

# Epigenetic regulation of estrogen signaling in breast cancer

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**Abbreviations:** BC, breast cancer; BpA, bisphenol A; Dnmt, DNA methyl transferase; ER, estrogen receptor; ER<sup>-</sup>, estrogen receptor negative; ER<sup>+</sup>, estrogen receptor positive; ESR1, gene encoding ER $\alpha$ ; ESR2, gene encoding ER $\beta$ ; EZH2, enhancer of zeste homolog 2; HMT, histone methylase; HDM, histone demethylase; HAT, histone acetylase; HDAC, histone deacetylase; JMJD, Jumonji domain-containing protein; LSD1, lysine specific demethylase 1; PR, progesterone receptor; TF, transcription factor; TSG, tumor suppressor gene

Estrogen signaling is mediated by ER $\alpha$  and ER $\beta$  in hormone dependent breast cancer (BC). Over the last decade the implication of epigenetic pathways in BC tumorigenesis has emerged: cancer-related epigenetic modifications are implicated in both gene expression regulation and chromosomal instability. In this review, the epigenetic-mediated estrogen signaling, controlling both ER level and ER-targeted gene expression in BC, are discussed: (1) ER silencing is frequently observed in BC and is often associated with epigenetic regulations while chemical epigenetic modulators restore ER expression and increase response to treatment; (2) ER-targeted gene expression is tightly regulated by co-recruitment of ER and both co-activators/co-repressors including HATs, HDACs, HMTs, Dnmts and Polycomb proteins.

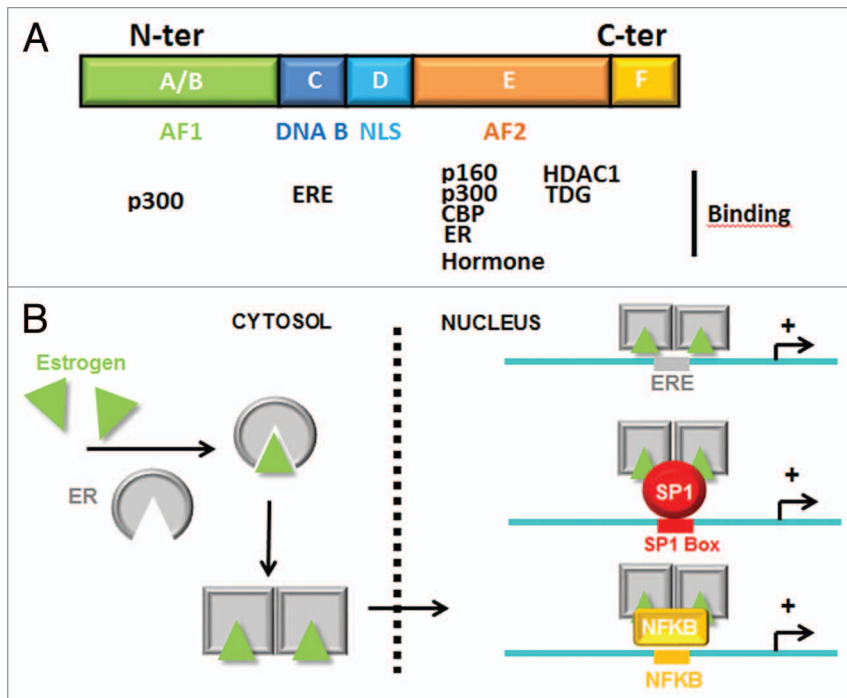
and ER $\beta$ ) that belong to the family of transcriptional receptors and recognize and bind to a specific DNA consensus sequence to facilitate the transcriptional initiation of hundreds of target genes.<sup>2</sup> Following estrogen treatment, the hormone binds to the E-domain of ERs, induces ER dimerization and favors its nuclear translocation, where the dimer finally interacts with DNA on the estrogen response element (ERE) and induces the activation of estrogen regulated genes (Fig. 1). However, following estrogen stimulation, the transcription of additional genes lacking an evident ERE is also activated in response to ER $\alpha$  interaction with particular transcriptional factors (TFs) such as AP-1, SP1 or NF $\kappa$ B. In the latter cases, mechanisms of ER-dependent transcriptional activation are indirect and mediated by the recruitment of ER $\alpha$  on TF boxes.<sup>3,4</sup>

Changes in gene expression caused by genetic mutations, which lead to oncogene activation or tumor suppressor gene silencing, have been studied in BC etiology and correlated with BC risk in a recent meta-analysis.<sup>5</sup> For example, mutations in *BRCAl/2* genes were frequently observed in hereditary BC. Over the last two decades, the idea of an epigenetic control of gene expression in diseases other than genetic disorders has emerged. This includes the deregulation of genes that participate in tumorigenesis initiation and progression. In the latter case, the outcome of both genetic and epigenetic modifications is an aberrant over-expression and/or silencing of genes implicated in cell proliferation and/or in the control of cell death. Epigenetic pathways regulate gene transcription by two different mechanisms that are not mutually exclusive: DNA methylation and post-translational modification of histones. DNA methylation occurs in 2–3% of cytosines in CpG islands and is not randomly distributed throughout the DNA as these sequences are mostly located in the upstream region of promoters. DNA methylation is implemented by a family of enzymes referred to as DNA methyl transferases (Dnmts) 1, 2, 3a, 3b and 3L. Promoters with a high density of CpGs are defined as CG-rich areas and are predominantly subject to DNA methylation. Methylated DNA is generally associated with a decreased TF binding capacity that diminishes/abolishes

