Expanded View Figures

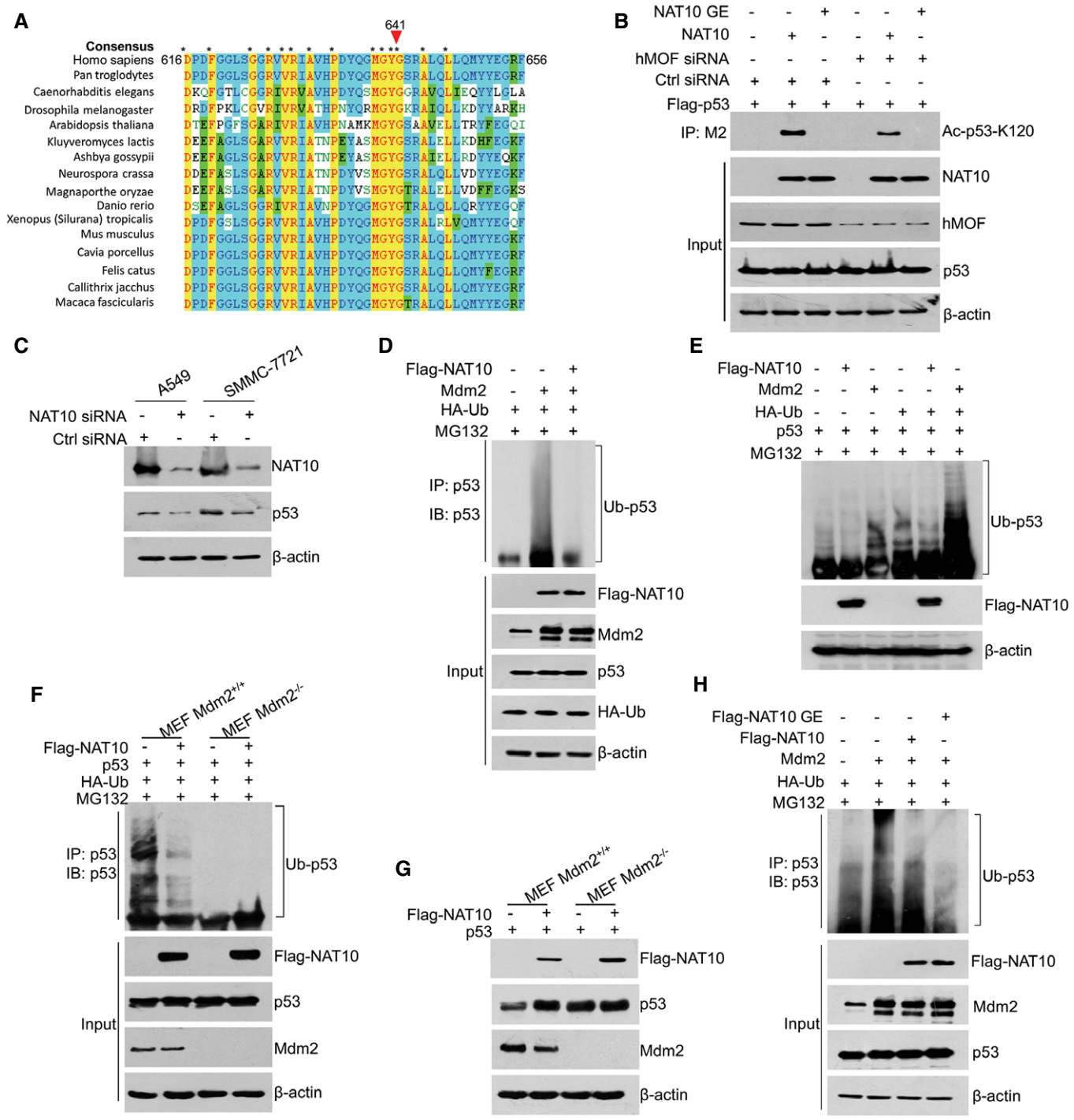


Figure EV1.

Figure EV1. NAT10 inhibits ubiquitination of p53.

A Alignment of NAT10 GNAT (Gcn5-related N-acetyltransferase) domain showing the conservation of G641.

B H1299 cells treated with the indicated siRNAs were transfected with the indicated constructs. Forty-eight hours later, cells were harvested and the acetylated p53 at K120 was analyzed.

