

**ER α and ER β expression and transcriptional activity
are differentially regulated by HDAC inhibitors**

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Running title: Estrogen receptors and HDAC inhibition

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Abbreviations: ER:estrogen receptor; HDAC: histone deacetylase; HDI: histone deacetylase
inhibitor; E2: 17 β -estradiol

ABSTRACT

The proliferative action of ER α largely accounts for the carcinogenic activity of estrogens. By contrast, recent data show that ER β displays tumor-suppressor properties, thus supporting the interest to identify compounds which could increase its activity. Here, we show that histone deacetylase inhibitors (HDI) up-regulated ER β protein levels, whereas it decreased ER α expression. Part of this regulation took place at the mRNA level through a mechanism independent of de novo protein synthesis. In addition, we found that, in various cancer cells, the treatment with different HDI enhanced the ligand-dependent activity of ER β more strongly than that of ER α . On the other hand, in MDA-MB231 and HeLa cells, the expression of ERs modified the transcriptional response to HDI. The use of deletion mutants of both receptors demonstrated that AF1 domain of the receptors was required. Finally, we show that ER β expression led to a dramatic increased in the antiproliferative activity of HDI, which correlated with a modification of the transcription of genes involved in cell cycle control by HDI. Altogether, these data demonstrate that the interference of ER β and HDAC on the control of transcription and cell proliferation constitute a promising approach for cancer therapy.

INTRODUCTION

It is well documented that the mitogenic action of estrogens is critical in the etiology and progression of human breast and gynecological cancers (Henderson et al., 1988). The biological actions of estrogens are mediated via two distinct nuclear estrogen receptor (ER) proteins, ER α and ER β , which belong to a large conserved superfamily of nuclear receptors (Pettersson & Gustafsson, 2001).

The two ERs share a similar primary structure. The N-terminal A/B domain contains the ligand-independent transactivation function AF-1. The DNA-binding or C domain contains a dimerization interface that mediates cooperativity in DNA binding. The E/F domain is involved in ligand binding, dimerization, cofactor binding and transactivation through the transactivation function (AF-2). ER α and ER β differ mostly in the N-terminal A/B domain and to a lesser extent in the ligand-binding domain. These differences suggest that the two receptors could serve distinct actions.

The transcriptional activities of ERs rely on their interactions with transcription coregulators broadly defined as coactivators that increase transcriptional activation when recruited, and as corepressors that attenuate promoter activity. Several coregulators either possess histone acetyltransferase activity (HAT) or recruit histone deacetylase (HDAC), both enzymes being involved in chromatin remodeling (McKenna & O'Malley, 2002) and subsequent access of the transcriptional machinery to promoters. Several HDAC inhibitors (HDI) such as trichostatin A (TSA) have been shown to modify reversibly or irreversibly the balance between HAT and HDAC activities (Marks et al., 2000). HDI induce growth arrest, differentiation, and/or apoptosis in a variety of transformed cell lines and inhibit tumor development in rodents (Cohen et al., 1999; Margueron et al., 2004). Several studies in animal models have reported the efficacy of some of these inhibitors in blocking tumor growth (Marks et al., 2000),

mammary tumors in particular (Vigushin et al., 2001). Phase I and II clinical trials are currently under way for several of these molecules (Kramer et al., 2001) to test whether HDI might provide an alternative therapeutic approach for the treatment of breast cancer.

The aim of the present study was to analyze the interferences between ERs and HDI on different parameters in human cancer cells. We report that ER α and ER β were differentially regulated by HDI at both the RNA and protein levels. HDI preferentially increased the estrogen-dependent transactivation of ER β as compared to ER α . On the other hand, ER β expression modulated the transcriptional effects of HDI on several genes involved in the control of cell proliferation. We propose that these effects could account, at least in part, for the synergistic effects of HDI and ER β on breast cancer cell proliferation.

MATERIALS AND METHODS

Materials

Propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) were from Tocris. Estradiol-17 β (E2) and trichostatin A were from Sigma.

