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1 Mdm2 and stress-inducing agents regulate
2 estrogen receptor α turn-over and transactivation.

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4 Running title: Stress and estrogen signaling

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1 **ABSTRACT**

2 In mammalian cells, the level of estrogen receptor α (ER α) is rapidly decreased upon
3 estrogen treatment and this regulation involves proteasome degradation. Using different
4 approaches, we showed that the Mdm2 oncogenic ubiquitin-ligase directly interacted with ER α
5 and is involved in the regulation of ER α turn-over. We showed that Mdm2 overexpression
6 decreased ER α stability (both in the absence or presence of estrogens) and deletion of the
7 ubiquitin-ligase domain abolished the negative regulation of ER α expression. Interestingly, our
8 results indicated that ligand-dependent receptor turn-over was not required for efficient
9 transactivation. Moreover, several lines of evidence suggested that Mdm2 interact with ER α in a
10 ternary complex with p53. In MCF-7 human breast cancer cells, various p53-inducing agents
11 (such as UV irradiation) or treatment with RITA (which inhibits the interaction of p53 with
12 Mdm2) stabilized ER α and abolished its E2-dependent turn-over. Finally, in addition to this
13 early positive effect on ER α stability, we showed that cellular stress inhibited ER α mRNA
14 accumulation leading to a strong delayed inhibition of receptor expression. Altogether, our
15 results indicate that ER α expression is complexly regulated by cellular stress, acting both on
16 gene transcription and protein stability by targeting the Mdm2 protein.

1 INTRODUCTION

2 Estrogens are key regulators of cell differentiation and proliferation and these hormones play
3 important roles in female reproduction physiology and tissue homeostasis. They exert their
4 biological action *via* specific receptors (ER α and ER β) which are members of a superfamily of
5 hormone nuclear receptors, acting as gene regulatory transcription factors (43). Upon ligand
6 binding, ERs regulate gene expression through the binding to their cognate estrogen response
7 elements (ERE) or *via* protein-protein interactions with transcription factors such as AP-1 or
8 Sp1. In the presence of hormone, ERs undergo a major conformational change allowing the
9 recruitment of transcriptional cofactor complexes, which in turn engage the basal transcription
10 machinery and/or act to locally modify chromatin structure and subsequently stimulate
11 expression of estrogen-responsive genes.

12 Liganded-nuclear receptors recruit various types of enzymatic activities that participate in
13 gene expression regulation (34). Previous studies have suggested that ubiquitin-conjugating
14 enzymes or ubiquitin-protein ligases such as UbcH5/UbcH7 (40, 45), RPF1/RSP5 (20) or E6-AP
15 (41) interact with members of the nuclear receptor superfamily and modulate their
16 transactivation functions. Similarly, ATPase subunits of the proteasome complex such as
17 TRIP1/SUG1 (27) or TBP1 (21) also bind nuclear receptors and modulate their functions.

18 More than 30 years ago, Jensen and collaborators have demonstrated that estradiol (E2)
19 treatment significantly reduces ER α levels in the uterus of ovariectomized rats (23). More
20 recently, several studies have shown that binding of E2 to ER α significantly decreases its
21 stability. This shorter half-life in the presence of hormone appears to implicate the
22 ubiquitin/proteasome pathway since ER α has been shown to be ubiquitinated (58) and the
23 ligand-dependent down-regulation is blocked by proteasome inhibitors (1, 12, 40). Interestingly,

1 although partial antiestrogens such as tamoxifene also increase ER α accumulation, pure
2 antihormones such as the ICI182,780 compound strongly decrease receptor stability (10, 58).
3 Several reports have suggested that the proteasome may control not only ER α protein levels but
4 also hormone-dependent transcription (32). This effect is associated with the immobilization of
5 ER α on the nuclear matrix as determined by fluorescence recovery after photobleaching (FRAP)
6 (54). Although several candidates have been proposed to account for the E2-dependent
7 regulation of ER α expression, the exact molecular mechanisms still remain unraveled.

8 The Mdm2 oncogene is overexpressed in a wide variety of human cancers (38) and its role in
9 tumorigenesis is linked to its ability to act as an E3 ubiquitin-ligase (18) which mediates the
10 ubiquitination and proteasome-dependent degradation of several growth regulatory proteins
11 including p53 (16, 37). Interestingly, it has been previously suggested that Mdm2 could directly
12 interact with ER α (31, 49) and a positive effect of Mdm2 overexpression on ER α activity has
13 also been reported (49).

14 In the present study, we show that the Mdm2 oncoprotein interacts with ER α and is involved
15 in the ligand-dependent decrease of receptor stability. Our data indicate that Mdm2 regulates
16 ER α expression as a ternary complex with p53 and, in support of this observation, we show that
17 various stress-inducing agents (which stabilize p53) block E2-dependent regulation of ER α
18 stability in MCF-7 human breast cancer cells. Finally, this study provides several lines of
19 evidence showing that the E2-dependent turnover of the receptor is not necessary for ERE-
20 mediated transactivation.

1 MATERIALS AND METHODS

2 **Plasmids and reagents.** The ER α expression vectors (wild-type and deletion mutants) were
3 given by P. Chambon (IGBMC, Strasbourg, France). The GST-AF2wt and GST-AF2mut vectors
4 (9), the plasmids encoding GST-C/D and ER-VP16 (56) and the ERE- β Glob-Luc and
5 17M5 β Glob-Luc (5) reporter constructs were described elsewhere. The GST-p53 and the
6 pcDNA3 plasmids encoding full-length p53 (26) were obtained from U. Hibner (IGMM,
7 Montpellier). The pXJ-mdm2 vector was obtained from B. Wasylyk (IGBMC, Strasbourg,
8 France) and the pcMI-Hdm2 contained the Mdm2 cDNA (BamHI/EcoRI fragment) subcloned
9 into the pCMI1 vector (42). The pcMI-Hdm2 Δ RING was obtained by inserting into the pCMI1
10 vector a PCR-amplified BamHI/EcoRI fragment corresponding to amino acids 1 to 435. The
11 GST-mdm2 was constructed by cloning a XhoI/Not1 fragment of the human Mdm2 cDNA into
12 the pGEX4T3 vector (Amersham Biosciences, Saclay, France). The Gal-Mdm2 was generated
13 by introducing a blunted NcoI/BglII fragment from pXJMdm2 into the SmaI site of the pSG424
14 plasmid (48). The RITA compound (NSC652287) was obtained from Dr R.J. Schultz (NIH,
15 DTP, DSCB, Rockville, MD, USA).

