



RAPID COMMUNICATION

A complex signature network that controls the upregulation of PRMT5 in colorectal cancer



Protein arginine methyltransferase 5 (PRMT5) deregulation has emerged as an important prognostic indicator in human cancers. Through aberrant methylation-mediated epigenetic modification of signaling molecules, PRMT5 overexpression contributes to dysregulation of a variety of cellular processes related to cancer progression.¹ However, the mechanisms governing PRMT5 expression levels in cancer remain largely unknown. In this study, we examined factors that regulate PRMT5 expression at multiple levels. We mapped three regions of the proximal promoter of PRMT5 and identified NF-Ya, SMAD3, and ZNF143 as part of a key signature network node regulating PRMT5 expression in HT29 colorectal cancer (CRC) cells. Importantly, we provide evidence that knockdown or ligand induced activation of SMAD3, and ZNF143 led to changes in PRMT5 transcript and protein levels, respectively. We showed that PRMT5 expression positively correlates with both TGF- β 2 and ZNF143 expression, suggesting that activation and/or upregulation of these proteins may be partly responsible for PRMT5 overexpression in a subset of CRC patients. Collectively, our data present a complex model that involves cell-autonomous induction of PRMT5 in CRC cells by transcriptional mechanisms and upregulation of PRMT5 mRNA and protein, which encompasses processes involving gene amplification and increased transcription and protein turnover rates.

Previously, we showed that PRMT5 protein levels are significantly upregulated in CRC.² However, the mechanisms underlying PRMT5 overexpression in CRC remain elusive. Hence, we considered several possibilities, including increased copy number, transcriptional upregulation, and altered mRNA/protein turnover of PRMT5. To test these possibilities, first we used genomic quantitative polymerase chain reaction (qPCR) and quantitative reverse transcription PCR (RT-qPCR) to assess the gene copy number and mRNA levels of PRMT5 respectively. Our data revealed an increase in the levels of PRMT5 gene and transcripts in CRC cell lines compared to the control (Fig. 1A, S1A). As mRNA stability could cause increased levels of PRMT5

mRNA, we treated the cells with actinomycin D (ActD) for the indicated times and PRMT5 mRNAs levels were measured. As shown in Figure S1B, the PRMT5 mRNA half-life was significantly increased in CRC cells compared to FHC normal control cells, suggesting that PRMT5 mRNA is more stable in CRC cells than in normal colon cells. Additionally, altered protein stability could constitute another mechanism to account for the differences in protein abundance in cancer cells. Thus, cells were treated with cycloheximide (CHX) for indicated days. Western blot analysis showed that in CRC cells, the half-life of PRMT5 protein is higher than that of the normal colon cells, suggesting that PRMT5 protein is more stable in CRC than in normal colon cells (Fig. 1B).

To date, the identification of upstream factors and signaling mechanisms that drive transcriptional activation of PRMT5 is significantly lacking. This knowledge is critical for improving our understanding of the signaling molecules that contributes to PRMT5 overexpression. Through the promoter bashing approach, we identified four putative transcription factor binding sites at PRMT5 promoter. These binding sites were inserted into a promoterless luciferase reporter and a pGluc Basic vector and transfected into HT29 cells. As we observed a significant decrease in PRMT5 luciferase activity within the $-400/0$ truncated region, we carried out further 5'-truncations of this $-400/0$ region and identified $-110/-120$ and $-190/-200$ as being important for PRMT5 promoter activity (Fig. S1C). Next, we sought to identify whether the $-110/-120$ and $-190/-200$ regions mapped to any known transcription factor (TF) consensus binding sites. Interestingly, we identified consensus binding sites for NF-Ya, SMAD3, and ZNF143 corresponding to regions $-103/-106$, $-112/-122$, and $-183/-202$, respectively (Fig. S1C). Using site-directed mutagenesis within the NF-Ya, SMAD3, and ZNF143, we observed that the consensus sequence motifs resulted in a significant reduction in luciferase reporter PRMT5 activity by 33%, 55%, and 50%, respectively (Fig. 1C). Collectively, these data provide new evidence that SMAD3 and ZNF143 can positively regulate the transcriptional activation of PRMT5.

