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Christèle Maison, Kelly Romeo, Delphine Bailly, Marion Dubarry, Jean-Pierre Quivy, et al.. The SUMO protease SENP7 is a critical component to ensure HP1 enrichment at pericentric heterochromatin.. Nature Structural and Molecular Biology, 2012, 19 (4), pp.458-60. 10.1038/nsmb.2244 . hal-00765968

HAL Id: hal-00765968

<https://hal.science/hal-00765968>

Submitted on 17 Dec 2012

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The SUMO protease SENP7 is a critical component to ensure HP1 enrichment at pericentric heterochromatin

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SUMOylation promotes targeting of HP1 α to pericentric heterochromatin. Here we identify the SUMO protease SENP7 as a maintenance factor for HP1 α accumulation at pericentric heterochromatin. SENP7 interacts directly with HP1 α , localizes at HP1-enriched pericentric domains and can deconjugate sumoylated HP1 α *in vivo*. Depletion of SENP7 delocalizes HP1 α from pericentric heterochromatin without affecting H3K9me3. We propose that following targeting of HP1 α , a subsequent desumoylation event enables HP1 α retention at these domains.

HP1, a protein highly conserved throughout evolution^{1,2}, interacts with many partners^{2,3} that contribute to its enrichment at pericentric heterochromatin⁴. Our recent discovery that HP1 α sumoylation promotes its targeting to pericentric heterochromatin⁵ in mouse cells raised the possibility that following this step, desumoylation could favour HP1 α maintenance at these domains. Sumoylation is rapidly reversed by the action of SUMO-specific proteases, known as SENPs⁶⁻¹⁰. There are six SENPs in mammals (SEN1-3, SEN5-7)^{8,9}, whose exact targets are not defined^{7,9}. The presence of SENP7 in the recent list of 82 candidate interactors of the human HP1 α ³ prompted us to further explore a role for this SENP as a SUMO regulator impacting HP1 stability at pericentric heterochromatin.

First, we verified that the association of SENP7 with HP1 α was conserved in mouse. We found that both wild type GFP-tagged mouse SENP7 (e-SENP7) and catalytically-dead C979S¹¹ (e-SENP7M) coimmunoprecipitated with endogenous HP1 α but not with KAP1 or SetDB1 (Fig. 1a and Supplementary Fig. 1a). We further confirmed that GST-SENP7 pulled down the three endogenous HP1 isoforms (Supplementary Fig. 1b). Conversely, HA-tagged HP1 α (e-HP1 α) retrieved endogenous SENP7 but not the most closely related SENP, SENP6 (Fig. 1b), indicating that HP1 α associates specifically with SENP7 regardless of its protease activity. Finally using recombinant proteins, we determined that His-SENP7 interacted directly with purified GST-HP1 α (Fig. 1c). At a cellular level, endogenous SENP7 colocalizes with HP1 α at pericentric heterochromatin and remains there after extraction of soluble material with Triton as shown by immunofluorescence (Fig. 2a). In an asynchronous

cell population, 70% of the cells showed a co-enrichment of HP1 α and SENP7 at the pericentric domain while the remaining 30% of cells showed diffuse staining for both proteins (Fig. 2b). We then investigated SENP7 localization at pericentric heterochromatin under conditions impairing localization of HP1 isoforms at these domains (after RNase A treatment on NIH3T3 cells¹² or in histone methyltransferase *Suv39h* double-null MEFs^{12,13}). Notably, both situations erased SENP7 staining at pericentric domains (Fig. 2c,d) without affecting the total amount of SENP7 and HP1 protein. These data demonstrate that the localization of SENP7 parallels that of HP1 α .

Since HP1 α directly interacts with SENP7 and is modified by SUMO-1 *in vivo*⁵, we examined whether SENP7 could affect the levels of SUMO-1-HP1 α *in vivo*. To obtain detectable SUMO-1-HP1 levels, we co-transfected GFP-SUMO-1 and HP1 α -HA into NIH3T3 cells. Under conditions preserving SUMO modification and after immunoprecipitation of HP1 α -HA, we revealed a 70 kD diagnostic band corresponding to GFP-SUMO-1-HP1 α -HA (Fig. 2e and Supplementary Figs. 2 and 3). Remarkably, this band decreased in intensity upon introduction of an active GFP-SENP7 (Fig. 2e and Supplementary Figs. 2 and 3) and remains identical to control when using the catalytic-dead mutant SENP7C979S¹¹ expressed at levels comparable to GFP-SENP7 (Fig. 2e). Thus, under our experimental conditions, SENP7 can deconjugate SUMO-1-modified HP1 α *in vivo*.

