

1 **Transcriptional repression of estrogen receptor α signaling by SENP2 in breast cancer cells**

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20

21 **ABSTRACT**

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

39

40 **Keywords:** estrogen signaling, SUMO protease, transcription factor, breast cancer cell, proliferation

41

42 INTRODUCTION

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

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

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

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

78

79 **MATERIALS AND METHODS**

80

81 **Plasmids**

82

83 pRL-CMV-renilla was obtained from Promega. GST-LBD (AF2) and GST-D have been described
84 elsewhere (19) and (7) respectively. p3XFlag-SUMO1 was created as follows: pGEX-SUMO1 was
85 digested with BamHI and the resulting fragment inserted into p3XFLAG-myc-CMV-24 (Sigma-
86 Aldrich). pFlag-SEN1 and pFlag-SEN2 (a kind gift of Dr Yeh, MD Anderson, Houston) were
87 subcloned into p3XFLAG-myc-CMV-24 to create p3XFlag-SEN1 and p3XFlag-SEN2. Wild type
88 SEN1 was subcloned into pECFP and wild-type SEN2 into p3XFLAG-myc-CMV, pECFP
89 (Clontech), pM (Clontech), pGEX (Pharmacia) or pcDNA3.1(-) (Invitrogen). All SEN2 fragments and
90 mutants were engineered by PCR, sequenced and then subcloned into the above mentioned vectors.

91

92 **Cell culture, transient transfections and luciferase assays**

93

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

111

112 **Generation of stable cell lines and siRNA transfections**

113

114 JetPEI (Polyplus) was used to stably transfect MCF7 cells according to the manufacturer's protocol On
115 the one hand, cells were transfected with either the parental pECFP vector (Clontech) or the pECFP-

116 SENP2 vector and treated with G418 (1 mg/ml) (GIBCO). On the other hand, cells were transfected
117 with either the non-target shRNA control vector (pLKO.1-NTshRNA-puro) or the SENP2 shRNA
118 vector (pLKO.1-SENP2shRNA-puro) (MISSION, Sigma) and treated with puromycin (1 µg/ml)
119 (Sigma). The DharmaFECT 2 (Dharmacon) or INTERFERin (Polyplus) transfection agent was used for
120 transfections with non-targeting (Ctrl), SENP2-specific or HDAC3-specific siRNA (Dharmacon). Each
121 transfection was performed in triplicates and interference efficiencies were tested by qPCR and western
122 blotting.

123

124 **Immunoprecipitation and immunoblotting assays**

125

126 Immunoprecipitation assays were performed as previously described (20). Cells were harvested in
127 modified RIPA buffer (50mM Tris-HCL, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0,25%
128 sodium deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals)
129 and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄, 1 mM β-glycerophosphate) in the presence of
130 20 mM N-Ethylmaleimide (NEM) whenever stated. Western-blot analyses were carried out with
131 primary antibodies against ERα 60C (Millipore), GAPDH (Life Science), Flag (Sigma), GFP, HDAC3
132 (N-19) or SENP2 (Santa Cruz), or Actin A2066 (Sigma). For the immunoprecipitation assays, cell
133 extracts were incubated with an anti-ERα rabbit polyclonal antibody HC20 (TEBU) or a mouse IgG.
134 Protein A/G agarose beads were added, and bound proteins were released and subjected to western-blot
135 analysis to detect coimmunoprecipitated proteins with a rabbit monoclonal anti-ERα 1C60 (Millipore)
136 and a rabbit polyclonal anti-GFP (Santa Cruz Biotechnology). A peroxidase-conjugated donkey anti-
137 rabbit antibody was used as secondary antibody.

138

139 **GST pull-down assays**

140

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

143

144 **Quantitative PCR**

145

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

148

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

150

151 The Duolink II Proximity Ligation Assay kit (Eurogentec, France) was used according to the
152 manufacturer's instructions (Olink). Briefly, the principle can be described as follows. Two primary

153 antibodies raised in different species recognize the target antigen or antigens of interest. PLA
154 probes are species-specific secondary antibodies that bind to the primary antibodies. When the
155 PLA probes are in close proximity (<40 nm), the two short DNA strands, attached to it, form a
156 circle oligonucleotide DNA that is ligated by enzymatic ligation. After the amplification
157 reaction, labeled complementary oligonucleotide probes highlight the product. The resulting
158 high concentration of fluorescence in each single-molecule amplification product is easily
159 visible as a distinct bright dot when viewed with a fluorescence microscope.

