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Protein arginine methyltransferase 6 regulates multiple aspects of gene expression

Matthew J. Harrison, Yue Hang Tang and Dennis H. Dowhan*

The University of Queensland, Diamantina Institute for Cancer, Immunology and Metabolic Medicine, Princess Alexandra Hospital, Woolloongabba, QLD 4102, Australia

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ABSTRACT

It is well established that transcription and alternative splicing events are functionally coupled during gene expression. Here, we report that protein arginine *N*-methyltransferase 6 (PRMT6) may play a key role in this coupling process by functioning as a transcriptional coactivator that can also regulate alternative splicing. PRMT6 coactivates the progesterone, glucocorticoid and oestrogen receptors in luciferase reporter assays in a hormone-dependent manner. In addition, small interfering RNA (siRNA) oligonucleotide duplex knockdown of PRMT6 disrupts oestrogen-stimulated transcription of endogenous GREB1 and progesterone receptor in MCF-7 breast cancer cells, demonstrating the importance of PRMT6 in hormone-dependent transcription. In contrast, the regulation of alternative splicing by PRMT6 is hormone independent. siRNA knockdown of PRMT6 increases the exon inclusion:skipping ratio of alternatively spliced exons in endogenous vascular endothelial growth factor and spleen tyrosine kinase RNA transcripts in both the presence and absence of oestrogen. These results demonstrate that PRMT6 has a dual role in regulating gene expression and that these two functions can occur independently of each other.

INTRODUCTION

Steroid hormone receptors (SHRs) and nuclear receptors (NRs) are members of a large superfamily of ligand-inducible transcription factors (1,2). SHRs/NRs activate transcription in a ligand-dependent manner by binding to hormone response elements in promoters and enhancer regions of target genes. DNA-bound SHRs/NRs recruit coactivators, which are often recruited to gene promoters in multi-protein complexes where they help to reorganize chromatin into a transcriptionally active state by remodelling nucleosomes or modifying histone tails (3,4). The p160/steroid receptor coactivator (SRC) family of SHR/NR coactivators is comprised of three related proteins: SRC-1, SRC-2/GRIP1/TIF2 and SRC-3/pCIP/ACTR/AIB1/RAC3/TRAM1. The p160/SRC coactivators bind directly to the carboxyl terminal activation function-2 domain of SHRs/NRs via three NR boxes, each consisting of an LXXLL motif (where L is a leucine and X is any amino acid), and recruit secondary coactivators via their activation domains (ADs) (5). The basic-helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) domain of p160/SRC proteins binds to the secondary coactivators CoCoA, GAC63, hMMS19 and Fli-I (6–9); the AD1 binds to the acetyltransferases CREB-binding protein (CBP) and p300 (10,11); and the AD2 recruits the protein *N*-arginine methyltransferases (PRMTs) PRMT1 and coactivator associated arginine methyltransferase 1 (CARM1/PRMT4) (12,13).

PRMT1 and CARM1 both function as secondary coactivators for the oestrogen receptor-α (ERα), thyroid hormone receptor (TR) and androgen receptor (AR) (12,13). They require their enzymatic activity for this process, which results in the transfer of either one or two methyl groups from the methyl donor *S*-adenosyl-L-methionine to guanidino nitrogen atoms in arginine residues. (14,15). Methylation of arginine residues on histone tails is believed to play a major role in transcriptional activation by PRMTs (16–18). In addition, PRMTs have been demonstrated to methylate an increasing number of non-histone proteins involved in transcription (19,20). For example, methylation of various sites of CBP, p300 and RAC3 by CARM1 regulates the ability of these proteins to bind to other coactivators, and thus may play a role in controlling the assembly and disassembly of coactivator complexes on an active promoter (21–23). This regulation of coactivator complex formation means that CARM1 activity enhances the ability of other coactivators to promote transcription. Indeed, CARM1 acts synergistically with

*To whom correspondence should be addressed. Tel: +61 7 3240 5285; Fax: +61 7 3240 5946; Email: d.dowhan@uq.edu.au

PRMT1, Fli-I, CoCoA, TIF1α, CCAR1, SRCAP, GAC63 or any of the three acetyltransferases p300, CBP or p/CAF to stimulate steroid hormone-dependent transcription (6,8,9,13,24–28). This demonstrates that PRMTs are an integral component of the steroid-hormone-signalling pathway and are required to fully activate steroid-hormone-regulated genes.

Transcriptional coactivators, including CoAA, p72, CAPERα and CAPERβ, Sam68, p68, ASC-1, ASC-2 and CARM1, have been found to also regulate pre-mRNA processing (29–34). Indeed, over 20 transcriptional coregulators are structurally or functionally related to known splicing factors (35). CARM1 is the only PRMT previously shown to directly regulate alternative splicing. CARM1 methylates the splicing factors CA150, SAP49, SmB and U1C and promotes skipping of both a splicing reporter and the endogenous CD44 gene in a methylation-dependent manner (31). The exact mechanism behind splicing regulation by CARM1 is unknown. However, arginine methylation can promote the assembly of small nuclear ribonucleoproteins (snRNPs), and regulate the localization of many RNA-binding proteins, such as heterogeneous nuclear ribonucleoproteins (hnRNPs) (36–39).

Protein arginine *N*-methyltransferase 6 (PRMT6) is a predominantly nuclear, type-I arginine methyltransferase, that has been demonstrated to methylate Histone 3 on arginine 2 (H3R2), H4R3 and H2AR3 (40–42). PRMT6 has also been found to methylate the HIV Tat, nucleocapsid and Rev proteins, which interferes with the ability of these proteins to interact with RNA (43–45). PRMT6 can also methylate the splicing factors RDA288 and hnRNP D (31). As PRMT6 targets are involved in both transcription and RNA processing, we investigated the ability of PRMT6 to regulate these events. Here, we report that PRMT6 coactivates SHR-dependent transcription in a hormone-dependent manner, and regulates alternative splicing by means of a hormone-independent mechanism.

MATERIALS AND METHODS

Plasmids

The pCDNA3.1-PR, pCDNA3.1-ERα, pCDNA3.1-ERβ, pCMX-β-galactosidase, pCMX-VP16-N, pCMX-GAL4-N, pG5E1b-luciferase, pGL3-E1b-luciferase, pGL3-PRE-E1b-luciferase, pGL3-ERE-E1b-luciferase, pSG5 (Stratagene), pCMX and pCM5 expression and reporter plasmids have all been described previously (29,30,46). The RARE-E1b-luciferase was constructed by inserting the sequence 5′-GTACCGGGTAGGGTTCAC CGAAAGT TCACTCGACGGGTAGGGTTCACCGA AAGTTCACTCGAG-3′ containing two retinoic acid receptor (RAR) response elements and its corresponding complementary sequence into the Asp718/Nhe1 sites of pGL3-E1b-luciferase. The human SRC-1 complementary DNA (cDNA) was a gift from Dr William W. Chin and was cloned into a pCMX, pCMX-GAL4-N or pSG5-based expression vectors. The pSV-GR and pCMX-RARα plasmids were gifts from Dr David D. Moore. The pSG5-HA-CARM1 was a gift from Dr Michael R. Stallcup (12). The peroxisome proliferator activated receptor-γ (PPARγ) and the PPAR-TK-luciferase vectors were a gift from Dr Jon Whitehead. The cDNAs for human PRMT6 and human PRMT1 were obtained from the I.M.A.G.E. consortium (47). The PRMT6 and PRMT1 cDNA clones were sequenced and the cDNAs were cloned into a pCMX or pCM5-based expression vectors. The PRMT6 mutant containing the open reading frame of PRMT6 with amino acids 86 and 88 changed from valine/aspartic acid to lysine/alanine respectively (V86K/D88A), was produced by low-cycle polymerase chain reaction (PCR) using *pfu*-turbo DNA polymerase (Stratagene). A wild-type PRMT6 clone containing only the PRMT6 open reading frame was also produced by low-cycle PCR using *pfu*-turbo DNA polymerase and cloned into pCMX-VP16-N and pCMX or pCM5-based expression vectors. All PCR-generated clones were verified by sequencing and were tested by transfection and western blotting to ensure that they generate similar expression levels as vectors expressing wild-type proteins. The precise cloning steps for the clones used are available on request. The RHCglo minigene was a gift from Dr Thomas A. Cooper (48). To make the RHCglo-SYK-Exon 7 minigene, PCR primers targeting intron 6 (5′-GCGCGTCGACGCTCG GTGAGACAGATCCATA-3′) and intron 7 (5′-GCGC GGCTAGCCTCAGATTACACCTCTTCTTTAC-3′) were used with HeLa cell genomic DNA, PfuUltra II Fusion HS DNA polymerase and PCR to produce a human genomic DNA fragment encompassing 602 bp of intronic sequence upstream of Syk Exon 7, and 532 bp of intronic sequence downstream of Exon 7. This PCR product was digested with Sal1/Nhe1, agarose gel isolated and cloned into the Sal1/Xba1 sites of the RHCglo minigene reporter. The cloned Syk PCR sequence was confirmed by sequencing.

