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Protein Arginine Methyltransferase 6 Enhances Ligand-dependent and -independent Activity of Estrogen Receptor α via Distinct Mechanisms

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Abstract

Recent studies reported that protein arginine methyltransferase 6 (PRMT6) enhances estrogen-induced activity of estrogen receptor α (ERα) and dysfunction of PRMT6 is associated with overall better survival for ERα-positive breast cancer patients. However, it is unclear how PRMT6 promotes ERα activity. Here we report that PRMT6 specifically interacts with ERα at its ligand-binding domain. PRMT6 also methylates ERα both *in vitro* and *in vivo*. In addition to enhancing estrogen-induced ERα activity, PRMT6 over-expression up-regulates estrogen-independent activity of ERα and PRMT6 gene silencing in MCF7 cells inhibits ligand-independent ERα activation. More interestingly, the effect of PRMT6 on the ligand-independent ERα activity does not require its methyltransferase activity. Instead, PRMT6 competes with Hsp90 for ERα binding: PRMT6 and Hsp90 binding to ERα are mutually exclusive and PRMT6 over-expression reduces ERα interaction with Hsp90. In conclusion, PRMT6 requires its methyltransferase activity to enhance ERα's ligand-induced activity, but its effect on ligand-independent activity is likely mediated through competing with Hsp90 for binding to the C-terminal domain of ERα. PRMT6-ERα interaction would prevent ERα-Hsp90 association. Since Hsp90 and associated chaperones serve to maintain ERα conformation for ligand-binding yet functionally inactive, inhibition of ERα-Hsp90 interaction would relieve ERα from the constraint of chaperone complex.

1. Introduction

Estrogen receptor (ER) is a member of the nuclear receptor superfamily and it is essential for the development of reproductive tract, mammary gland, skeletal and central nervous system [1, 2]. There are two isoforms of ER, ERα and ERβ. Despite the high level of homology in sequence and structure between these two isoforms, selective knock out of ERα or ERβ in mice revealed that they have distinct biological functions in different tissues [1, 3, 4]. In the mammary gland, ERα promotes breast cell proliferation whereas ERβ is anti-proliferative [5, 6]. Over-expression of ERα in the mammary gland of transgenic mice model results in hyperplasia [7].

ERα has a conserved domain structure common to nuclear receptors, consisting of the N-terminal variable region (VR), a central DNA-binding domain (DBD), a hinge region linking DBD and the C-terminal ligand binding domain (LBD). There is also an additional region F at the C-terminal end of ERα which may be involved in the interaction with antagonist [8]. Activity of ERα is regulated by two activation functions (AF), AF-1 in the VR is constitutively active and AF-2 in LBD is activated by ligand binding. These two activation functions exhibit both independent and synergistic activities [9]. The mechanism of ligand-induced ERα activation involving AF-2 has been studied extensively. Unliganded ERα associates with chaperone protein complex consisting of the Heat shock protein 90 (Hsp90), p23 and several other molecules. These chaperone proteins are essential for the proper folding of ERα, keeping it inactive while maintaining it in a conformation favorable for ligand binding [10-12]. Upon estrogen binding to the LBD of ERα, activated ERα undergoes conformational changes and is released from the Hsp90 chaperone complex. It can then bind to specific DNA sequences and recruit coregulators on the target promoter to modulate gene transcription [13]. In addition, the liganded ERα can also regulate gene transcription by tethering to other DNA binding proteins such as activator protein 1 (AP-1) and stimulating protein 1 (Sp1) [14, 15].

Activation of ERα by estrogen or cell signaling molecules sustains breast cancer progression in at least 50% of breast cancers. Current treatment for the ERα positive breast cancer mainly targets its hormone induced signaling pathway; either through the use of selective estrogen receptor modulators (SERMs) or the direct inhibition of estrogen production by targeting aromatase [16, 17]. SERMs such as tamoxifen can act as ERα antagonist in the mammary gland, compete with estrogen for the ligand binding pocket and hence block AF-2 activation [18]. However, it has been found that the efficacy for endocrine treatment is often limited by intrinsic or acquired anti-estrogen resistance which can be due to the increase in ligand-independent activity of ERα involving AF-1 [19]. This activation of ERα in the absence of hormone can occur either by post-translational modification (PTM) or by direct cofactor binding [20-22]. For instance, epidermal growth factor (EGF) activates ERα through the phosphorylation of serine 118 by mitogen-activated protein kinase (MAPK) and

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phosphorylation of serine 167 by 90kDa ribosomal S6 kinase (RSK) [23-25]. The increase in MAPK activity has been observed for both estrogen-independent breast cancer cell lines models [26] and human breast cancers [27]. Higher MAPK activity also correlates with poorer response to endocrine treatments and shorter survival for breast cancer patients [28].

The activation of ERα by direct cofactor binding also plays an important role in breast cancer development. Cyclin D1 binding to ERα LBD has been found to promote the recruitment of steroid receptor coactivators to ERα and ERα binding to DNA, hence activating transcription in the absence of estrogen [21, 29]. A meta-analysis involving 2580 breast cancer patients showed that over-expression of cyclin D1 predicts worse survival in the ERα-positive breast cancer subgroup [30]. Human X box-binding protein 1 (XBP-1) can also interact with ERα DBD to enhance the ligand-independent activity of ERα [22]. It is highly expressed in breast tumors [31] and ectopic over-expression of XBP-1 in breast cancer cell line model showed increased estrogen-independent growth and decreased sensitivity to anti-estrogen [32]. Further understanding on this complex ERα regulatory network is critical in the development of new therapy for anti-estrogen resistance.

Recent studies showed that protein arginine methyltransferases (PRMTs) act on multiple aspects of ERα signaling through different mechanisms. PRMT1, 2, 4 and 6 enhance the ligand induced activity of ERα, most probably by methylating and potentiating the activity of ERα coactivators [33-35]. PRMT1 can also enhance the cytoplasmic ERα signaling by directly methylating ERα at arginine 260 [36]. In addition, PRMT6 regulates breast cancer cell proliferation directly; depletion of PRMT6 in breast cancer cells completely inhibits their ability to form breast tumors after injection into mouse mammary fat pad [37]. This effect of PRMT6 on cells growth may be partly attributed to its negative regulatory action on the expression of tumor suppressors p53 and p21 [37-40]. However, microarray analysis of breast cancer cell lines and breast tumors gene expression profile showed that PRMT6 dysfunction associates specifically with gene expression signature for better overall relapse-free and distant metastasis-free survival in the ERα-positive breast cancer subgroup [41], suggesting that the oncogenic activity of PRMT6 is also mediated through ERα signaling. In this study, we demonstrate that PRMT6 interacts with and methylates several steroid hormone receptors, ERα, PRB and AR. More importantly, PRMT6 enhances the ligand-independent activity of ERα and this effect is methyltransferase activity-independent. We also provide evidence suggesting that PRMT6 enhances the ligand-independent activity of ERα by reducing the pool of ERα that is associated with Hsp90 chaperone complex which keeps ERα inactive in the absence of ligand. Our results suggest that PRMT6 may contribute to worse prognosis by promoting estrogen-independent growth of ERα-positive breast cancer.