To further explore a functional relationship between the two proteins, we tested whether the SENP7-HP1 α interaction could impact HP1 α maintenance at pericentric domains. We depleted SENP7 from NIH3T3 cells by transfecting a plasmid encoding both a miRNA to downregulate SENP7 (miSENP7) and a GFP mRNA to enable identification of the transfected cells (Fig. 3a and see Supplementary Fig. 4a,b for depletion efficiency). Cell viability and FACS profiles based on DNA staining remained comparable between control and downregulated cells (Supplementary Fig. 4c). However, only 44% of GFP-positive SENP7-depleted cells showed HP1 α localization at pericentric heterochromatin compared to 70% of GFP-positive control cells (Fig. 3b,c), indicating a significant decrease of HP1 α localization at pericentric domains ($p_{\text{value}} = 0.004$). In these SENP7-depleted cells, HP1 α

showed faint staining at pericentric domains and increased diffuse nuclear staining (compare top rows of miSENP7 and micont in Fig. 3b) while keeping a normal amount of HP1 protein and a dense DAPI staining corresponding to pericentric regions (Supplementary Fig. 4a,b). Remarkably, although SENP7 depletion leads to the loss of local HP1 α enrichment at pericentric heterochromatin, H3K9me3 remained present at these domains (Fig. 3d,e), as found when depleting Orc proteins¹⁴. We confirmed these results by siRNA depletion (Supplementary Fig. 4d-f). Next, to evaluate if this effect on HP1 α was specific to SENP7, we examined SENP6, which did not colocalize with HP1 α and SENP6 depletion did not alter HP1 α localization (Fig. 3f,g and Supplementary Fig. 4g). However, we noted that SENP6 was able to deconjugate sumoylated HP1 α in the transfection assay (Supplementary Fig. 5). Taken together, these data underline the fact that the importance of SENP7 in maintaining HP1 α enrichment at pericentric domains likely involves the specificity of SENP7 localization. The loss of SENP7 and HP1 without affecting the H3K9me3 mark nor the overall organization of the domains emphasizes the existence of distinct steps for HP1 accumulation at pericentric domains.

In conclusion, we propose that SENP7 is a novel maintenance factor for HP1 α accumulation at pericentric heterochromatin based on the following arguments : (i) its direct interaction with HP1, (ii) the common behaviour and localization of the two proteins at pericentric heterochromatin and (iii) loss of HP1 α enrichment at pericentric heterochromatin after SENP7 depletion. Moreover, we identify SUMO-1-modified HP1 α as a substrate for SENP7. Although sumoylation is a post-translational modification involved in many cellular processes, surprisingly, only a few putative substrates have been identified for the SENPs^{7,9}, and to our knowledge, none have been identified for SENP7. While previous studies *in vitro* indicated a SENP7 deconjugating activity for SUMO2/3¹⁵ and a limited activity for SUMO-1¹¹, we provide evidence that SENP7 could equally remove SUMO-1 or SUMO-2 *in vivo* using exogenously expressed SUMO (Supplementary Fig. 6). Thus, given that endogenous HP1 α is modified by SUMO-1, but not SUMO-2/3, when associated with major RNA as recently detected⁵, it is tempting to consider that SENP7 role in regulating HP1 α accumulation at

pericentric heterochromatin could serve to remove SUMO-1. We do not exclude however possible role to remove SUMO-2/3. To target the activity of SENP7 on SUMO-1-HP1 α , the simplest options to consider are the direct interaction between SENP7 and HP1 α combined with the localization of SENP7 at HP1-enriched domains. However, we do not exclude the possibility that SENP7 may deconjugate other sumoylated factors present at pericentric domains, such as KAP1, a known HP1 interacting partner that is SUMO-1-modified *in vivo*¹⁶.

Our data place the cycle of sumoylation/desumoylation as an important new player in regulating efficient accumulation of HP1 α at pericentric domains. After a SUMO-dependent targeting of HP1 α to pericentric heterochromatin⁵, a subsequent retention step would be provided by SENP7 desumoylation activity. Interestingly, an HP1 α -SUMO-1 fusion protein that remains constitutively sumoylated can be rapidly and efficiently targeted to pericentric heterochromatin, yet it proved toxic to the cells⁵. This indicates that persistence of the SUMO modification on HP1 should be avoided. While SENP7 is likely to be the specific SUMO protease acting at these domains on modified HP1, future work should aim to identify the HP1 specific SUMO ligase machinery in order to decipher how this cycle can be regulated.

