Supporting information

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SI Materials and Methods

Antibodies. Antibodies used in this study include: anti-Cul4 (Santa Cruz), anti-Cyclin D1(Santa Cruz), anti-Myc (Santa Cruz), anti-Skp2 (Santa Cruz), anti-Ubiquitin (Santa Cruz), anti-Myc (Cell Signaling), anti-FOXO3 (Cell Signaling), anti-phospho-Chk2 (Thr68, Cell Signaling), anti-phospho-p53 (Ser15, Cell Signaling), anti-Cyclin A (sigma), anti-Flag (M2, Sigma), anti-His (Sigma), anti-Actin (Sigma), anti-DDB1 (Bethyl), anti-p27 (Upstate), anti-PCNA (AB29, Abcam), anti-p53 (Santa Cruz), and anti-phospho-H2AX (Ser139, Upstate). Anti-Cdt1 and anti-geminin antibodies were generated by immunizing rabbits with Cdt1 and geminin proteins fused to GST according to standard procedure.

Immunofluorescence. MCF-7 cells expressing Myc-Cdt1 and Flag-FOXO3 were fixed in 4.0% paraformaldehyde in PBS for 10 min at room temperature, washed twice with 0.4% Triton X-100 in PBS, and stained with monoclonal anti-myc (1:500) and rabbit polyclonal anti-Flag (1: 500) antibodies. Anti-mouse Alexa-Fluor 568 (Molecular Probes) and anti-rabbit Alexa-Fluor 488 (Molecular Probes) were used as secondary antibodies. Cells were then mounted with 70% glycerol mounting medium containing DAPI. Stained cells were visualized using the Leica Confocal Scanner TCS SP II.

In Vitro Binding Assay. GST, GST-Cdt1, His-FOXO3, and His-Cdt1 were produced in *Escherichia coli* strain BL21 and purified on glutathione-Sepharose beads or Ni-NTA. Purified His-FOXO3 or His-Cdt1 proteins were incubated with HEK293T total cell lysates expressing Myc-Cdt1 or Flag-FOXO3. Bound proteins were washed four times with triton lysis buffer and analyzed by SDS/PAGE, followed by Western blotting.

In Vivo Ubiquitination Assay. MCF-7 cells stably expressing indicated shRNAs were transiently transfected with HA-ubiquitin. Cell lysates were then harvested in modified RIPA buffer, and the ubiquitination assay was performed as previously described (1).

QRT-PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen). cDNAs were synthesized using reverse transcriptase followed by quantitative real-time PCR (QRT-PCR) using SYBR Green (Applied Biosystems). Primer sequences used were the following: Cdt1—GGCTGCAGCGCTTAGAAC (forward) and CAGCAGGTGCTTCTCCATTT (reverse); FOXO3—TCAATCAGAACTTGCTCCACCA (forward) and GGACTCACTCAAGCCCATGTTG (reverse); and TBP—GCACAGGAGCCAAGAGTGAA (forward) and GCACAGGAGCCAAGAGTGAA (reverse).

1. Lovejoy CA, Lock K, Yenamandra A, Cortez D (2006) DDB1 maintains genome integrity through regulation of Cdt1. *Mol Cell Biol* 26:7977–7990.

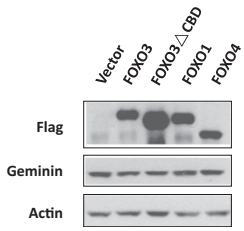

Fig. S1. Geminin protein levels remain unchanged in cells ectopically expressing indicated FOXO constructs. 293T cells transfected with indicated constructs were analyzed by Western blotting with anti-Flag, anti-geminin, and anti–β-actin antibodies.

