Supporting Information

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SI Materials and Methods

Plasmids, Antibodies, and Reagents. Full-length protein kinase D1 (PKD1), PKD1 constitutive active mutant (PKD1-CA) and PKD1 dominant negative (PKD1-DN) were kindly provided by Q.J.W. and Yun Wang (Peking University Health Science Center, Beijing), respectively, and then cloned into pWZL-Hygro and pLPC-puro vectors. The shRNA was designed according to the pMSCV instruction manual (Clontech). The double strand RNAs were synthesized by GENEray Biotechnology of Shanghai and then inserted into the EcoRI and XhoI sites of the pMCV-puro-miR30 vector. The two independent human PKD1-specific shRNA target sequences used were: miR30-PKD1: 5′-GTCGAGAGAAGAGGTCAAATT-3′, which target human PKD1 725–745bp (1); miR30-PKD1#2: 5′-CAAUCCUCAUUGUUUCGAAAT-3′, which target human PKD1 1461–1481bp (2).

The primary antibodies used for Western blot analysis were as follows: anti-PKD1, anti-phospho-PKD1 (ser744/ser748), anti-phospho-PKD1 (ser916), anti-p65, anti-IκBα, anti-phospho-ERK (Thr202/Tyr204), anti-IκB kinase (IKK), anti-MMP2, and anti-MMP9 were from Cell Signaling Technology. Anti-protein kinase Cδ (PKCδ), anti-p16INK4a, anti-RAS, anti-E1A, anti-phospho-IKK, and anti-Cytochrome C were from Santa Cruz Biotechnology. Anti-human IL-6 and human IL-8 were from R&D Systems. Anti-PKD2 and GAPDH were from Abcam and Tianjin Sungene Biotech, respectively.

Gö6976 (Sigma), Gö6983 (Sigma), Phorbol 12-myristate 13-acetate (PMA; Sigma), Ro 31-8425 methanesulfonate salt (Sigma), BAY 11-7082 (EMD), SB203580 (SB; CST), and EU.K.-134 (EU.K.; BioVision) were dissolved in DMSO (Sigma); Tamoxifen (Sigma) was dissolved in methanol; and *N*-Acetyl-L-cysteine (NAC) (Sigma) was dissolved in sterile water.

Cell Lines and Viral Infections. IMR90 human diploid fibroblasts (HDFs) transduced with an ER:RAS (kindly provided by Masashi Narita, Cancer Research U.K., Cambridge Research Institute), phoenix cells (provided by Masashi Narita) and human diploid 2BS fibroblasts cells (National Institute of Biological Products, Beijing, China) were cultured in DMEM supplemented with 10% FBS. To induce senescence, IMR90 cells expressing ER:H-RasV12 (ER:Ras-IMR90 cells) were given 100 nM 4-hydroxytamoxifen (4-OHT). The infections were performed as described (3).

Real-Time PCR. Total RNA was isolated from IMR90 cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the StarScript first strand cDNA synthesis kit (GenStar Biosolutions). Real-time PCR was performed in triplicate using the SYBR Green PCR Master Mix (Invitrogen) on an ABI Prism 7300 Sequence Detector (Applied Biosystems). Gene-specific primers were as follows:

IL-6 forward: 5′-TGACCCAACCACAAATGC-3′
and reverse: 5′-CTGGCTCTGAAACAAAGGAT;
IL-8 forward: 5′-TGTGGGTCTGTTGTAGGG-3′
and reverse: 5′-GTGAGGTAAGATGGTGGC-3′;
β-actin forward: 5′-GTGGACATCCGCAAAGAC-3′
and reverse: 5′-AAAGGGTGTAACGCAACTAA-3′.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP Assay kit (Upstate) according to the manufacturer's instruction. DNA released from the precipitated complexes was amplified by Real-time PCR using sequence specific primers. The primer sequences were as follows:

IL-6 promoter, forward: 5′-CACAGAAGAACTCAGATGACTGG-3′
and reverse: 5′-AAAACCAAAGATGTTCTGAACTGA-3′;
IL-8 promoter, forward: 5′-CATCAGTTGCAAATCGTGGA-3′
and reverse: 5′-GAACTTATGCACCCTCATCTTTTC-3′.

