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SHORT COMMUNICATION

Disruption of CTCF/cohesin-mediated high-order chromatin structures by DNA methylation downregulates *PTGS2* expression

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The CCCTC-binding factor (CTCF)/cohesin complex regulates gene transcription *via* high-order chromatin organization of the genome. *De novo* methylation of CpG islands in the promoter region is an epigenetic hallmark of gene silencing in cancer. Although the CTCF/cohesin complex preferentially targets hypomethylated DNA, it remains unclear whether the CTCF/cohesin-mediated high-order chromatin structure is affected by DNA methylation during tumorigenesis. We found that DNA methylation downregulates the expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*), which is an inducible, rate-limiting enzyme for prostaglandin synthesis, by disrupting CTCF/cohesin-mediated chromatin looping. We show that the CTCF/cohesin complex is enriched near a CpG island associated with *PTGS2* and that the *PTGS2* locus forms chromatin loops through methylation-sensitive binding of the CTCF/cohesin complex. DNA methylation abolishes the association of the CTCF/cohesin complex with the *PTGS2* CpG island. Disruption of chromatin looping by DNA methylation abrogates the enrichment of transcriptional components, such as positive elongation factor b, at the transcriptional start site of the *PTGS2* locus. These alterations result in the downregulation of *PTGS2*. Our results provide evidence that CTCF/cohesin-mediated chromatin looping of the *PTGS2* locus is dynamically influenced by the DNA methylation status.

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INTRODUCTION

Genomes are dynamically packaged into a compact three-dimensional nuclear space to form high-order chromatin structures. This packaging occurs in a highly specific manner to facilitate nuclear functions, such as DNA replication, DNA repair, and transcription.[1–4] Many high-order chromatin structures are established by a special class of architectural proteins, of which CCCTC-binding factor (CTCF) and cohesin are the best characterized.[5] CTCF forms long-range chromatin interactions between enhancers and promoters.[6] Half of the cohesin molecule colocalizes to the same regions with the CTCF-binding sites in the mammalian genome, and prior studies have shown that CTCF is required for the recruitment of cohesin to these binding sites.[7–9] Apart from its major function in sister chromatid cohesion,[10] cohesin affects gene transcription by facilitating long-range chromatin interactions along with CTCF between the members of many developmentally regulated gene families.[11–13]

Epigenetic modification is a heritable change in gene expression without alterations in DNA sequence and is a hallmark of cancer.[14,15] DNA methylation of CpG islands in promoter regions is a predominant epigenetic mechanism by which various genes are inactivated during tumorigenesis.[16] CTCF is associated with the transcriptional regulation of several imprinted genes, such as the *IGF2/H19* locus, by preferentially targeting hypomethylated DNA.[17–19] Thus, aberrant *de novo* DNA methylation may prevent the binding of CTCF to the promoter region, facilitating transcriptional silencing of the gene. Although genes that may be influenced by this type of regulation have been proposed,[20–23] there is no direct evidence that CTCF/cohesin-mediated long-range chromatin interactions are disrupted by DNA methylation during tumorigenesis.

Prostaglandin-endoperoxide synthase 2 (*PTGS2*, also known as cyclooxygenase-2 or prostaglandin G/H synthase 2) is the inducible form of the rate-limiting enzyme for prostaglandin production.[24] An aberrantly high level of *PTGS2* expression is frequently detected in numerous cancers and other diseases.[24] Conversely, we and others have reported that hypermethylation of the *PTGS2* CpG island directly downregulates the *PTGS2* expression in many human cancers, including gastric and colorectal cancers.[25,26] Considering that gastric cancers with *PTGS2* CpG island methylation show significantly lower recurrence and improved overall survival,[27] it is important to understand the underlying mechanisms leading to transcriptional silencing of *PTGS2* by DNA methylation.

In the present investigation, we examined whether DNA methylation can downregulate *PTGS2* expression by disrupting CTCF/cohesin-mediated chromatin looping of the *PTGS2* locus. Our data show that enrichment of the CTCF/cohesin complex near the *PTGS2* CpG island is prohibited by DNA methylation. Reduced

