Supplementary Figure Legends and Figures:

PROTEIN ARGININE METHYLTRANSFERASE 6 REGULATES MULTIPLE ASPECTS OF GENE EXPRESSION

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Supplementary Figure 1. PRMT6 coactivates SHRs in CV-1 cells. (A) PRMT6 coactivates ERα transcriptional activity from an estrogen response element linked to a minimal promoter. CV-1 cells were cotransfected with ERE-E1b-luciferase reporter along with expression vectors for ERα alone, or with PRMT6 or PRMT6 mutant (V86K/D88A) as indicated. Cells were treated with vehicle (ethanol) or 10^{-9}M E2 as indicated and tested for luciferase activity (see Experimental Procedures). (B) PRMT6 coactivates ERβ transcriptional activity from the ERE-E1b-luciferase reporter. CV-1 cells were cotransfected with ERβ expression plasmid as in (A). (C) PRMT6 enhances the transcriptional activity of PR. CV-1 cells were cotransfected with PRE-E1b-luciferase reporter along with expression vectors for PR alone, or with PRMT6 or PRMT6 mutant and treated with vehicle (ethanol) or 10^{-8}M Pg as indicated. (D) PRMT6 enhances the transcriptional activity of GR. CV-1 cells were cotransfected with PRE-E1b-luciferase reporter along with expression vectors for GR alone, or with PRMT6 or PRMT6 mutant and treated with vehicle (ethanol) or 10^{-8}M Dex as indicated. Each data point represents the mean and S.D. of results from four transfected cultures. Results shown are from a single experiment, which is representative of three independent experiments. * $p<0.001$, # $p<0.005$.

Supplementary Figure 2. PRMT6 does not coactivate non-steroidal NRs. (A) PRMT6 does not coactivate RARα transcriptional activity. HeLa cells were co-transfected with a RARE-E1b-luciferase reporter along with expression vectors for RARα alone, or with PRMT6 or SRC-1 and treated with vehicle (ethanol) or 10^{-7}M RA as indicated. Cells were then tested for luciferase activity (B) PRMT6 does not coactivate PPARγ transcriptional activity. The pSG5-TIF2 expression plasmid was a gift from Dr. Pierre Chambon**.** HeLa cells were co-transfected with a PPAR-TK-luciferase reporter along with expression vectors for PPARγ alone, or with PRMT6 or TIF2 and treated with vehicle (ethanol) or 10^{-5}M Cig as indicated. Cells were then tested for luciferase activity.

Supplementary Figure 3. PRMT6 does not regulate the expression of endogenous estrogen receptor-α. (A) Knock down of PRMT6 in MCF-7 cells does not affect the expression of ERα. MCF-7 cells were transfected with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-1). Following treatment with 10^{-9}M E2 for 12 hours, RNA was harvested and ERα levels were determined by Q-RT-PCR. (B) MCF-7 cells were transfected with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-2). P6-siRNA-2, sense 5'- GCACUUGUAUUUCCGUAUA-3' and PRMT6-siRNA-2, antisense 5'-UAUACGGAAAUACAAGUGCTT-3'.

Following treatment with 10^{-9}M E2 for 12 hours, RNA was harvested and ERα levels were determined by Q-RT-PCR. (C). Knock down of PRMT6 does not regulate ERα protein levels. Western blot showing PRMT6, ERα and β-tubulin expression in MCF-7 cells following transfection with control siRNA or siRNA targeting PRMT6 (P6-siRNA-1 and P6-siRNA-2) and treatment with or without 10^{-9}M E2 for 12 hours as indicated.

Supplementary Figure 4. Knockdown of PRMT6 expression disrupts estrogen signaling. (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-2, see Supplementary Fig. 3) and treatment with or without 10^{-9}M E2 for 12 hours 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 hours 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 analysis for expression of GREB1 as in (C). (G) Progesterone receptor (PR) expression levels following PRMT6 knockdown. PR levels were examined as in (C). Each data point represents the mean and S.D. of results from four transfected cultures. Results shown are from a single experiment, which is representative of two independent experiments. *$p<0.005$, # $p<0.05$; NS, no significant change compared to treatment with control siRNA.

Supplementary Figure 5. Knockdown of PRMT6 and CARM1 expression inhibits estrogen-stimulated proliferation of breast cancer cells. (A) Q-RT-PCR analysis of CARM1 RNA levels in MCF-7 cells following transfection by control siRNA (Ctrl-siRNA) or siRNA targeting CARM1 (C1-siRNA-1) and treatment with or without 10^{-9}M E2 for 12 hours as indicated. (B) PRMT1 expression as detected in (A). (C) PRMT6 expression as detected in (A). (D) MCF-7 cells were transfected with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-2, see Supplementary Fig. 3), CARM1 (C1-siRNA-1) or both PRMT6 and CARM1 (P6-siRNA-2 and C1-siRNA-1). Following treatment with 10^{-9}M E2, cell proliferation was determined by ^{3}H-thymidine incorporation. (E) MCF-7 cells were transfected as in (D) 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 S.D of results from 8 individual cultures. Results are shown from a single experiment, which is representative of two independent experiments. * $p<0.001$.

Supplementary Figure 6. PRMT6 regulates alternative splicing of endogenous VEGF. (A) Schematic representation of PCR strategy to detect full length VEGF (VEGFTotal) and the three major spliced isoforms of VEGF (VEGF189, VEGF165 and VEGF121). Diagram amended from (1). (B) Q-RT-PCR analysis of PRMT6 RNA levels in MCF-7 cells following transfection by control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-1) and treatment with or without 10^{-9}M E2 for 12 hours as indicated. (C) CARM1 expression as detected in (B). (D) PRMT1 expression as detected in (B). (E) Q-RT-PCR analysis of PRMT6 RNA levels in MCF-7 cells following transfection by control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-2, see Supplementary Fig. 3) and treatment with or without 10^{-9}M E2 for 12 hours as indicated. (F) CARM1 expression as detected in

(E). (G) PRMT1 expression as detected in (E). (H) VEGF expression levels as detected in (B). (I) VEGF expression levels as detected in (E). (J) MCF-7 cells were transfected either with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-2, see Supplementary Fig. 3) and treated either with or without 10^{-9}M E2 as indicated. RNA was harvested from the cells and the relative level of each alternatively spliced VEGF isoform was determined by Q-RT-PCR analysis as detailed in Experimental Procedures. (K) MCF-7 cells were transfected either with control siRNA (Ctrl-siRNA) or siRNA targeting PRMT6 (P6-siRNA-2, see Supplementary Fig.3) and treated either with or without estrogen for 12 hours as indicated. RNA was harvested from the cells and the relative level of each spliced isoform was determined by Q-RT-PCR analysis as detailed in Experimental Procedures. 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. Each data point represents the mean and S.D. of results from four transfected cultures. Results shown are from a single experiment, which is representative of two independent experiments * $p<0.005$; # $p<0.05$ compared to the corresponding transfection with control siRNA.