Immunoblot Analysis. Cells were washed twice with ice-cold 1× PBS, harvested, and lysed in radioimmune precipitation assay buffer (RIPA buffer; Applygen Technologies) with phosphatase inhibitor tablet (Roche Diagnostics) and protease inhibitor mixture (Fermentas). Cell lysates were then centrifuged for 15 min at 15,000 × *g* at 4 °C, and the insoluble debris was discarded. Protein concentration was determined by BCA Protein Assay Reagent (Pierce). Cell lysate (20–40 μg) was subjected to 8–15% SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to nitrocellulose membranes (Millipore). The primary antibodies used for Western blot analysis were described as above.

Isolation of Mitochondria. Mitochondrial isolation was performed using Mitochondrial Isolation Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. Briefly, cells were washed twice with ice-cold 1× PBS and resuspended in 1 mL of mitochondrial isolation buffer plus protease inhibitor mixture (Fermentas). Cells were homogenized with 12 strokes in a glass homogenizer. Lysates were centrifuged twice at 600 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 11,000 × *g* for 10 min at 4 °C to pellet mitochondria. Mitochondrial pellets were resuspended in lysis buffer and subjected to immunoblot analysis. Supernatants were spun for 1 h at 10,000 × *g* at 4 °C to pellet nuclei. Nuclear pellets were resuspended in lysis buffer and subjected to immunoblot analysis. Supernatants were cytoplasmic fraction.

SA-β-Gal and Colony Formation. For SA-β-gal staining, cells were washed twice with ice-cold 1× PBS, fixed for 10 min at room temperature in 3% formaldehyde, and washed twice with 1× PBS. The cells were then incubated overnight at 37 °C without CO_2 in a freshly prepared SA-β-gal staining solution.

To determine the colony formation, 1×10^3, 3×10^3, and 1×10^4 cells were cultured in six-well plate. Several days later, cells were fixed in 3% (wt/vol) formaldehyde at 37 °C for 30 min and washed twice with 1× PBS, then stained with crystal violet for 1 h and washed with 1× PBS twice.

Growth Curves. Cell proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were seeded into 96-well plate at a density 2×10^3 cells per well and cultured for periods ranging from 1 to 7 d. The medium was changed every 24 h. At the indicated times, an aliquot of cells were stained with 25 μL of MTT solution (5 mg/mL in 1× PBS; Sigma) for 4 h and then dissolved with DMSO. The optical density at 570 nm was determined.

Cell Cycle Analysis. When cells reached 70–80% confluence, they were washed with PBS, detached with 0.25% trypsin, and fixed with 75% ethanol overnight. After treatment with 1 mg/mL RNase A (Sigma) at 37 °C for 30 min, cells were resuspended in 0.5 mL of PBS and stained with propidium iodide in the dark for 30 min. Fluorescence was measured with a FACScan flow cytometry system (BD Biosciences).

ELISA. The supernatants of IMR90 cells were collected after treatment as indicated. Human IL-6 level was measured by an ELISA kit from Boster.

ROS Measurement. Cells were incubated with 10 μM dichlorodihydrofluorescein diacetate (DCF-DA; Applygen Technologies) for 30 min at 37 °C. Cells were then washed with PBS and trypsinized. Trypsinized cells were resuspended in ice-cold PBS and kept as a single cell suspension on ice until rapid analysis by FACS. The data are reported as the fold changes in mean fluorescence intensity normalized to the fluorescence intensity of untreated control cells.

Soft Agar Assay. The transformation cells and control cells were cultured in 0.3% agar on a cushion of 0.6% agar in six-well plate for 14 d, then the colonies were observed under a light microscope.

Tumorigenic Assay. In the experiment, NOD/SCID mice at age of 7 wk were injected with 5×10^6 cells in 100 μL of 1× PBS into forelimb armpit. Control and transformed cells were injected to the left and right, respectively. Eight animals were used and were maintained on regular food and water.

Statistical Analysis. Statistical significance was determined by a two-tailed unpaired Student t test. $^*P < 0.05$, $^{**}P < 0.01$.

1. Qureshi A, et al. (2010) Role of sphingomyelin synthase in controlling the antimicrobial activity of neutrophils against Cryptococcus neoformans. *PLoS ONE* 5(12):e15587.
2. Ding G, et al. (2007) Protein kinase D-mediated phosphorylation and nuclear export of sphingosine kinase 2. *J Biol Chem* 282(37):27493–27502.
3. Chen T, et al. (2012) The retinoblastoma protein selectively represses E2F1 targets via a TAAC DNA element during cellular senescence. *J Biol Chem* 287(44):37540–37551.