2. Materials and methods

2.1. Plasmid constructs

PRMT1 to PRMT8 were cloned into pcDNA3.1+/hygro plasmid using primers containing 1X Flag tag in the forward sequence. PRMT2, 4, 5, 6 and 8 in GFP vector were gifts from Dr. Mark Bedford (The University of Texas M.D. Anderson Cancer Center), PRMT1, 3 and 7 MGC clones were given by Dr. Tobias Cornvik (Nanyang Technological University, Singapore). pSG5-GR plasmid was a gift from Dr. Ravi Kambadur (Nanyang Technological University, Singapore). pCMV5-Flag-ERα and pCMV5-Flag-AR were gifts from Dr. Edwin Cheung (Genome Institute of Singapore). pCR3.1-SRC-1 plasmid was a gift from Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, Texas). Various ERα truncation constructs were generated by amplifying different ERα segments using specific primers targeting each region. ERα (R260K) mutant was generated using site-directed mutagenesis XL II kit with the primer set 5'-gtgggatacgaaaagaccgaaagggagggagaatgttgaaa-3' and 5'-tttcaacattctccctccctttcggtcttttcgtatcccac-3' (Stratagene, La Jolla, CA, USA).

2.2. Cell culture and transfection

All cancer cell lines were routinely maintained in phenol red-containing Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories Ltd., Somerset, UK) supplemented with 7.5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine (PAA Laboratories Ltd). Cells were plated in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS) for 48 hrs before hormone treatment to remove the residual effect of hormones from serum.

Plasmid transfection was carried out using Polyethyleneimine (PEI) (Polysciences, Warrington, PA, USA) according to the cellntec advanced cell systems transfection protocol for PEI (1 μg plasmid: 1.5 μl PEI for each 35mm dish). Transfection of siRNA was carried out using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instruction. siRNAs were purchased from Ambion (Austin, TX, USA, negative control:4390844. PRMT6 siRNA1: s30337, PRMT6 siRNA2: s30338).

2.3. Co-immunoprecipitation (Co-IP) cellular fractionation and Western blotting

Total cell lysates were collected as described in [42] and incubated with 1 μg of antibody plus protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or incubated with anti-Flag affinity gel (Flag beads) (Sigma-Aldrich). Both beads and supernatant were loaded for Western blotting analysis as described earlier [42]. Nitrocellular membranes were cut horizontally into strips to probe for proteins of interest with different molecular weights and aligned back for developing in some experiments.

For cellular fractionation, COS7 cells were pelleted and re-suspended in buffer C1 (10 mM Hepes pH7.9, 1.5 mM MgCl2, 10 mM KCl, 0.1% NP40, 10 mM DTT, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 µg/ml aprotinin), incubated on ice for 10 mins and passed through 21G syringe for 10 times. The supernatant after centrifugation at 1000 g was kept as the cytoplasmic fraction. Pellet was washed once in buffer C1, re-suspended in buffer N1 (10 mM Hepes pH7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5% NP40, 10 mM DTT, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 µg/ml aprotinin) and nuclei were broken down using a 29G syringe, followed by centrifugation (12000 g, 10 mins) to obtain the nuclear soluble fraction in the supernatant. Final pellet were re-suspended in buffer N2 (10 mM Hepes pH7.9, 1.5 mM MgCl2, 0.4 M KCl, 0.3 M sucrose, 0.5% NP40, 10 mM DTT, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 µg/ml aprotinin) and passed through 29G syringe for 10 times, spun down at 20000 g for 15 mins to obtain the chromatin-bound fraction.

Antibodies used in this study are anti-Flag (F1804, Sigma-Aldrich), anti-GAPDH (AM4300, Ambion), anti-Histone H3 (#9715, Cell signaling). Anti-ERα (sc-002, sc-543), anti-GR (sc-8992), anti-Hsp90 (sc-13119), anti-PRMT6 (sc-271744) and anti-PRB (sc-7208) were purchased from Santa Cruz Biotechnology.

2.4. Immunostaining and proximity ligation assay (PLA)
Cells on cover slip were fixed with 3.7% formaldehyde and permeabilized with 0.2% triton-X 100 (Sigma-Aldrich). After blocking with 2% FBS/PBS, they were incubated with primary antibodies (anti-ERα and anti-Flag) followed by secondary antibodies (Dylight 594 Goat anti-mouse IgG (H+L) (115-515-166): from Jackson ImmunoResearch, Suffolk, UK and Alexa Fluor 488 nm anti-rabbit IgG (H+L) (A11034) with 4',6-diamidino-2-phenylindole (DAPI) (0.1 µg/ml) (Life Technologies)).

For PLA, cells were plated on Nunc latek chamber slide (Nalge Nunc International, Rochester, NY, USA). After overnight incubation at 4°C with primary antibodies, samples were processed using Duolink II PLA kit (Olink Bioscience, Uppsala, Sweden) according to manufacturer's manual. Briefly, samples were incubated with anti-mouse and anti-rabbit PLA probes for 1 hr at 37°C, followed by ligation reaction mix incubation for additional 30 mins at 37°C. Signal was amplified using amplification-polymerase for 100 mins at 37°C. The slide was then washed and mounted for viewing. Detection reagent orange (ex/em 554/579) was used in this experiment.

2.5. Luciferase reporter assay
For luciferase reporter assay, HeLa cells in 60 mm dishes were transfected with 1.5 µg of PRE-luciferase or ERE-luciferase, 5 ng of receptor coding plasmids, with 25-50 ng of PRMT6 plasmids (the amount of plasmids was scaled down to 1/3 if transfection was done in 6-well plate, i.e. 1.6ng of receptor plasmid and

16ng of PRMT6 plasmid). Cells were treated with 10 nM of the respective hormone (17β-estradiol (E2) for ERα, progestin R5020 for PRB, Dihydroxytestosterone (DHT) for AR and dexamethasone (Dex) for GR) for 24 hrs after being transfected for 24 hrs. Lysate was collected for analysis using Promega Luciferase assay kit (Promega, Madison, WI, USA). For MCF7 cells, they were first transfected with PRMT6 siRNA for 24 hrs, followed by another 24 hrs transfection with ERE-Luciferase before treated with hormone for 24 hrs. Luciferase signals were detected using Tecan Safire II Plate Reader and normalized with protein concentration quantified by Pierce BCA Protein Assay Kit (Pierce Biotechnology Inc, Rockford, IL, USA). Student's t test was conducted and p value less than 0.05 is considered significant.

2.6. Reverse transcription and real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies), chloroform:isoamyl-ethanol (24:1) and phenol:chloroform:isoamyl-ethanol (50:24:1, Sigma-Aldrich), precipitated using isopropanol and washed with 75% ethanol in DEPC-treated water (Sigma-Aldrich) before re-suspending in DEPC-treated water. cDNA was synthesized from 1-5 μg of total RNA using random primer (Promega) and SuperScript II ™ reverse transcriptase (Life Technologies). Real-time PCR was performed using KAPA SYBR Green PCR reagents on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR for each gene was performed in triplicates. The relative amount of PCR products generated from each primer set was determined on the basis of the threshold cycle (Ct) number. Housekeeping gene 36B4 was used as control to normalize the amount of cDNA used. Relative expression = 2 [[Ct(control)gene X – Ct (treatment)gene X] – [Ct(control)36B4 – Ct(treatment)36B4]]. Primer sequences are available upon request.