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

165

166 **Chromatin Immunoprecipitation**

167

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

177

178 **Cell proliferation analysis**

179

180 Real time growth kinetics of stable cell lines was examined with an impedance-based Real-Time Cell
181 Analysis (RTCA) system (Roche Applied Science, Meylan, France). Background measurement of 16-
182 well E-plates was performed with 50 μ l phenol red-free Dulbecco's modified Eagle medium containing
183 1% dextran-charcoal treated FCS and 1mg/ml G418 or 1 μ g/ml puromycin. Then, 2500 cells/well were
184 seeded in 100 μ l of additional culture medium. Cell proliferation was monitored every 24h for 6 days.
185 Cell sensor impedance was expressed as a dimensionless parameter called the Cell Index (CI). Data
186 analysis was carried out with the RTCA Software 1.2 supplied with the instrument.

187

188 **Cell cycle analysis**

189
190 Cells were incubated for 4 h with 30 μ M BrdU, trypsinized, centrifuged, fixed by addition of 1.5 ml ice-
191 cold 100% ethanol, digested at room temperature (RT) in 0.05% pepsin/30mM HCl and 2 N HCl and
192 incubated with primary rat anti-BrdU antibody (clone BU1/75, AbC117-7517) and secondary goat anti-
193 rat-FITC conjugate antibody (Southern Biotech 3030-02). The cells were stained with propidium
194 iodide. The cell cycle was analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter). Data
195 analysis was carried out with FlowJo Software.

196
197 **Statistical analysis**

198
199 Data are presented as means \pm standard deviations. Statistical analyses were performed with Student's t-
200 test. Differences were considered statistically significant at $p < 0.05$. (* $p < 0.05$; ** $p < 0.01$ and ***
201 $p < 0.001$).

202

203 RESULTS

204 **SEN2 represses estrogen-dependent transcriptional activity**

205
206 In an attempt to define the role of protein sumoylation in estrogen signaling, we first investigated the
207 effect of SUMO1 on estrogen-dependent activity in ER α -positive MCF7 breast cancer cells (Figure
208 1A). In this model, in contrast to previous data obtained on HeLa cells (7), ectopic expression of
209 SUMO1 led to significant repression of estradiol-dependent transcriptional activity. We then analyzed
210 how the most-studied SUMO proteases, SENP1 and SENP2, might affect this repression by SUMO1.
211 Interestingly, when ectopically expressed, SENP1 was found to decrease SUMO1-triggered repression,
212 whereas SENP2 increased it.

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

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

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

233 In order to demonstrate that the repressive effect of SENP2 was not specific to MCF7 cells, we also
234 transfected T47D breast cancer cells with the CFP-SEN2 expression plasmid. As shown in
235 supplementary Figure S4 A and B, SEN2 repressed estradiol-dependent transcriptional activity both
236 on a reporter plasmid and on the same endogenous genes as in MCF7 cells. Altogether, these results
237 evidence that SEN2 represses ER-dependent transactivation in breast cancer cells.

238

239 **SEN2 is recruited to ER target promoters**

240
241 We then wondered whether SEN2 might be recruited to estrogen-responsive gene promoters. To
242 answer this question, we performed chromatin immunoprecipitation (ChIP) assays on MCF7 cells
243 overexpressing CFP-SEN2 (Figure 1D) and chose to amplify either the *TFF1* (top) or the *CTSD*
244 (bottom) promoter. As a positive control we used the anti-ER α antibody which allowed the
245 immunoprecipitation and the amplification of both *TFF1* and *CTSD* promoters in an estradiol-
246 dependent manner. Interestingly, the same results were obtained after immunoprecipitation of CFP-
247 SEN2 whereas no amplification could be observed in the presence of unrelated IgG. We failed to
248 amplify either promoter when the anti-CFP antibody was used on extracts of CFP-expressing cells (data
249 not shown). Secondly, when CFP-SEN2 was immunoprecipitated, no amplification was observed with
250 primers specific to the SEN2 coding sequence (data not shown). These data strongly suggest that
251 SEN2 exerts its repressive effects through a direct recruitment to target gene promoters.

