WOMEN IN CANCER THEMATIC REVIEW

Diverse functions of DNA methylation: implications for prostate cancer and beyond

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Abstract

Prostate cancer is one of the most common malignancies in men worldwide. Current clinical screening ensures that most prostate cancers are diagnosed while still organ confined, but disease outcome is highly variable. Thus, a better understanding of the molecular features contributing to prostate cancer aggressiveness is being sought. For many cancers, aberrant genome-wide patterns of cytosine DNA methylation in CpG dinucleotides distinguish tumor from normal tissue and contribute to disease progression by altering the transcriptome. In prostate cancer, recent genomic studies identified cancer and high grade-specific differential DNA methylation in gene promoters, gene bodies, gene 3′ ends and at distal regulatory elements. Using examples from developmental and disease systems, we will discuss how DNA methylation in each of these genomic contexts can contribute to transcriptome diversity by modulating transcription initiation, alternative transcription start site selection, alternative pre-mRNA splicing and alternative polyadenylation. Alternative transcripts from the same gene often exhibit altered protein-coding potential, translatability, stability and/or localization. All of these can have functional consequences in cells. In future work, it will be important to determine if DNA methylation abnormalities in prostate cancer modify the transcriptome through some or all of these mechanisms and if these DNA methylation-mediated transcriptome alterations impact prostate tumorigenesis and aggressiveness.

Key Words
- prostate cancer
- DNA methylation
- gene expression

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Introduction

Prostate cancer genomics

Prostate cancer is the most common non-skin cancer malignancy among American men and the second most common among men worldwide (Siegel *et al*. 2015). Both genetic and demographic factors contribute to the high incidence of prostate cancer (Al Olama *et al*. 2014). Clinically, elevated prostate-specific antigen (PSA) in the blood serves as an early marker for prostate cancer; early detection has led to approximately 90% of prostate cancers still being localized to the prostate at the time of diagnosis (Penney *et al*. 2013). However, disease outcome is highly variable on a spectrum ranging from indolent disease that can be monitored by active surveillance to highly aggressive disease that leads to death.

A useful predictor of prostate cancer aggressiveness is the Gleason grade (Epstein *et al*. 1996, Albertsen *et al*. 1998, Pan *et al*. 2000, Penney *et al*. 2013). The presence of Gleason pattern 4/5 cancers is associated with increased development of metastatic disease, morbidity and

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mortality compared with Gleason 3 cancers (Stamey *et al.* 1999, Humphrey 2004). However, the majority of cases contain a mixture of low- and high-grade cancers making it difficult to define the risks. In the absence of accurate prediction of aggressiveness, patients and clinicians justifiably err on the side of caution; recent estimates are that 37 patients still need treatment to prevent 1 death (Schroder *et al.* 2012). Such over-treatment is of concern because radical therapies like prostatectomy and radiation have serious side effects that significantly impair the quality of life. Thus, a major goal of prostate cancer research is to find other parameters that inform on disease aggressiveness.

Molecular features are one promising avenue that is being explored. Using genome-wide mutation, somatic copy number alterations, DNA methylation, mRNA expression and miRNA expression data from 333 prostate cancer specimens, The Cancer Genome Atlas (TCGA) identified a number of mutations that could stratify prostate cancer into subtypes for potential targeted therapies (Cancer Genome Atlas Research 2015). For example, 19% of prostate cancers had defects in DNA repair genes, and this subset of cancers may respond to PARP inhibitors as suggested by an independent study (Robinson *et al.* 2015). Further, genes that affect MAP kinase or phosphatidylinositol 3-kinase pathways were mutated in roughly 25% of prostate cancers, for which some lesions are predicted to confer sensitivity to particular pathway inhibitors (Dahlman *et al.* 2012, Bowyer *et al.* 2014). The TCGA study also found associations between molecular features and aggressiveness as categorized by the Gleason score, which is the sum of the most dominant and second most dominant Gleason grade patterns seen in a prostate cancer specimen. In addition to confirming a known positive correlation between DNA copy number variation and Gleason score, integration of DNA and RNA features revealed particular clusters of disease that correlate with Gleason score. Although this study ultimately concluded that no single parameter can accurately predict aggressiveness, it does raise the intriguing possibility that integrating several levels of molecular data, including DNA methylation, may provide better biological insights.