Cell culture

Cells were maintained in media recommended by the ATCC supplemented with 10% fetal calf serum (FCS) and gentamycin. To wean off steroids, the cells were cultured in phenol red-free DMEM/F12 medium supplemented with 10% CDFCS (charcoal dextran-treated FCS) for 4 days.

Plasmids

The luciferase reporter plasmid ERE2-TK-LUC contains two copies of the consensus estrogen-responsive element (ERE) cloned upstream of the thymidine kinase promoter. CMV-ER α and CMV-ER β correspond to the wild-type and mutant human ER α and ER β cDNAs cloned into CMV5 plasmid under the control of the cytomegalovirus (CMV) promoter. A CMV-Gal reporter was used as an internal control and corresponds to the β -galactosidase gene cloned into CMV5.

Transient transfection

3.10⁵ cells were plated in 12-well plates in phenol red-free DMEM-F12 supplemented with 10% CDFCS 24 h before transfection. Transfections were performed using lipofectamine according to the manufacturer's recommendations using 2 μ g of luciferase reporter along with 150 ng of each expression vector and 0.5 μ g of the internal reference reporter plasmid (CMV-

Gal) per well. After overnight incubation, the medium was removed and the cells were placed into fresh medium supplemented with control vehicle (ethanol) or TSA. 24 h later, cells were harvested and assayed for luciferase activity on a Centro LB960 Berthold luminometer. β -galactosidase was determined as previously described (Lazennec et al., 2001).

Adenovirus infection

The adenoviruses Ad5, Ad-hER α , Ad-hER β used in this study and their propagation have been described previously (He et al., 1998; Lazennec et al., 2001). The optimal infection conditions were determined for the different cell lines using a β -galactosidase encoding virus to determine the optimal MOI (multiplicity of infection).

RNA extraction and quantitative PCR

Total RNA was extracted using the TRIzol reagent. For RT-PCR, 1.5 μ g of total RNA was subjected to a reverse transcription step using the Omniscript Reverse Transcriptase kit (Qiagen, Valencia, California). Real-time PCR quantification was then performed using a SYBR Green approach (Light Cycler; Roche). For each sample, ER mRNA levels were corrected for HPRT mRNA levels (reference gene) and normalized to a calibrator sample.

The primers for the ER α/β and HPRT mRNA have been published elsewhere (de Cremoux et al., 2002).

Western blot analysis

Cells were resuspended in 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 10% glycerol containing a cocktail of protease inhibitors. Then cells were lysed by cycles of freezing/thawing and the cellular debris were pelleted by centrifugation at 13,000 X g for 20 min. Whole-cell extract proteins (30 μ g) were subjected to SDS-PAGE followed by

electrotransfer onto a nitrocellulose membrane. The blot was probed with anti-hER α (SRA-1000, Stressgen), hER β antibody (CWK-F12) (Lazennec et al., 2001), cyclin E (Santa-Cruz) or p21^{WAF1/CIP1} (Oncogene Research) at a 1:500 to 1:1000 dilution and then incubated according to the primary antibodies with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugated antibodies (Sigma-Aldrich, St Quentin Fallavier, France) (1 μ g/ml). An ECL kit (Amersham Pharmacia Biotech, Arlington, IL) was used for detection.

Cell proliferation studies

Cells were maintained for 5 days in 10% CDFCS phenol red-free medium and then seeded at 20,000 cells/ well in 24-well dishes. Cells were infected overnight with the different viruses. The next morning, the medium was removed and replaced with fresh medium. Treatment with E2 or TSA began at the same time. After 4 days of treatment, the total cell DNA was quantified by diaminobenzoic acid assay as described earlier (Lazennec et al., 2001).

Apoptosis

MDA-MB-231 cells were plated in 6-well plates (50 000 cells/well) and infected 24h later with adenoviruses (Ad5, Ad-hER α or Ad-hER β). After 48hrs, cells were treated or not with E2 (10 nM) and/or TSA (50 ng/ml) for 2 days. Apoptosis was then quantified using the Cell death detection kit (Roche Molecular Biochemicals), according to the manufacturer's conditions and corrected using DNA quantification in separate wells treated in parallel.

