-DHET, 12,13-EpOME, 12,13-DiHOME (each 10 μmol/L), or LiCl (3 mmol/L) for 4 h on ice before transplantation.

For the in vivo β-catenin reporter gene assay, unfractionated bone marrow-derived cells (1.5×10^5) from TOP-Gal/C57Bl6 mice were transplanted via tail vein injection into sublethally total-body irradiated (8 Gy) syngeneic WT recipients. *t*-AUCB (4 mg/L) was given via the drinking water over the entire 12-d transplantation period; 12,13-EpOME/DiHOME, 11,12-EET/DHET (each 20 mg/kg), and 6-bromoindirubin-3′-oxime (Bio, 50 μg/kg i.p.) were injected 12 h before harvesting. Thereafter, the spleen was removed and cells isolated by density centrifugation as described above. LacZ activity of the lysed cells was then measured using the Galacto-Light Plus Kit as suggested by the manufacturer (Applied Biosystems).

Proliferation Assays. Proliferation was assessed in cryo-sectioned (10 μm) spleens embedded in tissue tech (Sakura Finetec) and stained for phosphorylated histone H3 (Ser10; Millipore). Nuclei were counterstained with DAPI and imaged by confocal microscopy. To determine proliferation in CFU-S colonies, animals were treated with 11,12-EET/DHET or 12,13-EpOME/DiHOME (20 mg/kg i.p., 12 h) and then BrdU (10 mg per mouse) for 1 h before killing. Spleen cells were isolated and BrdU visualized using a BrDU flow kit according to the manufacturer's instructions (BD Biosciences). Flow cytometric analyses were performed on a FACSCalibur flow cytometer equipped with CellQuest software (version 3.3; BD Biosciences).

Matrigel Plug Assay. WT C57BL/6 or $sEH^{-/-}$ mice (8 wk old) were lightly anesthetized and then injected with 0.5 mL of Matrigel impregnated with stromal cell-derived factor-1 (SDF-1; 100 ng/mL) along the dorsal midline on each side of the spine, as previously described (16). Mice were treated for the first 5 d after implantation with G-CSF (250 μg·kg·d s.c.). After 10 d the mice were killed, the Matrigel plugs removed, embedded in tissue tech (Sakura Finetec), and frozen before being cryo-sectioned (10 μm) and processed for staining for CD31 (BD Biosciences) and α-actin (Sigma Aldrich). Vessel formation was quantified by analyzing pixel/area of at least five sections per plug using a computer-assisted program (AxioVision; Zeiss). The data obtained were normalized with respect to values recorded in the respective control.

Hindlimb Ischemia. Neovascularization capacity was investigated in a murine model of hindlimb ischemia using 6- to 8-wk-old WT and $sEH^{-/-}$ mice. The deep femoral artery was ligated using an electric coagulator (ERBOTOM ICC50; ERBE); afterward the superficial femoral artery and vein, as well as the epigastric arteries, were completely excised. The overlying skin was closed with three surgical staples. Relative blood flow was determined by laser Doppler imaging (Laser Doppler Perfusion Imager System) at regular intervals for up to 24 d postligation. The perfusion of the ischemic and nonischemic limb was calculated on the basis of colored histogram pixels. To minimize variables, including ambient light and temperature, and to maintain a constant body temperature, mice were exposed to infrared light for 10 min before laser Doppler scans. The calculated perfusion was expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

For the rescue experiments, unfractionated bone marrow-derived cells (1×10^6 cells) from WT or $sEH^{-/-}$ mice were transplanted via tail vein injection into sublethally total-body irradiated (8 Gy) syngeneic WT or $sEH^{-/-}$ recipients. Mice were allowed to recover for 16 wk and restoration was monitored by determining sEH activity and plasma lipid profiling. Thereafter, femoral artery ligation was performed and the recovery of blood flow was monitored over 24 d. For metabolite rescue experiments 12,13-EpOME/DiHOME (30 μg·kg·d) as well as solvent (PBS with DMSO) were administered via osmotic-minipumps (delivery duration 28 d; Alzet). Pumps were implanted subcutaneously 1 wk before femoral artery ligation, as described above.

Immunohistochemistry. ***Skeletal muscle.*** Cross-sections were stained using adductor and semimembranous muscles imbedded in Tissue Tek (Sakura). After fixation in phosphate buffer (100 mmol/L, pH 7.3) containing 4% (vol/vol) formalin, the tissue was blocked with 5% (vol/vol) goat serum and permeabilized with 0.5% Triton X-100, followed by incubation with lectin-B4-FITC and anti–α-smooth muscle actin Cy 3 and imaged by confocal microscopy. The polyclonal rabbit anti-rat sEH antibody used for immunohistochemistry was kindly provided by Michael Arand (University of Zurich, Zurich, Switzerland).

