PRMT1 Is Critical for the Transcriptional Activity and the Stability of the Progesterone Receptor

HIGHLIGHTS
The progesterone receptor (PR) is methylated by PRMT1 upon progestin treatment.
PRMT1-dependent methylation controls the stability of PR.
PRMT1 activates genes involved in the regulation of cell migration and invasion.
PRMT1 expression influences the survival of patients with PR-positive breast cancer.
PRMT1 Is Critical for the Transcriptional Activity and the Stability of the Progesterone Receptor

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SUMMARY

The progesterone receptor (PR) is an inducible transcription factor that plays critical roles in female reproductive processes and in several aspects of breast cancer tumorigenesis. Our report describes the type I protein arginine methyltransferase 1 (PRMT1) as a cofactor controlling progesterone pathway, through the direct methylation of PR. Mechanistic assays in breast cancer cells indicate that PRMT1 methylates PR at the arginine 637 and reduces the stability of the receptor, thereby accelerating its recycling and finally its transcriptional activity. Depletion of PRMT1 decreases the expression of a subset of progesterone-inducible genes, controlling breast cancer cells proliferation and migration. Consistently, Kaplan-Meier analysis revealed that low expression of PRMT1 predicts a longer survival among the subgroup with high PR. Our study highlights PR methylation as a molecular switch adapting the transcription requirement of breast cells during tumorigenesis.

INTRODUCTION

The progesterone receptor (PR) is a member of the nuclear hormone receptor family of ligand-dependent transcription factors (Mangelsdorf et al., 1995). Acting through its cognate steroid hormone progesterone, PR regulates the expression of gene networks to control development, differentiation, and proliferation of female reproductive tissues during the ovarian cycle and pregnancy (Brisken and O’Malley, 2010). Furthermore, numerous studies have established that PR is an important regulator of several aspects of breast cancer tumorigenesis and progression, including cell migration and invasiveness (Gnim et al., 2016; Knutson and Lange, 2014). Two major isoforms of PR exist across species, the longer PR-B and the shorter PR-A, which differ in promoter usage (Kastner et al., 1990). They are differentially expressed and exhibit distinct functions in vivo; PR-A is more responsible for progesterone actions in uterus and ovary, whereas PR-B is required for mammary gland development (Mulac-Jericevic et al., 2003), mediating the proliferative actions of progestins (Boonyaratanakornkit et al., 2001; Faire and Lange, 2007).

PR activity is regulated by extensive post-translational modifications (PTMs) that include phosphorylation, acetylation, ubiquitination, SUMOylation, and methylation (reviewed in Abdel-Hafiz and Horwitz, 2014; Knutson and Lange, 2014). These modifications affect PR hormone sensitivity, subcellular localization, protein stability, or interactions with cofactors. For example, progesterone stimulation induces MAPK-dependent phosphorylation of its receptor on the serine 294 (Ser-294 or S294), a key modification essential for the activation and the enhancement of PR transcriptional activity. Paradoxically, this event is also a signal for the ligand-dependent degradation of the receptor, essential for its regulated transcriptional functions (Lange et al., 2000; Shen et al., 2001).

The molecular mechanisms underlying PR-dependent transcription have been extensively studied, both in the absence of, and upon exposure to progesterone (Beato and Vicent, 2012; Jacobsen and Horwitz, 2012). These studies converge to a simplified model of gene expression induced by progestins: before hormonal treatment, PR binds genomic sites within a repressive complex maintaining these genes silenced prior to hormone treatment (Vicent et al., 2013). Progestin stimulation leads to the activation of cytoplasmic downstream kinase cascades, including ERK1/2, and finally to PR phosphorylation, especially on Ser-294 (Migliaccio et al., 1998). This active phosphorylated form of PR, associated with kinases, induces the
Figure 1. PRMT1 Interacts with PR in R5020-Stimulated T47D Breast Cancer Cells

(A) GST pull-down experiment. ^35S-labeled in vitro translated PR-B, and ERα used as a positive control, were incubated with GST and GST-PRMT1 bound to glutathione Sepharose beads. The eluted proteins were analyzed by SDS-PAGE and visualized by autoradiography. Autoradiograph (upper) and Coomassie staining (lower) are shown.

(B) Whole-cell extracts (WCE) of T47D were subjected to immunoprecipitation (IP) using anti-PRMT1 antibody, or control IgG, and immunoblotted (IB) with anti-PR antibody.

(C) Proximity ligation assay (PLA) was used to detect the endogenous interaction between PRMT1 and PR in T47D cells, using anti-PR and anti-PRMT1 antibodies. T47D cells were transfected with control siRNA (siCT) or with anti-PRMT1 siRNAs (siPRMT1) and were cultured in medium deprived of steroids for

| R5020 (Time) | 0 | 15' | 1h | 3h | 6h |
|-------------|---|-----|----|----|----|
| siCT        |   |     |    |    |    |
| siPRMT1     |   |     |    |    |    |

**PR - PRMT1**

- **Bar Graph**: Comparison of PR expression levels after R5020 treatment with or without siRNA.

- **Graph**: Quantitative analysis of PR expression levels over time with significant differences indicated.

**Table**: Summary of PRMT1 knockdown effects on PR expression.

**Figure**: Visual representation of PRMT1 interaction with PR in T47D cells under R5020 stimulation conditions.
recruitment of histone-modifying enzymes. This is followed by a local chromatin remodeling and the assembly of the transcription initiation complex on progestin-regulated genes (Beato and Vicent, 2012).

Protein arginine methyltransferases (PRMTs) are one such class of histone-modifying enzymes that regulate transcription. Two primary transcriptional coactivators in this family are PRMT1 and CARM1 (Coactivator Associated arginine (R) Methyltransferase 1), recruited as coregulators on promoters of genes targeted by nuclear receptors (Koh et al., 2001; Wang et al., 2001). PRMT1 is the predominant asymmetric arginine methyltransferase in humans and functions as a general transcriptional coactivator, by depositing dimethylarginines on histone 4 (H4R3me2as). However, PRMT1 also dimethylates a large variety of non-histone substrates, regulating many cellular processes required for tissue homeostasis, including RNA processing, transcriptional regulation, signal transduction, DNA repair, and protein-protein interactions (Bedford and Clarke, 2009; Blanc and Richard, 2017).

PRMT1 Interacts with the Progesterone Receptor in T47D Cancer Cells

RESULTS

PRMT1 Interacts with the Progesterone Receptor in T47D Cancer Cells

To test whether PR might be regulated by arginine methylation, we initially examined a physical association between PR and PRMT1 in vitro and in T47D mammary carcinoma cells, which contain constitutive high levels of PR (Smith et al., 2017). Using a GST-binding assay, we showed the direct binding between PR and PRMT1 (Figure 1A). When T47D cell extracts were immunoprecipitated using an anti-PRMT1 antibody, coimmunoprecipitation (coIP) of both PR isoforms were observed (Figure 1B). Given the functional specificity of PR-B in breast (Boonyaratanakornkit et al., 2001; Faivre and Lange, 2007), we focused our study essentially on this isoform (called PR). As PR is a ligand-regulated nuclear transcription factor, we investigated whether the PR-PRMT1 interaction was hormone dependent and in which cellular compartment it occurred. For that, T47D cells were starved in medium deprived of steroids for 48 h (time 0) prior to the treatment for the indicated times with R5020 (also known as Promegestone), a synthetic agonist of progesterone used in scientific studies because of its stability (Read et al., 1988; Vignon et al., 1983). To localize and quantify these interactions more precisely, we used the in situ proximity ligation assay (PLA) (Pouard et al., 2014; Soderberg et al., 2006). The presence of red dots indicates interactions between endogenous PR and PRMT1 that occurred mainly in the nucleus and varied during the course of R5020 induction (Figure 1C). The graph representing the counting of dots per 100 cells indicated a high number of interactions between the two proteins in the absence of hormonal induction (Figure 1C, lower left panel). Notably, 15 min of R5020 treatment engendered a significant reduction in the signal abundancy, reflecting the dissociation of the PR-PRMT1 complex; then a second interaction peak was detected after 1 h of treatment (Figure 1C, lower left panel). A strong decrease in dot numbers was observed when the expression of PRMT1 or PR was knocked down using a pool of siRNAs, compared with mock T47D cells transfected with scramble siRNA (siCT) (Figure 1C, lower right panel and Figures S1A–S1C), validating the specificity of the PR-PRMT1 interaction, which is nuclear, dynamic, and progesterone-regulated.