Cell culture and transfection

HeLa and CV-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. For luciferase assays, HeLa or CV-1 cells were plated in 24-well plates and cultured for 24 h in DMEM without phenol red, supplemented with 10% charcoal-stripped FBS before transfection. Cells were transiently transfected at 60–70% confluence by using calcium phosphate precipitation with 100 ng reporter, 0.15–15 ng individual SHR/NR (15 ng ERα, 15 ng ERβ, 12 ng PR, 15 ng GR, 2.5 ng RARα or 15 ng PPARγ unless stated otherwise), 50 ng PRMT6, 50 ng PRMT6 V86K/D88A, 50 ng PRMT1, 50 ng CARM1, 100 ng SRC-1 as indicated and 10 ng β-galactosidase expression vectors per well. An appropriate amount of empty expression vector was used in assays without cofactor and the total amount of DNA was brought to 1 μg/well using pGEM4. Post-transfection, cells were washed with phosphate-buffered saline (PBS) and maintained in DMEM without phenol red plus 10% charcoal-stripped serum. The media was supplemented with the appropriate ligand as indicated: 10^{-8} M

progesterone (Pg), 10^{-9} M estradiol (E2), 10^{-7} M *all-trans* retinoic acid (RA), 10^{-8} M Dexamethasone (Dex), 10^{-5} M Ciglitazone (Cig) or vehicle alone (ethanol). After 24–36 h incubation, cells were harvested and cell extracts assayed for luciferase and β-galactosidase activity on a Berthold luminometer. Mammalian-2-hybrid assays were performed in HeLa cells cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Mammalian-2-hybrid transfections were carried out using 100 ng pG5E1b-luciferase reporter, 25 ng Gal4 or Gal4-SRC-1, 300 ng VP16 or VP16-PRMT6 and 10 ng β-galactosidase expression vectors per well. After 24–36 h incubation, cells were harvested and cell extracts assayed for luciferase and β-galactosidase activity. The luciferase assay and Tropix β-galactosidase chemiluminescent assay were done according to the manufacturer's directions (Applied Biosystems) and β-galactosidase activity was used as an internal control to monitor for transfection efficiency. For luciferase assays, data shown are the means and standard deviations (SD) of results from four transfected cultures.

Splicing assays using the RHCglo-Syk-Exon 7 minigene were performed by transiently transfecting HeLa cells grown to 50% confluency in 6-well plates with 400 ng of RHCglo-Syk-Exon 7 reporter, 75 ng of PRMT6, PRMT6 V86K/D88A, CARM1 or empty expression vector, and pGEM4 to bring up total DNA to 1.5 μg/well. Transfections were performed by using Lipofectamine™ 2000 (Invitrogen) as described by manufacturer's instructions, and the cells were harvested 24 h post-transfection. RNA transcript quantification using γ-32P labelled primers and real-time (RT)-PCR were performed as previously described (29,33), using the RHCglo specific primers RSV5U and RTRHC as previously described (48) and RT-PCR without reverse transcriptase did not produce any detectable PCR products (data not shown). An autoradiograph of the radiolabeled RT-PCR products from a representative experiment is shown. Six replicate wells of cells were used for each experimental condition and all experiments were repeated twice.

MCF-7 cells were maintained in DMEM nutrient mixture F-12 plus 10% FBS. During experiments utilising hormone, cells were plated and maintained in phenol red-free DMEM nutrient mixture F-12 containing 10% charcoal-stripped serum at 37°C and 5% CO_2. To knock down PRMT6 expression levels, MCF-7 cells were transfected with small interfering RNA (siRNA) oligonucleotide duplex at a final concentration of 10 nM using RNAiMAX (Invitrogen) according to manufacturer's instructions. The annealed siRNA duplex sequences used were: PRMT6-siRNA-1, sense 5′-CGGG ACCAGCUGUACUACGTT-3′ and PRMT6-siRNA-1, anti-sense 5′-CGUAGUACAGCUGGUCCCGTT-3′. A scrambled control was used as a negative control: Control-siRNA, sense 5′-CAGCGACUAAACACAUCA ATT-3′ and Control-siRNA, anti-sense 5′-UUGAUGUG UUUAGUCGCUGTT-3′. After 48 h post-transfection, cells were treated for 12 h with 10^{-9} M E2 or vehicle control (ethanol) prior to harvesting RNA.

RNA extraction and quantitative RT–PCR

Total RNA was extracted from MCF-7 cells using Trizol Reagent (Invitrogen), according to manufacturer's protocol. Total RNA for quantitative (Q)-RT-PCR was further purified by using RNeasy RNA mini-purification columns with on column DNase treatment according to manufacturer's instructions (Qiagen). RNA was normalized using UV spectrophotometry and agarose gel electrophoresis. cDNA was synthesized from 600 ng of total RNA using TaqMan Reverse Transcription Reagents, according to the manufacturer's instructions (Applied Biosystems). Gene expression levels were determined by Q-RT-PCR on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Assay on Demand Taqman primer/probes (Applied Biosystems); PRMT6 (Hs00250803_s1), CARM1/PRMT4 (Hs00406354_m1), PRMT1 (Hs01587651_g1), GREB1 (Hs00536409_m1), PR (Hs01556701_m1), ERα (Hs01046818_m1) and normalised to GAPDH (Cat#4326317E) and RPLP0 (Cat#4326314E) expression levels. Target cDNA levels were analyzed by Q-RT-PCR in 10 μl reactions using Taqman PCR master mix, Taqman probe/primer sets and cDNA (5% of the starting 600 ng of RNA). PCR was initiated at 95°C for 10 min to activate Amplitaq Gold DNA polymerase, followed by 45 cycles of 95°C for 15 s and 60°C for a 1-min two-step thermal cycling. Relative changes in gene expression were calculated using the ΔΔCt method. For each experiment, four replicate wells of cells were used for each experimental condition and all experiments were repeated twice.

In order to determine the effects of PRMT6 on splicing of the endogenous vascular endothelial growth factor (VEGF) nascent RNA, a modified version of the protocol described by Wellmann *et al.* (49) was used. In order to detect levels of total VEGF transcript ($VEGF_{total}$) and three splice products $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, a common forward primer (5′-CCCTGATGAGATCGAGTACATCTT-3′) and common fluorescent hydrolysation probe (5′-ATCCTGT GTGCCCCTGATGCGATGCGGT-3′) both located on exon 3 were used (Supplementary Figure S6A). Specific amplification of each spliced product was achieved by using reverse primers spanning exon boundaries specific for the relevant product. Reverse primers used were: $VEGF_{121}$ (5′-GCCTCGGCTTGTCACATTTT-3′), $VEGF_{165}$ (5′-AGCAAGGCCCACAGGGATTT-3′), $VEGF_{189}$ (5′-AACGCTCCAGGACTTATACCG-3′) and $VEGF_{total}$ (5′-ACCGCCTCGGCTTGTCAC-3′). Expression levels were normalized to GAPDH and RPLP0, and changes in the expression of the spliced variants was normalized to $VEGF_{total}$. Relative changes in gene expression were calculated using the ΔΔCt method. Four replicate wells of cells were used for each experimental condition and all experiments were repeated twice.

Alterations in the splicing of spleen tyrosine kinase (Syk) were detected using a modified protocol described by Wang *et al.* (50). The primers used to amplify Syk (Syk[L]) and the shorter alternatively spliced isoform (Syk[S]) were Syk-for 5′-AATCGGCACACAGGGAAA TG-3′ and Syk-rev 5′-AGCTTTCGGTCCAGGTAA

AC-3′, and to amplify β2-microglobulin were β2-for 5′-GATGAGTATGCCTGCCGTGTG-3′ and β2-rev 5′-CAATCCAAATGCGGCATCT-3′. The primers were radiolabelled using ^{32}P-γATP (3000 Ci/mmol) and T4 kinase (Invitrogen). To amplify the Syk and β2-microglobulin cDNA, PCR was conducted for 30 cycles (30 s at 94°C, 45 s at 56°C and 1 min at 68°C) and radioactive PCR products were separated on non-denaturing 5% polyacrylamide gels. Dried gels were subject to autoradiography and phosphorimaging using a Storm 860 phosphorimager and ImageQuant software (Molecular Dynamics). Levels of Syk alternatively spliced products were normalized to β2-microglobulin expression levels. An autoradiograph of the radiolabelled PCR products from a representative experiment is shown. Four replicate wells of cells were used for each experimental condition and all experiments were repeated twice. DNA products from the alternative splicing experiments were sub-cloned into pCR2.1 using the TOPO-TA cloning kit (Invitrogen) and DNA sequences confirmed by DNA sequencing on a 3730*xl* DNA Analyzer (Applied Biosystems).

Cell proliferation assays

Cell proliferation assays were performed in 96-well plates in phenol red-free DMEM nutrient mixture F-12 containing 10% charcoal-stripped serum. siRNA was transfected into MCF-7 cells (10 000 cells/well) using RNAiMAX (Invitrogen) according to manufacturer's instructions. Cells were transfected either with 20 nM control siRNA, 10 nM siRNA targeting PRMT6 and/or 10 nM siRNA targeting CARM1; CARM1-siRNA-1, sense 5′-ACCAA AUGAUGUCCCUGCCTT-3′ and CARM1-siRNA-1, anti-sense 5′-GGCAGGGACAUCAUUUGGUTT-3′. When transfecting siRNA targeting either PRMT6 or CARM1, 10 nM control siRNA was added to make a final siRNA concentration of 20 nM. After 24 h, cells were treated with 10^{-9} M E2 or vehicle control (ethanol). The proliferation assay was conducted by incubating the cells with a final concentration of 10 μCi/ml ^{3}H-thymidine for 19 h. The cells were then lysed and incorporated ^{3}H-thymidine was harvested onto a filter mat (Wallac). The ^{3}H-thymidine levels were counted using a 1450 MicroBeta TriLux scintillation counter (Wallac) according to manufacturer's instructions.

