Supporting Information

Zhang et al. 10.1073/pnas.1411026111

SI Materials and Methods

Cell Culture. HEPG2 (human hepatic cancer), MEF (murine embryonic fibroblast), HCT116$^{(+/+)}$ and HCT116$^{(-/-)}$ (human colon cancer) cell lines were grown in DMEM supplemented with 10% (vol/vol) FBS (heat inactivated at 56 °C for 45 min) and the appropriate amount of penicillin/streptomycin in a 37 °C incubator with a humidified, 5% (vol/vol) CO_2 atmosphere.

Protein Extraction. DNA damaging agents such as adriamycin induce a tight association of sirtuin 6 (SIRT6) with chromatin that makes it difficult to extract SIRT6 using normal protein extraction methods. Therefore, for the present studies, the same numbers of cells (1×10^6) were collected and digested in 2× Sample Buffer (950 μL of Laemmli buffer plus 50 μL of 2-mecaptoethanol).

Immunoblot and Immunoprecipitation Assays. Equal amounts (100–150 μg) of proteins were size-fractionated by 7.5–15% (wt/vol) SDS/PAGE. The antibodies used were anti-FoxO1, anti-FoxO3, anti-SIRT6, and anti-acetyl-lysine (Cell Signaling); anti-SIRT1, anti-p53 (DO-1), anti-β-actin, anti-HDAC1, and anti-tubulin (Santa Cruz); anti-PCAF (Bethyl); anti-GAPDH (Genetex); and anti-Flag (Sigma). For immunoprecipitation, cells were harvested and then lysed in a Nonidet P-40 buffer supplemented with a complete protease inhibitor mixture (Roche). Whole-cell lysates or nuclear proteins were used for immunoprecipitation with the indicated antibodies. Generally, 1–4 μg of antibody was added to 1 mL of cell lysate, which was then incubated at 4 °C for 8–12 h. After the addition of Protein A/G-agarose beads, the incubation was continued for 1 h. The resulting immunoprecipitates were extensively washed with lysis buffer and eluted with SDS loading buffer by boiling for 5 min. Data were collected from at least three independent experiments.

RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen). cDNA was synthesized from 2 μg of RNA with oligo$(dT)_{18}$ primers using the SuperScript kit (Invitrogen). The primer sequences used for the amplification of glucose-6-phosphatase (G6PC), phosphoenolpyruvate carboxykinase (PCK1), forkhead box protein O1 (FoxO1), CREB, HNF4, p21, and SIRT6 for RT-PCR were as follows:

G6PC F: GGCTCAACCTCGTCTTTAAGTG, R: CTCCCTGGTCCAGTCTCACA;

PCK1 F: ACGGATTCACCCTACGTGGT, R: CCCCACAGAATGGAGGCATTT;

FoxO1 F: CTCCCATACCCACCCTG, R: AATGAACATGCCATCCAAG;

CREB F: TTAACCATGACCAATGCAGCA, R: TGGTATGTTTGTACGTCTCCAGA;

HNF4 F: CGAAGGTCAAGCTATGAGGACA, R: ATCTGCGATGCTGGCAATCT;

p21 F: CTGGAGACTCTCAGGGTCGAAA, R: GATTAGGGCTTCCTCTTGGAGAA;

SIRT6 F: AATTACGCGGCGGGGCT, R: CGCGCGCTCTCAAAGGT.

GST Pull-Down Assay. GST or GST fusion proteins were expressed in bacteria, induced with isopropyl-β-D-thio-galactoside, and purified. Equal amounts of GST or GST fusion proteins were incubated with glutathione-Sepharose 4B beads (GE Healthcare) and then washed three times with buffer [20 mM Tris (pH 7.4), 0.1 mM EDTA, and 100 mM NaCl]. The resulting GST-FoxO1 was incubated with cell extracts. The beads were then washed three times with TENT buffer [0.5% Nonidet P-40, 20 mM Tris (pH 7.4), 0.1 mM EDTA, and 300 mM NaCl] and analyzed by immunoblotting with anti-SIRT6 or anti-SIRT1 antibodies.

Chromatin Immunoprecipitation. Briefly, 2×10^7 cells were cross-linked with 1% formaldehyde, resuspended in lysis buffer on ice for 15 min, and fragmented by sonication. Soluble chromatin was then diluted and subjected to immunoprecipitation with the indicated antibodies [anti-FoxO1A (Bethyl), anti-CREB (Cell Signaling), anti-HNF4 (Cell Signaling), and anti-p53 (Santa Cruz)]. Immune complexes were then precipitated with Protein A/G Sepharose beads, washed sequentially with low-salt, high-salt, LiCl, and TE buffer, and eluted with elution buffer (1% SDS and 0.1 M $NaHCO_3$). The cross-link was reversed, and DNA was purified with a DNA extraction kit (Qiagen). Finally, the DNA was subjected to real-time PCR.