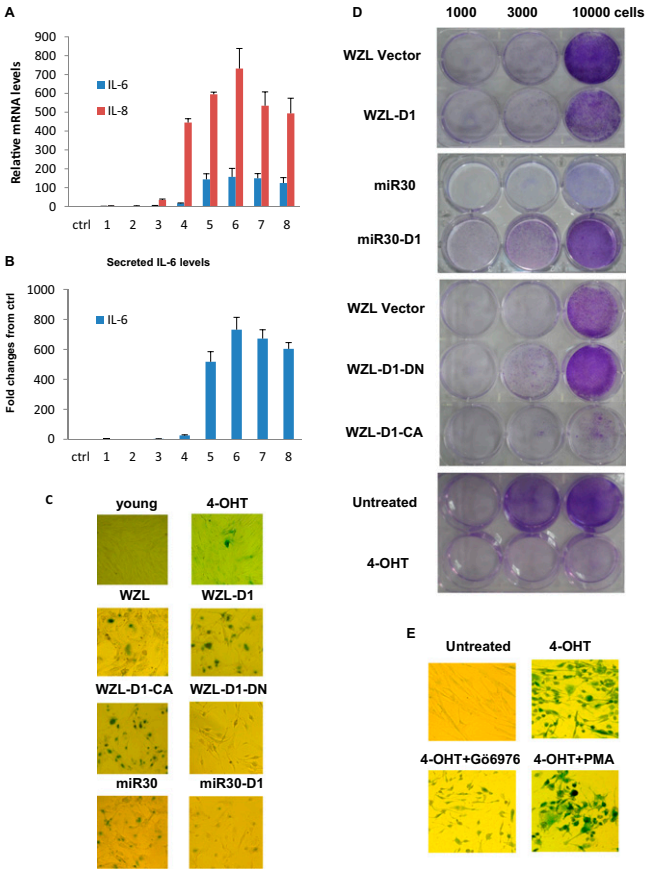

Fig. S1. (*A* and *B*) ER:Ras IMR90 cells were given 100 nM 4-OHT for the indicated number of days, and the relative mRNA levels of IL-6 and IL-8 were analyzed by quantitative PCR (*A*) or the secretory levels of IL-6 were measured by ELISA (*B*). (*C*) ER:Ras IMR90 cells expressing the indicated genes and shRNAs were induced to express Ras for 6 d, then, cells were stained for SA-β-gal activity. (*D*) ER:Ras IMR90 cells expressing the indicated genes and shRNAs were induced to express Ras for several days, then colony formation assays were performed. (*E*) PKD1 inhibition delays Ras OIS, whereas PKD1 activation promotes Ras OIS. ER:Ras IMR90 cells were cultured with solvent only, Gö6976 (2 μM), or PMA (100 nM) in the presence of 4-OHT for 6 d, then assessed for SA-β-gal activity.