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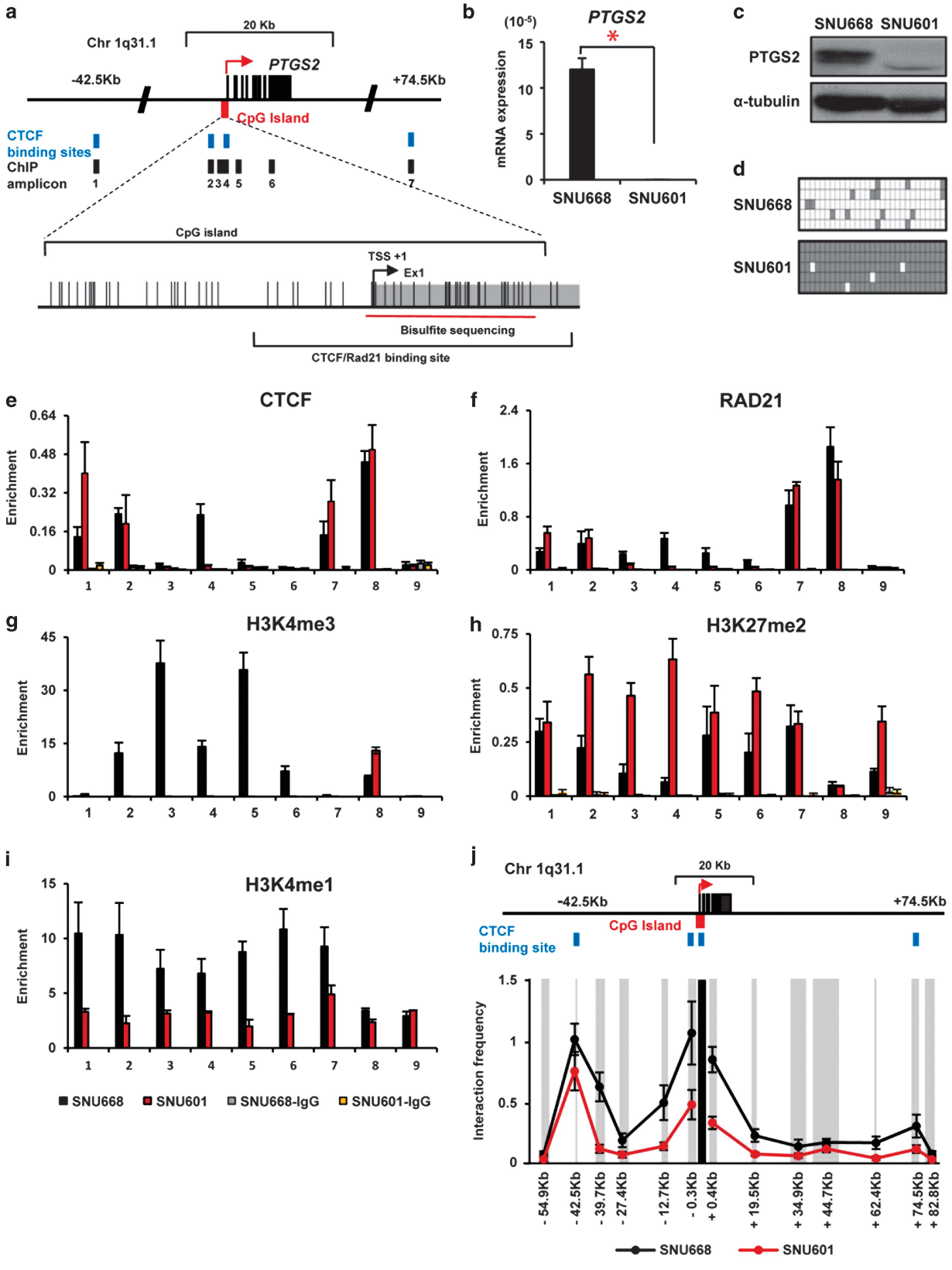
CTCF/cohesin binding abolishes the spatial proximity between CTCF/cohesin-binding sites. As a result, the recruitment of key transcriptional components, such as positive elongation factor b (P-TEFb), is diminished, leading to decreased transcriptional elongation and a low level of *PTGS2* expression. Our findings strongly suggest that DNA methylation downregulates *PTGS2* expression by disrupting CTCF/cohesin-mediated high-order chromatin structures.



RESULTS AND DISCUSSION

The *PTGS2* locus forms spatial chromatin organization in a methylation-sensitive manner

A bioinformatics search of the UCSC Genome database (http://genome.ucsc.edu/) for CTCF/cohesin-binding motifs across the *PTGS2* locus revealed four putative candidate sites (Figure 1a). One of the CTCF/cohesin-binding motifs was located at the transcriptional start site in the *PTGS2* CpG island (amplicons 3 and 4); thus, we speculated that the binding of CTCF/cohesin to the *PTGS2* locus may be influenced by DNA methylation.

