The Deubiquitinase USP10 Antagonizes c-Myc Transcriptional Activation through SIRT6 Stabilization to Suppress Tumor Formation

Zhenghong Lin[#], Heeyoung Yang[#], Can Tan[#], Jinping Li[#], Zhaojian Liu, Qiu Quan, Sinyi Kong, Junsheng Ye, Beixue Gao and Deyu Fang[*]

1. **Supplemental Materials and Methods**
2. **Supplemental Figures 1-4 and their legends**

Supplemental Materials and Methods

Cells, plasmids, and antibodies. Human colon cancer p53 wildtype and $p53^{-/-}$ HCT116 cells were cultured in DMEM containing 10% FBS. *SIRT6*$^{+/+}$ and *SIRT6*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were isolated and used as described (Lin et al., 2012). Plasmid DNA that expresses USP10, USP22, as well as SIRT6 and c-Myc were purchased from Addgene. The mutants of USP10 and SIRT6 were generated using PCR-based methods and subcloned into a pCMV empty vector. Antibodies (Abs, and their sources) used in this study included anti-USP10 (Cell Signaling and Santa Cruz), anti-CDC2, anti-CCNA2 (Cell Signaling), anti-SIRT6 (Sigma Aldrich), anti-HA, anti-Flag, and anti-Myc (Santa Cruz Biotechnology). shRNAs that specifically knock down USP10, USP22, P53, SIRT6, and control shRNA were purchased from Open Biosystems.

Transfection, co-immunoprecipitation (co-IP), and western blotting. HEK293, HCT116, or RKO cells grown in 60-mm dishes were transfected with 1 to 2 μg of plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). The transiently transfected cells were collected and lysed with RIPA buffer with protease inhibitor and incubated on ice for 15 minutes. Insoluble fractions were removed by centrifugation (15,000 × *g* for 15 min). After a 15-min precleaning with protein G-Sepharose at 4^{o}C, the supernatants were incubated with the indicated Ab (1μg/ml) for 1 hour followed by incubation with protein G-Sepharose beads for 2 more hours. The protein G-Sepharose beads were washed 4 times with lysis buffer, dissolved with 4× loading buffer, and boiled for 5 min. Supernatants were subjected to SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% (w/v) skim milk in Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated overnight at 4^{o}C with the indicated primary Abs followed by horse radish peroxidase-conjugated secondary Ab. Membranes were then washed and visualized with enhanced chemiluminescence. When necessary, membranes were stripped using stripping buffer (Bio-Rad, Hercules, CA) and reprobed with corresponding Abs.