Western blotting

Cellular extracts (20 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted/transferred on to polyvinylidene fluoride membrane (Sigma–Aldrich). Non-specific binding sites were blocked by immersing the membranes in 5% skim milk/TBS buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.5% Tween 20) for 1–2 h at room temperature and the membrane was subsequently incubated with polyclonal PRMT6 (Sigma–Aldrich), CARM1 (Bethyl Laboratories), ERα (Santa Cruz Biotechnology) or β-tubulin (Sigma–Aldrich) antibodies in 1% skim milk/TBS at a concentration of 1 μg/ml O/N at 4°C, then washed three times in TBS containing 0.05% Tween 20. Further steps involving goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Invitrogen) as required and the catalysed oxidation of luminal were carried out with Pierce ECL western blotting detection reagents according to the manufacturer's protocols. Western blot images were obtained and recorded using a CHEMI5000 chemiluminescence documentation system and Chemi-Capt software. Densitrometry was performed using Bio-profil Bio-1D software.

In vitro binding assay and chromatin immunoprecipitation assay

Glutathione S-transferase (GST) and GST-PRMT6 were isolated from *Escherichia coli* BL21 or JM109 cells following 5 h induction with 0.1 mM isopropyl thio-β-D-galactosidase. Cells were harvested in MTPBS buffer [150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 (pH 7.3) plus protease inhibitors (Roche)], sonicated and centrifuged at 3000*g* to remove cell debris. Five-hundred nanograms of GST-fusion proteins were isolated by incubation with GST beads (Amersham Biosciences) for 3 h with rotation at 4°C. Beads were washed five times in buffer C [20 mM HEPES (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1 mM DTT plus protease inhibitors (Roche)] and resuspended in 200 μl HEMG buffer [100 mM KCl, 40 mM HEPES (pH 7.5), 0.2 mM EDTA, 0.1% NP-40 and 10% glycerol] plus 5%w/v glycine and 0.4 mg/ml BSA. ^{35}S-methione labelled proteins were synthesized using TNT Quick Coupled Transcription/Translation System (Promega). *In vitro* binding assays were performed by incubating 10–20 μl ^{35}S-methione labelled protein with GST-protein bound beads for 16 h at 4°C with rotation. Beads were then washed three times with HEMG buffer plus 5% w/v glycine and 0.4 mg/ml BSA and then twice in HEMG buffer alone. Proteins were eluted from the beads with Laemmli sample buffer and analysed by SDS-PAGE and autoradiography. All experiments were repeated twice. Chromatin immunoprecipitation (ChIP) was performed as previously described (51), using the MCF-7 breast cancer cell line and anti-PRMT6 monoclonal antibody (Sigma–Aldrich). To analyse the levels of recruitment of PRMT6 to the endogenous GREB1 and PR genes, immunoprecipitated chromatin was quantified by SYBR-green RT-PCR using previously described primer sequences (52–54).

Statistical analysis

Statistical analysis was performed using either one- or two-tailed Student's *t*-tests as appropriate.

RESULTS

PRMT6 enhances steroid-hormone-dependent transcription

PRMT6 is a nuclear protein that is related to the SHR/NR coactivators PRMT1 and CARM1 (55). We therefore investigated whether PRMT6 can enhance the

transcriptional activity of SHRs in transient transfection assays in mammalian cells (Supplementary Figure 1 and Figure 1). HeLa cells were transfected with a SHR/NR expression vector, an appropriate luciferase reporter and an expression vector for PRMT6 as indicated. As shown in Figure 1A, PRMT6 significantly increased the activity of ERα on an ERE-E1b-luciferase reporter in a ligand-dependent manner by ~1.5-fold. In a similar experiment investigating the activity of ERβ, PRMT6 increased transcriptional activity by ~2.1-fold (Figure 1B). Furthermore, PRMT6 enhanced the transcriptional activity of the progesterone receptor (PR) by ~3.7-fold and the glucocorticoid receptor (GR) by ~1.7-fold (Figures 1C and D). Similar results were also obtained in CV-1 cells (Supplementary Figure S1A–D). All increases in transcriptional activity were ligand-dependent, showing that PRMT6 acts as a coactivator for SHRs rather than a general transcription enhancer. In contrast, PRMT6 was unable to coactivate several non-steroidal NRs tested, including the RARα, PPARγ and TRβ (Figure 1E and F, and data not shown). These NRs were still responsive to other known coactivators, demonstrating that the reporter had not reached its maximal level in the presence of ligand-bound receptor (Supplementary Figure S2). Therefore, transcriptional coactivation stimulated by PRMT6 has specificity in relation to certain SHR/NRs.

PRMT6 requires its enzymatic activity to function as a coactivator

In order to determine the importance of arginine methylation on the ability of PRMT6 to function as a transcriptional coactivator, we engineered a previously described PRMT6 methylation-deficient mutant (VLD/KLA at amino acids 86/88) (43). In HeLa and CV-1 cells, the PRMT6 V86K/D88A mutant was unable to function as a coactivator for SHRs/NRs and did not coactivate ERα, ERβ, PR or GR (Figure 1A–D and Supplementary Figure S1A–D). In both HeLa and CV-1 cells, the PRMT6 V86K/D88A mutant functioned in a dominant-negative fashion by decreasing the transcriptional activity of the PR to lower than observed with the receptor alone. From these experiments, we determined that PRMT6 requires its enzymatic activity to function as a transcriptional coactivator for SHRs.

Mechanism of recruitment of PRMT6 to SHRs

PRMT1 and CARM1 have both been shown to be secondary coactivators that are recruited to SHRs/NRs via the AD2 domains of p160/SRC proteins. It has been demonstrated that this leads to a synergistic response in transcriptional activation when either PRMT1 and CARM1 is co-transfected with a member of the p160/SRC family (12,13). In order to determine whether PRMT6 is also a secondary coactivator, we investigated the transcriptional response of ERα on an ERE-E1b-luciferase reporter in the presence of both PRMT6 and SRC-1 in CV-1 cells (Figure 2A). For this experiment, we reduced the levels of ERα to 5 ng as synergy between multiple PRMTs and p160/SRC proteins has been demonstrated to require low levels of SHR/NRs (13). This lower ERα level reduces the response of the reporter to ERα and E2 alone, while allowing greater levels of coactivation to be observed by exogenous coactivators. At this lower ERα level, SRC-1 increased transcriptional activity by ~7.1-fold and PRMT6 by ~4.1-fold compared to the response to ERα alone. However, SRC-1 and PRMT6 together lead to a synergistic enhancement of transcription of ~44.7-fold. We therefore investigated the binding of PRMT6 to SRC-1 using a mammalian-two-hybrid assay. PRMT6 was cloned into pCMX-VP16-N to produce a VP16 transcriptional activation domain/PRMT6 chimera (VP16-PRMT6) and SRC-1 was cloned into pCMX-GAL4-N to produce a Gal4 DNA-binding domain/SRC-1 chimera (Gal4-SRC-1). Co-transfection of VP16-PRMT6 and Gal4-SRC-1 lead to a significant increase in luciferase activity compared to transfection of either plasmid alone, demonstrating that PRMT6 is able to associate with full-length SRC-1 within the context of mammalian cells (Figure 2B). This interaction was confirmed using GST-pull-down assays with GST-tagged PRMT6 and ^{35}S-labelled SRC-1 (Figure 2C). Luciferase, used as a negative control, showed no interaction with PRMT6 in GST-pull-down assays. In order to determine which regions of SRC-1 interact with PRMT6, we divided the SRC-1 protein into three regions (Figure 2C). An amino-terminal region of SRC-1, containing the bHLH/PAS domain and the NR boxes, weakly interacted with PRMT6. The central region of SRC-1, from amino acids 620–1006, containing the NR boxes and AD1 domain, showed no interaction with PRMT6. However, PRMT6 did bind strongly to the carboxyl-terminal region of SRC-1 composed of amino acids 1006–1441, which contains the AD2 domain.

We then investigated whether PRMT6 is recruited to an active oestrogen-dependent promoter in response to oestrogen. ERα binds to three EREs to activate transcription of the GREB1 gene, but does not bind at a region 6 kb downstream of the GREB1a transcription start site (52). We, therefore, investigated whether PRMT6 is recruited to these sites in response to oestrogen treatment using ChIP assays. Following 15 min oestrogen stimulation, PRMT6 was recruited to EREs in the GREB1 promoter (Figure 2D). This recruitment was cyclical and returned to baseline levels after 45 min oestrogen treatment. There was no recruitment of PRMT6 to the 6 kb downstream site, which served as a negative control. We then examined PRMT6 recruitment to a second oestrogen-dependent gene, the progesterone receptor. ERα binds to an enhancer located +169 kb downstream of the PR transcriptional start site (53,56). ChIP assays demonstrated that PRMT6 is recruited to this enhancer region following 15 min oestrogen stimulation (Figure 2E). Again, PRMT6 occupancy returned to baseline levels after 45 min oestrogen treatment. A region 5 kb downstream of the PR transcriptional start site, that does not show ERα binding (54), served as a negative control and showed no PRMT6 occupancy.