To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) assays using two gastric cancer cells with different DNA methylation patterns at the *PTGS2* CpG island. We previously showed that SNU668 cells express a high level of *PTGS2* and are devoid of DNA methylation at the *PTGS2* CpG island. In contrast, SNU601 cells have undetectable *PTGS2* expression and are hypermethylated at the same region (Figures 1b, c and d).[25] High-resolution ChIP assays showed that CTCF binding occurred at four potential CTCF/cohesin-binding sites in the *PTGS2* locus in SNU668 cells with an unmethylated *PTGS2* CpG island (black bars; amplicons 1, 2, 4 and 7) (Figure 1e). Prominent binding of RAD21, a core subunit of the cohesin complex,[10] colocalized with CTCF at the *PTGS2* locus in SNU668 cells (Figure 1f). However, despite similar binding patterns at most putative CTCF/cohesin-binding sites in the *PTGS2* locus, recruitment of CTCF and RAD21 near the transcriptional start site of the *PTGS2* locus in SNU601 cells (red bars; amplicons 3, 4 and 5) was significantly less than that of SNU668 cells (Figures 1e and f). This difference in recruitment suggests that hypermethylation of the *PTGS2* CpG island disrupted the binding of CTCF/cohesin at these sites in SNU601 cells. Consistent with active transcription of *PTGS2* in SNU668 cells (Figures 1b and c), the binding of CTCF and cohesin at the transcriptional start site in the *PTGS2* CpG island coincided with the enrichment of the active histone marker, H3K4 trimethylation (H3K4me3)[15] (Figure 1g). Repressive histone H3K27 dimethylation (H3K27me2)[15] was enriched in SNU601 cells with a methylated *PTGS2* CpG island (Figure 1h). Similarly, CTCF/cohesin-binding sites were strongly enriched for histone H3K4 monomethylation (H3K4me1), which is a predictive marker of enhancers,[28] in SNU668 cells (Figure 1i). These data demonstrate that the CTCF/cohesin complex is localized at the CTCF/cohesin-binding sites of the *PTGS2* locus in SNU668 cells expressing a high level of *PTGS2*. However, hypermethylation of the *PTGS2* CpG island in SNU601 disrupts the binding of the CTCF/cohesin complex on these sites in methylation sensitive manner.

Recent evidence indicates that CTCF and cohesin mediate long-range chromatin interactions at CTCF/cohesin-binding sites at the *IFNG*, *IGF2-H19* and *β-globin* loci.[29–31] To test whether the *PTGS2* locus can form long-range chromatin interactions through CTCF/cohesin binding, we performed chromosome conformation capture (3C) assays[32] with the EcoRI restriction enzyme to assess chromatin interactions across the *PTGS2* locus (Figure 1j). The transcriptional start site of the *PTGS2* locus strongly interacted with the CTCF/cohesin-binding sites at − 42.5 kb and +74.5 kb in SNU668 cells, which display a high level of *PTGS2* expression (black line, Figure 1j); no interactions were detected at other sites without CTCF/cohesin enrichment. The physical proximity of CTCF/cohesin-binding sites in SNU601 cells (red line, Figure 1j) was significantly lower than that of SNU668 cells. Thus, CTCF/cohesin may form long-range chromatin interactions at the *PTGS2* locus to facilitate transcription and a high level of *PTGS2* expression in SNU668 cells. In contrast, DNA methylation of the *PTGS2* CpG island in SNU601 cells may abolish CTCF/cohesin-mediated spatial organization, resulting in transcriptional inefficiency.

We also compared the methylation status, CTCF/cohesin binding and spatial chromatin organization of the *PTGS2* locus in A549 and SNU719 cells. The A549 cells exhibit a methylation pattern similar to that of SNU668 cell, whereas SNU719 cells demonstrate a methylation pattern similar to that of SNU601 cells. As expected, our results in A549 and SNU719 cells were similar to the previous results obtained from SNU668 and SNU601 cells (Supplementary Figure S1). These data suggest that the inhibitory effect of DNA methylation on CTCF/cohesin-mediated high-order chromatin structures at the *PTGS2* locus may be generalized to other cell lines.