PRMT1 Methylates PR at a Conserved Site In Vitro and in Cells

We then investigated whether PR was methylated in T47D cells using two complementary approaches. First, extracts from V5-tagged PR T47D-transfected cells stimulated with R5020 for 1 h were
immunoprecipitated using a pan-methylarginine antibody against asymmetric dimethyl-arginine (adme-R, note pan-methyl-R), the type of methylation that marks PRMT1 deposits, and probed with anti-V5 antibody. We found that PR was dimethylated in cells mostly after hormonal treatment (Figure 2A). To confirm, we used PLA analysis with anti-PR and anti-pan-methyl-R antibodies to detect asymmetrically dimethylated forms of PR (Poulard et al., 2019). We observed a nuclear signal, increased after R5020 stimulation in T47D cells (Figure 2B). The methylation signal was significantly decreased after PR knockdown and in presence of MS 023, a selective inhibitor of type I PRMT-dependent methylation (Eram et al., 2016), validating the signal specificity (Figure 2B). The effect and the optimal concentration of this inhibitor on PRMT1 activity was validated on H4R3 dimethylation, a known target of PRMT1, by immunofluorescence (IF) and immunoblot (IB), and the concentration of 60 nM was selected for our experiments (Figures S2A and S2B). Collectively, these data identify PR as a potential novel substrate for PRMT1.

Then, we performed in vitro methylation assays using the purified GST-tagged PR fragments illustrated in Figure S2C, incubated with recombinant GST-PRMT1 (Figure S2D), in order to identify the region(s) of PR that are methylated by PRMT1. Among the various functional domains of PR, PRMT1 specifically dimethylated only the fragments 3 and 4, spanning in the DNA-binding domain (DBD) and the hinge region, suggesting that arginine methylation by PRMT1 mainly occurs in the 587–687 amino acids region (Figures S2C and S2D). Of interest, this region encompasses the C-terminal extension (CTE) region, previously described as a dynamic region involved in DNA binding, nuclear localization, interaction with coregulators of the receptor (Daniel et al., 2010; Hill et al., 2009), as well as a hot spot for PTMs, including the PRMT1-dependent methylation of ERα (Le Romancer et al., 2008). Sequence alignment of steroid receptor CTEs showed a conserved position for the arginine 637 (Arg-637 or R637) residue, within a GGR motif that PRMT1 preferentially targets, localized in the PR-3 fragment (Figure 2C). To assess whether this motif was a direct target for the enzyme, we mutated the R637 to alanine residue (R637A) into the GST-PR3 fragment, leading to a complete loss of the methylation signal in vitro (Figure 2D).

Further analyses were conducted in the context of full-length PR in T47D cells. For in vivo studies, the R637 was substituted to lysine (R637K) in order to preserve its positive charge. The wild-type (PR(B)-WT) and mutated versions (PR(B)-R637K) of V5-tagged PR were expressed into T47D cells, immunoprecipitated with the pan-methyl-R antibody, and revealed with anti-V5 antibody. Figure 2E shows that PR methylation was induced after hormonal stimulation. The R637K mutation strongly impaired PR modification, confirming this residue as the principal site of arginine methylation on PR.

We therefore focused on PR-R637 methylation and analyzed its role in progesterone signaling. We generated an antibody recognizing the asymmetric dimethylation of PR on R637 (R637me2(as), named anti-met-R637-PR). As an epitope, we used a branched peptide that contains the asymmetric dimethyl arginine (Figure 2F). The functionality and specificity of the antibody were established using several approaches in vitro and in cells. By western blot analysis, the anti-met-R637-PR specifically detected only the in vitro
PRMT1 Acts as a PR Coregulator in Breast Cancer Cells

Because PRMT1 has been previously described as a coactivator of several nuclear receptors (Koh et al., 2001; Stallcup et al., 2000), we addressed its role in regulating the transcriptional activity of PR. A
Figure 4. PRMT1 Acts as a PR Coregulator in Breast Cancer Cells

(A) HeLa cells were transfected with MMTV-LUC reporter plasmid and expression vectors encoding PR and wild-type or catalytic-mutant PRMT1 (E/Q). After R5020 treatment (10 nM), cell extracts were tested for luciferase activity using the Promega luciferase assay kit. The p value was determined using the Student’s t test: * indicates p \leq 0.05.

(B–D) Starved T47D cells untreated (Eth) or treated with R5020 (10 nM for 1 h) were collected and subjected to chromatin immunoprecipitation (ChIP) with (B) anti-PRMT1, (C) anti-PR, and (D) anti-H3K4me3 antibodies, or control IgG. The precipitated DNA fragments were used for qPCR analysis using specific primers with respect to the input DNA and normalized to a reference locus (human chromosome 1 gene). The p value was calculated using a paired t test: * indicates p \leq 0.05 and **p \leq 0.01.
luciferase-based transcription assay established that PRMT1 enhanced reporter gene activity of PR and that its enzymatic function was required for this effect (Figure 4A). We next asked whether PRMT1 was recruited to some PR-binding sites, identified by genome-wide mapping of, in T47D cells stimulated with R5020 (Ballaré et al., 2013; Kougioumtzi et al., 2014). Chromatin immunoprecipitation experiments followed by qPCR analysis (ChIP-qPCR) were performed in T47D cells stimulated with R5020 (or vehicle ethanol) for 1 h. Figures 4B–4D illustrate the recruitment of PRMT1, as well as PR and the activation mark H3K4me3, to chromatin binding sites of three endogenous well-characterized progesterone target genes, EGFR, STAT5, and FKBP5 (Ballaré et al., 2013; Vicent et al., 2013). To analyze the expression of these genes, starved T47D cells were treated with R5020 and mRNAs were collected several times after the treatment.

By quantitative real-time RT-PCR (RT-qPCR), we found a robust mRNA expression at 6 h (Figure S3A), and we chose this time for the remaining experiments. To determine whether PRMT1 was a coregulator of endogenous PR, we knocked down PRMT1 in T47D cells using a pool of three specific siRNAs and examined the expression of these PR target genes. RT-qPCR measurements revealed that, compared with the untreated cells, PRMT1 depletion differentially affected the expression of these PR-target genes: EGFR gene expression was decreased, STAT5 mRNA level was increased, whereas FKBP5 expression was not significantly changed (Figure 4E). As such, the impact of PRMT1 on the PR-dependent transcription appears to be gene specific. We confirmed the effect of PRMT1 depletion on the PR-dependent transcription in a second PR-positive breast cancer cell line, ZR-75 (Figures S3B and S3C).

**PRMT1 Is Required for Progestin-Induced Expression of PR Target Genes Regulating Cell Growth and Migration and Predicts Poor Survival in Patients with Breast Cancer**

To address the relevance and the functional significance of the above results, we performed RNA sequencing (RNA-seq) analyses using PRMT1-depleted T47D cells by siRNAs, stimulated 6 h with R5020 treatment (Figure 5A, left panel). The efficacy of hormonal treatment and PRMT1 knockdown were confirmed by IB and RT-qPCR (Figures S4A–S4C). RNA-seq results indicated that, among the 795 genes activated after hormonal exposure (Figure 5A, right panel, pink color), 235 genes were impacted by siPRMT1 (about 30% of the total R5020-regulated genes) (Figure 5A, red color). Among those, 64% of genes were downregulated when PRMT1 expression was decreased, indicating that their R5020-induced activation required PRMT1 (PRMT1-activated genes). Conversely, 36% are characterized as PRMT1-repressed genes (Figure 5B). RT-qPCR analysis of genes randomly selected from the list of PRMT1-dependent genes confirmed that PRMT1 is required for their R5020-induced transcription (Figure S4D). Among the 235 genes regulated by R5020 and PRMT1, we analyzed EGFR, EGR1, SGK1, and CD44, which are functionally connected and described for their roles in the regulation of mammary epithelium differentiation under normal physiology and in cell migration and invasion during breast cancer progression (Figure S4E) (Kovacevic et al., 2016; Menezes et al., 2017). RT-qPCR and IB analysis confirmed that depletion of PRMT1 significantly reduced the R5020-dependent expression of these key targets (Figures 4E and 5C). Of interest, PRMT1 knockdown also inhibited the phosphorylation of PDK1 and p38 MAPK, two enzymes involved in the activation of SGK1 (Liu et al., 2019), leading to the activation of downstream targets, such as NDRG1 (Castel et al., 2016; Godbole et al., 2018; McCaig et al., 2011) (Figures 5D and S4F). To rule out the possibility of off-target effects and further confirm the specificity of the PRMT1 silencing, we expressed a tagged form of rat PRMT1 cDNA (flag-PRMT1, resistant to siRNAs silencing) in PRMT1-silenced cells. In this rescue experiment, we found that the expression of PRMT1 restored the expression of previously tested genes affected by PRMT1 knockdown, almost to the levels of control cells (Figures 5E, S4G, and S4H). Altogether, these data strongly indicate that PRMT1 silencing is specific and reversible.

