Cancer Cell, Volume *14*

Supplemental Data

Article

Akt Determines Replicative Senescence and Oxidative or Oncogenic Premature Senescence and Sensitizes Cells to Oxidative Apoptosis

Veronique Nogueira, Youngkyu Park, Chia-Chen Chen, Pei-Zhang Xu, Mei-Ling Chen, Ivana Tonic, Terry Unterman, and Nissim Hay

Supplemental Experimental Procedures

Retroviruses and Lentiviruses Infection and Generation and Knockdown Procedures

Retroviruses were generated following transient transfection of retroviral vectors in Phoenix packaging cell lines as previously described (Skeen et al., 2006). Retrovirus infection was carried out for 16 to 24 h. Antibiotic selection was carried out 48 h later until complete loss of uninfected cells, before the different treatments were performed. The following retroviral vectors were used: pBabePuro-GSE56, expressing DN-p53 (Skeen et al., 2006); pLPCX-GFP and pLPCXGFP-DN-FoxO, expressing the amino acids 1-280 of FOXO1 (with Thr24 and Ser256 replaced with alanines) fused in frame with eGFP (Zhao et al., 2004); pBabe-Puro-FoxO(AAA) -ER, expressing FoxO1, with a triple mutation (T24A/S256A/S319A) on the sites phosphorylated by Akt, which is fused in frame with ligand binding domain of estrogen receptor (ER) (Cichy et al., 1998); pBabe-hygro and pBabe-hygro H-Ras^{val12} were previously described (Serrano et al., 1997); pBabeGFP-mAkt and pBabe-Puro-mAkt-ER were previously described (Gottlob et al., 2001; Hahn-Windgassen et al., 2005), pBabe-Puro-*Pten*-HA, and pBabe-Puro-*Pten*G129R-HA (Furnari et al., 1997). Block-it Lentiviral expression system (Invitrogen) was used to establish TOV21G cell lines harboring small hairpin RNAs (shRNAs) to downregulate the expression of Akt1 and/or Akt2. Double-stranded oligonucleotides for the construction of *Akt1*- and *Akt*- shRNAs were subcloned into the entry vector (pEnter/U6). The hairpin sequences used for *Akt1* and *Akt2* were as described (Irie et al., 2005). *Akt1*-shRNA and *Akt2*-shRNA were expressed by lentiviral vectors p-Lenti4/Block-it and p-Lenti6/Block-it, respectively. Stable populations of cells expressing *Akt1*-shRNA or *Akt2*-shRNA or both were generated following lentivirus infection and selection using 1.0 mg/ml of zeocin or 10 μg/ml of blasticidin, respectively, or both. Lipofectamine 2000 (Invitrogen) was used to transfect small interference RNAs (siRNAs) to downregulate the expression of *sestrin-3* (*Sesn3*) *FoxO3a*, *FoxO1* or *catalase*. All siRNAs were obtained from Dharmacon.

PEITC/Rapamycin-Induced Apoptosis in Rat1a-mAktGFP Cells

Rat1a fibroblasts (5×10^5) were plated in 10-cm dishes for infection with pBabe-mAkt-GFP retrovirus to achieve about 50% infection. A mixed population of cells was obtained with 53.48% ± 1.33% of Rat1a cells expressing mAkt-GFP. Cells were allowed to grow for 72 h before plating for experiments. The mixed population of Rat1a fibroblasts was plated (7.5×10^5 cells per 10-cm culture dish) and treated for 3 h with 100 nM rapamycin prior to the addition of 6 μM PEITC for 6 h. At the end of the treatment, floating cells were collected and combined with trypsinized, adherent cells. Cells were washed and fixed in iced-cold 75% ethanol for 10 to 24 h. Following fixation, cells were stained with propidium iodide (50 mg/ml) in PBS/RNase/Triton x-100 for 20 min before FACS analysis. Due to DNA fragmentation occurring during apoptosis, dead cells appeared as a sub-G1 population peak. Using the doublet discrimination module of the FACS, the population of dead cells was quantified within the entire population of cells as well as within the GFP-positive and -negative populations. The percentage of cell death was then calculated within each population.

Measurement of ROS

Cells (5×10^4) were plated into 30 mm plates and treated under various conditions described in the figure legends. Twenty minutes prior to the end of the treatment, a dye, dichlorofluorescein diacetate (10 μM, H_2DCFDA) was added to the media. The dye is cleaved to generate 2-7-dichlorodihydrofluorescein ($DCFH_2$) by esterase in cells. Intracellular ROS causes oxidation of $DCFH_2$ to yield the fluorescent product 2', 7'-dichlorofluorescein (DCF), which is proportional to the level of ROS. Cells were quickly washed with PBS, and photos of representative fluorescent fields were taken under an inverted microscope. In parallel samples, DCF fluorescence was measured in the supernatant of cell lysates after lysing the cells in lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreitol; 10% monothioglycerol; 1% Triton X-100), using a spectrofluorimeter (excitation, 485 nm; emission, 535 nm). To normalize the data, cells were also incubated with 1 μM MitoTrackerRed (Molecular Probes; excitation, 585 nm; emission, 610 nm) to estimate the number of mitochondria. Data were normalized to the mitochondria content, and fluorescence intensity was expressed as arbitrary units. For cells overexpressing GFP proteins, nonfluorescent dihydrorhodamine123 (Molecular Probes) was used instead of H_2DCFDA to measure the fluorescent emission at the different wavelength. Following the same protocol, this dye can be oxidized by ROS to generate the fluorescent chromophore rhodamine123 that is detected by spectrofluorimetry (excitation, 570 nm; emission, 600 nm). Data were normalized to the amount of protein, and fluorescence intensity was expressed as arbitrary units per mg protein. Alternatively, DCF fluorescence was measured by FACS analysis. Briefly, after incubation with H_2DCFDA, cells were rinsed twice with cold PBS, then trypsinized and subjected to FACScan flow cytometer. DCF fluorescence intensity was plotted against the number of cells.

Real-Time PCR and Primers

Total RNA were isolated at according time points and converted to cDNA using Invitrogen SuperScript III reverse transcriptase following standard protocol. Relative quantification of real-time PCR products was performed using Bio-RAD iQ SYBR green super-mix and related system. Samples were assayed in triplicate and data were normalized to actin mRNA levels. Primer sequences: for *Sesn1* (5' GGA CGA GGA ACT TGG AAT CA 3'and 5' ATG CAT CTG TGC GTC TTC AC 3'), for *Sesn2* (5' TAG CCT GCA GCC TCA CCT AT 3' and 5' TAT CTG ATG

CCA AAG ACG CA 3'), for *Sesn3* (5' CAT GCG TTT CCT CAC TCA GA 3' and 5' GGC AAA GTC TTC GTA CCC AA 3').

Xenograft Studies

Before administration, all the drugs were dissolved in solvent containing ethanol, cremophor-EL (sigma), and PBS (1:1:8 volume ratio). Control mice were injected with an equal volume of solvent as a control. Body weights and tumor size of the mice were measured and recorded once every week. Tumor size was calculated as follows: volume=(width^{2}xlength)/2. When tumor size reached end point criteria (for example, diameter of tumor mass >1.5cm), mice were euthanized. Tumor tissues from representative mice from each group were sectioned, embedded in paraffin, and stained.

Cells

Primary human foreskin fibroblasts (BJ cell line) were purchased from ATCC and were used for senescence studies. Cells were maintained in exponential phase of growth in MEM culture medium (with Earle's salts, L-Glutamine and Nonessential amino-acids) supplemented with 1mM sodium pyruvate, 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. BJ cells were infected with the same lentiviruses used for TOV21G cells to knockdown the expression of Akt1 and/or Akt2 as described above.

Annexin-V/Propidium Iodide Double Staining and FACS Analysis

To evaluate apoptosis, cells were double stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA). Briefly, cells (1x10^6) were washed twice with cold PBS, and stained with 5 μl of annexin V-FITC and 10 μl of PI (5 μg/ml) in 1mL binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. The apoptotic cells were determined using a FACScan cytofluorometer. Annexin V-positive/PI-negative, annexin V-positive/PI-positive, and annexin V-negative/PI-positive cells represent cells in early apoptosis, late apoptosis, and necrosis, respectively.

Measurement of Intracellular ROS by FACS Analysis

For measurement of intracellular ROS, cells were incubated with H_2DCF-DA (10 μM) for 30 min, washed twice with cold PBS and analyzed for intracellular ROS production within 1 h using a FACScan flow cytometer. DCF fluorescence distribution of cells was detected by FACS analysis.

Modulation of Oxygen Production and ROS Production by Galactose

MEFs (3x10^5 cells/plate) were plated in 150 mm plate in DMEM/25 mM D-glucose. The next day, the medium was replaced with either DMEM/25 mM D-galactose or DMEM/25 mM D-glucose. Forty-eight hours later, ROS production and oxygen consumption rates were measured as described above.

Transient Transfection and Luciferase Assays

MEFs (1 X 10^5 cells/well) were plated in 12-well plates in DMEM with 10 % FBS. The next day, cells were transfected with DNAs (1 μg of IRS3-tk-luciferase, 0.05 μg of pcDNA3-FOXO1-WT), 0.01 μg of pRL-CMV as an internal control (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Twenty four hours after transfection, luciferase

activities were measured by the dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to the constitutive *Renilla* luciferase activity. Results are expressed relative to the level of activity in WT-MEF. Data are presented as the mean ± SD of triplicate samples. All transfection experiments were repeated 5 times with similar results. Results were analyzed using Student's t-test.

siRNA Transfection

MEFs (8x 10^4 cells/well) were plated in 6-well plates in DMEM with 10 % FBS. The next day, cells were transfected with 50 nM control-siRNA, *FoxO3a*-siRNA or *Sestrin3*-siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Seventy two hours after transfection, cells were treated for 2h with increasing concentrations of H_2O_2 in DMEM to induce apoptosis. Apoptosis was quantified by DAPI staining as described in Experimental Procedures. The knockdown efficiency was analyzed by either immunoblotting or real-time PCR. All siRNA smartpools (targeting *FoxO3a*, *FoxO1, sestrin3, catalase*) and a control nontargeting siRNA were purchased from Dharmacon.

Adenovirus Infection

MEFs (8x 10^4 cells/well) were plated in 6-well plates in DMEM with 10 % FBS. The next day, cells were infected with adenovirus carrying β-galactosidase (LacZ as a control) or catalase (Ad5CMVCatalase, Gene Transfer Vector Core, U of Iowa) at a multiplicity of infection (MOI) of 100 for 4.5 h. Adenovirus was subsequently washed off with PBS and DMEM with 10% FBS was added to the cells. Seventy-two hours post-infection, cells were treated for 2h with increasing concentrations of H_2O_2 in DMEM to induce apoptosis. Apoptosis was quantified by DAPI staining as described in Experimental Procedures. Adenovirus-mediated expression of catalase was confirmed by immunoblotting.