252
253 **SEN2 interacts with ER α**

254
255 In order to support the ChIP data, we then used various means to investigate the interaction between
256 SEN2 and ER α . We first performed immunoprecipitation from transfected COS7 cells using an anti-
257 ER α antibody. As shown in Figure 2A, SEN2 co-immunoprecipitated with ER α only in the presence
258 of estradiol. The different inputs indicated that protein levels were constant under the different
259 conditions (lower panels).

260 We next performed proximity ligation assays (PLA) in MCF7 breast cancer cells, to evidence
261 interactions between endogenous ER α and SEN2 (Figure 2B). The number of dots revealing
262 complexes containing ER α and SEN2 was significantly higher in the presence of estradiol than with
263 ethanol alone, further confirming the first set of experiments. Control experiments for PLA assays are
264 shown in supplementary Figure S5.

265 We then used GST pull-downs to dissect the interaction. Using GST-SEN2 as a bait, we investigated
266 the interaction between SEN2 and various ER α fragments (Figure 2C). The results confirmed the
267 estradiol-dependent interaction between full-length ER α and GST-SEN2. Mutants lacking the hinge
268 domain (referred to as the D domain) (fragments HE39 and HE241G) showed no interaction with
269 SEN2, whereas fragment HE15, containing this region, displayed a clear interaction. Conversely,
270 GST-D was also found to interact with the protease. We conclude that the hinge region of ER α mediates
271 its interaction with SEN2.

272 We also generated GST-fused SEN2 deletion mutants to identify interacting domains on SEN2 (see
273 Figure 2D). The catalytic domain of SEN2 (aa 366-589) displayed no interaction, whereas the amino-
274 terminal part (aa 1-365) clearly bound ER α in an estradiol-dependent way. Interestingly, the SEN2
275 region responsible for the interaction with nucleoporin Nup153 (23) (aa 1-70) proved unable to pull

276 down ER α . Different fragments of the amino-terminal region of SENP2 were also tested. Among them,
277 only N4 (aa 132-251) and N5 (aa 192-311) were found to interact with ER α . These data indicate that
278 the ER α -interacting domain (ERID) of SENP2 encompasses the region located between amino acids
279 192 and 251.

280

281 **The repressive effect of SENP2 is independent of its catalytic activity**

282

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

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

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

307

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

309

310 We next sought to determine precisely which region of SENP2 mediates its repressive activity. To do
311 so, we generated a series of Gal4DBD-fused fragments (Figure 4A). The relative expression of the

312 mutants was checked in real time PCR assays (data not shown). As observed in Figure 4B, the amino-
313 terminal domain (1-365) (Nter) exerted a stronger repressive effect than the full-length protein, whereas
314 the carboxy-terminal domain (365-589) (Cter) displayed no repressive activity. Of the different
315 fragments tested (N1 to N11), only N8 (132-191) exhibited significant repressive activity (Figure 4C).
316 Remarkably, the N8 repressive domain displayed an inhibitory potential very similar to that of the Nter
317 domain.

318 To confirm the importance of the N8 fragment, we deleted the corresponding coding region from the
319 wild-type SENP2. The SENP2(Δ N8) fragment proved able to pull down *in vitro* translated ER α
320 effectively, indicating that the N8 region is not necessary for the interaction with the receptor (Figure
321 4D). We next generated Gal4DBD-SENP2(Δ N8) and compared its activity with that of the wild-type
322 construct Gal4DBD-SENP2 (Figure 4E). In contrast to Gal4DBD-SENP2, the Gal4DBD-SENP2(Δ N8)
323 protein proved unable to exert any repression. Finally, the mutant was then tested for the ability to
324 repress estradiol-dependent transcriptional activity in MCF7 cells (Figure 4F). While wild-type SENP2
325 repressed estradiol-dependent transcriptional activity as expected, SENP2(Δ N8) displayed no effect.
326 Altogether, these results clearly establish that SENP2(N8) is necessary for repression of estradiol-
327 dependent transcriptional activity and for the intrinsic repressive activity of SENP2.