Moreover, a pathway analysis of the 235 differentially regulated genes obtained by RNA-seq (list given in Table S1) revealed an enrichment of genes involved in cell movement, morphology, and proliferation (Figure 6A). Consistently, depletion of PRMT1 significantly reduced the proliferation and the cellular migration of R5020-stimulated T47D cells, compared with the vehicle (eth)-treated cells, both analyzed with Incucyte technology (Figures 6B, 6C, and S5A–S5C). To support these phenotypes, we then investigated the...
correlation between PR and PRMT1 expression and patient survival, in a cohort of 1,764 breast tumors expression, by Kaplan-Meier survival analysis (Gyorffy et al., 2010). After confirming that high-PR expression tumors depicted a better relapse-free survival (RFS) in this cohort, compared with low-PR expression ones (Figure 6Da), patients were separated into two groups according to PRMT1 expression (low and high PRMT1 following the median). Interestingly, patients with high expression of PR but low expression of PRMT1 reflected a significantly longer RFS ($p = 1 \times 10^{-11}$) (Figure 6Db). Similar observations were done with overall (OS) ($p = 0.0012$) and distal metastasis-free survival (DMFS) ($p = 1 \times 10^{-9}$) (Figures S5Db and S5Eb). Collectively, these results underscore that, in PR-positive tumors, low level of PRMT1, causing a decreased expression of some PR target genes including EGFR and EGR1, can inhibit cell proliferation and migration in hormonally responsive breast cancer, leading to a better survival outcome for patients (Figures 6D, S5D, and S5E).

**Loss of PR Methylation at R637 Affects PR Degradation and T47D Cell Proliferation**

To directly investigate the function of PRMT1-dependant PR methylation in cells, we used the CRISPR-Cas9 technology in T47D cells. We first knocked out endogenous PR, and the characterization of representative PR KO clones (T47D PRKO) is illustrated in Figures S6A and S6B. Next, we stably re-expressed the wild-type (WT) and mutated (R637K) forms of PR-B in the T47D PRKO cells and named these derived cell lines T47DWT and T47DR637K, respectively. The cellular localization of WT- and R637K-PR proteins was analyzed by immunofluorescence using an anti-PR antibody (Figure 7A). We showed that, in both T47DWT and T47DR637K cell lines, the receptor was correctly localized in the nucleus (Figure 7A). Importantly, we also confirmed that PR was dimethylated after hormonal treatment in T47DWT cells, whereas in T47DR637K cells, the methylation signal was strongly reduced (Figure 7B). Together, these results confirm not only that these cell lines are functionally comparable with the native T47D cells but also that the R637 is the major methylated residue of PR.

Using these two cell lines, we analyzed the impact of the R637K mutation on progesterone signaling. As expected, treatment of T47DWT and T47DR637K cells with R5020 induced a rapid and transient activation of ERK kinases (Figure 7C). However, as observed after PRMT1 depletion, the mutation affected ERK activation, even if to a lesser extent (see Figure 3A). As with PRMT1 inhibition, loss of PR methylation at R637 resulted in a higher basal PR protein level, which appeared stable after R5020 stimulation (Figures 7A–7C). Cycloheximide treatment showed that more than 90% of PR-R637K was still present after 6 h of R5020 exposure, compared with less than 50% in T47DWT cells (Figure 7D). The difference in protein level is caused by the shorter half-life of the wild-type PR compared with the mutant, which corresponds to the increased steady-state level of PR-R637K. Thus, PRMT1-mediated methylation seems to participate in the regulation of PR turnover, required for active hormonal-dependent transcription (Métiéver et al., 2003). In line with these results, absence of PR methylation at R637 markedly decreased oncogenic PR functions, leading to reduced cell growth and colony formation of T47DR637K cells, compared with T47DWT cells, when treated with R5020 (Figures 7E, 7F, and S6C). Importantly, expression of some PRMT1-dependent PR-downstream targets identified in Figure 5, as EGFR and EGR1, appeared lower in T47DR637K cells, after progesterone induction (Figures 7G and 7H).
Figure 6. Low PRMT1 Reduces R5020-Induced Proliferation and Migration of T47D Breast Cancer Cells and Predicts Improved Survival of Patients with Breast Cancer

(A) Ingenuity Pathway Analysis (IPA) of cellular functions for the 235 R5020-regulated genes dependent on PRMT1. The orange vertical line represents the fold of statistical significance.

(B) Analysis of T47D cell proliferation by Incucyte technology. Cells expressing the indicated siRNAs (PRMT1 or CT) were stimulated with R5020 (10 nM) every 48 h for 7 days. Image acquisition was conducted every hour using the Incucyte software, which calculates the percentage of cell confluence according to time over 7 days. Results are represented as a graph showing the proliferation rate every 24 h.

(C) Migration of T47D cells expressing the indicated siRNAs (PRMT1 or CT) and treated with R5020 (10 nM) for 12, 24, and 36 h was analyzed in a wound scratch assay with the Incucyte Live-Cell Imaging System and dedicated software (Essen Bioscience), as reported in the Transparent Methods section. Left panel: The bar plots indicate the cellular migration rate, with a direct comparison between control and PRMT1-depleted cells. Right panel: Images of cells 24 h after the scratch wound. The blue line corresponds to the initial area of the wound. White arrows indicate cell migration areas. Both (B) and (C) graphs show the mean ± SD of one experiment representative of three. The p value was determined using the Student’s t test: * indicates p < 0.05, **p ≤ 0.01, and ***p ≤ 0.001.

(D) Kaplan-Meier estimates relapse-free survival in patients, in GEO, EGA, TCGA datasets, with low (black) or high (red) PR expression, as indicated using KM-plotter in a cohort of 1,764 breast tumors (a), or stratified in 2 groups following low-PRMT1 (b) or high-PRMT1 expression (c).
Figure 7. Inhibiting PR Methylation Affects Breast Cancer Cell Proliferation and PR Stability

(A) Immunofluorescence of T47D PRKO, T47D WT, and T47D R637K cells, before and after stimulation with 10 nM of R5020, stained with anti-PR antibody. The nuclei were counterstained with DAPI (blue) (Obj: X40).

(B) WCE of T47D WT or T47D R637K, stimulated with R5020 (10 nM) for 1 h, were used for IP with anti-PR antibody or control IgG and immunoblotted with pan-meth-R and PR antibodies.

(C) WCE from T47DWT and T47D R637K cells stimulated with R5020 for the indicated times were immunoblotted.

(D) Half-life of the endogenous PR in T47D WT and T47D R637K cells. Left panel: WCE from T47D WT and T47D R637K cells treated with cycloheximide before the stimulation with R5020 were immunoblotted with the indicated antibodies. The amount of PR was quantified by densitometry; two different expositions are shown to well quantify the PR band intensity.
Interestingly, the loss of R637 methylation consequences observed under progesterone treatment are similar to those obtained with PRMT1-knockdown cells, suggesting that PRMT1 effects on the progesterone pathway occur, at least in part, through the methylation of PR.

**DISCUSSION**

Our study highlights the direct and functional cross talk between PRMT1, arginine methylation, and progesterone signaling, uncovering the molecular mechanisms by which PRMT1 functions as an important modulator of the progesterone response pathway. In the context of hormonal activation, we demonstrated that PRMT1 is recruited on PR-associated chromatin regions of some progestin-regulated genes. RNA-seq data support these results, showing that PRMT1 knockdown in breast cancer cells can affect the expression of thousand progesterone-targeted genes, positively or negatively. Moreover, PRMT1-regulated genes are involved in relevant cell functions including cell movement, morphology, and proliferation. We notably showed that PRMT1 positively regulates the expression of a subset of genes involved in the regulation of cell migration and invasion during breast cancer progression. Consistently, silencing of PRMT1 in T47D cells leads to a significant reduced ability to proliferate and migrate, supporting the involvement of PRMT1 in breast tumorigenesis. Therefore, it is possible that high-level expression of PRMT1 facilitates oncogenesis by providing tumor cells with a survival advantage, in part by enhancing the progestin-dependent receptor degradation and thereby maintaining cells in a proliferative mode. In line with this idea, the analysis of PRMT1 prognostic value in patients with breast cancer showed that high expression of PRMT1 associated with high expression of PR predicts poor relapse-free (RFS), overall (OS), and distal metastasis-free survival (DMFS). This suggests that PRMT1 expression influences the survival of patients with PR-positive breast cancer and that the prognostic value and pathophysiological role of PR depend on PRMT1 expression.

