Supplemental Data:

Materials and Methods

Animals

Mice were housed under specific pathogen-free (SPF) conditions at the animal care facility at Weill Cornell Medical College. All studies were performed under animal protocols approved by the IACUC of Weill Cornell Medical College. We obtained *C57BL/6* mice from Jackson Laboratory. *Sphk1*$^{f/f}$ mice (1) were from MMRRC (stock no.030038-UCD). *Sphk2*$^{-/-}$ mice were described previously(2). *Sphk1*$^{f/f}$ *Sphk2*$^{-/-}$ mice are crossed to *VE-Cadherin-Cre-ER*^{T2} (a gift from Dr. Ralf Adams, Max Planck Institute, Muenster, Germany) (3), *EpoR-Cre* mice (a gift from Dr. Ursula Klingmüller, German Cancer Research Center, Heidelberg, Germany) (4). Male and female mice were used equally in all experiments and littermates were always used as controls. In all of the figures, *Sphk1*$^{f/f}$ *Sphk2*$^{-/\pm}$ mice were used as controls.

Collection of circulating erythrocytes from E12 embryos

Experiments were performed as previously described (5). Pregnant female mice were sacrificed at E12, and uteri were removed. Intact embryo-yolk sac placenta tissue blocks were dissected, rinsed with PBS, and examined by microscopy for beating hearts. Viable, non-bleeding embryos and yolk sacs were cut from placenta directly into 1 ml heparinized Tyrode's buffer (Sigma) at 38°C and were allowed to hemorrhage until the embryo was exsanguinated. Whole blood in buffer was collected. Small pieces of embryo or yolk sac harvested after exsanguination were used for genotyping.

SEW2781 treatment

SEW2871 (Cayman) and vehicle (10% DMSO/25% Tween 20 v/v) were prepared (6). Pregnant female mice at E8.5 were administered 10 mg/kg/day SEW2871 via oral gavage continuously for 5 consecutive days. Embryos were harvested at E13.5 and immediately scored as alive or dead by the presence or absence of heartbeat and/or necrosis.

Tamoxifen treatment

Tamoxifen (Sigma) were dissolved in corn oil with 10% ethanol at 20 mg/ml. Pregnant female mice at E10 were administered 2 mg/day tamoxifen via oral gavage for 3 consecutive days (E10 - E12). Embryos were harvested at E13.5 and immediately scored as alive or dead by the presence or absence of a heartbeat and/or necrosis.

Immunofluorescence staining and imaging

For paraffin sections, samples were blocked in 10% donkey sera (Jackson ImmunoResearch)/0.2% Triton X-100/PBS for 30 min, then incubated overnight in Isolectin IB4 (Invitrogen) and α-SMA (Abcam) antibodies diluted in blocking solution and incubated in fluorescent conjugated secondary antibody for 1 hour. For whole-mount yolk sac and embryo staining, embryos with attached yolk sacs were dissected from maternal tissue, fixed for overnight in 4% paraformaldehyde. Yolk sacs were dissected away from their attached embryos and were permeabilized overnight in 0.2% Triton X-100/ PBS, and blocked in 10% donkey sera/0.2% Triton X-100/PBS. Samples were then incubated overnight in PECAM1 (BD Pharmingen) and α-SMA antibodies diluted in blocking solution. After washing for 1 hour, samples were incubated in fluorescent conjugated secondary antibody and flat-mounted on glass

slides for microscopic examination. Images were taken by Olympus FV1000 confocal microscope or Olympus fluoView FV1000MPE multiphoton microscope.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 10 μm tissue sections were deparaffinized in xylene and rehydrated through a graded series of alcohol incubations. Sections were boiled in 10mM citric acid pH 6.0 for 10 min for antigen retrieval, then incubated with blocking solution containing PBS, 0.1% Tween-20 and 5% donkey serum for 30 min and then with anti caspase-3 (Cell Signaling Technology) or anti Ki67 (Abcam) primary antibodies in blocking solution at 4 ^{o}C overnight. Sections were then washed with PBS and incubated with affinity-purified secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch) for 1-2 hours. After washing, the sections were developed using DAB kit (Vector, Burlingame, CA). The slides were counterstained with hematoxylin and dehydrated through graded alcohols to xylene.

