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## Supplemental information

# Aging-dependent mitochondrial dysfunction mediated by ceramide signaling inhibits antitumor T cell response

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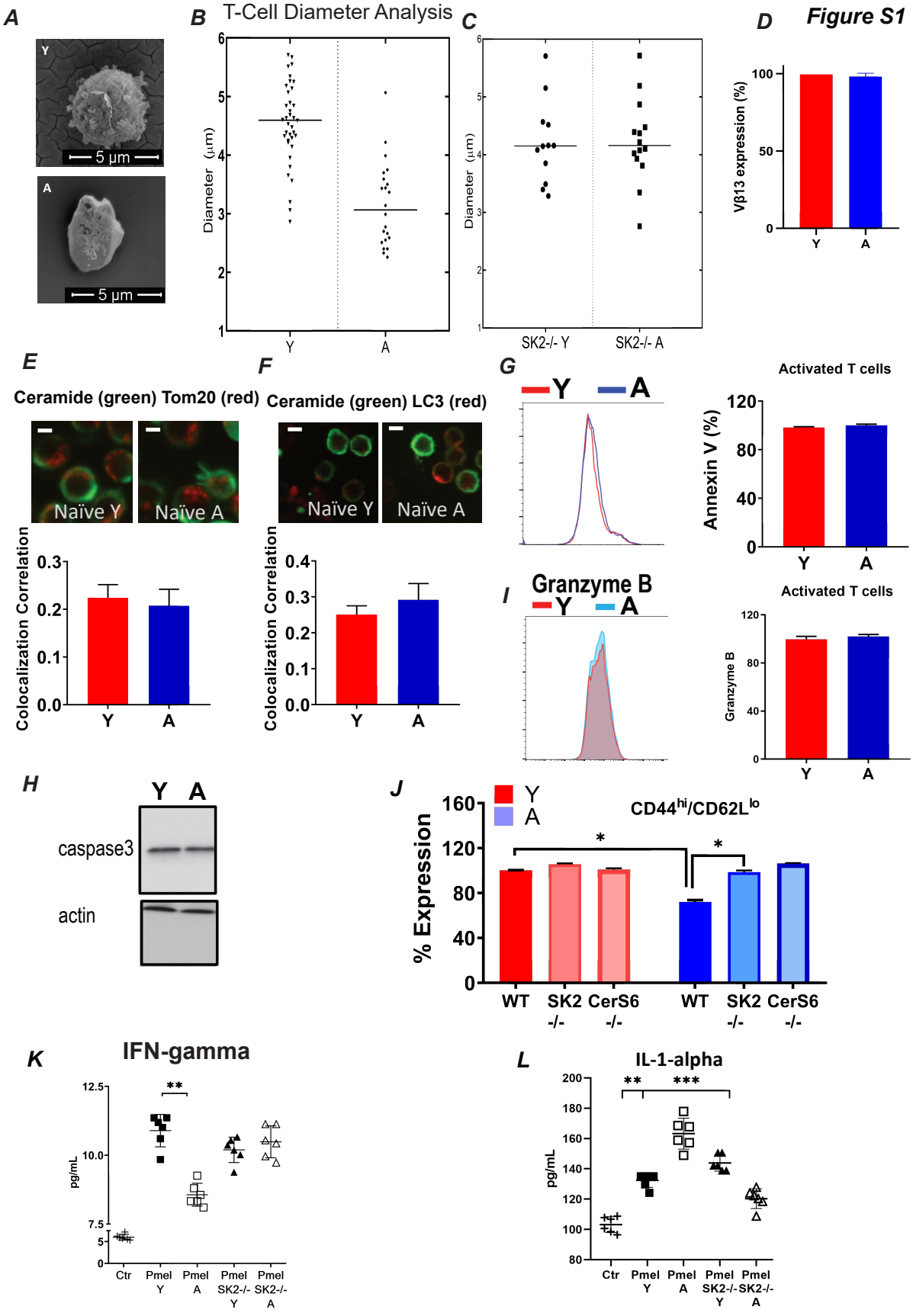
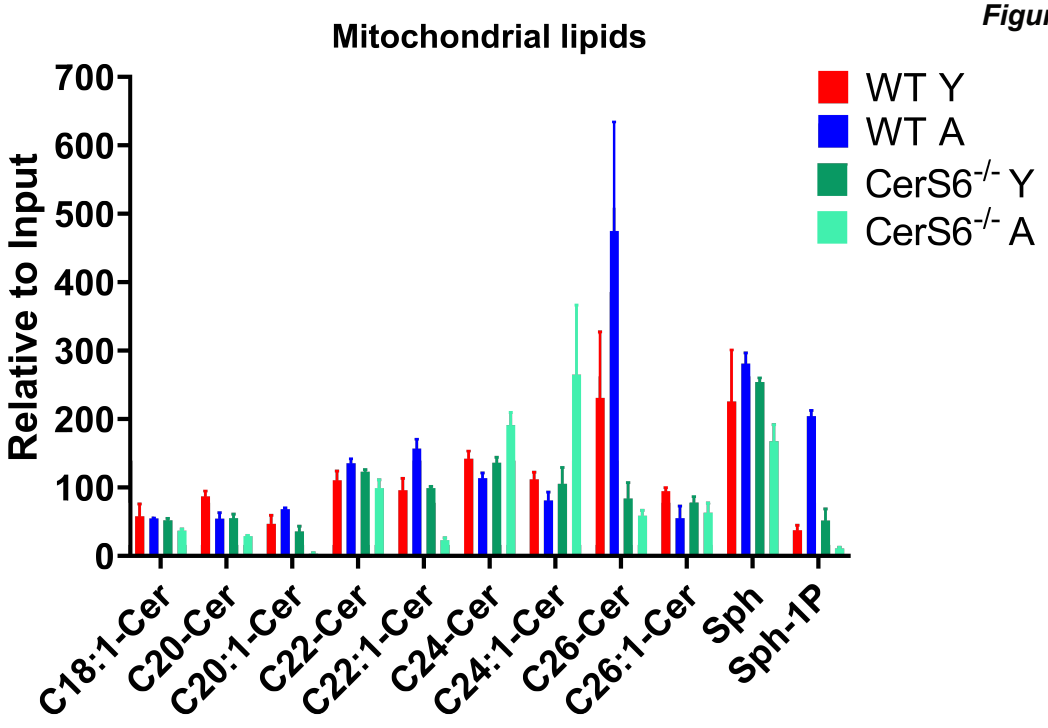

