Defining a role for sphingosine kinase 1 in p53-dependent tumors

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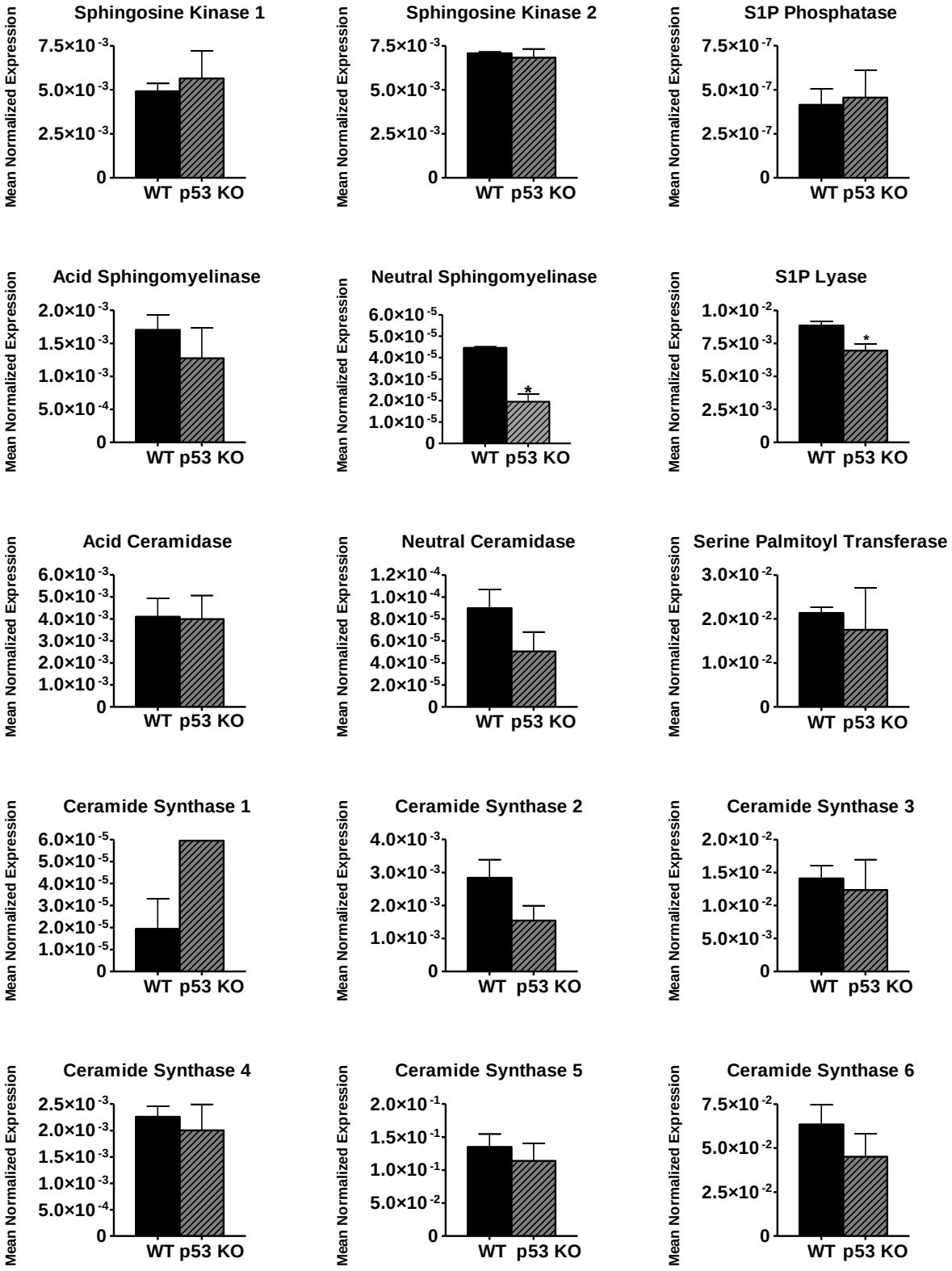
This file includes:

- Supplementary Figures 1-4
- Supplementary Materials and Methods
- Supplementary Acknowledgements
- Supplementary References

Supplementary Figures

Supplementary Figure 1 Expression of sphingolipid metabolizing enzymes in p53 KO tissues. Three male mice of each genotype were sacrificed at 60 days-of-age. Thymus glands were dissected and equivalent sections of each were homogenized in RLT buffer for processing and analysis of mRNA of the indicated enzymes by qRT-PCR and normalized to β-actin (n=3, except CerS1 which was below detection limits in some samples, and *p <0.04 by Student's t-test).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5.0×10-3
2.5×10-3
0
WT p53 KO
Sphingosine Kinase 2
Mean Normalized Expression
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5.0×10-3
2.5×10-3
0
WT p53 KO
S1P Phosphatase
Mean Normalized Expression
7.5×10-7
5.0×10-7
2.5×10-7
0
WT p53 KO
Acid Sphingomyelinase
Mean Normalized Expression
2.0×10-3
1.5×10-3
1.0×10-3
5.0×10-4
0
WT p53 KO
Neutral Sphingomyelinase
Mean Normalized Expression
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5.0×10-5
4.0×10-5
3.0×10-5
2.0×10-5
1.0×10-5
0
*
WT p53 KO
S1P Lyase
Mean Normalized Expression
1.0×10-2
7.5×10-3
5.0×10-3
2.5×10-3
0
*
WT p53 KO
Acid Ceramidase