So far, we have shown that site mutagenesis within the NF-Ya, SMAD3, and ZNF143 binding sequences of the PRMT5

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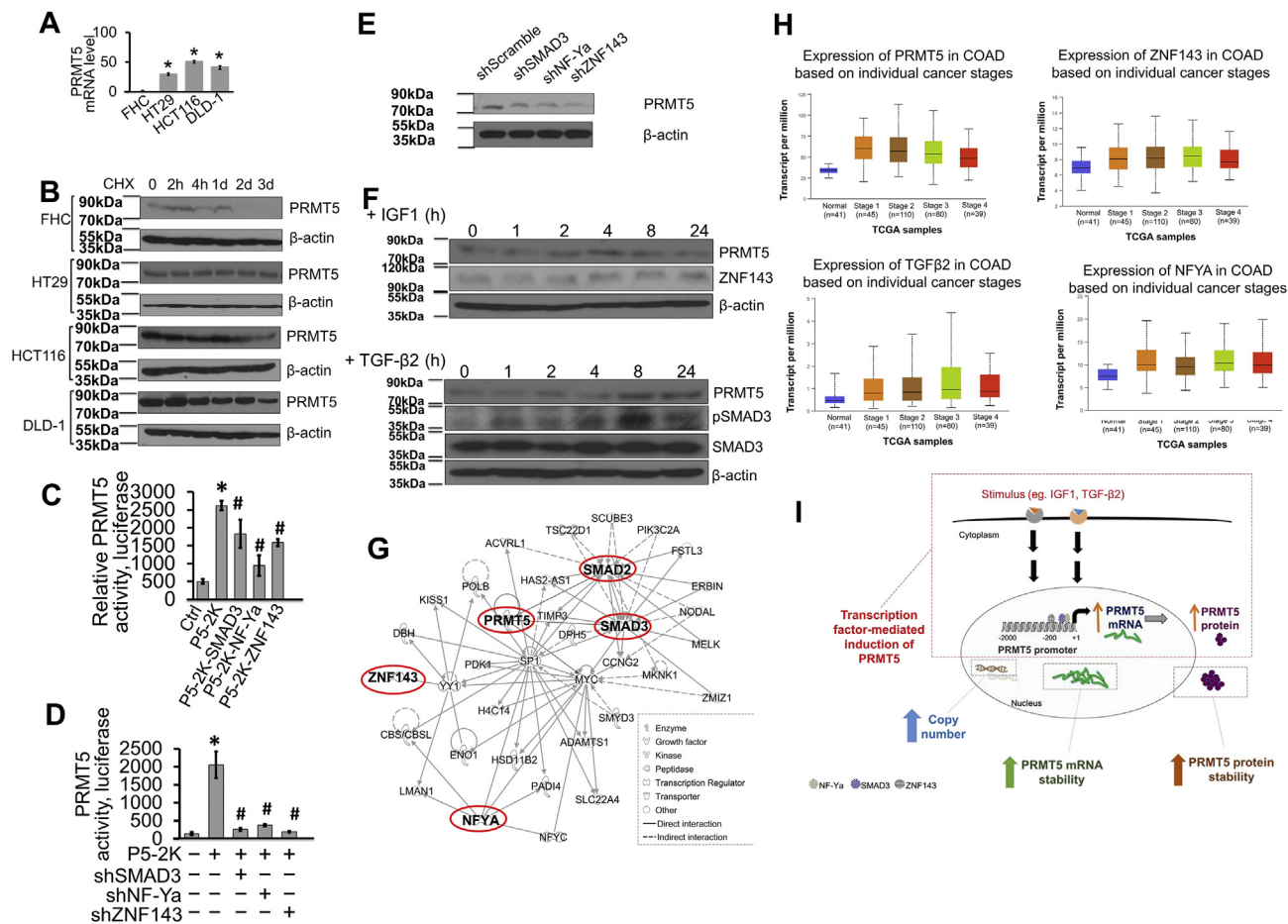


Figure 1 (A) qPCR was used to determine relative PRMT5 mRNA transcript levels in normal (FHC) colon cells compared to CRC cell lines (HT29, HCT116 and DLD-1). (B) FHC control cells and CRC cells HT29, HCT116 and DLD-1 were treated with CHX (60 μg/ml) for indicated times. Results from three independent experiments are presented as the mean ± SD. * $P \leq 0.05$ HT29, HCT116, DLD-1 vs. FHC. (C) Luciferase reporter assay for single mutations generated within predicted binding regions for SMAD3, NF-Ya and ZNF143 using the PRMT5 2k promoter sequence (denoted as P5-2K). (D) Luciferase PRMT5 reporter assay in HT29 cells with P5-2K construct and cells with knockdown of SMAD3, NF-Ya, and ZNF143. (E) Western analyses of PRMT5 protein levels in shScramble, shSMAD3, shNF-Ya, and shZNF143 HT29 cell lines. (F) Western analyses for detection of PRMT5 protein in HT29 cells treated with IGF-1 and TGF-β2 at various time points. (G) Ingenuity pathway analysis (IPA), showing gene network links among PRMT5, NF-Ya, SMAD3 and ZNF143. (H) Box-whisker plots showing transcript levels of PRMT5, TGF-β2, ZNF143, and NF-Ya across colorectal adenocarcinoma (COAD) tumors and normal based on individual cancer stages. Individual cancer stages were based on AJCC (American Joint Committee on Cancer) pathologic tumor stage information and samples were divided into stage I, stage II, stage III and stage IV group. Courtesy of UALCAN web-portal, publicly available at <http://ualcan.path.uab.edu>. (I) Schematic diagram depicts a complex multiple-mechanism regulation network that could lead to upregulation of PRMT5. PRMT5 proximal promoter is positively regulated by NF-Ya, ZNF143 and SMAD3. Ligand-induced (IGF-1 and TGF-β2) activation of PRMT5 mRNA transcript levels correlates with its increased protein expression through transcription factors ZNF143 and SMAD3, respectively. Moreover, copy number amplification, increased mRNA and protein stability may also account for increase the PRMT5 protein pool frequently observed in CRC cells. **Statistics:** Results from three independent experiments are presented as mean ± SD. * $P \leq 0.05$.