RNA Interference. One OD unit of siRNA was dissolved in 125 μL of H_2O to get a 10 μM siRNA stock solution, which was kept in small aliquots at −20 °C. When cells reached about 20~30% confluency, they were transfected with siRNA using Lipo2000 (Invitrogen). The final siRNA concentration was 50 nM. SIRT6 target sequences were as follows: siRNAi-SIRT6(1), 5′-AAGCTGGAGCCCAAGGAGGAA-3′; siRNAi-SIRT6(2), 5′-AAGAATGTGCCAAGTGTAAGA-3′; SIRT1 target sequence, 5′-GTTGGATGATATGACACTG-3′; HDAC1 target sequence, 5′-CAGCGACUGUUUGAGAACCTT-3′. The nonspecific small interfering RNA sequence was UUCUCCGAACGUGUCACGU. All RNAi oligonucleotides were purchased from Shanghai GenePharma Company.

Overexpression of Tumor Suppressor p53 and Knockdown of SIRT6 in Mice and Sirt6 Knockout Mice. Overexpression of p53 was achieved by tail-vein injection of a p53-expressing adenovirus vector (Ad-WTp53) in normal C57BL/6J mice at a concentration of 1.5×10^9 plaque-forming units. *Sirt6* conditional knockout mice were used as previously described (1).

Pyruvate Tolerance Test. Mice were fasted for 18–22 h before the pyruvate tolerance test. Blood glucose levels were measured immediately before pyruvate injection or at 0 min, 15 min, 30 min, 45 min, 60 min, and 90 min after pyruvate injection (2 g/kg body weight). Blood glucose levels were determined using a Freestyle Brand Glucometer (Roche) with blood collected from the tail vein.

