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**Eliminating epigenetic barriers induces transient hormone-regulated gene expression in estrogen receptor negative breast cancer cells**

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## Abstract

In breast cancer, approximately one third of tumors express neither the estrogen receptor ( $ER\alpha$ ) nor estrogen regulated genes such as the Progesterone Receptor gene (PR). Our study provides new insights into the mechanism allowing hormone-activated expression of  $ER\alpha$  target genes silenced in  $ER\alpha$ -negative mammary tumor cells. In cell lines derived from  $ER\alpha$ -negative MDA-MB231 cells, stable expression of different levels of  $ER\alpha$  from a transgene did not result in transcription of PR. A quantitative comparative analysis demonstrates that inhibiting DNA methyltransferases using 5-aza-2'-deoxycytidine or specific disruption of DNMT1 by small interfering RNAs and treatment with the histone-deacetylase inhibitor Trichostatin A enabled  $ER\alpha$ -mediated hormone-dependent expression of endogenous PR. We show that demethylation of a CpG island located in the first exon of PR was a prerequisite for  $ER\alpha$  binding to these regulatory sequences. Although not a general requirement, DNA demethylation is also necessary for derepression of a subset of  $ER\alpha$  target genes involved in tumorigenesis. PR transcription did not subsist four days after removal of the DNA methyltransferase blocking agents, suggesting that hormone-induced expression of  $ER\alpha$  target genes in  $ER\alpha$ -negative tumor cells is transient. Our observations support a model where an epigenetic mark confers stable silencing by precluding  $ER\alpha$  access to promoters.

## Introduction

Estradiol is a key regulator for normal growth and differentiation of mammary glands as well as for the malignant progression of breast cancer (Platet et al., 2004). Estrogens exert their function by binding to the estrogen receptor  $\alpha$  (ER $\alpha$ ), a transcription factor controlling the expression of genes involved in cell proliferation and differentiation. While two thirds of breast cancers are ER $\alpha$  positive, nearly one third of breast tumor cells do not express ER $\alpha$ . ER $\alpha$ -negative tumors are of higher grade and highly invasive (Sorlie et al., 2001) and are not responsive to endocrine therapies designed to block ER $\alpha$  function.

The absence of ER $\alpha$  does not appear to be a result of mutations that are found in less than 1% of ER-negative tumors (Lacroix et al., 2004; Roodi et al., 1995). Moreover, in ER $\alpha$ -negative breast cancer cells, silencing of the ER $\alpha$  gene is accompanied by alteration of estrogen regulated gene expression. Indeed, some genes such as the trefoil factor 1 gene (pS2/TFF1) or the Progesterone Receptor gene (PR) are silenced while other genes become constitutively expressed (such as Cathepsin D (Cat-D)) (Touitou et al., 1991). Whether or not the silencing of a subset of estrogen-regulated genes is a direct consequence of the absence of ER $\alpha$  remains debated.

Furthermore, the consequences of de novo ER $\alpha$  expression for the expression of hormone-regulated genes that are normally silenced in these cells remain controversial. Indeed, transient vector-based expression of ER $\alpha$  under the control of a SV40 promoter in ER $\alpha$ -negative mammary tumor cell lines such as MDA-MB231 did not lead to stimulated transcription of either pS2/TFF1 or PR. Yet, hormone-induced expression of pS2/TFF1 was observed after infection of MDA-MB231 with an adenovirus carrying the ER $\alpha$  gene (Lazennec et al., 1999) or stable expression of ER $\alpha$  under the control of a

CMV promoter (Metivier et al., 2003). Remarkably, hormone-induced expression of PR has never been observed in any of these systems.

Complete loss of ER $\alpha$  expression in ER $\alpha$ -negative mammary tumor cell lines has been linked to aberrant hypermethylation of a CpG island contained in its promoter (Ottaviano et al., 1994; Roodi et al., 1995). In recent years, epigenetic mechanisms of gene regulation have been increasingly associated with establishment and progression of cancer (Jones and Baylin, 2002; Momparler, 2003; Ting et al., 2006). DNA cytosine methyltransferase 1 (DNMT1) is the most abundant and catalytically active DNA methyltransferase. It induces the covalent addition of a methyl group to the 5' position of cytosine, predominantly within CpG dinucleotides whose occurrence in the human genome is generally increased in promoter regions (Lande-Diner et al., 2007). DNMTs can be inhibited by the nucleoside antimetabolite 5-aza-2'-deoxycytidine (AZA) (Jones and Taylor, 1980). In addition, chromatin at inactive promoters is generally hypoacetylated by Histone deacetylases (HDAC) that can be inhibited by HDAC inhibitors such as Trichostatin A (TSA). Because DNMT1 has been found to interact physically with HDACs, DNA-methylation and histone deacetylation may function through a common mechanistic pathway to repress transcription (Dobosy and Selker, 2001; Robertson, 2002; Rountree et al., 2000). The use of both TSA and AZA has synergistic effects in activating epigenetically silenced genes (Cameron et al., 1999). In particular, a number of tumor suppressor genes that are specifically silenced in various cancers have been re-activated using a combination of both drugs (Bachman et al., 2003; Lopez-Serra et al., 2006).

The goal of the study described here was to investigate the role of ER $\alpha$  and chromatin structure in regulating expression of the progesterone receptor gene. Here, we used two MDA-MB231-derived cell lines that stably express different levels of

ER $\alpha$  to quantify PR expression in response to general DNA methyltransferase inhibition, to specific disruption of DNMT1 by small interfering RNA, and to inhibitors of HDACs. We combined Chromatin Immuno-Precipitation (ChIP) analysis and bisulfite sequencing to determine whether the extent of ER $\alpha$  binding correlates with the methylation status of regulatory sequences of the PR gene. Finally, we demonstrate that the epigenetic mechanism involved in repression of PR can be extended to a subset of ER target genes silenced in ER negative cells.

## Results

### **Stable ER $\alpha$ expression is not sufficient to promote hormone-induced PR expression in ER $\alpha$ -negative breast cancer cell lines**

We analysed the expression levels of ER $\alpha$  target genes that are silenced in ER $\alpha$ -negative cell lines after ectopic, stable expression of ER $\alpha$  at different levels. This approach allowed us to investigate the effect of ER $\alpha$  expression in a homogenous and controlled background. Steady state ER $\alpha$  protein levels in HE-5 (Touitou et al., 1991) and MDA-66 cell lines (Metivier et al., 2004) treated with Estradiol (E2) were 10% and 40% respectively of the one found in MCF-7 cells (Figure 1A). Hormone-induced expression of three estradiol-regulated genes, PR, pS2/TFF1 and Cat-D, was analysed by quantitative RT-PCR in these cell lines (Figure 1 B-C). In MCF-7 cells, estrogens stimulated PR expression 2.5-fold, pS2/TFF1 expression nearly 10-fold and Cat-D twofold. In ER $\alpha$ -negative MDA-MB231 cells these genes are not induced by E2 (Figure B-C). Surprisingly, we did not observe hormone-regulation of PR expression in HE-5 or MDA-66 (Figure 1B). While we observe a small increase (<2 fold) in pS2/TFF1 transcription in MDA-66 cells, this level of expression is smaller than the

Figure 1

one reported previously (Metivier et al., 2003) and negligible relative to the one observed in MCF-7 cells. Here, we provide a quantitative analysis using quantitative RT-PCR and ChIP followed by quantitative PCR. All studies were performed in parallel, the results for different genes and under different conditions can be compared quantitatively. Although we cannot exclude that the ER $\alpha$  transgene bearing cell lines provided by F. Gannon (MDA-66) may have changed phenotype over time and cultures, we did not observe any differences in mRNA levels from cells before and after dozens of passages in our laboratory (data not shown).