A
Y
5 µm
A
5 µm
B
T-Cell Diameter Analysis
Diameter (µm)
Y
A
C
Diameter (µm)
SK2-/- Y
SK2-/- A
D
Figure S1
Vβ13 expression (%)
Y
A
E
Ceramide (green) Tom20 (red)
Naïve Y
Naïve A
Colocalization Correlation
Y
A
F
Ceramide (green) LC3 (red)
Naïve Y
Naïve A
Colocalization Correlation
Y
A
G
Y
A
Activated T cells
Annexin V (%)
Y
A
I
Granzyme B
Y
A
Activated T cells
Granzyme B
Y
A
H
Y
A
caspase3
actin
J
Y
A
CD44hi/CD62Llo
% Expression
WT
SK2 -/-
CerS6 -/-
WT
SK2 -/-
CerS6 -/-
K
IFN-gamma
pg/mL
Ctr
Pmel Y
Pmel A
Pmel SK2-/- Y
Pmel SK2-/- A
L
IL-1-alpha
pg/mL
Ctr
Pmel Y
Pmel A
Pmel SK2-/- Y
Pmel SK2-/- A

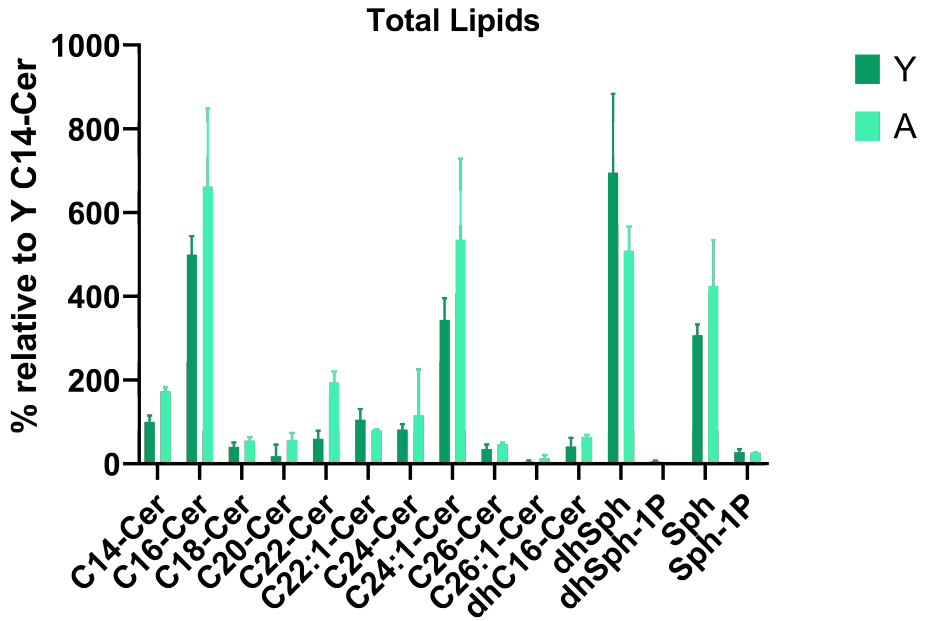
**Figure S1 (Related to Figure 1). Aging T cells exhibit morphological and functional alterations. A-C.** Scanning electron microscopy (SEM) micrographs (A) of ex vivo activated T cells isolated from Y and A WT or SphK2$^{-/-}$ (SK2) mice were acquired, and cell diameters (B-C) were quantified (n=15-30). **D**. Flow cytometry Vβ13 T-cell receptor analysis on CD8+ T-cells from Y and A Pmel mice. **E-F.** Confocal microscopy for Y and A naïve T cells dual labeled with TOM20 (red, mitochondrial marker), and ceramide (green) (E), as well as ceramide (green) and LC3 (red) fluorescent antibodies (F). Scale bars, 1 μm. Images represent at least three independent experiments. The bottom panel shows the quantification of colocalization extracted from the coefficient of colocalization (*Rc*) **G**. Flow cytometry Annexin V analyses of activated T cells obtained from Y and A mice. **H.** Western blotting measuring pro-caspase 3 in activated T cells obtained from Y and A mice. **I.** Flow cytometry for the detection of granzyme B in activated T cells obtained from Y and A mice. **J.** Measurement of CD44 and CD62L in activated T cells obtained from Y and A WT, SphK2$^{-/-}$ (SK2) and CerS6$^{-/-}$ mice using flow cytometry. **K-L**. Levels of IFN-gamma and IL-1alpha were measured by ELISA in activated T cells obtained from Y and A WT or SphK2$^{-/-}$ (SK2) mice. These data represent three independent experiments (n=3). *$p$< 0.05, **$p$< 0.005 or ***p< 0.0005 as determined by Student's *t-test*.