ACKNOWLEDGEMENTS

We thank Ron Hay for reagents, Claude Gazin for early investment, Sylvain Cantaloube for technical help, Alysia vandenBerg for critical reading of the manuscript, members of the unit for discussions and the Curie Imaging platform for microscopy facility. K. Romeo received support from University Pierre et Marie Curie (UPMC), Paris, France/Ministère de l'Enseignement Supérieur et de la Recherche. This work was supported by la Ligue Nationale contre le Cancer (Equipe labellisée Ligue 2010), the European Commission Network of Excellence EpiGeneSys (HEALTH-F4-2010-257082), ERC Advanced Grant 2009-AdG_20090506 "Eccentric" and ANR "ECenS" ANR-09-BLAN-0257-01.

AUTHOR CONTRIBUTIONS

JPQ, CM and GA conceived and designed the experiments. KR, CM, DB, MD and JPQ performed the experiments. JPQ, CM, KR and GA analysed the data. JPQ, CM and GA wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1 SENP7 associates with HP1 α . **(a)** Immunoprecipitation (IP) of GFP (control), GFP-SENP7 wild-type (e-SENP7) and GFP-SENP7C979S (e-SENP7M). Input (Inp) is 5, 2.5 and 1.25% of total cell extract. **(b)** Immunoprecipitation (IP) of hnRNPC-HA (e-hnRNPC) and HP1 α -HA (e-HP1 α). Input (Inp) is 10 and 5% of total cell extract. Asterisks indicate unspecific bands. **(c)** Direct interaction between His-SENP7 bound to beads and purified GST-HP1 α .

Figure 2 SENP7 localizes with HP1 α . **(a)** Endogenous SENP7 (green) and HP1 α (red) localization in NIH3T3 cells extracted with Triton as indicated with x3 magnification of selected chromocenters (arrows). Scale bar, 10 μ m. **(b)** Quantitative analysis of the percentage of cells with SENP7 and HP1 α localized at pericentric domains or diffuse. Bars represent the mean and error bars indicate s.d. from 3 different experiments. **(c)** Left, SENP7 and HP1 α detection in NIH3T3 cells treated with RNase A as indicated. Right, Western blot analysis of total proteins. **(d)** Left, SENP7 and HP1 α detection in wild type and *Suv39h* double-null (dn) MEFs. Right, Western blot analysis of total proteins. Asterisks indicate unspecific bands. **(e)** Left, analysis of sumoylated HP1 α -HA immunoprecipitate (GFP-SUMO-1-HP1 α -HA, black arrow and box), Asterisks indicate unspecific bands. Right, comparison of protein expression by Western blot.

Figure 3 SENP7 depletion disrupts HP1 α localization at pericentric domains. **(a)** Experimental scheme. **(b)** Endogenous HP1 α (blue) and SENP7 (red) localization in control (micont) and SENP7-depleted (miSENP7) GFP positive cells (green). Scale bar, 10 μ m. **(c)** Quantitative analysis of the percentage of GFP positive cells with HP1 α localized at pericentric domains or diffuse. Bars represent the mean and error bars indicate s.d. from 7 different experiments. $p_{\text{value}} = 0.004$. **(d)** Endogenous HP1 α (blue) and H3K9me3 (red) localization in GFP positive cells (green). **(e)** Quantitative analysis as in c. Bars represent the mean and error bars indicate s.d. from 3 different experiments. **(f)** Endogenous HP1 α (green) and SENP6 (red) localization in control (sicont) and siSENP6-treated (siSENP6) cells. **(g)** Quantitative analysis as in c. Bars represent the mean and error bars indicate s.d. from 3 different experiments.

ONLINE METHODS

Cells, miRNA and siRNA treatment, extracts. We cultured NIH3T3 cells and MEFs (provided by T. Jenuwein)¹³ in DMEM (Gibco BRL) containing 10% (v/v) FCS at 37°C and 5% CO₂. We performed RNase treatment as described in¹². We transfected NIH3T3 cells with Lipofectamin 2000 (Invitrogen) and performed analyses 72 h after transfection. We prepared total cell extract by resuspending cells in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 1% (v/v) Nonidet P-40, and 0.75% (w/v) sodium deoxycholate, supplemented with protease and phosphatase inhibitors, and 20 mM NEM) and nuclear extracts as described in¹⁷.