C The A549 or SMMC-7721 cells were transfected with the indicated siRNAs. Seventy-two hours later, cells were harvested and the total proteins were subjected to Western blot using the indicated antibodies.

D HCT116 cells were transfected with the indicated plasmids and treated with MG132 for 4 h before harvest. Total proteins were subjected to immunoprecipitation using p53 polyclonal antibody. The ubiquitination levels of p53 were analyzed by Western blot using anti-p53 antibody (DO-1).

E H1299 cells were transfected with the vectors as indicated. Twenty-four hours later, cells were treated with MG132 for 4 h before harvest and Western blot was performed for the indicated proteins.

F MEF Mdm2-expressing or Mdm2-null cells were transfected with the indicated plasmids and treated with MG132 for 4 h before harvest. Total proteins were subjected to immunoprecipitation using p53 polyclonal antibody. The ubiquitination levels of p53 were analyzed by Western blot using anti-p53 antibody (DO-1).

G MEF Mdm2-expressing or Mdm2-null cells were transfected with the vectors as indicated. Twenty-four hours later, cells were harvested and Western blot was performed for the indicated proteins.

H HCT116 cells were transfected with the indicated plasmids and treated with MG132 for 4 h before harvest. Total proteins were subjected to immunoprecipitation using p53 polyclonal antibody. The ubiquitination levels of p53 were analyzed by Western blot using anti-p53 antibody (DO-1).