2.7. *In vitro* and *in vivo* methyltransferase assay

In vitro methyltransferase assay for PRMT6 was performed in methylation buffer containing 100 mM of Tris pH 8.0, 25 mM $MgCl_2$ and 0.004 M DTT. 0.55 μCi of H3-S-adenosyl L-methionine (H3-SAM, PerkinElmer, Waltham, MA, USA) was used for each reaction. Flag tagged receptors and PRMT6 plasmids were transfected into COS7 cells and pulled down with Flag beads. Beads were incubated in the methylation buffer for 1 hr at 37 $^{\circ}$C with shaking. Reaction was quenched using protein loading dye. Samples were separated by SDS-PAGE and stained with coomassie blue. Signal was enhance using ELIGHTENING solution (PerkinElmer). Gels were dried in GelAir dryer (Bio-Rad, Richmond, CA, USA) and dried gels were kept with hyperfilm MP (GE Healthcare Biosciences, Pittsburgh, PA, USA) for 1-3 weeks at -80 $^{\circ}$C to detect the radioactivity.

In vivo methyltransferase assay was carried out by over-expressing Flag-ERα in the presence and absence of Flag-PRMT6. 24 hrs after transfection, cells were treated with 20 μM Adenosine, periodate oxidised (AdOx, Sigma-Aldrich) for 24 hrs to inhibit protein methylation. Cells were then pretreated with 100 μg/ml of cycloheximide and 40 μg/ml of chloramphenicol (Sigma-Aldrich) for 30 mins to inhibit protein synthesis followed by labeling with H3-methione (PerkinElmer) for additional 3 hrs. Lysates were collected for immunoprecipitation with Flag beads and processed as mentioned in the paragraph above.

3. Results

3.1. PRMT6 interacts with ERα, PR and AR independent of ligand binding

To search for the PRMT that can interact with ERα directly and hence may target ERα for methylation, Flag tagged PRMT1 to PRMT8 co-expressed with ERα in COS7 cells were immunoprecipitated with anti-Flag antibody. Although anti-Flag antibody was able to pull down all eight PRMTs specifically (note that PRMT2 band partially overlaps with Ig Heavy chain at 50kDa), ERα was only co-immunoprecipitated with PRMT2 and PRMT6 in the absence of hormone treatment (Fig. 1A). While this finding is consistent with a previous report that PRMT2 coactivates and interacts with ERα [34], it is the first demonstration that PRMT6 interacts specifically with ERα. Further validation for this interaction was carried out with reverse IP using anti-ERα antibody. As shown in Fig. 1B, PRMT6 was pulled down by anti-ERα antibody specifically.

We then examined if PRMT6 can interact with multiple steroid hormone receptors and if the interaction is regulated by hormone treatment. Co-IP was performed using COS7 cells over-expressing ERα, PRB, AR or GR together with PRMT6. Transfection with pcDNA3.1 vector in place of PRMT6 was used as a control. As shown in Fig. 1C to 1F, PRMT6 co-immunoprecipitated ERα, PRB and AR. However, no GR protein could be pulled down by PRMT6. In addition, the amount of ERα/PRB/AR protein pulled down by PRMT6 was down-regulated in the presence of hormone treatment, although there was no significant decrease in the input protein level after 6 hrs of hormone treatment, suggesting that PRMT6 preferentially binds to unliganded receptors.

3.2. PRMT6 methylates ERα, PRB and AR

While we showed that PRMT6 interacts with ERα, PRB and AR, it has not been demonstrated if PRMT6 can methylate these receptors. We tested this by *in vitro* methyltransferase assay using Flag-tagged PRMT6 and Flag-tagged ERα/PRB/AR co-expressed in COS7 and pulled down by anti-Flag affinity gel (Flag beads). Proteins attached to the Flag beads were at close proximity which facilitated the methylation reaction. Flag-PRMT6, Flag-ERα and Flag-AR were pulled down well as shown by the bands on coomassie blue stained gel but less Flag-PRB was pulled down and it appeared close to a background band (Fig. 2A). Autoradiogram showed that PRMT6 was capable of self-methylation as reported [43], indicating that the methylation reaction occurred successfully. Prominent autoradiograph bands corresponding to the size of PRB, ERα and AR were seen. ERα methylation band was the strongest and PRB methylation band was much weaker and appeared just below a background band, maybe due to the lower amount of proteins pulled down. Flag-PRMT2 was also included in the assay, showing that PRMT2 can also self-methylate, but it was unable to methylate the other co-immunoprecipitated proteins showed on the coomassie blue stained gel (less bands on the autoradiogram as compared to PRMT6), further confirming that the methylation carried out by PRMT6 was highly specific (Fig. 2A).

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As *in vitro* methylation labels target proteins after they are isolated from the cells, we also determined if PRMT6 can methylate ERα in live cells. *In vivo* labeling with H3-methionine in the presence of protein synthesis inhibitor was carried out for ERα with and without PRMT6 over-expression. The amount of ERα and PRMT6 proteins detected was lower than *in vitro* methylation assay due to the treatments that inhibited protein synthesis. Nonetheless, ERα was clearly methylated in the presence of PRMT6 with or without hormone treatment (Fig. 2B). In contrast, ERα was not methylated in the absence of PRMT6 over-expression. As a positive control, PRMT6 was also methylated, likely by itself (Fig. 2B).

3.3. PRMT6 methylates ERα at arginine 260

Amino acid sequence analysis for ERα revealed that arginine 260 is present in the 'RGG' recognition motif and is possible target of PRMT6. *In vitro* methylation of ERα(R260K) mutant showed significant decrease in the methylation signal as compared to the wild-type ERα. As shown in Fig. 2C, although PRMT6 could still methylate ERα(R260K), the methylation signal was weakened by more than 50% after normalization with protein level, suggesting that ERα is methylated by PRMT6 at more than one arginine sites and R260 is one of the target site. In addition, the specificity of PRMT6 methylation on ERα was further demonstrated using the enzymatically inactive mutant mPRMT6 (V86K/D88A). Although both ERα and mPRMT6 protein levels were high in the coomassie stained gel, no ERα methylation band could be seen in the autoradiogram, confirming that presence of active PRMT6 was needed for the labeling on ERα to occur (Fig. 2C). When ERα(R260K) mutant's activity was analyzed by ERE-Luciferase reporter assay, no significant change was detected as compared to wild-type ERα (See Supplementary Figure 1), suggesting that PRMT6 methylation of R260 may not be involved in regulating the genomic signaling of ERα.

3.4. PRMT6 binds to ERα in the nucleus *in vivo*

It has been reported [35] that PRMT6 interacts with SRC-1 to act as a secondary coactivator for ERα. Consistent with the finding, we also found that PRMT6 interacts with SRC-1 in the absence of ERα (Fig. 3A). However, SRC-1 and ERα interaction is largely E2-dependent (Fig. 3B). We also observed that PRMT6 preferentially bound to ERα when both ERα and SRC-1 were present (Fig. 3C), suggesting that PRMT6 has higher affinity for unliganded ERα than for SRC-1. It is possible that PRMT6 exhibits two modes of interaction with ERα: it binds to unliganded ERα directly but binds to liganded ERα through SRC-1. Hence PRMT6 may be involved in multiple aspects of ERα signaling.