We confirmed that the presence of ER $\alpha$  in the cell is sufficient to confer hormone-regulated Cat-D expression, since both HE-5 and MDA-66 exhibit a 1.8-fold increase in Cat-D mRNA levels similar to the twofold stimulation observed in MCF-7 cells (Figure 1C). These results taken together with previous observations that the chromatin structure of the PR and pS2/TFF1 promoters remains insensitive to DNaseI cleavage in the HE-5 cell line (Giamarchi et al., 1999) led us to postulate that promoter accessibility may hamper ER $\alpha$  binding to PR and, to some extent, pS2/TFF1 in MDA-MB231 derived cell lines.

**DNA demethylation and histone deacetylation trigger hormone-dependent expression of PR and pS2/TFF1 in MDA-MB231-derived cell lines, but do not affect hormone-regulation of Cat-D expression.**

Limited access of ER $\alpha$  to PR and pS2/TFF1 regulatory sequences may result from epigenetic mechanisms, involving DNA methylation, as has been demonstrated for the ER $\alpha$  gene itself (Yang et al., 2001). Thus, we quantified expression levels of PR, pS2/TFF1 and Cat-D genes following AZA, TSA or combination treatments (Figure 2A). Treatment of MDA-66 cells with TSA, led to a twofold and a 10-fold induction of PR and pS2/TFF1 mRNAs respectively. Cat-D gene expression was not

Figure 2

affected by these treatments. Exposure to AZA resulted in a dramatic increase in hormone stimulated expression of PR (10-fold) and pS2/TFF1 (35-fold) that was significantly higher than that observed after TSA treatment. Combining AZA and TSA treatments resulted in even greater hormone-stimulated PR and pS2/TFF1 mRNA synthesis. Simultaneous treatment of the cells that had been exposed to TSA, AZA or a combination treatment with estradiol and the pure anti-estrogen ICI 182 780, that binds to ER $\alpha$  and induces its degradation (Wijayaratne and McDonnell, 2001), abolished the hormone-induced increase of pS2/TFF1 or PR transcription (Figure 2B). This inhibition demonstrates that hormone-regulated activation of ER $\alpha$  target gene expression is dependent on ER $\alpha$ . We noted, however, that in the absence of estradiol, expression levels of both PR and pS2/TFF1 were significantly higher in AZA treated cells compared to untreated cells and identical to the levels observed for cells treated with E2/ICI (a 5-fold or 15-fold induction; Figure 2B). These results suggest that DNA demethylation directly affects target gene promoter accessibility by increasing basal transcription through an ER $\alpha$ -independent mechanism.

### **DNA demethylation is pivotal in PR transcription regulation**

Methylation of CpG dinucleotides is maintained by the action of DNMT1 (Pradhan et al., 1999). We investigated the role of DNMT1 in PR and pS2/TFF1 gene silencing in MDA-66 cells using a siRNA based approach to deplete DNMT1 protein. Western Blot analysis (Figure 3A) demonstrates significant reduction of DNMT1 protein levels present in MDA-66 transfected with two distinct siRNAs directed against DNMT1. Electroporation with a combination of the two siRNAs had the same effect as each siRNA alone. 48 hours post siDNMT1 transfection we observed a significant increase in hormone-stimulated accumulation of PR (5-fold) and pS2/TFF1 (7-fold) mRNAs (Figure 3B). In contrast, upon siDNMT1 transfection Cat-D expression

Figure 3



remained inducible and unchanged compared to expression levels found in mock-transfected cells. Furthermore, in MDA-66 cells transfected with siDNMT1 treated with E2 in combination with ICI the estradiol-induced increase in PR and pS2/TFF1 expression was abolished (Figure 3C). Thus, the observed transcription after DNMT1 depletion is mediated by ER $\alpha$ . Steady state levels of mRNA after AZA treatment compared to siDNMT1 transfection were roughly twofold for PR and 4-fold for pS2/TFF1 (Figure 3D). This could stem from the consequence of the more general AZA treatment that affects all DNA-methyltransferases, and thus indirectly from transcription of a large number of other genes. When quantifying PR and pS2/TFF1 mRNAs 4 days after complete removal of AZA or TSA treatments we found that neither PR nor pS2/TFF1 were expressed. This indicates that DNA demethylation causes transient re-expression of these two genes. The reversibility of the repressive chromatin structure suggests that an underlying signal independent of the DNA methylation status may render these promoters prone to silencing in MDA-MB231-derived cell lines.

**DNA demethylation and histone deacetylation trigger hormone-dependent expression of ER $\alpha$  in MDA-MB231-derived cells.**

We observed an important increase in hormone-regulated PR and pS2/TFF1 gene expression after AZA and AZA/TSA treatments. Knowing that such treatments also affect endogenous ER $\alpha$  gene expression, we investigated how intracellular ER $\alpha$  levels influence the extent of estrogen-regulated target gene expression. PR and pS2/TFF1 expression strongly increased reaching respectively a 8- and 12-fold stimulation in HE-5 cells and a 20-fold stimulation in MDA-66 cells (Figure 4A). Concomitantly, an increase in ER $\alpha$  protein levels was also noted (Figure 4B). Quantification of Western blots from three independent experiments revealed a

maximum 5-fold increase in ER $\alpha$  protein levels present in HE-5 cells and a maximum 20-fold increase in MDA-66 cells (Figure 4C). Thus, stimulation of ER $\alpha$  target gene transcription appears to tightly correlate with the quantity of ER $\alpha$  protein available in the cell. However, expression of PR and pS2/TFF1 is not dictated by ER $\alpha$  protein thresholds in the absence of treatments modifying chromatin structure. Indeed, transient transfection of MDA-66 cells with increasing quantities of a vector bearing the ER $\alpha$  gene resulting in ER $\alpha$  protein levels up to 20 times the one found in MCF-7 cells did not allow target gene transcription (data not shown).

### **CpG methylation of the PR promoter impedes ER $\alpha$ fixation in MDA-MB231-derived breast cancer cell lines expressing ER $\alpha$**

We next wanted to know whether epigenetic modifications of regulatory sequences of PR and pS2/TFF1 impede ER $\alpha$  binding. We analysed ER $\alpha$  binding using Chromatin Immunoprecipitation (ChIP) to regulatory sequences of PR known to lead to estrogen activation using the B promoter (Figure 5A). This region located in the first exon contains a half ERE, is associated with an AP1 site and contains a CpG island (Petz et al., 2004). We observed hormone-induced binding of ER $\alpha$  to pS2/TFF1 promoter (14-fold increase) and PR regulatory regions (12-fold increase) in MCF-7 cells, while ER $\alpha$  was never recruited to these regions in MDA-MB231-derived cells (Figure 5B). In contrast to the lack of ER $\alpha$  binding to these promoters observed in HE-5 and MDA-66 cells, siDNMT1 or AZA treatments enabled estrogen stimulated ER $\alpha$  binding to PR and pS2/TFF1 regulatory regions in MDA-66 cells (Figure 5C). Simultaneous treatment with E2 and ICI abolished hormone-dependent recruitment of ER $\alpha$ . To ascertain that the methylation status of the regulatory regions of PR was the cause for the lack of hormone-induced ER $\alpha$  recruitment, we determined the

Figure 5

methylation status of a CpG island located within the PR regulatory region (Figure 5D) using bisulfite sequencing in MDA-66 cells before and after exposure to AZA (Figure 5A and 5D). Amplified DNA sequences from AZA treated cells exhibited a significantly lower percentage of methylated CpGs than sequences from untreated cells. Taken together, ChIP and bisulfite sequencing results suggest that ER $\alpha$  access to the PR regulatory regions is blocked by CpG methylation.