Many studies have already revealed the impact of PRMT1 in breast tumorigenesis (Liu et al., 2019; Morettin et al., 2015). Its expression is often upregulated in tumor samples compared with adjacent normal tissue. Moreover, these studies have highlighted different mechanisms by which PRMT1 regulates the proliferation of tumor cells (regulation of the epithelio-mesenchymal transition EMT, sensitization of cells to a therapy, etc.) (Yang and Bedford, 2013). Here, we report that the direct methylation of the progesterone receptor, a key driver of breast cells proliferation, affects breast cancer progression. Since PRMT1 enhances the transcripational activity of PR (Figure 4A), we can consider that PRMT1-mediated transcription is mainly due to direct methylation of PR, leading to increased transcription via the tight control of PR turnover. This is supported by the biological consequences of PR methylation at R637 under physiological conditions, using the T47DWT and T47D R637K cell lines, engineered to stably express the wild-type and mutant forms of PR-B in PRKO T47D cells. We established that the effects of the methylation loss at R637 are consistent with the results obtained in PRMT1 knocked-down cells: T47D R637K cells display decreased oncogenic PR functions, such as a retarded cell growth and a reduced expression of some PRMT1-dependent PR downstream targets identified by RNA-seq (Figures 5 and 7). Moreover, the methylation of R637 directly affects PR stability (Figure 7), suggesting that PRMT1 effects on progesterone pathway occur, at least in part, through the methylation of PR on this conserved residue. Notably, the arginine R637 is located within the hinge region of the steroid receptor, which contains sites for posttranslational modifications, like phosphorylation and acetylation. Precisely, this conserved R637 flanks an acetylation consensus site, in which three lysine residues K638-640-641 are modified following progesterone stimulation (Daniel et al., 2010). It is interesting to note that non-acetylatable mutants (PRK-A) exhibit defective transcriptional activation and are more stable than wild-type receptors, namely, a phenotype similar to the non-methylable mutant PRR637K (Figure 7). Thus, it could exist as a functional communication...
between these two modifications, as we currently observed with the histone tails on chromatin (Bannister and Kouzarides, 2011).

In response to ligand binding, MAPK activation modulates PR activity by phosphorylating the receptor on serine 294 (Lange et al., 2000; Shen et al., 2001). This modification is crucial for PR nuclear activity, priming the receptor for robust transcriptional activation, also influencing its promoter selectivity (Daniel et al., 2009). Our report demonstrates that progestin treatment induces the recruitment of PRMT1 on several PR-target genes, implying that PRMT1 acts as a coregulator of PR. Furthermore, we also describe that PRMT1 directly dimethylates PR under progestin treatment, primarily at the conserved R637 residue on an RGG methylation consensus motif, in vitro and in vivo (Figures 2D–2I). This methylation occurs in the nucleus and facilitates PR degradation, which in turn speeds up its transcriptional properties. Previous studies have demonstrated the critical role of PR degradation/re-synthesis in the active transcription of the receptor (Dennis et al., 2005). Indeed, the degradation constitutes a stimulatory switch that accelerates the recycling of receptors from pre-initiation complexes, required for active hormonal-dependent transcription (Mé tivier et al., 2003). Our results show that this mechanism involves the asymmetric dimethylation of PR by PRMT1, which reduces PR stability by affecting the proteasome machinery, thereby accelerating its transcriptional activity. It is tempting to speculate that the effects of PRMT1 on PR degradation/stability can be mediated by a cross talk with PR ubiquitination. Interestingly, recent data have shown that the RNA-binding protein RBM15 is methylated by PRMT1, which triggers its ubiquitination and degradation by the E3 ligase CNOT4 (Zhang et al., 2015). CNOT4 is a subunit of the CCR4-NOT complex (Albert et al., 2002), and we previously showed that PRMT1 physically interacts with the CCR4-NOT complex, regulating its methyltransferase activity (Chapat et al., 2017; Robin-Lespinasse et al., 2007). In future studies, it will be interesting to investigate whether PRMT1-dependent methylation of PR induces its degradation through the recruitment of the E3 ligase CNOT4.

In conclusion, our findings reveal important insights linking PRMT1-dependent arginine methylation to the maintenance of the balance between transcriptional activity and degradation of PR. This result implies that altered methylation of the receptor can induce aberrant cellular response to hormonal stimuli contributing to pathogenesis. However, most of the mechanisms described above are based on in vitro experiments, obtained using a breast cancer cell line with a phenotype particularly useful to dissect the regulatory steps of progesterone signaling, independently of the estrogen receptor (ER\(\alpha\)). Future studies will be needed in preclinical models, notably mouse and patient-derived xenografts or directly in patient samples, to determine the clinical relevance of our in vitro mechanistic findings. In addition, improved knowledge of these mechanisms could have a significant impact on long-term outcome for patients with breast cancer, considering the differential effects of progesterone in breast cancers. In fact, recent studies (Finlay-Schultz et al., 2017; Mohammed et al., 2015) indicate that functional relationships between ER\(\alpha\) and PR signaling occur in ER\(+\)/PR\(\ast\) breast cancers. Indeed, PR can inhibit the growth-promoting functions of estrogen by directly reprogramming chromatin binding of ER\(\alpha\) or indirectly, by reducing the bio-availability of molecules needed for tumor growth. These new findings may provide additional strategies to treat ER\(\alpha\)-positive breast cancer, by modulating the interactions between ER\(\alpha\) and PR. Given that both receptors are regulated by PRMT1 via their direct methylation, and that PRMT1 clearly influences the clinical outcome of breast cancer patients according to PR expression, we speculate that PRMT1 can be involved in the ER\(\alpha\)/PR cross talk, whose mechanistic details are not yet well understood. Therefore, targeting PRMT1-mediated methylation may be a promising strategy for breast cancer treatments; the recent development of pre-clinical small molecules targeting PRMT1 (Fedoriw et al., 2019) may offer a path toward that goal.

**Limitations of the Study**

Our study depicts a new post-translational modification in the DNA-binding domain of the progesterone receptor, the arginine methylation, deposited by the enzyme PRMT1. Although our data highlight PRMT1 as a transcriptional coregulator of a subset of PR-target genes, involved in breast cancer cell proliferation and migration, we were unable to directly link the arginine methylation to this transcriptional effect. In fact, our home-made antibody specifically directed against the methylated form of PR did not give specific signal in chromatin immunoprecipitation (ChIP) experiment. Thus, we actually do not know if the methylated form of the receptor is bound to DNA and if the methylation affects the association between PR and its coregulators. Most of the molecular mechanisms described in this article come from in vitro studies using the PR-positive cell line T47D, which permits to dissect the PR progesterone...
signaling in the presence of progestin only and thus independently of ERα. On the contrary, recent studies have shown that estrogen and progestin have different biological consequences when analyzed individually or when both hormones are present, as these hormonal receptors are functionally interconnected. Therefore, these mechanistic studies need to be validated using more physiological preclinical models, such as mouse and patient-derived xenografts, or directly in patient samples, to assess their clinical relevance.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Laura Corbo (laura.corbo@lyon.unicancer.fr).

Materials Availability

All unique reagents generated in this study (CRISPR cell lines, primers, plasmids) are available from the Lead Contact without restriction. There are restrictions for the availability of the methylated-R637-PR antibody, owing to our inability to produce it and our need to maintain the stock.

Data and Code Availability

RNA-seq data have been submitted to Gene Expression Omnibus (GEO) and are available with the GSE134194 submission number.

Original membranes for all the immunoblots presented in the paper have been deposited to Mendeley Data: [https://doi.org/10.17632/rgctdkyjzx.2].

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101236.

ACKNOWLEDGMENTS

We thank Helene Joly for starting the project and Julien Jacquemetton, Farida Nasri, Maëva Ruel, and Aurore Souef for helpful technical assistance. We are grateful to Dr. Loredana D’Amato, Dr. Samuele Gherardi, and Dr. Romain Teinturier for their valuable advice and discussions. We also thank Prof. Pierre Chambon for kindly providing the pSGSPR-B and MMTV-Luc plasmids, Dr. Catherine Teyssier for the PRMT1 constructs, and Dr. Stéphane Ansieau for the plasmid pPRUpu. We are also grateful to Benjamin Gillet and Sandrine Hughes for performing the RNA sequencing at the IGFL platform in Lyon. A special thanks to Brigitte Manship and Clément Chapat for reading the manuscript. This study was partially supported by the “Ligue Nationale Contre le Cancer,” the “Association pour la Recherche sur le Cancer,” and the association “Odyssea Chambry.” L.M. is supported by a fellowship from the French Ministry of Research and by the “Fondation ARC pour la Recherche sur le Cancer” and C.P. by the “Fondation de France.”