Mean Normalized Expression
6.0×10-3
5.0×10-3
4.0×10-3
3.0×10-3
2.0×10-3
1.0×10-3
0
WT p53 KO
Neutral Ceramidase
Mean Normalized Expression
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1.0×10-4
8.0×10-5
6.0×10-5
4.0×10-5
2.0×10-5
0
WT p53 KO
Serine Palmitoyl Transferase
Mean Normalized Expression
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1.0×10-2
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WT p53 KO
Ceramide Synthase 1
Mean Normalized Expression
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5.0×10-5
4.0×10-5
3.0×10-5
2.0×10-5
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0
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Ceramide Synthase 2
Mean Normalized Expression
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1.0×10-3
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Ceramide Synthase 3
Mean Normalized Expression
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Mean Normalized Expression
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Ceramide Synthase 5
Mean Normalized Expression
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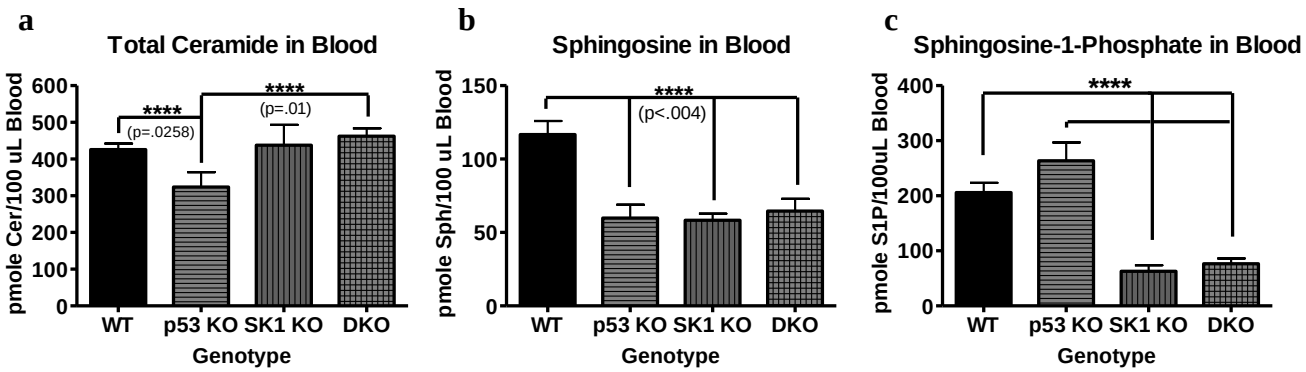
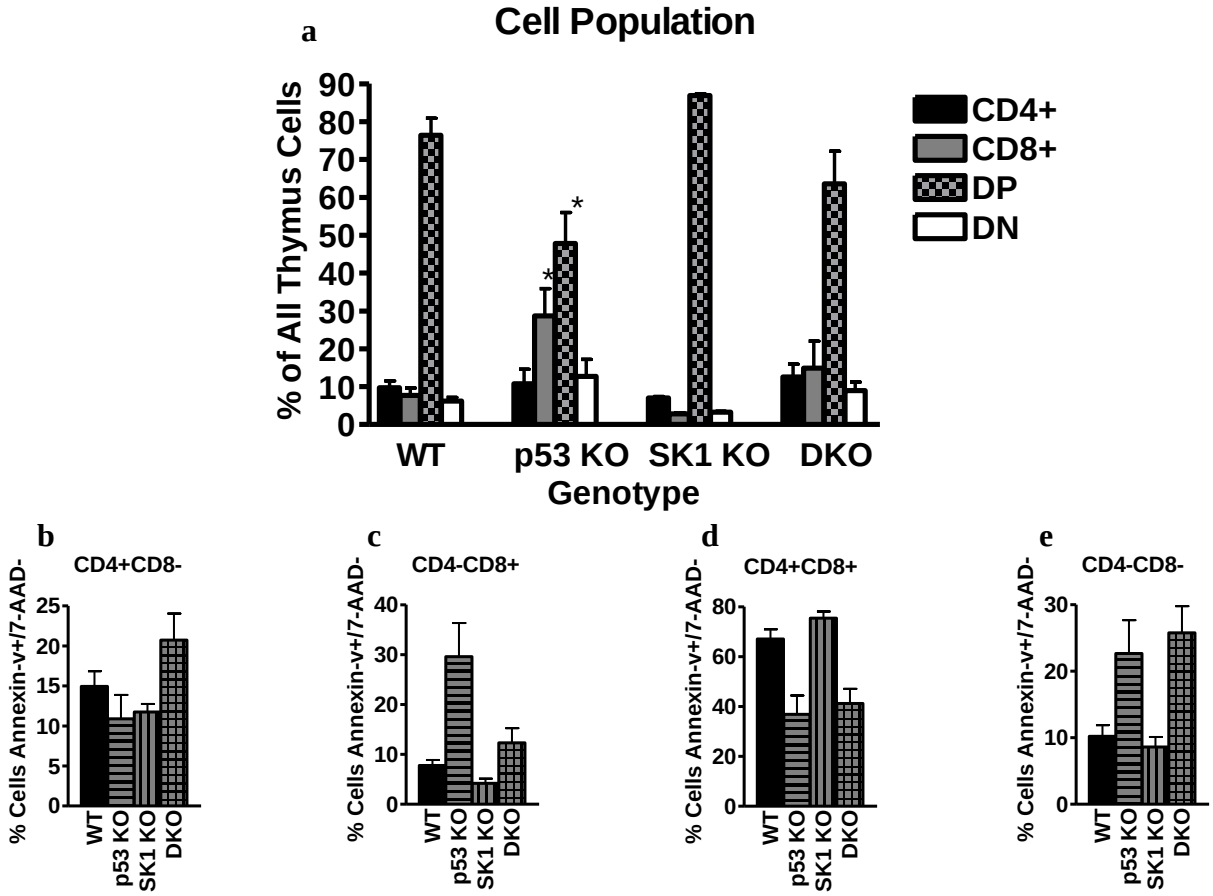
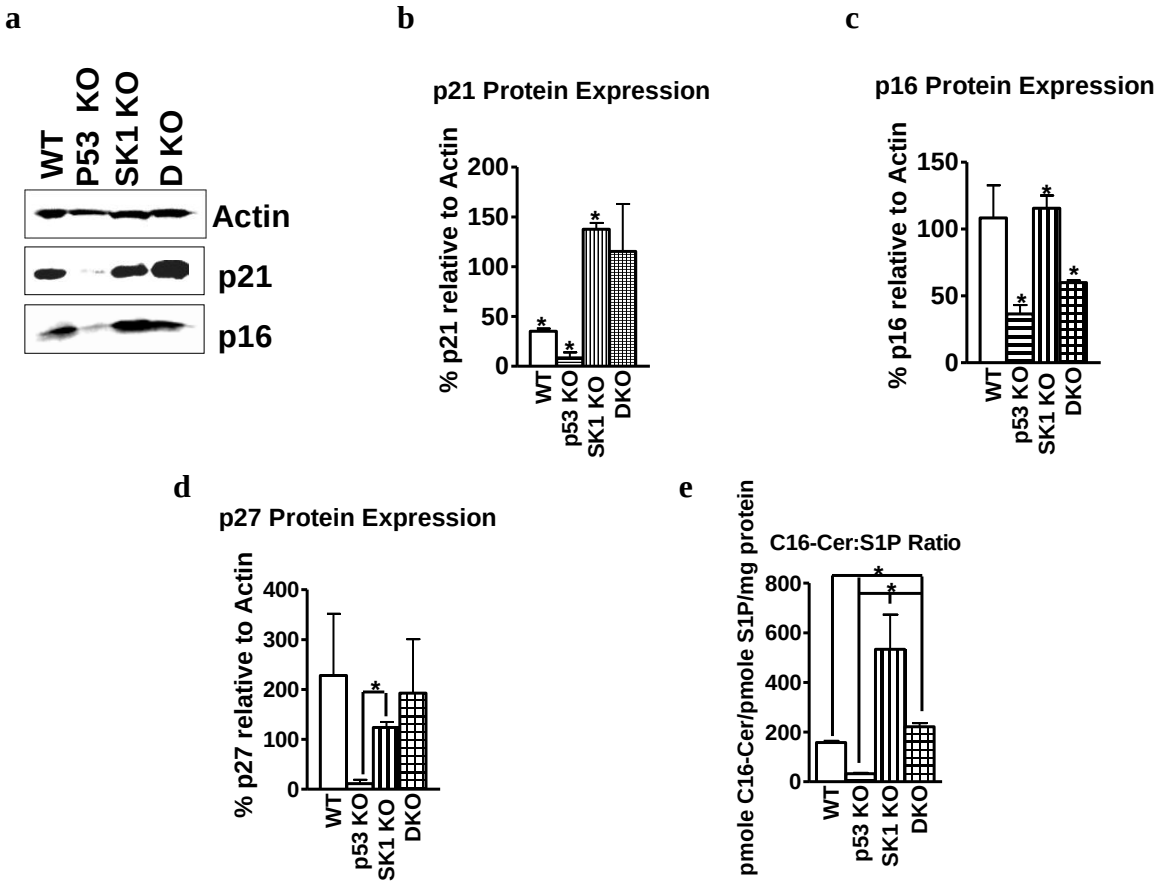
WT p53 KO