## Introduction

Breast cancer (BC) is the most common malignant tumor among women in the world and is the second cause of death in women between the ages of 35–55 in developed countries. BC can be divided based on molecular criteria into distinct phenotypes: the molecular subtypes are classified by (1) expression of estrogen receptors (ERs) and/or progesterone receptors (PRs), (2) human epidermal receptor 2 (*HER2/ERBB2*) amplification and (3) a triple negative type (ER<sup>-</sup>/PR<sup>-</sup> and normal expression of HER2).<sup>1</sup> While estrogen has normal biological roles, such as reproduction, brain development and additional protective effects of sexual steroid hormones, prolonged exposure, combined with high levels of hormone increases the risk of BC by constitutively activating the transcription of genes predominantly implicated in metabolism and cell cycle regulation. ER-mediated mechanisms of gene regulation are well documented. ERs exist as two isoforms (ER $\alpha$

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**Figure 1.** Structure and activation of Estrogen Receptor. (A) Schematic representation of ER $\alpha$  domains and their potential interaction with co-activators/co-repressors and ERE. (B) Mechanisms of ER $\alpha$  activation by estrogens. Recruitment of liganded ER $\alpha$  on DNA is mediated directly on ERE or not directly via SP1 or NFKB interaction.

transcriptional expression of the corresponding gene. Two distinct forms of DNA methylation processes have been described, the first is inherited DNA methylation or maintenance DNA methylation and is predominantly catalyzed by Dnmt1, the second is de novo DNA methylation and is performed mainly by Dnmt3a and Dnmt3b. Maintenance DNA methylation permits the conservation of DNA methylation patterns after DNA replication by copying methylation on the newly synthesized strand using the hemi-methylated DNA as a matrix. Conversely, de novo DNA methylation occurs on both strands of unmethylated DNA (for a review see ref. 6). Global DNA hypomethylation has been observed in many cancers including BC and prostate tumors.<sup>7-9</sup> Artificial disruption of DNA methylation complexes or invalidation of Dnmt1 in normal cells leads to a decrease in global DNA methylation and induces tumor formation in nude mice.<sup>10,11</sup> This phenomenon is promoted by reactivation of non-coding repetitive elements leading to chromosomal instability and abnormal gene expression. Besides global DNA hypomethylation, both local hypo and hypermethylation of promoters have also been reported and result in specific gene activation or silencing in cancers.

Nucleosomes are made up of a duplicate of histones H2A, H2B, H3 and H4 enclosed in a DNA loop and regulate chromatin compaction as well as TF accessibility for transcription initiation. Histones are subject to post-translational modifications such as acetylation, methylation and phosphorylation. The “histone code” refers to the sum of these modifications and allows a prediction of a favorable or unfavorable chromatin status for

gene transcription. Acetylation of lysines in histones is associated with an uncondensed chromatin status, accessibility of TFs, and is processed by histone acetyltransferase (HATs), while these acetyl groups are removed by histone deacetylases (HDACs). Histone methyltransferase (HMT) or histone demethylases (HDM), respectively, catalyze the methylation or demethylation of lysine or arginine in histones and these modifications favor the compaction or relaxation of chromatin, depending of the methylated residue (for a review see ref. 12).

### Epigenetic Silencing of ESR1 and ESR2 Genes in Cancer

Anti-estrogen therapies are used in treatment of BC but are inefficient in ER negative patients. In these therapies, the most used drug over the past 50 years is tamoxifen, a competitive inhibitor of estradiol that binds to ER $\alpha$ . More recent pharmacological molecules include selective ER down-regulators (SEDRs), which inhibit ER $\alpha$  dimerization and nuclear translocation; or aromatase inhibitors, which target the enzyme responsible for estrogen synthesis.