RESULTS

HDI differentially regulate the endogenous expression of ER α and ER β

We first wanted to determine how ER α and ER β RNA and protein levels were modulated by TSA in a physiological context. Endogenous ER α RNA found in MCF-7 cells was strongly down-regulated by TSA, whereas the levels of ER β RNA were slightly induced (Fig. 1A and B). The regulation was also observed at the protein level for ER α (Fig. 1A), whereas no ER β protein could be detected in these cells (data not shown). To assess whether the modulation of ER α and ER β mRNA levels was direct, we used a synthetic inhibitor of protein synthesis (CHX) (Fig. 1A and B). We observed that CHX was unable to block either ER α RNA down-regulation or ER β up-regulation by TSA, suggesting that the modulation of both RNA levels by HDI does not require de novo protein synthesis. To confirm that ER β expression was increased at the protein level, we used the OVCAR-3 ovarian cell line which expresses high levels of ER β . In these cells, both endogenous ER β RNA and protein levels were strongly increased upon TSA treatment (Fig. 1C), thus confirming the differential regulation of the two ERs upon inhibition of HDAC activity.

E2 and HDI synergistically activate ER β

We then analyzed the effect of HDI on the transactivation of ER α and ER β in different cancer cell lines by transient transfection assays using an estrogen-responsive reporter construct (Fig. 2A). The cell lines chosen were all ER-negative and subsequently transfected with an empty expression vector (CMV5) or with vectors encoding ER α or ER β . In MDA-MB-231 breast cancer cells, TSA alone had a slight effect on the regulation of ER α activity by E2. By contrast, TSA increased by more than 2-fold the E2-dependent activity of ER β . To assess whether this result could be generalized to other cell types, we performed the same

experiments in HeLa (cervix), PEO-14 (ovary) and HEK-293 (kidney) human cell lines (Fig. 2A). We observed the same general trend of greater activation for ER β than for ER α by TSA with a maximal effect in HeLa cells and an opposite effect of TSA on ER α and ER β transactivation in HEK-293 cells. Interestingly, in transfected cells, we also observed that ER β levels were up-regulated by HDI treatment, whereas ER α levels were poorly affected, except in HEK-293 cells. This suggests that the increased expression of ER β upon HDI treatment is at least partially involved in the higher transactivation of the reporter.

To ensure that these regulations could also be detected on a natural E2-regulated gene, we performed the same experiment using the pS2 promoter fused to the luciferase reporter gene. As shown in Fig 2B, we obtained again a significant induction of ER β transactivation, whereas in parallel, ER α transcriptional activity was decreased upon TSA treatment. Finally, to assess that the synergism between ER β and HDAC inhibition was not specific of TSA, we used two other HDI structurally unrelated to TSA (sodium butyrate and SAHA). As shown in Fig. 2C, these two HDI were also more potent to increase the activity of ER β in the presence of E2, than the one of ER α , confirming that ER β E2-dependent transactivation is more affected by HDI than that of ER α .

It was also of interest to assess whether the increased sensitivity of ER β to TSA was dependent or not on the concentration of hormone used in the assay. The E2 dose response performed in the absence or the presence of TSA showed that the preferential response of ER β to TSA was observed for concentrations ranging from 0.1nM to 1 μ M of E2 (Fig. 3A). We then investigated whether the same preferential regulation of ER β also occurred with specific agonist of each receptor. We used PPT or DPN to specifically activate ER α and ER β , respectively (Harrington et al., 2003). As obtained with E2, we noticed that TSA treatment increased only by 1.6-fold the activity of ER α in the presence of PPT, whereas the activity of ER β in the presence of DPN was increased by more than 6.5 -fold (Fig. 3B). In the same line,

we also analyzed if TSA could modulate ER α and ER β activities in the presence of either pure or partial anti-estrogens. As shown in Fig. 3C, TSA treatment did not significantly modify the antagonist effect of the two types of antihormones on either ER α or ER β in conditions where E2 effect was increased by 1.7 - and 5.6 -fold respectively.

