Supplementary data

Supplementary information for Materials and Methods

Constructs, truncation and mutagenesis

The Myc-CHIP wild-type, TPR and U-box constructs were kindly provided by Dr. Xiao-Dan Yu (Institute of Basic Medical Sciences, Beijing, China). Flag-Hsp90 was kindly offered by Dr. Shu-Bing Qian (Cornell University, NY, USA). CHIP K30A mutant construct was a gift of Dr. Chen Wang (Institute of Biochemistry and Cell Biology , Chinese Academy of Science, Shanghai, China). The RGS-SENP3 was constructed as described previously (Gong and Yeh, 2006). Fluorescence-labeled SENP3 was made by cutting full-length from RGS-SENP3 and subcloning into BamHI and XbaI sites of either pEGFP-C1 or pDsRed-C1 vectors (Clontech Laboratories Inc., Mountain View, CA, USA). *dam-/dcm-* competent *E. coli* (NEB, Ipswich, MA, USA) was used. Restriction enzymes and T4 ligase were purchased from NEB, and HS DNA polymerase was obtained from TAKARA BIO Inc. (Shiga, Japan). SENP3 truncates were made by PCR using specific primers and subcloning the PCR products into SalI and BamHI restriction sites of pEGFP-C1. TA Cloning System (TOYOBO Co., Ltd. Osaka, Japan) was used for cloning PCR products. The primers for truncate C1 were 5'-GCG CGT CGA CAA TGA CCA GGT GAT GAA C-3' and 5'-CGG GAT CCT CAC ACA GTG AGT TTG CAG TG-3'. The primers for C2 were 5'-GCG CGT CGA CGT ACA GAG CAT CTT GGA CG-3'and 5'-CGG GAT CCT CAC ACA GTG AGT TTG CAG TG-3'. The primers for C3 were 5'-GCG

CGT CGA CAA CCA TCT TTC ACC CCA GC-3' and 5'-CGG GAT CCT CAC ACA GTG AGT TTG CAG TG-3'. The primers for N1 were 5'-GCG CGT CGA CAT GAA AGA GAC TAT ACA AGG G-3' and 5'-CGG GAT CCG AGC CAG TTC TGT CCA TAC-3'. The primers for N2 were 5'-GCG CGT CGA CAT GAA AGA GAC TAT ACA AGG G-3' and 5'-CGG GAT CCG CAG GTC ACA TGC TCC TCT-3'. The primers for N3 were 5'-GCG CGT CGA CAT GAA AGA GAC TAT ACA AGG G-3' and 5'-CGG GAT CCC TGG GGT GAA AGA TGG TT-3'. The pEGFP-C1-SENP3 mutant constructs designed to mutate cysteine to serine were made by site-directed mutagenesis based on the pEGFP-C1-SENP3 wild-type construct using a QuikChange mutagenesis Kit (Stratagene, La Jolla, CA, USA).The primers for four sites of mutation (C183/184S, C243S, C274S and C316S) were as below. The primers of C183/184S were 5'-AGG TGC CAT CCC CCT CTA GTC GTT TTG ACT CCC-3' and 5'-GGG AGT CAA AAC GAC TAG AGG GGG ATG GCA CCT-3'. The primers of C243S were 5'-ACT CGG GCC TCC TTT CAT CTA CTC TGC CC-3' and 5'-GGG CAG AGT AGA TGA AAG GAG GCC CGA GT-3'.The primers of C274S were 5'-CTC ATC AGC AAT GTG AGC AGC ATC GGG GAC C-3' and 5'-GGT CCC CGA TGC TGC TCA CAT TGC TGA TGA G-3'. The primers of C316S were 5'-GAG GAG CAT GTG ACC AGC GTA CAG AGC ATC T-3' and 5'-AGA TGC TCT GTA CGC TGG TCA CAT GCT CCTC-3'.