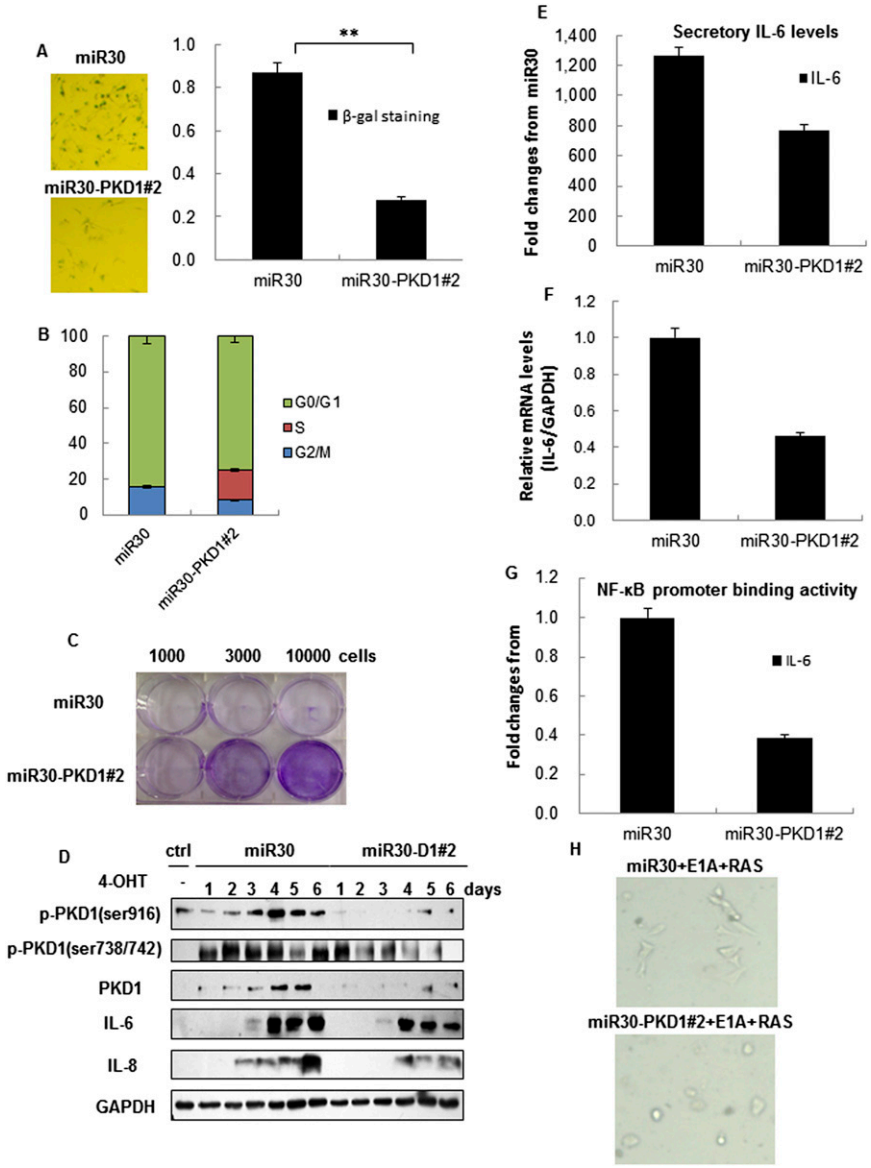

Fig. S2. PKD1 silencing by miR30-PKD1#2 prevents Ras OIS. (*A*–*C*) ER:Ras IMR90 cells stably expressing miR30 empty vector and miR30-PKD1#2 construct were induced to express Ras for 6 d. (*A*) After 6 d, cells were stained for SA-β-gal. (*A*, *Right*) The percentage of cells positive for SA-β-gal in each sample. At least 300 cells were counted for each sample. (*B*) Cell cycle was measured. (*C*) Colony formation assays were performed. Values are mean ± SD of triplicate points from a representative experiment ($n = 3$), which was repeated three times with similar results. (*D*) PKD1 deletion by miR30-PKD1#2 reduces IL-6/IL-8 induction. ER:Ras IMR90 cells stably transduced with miR30 and miR30-PKD1#2 constructs were given 4-OHT for the indicated days, fresh medium with 4-OHT was changed every other day. Cell lysates were then subjected to Western blot analysis for the indicated proteins. (*E*) Supernatants collected from above cells at day 6 after Ras induction were assessed secretory levels of IL-6 measured by ELISA. Three independent experiments were analyzed. Error bars represent means + SD ($n = 3$). (*F*) The relative mRNA levels of IL-6 were analyzed by qPCR. (*G*) Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding activities to IL-6 promoter were assessed by ChIP assays. (*H*) PKD1 depletion by miR30-PKD1#2 promotes cell transformation. Normal IMR90 cells were transduced with Ras, E1A, and miR30 vector or miR30-PKD1#2. The two stable cell lines grew in soft agar as described.

