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# Synergistic chromatin repression of the tumor suppressor gene *RARB* in human prostate cancers

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**Keywords:** retinoic acid receptor beta (*RAR*β), DNA hypermethylation, polycomb protein, H3K27 trimethylation, EZH2, prostate cancer

**Abbreviations:** ChIP, chromatin immunoprecipitation; ChIP-BS-pyro, ChIP bisulfite pyrosequencing; ChIP-BS-seq, ChIP bisulfite sequencing; DNMT, DNA methyltransferase; ES cells, embryonic stem cells; EZH2, enhancer of zeste homolog 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3K9me3, trimethylated lysine 9 of histone H3; H3K27me3, trimethylated lysine 27 of histone H3; MYT1, myelin transcription factor 1; ncRNA, non-coding RNA; PRC2, polycomb repressive complex 2; RA, retinoic acid; RARβ, retinoic acid receptor beta; RNAP II, RNA polymerase II

DNA methylation and polycomb proteins are well-known mediators of epigenetic silencing in mammalian cells. Usually described as mutually exclusive, this statement is today controversial and recent in vitro studies suggest the co-existence of both repressor systems. We addressed this issue in the study of *Retinoic Acid Receptor* β *(RAR*β*)*, a tumor suppressor gene frequently silenced in prostate cancer. We found that the *RAR*β promoter is hypermethylated in all studied prostate tumors and methylation levels are positively correlated with H3K27me3 enrichments. Thus, by using bisulfite conversion and pyrosequencing of immunoprecipitated H3K27me3 chromatin, we demonstrated that DNA methylation and polycomb repression co-exist in vivo at this locus. We found this repressive association in 6/6 patient tumor samples of different Gleason score, suggesting a strong interplay of DNA methylation and EZH2 to silence *RAR*β during prostate tumorigenesis.

## Introduction

Epigenetic repression is a common cellular mechanism to silence gene expression. To this goal, two major actors are DNA methylation and polycomb proteins. While these two repressing systems are well described, the interplay between them is still a key question in cellular biology in order to better understand both cell differentiation and tumorigenesis. Several reports have shown that DNA methylation and H3K27me3, catalyzed by EZH2 as part of the Polycomb Repressive Complex 2 (PRC2), are mutually exclusive.[1-3] Brinkman et al. addressed the overlap of these two marks at the genome-wide scale using ChIP-bisulfite-sequencing (ChIP-BS-seq) on the HCT116 tumor cell line and mouse embryonic stem (ES) cells.[4] This work concluded that DNA methylation and H3K27me3 can co-exist, except for CpG-dense regions where they are mutually exclusive. Meanwhile, with the same experimental strategy, Statham et al. described that in normal and prostate cancer cells DNA methylation and H3K27me3 are not always mutually exclusive and that they can co-occur according to the genomic context. Further, they showed for the first time that silenced genes with CpG island promoters can display both epigenetic silencing marks.[5]

In parallel to these studies we have characterized the two marks, DNA methylation and H3K27me3, on the *Retinoic Acid Receptor Beta* (*RAR*β) promoter in prostate cancer cell lines.[6] *RAR*β gene encodes several isoforms from two distinct promoters named P1 and P2. Among them, the *RAR*β*2* isoform is transcribed from the CpG-rich *RAR*β P2 promoter and act as a tumor suppressor. Its expression is frequently silenced leading to treatment resistance by Retinoic Acid (RA).[7,8] In our recent study, we observed and characterized distinct epigenetic silencing profiles at the *RAR*β P2 promoter in three prostate cancer cell lines: DU145 cells showed moderate DNA methylation concomitant with the H3K27me3 mark, while LNCaP and VCaP cells displayed high DNA methylation levels and no H3K27me3.[6]

It has been shown that, in specific cases, DNA methylation and polycomb proteins may cooperate to repress genes. For example, target genes of polycomb proteins are known to be 12 times more likely to undergo DNA hypermethylation during tumorigenesis[9-11] and polycomb proteins may directly interact with DNA methyltransferases (DNMTs).[12,13] Because of their