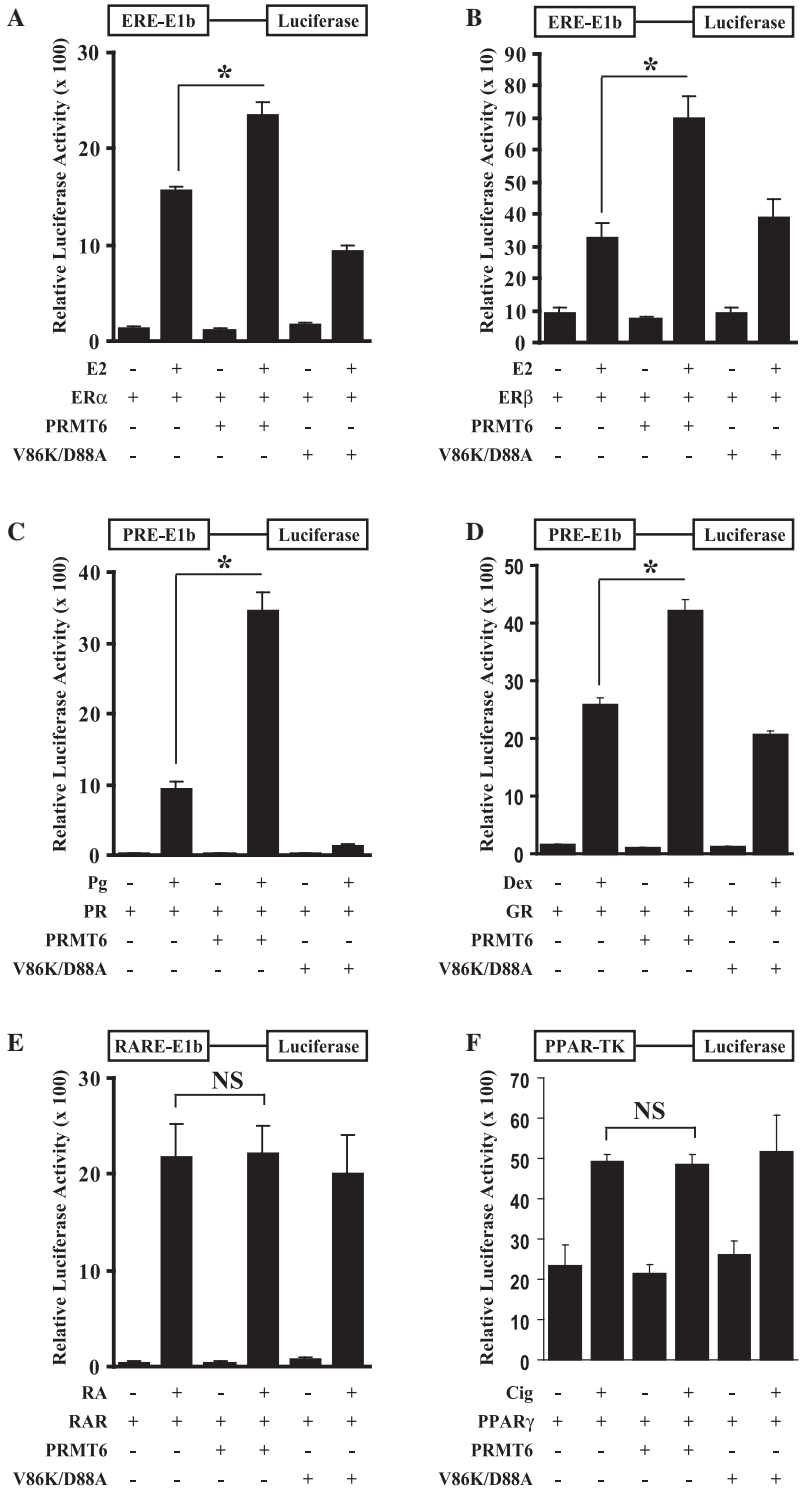

Figure 1. PRMT6 coactivates SHRs in HeLa cells. (**A**) PRMT6 coactivates ERα transcriptional activity from an oestrogen response element linked to a minimal promoter. HeLa cells were co-transfected with an ERE-E1b-luciferase reporter along with expression vectors for ERα alone, or with PRMT6 or PRMT6 V86K/D88A as indicated. Cells were treated with vehicle (ethanol) or 10^{-9} M E2 as indicated and tested for luciferase activity (see 'Materials and Methods' section). (**B**) PRMT6 coactivates ERβ transcriptional activity from the ERE-E1b-luciferase reporter. HeLa cells were co-transfected with an ERβ expression plasmid as in (A). (**C**) PRMT6 enhances the transcriptional activity of PR. HeLa cells were co-transfected with a PRE-E1b-luciferase reporter along with expression vectors for PR alone, or with PRMT6 or PRMT6 V86K/D88A mutant and treated with vehicle (ethanol) or 10^{-8} M Pg as indicated. (**D**) PRMT6 enhances the transcriptional activity of GR. HeLa cells were co-transfected with PRE-E1b-luciferase reporter along with expression vectors for GR alone, or PRMT6 or PRMT6 V86K/D88A mutant and treated with vehicle (ethanol) or 10^{-8} M Dex as indicated. (**E**) PRMT6 does not coactivate the RARα. HeLa cells were co-transfected with RARE-E1b-luciferase reporter along with expression vectors for RARα alone, or with PRMT6 or PRMT6 V86K/D88A mutant and treated with vehicle (ethanol) or 10^{-7} M RA as indicated. (**F**) PRMT6 does not coactivate PPARγ transcriptional activity. HeLa cells were co-transfected with a PPAR-TK-luciferase reporter along with expression vectors for PPARγ alone, or with PRMT6 or PRMT6 V86K/D88A mutant and treated with vehicle (ethanol) or with 10^{-5} M Cig as indicated. Each data point represents the mean and standard deviation (SD) of results from four transfected cultures. Results shown are from a single experiment, which is representative of three independent experiments. $^{*}P < 0.001$; NS, no significant change.

PRMT6 enhances transcriptional activation with CARM1 in the presence of SRC-1

It has been previously demonstrated that transient transfection experiments using very low levels of SHR display a greater requirement for several coactivators (13). Under these conditions, CARM1 and PRMT1 cooperate to synergistically enhance transcription in the presence of a p160/SRC protein. In order to determine whether PRMT6 is also capable of cooperating with other PRMTs, we transfected CV-1 cells with a very low level of ERα (0.15 ng) and various combinations of PRMT1, CARM1, PRMT6 and SRC-1 in luciferase reporter assays (Figure 3). At this low level of SHR, none of the PRMTs alone could stimulate transcription, as observed in previous studies (13). This is due to the requirement of several coactivators to stimulate transcription at this level of SHR. However, co-transfection of PRMTs with SRC-1 led to an increase in ERα transcriptional activity, confirming the roles of PRMTs as secondary coactivators (Figure 3; compare lanes 5 to 2, 6 to 3 and 7 to 4). Co-transfecting combinations of two of the PRMTs together in the absence of SRC-1 had little effect on transcriptional activation. However, PRMT1 and CARM1 cooperated to stimulate ERα activity in the presence of SRC-1 (Figure 3, lane 12). Similar results were obtained when PRMT6 was substituted for PRMT1, indicating that PRMT6 acts cooperatively with CARM1 and SRC-1 to promote ERα-dependent transcription (Figure 3, lane 14). Interestingly, CARM1 was necessary for this cooperative effect, as the same stimulation was not seen when SRC-1, PRMT1 and PRMT6 were co-transfected (Figure 3, lane 13). In addition, transfecting all three PRMTs together gave a similar transcriptional response as transfecting CARM1 with either PRMT1 or PRMT6 (Figure 3, lane 15). Therefore, both PRMT1 and PRMT6 cooperate with CARM1 to stimulate ERα-dependent transcription, but do not cooperate together. Furthermore, this enhanced transcriptional response is dependent on the presence of SRC-1.

PRMT6 is an integral component of the oestrogen-signalling pathway

In order to determine the importance of PRMT6 in the transcription of hormone-responsive genes within mammalian cells, we used siRNA interference to knockdown PRMT6 in MCF-7 cells and determined the effects on transcription of endogenous oestrogen-regulated genes. Transfection of PRMT6-siRNA-1 into MCF-7 cells reduced PRMT6 levels by ~80% at both the RNA and protein levels (Figure 4A–C). Importantly, knockdown by PRMT6-siRNA-1 was specific for PRMT6 and had no effect on the RNA levels of CARM1 and PRMT1, and no effect on the protein levels of CARM1 (Figure 4A, D and E). Knockdown of PRMT6 also had no effect on the levels of ERα at either the RNA or the protein level (Supplementary Figure S3). As PRMT6 is recruited to the promoter and enhancer regions of the GREB1 and PR genes (Figure 2D and E), we determined whether PRMT6 is required for the activation of these genes. Compared to treatment with a control siRNA, knockdown of PRMT6 had no significant effect on the transcription of GREB1 and PR in the absence of oestrogen (Figure 4F and G). However, the oestrogen-activated expression of both GREB1 and PR was significantly reduced by PRMT6 knockdown. This result establishes a role for PRMT6 in the activation of oestrogen-dependent transcription and the expression of endogenous oestrogen-regulated genes. A second PRMT6 siRNA targeting the 3′UTR of PRMT6 (PRMT6-siRNA-2) was also tested and produced similar results (Supplementary Figure S4).