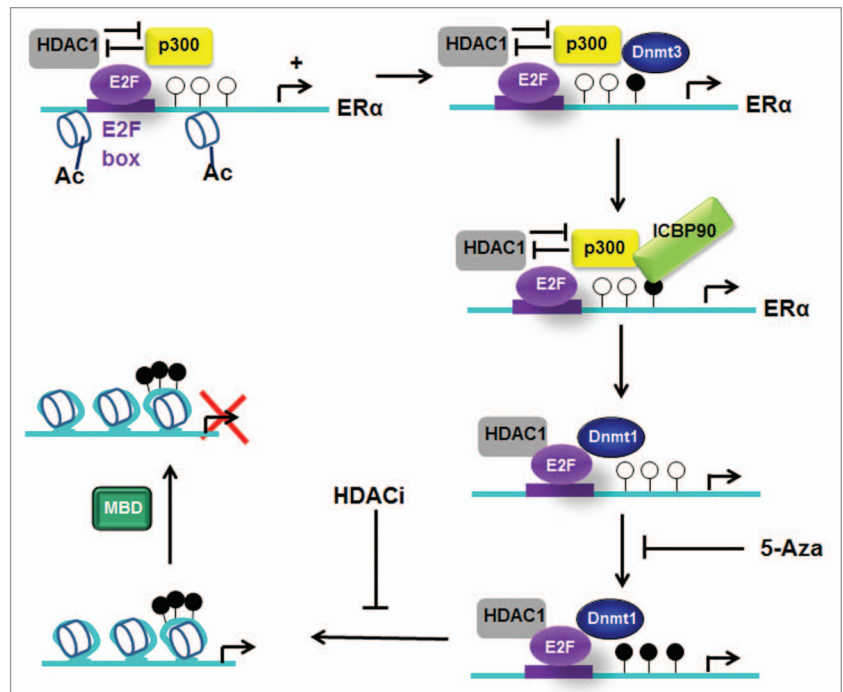
As described above, estrogen dependent genes are controlled by ER $\alpha$  and ER $\beta$ . However, a frequent decrease in ER $\alpha$  expression was observed in BC and may occur during the course of the disease. ER<sup>-</sup> breast cancers were observed in 20% of low and 50% of high grade BC patients.<sup>13</sup> ER expression status is paradoxical in BC. High ER $\alpha$  expression in high grade BC correlates with a better outcome, a lower aggressiveness and a better response to anti-estrogen therapies compared with ER<sup>-</sup> patients. However, estrogen stimulation in healthy cells increases BC risks. This may be explained by the dual role of ER $\alpha$  in both proliferation and differentiation. Some studies also suggest that DNA methylation-mediated promoter *ESR1* (estrogen receptor 1 gene) silencing is found frequently and may participate in tumorigenesis or progression of the disease in other cancers, such as leukemia or colon tumors.<sup>14</sup> The etiology of the loss of expression of ER $\alpha$  (about 30% of BC patients are ER $\alpha$ <sup>-</sup>) is due to DNA hypermethylation in 41% of cases, which correlates with tumor size and histological grade.<sup>15-17</sup> Moreover, a recent study on BC patients in India revealed that the proportion of *ESR1* hypermethylation was highly increased in triple negative tumors.<sup>18</sup> Manipulation of *ESR1* hypermethylation can also affect ER $\alpha$  expression. For example, a 5 d Bisphenol A (BpA) exposure in neonatal male rats induces persistent *ESR1* promoter hypermethylation in adults, associated with increasing levels of Dnmts.<sup>19</sup> Inactivation of Dnmt1 using siRNA or treatment with DNA methylation inhibitors such as 5-azadeoxycytidine, restores ER $\alpha$  expression in ER $\alpha$  negative BC cells.<sup>20</sup> As such, in addition to immunodetection of ER $\alpha$ , detection of *ESR1* methylation status

may aid in predicting a response to anti-estrogen therapies in BC patients.

Overexpression of HDAC1 abolishes *ESR1* expression in MCF7 cells.<sup>21</sup> Macaluso et al. proposed a model of epigenetic inactivation of the *ERα* promoter (Fig. 2).<sup>22,23</sup> In ER<sup>+</sup> BC cells such as the MCF7 cell line, an activator complex composed of pRb2/E2F4/5/HDAC1/SUV39H1/p300 binds to a region containing E2F boxes close to the initial transcription site in the *ESR1* promoter. The authors proposed that repressor activity of both HDAC1 and the HMT SUV39H1 might be overcome by the HAT activity of p300. Methylation of CpG by Dnmt3a/3b in this promoter may induce the recruitment of ICBP90 (inverted CCAAT box binding protein of 90 kDa) and consequently facilitate the replacement of p300 by Dnmt1 in the repressor complex pRb2/E2F4/5/HDAC1/SUV39H1/Dnmt1 found in ER<sup>-</sup> BC, MDA MB231 cells. A further recruitment of MeCP2 to an *ERα* methylated promoter may also participate in complete *ERα* repression, as illustrated in Figure 2.<sup>24</sup> These epigenetic signals, in particular DNA methylation near the AP-2 binding site, induce a repressive chromatin, blocking the loading of TFAP2C, further RNAP II recruitment and thus transcription of *ESR1*.<sup>25</sup> A recent study in male tissues revealed that among the methylated CpGs close to the *ESR1* promoter, the methylation of one particular CpG (located in the +1 kb intragenic region of *ESR1*) correlates with low *ESR1* expression. This CpG is included in a TGIF box, and its methylation provokes the recruitment of the repressor TGIF, targeting of HDAC1 and *ESR1* silencing. Interestingly, methylation status of *ESR1* in these tissues was not sensitive to estrogen exposure.<sup>26</sup> Moreover, in MCF7 cells, estrogen treatment induces *ESR1* repression in an ER<sup>α</sup>-mediated mechanism: while co-activators and ER<sup>α</sup> are found at both distal and proximal *ESR1* promoters, Sin3A/ER<sup>α</sup> complex is specifically recruited on the proximal promoter and represses *ESR1* transcription.<sup>27</sup>