Supplemental References

Cichy, S. B., Uddin, S., Danilkovich, A., Guo, S., Klippel, A., and Unterman, T. G. (1998). Protein kinase B/Akt mediates effects of insulin on hepatic insulin- like growth factor-binding protein-1 gene expression through a conserved insulin response sequence. J Biol Chem *273*, 6482-6487.

Furnari, F. B., Lin, H., Huang, H. S., and Cavenee, W. K. (1997). Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. Proc Natl Acad Sci U S A *94*, 12479-12484.

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., and Hay, N. (2001). Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev *15*, 1406-1418.

Irie, H. Y., Pearline, R. V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J. S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J Cell Biol *171*, 1023-1034.

Skeen, J. E., Bhaskar, P. T., Chen, C. C., Chen, W. S., Peng, X. D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H., and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell *10*, 269-280.

Zhao, X., Gan, L., Pan, H., Kan, D., Majeski, M., Adam, S. A., and Unterman, T. G. (2004). Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. Biochem J *378*, 839-849.

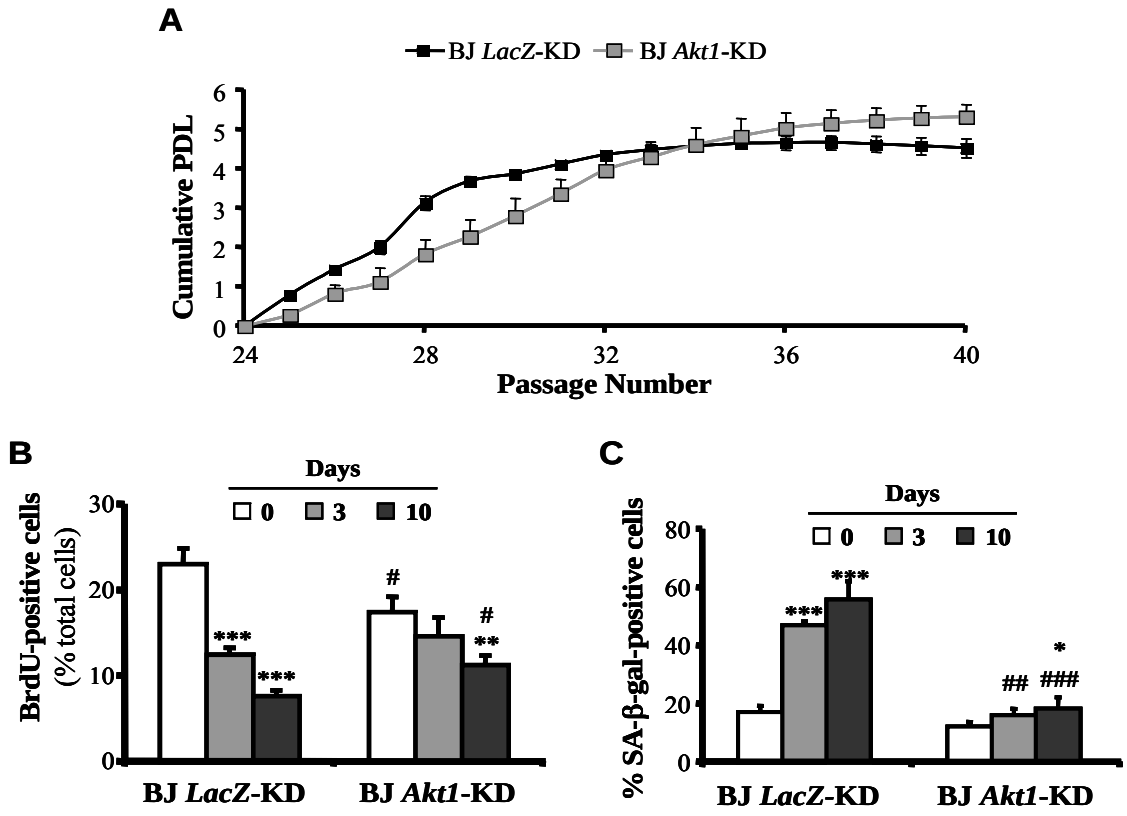

Figure S1. Akt Regulates Replicative Senescence and Premature Senescence in Human Diploid Fibroblasts (HDF)

(A) Cells were subjected to 3T3 protocol as described in Experimental Procedures. BJ cells (HDFs) ($2X10^5$) were split every 3 days at each passage and replated at the same density. The cumulative population doubling levels (PDLs) were calculated after passage 24. The cumulative PDLs of control (BJ *LacZ*-shRNA), *Aktl*-KD (BJ *Akt1*-shRNA) primary HDFs were then plotted. (B and C) Premature senescence of *Akt1*-KD, and *LacZ*-KD in HDFs was induced with consecutive treatment of 75μM H_2O_2 as described in Experimental Procedures. At days 0, 3 and 10 post-treatment, cells were analyzed for BrdU incorporation (B) and SA-β-gal activity (C). *, **, ***$p < 0.05, 0.01, 0.001$ vs. Day 0. #, ##, ###$p < 0.05, 0.01, 0.001$ vs. corresponding day values in BJ *LacZ*-KD group.

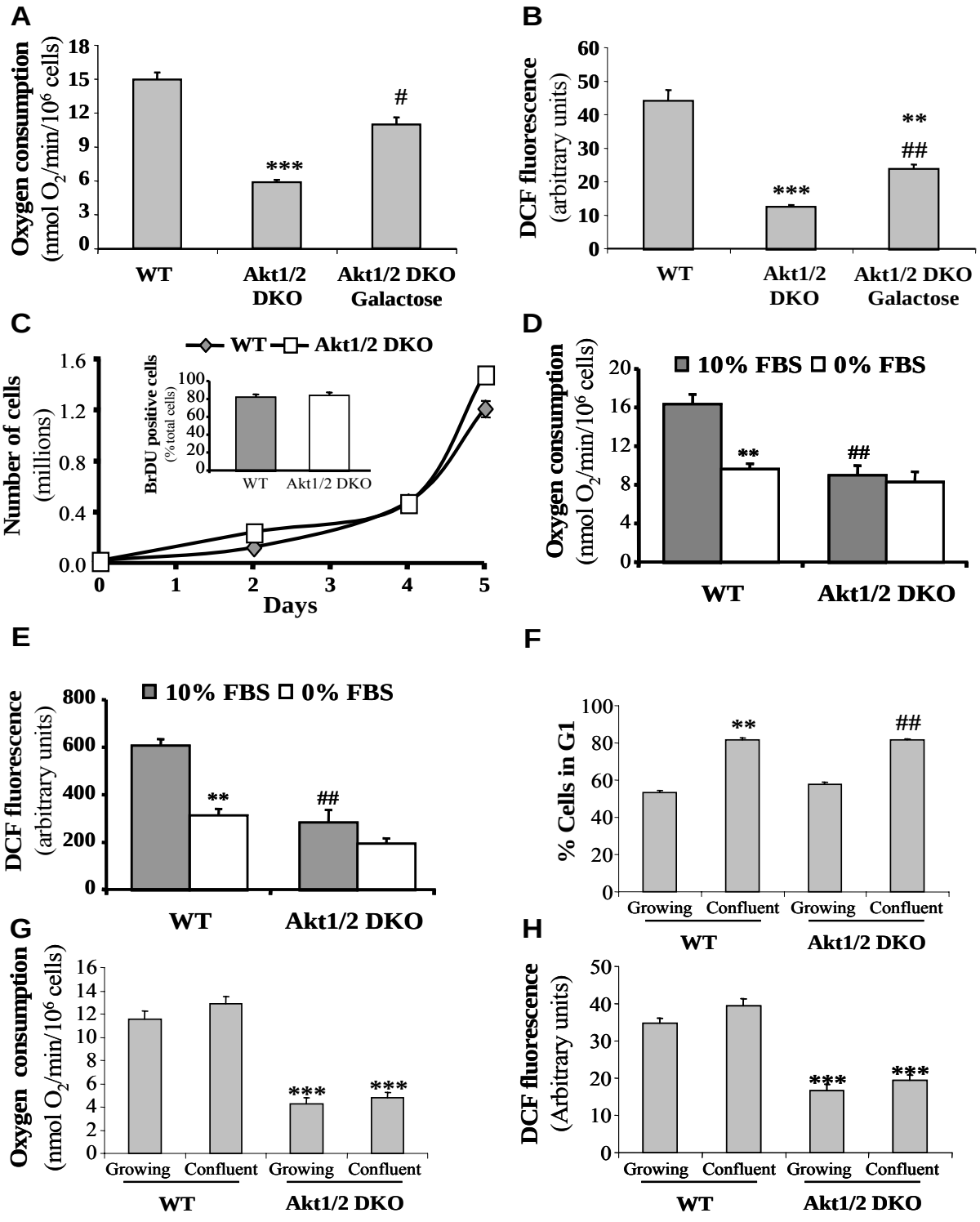
A
Oxygen consumption (nmol O2/min/10^6 cells)
18
15
12
9
6
3
0
***
#
WT
Akt1/2 DKO
Akt1/2 DKO Galactose
B
DCF fluorescence (arbitrary units)
60
50
40
30
20
10
0
***
**
##
WT
Akt1/2 DKO
Akt1/2 DKO Galactose
C
WT
Akt1/2 DKO
Number of cells (millions)
1.6
1.2
0.8
0.4
0.0
0
1
2
3
4
5
Days
BrDU positive cells (% total cells)
100
80
60
40
20
0
WT
Akt1/2 DKO
D
10% FBS
0% FBS
Oxygen consumption (nmol O2/min/10^6 cells)
20
16
12
8
4
0
**
##
WT
Akt1/2 DKO
E
10% FBS
0% FBS
DCF fluorescence (arbitrary units)
800
600
400
200
0
**
##
WT
Akt1/2 DKO
F
% Cells in G1
100
80
60
40
20
0
**
##
Growing
Confluent
Growing
Confluent
WT
Akt1/2 DKO
G
Oxygen consumption (nmol O2/min/10^6 cells)
16
14
12
10
8
6
4
2
0
***
***
Growing
Confluent
Growing
Confluent
WT
Akt1/2 DKO
H
DCF fluorescence (Arbitrary units)
50
40
30
20
10
0
***
***
Growing
Confluent
Growing
Confluent
WT
Akt1/2 DKO

Figure S2.