1. Kim HS, et al. (2010) Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab* 12(3): 224–236.

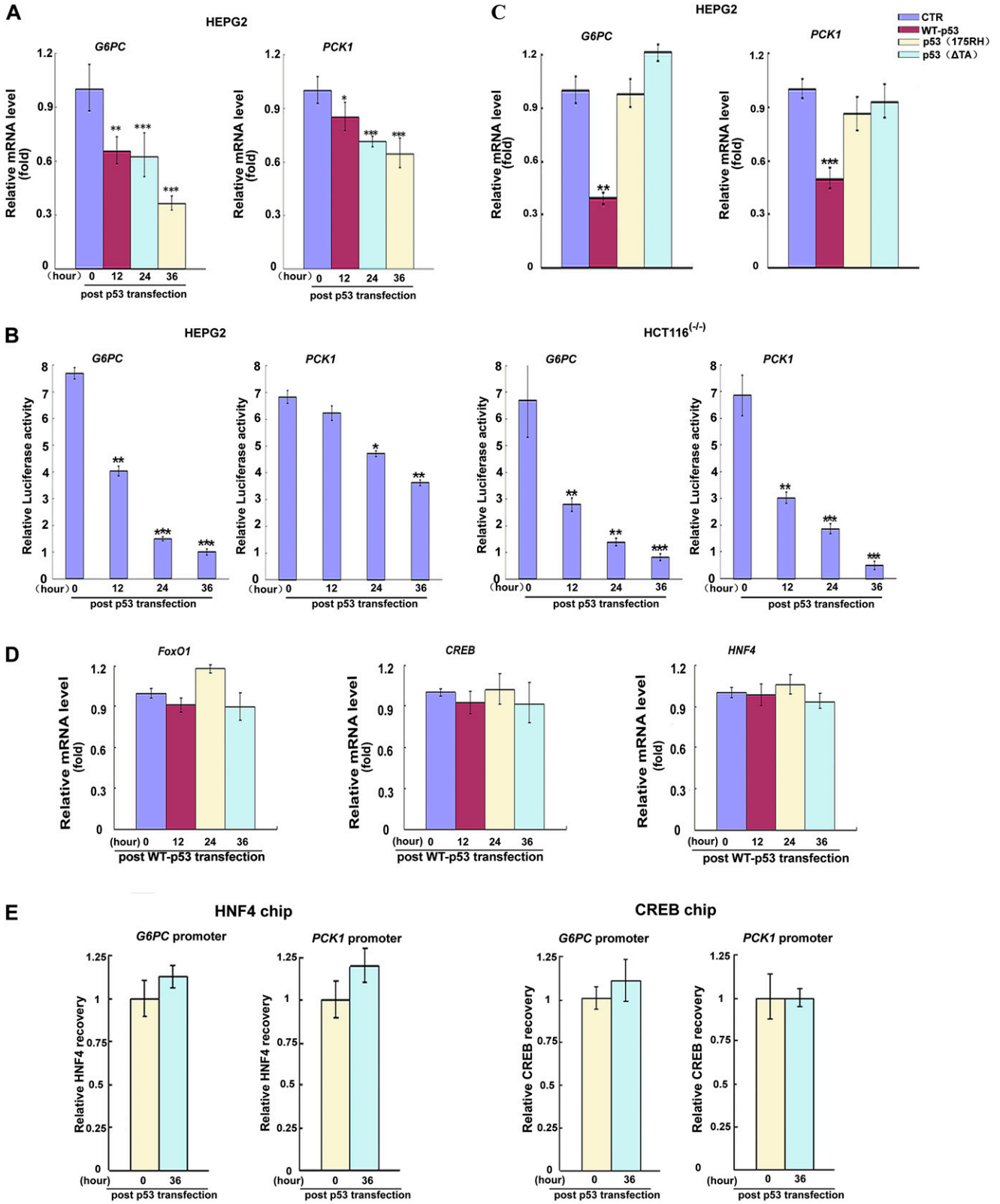

Fig. S1. p53 down-regulates expression of *G6PC* and *PCK1*. (*A*) Quantitative PCR (qPCR) analysis of *G6PC* and *PCK1* expression in HEPG2 cells. HEPG2 cells were transfected with an empty vector as a control (hereafter all transfected empty plasmids served as controls) or a plasmid expressing p53, and mRNA was extracted at 12 h, 24 h, or 36 h after p53 transfection to detect expression of *G6PC* and *PCK1*. mRNA levels of the CTR sample were set as 1, and relative mRNA levels of other samples were normalized to this control. (*B*) p53 down-regulates expression of *G6PC* and *PCK1* in a luciferase reporter assay. Twelve hours after transfection of luciferase reporters for *G6PC* and *PCK1* promoters, HEPG2 (*Left*) and HCT116p53$^{-/-}$ (*Right*) cells were transfected with an empty plasmid or a plasmid expressing p53. After 12 h, 24 h, or 36 h, cells were harvested, and their relative luciferase activities were measured and normalized to protein concentrations. (*C*) p53 transcription activity is required for down-regulation of *G6PC* and *PCK1*. HEPG2 cells were transfected with an empty plasmid or

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plasmids expressing p53, p53(175RH), or p53(ΔTA). After 36 h, relative mRNA expression levels were measured by qPCR. (*D*) Effect of ectopic p53 on expression of *FoxO1*, *HNF4*, and *CREB*. RNA was extracted from the HCT116$^{(-/-)}$ cells at different times after transfection with an empty plasmid or with a plasmid expressing p53, and qPCR was performed to analyze mRNA levels. (*E*) Effect of p53 on CREB and HNF4 binding to the promoters of *G6PC* or *PCK1*. A binding activity of CREB and HNF4 to the promoters of *G6PC* or *PCK1* in HCT116$^{(-/-)}$ cells was analyzed by qChIP 36 h after transfection with empty plasmid or with a plasmid expressing p53. The relative recovery of protein binding to the promoters in samples with empty plasmid transfection was set as 1, and the relative recovery of the other samples was normalized to this control. All experiments above were repeated at least three times. The data are shown as the mean ± SD with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