328

329 **HDAC3 partly mediates SENP2 repressive activity**

330

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

342 We next investigated whether HDAC3 might affect SENP2 activity. To this end, we knocked-down
343 HDAC3 expression with a specific siRNA (Supplementary Figure S8A). Remarkably, HDAC3 knock-
344 down significantly increased luciferase activity indicating a loss in the intrinsic repressive potential of
345 SENP2 (Figure 5D). A similar observation (although to a lesser extent) was made with the repressive
346 activity of the isolated N8 domain whereas no significant effect was observed with pM-SENP2(Δ N8)
347 (Figure 5D). Interestingly, transfection of HDAC3 siRNA affected the dose-dependent repression
348 mediated by both wild-type pM-SENP2 and pM-N8 alone (Supplementary Figures S8B and C).

349 Importantly, in T47D, HDAC3 proved equally important for the activity of SENP2 (Supplementary
350 Figure S4C), further reinforcing above described results. We then investigated the role of HDAC3 on
351 SENP2-mediated repression of ER α transcriptional activity. As shown in Figure 5E, SENP2 repression
352 of ER α activity was significantly weaker in the presence of HDAC3 siRNA. Altogether, these results
353 evidence that HDAC3 partly mediates SENP2 transcriptional repression.

354

355 **SENP2 inhibits MCF7 cell proliferation in a protease-dependent manner**

356

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

365 We then used impedance-based real-time cell analysis (xCelligence, Roche) to monitor proliferation of
366 the different cell lines in the presence of estradiol (Figure 6B). Control cells and the parental MCF7
367 cells grew similarly. In sharp contrast, MCF7 cells overexpressing SENP2 displayed very significantly
368 reduced proliferation in the presence of estradiol whereas shSENP2 cells grew significantly faster.

369 These results were supported by cell cycle analysis of the CFP-SENP2 and shSENP2 expressing cell
370 lines in the presence of estradiol. Indeed, as shown in Figure 6C, BrDU incorporation revealed a very
371 similar percentage of cells in the S phase in control cells and parental MCF7 cells. Strikingly, only 8%
372 of the CFP-SENP2-expressing cells were found in the S phase, and the percentage of cells in the G0/G1
373 was increased. ShSENP2 cells had an increase in S phase (39.6 %) and a decrease in G0/G1 phase.
374 SENP2 expression in MCF7 cells thus drastically affects the cell cycle at the G1/S transition by
375 significantly reducing the proportion of cells in the S phase.

376 In order to define whether the transcription repressive domain was necessary for the antiproliferative
377 effect of SENP2, the same experiments were performed with cells stably transfected with CFP-
378 SENP2(Δ N8) (Figure 6D). Interestingly, cells expressing the SENP2 mutant grew like the CFP-
379 expressing line indicating that the repressive domain is necessary for this property of SENP2.
380 Surprisingly, when using the CFP-SENP2 and shSENP2 expressing cell lines, we also noticed an
381 inhibition of cell proliferation in the absence of estradiol (Supplementary Figure S9) suggesting that the
382 effect of SENP2 on cell proliferation might involve a more complex mechanism. In support of this
383 observation, the isopeptidase activity mutant CFP-SENP2(W375A) was also ineffective to inhibit cell
384 proliferation both in the absence or presence of estradiol (Figure 6D and Supplementary Figure S9).

385 These data suggest that SENP2 is able to repress both estrogen-dependent and independent proliferation
386 of MCF7 cells and that this effect require both the proteolytic and transcriptional activities of SENP2.
387

388 **Discussion**

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

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

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

413 In the present work, we provide evidence that SENP2 interacts with the hinge domain of ER α , a region
414 targeted by many different post-translational modifications, including sumoylation. Even though we
415 clearly show that the proteolytic activity of SENP2 plays no role in the repression of ER α -dependent
416 transcriptional activity, we also demonstrate that SENP2 efficiently desumoylates ER α . Thus,
417 desumoylation of ER α by SENP2 might make possible or prevent other post-translational
418 modifications. If we take lysines 302 and 303 as an example, desumoylation could allow acetylation,
419 involved in repression of ER α transcriptional activity. In addition, the interaction between SENP2 and
420 ER α might reinforce this repression when needed.