AUTHOR CONTRIBUTIONS

L.C., M.L.R., and L.M. conceived the study, designed the experiments, and analyzed data. L.M. carried out most of the experiments, C.L. assisted with the experiments, I.M. performed the CRISPR experiments, C.P. and F.F. helped L.M. with bioinformatics analysis of RNA-seq data. M.L.R. and I.M. contributed to result discussions and provided material. L.C. coordinated the project. The manuscript was written by L.C., with a strong contribution from L.M.

DECLARATION OF INTERESTS

The authors have declared that no competing interest exist.
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Supplemental Information

PRMT1 Is Critical for the Transcriptional Activity and the Stability of the Progesterone Receptor

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Figure S1, related to Fig. 1 | PRMT1 and PR interact in the nucleus of T47D breast cancer cells.

Validation of the specificity of PRMT1-PR association detected by Ligation Assay (PLA). T47D were grown on coverslips in 12-well plates and transfected with siRNAs control (siCT) or against PR (siPR) or PRMT1 (siPRMT1). PLA was used to detect the cellular interaction of endogenous PRMT1 and PR A. in unstimulated cells or B. after R5020 treatment (1h). The interactions are represented by red dots. The nuclei were counterstained with DAPI (blue) (Obj: X60). Quantification of the number of signals per cell was performed by computer-assisted analysis, as reported in the Transparent Methods section, and is shown in the right panels. The mean ± SD of one experiment representative of three experiments is shown. The p-value was determined using the Student's t-test. ** indicates a $p \leq 0.01$ and *** indicates a $p \leq 0.001$. C. The efficacy of PRMT1- and PR-siRNAs treatments were analyzed by immunoblot.
A. **MS 023**

| kDa | Unl | DMSO | 40µM | 60µM | 80µM | 150µM |
|-----|-----|------|------|------|------|-------|
| 11  |     |      |      |      |      |       |
| 11  |     |      |      |      |      |       |
| 42  |     |      |      |      |      |       |
| 55  |     |      |      |      |      |       |

- H4R3me2(as)
- Total H4
- PRMT1
- Tubulin α

B. **R5020**

|               | DMSO | MS 023 |
|---------------|------|--------|
| Merge         |      |        |
| H4R3me2(as)   |      |        |
| DAPI          |      |        |

C. **N-terminal domain**

- DNA-BD
- Hinge
- Hormone-BD

D. **kDa**

- Tag
- PR-1
- PR-2
- PR-3
- PR-4
- PR-5
- Hinge-E60

E. **Peptide sequences**

- Methyl-peptide (628-640)[M2E2AS]R637: NH2-CQAGMVLLGG(M2e2as)RKFK-CONH2
- Non-met-peptide (628-640): NH2-CQAGVMLGRGKRK-CONH2

F. **R5020**

|               | DMSO | MS 023 |
|---------------|------|--------|
| Merge         |      |        |
| PR            |      |        |
| Met-PR        |      |        |
Figure S2, related to Fig. 2 | PR is methylated on arginine residues in R5020-stimulated T47D breast cancer cells. A-B. Analysis of MS 023 inhibitor specificity. A. Immunoblot of R5020-stimulated T47D cells (1h), treated or not (DMSO) with different quantities of MS 023. Expression of indicated proteins were analyzed. B. Immunofluorescence of T47D cells, treated with 60 nM of MS 023 (or DMSO) and then stimulated 1h with 10 nM of R5020, using anti-H4R3me2(as) primary antibody. The nuclei were counterstained with DAPI (blue) (Obj: X40). C. Schematic representation of human GST-tagged PR fragments used for the in vitro methylation assays. D. An in vitro methylation assay was conducted by incubating different recombinant GST-PR fragments, GST-ER hinge used as a positive control and GST (tag) as a negative control, with recombinant GST-PRMT1, in the presence of [methyl-3H] SAM. Reaction products were analyzed by SDS-PAGE followed by fluorography. The migration and the quality of recombinant GST-fragments used as substrates were verified by a Coomassie-stained SDS-PAGE gel, shown in the right panel. The methylated proteins were visualized by autoradiography. Red stars indicate the methylated fragments of PR. Black star indicates a bacteria associated contaminant, as the signal did not correspond to any detectable fragment by Coomassie blue staining gel, shown in the right panel. E. Dot-blot was performed using increasing amounts of the indicated peptides, asymmetrically methylated or not on the arginine 637, and immunoblotted with the met-R637-PR antibody. Peptide sequences were shown in the upper panel. F. Immunofluorescence assay performed on T47D cells treated with 60 nM of MS 023 inhibitor (or DMSO) and stimulated with 10 nM of R5020 for 1h using the met-R637-PR and anti-PR antibodies. The nuclei were counterstained with DAPI (blue) (Obj: X40).
**Figure S3, related to Fig. 4 | PRMT1 acts as a PR coregulator in breast cancer cells.**

A. RNAs were extracted for T47D cells, stimulated with R5020 for 3h or 6h (or with vehicle ethanol) and the expression of three endogenous PR-target genes was analyzed by RT-qPCR using specific primers. Results shown are mean ± SEM for three independent experiments. The p-value was calculated using a paired $t$-test: ** indicates $p \leq 0.01$ and ***$p \leq 0.001$. B. The expression of the indicated genes was analyzed by RT-qPCR using total RNA extracts from ZR-75 or C. T47D cells, previously transfected with siCT or siPRMT1 and stimulated with R5020 for 6h. The mean ± SEM of, at least, three independent experiments is shown. The $p$-value was calculated using a paired $t$-test: * indicates $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. 
**Figure S4**, related to Fig. 5 | PRMT1 affects the expression of endogenous PR target genes in T47D cells.  
**A-C.** PRMT1 knockdown and hormonal treatment of T47D, stimulated for 6h of R5020 and used for RNA-seq analysis described in fig. 5.  
**A.** RT-qPCR analysis using *PRMT1* specific primers.  
**B.** Immunoblot using the indicated antibodies.  
**C.** RT-qPCR analysis using primers of *FKBP5* and *STAT5*, two well-characterized PR target genes.  
**D.** Genes randomly selected from the list of PRMT1-dependent genes identified by the RNA-seq analysis, were analyzed by RT-qPCR using total RNA extracts from T47D, transfected with either siCT or siPRMT1 and stimulated for 6h of R5020. The mean ± SEM of at least three independent experiments is shown. The *p*-value was calculated using a paired *t*-test: * indicates *p* ≤ 0.05, ** *p* ≤ 0.01 and *** *p* ≤ 0.001.  
**E.** Scheme of the regulation of proteins involved in migration and proliferation pathways dependent on PR, according to the literature.  
**F.** Whole-cell extracts of T47D, depleted or not for PRMT1 using siRNA and treated with R5020 for the indicated times, were collected and subjected to immunoblot analysis using the listed antibodies.  
**G-H.** T47D cells were transfected with siRNAs (control or PRMT1) or co-transfected with siRNA plus a plasmid expressing the flag-tagged rat PRMT1 for 48h, and then treated with 10 nM of R5020 (6h).  
**G.** The expression of endogenous PRMT1 mRNA was analyzed by RT-qPCR using human *PRMT1* primers. Results shown are mean ± SEM for three independent experiments. The *p*-value was calculated using a paired *t*-test: ** indicates *p* ≤ 0.01 and *** *p* ≤ 0.001.  
**H.** The transfected rat PRMT1 was detected by immunoblot using anti-PRMT1 antibody.
Figure S5, related to Fig. 6 | Low PRMT1 reduces R5020-induced proliferation and migration of T47D cells and predicts improved survival of breast cancer patients. **A.** T47D cells were transfected with siCT or siPRMT1 and used for proliferation and migration assays. Whole cell extracts were assessed for PRMT1 inhibition by IB. **B-C.** T47D cells expressing the indicated siRNAs (PRMT1 or CT) were analyzed by IncuCyte technology. **B.** Cells were stimulated with R5020 (10 nM) or vehicle ethanol every 48h for 7 days and their proliferation rate was assessed. Image acquisition was conducted as explained in fig. 6B. **C.** T47D cells were kept unstimulated (vehicle ethanol) and their migration was analyzed in a wound scratch assay with the IncuCyte Live-Cell Imaging System and dedicated software (Essen Bioscience), as reported in the Transparent Methods section. Both **B.** and **C.** graphs show the mean ± SD of one experiment representative of three. The *p*-value was determined using the Student’s t-test: ** indicates *p* ≤ 0.01 and *** *p* ≤ 0.001. **D-E.** Kaplan-Meier estimates **D.** overall survival and **E.** distant metastasis free survival in patients, in GEO, EGA, TCGA datasets with low (black) or high (red) PR expression as indicated using KM-plotter in a cohort of 1764 breast tumors **(a)**, or stratified in 2 groups following low PRMT1 **(b)** or high PRMT1 expression **(c)**.
Figure S6, related to Fig. 7 | Inhibiting PR methylation decreases breast cancer cell proliferation and PR turnover. A. Chromatograms showing DNA sequences from mock and CRISPR-Cas9 cells. We performed genotyping PCRs. The amplified fragments were sequenced using an oligo targeting a sequence inside the fragment and analyzed using CRISP-ID, a web application. This tool allows the detection of the exact indel size and the location of a CRISPR-Cas9 targeted region, based on direct Sanger sequencing (as described in the Transparent Methods section). B. Whole-cell extracts of different clones of T47D PRKO were collected and analyzed by immunoblot for their expression of PR, using PR-antibody, as well as ERα. The clone #2 (underlined) was chosen for stably re-expressing PRWT or PRR637K, as observed in experiences of fig. 7. T47D (PR-positive) cell line was used as control. C. Cell lysates of PRWT and PRR637K, used for cell proliferation test shown in fig. 7E and for the colony growth assay (fig. 7F), were collected and analyzed by immunoblot for their expression of PR-V5.
Transparent Methods