Supplementary Figure 2 Circulating sphingolipids in different p53 and SK1 genotypic mice. After anesthesia and euthanasia with isoflurane and CO_2, respectively, blood was obtained from 120 day-old mice via cardiac puncture with a heparinized 1-cc insulin syringe and 100 μL of that was aliquoted into a glass tube, snap frozen, and submitted for lipid analysis. Sphingolipids were measured by mass spectrometry and are represented as pmole lipid/100 μL blood submitted. Asterisks indicate statistically significant ($p<0.05$) differences as assessed by the Student's t-test and exact p values are specified (n=3-7). (a) Total ceramide, (b) sphingosine, and (c) sphingosine-1-phosphate were measured.



Supplementary Figure 3 Thymic cell population in different p53 and SK1 genotypic mice. Five male mice of each genotype were sacrificed at 120 days-of-age. Thymi were dissected, dissociated into single-cell suspensions, and stained before being analyzed by flow cytometry. (a) Cell population based on the presence of one, both, or neither cell surface marker in thymus glands from mice of the indicated genotypes. (b-e) The percentage of apoptotic cells present in each sample is presented in Fig. 3B and the relative contribution of each cell population within the apoptotic population represented in Fig. 3B is described by (b-e) in this figure. The percentage of all cells staining Annexin-V positive and 7-AAD negative that also stain: (b) CD4+CD8-, (c) CD4-CD8+, (d) CD4+CD8+, or (e) CD4-CD8- (n=5, *$p<0.02$ by Student's t-test for comparisons between the specified cell populations of p53 KO vs. WT and p53 KO vs. SK1 KO).