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**Table 1.** *RARβ2* promoter is hypermethylated in six human prostate tumors

| Patient sample | Gleason score | % Tumor | % *RARβ2* methylation | Patient Age | Relapse | Survival outcome |
|---|---|---|---|---|---|---|
| T1 | 5 (3+2) | 95 | 34.9 | 71.5 | No | Alive |
| T2 | 6 (2+4) | 95 | 42.2 | 61.1 | Yes | Alive |
| T3 | 9 (4+5) | 80 | 52.4 | 62.5 | No | Alive |
| T4 | 5 (3+2) | 90 | 59.8 | 65.7 | No | Alive |
| T5 | 7 (3+4) | 95 | 65.3 | 64.3 | No | Alive |
| T6 | 8 (4+4) | 95 | 72.7 | 64.4 | No | Alive |

For each patient sample named T1 to T6, the Gleason score, the percentage (%) of tumor tissue within the sample, the average percentage of genomic DNA methylation at the *RARβ2* promoter, the patient age and informations about relapse and survival outcome are indicated. DNA methylation levels of *RARβ2* promoter were measured by bisulfite pyrosequencing on 10 CpGs located downstream of the TSS, between +1 and +100 (each CpG site methylation value is available in **Table S1**).

involvement in cancer, it is of great interest to understand how their deregulation leads to inappropriate gene silencing and the above cited contradictory reports require more investigation to clarify the crosstalk between DNA methylation and polycomb repression, particularly in biologically relevant material such as tumor specimens. Here we studied this interplay at the *RARβ* locus in six prostate tumor samples. We focused on the P2 promoter containing a CpG island and driving *RARβ2* tumor suppressor expression (referred as *RARβ2* promoter herein).

## Results and Discussion

*RARβ2* is a tumor suppressor gene frequently hypermethylated during breast and prostate tumorigenesis.[14-17] Previously, we reported its hypermethylation in four prostate tumor cell lines (DU145, PC3, LNCaP, and VCaP) and found that *RARβ2* promoter hypermethylation is associated with H3K27me3 in DU145 prostate cancer cells but not in LNCaP and VCaP cells.[6]

The relevance of studies using in vitro cell lines is always of debate, especially in the epigenetic field, as microenvironment and culture conditions differ dramatically from in vivo conditions and can modify the chromatin profile of cells.[18] Thus, we investigated the chromatin patterns of the *RARβ2* promoter in six human prostate tumors (T1 to T6) with various Gleason scores. We analyzed the *RARβ2* DNA methylation level in each tumor by bisulfite-pyrosequencing of 10 CpGs located in the gene promoter (downstream of the TSS, between +1 and +100). All of the tumors displayed *RARβ2* promoter hypermethylation with average levels ranging from 34.9% to 72.7% (**Table 1**; **Table S1**). In comparison, the non-malignant cell line named EPT2,[19,20] derived from primary prostate epithelial cells, showed a 2.4% DNA methylation level. This finding in our cohort of six patient tumors, representing low- and high-grade cancers (Gleason score from 5 to 9, **Table 1**), confirmed that *RARβ2* CpG island is targeted by DNA methylation in prostate cancer. Noteworthy, *RARβ2* methylation levels are not correlated with patient age (Pearson's r = -0.35) nor with tumor grade (Pearson's r = 0.47) in our samples.