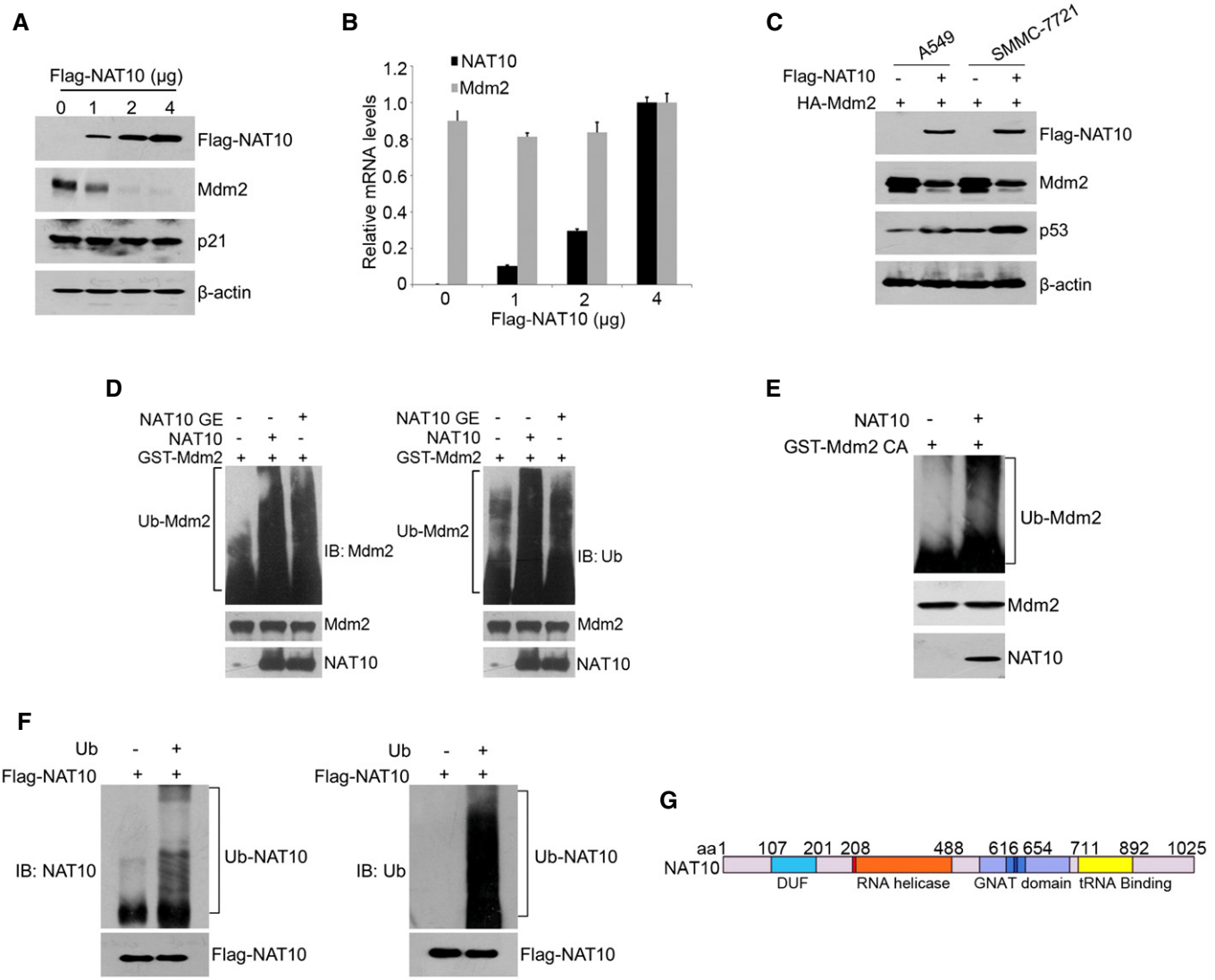


Figure EV2.

Figure EV2. NAT10 is a novel E3 ligase for Mdm2.

A HCT116 $p53^{-/-}$ cells were transfected with increasing amounts of NAT10 plasmids. Forty-eight hours later, cells were harvested and the total proteins were subjected to Western blot using the indicated antibodies.

B HCT116 $p53^{+/+}$ cells were transfected with the indicated vectors. Twenty-four hours later, cells were harvested after treatment with MG132 for 4 h. Relative mRNA levels were analyzed by RT–qPCR for the indicated genes.

C A549 or SMMC-7721 cells were transfected with the indicated plasmids. Forty-eight hours later, cells were harvested and lysed. Western blot was performed for the indicated proteins.

D *In vitro* ubiquitination of Mdm2 by NAT10. Recombinant GST-Mdm2 was incubated with Flag-NAT10 or Flag-NAT10 GE in the presence of E1, E2, and Ub *in vitro* as described in Materials and Methods. The reaction products were resolved by SDS–PAGE, followed by Western blot for the indicated proteins.

E *In vitro* ubiquitination of Mdm2 by bacterial NAT10. Recombinant GST-Mdm2 CA was incubated with bacterial NAT10 in the absence or presence of Ub *in vitro* as described in Materials and Methods. The reaction products were resolved by SDS–PAGE, followed by Western blot for the indicated proteins.

F *In vitro* auto-ubiquitination of NAT10 was carried out as described in Materials and Methods. Purified NAT10 were incubated with or without Ub in the presence of E1 and E2. The reaction products were resolved by SDS–PAGE, followed by Western blot for the indicated proteins.

G Representation of NAT10 with its known domains.

Figure EV3. NAT10 E3 ligase activity is located at residues 456–558.

A U2OS cells were transfected with the indicated siRNAs or plasmids. Seventy-two hours later, cells were harvested and Western blot was then performed for the indicated proteins.

B U2OS cells were transfected with the indicated constructs. Cells were harvested after CHX treatment for the indicated times and lysed. The p53 protein levels were analyzed by Western blot. The lower panel shows quantification of p53 protein levels relative to β-actin. Error bars represent the SEM from three independent experiments in triplicates.