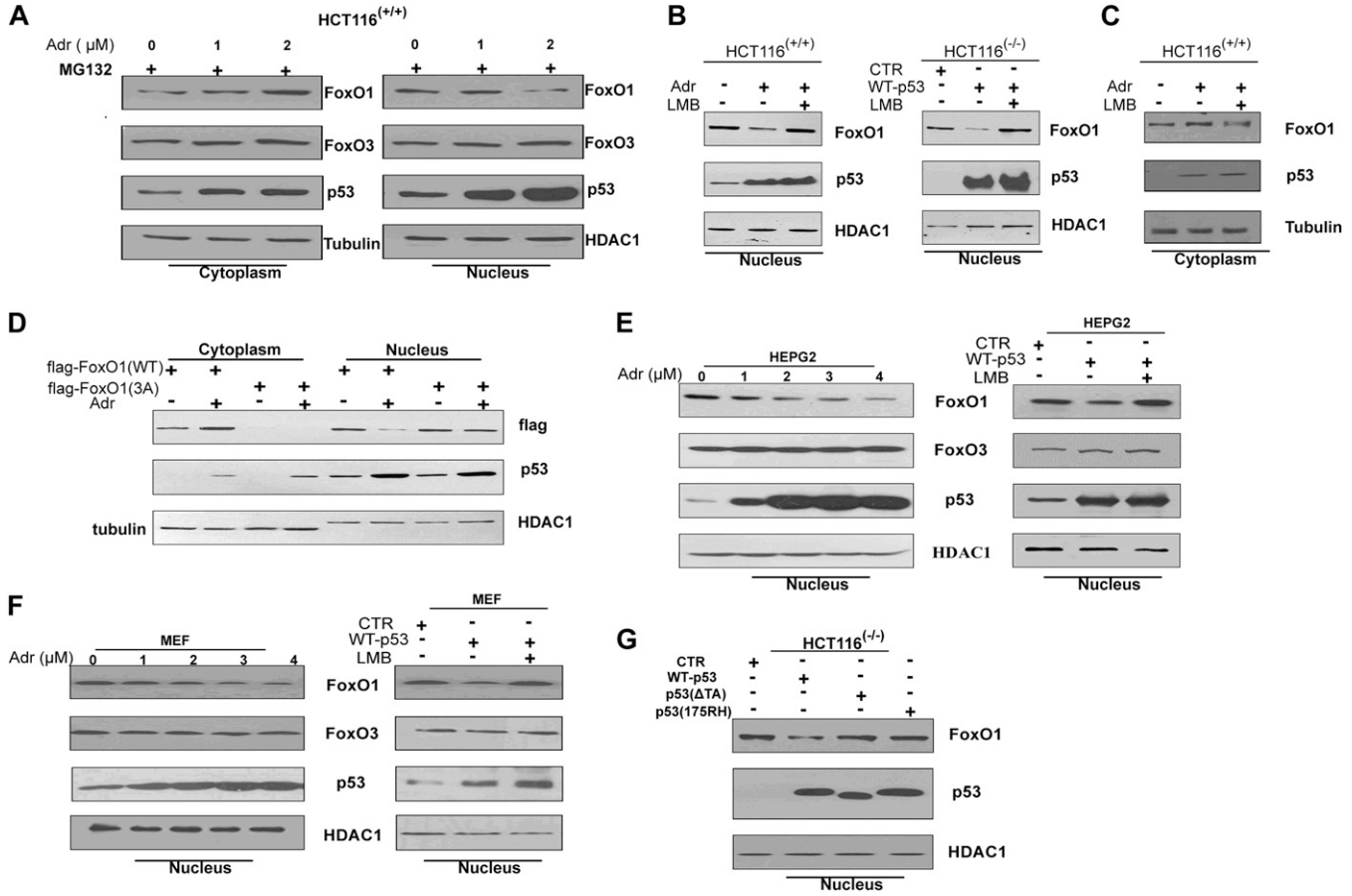

Fig. S2. p53 activation elicits FoxO1 nuclear exclusion. (*A*) Translocation of endogenous FoxO1 from nucleus to cytoplasm in response to adriamycin treatment. Cytoplasmic and nuclear lysates were prepared from HCT116$^{(+/+)}$ cells after adriamycin treatment in the presence of MG132 (2 μM for 12 h), and immunoblotting was performed to detect levels of FoxO1, FoxO3, or p53. HDAC1 and tubulin were used as loading controls for nuclear and cytoplasmic proteins, respectively. (*B*) Nuclear exclusion of endogenous FoxO1 in HCT116 cells in response to adriamycin treatment. Nuclear FoxO1 levels in HCT116$^{(+/+)}$ cells after adriamycin treatment (1 μM for 12 h) or in HCT116$^{(-/-)}$ cells transfected with p53 for 36 h in the presence or absence of LMB (0.5 ng/mL, 6 h before harvest; the same for experiments with LMB below). (*C*) HCT116$^{(+/+)}$ cells were treated with adriamycin at 1 μM for 12 h in the presence of LMB, and extracted cytoplasmic proteins were subjected to Western blotting. (*D*) Nuclear exclusion of ectopic FoxO1 in HCT116$^{(+/+)}$ cells in response to adriamycin treatment. HCT116$^{(+/+)}$ cells were transfected with flag-tagged FoxO1 (WT) or flag-tagged FoxO1 (3A), in the absence or presence of adriamycin. The levels of cytosolic or nuclear flag-FoxO1 or p53 were determined. (*E*) FoxO1 nuclear exclusion in HEPG2 cells. Nuclear protein from HEPG2 cells treated with adriamycin at up to 4 μM for 12 h (*Left*) or from HEPG2 cells transfected with plasmid expressing p53 (for 36 h) in the absence or presence of LMB (0.5 ng/mL, for 6 h) (*Right*) determined by immunoblotting with an anti-FoxO1, an anti-FoxO3, or an anti-p53 antibody. (*F*) p53 induction of FoxO1 nuclear exclusion in MEFs. MEF cells were treated as in *E*, and nuclear FoxO1, FoxO3, and p53 were determined by immunoblotting. (*G*) p53 induction of FoxO1 nuclear exclusion dependent on its transcription activity. HCT116$^{(-/-)}$ cells were transfected with an empty plasmid, a plasmid expressing p53, a plasmid expressing p53(175RH), or a plasmid expressing p53 (ΔTA), and nuclear protein was then extracted 36 h after transfection for immunoblotting using an anti-FoxO1 or an anti-p53 antibody.

