Supplemental Methods, Tables, and Figures

Supplement to:

"Radiation-induced acid ceramidase upregulation confers resistance and tumor relapse." Cheng *et al.* 2013.

Supplemental Materials & Methods

Cell culture and reagents.

HeLa, ACHN, A549, AsPC-1, and MCF-7 cell lines (ATCC) were grown according to ATCC instructions. OSCC-3 and UM-SCC74B, kind gifts of K.L. Kirkwood and X. Zhang, were grown in DMEM supplemented with bovine growth serum (10%). Desipramine (Sigma-Aldrich #D3900) was dissolved in ethanol at a concentration of 10 mM. Sphingosine kinase inhibitor (SKI-II; Calbiochem #567731) was dissolved in DMSO at a concentration of 10 mM. Solvent/vehicle administrations to cell cultures were conducted at dilutions of 1:1,000 or higher. Paclitaxel (Fisher #NC9441142) was dissolved in DMSO at stock concentration 10 mM.

Reverse transcription PCR (RT-PCR).

Semi-quantitative or real-time RT-PCR was performed with primers listed in Supplemental Table 1. GoTaq mastermix (Promega) or Sybr Green Supermix (Bio-rad) was used for thermocycling reactions per manufacturers' instructions. Cycling conditions were as follows: pre-incubation, 50º C for 10 minutes, 95º C for 3 minutes, followed by 25-35 cycles of denaturation at 95º C, 30 seconds; annealing/extension between 52 and 60º C, 45 seconds.

pGL3-Basic Luciferase reporter constructs.

–2739, –1430, –906, –496, –205, and –120 bp reporter constructs of the human *ASAH1* promoter (1, 2) were kind gifts of Dr. Marion B. Sewer (Skaggs School of Pharmacy and Pharmaceutical Sciences) and Dr. Natasha Lucki (Georgia Institute of Technology). Four mutant constructions bearing TCA→TTG mutations at AP-1 consensus binding sequences identified by TFBIND (3) analysis within the -496 bp upstream promoter of *ASAH1* (-494/-486 bp; -475/-468 bp; -274/-267 bp; and the three sites altogether) were developed by GeneWiz (South Plainfield, NJ).

ASAH1 *promoter analyses and chromatin immunoprecipitation (ChIP).*

Putative consensus *cis*-binding sequences upstream of the *ASAH1* transcription start site were identified *in silico* by TESS: Transcription Element Search System (4), or TFBIND (3). Primer sequences to cull out regions of interest were based on frequency and commonality of hits from both algorithms. Chromatin was prepared using the chromatin immunoprecipitation (ChIP) assay kit (Millipore #17-295) per manufacturer's instructions, and immunoprecipitated using antibodies against - c-Jun (Santa Cruz Biotechnology #sc-1694X), c-Fos (#sc-52X), or JUNB (#sc-73X). Cells are fixed with formaldehyde (1.0% for 20 minutes at 37º C), washed in ice-cold PBS, and sheared using a Branson Digital Sonifier (model 450) at 30% amplitude for 10 seconds on ice. Protein-DNA complexes are immunoprecipitated using 5.0 µg of antibody. Upon of reverse-crosslinking immunoprecipates, four microliters from 0.2 ml of eluted DNA was used in each real-time reaction, which was conducted using an iCycler with iQ SYBR Green Supermix (Bio-rad) per the manufacturer's instructions. Primers were used at a concentration of 250 nM. Cycling conditions were as follows: pre-incubation, 50º C for 10 minutes, 95º C for 3 minutes, followed by 50 cycles of denaturation at 95º C, 30 seconds; annealing/extension at 60º C, 45 seconds; and data acquisition at the end of each extension. Melting curve and data analysis was carried out per the manufacturer's recommendations using the accompanying software (Bio-Rad).

siRNA transfection.

Transient transfection of siRNA targeting Jun/Fos genes was conducted using commercially available siRNA cocktails (Santa Cruz Biotechnology) and Oligofectamine (Invitrogen/Life Technologies) per manufacturer's instructions.

Caspase-3/7 activity assay.

Assays were performed as previously described (5). Briefly, cells were seeded in 96-well plates at a density of 5×10^3 cells/well. Following treatment, caspase-3/7 activity was determined as per the manufacturer using the Apo-ONE assay (Promega, Madison, WI) according to the manufacturer's recommendations.

Trypan blue exclusion assay.

Assays were performed using Trypan blue 0.4% (Fisher #MT25900LI). Cells suspensions of buffered isotonic solutions were admixed 1:1 with Trypan blue and loaded onto a hemacytometer for examination. Blue staining cells, considered representative of dead/dying cells were compared to the total number of cells visualized.

Crystal violet staining.

Cell viability was assessed with fixation using 3.7% formaldehyde and staining with 1.0% crystal violet and 0.5% acetic acid. After serial washes in isotonic buffered saline, cells stained with crystal violet were washed with 2% ethanol and 1% SDS to completely de-stain cells. These solutions were measured by spectrophotometry at 620 nM.

Statistical considerations.

As stipulated in the main article and unless otherwise indicated, data represent means ± SEM of three independent experiments (n=3). Multiple data groups were tested for statistical significance by one-way ANOVA and Tukey-Kramer post-hoc analysis, assuming α=0.05, with GraphPad Instat 3; pairwise

comparisons of only two data groups were subjected to nonparametric analysis of statistical significance (Mann-Whitney U test).

Supplemental Table 1. Primer nucleotide sequences used in this study.