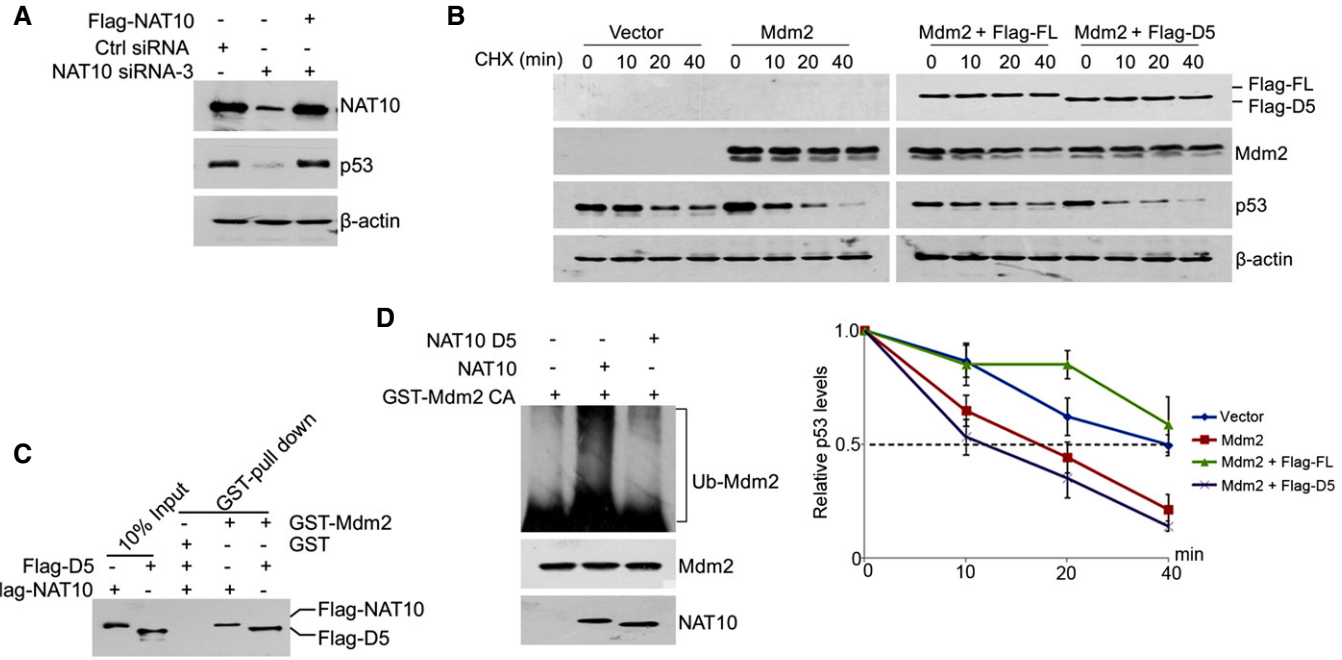
C GST pull-down assay was performed with GST or GST-Mdm2 fusion proteins and purified Flag-NAT10 or its mutant (Flag-D5).

D *In vitro* ubiquitination of Mdm2 by bacterial NAT10. Recombinant GST-Mdm2 CA was incubated with bacterial NAT10 or D5 mutant in the presence of E1, E2, and Ub *in vitro* as described in Materials and Methods. The reaction products were resolved by SDS–PAGE, followed by Western blot for the indicated proteins.

E The potential NAT10 interacting proteins (UPS-associated) were predicated via UniProtKB.

F GST pull-down assay was performed with GST or GST-NAT10 RFL fusion proteins and purified His-Ub (RFL is the abbreviation of "RING finger-like", representing residues 456–558.)

G The sequence of NAT10 RING finger-like motif. RING finger consensus sequence and schematic representation of RING finger (left). The sequence of NAT10 RING finger-like motif and the potential zinc coordination residues are in red and numbered (top right). Alignment of NAT10 RING finger-like domain showing the conservation of the potential zinc coordination residues (bottom right).



E

NAT10 interacting proteins, predication via MINT STING UniProtKB I2D

Symbol	Internal ID (s)	Details
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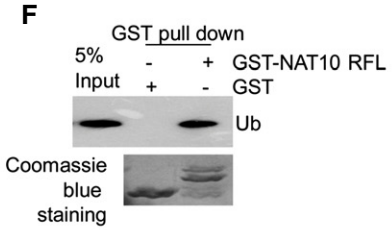