Epigenetic regulation of *ESR2* (gene coding for ER<sup>β</sup>) has been poorly investigated. However, one study has demonstrated a frequent occurrence of *ESR2* promoter methylation in ER<sup>β</sup> BC in Chinese women.<sup>28</sup> Indeed, *ESR2* methylation was significantly higher in high grade BC (45%) than in starting neoplasia and was strongly correlated with *ESR1* methylation, suggesting common epigenetic mechanisms of regulation.<sup>28</sup> Overexpression of ER<sup>β</sup> in MCF7 cells strongly decreased cell proliferation. Similarly, hypermethylation of *ESR2* was also identified in prostate tumors and present on 3 CpG islands during disease progression.<sup>29</sup> All of these observations strongly suggest a role of epigenetics in the inactivation of *ESR* genes in hormone dependent cancers.

HDAC inhibitors (HDACi) such as Entinostat or valproic acid, have been tested in BC cells and efficiently restored both ER<sup>α</sup> expression and Letrozole sensibility in ER<sup>+</sup> BC in vitro and in vivo.<sup>30,31</sup> The association of HDACi or 5-azadeoxycytidine with



**Figure 2.** Model of epigenetic inactivation of *ESR1*. Primary methylation and recruitment of ICBP90 on *ERα* promoter, provoke histone deacetylation and a large secondary methylation and *ERα* silencing. Ac, acetylation of histones; white circles symbolize unmethylated CpGs and black circles symbolize methylated CpGs.

a treatment inducing overexpression of TFAP2C might improve *ESR1* expression in ER<sup>-</sup> patients. A combined HDACi and 5-azadeoxycytidine treatment induces the most significant increase in ER<sup>α</sup> content. Surprisingly however, addition of tamoxifen does not produce a tumorigenic response in ER<sup>+</sup> BC cells. Hoestetter et al. demonstrated that a better response to tamoxifen in BC cells, correlated with a lower level of the RNA-stabilizing HuR protein. Tamoxifen treatment increased HuR content, and contributed to its own resistance while HDACi/5-azadeoxycytidine decreased HuR. Preliminary treatment with HDACi/5-azadeoxycytidine was given before delivering tamoxifen to attempt to obtain the best tamoxifen sensitivity.<sup>32</sup> The precise roles of tamoxifen are complex: although it competes with 17 $\beta$ -estradiol to bind to ER<sup>α</sup>, ER<sup>α</sup> bound to tamoxifen is still able to target the *TFF1* (also called pS2) promoter without constitutive activation of gene transcription. The loss of transcriptional activity of the tamoxifen-ER<sup>α</sup> complex is mediated by changes in the balance of co-activators/co-repressors and ER<sup>α</sup>-interacting partners.

### Epigenetic Regulation of Estrogen-Responsive Genes by Estrogen Receptors

**Regulation via ER and co-activators.** How epigenetic changes affect the transcriptional response of estrogen stimulation in cancer, and particularly in BC, is still poorly understood. However, several groups have shown a connection of both estrogen and ER in epigenetic regulation. Several reports suggest that ER<sup>α</sup> cooperates with co-activators to epigenetically regulate estrogen



responsive genes. Only a small percentage of genes with putative ERE are really activated following estrogen stimulation, suggesting that additional proteins could specifically control ER-responsive gene pathways. Maximal ER $\alpha$ -mediated transcription requires the addition of some epigenetic changes and the removal of others. Estrogen bound ER $\alpha$  orchestrates the recruitment of HATs (p300 and CBP) and HAT co-activators of the p160 family (SRC1/SRC2/SRC3) to modulate chromatin status and allow RNAP II recruitment.<sup>33,34</sup> Indeed, overexpression of SRC3 increases BC cell proliferation, while inhibition of SRC1/SRC2 blocks their proliferation. Moreover, in the absence of estrogen stimulation, a direct interaction between HDAC1 and unbound ER $\alpha$ , via its AF2 and DNA binding domains, is constitutive in BC and inhibits its activity.<sup>21</sup>

Methylation of histones and the enzymes that control this methylation are highly implicated in estrogen signaling. An increase in the epigenetic mark H3K4me3 is generally associated with positive effects on transcription and such an increase on the *TFPI* promoter is due to a direct interaction between ER $\alpha$  and the protein linker MEN1. This interaction recruits the co-activators H3K4 methylase MLL1/2 (Mixed Lineage leukemia).<sup>35</sup> Based on studies done on JMJD2B/MLL2/ER $\alpha$  interactions, a model was developed in which demethylation of H3K9me by the HDM JMJD2B (Jumonji domain-containing protein 2B) is first required for the further methylation of H3K4 by MLL2.<sup>36</sup> An increase of H3K4me3 after direct interaction between ER $\alpha$  and MLL 2–4, via its LxxLL domain, was required for activation of *cathepsin*, *liver x-receptor* genes.

