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Transcriptional Repression of Estrogen Receptor α Signaling by SENP2 in Breast Cancer Cells

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Abstract

Estrogen receptors (ERs) are ligand-activated transcription factors involved in many physiological and pathological processes, including breast cancer. Their activity is fine-tuned by post-translational modifications notably sumoylation. In the present study, we investigated the role of the SUMO protease SENP2 in the regulation of ER α activity. We first found SENP2 to significantly repress estradiol-induced transcriptional activity in breast cancer cells (MCF7 and T47D). This effect was observed with a reporter plasmid and on endogenous genes such as *TFF1* and *CTSD* which were shown to recruit SENP2 in chromatin immunoprecipitation experiments. Using GST pull-down, coimmunoprecipitation and proximity ligation assays, SENP2 was found to interact with ER α and this interaction to be mediated by the amino-terminal region of the protease and the hinge region of the receptor. Interestingly, we demonstrated that ER α repression by SENP2 is independent of its SUMO protease activity and requires a transcriptional repressive domain located in the amino-terminal end of the protease. Using siRNA assays, we evidenced that this domain recruits the histone deacetylase, HDAC3 to be fully active. Furthermore, using both overexpression and knock-down strategies, we showed that SENP2 robustly represses estrogen-dependent and independent proliferation of MCF7 cells. We provided evidence that this effect requires both the proteolytic and transcriptional activities of SENP2. Altogether, our study unravels a new property for a SUMO protease and identifies SENP2 as a classical transcription coregulator.

Author Keywords estrogen signaling ; SUMO protease ; transcription factor ; breast cancer cell ; proliferation

INTRODUCTION

Estrogens play a major role in diverse physiological functions, including control of development, sexual behavior and reproduction (1). These hormones are also directly implicated in various pathological processes, notably hormone-dependent cancers such as breast, endometrial and ovarian cancers (2). Estrogens act through binding to two members of the nuclear receptor superfamily (3), ER α and ER β , which can function through a genomic or a non-genomic pathway (4). In the genomic pathway, ER-dependent transcription involves sequential recruitment of an assortment of coregulators, including coactivators and corepressors (5).

It is largely accepted that ER α can undergo a wide variety of post-translational modifications which regulate its activity (6). A few years ago, Sentis *et al.* reported on modification of ER α by SUMO1 and its role in ER α transcriptional activity (7). The initial step in sumoylation is activation of a SUMO peptide by the hydrolase activity of a SUMO-specific cysteine protease called "sentrin proteases" or SENPs (8). Three enzymes (E1, E2 also known as ubc9 and an E3 ligase) are involved in this modification in a process closely related to ubiquitylation. Sumoylation is a reversible process because of the isopeptidase activity of SENPs that cleaves the isopeptide bond between the glycine residue of SUMO and the lysine of the substrate (9). The catalytic site of such proteases is located within a highly conserved 20 amino acid region in the carboxy-terminal part of the protease. SENPs are crucial for maintaining the level of sumoylated and unsumoylated substrates required for normal physiology.

The SENP family comprises six members. SENP1 and SENP2 can process all three SUMO isoforms and can desumoylate both monosumoylated proteins and polymeric SUMO side chains. SENP3 and SENP5 exert endopeptidase activity only on SUMO2 and SUMO3, while SENP6 and SENP7 display only low hydrolase activity (10). In SENP1 or SENP2 knockout mice, both proteases are essential to embryo viability (11, 12). Furthermore, elevated levels of SENP1 have been observed in thyroid adenocarcinoma and prostate cancer (13, 14). SENP2 is essential to trophoblast development, through modulation of the p53/Mdm2 pathway (11). Knockout mouse models have also evidenced a crucial role for SENP2 in regulating adipogenesis by targeting C/EBP β (15), whereas mice overexpressing SENP2 display severe cardiac dysfunction (16). SENPs, including SENP2, are reported to modulate the activity of transcription factors, notably the androgen receptor (AR) (17) and the progesterone receptor (PR) (18).

In the present work, we describe a repressive activity of SENP2 on estradiol-induced gene expression and breast cancer cell proliferation. We evidence and decipher the interaction between the SENP2 and ER α proteins. Although SENP2 has the potential to desumoylate ER α , we clearly demonstrate that the effect of SENP2 on estrogen signalling is independent of its desumoylase activity. Indeed, the inhibition is mediated by a repressive domain located in the amino-terminal region of SENP2 which recruits the histone deacetylase HDAC3. Altogether, our study reveals a new property for SUMO proteases and identifies SENP2 as a classical ER α transcriptional corepressor.

MATERIALS AND METHODS

Plasmids

pRL-CMV-renilla was obtained from Promega. GST-LBD (AF2) and GST-D have been described elsewhere (19) and (7) respectively. p3XFlag-SUMO1 was created as follows: pGEX-SUMO1 was digested with BamHI and the resulting fragment inserted into p3XFLAG-myc-CMV-24 (Sigma- Aldrich). pFlag-SENP1 and pFlag-SENP2 (a kind gift of Dr Yeh, MD Anderson, Houston) were subcloned into p3XFLAG-myc-CMV-24 to create p3XFlag-SENP1 and p3XFlag-SENP2. Wild type SENP1 was subcloned into pECFP and wild-type SENP2 into p3XFLAG-myc-CMV, pECFP (Clontech), pM (Clontech), pGEX (Pharmacia) or pcDNA3.1(-) (Invitrogen). All SENP2 fragments and mutants were engineered by PCR, sequenced and then subcloned into the above mentioned vectors.

Cell culture, transient transfections and luciferase assays

MCF7 and COS7 cells were cultured in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) (F12/DMEM) or in DMEM supplemented with 10 % fetal calf serum (FCS) (Invitrogen) and antibiotics (Gibco). 96 h before transfection, MCF7 cells were cultured under sterol-depletion in fresh phenol red- free medium containing 5% dextran-charcoal-treated FCS. For the luciferase assays, cell transfections were carried out with JetPEI (Polyplus) according to the manufacturer's protocol. Six hours after transfection, MCF7 cells were treated with 10^{-8} M estradiol, 10^{-8} M OH-tamoxifen or vehicle, and then harvested 18 h later with 0.1 ml lysis buffer (25mM Tris pH 7.8, 2mM EDTA, 10% glycerol, 1% Triton X-100). Luciferase (firefly and renilla) values were measured and the firefly luciferase data were normalized with respect to the renilla activity and expressed as relative luciferase activity. For the sumoylation and immunoprecipitation assays, the cells were treated 12 hours after transfection, when indicated, with 100 nM estradiol (for the sumoylation assay) or 10 nM estradiol (for the immunoprecipitation assay), and harvested 18 h later. Protein expression was analyzed by western blotting. For proliferation assays and cell cycle analysis, stable cell lines were cultured in phenol red- free Dulbecco's modified Eagle medium containing 5% dextran-charcoal treated FCS and 1mg/ml G418 or 1 μ g/ml Puromycin for 96 hours. To evaluate estradiol effect, cells were provided with fresh medium supplemented with 1% dextran-charcoal treated FCS for 48 hours and treated with 10^{-8} M E2 or vehicle for 24 hours for cell cycle analysis and as indicated for proliferation assays.

