Supplemental Information

Kumar et al: S1P lyase regulates DNA damage responses through a novel sphingolipid feedback mechanism

Materials

Caspase-3, -8 and -9 inhibitors, Z-DEVD-fmk, Z-IETD-fmk and Z-LEHD-fmk, respectively were from BD Biosciences (San Jose, CA), Caspase-2, and pan-caspase inhibitors AC-VDVAD-CHO and AC-VAD-CHO, respectively were from Enzo Life Science Inc (Plymouth Meeting, PA). Propidium iodide, paraformaldehyde and nocodazole were obtained from Sigma. Aurora Kinase inhibitor II was from EMD Bioscience, Inc. (La Jolla, CA). Antibody against cytochrome c and Alexa Fluor-555 conjugated goat-anti rabbit antibody were from Invitrogen.

Supplemental Methods

Immunofluorescent detection of mitotic Cells

Cells were grown in amine coated 24-well plate ((Becton Dickinson). Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing, cells were fixed in ice-cold 100% methanol for 10 min at -20 °C. Then, the cells were stained with antibody against phosphorylated histone H3 Ser 10 (Millipore) overnight at 4 °C, followed by incubation with Alexa Flour 555 conjugated goat anti-rabbit antibody. Cells were visualized under an inverted microscope (Axiovert 200, Carl Zeiss Inc.).

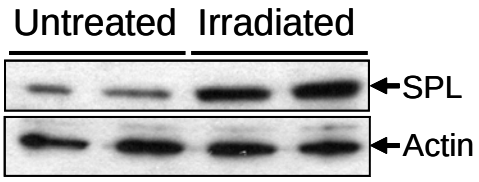
Preparation of cytosolic extracts

Control and SPL^{hi} were plated in 10 cm culture dishes. Cells were were collected by centrifugation at 200 *g* for 5 min and at 4°C. The cells were then washed twice with ice-cold PBS, pH 7.4, followed by centrifugation at 200 *g* for 5 min. The cell pellet was resuspended in 200 μl of extraction buffer, containing 250 mM sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM PMSF and protease inhibitors cocktail (Roche Applied Sciences, Indianapolis, IN). After 30 min incubation on ice, cells were homogenized with a glass dounce and a B pestle (40 strokes). Cell homogenates were spun at 14 000 *g* for 15 min and supernatants were removed and stored at -70°C, until analysis by gel electrophoresis.

RT-PCR

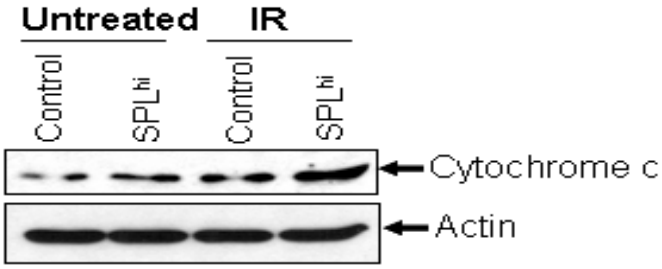
Total RNA was isolated from control and SPL^{hi} cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Primer pairs for human acid sphingomyelinase *(SMPD1)* and actin were follows: *SMPD1* Forward 5' CTATTCACCGCCATCAACCT3', *SMPD1* Reverse 5' TCCACCATGTCATCCTCAAA 3', Actin Forward 5' AGAAAATCTGGCACCACACC 3', Actin Reverse AGAGGCGTACAGGGATAGCA. RT-PCR was performed using the one-step RT-PCR kit (Qiagen) according to the manufacturer's instructions.