16 **Cell culture.** Monolayer cell cultures (MCF-7, HeLa, U2OS) were grown respectively in
17 Ham's F-12/Dulbecco's modified Eagle's medium (1:1) (F12/DMEM) or DMEM alone
18 supplemented with 10% fetal calf serum (FCS) (Invitrogen, Cergy-Pontoise, France) and
19 antibiotics. Before hormonal treatments, cells were stripped of endogenous steroids by passage
20 in medium without phenol red containing 3% charcoal-stripped FCS (FCS/DCC). Control cells
21 were grown under the same conditions and complemented with vehicle alone (ethanol). When
22 indicated, cells were irradiated using a Stratalinker UV crosslinker (Model 1800) from
23 Stratagene (Montigny-le-Bretonneux, France). The MELN cell line derived from MCF-7 cells

1 stably transfected with the ERE- β Glob-Luc-SVneo plasmid (4). Mouse embryo fibroblasts null
2 for both p53 and Mdm2 (MEFdKO) (35) were obtained from G. Lozano (Houston, Texas) and
3 cultured in F12/DMEM.

4 **Transient transfection and luciferase assays.** HeLa cells and MEFdKO were plated in 6-
5 well plates (10^5 cells per well) 24h prior to DNA transfection (4 μ g of total DNA) by the calcium
6 phosphate method using CMV- β Gal expression vector as an internal control. For measurement
7 of E2-dependent transactivation and protein accumulation in MELN cells, cells were plated in 6-
8 well plates ($5 \cdot 10^5$ cells per well) and were treated in parallel for luciferase assay and Western-
9 blot analysis. To quantify luciferase activity, cells were lysed at 4°C for 10min in 400 μ l of lysis
10 buffer (25mM Tris pH 7.8, 2mM EDTA, 10% glycerol, 1% Triton X-100). Luciferase activity
11 was measured on 100 μ l of supernatant aliquots by integrating photons emitted during 2s after
12 injection of 100 μ l of luciferase detection solution using a luminometer (LB 960 Berthold).
13 Luciferase values from transient transfection were normalized by the β -galactosidase activities
14 as described (8) and all data were expressed as mean \pm SD.

15 **Western-blot analysis.** Whole cells extracts were prepared in high-salt lysis buffer (HSB)
16 containing 500mM NaCl, 50mM Tris pH8, 1% Nonidet P-40, 1mM dithiothreitol and proteases
17 inhibitors (Roche Diagnostics, Meylan, France). Insoluble material was removed by
18 centrifugation. Proteins were quantified using the Bradford assay (Bio-Rad Laboratories,
19 Marnes, France) and 30 μ g were usually loaded on SDS-PAGE and transferred to PVDF
20 membrane. Blots were saturated in TBST buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% tween
21 20 (v/v), 5% nonfat dehydrated milk (w/v)), incubated with specific primary antibodies for ER α
22 (sc-543 from Tebu, Le Perray, France), p53 (sc-126 from Tebu, Le Perray, France) Mdm2 (clone
23 2A10, gift from J. Piette, IGMM, Montpellier) or actin (Sigma) and probed with the appropriate

1 secondary antibody (Sigma). Detection was done using the Chemiluminescence Reagent Plus kit
2 (Perkin Elmer Life Science, Courtaboeuf, France).

3 **Coimmunoprecipitation experiments.** Transiently transfected cells were washed in PBS,
4 centrifuged and frozen at -80°C. After thawing, cells were lysed and sonicated in 50mM Tris
5 pH8, 5mM EDTA, 150mM NaCl, 0,5% NP40 supplemented with 1mM PMSF. Preclearing of
6 cell extracts was realized by incubation with protein A sepharose for 10 mn at 4°C. After
7 centrifugation, precipitation of Mdm2 was carried out for 2 h at 4°C using the anti-Mdm2
8 antibody (clone 4B11, gift from J. Piette, IGMM, Montpellier) in a final volume of 500µl. Beads
9 were washed 3 times in 0.5 ml of SNTE buffer (50mM Tris pH7.4, 500mM NaCl, 5mM
10 EDTA, 5% sucrose, 1% NP40) and once in RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1%
11 Triton X100, 0.1% SDS, 1% Na deoxycholate). Finally, the beads were resuspended in 30µl of
12 loading sample buffer and immunoprecipitated proteins were analyzed by SDS-PAGE.

13 **GST-pull down assays.** *In vitro* translation and GST pull-down assays were performed as
14 previously described (8). Briefly, ³⁵S-labeled proteins were cell-free-synthesized using the TNT
15 lysate system (Promega) and incubated with purified GST fusion proteins overnight at 4°C in
16 NETN buffer containing 0.5% Nonidet P-40, 1mM EDTA, 20mM Tris pH8, 100mM NaCl,
17 10mM DTT and proteases inhibitors cocktail (Roche Diagnostics, Meylan, France). Protein
18 interactions were analyzed by SDS-PAGE followed by quantification using a Phosphorimager
19 (Fujix BAS1000). Gels were stained with Coomassie Brilliant Blue (BioRad) to visualize the
20 GST fusion proteins present in each track.