*Figure S2*

*A*




*B*




*C*

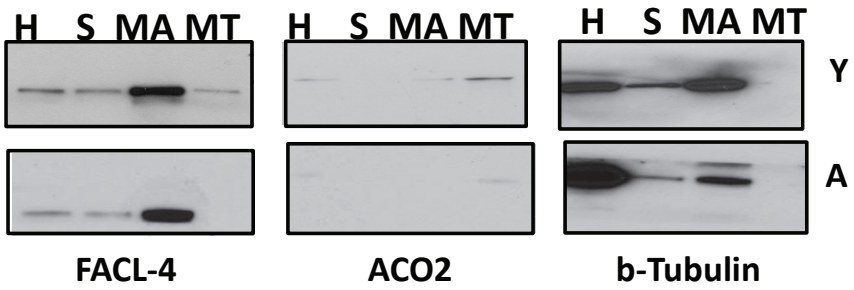
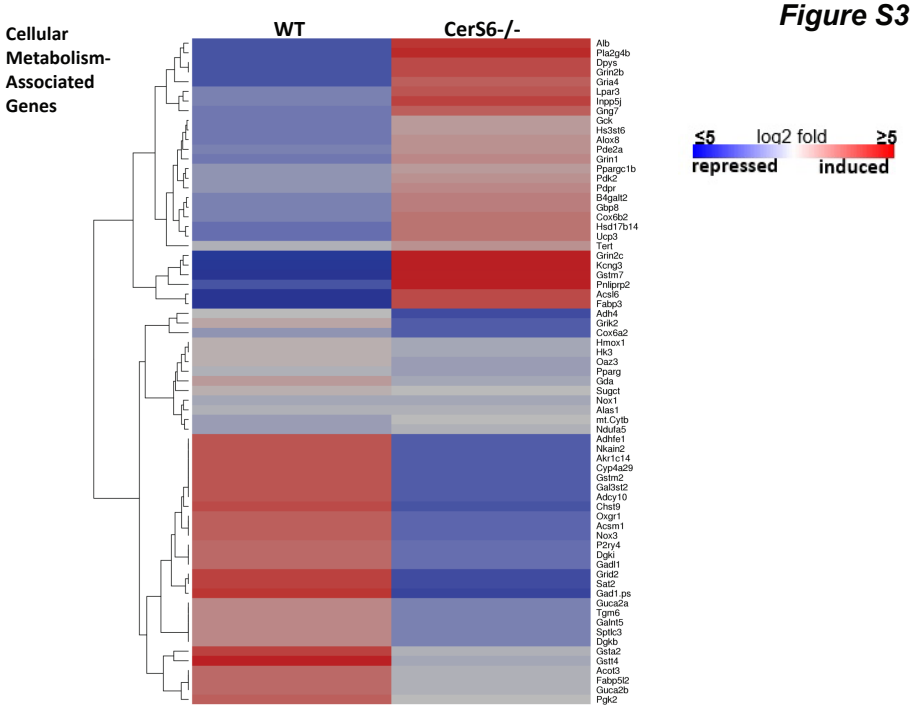


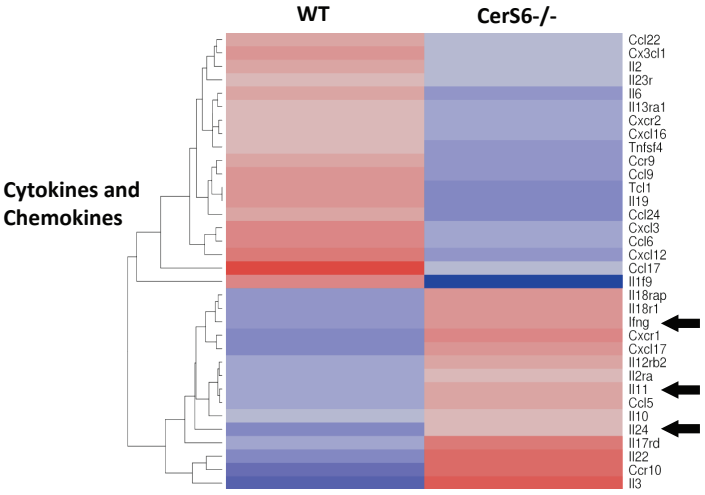
**Figure S2 (Related to Figure 2). Mitochondrial accumulation of sphingolipids in young and aging T cells. A-B.** HPLC-MS-MS and lipidomics-based measurements of sphingolipids in mitochondria (MITO) enriched gradient fractions (A) or total cell lysates (B) from T cells obtained from Y and A WT or CerS6$^{-/-}$ mice. Data are means ± SD from at least three experiments. **C.** Western blots showing enrichment of resident proteins for fractions (H, total homogenates, S, supernatant, MA, MAMs, MITO, mitochondrial fractions) obtained during the purification procedures in T cells shown in A for FACL4, ACO2 and beta-tubulin. These data represent three independent experiments (n=3).

*Figure S3*

*A*




*B*




*C*

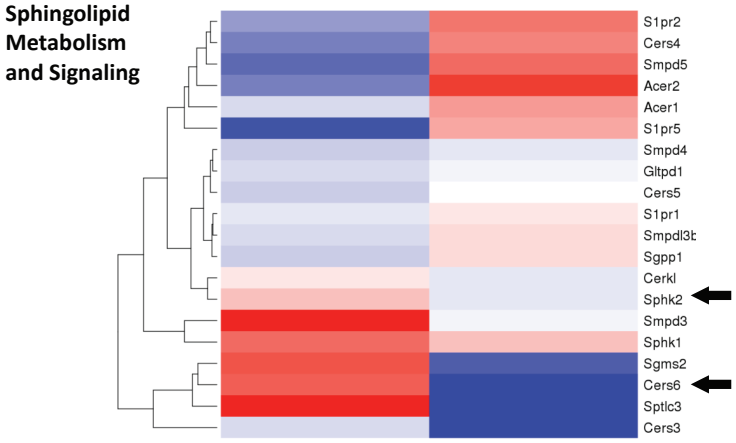


**Figure S3 (Related to Figures 2 and 3). RNAseq analyses of activated T cells obtained from Y and A WT and CerS6$^{-/-}$ mice. A-C.** Genes that are involved in the regulation of cellular metabolism (A), cytokines/ chemokines (B) or sphingolipid metabolism and signaling (C) were analyzed. The gene expression (upregulated or downregulated) levels were depicted into heatmaps in T cells from A compared to Y WT or CerS6$^{-/-}$ mice.