Sphingolipid analysis by LC/MS/MS

Plasma, RBC and tissues of E12 Embryos were used for quantitation of sphingolipids by LC/MS/MS. Levels of ceramide (Cer) species, sphingosine (Sph) and S1P were analyzed by the Lipidomics Analytical Core at the Medical University of South Carolina as previously described (7). Ceramide species were compiled as total ceramide levels.

Electron microscopy

Tissues were fixed with a modified Karnovsky's fixative and a secondary fixation in reduced osmium tetroxide. Following dehydration, samples were embedded in an epon analog resin.

Ultrathin sections (65 nm) were contrasted with lead citrate and viewed on a JEM 1400 electron microscope (JEOL, USA, Inc., Peabody, MA) operated at 120 kV. Digital images were captured on a Veleta 2K x 2K CCD camera (Olympus-SIS, Germany).

Fetal liver transplantion

Pregnant female mice were administered SEW2871 and fetal liver cells were collected at E13.5. For transplantation studies, adult wild-type B6.SJL (CD45.1) recipient mice on the C57BL/6 background (8 weeks of age) were lethally irradiated (9.5 Gy) and were then transplanted with 2×10^6 of fetal liver cells by lateral tail vein injection. Engraftment efficiency in recipients was monitored by donor contribution of $CD45.2^+$ cells using flow cytometry analysis.

FACS and Flow cytometry

For $CD31^+$ cell isolation, embryos were digested with collagenase A (Roche) at 37 ^oC for 30 min. Digested tissues was vigorously mixed to ensure disruption of capsule and filtered through a 70μm disposable cell strainer. Cells were incubated with CD31-PE antibody (eBioscience) for 30 min on ice and washed. For $Ter119^+$ cell isolation, circulating cells were washed and incubated with Ter119-PE antibody (eBioscience) for 30 min on ice. Cells were then isolated with a Becton-Dickinson Aria II platform (BD Biosciences) FACS and stored at -80 ^oC for later extraction of RNA. For engraftment efficiency analysis, the whole blood were collected and incubated with CD45.1-PE and CD45.2-APC antibodies (eBioscience) for 30 min on ice. Stained cells were subsequently treated with ACK red cell lysis buffer, washed and analyzed using an LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (version 8; Tree Star, Inc.).

RNA isolation and qRT-PCR analysis

Total RNA was extracted from cells using *TRIzol® Reagent* (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out using iScript™ Select cDNA Synthesis Kit (Bio-Rad) for reverse transcription-PCR using random primers. Real time PCR was performed on a 7500 real time PCR system (Applied Biosystems) using Fast SYBR® Green Master Mix (Applied Biosystems), relative RNA levels were calculated using the ΔΔCT method (8). Primer sets for qRT-PCR were

Sphk1 forward 5'- AGGTGGTGAATGGGCTAATG -3'

Sphk1 reverse 5'- TGCTCGTACCCAGCATAGTG -3'

Mef2c forward 5'- ATCCCGATGCAGACGATTCAG -3'

Mef2c reverse 5'- AACAGCACACAATCTTTGCCT -3'

Pitx2 forward 5'- ACCCCGGCTATTCGTACAAC -3'

Pitx2 reverse 5'- GAGGACAGGGGATTGACGTTC -3'

Gata4 forward 5'- CCCTACCCAGCCTACATGG -3'

Gata4 reverse 5'- ACATATCGAGATTGGGGTGTCT -3'

Nkx2.5 forward 5'- GACAAAGCCGAGACGGATGG -3'

Nkx2.5 reverse 5'- CTGTCGCTTGCACTTGTAGC -3'

Tbx5 forward 5'- ATGGCCGATACAGATGAGGG -3'

Tbx5 reverse 5'- TTCGTGGAACTTCAGCCACAG -3'

Hand1 forward 5'- CTACCAGTTACATCGCCTACTTG -3'

Hand1 reverse 5'- ACCACCATCCGTCTTTTTGAG -3'

Irx4 forward 5'- GGATACCCCTATTCCTCTGCTC -3'

Irx4 reverse 5'- CTCTCGTAGACAGGGCAGT -3'

Bmp10 forward 5'- ATGGGGTCTCTGGTTCTGC -3'

Bmp10 reverse 5'- CAATACCATCTTGCTCCGTGAA -3'