Cell culture and treatments

T47D (ATCC) were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2% penicillin-streptomycin (Life Technologies) and insulin (10 μg/ml). Cos7 and HeLa cells (ATCC) were maintained in DMEM, supplemented with 10% FBS and 2% penicillin-streptomycin (Life Technologies). All cell lines were grown in a humidified atmosphere with 5% CO₂ at 37°C, authenticated by Eurofins and tested for Mycoplasma infection by the MycoAlert Mycoplasma Detection Assay (Lonza, Rockland, ME USA).

Prior to experiments, T47D cells were grown in phenol red-free medium supplemented with 10% charcoal-stripped serum (Biowest). 48h later, medium was replaced by fresh serum-free medium. After 48h in serum-free conditions, cells were treated with 10 nM of R5020 (Perkin Elmer) or an equivalent amount of ethanol vehicle for the indicated time. When indicated, inhibitors (or DMSO vehicle) were added to cells: MS 023 Type I PRMT inhibitor (Tocris) for 48h, at the indicated concentration, cycloheximide (Sigma, 50 μM) or MG132 proteasome inhibitor (Sigma, 10 μM) for 16h or 8h respectively, before R5020 treatment.

Plasmids and constructions

The GST-PR vectors (GST-PR-1, -PR-2, -PR-3, -PR-4, -PR-5 and -PR-3-R637A mutant) were constructed by inserting the cDNA fragments illustrated in Figure S2C, obtained by PCR amplification, into the pET41a vector (Novagen). All of the PR mutants were generated using a QuikChange XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions and the sequences were verified by DNA sequencing. The mammalian expression vector pSG5-PR(B) was a gift from Pr. P. Chambon (Kastner et al., 1990). pSG5V5-PR(B) was obtained by inserting the V5-TAG coding sequence in frame with the coding sequence of PR(B) by PCR.
pSG5V5-PR(B) plasmid was used to generate PR-R637A. To rescue the phenotype of PR$_{KO}$ clones, a guide-resistant mutant of the PR(B) isoform was created (pSG5V5gr) by substituting four nucleotides in the PR guide 1 (G633A, G6364, T639C and G641A) and four nucleotides in the PR guide 2 (C715T, T717A, C718T and G720A) targeting regions. This guide-resistant PR(B) was cloned into the stable mammalian expression vector pPRUpu. pSG5V5gr PR(B) and pPRUpuV5gr-PR(B) were used to obtain the PR-R637K. Sequences of the primers used for the constructions are listed in Table S2. The human HA-PRMT1 and HA-PRMT1(E153Q) in pSG5 vector were a gift of C. Teyssier (Teyssier et al., 2005) and the rat flag-PRMT1 in pSG5 vector was previously described (Robin-Lespinasse et al., 2007).

**Generation of CRISPR cell lines: PR$_{KO}$**

To knockout PR genes in T47D cells, we used the pLCV2 plasmid (a gift from F. Zhang, Addgene plasmid #52961). Oligonucleotides pairs were hybridized and cloned into the LentiCRISPR V2 vector linearized with BsmB1 to generate T47D clones KO: PR Guide#1 – Fwd. CACCgccagttgaacgcttcgcg / Rev. AAACgggagaggtcgtcactgggC; PR Guide #2 – Fwd. CACCgtcgggtgcttcgtgaa / Rev. AAACttcagaagggaccgcagaC, oligonucleotides targeting the regions 624-637 and 693-713 of the PR(B) coding sequence. T47D cells were transfected with the corresponding LentiCRISPR V2 plasmids and selected with blasticidin (5 μg/ml, Invitrogen, Grand Island, NY, USA) for 1 week. Cells were then cloned in 96-well plates by limiting dilution. Isolated clones were characterized by immunoblotting. For five different clones, we confirmed the knockout at DNA and protein levels. We performed genotyping PCRs using a forward primer upstream (5’-GGGGAGTCCAGTGTCGTA-3’) and a reverse primer downstream (5’-ACTTTGCTTTCCACGACG-3’) of the sgRNA cleavage site. The amplified fragments were then sequenced using an oligo targeting a sequence inside the fragment (5’-CCAGAAAAGGACACGGAC-3’) and analyzed using CRISP-ID, a web application that allows the detection of the exact indel size and location of a CRISPR-Cas9 targeted region, based on direct Sanger sequencing (Dehairs et al., 2016).
Generation of two stable cell lines: $T47D_{WT}$ and $T47D_{R637K}$

For the production of the rescue cell lines, CRISPR PR$_{KO}$ cells were transfected with pPRUpuPR(B)-WT and -R637K plasmids using Jetprime (Polyplus-transfection). Screening of stable transfected cells was performed using puromycin dihydrochloride, 1 µg/ml. Stable cell populations, called $T47D_{WT}$ and $T47D_{R637K}$, were maintained in complete RPMI-1640 medium containing 0.5 µg/ml of puromycin.

Generation of methylated-R637-PR antibody (anti-met-R637-PR)

Rabbits were immunized with a peptide corresponding to PR amino acids 628-640 (NH2-CQAGMVLGG([Me2as]R)KFK-CONH2), in which R637 was asymmetrically dimethylated by Covalab (Villeurbanne, France). To purify the met-R637-PR antibody, the dimethyl peptide used for the immunization and the corresponding control peptide (non-methyl) were coupled separately to cyanogen bromide activated agarose beads. The antisera were first bound on the non-methylated peptide column. The unbound antiserum was then applied to the methylated peptide column and eluted. The titre and the specificity of the purified antibody were then tested by enzyme-linked immunosorbent assay (ELISA).

Glutathione transferase (GST) pull-down assay

The GST-PRMT1 and GST-PR fusion proteins were expressed in BL21 competent cells and purified using glutathione-sepharose 4B resin (GE Healthcare Life Sciences). SDS-PAGE and Coomassie staining were used to confirm the integrity of the fusion proteins. PR-B or ERα expression plasmids were transcribed and translated in vitro using T7-coupled reticulocyte lysate in the presence of $^{35}$S methionine. For in vitro protein-protein interaction assays, GST fusion proteins were incubated for 2h at room temperature with 200 µl of binding buffer (Tris 20 mM pH 7.4, NaCl 0.1 M, EDTA 1 mM, glycerol 10%, Igepal 0.25% with 1 mM dithiothreitol and 1% milk) and packed in minicolumns. After washing, the retained proteins were eluted and analyzed on a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.
**In vitro methylation assays**

GST-PRMT1 (Upstate Biotechnology) was incubated with different GST-tagged PR fragments, or with a GST-tagged hinge ERα as a positive control (Le Romancer et al., 2008), in the presence of S-adenosyl-l [methyl-³H] methionine ([³H] SAM 85 Ci/mmol from a 10.4 mM stock solution in dilute HCl/ethanol 9/1 (pH 2.0–2.5); Perkin Elmer) for 90 min at 37°C. Methylation reactions were quenched by the addition of 2x Laemmli sample buffer, heated at 95°C for 5 min and separated on SDS-PAGE. Following electrophoresis, gels were soaked in Amplify fluorographic reagent (Sigma) according to the manufacturer’s instructions and visualized by autoradiography. The cold methylation assays were performed in the same experimental conditions, using 0.5 mM of nonradiolabeled AdoMet (SAM). The reaction mixtures were subjected to SDS-PAGE and then analyzed by western blot using the anti-met-R637-PR (homemade) antibody.