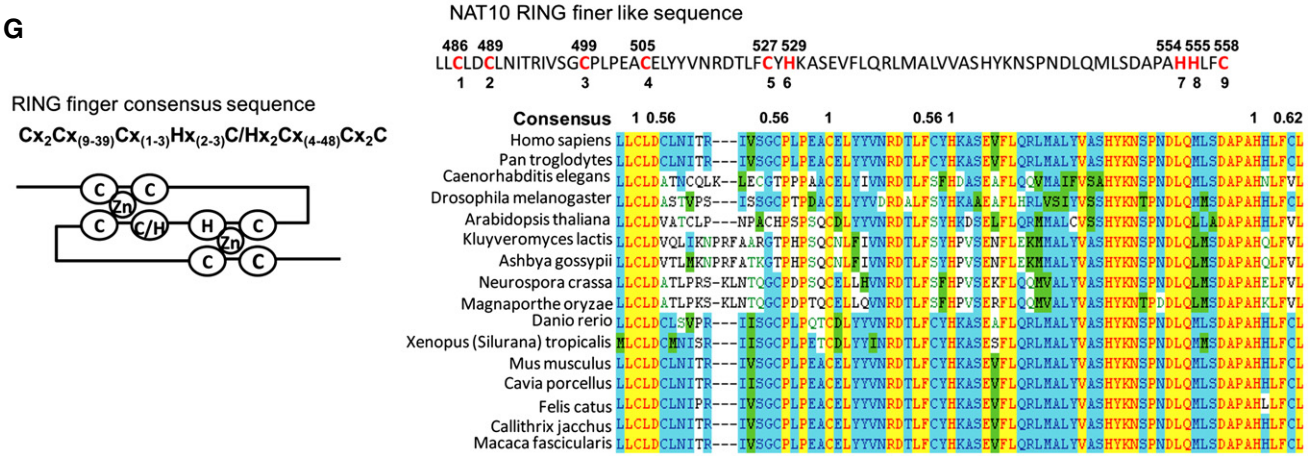

Figure EV3.

Figure EV4. NAT10 regulates p53 activity in DNA damage response.

A U2OS cells were treated with etoposide (20 μM) or Dox (4 μM) for the indicated times. Cells were harvested and lysed. Western blot was then performed for the indicated proteins.

B A549 and SMMC-7721 cells were treated with actinomycin D (10 nM). Twelve hours later, cells were harvested and the whole-cell extracts were subjected to Western blot using the indicated antibodies.

C HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with actinomycin D (10 nM). Twelve hours later, cells were harvested and the whole-cell extracts were subjected to Western blot using the indicated antibodies.

D U2OS cells were transfected with the indicated siRNAs and treated with actinomycin D (10 nM) for 12 h before harvest. The cells were then lysed, and the whole-cell extracts were subjected to Western blot using the indicated antibodies.

E A549 and SMMC-7721 cells were transfected with the indicated siRNAs. Seventy-two hours later, cells were harvested after treatment with actinomycin D (10 nM) for 4 h. The total proteins were extracted, and Western blot was performed for the indicated proteins.

F U2OS cells were transfected with the indicated siRNAs. Seventy-two hours later, cells were harvested after treatment with actinomycin D (10 nM), UV (50 J/m^2), etoposide (20 μM), or Dox (4 μM) for 4 h. The cells were lysed, and Western blot was performed for the indicated proteins. Right panel: Quantification of p53 protein levels relative to β-actin. Error bars represent the SEM of the experiments in triplicate.

G U2OS cells were transfected with the indicated siRNAs. Seventy-two hours later, cells were fixed and stained with anti-NAT10 antibodies, and nuclei were stained with DAPI. Scale bar represents 10 μm.

H U2OS cells were fixed and immunostained with the indicated antibodies and DAPI. Scale bar represents 10 μm.

I U2OS and H1299 cells were treated with actinomycin D (10 nM) for the indicated times. Then, cells were fixed and stained with anti-NAT10 antibodies and DAPI. Scale bar represents 10 μm.