21 **Apoptosis assay.** MCF-7 cells were plated in 6-well plates (50 000 cells/well) and treated or
22 not with MG132 (4µM), actinomycin D (4nM) or UV irradiated. Apoptosis was quantified 24h
23 later using the Cell Death Detection ELISA (Roche Molecular Biochemicals, Meylan, France),

1 according to the manufacturer's conditions. Values from absorbance measurements at 405nm
2 were corrected using DNA quantification in separate wells treated in parallel.

3 **RNA extraction and quantitative PCR.** Total RNA was extracted using RNeasy kit
4 (Qiagen, Courtaboeuf, France) according to the manufacturer's conditions. For RT-PCR, 1.5µg
5 of total RNA was subjected to reverse transcription using the Omniscript Reverse Transcriptase
6 kit (Qiagen, Coutaboeuf, France). Real-time PCR quantification was then performed using a
7 SYBR Green technology (Light Cycler Roche). For each sample, ERα mRNA levels were
8 corrected for HPRT mRNA levels used as a reference gene and normalized to a calibrator
9 sample. The primers for ERα and HPRT mRNAs have been described elsewhere (11).

1 **RESULTS**

2 **ER α is involved in a ternary complex with p53 and Mdm2.** In order to characterize
3 protein-protein interactions between Mdm2 and ER α , we first performed GST-pull down
4 experiments. As shown in Figure 1A (left panel), we observed a ligand-independent interaction
5 of *in vitro* translated ER α with Mdm2 expressed as a fusion protein with GST, whereas no
6 detectable interaction was obtained with GST alone (data not shown). The binding of Mdm2 to
7 ER α was mediated by the ligand binding domain (LBD) of the receptor, as shown by the
8 recruitment of *in vitro* expressed Mdm2 with the GST-LBD protein, which was also unaffected
9 by E2 or by the partial antiestrogen 4-hydroxytamoxifen (right panel). Moreover, the use of a
10 GST-LBD mutated in the conserved AF2 activation domain indicated that this interaction did not
11 require an intact AF2 transactivation domain (data not shown). The use of ER α deletion mutants
12 (shown in Figure 1B, left panel) confirmed that both the DBD (HE11 mutant) and the N-terminal
13 region (HE19 mutant) were not necessary for the binding to Mdm2 (Figure 1B, right panel). By
14 contrast, our data confirmed that the C-terminal LBD of ER α was required for the *in vitro*
15 interaction since the HE15 mutant poorly associated with GST-Mdm2. Altogether, these results
16 demonstrated a direct ligand-independent interaction of ER α with Mdm2.

17 In order to confirm that these interactions also occurred in intact cells, we performed two
18 types of experiments. We first set up coimmunoprecipitation experiments using an anti-Mdm2
19 antibody to precipitate overexpressed Mdm2 and we analyzed co-precipitated proteins for the
20 presence of transfected ER α . As illustrated in Figure 1C, endogenous Mdm2 was efficiently
21 precipitated by the anti-Mdm2 specific antibody (clone 4B11) and ER α was associated with
22 immunoprecipitated Mdm2, as determined by probing the Western blot with a polyclonal
23 antibody specific for the receptor (lanes 1 and 2). Interestingly, when the experiment was carried

1 out in MEFdKO embryonic fibroblasts (39) derived from p53^{-/-} and Mdm2^{-/-} mice (lanes 3 and
2 4), ER α was not coprecipitated with Mdm2 suggesting that p53 could be required for the
3 interaction between ER α and Mdm2. Such a hypothesis was supported by the fact that p53 also
4 directly interacted with ER α in a ligand-independent manner (31). Interestingly, in support of
5 this study, we found that the p53/ER α interaction domain encompassed the central region of the
6 receptor (GST-C/D) *i.e.* different from that required for Mdm2 binding (Figure 1D).

7 The hypothesis that ER α could be engaged in a ternary complex with p53 and Mdm2 was
8 confirmed using a modified mammalian two-hybrid system (Figure 1E). An expression plasmid
9 coding for Mdm2 fused to the Gal4 DBD (Gal-Mdm2) was cotransfected in MCF-7 cells
10 together with a Gal4 responsive reporter plasmid and an expression vector coding for ER α fused
11 to the VP16 activation domain or for VP16 alone. In our experimental conditions, a slight but
12 significant increase in luciferase activity (more than 2-fold) was obtained when ER-VP16 was
13 coexpressed with Gal-Mdm2 (as compared to the activity obtained with VP16 alone).
14 Interestingly, when a p53 expression plasmid was cotransfected with Gal-Mdm2 and ER-VP16,
15 we observed a significant increase in luciferase activity (more than 10-fold) suggesting that p53
16 may indeed stabilize the interaction between ER α and Mdm2. Altogether, these results suggest
17 that ER α , p53 and Mdm2 coexist within the same protein complex in intact cells.

18 **p53 and Mdm2 are required for ligand-dependent ER α turn-over.** In order to evaluate the
19 role of p53 and Mdm2 in ligand-dependent down-regulation of ER α , we first used the MEFdKO
20 model (39). Interestingly, in this p53/Mdm2^{-/-} background, estrogen treatment did not decrease
21 ER α accumulation (as observed in parallel in p53/Mdm2^{+/+} wild-type MEFs) but instead
22 slightly increased receptor levels (Figure 2A). The same effect was observed whatever the

1 concentration of ER α expression vector used to transiently transfect these p53/Mdm2 -/- cells
2 (data not shown). To demonstrate that the expression of p53 and Mdm2 was important for the
3 E2-dependent inhibition of ER α accumulation, we transiently transfected increasing
4 concentrations of expression vectors for both p53 and Mdm2 together with the expression vector
5 for ER α in p53/Mdm2-/- MEFs (Figure 2B). The positive effect of E2 on ER α accumulation that
6 we observed in control p53/Mdm2-/- cells was progressively diminished and, at the highest
7 concentration of expression vectors for p53 and Mdm2, we restored the negative hormonal
8 regulation.