Supplementary Figure 7. CARM1 regulates splicing of VEGF 165 isoform. (A) Q-RT-PCR analysis of CARM1 RNA levels in MCF-7 cells following transfection by control siRNA (Ctrl-siRNA) or two siRNAs targeting CARM1 (C1-siRNA-1 and C2-siRNA-2; sense 5'-UCACAGCUCUCUUUGCUAUTT-3' and C1-siRNA-2, antisense 5'-AUAGCAAAGAGAGCUGUGATT-3') and treatment with or without 10^{-9}M E2 for 12 hours as indicated. (B) PRMT6 expression as detected in (A). (C) PRMT1 expression as detected in (A). (D) VEGF expression as detected in (A). (E) MCF-7 cells were transfected either with control siRNA (Ctrl-siRNA) or siRNA targeting CARM1 (C1-siRNA-1 or C1-siRNA-2) and treated either with or without estrogen for 12 hours as indicated. RNA was harvested from the cells and the relative level of each spliced isoform was determined by Q-RT-PCR analysis as detailed in Experimental Procedures. Each data point represents the mean and S.D. of results from four transfected cultures. Results shown are from a single experiment, which is representative of two independent experiments *$p<0.005$; #$p<0.05$ compared to the corresponding transfection with control siRNA.

Supplementary Figure 8. PRMT6 regulates alternative splicing of Syk. (A) MCF-7 cells were transfected with control siRNA or siRNA targeting PRMT6 and treated with or without 10^{-9}M E2 for 12 hours as indicated. RNA was harvested and the levels of the alternatively spliced Syk RNA transcripts were determined by RT-PCR. The graph shows the relative expression of each Syk alternatively spliced isoform. (B) MCF-7 cells were transfected either with control siRNA or siRNA targeting PRMT6 (P6-siRNA-2, see Supplementary Fig. 3) and treated either with or without 10^{-9}M E2 for 12 hours as indicated. RNA was harvested from the cells and Syk splicing was determined by RT-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 condition. An autoradiograph of the radiolabeled Syk and β-2-microglobulin RT-PCR products from a representative experiment is shown , Lane 1; Radiolabeled 100 base pair 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 (C) MCF-7 cells were transfected with control siRNA or siRNA targeting PRMT6 (P6-siRNA-1) and treated with or without 10^{-9}M E2 for 12

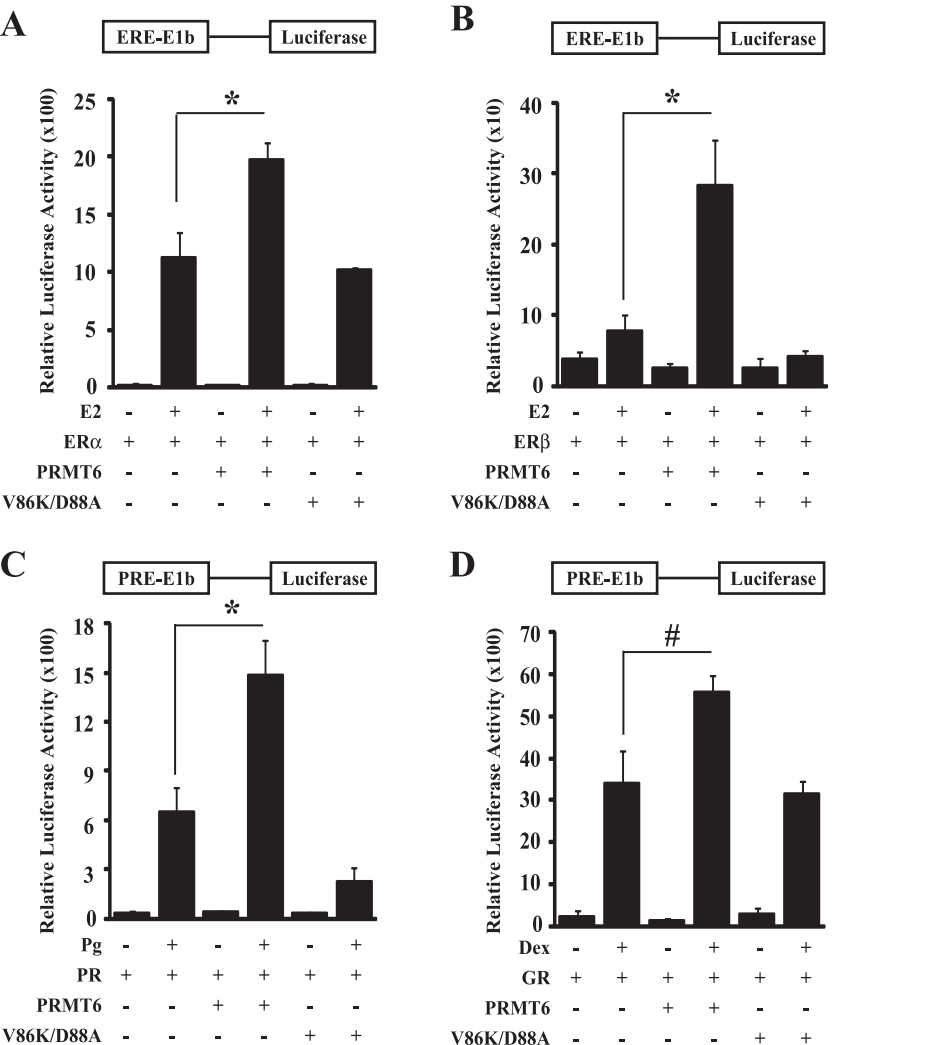
hours as indicated. RNA was harvested from the cells and the expression of Syk[L] and Syk[S] were determined by Q-RT-PCR using custom designed Taqman probe/primer sets;

SYK-L-For:5'-TCAGCGGGTGGAATAATCTC-3'
SYK-L-Rev:5'-TGACACAGTACTCTCTTGCCG-3'
SYK-L Probe: 5'-6-FAM-GCCACAGAAAGTCCTCCCCTG-MGB-3'
SYK-S-For:5'-GGCACACAGGGAAATGTTAAT-3'
SYK-S-Rev: 5'-TGGCTCATACGGATTGAATG-3'
SYK-S Probe: 5'-6-FAM-CCATCCTGCGTCCTCCC-MGB-3'

