Supporting Information

Pan *et al.* 10.1073/pnas.0810147106

SI Materials and Methods

ChIP Analysis. Cells (1×10^7) seeded in 10-cm plates were treated with 1 μg/mL doxorucin for 6 h, cross-linked with 1% formaldehyde, and then lysed in SDS lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris (pH 8.1)]. The ChIP procedure was performed as described in ref. 1 by using anti-p53 (DO-1) and anti-Myc antibodies. A control IgG also was included. The primers used in this study were 5′-CAGGCTGTGGCTCTGATTGG-3′ and 5′-TTCAGAGTAACAGGCTAAGG-3′ for p21 promoter; and 5′-CGGTGCGTGCCCAGTTG-3′, 5′- GCGACGCAAAAGAAGATG-3′ for GAPDH primers.

Flow Cytometry. Cells cotransfected with GFP and Myc-SOX4 or empty vector were harvested and resuspended in 0.3 mL of PBS, containing 10% FBS, and ice-cold 100% ethanol (0.7 mL). Then, cells were incubated with RNase A (10 mg/mL) and 50 mg/mL PI (Sigma). GFP-positive cells were sorted and analyzed for DNA content with a FACScan flow cytometer (Becton Dickinson).

RNA Interference and Stable Cell Line Construction. The small-interfering RNA (siRNA) targeting the SOX4 gene was chemosynthesized and was used as a pool of 2 SOX4-specific siRNAs. The target sequences are 5′-GCGACAAGATCCCTTTCAT-3′ and 5′-ACGCTGGAAGCTGCTCAAA-3′. The nonspecific siRNA (5′-GACGGTCCAGATTCGTGTA-3′) was used as a control. For stable knockdown of SOX4, pSuper-retro RNAi retrovirus vector expressing SOX4 siRNA (target sequences: 5′-GACGACCTGCTCGACCTGA-3′; 5′-GCGACAAGATCCCTTTCAT-3′; 5′-AGACAGCGACAAGATCCCT-3′; and 5′-CTCCAAACCGGCGCAGAAA-3′) were constructed by recombinant DNA technology. The packaging cell Phoenix (from American Type Culture Collection) was transfected with these combinant plasmids by using liposome-based transfection method, and the virus supernatant was collected and then infected HCT116 cells. The stable integrant was selected by using puromycin, and the stable cell lines that silenced of SOX4 gene were obtained. For the stable cells expressing SOX4, SOX4 cDNA was inserted into pBabe-retro-puro retrovirus vector. HCT116 cells were infected with virus supernatant from Phoenix cells transfected with pBabe-SOX4 and selected with puromycin.

Fluorescence Microscopy. For endogenous p53 and SOX4 location, H460 cells were seeded in 6-well plates onto glass coverslips. At 24 h later, the cells were fixed and analyzed by fluorescence microscopy as described by Man *et al.* [(2006) Pias3 induction of prb sumoylation represses prb transactivation by destabilizing its retention in the nucleus. *Nucleic Acids Res* 34:5552–5566].

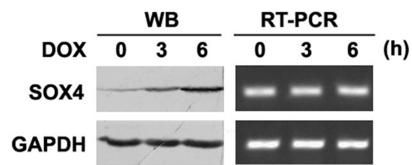

Fig. S1. DOX treatment does not increase SOX4 mRNA level. Lysates from H460 cells treated with DOX at the indicated times were subjected to immunoblotting (*Left*), and the total RNAs from the same cells were extracted and analyzed by RT-PCR (*Right*)