*Figure S4*

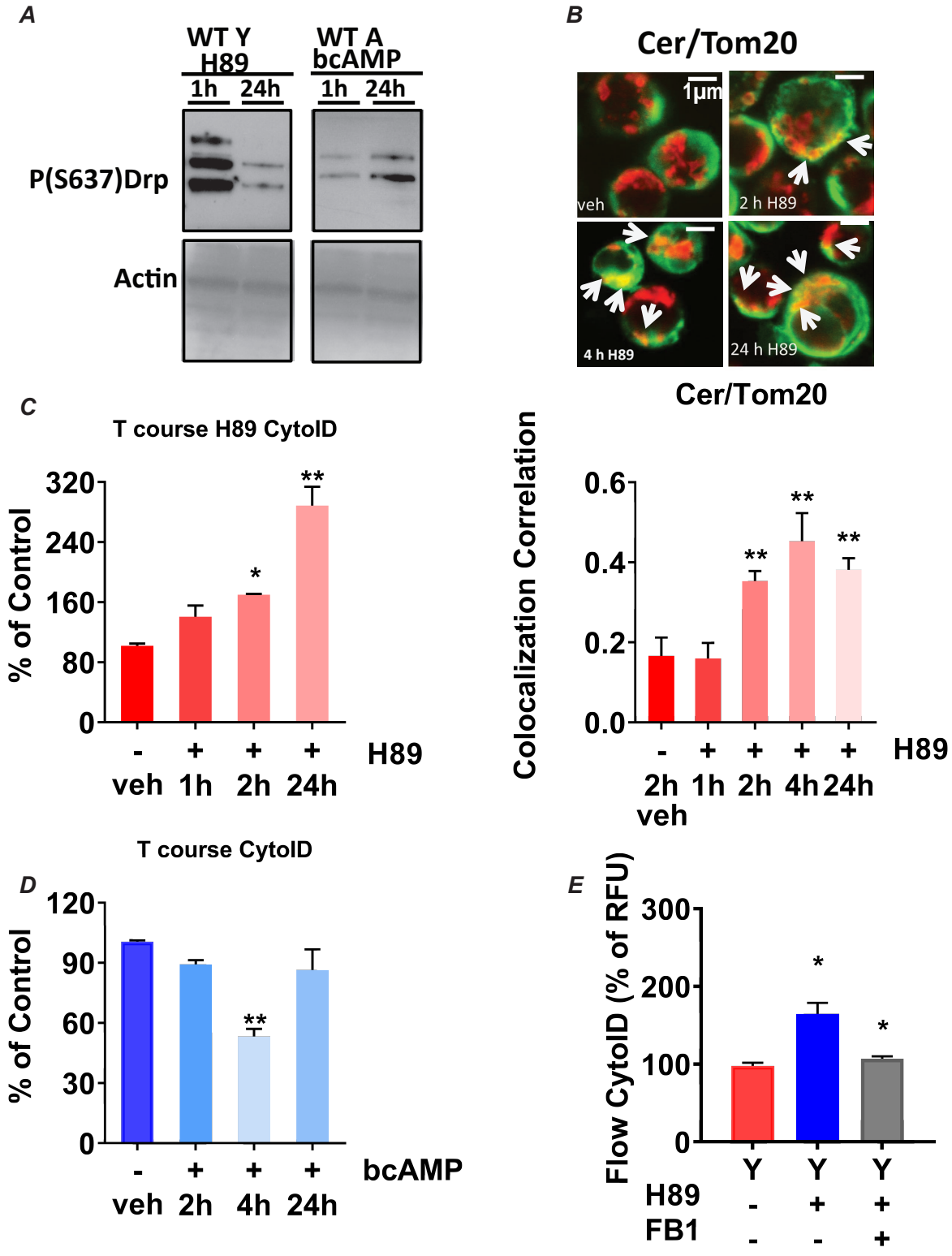

A
WT Y H89
WT A bcAMP
1h 24h 1h 24h
P(S637)Drp
Actin
B
Cer/Tom20
1µm
veh
2 h H89
4 h H89
24 h H89
C
T course H89 CytoID
% of Control
320
240
160
80
0
**
*
- + + + H89
veh 1h 2h 24h
Cer/Tom20
Colocalization Correlation
0.6
0.4
0.2
0.0
**
**
**
- + + + + H89
2h 1h 2h 4h 24h
veh
D
T course CytoID
% of Control
120
90
60
30
0
**
- + + + bcAMP
veh 2h 4h 24h
E
Flow CytoID (% of RFU)
300
200
100
0
*
*
Y Y Y
H89 - + +
FB1 - - +

**Figure S4 (Related to Figures 4 and 5). Modulation of PKA activity affects mitophagy in Y and A T cells. A.** Western blot to detect P-(S637)-Drp1 in activated T cells isolated from Y with/without H89 (PKA inhibitor) and A with/without PKA activator (bcAMP). Blots represent at least three independent experiments. **B.** Co-localization of ceramide (Cer) and TOM20 was measured in activated T cells isolated from Y mice in the absence/presence of H89 at 1, 2, 4, and 24 h. The lower panel shows the quantification of co-localization extracted from the coefficient of colocalization (*Rc*). Arrows indicate merged, yellow. Scale bars, 1 μm. Quantification of colocalization extracted from the coefficient of colocalization (*Rc*) using the Fiji software. **C-D.** LC3 activation was measured using cyto-ID by flow cytometry in activated T cells isolated from Y mice (C) in the absence/presence of H89 or A mice in the absence/presence of bcAMP at 0, 1, 2 and 24 h or 0, 2, 4 or 24 h (D). **E.** Effects of FB1 in the absence/presence of H89 on LC3 activation were measured by flow cytometry (cyto-ID) in activated T cells isolated from Y WT mice. These data represent three independent experiments (n=3). *$p< 0.05$ and **$p< 0.005$ as determined by Student's *t-test*