In addition, ER $\alpha$  fixation to PR regulatory sequences in MDA-MB231, HE-5 or MDA-66 cells treated with AZA increases with intracellular ER $\alpha$  protein levels (compare Figure 6 and 4). We conclude that DNA-demethylation allows quantitative binding of ER $\alpha$  to promoter sequences of ER $\alpha$  target genes leading to hormone-stimulated gene expression.

Figure 6

**The epigenetic mechanism of PR silencing is common to some but not all ER target genes that are repressed in ER negative cell lines**

Finally, we analysed the expression levels of several, newly identified estrogen receptor target genes to test whether our observations could be extended to genes distinct from PR and PS2/TFF1. We crossed several studies from the recent literature based on micro-array transcriptome analyses and ChIP-chip, ChIP-DSL or ChIP-paired end di-tag data obtained from mammary tumor cell lines (Kininis et al., 2007; Kwon et al., 2007; Laganier et al., 2005; Lin et al., 2004; Lin et al., 2007) to select a group of relevant genes based on the following criteria: hormone-stimulated gene expression in ER $\alpha$ -positive mammary tumor cell lines, complete lack of expression in ER $\alpha$ -negative breast cancer cell lines (Nagaraja et al., 2006) and identified as an ER $\alpha$  target by ChIP. Thus, we investigated gene regulation of Annexin A9 (ANXA9), a gene coding for a protein of the annexin superfamily (Gerke and Moss, 2002; Raynal and Pollard, 1994), the RET proto-oncogene, which encodes a protein receptor tyrosine kinase with a zinc-finger domain associated with dominantly inherited cancer syndromes (Goodfellow and

Wells, 1995), the tumor protein D52-like 1 (TPD52L1 (D53)) upregulated in human breast and prostate cancers (Ahram et al., 2002; Balleine et al., 2000; Pollack et al., 2002; Rubin et al., 2004), bone morphogenetic protein-6 (BMP6), a multifunctional molecule of the transforming growth factor- $\beta$  superfamily overexpressed in breast, prostate and salivary gland cancers (Hamdy et al., 1997; Heikinheimo et al., 1999), and the gene regulated in breast cancer 1 (GREB1), whose product contributes to the growth-promoting effects of estrogens in MCF-7 cells (Rae et al., 2005). We confirmed that expression of these genes was stimulated by estradiol in MCF7 cells (Figure 7A) and assessed changes in relative expression levels in the MDA-66 cells in the absence and in the presence of AZA.

Similarly to PR, the expression of ER $\alpha$  is not sufficient to induce estrogen regulated expression of ANXA9, and, to a lesser extent, of the RET proto-oncogene in MDA-66 cells (Figure 7B). Hormone-dependent stimulation of transcription of these genes required DNA demethylation (Figure 7B). In contrast, expression of D53 was weakly hormone-regulated in the presence of ectopic ER $\alpha$  in MDA-66 cells (Figure 7B). Transcription of D53 further increased in the presence of AZA. For BMP6 and for GREB1 the presence of ectopic ER $\alpha$  is also sufficient for hormone-regulated expression (Figure 7B). Exposure to AZA did not increase BMP6 or GREB1 expression levels suggesting that DNA methylation is not directly involved in downregulation of these genes in MDA-MB231 cells. Previously demonstrated up-regulation of BMP6 gene expression in AZA treated MDA-MB231 (Zhang et al., 2007) was thus due directly to re-expression of ER $\alpha$ .

## Discussion

Our study provides new insights into the mechanisms allowing hormone-activated expression of ER $\alpha$  target genes that are silenced in ER $\alpha$ -negative mammary tumor cell lines. We demonstrate that the first step needed to stimulate estrogen regulated gene expression of PR and a subset of estrogen target genes silenced in ER $\alpha$ -negative mammary tumor cell lines is to eliminate epigenetic barriers to allow ER $\alpha$  access to regulatory sequences. In particular, we demonstrate that ER $\alpha$  binding to PR was specifically impaired by DNA hypermethylation of CpGs downstream of the PR B promoter. In addition, we show that the level of ER $\alpha$  target gene expression correlates positively with ER $\alpha$  protein levels and binding present in breast cancer cells (Figure 4 and Figure 6).

Some reports claim that genes such as PR or pS2/TFF1 could be expressed simply by ectopically introducing ER $\alpha$  into these cell lines (Lazennec et al., 1999; Metivier et al., 2003), while other reports indicate that expression of ER $\alpha$  is not sufficient to transcribe these silenced genes (Giamarchi et al., 1999; Touthou et al., 1991). However, it was previously shown that treatment of ER $\alpha$ -negative breast cancer cell lines such as MDA-MB231 or MDA-MB435 with DNA methylation inhibitors can lead to expression of ER $\alpha$  protein (Yang et al., 2001). Our data demonstrate that although some ER $\alpha$  target genes that are silenced in MDA-MB231 cells, including D53, BMP6 and GREB1, can be expressed by re-introducing ER $\alpha$  in ER $\alpha$ -negative cell lines, a subset of ER $\alpha$  target genes, including PR, ANXA9 and RET, require DNA demethylation of their regulatory regions in addition to the presence of ER $\alpha$  for transcription in MDA-MB231 derived cells. In contrast to PR, AZA induced overexpression of ER $\alpha$  did not lead to increased transcription of BMP6 and GREB1.

To induce transcription of these genes, it is not necessary to eliminate epigenetic barriers for ER $\alpha$  binding even at low local ER $\alpha$  protein concentrations.

The observed heterogeneity of repression mechanisms may be related to the structure of regulatory sequences of these genes. The interplay between ER $\alpha$  and other transcription activators that modulate the ability of ER $\alpha$  to activate gene expression as shown recently for the pS2/TFF1 promoter (Baron et al., 2007) could be altered in ER $\alpha$ -negative breast cancer cells. We postulate that a distinct chromatin environment that surrounds ER $\alpha$  target genes in their silent conformation may translate into distinct requirements for cofactors that are necessary to overcome the repressive chromatin structure during gene activation.

Finally, our observation that **hormone-induced expression of ER $\alpha$  target genes in ER $\alpha$ -negative tumor cells is transient** supports a model where an epigenetic mark confers stable silencing by precluding ER $\alpha$  access to promoters. Silent ER $\alpha$  target gene promoters have also been shown to accumulate DNA methylation following induced depletion of ER $\alpha$  (Leu et al., 2004). Nevertheless the opposite is not true. In fact, we show that the presence of ER $\alpha$  in cells expressing PR as result of AZA treatment is not sufficient to maintain an open chromatin conformation and to sustain PR expression (Figure 3). In a different approach, DNA methylation was erased through mutating DNMT1 and DNMT3 which allowed activation of the tumor suppressor gene p16INK4 normally found to be silenced in colorectal cancer cells (Bachman et al., 2003). However, a week later this gene was again completely silenced indicating that re-expression was transient. Here, silencing was possible through epigenetic modifications in the absence of DNA methylation, thus suggesting that a distinct mark specifically identifies genes targeted for long term repression as may be the cause for ER $\alpha$ -negative breast cancer cells. The identification of the signal that

imposes selective pressure to silence a subset of genes may open new avenues for the development of therapeutic agents.