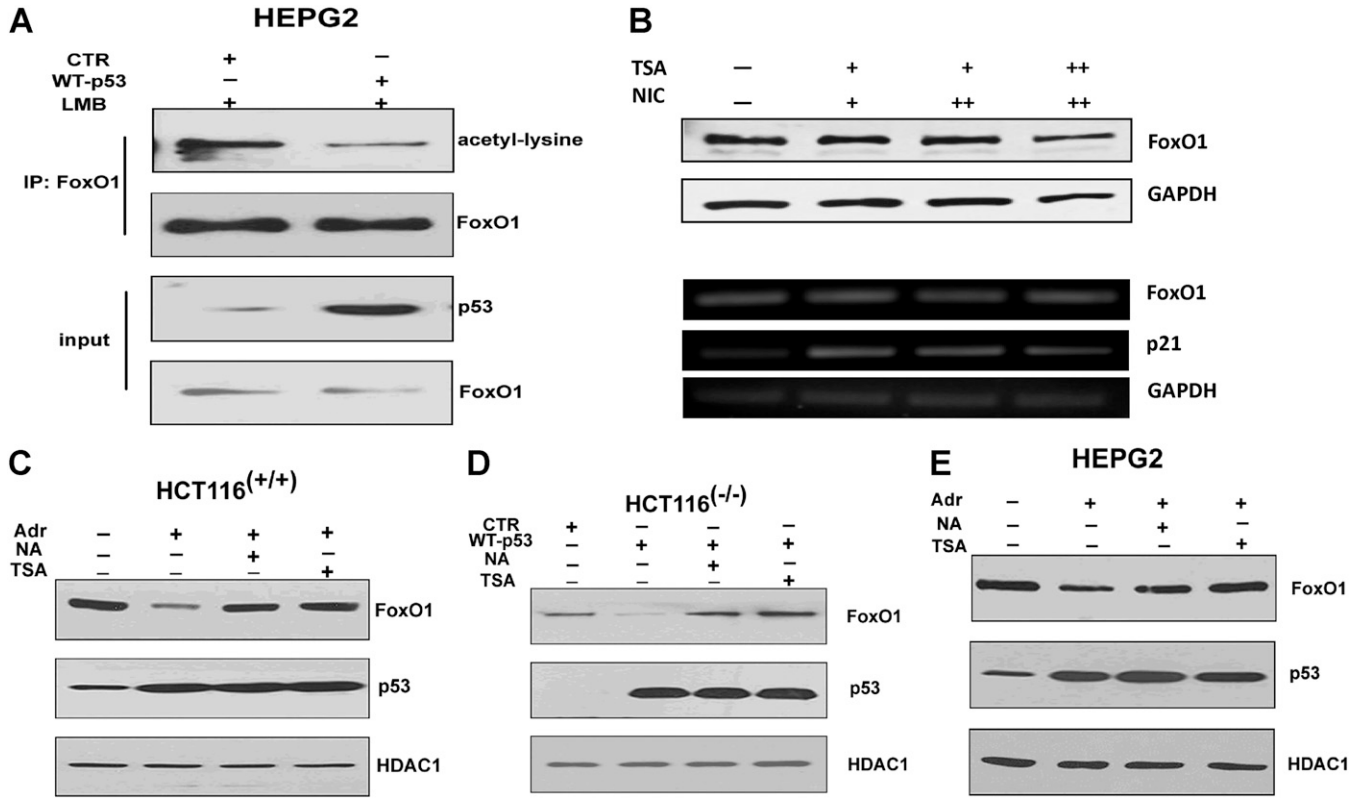

Fig. S3. p53 activation is associated with deacetylation of FoxO1. (*A*) Induction of FoxO1 deacetylation by overexpression of p53 in HEPG2 cells. HEPG2 cells were transfected with an empty plasmid or a plasmid expressing p53 in the presence of LMB (0.5 ng/mL for 6 h). Nuclear proteins were extracted, immunoprecipitated with anti-FoxO1, and immunoblotted with anti–acetyl-lysine antibody. (*B*) HEK293 cells were treated with TSA or NIC at different concentrations for 12 h, and proteins or mRNA were extracted for Western blotting or RT-PCR, respectively, to detect expression of FoxO1. (*C*–*E*) Suppression of FoxO1 nuclear exclusion by HDAC inhibitors. Immunoblotting was performed to determine the nuclear FoxO1 levels in the $HCT116^{(+/+)}$ (*C*) and HEPG2 (*E*) cells in response to adriamycin treatment (1 μM for 12 h) or in $HCT116^{(-/-)}$ cells transfected with p53 for 36 h in the presence of TSA (1 μM for 6 h) or NA (5 mM for 6 h) (*D*).