Generation of stable cell lines and siRNA transfections

JetPEI (Polyplus) was used to stably transfect MCF7 cells according to the manufacturer's protocol. On the one hand, cells were transfected with either the parental pECFP vector (Clontech) or the pECFP-SENP2 vector and treated with G418 (1 mg/ml) (GIBCO). On the other hand, cells were transfected with either the non-target shRNA control vector (pLKO.1-NTshRNA-puro) or the SENP2 shRNA vector (pLKO.1-SENP2shRNA-puro) (MISSION, Sigma) and treated with puromycin (1 μ g/ml) (Sigma). The DharmaFECT 2 (Dharmacon) or INTERFERin (Polyplus) transfection agent was used for transfections with non-targeting (Ctrl), SENP2-specific or HDAC3-specific siRNA (Dharmacon). Each transfection was performed in triplicates and interference efficiencies were tested by qPCR and western blotting.

Immunoprecipitation and immunoblotting assays

Immunoprecipitation assays were performed as previously described (20). Cells were harvested in modified RIPA buffer (50mM Tris-HCL, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaF, 1 mM Na₃ VO₄, 1 mM β -glycerophosphate) in the presence of 20 mM N-Ethylmaleimide (NEM) whenever stated. Western-blot analyses were carried out with primary antibodies against ER α 60C (Millipore), GAPDH (Life Science), Flag (Sigma), GFP, HDAC3 (N-19) or SENP2 (Santa Cruz), or Actin A2066 (Sigma). For the immunoprecipitation assays, cell extracts were incubated with an anti-ER α rabbit polyclonal antibody HC20 (TEBU) or a mouse IgG. Protein A/G agarose beads were added, and bound proteins were released and subjected to western-blot analysis to detect coimmunoprecipitated proteins with a rabbit monoclonal anti-ER α 1C60 (Millipore) and a rabbit polyclonal anti-GFP (Santa Cruz Biotechnology). A peroxidase-conjugated donkey anti-rabbit antibody was used as secondary antibody.

GST pull-down assays

GST pull-down assays were performed as previously described (21). Radioactivity detection was done with the Fujix BAS5000 phosphoimager (Fujifilm).

Quantitative PCR

Quantitative PCR was performed as previously described (22). Results were normalized with respect to the 28S rRNA levels (endogenous control).

DuoLink *in situ* Proximity Ligation Assay (PLA) for protein-protein interactions

The Duolink II Proximity Ligation Assay kit (Eurogentec, France) was used according to the manufacturer's instructions (Olink). Briefly, the principle can be described as follows. Two primary antibodies raised in different species recognize the target antigen or antigens of interest. PLA probes are species-specific secondary antibodies that bind to the primary antibodies. When the PLA probes are in close proximity (<40 nm), the two short DNA strands, attached to it, form a circle oligonucleotide DNA that is ligated by enzymatic ligation. After the amplification reaction, labeled complementary oligonucleotide probes highlight the product. The resulting high concentration of fluorescence in each single-molecule amplification product is easily visible as a distinct bright dot when viewed with a fluorescence microscope.

MCF7 cells were fixed in 3,7% formaldehyde, permeabilized with PBS-1% Triton-X100 and blocked with PBS-1% BSA. Nuclear staining was performed with Hoechst 33342. The primary antibodies used targeted ER α (1D5, Dako), SENP2 (Santa Cruz) or HDAC3 (N19, Santa Cruz). Signal detection was carried out by red fluorescence imaging performed on a Carl Zeiss Axioplan 2 imaging microscope equipped with a 40 \times objective.

Chromatin Immunoprecipitation

Transfected MCF7 cells were treated with 10⁻⁸ M estradiol or vehicle for 45 min. Chromatin was purified from the cells after formaldehyde cross-linking (1% final concentration). ChIP assays were performed with the ChIP kit according to the manufacturer's instructions (Cell Signaling) and with the indicated antibodies and primers. The antibodies used were rabbit polyclonal anti-ER α H-184 (Santa Cruz Biotechnology), rabbit polyclonal anti-GFP Ab290 (Abcam) and rabbit IgG (Cell Signaling). The primers were selected to detect the human *TFF1* promoter region (-353 to -30) (forward: ATGGCCACCATGGAGAACAA, reverse: TAAAACAGTGGCTCCTGGCG) and the human *CTSD* promoter region (-295 to -54) (forward: TCCAGACATCCTCTCTGGAA, reverse: GGAGCGGAGGGTCCATTC).

Cell proliferation analysis

Real time growth kinetics of stable cell lines was examined with an impedance-based Real-Time Cell Analysis (RTCA) system (Roche Applied Science, Meylan, France). Background measurement of 16-well E-plates was performed with 50 μ l phenol red-free Dulbecco's modified Eagle medium containing 1% dextran-charcoal treated FCS and 1mg/ml G418 or 1 μ g/ml puromycin. Then, 2500 cells/well were seeded in 100 μ l of additional culture medium. Cell proliferation was monitored every 24h for 6 days. Cell sensor impedance was expressed as a dimensionless parameter called the Cell Index (CI). Data analysis was carried out with the RTCA Software 1.2 supplied with the instrument.

Cell cycle analysis

Cells were incubated for 4 h with 30 μ M BrdU, trypsinized, centrifuged, fixed by addition of 1.5 ml ice-cold 100% ethanol, digested at room temperature (RT) in 0.05% pepsin/30mM HCl and 2 N HCl and incubated with primary rat anti-BrdU antibody (clone BU1/75, AbC117-7517) and secondary goat anti-rat-FITC conjugate antibody (Southern Biotech 3030-02). The cells were stained with propidium iodide. The cell cycle was analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter). Data analysis was carried out with FlowJo Software.

Statistical analysis

Data are presented as means \pm standard deviations. Statistical analyses were performed with Student's t-test. Differences were considered statistically significant at $p < 0.05$. (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$).

RESULTS

SENP2 represses estrogen-dependent transcriptional activity

In an attempt to define the role of protein sumoylation in estrogen signaling, we first investigated the effect of SUMO1 on estrogen-dependent activity in ER α -positive MCF7 breast cancer cells (Figure 1A). In this model, in contrast to previous data obtained on HeLa cells (7), ectopic expression of SUMO1 led to significant repression of estradiol-dependent transcriptional activity. We then analyzed how the most-studied SUMO proteases, SENP1 and SENP2, might affect this repression by SUMO1. Interestingly, when ectopically expressed, SENP1 was found to decrease SUMO1-triggered repression, whereas SENP2 increased it.

In order to further decipher the mechanism of action of SENP2, we first transfected MCF7 cells with increasing doses of SENP2 expression vector (Figure 1B, left panel). We observed a significant and dose-dependent inhibition of estradiol-induced transcriptional activity while OHT-dependent activity was not affected. Similar results were also obtained with HeLa (Supplementary Figure S1) and COS cells (data not shown). In parallel, we checked that SENP2 did not significantly affect ER α expression levels (Supplementary Figure S2).