Lin^- cells. Lineage-depleted cells from bone marrow were seeded on fibronectin-coated slides and incubated as described before for the CFU-S assay. After fixation with 4% (wt/vol) PFA in PBS, cells were permeabilized with 0.2% Triton X-100 in PBS and labeled with anti–β-catenin antibodies (BD Bioscience). The control included the appropriate isotype control antibodies. Nuclei were counterstained with DAPI and imaged by confocal microscopy.

Murine femurs. Paraffin sections of femurs were prepared for immunohistochemistry as previously described (17). After deparaffinisation and rehydration, 5-μm sections were incubated with trypsin (Digest All; Zytomed) to unmask the antigens. Unspecific binding sites were blocked using goat serum (Alexis). Samples were then incubated overnight (4 °C) with a polyclonal rabbit anti-rat sEH antibody (dilution 1:2,000). A 3,3′diaminobenzidine and horseradish peroxidase cell and tissue staining kit was then used to visualize the sEH, according to the manufacturer's instructions (R&D Systems). In some cases a streptavidin con-

jugated alkaline phosphatase and NBT/BCIP as substrate were used and endogenous phosphatase activity was blocked using Tetramisole hydrochloride. Nuclear counterstaining was performed with hematoxylin (Zytomed).

Statistical Analysis. Data are expressed as mean ± SEM and statistical evaluation was performed using Student *t* test for unpaired data or one-way ANOVA followed by a Bonferroni *t* test when appropriate. Values of $P < 0.05$ were considered statistically significant.

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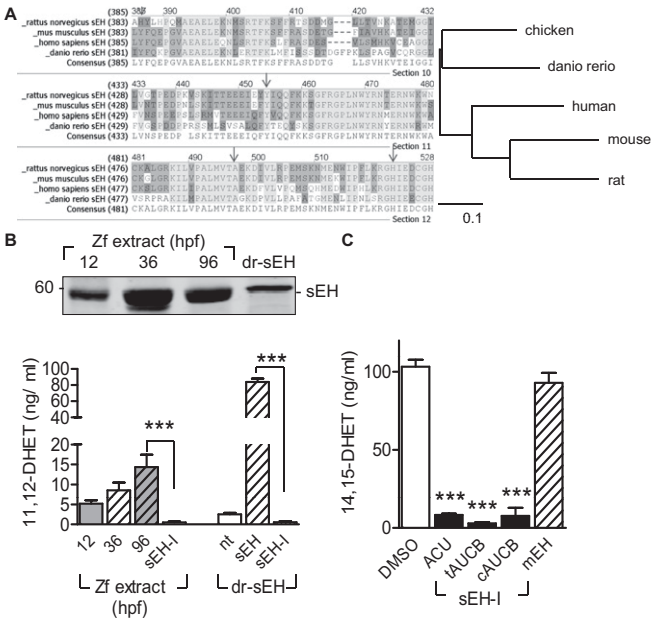

Fig. S1. Expression and activity of the recombinant *Danio rerio* sEH and the sEH in Zebrafish extracts. (*A*) Comparison of orthologous sEH hydrolase domain protein sequences. Dark gray boxes indicate identical amino acids, light gray boxes indicate similar amino acids. Arrows highlight amino acids essential for enzymatic activity. The distance of the branches from each node in the phylogenetic tree indicates increased divergence in sequence similarity among the orthologs; the scale represents the fraction of nonidentical amino acids along each branch. (*B*) sEH activity in zebrafish (Zf) extracts [12–96 hours post-fertilization (hpf)] and in nontransfected (nt) and *Danio rerio* sEH (dr-sEH) overexpressing SF9 cells. Assays were performed in the absence and presence of the sEH inhibitor (sEH-I) ACU. (*C*) Sensitivity of dr-sEH activity to the sEH inhibitors; ACU, *t*-AUCB, or *c*-AUCB, and insensitivity to the microsomal epoxide hydrolase (mEH) inhibitor elaidamide. The bar graphs summarize the data from three to four independent experiments: ***$P < 0.001$.

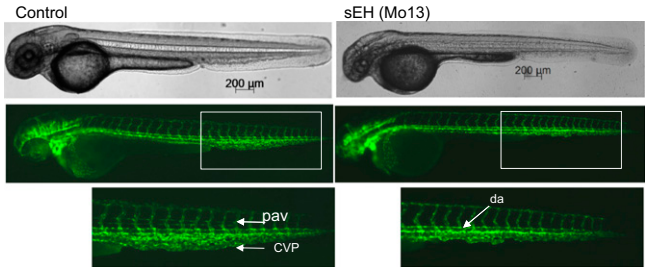

Fig. S2. Effect of sEH down-regulation on zebrafish morphology. Phase-contrast and fluorescent images showing the effect of control morpholinos and sEH splice morpholinos (against exon 13) on vascular morphology in*Tg(kdrl:EGFP)* zebrafish embryos 48 hpf. CVP, caudal vein plexus; da, dorsal aorta; pav, parachordal vessel.