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 condition. (D) MCF-7 cells were transfected with control-siRNA or siRNA targeting PRMT6 (P6-siRNA-2, see Supplementary Fig. 3) and treated either with or without 10^{-9}M E2 for 12 hours as indicated. Syk splicing was determined as in (C). (E) MCF-7 cells were transfected with control-siRNA or siRNA targeting CARM1 (C1-siRNA-1 or C1-siRNA-2, see Supplementary Fig. 7) and treated either with or without 10^{-9}M E2 for 12 hours as indicated. Syk splicing was determined as in (C).Each data point represents the mean and S.D. of results from four transfected cultures. Results shown are from a single experiment, which is representative of two independent experiments * $p<0.005$; # $p<0.05$ compared to the corresponding transfection with control siRNA. (F) PRMT6 does not regulate splicing of CD44 from an RHCglo-CD44-minigene. To make the RHCglo-CD44 minigene, PCR primers CD44-for (5'-GCATCGGTCGACGCAGATCCTCATGATCCTTC-3') and CD44-rev (5'-GCATCGGCTAGCTCAAATGAAGACCATAAGAGC-3') were used with PfuUltra II Fusion HS DNA polymerase and PCR to produce a DNA fragment from a CD44 plasmid encompassing CD44 exons V4 and V5. 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 CD44 PCR sequence was confirmed by sequencing. HeLa cells were co-transfected with an RHCglo-CD44-minigene along with an expression vector for PRMT6 as indicated. RNA was harvested from the cells after 24 and 48 hours and CD44 alternative splicing determined by using the RHCglo specific primers RSV5U and RTRHC. RT-PCR without reverse transcriptase did not produce any detectable PCR products (data not shown). The relative Skipping:Inclusion ratio was obtained by dividing the relative Skipping cDNA level by the relative inclusion cDNA level for each experimental condition. A representative autoradiograph of the radiolabeled CD44 products is shown. Each data point represents the mean and S.D. of results from four transfected cultures. Results shown are from a single experiment, which is representative of two independent experiments.