9 Since previous studies proposed that the E2-dependent decrease of ER α accumulation was
10 required for transcriptional activity of the receptor (32), we measured in parallel, the ability of
11 the transiently transfected ER α to increase the transcription of a reporter gene in MEFs
12 expressing or not p53/Mdm2. As shown in Figure 2C, we found that ER α strongly activated
13 transcription in conditions where E2 up-regulated its accumulation. Very interestingly, we found
14 that overexpression of p53/Mdm2 only slightly modify ER α transactivation although it
15 completely inversed the effect of E2 on its accumulation.

16 Finally, to emphasize the importance of Mdm2 in ligand-dependant turn-over of ER α , we
17 measured the effect of Mdm2 overexpression on the apparent stability of the ER α protein. This
18 experiment was performed in E2-treated HeLa cells (which are p53 positive cells), by chase
19 experiments using cycloheximide as a protein synthesis inhibitor. As shown in Figure 2D, we
20 noticed a significant decrease in ER α apparent stability, thus emphasizing the role of Mdm2 in
21 the post-translational regulation of receptor expression. Altogether these data suggest that i)

1 Mdm2 is involved in E2-dependent turn-over of ER α ii) a negative effect of E2 on ER α
2 expression is not required for efficient transactivation.

3 **Mdm2 regulates ligand-independent expression of ER α .** Since some of the data shown in
4 Figure 1 suggested that Mdm2 could also bind ER α in an E2-independent manner, we
5 investigated the effect of Mdm2 overexpression on ER α accumulation in the absence of ligand.
6 As shown in Figure 3A, when Mdm2 and ER α expression vectors were transiently cotransfected
7 in HeLa cells, the accumulation of the receptor was strongly decreased. This effect depended on
8 the amount of cotransfected expression vector for Mdm2 (Figure 3B). As expected, this decrease
9 in the level of ER α accumulation correlated with a shorter apparent half-life of the protein
10 (Figure 3C) and led to a lower ligand-independent ER α transactivation (Figure 3D).
11 Interestingly, overexpression of Mdm2 in p53/Mdm2^{-/-} MEFs also led to a 2- to 3-fold decrease
12 in the basal level of ER α transactivation suggesting that this effect was p53 independent (data
13 not shown).

14 We then tried to further decipher the molecular mechanisms involved in ER α degradation by
15 Mdm2. We used a mutant of Mdm2 (Mdm2 Δ RING) deleted in the C-terminal part of the protein
16 which contains the RING domain required for its ubiquitin-ligase activity (19). As shown in
17 Figure 4A, this mutant still interacted with ER α in GST-pull down experiment. Interestingly,
18 overexpression of the Mdm2 Δ RING mutant did not decrease ER α accumulation as compared
19 with the effect of its wild-type counterpart (Figure 4B) suggesting that the E3 ubiquitin-ligase
20 activity of Mdm2 is directly involved in ER α degradation.

21 Finally, in an attempt to compare the E2-dependent turn-over of the receptor to the ligand-
22 independent degradation observed upon Mdm2 overexpression, we used a mutant of ER α

1 deleted in the central DNA-binding domain involved in the binding of p53 (see Figure 1B and
2 D). This ER α - Δ DBD mutant (HE11) was no longer degraded upon E2 stimulation (Figure 4C,
3 right panel lane 5) in support with the role of this domain in recruiting p53 and consistent with
4 the formation of a ternary complex between the receptor, p53 and Mdm2 (Figure 1). By contrast,
5 the ligand-independent increase in ER α turn-over observed upon Mdm2 overexpression was
6 comparable for the wild-type protein and the DBD-deleted mutant (Figure 4C, lane 3 for ER α
7 WT and lane 6 for HE11). Altogether, these data support the hypothesis that different
8 mechanisms might be involved in the regulation of ER α turn-over upon E2 treatment or Mdm2
9 overexpression.

10 **Stress-inducing agents block ligand-dependent turn-over of ER α .** Previous studies
11 reported that, in MCF-7 human breast cancer cells, the E2-dependent decrease of ER α
12 accumulation was abolished by the MG132 proteasome inhibitor (1, 12, 40). Results shown in
13 Figure 5A confirmed that MG132 was able to reverse the effect of various ligands such as E2,
14 estrone (E1) or estriol (E3) on ER α levels in MCF-7 cells. Interestingly, proteasome blockade is
15 a cellular stress which increased accumulation of p53 (Figure 5A) and as a consequence, induced
16 apoptosis, as shown by quantification of cytoplasmic nucleosomes (Figure 5B).

17 Based on our results concerning the role of Mdm2 in the regulation of ER α turn-over, we
18 thought that the effect of MG132 could be linked (at least in part) to its ability to dissociate
19 Mdm2 from p53 and/or ER α . We therefore analyzed the effect of other stress-inducing agents
20 (genotoxic or non-genotoxic) for their ability to regulate E2-dependent turn-over. As shown in
21 Figure 5B, both UV irradiation and inhibition of transcription (actinomycin D treatment)
22 increased significantly programmed cell death in MCF-7 cells. In all conditions, the
23 accumulation of p53 was significantly increased to levels comparable to those obtained after

1 MG132 treatment and, very interestingly, both treatments that increase p53 levels concomitantly
2 suppressed the hormone-dependent down-regulation of ER α (Figure 5C).

3 We then characterized the effect of UV irradiation on the regulation of ER α accumulation.
4 We first showed that the blockade of E2-dependent turn-over after UV exposition was rapid
5 since observed 4h after irradiation and still detectable 24h later (data not shown). We then
6 investigated the effect of UV irradiation on the response to antiestrogens which have also been
7 shown to regulate ER α expression. Indeed, as previously reported, incubation with the partial
8 antagonist 4-hydroxy tamoxifen (OHT) increased the level of ER α whereas pure antiestrogens
9 such as the ICI182,780 molecule (ICI) negatively regulated the accumulation of the receptor
10 (58). As shown in Figure 5D, UV irradiation did not affect the stabilization of ER α upon
11 OHTam treatment. Very interestingly, it did not antagonize the effect of ICI182,780 whereas it
12 completely reversed the agonist-dependent decrease of ER α levels. This suggested that
13 degradation of ER α by pure antiestrogens involved different mechanisms than those required for
14 hormone-dependent degradation.