Supplemental Figure 1



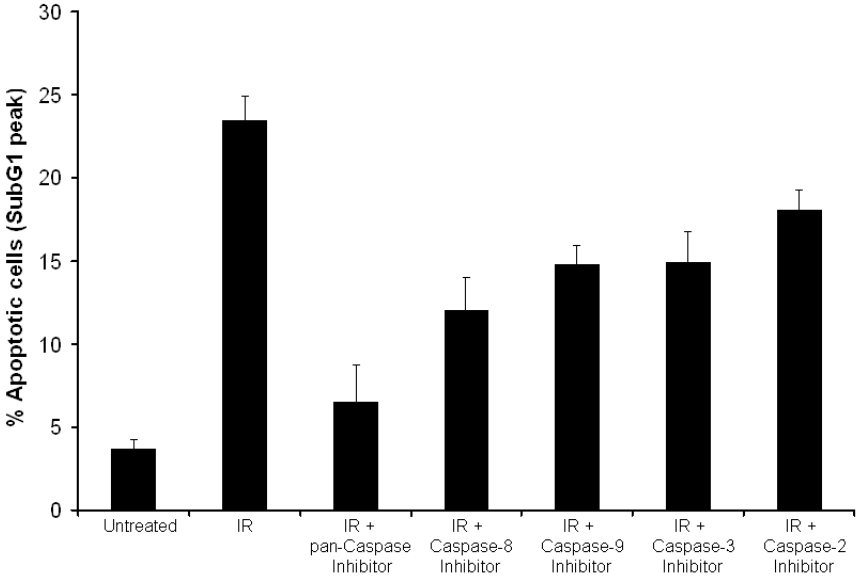
Supplemental Figure 1: HEK293 cells were left untreated or irradiated with 20 Gy of X-rays. Cells were harvested 20h after radiation exposure and whole cell lysate was immunoblotted with anti-human SPL or actin antibodies.

Supplement Figure 2



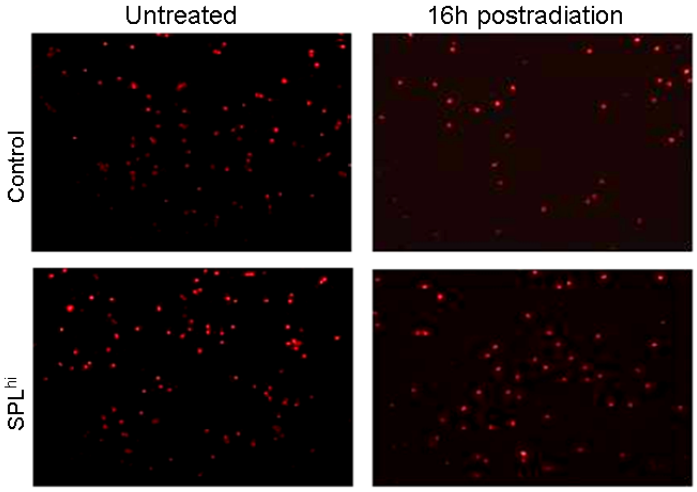
Supplemental Figure 2: SPL promotes cytochrome c release into cytosol. Control and SPL^{hi} cells were left untreated or irradiated with 10 Gy of X-rays. Then, cells were harvested and cytosolic fractions were immunoblotted with cytochrome c or actin antibody.