Plasmids. To generate GST-SENP7 and His6-SENP7, we cloned mouse SENP7 cDNA obtained from I.M.A.G.E clone collection (isoform 1, clone ID IRAVp968H0599D6, RZPD) into pGEX-4T1 and pET-30a vector (Novagen). We made GFP-SENP7 by inserting SENP7 cDNA in the pEGFP-C3 vector (Invitrogen). The catalytic-mutant GFP-SENP7C979S was

generated from GFP-SEN7 by site directed mutagenesis (Genescript). We used the pEGFP-C3 plasmid to express GFP controls. HP1 α -HA expressing plasmid was as described in⁵. GFP-SUMO1, GFP-SUMO-2, GFP-SEN6 and GFP-SEN6C1030S¹⁸ were kindly provided by R. Hay. We constructed the plasmid expressing the miRNA targeting SEN7 (miSEN7) according to the Block-iT™ Pol II miR RNAi expression vector kit (Invitrogen) with the sequence 5'-TGATGAAGAAAGTTGCTCTGA-3'. Control miRNA (micont) with the sequence 5'-GAAATGTACTGCGCTGGAGA-3' predicted not to target known vertebrate genes was provided with the Block-iT™ Pol II miR RNAi system (Invitrogen). The sequences of the siRNA were as follows: siSEN7, 5'-ACAAGAAGCCUAAGAAUA-3'; siSEN6, ON-TARGETplus SMARTpool J-062052-05, 06, 07 and 08 (Dharmacon); sicontrol, 5'-CGUACGCGGAAUACUUCGA-3'.

Immunofluorescence and image acquisition. We processed cells for immunostaining as described^{17,19}. For simultaneous visualisation of GFP with SEN7 and HP1 α or H3K9me3 and HP1 α , cells were not extracted prior to fixation. HP1 α was detected with Alexa Fluor 647 secondary antibodies and SEN7 or H3K9me3 with Alexa Fluor 594 secondary antibodies. We used a Zeiss Z1 epifluorescence microscope equipped with a x63 objective lens and a chilled CCD camera (H2Q2, Roper) for image acquisition.

Statistical analysis. The p_{value} were calculated in R program. We used the Student paired-t test to compare the % of cells with HP1 localization at pericentric domains between miSEN7 and micont from 7 independent experiments. We counted more than 100 nuclei for each case per experiment. Differences were considered significant when $p_{\text{value}} < 0.05$.

Antibodies and immunoprecipitation. We generated a rabbit polyclonal anti-mSEN7 by injecting recombinant full-length His6-mSEN7 (Agro-Bio). We tested its specificity for SEN7 by immunofluorescence and Western blot after depletion of SEN7 (Supplementary Fig. 2a,b). Rabbit serum was used at 1:1,000 dilution for Western blot. For

immunofluorescence we affinity purified the antibodies by incubating 200µl of serum with 5 µg of purified His6-mSENP7 present on a nitrocellulose membrane according to²⁰, and used this purified antibody at 1:10 dilution. We used the following antibodies: for immunofluorescence, mouse monoclonal anti-HP1α (2HP-1H5-AS, Euromedex ; 1:1,000), rabbit polyclonal anti-H3K9me3 (#07-442, Upstate; 1:1,000), goat polyclonal anti-SENP6 (#ab77619, Abcam; 1:50) and secondary antibodies Alexa Fluor 568, 594 or 647 (Invitrogen); for Western blot, rabbit polyclonal anti-HP1α (C7F11, Cell signaling; 1:1,000), mouse monoclonal anti-HP1α (2HP-2G9-AS, Euromedex ; 1:1,000), mouse monoclonal anti-HP1β (1MOD-1A9-AS, Euromedex; 1:1,000), mouse monoclonal anti-HP1γ (2MOD-1G6, Euromedex; 1:1,000), goat polyclonal anti-SENP6 (#ab77619, Abcam; 1:250), mouse monoclonal anti-GST (3G10/1B3, Abcam, 1/1,000), mouse monoclonal anti-β-actin (AC-15, Sigma; 1:20,000), rat monoclonal anti-HA (#1867423, Roche; 1:2,000), mouse monoclonal anti-GFP (7.1 and 13.1 mix, Roche; 1:1,000), rabbit polyclonal anti-KAP1 (#A300-274A, Bethyl; 1:1,000), rabbit polyclonal anti-SetDB1 (#07-378, Upstate ; 1:1,000) and secondary antibodies coupled to horseradish peroxidase (Jackson Immuno Research Laboratories, Inc). We performed Western blot immunodetection on the membrane with the Super Signal kit (Pierce). We performed GFP and HP1α immunoprecipitation with anti-HA agarose-conjugated beads (Roche) and GFP-trap_A beads (Chromotek). We performed HA immunoprecipitations in RIPA buffer as in⁵. We performed GFP precipitations and washes in 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 15 mM MgCl₂ and 0.1% (v/v) Nonidet P-40 supplemented with protease inhibitors. We quantified HP1α-HA and GFP-SUMO-1-HP1α-HA from Western blots with a Chemi-doc XRS system and the QuantityOne software (Biorad).