proximal promoter resulted in a decreased PRMT5 transcriptional activation. Thus, we reasoned that shRNA-mediated knockdown of these TFs should show a similar result. We successfully generated stable TF knockdown cell lines (Fig. S1D). Next, we investigated the effect of depleting these TFs on promoter transactivation, mRNA transcripts, and protein expression of PRMT5. Compared to cells expressing full-length PRMT5 promoter sequence (P5-2K), the NF-Ya, SMAD3, and ZNF143 knockdown cell lines showed a significant decrease in the PRMT5 luciferase promoter activity (Fig. 1D). Likewise, we observed a reduction in both the mRNA transcript and protein levels of

PRMT5 upon depletion of the TFs (Fig. 1E, S1E), confirming that these TFs are important positive regulators of PRMT5 promoter activation, transcription and protein expression.

As a corollary experiment to Figure 1D and E, we next investigated if signal-induced activation of these TFs would increase PRMT5 transcript and protein levels. Given that transforming growth factor beta 2 (TGF-β2) and insulin-like growth factor 1 (IGF-1) can activate SMAD3 and ZNF143, respectively, we assessed PRMT5 mRNA and protein levels, following treatment of HT29 cells with these ligands. Interestingly, with increasing time points of IGF-1 and TGF-β2 treatment, PRMT5 mRNA transcripts and protein levels are

significantly increased (Fig. 1F, S1F). The induction of PRMT5 protein levels corresponded with IGF-1-mediated increase in ZNF143 expression as well as TGF- β 2-induced phosphorylation of SMAD3. Taken together, these data suggest that induction of SMAD3 and ZNF143 with TGF- β 2 and IGF-1, respectively, leads to the upregulation of PRMT5 at both the transcriptional and protein level. Furthermore, using Ingenuity Pathway Analysis (IPA), we identified that NF- κ B, SMAD3 and ZNF143 are among key transcription factors involved in the same transcriptional regulatory network with PRMT5 (Fig. 1G). This network showed that PRMT5 can be regulated by multiple transcription factors such as NF- κ B, SMAD2/3, and ZNF143, directly or indirectly, leading to the activation of various downstream effectors, such as Myc.

To provide a clinical perspective to the lists of data above, we assessed the levels of PRMT5, ZNF143, TGF- β 2 and NF- κ B in CRC patients. In the assessment of colon adenocarcinoma (COAD) data samples, across all stages and compared to normal tissue matched samples, PRMT5, ZNF143, TGF- β 2 and NF- κ B are increased (Fig. 1H). Moreover, the protein levels of ZNF143 and TGF- β 2 were positively correlated with that of PRMT5 in COAD samples. The TFs were also positively correlated, further supporting the cooperativity of these proteins in regulating PRMT5 and promoting colorectal cancer progression (Fig. S1G). Notably, the low ZNF143 was shown to reduce p53 stability in breast cancer.³ Another study showed that NF- κ B increases PRMT5 levels, whose levels can be further lowered by the carboxyl terminus of heat shock cognate 70-interacting protein via ubiquitin-dependent proteasomal degradation in prostate cancer.⁴ Similarly, TGF- β regulates the mRNA stability of several critical oncogenes.⁵ Thus, these mechanisms of mRNA and protein regulation may translate well to defining how high stability PRMT5 is achieved in various cancer types.

In summary, based on the data obtained, we hypothesize that the upregulation of PRMT5 is regulated by systematically coordinated complex mechanisms (Fig. 1I). This model supports the notion that, instead of using a simple regulation mechanism, the upregulation of PRMT5 in CRC is the consequence of a complex key signature network depicted above, making this network a significant and novel diagnostic and therapeutic focus in CRC.

Author contributions

TL supervised the project and revised the manuscript. HW, AVH and AM participated in the experimental work and writing of the manuscript. AS contributed to scientific discussion and revised the manuscript. GJ, LP and YL contributed to some of the experimental work and data analysis.

Conflict of interests

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2021.11.001>.

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