**Dot immunoblot assay**

A peptide corresponding to PR amino acids 628-640 (NH2-CQAGMVLGG([Me2as]R)FKF-CONH2), in which R637 was asymmetrically dimethylated, and the corresponding control peptide (non-methyl), were produced by Covalab (Villeurbanne, France). For dot blot assays, peptides were spotted onto nitrocellulose membrane allowing solution to penetrate (usually 3–4 mm diameter) by applying it slowly as a volume of 1 μL. The membrane was dried and analyzed by immunoblot using the anti-met-R637-PR (homemade) antibody.

**siRNA and plasmid transfection**

siRNA transfections were performed using Lipofectamine 2000 (Invitrogen, Thermofisher) according to the manufacturer’s protocol. After 72h, the down-regulation was analyzed by immunoblot or by RT-qPCR. If requested, after 48h the medium was replaced by fresh medium without serum and cells were treated with R5020 (10 nM) or ethanol for different times.
Plasmid transfections were done using the JetPRIME reagent (Ozyme) according to the manufacturer’s protocol. Cells were analyzed after the indicated times (48h or 72h). SiRNAs pool against PR, PRMT1, PRMT4 and PRMT6 were purchased from Thermofischer Scientific (catalog # AM16708) and the siRNA negative control from Eurogentec (catalog # SR-CL000-00). For the rescue experiments, the plasmid expressing rat-PRMT1 was co-transfected with siRNAs pool against PRMT1 using the JetPRIME reagent (Ozyme) according to the manufacturer’s protocol.

**Luciferase reporter assay**

HeLa cells were plated in 96-well plates the day before transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen) with the indicated plasmids according to the manufacturer’s protocol. After transfection, the cells were grown for 48h in the presence or absence of 10 nM of R5020. Cell lysis and luciferase assays were performed with Promega luciferase assay kit. The results were normalized as indicated and presented as the mean ± SEM of three independent experiments.

**Immunoprecipitation (IP), immunoblot (IB) and antibodies**

After treatment, cells were lysed with RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1 mM ethylenediamine tetra-acetic acid (EDTA), 1% NP-40 and 0.25% deoxycholate) supplemented with protease inhibitors (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM Na3VO4 and 1 mM β-glycerophosphate). For immunoprecipitation (IP), 1 mg of protein extracts were incubated with primary antibodies at 4°C overnight. Protein A-Agorase beads were added in the medium for 2h at 4°C on a rotating wheel. After washing, the immunoprecipitates and their corresponding inputs (30 μg) were denatured by boiling in Laemmlili sample buffer and separated on SDS-PAGE. For immunoblot (IB), the gels were electroblotted onto a PVDF membrane and incubated with primary antibodies overnight, at 4°C. The following day, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulins (Jackson ImmunoResearch) and proteins were visualized by chemiluminescence (Clarity Western ECL Substrate,
BioRad) following the manufacturer's instructions. Quantification of the immunoblot band intensity was performed with Image J software. The primary antibodies used in the study, and their dilutions, are listed in Table S3.

**Cycloheximide chase assay**

$3 \times 10^5$ cells were grown in complete RPMI medium for 24h and then in phenol red-free medium supplemented with 10% charcoal-stripped serum for 48h. 16h before starting the R5020 stimulation, 50 \( \mu \text{g/mL} \) cycloheximide (Sigma) or vehicle DMSO were added. After each R5020-time point, cells were lysed with protein lysis buffer with freshly-added protease inhibitors and analyzed by IB.

**Immunofluorescence (IF)**

T47D cells ($3 \times 10^5$) and T47D$_{WT}$ or T47D$_{R637K}$ stable cells ($2 \times 10^5$) were grown on coverslips in 12-well plates. After chemical treatment(s), cells were fixed in methanol for 2 min and washed twice in PBS. Non-specific binding was blocked using a 1% gelatin solution for 30 min at room temperature. Cells were incubated with the different primary antibodies (listed in Table S3) for 1h at 37°C and then with the secondary antibodies [Alexa Fluor 488 anti-mouse (Jackson ImmunoResearch, Cambridge, UK) (1/2000)]) or Alexa Fluor 568 anti-rabbit (Invitrogen, Carlsbad, USA) (1/1000)] in Dako diluent for 1h. To finish, coverslips were mounted on glass slides in mounting solution (Dako, Carpinteria, CA, USA). The fluorescent slides were viewed with the Nikon Eclipse Ni microscope.

**Proximity ligation assays (PLA), image acquisition and analysis**

PLA assays were performed to visualize protein/protein interactions *in situ*, using the Duolink kit (Sigma) according to the manufacturer’s instructions. Cells ($3 \times 10^5$) were grown on coverslips in 12-well plates and treated (as explained in figures and legends), before fixation with methanol for 2 min. After saturation in the blocking solution, seeded cells were incubated with different pairs of primary antibodies at 37°C for 1h. The PLA probes consisting of secondary antibodies conjugated with
complementary oligonucleotides were then incubated for the same conditions. The step of nucleotides ligation (30 min at 37°C) is followed by the amplification phase, for 100 min at 37°C in a dark and humidified chamber. At the end, coverslips were mounted on glass slides in mounting solution (Dako, Carpinteria, CA, USA) and were analyzed under fluorescence microscopy on a Nikon Eclipse Ni microscope. Images were acquired under identical conditions at 60X magnification. Image acquisition was performed by imaging DAPI staining at a fixed Z-Position, while a Z-stack of ± 5 μm at 1 μm intervals was carried out. The final image was stacked to a single level before further quantification. On each sample, at least one hundred cells were counted. Analysis and quantifications of these samples were performed using the Image J software (Version 1.52, NIH, Bethesda, MD, USA). The primary antibodies used in the study, and their dilutions, are listed in Table S3.

RNA extraction and real-time qPCR analysis

Total RNA (1 μg) was extracted and purified using TRI-Reagent (Sigma-Aldrich, USA), prior to being reverse-transcribed using 100 ng of random primers following the Superscript II (ThermoFisher, USA) protocol. Real time PCR was performed with SYBR Green qPCR master mix (BioRad) in a Step One plus real-time PCR detection system (Applied Biosystems). Mean values of measurements were calculated according to the -ΔΔCt quantification method and were normalized against the expression of 28S ribosomal mRNA as reference. Results shown are mean ± SEM for, at least, three independent experiments. The p-value was calculated using a paired t-test comparing results for R5020-treated cells expressing siRNA against PRMT1 to the R5020-treated siCT sample: * indicates a p ≤ 0.05, ** for p ≤ 0.01 and *** for p ≤ 0.001. Sequences of the oligonucleotides are listed in Table S4.

Chromatin ImmunoPrecipitation (ChIP)

Chromatin was prepared from 5x106 of T47D cells (untreated or treated with 10 nM of R5020 for 1h). Cells were crosslinked with 1% formaldehyde (Sigma-Aldrich, USA) for 10 min at room temperature and then treated with 0.125 M glycine for 5 min under a gentle shaking. Nuclei were lysed in 300 μL of
ice-cold RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% NaDoc and 0.1% SDS) prior to Chromatin-DNA shearing with a Diogene Bioruptor. ChIP was performed with the primary antibodies listed in Table S3. The antibody–chromatin complexes were precipitated with salmon sperm DNA/protein A agarose beads for 3h by rotation. Samples were extracted and heated at 65°C for 5h to reverse cross-links. After DNA purification, 2 ng of input DNA were used for qPCR analysis to quantify co-precipitated chromatin-DNA. Relative enrichment of a given promoter region obtained with a specific antibody was compared with Input DNA, normalized to a reference locus (human chromosome 1 in which no histone modification was reported). Sequences of the primers used to amplify ChIP-enriched DNA are listed in Table S4.