Supplementary References

1. Wellmann, S., Taube, T., Paal, K., Graf v. Einsiedel, H., Geilen, W., Seifert, G., Eckert, C., Henze, G. and Seeger, K. (2001) Specific Reverse Transcription-PCR Quantification of Vascular Endothelial Growth Factor (VEGF) Splice Variants by LightCycler Technology. *Clin. Chem.*, 47, 654-660.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ERE-E1b
Luciferase
Relative Luciferase Activity (x100)
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20
15
10
5
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*
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ERα + + + + + +
PRMT6 - - + + - -
V86K/D88A - - - - + +
B
ERE-E1b
Luciferase
Relative Luciferase Activity (x10)
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30
20
10
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*
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ERβ + + + + + +
PRMT6 - - + + - -
V86K/D88A - - - - + +
C
PRE-E1b
Luciferase
Relative Luciferase Activity (x100)
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15
12
9
6
3
0
*
Pg - + - + - +
PR + + + + + +
PRMT6 - - + + - -
V86K/D88A - - - - + +
D
PRE-E1b
Luciferase
Relative Luciferase Activity (x100)
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60
50
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GR + + + + + +
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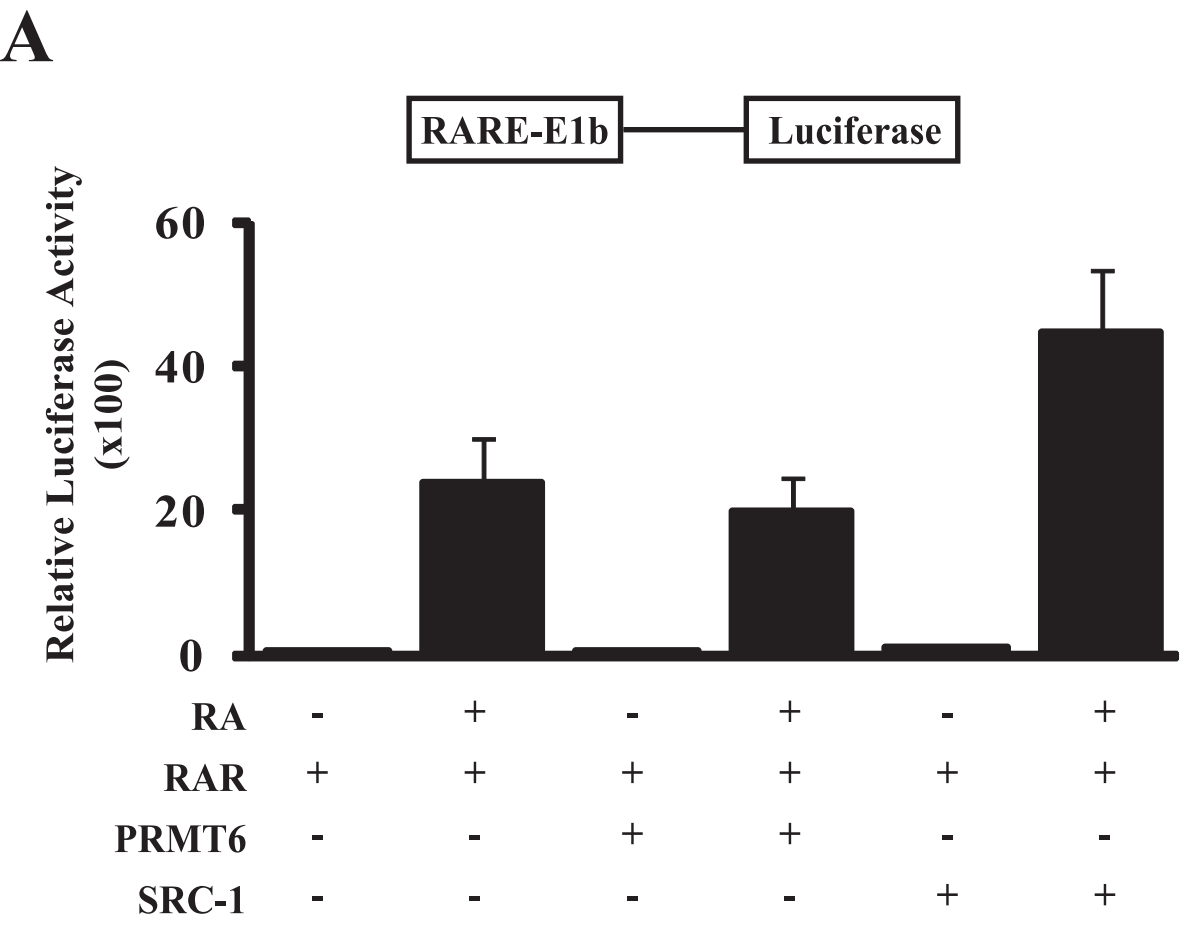
V86K/D88A - - - - + +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RAR + + + + + +
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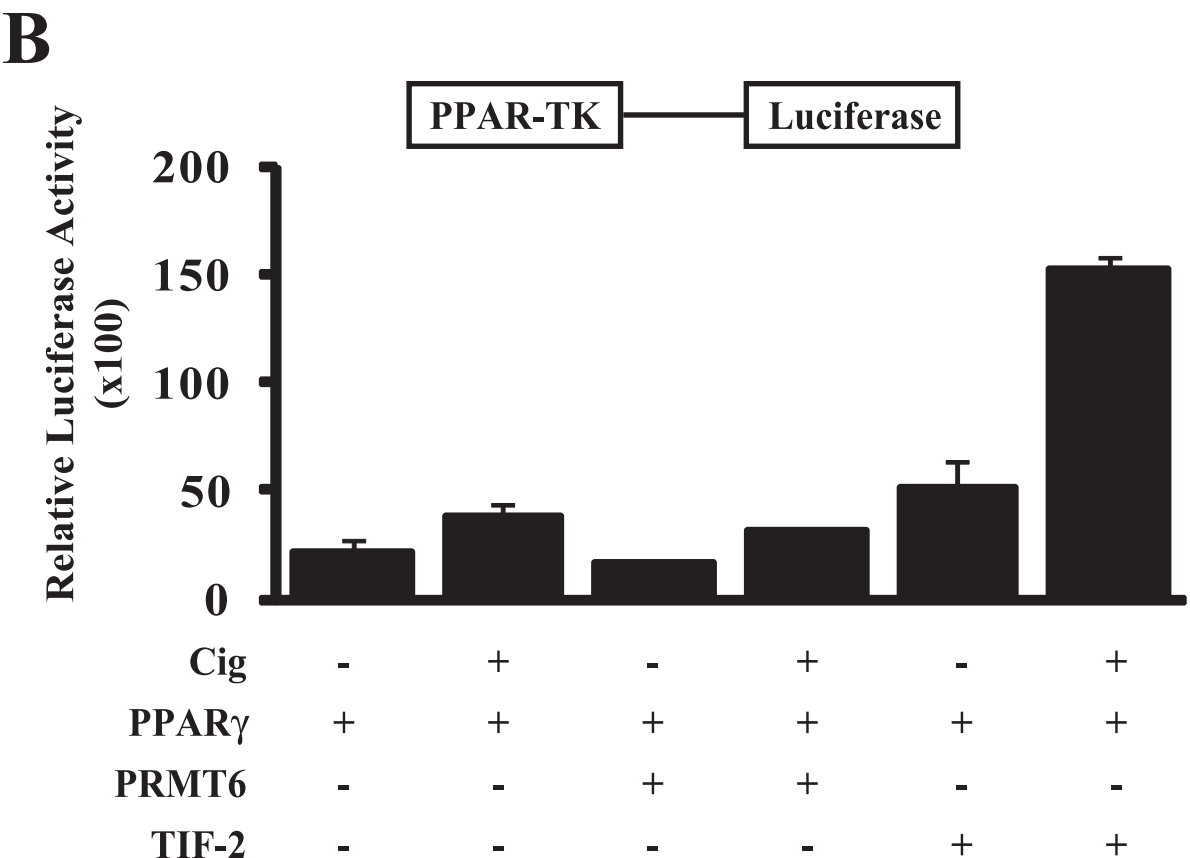
SRC-1 - - - - + +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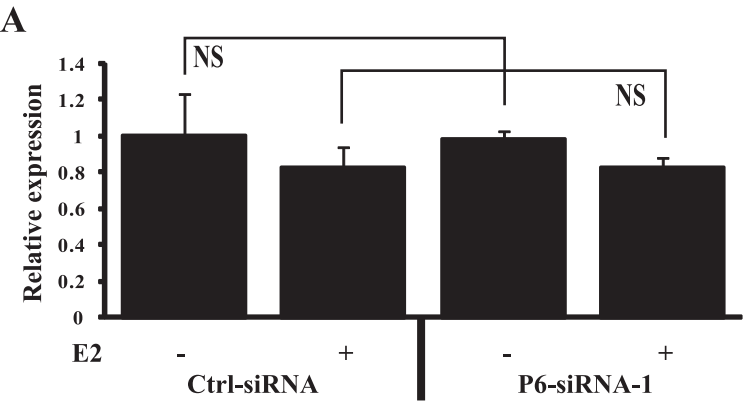
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NS
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+
-
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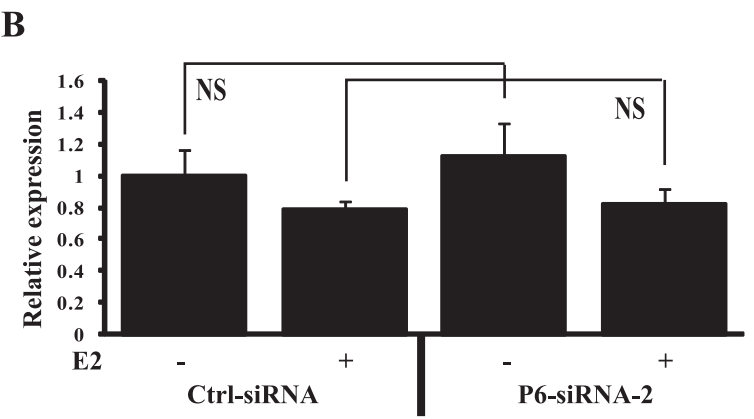
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NS
Relative expression
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1.4
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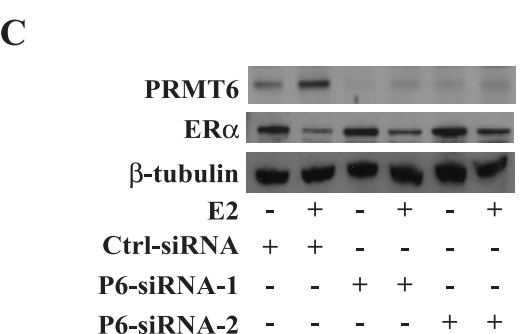
-
+
-
+
Ctrl-siRNA
P6-siRNA-2