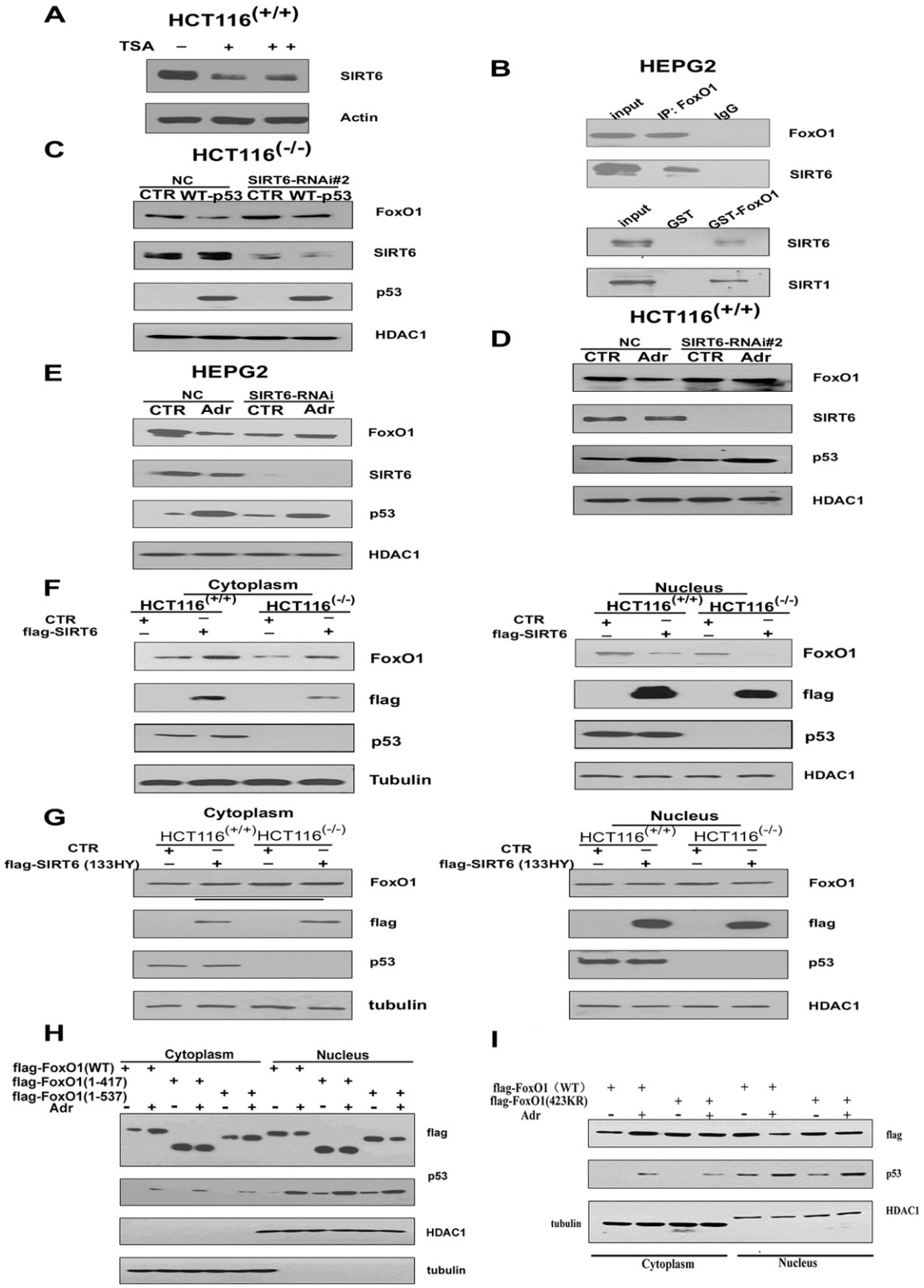

Fig. S4. p53 activation enhances the interaction of SIRT6 and FoxO1, which may induce FoxO1 deacetylation at K423. (*A*) HCT116$^{(+/+)}$ cells were treated with TSA at 1 μM or 2 μM for 12 h, and proteins were extracted for Western blotting by using anti-SIRT6 antibody. (*B*) Interaction between FoxO1 and SIRT6 in HEPG2 cells. A Co-IP assay (*Upper*) was performed to detect interaction of FoxO1 and SIRT6 in HEPG2 cells. A GST pulldown assay (*Lower*) was performed to verify the interaction between FoxO1 and SIRT6 or SIRT1 in HEPG2 cells. (*C*) SIRT6 knockdown blocks FoxO1 nuclear exclusion. Both nonspecific siRNA- and SIRT6-RNAi-pretreated HCT116$^{(-/-)}$ cells were transfected with p53 or with an empty plasmid. Nuclear extracts were then blotted with an anti-FoxO1 antibody, an anti-SIRT6 antibody, or an anti-p53 antibody. HDAC1 was used as a loading control for nuclear proteins. (*D*) SIRT6 knockdown blocks FoxO1 nuclear exclusion in HCT116$^{(+/+)}$ cells in response to adriamycin treatment. Both nonspecific siRNA$^{\#2}$ fragment and SIRT6RNAi$^{\#2}$ fragment pretreated HCT116$^{(+/+)}$ cells

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were cultured with or without adriamycin (1 μM for 12 h). Nuclear extracts were then blotted with an anti-FoxO1 antibody, an anti-SIRT6 antibody, or an anti-p53 antibody. HDAC1 was used as a loading control for nuclear proteins. (*E*) SIRT6 knockdown blocks FoxO1 nuclear exclusion in HEPG2 cells. Both nonspecific siRNA- and SIRT6-RNAi-pretreated HEPG2 cells were cultured with or without adriamycin (1 μM for 12 h). Nuclear extracts were then blotted with an anti-FoxO1 antibody, an anti-SIRT6 antibody, or an anti-p53 antibody. HDAC1 was used as a loading control for nuclear proteins. (*F*) Overexpression of SIRT6 elicits FoxO1 nuclear exclusion. $HCT116^{(+/+)}$ or $HCT116^{(-/-)}$ cells were transfected with an empty plasmid or a flag-tagged SIRT6 plasmid. After 48 h, the cytoplasmic (*Left*) or nuclear (*Right*) lysates were separately blotted with an anti-FoxO1 antibody, an anti-flag antibody, or an anti-p53 antibody. HDAC1 and tubulin were used as the loading controls for the nuclear protein and cytoplasmic protein, respectively. (*G*) SIRT6 deacetylase activity is required for FoxO1 nuclear exclusion. $HCT116^{(+/+)}$ or $HCT116^{(-/-)}$ cells were transfected with an empty plasmid or a flag-tagged SIRT6 (133HY) plasmid. After 48 h, cytoplasmic or nuclear lysates were separately blotted with anti-FoxO1 antibody, anti-flag antibody, or anti-p53 antibody. (*H*) Identification of a FoxO1 fragment deacetylated by SIRT6. $HCT116^{(+/+)}$ cells were transfected with a flag-tagged FoxO1 (WT), a flag-tagged FoxO1 (1-417), or a flag-tagged FoxO1 (1-537) plasmid with or without adriamycin treatment (1 μM, for 12 h). After 36 h, cytosolic and nuclear extracts were separately immunoblotted with anti-flag antibody or anti-p53 antibody. (*I*) Lysine 423 of FoxO1 is a potential deacetylated site for SIRT6. $HCT116^{(+/+)}$ cells were transfected with a flag-tagged FoxO1 (WT) or a flag-tagged FoxO1 (423KR) vector with or without adriamycin treatment (1 μM, for 12 h). After 36 h, the cytosolic and nuclear extracts were separately immunoblotted with an anti-Flag antibody or an anti-p53 antibody.