PRMT6 mediates oestrogen-dependent proliferation

As PRMT6 plays a role in oestrogen signalling, we investigated whether PRMT6 is involved in the oestrogen-stimulated proliferation of ERα-expressing breast cancer cells. We used PRMT6-siRNA-1 to knockdown PRMT6 expression in MCF-7 cells and measured cellular proliferation following oestrogen treatment. As CARM1 is known to play a role in MCF-7 cell proliferation in response to oestrogen (57), we also knocked down CARM1 expression to allow the effects of these two PRMTs to be compared. In addition, as PRMT6 and CARM1 can function synergistically in oestrogen signalling (Figure 3), we also knocked down the two proteins together to see if they function cooperatively in regulating oestrogen-induced proliferation. The siRNA trigger targeting CARM1 reduced CARM1 expression by ~80% without affecting PRMT6 or PRMT1 expression (Supplementary Figure S5A–C). Knockdown of either PRMT6 or CARM1 levels significantly reduced the proliferation of MCF-7 cells 72 and 96 h after oestrogen treatment compared to treatment with control siRNA. Reducing the levels of either PRMT6 or CARM1 resulted in a similar inhibition of oestrogen-stimulated proliferation (Figure 5A). In addition, treating the cells with siRNAs targeting both PRMT6 and CARM1 together had an additive effect, significantly lowering proliferation when compared to treatment with either siRNA individually. These effects were oestrogen dependent, as knocking down PRMT6 and CARM1 either individually or together had no effect on MCF-7 proliferation in the absence of oestrogen (Figure 5B). Similar results were also obtained using P6-siRNA-2 (Supplementary Figure S5D and E). Therefore, PRMT6 is an integral component of the oestrogen-signalling pathway, and is required to stimulate both oestrogen-dependent transcription and cellular proliferation.

PRMT6 regulates alternative splicing

To investigate the ability of PRMT6 to regulate alternative splicing, we examined the splicing of the VEGF gene following knockdown of PRMT6 in MCF-7 cells. Alternative splicing of VEGF produces several major spliced forms of mRNA (58). We used Q-RT-PCR analysis to determine the relative levels of three of these isoforms: full-length VEGF ($VEGF_{189}$), VEGF following skipping of exon 6 ($VEGF_{165}$) and VEGF following skipping of exons 6 and 7 ($VEGF_{121}$) (Figure 6A). MCF-7 cells were transfected with either control siRNA

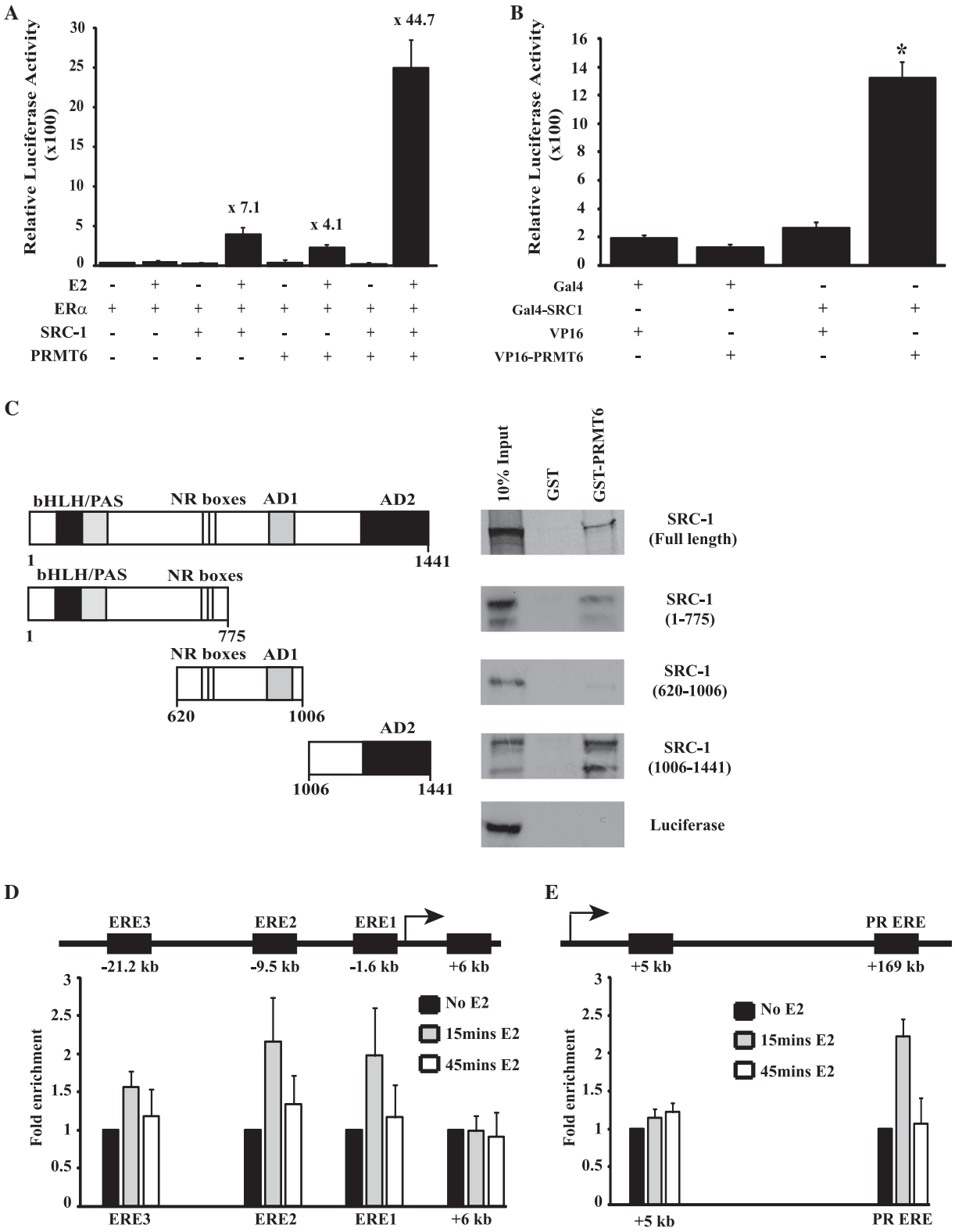

Figure 2. PRMT6 synergistically coactivates ERα transcriptional activity in the presence of SRC-1. (**A**) CV-1 cells were transiently co-transfected with an ERE-E1b-luciferase reporter, 5-ng expression vector for ERα, along with expression vectors for PRMT6 or SRC-1 as indicated. Following treatment with vehicle (ethanol) or 10^{-9} M E2, cells were assayed for luciferase activity. Numbers above bars show fold increase in luciferase activity compared to transfection with ERα alone and 10^{-9}M E2 stimulation. Each data point represents the mean and SD of results from four transfected cultures. Results shown are from a single experiment, which is representative of three independent experiments. (**B**) Mammalian-2-hybrid analysis demonstrates that PRMT6 interacts with full-length SRC-1. HeLa cells were co-transfected with pG5-E1b-luciferase reporter plasmid along with expression vectors for the Gal4 DNA-binding domain alone (Gal4), Gal4-SRC-1 chimera, the VP16 transcriptional activation domain alone (VP16) or VP16-PRMT6 chimera as indicated. Cell extracts were tested for luciferase activity. Each data point represents the mean and SD of results from

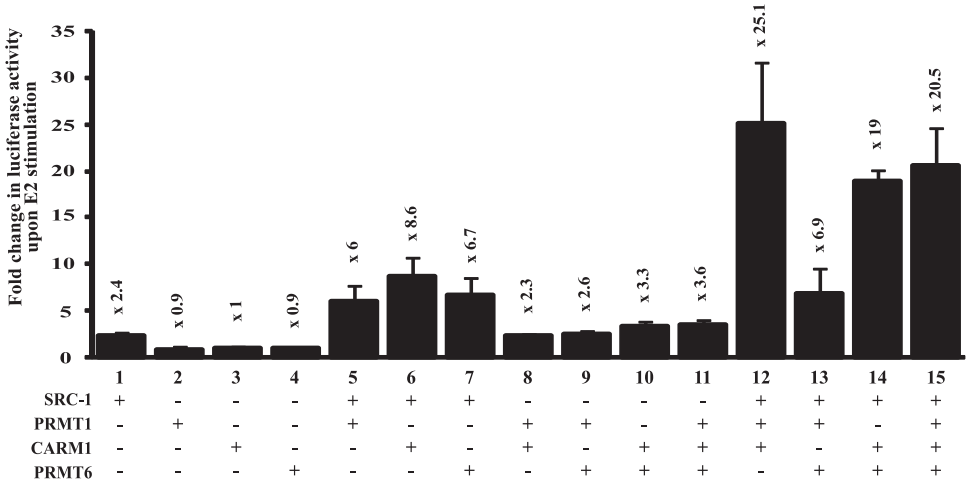

Figure 3. PRMT6 and CARM1 synergistically coactivate ERα transcriptional activity in the presence of SRC-1. CV-1 cells were co-transfected with an ERE-E1b-luciferase reporter, 0.15-ng expression vector for ERα, and combinations of expression vectors for PRMT6, CARM1, PRMT1 or SRC-1 as indicated. Following oestrogen treatment, cells were assayed for luciferase activity. Numbers above bars show fold increase in luciferase activity upon 10^{-9} M E2 stimulation. Each data point represents the mean and SD of results from four transfected cultures. Results shown are from a single experiment, which is representative of three independent experiments.