Target	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)	Notes (Refs.)
ASAH1	TGTGGATAGGGTTCCTCACTAGA	TTGTGTATACGGTCAGCTTGTTG	AC (6)
ASAH2	TTACCCTTGGGTCCTTGGCCATAA	TCTGCCACGATGTTGAAGTAGCCT	
ACER1	GCCTAGCATCTTCGCCTATCAG	GGAAGTTGCTCTCACACCAGTC	(7)
ACER2	AGTGTCCTGTCTGCGGTTACG	TGTTGTTGATGGCAGGCTTGAC	(7)
ACER3	TGGACTGGTGCGAGGAGAAC	TCCAGAACTCGGCGATGTACC	(7)
SMPD1	GAAAGCACACCTGTCAATAGCTT	AGAAGACCTCAAATTCATCCACA	ASMase
JUN	GCAAACCTCAGCAACTTCAACCCA	AGGCAGGCCAGAAAGAGTTCATCT	c-Jun
JUNB	AATGGAACAGCCCTTCTACCACGA	ATGCGCTCTTGGTCTTCCATGTTG	
JUND	ATGGAAACACCCTTCTACGGCGAT	TGTTGACGTGGCTGAGGACTTTCT	
FOS	ACTTCCTGTTCCCAGCATCATCCA	AGGEELGGCTEAAEATGETAETAA	c-Fos
FOSB	ACTTTGCCATTGTTGGAACGGGAC	AAAGCCTGAAACATTTCCCTGGGC	
FOSL1	TTGAGCTGGCCTCTCTAGCACAAT	TGGGTAAAGTGGCACCTTCTGTCA	Fra-1
FOSL2	AAGTCAGGCCTGCAGAAGGAGATT	GCAATGCTGATGGGCTTGATGACA	Fra-2
RPS15	TTCCGCAAGTTCACCTACC	CGGGCCGGCCATGCTTTACG	(8)
GAPDH	CAATGACCCCTTCATTGACC	GATCTCGCTCCTGGAAGATG	(9)
ASAH1 promoter	GATCCTCGAGCTCCACTGCATTTGTCAC	GATCTGATCAATCGCTCTAGCAGCCAAC	~1.5 kb upstream of *ASAH1* transcription start site
ASAH1 promoter	TAGCCTGGAAGGCTCTCTCTCTTT	GGACTCCAGCAGAGGCAAAGAA	ChIP at -227/-26
ASAH1 promoter	CAGCCGCTTAATGAACTGCTGCAT	ACCCACGAAACGGATCCAAAGAGT	ChIP at -753/-418
ASAH1 promoter	GCGTGCTGAGCTTCATCAAAGCAT	CAGCAAAGCTGCCAAATCCCAGAA	ChIP at -1004/-628
ASAH1 promoter	ACCACTGCAAAGTCTCCTACACAC	AGTGTGCGAAGAAGAGGATTCCCA	ChIP at -2358/-1918
ASAH1 intron 5	AGGGAGAAGACCTCACATGCCATT	TGACACTACTGCTTTGCTAGCTT	ChIP negative control at ~19.3kb (163bp)
MMP1 promoter	GCACTTTATGACCATCAGAACCAGCC	AAAGCATGAGTCAGACAGCCTCTG	ChIP positive control at -243/-3 (10)

Supplemental Table 2. RNAi nucleotide sequences used in this study.

Gene target	Target sequence (5′ → 3′)	Product no. (Ref.)
ASAH1 (AC)	AATCAACCTATCCTCCTTCAG	Qiagen (5)
SCRambled-sequence control	AATTCTCCGAACGTGTCACGT	Qiagen (5)
JUN (c-Jun)	GTGACGGACTGTTCTATGA	SCBT sc-29223A
JUN (c-Jun)	CCAGAAAGGATATTTAAGA	SCBT sc-29223B
JUN (c-Jun)	GATGGCCTTTGCTTATGAA	SCBT sc-29223C
JUN (c-Jun)	GCATCATCTGTAGATACTA	SCBT sc-29223D
JUNB (Jun-B)	AATGGAACAGCCCTTCTAC	SCBT sc-35726A
JUNB (Jun-B)	AAGATGAACCACGTGACAC	SCBT sc-35726B
JUNB (Jun-B)	AAACAGAAGGTCATGACCC	SCBT sc-35726C
JUND (Jun-D)	GGATGGAAACACCCTTCTA	SCBT sc-35728A
JUND (Jun-D)	CGAGCTCACAGTTCCTCTA	SCBT sc-35728B
JUND (Jun-D)	CCCTATTTATGTTTCTACT	SCBT sc-35728C
FOS (c-Fos)	CAAGGTGGAACAGTTATCT	SCBT sc-29221A
FOS (c-Fos)	GGCTTCCCTTGATCTGACT	SCBT sc-29221B
FOS (c-Fos)	CCGAGCCCTTTGATGACTT	SCBT sc-29221C
FOS (c-Fos)	GGCAATAGTGTGTTCTGAT	SCBT sc-29221D
FOSB (FosB)	CTGGAGTGATTTATACTGT	SCBT sc-35403A
FOSB (FosB)	CGAGCCGTTGAATTGGAAA	SCBT sc-35403B
FOSB (FosB)	CCAACCACAATTCAATGAA	SCBT sc-35403C
FOSL1 (Fra-1)	CCAGCAACTTCTTCTCCAT	SCBT sc-35405A
FOSL1 (Fra-1)	CTGACCATATTGTGCTTCA	SCBT sc-35405B
FOSL1 (Fra-1)	CGGATCTCAGCTTTGAGAA	SCBT sc-35405C
FOSL2 (Fra-2)	GAACCTCGTCTTCACCTAT	SCBT sc-35407A
FOSL2 (Fra-2)	CAGCGGGGACCAATCATCA	SCBT sc-35407B
FOSL2 (Fra-2)	CACTCTGCTGGCTCTGTAA	SCBT sc-35407C
Adsh*ASAH1*$_{ORF}$-GFP	GTACCGGGGTCATAACTGAGCAACTAAACTCGAGTTTAGTTGCTCAGTTATGACCTTTTTTG	Vector Biolabs shADV-201505
Adsh*ASAH1*$_{3'UTR}$-GFP	CCGGGCTGTTATTGACAGCGATATACTCGAGTATATCGCTGTCAATAACAGCTTTTT	Vector Biolabs custom design

