DNA and Histone Methylation in Prostate Cancer

Kexin Xu

Abstract As a model of "epigenetic catastrophe", prostate cancer is driven by progressive epigenetic changes that arise early in carcinogenesis and persist throughout disease progression. In this chapter, two common epigenetic modifications, DNA methylation and histone methylation, are reviewed regarding their up-to-date roles in the disease. DNA hypermethylation at certain promoter regions is an early event during prostate tumorigenesis and epigenetically silences tumor suppressor genes. Genome-wide DNA hypomethylation is thought to activate oncogenes and becomes more extensive as the tumors become metastatic and aggressive. Dynamic regulation of histone methylation patterns leads to cancer-specific transcriptional profiles, and histone-modifying enzymes closely crosstalk with critical biological pathways such as the androgen receptor (AR) signaling. The functions and features of these two epigenetic programs make them highly promising as diagnostic and prognostic biomarkers or new therapeutic targets for prostate cancer. However, epigenetic therapy is still in its infancy and imposes a lot of challenging issues such as specificity, toxicity and potency. Therefore, we need to comprehensively understand the epigenetic regulatory mechanisms of prostate cancer development and progression, identify the pharmacodynamics and biomarkers of the epigenetic drugs targeting DNA methylation or histone methylation to better stratify patient populations who will likely benefit from the precision medicine.

Keywords Prostate cancer • Epigenetics • DNA methylation • Histone methylation • AR signaling • Gene expression regulation • Diagnostic/prognostic/predictive biomarkers • Epigenetic therapy

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1 The Prostate and Prostate Cancer

1.1 The Prostate and Prostate Epithelial Cells

The prostate is part of the mammalian reproductive system in males. It is a walnut-sized exocrine gland located in front of the rectum and just below the bladder. The main function of the organ is to discharge a clear, slightly alkaline solution that nourishes and protects sperm cells produced in the testicles [1]. During ejaculation, the muscles of the prostate help to squeeze this fluid into the urethra and expel it, together with sperms and fluids from other glands, as semen. Although the protein content of human prostatic secretions is less than 1%, it contains a very important clinical index for the pathological status of the organ, prostate-specific antigen (PSA). Male hormones, testosterone and predominantly its metabolite dihydrotestosterone (DHT), regulate the normal development, proper function as well as neoplastic transformation of the prostate cells through binding to and activating the nuclear receptor, the androgen receptor (AR).

There are two generic types of cells that form the prostate gland: epithelial and stromal [2]. The epithelial cells line the surfaces of the glandular ducts, and they are exclusively essential for the secretory activity and structural integrity of the gland. It goes without saying that the epithelium compartment is very important to the biology of prostate considering the fact that over 90% of the prostate tumors are adenocarcinomas [3]. Three prominent cell populations have been identified in prostate epithelium, which are the columnar luminal cells, the cuboidal basal cells, and the neuroendocrine (NE) cells [4]. These three cell types are quite distinguished in terms of their morphologies, molecular characteristics, functional significance, and relevance to carcinogenesis. The tall luminal cells are aligned along the inner layer of the prostate ducts and project inwards into the gland lumen. They express high levels of AR, so these cells require androgens for their survival and secret the AR-produced PSA into the fluid. The outer layer of the prostate ducts is lined up by the stretched basal cells that, together with an underlying basement membrane, divide the prostatic glands from the surrounding connective tissues. AR level in basal cells is low and even undetectable, so androgens are not essential for the growth of basal epithelial cells. It is generally believed that within the basal cell layer exist the prostate stem cells, which give rise to the terminally differentiated secretory cells [5]. Neuroendocrine (NE) cells constitute a small portion of cells within prostatic epithelium compartment. They are irregularly and sparsely scattered throughout the basal layer. Little is known about this type of cells, only that they are androgen-independent, non-proliferating and terminally differentiated. The exact origin and physiological function of the NE cells are not completely understood, but it is believed that they may play a role in the differentiation of growing prostate and have been implicated in the development of carcinogenesis. The stromal compartment is mainly composed of smooth muscle cells and also includes fibroblasts, nerves, blood vessels and various infiltrating immune and inflammatory cell types. Crosstalk between prostatic epithelium and the surrounding stroma has a profound effect on prostate organogenesis and development, maintenance of homeostasis of the organ, as well as the evolution of prostatic carcinogenesis and cancer progression [6].