Supplementary Figure 4 Expression of senescence associated proteins in thymus homogenates from different p53 and SK1 genotypic mice. Homogenized thymus glands from three 120 day-old male mice of each genotype were analyzed by Western blotting for proteins known to inhibit cell cycle progression. (a) A representative Western blot of what was observed for p21 and p16 in the samples analyzed, due to the variable nature of p27 protein in WT and DKO thymi a representative blot cannot be shown. Western blots were quantified by densitometry and normalized to actin using the Image J software for (b) p21, (c) p16, and (d) p27. (e) The C_{16}-ceramide:S1P ratio found in thymus homogenates was found to correlate with their p21 protein expression. (n=3 and *$p<0.05$ by Student's t-test as compared to each other * column (b,c) or between the indicated columns (d,e)).

Supplementary Materials and Methods

Breeding. From the breeding of the originally acquired p53 heterozygote and SK1 KO mice, F1, double heterozygote mice (p53 HZ and SK1 HZ), F2, were organized into mating pairs to eventually obtain p53 KO and p53 HZ mice that were WT, HZ, or KO for SK1. Also, new p53 heterozygotes obtained from Jackson labs and new SK1 KO breeders were periodically utilized to prevent genetic drift within the colony. While this breeding scheme was effective for the p53 HZ colony and even established an adequate number of p53 KO, SK1 HZ mice, both female p53 KO, SK1 WT and double p53 and SK1 knockout (DKO) mice proved particularly difficult to obtain. Thus, we bred p53 HZ, SK1 KO offspring to obtain the p53 and SK1 DKO mice and added a p53 HZ, SK1 WT mating pair to produce p53 KO SK1 WT mice. Even with this modified breeding scheme, only about one in every thirty pups produced were DKO. Just as smaller litter size and low female:male ratios have been reported for the p53 KO mice (Sah *et al.*, 1995) we found the DKO mice to be challenged at reproduction.

Genotyping. Mice were tagged with an identification number after weaning at 1 month-of-age and a 5-mm section of distal tail was snipped and digested in 180 µL of ALT buffer and 20 µL proteinase K overnight at 56 °C (Qiagen). Genomic DNA was harvested from each sample using a Qiagen DNAeasy Blood and Tissue kit. Briefly, digested samples were diluted in a 1:1 mix of ethanol:AL buffer, centrifuged in a spin column, washed twice via centrifugation, and then DNA was eluted from the filter of the spin column with 100 µL AE buffer and a 1-min spin at 8,000 rpm. For each tail sample, 2 µL of DNA was combined with 1 µL each of three primers for SK1, or 0.8 µL each of three primers for p53, and 20 or 16.6 µL, respectively, of PCR Platinum SuperMIx (Invitrogen) for a total reaction volume of 25 or 21 µL, respectively, for PCR. The following primers (Integrated DNA Technologies) at a concentration of 10 µM were used for