421 The SENP2 repressive activity is not limited to MCF7 cells but would be more widely extended to
422 breast cancer cells. Indeed, in T47D, as shown in supplementary Figure S4, SENP2 not only repressed
423 ER α -dependent transcriptional activity on a reporter plasmid but also on endogenous ER-dependent

424 genes (TFF1, PGR, CCND1 and CTSD). We also demonstrated that in T47D, repression by SENP2 is
425 equally dependent on HDAC3.

426 Altogether, our data on ER α signaling in breast cancer cells appear novel and original as compare to
427 previous studies which describe a positive effect of SENP2 on the transcriptional activity of other
428 nuclear receptors such as AR (17, 25), ERR γ 2 (estrogen-receptor-related receptor γ 2) (38), and PR (18).
429 In our hands, SENP2 also displayed an activating effect on AR and ERR γ -dependent transactivation as
430 mentioned above while it acted as a repressor of PR and ER β -dependent transcriptional activity
431 (supplementary Figure S3). Our data, together with the above described papers suggest that the effect of
432 SENP2 on receptor transcriptional activity may depend on the receptor concerned. It must be underlined
433 that while transcriptional repression of ER α was robustly described as independent of SENP2
434 enzymatic activity, the mechanism of SENP2 effect on AR, ERR γ or PR activity is totally unknown. It
435 would be of great interest to study whether the specificity of SENP2 activity could differentially
436 implicate its enzymatic activity. In experiments focusing on these receptors, the enzyme PIAS1, also
437 involved in the SUMO pathway, likewise appeared to exert opposite effects according to the receptor
438 (7, 27, 39). In the light of our study, one might hypothesize that according to the targeted protein, the
439 SENP2 repressive domain might be differently exposed and therefore either silenced or activated. One
440 should also note that the above-mentioned effects of SENP2 were observed in different cell contexts.
441 Accordingly, SENP2 might exert very different transcriptional effects depending on the targeted nuclear
442 receptor.

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

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

460

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470 SUMO1, to Dr Hay (University of Dundee, Dundee) for pGEX-SUMO1 and to Dr Shuai (University of
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472

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- 579

580 **FIGURE LEGENDS**

581

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

604

605 **Figure 2.** Interaction between ER α and SENP2. (A) COS7 cells were transfected with 1 μ g of each
606 indicated expression vector (encoding ER α or CFP-SENP2) and treated with vehicle (EtOH) or
607 estradiol (10^{-8} M). Whole-cell lysates were then immunoprecipitated with either anti-ER α antibody or
608 the control mouse IgG and analyzed by western blotting with anti-GFP antibody or anti-ER α . An
609 aliquot of each whole-cell extract was also immunoblotted to evaluate the expression levels of ER α ,
610 CFP-SENP2 and GAPDH (Input) (B) In situ proximity ligation assays between ER α and SENP2 in
611 MCF7 cells. Cells were treated with vehicle (EtOH) or estradiol (10^{-8} M) and processed as described
612 under Materials and Methods. Anti-ER α and anti-SENP2 were used as primary antibodies. Graph
613 results are averages of dots per cell from ten microscope fields for each condition with error bars
614 indicating standard deviations. (C) Schematic representation of full-length ER α and various ER α
615 deletion mutants (left). GST pull-down assays were carried out with bacterially expressed GST, GST-

616 SENP2 and GST-D fusion proteins and ³⁵S-labeled ER α , HE15, HE39, HE241G and SENP2 in the
617 presence of vehicle (-) or 10⁻⁶ M estradiol (+) (right). (D) Schematic representation of full-length
618 SENP2 and various SENP2 deletion mutants. The ER α -interacting domain (ERID) is also shown (left).
619 GST pull-down assays were carried out with bacterially expressed GST and GST-fused fragments of
620 SENP2 and ³⁵S-labeled ER α in the presence of vehicle (-) or 10⁻⁶ M estradiol (+) (right).