We then investigated the histone marks associated with the *RARβ2* promoter hypermethylation in these six prostate cancer samples (**Fig. 1**). Using small amounts of fresh-frozen tumor samples, we performed ChIP experiments to detect the heterochromatin H3K9 trimethylation mark (H3K9me3) and the polycomb H3K27me3 mark. As negative control, we chose the *GAPDH* promoter at which these two repressive marks were absent, while RNA polymerase II (RNAP II) was enriched, consistently with the expression of this housekeeping gene (**Fig. 1**, hatched bars). *MYT1* promoter has been previously reported as repressed by polycomb proteins[21] and thus was here used as positive control for the polycomb mark H3K27me3. Accordingly, we detected a strong enrichment of H3K27me3 together with variable levels of H3K9me3 and absence of RNAP II (**Fig. 1**, black bars). Interestingly, at the *RARβ2* promoter, the H3K27me3 repressive mark was present in all tumor samples and H3K9me3 was detected in four of them (**Fig. 1**, gray bars). The weak enrichment for RNAP II measured in some of the samples might suggests residual low expression of the gene. These results demonstrate that repressive histone marks are associated with hypermethylated *RARβ2* promoter, and in particular with the polycomb mark H3K27me3. Indeed, we found a positive correlation between *RARβ2* promoter methylation levels and H3K27me3 enrichments (Pearson's r = 0.84), while no such correlation is observed with H3K9me3 (Pearson's r = 0.15). These results suggest that an interplay between DNA methylation and polycomb repression occur at *RARβ2* CpG-rich locus in prostate tumorigenesis. For comparison, in the EPT2 cell line derived from primary prostate epithelial cells, the non-methylated *RARβ2* promoter did not display enrichment for H3K27me3 mark (data not shown).

To directly assess the co-existence of DNA methylation and H3K27me3 histone mark on the same DNA molecule, bisulfite conversion and pyrosequencing was performed on the immunoprecipitated ChIP samples analyzed in **Figure 1**. ChIP-bisulfite-pyrosequencing (ChIP-BS-pyro) allowed us to measure the DNA methylation level associated with a chromatin modification of interest (**Fig. 2A**). As expected, ChIP input samples T1 to T6 showed a similar *RARβ2* methylation level (**Fig. 2B**) to that performed directly on genomic DNA (**Table 1**) (Pearson's r = 0.94). H3K9me3 immunoprecipitated samples showed levels of methylation equal or higher to input controls, confirming that H3K9me3 mark is tightly associated with DNA methylation. Remarkably, similar results were also observed in the H3K27me3 immunoprecipitated samples—and not in the RNAP II one—demonstrating that DNA methylation and polycomb repression co-exist at the same locus in tumor patients' samples (**Fig. 2B**).

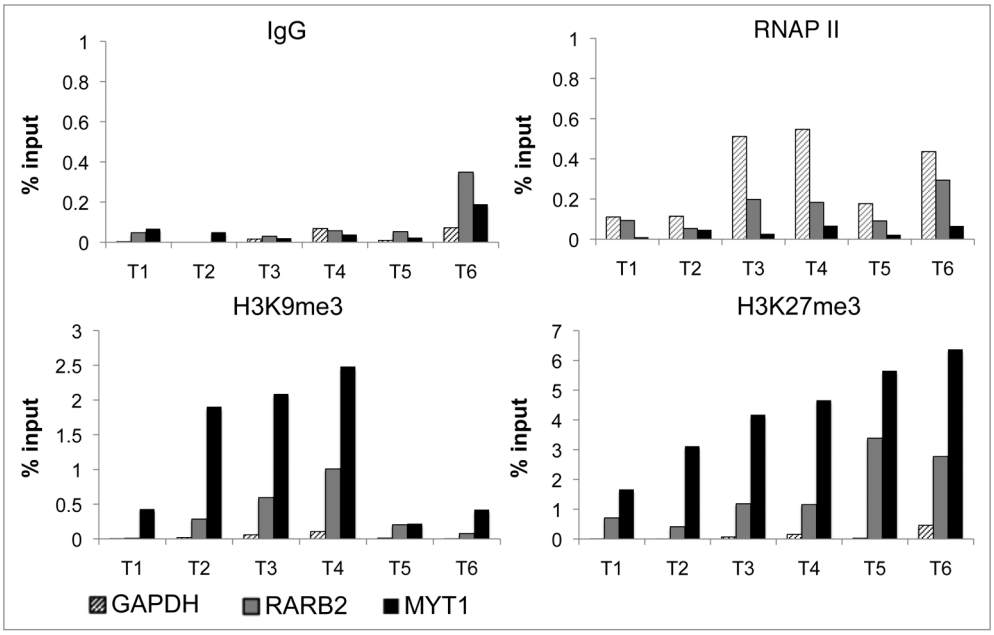


**Figure 1.** Repressive chromatin pattern at the *RARβ2* promoter in prostate cancer. ChIP analysis of RNAP II, H3K9me3 and H3K27me3 enrichment in six prostate tumors (T1–T6). IgG is used as control to measure non-specific immunoprecipitation. Enrichments were analyzed by qPCR at the *RARβ2*, *GAPDH* and *MYT1* promoters.