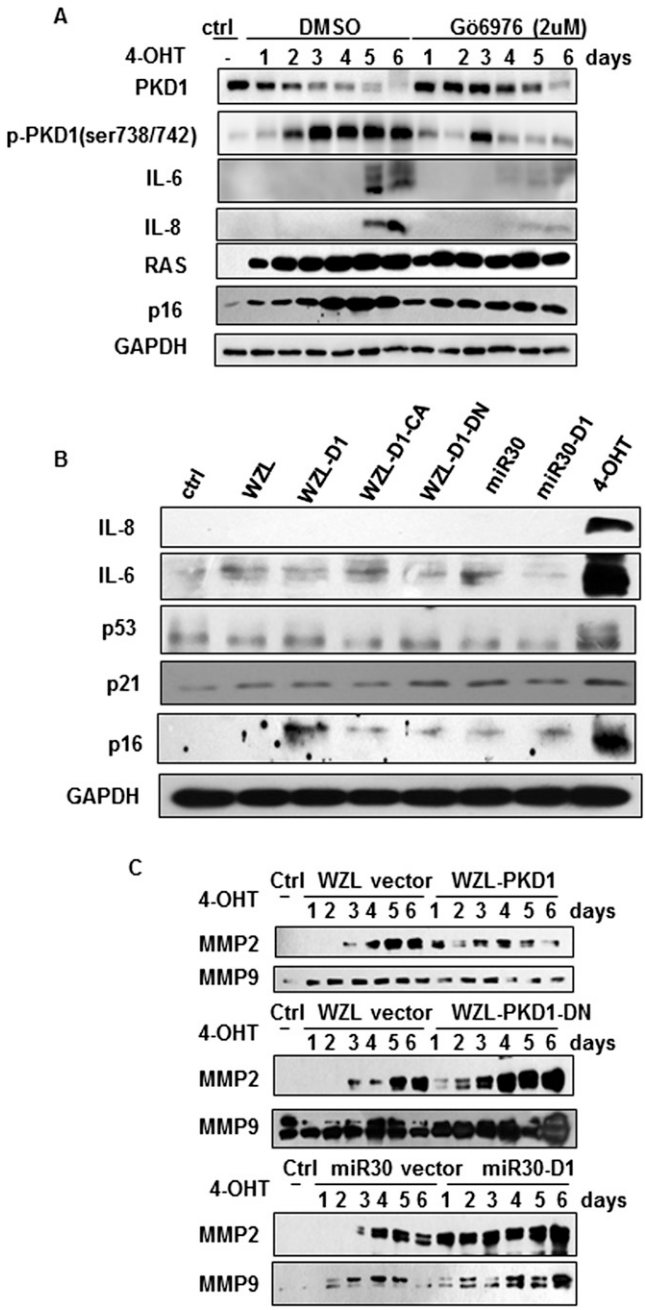

Fig. S3. (*A*) PKD1 inhibition blocks IL-6/IL-8 expression. ER:Ras IMR90 cells were treated with solvent or Gö6976 (2 μM) in the presence of 4-OHT, fresh medium with 4-OHT and Gö6976 was changed every other day, and whole cell lysates were collected at the indicated days thereafter. Western blot analyzes of indicated proteins. (*B*) PKD1 does not trigger IL-6/IL-8 expression and alter p53-p21 as well as p16 pathways without Ras induction. ER:Ras IMR90 cells were infected by the indicated genes and shRNAs. After 2 d of infection and 2 d of selection, cell lysates were collected for immunoblotting of indicated proteins. (*C*) PKD1 negatively regulates MMPs expression in Ras OIS. ER:Ras IMR90 cells expressing indicated genes and shRNAs were given 4-OHT for the indicated days; fresh medium with 4-OHT was changed every other day. Cell lysates were collected every day for total 6 d, then the expression of MMP2 and MMP9 proteins was analyzed.