15 We then analyzed the effect of UV irradiation on endogenous ER α activity. To this aim, we
16 used a cell line established in our laboratory (namely the MELN cells) which are MCF-7 cells
17 stably transfected with an E2-regulated luciferase reporter gene allowing easy monitoring of
18 endogenous ER α activity (5). MELN cells were treated with E2 at various time post irradiation
19 by UV (*i.e.* when E2-dependent turn-over of ER α was no longer detectable). As shown in Figure
20 5E, the transcriptional activity of ER α was significantly increased both in the absence of ligand
21 or in the presence of E2 or antiestrogens. In all conditions, the induction by E2 was detectable

1 confirming that E2-dependent turn-over was not a prerequisite for transactivation even on
2 endogenous ER α .

3 **Concomitant stabilization of ER α and p53 by stress-inducing agents.** To confirm that UV
4 irradiation modulated the stability of the ER α protein, we performed chase experiments with
5 cycloheximide. As shown in Figure 6A and B, the stability of the receptor in the absence of *de*
6 *novo* protein synthesis decreased upon E2 treatment and UV irradiation completely inhibited the
7 hormone-dependent degradation. As expected, upon UV treatment, p53 appeared very stable and
8 its accumulation was strongly increased (Figure 6A). Altogether, these data suggest that stress-
9 inducing agents induce a co-stabilization of p53 and ER α in MCF-7 breast cancer cells. Our
10 hypothesis was that disruption of the p53/Mdm2/ER α complex upon cellular stress was at the
11 basis of the loss of E2-dependent turn-over of the receptor.

12 To further emphasize this point, we used the NSC652287 compound (also known as RITA
13 which stands for *Reactivation of p53 and Induction of Tumor cell Apoptosis*), and recently
14 described as an inhibitor of the p53/Mdm2 interaction (22). As shown in Figure 6C, treatment of
15 MCF-7 cells with RITA at 1 μ M significantly abolished the ligand-dependent decrease in ER α
16 accumulation. This effect was detectable 4h after the beginning of the treatment and very
17 interestingly, it correlated again with the increase in p53 accumulation. Finally, when tested over
18 the same period of time, we found no significant decrease in ER α transactivation in the presence
19 of RITA (Figure 6D), thus supporting the data obtained after UV irradiation (Figure 5E).
20 Altogether, these data suggest that dissociation of the p53/Mdm2/ER α complex upon cellular
21 stress leads to a co-stabilisation of p53 and ER α proteins without significantly decreasing
22 receptor transactivation.

1 **Cellular stress inhibits ER α transcription.** As a control of UV irradiation and RITA
2 treatment, we also measured the levels of ER α mRNA by quantitative RT-PCR in order to check
3 that no up-regulation of the mRNA was associated with the blockade of the E2-dependent
4 decrease observed at the protein level. We were very surprised to find that both UV irradiation
5 and RITA treatment strongly decreased ER α mRNA levels in MCF-7 cells both in the absence
6 or presence of E2. Indeed, as shown in Figure 7A, the levels were lowered by 3- to 5-fold after
7 12h treatment with RITA in the presence or absence of E2, respectively.

8 We then verified that the accumulation of ER α protein was decreased after a long term
9 treatment with stress-inducing agents. Western-blot analyses confirmed that both UV irradiation
10 and RITA treatment (24h or 48h) strongly inhibited ER α expression (Figure 7B). As expected,
11 this was associated with a significant decrease in ER α -dependent transactivation which was
12 clearly observed 48h after UV irradiation or RITA treatment and already detectable for RITA
13 after 24h of treatment (Figure 7C). Altogether, this suggested that stress-inducing agents exerted
14 a dual effect on ER α expression and activity: over a short period of time, they stabilized the
15 protein and increased its transactivation. By contrast, over a longer period, they decreased ER α
16 expression at the transcriptional level, resulting in a strong repression of ER α -dependent
17 transcriptional regulation.

1 **DISCUSSION**

2 Modulation of ER α levels is a critical parameter in determining the hormonal response of
3 breast cancer cell proliferation. The control of ER α expression is under a complex regulation
4 which takes place both at the transcriptional and post-transcriptional levels. Previous data
5 suggested that ER α was down-regulated in the presence of E2 through a proteasome-dependent
6 mechanism (1, 12, 40). In this study, we have investigated the mechanisms regulating the
7 hormone-induced ER α turn-over and several lines of evidence indicate that Mdm2, an oncogenic
8 ubiquitin-ligase, plays an important role in the regulation of ER α accumulation by E2.

9 First, using a modified two hybrid assay we show that Mdm2 interacted with ER α in a ternary
10 complex with p53. The direct *in vitro* interaction between the receptor and both p53 and Mdm2
11 was also demonstrated using GST pull-down assays. Our data suggested that Mdm2 binding
12 involved the LBD of ER α but did not require the ligand-dependent AF2 interface. Interestingly,
13 p53 interacted with a different region of the receptor (*i.e.* the central region) thus supporting the
14 formation of a ternary complex.

15 The use of p53/Mdm2^{-/-} cells demonstrated that the two proteins were required for the E2-
16 dependent down-regulation of ER α . Moreover, we showed that the C-terminal region of Mdm2
17 which encompassed the E3 activity was required for the degradation of ER α . Previous studies
18 highlighted the role of the Mdm2 ubiquitin-ligase in the degradation of steroid hormone nuclear
19 receptors. In the case of the glucocorticoid receptor (GR), Sengupta and Wasylyk showed that
20 disruption of the p53/Mdm2 interaction prevented ubiquitination of GR and that the ligand-
21 dependent trimeric complex between GR, p53 and Mdm2 enhanced proteasomal degradation of
22 the receptor (51). Moreover, the E3 ligase activity of Mdm2 was also necessary for the
23 ubiquitination and degradation of the androgen receptor (30).