Primers and reverse transcriptional polymerase chain reaction (RT-PCR) and quantitative PCR

The primers for c-Cbl gene were 5'-GGA CCA GTG AGT TGG GAG TTA TTA

CT-3’and 5’-GGC AAG ACT TCA CTG TGA AGT CA-3’. The primers for CHIP gene were 5’-TGT GCT ACC TGA AGA TGC AG-3’ and 5’-TGT TCC AGC GCT TCT TCT TC-3’. The primers for E6AP gene were 5’- AGG AGC AAG CTC AGC TTA CCT-3’and5’-CAG CAG CAG AAC ATG CAG C-3’. The primers for HHARI gene were 5’-GCT ACG AGG TGC TCA CG-3’and5’-ATC CTG TGC TGA TGA CCT TG-3’. The primers for parkin gene were 5’- CCA GAT TGC CAC CAT GTT GT-3’ and 5’-CAC TGT CAT CAT CAC ACT-3’. The primers for G3PDH gene were 5’- TCT CCA GAA CAT CAT CCC TGC CTC -3’ and 5’- GCC TGC TTC ACC ACC TTC TTG ATG-3’. For PCR, up to 1 μl of cDNA were used as template. Thermal cycling conditions were 95°C for 5 min, followed by 25 cycles of 95°C for 30s, 56°C for 30s and 72°C for 30s. Final extension was at 72°C for 5 min.

For quantitative RT-PCR, 48 h post-transfection with the constructs of GFP-SENP3WT or GFP-SENP3C243/274S, HeLa cells were treated with H_2O_2 100 μM for 8 h and quantitative PCR was performed on the ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA) using SYBR Green (Roche, Mannheim, Germany). The primers for the VEGF gene were 5’-TTT CTG CTG TCT TGG GTG CAT TGG-3’, and 5’-ACC ACT TCG TGA TGA TTC TGC CCT-3’. The primers for actin were 5’-ACC AAC TGG GAC GAC ATG GAG AAA-3’ and 5’-TAG CAC AGC CTG GAT AGC AAC GTA-3’.Thermal cycling conditions were 95°C for 10min, followed by 40 cycles of 95°C for 15s, 60°C for 60s. Primer efficiency of > 90% was confirmed with a standard curve spanning four orders of magnitude. Following the reactions, the raw data were exported using the 7300 System Software

v1.3.0 (Applied Biosystems) and analyzed.

Sequential two-dimensional non-reducing/reducing SDS PAGE

This method has been referred to as redox diagonal electrophoresis. Briefly, samples were prepared in buffer free of reducing agent (i.e., neither βmereaptoethanol nor DTT) and loaded onto SDS-PAGE gels. After the first dimension electrophoresis, the entire lane containing the separated proteins was excised and incubated in SDS sample buffer containing 100mM DTT for 20 min. The lane was then rotated 90° and laid horizontally on top of a large-format 12% acrylamide gel. Proteins that were linked together via disulfide bonds would be presented as a complex at a single position in the first dimension, but would separate and move to their monomeric positions in the second dimension. Finally, immunoblot was performed to display the dots of CHIP.

Assessment of thiol reduction by fluorescein-5-maleimide (F5M)

HeLa cells transfected with pEGFP-SENP3 were incubated in fresh medium containing 10 mM F5M for 30 min at 37°C after H_2O_2 treatments. The cells were lysed with cold 1× IP buffer containing 1 mM F5M at 4°C for 30 min, and then lysates centrifuged at 4°C for 15 min. The supernatant were incubated with a rabbit antibody against GFP (ABcam) overnight at 4°C and the protein A/G beads (Merck) for another 4 hours. pEGFP-SENP3 was then separated from the antibody-proteinA/G bead complex using 1× SDS sample buffer with 0.5 mM F5M. After the denatured proteins were subjected to SDS-PAGE, the F5M fluorescence of the pEGFP-SENP3 bands on the fresh gel were visualized and photographed using the FLA-5000

Imaging System (Fujifilm, Japan) at an excitation wavelength of 472 nm. An immunoblotting was performed to assess total SENP3 in the identical positions blotted from the same gel.