Supplemental References

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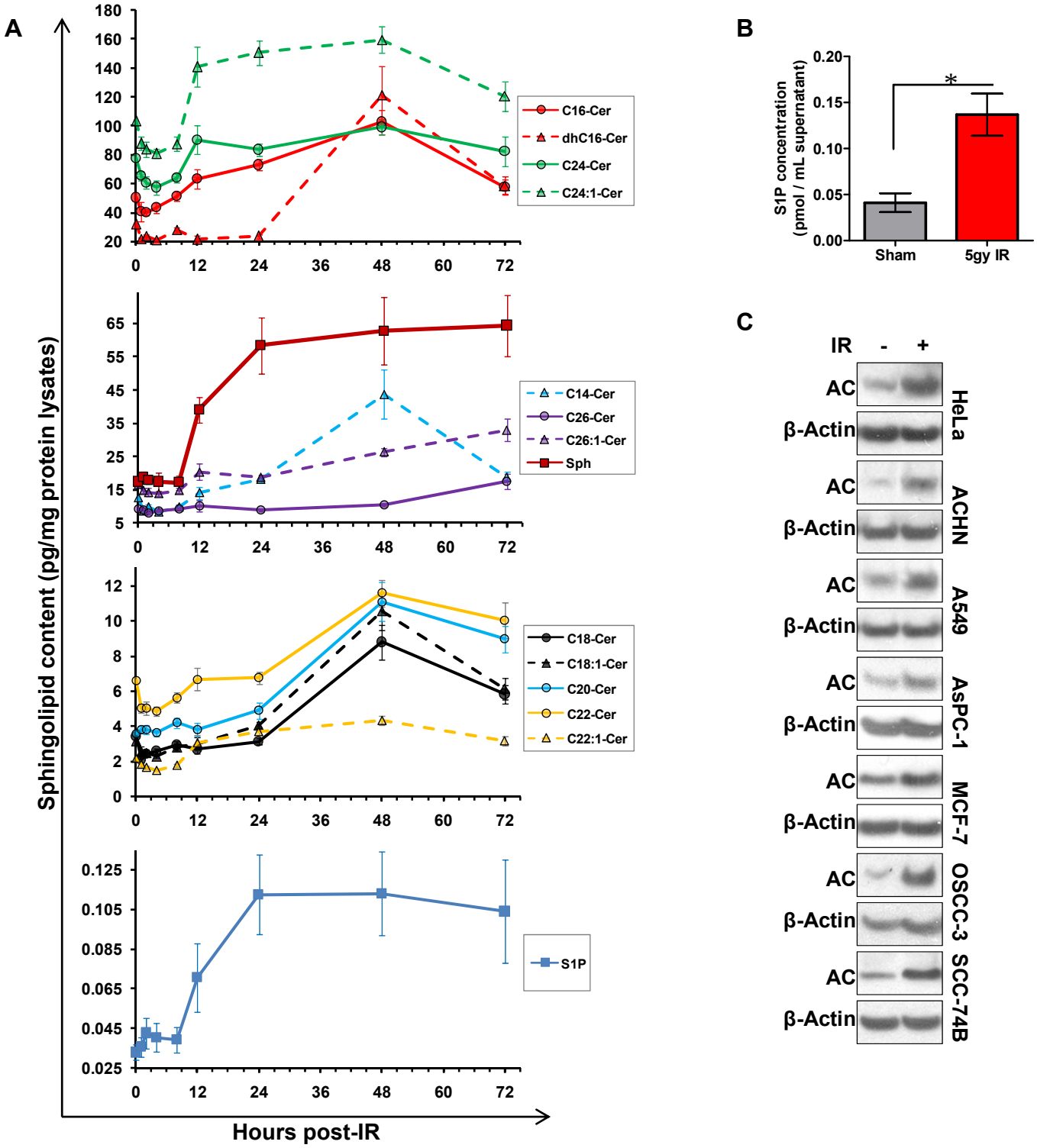
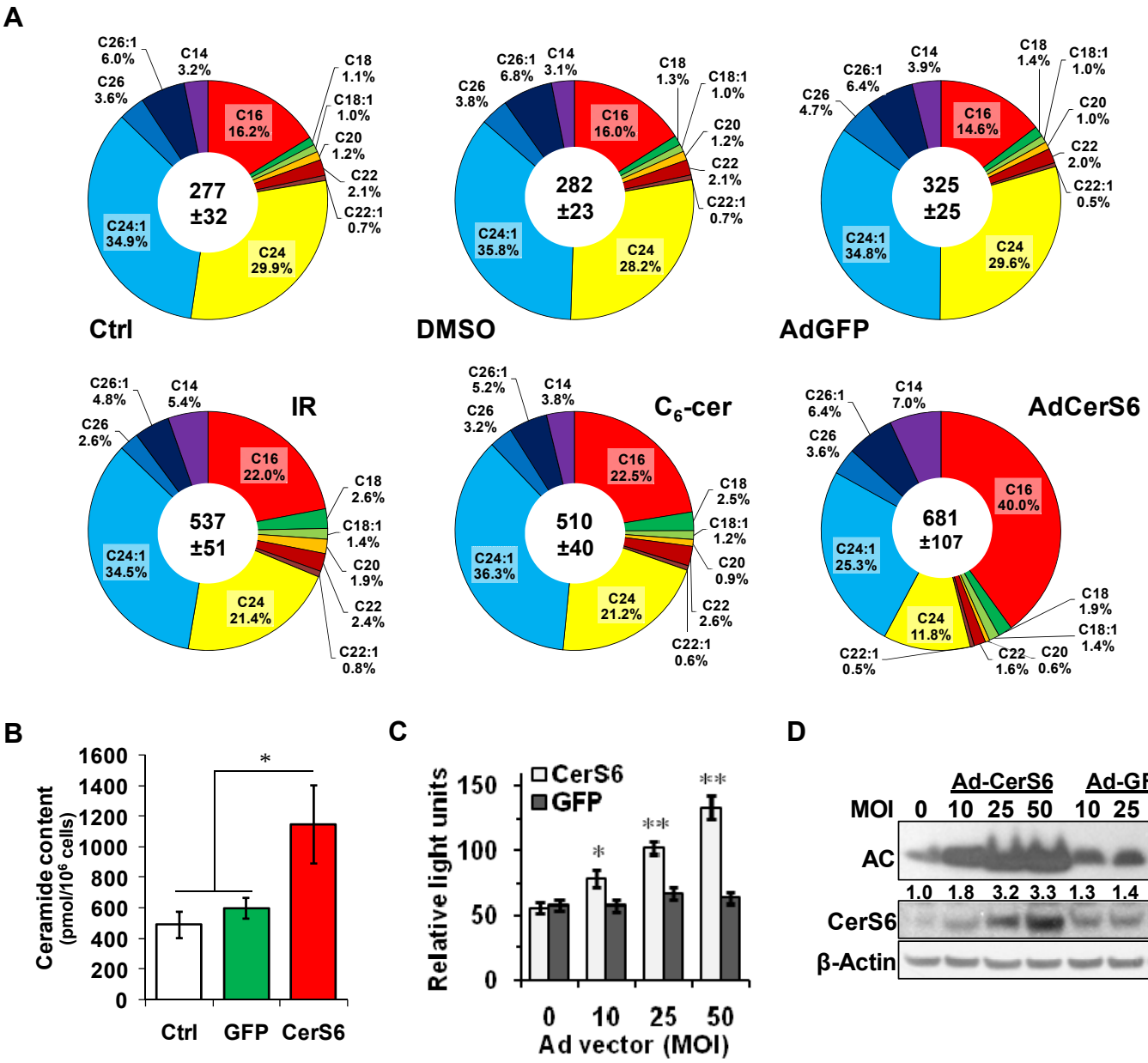
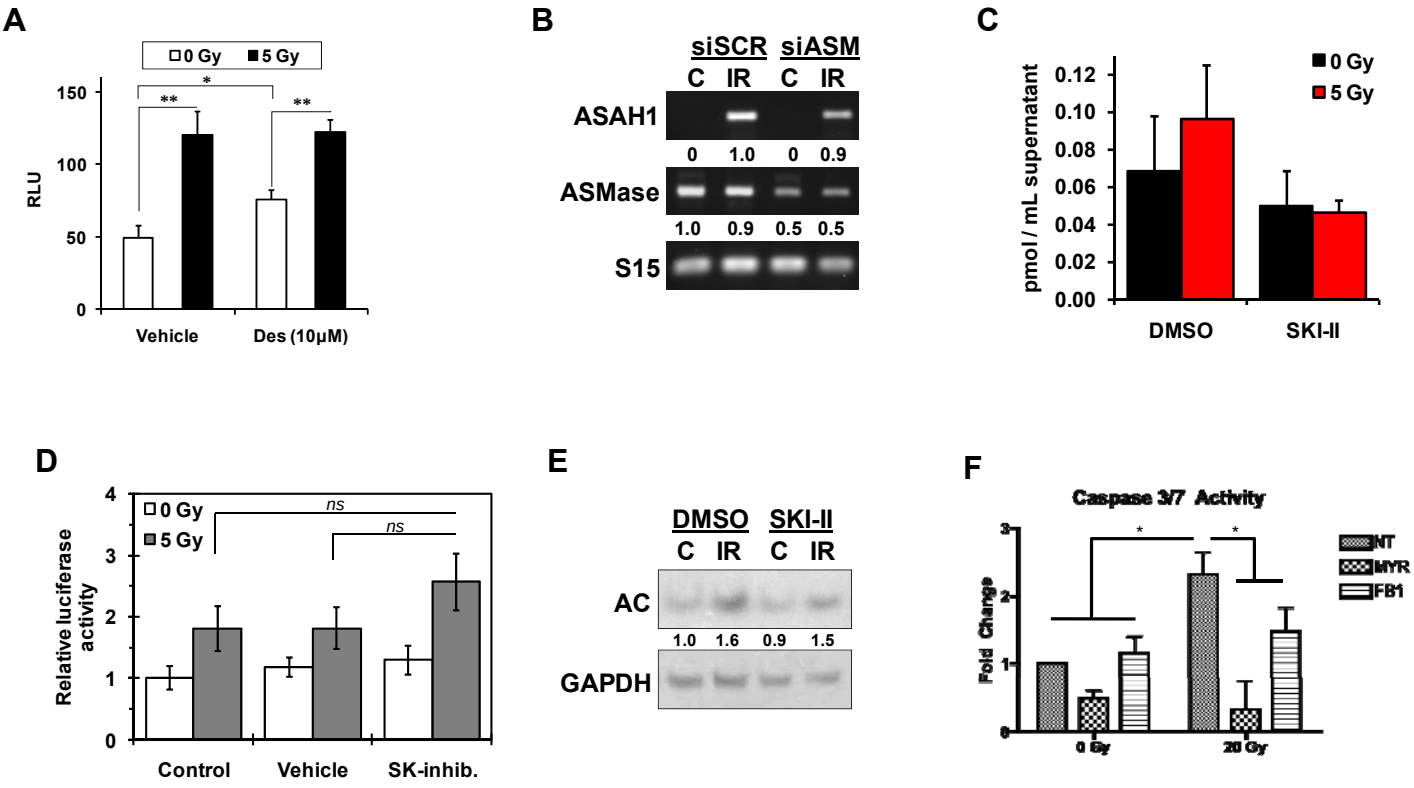


