Supplemental Materials and Methods

Plasmid construction and site-directed mutagenesis

Constructs of pCMV-EGFP-SUMO1aa (an inactive form of SUMO1), pCMV-EGFP-SUMO1gg (an active form of SUMO1), pTY-EGFP-SUMO1aa, pTY-EGFP-SUMO1gg, pCMV-HA-SENP1, and pCMV-HA-UBC9 were kindly provided by Dr. Hsiu-Ming Shih at the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan. The human POU5F1 gene (known as OCT4, NM_002701) was cloned into the pcDNA3-HA vector. The human SENP1 gene (NM_014554) was cloned into the pLKO_AS3w.tRFP-C lentiviral vector (RNAi consortium, Academia Sinica, Taipei, Taiwan). Site-directed mutagenesis of 123/222 lysine residues to arginine was performed using the GeneTailor Site-Directed Mutagenesis kit (Invitrogen, Frederick, MD, USA) according to the manufacturer's instructions.

Short hairpin RNA and lentivirus production

The packaging pCMVΔR8.91 and the envelope VSV-G pMD.G plasmids were co-transfected with pLKO_AS3w-IRES-tRFP-C, pLKO_AS3w-HA-SENP1-IRES-tRFP-C, pTY-EGFP-SUMO1aa, pTY-EGFP-SUMO1gg, shLuc (TRCN0000072246, RNAi consortium, Taiwan), shUBC9#1 (TRCN0000320448), shUBC9#2 (TRCN0000320375), shOCT4#1 (TRCN00004879), or shOCT4#2 (TRCN00004881) plasmid into HEK293T cells by a

calcium-phosphate transfection protocol. The medium was collected at 72 h post-transfection and ultracentrifuged at $1.4 \times 10^5 \times g$ for 2.5 h to concentrate the virus. Cells were infected with the lentivirus in the presence of 8 μg/ml polybrene (Sigma-Aldrich, Frederick, MD, USA).

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction

Total RNA was extracted by a Trizol Kit (Invitrogen) according to the manufacturer's instructions. For cDNA synthesis, oligo-dT primers (Invitrogen) and 5 μg total RNA were used with MMLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The real-time PCR was performed using Light Cycler-DNA Master SYBR Green I (Hoffman-La Roche, Basel, Switzerland). Primer sequences are listed in Supplementary Table S1. Beta-2 microglobulin expression was used for normalization. Data were analyzed on a LightCycler 480 (Roche). Each sample was analyzed in duplicate, and three different experiments were performed for each experimental condition.

Immunoprecipitation and Western blot analysis

Cell extracts were prepared using cold protein lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.1% sodium deoxycholate, 0.5% NP-40, and 1% SDS) supplemented with 20 mM N-ethylmaleimide and protease inhibitor cocktail for 30 min. The cell extracts were centrifugated at >15,000 ×g for 15 min, and the cell suspension was collected.

For immunoprecipitation, total cell lysates (500 μg) were diluted by adding 20-fold PBS for use. For OCT4 immunoprecipitation assay, the cell lysates were incubated with primary monoclonal OCT4 antibodies (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4℃ on a rocking platform, and then fresh protein G beads were added and the mixture was incubated at 4℃ for another 2 h. For FLAG-SUMO1 immunoprecipitation assay, the cell lysates were treated with anti-FLAG M1 agarose affinity gel (A4596, Sigma-Aldrich) at 4℃ overnight on a rocking platform. Thereafter, these beads were collected and washed three times with PBS containing 0.4% NP-40. After washes, the beads were resuspended in 2× SDS loading buffer (0.125 m Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromphenol blue, 0.2 m DTT) and boiled for 5 min for SDS-PAGE and Western blot analysis.

For Western blotting, total cell lysates (30 ~100 μg) or immunoprecipitates were subjected to 10% SDS-PAGE and then transferred to a PVDF membrane. The primary antibodies are listed in Supplementary Table S2, and horseradish peroxidase

(HRP)-conjugated anti-mouse/rabbit IgG served as the secondary antibody. The enzyme activity of HRP was detected by an enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, USA).

Immunostaining

The primary antibodies used for immunostaining are listed in Supplementary Table S2. For immunofluorescence staining, cells were fixed in 4% paraformaldehyde at room temperature for 30 min. After fixation, cells were rinsed with PBS twice at room temperature for 30 min and blocked with bovine serum albumin (BSA; 50 mg/ml) and 0.5% triton X-100 in PBS for 1 h at room temperature. Cells were then incubated with a primary antibody, and specific labeling of the primary antibody was detected with Cy3- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, Bar Harbor, ME, USA). Nuclei of all cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). All cells were covered with an anti-fading reagent (Vector Laboratories, Burlingame, CA, USA) and analyzed with a fluorescence microscope or a confocal laser scanning microscope (Leica, Buffalo Grove, IL, USA). For immunohistochemical staining, slides of the tissues were probed with primary antibodies for the microscope analysis. Sections were evaluated by Image Scope software (Aperio Technologies, Vista, CA, USA) for quantitative

analysis. The ratio of positive cells to total cells was shown as a percentage. The intensity of positive staining was presented as the total intensity per total pixels. Three randomly selected high-power fields were analyzed for each tumor section.

Cell viability assay

Cells in various experimental conditions, including with or without infection of lentivirus carrying empty vector, HA-SENP1, shLuc, or shOCT4 plasmids, were seeded at 10^4 cells per well in 96-well plates and incubated in 21% O_2 for 24 h. Cells were then treated with cisplatin (P4394, Sigma-Aldrich) or bleomycin (Nippon Kayaku, Tokyo, Japan) at various concentrations, and cultivated in 1% O_2 (hypoxic) or 21% O_2 (normoxic) conditions. After 24 h, a WST-1 assay (Roche) was used to detect cell proliferation according to the manufacturer’s instructions. Three different experiments were performed for each experimental condition. Cell viability is expressed as a percent of the non-treated group, and the IC50 values were determined