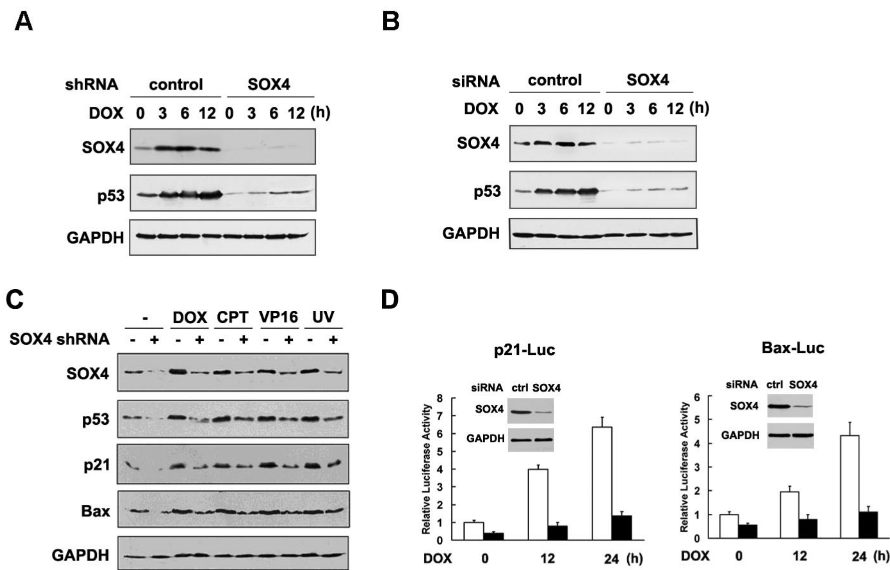

Fig. S2. Knockdown of SOX4 resulted in subdued activation of p53 target proteins by DNA damage. (*A*) An additional clone of HCT116 $p53^{+/+}$ SOX4 shRNA-stable cells were treated with DOX for the indicated times, and cell lysates were analyzed by immunoblotting. (*B*) HCT116 $p53^{+/+}$ cells transfected with control or SOX4-specific siRNAs were treated with DOX for the indicated times, and the cell lysates were analyzed by immunoblotting. (*C*) HCT116 $p53^{+/+}$ control or SOX4 shRNA-stable cells were treated with DNA damage agents as indicated. At 6 h after treatment, cells were harvested, and cell lysates were analyzed by immunoblotting. (*D*) HCT116 $p53^{+/+}$ cells were transfected with control siRNA (white bars) or SOX4 siRNA (black bars). At 48 h after the transfection, the cells were transfected with p21-Luc (*Left*) or Bax-Luc (*Right*) together with Renilla reporters. Then, the cells were treated with DOX for the indicated times, and luciferase assays were performed. The knockdown efficiencies of SOX4 at 0 h are shown.

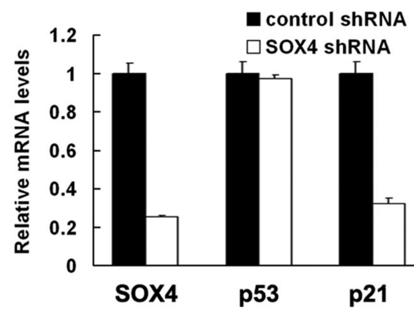

Fig. S3. Knockdown of SOX4 decreases p21 but not p53 mRNA level. Total RNAs from HCT116 $p53^{+/+}$ control or SOX4 shRNA cells were extracted and analyzed by real-time PCR.

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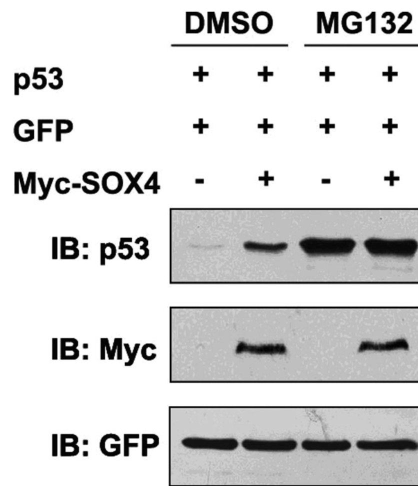

Fig. S4. The stabilization of p53 by SOX4 is blocked by MG132 treatment. HCT116 p53$^{-/-}$ cells were transfected with the indicated plasmids. Cells were treated with DMSO or 20 μM MG132 for 6 h before harvest, and the lysates were analyzed by immunoblotting.