Figure S5

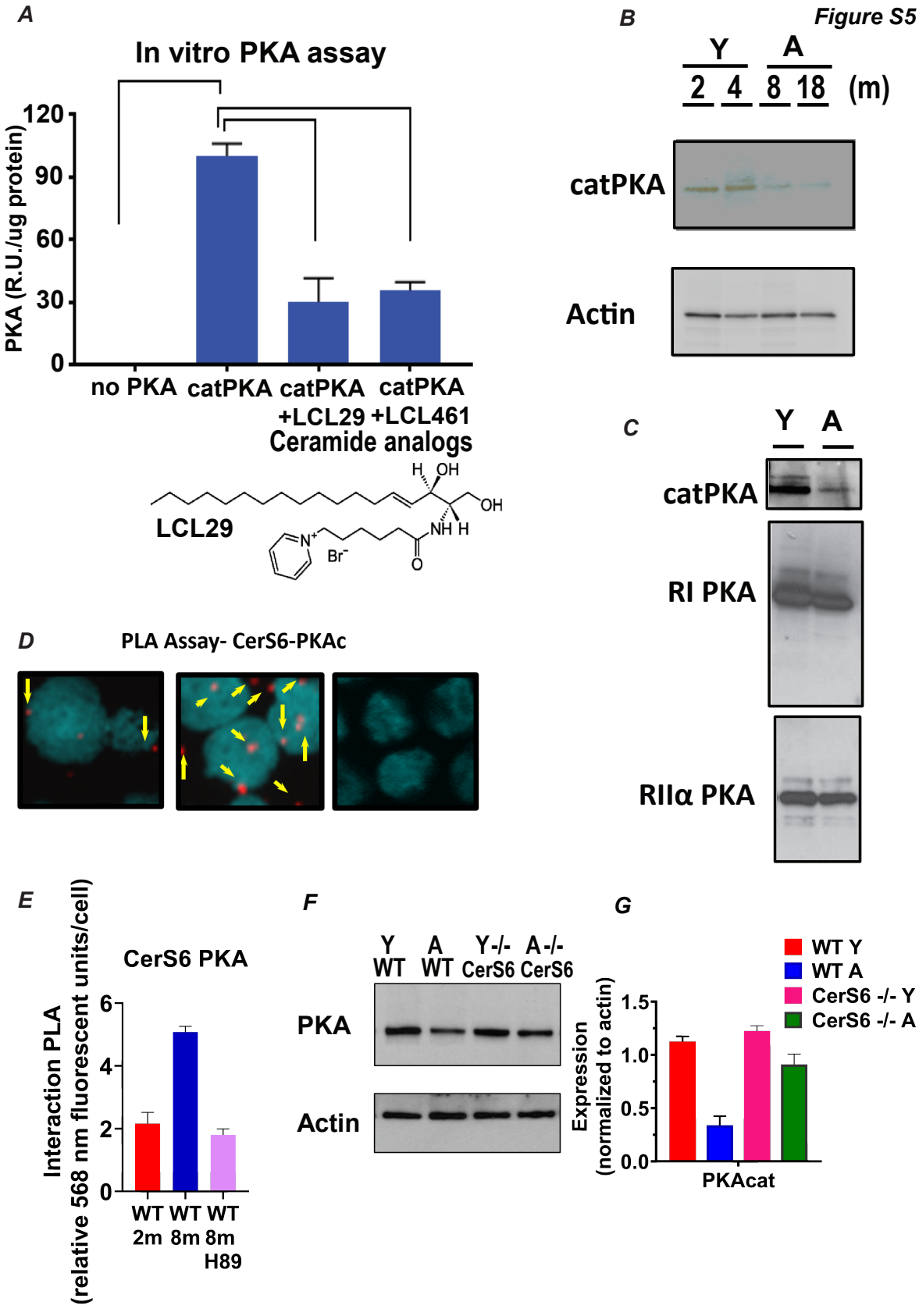

A
In vitro PKA assay
PKA (R.U./ug protein)
120
90
60
30
0
no PKA
catPKA
catPKA +LCL29
catPKA +LCL461
Ceramide analogs
LCL29
B
Y
A
2 4 8 18 (m)
catPKA
Actin
C
Y A
catPKA
RI PKA
RIIα PKA
D
PLA Assay- CerS6-PKAc
E
CerS6 PKA
Interaction PLA
(relative 568 nm fluorescent units/cell)
6
4
2
0
WT 2m
WT 8m
WT 8m H89
F
Y WT
A WT
Y-/- CerS6
A-/- CerS6
PKA
Actin
G
WT Y
WT A
CerS6 -/- Y
CerS6 -/- A
Expression (normalized to actin)
1.5
1.0
0.5
0.0
PKAcat

**Figure S5 (Related to Figures 5 and 6). Ceramide inhibits PKA in vitro and in T cells. A.** Effects of ceramide analogs, LCL-29 and LCL461, on PKA catalytic activity was measured in vitro using purified recombinant PKAc. **B.** Western blot measuring the abundance of PKAc protein in activated T cells from Y (2- 4 months old) and A (8-18-months old) mice. Actin was used as a loading control. **C.** Western blot measuring the levels of PKA regulatory I and II subunits (RI and RIIalpha PKA), along with catalytic PKA (catPKA) in activated T cells obtained from Y and A mice. **D-E.** Co-localization and association of CerS6 and PKAc in activated T cells isolated from Y and A mice in the absence/presence of H89 were measured by proximity ligation assay (PLA). Red signal shows close proximity of two proteins (<40 nm) in T cells (D). The quantification of PLA signals was performed as described by the manufacturer. These data represent three independent experiments (n=3). **F-G.** Western blot measuring the abundance of PKAc protein in activated T cells from Y (2- 4 months old) and A (8-18-months old) WT and CerS6$^{-/-}$ mice. Actin was used as a loading control (F). Quantification of the expression levels of PKAc normalized to actin is shown (G). These data represent three independent experiments (n=3). *$p$< 0.05 as determined by Student's *t-test*.