In prostate cancer, DNA methylation has been evaluated as a means to identify cancerous tissue and to discern the aggressiveness of tumors. Early work discovered that assaying tumor DNA for methylation of glutathione S-transferase pi 1 (*GSTP1*) promoter can identify prostate cancer (Lin *et al.* 2001, Gonzalgo *et al.* 2004). More recently, multigene panels of DNA methylation are suggested to provide information on aggressiveness (Valdes-Mora & Clark 2015). One limitation of the TCGA work described previously is that it used DNA methylation data from the Infinium Human Methylation 450 Bead Chip array that only assays a small portion of all possible DNA methylation sites in the human genome. Using methyl-CpG-binding domain (MBD)-isolated genome sequencing, a technique which provides more comprehensive coverage of the mappable genome (Serre *et al.* 2010), we more fully explored the relationship between prostate cancer aggressiveness and genome-wide DNA methylation patterns in a cohort consisting of benign prostates, low-grade cancers (exclusively Gleason grade 3) and high-grade cancers (exclusively Gleason grade 4/5) (Bhasin *et al.* 2015). We identified both cancer-specific and high Gleason grade-specific DNA methylation. These differentially methylated regions (DMRs) are observed throughout the genome in both coding and non-coding sequences and prompted us to consider the functions of DNA methylation in the different genomic contexts to help shed light on prostate cancer biology.

DNA methylation

The DNA methylation discussed in this review refers to the covalent addition of a methyl group to the 5th position of cytosine within CpG dinucleoties (Fig. 1). In mice and men, DNA methylation is catalyzed by DNA methyltransferases (DNMTs), where *de novo* activity is largely conferred by DNMT3a and DNMT3b, and maintenance function during DNA replication is carried out by DNMT1 (Bestor 1988, Li *et al.* 1992, Lei *et al.* 1996, Okano *et al.* 1998, Kaneda *et al.* 2004). On the other hand, active erasure of DNA methylation is achieved by ten-eleven translocation (tet) methylcytosine dioxygenases (TETs). TET enzymes convert 5-methylcytosine to 5-hydroxymethylcytosine and subsequently to formyl or carboxyl cytosine, which is excised by thymine-DNA glycosylase (TDG) and repaired with an unmodified cytosine (Tahiliani *et al.* 2009, He *et al.* 2011, Ito *et al.* 2011). Whether the oxidized derivatives of methylcytosine harbor distinct biological functions and the cues that direct these methyltransferases and demethylases to particular genomic regions to regulate DNA methylation are areas of active research.

CpG-methylated DNA has different properties than unmethylated DNA. For example, binding of a wide range of transcription factors, chromatin modifiers and DNA repair enzymes to DNA is sensitive to DNA methylation status (Hu *et al.* 2013). Functionally, this has profound consequences, as DNA methylation status in turn influences transcription and associated processes

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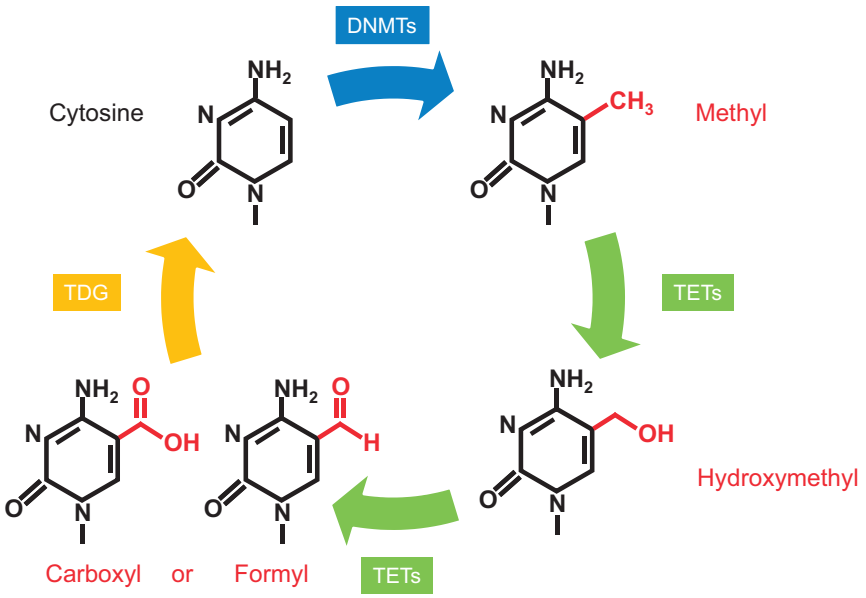