Supplemental Figures 1-4 and their legends

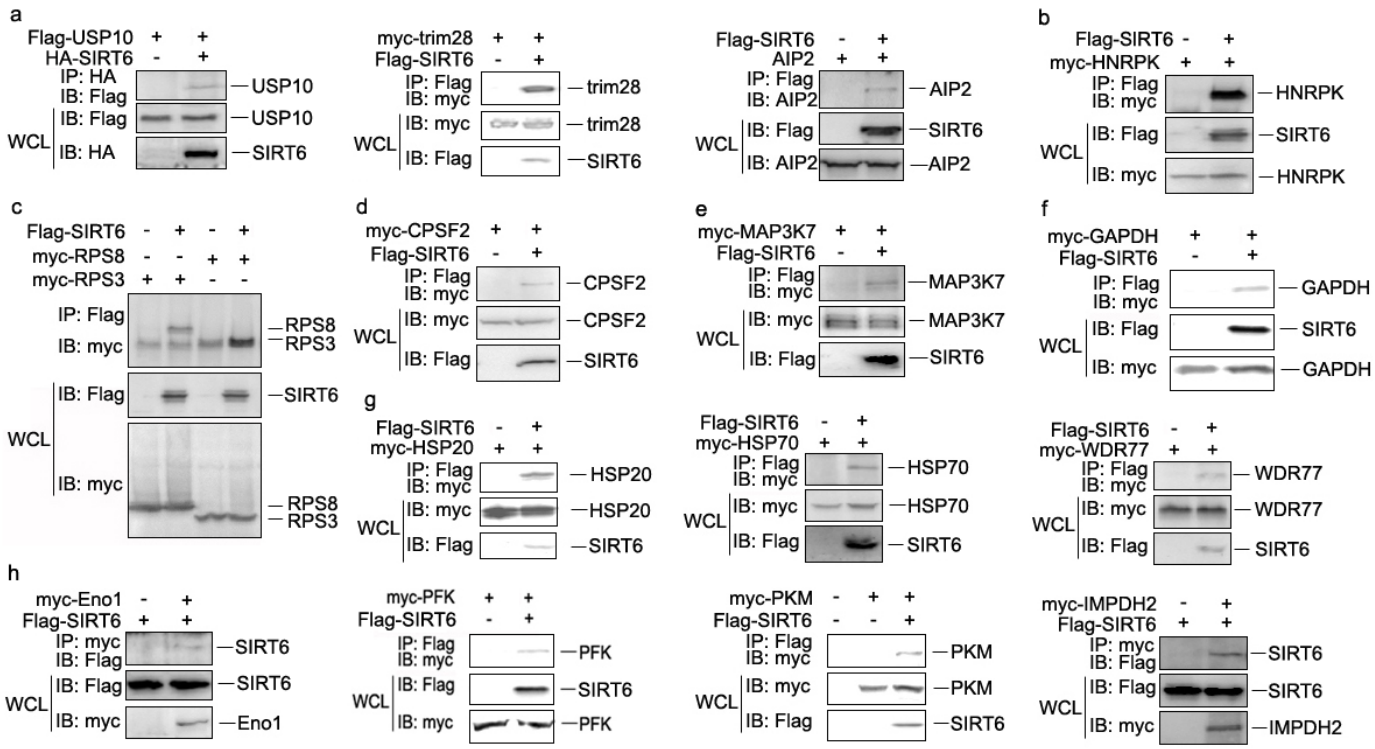

Fig.S1 Validation of SIRT6-interacting proteins. a-h. Validation of SIRT6 interaction candidates. Either Flag- or HA-tagged SIRT6 expression plasmids were cotransfected with the expression plasmid of each indicated candidate into HEK293 cells. Their interactions in the transfected cells were determined by co-IP and western blotting using each of the indicated Abs (top panels). The expression levels of both transfected proteins in whole-cell lysates were confirmed by western blotting (bottom two panels).