Besides methylated marks, removal of other methylation may also be implicated in estrogen responsive gene regulation. Recruitment of the HMT SMYD3, whose levels increase in BC, was also able to produce the tri-methylation of H3K4me3 and was mediated by both a direct ER $\alpha$ /SMYD3 interaction on the ERE of the *TFPI* promoter and/or by the identification of the Ser10 phosphorylation mark on histone H3.<sup>37,38</sup> The HDM LSD1 (lysine specific demethylase, also called KDM1) also contributes to H3K9 demethylation on ER targets genes and recruitment of co-activators, but this required the presence of activated ER $\alpha$ .<sup>39</sup> In some other genes, however, recruitment of LSD1 also follows H3K9 deacetylation and provokes H3K4 demethylation, which is unfavorable to transcription.<sup>40</sup> The specificity of H3 methylated substrate on ER target loci such as *TFPI* promoter, is orchestrated by the co-recruitment of PELP1/activated ER $\alpha$ /LSD1. PELP1 (proline glutamic acid and leucine rich protein 1) is a reader of methylation marks that recognizes both H3K4me2 and H3K9me2 but its interaction with ER $\alpha$  and LSD1 decreases the LSD1-mediated HDM activity on H3K4me2 in favor of H3K9me2 demethylation and increased ER target gene expression.<sup>41</sup> Moreover, the early engagement of some factors on condensed chromatin, in a specific sequence that is dependent of an epigenetic signature, refers to a class called competence or pioneer factors. Pioneer factors are implicated in the opening and activation of transcription. Magnani et al. reported that the association of activated ER $\alpha$  with the pioneer factors PBX1 (pre-B-cell leukemia homeobox 1), and FOXA1 (forkhead box A1) considerably increased estrogen dependent transcriptional response

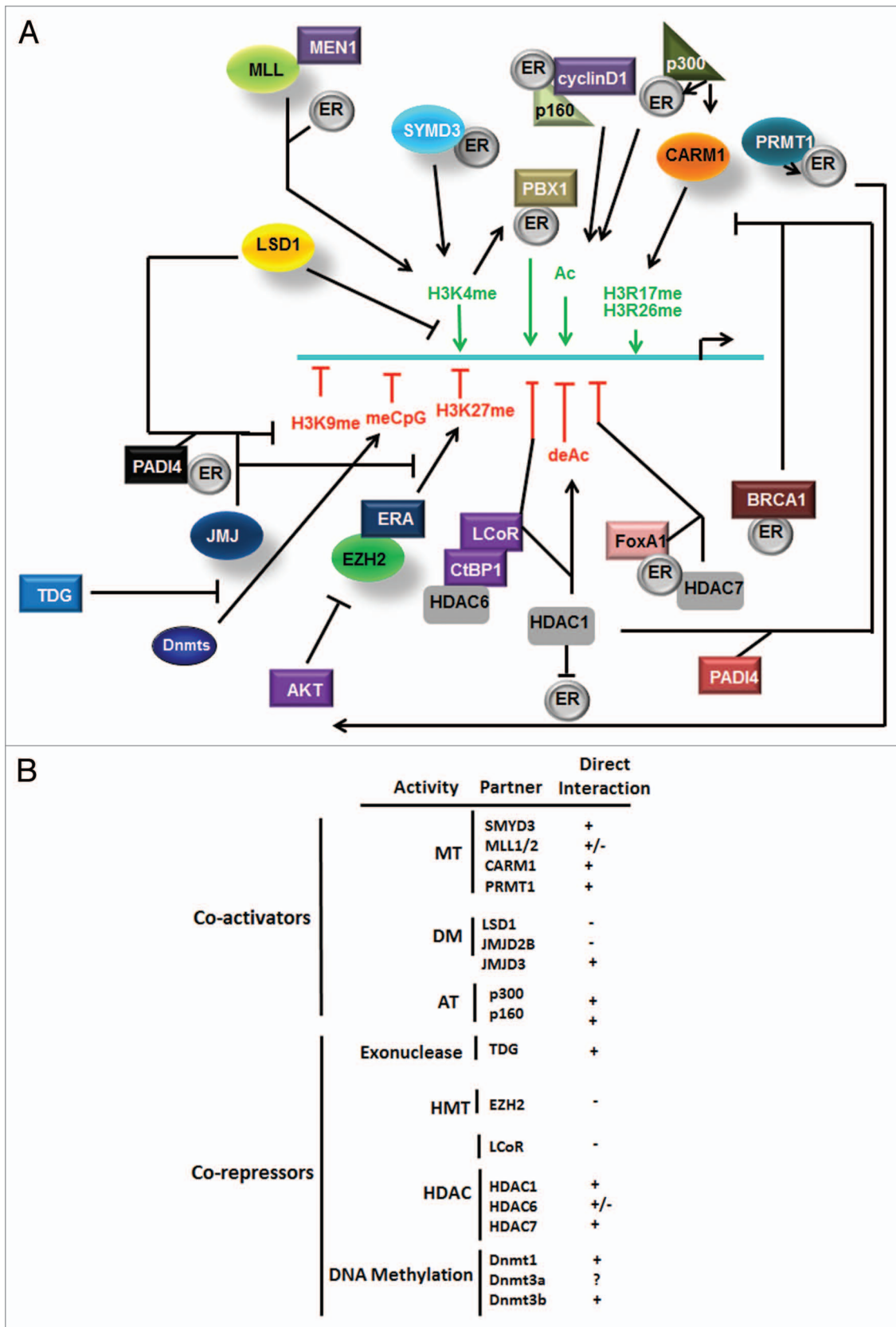
via PBX1-dependent identification of H3K4me2 and chromatin remodeling.<sup>42</sup>