Figure 1
The cycle of DNA methylation addition and removal. A model for how DNA methylation is added and removed from the 5th position of cytosine is presented. Clockwise from cytosine: cytosine is converted to methylcytosine by DNA methyltransferases (DNMTs), and demethylated by successive conversion to hydroxymethylcytosine then formylcytosine or carboxycytosine carried out by ten-eleven translocation (TET) methylcytosine dioxygenases. Then as part of the base excision repair pathway, thymine-DNA glycosylase (TDG) excises formylcytosine or carboxycytosine from the DNA. The DNA is repaired with an unmodified cytosine to complete the demethylation cycle.

such as RNA expression, RNA processing, higher order genome structure and genomic stability. Underscoring the regulatory potential of DNA methylation, different cell types exhibit different genome-wide patterns of DNA methylation within the same tissue, across different tissues and between diseased states and their normal counterparts. With respect to mRNA expression, DNA methylation has been most extensively studied as a mechanism to silence gene promoters. However, DNA methylation is also present throughout the genome, and recent work shows widely varied functions dependent upon the genomic context of DNA methylation (Fig. 2).

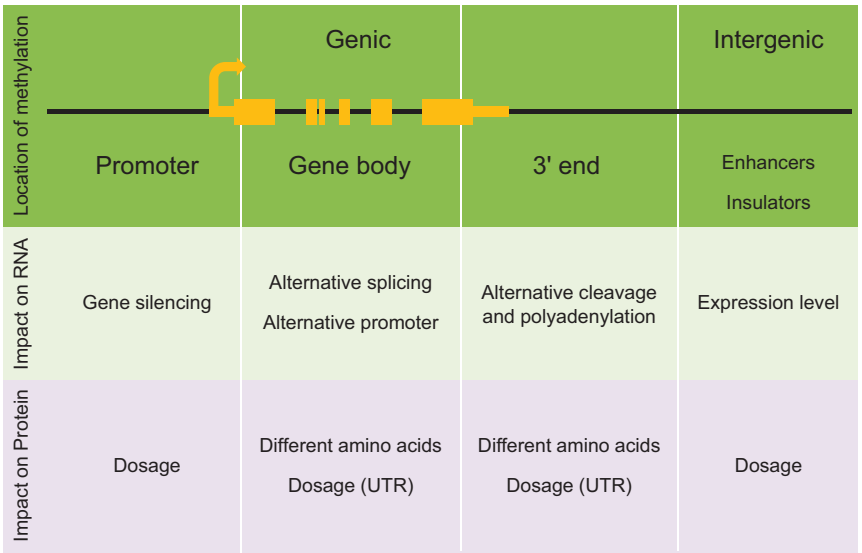

Figure 2
DNA methylation has diverse gene regulatory functions depending upon genomic context. A simplified model of the genome is depicted at the top (green box). The yellow rectangles are exons of a generic mRNA-producing gene, and the black lines between exons are introns. Thin yellow exonic regions correspond to untranslated regions (UTR) of mRNA, and thick yellow exonic regions correspond to protein coding mRNA sequences. The black lines outside of the genic region depict intergenic DNA. Within each genomic context, the first row below indicates known, context-specific functions of DNA methylation on mRNA expression (light green box). The second row below indicates how these effects on mRNA expression translate to altered protein expression (pink box). It is important to note that this figure does not imply evidence exists for each of these mechanisms specifically in prostate cancer. Instead, it serves to summarize known functions of DNA methylation studied in a wide range of biological systems and suggests that prostate cancer-specific DNA methylation found in each of these contexts may function through some or all of these mechanisms.