Antibodies

The mouse monoclonal antibodies against RGS (34610, Qiagen, Germany), the rabbit polyclonal antibodies against HA (ab9110, Abcam, UK), the rabbit polyclonal antibodies against Myc (ab9106, Abcam), the mouse monoclonal antibodies against Flag (F1804,Sigma), the mouse monoclonal antibodies against GFP (ab38689, Abcam), the mouse monoclonal antibodies against HSP90 (ab13492, Abcam), the rabbit polyclonal antibodies against CHIP (ab2917, Abcam), the mouse monoclonal antibodies against β-actin (ab6276, Abcam), the rabbit polyclonal antibodies against SENP3(Protein Techgroup, Chicago,USA) were used.

siRNA

siRNA oligonucleotides were synthesized (RIBOBIO, China). The sequences of the siRNA oligonucleotides for c-Cbl gene were 5'-GGA GAC ACA UUU CGG AUU A dTdT-3' and 3'-dTdT CCU CUG UGU AAA GCC UAA U-5'. The sequences of the siRNA oligonucleotides for E6AP gene were 5'- GUA GAG AAA GAG AGG AUU A dTdT-3' and 3'- dTdT CAU CUC UUU CUC UCC UAA U-5'. The sequences of the siRNA oligonucleotides for HHARI gene were 5’-CGA ACA CGC CAG AUG AAU A dTdT-3' and 3'- dTdT GCU UGU GCG GUC UAC UUA U-5'. The sequences of the siRNA oligonucleotides for CHIP gene were 5'-AGG CCA AGC ACG ACA AGU A dTdT-3' and 3'- dTdT UCC GGU UCG UGC UGU UCA U-5'. The sequences of

the siRNA oligonucleotides for parkin gene were 5'-GGA AGG AGC UGA GGA AUG A dTdT-3' and 3'- dTdT CCU UCC UCG ACU CCU UAC U-5'. The sequences of the siRNA oligonucleotides for HSP90 gene were 5'-GGA ACG UGA UAA AGA AGU A dTdT-3' and 3'- dTdT CCU UGC ACU AUU UCU UCA U-5'. The sequences of the siRNA oligonucleotides for CHIP (3'UTR) gene were 5'-GAC GUG CUG GUG UGU GAA A dTdT-3' and 3'-dTdT CUG CAC GAC CAC ACA CUU U-5'.

Immunoblotting (IB)

Cells were lysed in sample solution. Proteins were separated on 8, 10 or 12% SDS–PAGE gels, transferred to nitrocellulose membranes, 5% milk powder was used to reduce nonspecific background. Bands were detected using various antibodies as indicated. The antibodies were described in Supplemental data. The membranes were incubated with the primary antibodies at 4℃overnight and horseradish peroxidase-conjugated secondary antibodies (SantaCruz Biotechnology, Santa Cruz, CA) for 2 h at the room temperature (RT) before detection using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL).

Co-immunoprecipitation (co-IP) and, **denaturing co-immunoprecipitation (denaturing co-IP)**

Cells were harvested using the IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, 1 mM PMSF, 0.5% protease inhibitor cocktail, 0.5% IGEPAL CA-630). Cell lysates were centrifuged at 16,000 ×g for 30 min at 4°C and supernatants were incubated with specific antibodies as indicated overnight at 4°C, followed by incubation with

Protein A/ Protein G-coated agarose beads (Merck) for another 4 h at 4°C. After samples were washed three times with ice-cold IP buffer and supernatants were removed by centrifugation at 2,000×g for 1 min, proteins were precipitated individually or co-precipitated. The proteins were then separated from the beads using IB loading buffer for 5 min at 95°C. The supernatants were collected for IB. Proteins were detected with various antibodies by blotting and re-blotting as indicated.

For co-IP using two fresh hepatocarcinoma and the adjacent normal liver tissue, samples were collected from surgically resected tissues in Ren Ji Hospital following an institute-approved protocol. Normal liver tissues were the tissues 3 cm adjacent to the edges of carcinoma, having normal appearance. A mixture of 100 mg tissue evenly derived from two hepatocarcinoma or their adjacent normal regions were respectively homogenized using glass dounce. The buffer contained 0.1% SDS, 15mM Tris, pH7.6, 1mM DTT, 0.25mM sucrose, 1mM $MgCl_2$, 0.5mM PMSF, 2.5mM EDTA, 1mM EGTA, 0.25M Na_3VO_4, 25mM NaF, 1g/mL prostatin, 5g/mL leupeptin, 2.5g/mL aproptonin, and 2mM sodium pyrophosphate. The homogenates were further sonicated on ice for 40s and centrifuged at 20,000×g for 30min at 4°C. The supernatant was taken for co-IP.