*Figure S6*

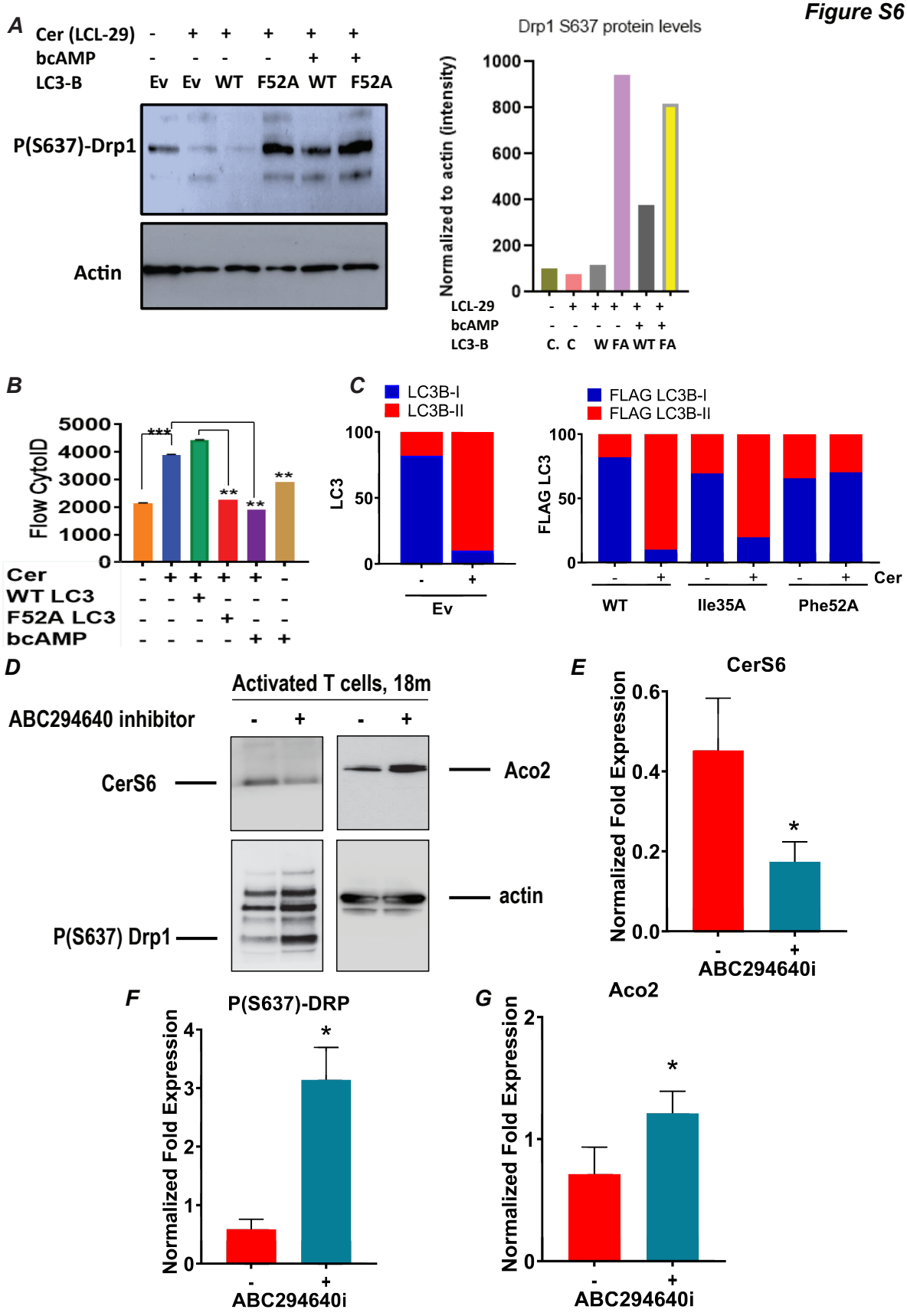

A
Cer (LCL-29) - + + + + +
bcAMP - - - - + +
LC3-B Ev Ev WT F52A WT F52A
P(S637)-Drp1
Actin
Drp1 S637 protein levels
Normalized to actin (intensity)
1000
800
600
400
200
0
LCL-29 - + + + + +
bcAMP - - - - + +
LC3-B C. C W FA WT FA
B
Flow CytoID
5000
4000
3000
2000
1000
0
***
**
**
**
Cer - + + + + -
WT LC3 - - + - - -
F52A LC3 - - - + - -
bcAMP - - - - + +
C
LC3B-I
LC3B-II
LC3
100
50
0
- +
Ev
FLAG LC3B-I
FLAG LC3B-II
FLAG LC3
100
50
0
- + - + - + Cer
WT
Ile35A
Phe52A
D
Activated T cells, 18m
ABC294640 inhibitor - + - +
CerS6
Aco2
P(S637) Drp1
actin
E
CerS6
Normalized Fold Expression
0.6
0.4
0.2
0.0
*
- +
ABC294640i
F
P(S637)-DRP
Normalized Fold Expression
4
3
2
1
0
*
- +
ABC294640i
G
Aco2
Normalized Fold Expression
2
1
0
*
- +
ABC294640i