Supplemental Figure 3



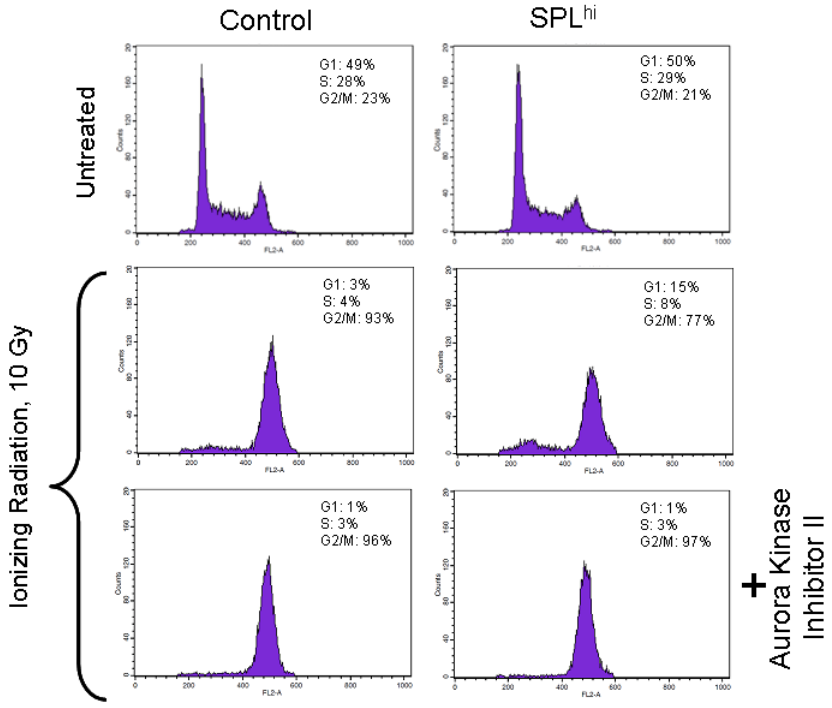
Supplemental Figure 3. SPL mediates apoptosis in caspase-dependent manner. SPLhi cells were pretreated with a pan-caspase inhibitor (AC-VAD-CHO, 70 μM), a caspase-8 inhibitor (Z-IETD-fmk, 80 μM), a caspase-9 inhibitor (Z-LEHD-fmk, 50 μM), a caspase-2 inhibitor (AC-VDVAD-CHO, 80 μM), or a caspase-3 inhibitor (Z-DEVD-fmk, 80 μM) for 3h. Cells were then subjected to 10 Gy of ionizing radiation (IR) and harvested after 24 h. Apoptosis was determined by flow cytometry using propidium iodide staining. Data shown is mean ± SD of three independent experiments.

Supplement Figure 4



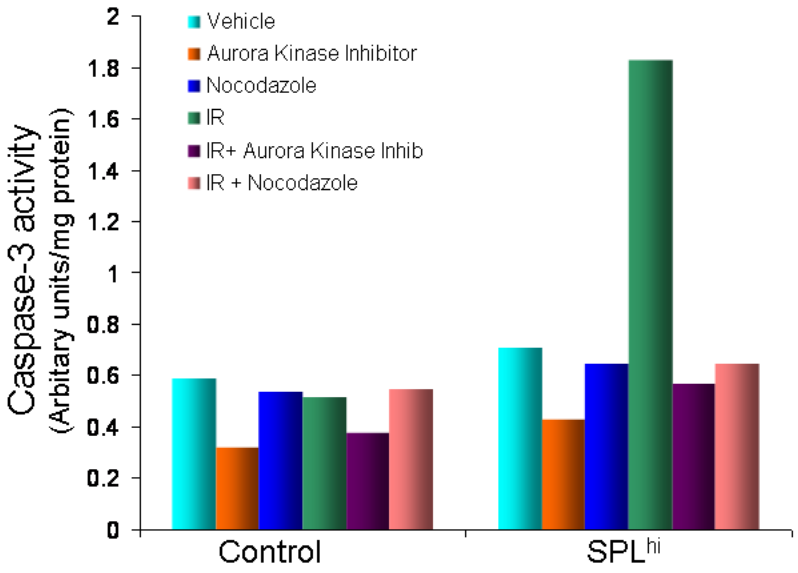
Supplemental Figure 4. SPL^{hi} cells exhibits increased mitosis after DNA damage. Control and SPLhi cells were left untreated or irradiated and harvested after 16h radiation exposure. Immunofluorescence staining for phospho-histone H3 (Ser10) was performed as described in supplemental method section.