To further characterize the interaction between PRMT6 and ERα in breast cancer cells, MCF7 cells stably expressing either pcDNA3.1 vector or Flag-PRMT6 vector were established for immunostaining and

PLA. Consistent with a previous study [43], immunostaining showed that PRMT6 protein mainly localizes in the nucleus. Majority of ERα protein was also present in the nucleus (Fig. 4A, top). After E2 treatment, PRMT6 remained in the nucleus, whereas ERα protein showed decreased cytoplasmic staining (Fig. 7A, bottom). The co-localization of PRMT6 and ERα was further demonstrated by PLA. Red dots represent positive signal amplification that occurs if the two proteins are in close proximity (<40 nm). In the negative control with pcDNA3.1 transfected, none or very few red dots were seen after PLA staining, showing that the assay is highly specific. In the presence of Flag-PRMT6, numerous red dots were seen in the nucleus of both ethanol- and E2-treated samples, indicating that Flag-PRMT6 interacted with endogenous ERα specifically in the nucleus (Fig. 4B). Furthermore, E2-treated cells contain fewer but larger ERα-PRMT6 interaction spots than the ethanol treated cells. The larger PLA signal dots in E2-treated cells compared to the ethanol treated controls (also see supplementary Figure 2) suggest more concentrated ERα-PRMT6 interactions and hence E2-induced ERα re-organization of its nuclear loci [44]. It has been well documented that transcription regulation involves dynamic chromatin configuration with RNA polymerase II clustered into discrete transcription loci known as transcription factories [45, 46]. Ligand-activated ERα has been reported to instigate reorganization of these transcription factories by recruiting its target genes [44]. It is plausible that these ERα-PRMT6 interaction spots are concentrated in the transcription factories organized by ligand-activated ERα.

Although co-immunoprecipitation experiment showed decrease of ERα and PRMT6 interaction following E2 treatment, it is not clear from the PLA images if there is a decreased interaction between ERα and PRMT6 upon E2 treatment. Cell fractionation experiment (Fig. 4D) showed that E2 treatment increased the proportion of ERα binding to chromatin, suggesting that the decrease in ERα and PRMT6 interaction by co-immunoprecipitation experiment following E2 treatment may be caused by the change in ERα localization. The chromatin associated ERα would not be extracted efficiently by the routine IP buffer, leading to decreases in PRMT6-ERα interaction. On the other hand, there is no significant increase of chromatin-bound PRMT6 in response to E2 treatment in spite of its co-localization with ERα (Fig. 4D). It is possible that PRMT6 is recruited to the transcription loci by associating with other co-regulatory proteins recruited by E2-activated ERα. But it is not stably associated with the chromatin and can be eluted with high salt buffer. This notion is consistent with an earlier report that PRMT6 acts as a secondary coactivator for the ligand-activated ERα [35].

As was expected, both ERα and PRMT6 are found mainly in the nuclear and chromatin fractions. But these proteins are also present in large amount in the cytoplasmic fraction (Fig. 4D), which can be explained by the fact that a large amount of ERα and PRMT6 proteins were continuously synthesized in the cytoplasm following transient transfection.

3.5. PRMT6 up-regulates ligand-independent activity of ERα independent of its enzymatic activity

In agreement with previous report [35], PRMT6 coactivates the hormone induced transcriptional activity of PRB, GR, AR and ERα in the luciferase reporter assay in HeLa cells (Fig. 5A to 5C, Fig. 6A). In addition, our study showed that PRMT6 doubled the ligand-independent activity of ERα (Fig. 6A). Surprisingly, this effect of PRMT6 on the ligand-independent activity of ERα is independent of its methyltransferase activity as the inactive mutant mPRMT6 enhanced the ligand-independent activity of ERα similarly. In contrast, the ligand-induced ERα activity was only up-regulated by wild-type PRMT6 but not mPRMT6, consistent with the earlier report by Harrison et al [35]. This suggests that PRMT6 regulations of the ligand-independent and ligand-dependent ERα activity are mediated through different mechanisms (Fig. 6A). It should be noted that the mutant PRMT6 protein expression level is consistently lower than that of the wild-type PRMT6 and the cause is not clear (Fig. 6D). In spite of this low expression level, mPRMT6 significantly enhanced the ligand-independent activity of ERα. It is also important to note that the ligand-independent ERE luciferase activity can be reduced by 70% using specific ERα antagonist ICI 182780 (Fig. 6B), confirming that the observed transcriptional activity is ERα specific. On the other hand, it is expected that there is some remaining ERα activity (30% in this case) depending on the dose and duration of ICI 182780 treatment. Interestingly, wild-type PRMT6 or mPRMT6 over-expression also enhanced the remaining ligand-independent activity of ERα following ICI 182780 treatment (Fig. 6B). Similarly, while tamoxifen decreased the ligand-independent activity of ERα by 50%, PRMT6 over-expression doubled the ERα activity in the presence of tamoxifen, to a level that is higher than the control ERE luciferase activity without tamoxifen treatment (Fig. 6C). Protein expression of ERα in HeLa cell was analyzed by Western blotting to show that the change in activity detected was not contributed by increase in the ERα protein level (Fig. 6D).

The positive effect of PRMT6 on the ligand-independent activity of ERα is also demonstrated in breast cancer cells MCF7. Knockdown of endogenous PRMT6 in MCF7 cells decreased both ligand-independent and ligand-dependent ERα activities without significant effect on ERα's expression level (Fig. 7A and 7D). ERα direct target genes pS2 and GREB1 were also down-regulated upon PRMT6 knockdown (Fig. 7B). When the effect of PRMT6 on MCF7 cell proliferation was analyzed by cell counting, depletion of PRMT6 by transient transfection of siRNA caused 40% decrease in cell number after 4 days, both with and without hormone treatment (Fig. 7C).

3.6. Competitive binding between PRMT6 and Hsp90

We next investigated the possible mechanisms by which PRMT6 enhances ligand-independent activity of ERα. It is well known that Hsp90 is an important component of the steroid receptor protein complex in the absence of ligand and this complex formation is critical for receptor folding, maturation and dimerization while

keeping the receptors in an inactive state [10, 11, 47]. Since PRMT6 binds to the unliganded ERα better, we examined if PRMT6 was also part of this pre-activation chaperone complex. Although ERα binds to both Hsp90 and PRMT6 (Fig. 8A), Hsp90 and PRMT6 seem to exist in distinct complexes with ERα. While both wild-type and mutant PRMT6 could pull down ERα well, no Hsp90 was pulled down concurrently (Fig. 8B). On the other hand, anti-Hsp90 antibody could co-immunoprecipitate ERα but not PRMT6 (Fig. 8C). These data suggest that PRMT6 and Hsp90 binding to ERα are mutually exclusive. The efficiency of Hsp90 pull down by ERα was then compared in the absence and presence of exogenous PRMT6. As shown in Fig. 8D, the amount of Hsp90 pulled down by ERα was drastically reduced in the presence of PRMT6, despite similar expression levels of Hsp90 and ERα. This demonstrates that PRMT6 can compete with Hsp90 directly for binding to ERα, increased PRMT6 binding to ERα results in a decrease in ERα/Hsp90 interaction.