Figure S2. Akt Increases ROS Generation through Increasing Oxygen Consumption Independently of Cell Proliferation

(A and B) Galactose stimulates oxygen consumption and ROS production in Akt1/2 DKO cells**.** Akt1/2 DKO cells were incubated in media supplemented with galactose instead of glucose and grown for 48 h. Oxygen consumption (A) and ROS production (B) were measured as described in Experimental Procedures. Values in Akt1/2 DKO cells cultured with galactose were compared with those in WT and Akt1/2 DKO cells incubated in DMEM media with glucose. **, ***$p < 0.01, 0.001$ as compared with WT cells. #, ##$p < 0.05, 0.01$ as compared with Akt1/2 DKO group.

(C) WT-MEFs or Akt1/2 DKO-MEFs were immortalized with SV40-LargeT antigen and cell proliferation rate was measured by counting number of cells in the plates for 5 consecutive days. Insert shows BrdU incorporation in SV40 large T immortalized WT and Akt1/2 DKO MEFs.

(D and E) Oxygen consumption and ROS generation in SV40 large T immortalized WT and Akt1/2 DKO MEFs. Cells were grown in the presence or absence of serum for 24 h. Oxygen consumption (D) and ROS production (E) were then measured. ##$p < 0.01$ as compared with WT cells; **$p < 0.01$ as compared with cells cultured in medium with 10% FBS.

(F-H) Oxygen consumption and ROS generation in proliferating or arrested WT and Akt1/2 DKO MEFs. MEFs were maintained in exponential growth phase or confluence. MEFs in exponential or confluent growth phase were subjected to PI staining and flow cytometry showing the fraction of cells in G1 phase of cell cycle (F). Oxygen consumption (G) and ROS measurement estimated by DCF fluorescence (H). Data obtained from cells in the exponential and confluence growth phase are labeled as “growing” and “”confluent” respectively in the graph. **, ***$p < 0.01, 0.001$ as compared with WT cells (Growing). ##$p < 0.01$ as compared with Akt1/2 DKO cells (Growing).

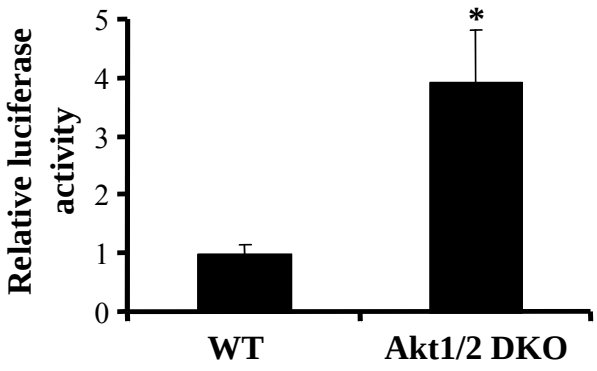

Figure S3. Transcriptional Activity of FoxO1 Is Increased in Akt1/2 DKO-MEFs

WT or Akt1/2 DKO MEFs were transfected with 3X-irs-tk-luciferase, FoxO1 expression vector, and renilla luciferase. Relative luciferase activities were measured as described in Experimental Procedures. Data are presented as the mean ± SD of triplicate samples and are representative of five independent experiments. Student *t* test for compared values: * indicates significant difference with $p \leq 0.005$.

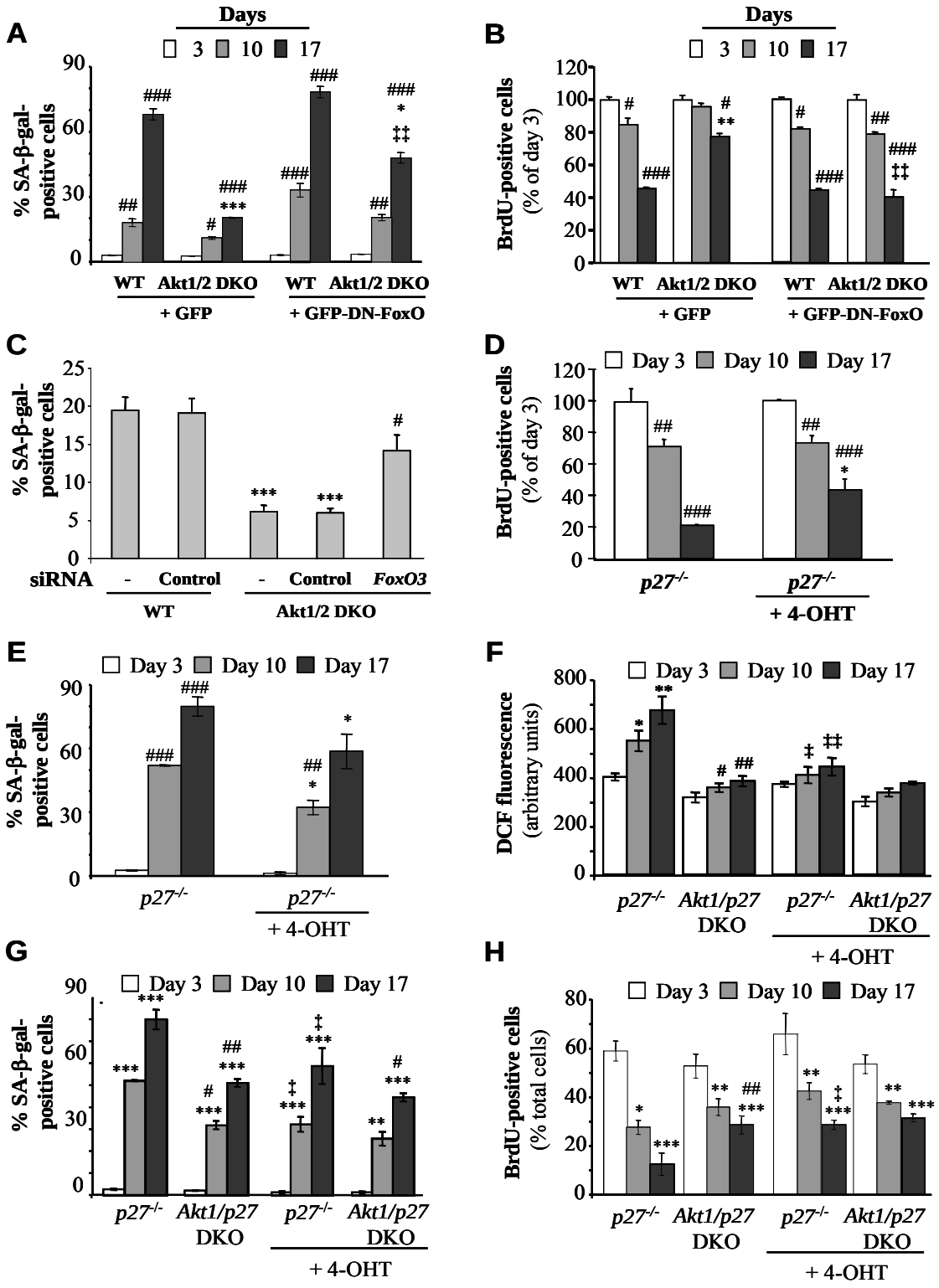
A
Days
3 10 17
% SA-β-gal-positive cells
WT Akt1/2 DKO WT Akt1/2 DKO
+ GFP
+ GFP-DN-FoxO
B
Days
3 10 17
BrdU-positive cells (% of day 3)
WT Akt1/2 DKO WT Akt1/2 DKO
+ GFP
+ GFP-DN-FoxO
C
% SA-β-gal-positive cells
siRNA - Control - Control FoxO3
WT
Akt1/2 DKO
D
Day 3 Day 10 Day 17
BrdU-positive cells (% of day 3)
p27-/- p27-/-
+ 4-OHT
E
Day 3 Day 10 Day 17
% SA-β-gal-positive cells
p27-/- p27-/-
+ 4-OHT
F
Day 3 Day 10 Day 17
DCF fluorescence (arbitrary units)
p27-/- Akt1/p27 DKO p27-/- Akt1/p27 DKO
+ 4-OHT
G
Day 3 Day 10 Day 17
% SA-β-gal-positive cells
p27-/- Akt1/p27 DKO p27-/- Akt1/p27 DKO
+ 4-OHT
H
Day 3 Day 10 Day 17
BrdU-positive cells (% total cells)
p27-/- Akt1/p27 DKO p27-/- Akt1/p27 DKO
+ 4-OHT

Figure S4.

Figure S4. Akt1 Deficiency Exerts Resistance to H_2O_2-Induced Premature Senescence, which Is Mediated by FoxO

(A) DN-FoxO restores sensitivity of Akt1/2 DKO MEFs to premature senescence induced by H_2O_2, as measured by SA-β-gal activity.

(B) DN-FoxO restores sensitivity of Akt1/2 DKO MEFs to premature senescence induced by H_2O_2, as measured by BrdU incorporation. The generation of cells expressing DN-FoxO is described in Experimental Procedures. At days 3, 10 and 17, following exposure to H_2O_2, cells were analyzed for SA-ß-gal activity and BrdU incorporation. *, **, ***$p < 0.05, 0.01, 0.001$ as compared with WT-GFP; ‡‡$p < 0.01$ as compared with DKO-GFP; #, ##, ###$p < 0.05, 0.01, 0.001$ as compared with Day 3.

(C) WT and Akt1/2 DKO primary MEFs were treated with H_2O_2 as described in Experimental procedures and then transfected with control siRNA or FOXO3a siRNA. At day 7 post-transfection, cells were analyzed for SA-β-gal activity. ***$p < 0.001$ as compared with corresponding siRNA group in WT cells. #$p < 0.05$ as compared with Akt1/2 DKO-siRNA control group.

(D and E) Expression of activated FoxO inhibits premature senescence induced by H_2O_2. The generation of p27-/- MEFs cells stably expressing FoxO (AAA) ER is described in Supplemental Experimental Procedures. The activity of FoxO (AAA) ER was induced by incubating cells with 300nM 4-hydroxy-Tamoxifen (4-OHT) for 48h as described in Supplemental Experimental Procedures. Cells were then treated with 2 x 75 μM H_2O_2. At days 3, 10 and 17 following treatment cells were analyzed for SA-β-gal activity (D) and BrdU incorporation (E). *$p < 0.05$ as compared with $p27^{-/-}$; ##. ###$p < 0.01, 0.001$ as compared with Day 3.

(F-H) *Akt1-/-, p27*-/- MEFs stably expressing FoxO(AAA)ER were treated for 48 h with 300 nM 4-hydroxy-Tamoxifen (4-OHT). Cells were then treated with 75 μM H_2O_2 as described in Experimental Procedures. At days 3, 10 and 17 post-treatment, cells were analyzed for ROS production (F), SA-β-gal activity (G) and BrdU incorporation (H). *, **, ***$p < 0.05, 0.001, 0.001$ as compared with Day3 group. #, ##$p < 0.05, 0.01$ as compared with $p27^{-/-}$ group. ‡, ‡‡$p < 0.05, 0.01$ as compared with group in the absence of 4-OHT.