## **Materials and Methods**

**Reagents** - Estradiol, 5-Aza-2'deoxyctidine (AZA) and Trichostatin A (TSA) were purchased from Sigma-Aldrich. ICI 182780 was purchased from TOCRIS. Antibodies: rabbit polyclonal anti-ER $\alpha$  antibodies (HC20) were purchased from SantaCruz, mouse monoclonal anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies from Chemicon International, and mouse monoclonal anti-DNMT1 antibodies from Imgenex.

**Cell lines and tissue culture** – MCF-7 cells were maintained in Dulbecco's modified Eagle's medium F-12 with Glutamax containing 50  $\mu$ g/ml gentamycin, 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum (Invitrogen). MDA-MB231 were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, 50  $\mu$ g/ml gentamicin, 1 mM sodium pyruvate, and 10% heat-inactivated fetal calf serum. HE-5 and MDA-66 cells are stable cell lines derived from MDA-MB 231 cell lines: the HE-5 cell line carries an ER $\alpha$  transgene under the control of a SV40 promoter (Touitou et al., 1991) and the MDA-66 cell line carries an ER $\alpha$  transgene under the control of a CMV promoter (Metivier et al., 2004). HE-5 and MDA-66 cells were maintained under the same conditions as MDA-MB231 cells with 100 $\mu$ g/mL and 600 $\mu$ g/mL hygromycin, respectively. All cells were grown at 37°C in a humidified atmosphere containing 10% CO<sub>2</sub>

To study the effects of estrogens and anti-estrogens, cells were grown for 2 days in media without phenol red, without gentamycin and 10% of serum stripped of

endogenous steroids. Cells were treated or not with 10 nM estradiol (E2), 5  $\mu$ M of 5-Aza-2'deoxyctidine (AZA), 100 nM ICI 184 780 (ICI), 50 $\mu$ g/mL Trichostatin A (TSA) for the indicated times.

**Western blot** - Samples were separated by SDS-PAGE and then blotted with the indicated antibody onto Hybond<sup>TM</sup>-P nitrocellulose smembrane (Amersham Biosciences). Anti-DNMT1 and HC20 antibodies were used at 1/200 dilution, anti-GAPDH was used at 1/1000 dilution. The membrane was washed and incubated with an alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG (Sigma-Aldrich). The membrane was washed again and ER $\alpha$  or DNMT1 were detected with ECF substrat (Amersham Biosciences) on a Fluorimager 595 (Molecular Dynamics). Quantification analysis was performed using Imagequant software. The quantity of extract loaded on gels was normalized to total protein content, assayed by the AmidoSchwartz technique.

**Chromatin immunoprecipitation assay** – Four millions of cells were seeded in 14 cm diameter plates. After AZA treatment or si DNMT1 electroporation, cells were treated for 1h with EtOH (as mock), E2 or ICI Chromatin immunoprecipitation (ChIP) assays were performed essentially as described in (Tyteca et al., 2006) using HC20 (4 $\mu$ g/30 $\mu$ g of chromatin) or no antibody as a control. Q-PCR analysis was performed on a i-Cycler device (Biorad) using the platinum SYBR Green qPCR SuperMix (Invitrogen), according to the manufacturer's instructions. The following primer pairs were used to amplify genomic DNA from ChIPs: for the pS2/TFF1 promoter fragment, 5'-GGCCATCTCTCACTATGAATCACTTCTGC-3' and 5'-GGCAGGCTCTGTTTGCTTAAAGAGCG-3'; for PR regulatory sequences DNA fragment, 5'-ATCTACAACCCGAGG CG-3' and 5'-CCCAGGAAGGGTCGGACTT-3' ; for the  $\beta$ -actin promoter, 5'-TGGACTTCGAGCAAGAGATG-3' and 5'-GAAGGAAGGCTGGAAGAGTG-3'.



Amplification conditions were 3 min at 95°C followed by 50 cycles (20 sec at 95°C, 30 sec at 58°C (for PR amplification) or 62.5°C (for pS2/TFF1 and  $\beta$ -actin amplifications), 20 sec at 72°C).

**RT-qPCR experiments** – quantitative RT-PCR experiments were performed as described in (Baron et al., 2007). The following primer pairs were used to amplify cDNAs after reverse transcription experiments: for GAPDH, 5'-ACAGCAACAGGGTGGTGGAC-3' and 5'-GACCATTGCTGGGGCTGGTG-3'; for pS2/TFF1, 5'-GTACACGGAGGCCAGACAGA-3' and 5'-AGGGCGTGACACCAGGAAA-3'; for PR, 5'-GGCCATACCTATCTCCCTGGA-3' and 5'-CTCCACGTCCGACAGCGACT-3'; for  $\beta$ -actin, 5'-GGTGACAGCAGTCGGTTGGA-3' and 5'-CACAATAGTCCTCGGCCACATT-3'. For ANXA9, , 5'-CCGCTGTACTTTGCTGACAA-3' and 5'-GTTTCAGCCAAACACGGAAAT-3'; for BMP6 5'-CCCTCTTCATGCTGGATCTG-3' and 5'-AGGGGAGAACTCCTTGTCGT-3'; TPD52L1 5'-AACCGTTGCAAGGAACAGAC-3' and 5'-ATGCCAGCTTTTGCTGAAGT-3'; RET 5'-TCCTGGGAGAAGCTCAGTGT-3' and 5'-GATGTTGGGGCACAAGAACT-3'; GREB1 5'-GTGGTAGCCGAGTGGACAAT-3' and 5'-AAACCCGTCTGTGGTACAGC-3';

Amplification conditions were 30 min at 45°C and 3 min at 95°C followed by 50 cycles (20 sec at 95°C, 30 sec at 58°C, 20 sec 72°C).

**Cell electroporation and RNA interference** – The transfection of small interfering RNA was performed by electroporation using a Gene Pulser Xcell apparatus (Bio-Rad Laboratories, Hercules, CA) set to 250 V, 950  $\mu$ F, in 4 mm cuvettes (Eurogentec). The final concentration of siRNA was 20 $\mu$ M for  $8 \times 10^6$  cells. After electroporation, cells were plated and grown for 2 days in phenol-red free. Then, cells were treated or not

with E2 or ICI for 16 hours for Q-PCR and for 1h for ChIP experiments. siRNA directed against DNMT1 were chosen as in (Suzuki et al., 2004) (Eurogentec).

**Bisulphite genomic sequencing (BGS)** - 340µg of genomic DNA were boiled for 1 min at 98°C and denaturated by 3M NaOH for 15min at 37°C. Bisulphite conversion was performed as indicated in MethylDetector™ kit manufacturer's instruction (Active Motif). The primer sequences used for BGS were 5'-TGTGGGTGGTATTTTAAATGAGAAT-3' and 5'-CCCCTCACTAAAACCCTAAAATA-3'. PCR products were diluted 1/500 fold and a second PCR was performed using identical conditions. Resulting amplicons were subcloned into the plasmid pGEM-T (Promega), and sequenced.