C
PRMT6
ERα
β-tubulin
E2 - + - + - +
Ctrl-siRNA + + - - - -
P6-siRNA-1 - - + + - -
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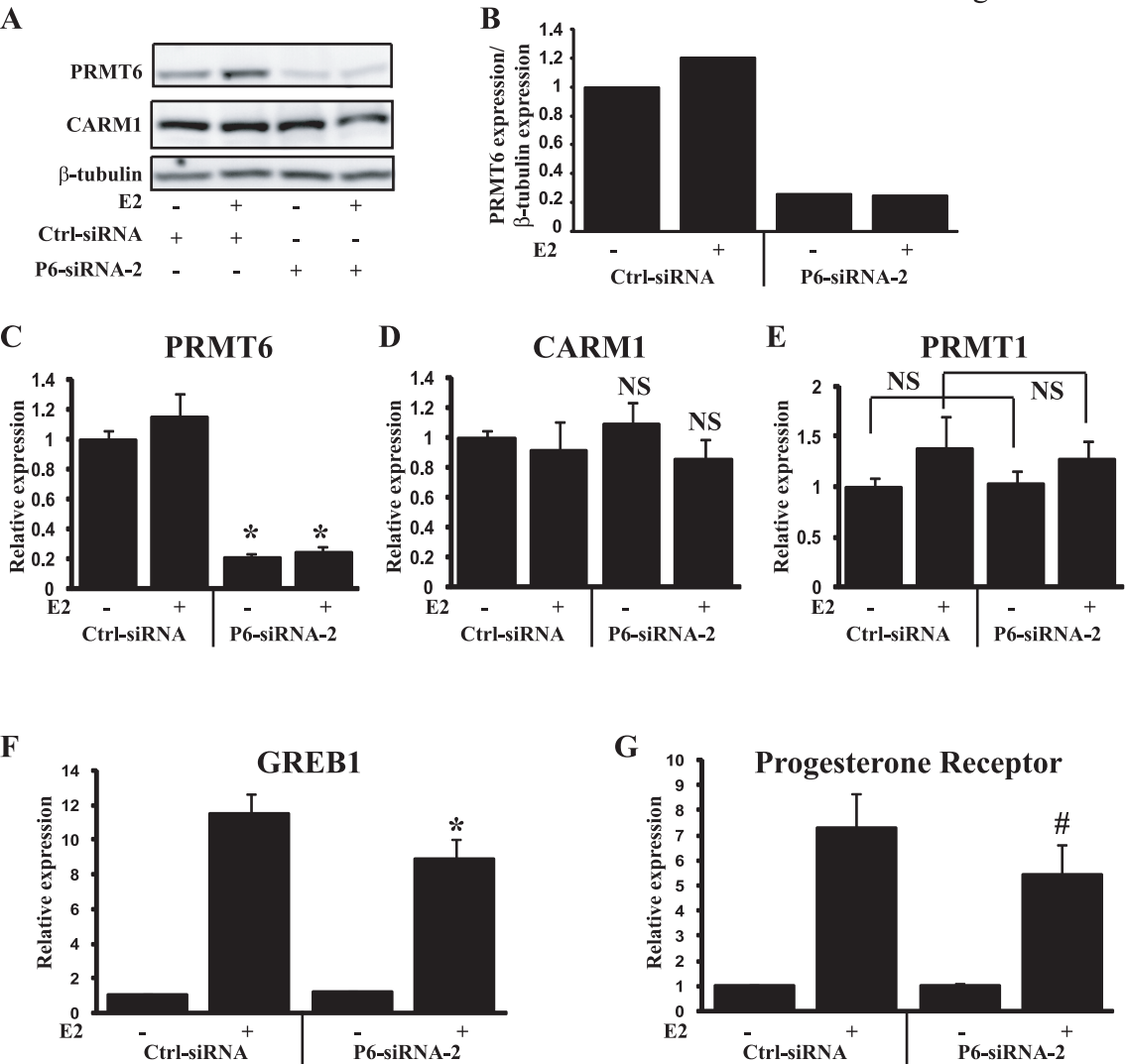
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CARM1
β-tubulin
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PRMT6 expression/ β-tubulin expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E2 - + - +
Ctrl-siRNA
P6-siRNA-2
C
PRMT6
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
E2 - + - +
Ctrl-siRNA
P6-siRNA-2
D
CARM1
Relative expression
1.4
1.2
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0.8
0.6
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0.2
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NS
NS
E2 - + - +
Ctrl-siRNA
P6-siRNA-2
E
PRMT1
Relative expression
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NS
E2 - + - +
Ctrl-siRNA
P6-siRNA-2
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GREB1
Relative expression
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12
10
8
6
4
2
0
*
E2 - + - +
Ctrl-siRNA
P6-siRNA-2
G
Progesterone Receptor
Relative expression
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3
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1
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#
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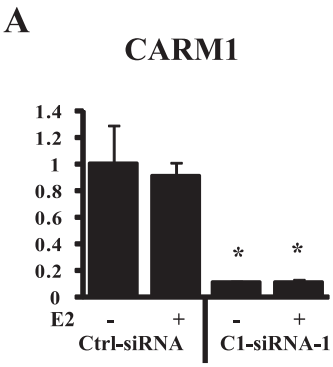
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CARM1
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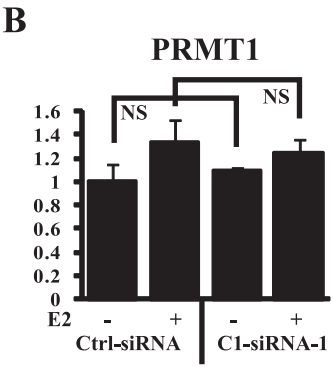
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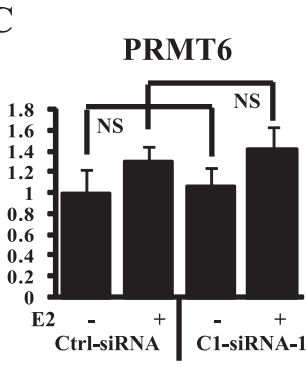
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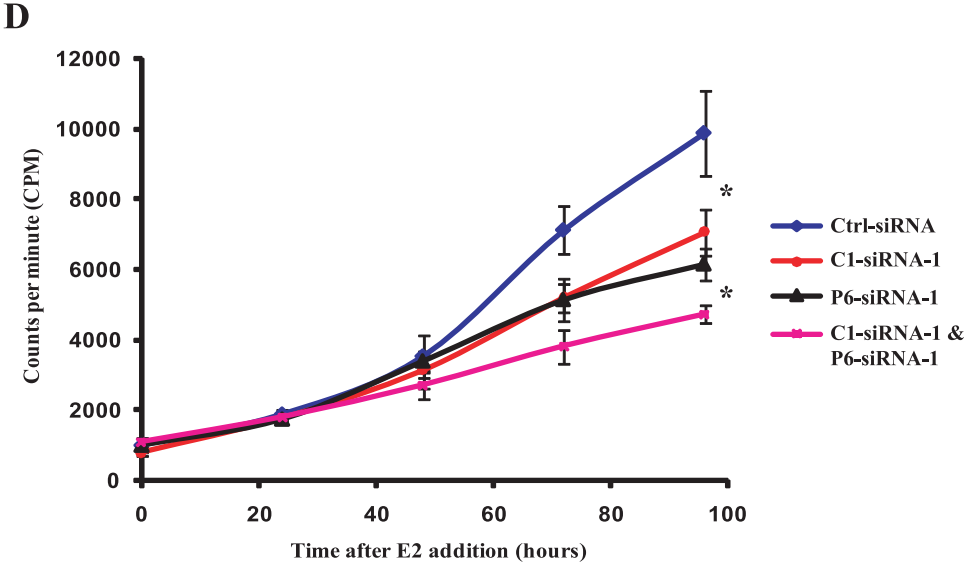
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12000
10000
8000
6000
4000
2000