Cell cycle/DNA content analysis by flow cytometry. After washes in PBS supplemented with 0.1% (w/v) BSA and 0.02% (w/v) sodium azide (PBA), we resuspended NIH3T3 cells with continuous gentle vortexing in 70% (v/v) ethanol at -20°C and incubated them on ice for 30 min. Then we stained cells with 20 µg.ml⁻¹ propidium iodide plus 10µg.ml⁻¹ RNase A in

PBA for 30 min. We performed analysis on a FACSCalibur (Becton Dickinson) or on a C6 (Accuri) cytometer and FlowJo software (Treestar). We selected cells for analysis on their cycle profile by first gating on live cells with a forward scatter (FSC) vs side scatter (SSC) gate, then excluding multiplets with a SSC-H vs SSC-W gate and finally selecting either GFP-positive or GFP-negative cells.

Recombinant proteins and pull down. We bacterially expressed GST, GST-HP1 α ¹², GST-SEN7, and His-SEN7 from *E. coli* BL21 (DE3) strain and immobilized proteins on glutathione (Amersham) or Ni-NTA beads (Novagen). We purified GST and GST-HP1 α by elution from the glutathione beads according to manufacturer's instructions in buffer A (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.05% (v/v) Nonidet P-40, supplemented with protease inhibitors). We performed GST pull-down by mixing 3T3 nuclear extract with GST or GST-SEN7 beads and incubating for 2 hours at 4°C on a rotating wheel in buffer A. After five washes in buffer A containing 500 mM NaCl, we recovered proteins by boiling in SDS-PAGE loading buffer and analyzed by Western-blot. We performed direct interaction assay by mixing GST or GST-HP1 α with His-SEN7 beads and proceeded as above.

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SUPPLEMENTARY DATA

**The SUMO protease SENP7 is a critical component to ensure HP1 enrichment
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Supplementary Figure 1 SENP7 interacts with HP1. **(a)** Immunoprecipitation (IP) of GFP (control), GFP-SEN7 wild-type (e-SEN7) and GFP-SEN7C979S (e-SEN7M). We performed Western blot using anti-GFP, anti-HP1 α , anti-KAP1 and anti-SetDB1 antibodies to reveal proteins in the input (Inp) and the immunoprecipitates (IP). We show 2 exposures for KAP1 detection. Input (Inp) is 5, 2.5, 1.25% of total cell extract. Asterisk indicates unspecific band. **(b)** We performed GST and GST-SEN7 pull-down from NIH3T3 nuclear extract. Western blot revealed HP1 α , HP1 β , HP1 γ , GST and GST-SEN7 in the input (I, 10%), unbound (U) and bound (B) fractions.