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## Figure legends

Figure 1. Absence of PR and pS2/TFF1 gene expression but not Cat-D in MDA-MB231-derived breast cancer cell lines. A) Analysis of ER $\alpha$  expression. Top panel: Western Blot analysis. Cells stimulated with  $10^{-8}$  M E2 were immunoblotted. Bottom panel: Western Blot quantification ER $\alpha$ /GAPDH ratio in MCF-7 cells set to 100%. B) RTqPCR using primer sets for PR, pS2/TFF1 or  $\beta$ -actin (as an internal control). C) RTqPCR using primer sets for Cat-D or  $\beta$ -actin. Expression levels from E2 treated relative to mock treated cell lines as indicated (see material and methods) are represented. Data shown are the average of three independent experiments, error bars represent  $\pm$  standard error mean.

Figure 2. DNA demethylation and histone deacetylation trigger hormone-dependent expression of PR and pS2/TFF1 in MDA-66 cells, but do not affect Cat-D expression.

A) RTqPCR using primer sets for PR, pS2/TFF1, Cat-D or  $\beta$ -actin in MDA-66 cells. Cells were treated with 5 $\mu$ M of 5-Aza-2'deoxyctidine (AZA) before addition of 50 $\mu$ g/mL of Trichostatin A (TSA) and E2 10<sup>-8</sup> M or EtOH. Expression levels from E2 treated relative to mock treated cells are represented . B) Expression of PR and pS2/TFF1 in MDA-66 cells is mediated by ER $\alpha$ . Cells were incubated with AZA and TSA as in A) before treatment with E2 and ICI 10<sup>-7</sup> M. RTqPCR analysis using primer sets for PR and pS2/TFF1.

Figure 3. DNA demethylation using AZA or siDNMT1 triggers hormone-dependent expression of PR and pS2/TFF1 in MDA-66 cells, but do not affect Cat-D expression.

A) Western Blot analysis. MDA-66 cells were transfected mock or two distinct siRNA against DNMT1. Cellular proteins from siRNA-treated and control cells were immunoblotted. B) RTqPCR analysis using primer sets for PR, pS2/TFF1, Cat-D,  $\beta$ -actin. C) Expression of PR and pS2/TFF1 in MDA-66 cells is mediated by ER $\alpha$ . Cells were transfected with both siDNMT1.1 and/or siDNMT1.2 as in A) before addition of E2 10<sup>-8</sup> M +/- ICI 10<sup>-7</sup> M. RT-PCR analysis using primer sets for PR, pS2/TFF1 and  $\beta$ -actin. Expression levels are represented relative to mock transfected, mock treated cells. D) Expression of PR and pS2/TFF1 in MDA-66 cells treated with AZA for 72h or transfected with siDNMT1 is transient. RTqPCR analysis using primers for PR, pS2/TFF1, Cat-D and  $\beta$ -actin. Expression levels were analysed immediately following treatment or 4 days after removal of treatments

Figure 4. The extent of PR and pS2/TFF1 gene activation correlates with ER $\alpha$  protein levels MDA-MB231, HE-5 and MDA-66 cells stimulated as in Figure 2. A) RTqPCR analysis using primers for PR and pS2/TFF1. Expression levels are represented relative

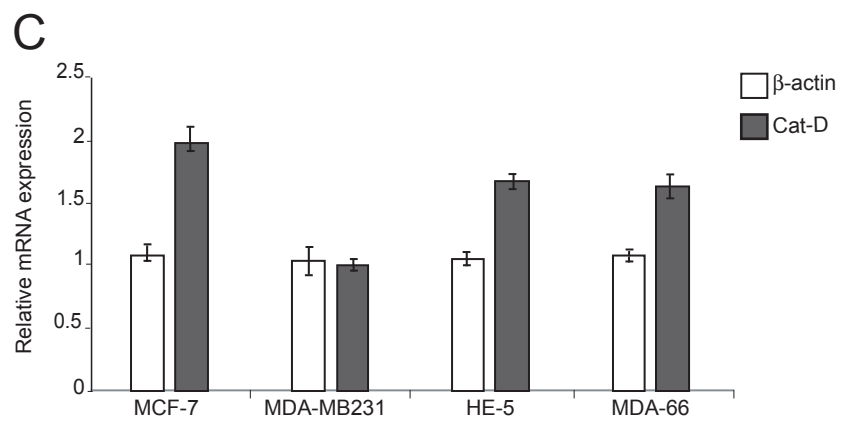
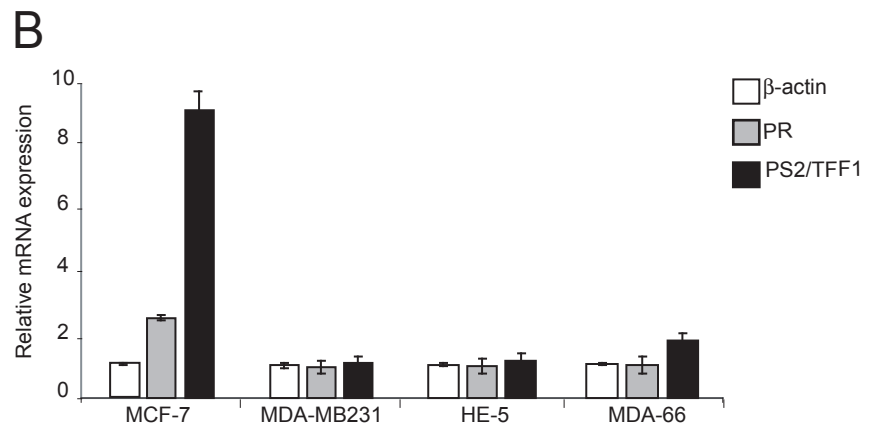
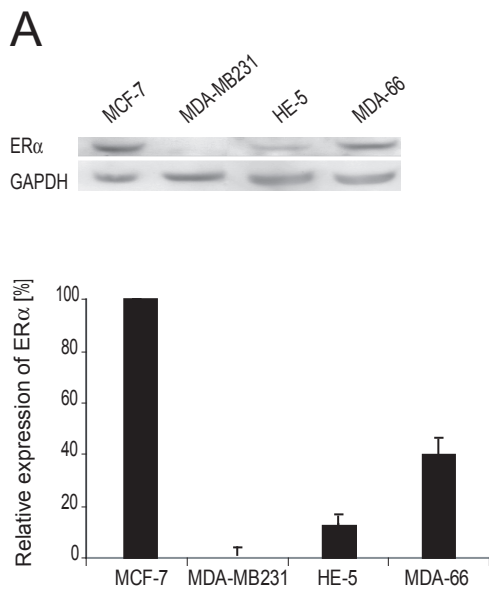
to mock treated cells. B) Top panel: Western Blot analysis of ER $\alpha$  and GAPDH after total protein extraction of cells treated as in A. Bottom panel: ImageQuant analysis of three independent Western blot analyses. Protein levels are represented relative to ER $\alpha$  protein concentration measured in untreated MCF7 cells.

Figure 5. DNA demethylation triggers hormone-dependent ER $\alpha$  recruitment to PR and pS2/TFF1 promoters in MDA-66 cells. A) Organisation of the PR gene regulatory region. The grey area is CpG rich. Fragments amplified for ChIP (B) and BSP (D) analyses are indicated. B) ChIP followed by qPCR analysis of ER $\alpha$  recruitment to PR, pS2/TFF1 and  $\beta$ -actin promoters. MCF-7, MDA-MB231, HE-5 and MDA-66 cells were stimulated with E2  $10^{-8}$  M. C) ChIP followed by qPCR analysis of ER $\alpha$  recruitment to PR, pS2/TFF1 and  $\beta$ -actin promoters. MDA-66 cells were stimulated with E2 +/- ICI following AZA treatment or siDNMT1.1 post-transfection. Results are shown as fold induction of ER $\alpha$  binding upon demethylation. D) Bisulfite Sequencing (BSP) analysis of the CpG methylation status of the CpG island containing PR regulatory region. Genomic DNA from MDA-66 cells treated with AZA or mock treated was exposed to bisulfite. PCR amplified fragments of the region of interest were cloned and sequenced. Methylation patterns are represented by a succession of circles symbolising the methylation status of cytosines part of CpG di-nucleotides; filled: methylated, open: unmethylated.