To strengthen these results, we investigated the effect of SENP2 expression knock-down in MCF7 cells. By means of siRNA transient transfection we obtained a 5-fold inhibition of SENP2 mRNA levels (data not shown). As expected, downregulation of SENP2 expression significantly increased estradiol-dependent transcriptional activity (Figure 1B, right panel), confirming the repressive activity of the protease. SENP2 was also observed to robustly repress the transcriptional activity of two other nuclear receptors (ER β and PR) while it displayed an activation effect on both AR and ERR γ -dependent transcriptional activity (Supplementary Figure S3).

We next investigated whether SENP2 might regulate endogenous estradiol-induced genes such as *PGR*, *TFF1*, *CCND1* or *CTSD* (Figure 1C). To do so, MCF7 cells were transfected with the CFP or CFP-SENP2 expression plasmid and treated with either vehicle or estradiol (10^{-8} M). As expected, the SENP2 transcript level increased significantly upon overexpression (upper panel). We then analysed mRNA expression levels of the different estrogen responsive genes. CFP-transfected cells displayed markedly increased *PGR*, *TFF1*, *CCND1* and *CTSD* mRNA levels in response to estradiol treatment, and this induction was strongly decreased upon SENP2 overexpression.

In order to demonstrate that the repressive effect of SENP2 was not specific to MCF7 cells, we also transfected T47D breast cancer cells with the CFP-SENP2 expression plasmid. As shown in supplementary Figure S4 A and B, SENP2 repressed estradiol-dependent transcriptional activity both on a reporter plasmid and on the same endogenous genes as in MCF7 cells. Altogether, these results evidence that SENP2 represses ER-dependent transactivation in breast cancer cells.

SENP2 is recruited to ER target promoters

We then wondered whether SENP2 might be recruited to estrogen-responsive gene promoters. To answer this question, we performed chromatin immunoprecipitation (ChIP) assays on MCF7 cells overexpressing CFP-SENP2 (Figure 1D) and chose to amplify either the *TFF1* (top) or the *CTSD* (bottom) promoter. As a positive control we used the anti-ER α antibody which allowed the immunoprecipitation and the amplification of both *TFF1* and *CTSD* promoters in an estradiol-dependent manner. Interestingly, the same results were obtained after immunoprecipitation of CFP-SENP2 whereas no amplification could be observed in the presence of unrelated IgG. We failed to amplify either promoter when the anti-CFP antibody was used on extracts of CFP-expressing cells (data not shown). Secondly, when CFP-SENP2 was immunoprecipitated, no amplification was observed with primers specific to the SENP2 coding sequence (data not shown). These data strongly suggest that SENP2 exerts its repressive effects through a direct recruitment to target gene promoters.

SENP2 interacts with ER α

In order to support the ChIP data, we then used various means to investigate the interaction between SENP2 and ER α . We first performed immunoprecipitation from transfected COS7 cells using an anti-ER α antibody. As shown in Figure 2A, SENP2 co-immunoprecipitated with ER α only in the presence of estradiol. The different inputs indicated that protein levels were constant under the different conditions (lower panels).

We next performed proximity ligation assays (PLA) in MCF7 breast cancer cells, to evidence interactions between endogenous ER α and SENP2 (Figure 2B). The number of dots revealing complexes containing ER α and SENP2 was significantly higher in the presence of estradiol than with ethanol alone, further confirming the first set of experiments. Control experiments for PLA assays are shown in supplementary Figure S5.

We then used GST pull-downs to dissect the interaction. Using GST-SENP2 as a bait, we investigated the interaction between SENP2 and various ER α fragments (Figure 2C). The results confirmed the estradiol-dependent interaction between full-length ER α and GST-SENP2. Mutants lacking the hinge domain (referred to as the D domain) (fragments HE39 and HE241G) showed no interaction with SENP2, whereas fragment HE15, containing this region, displayed a clear interaction. Conversely, GST-D was also found to interact with the protease. We conclude that the hinge region of ER α mediates its interaction with SENP2.

We also generated GST-fused SENP2 deletion mutants to identify interacting domains on SENP2 (see Figure 2D). The catalytic domain of SENP2 (aa 366-589) displayed no interaction, whereas the amino-terminal part (aa 1-365) clearly bound ER α in an estradiol-dependent way. Interestingly, the SENP2 region responsible for the interaction with nucleoporin Nup153 (23) (aa 1-70) proved unable to pull down ER α . Different fragments of the amino-terminal region of SENP2 were also tested. Among them, only N4 (aa 132-251) and N5 (aa 192-311) were found to interact with ER α . These data indicate that the ER α -interacting domain (ERID) of SENP2 encompasses the region located between amino acids 192 and 251.

The repressive effect of SENP2 is independent of its catalytic activity

To better comprehend the transcriptional effect of SENP2 on ER α activity, we generated mutants lacking isopeptidase activity and/or the capacity to bind SUMO peptides. On the basis of the study of Best *et al.* on the SUMO protease SuPr-1 (24), we introduced point mutations to generate SENP2(C466S) and SENP2(W375A). As shown in Supplementary Figure S6A, we evidenced that wild-type SENP2 can desumoylate ER α efficiently, whereas neither SENP2(C466S) nor SENP2(W375A) exhibits any isopeptidase activity. In GST pull-down assays, both wild-type SENP2 and SENP2(C466S) were found to bind SUMO1, while SENP2(W375A) failed to interact with this peptide (Supplementary Figure S6B). We next evidenced that an estradiol-dependent interaction between ER α and either SENP2(C466S) or SENP2(W375A) which was very similar to the interaction with wild-type SENP2 (Figure 3A).

We then performed transient transfections of MCF7 cells in the presence of increasing doses of wild-type or mutant SENP2 (Figure 3B). Wild-type SENP2 and both SENP2 mutants proved equally able to inhibit ERE-mediated transactivation, without affecting the basal activity. These results clearly indicate that the repressive action of SENP2 on ER α activity is independent of both its isopeptidase activity and its ability to interact with a SUMO peptide. We also tested the effect of SENP2 on ER α mutated at its SUMO sites (Supplementary Figure S7). As shown on the figure, SENP2 proved able to repress the transcriptional activity of the non-sumoylated form of ER α , strengthening the view that SENP2 represses ER α transcriptional activity independently of its enzymatic activity.

Since the action of SENP2 on ER α is independent of its proteolytic activity, we wondered whether the protease might have an intrinsic repressive function. We generated plasmids allowing the expression of wild-type or mutated SENP2 fused to the Gal4 DNA binding domain (pM-SENP2). Interestingly, when transfected in MCF7 cells, we observed a significant and dose dependent repression exerted by wild type or mutated SENP2 (pM-SENP2(C466S) and pM-SENP2(W375A)) (Figure 3C). We conclude that SENP2 has an intrinsic transcriptional repressive potential, independent of its catalytic potential.