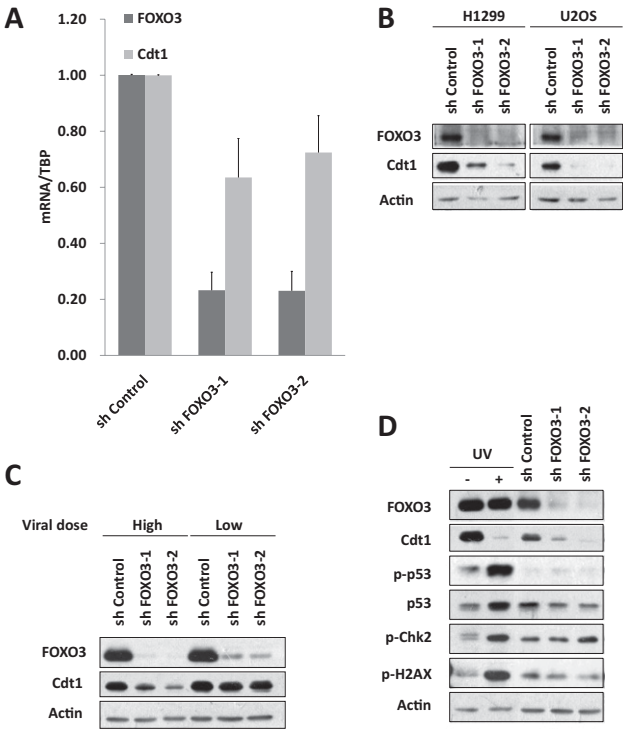

Fig. S2. FOXO3 interacts with Cdt1 and is required for cell proliferation. (*A*) RNA expression levels of FOXO3 and Cdt1 in FOXO3-depleting cells. Total RNA was prepared from MCF-7 cells infected with indicated shRNA lentivirus. FOXO3 and Cdt1 mRNA levels were then analyzed by QRT-PCR. Data shown are mean ± SD of three experiments. (*B*) Impaired Cdt1 protein expression levels upon FOXO3 depletion in human cancer cell lines. Cell lysates from H1299 and U2OS cells infected with the indicated shRNA lentivirus were analyzed by immunoblotting with anti-FOXO3 and anti-Cdt1 antibodies. Actin, loading control. (*C*) MCF-7 cells infected with different amounts of the indicated shRNA lentivirus were analyzed by immunoblotting with anti-FOXO3 and anti-Cdt1 antibodies. Actin, loading control. (*D*) FOXO3 depletion failed to activate DNA damage checkpoint. MCF-7 cells stably expressing FOXO3 shRNA or control shRNA were analyzed by immunoblotting with indicated antibodies. MCF-7 cells irradiated with UV (50 J/m^2) were analyzed as a positive control.

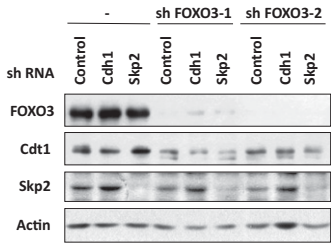

Fig. S3. Depletion of Skp2 or Cdh1 failed to block Cdt1 proteolysis in FOXO3-deficient cells. MCF-7 cells were infected with the indicated shRNAs. Cell extracts were prepared and analyzed by immunoblotting with anti-FOXO3, anti-Skp2, and anti-Cdt1 antibodies. Actin, loading control.

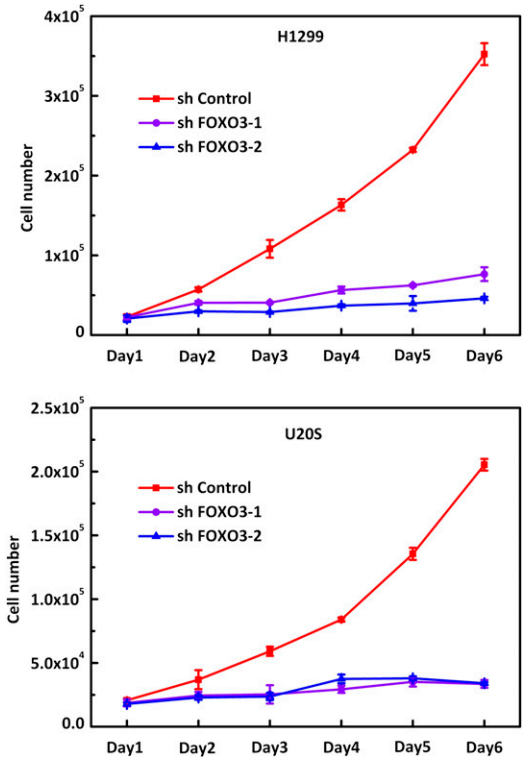

Fig. S4. Reduced cell proliferation in H1299 and U2OS cells upon FOXO3 depletion. Cells stably expressing FOXO3 or control shRNA were plated, and cell proliferation was then quantified at the indicated times. Data presented are the mean ± SD of three independent experiments.