SK1: 5’-TGT-CAC-CCA-TGA-ACC-TGC-TGT-CCC-TG-3’, 5’-AGA-AGG-CAC-TGG-CTC-CTC-CAG-AGG-A-3’, and 5’-TCG-TGC-TTT-ACG-GTA-TCG-CCG-CTC-CC-3’ and the following primers were used for p53: 5'-ACA-GCG- TGG-TGG-TAC-CTT-AT-3', 5'-TAT-ACT-CAG-AGC-CGG-CCT-3', and 5'-TCC-TCG-TGC-TTT-ACG-GTA-TC-3'. PCR was performed on a Biometra Thermocycler, T3000, with the following reaction conditions: for SK1, 94 °C, 0.5 min; 55 °C, 0.5 min; 72 °C, 6.5 min for 30 cycles, then stop/store at 4 °C, and for p53, 94 °C, 3 min; then 94 °C, 1 min; 60 °C, 2 min; 72 °C, 2 min for 30 cycles; then 72 °C, 5 min, then stop/store at 4 °C. Then, 5 µL 6X DNA loading buffer was added to each PCR reaction product and samples were run on an ethidium bromide stained 2% agarose gel and visualized via UV trans-illumination on a BioRad Imager. Genotype was determined based on band size with a band at 310 bp representing a WT SK1 allele, a band at 390 bp representing a KO or neo SK1 allele, a band at 430 bp representing WT p53, and a band at 590 representing a KO or neo p53 allele. For genotyping of MEF cell lines, each embryo harvested was numbered and the head was digested in 180 µL of ALT buffer and 20 µL proteinase K overnight at 56 °C (Qiagen). Genomic DNA was harvested from each sample using a Qiagen DNAeasy Blood and Tissue kit and analyzed as described above for mouse tail segments.

Tissue Collection. Prior to collection of tissue samples, mice were dispatched by 100% CO_2 inhalation for 5 min, followed by cervical dislocation to ensure death. The abdomen was incised along the midline and exploratory autopsy performed to identify and extract tumor and non-tumor tissue samples of interest. Samples were then either placed on ice and analyzed by flow cytometry, fixed in formalin or OCT for histological analysis, stored at -80 °C or snap frozen in a dry ice/methanol bath before being stored at -80 °C until further processing.

Analysis of Protein Levels in Mouse Cell Lines. MEF cells (2.5×10^5, passage 4–7) were plated in 60-mm dishes in RPMI media with 10% FBS and 1% antimycotic antibiotic. The next day, they were treated with the indicated dose of UVC radiation. Immediately after treatment, cells were returned to the incubator for the indicated amount of time before being harvested. Plates were put on ice and cells were washed with PBS twice, and then scraped on ice with Tris-Triton buffer with protease and phosphatase inhibitors. Samples were sonicated and analyzed by BCA protein assay (Thermo Scientific), then brought to equivalent concentrations with Laemmli sample buffer (Boston Bioproducts) boiled for 10 min and separated on a 4–20% precast Tris-HCl gel (BioRad). Routine Western blot procedures for most proteins analyzed included loading 28 mL (~40 mg protein) per lane, running the gel at 100 V for approximately 2.5 h, transferring to a nitrocellulose membrane at 100 V, at 4 °C for 30 min, blocking in 5% milk in PBS-T for 1 h at room temperature. Next, diluted primary antibodies (1:1,000) in 5% milk in PBS-T were incubated with the blot at 4 °C overnight. Blots were washed three times for 10 min with PBS-T at room temperature and incubated with the appropriate secondary antibody (Santa Cruz Biotechnology, Jackson Laboratories; 1:5,000) in 5% milk in PBS-T for 1.5 h at room temperature. After three 10-min washes with PBS-T, blots were covered in ECL (enhanced chemiluminescence) mixture or Super Signal from Thermo Scientific and exposed to film. For the best results when probing for mouse SK1 using our custom-made antibody from Biosource/Thermo Scientific rabbit 8350, the following specifications were used: a 4–20% gradient gel was pre-run at 50 V for 1 h prior to loading sample. Primary antibody was diluted 1:200. Anti-rabbit secondary antibody was from Santa Cruz Biotechnology (diluted 1:2,500). Blots were developed for 2 min using Super Signal (Thermo Scientific).

Protein Analysis and Western Blotting of Mouse Tissues. Protein analysis of tissue homogenates was performed in duplicate with the BCA protein assay (Thermo Scientific). Samples were then brought to equivalent concentrations with Laemmli sample buffer (Boston Bioproducts) boiled for 10 min and separated on a 4–20% precast Tris-HCl gel (BioRad). Routine Western blot procedures for most proteins analyzed included loading 28 mL (~60 mg protein) per lane, running the gel at 100 V for approximately 2.5 h, transferring to a nitrocellulose membrane at 100 V, at 4 °C for 30 min, blocking in 5% milk in PBS-T for 1 h at room temperature. Primary antibodies (p16, p21, p27, and β-actin from Santa Cruz Biotechnology; 1:1,000) in 5% milk in PBS-T was incubated with the blot at 4 °C overnight. Blots were washed 3 times for 10 min with PBS-T at room temperature and then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology, Jackson Laboratories; 1:5,000) in 5% milk in PBS-T for 1.5 h at room temperature. After three 10-min washes with PBS-T, blots were then covered in ECL (enhanced chemiluminescence) mixture or Super Signal from Thermo Scientific and exposed to film.