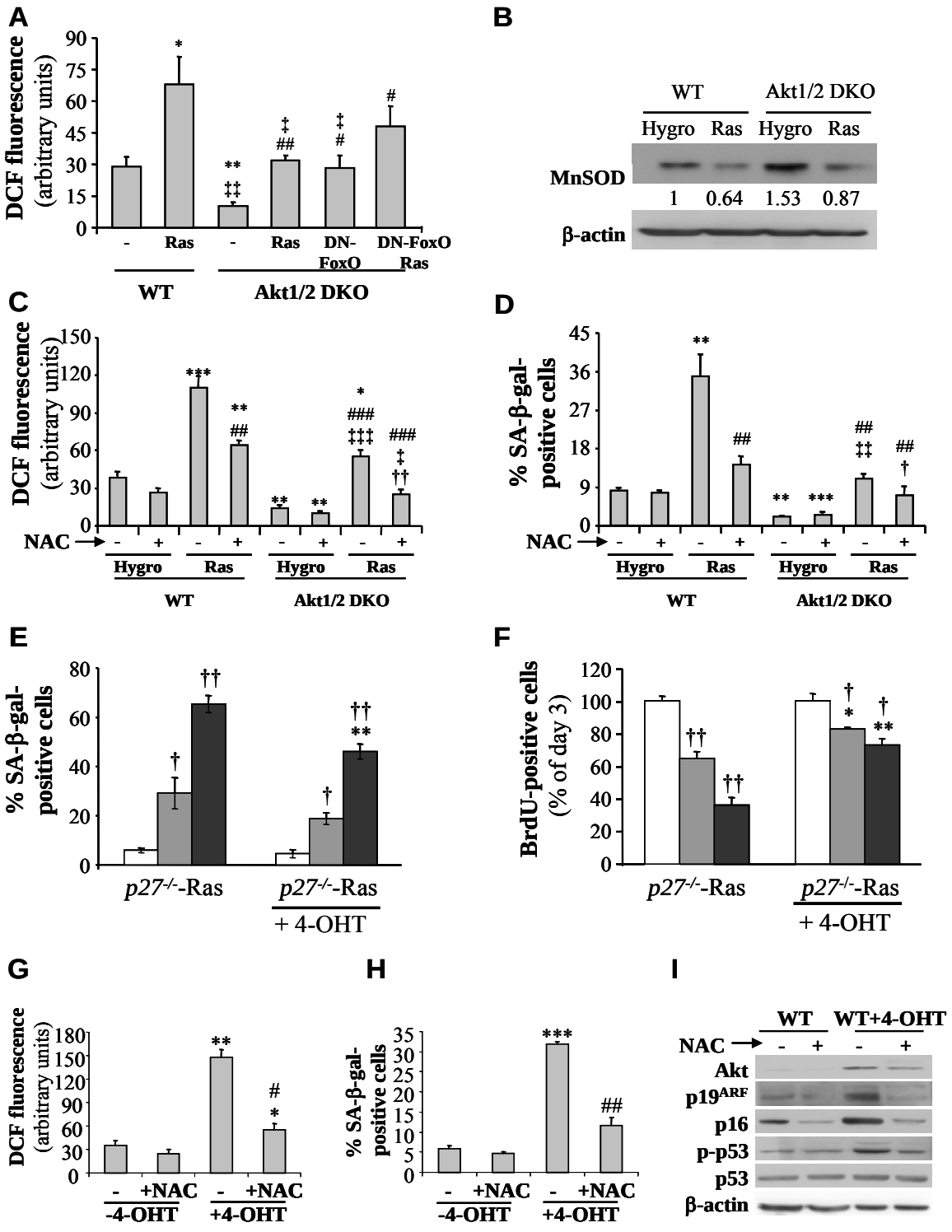
A
DCF fluorescence (arbitrary units)
90
75
60
45
30
15
0
-
Ras
-
Ras
DN-FoxO
DN-FoxO Ras
WT
Akt1/2 DKO
B
WT
Akt1/2 DKO
Hygro
Ras
Hygro
Ras
MnSOD
1
0.64
1.53
0.87
β-actin
C
DCF fluorescence (arbitrary units)
150
120
90
60
30
0
NAC
Hygro
Ras
Hygro
Ras
WT
Akt1/2 DKO
D
% SA-β-gal-positive cells
45
36
27
18
9
0
NAC
Hygro
Ras
Hygro
Ras
WT
Akt1/2 DKO
E
% SA-β-gal-positive cells
80
60
40
20
0
p27-/--Ras
p27-/--Ras
+ 4-OHT
F
BrdU-positive cells (% of day 3)
120
100
80
60
40
20
0
p27-/--Ras
p27-/--Ras
+ 4-OHT
G
DCF fluorescence (arbitrary units)
180
150
120
90
60
30
0
-
+NAC
-
+NAC
-4-OHT
+4-OHT
H
% SA-β-gal-positive cells
35
30
25
20
15
10
5
0
-
+NAC
-
+NAC
-4-OHT
+4-OHT
I
WT
WT+4-OHT
NAC
-
+
-
+
Akt
p19ARF
p16
p-p53
p53
β-actin

Figure S5.

Figure S5. ROS Mediates Ras and Akt-Induced Premature Senescence

(A) WT and Akt1/2 DKO MEFs expressing Ras, DN-FoxO or Ras/DN-FoxO were stained with H_2DCF-DA, and ROS production was analyzed by flow cytometry as described in Experimental Procedures. Results represent the intensity of DCF fluorescence in each group. *, **p < 0.05, 0.01 as compared with WT-Hygromycin group. #, ##p < 0.05, 0.01 as compared with Akt1/2 DKO-Hygromycin group. ‡, ‡‡p < 0.05, 0.01 as compared with WT-Ras group.

(B) Total cell extracts of WT or Akt1/2 DKO MEFs expressing Ras or none as a control were prepared and analyzed by immunoblotting. Blots were probed with antibodies specific for MnSOD, and β-actin as a control. The relative protein levels were quantified using the NIH ImageJ software program, and normalized to the densitometric signal for β-actin as a control protein load. Values are expressed relative to WT control cells.

(C and D) WT and Akt1/2 DKO primary MEFs were infected with retrovirus expressing Ras or none (hygromycin-resistant) as a control, and then treated with 2mM NAC as described in Experimental Procedures. At day 7 post-treatment, cells were analyzed for ROS accumulation (C) and SA- ß-gal activity (D). *, **, ***p < 0.05, 0.01, 0.001 as compared with WT group; ##, ###p < 0.01, 0.001 as compared with WT-Ras group; ‡, ‡‡, ‡‡‡p < 0.05, 0.01, 0.001 as compared with Akt1/2 DKO group; †, †† p < 0.05, 0.01 as compared with Akt1/2 DKO-Ras group.

(E and F) Expression of activated FoxO inhibits premature senescence induced by activated Ras. *p27*-/- MEFs co-expressing H-Ras^{v12} and FoxO(AAA)-ER were generated as described in Experimental Procedures. At days 3, 10 and 17 following FOXO activation by 4-OHT, cells were analyzed for SA-ß-gal activity (E) and BrdU incorporation (F). *, **p < 0.05, 0.01 as compared with *p27*$^{-/-}$-Ras; †, †† p < 0.05, 0.01 as compared with Day 3.

(G and H) WT primary MEFs were infected with retrovirus expressing mAkt-ER in pBabe-puro vector, then treated with 4-OHT for 24h to activate the mAkt and then treated with 2mM NAC as described in Experimental procedures. At day 7 post-treatment, cells were analyzed for ROS accumulation (G) and SA-β-gal activity (H) (. * **, ***p < 0.05, 0.01, 0.001 as compared with the corresponding vehicle-treated WT group; #, ##p < 0.05, 0.01 as compared with 4-OHT treated-WT group.

(I) WT primary MEFs were infected with retrovirus expressing mAkt-ER. Following infection, cells were treated with 4-OHT for 24h to activate the mAkt, and then treated with 2 mM NAC as described in Experimental Procedures. Total cell extracts were prepared and analyzed by immunoblotting. The blots were probed with the indicated antibodies.

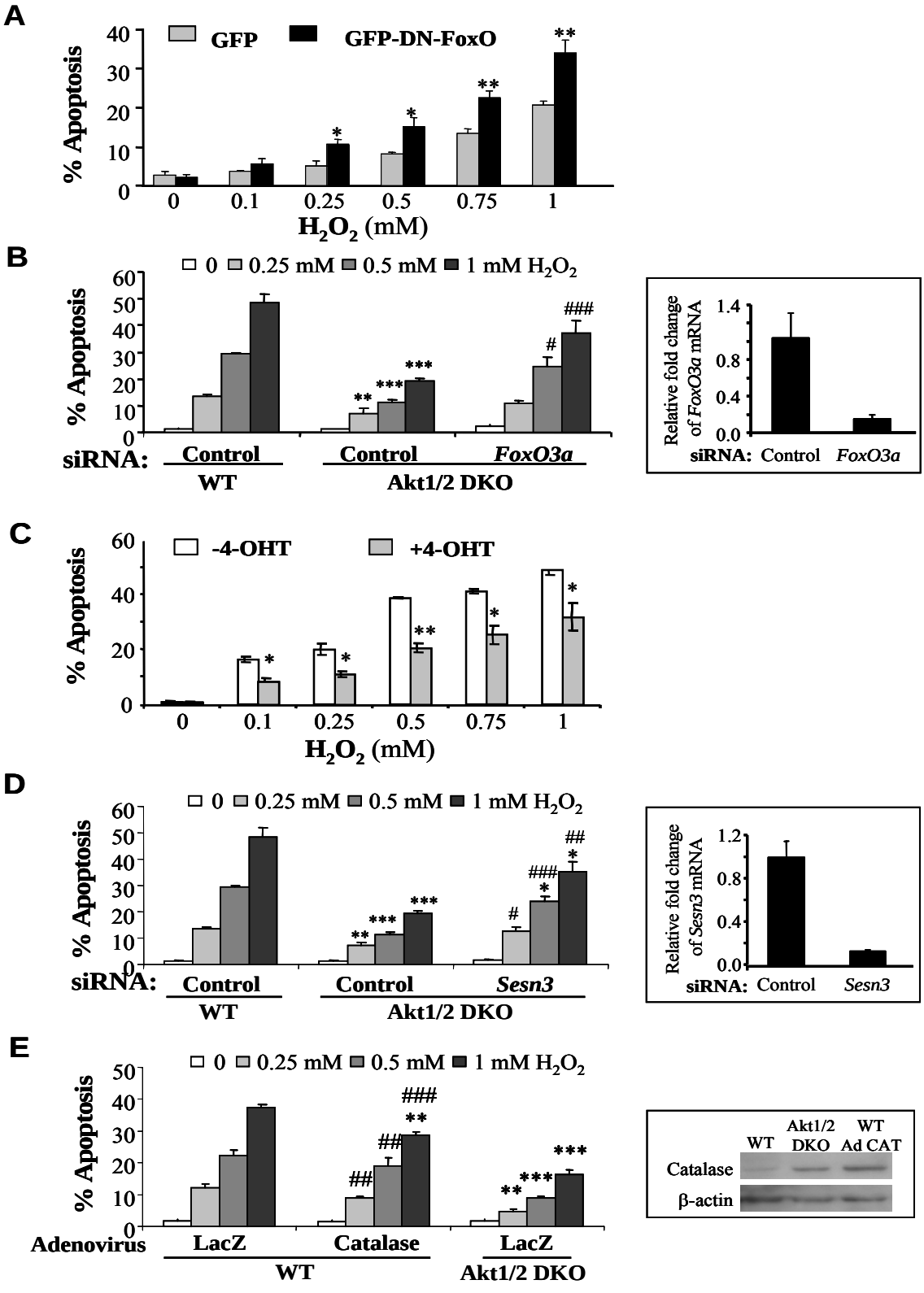
A
GFP
GFP-DN-FoxO
% Apoptosis
H_2O_2 (mM)
B
0 0.25 mM 0.5 mM 1 mM H_2O_2
% Apoptosis
siRNA: Control Control FoxO3a
WT
Akt1/2 DKO
Relative fold change of FoxO3a mRNA
siRNA: Control FoxO3a
C
-4-OHT
+4-OHT
% Apoptosis
H_2O_2 (mM)
D
0 0.25 mM 0.5 mM 1 mM H_2O_2
% Apoptosis
siRNA: Control Control Sesn3
WT
Akt1/2 DKO
Relative fold change of Sesn3 mRNA
siRNA: Control Sesn3
E
0 0.25 mM 0.5 mM 1 mM H_2O_2
% Apoptosis
Adenovirus LacZ Catalase LacZ
WT
Akt1/2 DKO
WT
Akt1/2 DKO
WT Ad CAT
Catalase
β-actin