Supplemental Figure 1. IR stimulates sphingolipid generation and AC upregulation. As described in Figure 1, PPC-1 cells were collected for sphingolipid analysis at various times post-IR (5 Gy). **A.** Graphs representing sphingolipid content, particularly ceramide species levels. (**B**) S1P concentrations measured in the cell culture supernatants of mock- and γ-irradiated PPC-1 cell cultures 24 hours post-IR. *p<0.05, Mann-Whitney U test. (**C**) Cancer cells lines derived from adenocarcinomas of the cervix (HeLa), kidney (ACHN), lung (A549), pancreas (AsPC-1), and breast (MCF-7), as well as oral squamous cell carcinomas (OSCC-3 and (SCC-74B) were γ-irradiated (5 Gy) and collected 48 hours post-IR for protein expression analysis by Western blot. Supplements to Figure 1.

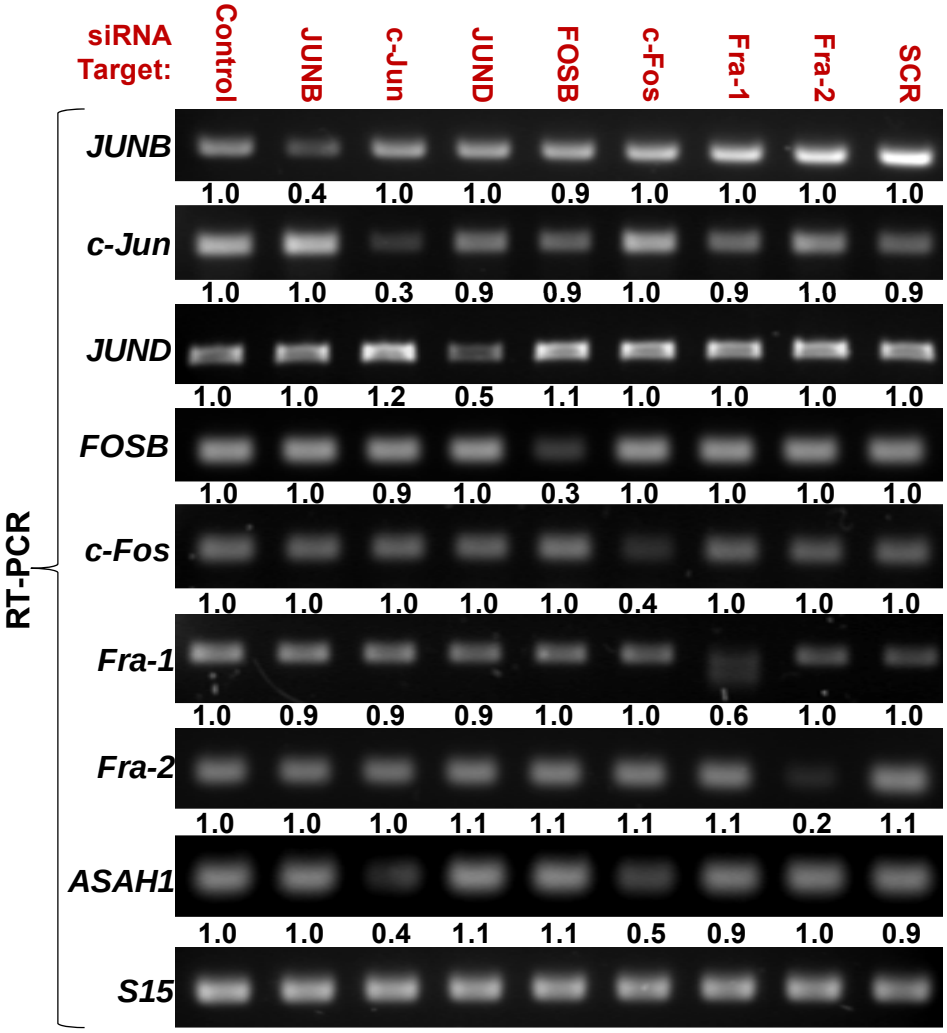


Supplemental Figure 2. Exogenous and endogenous ceramide induces AC expression. (**A**) Ceramide species profiles of PPC-1 cells treated with IR (5 Gy, 48 hours), exogenous C_6-ceramide (5 μM, 24 hours), or AdCerS6-GFP (20 MOI, 48 hours), versus cells under corresponding control conditions. Numbers encircled in each pie chart indicate mean picomolar mass of total ceramides per mg protein of whole cell lysates. PPC-1 cells were transduced with AdGFP or AdCerS6 (20 MOI, 48 hours), and collected for analysis of (**B**) cellular ceramide content, (**C**) *ASAH1* promoter luciferase-reporter, and (**D**) AC protein expression. Mean densitometry of normalized AC expression is shown below each band. *p<0.05, **p<0.01. Supplements to Figure 2.