Harvesting MEFS. Pregnant female mice were anesthetized with isoflurane and euthanatized by CO_2 inhalation followed by cervical dislocation. After superficial vertical incision of the abdomen, the bifid uterus containing the fetuses was removed and washed in PBS. Fetuses were then separated from the uterus and each embryo was dissected from the amnion and placenta and washed again in PBS. The embryo head was removed for genotyping as described below. The embryo heart and liver were then dissected out and discarded. The remaining carcasses were minced with scissors and treated with 2 mL 0.5% trypsin/0.02% EDTA solution (Gibco) in a 60-mm plate for 10 min at 37 °C and pipetted vigorously until the tissues were broken into single cells. After trypsin treatment, MEFs were resuspended in 8 mL high glucose DMEM

supplemented with 10% FBS and 1% antimycotic antibiotic, pipetted up and down several times, and then left to sit in a 15 mL conical tube at room temperature for ~5 min to allow large chunks of tissue to settle to the bottom. Then the supernatant solution was pipetted into a 10-cm plate and this was considered passage 1 for culturing the MEFs.

Sphingolipidomic Analysis Sample Preparation. After treatment, cells were incubated for the indicated amount of time with a media change at least 8 h prior to harvesting. At the time of harvest, adherent cells were washed twice with cold PBS on ice and lysed directly in 300 uL of buffer containing 1% Triton X-100, 50 mM Tris (pH 7.4), 0.15 M NaCl, 1.0 mM EDTA, supplemented with protease and phosphatase inhibitors (Sigma). 100 uL sample was put into a labeled 15-mL conical flask, and snap frozen in a dry ice/methanol bath and frozen at -80 °C prior to sphingolipidomic analysis.

^{17}C-Labeling. MEF cells (1 x 10^6; passage 4–7) were plated in 10-cm dishes in RPMI media with 10% FBS and 1% antimycotic antibiotic. The next day labeling was initiated by adding ^{17}C sphingosine, 1 mM stock in ethanol (final concentration = 1 nM) in the media of each dish. Cells incubated for 30 min; then, cell dishes were put on ice. Media was aspirated; cells were washed twice with PBS; and cells were harvested by scraping into ice-cold PBS and pelleted. Cells were then resuspended in 200 µL PBS. An aliquot (20 µL) was saved for BCA protein analysis and 180 µL was pipetted into a 15-mL conical flask and stored at -80 °C until extraction. Lipids were extracted and ^{17}C-containing S1P was analyzed using quantitative high performance liquid chromatography/mass spectrometry (HPLC/MS) at the MUSC Lipidomics Core facility as described previously (Spassieva *et al.*, 2007). ^{17}C-sphingolipids were normalized to protein for each sample.