Intrinsic SENP2 repressive activity is mediated by its amino-terminal domain

We next sought to determine precisely which region of SENP2 mediates its repressive activity. To do so, we generated a series of Gal4DBD-fused fragments (Figure 4A). The relative expression of the mutants was checked in real time PCR assays (data not shown). As observed in Figure 4B, the amino-terminal domain (1-365) (Nter) exerted a stronger repressive effect than the full-length protein, whereas the carboxy-terminal domain (365-589) (Cter) displayed no repressive activity. Of the different fragments tested (N1 to N11), only N8 (132-191) exhibited significant repressive activity (Figure 4C). Remarkably, the N8 repressive domain displayed an inhibitory potential very similar to that of the Nter domain.

To confirm the importance of the N8 fragment, we deleted the corresponding coding region from the wild-type SENP2. The SENP2(Δ N8) fragment proved able to pull down *in vitro* translated ER α effectively, indicating that the N8 region is not necessary for the interaction with the receptor (Figure 4D). We next generated Gal4DBD-SENP2(Δ N8) and compared its activity with that of the wild-type construct Gal4DBD-SENP2 (Figure 4E). In contrast to Gal4DBD-SENP2, the Gal4DBD-SENP2(Δ N8) protein proved unable to exert any repression. Finally, the mutant was then tested for the ability to repress estradiol-dependent transcriptional activity in MCF7 cells (Figure 4F). While wild-type SENP2 repressed estradiol-dependent transcriptional activity as expected, SENP2(Δ N8) displayed no effect. Altogether, these results clearly establish that SENP2(N8) is necessary for repression of estradiol-dependent transcriptional activity and for the intrinsic repressive activity of SENP2.

HDAC3 partly mediates SENP2 repressive activity

To identify a protein that might mediate SENP2 repressive activity, we tested the ability of SENP2 to interact with various histone deacetylases. Among these, HDAC3 not only displayed a robust interaction with SENP2 (Figure 5A) but showed virtually no interaction with SENP2(Δ N8). Moreover, GST-fused N8 was able to pull-down HDAC3. To further investigate the interaction between HDAC3 and SENP2, we performed coimmunoprecipitation experiments and showed that the anti-HDAC3 antibody efficiently pulled down Flag-SENP2 after transient transfection of COS7 cells (Figure 5B). To confirm the interaction between endogenous proteins, PLAs were performed. While anti-SENP2 (Figure 5C, left panel) or anti-HDAC3 (middle panel) antibody alone indicated expression levels of each protein, simultaneous addition of the two antibodies revealed that both proteins are present within the same complex, as shown by red dots (right panel). Negative controls for the assays are shown in supplementary Figure S4.

We next investigated whether HDAC3 might affect SENP2 activity. To this end, we knocked-down HDAC3 expression with a specific siRNA (Supplementary Figure S8A). Remarkably, HDAC3 knockdown significantly increased luciferase activity indicating a loss in the intrinsic repressive potential of SENP2 (Figure 5D). A similar observation (although to a lesser extent) was made with the repressive activity of the isolated N8 domain whereas no significant effect was observed with pM-SENP2(Δ N8) (Figure 5D). Interestingly, transfection of HDAC3 siRNA affected the dose-dependent repression mediated by both wild-type pM-SENP2 and pM-N8 alone (Supplementary Figures S8B and C). Importantly, in T47D, HDAC3 proved equally important for the activity of SENP2 (Supplementary Figure S4C), further reinforcing above described results. We then investigated the role of HDAC3 on SENP2-mediated repression of ER α

transcriptional activity. As shown in Figure 5E, SENP2 repression of ER α activity was significantly weaker in the presence of HDAC3 siRNA. Altogether, these results evidence that HDAC3 partly mediates SENP2 transcriptional repression.

SENP2 inhibits MCF7 cell proliferation in a protease-dependent manner

Since SENP2 clearly represses ER α transcriptional activity, we wondered whether this SUMO protease might affect E2-dependent proliferation of breast cancer cells. We thus generated MCF7 cells stably transfected with a vector expressing either CFP-SENP2 or a short hairpin RNA targeting SENP2 (shSENP2). As shown in Figure 6A, the transfected cells showed the expected increase (upper left panel) or decrease (upper right panel) in SENP2 mRNA and protein. When transfected with a Flag-SUMO1 expression vector, CFP-SENP2 expressing cells displayed strongly reduced levels of SUMO-conjugated proteins while shSENP2 expressing cells exhibited increased levels of sumoylated proteins (lower panels).

We then used impedance-based real-time cell analysis (xCelligence, Roche) to monitor proliferation of the different cell lines in the presence of estradiol (Figure 6B). Control cells and the parental MCF7 cells grew similarly. In sharp contrast, MCF7 cells overexpressing SENP2 displayed very significantly reduced proliferation in the presence of estradiol whereas shSENP2 cells grew significantly faster. These results were supported by cell cycle analysis of the CFP-SENP2 and shSENP2 expressing cell lines in the presence of estradiol. Indeed, as shown in Figure 6C, BrDU incorporation revealed a very similar percentage of cells in the S phase in control cells and parental MCF7 cells. Strikingly, only 8% of the CFP-SENP2-expressing cells were found in the S phase, and the percentage of cells in the G0/G1 was increased. ShSENP2 cells had an increase in S phase (39.6 %) and a decrease in G0/G1 phase. SENP2 expression in MCF7 cells thus drastically affects the cell cycle at the G1/S transition by significantly reducing the proportion of cells in the S phase.

In order to define whether the transcription repressive domain was necessary for the antiproliferative effect of SENP2, the same experiments were performed with cells stably transfected with CFP-SENP2(Δ N8) (Figure 6D). Interestingly, cells expressing the SENP2 mutant grew like the CFP-expressing line indicating that the repressive domain is necessary for this property of SENP2. Surprisingly, when using the CFP-SENP2 and shSENP2 expressing cell lines, we also noticed an inhibition of cell proliferation in the absence of estradiol (Supplementary Figure S9) suggesting that the effect of SENP2 on cell proliferation might involve a more complex mechanism. In support of this observation, the isopeptidase activity mutant CFP-SENP2(W375A) was also ineffective to inhibit cell proliferation both in the absence or presence of estradiol (Figure 6D and Supplementary Figure S9). These data suggest that SENP2 is able to repress both estrogen-dependent and independent proliferation of MCF7 cells and that this effect require both the proteolytic and transcriptional activities of SENP2.

Discussion

We have investigated here the role of the SUMO protease SENP2 in estrogen signaling in human breast cancer cells. We clearly show that SENP2 acts like a direct transcriptional repressor of ER α activity both on transiently transfected reporter and on several endogenous ER-target genes. Most interestingly, we demonstrate that this effect does not depend on the SUMO protease activity of SENP2, and thus evidence a new property for a member of the SENP family. Importantly, we also reveal an antiproliferative action of SENP2 in the MCF7 breast cancer cell line.