Hand2 forward 5'- TCAAGGCGGAGATCAAGAAG -3'

Hand2 reverse 5'- TGGTTTTCTTGTCGTTGCTG -3'

GAPDH forward 5'- AGAACATCATCCCTGCATCC -3'

GAPDH reverse 5'- CACATTGGGGGTAGGAACAC -3'

Red blood cell collection

Blood was collected in a tube containing anticoagulant and diluted with an equal volume of PBS. 800 µl of diluted blood was then added to a centrifuge tube. 600 µl Lympholyte®-Mammal (Cedarlane) solution was then added to the bottom of the diluted blood as a layer and centrifuged at 800 x g for 20 minutes room temperature. The RBC pellet were collected and used immediately or washed and stored at -80 ^{o}C.

Blood cell hematology

Whole blood was collected in EDTA-coated tubes. The peripheral blood smears were stained with Eosin. The complete blood counts (CBC) and differential counts were determined on an automated hematology analyzer. Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width-standard deviation (RDW-SD), red cell distribution width-coefficient of variation (RDW-CV), absolute reticulocytes value (RET#), reticulocytes percentage (RET%), platelets (PLT), platelet distribution width (PDW), mean platelet volume (MPV) were analyzed.

References:

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Fig S1

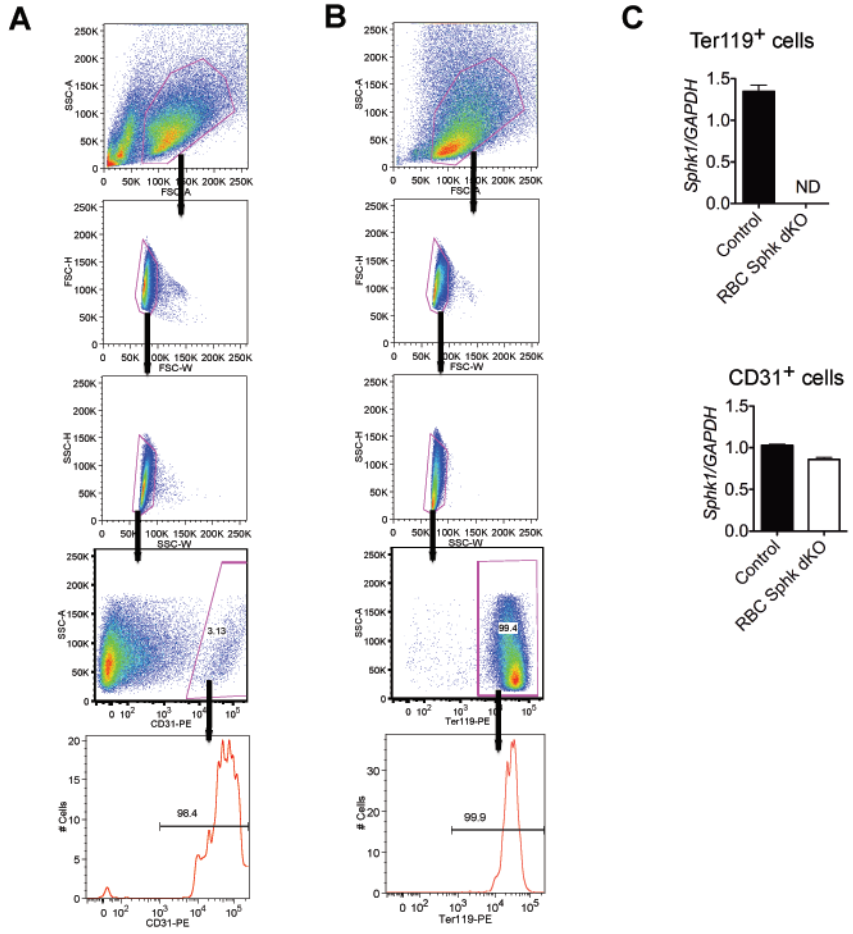

Figure S1. *Sphk1* gene specifically and efficiently deleted in *RBC Sphk dKO* embryo. Gating strategy for the isolation of CD 31^{+} (A) and $Ter119^{+}$ (B) cells from E12 embryo by FACS. (C) *Sphk1* mRNA levels in erythrocytes and endothelial cells from E12.5 *RBC Sphk dKO* embryo. ND; non detectable.