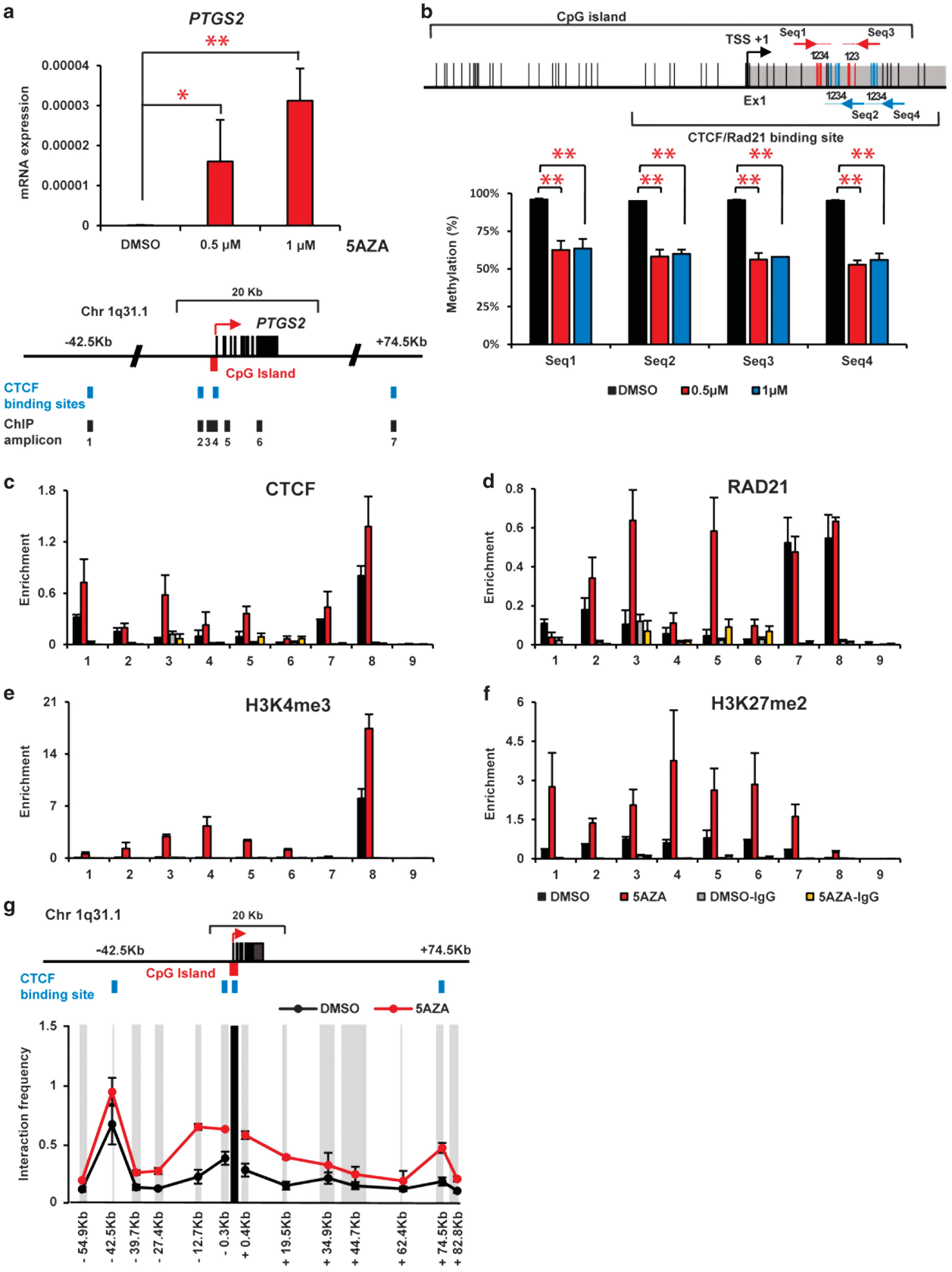
DNA methylation reduces the spatial organization of the *PTGS2* locus

We examined whether DNA methylation abrogates transcription by disrupting the binding of CTCF/cohesin to the *PTGS2* locus. We determined the dynamics of CTCF and cohesin binding after the treatment of SNU601 cells with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR).[33]

Treatment with 5-aza-CdR caused a dose-dependent reactivation of *PTGS2* mRNA expression in SNU601 cells (Figure 2a). Pyrosequencing analysis of SNU601 cells treated with 500 nM 5-aza-CdR demonstrated reduced DNA methylation at the *PTGS2* CpG island (Figure 2b), confirming the importance of DNA methylation in the transcriptional repression of *PTGS2*.[25] Next, ChIP assay with antibodies against CTCF and