**Figure S6 (Related to Figure 6). LC3B-ceramide binding is required for ceramide-dependent T cell mitophagy. A.** Levels of P-(S637)-Drp1 in activated T cells isolated from WT Y mice were measured by Western blotting. Activated T cells were electroporated with WT-LC3B and LC3B-F52A mutant, or empty vector (EV). Effects of LCL-29 in the absence/presence of bcAMP on P-(S637)-Drp1 abundance were then measured by Western blotting. Actin was used as a loading control. Quantification of the expression levels of P-Drp1 on Western blots normalized to actin was shown on the right panel. **B-C.** Effects of ceramide analog LCL-29 (Cer) on LC3 activation were measured by flow cytometry (B) or Western blotting (C) in activated T cells transfected with EV, FLAG-LC3B-WT, -I35A, and -F52A in the absence/presence of bcAMP. Quantifications correspond to three independent experiments (n=3). **D-G.** Effects of SphK2 inhibitor ABC294640 on mitophagy were detected by Western blotting (D) in A T cells isolated from 18 months old WT mice to detect the levels of CerS6 (E), P-Drp1-S637 (F), and ACO2 (G) compared to age-matched vehicle treated controls, and actin was used as a loading control (D, lower panel). Quantifications correspond to three independent experiments (n=3).

*Figure S7*

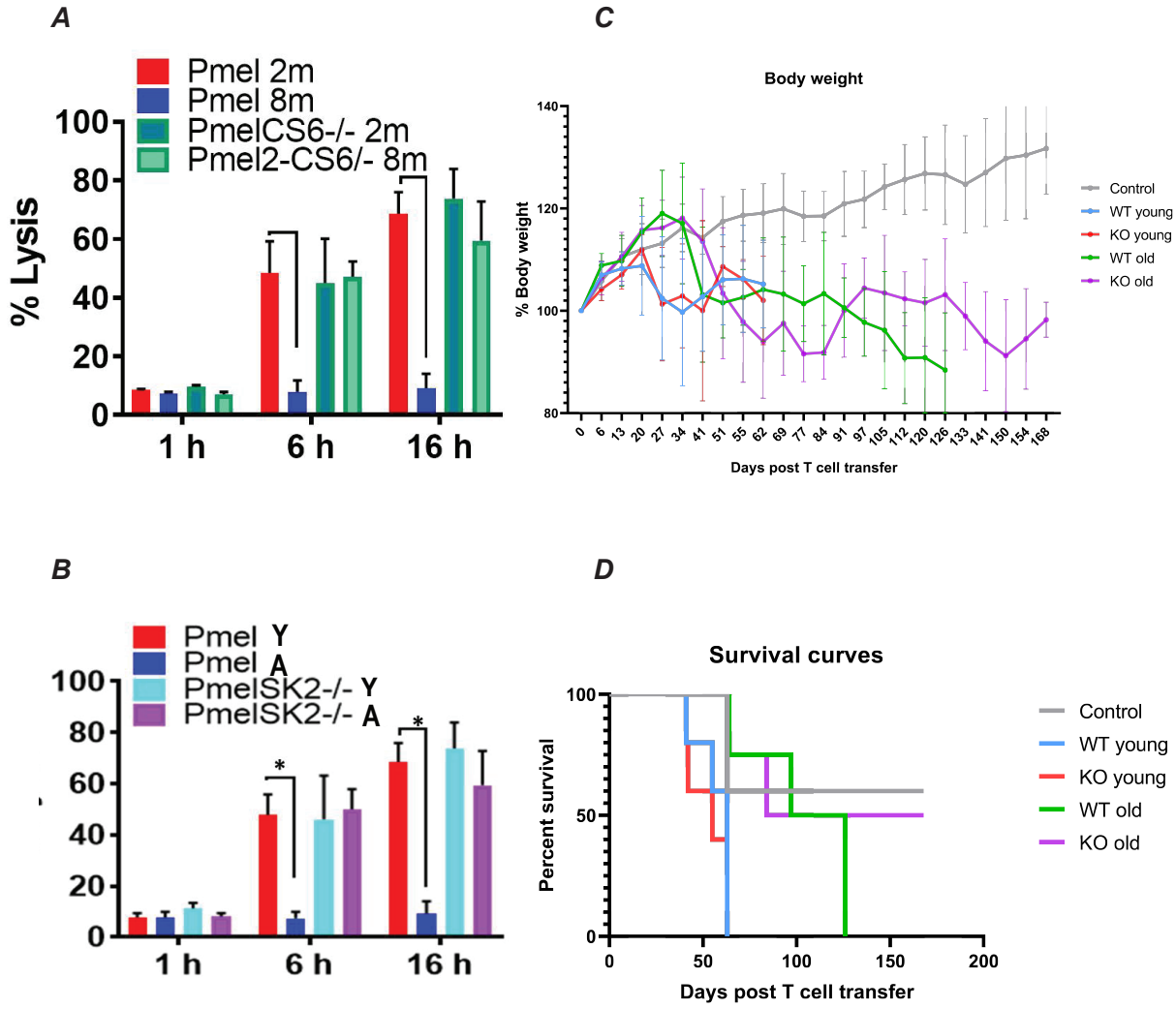
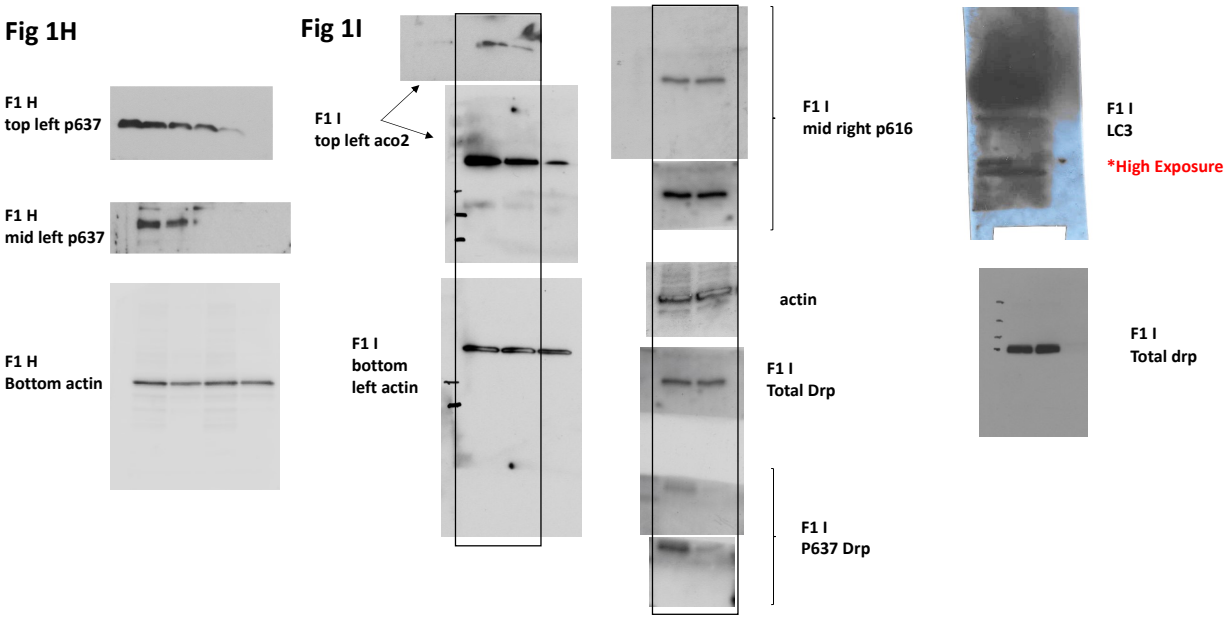