Real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). After the indicated treatment, plates were put on ice, and cells were washed twice with PBS and scraped with 350 µL of RLT buffer (Qiagen) containing 1% β-mercaptoethanol (BioRad) or tissues were harvested, put on ice, and homogenized in 350 µL of RLT buffer (Qiagen) containing 1% β-mercaptoethanol. Samples were homogenized using the QIAShredder kit and RNA was extracted using the RNeasy kit (Qiagen). cDNA was synthesized from 1.0 µg RNA using Oligo-dT primers and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. The standard RT-PCR reaction included 12.5 µL SYBR Green (BioRad), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 5.0 µL of diluted (1:10) cDNA adjusted to a final volume of 25.0 µL. The RT-PCR was performed using a BioRad iCycler as follows: 3 min at 95 °C, followed by 40 cycles consisting of a 10-sec melt at 95 °C, a 45-sec annealing at 54 °C, and an extension step of 45 sec at 68 °C. All reactions were performed in triplicate. Primers were designed using Beacon Primer Design Software and purchased from Integrated DNA Technologies. Primers used for real-time PCR are as follows: forward mouse SK1 5′-CCG-TAC-TTG-GTT-CAT-GTG-CCA-3′, reverse mouse SK1 5′-TCC-CCG-TCC-ACA-GAA-AAC-ACT-3′; forward mouse SK2 5'-GAC-AGA-ACG-ACA-GAA-CCA-TGC-3', reverse mouse SK2 5'-CAG-TCT-GGC-CGA-TCA-AGG-AG-3'; forward mouse β-actin 5′-TAA-GGC-CAA-CCG-TGA-AAA-GAT-G-3′, reverse mouse β-actin 5′-CTG-GAT-GGC-TAC-GTA-CAT-GGC-T-3′. Threshold cycle (C_t) values for target genes were normalized to the reference gene using Q-Gene software (Muller *et al.*, 2002) to determine mean normalized expression.

Dissociation of Thymi and Flow Cytometry. Immediately after harvest, thymi were homogenized in a Stomacher 80 homogenizer (Seward) set on medium for 120 sec. The

homogenate was rinsed with HBSS (Invitrogen), filtered to remove debris, and resuspended at 1 x 10^7 cells/ml in HBSS for immunostaining. Nonspecific staining was blocked by incubation of cells with FBS and 1 µl CD16/32 (BD Pharmingen) per 1 x 10^6 cells. Cells were then stained by 25 min incubation at 4 °C in the dark with 5 µl each of FITC-CD4 and PE-Cy7-CD8 (BD Pharmingen). Atfer incubation, cells were washed twice and resuspended in Annexin binding buffer for staining with the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen). Following the manufacturer's instructions, cell suspensions were stained by incubation for 15 min at room temperature in the dark with 5 µl each of PE-Annexin V and 7-AAD. Labeled cells were then analyzed using flow cytometry (FACSCanto; BD Bioscience).

Sphingolipidomic Analysis of Blood. Blood was acquired through cardiac puncture directly following euthanization of mice. Then 100 µL of whole blood was transferred to a 15-mL conical flask and flash frozen and kept at -80 °C until sphingolipidomic analysis. Sphingolipid masses were determined by ESI/MS/MS. Analysis of ceramides, sphingosine, and sphinosine-1-phosphate was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a multiple reaction-monitoring positive ionization mode, as described (Bielawski *et al.*, 2009; Bielawski *et al.*, 2006).

Supplementary Acknowledgements

This work was supported, in part, by Award Number I01BX000156 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development, NIH/NIGMS R01 GM062887, NIH/NCI P01 CA097132 - project 3 (to LMO). NIH/NCI P01 CA097132 - project 1 (to YAH). American Heart Association Pre-Doctoral Fellowship (AHA 081509E – to RWJ), NIH MSTP Training Grant (GM08716 – to RWJ, AMDC, LAHS), MUSC Hollings Cancer Center Abney Foundation Scholarship (to RWJ, LAHS), NIH/NIEHS TG T32 ES012878, and NIH/NRSA F30ES017379 (to LAHS).

LC-MS/MS analysis of sphingolipids was performed by Lipidomics Shared Resource, MUSC (*Methods* 2006, 39: 82-91) supported by NCI Grants: IPO1CA097132 and P30 CA 138313 and NIH/NCRR SC COBRE Grant P20 RR017677. Laboratory space in the CRI building of MUSC was supported by the NIH, Grant C06 RR018823 from the Extramural Research Facilities Program of the National Center for Research Resources.

The authors thank Dr. Mehmet Bilgen and Dr. Peter Chou for their assistance with animal imaging, Margaret Romano for her immunohistochemical expertise, Hollings Cancer Center for statistical assistance, Nancy Lewis and George Washington for animal care, Dr. Jennifer G. Schnellmann for her review of this work, and Ms. Kathy Wiita-Fisk for administrative assistance.

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