or PRMT6-siRNA-1 and knockdown of PRMT6 was verified by Q-RT-PCR (Supplementary Figure S6B–D). Whilst reduction of PRMT6 transcript had no effect on levels of $VEGF_{121}$, it did produce a significant increase in the levels of $VEGF_{189}$ and a decrease in the levels of $VEGF_{165}$ compared to treatment with control siRNA (Figure 6B). This leads to a >2-fold increase in the $VEGF_{189}$:$VEGF_{165}$ ratio (Figure 6C). This change was observed in both the presence and absence of oestrogen, despite the transcription of VEGF being oestrogen-regulated (Supplementary Figure S6H). Therefore, the regulation of alternative splicing of VEGF by PRMT6 does not require steroid hormone stimulation. A similar change in VEGF splicing was observed following PRMT6 knockdown by a different siRNA targeting PRMT6 (PRMT6-siRNA-2) (Supplementary Figure S6E–G and S6I-K). These results suggest that PRMT6 can play a role in the alternative splicing of endogenous genes in MCF-7 cells, and in contrast to its effects on transcription, the regulation of alternative splicing is hormone-independent. As CARM1 can also regulate alternative splicing in a steroid hormone-independent manner, we investigated whether PRMT6 and CARM1 are redundant for splicing of VEGF. Two siRNAs were designed to significantly reduce the levels of CARM1, but not PRMT6 or PRMT1 (Supplementary Figure S7A–C). Similar to PRMT6, knockdown of CARM1 in MCF-7 cells had no effect on $VEGF_{121}$ and significantly reduced the levels of $VEGF_{165}$ (Supplementary Figure S7E). However, in contrast to PRMT6, CARM1 knockdown had no effect on the levels of $VEGF_{189}$ (Supplementary Figure S7E). Therefore, PRMT6 and CARM1 display similar but not redundant effects on the splicing of VEGF.

To further investigate the ability of PRMT6 to regulate RNA processing, we examined the effect of PRMT6 on the alternative splicing of Syk. Syk is expressed in breast tissue and has been implicated to play a role in breast cancer (59). Two alternatively spliced isoforms of Syk have been documented (Figure 6D): full-length Syk (Syk[L]) and a shorter form that lacks a 69-bp sequence comprising exon 7 (Syk[S]) (60–62). In order to investigate the regulation of alternative splicing of the endogenous Syk gene by PRMT6, PRMT6 levels were reduced in MCF-7 cells using PRMT6-siRNA-1. The two different Syk transcripts were then detected by reverse transcriptase-PCR with ^{32}P-radiolabelled primers that span the alternative exon of Syk[L]. PRMT6 knockdown led to a significant increase in the Syk[L]:Syk[S] ratio (Figure 6F and Supplementary Figure S8A). A similar result was seen using a second siRNA directed against

four transfected cultures. Results shown are from a single experiment, which is representative of three independent experiments. *$P < 0.001$. (**C**) GST pull-down assays were used to test the ability of GST-PRMT6 to interact with full length or fragments of ^{35}S-radiolabelled SRC-1. GST alone and *in vitro* translated ^{35}S-radiolabelled luciferase served as negative controls. A schematic representation of the SRC-1 protein fragments used in the assays is shown in the panel, with amino acid positions of the SRC-1 protein or SRC-1 protein fragments indicated. The major functional domains of SRC-1 are indicated, bHLH/PAS, NR Boxes, AD1 and AD2. (**D**) Recruitment of PRMT6 to oestrogen response elements (EREs) located in promoter regions of the GREB1 gene. Following 0-, 15- and 45-min treatment of MCF-7 cells with 10^{-9} M E2, recruitment of PRMT6 to EREs in the GREB1 promoter and to a site downstream of the GREB1 transcriptional start site (+6 kb) was determined by chromatin immunoprecipitation as detailed in 'Materials and Methods' section. Results show the average and SD of four independent experiments. (**E**) Recruitment of PRMT6 to an oestrogen-receptor-binding enhancer region of the PR gene. Following 0-, 15- and 45-min treatment of MCF-7 cells with 10^{-9} M E2, recruitment of PRMT6 to a known oestrogen-receptor-binding enhancer region of the PR gene (PR ERE) and to a site downstream of the PR gene transcriptional start site (+5 kb) was determined by chromatin immunoprecipitation as detailed in 'Materials and methods' section. Results show the average and SD of four independent experiments.

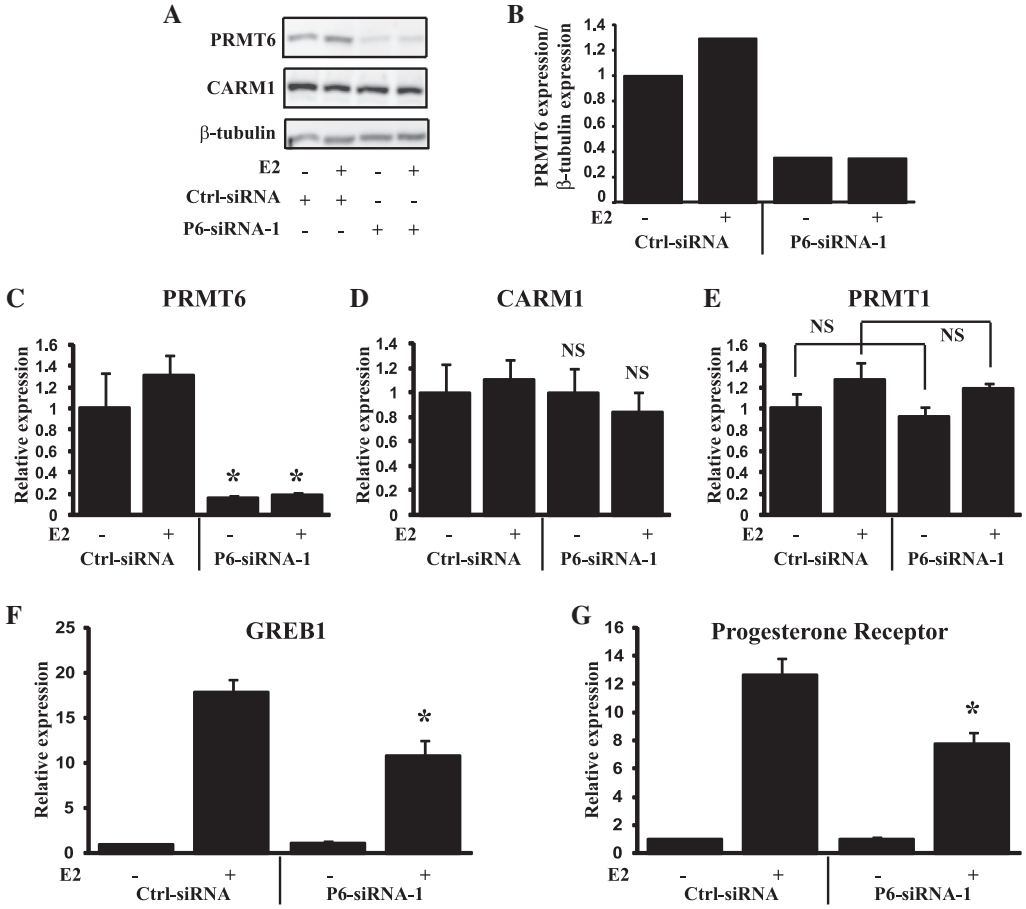

Figure 4. Knockdown of PRMT6 expression disrupts oestrogen signalling. (**A**) Western blot showing PRMT6, CARM1 and β-tubulin expression in MCF-7 cells following transfection with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-1) and treatment with or without 10^{-9} M E2 for 12 h as indicated. (**B**) Graphical representation of PRMT6 expression levels of western blot shown in (A). (**C**) Q-RT-PCR analysis of PRMT6 RNA levels in MCF-7 cells following transfection by control siRNA or siRNA targeting PRMT6 and treatment with or without 10^{-9} M E2 for 12 h as indicated. (**D**) CARM1 expression as detected in (C). (**E**) PRMT1 expression as detected in (C). (**F**) GREB1 expression levels following PRMT6 knockdown. RNA was analysed by Q-RT-PCR for expression of GREB1 as in (C). (**G**) PR expression levels following PRMT6 knockdown. PR levels were examined as in (C). Each data point represents the mean and SD of results from four transfected cultures. Results shown are from a single experiment, which is representative of two independent experiments. $^{*}P < 0.005$; NS, no significant change compared to treatment with control siRNA.

PRMT6 (PRMT6-siRNA-2) (Supplementary Figure S8B). Taqman Q-RT-PCR using primers and probes designed to detect the alternative isoforms of Syk gave similar results (Supplementary Figure S8C and D). As with the alternative splicing of VEGF, the effects of PRMT6 on alternative splicing of Syk were hormone independent. In order to investigate the effects of CARM1 on alternative splicing of endogenous Syk, two siRNAs were employed that specifically target CARM1 (Supplementary Figure S7A–C). Following reduction of CARM1 levels in MCF-7 cells, Taqman Q-RT-PCR was used to detect the endogenous levels of the Syk isoforms. CARM1 knockdown did not influence splicing of the endogenous Syk RNA transcripts (Supplementary Figure S8E). Therefore, as with the VEGF gene, PRMT6 and CARM1 are not redundant in the splicing of Syk.