A
Pmel 2m
Pmel 8m
PmelCS6-/- 2m
Pmel2-CS6/- 8m
% Lysis
100
80
60
40
20
0
1 h
6 h
16 h
C
Body weight
% Body weight
140
120
100
80
0 6 13 20 27 34 41 51 55 62 69 77 84 91 97 105 112 120 126 133 141 150 154 168
Days post T cell transfer
Control
WT young
KO young
WT old
KO old
B
Pmel Y
Pmel A
PmelSK2-/- Y
PmelSK2-/- A
100
80
60
40
20
0
*
*
1 h
6 h
16 h
D
Survival curves
Percent survival
100
50
0
0
50
100
150
200
Days post T cell transfer
Control
WT young
KO young
WT old
KO old

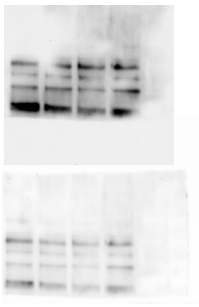
**Figure S7 (Related to Figure 7). Genetic loss of CerS6 or SphK2 improves anti-cancer functions of activated T cells in situ. A-B.** Effects of T cells isolated from young (Y) and aging (A) WT or CerS6$^{-/-}$ (CS6) (panel A) or SphK2-/- (SK2) (panel B) on B16 melanoma cell killing were measured in co-culture studies in situ at 1, 6 and 16 h. These data represent three independent experiments (n=3). *$p$< 0.05. **C-D.** Effects of CD4+ naïve T cells isolated from WT and CerS6$^{-/-}$ (KO) A and Y mice on colitis induction was evaluated by body weight (C) and overall survival (D) measurements of Rag$^{-/-}$ recipient mice.

**Data S1: Original blot images, related to Figures 1,2,4, and 6.**

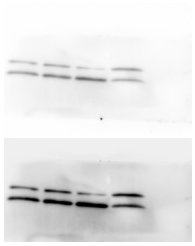


## Fig 2F

F2 F
Top P637drp

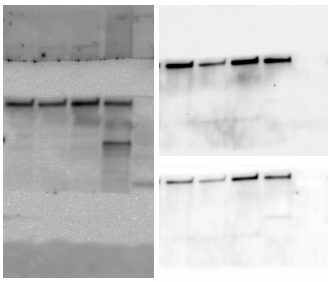


F2 F
Bottom LC3

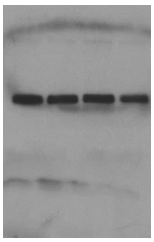


## Fig 2G

F2 G
top ACO2

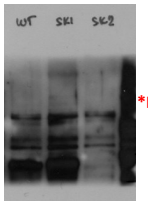


F2 G
Actin



*High Exposure

## Fig 4A

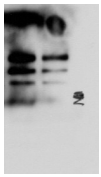



F4 A
top ACO2

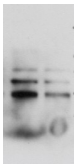
*High Exposure

## Fig 4C

F4 C
top left P637drp



F4 C
top right P637drp

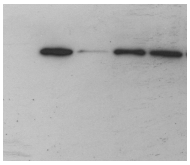


F4 C
Bottom left ACO2

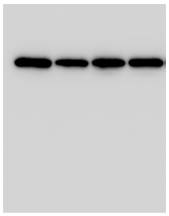
F4 C
Bottom right ACO2

*Original membrane was cut before imaging

## Fig 4E



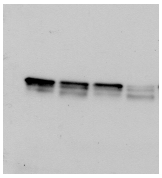
F4 E
top ACO2



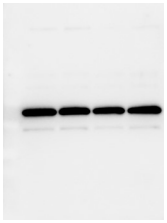
F4 E
Bottom actin

*High Exposure

**Fig 6B**



F6 B catPka top



F6 B actin bottom