Fig S2

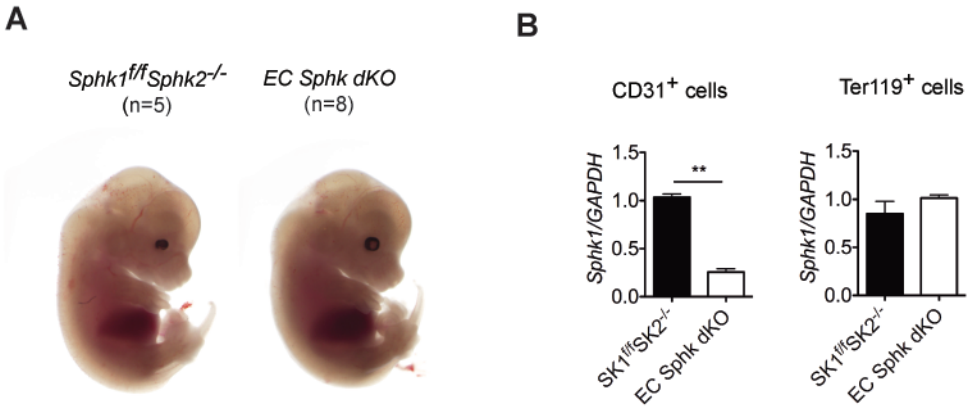

Figure S2. Deletion of *Sphk1* and *Sphk2* in the vascular endothelial cells during embryogenesis. (A) Representative E13.5 embryos of *Sphk1*$^{f/f}$ *Sphk2*$^{-/-}$ and *EC Sphk dKO* mice after tamoxifen administration. (B) *Sphk1* mRNA levels in FACS-sorted erythrocytes and endothelial cells from E13.5 *EC Sphk dKO* embryo after tamoxifen administration.