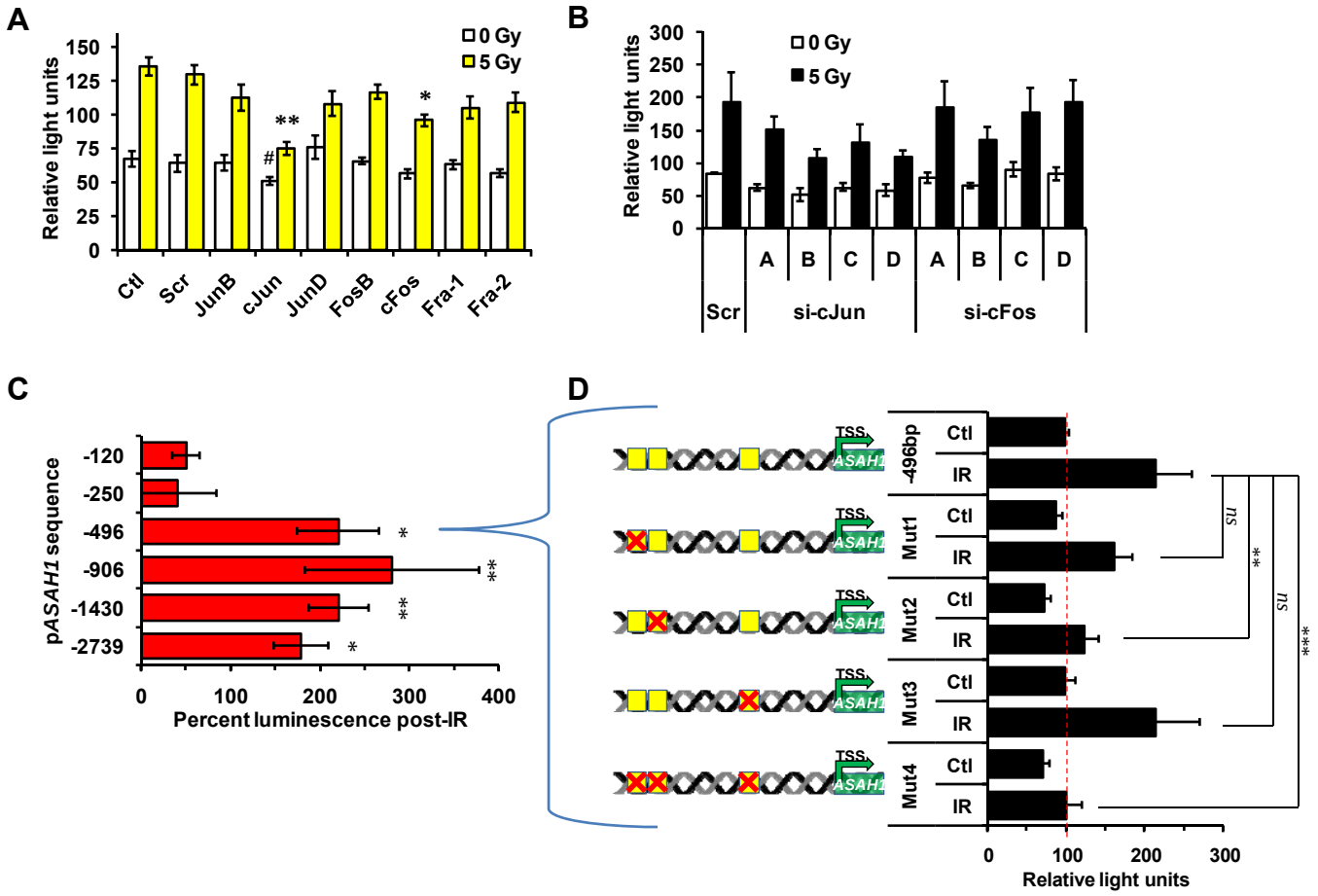
Supplemental Figure 3.

A. *ASAH1* promoter reporter activity of PPC-1 cells treated with the inhibitor of ASMase, desipramine (10 μM, 4 hours) or vehicle (ethanol) prior to IR exposure (5 Gy, 24 hours). **B.** RT-PCR of *ASAH1* mRNA in PPC-1 cells transfected with siRNA targeting ASMase or a scrambled-sequence control (50 nM) and γ-irradiated (5 Gy, 48 hours) prior to assessment. PPC-1 cells were pre-treated with the sphingosine kinase inhibitor, SKI-II (1 μM, 4 hours) or DMSO vehicle control, and γ-irradiated (5 Gy), and subsequently collected for (**C**) S1P measured by LC/MS in cell culture supernatant (24 hours post-IR), (**D**) *ASAH1* promoter reporter activity (24 hours post-IR), and (**E**) western blot analysis (48 hours post-IR). **F.** Caspase-3/7 assay of PPC-1 cells pre-treated with fumonisin B1 (FB1, 25 μM, 4 hours) and myriocin (MYR, 100 nM, 4 hours) versus non-treatment control (NT), and irradiated at 20 Gy, time. *p<0.05, **p<0.01.



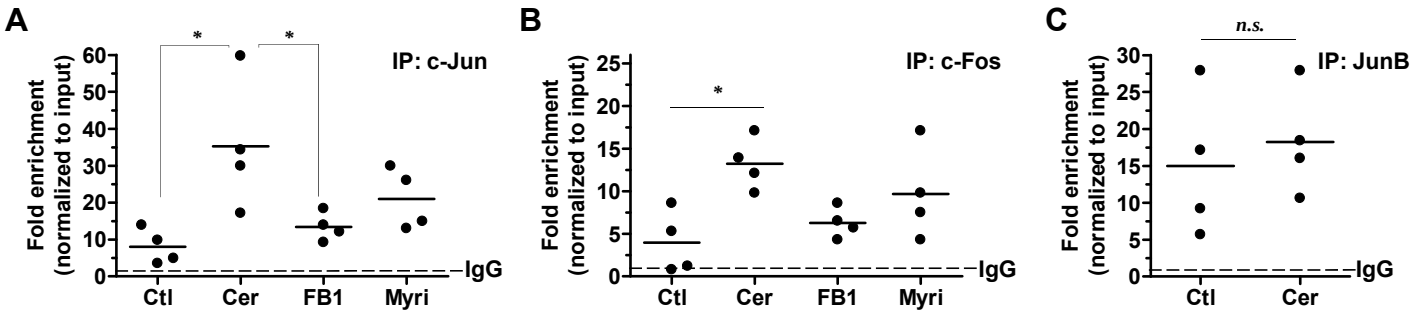
Supplemental Figure 4. c-Jun/AP-1 binding activity on the *ASAH1* promoter regulates post-IR expression.

RT-PCR of mRNA transcripts in PPC-1 cells transfected with commercial siRNA cocktails targeting Jun/Fos AP-1 family members (50 nM, 48 hours). Mean densitometry of amplicons is shown below each band.