In order to verify the observed effects of PRMT6 and CARM1 on the splicing of Syk, we developed a minigene containing the alternatively spliced exon 7 of the Syk gene (Figure 6E). We transfected this minigene into HeLa cells along with PRMT6, PRMT6 V86K/D88A mutant or CARM1 and determined the levels of the Syk isoforms by reverse transcriptase-PCR with ^{32}P-radiolabelled primers. Over-expression of PRMT6 significantly reduced the Syk Exon 7 Inclusion:Exclusion ratio to ~50% of control levels (Figure 6G). This is in agreement with our data showing that knocking down PRMT6 leads to an approximate 2-fold increase in the Syk[L]:Syk[S] ratio (Figure 6F). The PRMT6 V86K/D88A mutant had no effect on the splicing of the RHCglo-Syk-Exon 7 minigene, demonstrating that PRMT6 requires its enzymatic activity to regulate RNA processing (Figure 6G). In addition, CARM1 had no affect on the alternative splicing of the RHCglo-Syk-Exon 7 minigene, as observed with the splicing of the endogenous Syk gene (Figure 6G and Supplementary Figure S8E). This validates our findings on the regulation of the endogenous Syk gene, and demonstrates that PRMT6 and CARM1

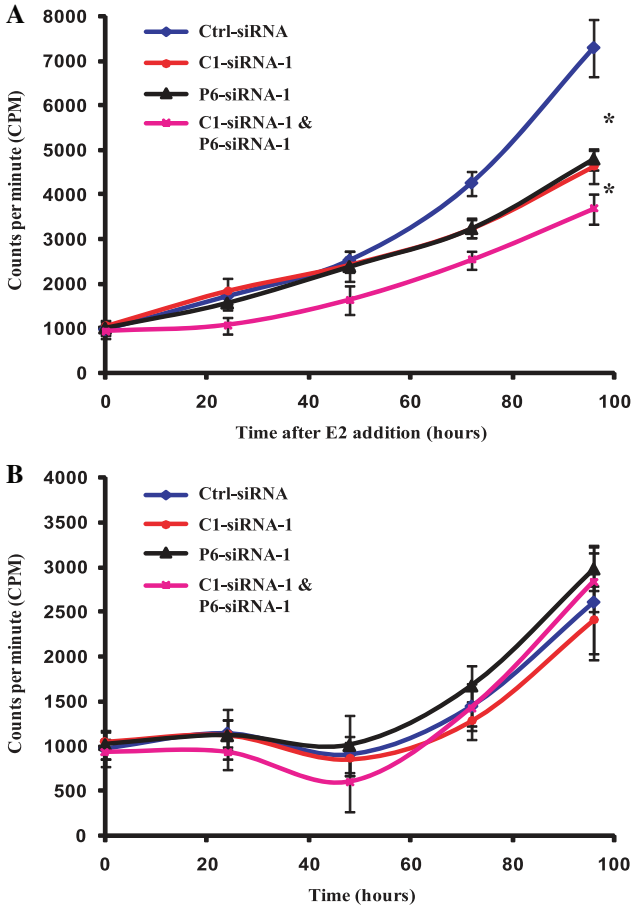

Figure 5. Knockdown of PRMT6 and CARM1 expression inhibits oestrogen-stimulated proliferation of breast cancer cells. (**A**) MCF-7 cells were transfected with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-1), CARM1 (C1-siRNA-1) or both PRMT6 and CARM1 (P6-siRNA-1 and C1-siRNA-1). Following treatment with 10^{-9} M E2, cell proliferation was determined by ^{3}H-thymidine incorporation. (**B**) MCF-7 cells were transfected as in (A) and cell proliferation was determined by ^{3}H-thymidine incorporation in the absence of 10^{-9} M E2. Each data point represents the mean and SD of results from eight individual cultures. Results are shown from a single experiment, which is representative of two independent experiments. $*P < 0.001$.

regulate the alternative splicing of a different subset of genes.

DISCUSSION

Coupling of RNA polymerase-II-mediated transcription and RNA processing is a well-established concept (63). These two processes can be functionally linked by the activity of SHR/NR coactivators. This was first demonstrated by the PPARγ coactivator PGC-1, which can regulate alternative splicing of the fibronectin minigene (64). Further investigations have demonstrated that other coactivators, including CoAA, p72, Sam68, p68, ASC-1 and ASC-2, can play this dual role, whilst the CAPER α and β proteins have been shown to regulate both transcription and alternative splicing in a SHR-dependent manner (29,30,34,65). Recently, CARM1, a PRMT known to function as a SHR/NR coactivator, has been shown to influence alternative splicing (31). Here, we report that another PRMT protein, PRMT6, is a SHR/NR coactivator that can regulate both hormone-dependent transcription and hormone-independent alternative splicing.

We have demonstrated that PRMT6 can function as a coactivator for ERα, ERβ, PR and GR (Figure 1 and Supplementary Figure S1). Similar to PRMT1 and CARM1, we also demonstrate that PRMT6 requires its enzymatic activity to function as a coactivator (Figure 1 and Supplementary Figure S1). We have further characterized the involvement of PRMT6 on the expression of two oestrogen-stimulated genes, GREB1 and PR. PRMT6 is cyclically recruited to the promoter and enhancer regions of these genes in response to oestrogen (Figure 2D and E). The recruitment of PRMT6 was detected at these sites following 15 min oestrogen treatment, but had returned to baseline levels after 45 min. PRMT6 is also required to fully activate these genes, as knockdown of PRMT6 leads to a decrease in their oestrogen-stimulated transcription (Figure 4). This demonstrates that PRMT6 is an integral component of the oestrogen-signalling pathway and is required to fully activate hormone-dependent transcription.

Even though PRMT6 can coactivate SHRs in the absence of other exogenous coactivators, the mode of action of PRMT6 is similar to that of PRMT1 and CARM1, in that it functions as a secondary coactivator to p160/SRC proteins. PRMT6 binds to the AD2 domain of SRC-1, and functions synergistically with SRC-1 to coactivate ERα (Figure 2A–C). Therefore, PRMT6 can be added to the list of SHR coactivators that are able to interact with SRC-1 to enhance transcriptional activation. Two PRMTs previously identified to interact with the p160/SRC proteins, PRMT1 and CARM1, also function synergistically to coactivate SHRs in luciferase reporter experiments (13). In order to determine whether PRMT6 also acts in concert with these PRMTs to stimulate gene expression, we transfected combinations of PRMT6, PRMT1, CARM1 and SRC-1 into CV-1 cells. We found that PRMT6 acts synergistically with CARM1 but not PRMT1, and synergy was dependent on the presence of SRC-1 (Figure 3). PRMT6 and PRMT1 share methylation targets, and so it is possible that methylation of a common target by either PRMT6 or PRMT1 could be required for CARM1 to further activate transcription. However, PRMT6 and PRMT1 are not redundant in stimulating hormone-induced gene activation, as siRNA knockdown of PRMT6 alone leads to a significant decrease in oestrogen-induced GREB1 and PR expression (Figure 4).

Potential methylation targets that may be responsible for PRMT6 coactivation include H4R3, a target that is shared with PRMT1 and is methylated as an early step in oestrogen-induced gene activation (40,66,67). Other targets include the high-mobility group (HMG) A1 proteins HMGA1a and HMGA1b (68–70). These non-histone chromosomal proteins are involved in chromatin structure organization and gene transcription, although the effect of arginine methylation on these