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For the rest of this review, we will discuss how DNA methylation impacts mRNA expression and pre-mRNA processing in each of these genomic contexts in multiple systems to better highlight the roles that cancer- and high grade-specific DNA methylation may play in prostate cancer biology.

Context-dependent functions of DNA methylation

Promoter methylation

Mammalian gene promoters are DNA sequences that stimulate transcription initiation. In this review, we will focus on gene promoters that produce messenger RNA by recruiting RNA polymerase II. Approximately half of the known mammalian promoters are GC rich and CpG dense relative to the genomic background. This CpG-dense configuration of DNA, known as a CpG island (CGI), helps promote a nucleosome-depleted state at promoters when CGIs are unmethylated (Illingworth & Bird 2009, Deaton & Bird 2011). CGIs and the 2 kilobases flanking CGIs (CGI shores) often contain DNA regulatory elements that can modulate transcription initiation by RNA polymerase II, and the methylation status of these elements can impact the transcriptional activity at these gene promoters.

Although unmethylated CGIs are most often associated with competency to initiate transcription, methylation of promoter CGI can silence the expression of genes. Convincing evidence that methylated CpGs near a transcription start site can directly participate in silencing was provided by site-specific alteration of DNA methylation in native chromatin (Maeder *et al.* 2013) and minimal promoter reporter constructs (Klug & Rehli 2006, Han *et al.* 2013). Promoter silencing by DNA methylation occurs by at least two paradigms. First, many transcription activators are known to preferentially bind to an unmethylated DNA-binding site (Hu *et al.* 2013). For example, methylation of n-Myc DNA-binding sites (E-boxes) within gene promoters limits the recruitment of n-Myc to activate transcription from several genes, including *EGFR* (Perini *et al.* 2005). A second mode of regulation involves increased binding of repressive methyl-CpG-binding domain (MBD) proteins to methylated CGI promoters (Parry & Clarke 2011). MBDs in turn can recruit proteins such as histone deacetylases (HDACs) that remove acetyl groups from histones, which allows for compaction of promoter chromatin and silencing of gene expression.

There are now countless examples of promoter silencing by DNA methylation, including silencing of germ cell- (Maatouk *et al.* 2006), pluripotency- and differentiation-associated genes (Boland *et al.* 2014). Additionally, in most cancer types, aberrant DNA methylation of gene promoters can effectively silence tumor suppressor genes. The list of tumor suppressor genes silenced by promoter DNA methylation is extensive and growing and includes classical tumor suppressor genes such as *RB*, *APC*, *BRCA1* and *MLH1* (Baylin 2005). Whether DNA methylation reinforces a pre-existing silenced state or plays a role in initial silencing remains to be solidified. Experiments involving DNA methylation-mediated inactivation of the X-chromosome (Chaligne & Heard 2014), of tumor suppressor genes in cancer cells (Bachman *et al.* 2003), and of genes during cellular reprogramming (Papp & Plath 2013) suggest that the silenced state occurs first, and DNA methylation serves to reinforce and maintain silencing.

Although repression of promoter CGI is the most well-documented function of DNA methylation in gene expression, it is not the only function of DNA methylation at promoters. Examples of promoter CGI methylation associated with gene activation also exist. In one case, cyclical methylation of the *pS2* gene promoter is associated with transcriptional activation of *pS2* by estrogen receptor alpha (Kangaspeska *et al.* 2008, Metivier *et al.* 2008). In another case, *FOXA2* gene activation is associated with DNA methylation-mediated block of repressor binding to its promoter, and this activation can be suppressed by an inhibitor of DNMT1 or by knockdown of DNMT3b (Bahar Halpern *et al.* 2014).