This is the first reported case of a SUMO protease exerting a transcriptional effect that does not depend on its isopeptidase activity. Indeed, former studies found that not only SENP2 but also SENP1 activating functions to depend on the protease activity of the enzyme (17, 25). The isopeptidase- activity-independent action of SENP2 is reminiscent of the action of PIAS proteins (26). It has been reported that a mutant form of PIASy, no longer acting as a SUMO E3 ligase, can still repress AR transcriptional activity (27). More recently, the ubiquitin-specific protease-like 1 (USPL1), a newly described SUMO protease, was shown to display essential functions including cell proliferation independently of its catalytic activity (28). These findings suggest a general paradigm in which enzymes involved in post-translational modifications, including SENPs, may require recruitment of classical transcription factors to fully exert their pleiotropic effects.

In support of this hypothesis, we have found HDAC3 recruitment by SENP2 to be required for full repression by the repressive domain. A tamoxifen-complexed ER α has been reported to recruit the NCoR-HDAC3 complex to the *TFF1* and *MYC* gene promoters (29). More recently, estradiol-activated ER α has been shown to recruit the NCoR-SMRT-HDAC3 complex to the *PROS1* promoter, causing chromatin hypoacetylation (30). As already shown with other classical transcription repressors, such as SMRT (31), NCoR (32), SHP (33) and more recently RIP140 (34), we hypothesize that SENP2 might recruit HDAC3 to induce histone hypoacetylation and less permissive transcription of estradiol- responsive genes.

In the present work, we provide evidence that SENP2 interacts with the hinge domain of ER α , a region targeted by many different post-translational modifications, including sumoylation. Even though we clearly show that the proteolytic activity of SENP2 plays no role in the repression of ER α -dependent transcriptional activity, we also demonstrate that SENP2 efficiently desumoylates ER α . Thus, desumoylation of ER α by SENP2 might make possible or prevent other post-translational modifications. If we take lysines 302 and 303 as

an example, desumoylation could allow acetylation, involved in repression of ER α transcriptional activity. In addition, the interaction between SENP2 and ER α might reinforce this repression when needed.

The SENP2 repressive activity is not limited to MCF7 cells but would be more widely extended to breast cancer cells. Indeed, in T47D, as shown in supplementary Figure S4, SENP2 not only repressed ER α -dependent transcriptional activity on a reporter plasmid but also on endogenous ER-dependent genes (TFF1, PGR, CCND1 and CTSD). We also demonstrated that in T47D, repression by SENP2 is equally dependent on HDAC3.

Altogether, our data on ER α signaling in breast cancer cells appear novel and original as compare to previous studies which describe a positive effect of SENP2 on the transcriptional activity of other nuclear receptors such as AR (17, 25), ERR γ 2 (estrogen-receptor-related receptor γ 2) (38), and PR (18). In our hands, SENP2 also displayed an activating effect on AR and ERR γ -dependent transactivation as mentioned above while it acted as a repressor of PR and ER β -dependent transcriptional activity (supplementary Figure S3). Our data, together with the above described papers suggest that the effect of SENP2 on receptor transcriptional activity may depend on the receptor concerned. It must be underlined that while transcriptional repression of ER α was robustly described as independent of SENP2 enzymatic activity, the mechanism of SENP2 effect on AR, ERR γ or PR activity is totally unknown. It would be of great interest to study whether the specificity of SENP2 activity could differentially implicate its enzymatic activity. In experiments focusing on these receptors, the enzyme PIAS1, also involved in the SUMO pathway, likewise appeared to exert opposite effects according to the receptor (7, 27, 39). In the light of our study, one might hypothesize that according to the targeted protein, the SENP2 repressive domain might be differently exposed and therefore either silenced or activated. One should also note that the above-mentioned effects of SENP2 were observed in different cell contexts. Accordingly, SENP2 might exert very different transcriptional effects depending on the targeted nuclear receptor.

By overexpressing or silencing SENP2 in MCF7 cells, we have highlighted yet another property of SENP2 dealing with repression of cell proliferation under both basal conditions and estradiol stimulation. In our cell cycle analysis, CFP-SENP2 expressing cells displayed a strongly decreased and shRNA SENP2 expressing cells an increased proportion of cells in the S phase. Interestingly, cells expressing the SENP2(W375A) or SENP2(Δ N8) mutant displayed the same proliferation as CFP-expressing MCF7 cells, indicating that both the isopeptidase activity and the transcriptional repressive domain are required for the antiproliferative activity of the protease. One might hypothesize that SENP2 targets different factors regulating cell proliferation in these two different situations.

From a clinical point of view, our data appear rather original since previous studies on SENP1 have shown it to have the opposite effect on prostate cancer cell proliferation (17, 35–37) and to behave as a promoter of prostate cancer (14, 35, 36, 40). In addition, high levels SENP3 expression have been found in various carcinomas, notably of the prostate, ovary, lung, rectum, and colon (41). In prostate cancer, it would seem that desumoylation is favoured by an increase in both SENP1 and SENP3 expression. By contrast, in breast cancer, global sumoylation is increased in response to downregulation of SENP6 (42). A parallel can be drawn with our work which describes increased MCF7 cell proliferation upon SENP2 downregulation. It would thus be of great interest to investigate whether SENP2 expression is altered in breast tumors and correlates with various clinical settings.

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Footnotes:

DISCLOSURE STATEMENT: The authors have nothing to disclose.

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Figure 1

SENP2 represses estradiol-dependent transcriptional activity in MCF7 cells. (A) MCF7 cells were transiently transfected with 50 ng of pEBL+ (encoding ERE-driven luciferase), 50 ng of pRL-CMV-renilla as an internal control, and 50 ng p3XFlag-SUMO1 together with 150 ng SENPs expression plasmids. Cells were treated with vehicle (EtOH) or estradiol (10^{-8} M) for 16 h. Luciferase values, normalized for transfection efficiency with respect to the internal renilla luciferase control, are expressed as percentages of the activity obtained with cells transfected only with EBL+ and treated with vehicle. (B) Left panel: MCF7 cells were transiently transfected with the same reporter genes as described in 1A, together with increasing amounts of CFP-SENP2 expression vector and treated with vehicle (EtOH), estradiol (10^{-8} M) or 4-hydroxytamoxifen (OHT 10^{-8} M). Luciferase values are expressed as described above. Right panel: MCF7 cells were first transfected with 3.5 nmol of either siCtrl or siSENP2 as indicated and then with the same reporter genes as described above. Cells were treated with vehicle (EtOH) or estradiol (10^{-8} M) for 16 h. Luciferase values are expressed as in 1A. (C) MCF7 cells were transiently transfected with either the CFP or the CFP-SENP2 expression vector and treated with vehicle (EtOH) or estradiol (10^{-8} M). mRNA extracts were subjected to real-time PCR assays with primers specific to SENP2, PGR, TFF1, CCND1 and CTSD. Values are expressed in relative units and plotted as means \pm SD of three independent experiments. (D) MCF7 cells were transiently transfected with the CFP-SENP2 expression vector and treated for 45 min with 10^{-8} M estradiol or vehicle. Chromatin immunoprecipitation assays were performed with the indicated antibodies and the *TFF1* and *CTSD* promoter regions were quantified by qPCR. The data are represented as percentages of total input before immunoprecipitation. All figures derived from a single assay representative of at least three independent experiments. Student's t test was used for statistical analysis: ** $p < 0.001$, *** $p < 0.01$ and * $p < 0.05$.