Denaturing co-IP was performed to detect the ubiquitin conjugations of SENP3 Cells were lysed in 100 μl of the denaturing buffer (50 mM Tris (pH 7.5), 1% SDS, 5 mM DTT) before harvested. After boiling for 10 min, cell lysates were centrifuged for 5 min at room temperature. Supernatants were mixed with denaturing IP buffer (50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.5% NP-40) and incubated with

specific antibodies as indicated. The proteins were separated from the beads using IB loading buffer specifically containing 50 mM DTT for 5 min at 95°C. The supernatants were collected for IB.

SENP3 fluorescence and immunofluorescence

HeLa cells were transiently transfected with pDsRed-tagged SENP3 (RFP-SENP3) and Myc-CHIP or Flag-Hsp90. At 48 hours post-transfection, cell monolayers were fixed with 4% paraformaldehyde, permeablized with 0.2% Triton X-100, and were blocked with 5% BSA before incubation respectively with antibodies against Myc or Flag for 2 h at 37°C. Subsequently, the cells were incubated with fluorescent Isothiocyanate (FITC)-conjugated secondary antibody (ZYMED, S. San Francisco, CA) for 2 h at 37°C. Cells were then examined by a laser confocal microscope (LSM510, Zeiss, Germany). Scale bars = 10μm.

Transient co-transfection with SENP3 3'UTR siRNA and wild-type/mutant SENP3 expressing constructs for cell proliferation assay

HeLa cells were seeded at 50% confluence and were co-transfected with SENP3 3'UTR siRNA and the constructs of GFP-SENP3-WT or GFP-SENP3-C243/274S using lipofectamine 2000. After 48 h, cells were re-seeded in 24-well plates at 30% confluence and were treated with 100 μM H_2O_2 once a day. Cell proliferation was then tested for 4 days.

Stable transfection with wild-type/mutant SENP3 expressing constructs followed by SENP3 3'UTR siRNA for colony formation assay

HepG2 cells were trypsinized and suspended at a density of 1×10^8/ml in

HEPES-buffered saline before chilled DNA constructs were added. Electroporation was immediately performed with a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA). After cultured for 36 h, cells were split into dishes containing medium supplemented with 600 μg/ml G418 (GibcoBRL) and were maintained in this condition. Individual colony were isolated after 30 days and expanded. Cells then transfected with SENP3 3'UTR siRNA for 72 h and then used for colony formation assay.

Figure titles and legends for the Supplementary Figures

S. Figure 1. Co-localization of CHIP and SENP3 occurs in the presence of MG132

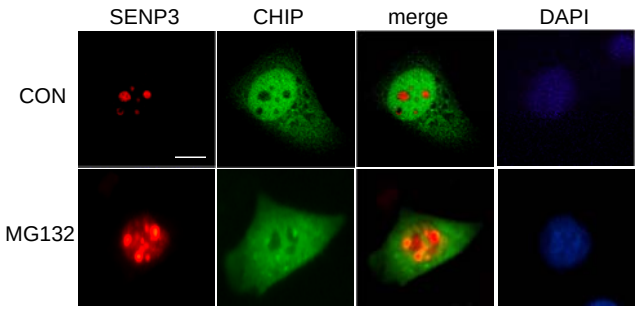
HeLa cells were transfected with RFP-SENP3 and Myc-CHIP for 48 h and treated with 10 μM MG132 for the later 12 h. Immunofluerescence was performed with Myc antibody. Cell nuclei were stained with DAPI. Scale bar=10μM.

S. Figure 2. SENP3 and Hsp90 can co-localize upon oxidative stress

HeLa cells were transfected with RFP-SENP3 and Flag-Hsp90 for 48 h and treated with 100 μM H_2O_2 for 1 h as indicated. Immunofluerescence was performed with Flag antibodies. Cell nuclei were stained with DAPI. Scale bar=10μM

Supplemental figures

S. Figure 1. Co-localization of CHIP and SENP3 occurs in the presence of the proteasome inhibitor



S. Figure 2. SENP3 and Hsp90 can co-localize upon oxidative stress