H3K9me3 and DNA methylation co-exist at the *RARβ2* locus but no correlation exist between these two marks in the six tumors (Pearson's r = 0.15). On the contrary, H3K27me3 co-exist with DNA methylation at the promoter and higher H3K27me3 ChIP enrichments are associated to higher DNA methylation with a positive correlation (Pearson's r = 0.84) (**Fig. 2C**).

All together, these results in patients' samples are in agreement with our previous observations in the DU145 cell line[6] and support in vitro data showing that these two silencing marks are not mutually exclusive at CpG islands.[5] Moreover, our findings at the *RARβ2* locus strongly suggest an interplay between DNA hypermethylation and the polycomb mark H3K27me3 during prostate tumorigenesis.

Understanding of the crosstalk between DNA methylation and polycomb repression is an important issue in both normal and cancer development. Further studies are required to address how methylation and polycomb profiles are established and cooperate to achieve abnormal silencing of specific tumor suppressor gene. In human embryonic fibroblasts, *RARβ2* has been shown to be a polycomb target gene[22] and polycomb proteins are deregulated during prostate tumorigenesis,[23] which could explain aberrant H3K27me3 tagging. Non-coding RNAs (ncRNA) could also play a part in recruiting polycomb to specific locus. We did not, however, identify ncRNAs associated with the *RARβ2* promoter in prostate cancer cell lines.[24] Interestingly, Zhao et al.[25] identified a PRC2-interacting antisense transcript to the 3′ of *RARβ2* gene in mouse ES cells suggesting that ncRNA might target polycomb repression at this specific locus. According to the "epigenetic switch" hypothesis, polycomb repression can be replaced by DNA methylation;[26] this could explain why polycomb target genes are predisposed to hypermethylation in cancers.[9-11] Moreover, it has been reported that modifications in the DNA methylome can alter polycomb binding and repressive pattern.[27-29] Future studies will need to clarify the interplay between DNMTs and polycomb proteins to understand which one triggers the upsetting of the other one and in which context.

In conclusion, the data presented here provide the first evidence that H3K27me3 and DNA methylation are related and co-exist in CpG rich regions in primary tissue samples from prostate cancer patients.

## Materials and Methods

### Prostate tumors

Patients' samples were obtained after informed consent in accordance with the Declaration of Helsinki and stored at the "CRB Cancer des Hôpitaux de Toulouse" collection. According to the French law, CRB Cancer collection has been declared to the Ministry of Higher Education and Research (DC-2008-463) and obtained a transfer agreement (AC-2008-820) after approbation by ethical committees. Clinical and biological annotations of the samples have been declared to the CNIL (Comité National Informatique et Libertés). None of the six patients have received radiotherapy or hormonotherapy before prostate surgery. The six prostate tumors were freshly frozen after surgery to guarantee in vivo condition of analysis.

### Bisulfite conversion followed by pyrosequencing

500 ng of genomic DNA was purified (DNeasy Blood and Tissue Kit, Qiagen) and bisulfite-converted (EZ DNA Methylation kit, Zymo Research) following the manufacturer's instructions. Quantitative DNA methylation analysis was performed by pyrosequencing on bisulfite treated DNA.[30]