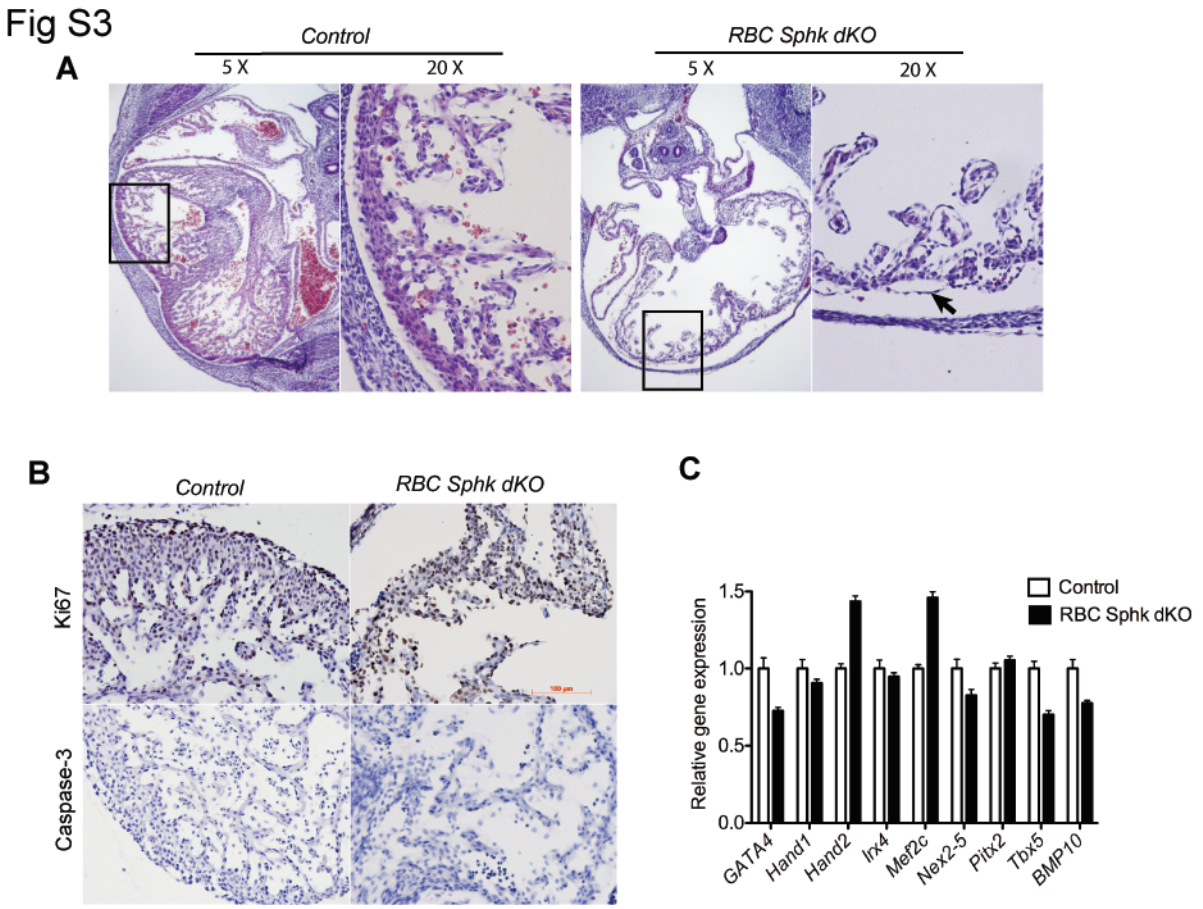

Figure S3. Cardiac defects in E12.5 RBC *Sphk* dKO embryos. (A) Histological evidence of cardiac abnormalities. Histological sections of the ventricular wall regions (boxed) reveal a marked reduction in ventricular wall thickness and detachment of the epicardium in *RBC Sphk dKO* hearts (indicated by arrowheads). Normal cardiomyocyte proliferation or apoptosis in the heart of E12.5 *RBC Sphk dKO* embryos revealed by Caspase-3 and Ki67 immunohistochemistry (B). Magnification is 20 ×. (C) Comparable mRNA levels of genes involved in the cardiogenic program in the heart of E12.5 *RBC Sphk dKO* embryos.

Fig S4

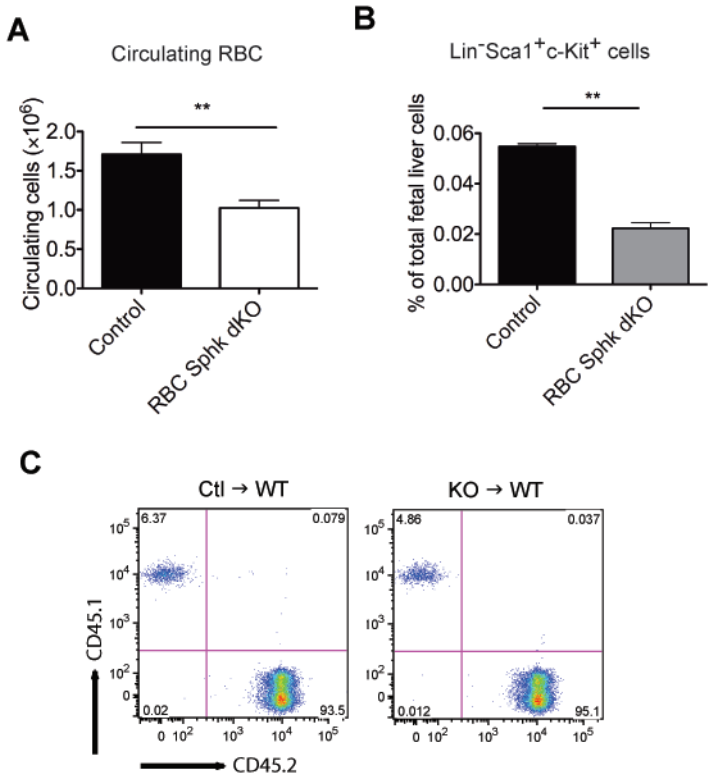

Figure S4. Sphk isoenzymes are not needed for adult erythropoiesis. Reduced (A) circulating blood cell numbers and (B) LSK hematopoietic progenitor cells in fetal livers of E12.5 RBC *Sphk* dKO embryos. (C) Engraftment efficiency of fetal liver stem cell-transplanted mice. Lethally irradiated wild-type (WT) were reconstituted with *Control* or *RBC Sphk dKO* (KO) fetal liver cells and % reconstitution was determined by flow cytometry of blood cells.