Both ER α and ER β interfere with the transcriptional activity of HDI

We then thought to investigate the transcriptional interference in the reverse way. In response to TSA, transcriptional activation of the thymidine kinase reporter occurred through Sp1-binding sites and is based on the inhibition of Sp1-recruited HDAC activity (Doetzlhofer et al., 1999). We therefore analyzed if the expression of ERs could modulate the TSA-regulation of the reporter. As shown in Fig 4B, expression of either wild-type ER α or ER β increased the transcriptional activation in response to TSA only in the presence of E2. This effect required the binding of the receptors to DNA since no modulation of TSA activity by ERs was observed on the reporter construct deleted of the ERE (data not shown). To define the role of the constitutive AF1 domain in the regulation of TSA activity by ERs, we used deletion mutants of both receptors (Fig. 4A). Interestingly, both in the absence or the presence of E2, the deletion of the A/B domain of ER α or ER β decreased by more than 2-fold the level of TSA-induced reporter activity obtained with wild-type receptors (Fig. 4B). Similar results were obtained in MDA-MB-231 cells (data not shown). This suggests that the modulation of HDI action by ERs requires the A/B domain.

The combination of ER expression and HDI treatment inhibits cancer cell proliferation

HDI are known to inhibit the proliferation of many cancer cells and to increase their apoptosis. It was thus of great interest to test whether the combination of ER introduction and HDI treatment could lead to a supra-additive inhibition of breast cancer cell proliferation. The

ERs were introduced into HeLa (Fig. 5A) and MDA-MB231 (Fig. 5B) cells using the adenovirus-based approach. In HeLa cells, Ad5 or Ad-hER α cells exhibited a similar decreased growth in the presence of TSA (Fig. 5A). Introduction of ER β in HeLa cells reduced their basal proliferation and this was further amplified by TSA treatment (Fig. 5A). In MDA-MB231 cells (Fig. 5B), when ER α -expressing cells were treated with TSA, the growth inhibition observed was more important to that of Ad5 cells, in accordance with our previous results (Margueron et al., 2003) (Fig. 5B). The results were even more striking when looking at ER β expressing cells, as addition of TSA strongly blocked cell proliferation (Fig. 5B).

These data suggest that the combination of ER β and HDI treatment constitutes a powerful way to inhibit cancer cell proliferation. The question could be raised whether the decreased proliferation observed was due at least in part to an increased apoptosis. When treating cells with TSA, ER α and ER β displayed a more pronounced apoptosis than control cells (Fig. 5C). As ER β cells do not display a higher apoptosis rate compared to their ER α counterparts, it is likely that apoptosis is not the major reason accounting for the differential sensitivity in terms of proliferation of both types of cells to HDI.

To understand how ER α and ER β were amplifying the anti-proliferative effect of TSA, we first analyzed the expression of synthetic reporter constructs corresponding to genes involved in the control of cell proliferation, such as p21^{WAF1/CIP1}, cyclin D1 and cyclin E (Fig. 6A). As shown in previous studies, HDI treatment increased the levels of p21^{WAF1/CIP1} and decreased that of cyclin D1. By contrast, despite its anti-proliferative activity, TSA strongly increased the transcription of cyclin E gene which positively controls cell proliferation (Sambucetti et al., 1999). Transfection assays in HeLa cells showed that, in the presence of ER β , TSA was both a better inducer of p21^{WAF1/CIP1} promoter and a stronger inhibitor of cyclin D1 transcription than when ER α was expressed (Fig. 6A). Very interestingly, we also observed that ER β significantly antagonized the TSA regulation of the cyclin E reporter. Similarly,

HDI treatment also increased an artificial construct containing an AP-1 response element (as a model for growth factor- regulated genes) and, as in the case of cyclin E, ER β strongly diminished the induction by TSA.