Fig. S5. SOX4 increases p53 transcriptional activity. (*A*) SOX4 increases activity of the p53-responsive luciferase reporter from the p21 and Bax promoter. H1299 cells were cotransfected with 50 ng of p53 expression vector, 0.5 μg of p21-Luc (*Left*) or Bax-Luc (*Right*) reporter, and increasing amounts of SOX4 expression vector as shown. Luciferase activity was measured at 24 h after the transfection. (*B*) Knockdown of SOX4 decreases endogenous p53-mediated transactivation. HCT116 $p53^{+/+}$ control or SOX4 shRNA cells were transfected with PG13-Luc reporter plasmid, and luciferase activity then was measured at 24 h later. The luciferase activity of PG13-Luc in control cells was arbitrarily set at 1. (*C*) SOX2 does not affect p53 activity. H460 cells were cotransfected with PG13-Luc and SOX4 or SOX2 expression vectors as indicated. Luciferase activity was measured at 24 h after transfection. All of the results are means ± SEM of 3 independent experiments.

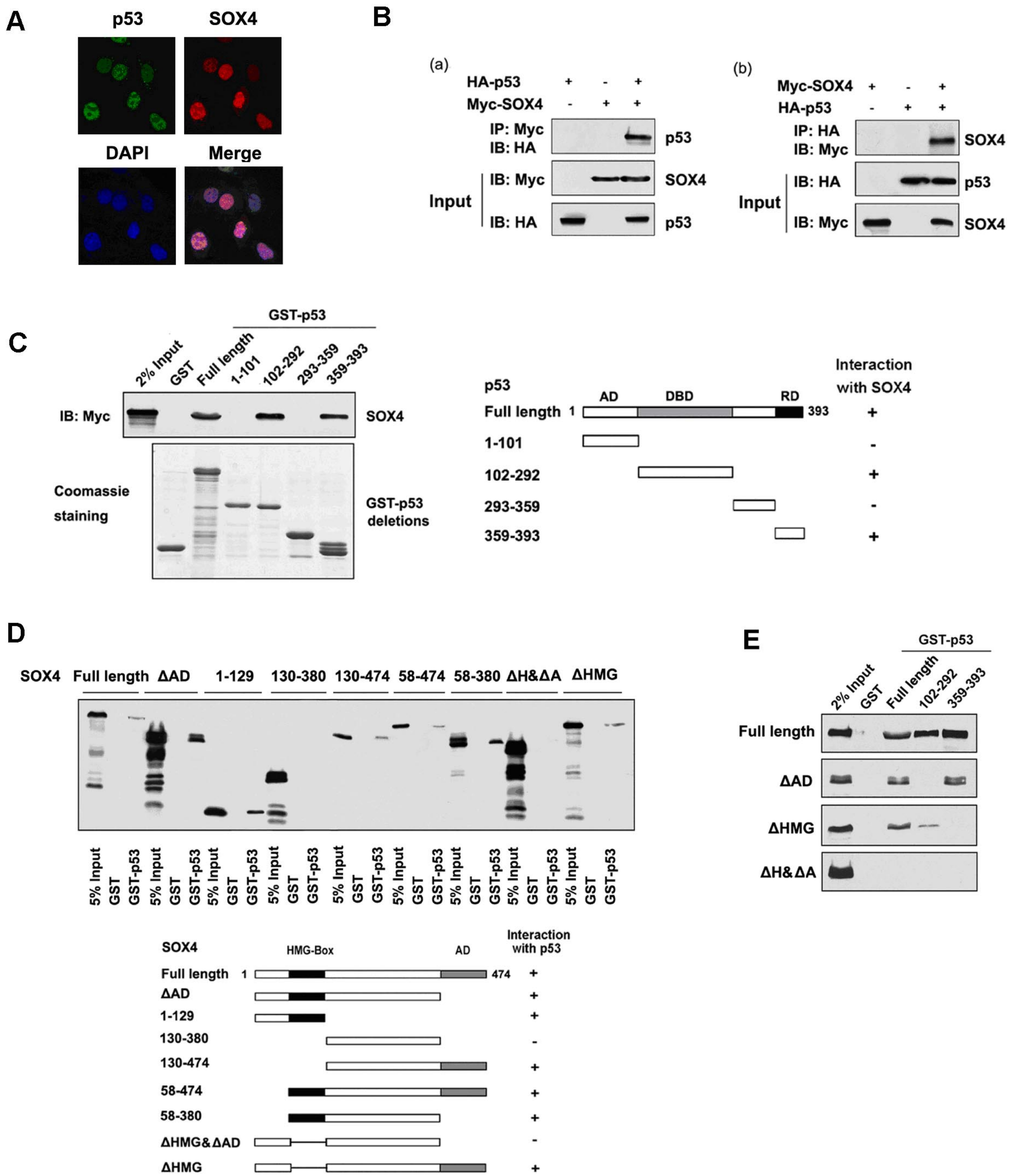

Fig. S6. SOX4 interacts with p53. (*A*) SOX4 and p53 colocalize in the nuclei of H460 cells. The localization of endogenous SOX4 and p53 was visualized by immunofluorescence using anti-SOX4 or anti-p53 (FL-393) antibodies. (*B*) SOX4 interacts with p53 in vivo. HEK293 cells were transfected with 2 μg of HA-p53 and/or Myc-SOX4 expression plasmids as indicated. Whole-cell lysates then were immunoprecipitated with anti-Myc (*Left*) or anti-HA (*Right*) monoclonal antibodies and immunoblotted with anti-HA polyclonal antibody or anti-Myc antibody, respectively. (*C*) Mapping of SOX4-binding regions in p53. (*Left*) Full-length or truncated GST–p53 fusion proteins were incubated with cell lysates from HEK293 cells