Figure S6.

Figure S6. Role of FoxO, Sesn3, and Catalase in Akt-Dependent Sensitivity to H_2O_2-Induced Apoptosis

(A) Expression of DN-FoxO inhibits resistance to oxidative stress caused by Akt-deficiency. Apoptosis of DNp53-immortalized and Akt1/2 DKO MEFs overexpressing GFP or DN-FoxO-GFP treated with increasing concentrations of H_2O_2 for 2 h. *, **p < 0.05, 0.01 as compared with GFP.

(B) The knockdown of *FoxO3a* inhibits resistance to oxidative stress caused by Akt-deficiency. WT and Akt1/2 DKO MEFs were transfected with control siRNA or *FoxO3*a-siRNA, using Lipofectamine 2000. Seventy two hours later, cells were treated with increasing concentration of H_2O_2 for 2h. Apoptosis was then quantitated as described in Experimental Procedures. **, ***p < 0.01, 0.001 as compared with control siRNA-transfected WT group. #, ###p < 0.05, 0.001 as compared with control siRNA-transfected Akt1/2 DKO group. Total RNAs from Akt1/2 DKO cells transfected with control siRNA or *FOXO3a* siRNA were isolated and analyzed for relative fold change of *FoxO3a* (right panel) mRNA levels by real-time RT-PCR as described in Experimental Procedures.

(C) Expression of activated FoxO renders cells more resistant to oxidative stress. Quantification of apoptosis of DN-p53-immortalized $p27^{-/-}$ MEFs overexpressing FoxO(AAA)-ER treated with increasing concentrations of H_2O_2 for 2 h. Prior to treatment with H_2O_2 cells were treated for 48 h with 300 nM 4-OHT. *, **p < 0.05, 0.01 as compared with no treatment.

(D) The knockdown of *Sesn3* inhibits resistance to oxidative stress caused by Akt-deficiency. Experiments were performed as in (B), except that *Sesn3* siRNA was used instead of *FoxO3* siRNA. **, ***p < 0.01, 0.001 as compared with control siRNA-transfected WT group. #, ## ###p < 0.05, 0.01, 0.001 as compared with control siRNA-trasfected Akt1/2 DKO group.

(E) The effect of catalase on premature senescence induced by oxidative stress. WT MEFs were infected with adenovirus carrying catalase or β-galactosidase (LacZ) as a control. Akt1/2 DKO MEFs were infected with adenovirus carrying LacZ. Seventy-two hours post infection, cells were treated with increasing concentration of H_2O_2 for 2h. Apoptosis was then quantitated as described in Experimental Procedures. **, ***p < 0.01, 0.001 as compared with WT Ad-LacZ group. ##, ###p < 0.01, 0.001 as compared with Akt1/2 DKO Ad-LacZ group. Total cell extracts from WT or Akt1/2 DKO MEFs infected with adenovirus carrying LacZ or catalase were prepared and analyzed by immunoblotting using antibodies specific to catalase or β-actin as a loading control (Insert).

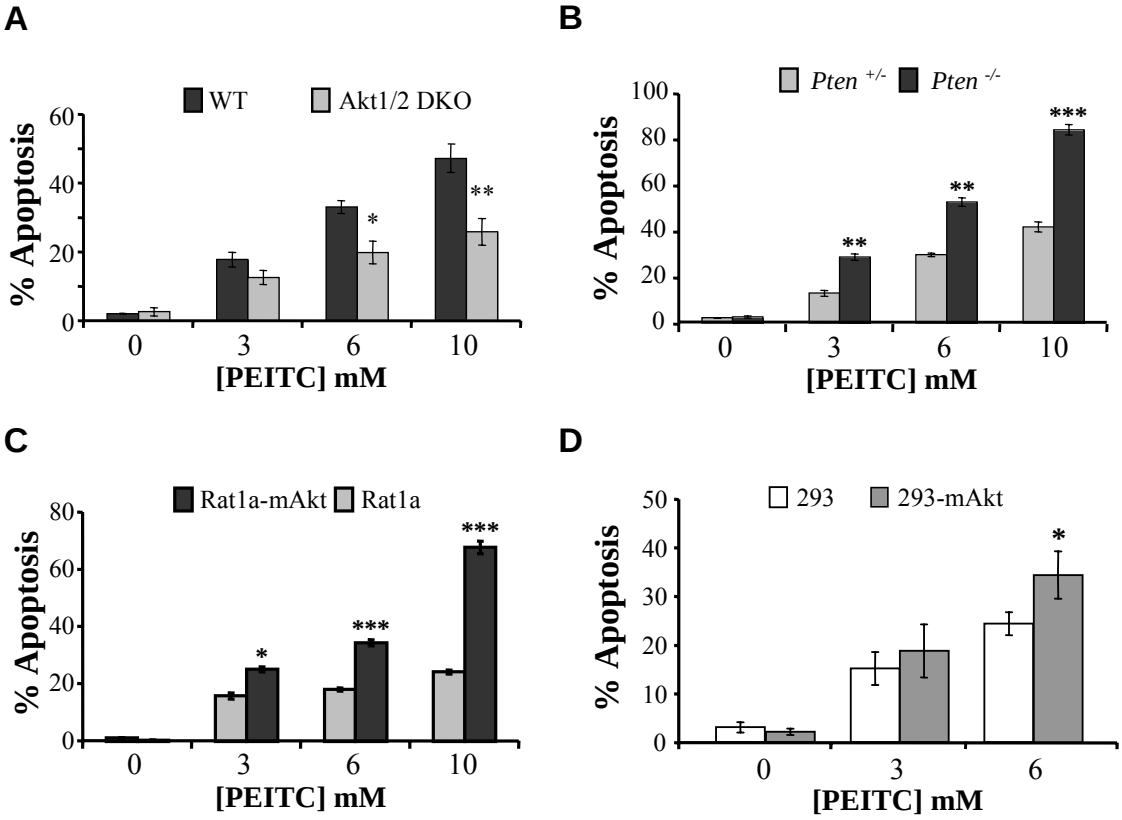

Figure S7. PEITC Induces Apoptosis in a Dose-Dependent Manner

Cells were treated for 5h with increasing doses of PEITC as indicated, and then stained with DAPI to assess apoptosis. Assays were performed in DN-p53 immortalized WT and Akt1/2 DKO MEFs (A), *Pten* $^{+/-}$ and *Pten* $^{-/-}$ immortalized MEFs (B), Rat1a and Rat1a-mAkt fibroblasts (C), and HEK 293 and HEK 293-mAkt cells (D). *, **, ***$p < 0.05, 0.01, 0.001$ as compared with (A) WT, (B) PTEN$^{+/-}$, (C) Rat1a, (D) 293 groups treated with the same dose of PEITC.

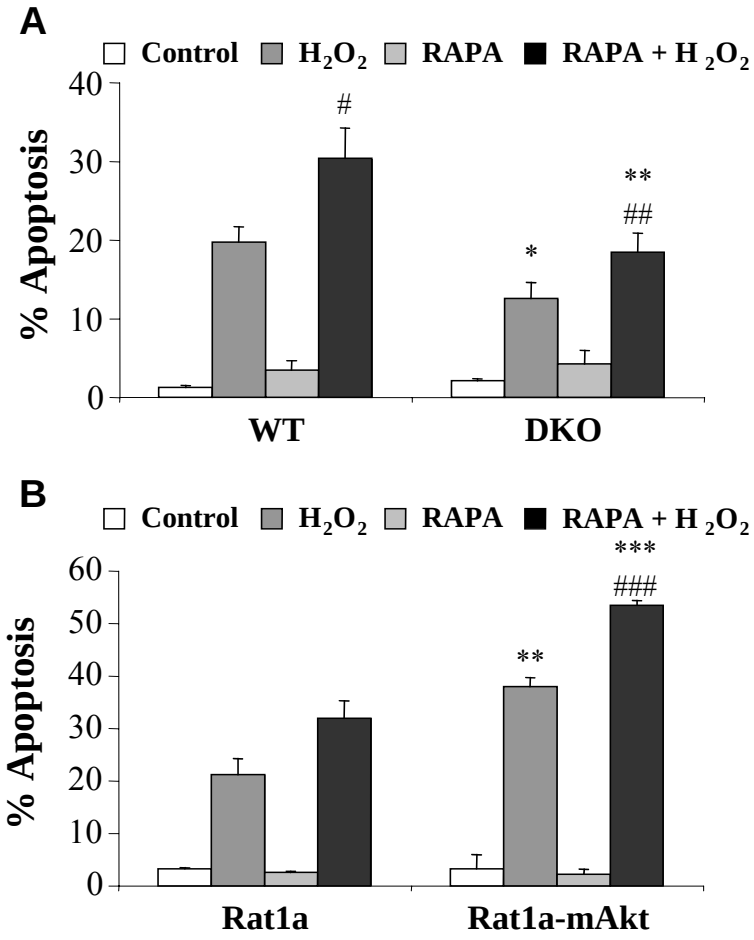

Figure S8. Rapamycin Augments Susceptibility to H_2O_2-Mediated Apoptosis

Cells were pretreated with 100 nM Rapamycin (RAPA) for 3h and then treated with 0.5 mM H_2O_2 for 5h. Apoptosis was assessed by DAPI staining. Assays were performed in DN-p53 immortalized WT and Akt1/2 DKO MEFs (A), and Rat1a and Rat1a-mAkt fibroblasts (B). Data are presented as the mean ± SEM of at least three independent experiments. *, **, ***$p < 0.05$, 0.01, 0.001 as compared with (A) WT, (B) Rat1a groups treated with the same reagents; #, ##, ###$p < 0.05$, 0.01, 0.001 as compared with groups treated with H_2O_2 in absence of Rapamycin in the same cell line.