621
622 **Figure 3.** The repressive potential SENP2 is independent of its catalytic activity. (A) GST pull-down
623 assays were carried out using bacterially expressed GST, GST-SENP2, GST-SENP2(C466S) and GST-
624 SENP2(W375A) and ³⁵S-labeled ER α , in the presence of vehicle (-) or 10⁻⁶ M estradiol (+). Input
625 represents 10% of the material used in each assay. (B) MCF7 cells were transiently transfected with the
626 same reporter genes as in 1A, together with increasing doses of a vector expressing CFP-SENP2, CFP-
627 SENP2(C466S) or CFP-SENP2(W375A). The cells were treated with vehicle (EtOH) or estradiol (10⁻⁸
628 M). Luciferase values are expressed as described in Figure 1A. (C) MCF7 cells were transiently
629 transfected with 50 ng pRL-CMV-renilla and 25 ng L8G5 reporter plasmids, 12.5 ng LexAVP16 and
630 increasing doses of SENP2-fused Gal4DBD expression plasmid. Luciferase values normalized with
631 respect to the renilla luciferase control are expressed as percentages of the activity obtained with
632 untransfected Gal4DBD-SENP2 cells. Figures are representative of at least three independent
633 experiments. Student's t test was used for statistical analysis: *p < 0.05 and ***p < 0.001.

634
635 **Figure 4.** The repressive domain of SENP2 is located in the amino-terminal region. (A) Schematic
636 representation of full-length SENP2 and various SENP2 deletion mutants. The repressive domain (RD)
637 is also shown. (B) (C) MCF7 cells were transiently transfected with 50 ng pRL-CMV-renilla and 25 ng
638 L8G5 reporter plasmid, 12.5 ng LexAVP16 and increasing doses of Gal4DBD-fused SENP2 expression
639 plasmids (Nter and Cter regions in (B) and different fragments of the Nter region in (C)). Luciferase
640 values are expressed as described in Figure 3C. (D) GST-SENP2 deleted of the repressive domain
641 (Δ N8) is represented on the upper panel. GST pull-down assays were carried out using bacterially
642 expressed GST and GST-SENP2(Δ N8) and ³⁵S-labeled ER α , in the presence of vehicle (-) or 10⁻⁶ M
643 estradiol (+) (lower panel). Input represents 10% of the material used for each assay. (E) MCF7 cells
644 were transiently transfected as indicated in Figure 4B and with increasing doses of SENP2 and
645 SENP2(Δ N8)-fused Gal4DBD expression plasmid. Luciferase values are expressed as in Figure 3C. (F)
646 MCF7 cells were transiently transfected with the same reporter genes as in 1A, together with increasing
647 doses of CFP-SENP2 or CFP-SENP2(Δ N8). The cells were treated with vehicle (EtOH) or estradiol
648 (10⁻⁸ M). Luciferase values are expressed as in Figure 1B. Figures are representative of at least three
649 independent experiments. Student's t test was used for statistical analysis: ***p < 0.001.

650

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

672
673 **Figure 6.** SENP2 inhibits MCF7 cell proliferation and cell cycle progression. (A) SENP2 expression
674 was assessed by real time PCR (upper panels) and western blotting with anti-SENP2 and anti-actin
675 antibodies (middle panels). SUMO protease activity was evaluated by transfecting stable cell lines with
676 5 μ g Flag-SUMO-1 encoding vector. Whole-cell lysates were then immunoblotted with anti-Flag and
677 anti-actin antibodies (CFP-SENP2 population, upper right panel and shRNA-SENP2 population, lower
678 panels). (B) Cell proliferation was measured over 6 days, under estradiol treatment (10^{-8} M). The
679 change in impedance as the cells spread on the E-plate (Roche) is displayed as a cell index value (CI).
680 CI values of quadruplicates are normalized to the last time point before addition of estradiol and plotted.
681 Relative proliferation values are expressed as percentages of the normalized CI observed on day 0 (D0).
682 Figures are representative of at least three independent experiments. (C) FACS analysis of S-phase
683 progression in stable cell lines. DNA synthesis was estimated by measuring BrdU incorporation with an
684 anti-BrdU FITC antibody. Total DNA was stained with propidium iodide (upper panel). The percentage
685 of cells in each cell-cycle phase is indicated for each cell line (lower panel). Graphs are representative
686 of three independent experiments. (D) Cell proliferation was measured as described in (B).