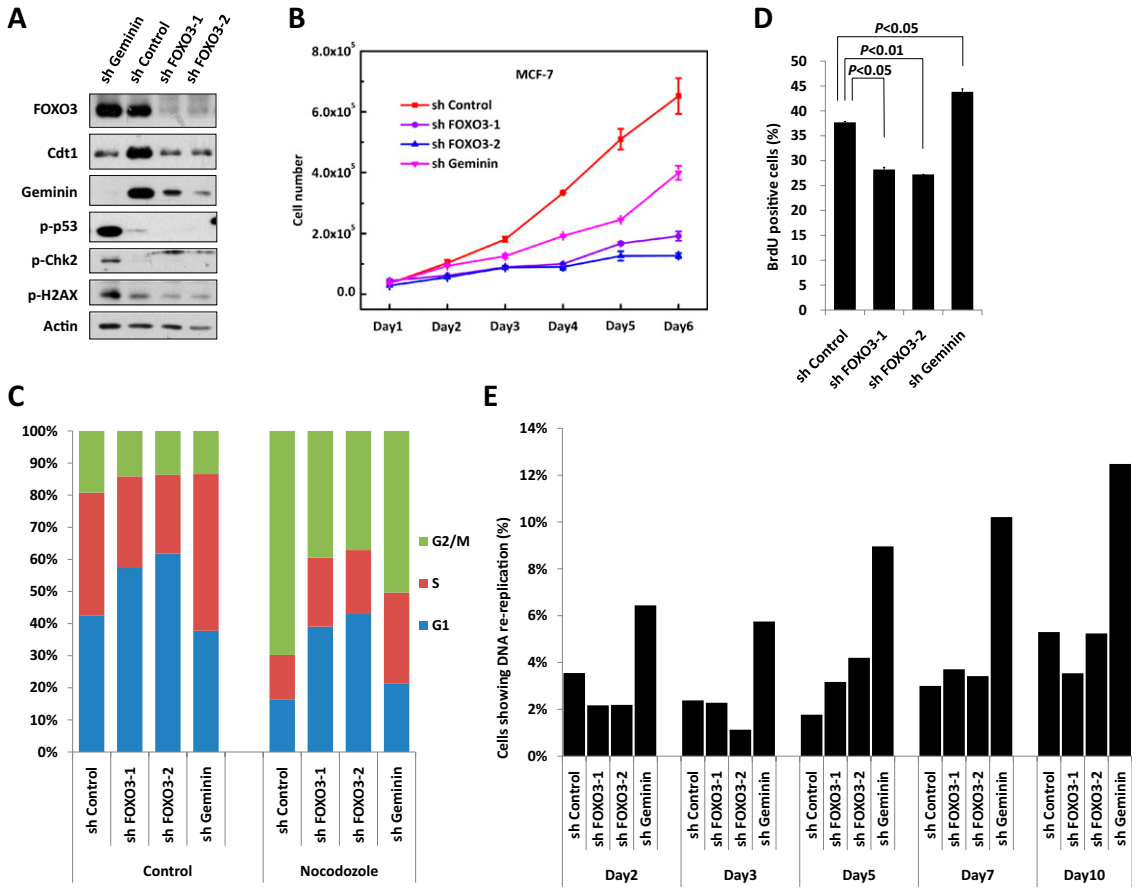

Fig. S5. Down-regulation of geminin upon FOXO3 depletion is secondary to Cdt1 destabilization. (*A*) MCF-7 cells were infected with indicated shRNA lentiviral vectors, and cell lysates were analyzed by immunoblotting with anti-geminin, anti-FOXO3, anti-p53Ser15, anti-Chk2Thr68, phosphor-H2AXSer139 antibodies. Actin, loading control. (*B*) Geminin knockdown reduced cell proliferation. MCF-7 cells stably expressing the indicated shRNAs were plated, and cell proliferation was then quantified at the indicated times. Data presented are the mean ± SD of three independent experiments. (*C*) Flow cytometry analysis by PI staining of the DNA content of cells in *A* before and after synchronization in mitosis with nocodazole (100 ng/mL) for 16 h. (*D*) Cells in *A* were pulsed with BrdU for 1 h and then stained with anti-BrdU antibody, followed by flow cytometry analysis for BrdU incorporation into DNA. Data represent mean ± SD of three independent experiments. (*E*) FOXO3-deficient cells failed to induce overreplication. Cells in *A* were collected at different time points after infection and subjected to PI staining, followed by FACS analysis.

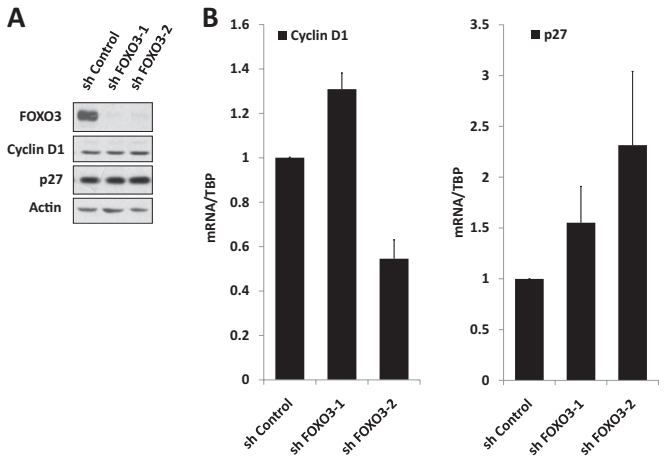

Fig. S6. p27 and cyclin D1 expression levels in FOXO3-deficient cells. (*A*) MCF-7 cells infected with the indicated shRNA lentivirus were lysed and analyzed by immunoblotting with the indicated antibodies. Actin, loading control. (*B*) RNA expression levels of cyclin D1 and p27 in FOXO3-depleting cells. Total RNA was prepared from MCF-7 cells infected with the indicated shRNA lentivirus. Cyclin D1 and p27 mRNA levels were then analyzed by QRT-PCR. Data shown are mean ± SD of three independent experiments.