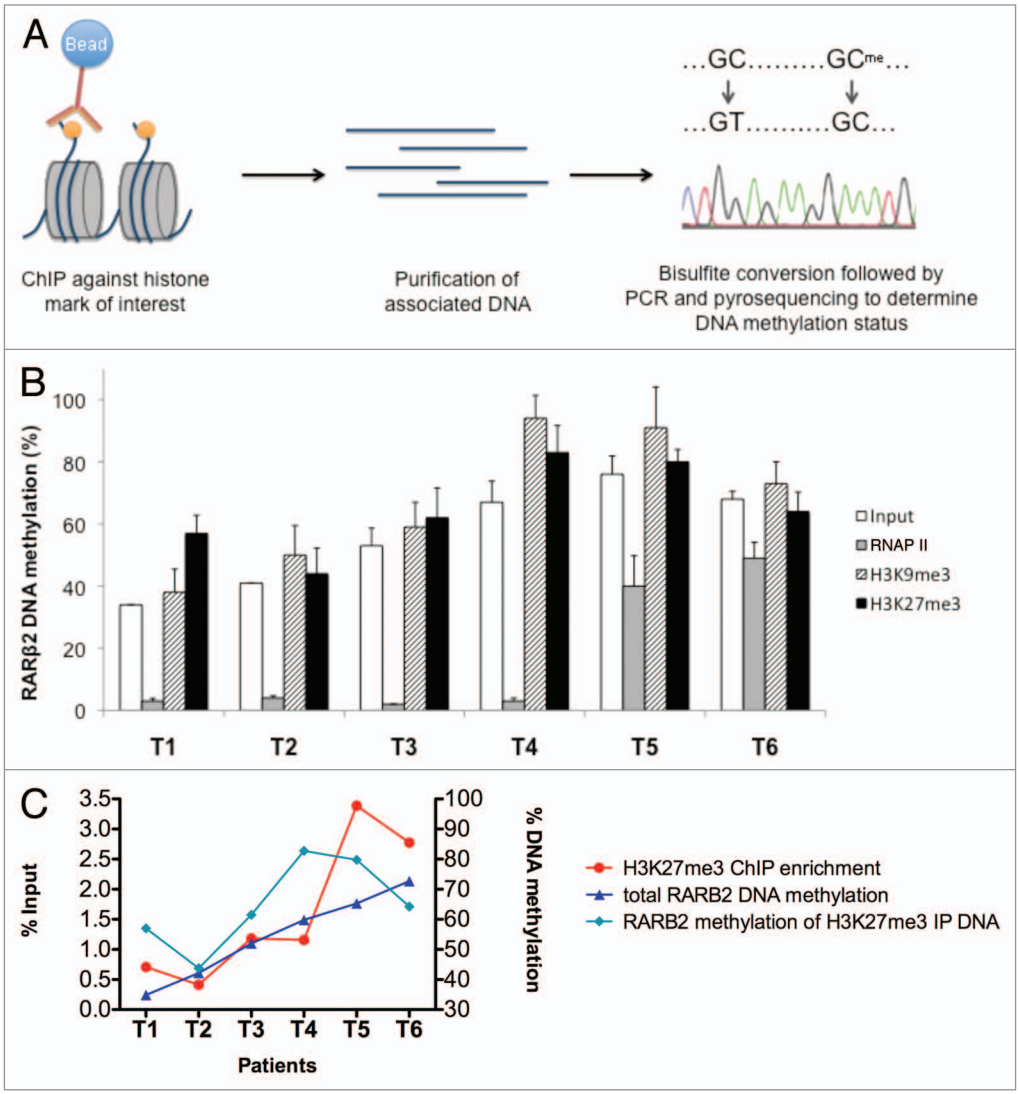


**Figure 2.** Co-existence of H3K27me3 and DNA methylation at the *RARβ2* promoter. (**A**) Schematic representation of the ChIP-BS-pyro experimental procedure. (**B**) Average percentage of *RARβ2* promoter methylation measured by bisulfite conversion-pyrosequencing on input samples, RNAP II, H3K9me3 and H3K27me3 immunoprecipitated DNA. Results are presented for samples named T1 to T6 as mean +/– SD of PCR technical replicates. (**C**) Graphic representation of H3K27me3 ChIP enrichments at the *RARβ2* promoter with *RARβ2* methylation levels on total DNA and after H3K27me3 immunoprecipitation (IP) in the six patient samples (expressed as average methylation percentage).