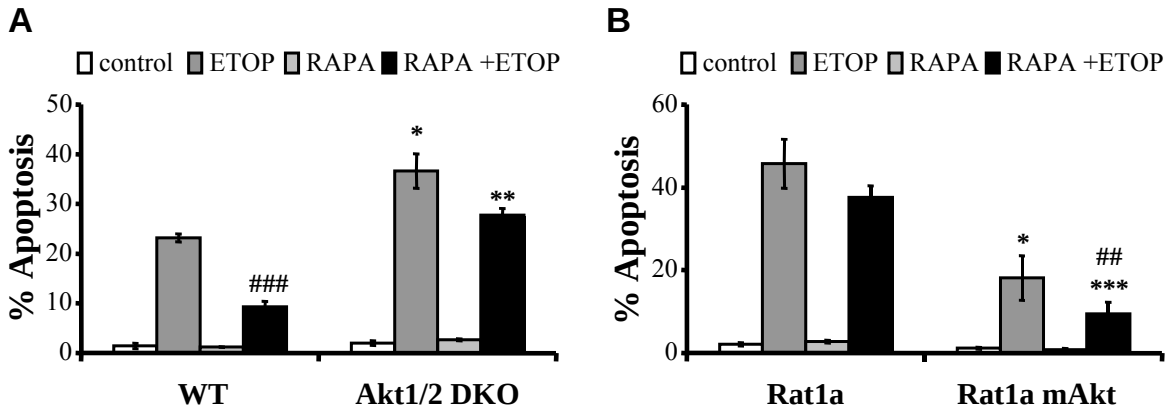

Figure S9. Rapamycin Augments Akt-Dependent Resistance to Etoposide-Induced Apoptosis

Cells were pretreated with 100 nM Rapamycin (RAPA) for 3h and then treated 50μM Etoposide (ETOP) for 5h. Apoptosis was quantified by DAPI staining. Assays were performed using WT and Akt1/2 DKO MEFs (A), and Rat1a and Rat1a-mAkt fibroblasts (B). Data are presented as mean ± SEM of at least three independent experiments. *, **, ***$p < 0.05, 0.01, 0.001$ as compared with (A) WT, (B) Rat1a groups treated with same drugs. ##, ###$p < 0.01, 0.001$ as compared with groups treated with ETOP in absence of Rapamycin in the same cell line.

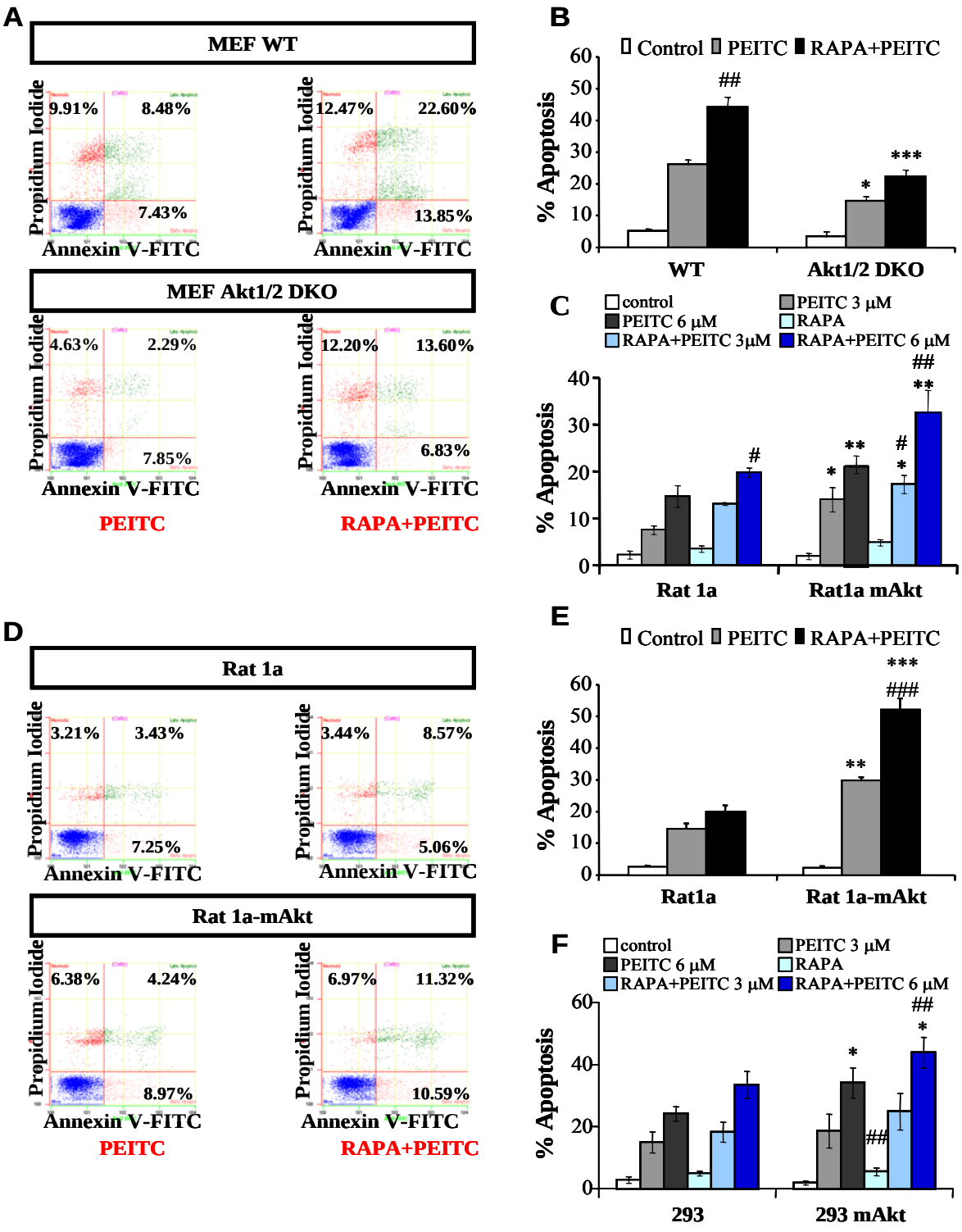
A
MEF WT
Propidium Iodide
9.91%
8.48%
7.43%
Annexin V-FITC
12.47%
22.60%
13.85%
Annexin V-FITC
MEF Akt1/2 DKO
4.63%
2.29%
7.85%
Annexin V-FITC
12.20%
13.60%
6.83%
Annexin V-FITC
PEITC
RAPA+PEITC
B
Control
PEITC
RAPA+PEITC
% Apoptosis
WT
Akt1/2 DKO
C
control
PEITC 3 μM
PEITC 6 μM
RAPA
RAPA+PEITC 3μM
RAPA+PEITC 6 μM
% Apoptosis
Rat 1a
Rat1a mAkt
D
Rat 1a
3.21%
3.43%
7.25%
Annexin V-FITC
3.44%
8.57%
5.06%
Annexin V-FITC
Rat 1a-mAkt
6.38%
4.24%
8.97%
Annexin V-FITC
6.97%
11.32%
10.59%
Annexin V-FITC
PEITC
RAPA+PEITC
E
Control
PEITC
RAPA+PEITC
% Apoptosis
Rat1a
Rat 1a-mAkt
F
control
PEITC 3 μM
PEITC 6 μM
RAPA
RAPA+PEITC 3 μM
RAPA+PEITC 6 μM
% Apoptosis
293
293 mAkt

Figure S10.

Figure S10. Akt Sensitizes Cells to PEITC- and PEITC + Rapamycin-Induced Apoptosis, as Assessed by Annexin-V-FITC/PI and PI Staining

(A) Cell death was as assessed by Annexin-V-FITC/PI staining**.** WT and Akt1/2 DKO MEFs, immortalized by DN-p53, were pretreated with (Right panels) and without (Left panels) 100 nM Rapamycin (RAPA) for 3h and then treated with 6 μM PEITC for 5h. Cells were then collected, double-stained with Annexin-FITC and PI, and subjected to FACS analysis. Results are displayed as a bivariate distribution of annexin V and PI fluorescence intensity. The results shown in each panel are representative of three independent experiments for (*Upper* panels) WT MEFs and (*Lower* panels) Akt1/2 DKO MEFs. Cells in the lower-left quadrant, unstained for both annexin V and PI, are defined as viable cells. Cells in the lower-right quadrant, stained for annexin V but negative for PI, are defined as early apoptotic cells. Cells in the upper-right quadrant, positive for both annexin V and PI, represent the late apoptotic population. Cells in the upper-left quadrant, negative for both annexin V but positive for PI, represent the necrotic population. The percentage of cells in each quadrant is shown at the top of each corresponding panel.

(B) Cell death was assessed by flow cytometry analysis after PI staining. DN-p53 immortalized WT and Akt1/2 DKO MEFs were treated with PEITC in the presence or absence of rapamycin as described in (A). After PI staining the cells were subjected to flow cytometry analysis. The percentage of apoptotic cells (sub-G1 population) is calculated within each group and presented as the mean ± SEM of at least three independent experiments. *, ***$p < 0.05$, 0.001 as compared with WT group; ##$p < 0.01$ compared with group treated with PEITC in absence of RAPA.

(C) Rapamycin sensitizes Rat1a and Rat1a-mAkt cells to PEITC-induced apoptosis. Cells were incubated for 3 h with 100 nM rapamycin (RAPA) prior to the addition of PEITC (3 or 6 μM). After 5 h of incubation with the PEITC, cells were fixed and apoptosis was quantified by DAPI staining. *, **$p < 0.05$, 0.01 as compared with Rat1a; #, ##$p < 0.05$, 0.01 as compared with PEITC in the absence of RAPA.