We next measured the regulation of cyclin E and p21^{WAF1/CIP1} endogenous genes by HDI at the protein level (Fig. 6B and C). We observed that cyclin E levels were lower in the presence of ER β compared to ER α cells (Fig. 6B). In addition, cyclin E expression was up-regulated by TSA only in ER α expressing cells. Concerning p21^{WAF1/CIP1}, we noticed that its expression was better induced by TSA in ER β expressing cells compared to ER α cells. Altogether, these data suggest that the differential effects of ER β on HDI-regulation of genes involved in proliferation, could account for the higher anti-proliferative action of TSA in cells expressing this receptor.

DISCUSSION

Research performed over the last decade has highlighted the role of HDI as modulators of transcriptional activity and as a new class of therapeutic agents. The aim of this study was to determine how HDI influenced the expression and transcriptional activity of ER α and ER β and how the expression of these receptors modulated the anti-proliferative properties of HDI. Our data first demonstrate a differential effect of HDAC inhibition on ER α and ER β expression. Indeed, in cells expressing high levels of ER α such as MCF-7 cells, ER α mRNA and protein are strongly reduced as previously shown by other studies (Stevens et al., 1984). However, the effects of HDI on ER α levels are complex since in ER α -negative cells, we observed that TSA treatment re-activated ER α expression (data not shown), which is in agreement with previous studies (Yang et al., 2000). Recent work has demonstrated that this up-regulation in ER α -negative cells involves histone hyperacetylation of ER α promoter and the release of HDAC1 and MeCP2 from ER α promoter (Sharma et al., 2005). On the contrary, ER β expression was up-regulated in all cell lines (ER α -negative or positive) that we tested. ER β protein enhanced expression by HDI is due at least in part to a direct increase in ER β RNA levels, but could also result from the regulation at the post-transcriptional level. We also show that HDI increase the ligand-induced activity of ER β in a more pronounced manner compared to the effects observed for ER α on both synthetic and natural promoters, when using different HDI. Very interestingly, the combination of both HDI and E2 enables ER β to be at least equal or more active than ER α . This is definitely important as ER β is generally a twice less potent transactivator than ER α in the presence of estrogens. The lower transactivation ability of ER β has been reported by several groups both on synthetic and endogenous genes (Cowley & Parker, 1999; Lazennec et al., 2001; McInerney et al., 1998). In the situation in which AF-1 is important for transactivation, ER α is a better activator than

ER β , whereas both receptors display equivalent potencies when only AF-2 is required. A supra-additive action of HDI and nuclear receptor ligands has been reported for ER α (Ruh et al., 1999) and also for PR (Liu et al., 1999), retinoid receptors (Minucci et al., 1997), PPAR γ (Fajas et al., 2003), AR (Shang et al., 2002) and TR (Stanley & Samuels, 1984), suggesting that this is a general phenomenon.

The enhanced ER β activity upon HDI treatment involves certainly an increase of ER β protein levels. However, we believe that regulatory events controlling the transcriptional activity of the receptor could also take place. These probably include effects on chromatin structure, as suggested by the group of Lee Kraus (Cheung et al., 2003). Using *in vitro* transcription assays, these authors have reported that ER β was a weak activator on chromatin templates, whereas it efficiently increased transcription on naked DNA. Moreover, the addition of TSA only weakly affected ER α activity on chromatin templates but strongly enhanced the one of ER β (Cheung et al., 2003). This difference has been attributed to the fact that ER α (but not ER β) contains a transferable activation function in its A/B region that facilitates transcription with chromatin templates.