**RNA-sequencing and RNA-seq analysis**

RNA-Sequencing experiment was performed using T47D cells. Cells were transfected with siCT or siPRMT1 (50 nM) for 72h and treated with R5020 (10 nM) for 6h before RNA extraction. Sequencing was done by the IGFL (Institute of Functional Genomic of Lyon) Sequencing Platform, to compare gene expression levels of R5020-induced genes between siCT and siPRMT1 conditions. cDNA libraries were prepared using the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen, Vienna Austria). Quality of the cDNA was assessed and RT-qPCR was performed for selected PR target genes as quality control. All libraries were sequenced on an Illumina Nextseq500 and mapped on the hg19 version of the human genome using Bowtie2 (Galaxy Version 2.3.2.2). Count tables were prepared using htseq-count (Galaxy Version 0.9.1galaxy3). Differential gene expression analysis was performed with DEseq2 (Galaxy Version 2.1.8.3) using different thresholds. For R5020-induced genes in siCT-cells: FDR < 0.05; \( p \)-adjusted value < 0.01; fold-change > 2; expression > 10 reads per million. For R5020-induced genes in siPRMT1-cells: FDR < 0.05; \( p \)-adjusted value < 0.01; fold-change > 1.5; expression > 10 reads per million. Experiments were performed three independent times for siRNA transfection and for RNA extraction. The list of genes obtained by RNA-Seq analysis is provided in Table S1.
Analysis of cell migration

6x10^5 T47D cells were plated on 96-well ImageLock plates (Essen BioScience) for 24h at 37°C and then scratched (800 μm width) with the Wound Maker (Essen BioScience). Addition of R5020 or vehicle ethanol were added in the medium just after scratching and wound closure was followed and evaluated with the Incucyte Live-Cell Imaging System and dedicated software (Essen Bioscience). Cell migration was evaluated by monitoring the evolution of the size of wound closure (μm) for 24h maximum, in order to assess the contribution of cell proliferation to gap filling. Since wound width decreases as cell migration progresses over time, we represented the results as graphs indicating the rate of migration, corresponding to the change in wound area over time, extrapolated from three independent experiments, each one performed in triplicate.

Analysis of cell proliferation

4x10^3 T47D and 2x10^3 T47D_{WT} or T47D_{R637K} stable cells were seeded onto a 96-well plate 5h before incubation with the different hormones (E2, R5020 or ethanol). Images were acquired using an IncuCyte ZOOM over 7 days and cell proliferation was measured as the percentage of cell density observed over this period. Results are represented as graphs indicating the rate of proliferation over time, extrapolated from at least three independent experiments, each one performed in triplicate.

Colony formation assay

Cells were seeded into 6-well plates (600 cells/well) and left for 8–12 days until formation of visible colonies. Colonies were washed with PBS and fixed with 10% acetic acid/10% methanol for 20 minutes, then stained with 0.4% crystal violet in 20% ethanol for 20 minutes. After staining, the plates were washed and air-dried, and colony numbers were counted.
## Table S2, Related to Methods | Plasmids sequences

| Name   | Forward sequence (5’ – 3’) | Reverse sequence (5’ – 3’) |
|--------|-----------------------------|-----------------------------|
| PR-1   | GATATCTGACTGAGCTGAAGGCAAGG | CTCGAGTTACTGCCTCCACCAAGGCGAC |
| PR-2   | GATATCTGGACGGCCGATGGCCGCCC | CTCGAGTTATGGGCTCGCTGGCTGCTTC |
| PR-3   | GATATCTGCAATACAGCTTGCAGTCATTACC | CTCGAGTTACTTTTTAAATTTCGACC |
| PR-4   | GATATCTGTCAATAAAGTCAGAGTTTGG | CTCGAGTTATGGGAAATCAACTG |
| PR-5   | GATATCTGCGATCAACCTGTATAATGAG | CTCGAGTTACTTTTTATGAAAGAGAAGG |
| PR-3/  | GCTGGCATGTCCTTGAGAAGTCGAACATTAATTTTTAAAGT AACTCGAG | CTCGAGTTACTTTTTAAATTGACCTCCAAGGACCACAT GCCAGC |
| PR-R637A | CAGGCTGGCATGGTCCTTGAGTAATTTTTAAAATTTAAAAGTGTCAT | GACCTTTATGAAACTTTTTAAATTITTACCTCCAAGGAC |
| PR-R637K | CAGGCTGGCATGGTCCTTGAGTAATTTTTAAAATTTAAAAGTGTCATTTTC | CATGCCAGGCTG |
| PROTEIN NAME | Company       | Reference     | IB     | IP     | IF     | PLA | ChIP | Species |
|-------------|---------------|---------------|--------|--------|--------|-----|------|---------|
| EGFR        | Ozyme (CST)   | #4267         | 1/500e |        |        |     |      | Rabbit  |
| EGR1        | SCBT          | Sc-515830     | 1/500e |        |        |     |      | Mouse   |
| ERK1/2      | CST           | #4695         | 1/2000e|        |        |     |      | R       |
| GAPDH       | SCBT          | Sc-4724       | 1/1000e|        |        |     |      | M       |
| H3K4me3     | Diagenode     | C15410003     |        |        |        |     | 4μg  | R       |
| H4          | CST           | #13919        | 1/1000e|        |        |     |      | R       |
| H4R3me2as   | ActiveMotif   | #39705        | 1/500e | 1/1000e|        |     |      | R       |
| IgG         | CST           | #2729         | 2 μg   |        |        |     | 4μg  | R       |
| Met-R637-PR | Home made     |               | 1/100e | 15μL   | 1/300e |     |      | R       |
| Pan-methyl-R| CST           | #13522        | 1/500e | 4μL    | 1/1000e|     |      | R       |
| p-ERK1/2    | CST           | #5726         | 1/2000e|        |        |     |      | R       |
| p-P38       | CST           | #9211         | 1/500e |        |        |     |      | R       |
| p-PDK1      | CST           | #3438         | 1/1000e|        |        |     |      | R       |
| p-PR        | CST           | #13736        | 1/500e |        |        |     |      | R       |
| PR          | SCBT          | Sc-7208       | 1/2000e| 2μg    |        |     | 4μg  | R       |
| PR          | ThermoFischer  | MA1-12626     | 1/1000e| 1/500e | 1/500e |     |      | M       |
| PRMT1       | Bethyl Lab.   | #A300-722A    | 2μg    |        |        |     | 4μg  | R       |
| PRMT1       | Millipore     | #07-404       | 1/2000e|        | 1/1000e|     |      | R       |
| PRMT1       | Sigma         | P1620         | 1/500e |        | 1/500e |     |      | M       |
| SGK1        | SCBT          | Sc-28338      | 1/300e |        |        |     |      | M       |
| Tubulin α   | Sigma         | T6074         | 1/10000e|       |        |     |      | M       |
| V5-tag      | Life Technol. | R920-25       | 1/1000e|        |        |     |      | M       |
| EXPERIMENT             | TARGET GENE | Forward sequence (5’ – 3’) | Reverse sequence (5’ – 3’) |
|------------------------|-------------|-----------------------------|---------------------------|
| **RT-qPCR**            | **CCND1**   | AAGCTCAAGTGGAACCT           | AGGAAGTTTTGGGGGC          |
|                        | **CD44**    | TTTGCAATGGGATGACAGTC        | GTTAACCCAAAATCTCTAGTCCAC  |
|                        | **EGFR**    | GACAGGGCCACCTCGTCCG         | CCGGCTCTCCCGATCAATAC      |
|                        | **EGR1**    | GGCGAGCACCCCTACGAGG         | GTATAAGTGATGGGGGAGGAGTC   |
|                        | **IGFBP5**  | GGTTCGCTCAAGAAAAGGA         | CGGTCTCTCTTCACTGCTCT      |
|                        | **IGF1R**   | TGTCCAGGCCCCAACAGGA         | CGGGTTCAGAGGCACTACA       |
|                        | **FKBP5**   | GGTATATCCCAACATGTTCA        | CCATTGCCTTAATGGCTCT       |
|                        | **NRDGI**   | GGAACACTGCACCTGATCAAT       | TGAGGAGATGGCTCTTGTGAGG    |
|                        | **PGR**     | TGCCTGAAAGTGTCGGCA          | CGCAACAGAGTGCTTCAAGA      |
|                        | **PRMT1**   | GCCTCTCTGAGAAGTCTCTCT       | GATGCCAAAGTGCTGCTAG       |
|                        | **STAT5A**  | AAGCCCCACTGGGAATGATGG       | GGAGTCTAACCTCCAGCGCA       |
|                        | **SGK1**    | CATAGGAGTTTTGCAAT           | CTTCATCTCCTACAACCA        |
|                        | **28S**     | CGATCCCATCACCCGCAATG        | AGCCAAAGCTCACGCAAC        |
| **ChIP-qPCR**          | **EGFR +1960** | GAATCTCTGGACTCTGTCAGGTA    | CTAGGACTATGTCATTACGATCA   |
|                        | **FKBP5 (PRE)** | TAATAGGGGCGAGAAGGCAGA    | GGTAAGTGGGTGCTCCGCTCA     |
|                        | **STAT5A +6896** | GGACTACTCTGAGATTTGCTGCT    | GCTTTCTTTCTGTTCTTTGA      |
|                        | hChr1 (NG)  | CGGGGTCTTTTTGGACCTT         | GAAACACGGCTGCCAGAAAC      |
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