(D and E) Same experiments were carried out as described in (A and B) using Rat1a and Rat1a-mAkt fibroblasts. **, ***$p < 0.01$, 0.001 as compared with Rat1a group. ###$p < 0.001$ as compared with group treated with PEITC in absence of RAPA.

(F) Rapamycin sensitizes HEK293 and HEK293-mAkt cells to PEITC-induced apoptosis. Cells were pretreated for 3 h with 100nM rapamycin (RAPA) and then treated for 5h with PEITC (3 or 6 μM). Cells were then fixed and stained with DAPI, for quantification of apoptosis. *$p < 0.05$ as compared with 293 group; ##$p < 0.01$ as compared with group treated with PEITC alone.

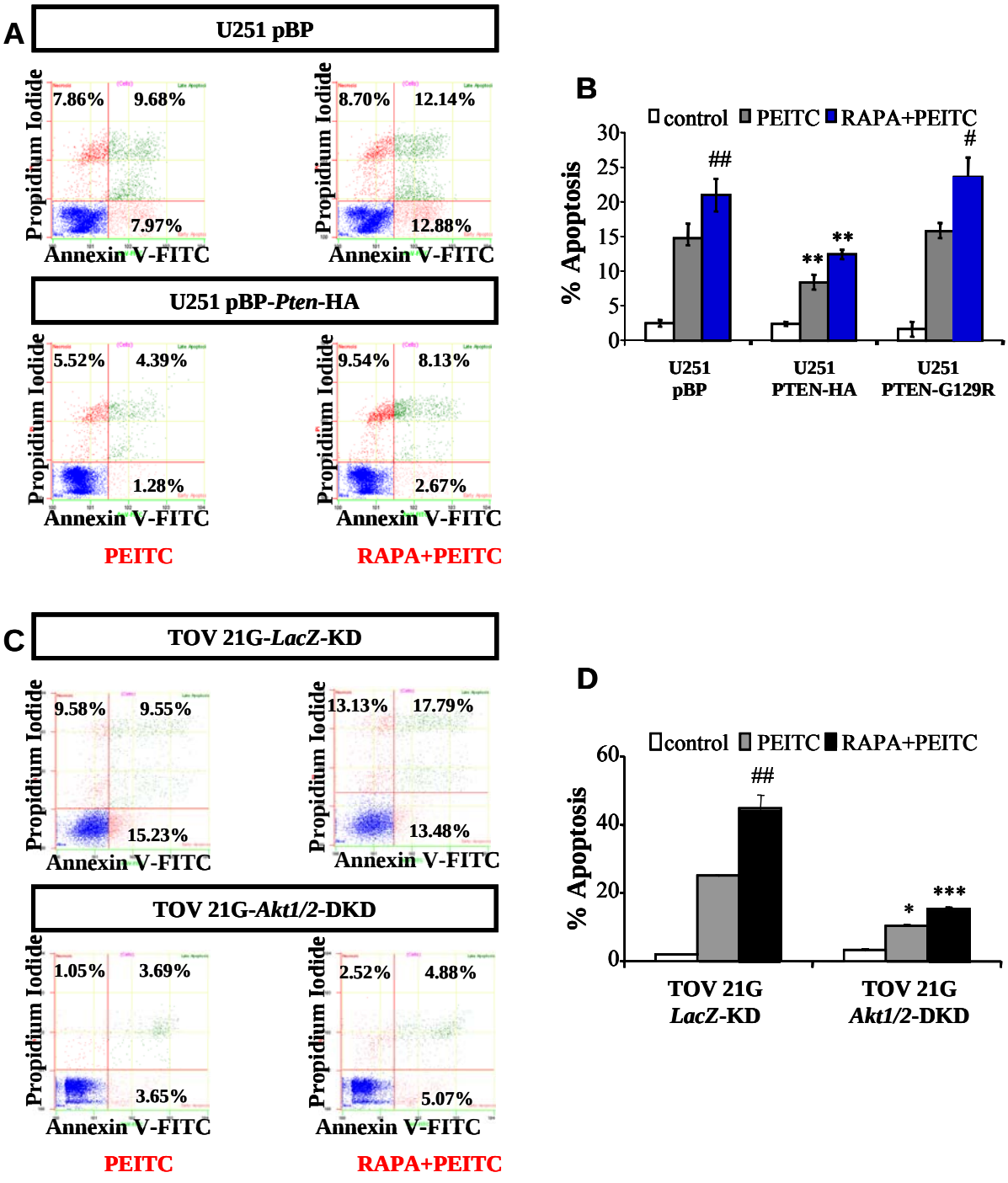
A
U251 pBP
Propidium Iodide
7.86%
9.68%
7.97%
Annexin V-FITC
8.70%
12.14%
12.88%
Annexin V-FITC
U251 pBP-Pten-HA
5.52%
4.39%
1.28%
Annexin V-FITC
9.54%
8.13%
2.67%
Annexin V-FITC
PEITC
RAPA+PEITC
B
control
PEITC
RAPA+PEITC
% Apoptosis
30
25
20
15
10
5
0
##
**
**
#
U251 pBP
U251 PTEN-HA
U251 PTEN-G129R
C
TOV 21G-LacZ-KD
Propidium Iodide
9.58%
9.55%
15.23%
Annexin V-FITC
13.13%
17.79%
13.48%
Annexin V-FITC
TOV 21G-Akt1/2-DKD
1.05%
3.69%
3.65%
Annexin V-FITC
2.52%
4.88%
5.07%
Annexin V-FITC
PEITC
RAPA+PEITC
D
control
PEITC
RAPA+PEITC
% Apoptosis
60
40
20
0
##
*
***
TOV 21G LacZ-KD
TOV 21G Akt1/2-DKD

Figure S11.

Figure S11. Akt Sensitizes U251 Glioblastoma and TOV21G Ovarian Cancer Cells to PEITC- and PEITC + Rapamycin-Induced Apoptosis, as Assessed by Annexin-V-FITC/PI and PI Staining

(A) Quantification of cell death by Annexin-FITC/PI double staining and flow cytometric analysis. U251-pBP (*Upper* panels) and U251 pBP-*Pten* HA (*Lower* panels) glioblastoma cells were incubated for 17h with 3μM PEITC with (Right panels) or without (Left panels) pre-incubation with 100 nM Rapamycin (RAPA). At the end of the incubation, all cells were collected and treated as described in Figure S10A. Results are displayed as a bivariate distribution of annexin V and PI fluorescence intensity. The results shown in each panel are representative of three independent experiments.

(B) Cell death was as assessed by flow cytometry analysis after PI staining. U251 and U251 expressing Pten-HA or Pten-G129R were used and treated with PEITC in the presence or absence of rapamycin as described above in (A). After PI staining, cells were subjected to flow cytometry analysis as described in Figure S10, B. Data are presented as the mean ± SEM of at least three independent experiments. **$p < 0.01$ as compared with U251-pBP group. #, ##$p < 0.05$, 0.01 as compared with group treated with PEITC without RAPA.

(C and D) The same experiments were carried out as described in (A) and (B) using TOV21G-*LacZ*-shRNA and TOV21G-*Akt1/2*-shRNA cells. *, ***$p < 0.05$, 0.001 as compared with TOV21G-*LacZ* group. ##$p < 0.01$ as compared with group treated with PEITC alone.

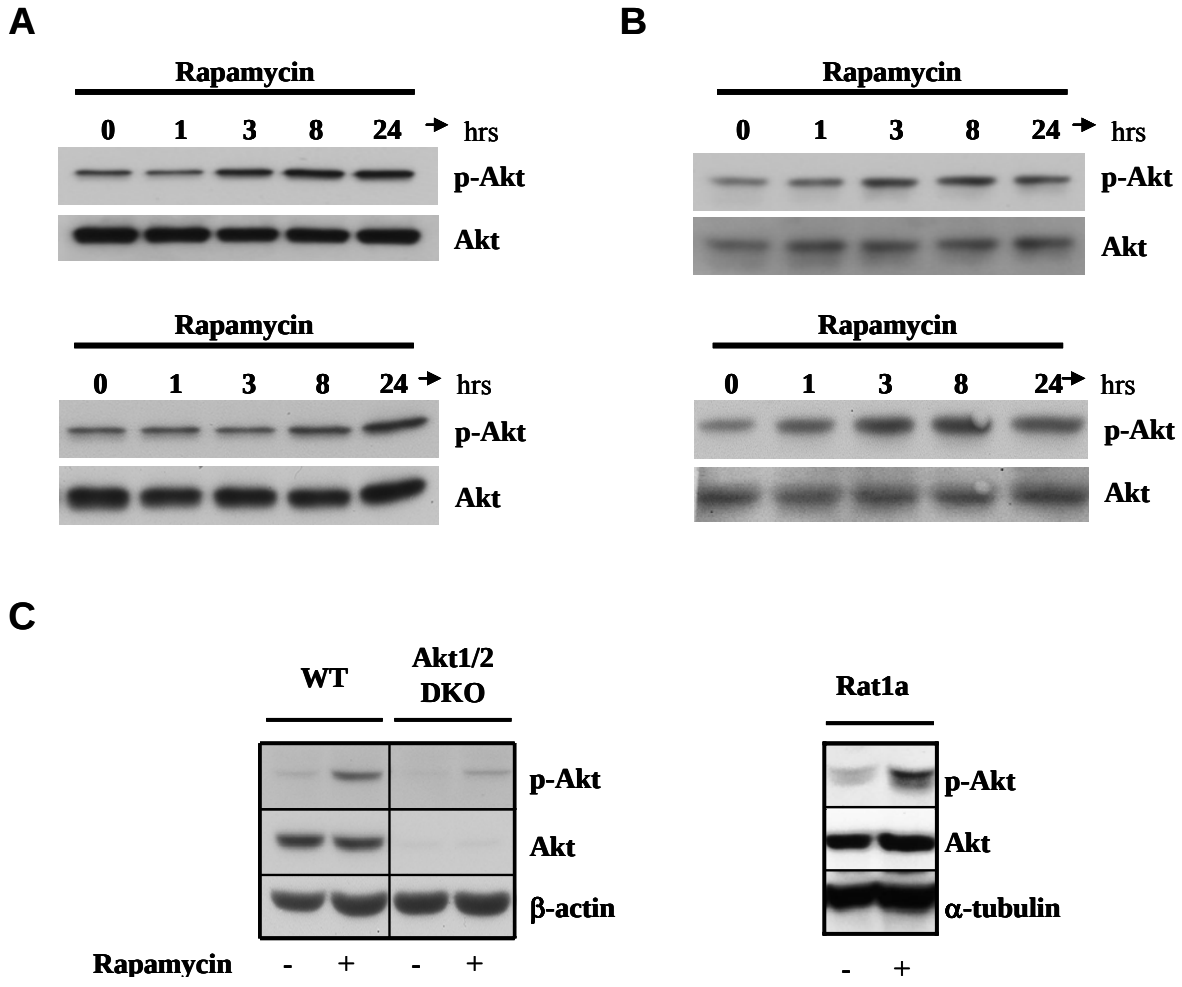

Figure S12. Rapamycin Elevates Akt Activity

(A and B) Rat1a-mAkt fibroblasts (A) and TOV21G cells (B) were grown in medium containing 10% (upper panels) or 2% FBS (bottom panels) for 16 h and then treated with rapamycin (100 nM). Total cell extracts were prepared at different time points as indicated and subjected to immunoblotting with antibodies specific for Akt and p-Akt.