In addition, it is tempting to speculate that post-translational modifications could differentially modulate ER activity in response to HDI treatment. Indeed, several studies have shown that nuclear receptors could be acetylated, which in turn could modulate their transactivation ability. This is the case of ER α and AR in D domain (Fu et al., 2000; Wang et al., 2001). It should be noted that the acetylated motif in ER α is poorly conserved in ER β suggesting that the two receptors could be differentially modified. However, using *in vitro* interaction assays, we have not observed a different ability of the two ER to interact with class I or II HDACs (data not shown). The present work demonstrates that the cross-talk also exist in the reverse way since expression of ERs strongly modulates the transcriptional response observed upon TSA treatment. One interesting observation concerning the regulation of an ERE-containing

reporter is that this synergy with HDI required the A/B domain of the receptors. Most notably, in the absence and the presence of E2, the AF1 deleted version of the two receptors exhibited a strong repressive activity on the regulation by TSA and it would be valuable to understand the underlying mechanisms of this negative regulation.

From a clinical point of view, several studies have shown that ER β expression was decreased when cells turn cancerous and suggest that ER β could play a tumor suppressor role. This holds true for breast, ovary, colon, and prostate cancers (Campbell-Thompson et al., 2001; Pujol et al., 1998; Roger et al., 2001). We and others have shown that ER β could inhibit the proliferation and invasion of breast, ovary and prostate cancers, while increasing apoptosis (Cheng et al., 2004; Lazennec et al., 2001; Paruthiyil et al., 2004). In addition, several studies have shown that ER α expressing cancer cells were more sensitive to HDI than ER α -negative cells (Jang et al., 2004; Margueron et al., 2003). On the other hand, as shown by our observation and recent data, ER β and to a lesser extent ER α strongly enhanced the anti-proliferative action of HDI (Jang et al., 2004). In addition, both receptors enhanced the pro-apoptotic action of HDI. The greater effects of ER β on proliferation compared to ER α could be the result of distinct cell cycle gene regulations. Indeed, we showed that HDI-induced p21^{WAF1/CIP1} promoter activity was higher in ER β compared to ER α cells. On the other hand, the decrease of cyclin D1 transcription by TSA was stronger when ER β was expressed instead of ER α . In addition, the positive effects of HDI on cyclin E promoter and on global AP-1 activity were lower in ER β compared to ER α expressing cells. Altogether, these data suggest that the differential effects of ER β and ER α on genes involved in cell proliferation account for the synergistic inhibition of proliferation by ER β and HDI. The higher sensitivity of ER β to HDI compared to ER α and the fact that HDI differentially regulate the expression of endogenous receptors could be a very valuable result. It would thus be of great interest to potentiate the overall tumor-suppressor properties by increasing its expression and activity to

design new strategies in the future. HDI are currently tested in several clinical trials at phase I or II (Vigushin & Coombes, 2002) and future work will determine whether part of their effects in cancers could arise from the increased expression of ER β .

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ACKNOWLEDGEMENTS

We are grateful to S. Bonnet and A. Lucas for their technical help. We thank the Vector Core of the University Hospital of Nantes supported by the Association Française contre les Myopathies (AFM) for the production of Adenoviruses. This work was supported by grants from ARC (Association pour la Recherche contre le Cancer, Grant No. 3582; La ligue Nationale Contre le Cancer and from the National Institutes of Health (NIH CA18119). V.D., R.M. and A.L. were recipient from the French Minister of Research. A.L. was also supported by the Ligue Nationale Contre le Cancer.

LEGENDS FOR FIGURES

Fig. 1. ER α and ER β levels are differentially regulated by HDI.

A and B. MCF-7 cells were pretreated or not with cycloheximide (CHX) at 20 μ g/ml for 2 h and then further stimulated for 4 hrs with TSA (500 ng/ml) with CHX still present or not. Control cells were treated in parallel with ethanol alone in the presence or absence of CHX. Total RNA was extracted and the expression of ER α (panel A) and ER β (panel B) mRNAs was quantified by real-time PCR. Results represent the mean of 2 independent quantifications. ER α protein levels were determined by western blot on cells treated or not with TSA. **C.** ER β mRNA and protein levels were determined in OVCAR-3 cells treated or not with TSA.

Fig. 2. TSA differentially regulates ER α and ER β activities.