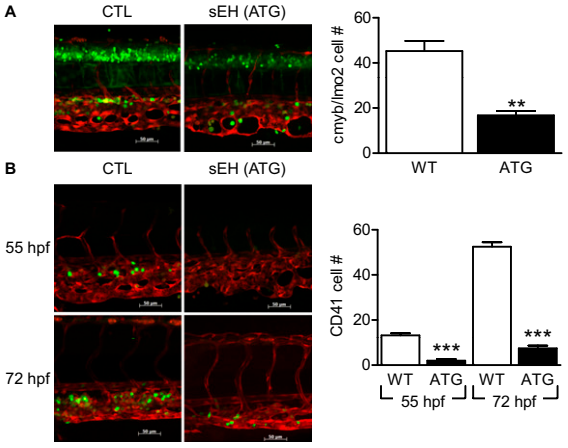

Fig. S3. Effect of sEH down-regulation (ATG morpholino) on the number of stem/progenitor cells in the CVP. (*A*) Confocal images of *cmyb*$^{+}$GFP/*lmo2*$^{+}$dsRED cells in the CVP in control (CTL) and sEH (ATG) morphants. (*B*) Confocal images of CD41–GFP (green); flk1–cherry (red) bigenic fish 55 and 72 hpf; cmyb-GFP (green), and lmo2–DsRed (red) after 55 hpf. The graphs summarize data obtained with four to five embryos per group; $^{**}P < 0.01$, $^{***}P < 0.001$.

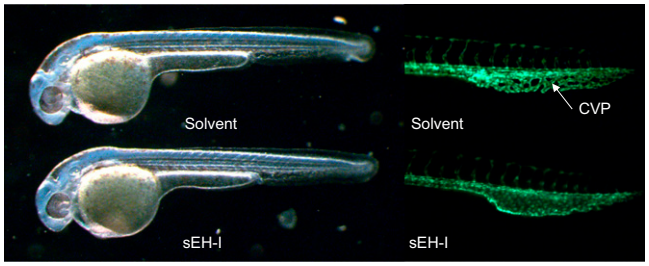

Fig. S4. Effect of pharmacological sEH inhibition on zebrafish morphology. Phase-contrast and fluorescent images showing the effect of solvent and the sEH inhibitor *t*-AUCB (sEH-I) on vascular morphology in*Tg(kdrl:EGFP)* zebrafish embryos. The inhibitor was added 22 hpf and images made 48 hpf. (Magnification: ×60.)

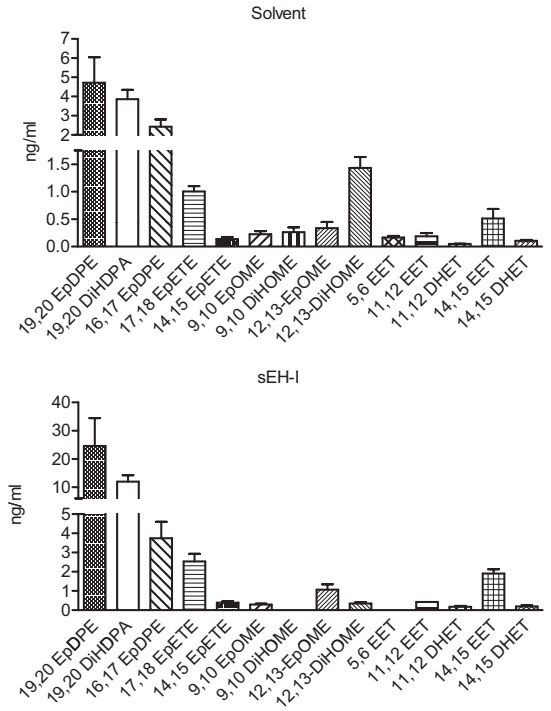

Fig. S5. Results of the LC-MS/MS analysis of the epoxide/diol profile in 4-d-old zebrafish treated with either solvent or the sEH inhibitor (sEH-I, *t*-AUCB) for 96 h. Epoxyoctadecenoic acid (EpOME), epoxyeicosatrienoic acids (EET), epoxydocosapentaenoic acids (EpDPE), epoxyeicosatetraenoic acids (EpETE), dihydroxyoctadecenoic acid (DiHOME), dihydroxyeicosatrienoic acid (DHET), dihydroxydocosapentaenoic acid (DiHDPA).