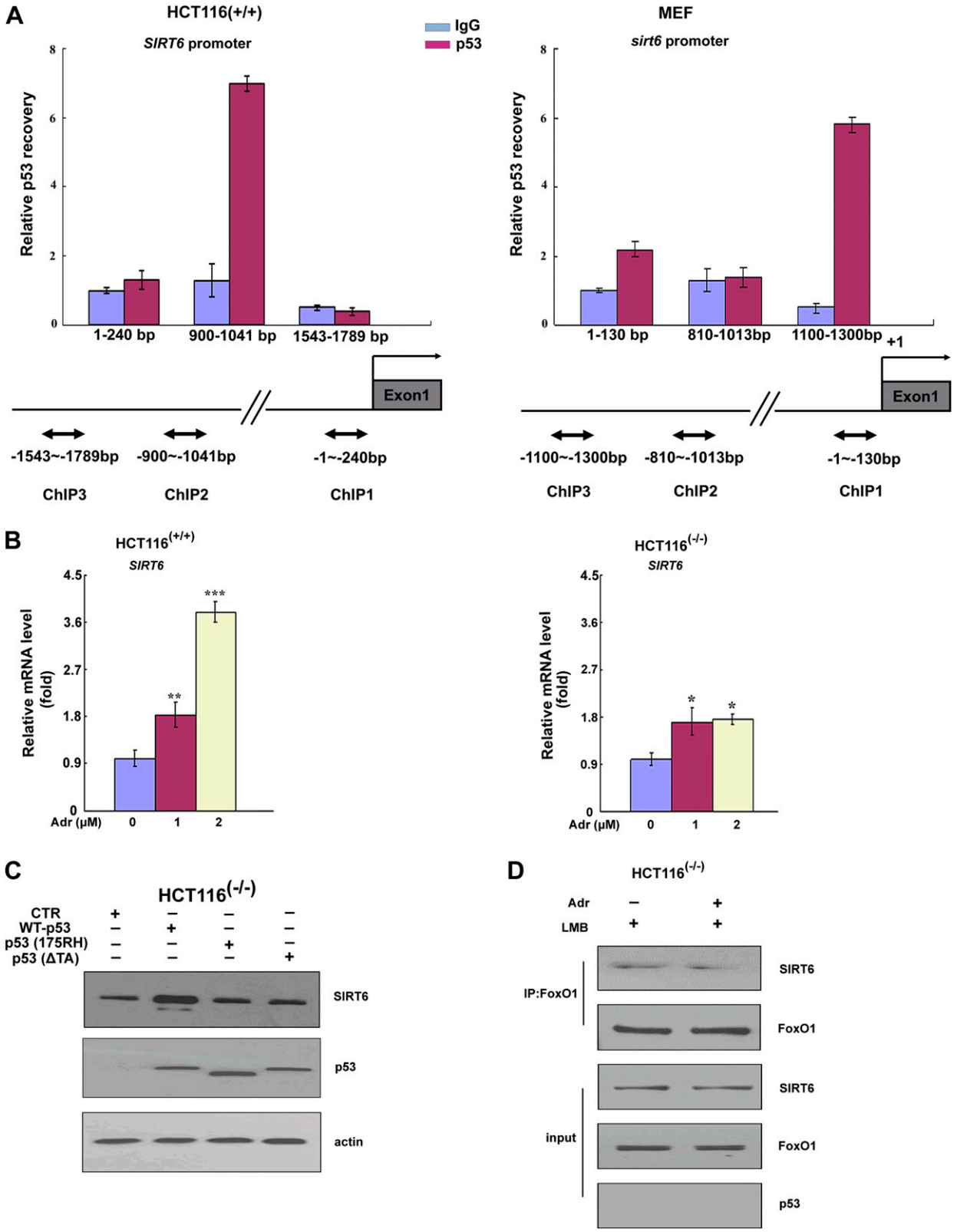

Fig. S5. p53 directly binds to the promoter of *SIRT6* and activates its expression. (*A*) qChIP analysis of p53 binding to *SIRT6*/*Sirt6* promoters in HCT116$^{(+/+)}$ cells (*Left* graph) or MEFs (*Right* graph). The fragment between 900 bp and 1,041 bp of the human *SIRT6* promoter contains one predicted p53 binding site as determined in HCT116$^{(+/+)}$ cells, and the fragment between 1,100 bp and 1,300 bp of the murine *Sirt6* promoter contains one predicted p53 binding site as determined in MEF cells. A diagram at the bottom shows the fragments for designing the qChIP primers. (*B*) qPCR analysis of *SIRT6* expression in HCT116$^{(+/+)}$ (*Left* graph) and HCT116$^{(-/-)}$ (*Right* graph) cells subjected to variable doses (0 μM, 1 μM, or 2 μM for 12 h) of adriamycin. Data are shown as the mean ± SD with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*C*) Requirement of p53 transcription activity for *SIRT6* expression. HCT116$^{(-/-)}$ cells were transfected separately with an empty plasmid, a plasmid expressing p53, a plasmid expressing p53(175RH), or a plasmid expressing p53(ΔTA), and, after 36 h, derived whole-cell lysates were