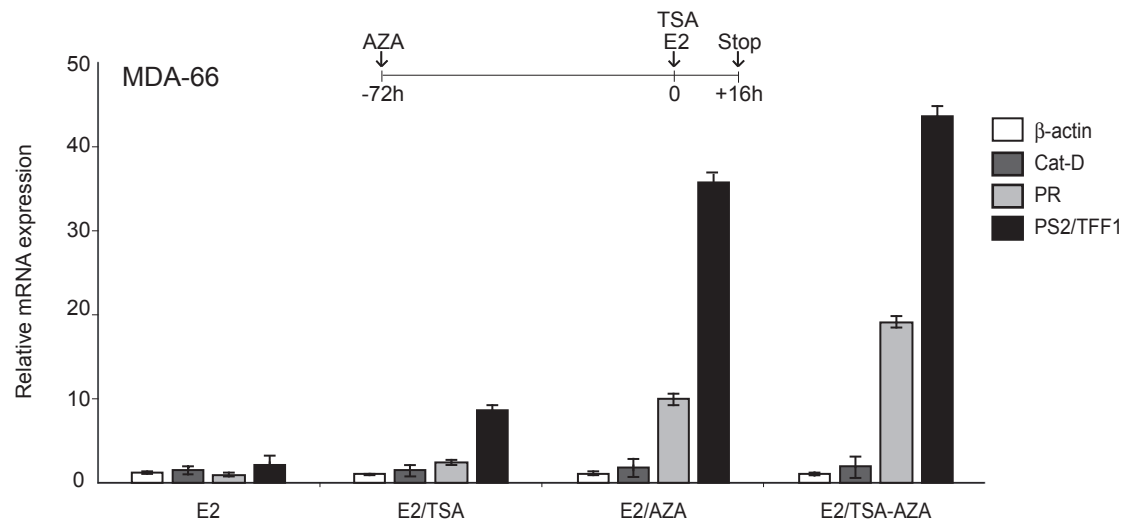
Figure 6. Quantitative ER $\alpha$  recruitment to demethylated PR regulatory sequences. ChIP followed by RTqPCR analysis of ER $\alpha$  recruitment to PR regulatory sequences in MDA-MB231, HE-5 and MDA-66 cells. Cells were stimulated following AZA treatment. Results are shown as fold induction of ER $\alpha$  binding upon E2 stimulation

Figure 7. The epigenetic mechanism of PR silencing is common to some but not all ER $\alpha$  target genes that are repressed in ER $\alpha$  negative cell lines A) RTqPCR using primer sets for PR, ANXA9, RET, TPD52L1, BMP6, GREB1,  $\beta$ -actin in MCF-7 cells. Cells were incubated in steroid-free medium, treated with E2  $10^{-8}$  M or EtOH for 16hours. Expression levels from E2 treated relative to mock treated cells are represented. B) RTqPCR using same primer as A) in MDA-66 cells. Cells were incubated in steroid-free medium, treated with 5 $\mu$ M of 5-Aza-2'deoxycytidine (AZA) for 72 hours before addition of E2  $10^{-8}$  M or EtOH for 16 hours. Expression levels from treated cells relative to mock treated cells are represented.