It is also important to note that the majority of genes exhibiting differential promoter methylation between cellular states do not exhibit differential gene expression (Moarii *et al.* 2015). These changes in promoter methylation could have more complex effects. As promoter state can modulate post-initiation events such as RNA processing (Ji *et al.* 2011), an interesting hypothesis is that promoter methylation can influence downstream steps of gene expression. Although we have a great deal of knowledge about how DNA methylation can silence promoters, there is still much to be learned about how DNA methylation can activate promoters and potentially affect other steps of gene expression.

The list of gene promoters that are found to be methylated in prostate cancer is long and has been extensively reviewed elsewhere (Day & Bianco-Miotto 2013). One classical example is methylation of glutathione

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S-transferase pi 1 (*GSTP1*) promoter, which occurs in the majority of prostate cancer samples (Lin *et al*. 2001). *GSTP1* encodes an enzyme that is involved in detoxification and metabolism of a wide range of carcinogens as well as steroid hormones (Katoh *et al*. 2008). Loss of GSTP1 function in the prostate may allow for increased carcinogenic insult and/or improper hormonal signaling, contributing to prostate cancer progression. Furthermore, *GSTP1* promoter methylation has shown utility for prostate cancer diagnosis and prognostication. As such, it is part of a multigene panel that is now clinically used (ConfirmMDx) on prostate cancer biopsies for diagnosing localized disease.

Gene body methylation

In the last section, we focused on the most well-studied case: promoter DNA methylation and its relationship with gene expression. However, DNA methylation also occurs throughout gene bodies. In contrast to promoter methylation, gene body methylation more often positively correlates with gene expression, but its functions are less characterized (Lister *et al*. 2009, Lou *et al*. 2014). Studies over the last several years have linked gene body DNA methylation to diversification of the transcriptome through mechanisms including alternative transcription start site selection, alternative splicing and alternative polyadenylation site selection. These alternative transcripts can greatly expand the proteome by encoding different polypeptides. Additionally, alternative transcripts can have unique untranslated regions, which can control translatability, stability and/or localization of mRNA. Major goals of gene expression research include how alternative transcript isoforms are generated and the functionality of unique transcript isoforms. In the following sections, we will discuss the current evidence from many systems that support the functional roles for gene body DNA methylation in modulating *alternative transcription start sites*, *alternative pre-mRNA splicing* and *alternative polyadenylation*.

Alternative transcription start sites

Several studies have detected m7G capped 5′ ends of transcripts from many more intragenic regions than would be predicted by known protein and non-coding gene databases (Yamashita *et al*. 2011, Consortium *et al*. 2014). Although some may be 'transcriptional noise', others reflect *bona fide* activity of previously unannotated promoters within gene bodies. Transcription initiations from these cryptic promoters yield mRNA transcripts with different 5′ end sequences. Many unmethylated gene body CGIs had histone H3 lysine 4 trimethylation (H3K4me3) and CAGE tags originating nearby, both of which are features of sequences surrounding transcription start sites (TSSs) (Maunakea *et al*. 2010). Subsequent experiments demonstrated that DNA methylation can influence alternative TSS selection through its influence on gene body CGI promoter activity, presumably via analogous mechanisms detailed for annotated gene promoters (Maunakea *et al*. 2010, Nagarajan *et al*. 2014). Studies of the *SHANK3* gene that is important for neural development and is defective in several neurological syndromes suggest that demethylation drives the upregulation of alternative TSS. When DNA methylation is removed from gene body CGIs in *SHANK3* using 5-aza-2′-deoxycytidine, transcription initiates from these CGIs, creating unique 5′ ends for *SHANK3* transcripts (Maunakea *et al*. 2010). It remains unknown whether these alternative transcripts are actually functional, simply serve to limit the production of full-length transcript or if both occurs. Interestingly, differential DNA methylation across tissues is more prevalent in gene body CGIs than at CGIs near the 5′ end of the genes, suggesting that the influence of DNA methylation on alternative TSS selection may have greater developmental consequences.

Alternative TSS regulation by DNA methylation at *RASSF1* and *APC* has been reported in prostate cancer tissue and shown by treatment of cells with 5-aza-2′-deoxycytidine (Massie *et al*. 2016). Another intriguing example is prostate cancer-specific demethylation of an internal promoter in *MCT2* (Pertega-Gomes *et al*. 2015). Utilization of this alternative promoter alters the 5′ untranslated region of *MCT2* mRNA and leads to MCT2 protein overexpression, which in turn contributes to prostate cancer cell growth.