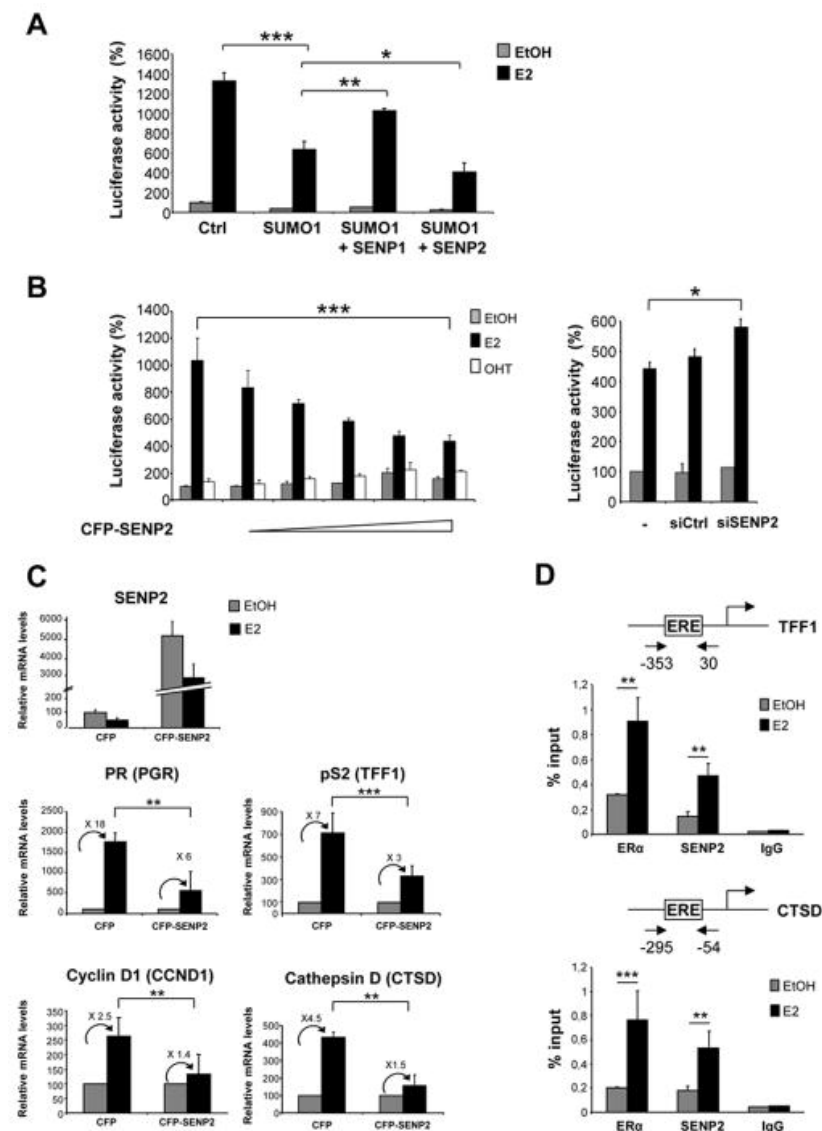


Figure 2

Interaction between ER α and SENP2. (A) COS7 cells were transfected with 1 μ g of each indicated expression vector (encoding ER α or CFP-SENP2) and treated with vehicle (EtOH) or estradiol (10^{-8} M). Whole-cell lysates were then immunoprecipitated with either anti-ER α antibody or the control mouse IgG and analyzed by western blotting with anti-GFP antibody or anti-ER α . An aliquot of each whole-cell extract was also immunoblotted to evaluate the expression levels of ER α , CFP-SENP2 and GAPDH (Input) (B) In situ proximity ligation assays between ER α and SENP2 in MCF7 cells. Cells were treated with vehicle (EtOH) or estradiol (10^{-8} M) and processed as described under Materials and Methods. Anti-ER α and anti-SENP2 were used as primary antibodies. Graph results are averages of dots per cell from ten microscope fields for each condition with error bars indicating standard deviations. (C) Schematic representation of full-length ER α and various ER α deletion mutants (left). GST pull-down assays were carried out with bacterially expressed GST, GST-SENP2 and GST-D fusion proteins and 35 S-labeled ER α , HE15, HE39, HE241G and SENP2 in the presence of vehicle (-) or 10^{-6} M estradiol (+) (right). (D) Schematic representation of full-length SENP2 and various SENP2 deletion mutants. The ER α -interacting domain (ERID) is also shown (left). GST pull-down assays were carried out with bacterially expressed GST and GST-fused fragments of SENP2 and 35 S-labeled ER α in the presence of vehicle (-) or 10^{-6} M estradiol (+) (right).