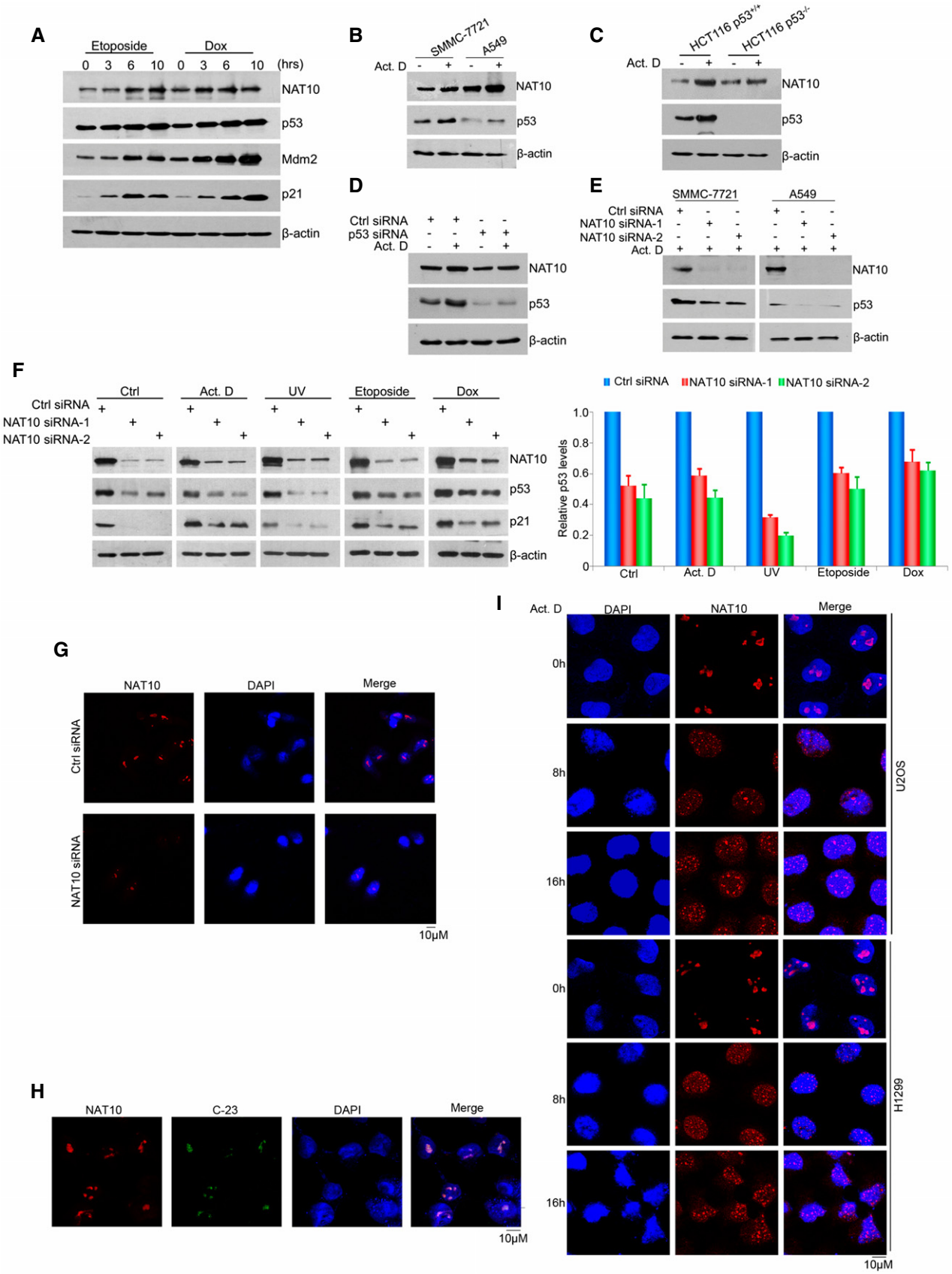

Figure EV4.