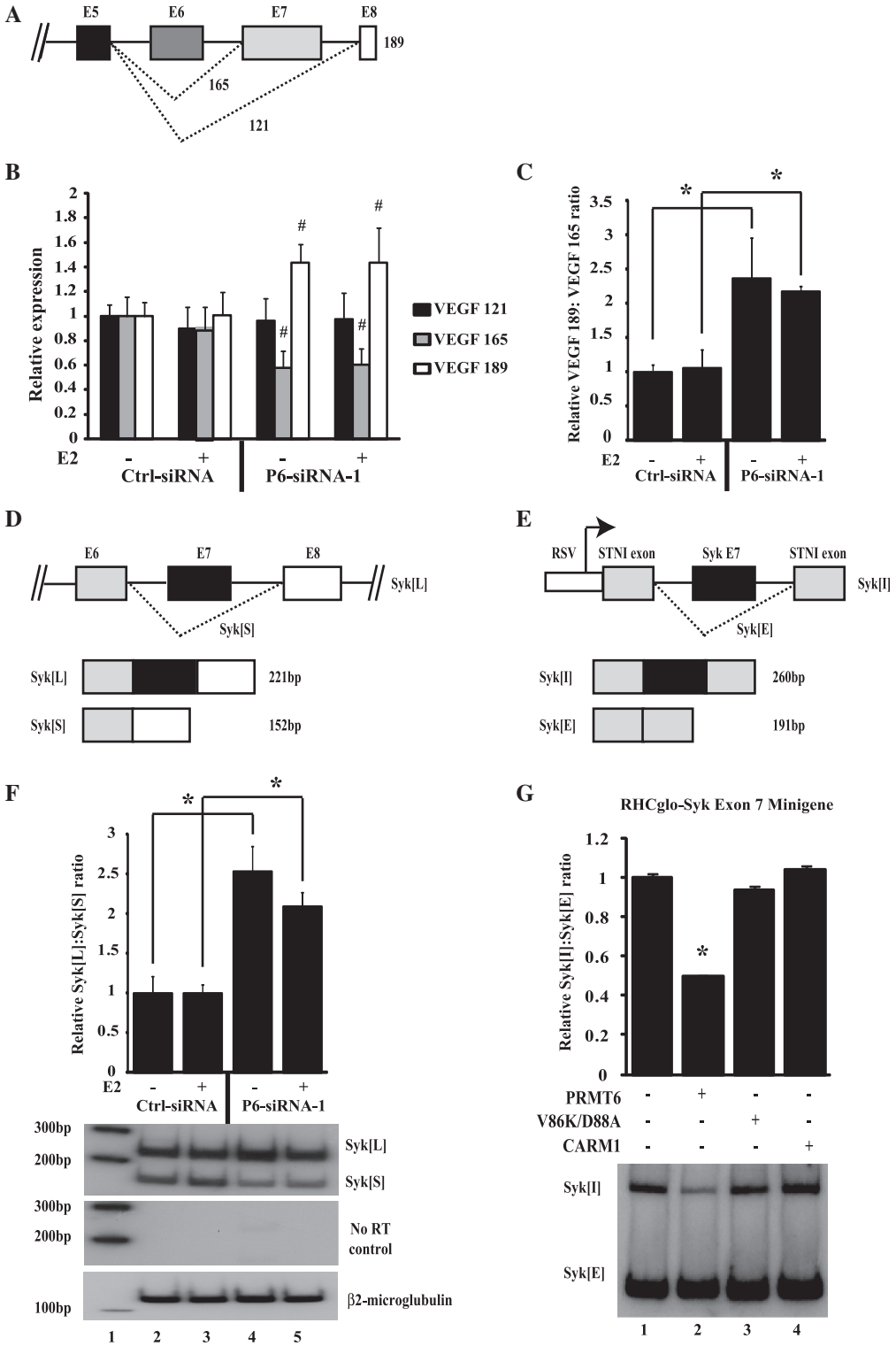

Figure 6. PRMT6 regulates alternative splicing of endogenous VEGF and Syk. (**A**) Schematic representation of the major spliced products of the VEGF gene (not to scale). (**B**) Effect of PRMT6 knockdown on alternative splicing of VEGF. MCF-7 cells were transfected either with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-1) and treated either with or without 10^{-9} M E2 for 12 h as indicated. RNA was harvested from the cells and the relative expression level of each spliced isoform was determined by Q-RT-PCR analysis as detailed in 'Materials and Methods' section. (**C**) The relative VEGF 189:VEGF 165 ratio was obtained by dividing the relative VEGF 189 cDNA level by the relative VEGF 165 cDNA level for each experimental condition. (**D**) Schematic representation of the major alternatively spliced products of the endogenous Syk gene (not to scale). (**E**) Representation of the RHCglo-Syk-Exon 7 minigene construct, not to scale. (**F**) Effect of PRMT6 knockdown on splicing of endogenous Syk. MCF-7 cells were transfected either with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-1) and treated either with or without 10^{-9} M E2 for 12 h as indicated. RNA was harvested from the cells and Syk splicing was determined by reverse transcriptase-PCR. The relative Syk[L]:Syk[S] ratio was obtained by dividing the relative Syk[L] cDNA level by the relative Syk[S] cDNA level for each experimental

proteins is not currently known. Another PRMT6 methylation target is H3R2, which has been reported to be a repressive histone mark (40–42); however, in certain conditions both of the known coactivators PRMT1 and CARM1 can methylate this histone residue (16,17,41,71). H3R2 methylation has been shown to prevent transcription primarily by preventing the mixed lineage leukaemia (MLL) K4 methyltransferase unit binding to and methylating H3K4 (40,42). However, this mechanism only represses a defined subset of genes that are regulated by the MLL complex (40). It is known that transcription of oestrogen-regulated genes can occur independently of MLL1, as siRNA targeting MLL1 has little effect on transcription of the prototypical oestrogen-regulated gene pS2 (72). Further evidence that H3K4 methylation is not required for steroid hormone-activated transcription is provided by the fact that the H3K4 demethylase LSD1 is recruited to the GREB1 enhancer site and is required for oestrogen-dependent GREB1 transcription (72). Indeed, 58% of ERα-enriched promoters examined by Garcia-Bassets *et al*. (72) showed recruitment of LSD1, and both glucocorticoid and DHT stimulation lead to a decrease in dimethylation of H3K4 on a tandem array of the mouse mammary tumour virus promoter and on the PSA promoter respectively (17,73). Therefore, in the case of steroid-hormone-induced gene expression it appears that H3K4 demethylation does not prevent, and may even promote transcription. One example of H3K4 demethylation being an activating histone mark has been provided by the autoimmune regulator, which binds to non-methylated H3K4 and promotes transcription (74). Further studies will have to be conducted to determine the complete role of H3R2 and H3K4 methylation in steroid-hormone-activated transcription.

As PRMT6 can regulate oestrogen-dependent transcription; we investigated whether it is also involved in the oestrogen-stimulated proliferation of ER + breast cancer cells. Reducing PRMT6 levels by siRNA interference significantly inhibited the oestrogen-dependent, but not oestrogen-independent, proliferation of MCF-7 cells (Figure 5). This demonstrates that PRMT6 is a fundamental component of the oestrogen-signalling pathway in breast cancer cells. This inhibition of proliferation was similar to that obtained by knocking down CARM1, which has previously been demonstrated to inhibit the oestrogen-stimulated proliferation of MCF-7 cells (57) (Figure 5). As PRMT6 and CARM1 can cooperate to stimulate ERα-dependent transcription (Figure 3), we investigated the effects of knocking down both PRMTs simultaneously on oestrogen-dependent proliferation. Reducing the levels of both PRMT6 and CARM1 together further reduced oestrogen-driven MCF-7 cell proliferation when compared to knocking down either PRMT alone. Therefore, both PRMT6 and CARM1 are required to stimulate oestrogen-dependent proliferation of breast cancer cells, and their roles are not redundant in this process.

As well as regulating transcriptional initiation, we have shown that PRMT6 can also affect aspects of RNA processing, specifically alternative splicing. The siRNA knockdown of PRMT6 altered the relative levels of alternatively spliced products of the endogenous VEGF and Syk genes (Figure 6). In both cases, PRMT6 knockdown leads to an increase in exon inclusion, and a decrease in exon skipping. Therefore, it would appear that one cellular function of PRMT6 is to increase skipping of alternative exons. The effects of PRMT6 on alternative splicing of both VEGF and Syk were steroid hormone independent, despite the transcription of VEGF being oestrogen-dependent (Supplementary Figure S6H). CARM1, the only other PRMT identified to have a direct role in alternative splicing, also regulates alternative splicing in a hormone-independent manner (31). Unlike several coactivators involved in coupling transcription and alternative splicing, such as PGC-1, CoAA and CAPERα/β, both PRMT6 and CARM1 have none of the domains characteristic of splicing factors such as RNA-recognition motifs and serine-arginine-rich domains. As PRMT6 requires its enzymatic activity to regulate the splicing of Syk (Figure 6G), the most likely method by which it regulates alternative splicing is by methylating other splicing proteins. In the study by Cheng *et al*. (31), CARM1 and PRMT6 were found to have different substrate specificities in relation to the methylation of various splicing-related proteins. Therefore, we investigated whether PRMT6 and CARM1 have non-redundant roles in the regulation of alternative splicing. Both PRMTs regulate the production of the $VEGF_{165}$ isoform. However, only PRMT6 regulates the production of the $VEGF_{189}$ alternatively spliced product (Figure 6B and Supplementary Figure S7E). In addition, CARM1 has no effect on the splicing of the Syk gene (Figures 6G and Supplementary Figure S8E). Therefore, CARM1 and PRMT6 play a similar yet non-redundant role in the regulation of alternative splicing. In conclusion, this study provides evidence that PRMT6 has a pleiotropic role in multiple aspects of gene expression, and is able to couple transcription and alternative splicing.

condition. An autoradiograph of the radiolabeled Syk and β-2-microglobulin reverse transcriptase-PCR products from a representative experiment is shown. Lane 1; radiolabelled 100-bp DNA marker; lane 2; Ctrl-siRNA without 10^{-9} M E2; lane 3; Ctrl-siRNA with 10^{-9} M E2, lane 4; P6-siRNA-1 without 10^{-9} M E2; lane 5; P6-siRNA-1 with 10^{-9} M E2. (**G**) Effect of PRMT6 on the splicing of an RHCglo-Syk-Exon 7 minigene. HeLa cells were transiently co-transfected with a RHCglo-Syk-Exon 7 minigene along with expression vectors for PRMT6, the PRMT6 V86K/D88A mutant or CARM1. RNA was harvested from the cells and Syk splicing determined by reversetranscriptase-PCR. The relative RHCglo-Syk-Exon 7 inclusion (Syk[I]): RHCglo-Syk-Exon 7 exclusion (Syk[E]) ratio was obtained by dividing the relative Syk[I] cDNA level by the relative Syk[E] cDNA level for each experimental condition. RT-PCR without reverse transcriptase did not produce any detectable PCR products (data not shown). A representative autoradiograph of the radiolabelled RHCglo-Syk-Exon 7 minigene products is shown. For all experiments, each data point represents the mean and SD of results from four transfected cultures (or six transfected cultures for the RHCglo-Syk-Exon 7 minigene experiment). Results shown are from a single experiment, which is representative of two independent experiments. $*P < 0.005$, $\#P < 0.05$.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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