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immunoblotted with anti-SIRT6 or anti-p53 antibody. (*D*) Requirement of p53 transcription activity for the interaction between SIRT6 and FoxO1. HCT116$^{(-/-)}$ cells were cultured with or without adriamycin (1 μM for 12 h) in the presence of LMB (0.5 ng/mL for 6 h before harvest). Nuclear proteins were then extracted for coimmunoprecipitation with anti-FoxO1 antibody and probed with anti-SIRT6 antibody.

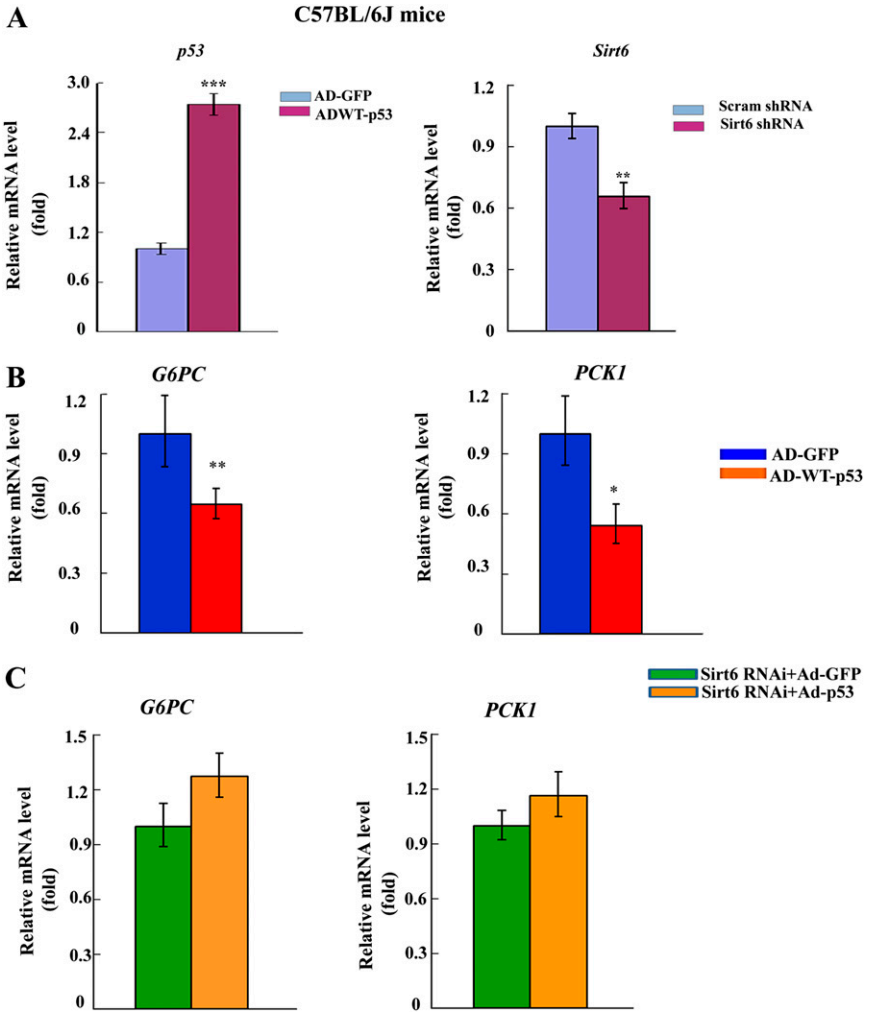

Fig.S6. Effect of p53 on *G6PC* and *PCK1* expression in liver tissues of C57BL/6J and *Sirt6* KO mice. (*A*) Expression of p53 and *Sirt6* in the liver of C57BL/6J and *Sirt6*-knockdown mice. mRNA was extracted from mice injected with a control (Ad-GFP) or a p53-expressing (Ad-WT-p53) adenovirus vector, and expression of p53 was assessed by qPCR (*Left* graph). The efficacy of *Sirt6* siRNA was also evaluated by qPCR in the liver tissue of Scram shRNA or Sirt6 shRNA-injected mice (*Right* graph). (*B*) Expression of *G6PC* and *PCK1* in C57BL/6J mice following SIRT6 knockdown. The same treatments described in *A* were applied to *Sirt6* siRNA-pretreated C57BL/6J mice, and *G6PC* and *PCK1* expression levels were assessed by qPCR. Twelve mice were separated into three groups, with four mice per group. Data are reported as the mean ± SD with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (*C*) Expression of *G6PC* and *PCK1* in C57BL/6J mice. A qPCR analysis was performed to assess *G6PC* and *PCK1* expression levels in the liver tissues of C57BL/6J mice injected with a control (Ad-GFP) or p53-expressing (Ad-WT-p53) adenovirus vector after 22 h of fasting.