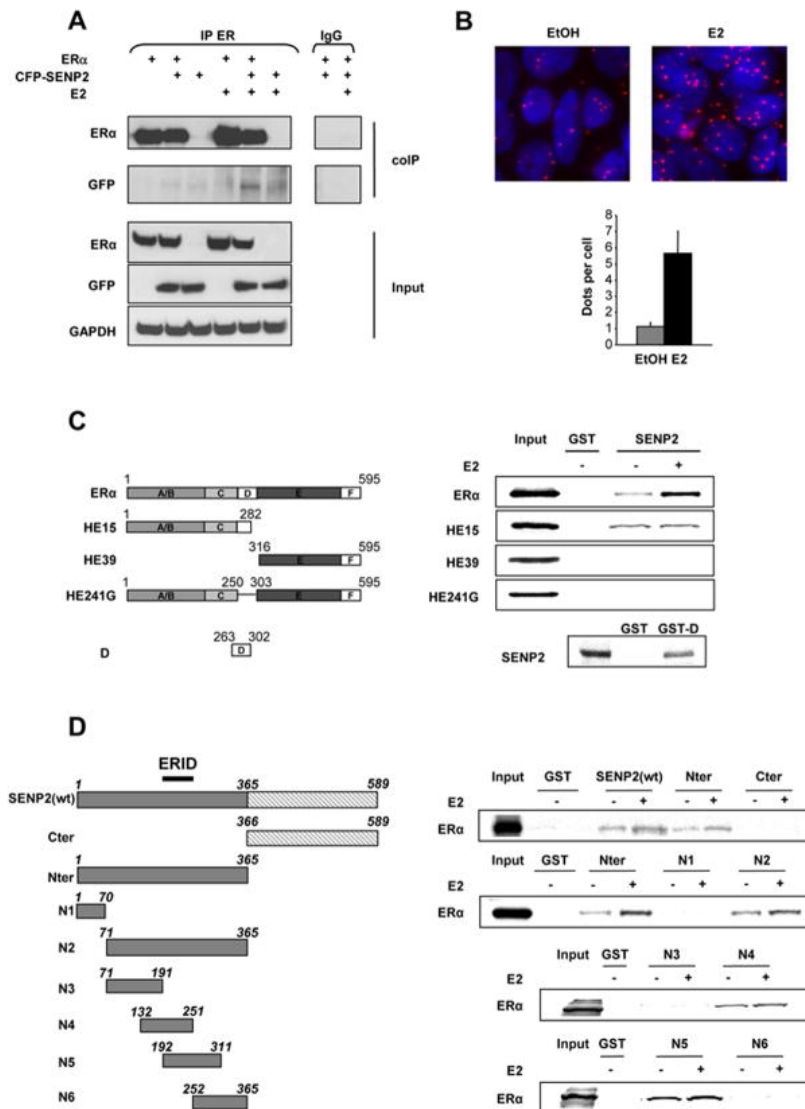


Figure 3

The repressive potential SENP2 is independent of its catalytic activity. (A) GST pull-down assays were carried out using bacterially expressed GST, GST-SENP2, GST-SENP2(C466S) and GST-SENP2(W375A) and 35 S-labeled ER α , in the presence of vehicle (-) or 10^{-6} M estradiol (+). Input represents 10% of the material used in each assay. (B) MCF7 cells were transiently transfected with the same reporter genes as in 1A, together with increasing doses of a vector expressing CFP-SENP2, CFP-SENP2(C466S) or CFP-SENP2(W375A). The cells were treated with vehicle (EtOH) or estradiol (10^{-8} M). Luciferase values are expressed as described in Figure 1A. (C) MCF7 cells were transiently transfected with 50 ng pRL-CMV-renilla and 25 ng L8G5 reporter plasmids, 12.5 ng LexAVP16 and increasing doses of SENP2-fused Gal4DBD expression plasmid. Luciferase values normalized with respect to the renilla luciferase control are expressed as percentages of the activity obtained with untransfected Gal4DBD-SENP2 cells. Figures are representative of at least three independent experiments. Student's t test was used for statistical analysis: * $p < 0.05$ and *** $p < 0.001$.

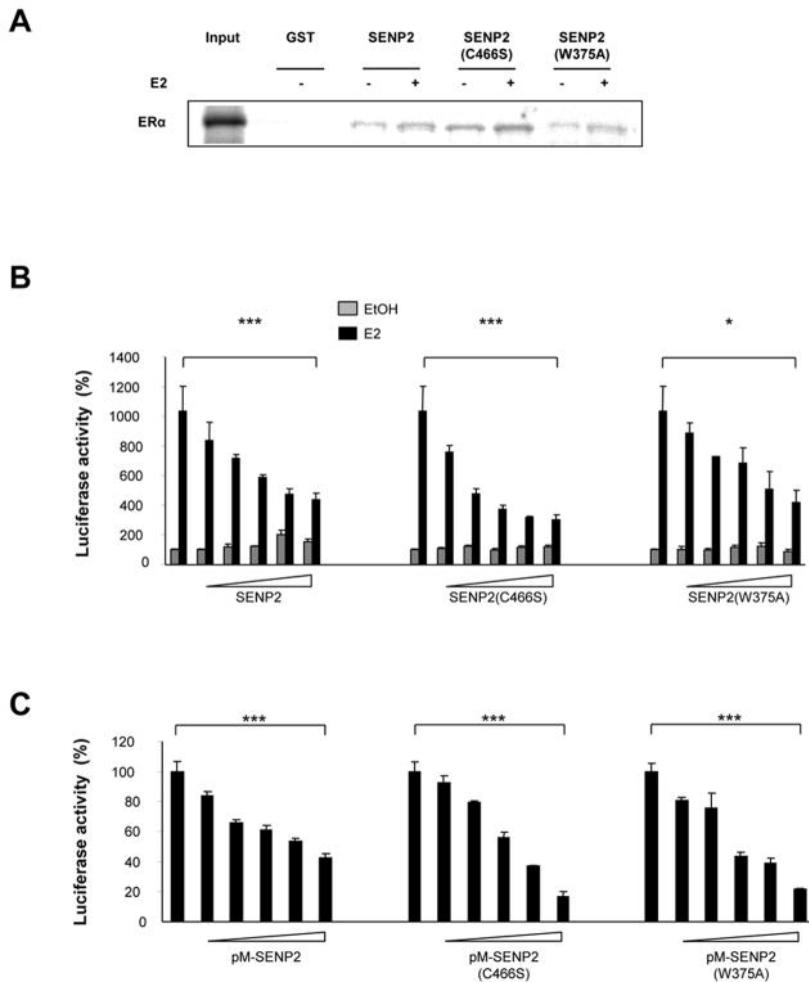


Figure 4

The repressive domain of SENP2 is located in the amino-terminal region. (A) Schematic representation of full-length SENP2 and various SENP2 deletion mutants. The repressive domain (RD) is also shown. (B) (C) MCF7 cells were transiently transfected with 50 ng pRL-CMV-renilla and 25 ng L8G5 reporter plasmid, 12.5 ng LexAVP16 and increasing doses of Gal4DBD-fused SENP2 expression plasmids (Nter and Cter regions in (B) and different fragments of the Nter region in (C)). Luciferase values are expressed as described in Figure 3C. (D) GST-SENP2 deleted of the repressive domain (Δ N8) is represented on the upper panel. GST pull-down assays were carried out using bacterially expressed GST and GST-SENP2(Δ N8) and 35 S-labeled ER α , in the presence of vehicle (-) or 10^{-6} M estradiol (+) (lower panel). Input represents 10% of the material used for each assay. (E) MCF7 cells were transiently transfected as indicated in Figure 4B and with increasing doses of SENP2 and SENP2(Δ N8)-fused Gal4DBD expression plasmid. Luciferase values are expressed as in Figure 3C. (F) MCF7 cells were transiently transfected with the same reporter genes as in 1A, together with increasing doses of CFP-SENP2 or CFP-SENP2(Δ N8). The cells were treated with vehicle (EtOH) or estradiol (10^{-8} M). Luciferase values are expressed as in Figure 1B. Figures are representative of at least three independent experiments. Student's t test was used for statistical analysis: ***p < 0.001.