Figure 1. The spatial chromatin organization of the *PTGS2* locus by methylation-sensitive binding of CTCF/cohesin. (**a**) The *PTGS2* locus at chromosome 1q31.1 is illustrated to scale. The location of putative CTCF/cohesin-binding sites and the primer pairs used for quantitative real-time PCR (qRT–PCR) are shown with labels below. The *PTGS2* CpG island is also shown. Exon1 is indicated by a gray box. Vertical bars represent each CpG site. TSS, transcriptional start site. (**b**) *PTGS2* mRNA expression in SNU668 and SNU601 cells was analyzed by qRT–PCR and normalized relative to 18S ribosomal RNA. Data are presented as the mean ± s.d. of triplicate independent RNA preparations. $*P < 0.05$, $**P < 0.001$. (**c**) Western blot analysis was performed with anti-PTGS2 antibody; α-tubulin served as a loading control. (**d**) Bisulfite-sequencing analysis of the *PTGS2* CpG island in SNU668 and SNU601 cells is shown. Each square denotes a CpG site within the PCR product amplified from bisulfite-treated DNA.[33] (Filled squares: methylated; open squares: unmethylated). (**e**–**i**) A ChIP assay was performed with SNU668 (black bars) or SNU601 (red bars) cells using antibodies against (**e**) CTCF, (**f**) RAD21, (**g**) H3K4me3, (**h**) H3K27me2 and (**i**) H3K4me1. qRT–PCR using SYBR Green was performed to detect enriched DNA. Enrichment of target DNA over input was calculated using the ΔΔCt method,[30,43] and results are presented as the mean ± s.e.m., $n = 3$. The +1775 site (amplicon 8) of the *P21* locus[44] and *NECDIN* (amplicon 9)[43] served as positive and negative controls, respectively, for CTCF/cohesin binding. (**j**) Relative crosslinking frequencies among CTCF/cohesin-binding sites in the *PTGS2* locus were measured with a 3C assay[32] in SNU668 (black line) and SNU601 (red line) cells. Chromatin crosslinked in 1% formaldehyde was digested with the restriction enzyme EcoRI overnight, followed by ligation with T4 DNA ligase at 16 °C for 4 h.[30,43,45] Crosslinking was reversed, and the DNA was purified as previously described.[43] Crosslinking frequencies and ligation efficiencies between different samples were normalized relative to the ligation frequency of two adjacent digested fragments in the *CalR* gene.[45] Quantification of data was performed by qRT–PCR using SYBR Green. EcoRI restriction sites in the *PTGS2* locus appear as gray, shaded bars. Black shading indicates the anchor fragment of the transcriptional start site of *PTGS2*. The maximum crosslinking frequency was set to 1 (mean ± s.e.m., $n = 4$). See the Supplementary information for further details.

cohesin was performed on SNU601 cells treated with 500 nM 5-aza-CdR (Figures 2c and d). Demethylation of the *PTGS2* CpG island by 5-aza-CdR treatment (Figures 2a and b) resulted in increased binding of CTCF and cohesin at the *PTGS2* CpG island (red bars; amplicons 3, 4 and 5 in Figures 2c and d). Binding of CTCF and cohesin at this region was undetectable in



dimethylsulfoxide-treated SNU601 cells (black bars; Figures 2c and d); thus, we concluded that CTCF and cohesin binding was influenced by the methylation status of the *PTGS2* CpG island (Supplementary Figures S2a and b).

Because G9A- and EZH2-mediated histone modification cooperates with DNA methyltransferases to silence *PTGS2* expression,[34] we asked whether 5-aza-CdR treatment changed the epigenetic signatures within the *PTGS2* locus. Upon 5-aza-CdR treatment, the binding of active H3K4me3 within the *PTGS2* locus was enhanced ~10-fold (Figure 2e and Supplementary Figure S2c) in parallel with increased transcription of *PTGS2* (Figure 2a). Interestingly, the repressive H3K27me2 mark throughout the *PTGS2* locus was also slightly enriched after 5-aza-CdR treatment (Figure 2f and Supplementary Figure S2d), consistent with the previous observation that promoter regions of demethylated genes are frequently marked by bivalent histone modifications in human cancer cells.[35,36] Taken together, the binding of CTCF and cohesin to their binding sites at the transcriptional start site of *PTGS2* was regulated by DNA methylation within the *PTGS2* CpG island.

Consistent with the increased enrichment of CTCF and cohesin (Figures 2c and d), the physical proximity of the *PTGS2* locus in the 5-aza-CdR-treated SNU601 cells (red line, Figure 2g) was significantly increased compared with that of control dimethylsulfoxide-treated cells (black line, Figure 2g). These results suggest that DNA methylation of the *PTGS2* CpG island in SNU601 cells abolishes CTCF/cohesin-mediated spatial organization of the *PTGS2* locus (Supplementary Figure S2e).

Cohesin binding is crucial for the spatial organization of the *PTGS2* locus

To test whether CTCF/cohesin binding is a prerequisite for efficient transcription of *PTGS2*, we reduced the expression of cohesin using *RAD21* knockdown (KD) in SNU668 cells. Two different *RAD21*-specific shRNAs reduced the RAD21 expression with a similar efficiency, decreasing *RAD21* mRNA and protein levels by ~90% in SNU668 cells (Figures 3a and c). Using fluorescence-activated cell sorting analysis we observed that *RAD21*-KD SNU668 cells exited mitosis, divided and survived well without pronounced levels of cell death (Supplementary Figure S3c), which is consistent with earlier data.[11,37] Notably, *PTGS2* mRNA and protein expression were significantly reduced by *RAD21*-KD in SNU668 cells (Figures 3b and c). We obtained similar results after *RAD21*-KD in A549, HeLa and N87 cancer cells, which have high levels of *PTGS2* expression (Supplementary Figure S4). Furthermore, although *PTGS2* expression was induced by phorbol 12-myristate 13-acetate (PMA) treatment in control SNU668 cells,[38] we found that PMA-induced *PTGS2* expression was completely abrogated in *RAD21*-KD SNU668 cells (Supplementary Figure S5); these data suggest that cohesin is crucial for optimal expression of *PTGS2* in human cancer cells.