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Counts per minute (CPM)
0
20
40
60
80
100
Time after E2 addition (hours)
*
*
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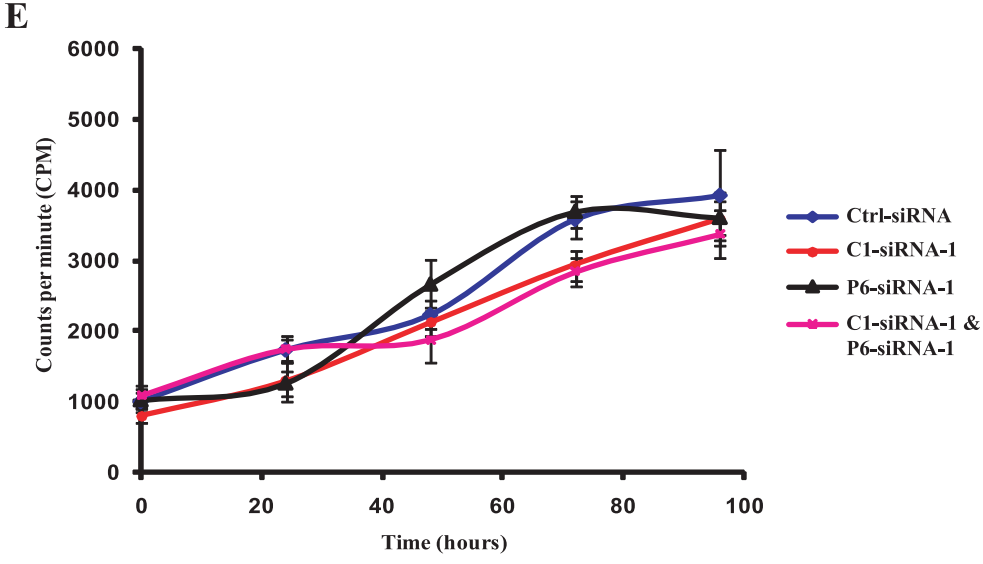
P6-siRNA-1
C1-siRNA-1 &
P6-siRNA-1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Time (hours)
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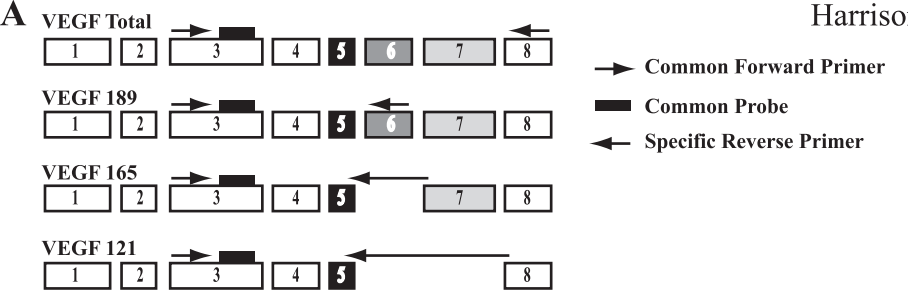
P6-siRNA-1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VEGF 165
VEGF 121
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2
3
4
5
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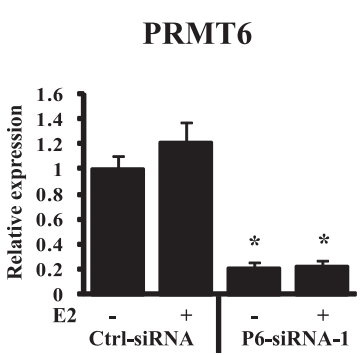
7
8
Common Forward Primer
Common Probe
Specific Reverse Primer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PRMT6
Relative expression
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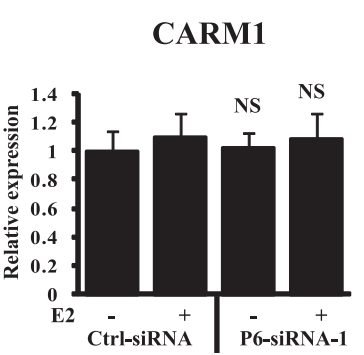
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Relative expression
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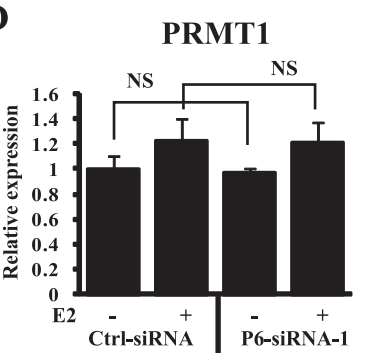
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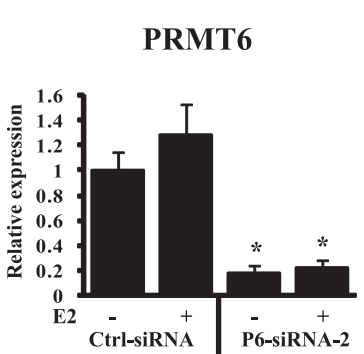
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PRMT6
Relative expression
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*
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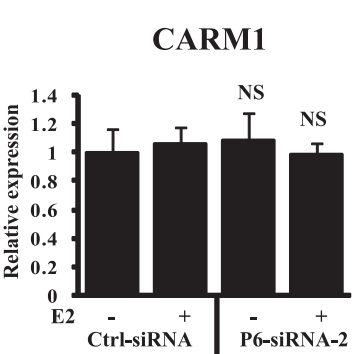
Ctrl-siRNA
P6-siRNA-2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CARM1
Relative expression
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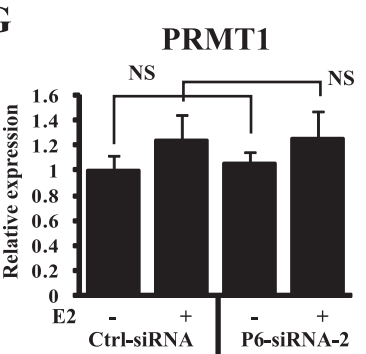
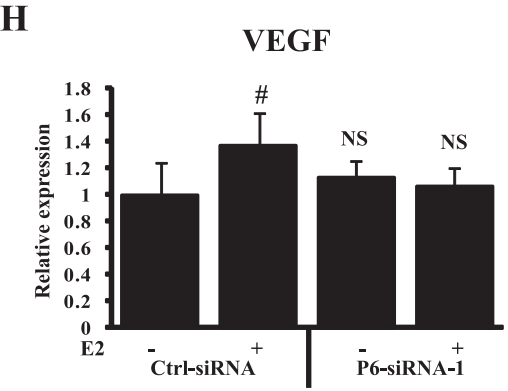
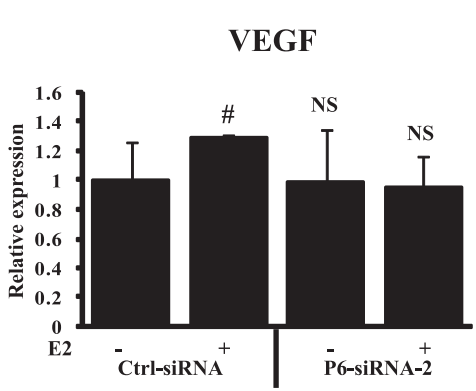
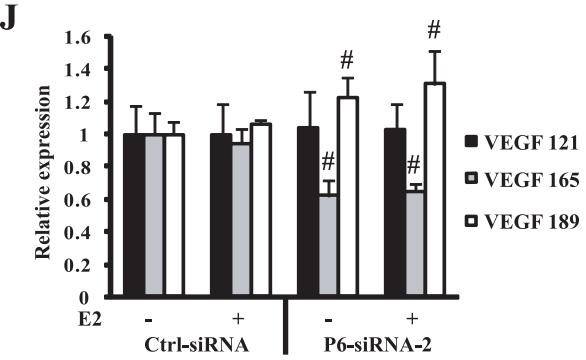
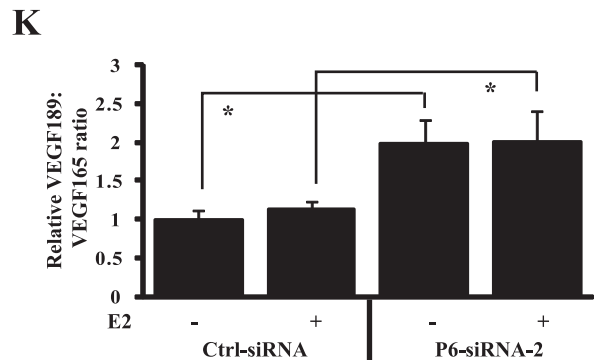
0.8
0.6
0.4
0.2
0
NS
NS
E2 - + - +
Ctrl-siRNA
P6-siRNA-2