transiently transfected with Myc-SOX4 vector. After extensive washes, bound proteins were analyzed by immunoblotting with anti-Myc antibody. The GST fusion proteins were resolved by SDS/PAGE and stained with Coomassie bright blue. (*Right*) A schematic diagram depicting different p53 deletion mutants used in the domain mapping experiments. (*D*) Mapping of p53-binding regions in SOX4. (*Upper*) GST or GST–p53 fusion protein was incubated with Myc-SOX4 or its truncation vectors. After extensive washes, the bound proteins were analyzed by immunoblotting with anti-Myc antibody. (*Lower*) A schematic diagram of the various SOX4 protein deletion mutants. (*E*) Mapping of interactive domains in p53 and SOX4. GST-fused full-length p53 protein or its DBD fragment (102–292) and C-terminal fragment (359–393) were incubated with lysates of HEK293 cells transfected with Myc-tagged, full-length SOX4 or deletion mutants as indicated. Bound proteins were analyzed by immunoblotting with anti-Myc antibody.

Fig. S7. SOX4 mutants lacking p53-binding domains fail to stabilize and activate p53. (*A*) HCT116 $p53^{-/-}$ cells transfected with p53, Myc-SOX4, or its truncation expression plasmids were treated with 20 μM CHX and were harvested at the indicated times. The lysates were subjected to immunoblotting (*Left*). Relative p53 levels were quantified by densitometry (*Right*). (*B*) Myc-tagged SOX4 or its truncation was transfected into HCT116 $p53^{+/+}$ cells. Immunoblotting was performed at 48 h after transfection. (*C*) HCT116 $p53^{+/+}$ cells were transfected with pp53-TA-Luc, increasing amounts of full-length SOX4, or its deletion mutant as indicated. Luciferase activities were measured at 24 h after transfection.