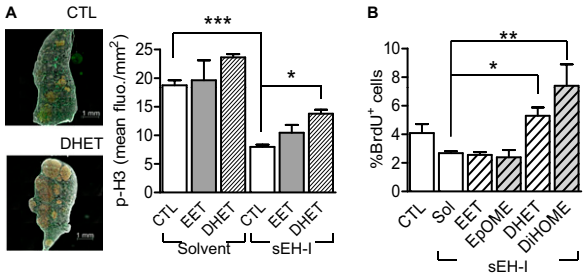

Fig. S6. Consequences of sEH inhibition and EET/DHET or EpOME/DiHOME supplementation on proliferation in short-term repopulating colonies in spleens from irradiated WT mice 12 d after transplantation. (*A*) Comparison of the ability of Lin^- cells from WT and $sEH^{-/-}$ mice (2×10^5 cells per recipient) to form colonies in the spleens of irradiated WT mice. Cells were pretreated with either solvent, 11,12-EET or –DHET for 4 h before transplantation and proliferation was quantified after 8 d by phospho-H3 immunohistochemistry. (*B*) Effect of 11,12-EET, 11,12-DHET, 12,13-EpOME, and 12,13-DiHOME (each 20 mg/kg i.p., 12 h before killing) on BrdU incorporation into spleen cells isolated from transplanted irradiated mice. Animals were treated with either vehicle or the sEH inhibitor, *t*-AUCB (sEH-I, 8 mg/mL in drinking water, 12 d). The graphs summarize data obtained with 6–10 animals per group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

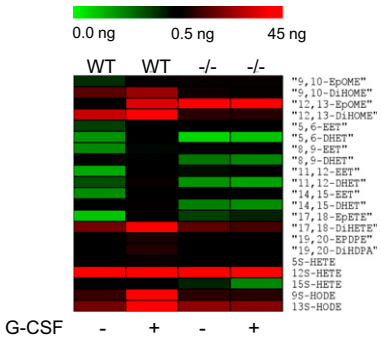

Fig. S7. Analysis of fatty acids in plasma from WT and $sEH^{-/-}$ mice treated with saline or G-CSF (5 d). EpETE, epoxyeicosatetraenoic acid; EpDPE, epoxydocosapentaenoic acid; DiHDPA, dihydroxydocosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; and HODE, hydroxyoctadecadienoic acid.

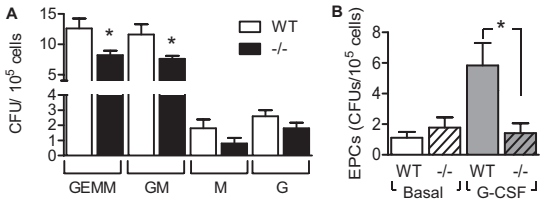

Fig. S8. Role of sEH in progenitor cell differentiation. (*A*) Effect of sEH expression on hematopoietic differentiation in a CFU-C assay with blood from G-CSF-treated WT and sEH$^{-/-}$ ($^{-/-}$) mice. The graph shows the numbers of multipotent (GEMM), mixed granulocyte/monocyte (GM), monocyte (M), and granulocyte (G) progenitor colonies. (*B*) Proangiogenic circulating cell (EPC) colony formation from 10^5 cells derived from the spleens of WT and sEH$^{-/-}$ mice under basal conditions and following treatment with G-CSF (3 d). The graph summarizes data obtained with 6–10 animals per group; $*P < 0.05$.

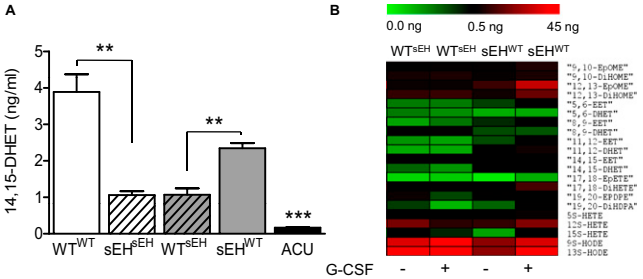

Fig. S9. sEH activity and plasma lipid profiles in chimeric mice 16 wk after transplantation. (*A*) sEH activity in WT^{WT} and sEH^{sEH} vs. chimeric WT^{sEH} and sEH^{WT} mice; $**P < 0.01$. The sEH inhibitor ACU served as a negative control. (*B*) Lipid class composition analysis of fatty acids in plasma from chimeric mice treated with saline or G-CSF (5 d). EpETE, epoxyeicosatetraenoic acid; EpDPE, epoxydocosapentaenoic acid; DiHDPA, dihydroxydocosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; and HODE, hydroxyoctadecadienoic acid.