1 From the data presented in our study, we propose that p53 and Mdm2 are involved in the
2 ligand-dependent degradation of ER α . Interestingly, in support of our data, it has been recently
3 demonstrated, by chromatin immunoprecipitation, that Mdm2 was recruited on the ER α -
4 regulated pS2 promoter upon E2 stimulation (47, 57). Moreover, both Mdm2 and p53 expression
5 levels are increased by E2 in MCF-7 breast cancer cells (24). This positive regulation could be of
6 importance in the E2-dependent down-regulation of the receptor.

7 Obviously, other factors associated with p53 or Mdm2 could be involved in such a
8 degradation complex. A strong candidate is the coactivator AIB1 which has been shown to
9 interact with p53 (28) and to be required for the E2-dependent turn-over of ER α (53). The
10 involvement of AIB1 could explain why the AF2 mutated ER α is no longer degraded upon
11 hormone stimulation (V. Duong, unpublished data). Interestingly, in breast cancer, AIB1
12 amplification correlates with that of Mdm2 (6). On the other hand, AIB1 expression is also
13 regulated by ubiquitination (59). However, other proteins that interact with p53 and/or ER α
14 could also play important roles in regulating the accumulation of the receptor in breast cancer
15 cells. A good candidate could be E6-AP, an E3 ubiquitin-ligase recruited by ER α (41, 29) which
16 also targets p53 (50).

17 Our results dealing with the effect of stress-inducing agents on ER α expression clearly
18 indicated that the increased turn-over of the receptor in the presence of the pure antiestrogen
19 ICI182,780 involves different mechanisms than those implicated in the E2-dependent
20 degradation. Similar dissociation between the effects of E2 and ICI182,780 were previously
21 reported (2) and other pathways involving for example the NEDD8 ubiquitin-like protein (13) or
22 CSN5/Jab1 (7) could account for the degradation in the presence of pure antihormones.

1 Previous data have highlighted the existence of a ligand-independent degradation of ER α and
2 demonstrated the role of CHIP (*Carboxyl terminus of Hsc70-interacting protein*) in the
3 ubiquitination of misfolded ER α (55). Our data suggest that, when overexpressed, Mdm2 is also
4 involved in the ligand-independent turn-over of ER α . In these conditions, several evidences
5 indicate that p53 could be dispensable for the effect of Mdm2 and this could reflect the fact that,
6 upon overexpression of Mdm2, the binding equilibrium between ER α and Mdm2 could be
7 strongly displaced towards complex formation thus avoiding the requirement for p53 to stabilize
8 the interaction. Finally, although ubiquitination of ER α has previously been evidenced (58), the
9 location of target residues is still unknown and further work will be require to define which
10 particular lysine residues are modified by Mdm2 in the presence or absence of E2.

11 The present work also highlights the complex regulation of ER α gene expression by cellular
12 stress. We first show that several stress-inducing agents which stabilize p53 also increase ER α
13 levels and block its E2-dependent down-regulation. The stabilization of p53 upon treatment with
14 stress-inducing agents results from its dissociation from Mdm2 due to post-translational
15 modifications such as phosphorylation (36). It is interesting to note that phosphorylation of
16 nuclear receptors has also been linked to their turn-over (25, 15 ,30). Concerning ER α , it has
17 very recently been reported that S118 is an essential determinant of ER α degradation (57) and a
18 previous work has shown that extracellular signal-regulated kinase 7 (ERK7) enhances the
19 destruction of the receptor in a ligand-independent manner (17). By contrast, another report
20 suggests that in MCF-7 cells, inhibition of MAPK results in an increased degradation of ER α
21 (33). Altogether, these data suggest that phosphorylation is directly or indirectly involved in
22 ER α turn-over. Interestingly we found that the degradation observed upon Mdm2 overexpression

1 was no longer detected when we tested ER α mutated on the phosphorylated serine 118 residues
2 (data not shown).

3 Our data clearly indicated that all the stress-inducing agents that we used (MG132, UV,
4 RITA) strongly inhibited ER α mRNA accumulation. Cellular stress produced therefore a dual
5 effect on ER α protein accumulation, *i.e.* an early positive effect on ER α stability followed by a
6 delayed negative regulation at the mRNA level (which led to a strong inhibition of receptor
7 activity). Additional experiments will be needed to decipher the mechanisms which take place at
8 the transcriptional or post-transcriptional levels and account for the negative regulation of ER α
9 mRNA accumulation. One hypothesis could be that the increase in p53 levels (which occurs
10 upon cellular stress) directly or indirectly participates in the repression of ER α mRNA
11 accumulation. However, it has recently been suggested using stable transfection of antisense p53
12 or overexpression of p53 in MCF-7 cells that ER α transcription was positively regulated by p53
13 (3). Further work will be required to precise the mechanisms of stress-induced inhibition of ER α
14 expression and to define its relevance in the efficacy of proteasome inhibitors in breast cancer
15 therapy (44).

16 It has been suggested that the E2-dependent turn-over of ER α was required for efficient
17 transactivation of the receptor (32). The data presented herein do not support such a conclusion.
18 We first show that in p53/Mdm2 $^{-/-}$ cells where E2 treatment led to an increase in ER α
19 accumulation, we observed a strong hormone-dependent transactivation. In addition, in MCF-7
20 cells stably transfected with an ERE-containing reporter gene, the blockade of ER α E2-
21 dependent degradation upon UV irradiation (or MG132, data not shown) did not abolish the
22 transcriptional response to E2. Moreover, the same results were obtained on the endogenous E2-

1 regulated pS2 gene (data not shown) thus confirming the dissociation of stress-inducing agents
2 on the accumulation of ER α and on its transactivation. Altogether, the present work dissociates
3 the effect of E2 on ER α turn-over from the effect of hormone on transactivation, thus supporting
4 previous studies performed using MG132 which suggested that the two events are not linked
5 (14).