Supplementary Figure 2 SENP7 deconjugates SUMO-1-HP1α in vivo. **(a)** Experimental scheme. **(b)** We transfected NIH3T3 cells with GFP, GFP-SUMO-1, GFP-SEN7, HP1α-HA as indicated. We verified the expression of the proteins in total cell extracts using anti-SEN7, anti-HA, anti-HP1α, anti-GFP and anti-β-actin antibodies. Asterisks indicate unspecific bands. **(c)** After anti-HA immunoprecipitation, Western blot using anti-HA, anti-HP1α and anti-GFP antibodies revealed sumoylated HP1α-HA (GFP-SUMO-1-HP1α-HA, black arrow and box) and unmodified HP1α-HA. We show low, medium and high exposures for each detection. IgG corresponds to the immunoglobulin light chain. Asterisks indicate unspecific bands. **(d)** Quantification of the decrease of GFP-SUMO1-HP1α-HA levels. An histogram showing comparison of the ratio between GFP-SUMO-1-HP1α-HA and unmodified HP1α-HA in the absence (-) or presence (+) of GFP-SEN7 is represented. We performed quantifications with anti-HA (top) or anti-HP1α (bottom) antibodies. The 100 % is set for the control (absence of GFP-SEN7). The bars represent the mean and error bars indicate the s.d. from 5 and 2 different experiments for anti-HA and anti-HP1α detections respectively.

Supplementary Figure 3 Detection of GFP-SUMO-1-HP1α-HA and HP1α-HA by western blot is linear. (a) We transfected NIH3T3 cells with GFP, GFP-SUMO-1, GFP-SEN7, HP1α-HA as indicated. After anti-HA immunoprecipitation, we loaded 1x, 2x and 3x amounts of the immunoprecipitates and performed Western blot using anti-HA antibodies. We show low and high exposures which allow detection of unmodified HP1α-HA and sumoylated HP1α-HA. The positions of sumoylated HP1α-HA (GFP-SUMO-1-HP1α-HA, black arrow and box), unmodified HP1α-HA and IgG light chains (IgG) are indicated. Asterisks indicate unspecific bands. (b) Quantitative analysis. Left: histogram showing the levels of unmodified HP1α-HA detected for each condition. The 100% is set for the 2x load of the GFP control and is represented by the dashed line. Right: as in left but for sumoylated HP1α-HA (GFP-SUMO-1-HP1α-HA). The bars represent the mean and the error bars indicate the s.d. from 3 independent experiments.

Supplementary Figure 4 SENP7 downregulation in NIH3T3 cells. **(a)** Endogenous SENP7 (red) localization in control (micont) and SENP7-depleted (miSENP7) GFP positive cells (green). Scale bar, 10 μ m. **(b)** Western blot of total proteins from cells transfected as in a. Since we did not select the GFP-positive cells, the presence of SENP7 (arrow) in the miSENP7 extract arises from non-transfected cells. Asterisks indicate unspecific bands. **(c)** Flow cytometry analysis of the DNA content of NIH3T3 cells transfected as in a. **(d)** Endogenous SENP7 (red) and HP1 α (green) localization in control (sicont) and siSENP7-treated (siSENP7) cells. Scale bar, 10 μ m. **(e)** Western blot of total proteins from cells transfected as in d. Asterisks indicate unspecific bands. **(f)** Quantitative analysis of the percentage of cells with HP1 α and H3K9me3 localized at pericentric domains or diffuse from 3 different experiments. **(g)** Western blot of total proteins from control (sicont) and SENP6-depleted (siSENP6) cells. Asterisk indicates unspecific band.

Supplementary Figure 5 SENP6 deconjugates SUMO-1-HP1α in vivo. **(a)** Experimental scheme. **(b)** We transfected NIH3T3 cells with GFP, GFP-SUMO-1, GFP-SEN6, GFP-SEN6C1030S and HP1α-HA as indicated. We verified the expression of the proteins in total cell extracts using anti-HA, anti-HP1α, anti-GFP and anti-β-actin antibodies. **(c)** After anti-HA immunoprecipitation, Western blot using anti-GFP, anti-HA and anti-HP1α antibodies revealed sumoylated HP1α-HA (GFP-SUMO-1-HP1α-HA, black arrow and box) and unmodified HP1α-HA.

Supplementary Figure 6 SENP7 deconjugates SUMO-2-HP1α in vivo. **(a)** Experimental scheme. **(b)** We transfected NIH3T3 cells with GFP, GFP-SUMO-2, GFP-SEN7, GFP-SEN7C979S and HP1α-HA as indicated. We verified the expression of the proteins in total cell extracts using anti-SEN7, anti-HA, anti-HP1α, anti-GFP and anti-β-actin antibodies. Asterisk indicates unspecific band. **(c)** After anti-HA immunoprecipitation, Western blot using anti-GFP, anti-HA and anti-HP1α antibodies revealed sumoylated HP1α-HA (GFP-SUMO-2-HP1α-HA, black arrow and box) and unmodified HP1α-HA.