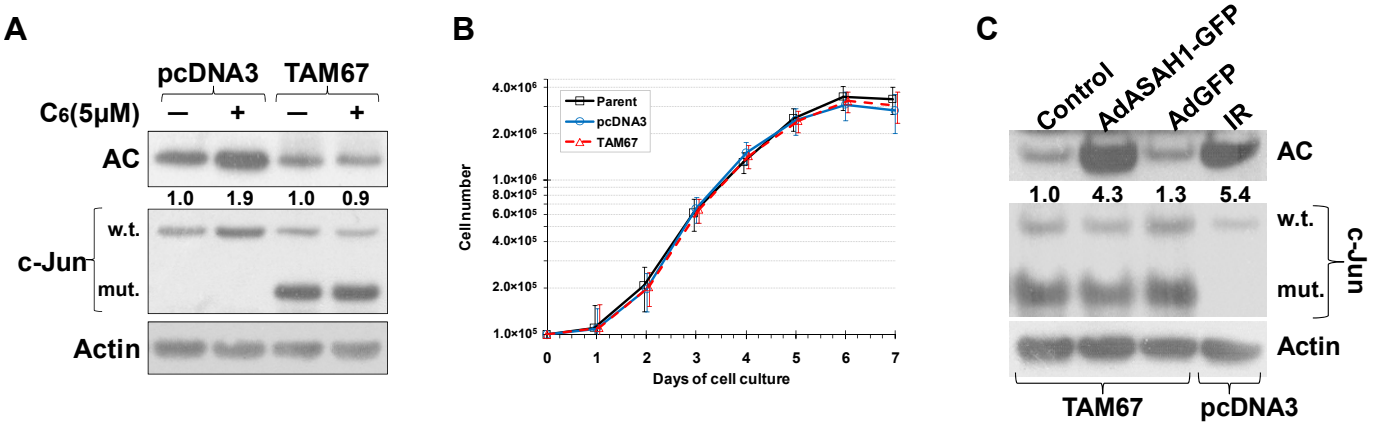
Supplemental Figure 5. c-Jun/AP-1 binding activity on the *ASAH1* promoter regulates post-IR expression.

PPC-1 cells were pre-treated with pooled siRNA targeting Jun/Fos members of the AP-1 family (panel **A**, 50 nM, 24 hours), or the singular siRNA constituent sequences from the cJun or cFos pooled siRNA (panel **B**, see Supplemental Table 2 for sequences), prior to irradiation (5 Gy, 24 hours) and were collected for *ASAH1* promoter-reporter activity assays. n=4; *p<0.05, **p<0.01 compared to non-irradiated controls; $^{\#}p$<0.05 compared to IR- and siRNA-naïve control cells. (**C**) Firefly luciferase assay of *ASAH1* proximal promoter reporters stably expressed by PPC-1 cells exposed to IR (5 Gy, 24 hours) compared to respective non-irradiated controls. (**D**) Luciferase assay of PPC-1 transfected with reporter constructs of the -496 bp upstream promoter of *ASAH1* with TCA→TTG mutations at three AP-1 consensus binding sequences identified by TFBind analysis: -494/-486 bp (Mut 1), -475/-468 bp (Mut 2), -274/-267 bp (Mut 3), and all three sites (Mut 4). *p<0.05, **p<0.01, ***p<0.001.

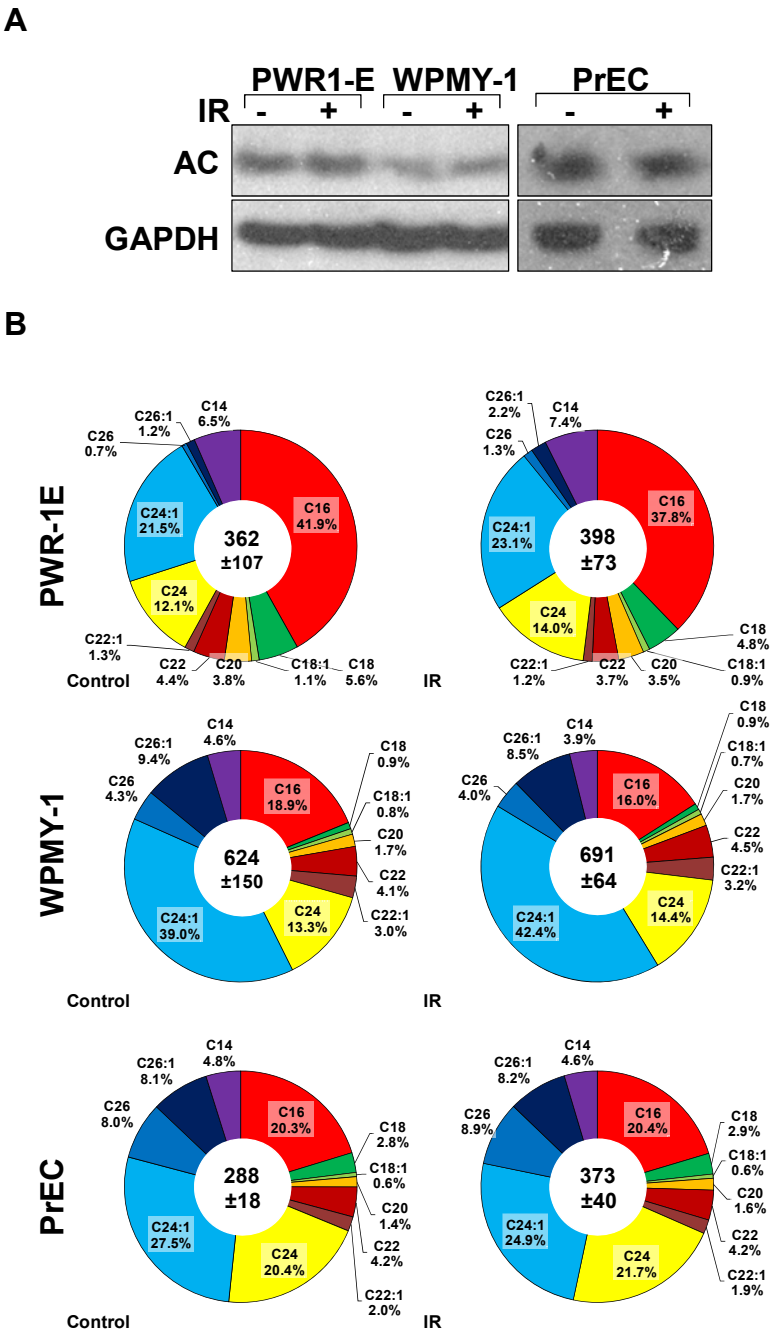


Supplemental Figure 6. c-Jun/AP-1 binding activity on the *ASAH1* promoter regulates post-IR expression.