Alternative pre-mRNA splicing

RNA polymerase II transcripts are initially generated as premature mRNA (pre-mRNA) containing both exons and introns. Introns are then removed, and exons are spliced together by the spliceosome to generate mature mRNA. Not all exons are constitutively included in mature mRNA; instead, exon inclusion is heavily regulated in tissue- and cell type-specific manners (Licatalosi & Darnell 2010). Alternative pre-mRNA splicing (AS) refers to the process of selecting exons that are to be included in a mature mRNA. The vast majority of genes undergo AS, and

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AS is a mechanism for greatly expanding the transcriptome and proteome. RNA factors, DNA factors, chromatin factors and RNA polymerase II elongation rate are known to influence splicing decisions either separately or in combination (Luco *et al*. 2011). Observations of enhanced DNA methylation across mammalian exons have led to the hypothesis that DNA methylation can influence splicing by recruiting either RNA or DNA factors, changing local chromatin, or influencing RNA polymerase II transit (Gelfman *et al*. 2013, Singer *et al*. 2015).

One example of a splicing event regulated by DNA methylation is *CD45* exon 5 inclusion (Shukla *et al*. 2011). Exon 5 of *CD45* encodes a glycosylated extracellular domain important for signaling, and exon 5 is retained in peripheral lymphocytes until late in development. Exon 5 is included in the mature mRNA when a downstream region is occupied by CCCTC-binding factor (CTCF). CTCF binding at this location likely causes RNA polymerase II pausing, and this pausing presumably allows time for recognizing and utilizing the 'weak' cis-acting splicing sequences at exon 5. Importantly, this only occurs when the DNA binding site for CTCF is unmethylated because CTCF binding to DNA is blocked by DNA methylation (Wang *et al*. 2012). Therefore, when this same region is methylated in B cells, CTCF cannot bind resulting in a relatively rapid RNA polymerase II transit and exon 5 exclusion. More recent genomic analyses after this initial observation estimated that about 200 genes had exons regulated by this mechanism (Marina *et al*. 2016).

Another example that links DNA methylation, chromatin state and transcription rate with alternative splicing involves methyl-CpG-binding protein 2 (MECP2), a protein that preferentially binds methylated DNA. Maunakea and coworkers demonstrated that inclusion of certain exons depends on DNA methylation-mediated recruitment of MECP2 and that MECP2 binding correlates with decreased histone acetylation and increased RNA polymerase II occupancy at these exons (Maunakea *et al*. 2013). Likely, MECP2 sets up a chromatin and transcriptional state conducive to exon inclusion. One final example of DNA methylation regulating alternative splicing implicates heterochromatin protein 1 (HP1)-mediated recruitment of a pre-mRNA-binding protein (Yearim *et al*. 2015). Here, DNA methylation promotes histone H3 lysine 9 trimethylation (H3K9me3), which in turn recruits HP1 to exons. HP1 then recruits SRSF3 RNA-binding protein to the pre-mRNA to exclude a nearby exon. Genomic surveys revealed this mechanism to regulate at least 84 exons that are present in genes enriched for cell differentiation processes. Perturbation of RNA polymerase II elongation rate at these loci was not assessed.

Although none has been linked to a DNA methylation-regulated mechanism, alternative splicing is widespread in prostate cancer (Lapuk *et al*. 2014). One clinically relevant example is the loss of the androgen receptor (AR) ligand-binding domain by splicing. This alternative splicing event renders prostate tumors insensitive to drugs that target the AR ligand-binding domain (Guo *et al*. 2009). Since prostate cancer and high grade-specific DMRs occur throughout gene bodies (Bhasin *et al*. 2015), an interesting future direction will be to test if DNA methylation regulates splicing.