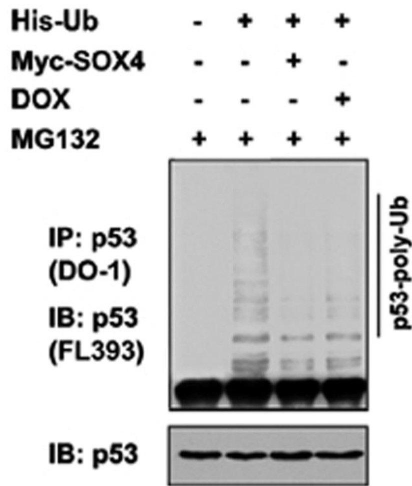

Fig. S8. Overexpression of SOX4 reduced the level of p53 ubiquitination. HCT116 p53$^{+/+}$ cells were transfected with Myc-SOX4 and His-ubiquitin-expressing plasmids as indicated. At 36 h after the transfection, cells were treated with or without DOX for 6 h, followed by the treatment of 20 μM MG132 for 6 h. Cell lysates were immunoprecipitated with anti-p53 (DO-1) antibody and analyzed by immunoblotting with anti-p53 (FL-393) antibody.

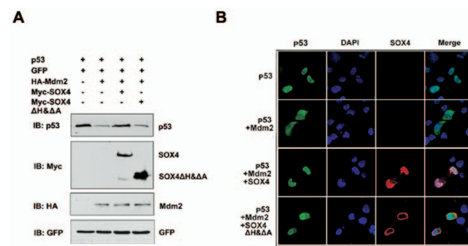

Fig. S9. SOX4 reverses Mdm2-mediated p53 degradation and nuclear export. (*A*) HCT116 p53$^{-/-}$ cells were transfected with p53, GFP, HA-Mdm2, and Myc-SOX4 or Myc-SOX4ΔH&ΔA expression vectors as indicated. Cell lysates were analyzed by immunoblotting. (*B*) SOX4 prevents Mdm2-mediated p53 nuclear export. H1299 cells were cotransfected with p53, HA-Mdm2, and Myc-SOX4 or Myc-SOX4ΔH&ΔA expression vectors in various combinations as indicated. At 48 h after the transfection, the cells were fixed and stained by using anti-p53 and anti-Myc antibodies for visualization by immunofluorescence. DAPI was used for staining of nucleus. (Original magnification: ×100.)

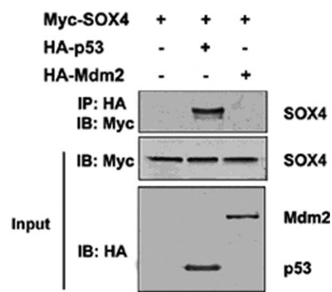

Fig. S10. SOX4 does not bind to Mdm2. HCT116 $p53^{-/-}$ cells were cotransfected with Myc-SOX4 and HA-Mdm2 or HA-p53-expressing plasmids. Cell lysates were immunoprecipitated with anti-HA antibody, and the immunoprecipitates and cell lysates were analyzed by immunoblotting.

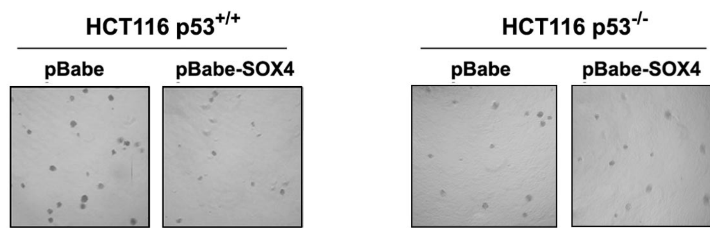

Fig. S11. SOX4 inhibits cell growth in soft agar. A total of 1×10^4 HCT116 p53$^{+/+}$ cells (*Left*) or p53$^{-/-}$ cells (*Right*) stably expressing empty vector (pBabe) or SOX4 (pBabe-SOX4) were seeded in semisolid medium. The photographs are representative fields of the soft agar colonies formed at day 14. (Original magnification: ×10.)