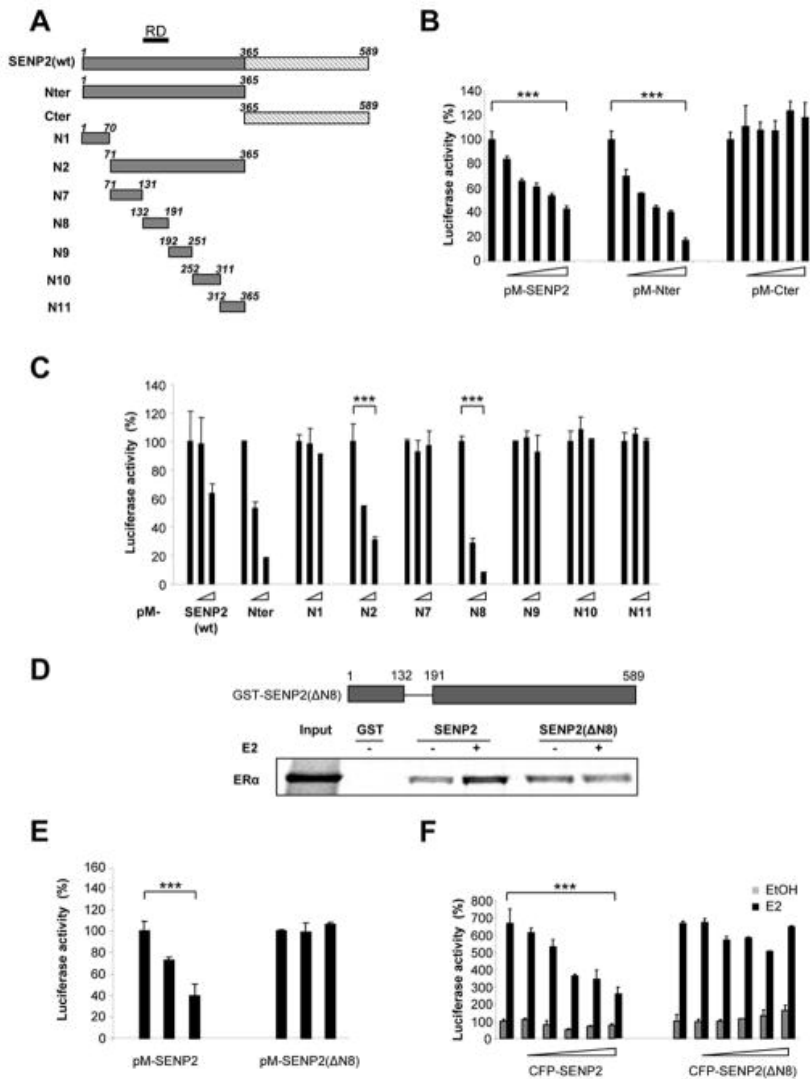


Figure 5

Role of HDAC3 in SENP2 repressive activity. (A) GST pull-down assays were carried out with bacterially expressed GST, GST-SENP2, GST-SENP2(Δ N8) and GST-N8 and with 35 S-labeled HDAC1, HDAC6, HDAC9 and HDAC3. (B) COS7 cells were transfected with 5 μ g Flag-SENP2 expression plasmid. Whole-cell lysates were then immunoprecipitated with either anti-HDAC3 antibody or a control mouse IgG and analyzed by western blotting with anti-HDAC3 and anti-Flag antibodies. An aliquot of each whole-cell extract was also immunoblotted to evaluate the levels of both HDAC3 and Flag-SENP2 (Input). (C) In situ proximity ligation assays between SENP2 and HDAC3 in MCF7 cells using primary antibodies diluted in PBS-1%BSA. Left panel: the expression of SENP2 was assessed with a rabbit primary antibody recognized by anti-rabbit PLA probes PLUS and MINUS. Middle panel: the expression of HDAC3 was assessed with a goat primary antibody recognized by anti-goat PLA probes PLUS and MINUS. Right panel: the SENP2-HDAC3 interaction was detected with one anti-rabbit PLA probe PLUS and one anti-goat PLA probe MINUS. Red dots represent individual proteins or protein-protein interactions. (D) MCF7 cells were first transfected with 7 pmol siCtrl or siHDAC3 as indicated and then with 50 ng pRL-CMV-renilla and 25 ng L8G5 reporter plasmids, 12.5 ng LexAVP16 and 50ng pM-SENP2 as indicated. Luciferase values are expressed as percentages of the activity in the presence of siCtrl. (E) MCF7 cells were first transfected with 7 pmol of either siCtrl or siHDAC3 as indicated and then with the same reporter genes described in 1B together with 150 ng CFP-SENP2 expression vector. Cells were treated with vehicle (EtOH) or estradiol (10^{-8} M). Luciferase values are expressed as percentages of the activity in the presence of estradiol and in the absence of SENP2. Figures are representative of at least three independent experiments. Student's t test was used for statistical analysis: ***p < 0.001 and *p < 0.05.

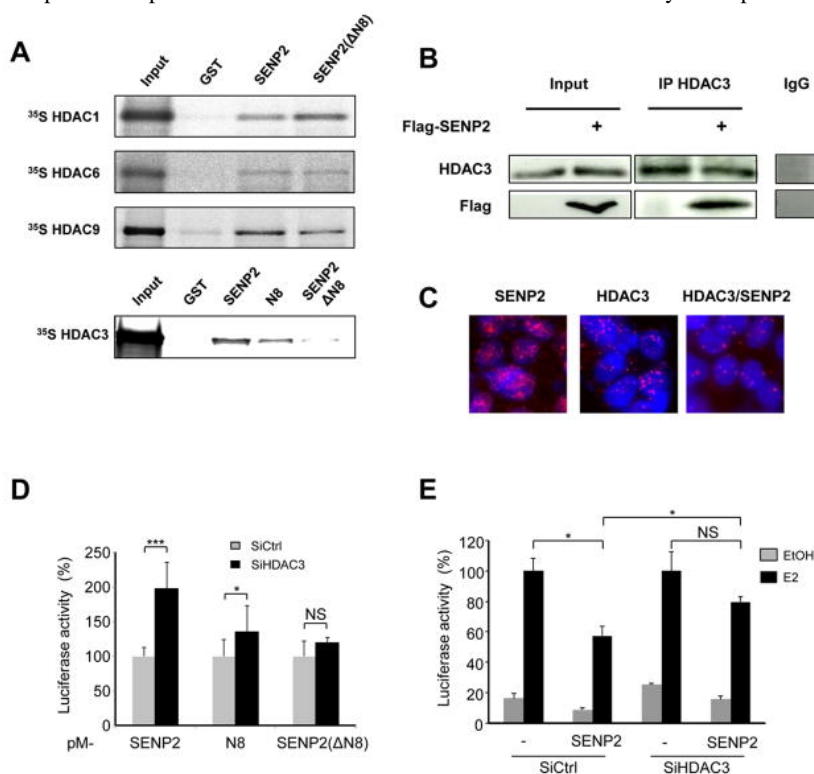


Figure 6

SENP2 inhibits MCF7 cell proliferation and cell cycle progression. (A) SENP2 expression was assessed by real time PCR (upper panels) and western blotting with anti-SENP2 and anti-actin antibodies (middle panels). SUMO protease activity was evaluated by transfecting stable cell lines with 5 μ g Flag-SUMO-1 encoding vector. Whole-cell lysates were then immunoblotted with anti-Flag and anti-actin antibodies (CFP-SENP2 population, upper right panel and shRNA-SENP2 population, lower panels). (B) Cell proliferation was measured over 6 days, under estradiol treatment (10^{-8} M). The change in impedance as the cells spread on the E-plate (Roche) is displayed as a cell index value (CI). CI values of quadruplicates are normalized to the last time point before addition of estradiol and plotted. Relative proliferation values are expressed as percentages of the normalized CI observed on day 0 (D0). Figures are representative of at least three independent experiments. (C) FACS analysis of S-phase progression in stable cell lines. DNA synthesis was estimated by measuring BrdU incorporation with an anti-BrdU FITC antibody. Total DNA was stained with propidium iodide (upper panel). The percentage of cells in each cell-cycle phase is indicated for each cell line (lower panel). Graphs are representative of three independent experiments. (D) Cell proliferation was measured as described in (B).