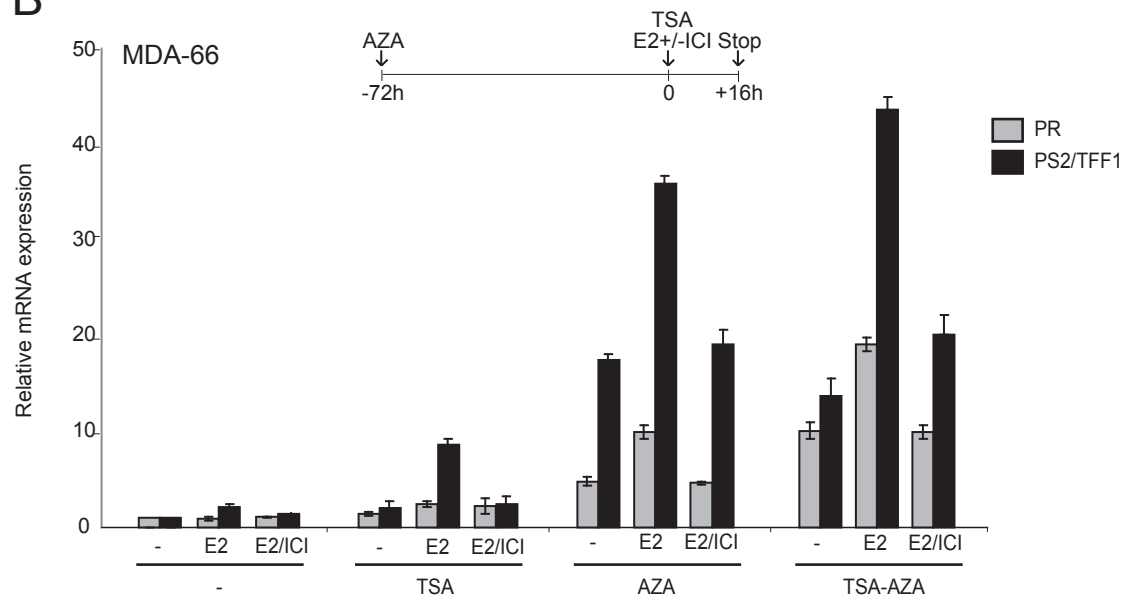


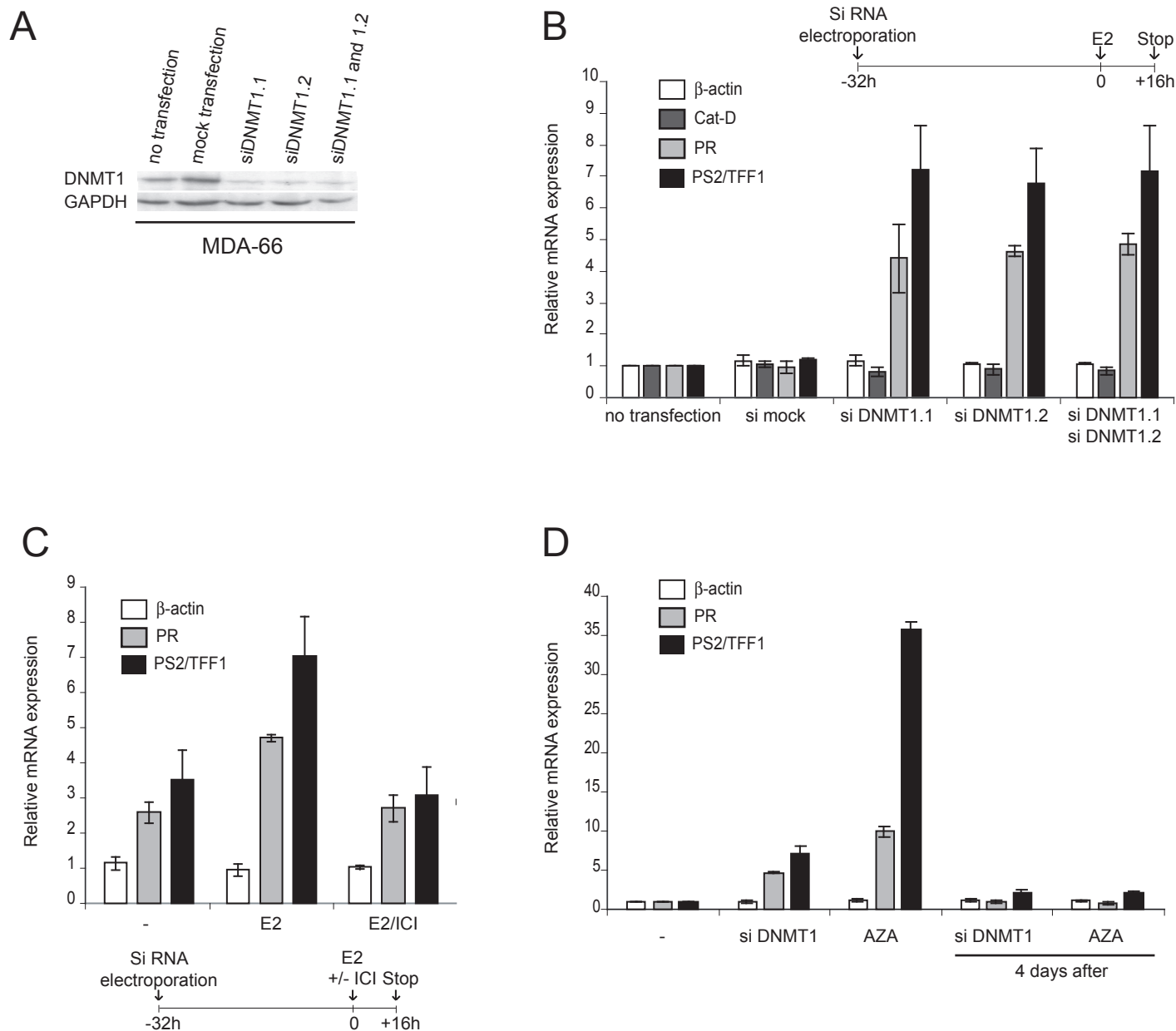


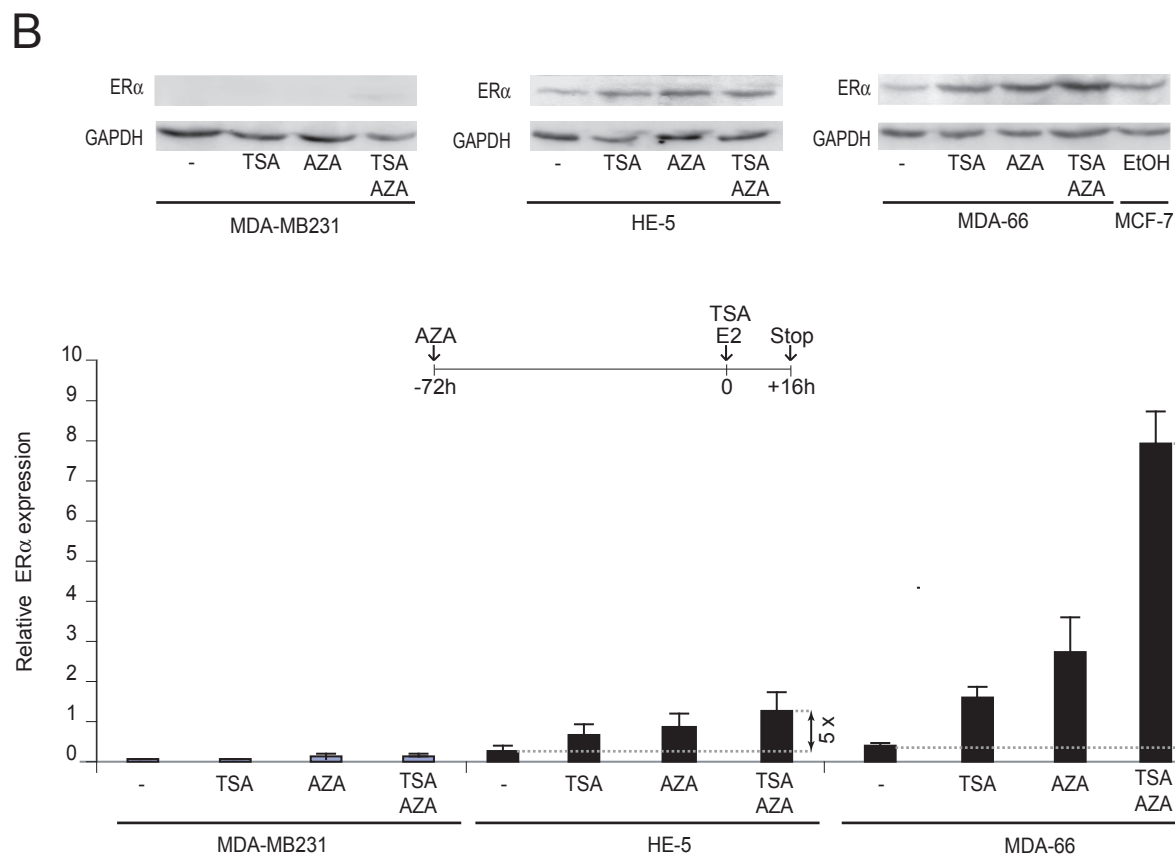
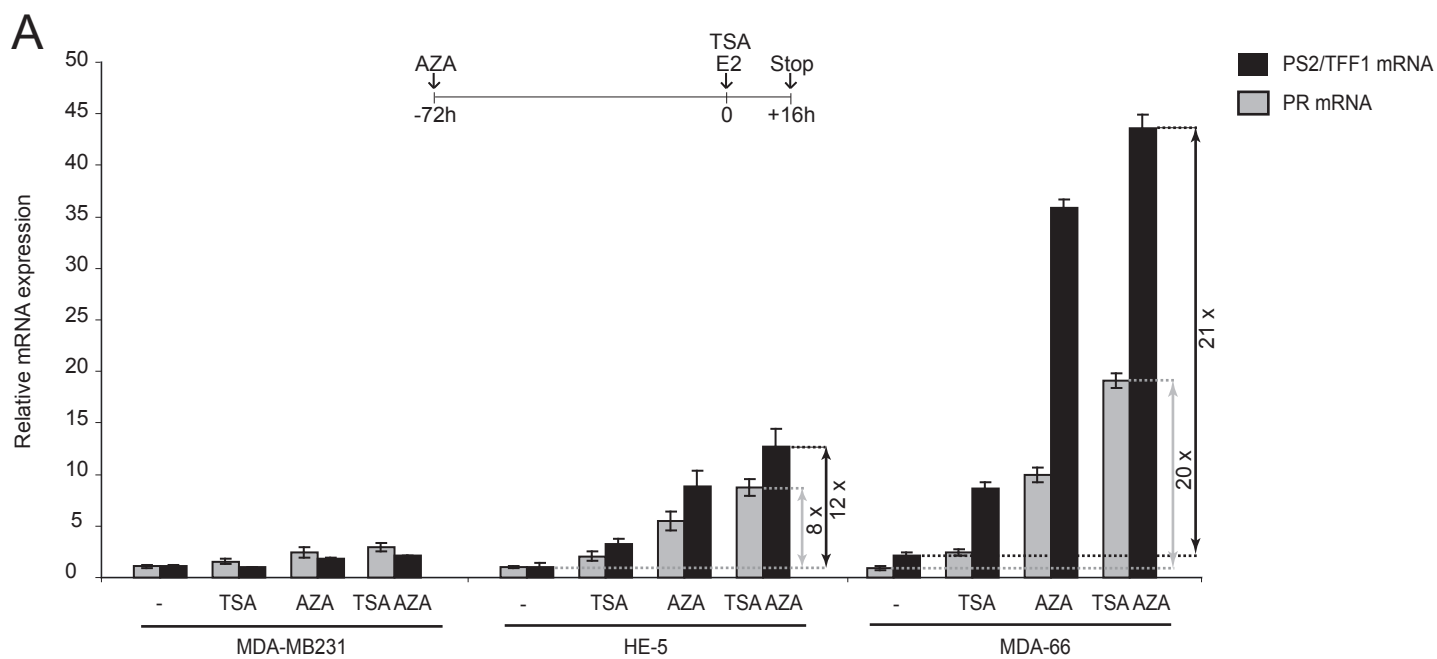
**A**