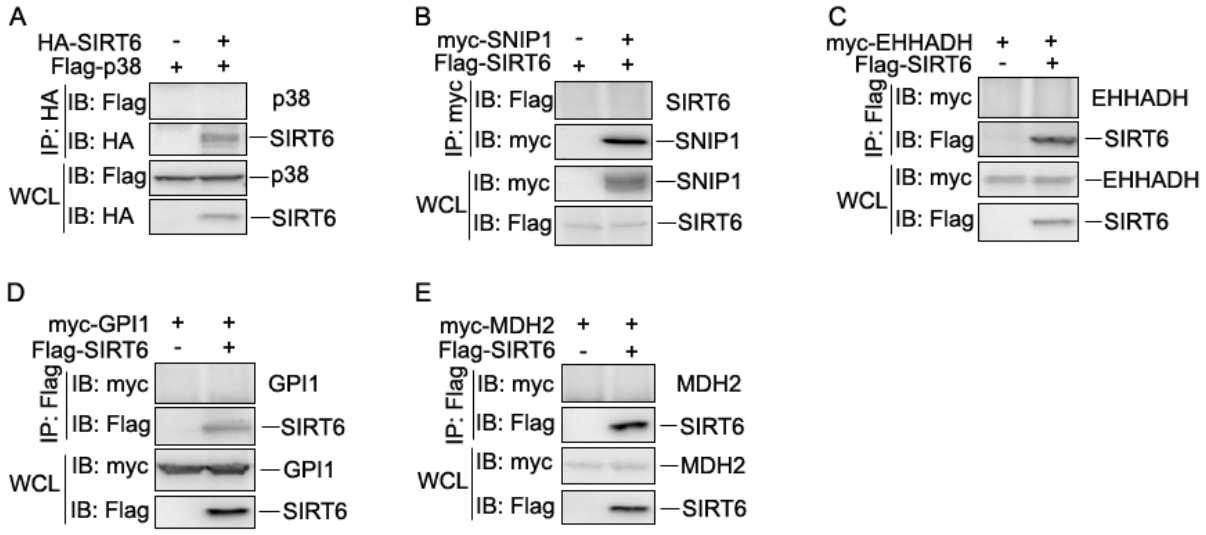

Fig.S2 Candidates do not interact with SIRT6 protein. (A) Flag-p38, (B) myc-SNIP1, (C) myc-EHHADH, (D) myc-GPI1, (E) myc-MDH2 and Flag- or HA-SIRT6 expression plasmids were co-transfected into HEK293 cells. The interactions of SIRT6 with each indicated protein in the transfected cells were determined by co-immunoprecipitation with anti-Flag, anti-HA or anti-myc and western blotting with each indicated antibody (top panels). The expression levels of SIRT6 and its co-transfected proteins in the whole cell lysates were analyzed by western blotting (middle and bottom panels).

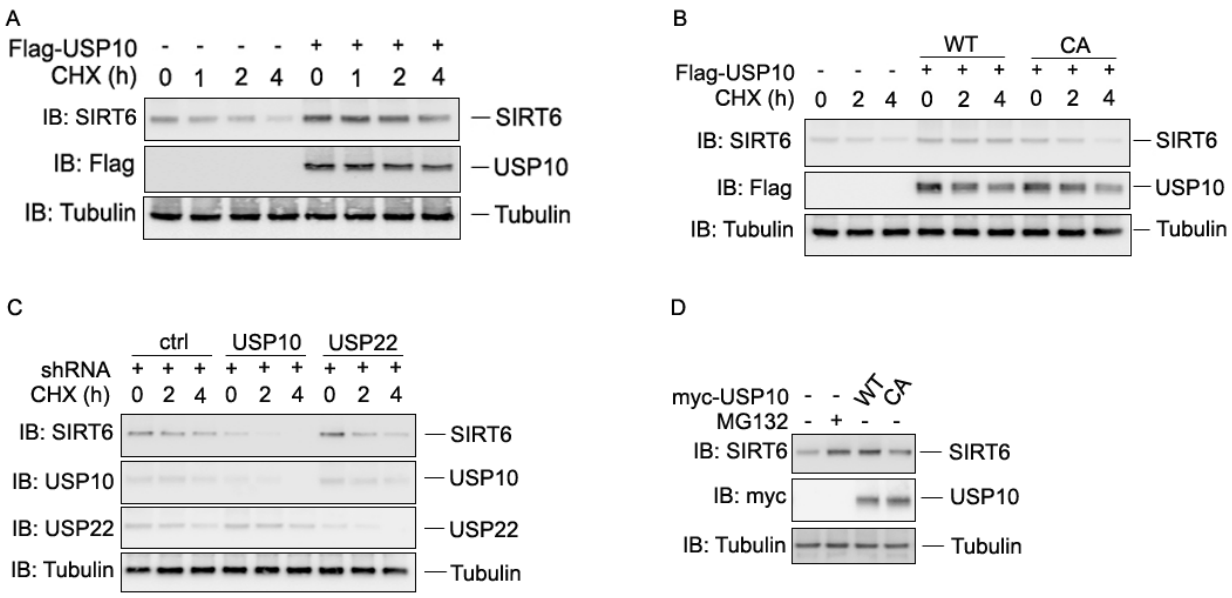

Fig.S3 USP10 protects SIRT6 protein from degradation in RKO cells. (A) USP10 expression plasmids were transfected into RKO cells. The transfected cells were treated with cycloheximide (CHX) for different times. The protein levels in the treated cells were determined by western blotting using indicated Abs. Tubulin was used as loading control (bottom panel). **(B)** USP10 expression plasmid or its CA mutant was transfected into RKO cells. The protein stabilities of SIRT6 in the transiently transfected RKO cells were examined as described in (A). **(C)** RKO cells were transfected with control shRNA or with shRNA specifically against USP10 and USP22. SIRT6 protein stabilities were analyzed as described in (A) (top panels). The expression levels of USP10 (2nd panel) and USP22 (3rd panel) were confirmed by western blotting using Tubulin as a loading control (bottom panel). **(D)** RKO cells were transfected with USP10 expression plasmid or its CA mutant. 48 hours later, cells were treated with the proteasome inhibitor MG132 as indicated. The protein levels of SIRT6 (top panel) and USP10 (middle panel) were determined by western blotting using Tubulin as loading control (bottom panel).