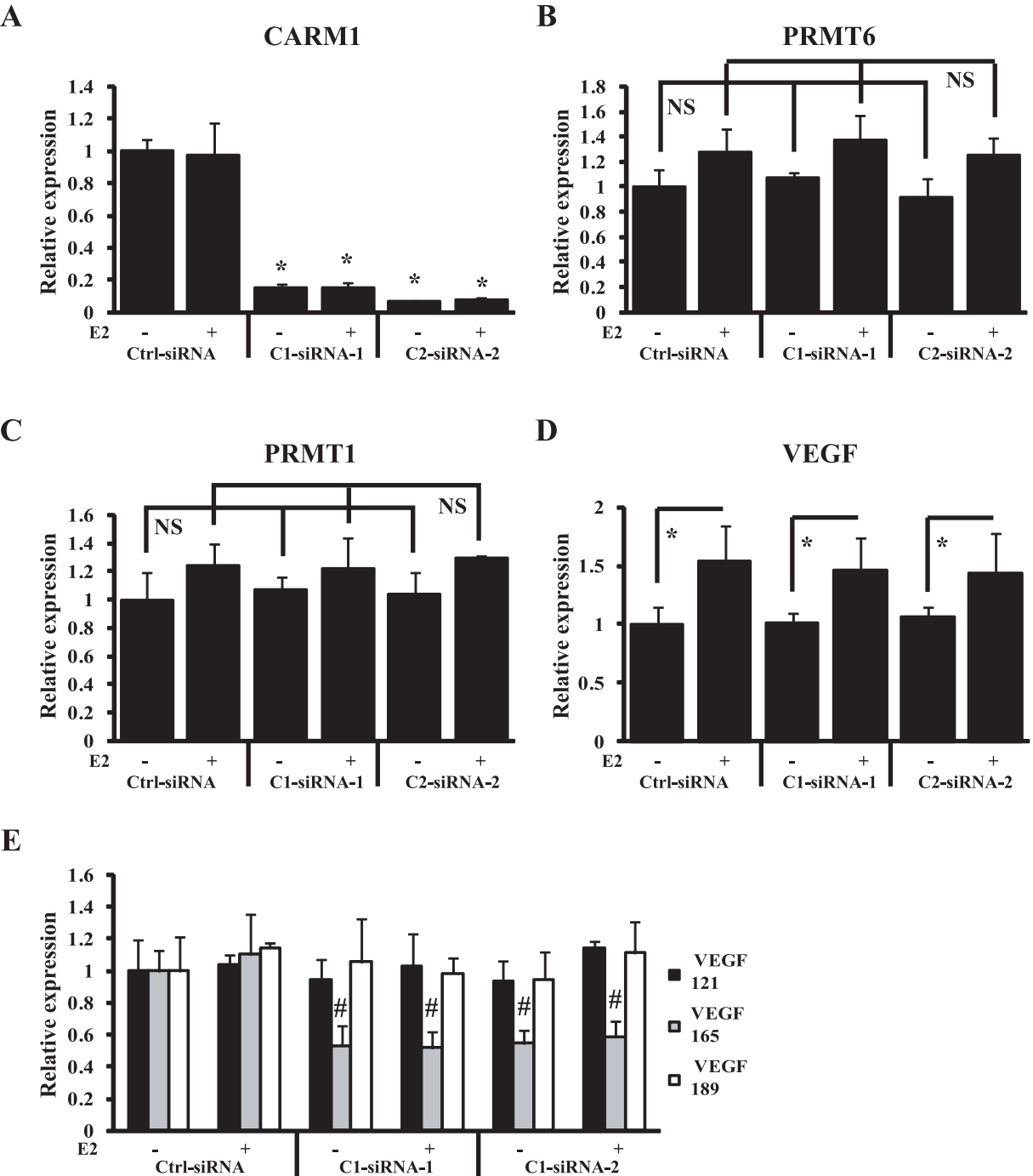

G
PRMT1
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
NS
E2 - + - +
Ctrl-siRNA
P6-siRNA-2

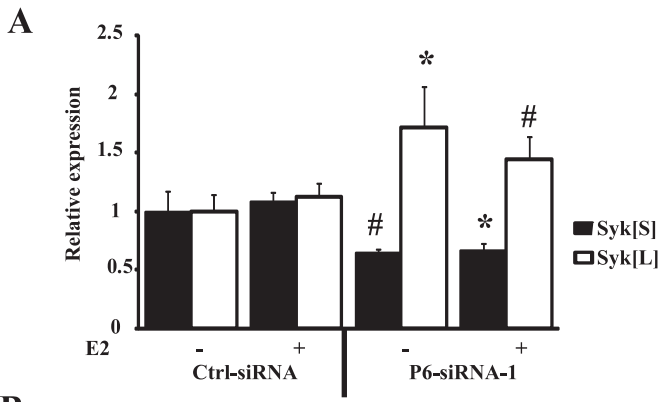

H
VEGF
Relative expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
NS
NS
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-1