3.7. PRMT6 binds to ERα at C-terminal LBD-F region

It has been reported that Hsp90 binds to the DBD-LBD region of ERα [48]. We asked the question if PRMT6 binds to ERα in this region as well. Co-immunoprecipitation showed that deletion of the variable region of ERα (amino acid 1-185) (ΔVR) did not reduce PRMT6-ERα interaction, suggesting that VR is not required for the interaction (Fig. 8E). Vectors containing VR only (amino acid 1-185), VR to hinge region (amino acid 1-355) and LBD to region F (amino acid 356-595) were then tested. As expected, VR alone could not interact with PRMT6. ERα ΔLBD also did not bind to PRMT6. However, LBD to region F alone was sufficient to interact with PRMT6. Although the protein expression level for LBD to region F was lower than the other domains, it could be pulled down by PRMT6 very well, at a level that was much higher than the input control (Fig. 8F). The findings that both Hsp90 and PRMT6 interact with ERα LBD may explain their mutually exclusive and competitive binding with ERα. We speculate that PRMT6 increases the ligand-independent activity of ERα by freeing ERα from the inactivating effect of Hsp90 chaperone complex.

4. Discussion

PRMT6 expression is generally low in normal tissue but over-expressed in a significant number of tumors including breast tumors [37, 49]. Serum level of asymmetrical dimethylarginine is also elevated in breast cancer patients, which may be partly caused by the higher PRMT6 activity in cancer and contribute to tumor progression [49]. Recent studies on the involvement of PRMT6 in breast cancer also reported that PRMT6 dysfunction is associated with better overall survival in the ERα-positive breast cancer [41] and PRMT6 silencing abolishes the tumor forming ability of breast cancer cells in mouse model [37]. Here we report that PRMT6 binds to ERα specifically using both co-immunoprecipitation and proximity ligation assay in the presence or absence of estrogen. PRMT6 also enhances both ligand-dependent and ligand-independent activity of ERα. We provide evidence to suggest that these two effects are mediated through distinct mechanisms. In agreement with a previous study [35], the effect of PRMT6 on ligand-induced ERα activity is methyltransferase activity-dependent. On the other hand, the stimulatory effect of PRMT6 on ligand-independent activity of ERα does not require the enzymatic activity. This effect of PRMT6 on the ligand-independent activity of ERα is very prominent in our assays but it was not reported [35]. One possible reason for this discrepancy is that we used significantly different amount of plasmid vector and ERα/PRMT6 plasmid ratios from the earlier study. *Harrison et al* used 15 ng ERα/50 ng PRMT6 vector in 24-well plates in the transfection [35]. We used 1.6 ng ERα/16 ng PRMT6 vector in 6-well plates. They and earlier study [33] also noted that a greater coactivating effect was observed with lower amount of nuclear receptor transfected. Our observation that ERα activity decreased after knockdown of endogenous PRMT6 in MCF7 cells further support that PRMT6 can enhance the ligand-independent activity of ERα specially.

Our data suggest that the ligand-independent PRMT6-ERα interaction plays a key part in enhancing the ligand-independent ERα activity. Unliganded ERα is associated with chaperone protein complex consisting of Hsp90, cyclophilin 40, p23 etc. Interaction with the chaperone proteins not only facilitates proper folding of the receptor for ligand binding, but also keeps ERα in an inactive state in the absence of ligand [10-12, 47]. We found that PRMT6-ERα and Hsp90-ERα bindings were mutually exclusive. While immunoprecipitation of ERα pulled down both PRMT6 and Hsp90, immunoprecipitated PRMT6 was only associated with ERα but not Hsp90. Similarly, immunoprecipitated Hsp90 was associated with ERα but not PRMT6. Moreover, over-expression of PRMT6 leads to a decrease in the interaction between ERα and Hsp90 directly. This implies that the ligand-independent PRMT6-ERα interaction may prevent ERα association with Hsp90 and other associated chaperone proteins that normally keep ERα inactive. Meanwhile, PRMT6-bound ERα can still be the functionally mature form from Hsp90 complex. Therefore, competitive binding of ERα between PRMT6 and Hsp90 does not reduce the pool of structurally mature ERα. Consequently, there is a greater propensity for ERα to be activated. It is also interesting to note that the enzymatically inactive PRMT6 mutant interacted with ERα

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equally well as the wild-type PRMT6, which is also consistent with its equally potent effect on enhancing the ligand-independent activation of ERα. This further supports the notion that the ligand-independent activation of ERα by PRMT6 is mediated through their ligand-independent interaction but not caused by the methyltransferase activity of PRMT6. In addition, the fact that PRMT6 mutant did not have any effects on the ligand-induced ERα activity confirmed that PRMT6's effect on the ligand-independent activity of ERα is different from its action on the liganded ERα and it is highly specific. This ruled out the possibility that the observed ligand-independent activity was a result of residual hormone in the medium.

Analysis of ERα domain truncations indicated that the site of its ligand-independent interaction with PRMT6 lies in the LBD-F region (amino acid 356-595). This interaction is attenuated in the presence of estrogen, suggesting that ligand binding alters the conformation of the interaction interface, resulting in the impaired interaction with PRMT6. Interestingly, ERα also interacts with Hsp90 via its C-terminus region including the LBD [48]. Since both PRMT6 and Hsp90 binds to ERα at its C-terminus [48], it is likely that PRMT6 can compete with Hsp90 for binding to ERα, resulting in the dissociation of ERα from chaperone complex to facilitate ERα activation in the absence of hormone. This makes PRMT6 the third cofactor which is found to be able to enhance ERα's ligand-independent activity through direct binding to ERα, after Cyclin D1 and XBP-1 [21, 22]. This is also the first report that PRMT6 can regulate cellular function independent of its enzymatic activity.

PRMT6 can also bind and methylate ERα, PRB and AR, but not GR, in the presence and absence of ligand. The absence of interaction between PRMT6 and GR may be due to the difference in cellular localization. PRMT6 localizes in the nucleus whereas GR is predominantly cytoplasmic in the absence of ligand [50]. On the other hand, while PRMT6 enhanced the ligand-induced effect of PRB, AR, and GR as reported [35], it did not exhibit significant effect on the ligand-independent activation of these receptors. Truncation analysis of PRB domains revealed that PRMT6 interacts with the N terminal domain-DBD instead of the LBD (Data not shown), which may explain why PRMT6 has no effect on the ligand-independent activity of PRB.