A. MDA-MB-231, HeLa, PEO-14, HEK-293 cells were transfected with ERE2-TK-LUC reporter construct along with CMV5, CMV-hER α or CMV-hER β expression vectors. Cells were then treated with control vehicle ethanol (C), estradiol (E2 10⁻⁸M), TSA (500 ng/ml) or the combination of E2 and TSA for 18h prior to harvesting cells for luciferase assays. Results show relative luciferase activities (expressed as percent of the values obtained in the absence of E2 for each conditions) after normalization for β -galactosidase activity (n=3 independent experiments). The levels of expressed receptors have been monitored by western blots. **B.** Same experiment as in A but in HeLa cells transfected with pS2 promoter **C.** HeLa cells transfected as in A were then treated with E2 (10⁻⁸M) and either ethanol vehicle (C), sodium butyrate (NaBu, 2 mM) or SAHA (5 μ M) for 18h. Results show relative luciferase activities (% of values without E2) after normalization for β -galactosidase activity (n=3 independent experiments).

Fig. 3. Regulation of ER activity by HDI is a general phenomenon.

A. HeLa cells were transfected with ERE2-TK-LUC reporter construct along with CMV-5 (●) CMV-hER α (■) or CMV-hER β (▲) expression vectors and treated with ethanol control vehicle (C) or increasing concentrations of E2, without or with TSA (500 ng/ml). Results are expressed as percent of values obtained in the absence of E2. **B** and **C.** Same conditions as in A to analyze the effects of E2 (10^{-8} M), propyl pyrazole triol (PPT, 10^{-8} M), diarylpropionitrile (DPN, 10^{-8} M), hydroxy-tamoxifen (OHTam, 10^{-8} M) or ICI 182,780 (ICI, 10^{-8} M).

Fig. 4. ER α and ER β modulate the transcriptional regulation by TSA

A. Representation of the deletion mutants used. **B.** Wild-type or deletion mutants of hER α and hER β were transfected in HeLa cells along with ERE2-TK-LUC construct. Cells were then treated with TSA (500 ng/ml) in the presence (upper panel) or absence (lower panel) of estradiol (E2 10^{-8} M) for 18h prior to harvesting cells for luciferase assays. Results show relative luciferase activities (expressed as percent of the values obtained in the absence of TSA for each conditions) after normalization for β -galactosidase activity (n=3 independent experiments). The dotted lines show the level of luciferase obtained in the absence or presence of TSA in CMV5-transfected cells.

Fig. 5. Expression of ER β drastically enhances the anti-proliferative effects of TSA.

A. HeLa cells were either infected with Ad5, Ad-hER α , or Ad-hER β viruses at MOI 100. The cells were treated with ethanol vehicle or TSA (50 ng/ml) 24 h after the beginning of the infection. Proliferation rate was determined by counting the cells at days 4. Results are express as % of Ad5-infected cells treated with ethanol alone and represent the mean \pm SD of four independent determinations. **B.** Same proliferation experiments performed in MDA-MB-231 cells. **C.** MDA-MB-231 cells which had been infected as in A, were then treated for 24 h

with control vehicle Ethanol (C) or TSA (50 ng/ml). Apoptosis was set to 100% in control cells (not treated with TSA) infected with Ad5, Ad-ER α or Ad-ER β viruses. Results represent the mean of \pm SD of three independent determinations.

Fig. 6. ER α and ER β differentially regulate genes involved in proliferation

A. HeLa cells were transfected with the following reporter constructs: p21^{WAF1/CIP1}-Luc, cycD1-Luc, cyclinE-Luc or (TRE)5-Luc (AP-1), along with hER α and hER β expression vectors. Cells were then treated (hatched boxes) or not (empty boxes) with TSA (500 ng/ml) for 18h prior to harvesting cells for luciferase assays. Results show relative luciferase activities (n=3 independent experiments) after normalization for β -galactosidase activity. **B-**

C. HeLa cells were transfected with CMV-hER α or CMV-hER β expression vectors. Cells were treated for 20h with ethanol vehicle or TSA (500 ng/ml). Cyclin E (B) and p21^{WAF1/CIP1} (C) protein expression was monitored by western blot.