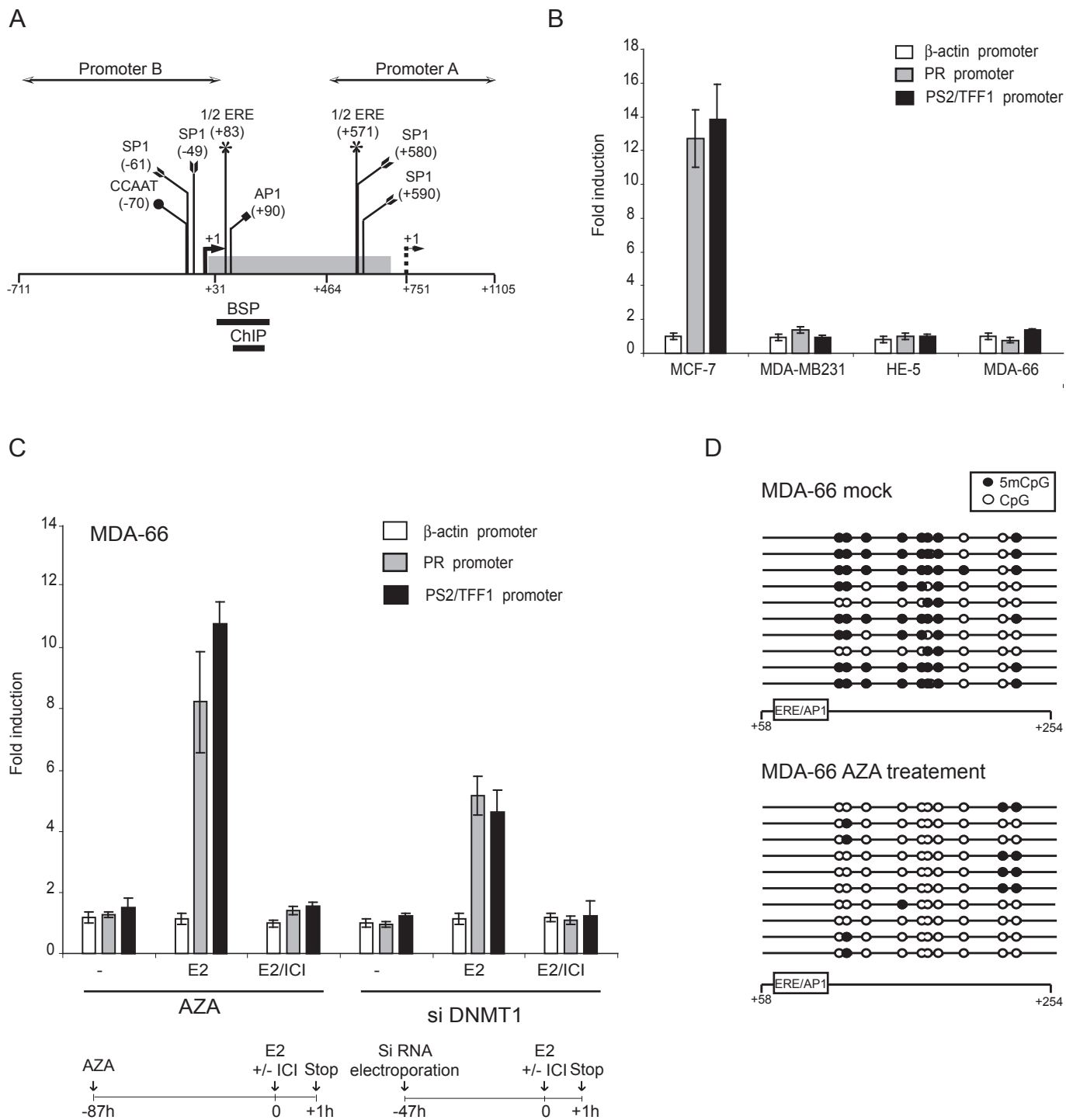
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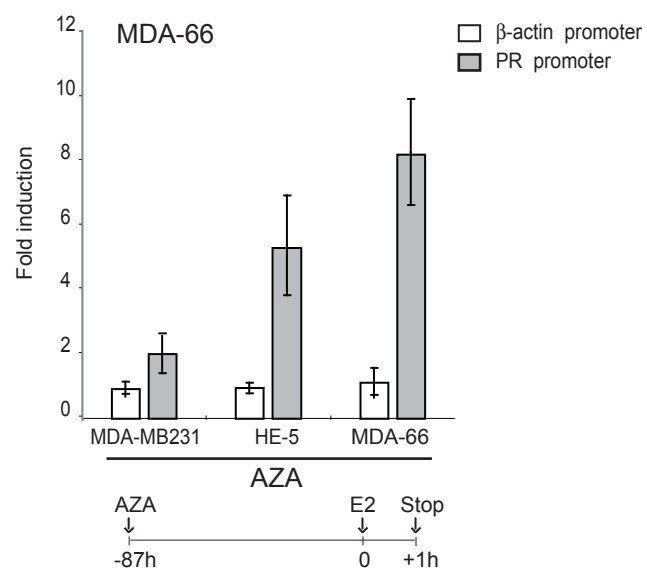




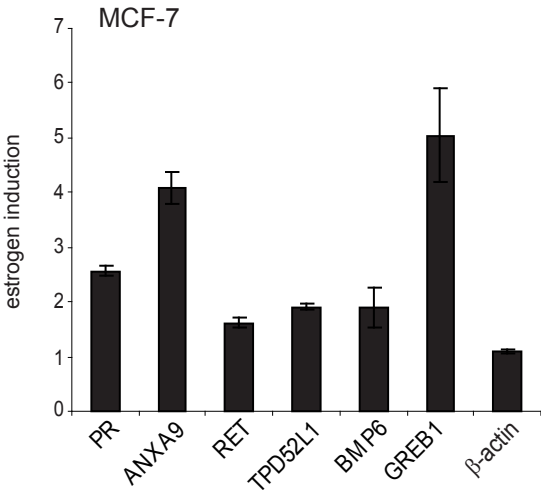


Fleury L et al., Figure 4





A



B