The study indicated that one of the arginine residues targeted by PRMT6 in ERα is R260, which is also targeted by PRMT1 [36]. The overlap in target specificity between PRMT1 and PRMT6 is not surprising given the fact that both of them recognize arginine/glycine rich RGG motif and histone H4R3 has been shown to be methylated equally well by PRMT1 and PRMT6 [51]. It seems that PRMT1 and PRMT6 regulate ERα activity through both overlapping and distinct mechanisms. They both act as secondary coactivators for the ligand-induced genomic activity of ERα by enhancing the activity of other co-regulators such as SRC1 [33, 35]. In addition, methylation of ERα at R260 by PRMT1 activates ERα non-genomic signaling by promoting ERα

binding to PI3K signaling molecules and activates Akt [36]. Although PRMT6 also methylates R260, we have not been able to demonstrate that PRMT6 exerts similar effects on the non-genomic ERα signaling. Since PRMT1 expression level is higher than PRMT6 in normal tissues [43], it may be the dominant regulator of this ERα non-genomic signaling pathway. Moreover, we demonstrate here that PRMT6 also exhibits unique stimulatory effect on the ligand-independent genomic activity of ERα, which has not been observed for PRMT1. Overall, PRMT1 and PRMT6 may work together to promote various aspects of ERα signaling - genomic and non-genomic, ligand-independent and ligand-dependent.

PRMT6 has been reported to repress the expression of several tumor suppressor genes, including p53, p21(CIP1/WAF1) and p27 by methylating H3R2 at their gene promoter sites [37-40]. PRMT6 also directly methylates and inhibits the activity of tumor suppressor p16 (INK4A) [52]. The present study together with an earlier study [35] further highlights that PRMT6 can also function as an oncogene in breast cancer by promoting both ligand-dependent and ligand-independent activity of ERα. PRMT6 gene silencing down-regulates breast cancer cell proliferation both in the presence and absence of estrogen. The effect of PRMT6 on the ligand-independent activity of ERα is especially significant for anti-estrogen resistant breast cancer. It is well established that ligand-independent activation of ERα through crosstalk with cellular signaling molecules plays a significant role in cancer progression [53, 54]. It is conceivable that PRMT6 over-expression observed in many cases of breast cancer [37, 55] can further bolster ligand-independent ERα activity, enhancing the aggressiveness of the disease [49]. Thus, small molecule inhibitors that disrupt the interaction between PRMT6 and ERα may be useful in treating anti-estrogen resistant breast cancer with PRMT6 over-expression.

In conclusion, the present study provides novel insight into the regulation of ERα activity by PRMT6. PRMT6 up-regulates both the ligand-dependent and -independent activity of ERα, but the effect on the ligand-independent ERα activity is independent of its arginine methyltransferase activity. This study provides evidence to support the notion that PRMT6 exerts the effect by competing with Hsp90 for binding to the C-terminal domain of ERα so as to relieve ERα from the constraint of chaperone complex. Meanwhile, PRMT6 could function as a co-activator to facilitate ERα activation. In view of the understanding that many cases of anti-estrogen resistant breast cancers are still fueled by ligand-independent ERα activity [56], the findings also raise an interesting possibility that PRMT6 over-expression may play a part in driving the progression of anti-estrogen resistant breast cancers via promoting estrogen-independent ERα activation.

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Figure Legends

Fig. 1. PRMT6 interacts directly with ER, PRB and AR but not GR. (A) Full length Flag tagged PRMT1 to PRMT8 were co-transfected with ERα in COS7 cells for co-IP with anti-Flag antibody. (B) Reverse IP of PRMT6 by anti-ERα antibody in COS7 cells. (C-F) COS7 cells were transfected with (C) ERα, (D) PRB, (E) AR or (F) GR, in combination with pcDNA3.1 vector control or Flag-PRMT6 plasmid. The cells were treated with 0.1% ethanol or 10 nM of their respective steroid hormones for 6 hrs before total cell lysate were collected for co-IP with anti-PRMT6 antibody. Protein band intensities were analyzed by densitometer and the relative protein intensities for ERα, PRB and AR were indicated at the bottom of the blots after normalizing with ethanol control.

Fig. 2. PRMT6 methylates ERα, PRB and AR. (A) *In vitro* methyltransferase assay showed PRMT6 methylates itself and PRB, ERα, AR. PRMT2 only methylated itself but not other co-immunoprecipitated proteins. Positions for the receptors and PRMT6 are marked with numbers. (B) *In vivo* methyltransferase assay showed that PRMT6 can methylate ERα in live cells. (C) PRMT6 methylates R260 in the RGG conserved PRMT recognition motif. Relative methylation signals for ERα and R260K mutant were analyzed by densitometer. Enzymatically inactive mPRMT6 was unable to methylate ERα.

Fig. 3. PRMT6 binds to unliganded ERα better than SRC-1. (A) PRMT6 pulled down SRC-1 in the absence of ERα and hormone treatment. (B) ERα only interacts with SRC-1 well in the presence of E2 treatment (10 nM, 1 hr). (C) PRMT6 preferentially pulled down ERα but not SRC-1 in the absence of E2 treatment. COS7 cells were transfected with PRMT6, ERα and SRC-1 for 48 hrs followed by 1 hr Ethanol (0.1%) or E2 treatment (10 nM) before lysate collection for co-IP.

Fig. 4. PRMT6 interacts with ERα in the nucleus *in vivo*. (A) Immunostaining showed the nuclear localization for both PRMT6 and ERα in MCF7 cells, with or without E2 treatment for 1 hr. (B) Positive PLA signal (red dots) demonstrating the direct interaction between PRMT6 and ERα *in vivo*. (C) Western blotting for ERα expression levels in MCF7 cells stably over-expressing pcDNA3.1 control or Flag-PRMT6. (D) Cellular fractionation showing that the amount of chromatin-bound ERα increased after E2 treatment while the amount of chromatin-bound PRMT6 remained similar. COS7 cells transfected with ERα and PRMT6 were treated with EtOH control or 10 nM E2 for 3 hr before fractionation into Cytoplasmic (C), nuclear soluble fraction (N) and chromatin-bound fraction (Ch). GAPDH was used as cytoplasmic marker and Histone H3 was used as the marker for both nuclear and the chromatin-bound fractions.

Fig. 5. Up-regulation of PRB, AR and GR transcriptional activities by PRMT6. Transcriptional activities of steroid hormone receptors in the presence of PRMT6 were examined using luciferase reporter assay. (A-C) HeLa cells were co-transfected with PRE-luciferase and PRB/AR expression vector (GR is expressed endogenously in HeLa) with different amount of PRMT6 (0, 25, 50 ng). Relative luciferase activity was calculated by normalizing with empty vector control. Results shown are from a single experiment using triplicates, which is representative of at least three independent experiments, error bar represents standard error of mean and the p values were obtained by Student's t-test. *$p<0.05$; **$p<0.01$. (D) Western blots showing that protein expressions of PRB/GR/AR were not affected by PRMT6.

Fig. 6. PRMT6 up-regulates ligand-independent activity of ERα independent of its enzymatic activity. (A) ERE-luciferase assay showing the enhancement of ERα ligand-independent activity by both wild-type and mutant PRMT6 (mPRMT6, V86K/D88A) in HeLa cells. Ligand-induced activity was only up-regulated by wild-type PRMT6 (10 nM E2, 24 hrs). (B) Treatment with ICI 182780 (100 nM, 24 hrs) down-regulates ERα's ligand-independent activity with and without PRMT6 over-expression. (C) PRMT6 increases ERα's ligand-independent activity in the presence of tamoxifen (OHT, 100 nM, 24 hrs). Relative luciferase activity was

calculated by normalizing with empty vector control. Results shown for panel A to C are from a single experiment using triplicates, which is representative of at least three independent experiments, error bar represents standard error of mean and the p values were obtained by Student's t-test. *p<0.05; **p<0.01. (D) Western blot showing that ERα expression level was not affected by PRMT6 over-expression.