Alternative polyadenylation (APA)

In concert with RNA polymerase II transcription termination, 3′ ends of mature mRNA transcripts are generated by cleavage and polyadenylation of pre-mRNA. These RNA processing steps are carried out co-transcriptionally by several protein complexes (Elkon *et al*. 2013). In recent years, it has become appreciated that most mammalian genes encode multiple possible sites of cleavage and polyadenylation. Selection of polyadenylation sites (alternative polyadenylation or APA) is tissue and cell type dependent and contributes to transcript diversity by changing protein coding sequences and/or 3′ untranslated regions (3′ UTRs), which can lead to changes in mRNA stability, translatability or localization (Di Giammartino *et al*. 2011). Similar to splicing, alternative polyadenylation is likely influenced by some combination of RNA factors, DNA factors, chromatin factors and RNA polymerase II elongation rate.

At the *H13* and *Herc3* loci in mouse, gene body DNA methylation has been shown to modulate APA (Wood *et al*. 2008, Cowley *et al*. 2012). *H13* has a retrogene embedded within an intron in the sense orientation, whereas *Herc3* has a retrogene embedded within an intron in the antisense orientation. On one allele, the retrogene's promoter (also encoded in the intron) is silenced by DNA methylation, and the most distal polyadenylation site in the host gene is used, producing full-length *H13* and *Herc3* mRNAs and proteins. On the other allele, the retrogene's promoter is unmethylated, transcription initiates within each host gene and polyadenylation sites upstream of the retrogene are used, generating truncated *H13* and *Herc3* mRNAs and proteins. Although the mechanistic details are yet to be worked out (i.e., whether transcription interference is occurring or differentially methylated DNA is helping to differentially recruit polyadenylation factors), these

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studies clearly show that DNA methylation can influence APA by linking allele-specific DNA methylation with allele-specific APA in the same cell.

Although cases of DNA methylation-regulated APA have not been reported in prostate cancer, APA does occur, and 3′ UTR lengths in select genes, including *RUNX1*, *MYC*, *KLK2* and *KLK3*, could cluster tumors into risk categories (Li *et al*. 2014). As prostate cancer and high grade-specific DMRs occur near 3′ ends of genes (Bhasin *et al*. 2015), determining if DNA methylation regulates alternative polyadenylation could provide novel disease insights.

Distal regulatory elements

It is now widely accepted that DNA elements far away on the linear chromosome can influence gene expression (Heintzman & Ren 2009). Classically, these elements are categorized as enhancers, which can come into close proximity to activate promoters, or insulators, which can keep enhancers away from promoters. Each of these regulatory elements can be regulated by DNA methylation to confer gene expression changes.

Enhancers

Enhancers are thought to loop into close spatial proximity to promoters to help activate transcription through a variety of mechanisms, including but not limited to recruitment of transcriptional activators, RNA polymerase II and chromatin-modifying enzymes (Blackwood & Kadonaga 1998, Shlyueva *et al*. 2014). Enhancer RNA (eRNA) is often produced from the enhancer region by RNA polymerase II, but whether eRNA is essential for enhancer function may be locus dependent. Super-enhancers are proposed to be clusters of enhancers, each of which is required in combination to activate genes, but the requirement that the combinatorial action of all enhancers in the cluster is needed for activation is still under investigation (Pott & Lieb 2015).

Several studies showed that enhancers can be regulated by DNA methylation. One study using The Cancer Genome Atlas datasets found inverse correlations between DNA methylation of enhancers and putative enhancer-regulated gene expression across many cancer types (Yao *et al*. 2015). This included potential for silencing many tumor suppressor genes by cancer-specific enhancer hypermethylation as well as activation of many oncogenes by cancer-specific enhancer hypomethylation. Independently, a comparison of a wide range of normal tissues, cancer tissues and cultured cells suggested that super-enhancer DNA is aberrantly hypomethylated across many cancer types, with potential activating effects on oncogenes such as *MYC* and *RNF43* (Heyn *et al*. 2016). Lastly, intragenic DNA methylation in HCT116 colon cancer cells was shown to silence intronic enhancers, leading to reduced expression of the gene harboring the silenced enhancer (Blattler *et al*. 2014). Although interesting, these studies were largely correlative, and it will be neccessary to emperically test enhancer and super-enhancer regions for the direct regulation of putative target genes and sensitivity to DNA methylation.