The region of interest validation was amplified using 30 ng of bisulfite treated human genomic DNA and 5 pmol of forward (5′-AGGAGGGTTT ATTTTTTGTT AAAGG) and reverse biotinylated primer (5′-Biotin–AAATTCTCCT TCCAAATAAA TACTTACAA). Reaction conditions were 1× HotStar Taq buffer supplemented with 1.6 mM $MgCl_2$, 100 μM dNTPs and 2.0 U HotStar Taq polymerase (Qiagen) in a 25 μL volume. The PCR program consisted of a denaturing step of 15 min at 95 °C followed by 50 cycles of 30 s at 95 °C, 30 s at 61 °C and 20 s at 72 °C, with a final extension of 5 min at 72 °C. Ten microliters of PCR product were rendered single-stranded as previously described[30] and 4 pmol of the respective sequencing primer (5′-TTGAGGATTG GGATGT for CpGs 1–5 and 5′-AGGGTTTGTT TGGGT for CpGs 6–10) were used for analysis. Quantitative DNA methylation analysis was performed on a PSQ 96MD system with the PyroGold SQA Reagent Kit and results were analyzed using the Q-CpG software (V.1.0.9, Pyrosequencing AB). For ChIP samples the immunoprecipitated DNA was bisulfite converted using the EpiTECT Plus kit (Qiagen) and the pyrosequencing was performed as previously described with the sole modification of using 3–4 μL of bisulfite converted DNA as input for PCR amplification. The average percentage of the methylation measured at the 10 CpGs located in the *RARβ2* promoter is reported.

**ChIP assays**

Tumor samples were thawed on ice and fixed in 1% formaldehyde for 10 min at RT. The crosslinking reaction was stopped by incubation with 0.125 M of glycine for 5 min at RT. After centrifugation, tumors were resuspended in dissociation buffer (50 mM Hepes-KOH pH7.5, 140 mM NaCl, 1 mM EDTA,

10% glycerol, 0.5% NP-40, 0.25% Triton X100 and protease inhibitor) and fully dissociated with a glass dounce homogenizer. After centrifugation, cell pellets were incubated 10 min at 4 °C in a lysis buffer (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8 and protease inhibitor). Then, tumors were sonicated for 20 min using cycles of 30 s ON–30 s OFF (Diagenode Bioruptor). Sheared chromatin was pre-cleared and incubated at 4 °C overnight with 1 μg of the following primary antibodies: anti-H3K9me3 (Abcam), anti-H3K27me3 (Abcam), anti-RNAP II (Millipore) and non-specific IgG (Millipore). Chromatin-antibody complexes were precipitated with agarose beads and washed four times (Low salt buffer: 20 mM Tris pH8, 0.1% SDS, 1% Triton X100, 2 mM EDTA and 150 mM NaCl. High salt buffer: 20 mM Tris pH8, 0.1% SDS, 1% Triton X100, 2 mM EDTA and 500 mM NaCl. Li buffer: 0.25 mM LiCl, 1% NP-40, 1% Na-Deoxycholate, 10 mM Tris pH8 and 1 mM EDTA. TE buffer: 50 mM Tris pH8, 50 mM NaCl and 1mM EDTA). Eluted and de-crosslinked samples were treated with RNase A (0.3 μg/μL) and Proteinase K (0.2 μg/μL) and DNA was purified by phenol-chloroform extraction. The immunoprecipitated DNA and input samples were analyzed by real-time qPCR using SYBR Green (Applied Biosystems) according to the manufacturer's instructions. Primers used were as follow: *RARβ2* 5′-ATCCTGGGAG TTGGTGATGT CAG-3′ and 5′-AAAGAATAGA CCCTCCTGCC TC-3′, *GAPDH* 5′-TACTAGCGGT TTTACGGGCG-3′ and 5′-TCGAACAGGA GGAGCAGAGA GCGA-3′, *MYT1* 5′-CCAAGGGTTC ATGGGTAGCG TATT-3′ and 5′-GTGCGAACTC CTAAGCCAGC TAAA-3′.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/27869

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