PPC-1 cells treated with IR and analyzed by ChIP-qPCR assay in **Figure 3** were similarly treated with C6-ceramide (5 μM, 24 hours) and subjected to ChIP-qPCR analysis for c-Jun (**A**), c-Fos (**B**), or JunB (**C**) binding to -753/-418 *ASAH1* promoter fragment. *p<0.05

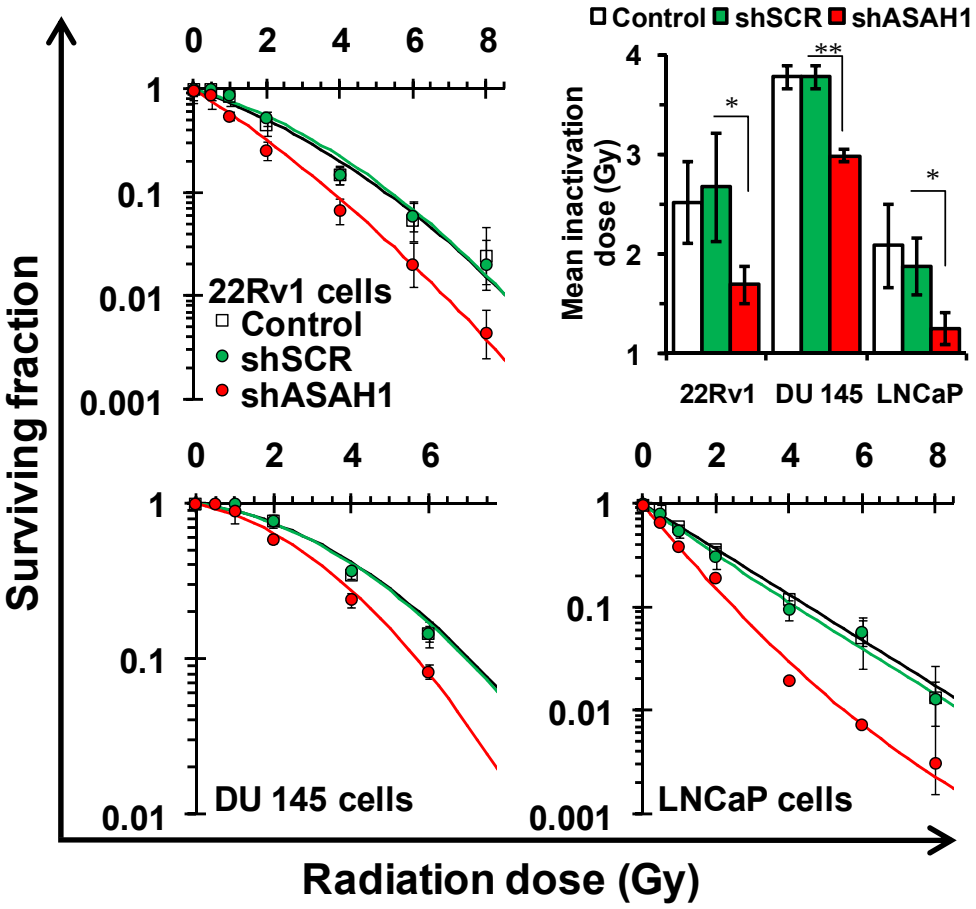


Supplemental Figure 7. AP-1 mediates PPC-1 cell radioresistance through AC.
A. Western blot analysis of PPC-1-TAM67 *versus* PPC-1-pcDNA3 (vector control) cells treated with C_6-ceramide (5 μM) or DMSO (vehicle control) for 24 hours. **B.** Parental, pcDNA3- and TAM67-stable PPC-1 cell transfectants were seeded for culture and quantified during one week of propagation in vitro. **C.** Immunoblot of PPC-1-TAM67 cells transduced with AdGFP or AdASAH1-GFP vectors in order to restore AC over-expression.

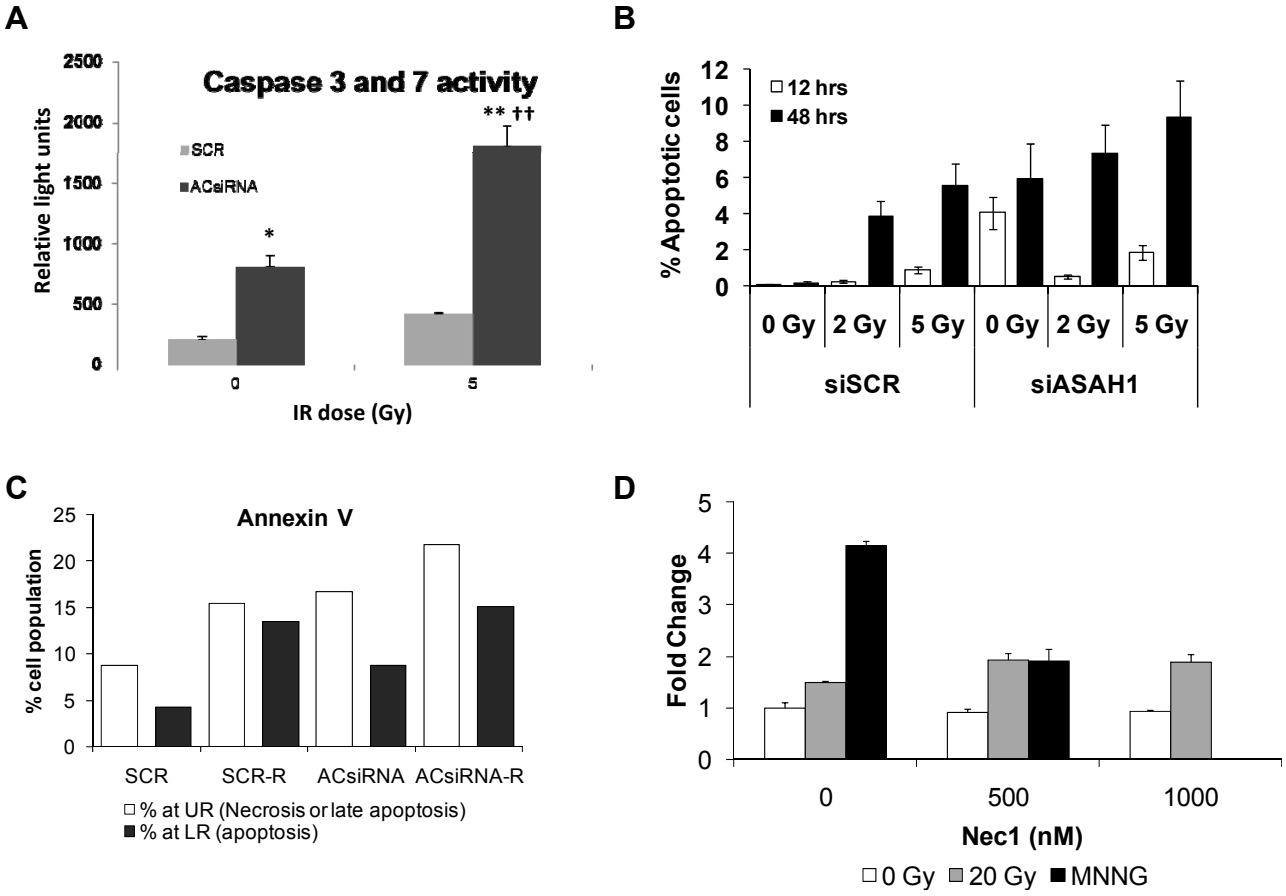


Supplemental Figure 8. Non-cancer prostate cell lines do not over-express AC or ceramide after IR exposure.