Expression of CARM1 (Co-activator-associated arginine methyltransferase), a co-activator of ER $\alpha$ , correlates with low grade BC and with a decrease in BC cell proliferation. CARM1 is believed to partially govern the proliferation/differentiation balance in BC by controlling 16% of estrogen dependent genes.<sup>43</sup> While mechanisms implicating CARM1 are complex and still under investigation, CARM-1-mediated H3R17me and H3R26me seems to be implicated in estrogen response, while methylation of p300 may regulate its activity.<sup>44</sup> A direct interaction between free ER $\alpha$  and phosphorylated CARM-1 may be used to recruit other co-activators, while association of CARM-1 with activated ER $\alpha$  may require a p160 co-activator SRC-2.<sup>45</sup> Indeed, CARM1-mediated CBP methylation is required for CBP recruitment to some ER target genes and increases its HAT activity.<sup>46</sup>

Fewer studies have been performed to identify ER $\beta$  co-activators. Indeed, as has been observed for ER $\alpha$ /MLL interactions, MLL1-4/ER $\beta$  complexes are implicated in *HOXC13* gene regulation.<sup>47</sup> However, ER $\beta$ /eNOS (endothelial nitric oxide synthase) complex was observed in prostate cancer and provoked the activation of *hTERT*, *MSH2*, *CyclinD1* and *TFPI*, 4 genes previously identified in prostate cancer grading.<sup>48</sup> On the other hand, this complex was also associated with the epigenetic repression of *GSTP1* expression, a gene frequently silenced in prostate tumors. Further investigation will be necessary for a better view of the mechanisms controlling epigenetic-mediated ER $\beta$  target gene expression.

**Regulation via ERs and co-repressors.** Although the link between ER $\alpha$  and upregulation of gene transcription is well studied, some transcriptome analyses have revealed that about 50% of ER $\alpha$  target genes are downregulated following estrogen treatment.<sup>49,50</sup> Indeed, estrogen exposure or ER $\alpha$  loss using both chemical mimetics or siRNA, leads to epigenetic modifications in ER target genes requiring both histone modifying enzymes and Dnmts.

HMT EZH2 (enhancer of zeste homolog 2) is a polycomb protein that catalyzes H3K27me3, a chromatin repressive mark. A high level of EZH2 has been reported in several cancers and is associated with malignancy and the grade in BC. Interestingly, an increase in EZH2 expression, both in MCF7 and in vivo, was also reported following estrogen-like exposure.<sup>51</sup> Overexpression of EZH2 induces a decrease in the expression of numerous genes, in particular in the ER responsive gene pathway. EZH2-mediated H3K27me3 on ER target genes in BC cells requires EZH2 interaction with REA (repressor of estrogen activity), which preferentially targets ERE and may also recruit HDACs for complete gene silencing.<sup>52</sup> On the other hand, *Bcl2* is an estrogen responsive gene encoding a major anti-apoptotic protein, upregulation of which is often observed in many cancers including BC. Both genetic and non-genetic ER pathways regulate the expression of *Bcl2*.<sup>53</sup> *Bcl2* is normally silenced by EZH2-mediated repressive mark H3K27me3 in its enhancer, promoting the recruitment of other polycomb group proteins (PRC1 and 2). Constitutive S21 phosphorylation-mediated inhibition of EZH2 following PI3K/



**Figure 3.** Model of ER-mediated epigenetic response in ER target genes. **(A)** Schematic representation of action of co-activators and co-repressors. **(B)** Direct and indirect interactions of ER $\alpha$  with epigenetic related proteins.