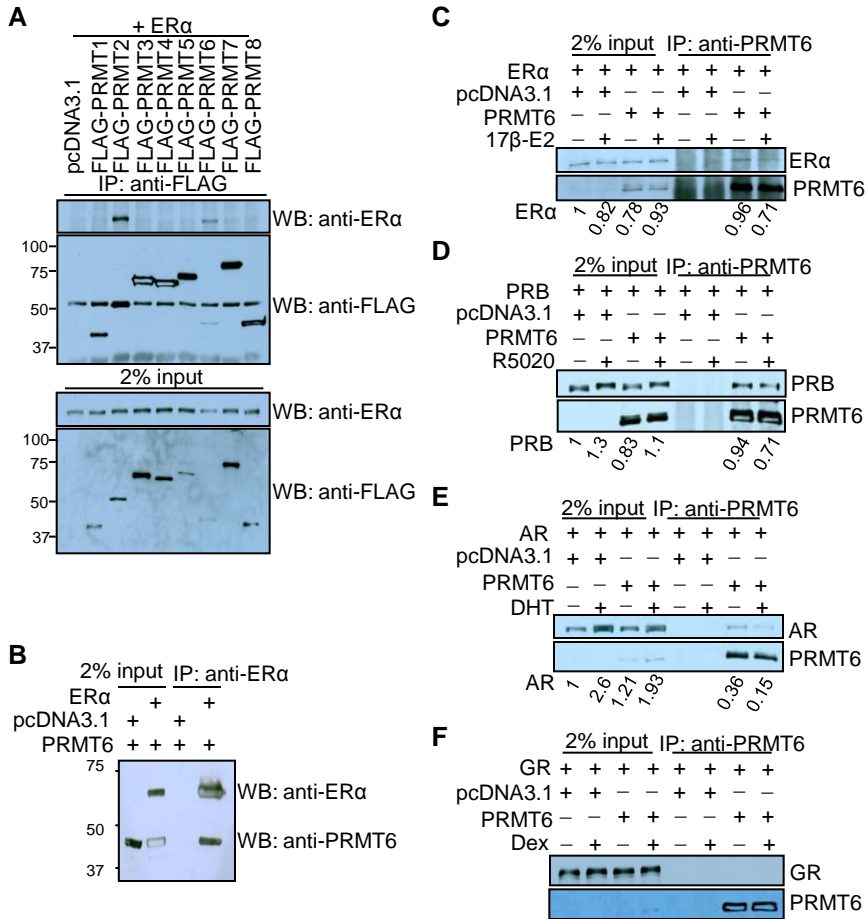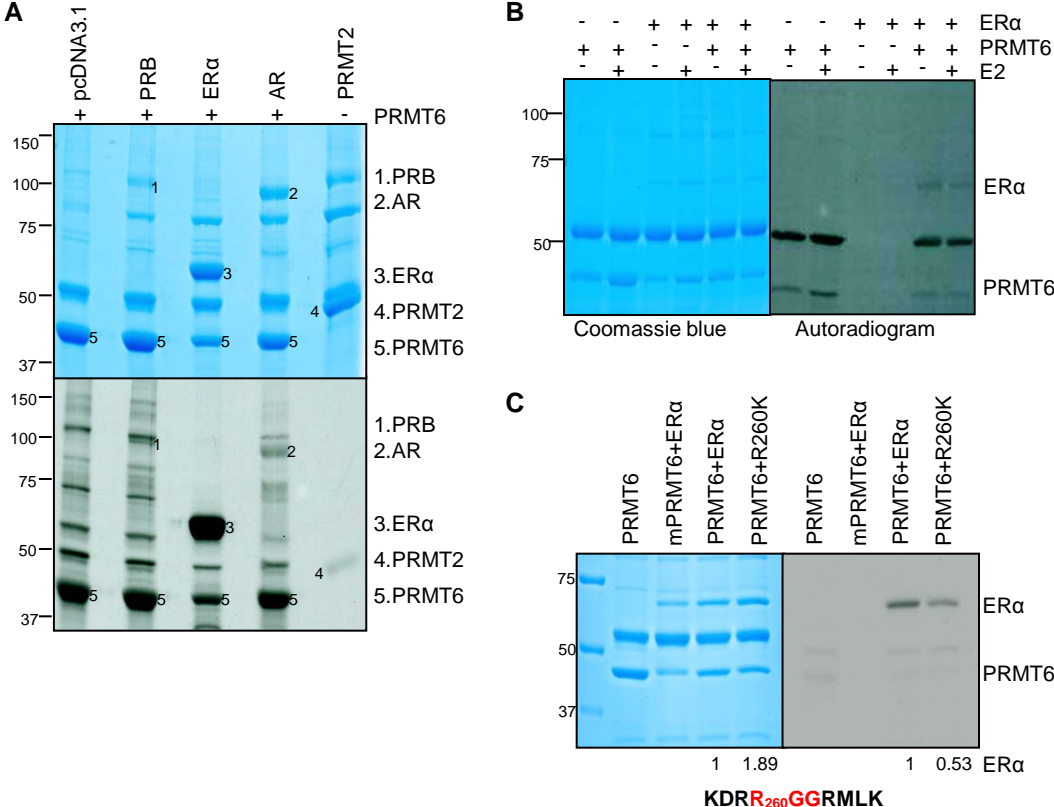**Fig. 7. PRMT6 gene silencing down-regulates both ERα transcriptional activity and breast cancer cell proliferation.** (A) ERα transcriptional activity decreased following PRMT6 knockdown in MCF7 for 72 hrs, treated with EtOH or 10nM E2 for the last 24 hrs. Results shown are from a single experiment using triplicates, which is representative of at least three independent experiments, error bar represents standard error of the mean and the p values were obtained by Student's t-test. *p<0.05; **p<0.01. (B) Real-time PCR showing that the expression level of ERα direct target genes pS2 and GREB1 decreased after PRMT6 knockdown. Error bar represents standard error of the mean and the p values were obtained by Student's t-test. **p<0.01. (C) Knockdown of PRMT6 for 4 days reduced MCF7 cell number by 40% as compared to control, with or without E2 treatment for the last 48 hrs. Results shown are from a single experiment using triplicates, which is representative of two independent experiments, error bar represents standard deviation and the p values were obtained by Student's t-test. *p<0.05; **p<0.01. (D) Western blot showing the ERα and PRMT6 expression level in MCF7 cells after 72 hrs knockdown.

Fig. 8. Mutually exclusive binding of Hsp90 and PRMT6 with ERα. (A) ERα or pCMV control was co-transfected with PRMT6 in COS7 cells. Anti-ERα antibody pulled down ERα well and co-IP both Hsp90 and PRMT6. (B) Both wild-type and mutant PRMT6 interact with ERα but not Hsp90. (C) ERα was co-immunoprecipitated by Hsp90 but not PRMT6. (D) ERα was co-transfected with control vector or GFP-PRMT6 in COS7 cells and pulled down using anti-ERα antibody. The amount of Hsp90 pulled down by ERα decreased in the presence of PRMT6. (E) Full length ERα or its truncation constructs were co-transfected with PRMT6 vector into COS7 cells for co-IP. Removal of VR did not affect the interaction between PRMT6 and ERα. (F) PRMT6 interacts with ERα at C-terminus LBD-region F. PRMT6 only pulled down LBD-region F but not N-terminus VR or VR-Hinge (1-355) domains. Bands were numbered and labeled at the side for easier identification.