A. Western blot analysis of prostate cell lines PWR-1E (immortalized epithelium), WPMY-1 (immortalized stroma), and PrEC (primary epithelium) 48 hours post-IR (5 Gy). **B.** Ceramide species profiles of these cell lines treated with IR (5 Gy, 48 hours), versus mock-irradiation. Numbers encircled in each pie chart indicate mean picomolar mass of total ceramides per mg protein of whole cell lysates.

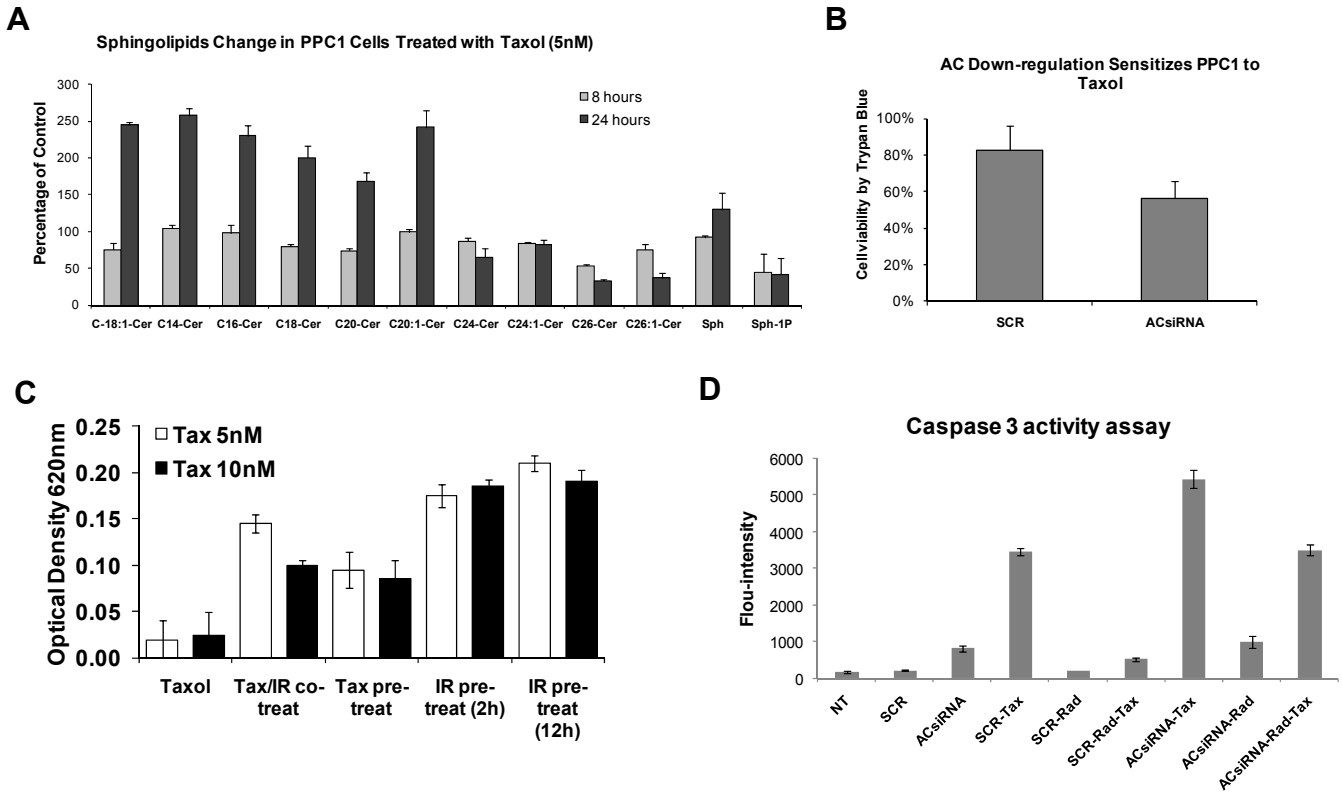


Supplemental Figure 9. Targeting *ASAH1* increases PCa cell susceptibility to IR. Clonogenic survival of PCa cell lines pre-treated with Adsh*ASAH1* or AdshSCR and γ-irradiated between 0 and 8.0 Gy. Results are shown as geometric means ± SE, and fitted to the linear-quadratic model to quantify mean inactivation dose (upper right panel). *p<0.05, **p<0.01.



Supplemental Figure 10. Mechanisms of death cell contributing to AC-mediated PPC-1 cell response to IR.

A. PPC-1 cells were transfected with siRNA (50 nM for 24 hours) targeting *ASAH1* or scrambled control sequence prior to IR exposure at 0 or 5 Gy. Cells were harvested at 24 post-IR for Caspase 3/7 assay. **p*<0.05, ***p*<0.01 compared to mock-irradiated conditions; †† *p*<0.01 compared to ACsiRNA treatment at 0 Gy. **B**. Propidium iodide staining and flow cytometry analysis at 24 hours post-IR of the apoptotic fraction of PPC-1 cells pre-treated with *ASAH1* or SCR siRNA. **C**. Flow cytometry analysis at 24 hours post-IR for Annexin V expression in PPC-1 cells pre-treated with *ASAH1* or SCR siRNA. **D**. PPC-1 cells were pre-treated with necrosis inhibitor Necrostatin-1 (Nec1) prior to IR exposure (20 Gy) or necrosis induction with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). At 12 hours of induction, cells were stained with membrane impermeant YOYO-1 iodide and scanned by Incucyte Zoom (Essen BioScience) for necrotic core quantitation. Data are presented as fold change versus untreated control cells.



Supplemental Figure 11. AC inhibition alone or in combination with IR sensitizes PCa cells to Taxol-induced cell killing.

A. Sphingolipid measurements of ceramide species, sphingosine, and sphingosine 1-phosphate in PPC-1 cells treated with Taxol (5 nM) for 8 hours (light gray bars) or 24 hours (dark gray bars), shown as percent of sphingolipids assessed in untreated control cells. **B.** PPC-1 cells were transfected with siRNA to *ASAH1* and to scrambled-control (SCR) sequence 24 hours prior to Taxol (5 nM) administration . Cell viability is quantified by crystal violet extraction and quantitation. Results are expressed as percent of crystal violet extracted from control untransfected cells under Taxol treatment. **C**. Cell viability, measured by crystal violet staining and extraction, is demonstrated in PPC-1 cells 48 hours after sequential treatments of Taxol (5 and 10 nM) and/or IR (5 Gy). Sequences of administration included Taxol alone, Taxol and IR simultaneous co-administration, Taxol pretreatment (12 hours), or IR pretreatment (2 or 12 hours). **D**. PPC-1 cells were transfected with siRNA to ASAH1 or SCR versus control mock-transfected (NT). 24 hours later, cells were exposed to IR (5 Gy), Taxol (5 nM), or both. Caspase-3 activity by fluorogenic assay at 24 hours of induction is shown as arbitrary light units.