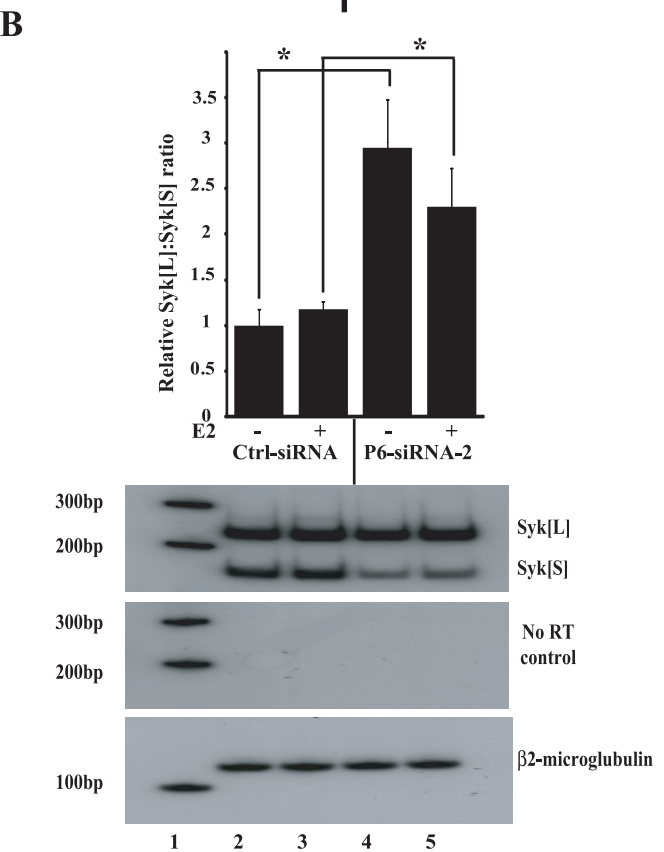

I
VEGF
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
NS
NS
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-2

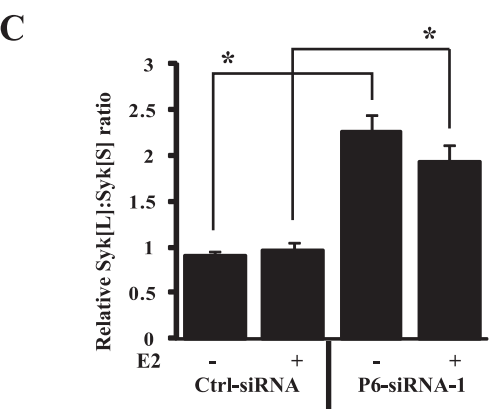

J
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
#
#
#
VEGF 121
VEGF 165
VEGF 189
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-2

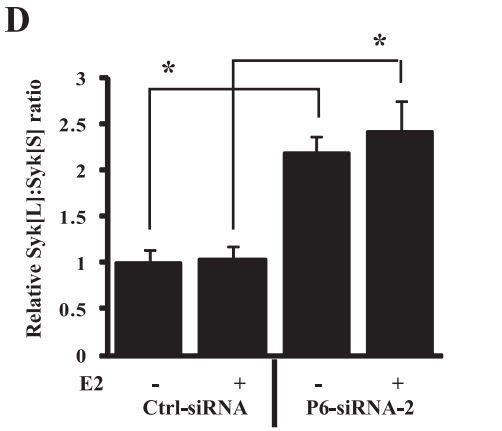

K
Relative VEGF189: VEGF165 ratio
3
2.5
2
1.5
1
0.5
0
*
*
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-2

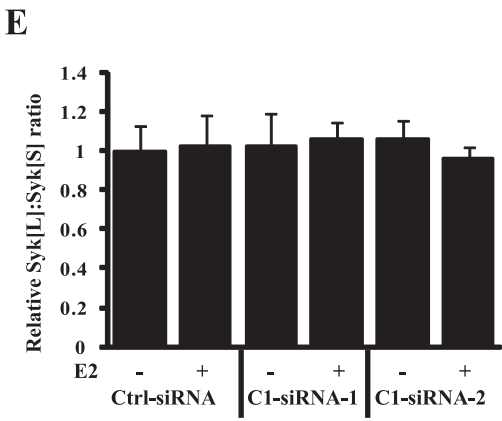

A
CARM1
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E2 - + - + - +
Ctrl-siRNA
C1-siRNA-1
C2-siRNA-2
B
PRMT6
NS
NS
C
PRMT1
NS
NS
D
VEGF
*
*
*
E
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
VEGF 121
VEGF 165
VEGF 189

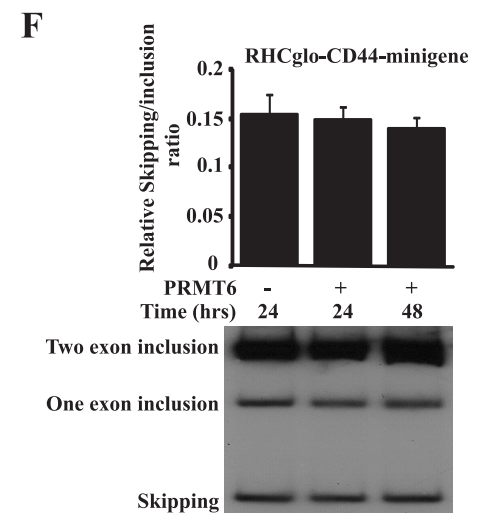

A
Relative expression
2.5
2
1.5
1
0.5
0
*
*
#
#
Syk[S]
Syk[L]
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-1


B
Relative Syk[L]:Syk[S] ratio
3.5
3
2.5
2
1.5
1
0.5
0
*
*
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-2
300bp
200bp
Syk[L]
Syk[S]
300bp
200bp
No RT
control
100bp
β2-microglubulin
1
2
3
4
5


C
Relative Syk[L]:Syk[S] ratio
3
2.5
2
1.5
1
0.5
0
*
*
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-1


D
Relative Syk[L]:Syk[S] ratio
3
2.5
2
1.5
1
0.5
0
*
*
E2
-
+
-
+
Ctrl-siRNA
P6-siRNA-2


E
Relative Syk[L]:Syk[S] ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E2
-
+
-
+
-
+
Ctrl-siRNA
C1-siRNA-1
C1-siRNA-2


F
RHCglo-CD44-minigene
Relative Skipping/inclusion ratio
0.2
0.15
0.1
0.05
0
PRMT6
-
+
+
Time (hrs)
24
24
48
Two exon inclusion
One exon inclusion
Skipping