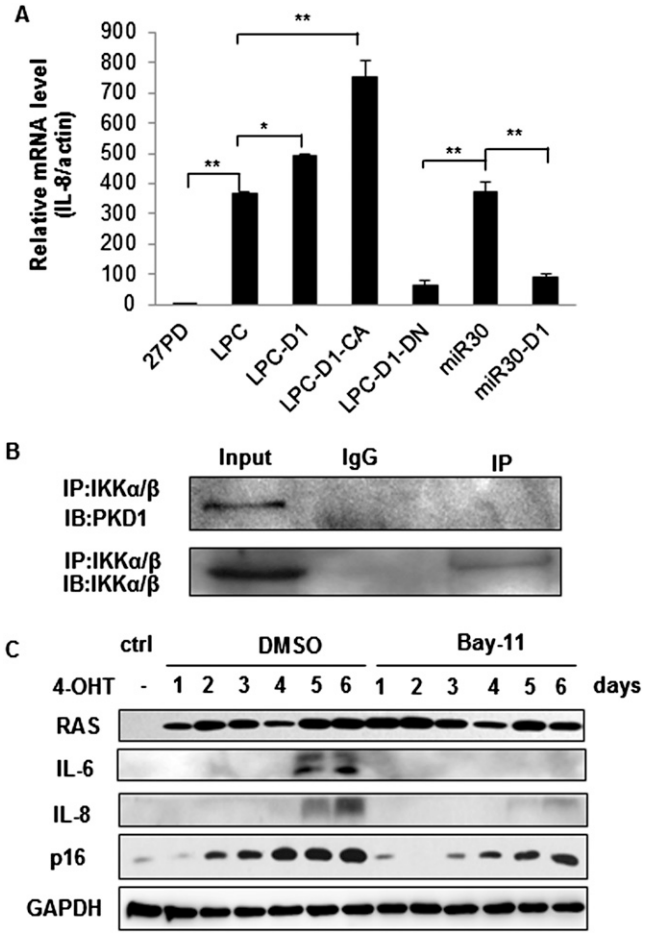

Fig. S4. (*A*) PKD1 regulates IL-8 at mRNA level. Total mRNA were extracted from ER:Ras IMR90 cells expressing indicated genes and shRNAs at day 6 after Ras-induction. Relative mRNA level of IL-8 was determined by qPCR. mRNA level in young IMR90 cells are considered as control. (*B*) ER:Ras IMR90 cells were given 100 nM 4-OHT for 5 d, then, proteins were extracted and immunoprecipitated by using anti-IKKα/β. Western blotting was performed with antibodies as indicated. (*C*) NF-κB inhibition blocks IL-6/IL-8 expression. ER:Ras IMR90 cells were incubated with solvent or IκB kinases inhibitor BAY 11-7082 (5 μM) in the presence of 4-OHT for the indicated times, then analyzed for expression of the indicated proteins.