6 In conclusion, this study emphasizes the relevance of protein-protein interactions between
7 nuclear receptors, the Mdm2 oncogene and the p53 tumor suppressor. Several physiological and
8 pathological consequences of these interactions have been proposed for the glucocorticoid
9 receptor (52). The involvement of an oncogenic E3-ligase in the regulation of ER α expression
10 might appear surprising since this receptor is believed to mediate proliferative signaling of
11 estrogens. However, our laboratory has shown that in the absence of ligand, ER α also exerts
12 anti-invasive activity in breast cancer cell lines (46) and this effect could therefore be lowered in
13 tumor cells overexpressing Mdm2. Mdm2 is frequently overexpressed in human cancers
14 including breast carcinoma and ER-positive breast cancer cell lines express high levels of Mdm2
15 mRNA and protein compared to ER-negative cells. (Gudas CCR, 1995). It would be of great
16 interest to quantify the level of Mdm2 protein in different breast tumors with changes in ER α
17 expression. Altogether, the present work highlights the role of Mdm2 on hormone signaling and
18 will be at the basis of future investigations to decipher its importance in various physio-
19 pathological situations.

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18

1 **FIGURE LEGENDS**

3 **Figure 1 - Interaction of ER α with p53 and Mdm2.**

4 (A) GST pull-down assays were carried out as described in material and methods using
5 bacterially expressed GST, GST-Mdm2 or GST-LBD proteins to retain ^{35}S -labelled ER α or
6 Mdm2 in the presence of vehicle (C), 17 β -estradiol (E2, 10^{-6}M) or 4-hydroxytamoxifen (OHT,
7 10^{-6}M). Inputs represent 10% of the material used in the assay. (B) Schematic representation of
8 the ER α mutants. *In vitro* translated full-length ER α or various deletion mutants were analyzed
9 for their interaction with the GST-Mdm2 fusion protein. (C) ER α and Mdm2 expression vectors
10 were transfected into p53/Mdm2 $^{+/+}$ U2OS cells (lanes 1 and 2) or p53/Mdm2 $^{-/-}$ mouse embryo
11 fibroblasts (MEFdKO, lanes 3 and 4). Whole cell extracts were subjected to
12 immunoprecipitation (lanes 2 and 4) as described in Materials and Methods using anti-Mdm2
13 antibody and Western-blotted using either anti-Mdm2 (upper panel) or anti-ER α antibody (lower
14 panel). Whole-cell extracts are shown in lanes 1 and 3. (D) GST pull-down assays were carried
15 out as in (A) using the bacterially expressed GST-C/D protein (containing the DBD of ER α) or
16 GST alone to retain ^{35}S -labelled p53. (E) Mammalian two-hybrid assay was carried out using
17 MCF-7 cells transfected with the 17M5-Luc reporter together with expressing vectors for Gal4
18 or Gal-Mdm2 with VP-16 or ER-VP16, in the presence or not of p53. Luciferase activity was
19 expressed as % of control in presence of Gal4 and was the mean of three values.

1 **Figure 2 - p53 and Mdm2 regulate E2-dependent expression of ER α .**

2 (A) ER α accumulation was analyzed by Western-blot using an anti-ER α antibody. MEF WT and
3 MEFdKO cells (p53/Mdm2^{-/-}) were transfected with an ER α expression vector and treated 20h
4 post-transfection with vehicle (C) or 17 β -estradiol (E2, 10⁻⁸M) for 20h. (B) Western-blot
5 analysis of ER α accumulation in MEFdKO cells transiently transfected with an ER α expression
6 vector (150ng) together with increasing amounts of p53 and Mdm2 expression plasmids (62.5,
7 125 and 250ng for each vector) or empty vectors alone. Cells were cultured 20h post-transfection
8 in the presence or absence of 17 β -estradiol (E2, 10⁻⁸M). The asterisk indicated a non- specific
9 band detected by anti-ER α antibody. (C) MEFdKO were transiently transfected either with the
10 17EB-Luc reporter plasmid (300ng) and ER α (100ng) alone or with increasing amounts of p53
11 and Mdm2 expression vectors (same transfection experiment as for the Western-Blot analysis
12 shown in 2B). Cells were treated with vehicle (C) or 17 β -estradiol (E2, 10⁻⁸M) and the luciferase
13 activity was quantified as described in Materials and Methods. Results were expressed relative to
14 control in presence of ER α alone (% of control) and were the mean of three values. (D) HeLa
15 cells were transfected either with ER α (1.5 μ g) or ER α and Mdm2 (1.5 μ g each) expression
16 vectors and treated with 17 β -estradiol (E2, 10⁻⁸M) for 1h. Cycloheximide (CHX, 50 μ M) was
17 added to inhibit ER α synthesis (time 0) and experiments were stopped at the indicated times.
18 Mdm2 overexpression and ER α half-life were analyzed by Western-blot.

1 **Figure 3 - Effect of Mdm2 on ligand-independent ER α expression.**

2 **(A)** Either ER α or ER α and Mdm2 expression vectors were transfected (1.5 μ g each) into HeLa
3 cells. ER α and Mdm2 levels were quantified by Western-Blot 24h after transfection. **(B)** HeLa
4 cells were transfected either with ER α expression vector alone (1.5 μ g) or with ER α and
5 increasing amounts of Mdm2 expression vector (150, 500 and 1500ng). ER α and Mdm2 levels
6 were analyzed by Western-blot. **(C)** The stability of ER α protein was measured in the absence of
7 ligand by pulse-chase assay with cycloheximide in HeLa cells transfected with ER α or with ER α
8 and Mdm2 expression vectors (1.5 μ g each). The presence of exogenous Mdm2 protein was
9 confirmed by Western-blot. **(D)** The 17EB-Luc reporter plasmid (500ng) was transiently
10 transfected in HeLa cells together with ER α expression vector (150ng) in the presence or not of
11 Mdm2 expression vector (1 μ g). The luciferase activity was quantified 20h after transfection.
12 Results, expressed relative to control in presence of ER α alone (% of control), were the mean of
13 three values.