Next, we determined whether cohesin is essential for long-range chromatin interactions (Figure 1j). Our ChIP experiments showed that *RAD21*-KD significantly reduced the binding of RAD21 and SMC1 (structural maintenance of chromosome 1), two members of the cohesin complex,[10] at the *PTGS2* locus (Figures 3d and e). These results suggest that *RAD21*-KD blocks the formation and enrichment of the cohesin complex at the *PTGS2* locus in SNU668 cells. However, *RAD21*-KD had no significant effect on the expression or binding of CTCF at the *PTGS2* locus (Supplementary Figures S3a and b); this finding supports the paradigm that CTCF binds to chromatin independently from cohesin.[8,39] Using the 3C assay with *RAD21*-KD SNU668 cells, we found that the spatial proximity in the *RAD21*-KD SNU668 cells (red line, Figure 3i) was significantly lower (twofold) than that in the control *GFP*-KD SNU668 cells (black line, Figure 3i). We also identified that re-establishment of CTCF/cohesin-mediated high-order chromatin structures after 5-aza-CdR treatment was significantly abolished after Rad21 knockdown. Consequently, re-expression of PTGS2 mRNA after 5-aza-CdR treatment was also abrogated (Supplementary Figure S6). These data strongly support that cohesin binding is required for the long-range chromatin interactions among CTCF/cohesin-binding sites in the *PTGS2* locus.

Next, we examined how cohesin-mediated spatial chromatin organization affects *PTGS2* expression. We examined enrichment of the elongation-competent form of RNA pol II (Pol II), which is phosphorylated at Ser2 of the C-terminal domain (Ser2P), in *RAD21*-KD SNU668 cells.[40] The binding of Ser2P Pol II was significantly decreased throughout the coding region of the *PTGS2* locus in *RAD21*-KD SNU668 cells (Figure 3f), which was consistent with the reduced expression of *PTGS2* (Figure 3b). We found that CDK9, a subunit of the P-TEFb complex that catalyzes phosphorylation of Ser2P,[40] was also reduced at these sites by approximately twofold in *RAD21*-KD SNU668 cells (Figure 3g). These data confirm previous observations that cohesin facilitates the transition of paused Pol II to elongation.[11] In addition, the binding of acetylated histone H3, a marker of active transcription,[15] was present within the *PTGS2* locus, although it was reduced by twofold at these sites in the *RAD21*-KD SNU668 cells (Figure 3h).

Together, these results suggest that cohesin-mediated spatial chromatin organization of the *PTGS2* locus is required for recruitment of the transcription regulator P-TEFb to enhance elongation of *PTGS2* transcripts.

DNA methylation during tumorigenesis abrogates CTCF/cohesin-mediated chromatin looping essential for *PTGS2* expression

In the present investigation, we found that CTCF/cohesin-mediated spatial chromatin organization is an essential mechanism for transcriptional regulation of *PTGS2* (Figure 4). Deregulation of CTCF/cohesin binding by DNA methylation may disrupt the proper spatial chromatin organization of the *PTGS2* locus, which subsequently reduces the overall *PTGS2* expression. We found that downregulation of cohesin abolished the proximity among the CTCF/cohesin-binding sites without disturbing CTCF enrichment in *RAD21*-KD cells (Figure 3i and Supplementary Figure S3b). CTCF, which positions cohesin at the cohesin-binding sites,[8,9] binds to the *PTGS2* CpG island in a DNA methylation-sensitive manner (Figure 1e), Therefore, we propose that DNA methylation

Figure 2. Demethylation of the *PTGS2* CpG island restores the spatial organization of the *PTGS2* locus. (**a**) SNU601 cells were treated with 500 nM or 1 μM 5-aza-CdR for 4 days, and *PTGS2* mRNA levels were analyzed with qRT–PCR and normalized to 18S ribosomal RNA (mean ± s.d., $n=4$); $^{*}P<0.05$, $^{**}P<0.001$. (**b**) SNU601 cells were treated with 500 nM or 1 μM 5-aza-CdR for 4 days, followed by genomic DNA preparation. For pyrosequencing analysis,[33] bisulfite-modified genomic DNA was amplified with specific biotinylated primers. Quantitative analysis of the *PTGS2* CpG island methylation was performed using a PyroMark ID pyrosequencer (Qiagen) as previously described.[33] Locations of sequences corresponding to the four primer sets used for pyrosequencing are indicated by arrows. The *y*-axis represents the percentage of CpG methylation of each sequence (mean ± s.d., $n=3$); $^{*}P<0.05$, $^{**}P<0.001$. (**c**–**f**) SNU601 cells were treated with dimethylsulfoxide (black bars) or 500 nM 5-aza-CdR (red bars) for 4 days, and the ChIP assay was performed using antibodies against (**c**) CTCF, (**d**) RAD21, (**e**) H3K4me3 and (**f**) H3K27me2 (mean ± s.e.m., $n=4$). (**g**) Relative crosslinking frequencies among CTCF/cohesin-binding sites in the *PTGS2* locus were measured with a 3C assay[32] in SNU601 cells after treatment with dimethylsulfoxide (black line) or 500 nM 5-aza-CdR (red line) for 4 days. EcoRI restriction sites in the *PTGS2* locus appear as gray, shaded bars. Black shading indicates the anchor fragment at the transcriptional start site of *PTGS2*. The maximum crosslinking frequency was set to 1 (mean ± s.e.m., $n=3$).