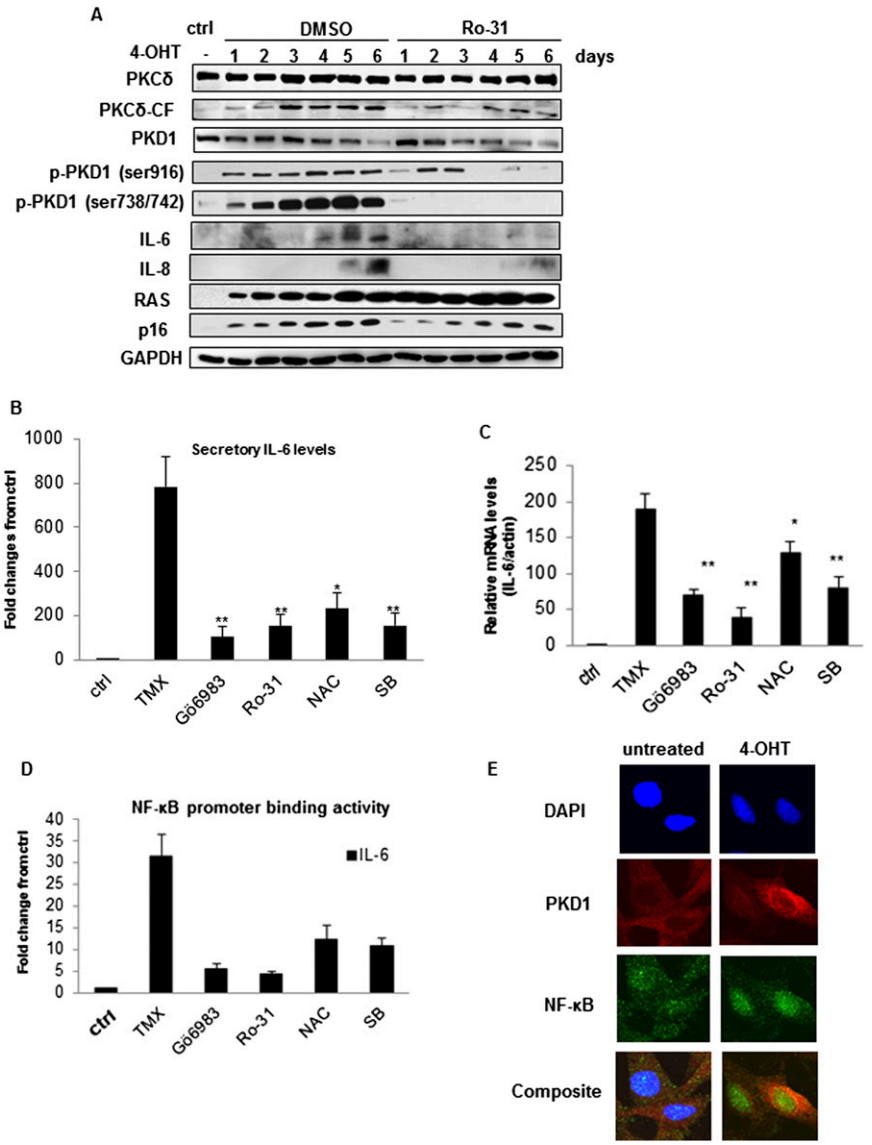

Fig. S5. (*A*) PKCδ inhibition suppresses PKD1 activation and IL-6/IL-8 induction. ER:Ras IMR90 cells were treated with solvent DMSO or PKCδ inhibitor Ro 31-8425 (2 μM) in the presence of 4-OHT for indicated times, then subjected to immunoblotting analysis for the indicated proteins. (*B–D*) Interfering ROS-PKCδ pathway decreases IL-6 levels. ER:Ras IMR90 cells were treated with Gö6983 (2 μM), or Ro 31-8425 (2 μM), or NAC (10 mM), or SB203580 (SB, 5 μM) for 6 d in the presence of 4-OHT, then the secretory levels of IL-6 were measured by ELISA (*B*); the relative mRNA levels of IL-6 were detected by qPCR (*C*); and the NF-κB binding activities to IL-6 promoter were assessed by ChIP assays (*D*). SB serves as a positive control. Three independent experiments were analyzed. Error bars represent means + SD $*P < 0.05$, $**P < 0.01$. (*E*) NF-κB partially localizes to the nucleus during Ras OIS. ER:Ras IMR90 cells were seeded on glass coverslips and cultured with or without 4-OHT for 4 d, and then stained with phospho-PKD1 (ser738/742), red; NF-κB, green; and nuclei, DAPI, blue; representative images are shown.