Akt activation in HER2 positive BC and/or demethylation of H3K27me via ER $\alpha$ /JMJD3 complex recruitment on *Bcl2*, induce gene expression and contribute to apoptosis resistance in BC. ER $\alpha$  methylation appears essential for non-nuclear functions of ER $\alpha$  such as activation of AKT following ER $\alpha$ /Src/PI3K interaction.<sup>52</sup> PRMT1-mediated R260 methylation of cytosolic ER $\alpha$  within its DNA interacting domain occurs rapidly after estrogen treatment and ER $\alpha$  hypermethylation has been reported in 55% of BC. This methylation also required p160 co-activators and is implicated in the non-genomic functions of ER $\alpha$ , leading to a constitutive activation of AKT signaling and a promotion of proliferation and survival signals.<sup>54,55</sup>

In fact, epigenetic silencing of ER target genes was most frequent in ER<sup>-</sup> than in ER<sup>+</sup> patients and was comparable to the panel of epigenetic modifications observed in MCF7 following ER $\alpha$  inactivation by RNAi.<sup>56</sup> According to the literature, DNA methylation and histone modification can cooperate to govern the sequence of epigenetic events leading to the silencing of one gene. DNA methylation and histone modification can be catalyzed within the same complex or successively by independent complexes. Investigations on the kinetics of the addition of epigenetic marks on ER target loci revealed that chromatin remodeling begins 36 h after ER $\alpha$  invalidation. First, HDAC1 and the polycomb co-repressors YY1 and EZH2 are recruited while addition of persistent heritable epigenetic marks via Dnmt1 recruitment occurs only at 168 h. Local hypermethylation in BC may be the consequence of an increase in Dnmts followed by MeCP2 induction, as was observed in rats treated with high amounts of estrogen.<sup>57</sup> Following estrogen exposure, a similar increase in both Dnmt3b expression and activity in endometrial cancer cells was reported and could be inhibited by an ER antagonist, suggesting a direct implication of ER in Dnmts regulation.<sup>58</sup> However, diethylstilbestrol exposure in mice provokes a decrease in Dnmts and SP3 on day 5 while SP1 levels only decreased at day 14, followed by demethylation of several DNA loci.<sup>59</sup> Dnmt3a/b expression is under estrogen regulation in normal female tissues.<sup>60</sup> To date, the effects of estrogen and the ER pathway on the recruitment of SP1/SP3 to *Dnmt* promoters have not been investigated. This could be studied with folate treatment, which preferentially permits the recruitment of SP3 in detriment to SP1 and increases *Dnmt* genes transcription.<sup>61</sup> Relative amounts and/or preferential recruitment of SP1/SP3 may explain the tissue-specific response to estrogen exposure. Indeed, hypermethylation of *ERCC1*, *XPC*, *OGG1* and *MLH1* genes, all involved in DNA repair, after estrogen treatment contributed to chromosomal instability and mutations that occur in BC.<sup>62</sup> Conversely, expression of *HOXA10* was increased following BpA exposure and ERE hypomethylation.<sup>63</sup>

Moreover, some experiments show that estrogen exposure of breast progenitor cells induces epigenetic modifications and confers a cancer-like methylome in these cells, suggesting a possible role of epigenetic modifications in breast progenitor cells in the initiation of BC.<sup>64</sup> Epigenetic modifications (global DNA hypomethylation and histone modifications) occurred as soon as 6 weeks in treated rats, while evident signs of neoplasia could be detected only after 12 weeks. Indeed, estrogen stimulation

provoked long-range epigenetic silencing (LRES) in a cluster of 14 genes located at 16p11.2 in normal breast cells.<sup>65</sup> The silencing is mediated by ER $\alpha$  translocation into the nucleus and addition of epigenetic marks such as H3K27me3. Prolonged estrogen exposure induces progressive DNA methylation, which confers a persistence of epigenetic modifications similar to those of neoplastic cells.

ER $\beta$  has recently been implicated in epigenetic control of estrogen responsive genes. Expression of *Glut-4* in MEF cells required the interaction between ER $\beta$  and the *Glut-4* promoter, which prevented methylation of CpG 11 and therefore allowed the recruitment of SP1 to this region and activation of transcription.<sup>65</sup> Epigenetic-mediated neoplastic transition following estrogen exposure has been clearly demonstrated.<sup>66,67</sup> Progression of pre-cancerous lesions provoked by estrogen exposure in neoplastic lesions required continuous exposure to estrogen in ACI rats. Indeed, removal of estrogen treatment after 4 weeks followed by 8 weeks of recovery induced a regression of hyperplasia in conjunction with modifications in Dnmts expression.<sup>56</sup>