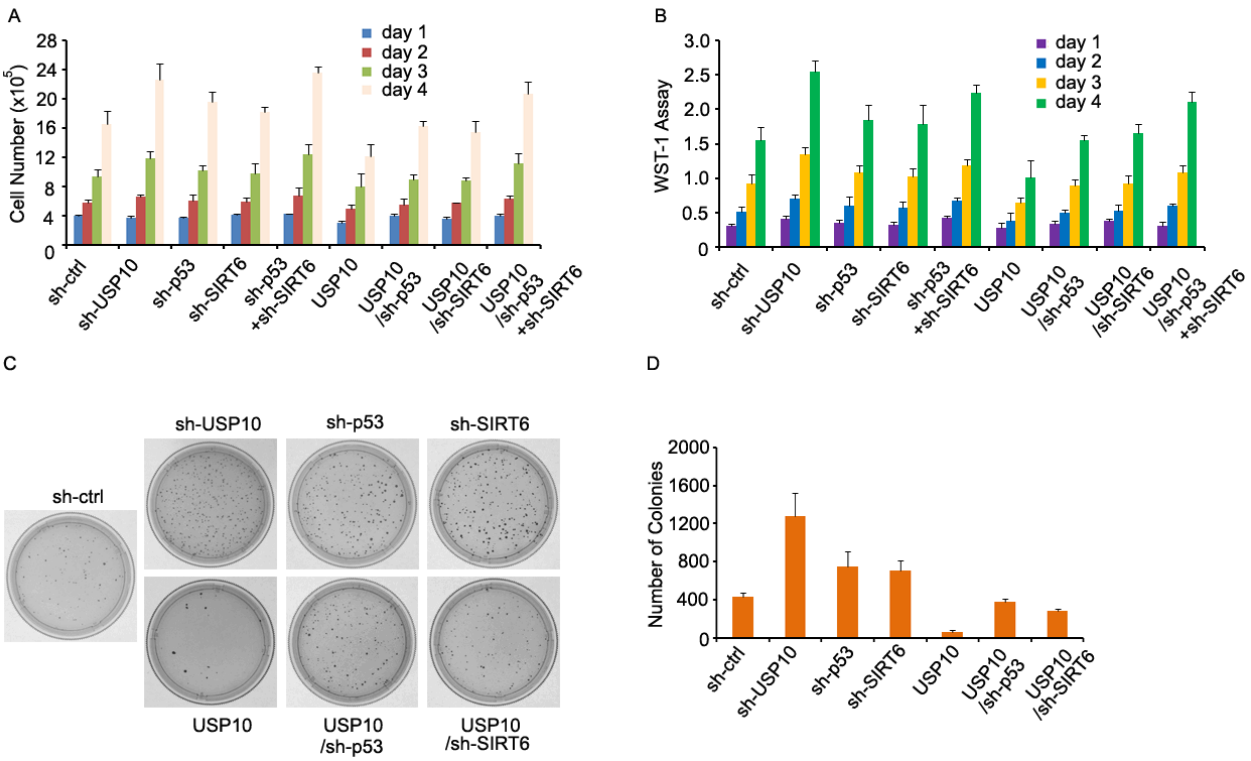

Fig.S4 Down-regulation of USP10 leads to enhanced cell proliferation and tumorigenesis. (A & B) The cell proliferation of RKO cells expressing indicated plasmid combinations were determined either by counting cell numbers (A) or by WST-1 assay (B). **(C & D)** Anchorage-independent colony formation of RKO cells stably expressing indicated plasmid combinations were determined by soft agar assay. Representative images from three experiments are shown (C). The average number of colonies from three experiments is indicated (D).