1 **Figure 4 - Mechanisms of Mdm2-dependent degradation of ER α .**

2 **(A)** A GST pull-down experiment was performed to determine the *in vitro* interaction between
3 ³⁵S-labelled Mdm2 or Mdm2 Δ RING (deleted of the C-terminal region) with the GST-AF2 fusion
4 protein which contains the ligand-binding domain of ER α . **(B)** HeLa cells were transfected either
5 with the ER α expression plasmid alone (1.5 μ g) or together with Mdm2 or Mdm2 Δ RING
6 expression vector (1.5 μ g). The accumulation of ER α and Mdm2 proteins (wild-type or
7 Mdm2 Δ RING) was analyzed by Western-Blot. **(C)** HeLa cells were transiently transfected with
8 ER α expression vector alone (500ng) (lanes 1, 2, 4 and 5) or together with the Mdm2 expression
9 vector (1 μ g) (lane 3 and 6). Cells were treated with 17 β -estradiol (E2, 10⁻⁸M, lanes 2 and 5) or
10 with vehicle alone (lanes 1, 3, 4 and 6). The expression of ER α and Mdm2 was analyzed by
11 immunoblotting. The same experiment was performed using the wild-type receptor or the HE11
12 ER α mutant (deleted of the DBD).

1 **Figure 5 - Effect of p53-inducing agents on ER α signaling.**

2 **(A)** MCF-7 cells were treated for 20h with 10^{-7} M estrone (E1), 17β -estradiol (E2), estriol (E3) or
3 diethylstilbestrol (DES) in the absence (control) or presence of MG132 (4 μ M). ER α and p53
4 protein levels were analyzed by Western-blot. **(B)** MCF-7 cells were cultured with MG132
5 (4 μ M), actinomycin D (10nM) or exposed to UV (150J/m²) for 20h and apoptosis was measured
6 by cytoplasmic nucleosomes quantification. Values were normalized by DNA quantification
7 assay (measurement of DNA with DABA). **(C)** MCF-7 cells were irradiated (UV), treated with
8 MG132 (4 μ M), actinomycin D (10nM) or untreated (control) in the presence of vehicle (C) or
9 17β -estradiol (E2, 10^{-8} M). Extracts were prepared and Western-blotted with antibodies for ER α
10 and p53. **(D)** MCF-7 cells were irradiated by UV or not (control) and cultured in the presence of
11 vehicle (C), 17β -estradiol (E2, 10^{-8} M), hydroxytamoxifen (OHT, 10^{-8} M) or ICI182,780 (ICI, 10^{-8}
12 M) for 20h. ER α and p53 accumulation were analyzed by Western-Blot. **(E)** Time course
13 experiment with UV was performed in MCF-7 cells stably transfected with ERE- β globin-
14 Luciferase (MELN cells). Cells were treated with vehicle (control), 17β -estradiol (E2, 10^{-8} M),
15 hydroxytamoxifen (OHT, 10^{-8} M) or ICI182,780 (ICI, 10^{-8} M) for 20h either 2, 4 or 8h post-
16 irradiation or without being irradiated (0). Luciferase activity was measured as described in
17 Materials and Methods and results expressed as relative activity (% of E2 without irradiation)
18 were the mean (\pm SD) of three values.

1 **Figure 6 - Effect of stress-inducing agents on ER α stability and activity.**
2 **(A)** The steady-state level of ER α was measured by pulse-chase assay with cycloheximide
3 (CHX). MCF-7 cells were irradiated with UV (lower panel) or not (upper panel), and treated
4 concomitantly with 17 β -estradiol for 4h. Cycloheximide was then added (time 0) during 2, 4 or
5 6h. ER α stability was measured by Western-Blot. **(B)** Quantification of the experiment shown in
6 A. The intensity of the bands corresponding to ER α levels was determined by PCBAS imaging.
7 Values were normalized by quantifying actin expression on the same blot. Results are expressed
8 as % of control (time 0). **(C)** Kinetics of ER α and p53 accumulation by Western-Blot in stably
9 transfected MELN cells treated or not with 17 β -estradiol (E2, 10⁻⁸M) in presence or not of RITA
10 (1 μ M) during 2, 4 or 8h. **(D)** MELN cells were treated exactly as described in panel C and the
11 luciferase activity corresponding to the stably integrated reporter gene was quantified. Results
12 expressed as relative luciferase activity (% of control) were the mean (\pm SD) of three values.

1 **Figure 7 – Stress-inducing agents decrease ER α expression at the mRNA level.**
2 (A) MELN cells were treated with vehicle or 17 β -estradiol (E2, 10⁻⁸M) in the presence or not of RITA (1 μ M) for
3 12h. ER α mRNA levels were quantified by real-time quantitative RT-PCR as described in Materials and Methods.
4 The results are expressed in arbitrary units after normalization using HPRT mRNA levels. Values are the means \pm
5 SD of three values. (B) Kinetics of ER α and p53 accumulation measured by Western-Blot in MELN cells treated
6 with vehicle or 17 β -estradiol (E2, 10⁻⁸M) in the presence or not of RITA (1 μ M) for 24 or 48h. (C) MELN cells
7 were treated exactly as in B, and the luciferase activity of the stably integrated reporter gene was quantified. Results
8 were expressed as relative luciferase activity (% of control without stress) and were the mean of three values.