(C) WT, Akt1/2 DKO MEFs and Rat1a fibroblasts were treated for 8h with 100 nM rapamycin. Total cell extracts were prepared and analyzed by immunoblotting with antibodies specific for Akt, p-Akt, and β-actin or α-tubulin as loading controls.

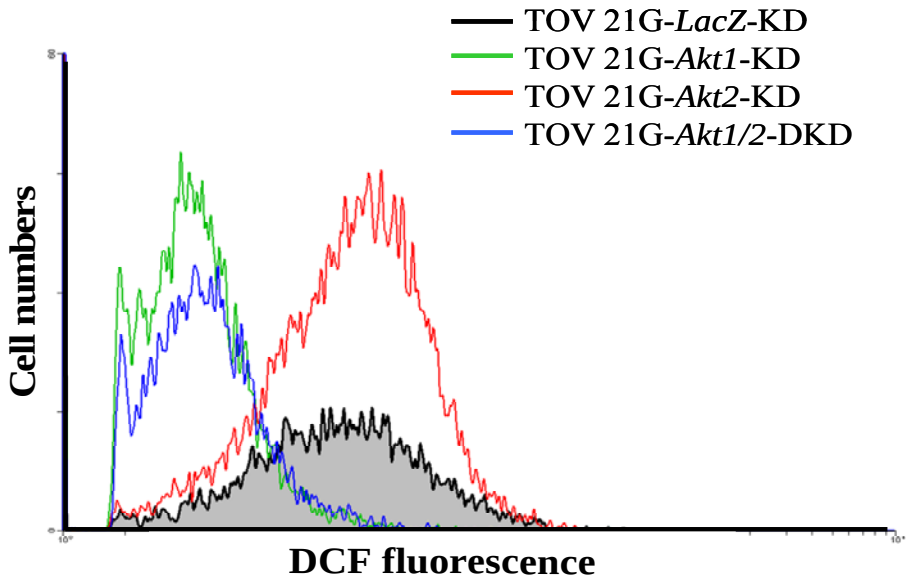

Figure S13. Knockdown of *Akt1* and *Akt2* in TOV21G Cells Decreases Intracellular Levels of ROS

ROS production was measured in TOV21G-*Akt1*-KD, TOV21G-*Akt2*-KD, TOV21G-*Akt1/2*-KD, and TOV21G-*LacZ*-KD cells as a control. Cells were stained with H_2DCF-DA, and ROS production was analyzed by flow cytometry as described in Experimental Procedures. Results represent the intensity of DCF fluorescence in each group.

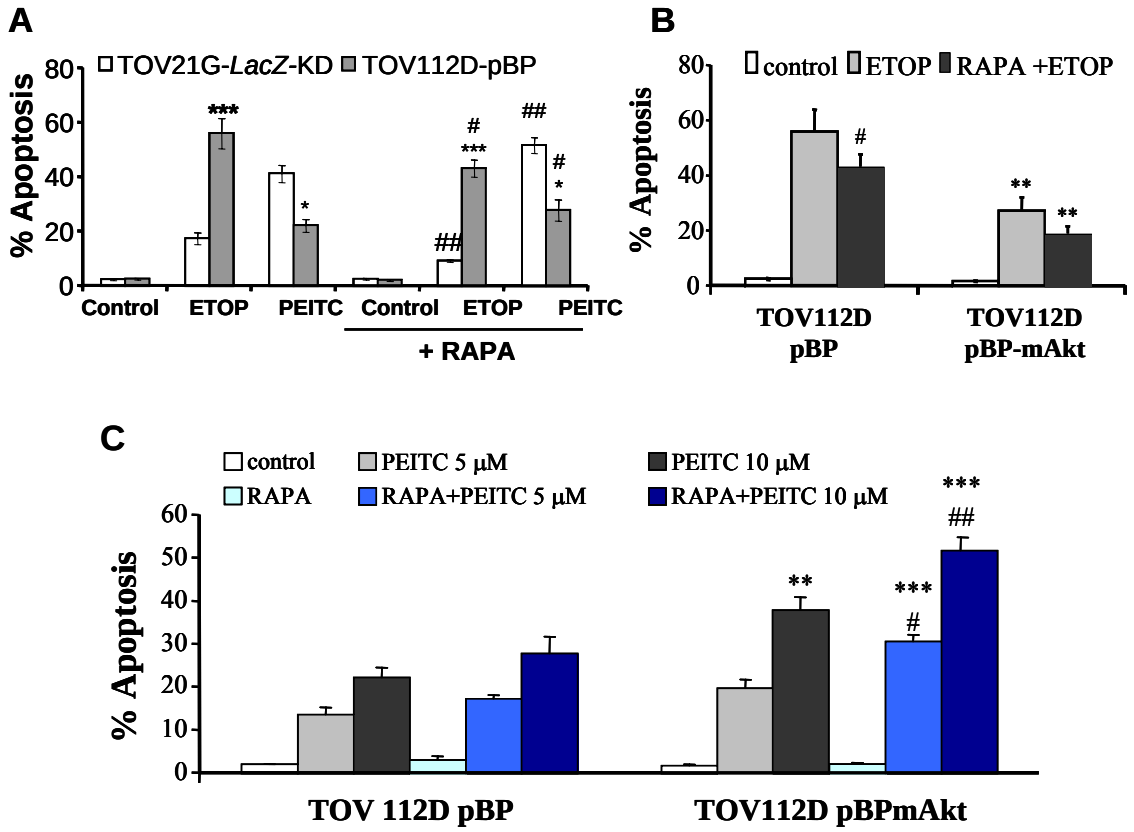

Figure S14. Rapamycin Increases the Sensitivity of Ovarian Cancer Cells to PEITC, and Decreases Their Sensitivity to Etoposide in an Akt-Dependent Manner

(A) TOV21G LacZ and TOV112D cells were incubated for 3 h with 100 nM rapamycin (RAPA) before the addition of PEITC (5 µM) or Etoposide (ETOP- 50 µM). After 17h of incubation with the drugs, cells were fixed and apoptosis was quantified by DAPI staining. *, ***$p < 0.05$, 0.001 as compared with control group. #, ##$p < 0.05$, 0.01 as compared with group treated with PEITC without RAPA.

(B) TOV112D and TOV112D cells expressing mAkt were incubated for 3 h with 100 nM rapamycin (RAPA) before the addition of Etoposide (ETOP- 50 µM). After 17h of incubation with ETOP, cells were fixed and apoptosis was quantified by DAPI staining. **$p < 0.01$ as compared with TOV112D pBP group. #$p < 0.05$ as compared with group treated with ETOP in absence of RAPA.

(C) TOV112D and TOV112D cells expressing mAkt were incubated for 3 h with 100nM rapamycin (RAPA) before the addition of PEITC (5 or 10 µM). After 17 h of incubation with PEITC, cells were fixed and apoptosis was quantified by DAPI staining. **, ***$p < 0.01$, 0.001 as compared with TOV112D pBP group. #, ##$p < 0.05$, 0.001 as compared with group treated with PEITC in absence of RAPA.

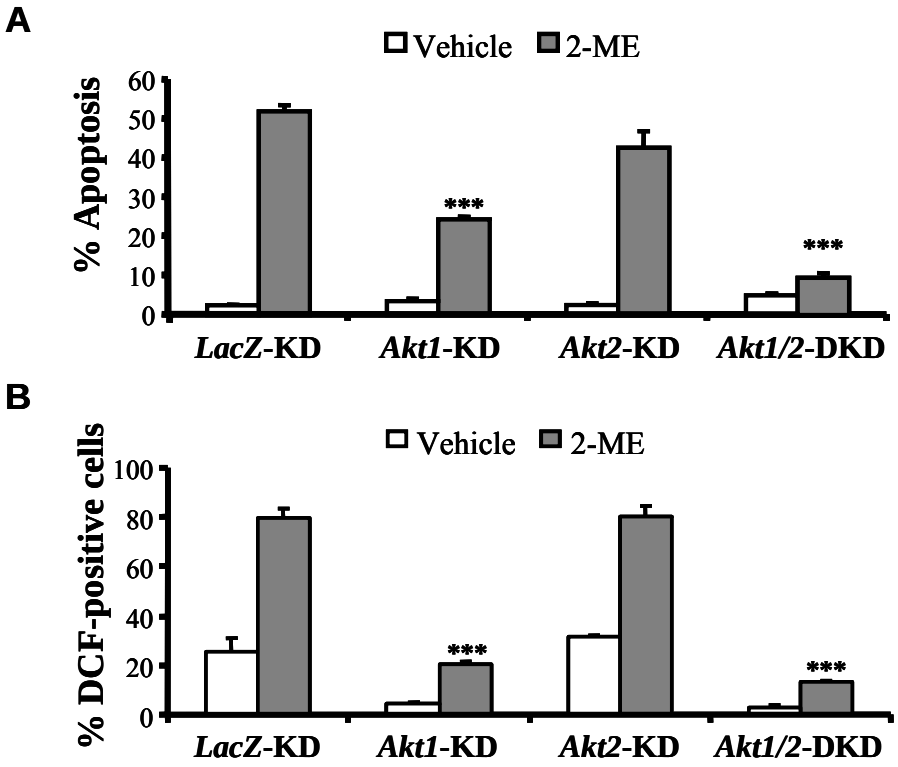

Figure S15. 2-Methoxyestradiol Increases ROS Production and Apoptosis of TOV21G Cells with High Akt Activity

TOV21G-*LacZ*-KD, *Akt1*-KD, *Akt2*-KD and *Akt1/2*-DKD cells were treated with 1 μM 2-Methoxyestradiol (2-ME) for 20 h and were then subjected to DAPI staining to determine apoptosis levels (A) and H_2DCF-DA staining to measure ROS production (B) described in Experimental Procedures. ***$p < 0.001$ as compared with *LacZ*-KD group.