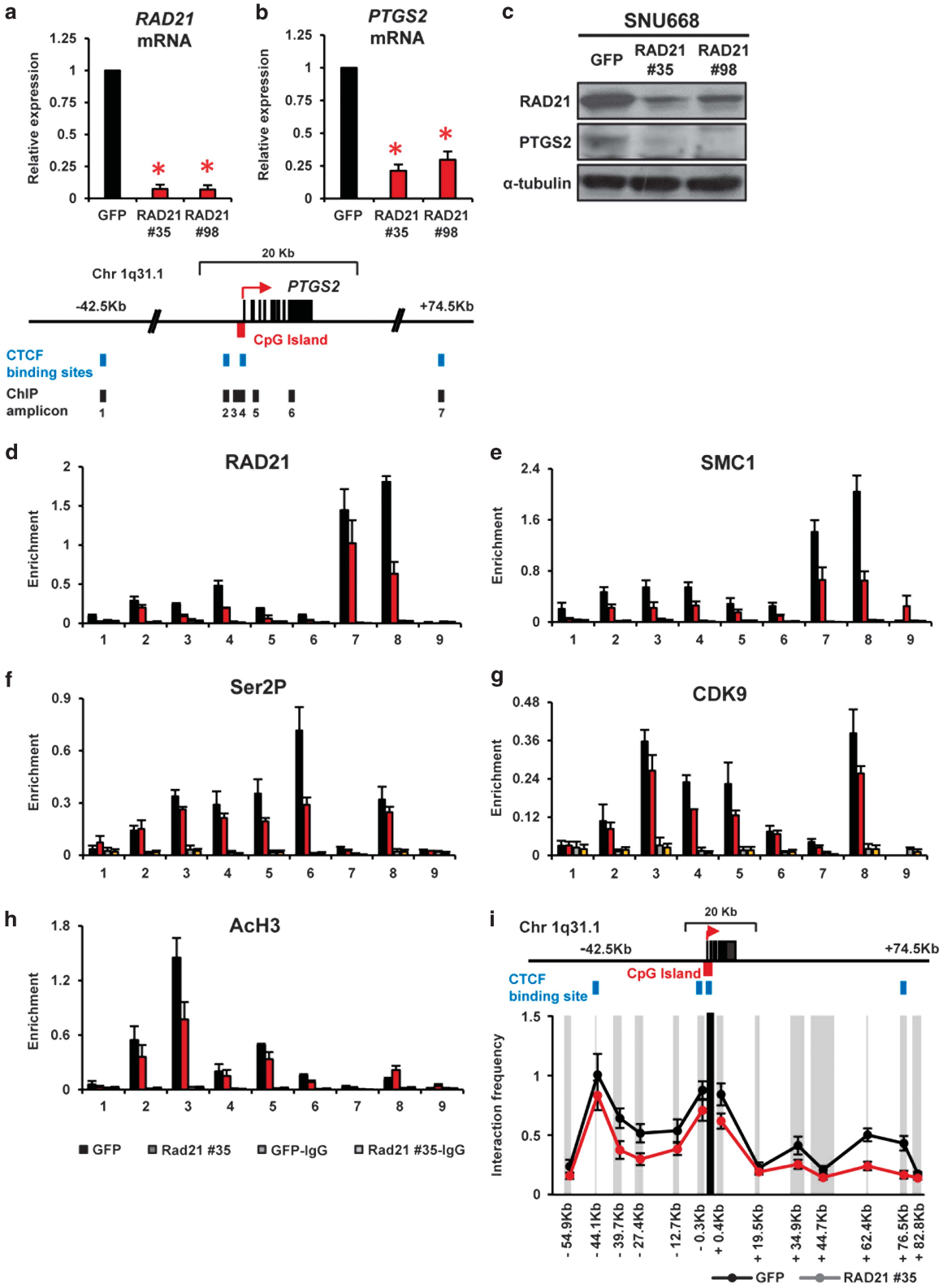


Figure 3. Cohesin depletion reduces *PTGS2* expression by abolishing proper spatial chromatin organization of the *PTGS2* locus. SNU668 cells were transduced with control (*GFP*) shRNA or two different *RAD21* shRNAs (#35 and #98) for 5 days. Lentiviruses were produced as previously described.[43] See Supplementary information for further details. (**a**) *RAD21* and (**b**) *PTGS2* mRNA expression levels were analyzed with qRT–PCR and normalized to that of 18S ribosomal RNA, expressed relative to control *GFP-shRNA*-expressing cells. Data are the mean ± s.d. of triplicate independent viral transduction experiments; $^{*}P < 0.05$, $^{**}P < 0.001$. (**c**) Western blot analysis was performed with anti-RAD21 and anti-PTGS2 antibodies on day 5 after *RAD21*-KD; α-tubulin served as a loading control. (**d**–**h**) A ChIP assay was performed with *GFP*-KD (black bars) or *RAD21*-KD SNU668 (#35; red bars) cells on day 5 after *RAD21*-KD using antibodies against (**d**) RAD21, (**e**) SMC1, (**f**) Ser2P, (**g**) CDK9 and (**h**) AcH3 (mean ± s.e.m., $n = 3$). (**i**) Long-range interactions at the *PTGS2* locus were assessed with a 3C assay using *GFP*-KD (black line) or *RAD21*-KD SNU668 (red line) cells (mean ± s.e.m., $n = 5$). AcH3, acetylated histone H3; SMC1, structural maintenance of chromosomes 1.

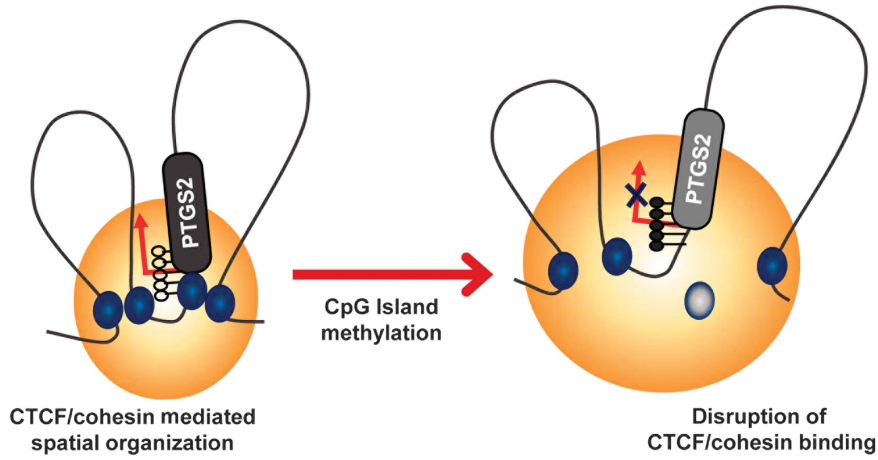


Figure 4. Cohesin is required for the formation of organized chromatin at the *PTGS2* locus. A proposed model for stabilizing high-level *PTGS2* expression by cohesin-mediated chromatin looping is shown. The *PTGS2* locus forms chromatin loops in cancer cells that express high levels of *PTGS2* (left). CTCF/cohesin (blue ovals) stabilizes long-range chromatin interactions through direct interactions among CTCF/cohesin localization sites. However, DNA methylation of the *PTGS2* CpG island abolishes the association among CTCF/cohesin-binding sites at the *PTGS2* locus by inhibiting the enrichment of CTCF/cohesin at these regions (right, gray oval). Deregulation of chromatin organization at the *PTGS2* locus decreases the association of the transcription regulator P-TEFb near these regions, reducing *PTGS2* expression in cancer cells with heavily methylated CpG islands.

abrogates spatial chromatin organization of the *PTGS2* locus by disrupting CTCF and cohesin binding, both of which are indispensable for direct interactions among CTCF/cohesin-binding sites. Considering the recent discovery that aberrant overexpression of the cohesin component is frequently detected in many human cancers,[41] this raises the possibility that activated CTCF/cohesin mediates *PTGS2* upregulation during tumor progression. Thus, hypomethylation of *PTGS2* may confer an obvious advantage to cancer cells by increasing and maintaining elevated levels of *PTGS2* during tumor development.[42]

Our results provide evidence that cohesin-mediated spatial chromatin organization can be abolished by DNA methylation at the *PTGS2* CpG island; this finding highlights a mechanistic linkage between high-order chromatin structures and DNA methylation during tumorigenesis. Our findings correlate with a previous result that gastric cancer patients with methylated *PTGS2* show lower recurrence and improved overall survival rates compared with patients with unmethylated *PTGS2*.[27] Thus, further studies should be performed to determine the detailed mechanism by which DNA methylation affects this dynamic transition of the high-order chromatin landscape during tumor progression; such studies will ultimately allow new, effective epigenetic therapies to be developed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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