Figure 1

A

+ ERα

pcDNA3.1
FLAG-PRMT1
FLAG-PRMT2
FLAG-PRMT3
FLAG-PRMT4
FLAG-PRMT5
FLAG-PRMT6
FLAG-PRMT7
FLAG-PRMT8

IP: anti-FLAG

WB: anti-ERα

100
75

50

37

WB: anti-FLAG

2% input

WB: anti-ERα

100
75

50

37

WB: anti-FLAG

B

2% input IP: anti-ERα

ERα   +   +
pcDNA3.1 +   +
PRMT6   + +   + +

75

WB: anti-ERα

50

WB: anti-PRMT6

37

C

2% input IP: anti-PRMT6

ERα   + + + +   + + + +
pcDNA3.1 + + − −   + + − −
PRMT6   − − + +   − − + +
17β-E2   − + − +   − + − +

ERα

PRMT6

ERα   *1* *0.82* *0.78* *0.93*   *0.96* *0.71*

D

2% input IP: anti-PRMT6

PRB   + + + +   + + + +
pcDNA3.1 + + − −   + + − −
PRMT6   − − + +   − − + +
R5020   − + − +   − + − +

PRB

PRMT6

PRB   *1* *1.3* *0.83* *1.1*   *0.94* *0.71*

E

2% input IP: anti-PRMT6

AR   + + + +   + + + +
pcDNA3.1 + + − −   + + − −
PRMT6   − − + +   − − + +
DHT   − + − +   − + − +

AR

PRMT6

AR   *1* *2.6* *1.21* *1.93*   *0.36* *0.15*

F

2% input IP: anti-PRMT6

GR   + + + +   + + + +
pcDNA3.1 + + − −   + + − −
PRMT6   − − + +   − − + +
Dex   − + − +   − + − +

GR

PRMT6

Figure 2

A

	+ pcDNA3.1	+ PRB	+ ERα	+ AR	- PRMT2	PRMT6

1.PRB
2.AR
3.ERα
4.PRMT2
5.PRMT6

B

Coomassie blue   Autoradiogram

ERα
PRMT6

C

PRMT6   mPRMT6+ERα   PRMT6+ERα   PRMT6+R260K

ERα
PRMT6

1   1.89   1   0.53   ERα

KDRR₂₆₀GGRMLK

Figure 3

A

	2% input	IP: anti-PRMT6

SRC-1   + +   + +
ERα   +   +
PRMT6   +   +

150 —
100 —
75 —

50 —

WB: anti-SRC-1

WB: anti-ERα

WB: anti-PRMT6

B

	2% input	IP: anti-ERα

SRC-1   + + +   + + +
ERα   + +   + +
17β-E2   +   +

150 —
100 —
75 —

50 —

WB: anti-SRC-1

WB: anti-ERα

C

	2% input	IP: anti-PRMT6

17β-E2   − +   − +

150 —
100 —
75 —

50 —

WB: anti-SRC-1

WB: anti-ERα

WB: anti-PRMT6

Figure 4

A

Flag-PRMT6 (594nm)	ERα (488nm)	DAPI	Merge

control

50 µm | 50 µm | 50 µm | 50 µm

E2

50 µm | 50 µm | 50 µm | 50 µm

B

1. pcDNA3.1-control | 2. pcDNA3.1-E2 | 3. PRMT6-control | 4. PRMT6-E2

PLA

20µm | 20µm | 20µm | 20µm

DAPI

20µm | 20µm | 20µm | 20µm

Merge

C

pcDNA3.1 Flag-PRMT6

E2   -   +   -   +

ERα

Flag-PRMT6

GAPDH

1   0.39   0.8   0.29   ERα/GAPDH

MCF7

D

EtOH   E2

C N Ch C N Ch

ERα

PRMT6

GAPDH

Histone H3

Figure 5

Figure 6

A

B

C

D

Figure 7

Figure 8

A 2% input   IP: anti-ERα

100 — Hsp90
75 — ERα
50 — PRMT6

B 2% input   IP: anti-PRMT6

100 — Hsp90
75 — ERα
50 — PRMT6

C 2% input   IP: anti-Hsp90

100 — Hsp90
75 — ERα
50 — PRMT6

D 2% input   IP: anti-ERα

Hsp90
ERα
PRMT6

E

Interaction

ERα   VR | DBD | Hinge | LBD | F   +
ER(ΔVR)   +

2% input   IP:anti-PRMT6

PRMT6 - + +   - + +

75 — ERα
50 — ER(ΔVR)
PRMT6

F

Interaction

ERα   FLAG-   VR | DBD | Hinge | LBD | F   +
ER(VR)   FLAG-   -
ER(1-355)   FLAG-   -
ER(356-595)   FLAG-   +

2% input   IP: anti-PRMT6

PRMT6 - ERα + ERα + ER(VR) + ER(356-595) + ER(1-355)   - ERα + ERα + ER(VR) + ER(356-595) + ER(1-355)

100
75 — 1
50
37
25
20

1.ERα
2.PRMT6
3.ER(1-355)
4.ER(356-595)
5.ER(VR)

WB: anti-FLAG

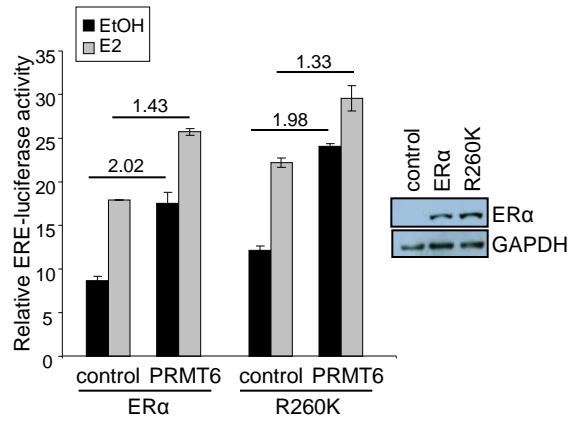**Supplementary Figure Legend**

Supplementary Fig. 1. PRMT6 enhances activity of both the wild-type ERα and ERα(R260K) mutant to a similar extent in HeLa cells. PRMT6 up-regulates ERE-luciferase activity for both wild-type and mutant ERα. The higher basal activity for R260K was most probably caused by the higher protein expression level of ERα(R260K) than wild-type ERα. Results shown is from a single experiment using triplicates, which is representative of two independent experiments, error bar represents standard error of mean. Fold induction by E2 treatment (10 nM, 24 hrs) is also indicated above the bars. Western blot showing the protein expression of wild-type ERα and R260K mutant is also included.

Supplementary Fig. 2. Additional PLA images showing that E2-treated MCF7-PRMT6 cells contain fewer but larger PLA dots representing ERα and PRMT6 interaction.

Supplementary Figure 1

Supplementary Figure 2