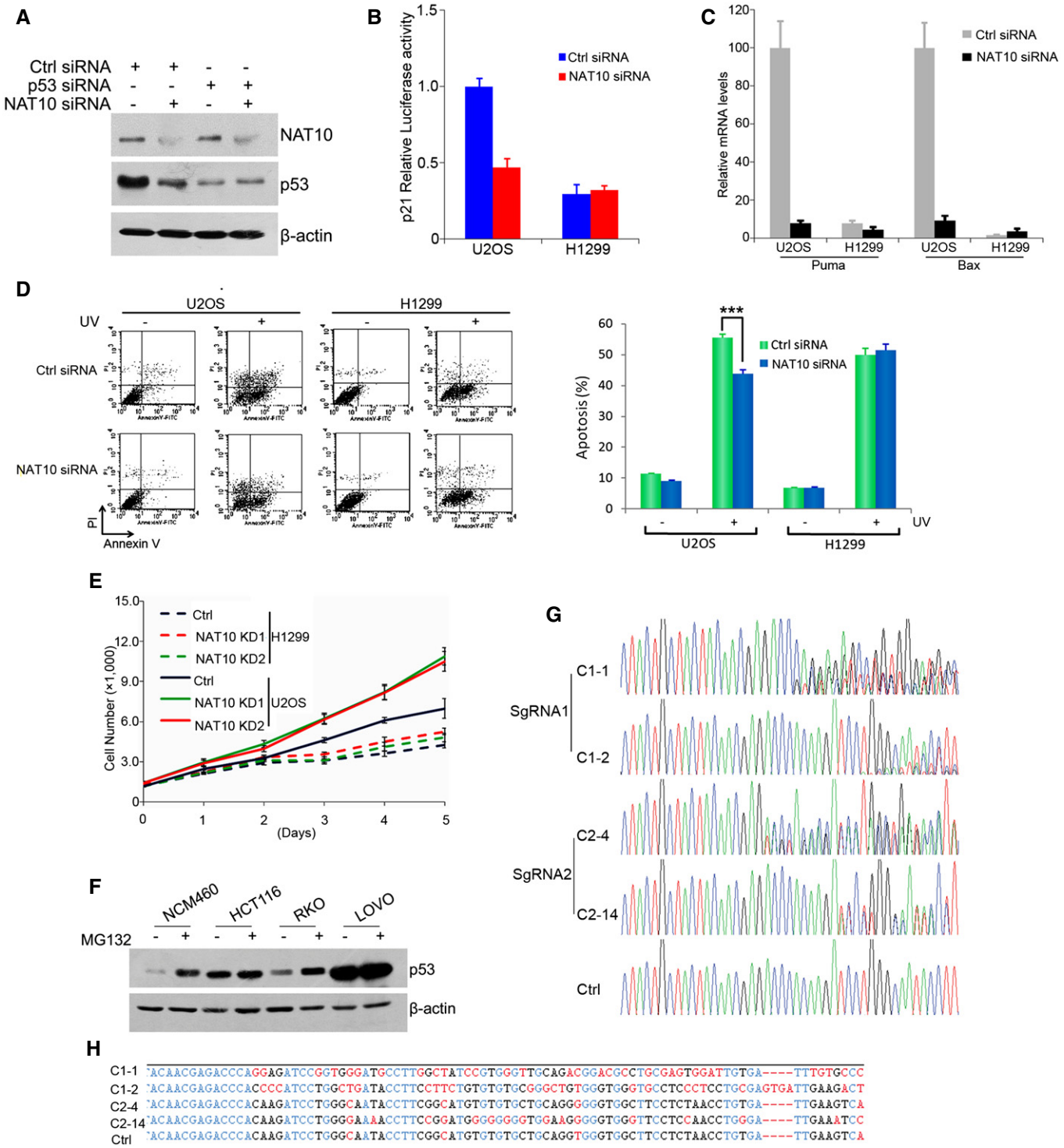

Figure EV5.

Figure EV5. NAT10 is downregulated in CRC samples.

A U2OS cells were transfected with the indicated siRNAs. Seventy-two hours later, cells were harvested and Western blot was performed for the indicated proteins.

B p21 promoter luciferase reporter plasmid was cotransfected with the indicated siRNAs into U2OS and H1299 cells. Luciferase activity was determined as described in Materials and Methods.

C U2OS cells or H1299 cells were transfected with siRNAs as indicated. Seventy-two hours later, cells were irradiated with 50 J/m^2 UV for 16 h. Relative mRNA levels for the indicated genes were analyzed by RT–qPCR.

D U2OS and H1299 cells were transfected with the indicated siRNAs. Cells were irradiated with 50 J/m^2 UV. Forty-eight hours later, apoptotic cells were determined. Quantification of apoptotic cells is shown in the right. ***$P < 0.001$ (two-tailed Student's *t*-test).

E U2OS and H1299 cells transfected with control or NAT10 siRNAs (NAT10 KD) were plated. Cell numbers were determined as described in Materials and Methods at the indicated time points.

F The CRC cell lines were harvested with or without MG132 treatment. Western blot was then performed for the indicated proteins.

G Verification of knockdown of *NAT10* gene by Cas9 technology. HCT116 $p53^{+/+}$ cells were cotransfected with the DNA expressing Cas9 and sgRNA. Single cell was plated into 96-well plates, and monoclone was obtained. Genomic DNA was extracted from HCT116 NAT10 knockdown cell lines, and *NAT10* gene was amplified by PCR. PCR products were subjected to DNA sequencing.

H Alignment of mutated *NAT10* genes in the NAT10 knockdown cells as described in (G) and wild-type *NAT10* gene.

Data information: (B, D, E) Error bars represent the SEM from three independent experiments in triplicates.