In prostate cancer, many enhancers are predicted to change activity (Taberlay *et al*. 2014), including the androgen-responsive enhancers that control the expression of *PSA* (Lawrence *et al*. 2012). Importantly, activity changes in a subset of enhancers correlate with the DNA methylation status over the enhancers (Taberlay *et al*. 2014), and work from our group detected cancer and high grade-specific DMRs at putative enhancers in prostate and prostate cancer (Bhasin *et al*. 2015). Additionally, TSS and CGI DNA methylation are relatively static throughout a prostate cancer, but enhancer DNA methylation is highly variable among different foci within the same cancer (Brocks *et al*. 2014). These observations suggest the intriguing possibility that DNA methylation contributes to the heterogeneity in prostate cancer biology and clonality via enhancer regulation.

Insulators

Insulators are DNA elements that define boundaries of enhancer reach. When functional, insulators keep enhancers from coming into proximity with gene promoters by changing higher order DNA structure. The classical example of insulator regulation by DNA methylation is the imprinting of the *IGF2/H19* region (Soejima & Higashimoto 2013). The paternal allele exhibits methylation that blocks CTCF-mediated insulation between *H19* and *IGF2*, allowing enhancers near *H19* to upregulate *IGF2*. On the maternal allele, CTCF occupies its unmethylated binding site between the genes and insulates *IGF2* from these same enhancers, resulting in the silencing of maternal *IGF2* allele and upregulation of *H19* instead. Loss of *IGF2/H19* imprinting, where both alleles are methylated, leads to the overexpression of IGF2 and IGF2-mediated cellular overgrowth, which are the molecular underpinnings of Beckwith–Wiedemann syndrome and Wilms' tumor (Leick *et al*. 2012).

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The methylation of insulators in cancer cells, including prostate cancer cells, has been shown to correlate with changes in their chromatin configuration (Taberlay *et al*. 2014). Specifically, insulators acquiring DNA methylation in both prostate and breast cancer cell systems exhibited chromatin parameters indicative of insulator silencing. Perhaps the most convincing example to date of DNA methylation-mediated insulator silencing is in gliomas. *IDH*-mutant gliomas acquire DNA methylation at insulator elements that prevent CTCF-mediated insulator function (Flavahan *et al*. 2016). One particular insulator that is silenced ultimately leads to the overexpression of oncogenic *PDGFRA*. The CTCF-binding site within the insulator sequence was mutated using CRISPR in *IDH* WT glioma cells, where this insulator is unmethylated and occupied by CTCF. This perturbation abolished CTCF binding, nullified insulator function and subsequently drove *PDGFRA* overexpression and cell proliferation. In other words, the silencing of CTCF-mediated insulator function by DNA methylation seen in *IDH*-mutant gliomas could be phenocopied by destroying the insulator's unmethylated, occupied CTCF-binding site in *IDH* WT cells.

Summary/future directions

DNA methylation is a versatile modification that is of great interest because genome-wide patterns of DNA methylation are implicated in 'locking in' of cellular fates. Indeed, aberrant methylation patterns seen in virtually every type of cancer, including prostate cancer, are thought to contribute to malignant phenotypes. In addition, aberrant methylation of particular gene subsets can serve as biomarkers. Despite the wealth of information about silencing tumor suppressor gene promoters in prostate cancer, there is much to be learned about DNA methylation in other genomic contexts. Indeed, our own work has identified prostate cancer-specific and high grade-specific DMRs enriched in other genomic compartments, including in gene bodies, and at gene 3′ ends, genic locations where alternative RNA isoform production is regulated via alternative TSS, splicing and 3′ end formation. Also, DMRs are enriched at enhancers that are active in prostate and prostate cancer. As alternative RNA isoform production and regulation of gene expression by distal elements have been shown to occur in prostate cancer cells or tissue, the challenge now is to establish the functional relevance and clinical utility of prostate cancer and high grade-specific DMRs in each genomic context (Fig. 2).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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