Supplemental Figure 5



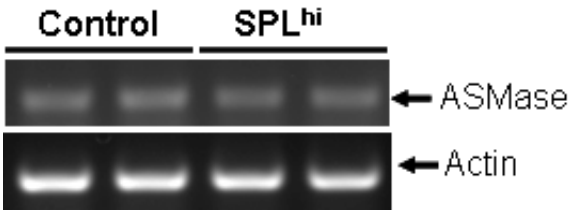
Supplemental Figure 5. An aurora kinase inhibitor prevents premature exit of SPLhi from G2 arrest caused by ionizing radiation. Control or SPL^{hi} cells were pretreated with a cell cycle inhibitor (Aurora Kinase Inhibitor I, 20 µM) for 3h. Cells were then subjected to 10 Gy of ionizing radiation (IR) and harvested after 24 h. Cell cycle distribution was determined by flow cytometry using propidium iodide staining.

Supplement Figure 6



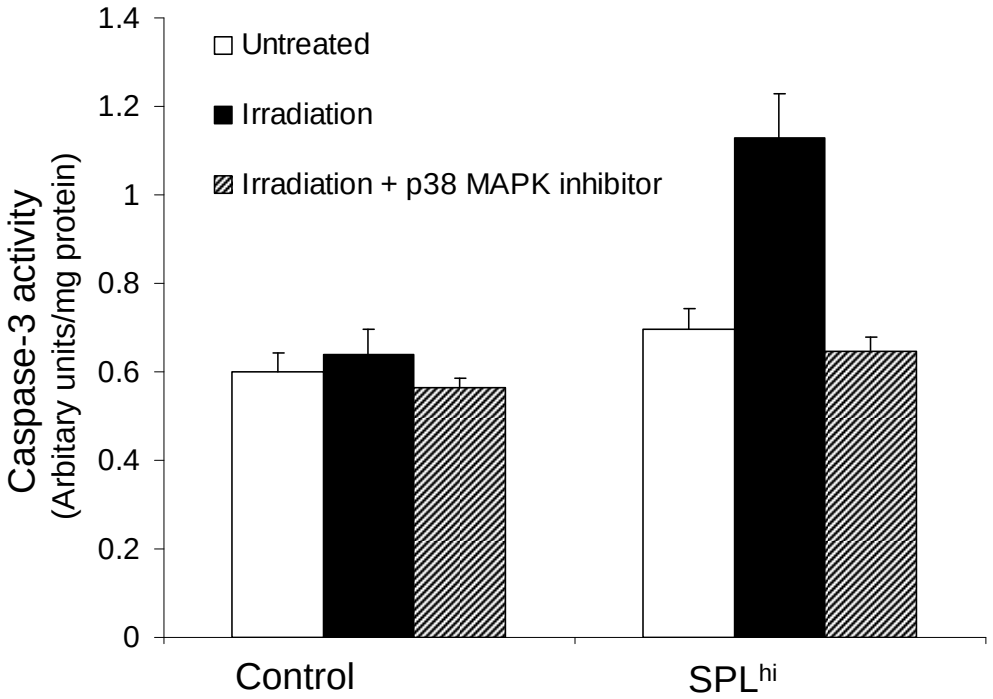
Supplemental Figure 6. Inhibition of cell cycle progression by an aurora kinases inhibitor or nocodazole abrogates the radiation-induced apoptosis in SPLhi cells. Control and SPL^{hi} cells were pretreated with Aurora Kinase Inhibitor II (10 μM, 3h) or nocodazole (100 ng/ml, 1h). Cells were then exposed to 10 Gy dose of X-rays. After 36 h incubation, cells were harvested, and caspase-3 activity was measured using DEVD-pNA as a substrate.

Supplemental Figure 7



Supplemental Figure 7. RT-PCR for acid sphingomyelinase (ASMase). Total RNA was isolated from control and SPL^{hi} cells and semiquantitative RT-PCR was performed for ASMase and actin.

Supplemental Figure 8



Supplemental Figure 8: Inhibition of p38MAPK abrogates SPL-mediated apoptosis. Control and SPL^{hi} cells were left untreated or pretreated with a p38MAPK inhibitor (SB203580, 10 μM) for 1h. Then, cells were irradiated and caspase-3 activity measured 24h after radiation exposure.