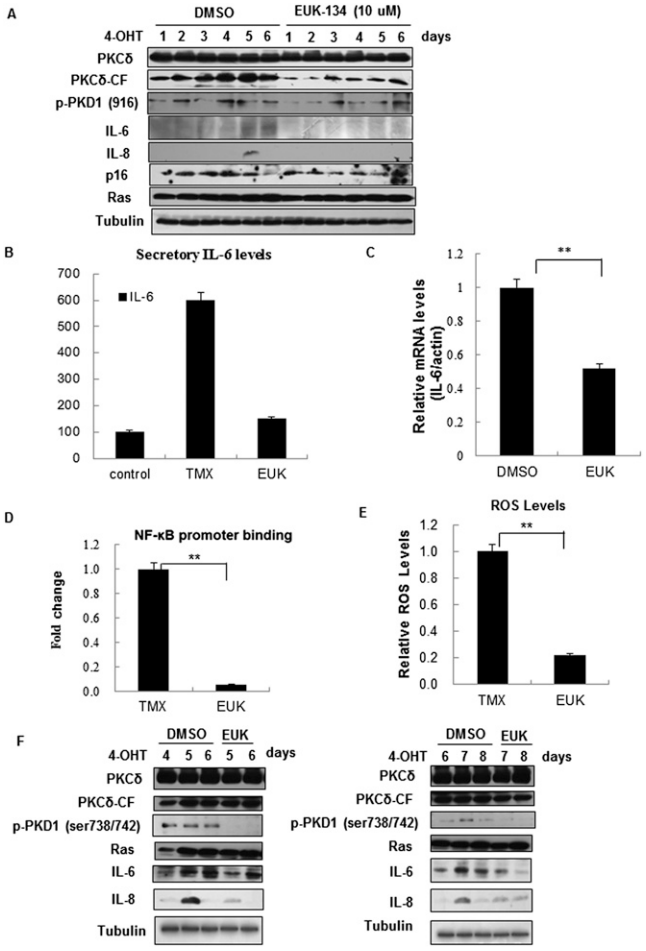

Fig. S6. EU.K.-134 reduces ROS level and inhibits PKCδ-PKD1 activation and IL-6/IL-8 induction in Ras OIS. (*A*) ER:Ras IMR90 cells were given 4-OHT in the presence of solvent DMSO or EU.K.-134 (10 μM) for the indicated times, fresh medium with 4-OHT and EU.K. was changed every other day. Cell lysates were collected every day for a total of 6 d, then subjected to Western blot analysis for the indicated proteins. (*B–E*) ER:Ras IMR90 cells were treated with EU.K.-134 (10 μM) for 6 d in the presence of 4-OHT, then the secretory levels of IL-6 were measured by ELISA (*B*), the relative mRNA levels of IL-6 were detected by qPCR (*C*); the NF-κB binding activities to IL-6 promoter were assessed by ChIP assays (*D*); the relative ROS levels were measured by DCF-DA staining (*E*). Three independent experiments were analyzed. Error bars represent means + SD. (*F*) EU.K.-134 suppresses the initial induction and sustained production of IL-6/IL-8. ER:Ras IMR90 cells were cultured with 4-OHT either for 4 d or 6 d, then EU.K.-134 (10 μM) was added for another 1 or 2 d in the presence of 4-OHT, respectively. The indicated proteins were analyzed by Western blot.

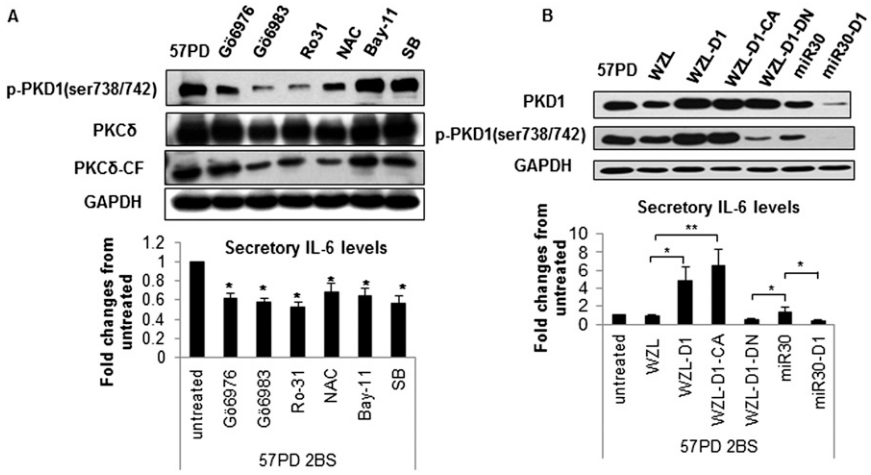

Fig. S7. (*A* and *B*) ROS-PKCδ-PKD1 axis regulates IL-6 expression in replicative senescent cells. (*A*) Replicative senescent 2BS cells were treated with indicated inhibitors for 24 h. (*B*) Replicative senescent 2BS cells were infected with indicated genes and shRNAs for 48 h. The indicated proteins were then subjected to Western blot analysis, and the secretory levels of IL-6 were measured by ELISA. Three independent experiments were analyzed. Error bars represent means + SD $*P < 0.05$, $**P < 0.01$.

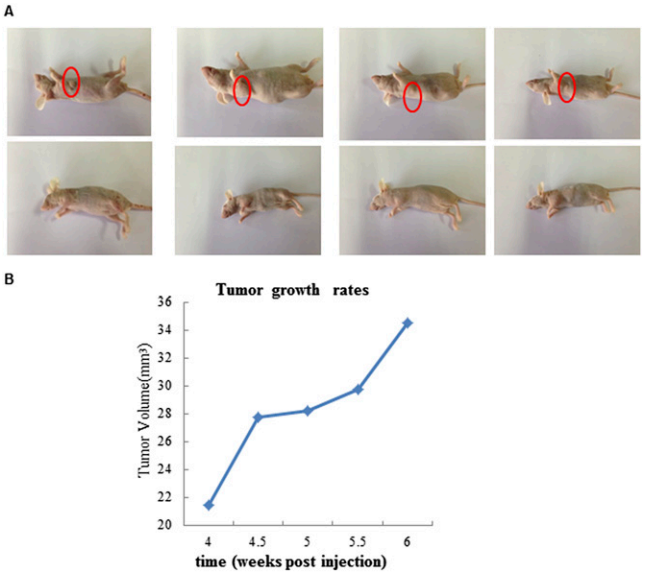

Fig. S8. (*A* and *B*) Additional injected animals and the representative kinetics of tumor growth.