Epigenetic mechanisms implicated in ER target gene silencing seem highly variable and require different co-repressors. Indeed, CTCF (CCCTC-binding factor) recruitment on the *CDKN1c* promoter following estrogen stimulation is implicated in *CDKN1c* silencing.<sup>68</sup> LCoR, a repressor able to bind to ligand-associated receptors to repress their transcriptional activity also interacts with nuclear HDAC6 and attenuates specific ER target genes, including *IGFBP4*, but not *TFE1* in BC cells. Moreover, the LCoR/CtBP1 repressor complex interacts with HDAC1 and ER on *TFE1* and other estrogen responsive promoters.<sup>69,70</sup> Malik et al. recently demonstrated a cooperation between HDAC7/FoxA1/ER $\alpha$  in *RPRM* repression.<sup>71</sup> Nevertheless, HDAC7-mediated gene silencing was not related to the weak HDAC activity of HDAC7 but rather to additional properties of this protein. This complex was recruited to both proximal and distal *RPRM* promoters. HDAC1/PADI4 (peptidylarginine deiminase IV) interaction was also associated with *TFE1* silencing. Indeed, this complex provoked H3R deimination, resulting in either a blockade of H3R methylation or a demethylation of monomethylated H3Rme and therefore inhibited the addition of the positive transcriptional mark H3Rme2, normally processed by CARM1 or PRMT1 (H3R17me and H4R3me). On the other hand, CARM1-mediated H3Rme2 blocked H3R deimination and allowed the recruitment of ER $\alpha$  to the active *TFE1* promoter.<sup>72</sup> Sin3A is frequently associated with HDAC1/2 in the Sin3 repressor complex and can also be involved in ER-mediated gene silencing (including *ESR1*) via its multiple interactions with both additional repressors and ER $\alpha$ .<sup>27</sup> Similarly, MTA1 (metastasis associated antigen 1), the expression of which correlates with BC progression, can also bind HDAC1/2 and ER $\alpha$  and participate in ER-mediated gene silencing such as *BRCA1* silencing.<sup>73</sup> BRCA1 was associated with a repression of a subset of estrogen responsive genes in 293T cells while its overexpression induces an almost 90% decrease in ER target gene expression, including *TFE1*, in MCF7 cells. This inhibition required a direct interaction between active ER $\alpha$  and BRCA1 (aa 338–379 of ER $\alpha$ ) and their co-recruitment on ERE, which blocked further ER $\alpha$

recognition by co-activators such as p300.<sup>74,75</sup> Increasing concentrations of estrogen or overexpression of cyclin D1, which antagonizes and excludes BRCA1, induces estrogen responsive genes. cyclin D1 is frequently overexpressed in BC and its interaction with ER $\alpha$  increases p160 recruitment and promote estrogen signaling. A strong correlation between FoxA1 and ER $\alpha$  recruitment on activated/silenced genes after estrogen exposure was reported following high scale ChIP analysis, suggesting a cooperation between TF and ER $\alpha$  not only in gene activation but also in their silencing. Co-recruitment, however, seemed limited to 7% of ER bound promoters.<sup>76,77</sup> Epigenetic mechanisms governing ER $\alpha$  target genes are summarized in Figure 3.

## Conclusion

Studies on ER $\alpha$  target gene regulation have introduced a new degree of complexity by reporting cycling of active/repressive states of the *TFF1* promoter.<sup>78</sup> A combination of interactions between ER $\alpha$  and HAT, HDAC, HMT, MDT, co-activators, co-repressors, TFs and RNAP II reveals a complex histone code that regulates competent or transcriptionally engaged *TFF1* and *CathepsinD* promoters with periodic waves of transcription interrupted by clearing of TFs from promoters.<sup>79</sup> Recently, a dynamic process of DNA methylation was also reported to be involved in the control of the cyclic expression of ER $\alpha$  target genes. Cycled methylation/demethylation of CpG in the *pTFF1* and *Wisp-2* promoters following estrogen stimulation revealed the importance of Dnmts control on estrogen dependent gene expression.<sup>80</sup> Each cycle, corresponding to an active transcription time, was determined by, first a demethylation of CpGs

catalyzed by Dnmt3a/Dnmt3b associated with TDG (thymine DNA glycosylase) complex, and a remethylation and gene silencing assumed by Dnmt1/Dnmt3a/Dnmt3b, in collaboration with NuRD complex. The recruitment of TDG via a direct interaction with the AF2 domain of ER $\alpha$  might occur in some specific targets. TDG is likely implicated in the co-recruitment of other co-activators for its glycosylase activity, as a TDG mutant, incapable of DNA repair, still increased ER responsive gene transcription.<sup>81</sup> Putative interactions between ER and Dnmts have also been reported.<sup>82,83</sup> As methylation and demethylation are cyclic, global methylation status on these promoters is conserved. Interestingly, as discussed above, ER $\beta$  seems implicated in the establishment of new and stable methylation.<sup>84</sup> All of these results provide strong evidence that estrogen target gene expression is tightly regulated by multiple and highly dynamic machineries implicating ERs, co-activators and co-repressors in a classical and epigenetic manner.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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