1.2 Pathological Conditions of Prostate

As the largest accessory sex gland in men, the prostate, however, is not required for viability. Still, this organ has elicited great attention from biomedical researchers because of the high occurrence of prostate diseases. There are several common categories in prostate-related disorders: prostatitis, benign prostatic hyperplasia (BPH), prostate intraepithelial neoplasia (PIN), and cancer [7]. Prostatitis is actually inflammation and swelling of the gland, which can be caused by bacterial infection and therefore treated with antibiotics. Prostatitis can happen in men of all ages, and does not have a clear link with an increased risk of prostate malignancy. BPH is a specific term used to describe the condition of an enlarged prostate. It is the most common aging-related prostate problem, which occurs in up to 90% of men older than 80. The symptoms can be relieved by lifestyle management, medications, or surgery that removes part of the prostate. Again, having BPH does not necessarily lead to prostate cancer. PIN, however, is considered as a preliminary step in the development of prostate cancer. In this case, the epithelial cells lining the acini and ducts become abnormally shaped, their nuclei get enlarged and nucleoli darkened. PIN is recognized as a continuum between low-grade (LG) and high-grade (HG) forms according to increasing degrees of abnormality, with high-grade PIN considered as the immediate precursor of early invasive carcinoma. Currently, the only way to detect and diagnose PIN is to use the technique of transrectal ultrasonography-guided biopsy. When HGPIN is identified, follow-up care is necessary. If the lesions are present in multiple areas on the initial and subsequent biopsies, patients may be treated with inhibitors of the enzymes involved in androgen and estrogen metabolism, anti-androgens, or selective estrogen receptor modulators to eliminate HGPIN and to decrease the incidence of prostate cancer.

Carcinoma of prostate is for sure the most deleterious situation of the organ. For decades, prostate cancer has been the most prevalent non-dermatologic type of cancer in men in the Western countries, with death rates second only to lung cancer [8]. According to the American Cancer Society, about 1 man in 7 will be diagnosed with prostate cancer at some point during his lifetime, and this ratio is even higher in men aged 65 years or older, about 6 cases in 10. Every 20 min another American man dies from the disease. In the US, an estimated 180,890 men will be newly diagnosed with prostate cancer in 2016, and approximately 26,120 men will die from it [9]. The only established factors that may increase the risk of developing prostate cancer are age, race/ethnicity and family history. There are other factors that may also influence the risk, which include diet, exposures to endocrine disrupting chemicals, occupation, etc. [10]. Prostate cancer is intimately associated with aging. Statistic reports indicate that prostate cancer affects 1 in 44 males at the ages of 40–59, 1 in 7 males at 60–79 and over half of men over 80 years of age. These data clearly demonstrate that the risk of developing prostate cancer is significantly influenced by age. Racial disparity is another element critical for prostate cancer incidence and mortality. For example, the frequency of prostate carcinoma occurrence is the highest among African-American men and in Caribbean men of African ancestry, while

Asian men living in Asia have the lowest risk. The exact reasons for these ethnic differences are still not well understood, but may involve with the differences in genetic variations, lifestyles, and socioeconomic statuses, etc. About 20% of prostate cancer runs in a family, and a man having a first-degree relative (father or brother) who was diagnosed with the cancer is at least twice as likely to develop the disease other men in general. Studies have found some inherited gene changes, like mutations in the *BRCA1*, *BRCA2* and *HOXB13* genes, but they only account for about 5–10% of all prostate cancer cases [11]. Therefore, besides the shared genetic makeup, the familial prostate cancer may also be inherited due to similar living environment (e.g., diet, lifestyle, carcinogen exposures, etc.). There are still incomplete knowledge and several misconceptions regarding the risk factors for prostate cancer, therefore future work in prostate cancer etiology, especially understanding the gene-environment interaction, is necessary and will help to make more informed health care choices and personalized treatment of the disease.

1.3 Evolving Biology and Treatment of Prostate Cancer

High rates of incidence and mortality of prostate carcinoma lead to great interests and tremendous efforts in both basic research and clinical trials. Nearly every prostate cancer is adenocarcinoma, which starts in the glandular epithelial cells lining the prostate. The tumors display mainly a luminal phenotype as most prostate cancer cells express the steroid hormone receptor AR, which is only present in the luminal layer. It is now widely accepted that androgens-AR axis plays a pivotal role in almost every step of prostate cancer initiation and progression [12]. The male hormone binds to a specific protein module on AR, which is called ligand-binding domain (LBD), and activates the nuclear receptor by promoting its translocation from the cytosol into the nucleus. Once activated, AR binds to target DNA sequences, also known as androgen response elements (AREs), and results in up- or down-regulation of specific gene transcription, which will stimulate proliferation, inhibit apoptosis or maintain dedifferentiation status. Activation of AR signaling is a predominant driving force for the uncontrolled growth of cancerous prostate cells, thus AR-expressing luminal cells are targets of tumorigenic transformation. Prostate cancer is very multi-focal, and different foci of the carcinoma are anatomically distinct. Compared with other epithelial cancers, prostate carcinoma is unique in that it is a relatively slow growing malignancy and follows a multistage process. Finally, metastatic cascade of the tumors may precede clinical detection of indicative parameters and happen even without capsule perforation. All these factors make the behaviors of prostate cancer cells highly unpredictable. The disease is usually detected and monitored by measuring the amount and velocity of serum PSA, which is a secretory prostatic protein circulating in the blood. The quantity of PSA generally rises when prostate cancer occurs, and the upper limit of a normal situation is clinically set at 4.0 ng/ml. Any tumors with PSA levels between 4 and 10 ng/ml are usually considered at intermediate stage, and may or may not need a biopsy. Cases

with PSA concentrations over 10 ng/ml in general indicate the presence of prostate cancer. The other diagnostic method to detect the tumor at its earliest stages is a digital rectal exam (DRE), which looks for any irregularities in size, shape and texture of the organ. If cancer is suspected, follow-up tests will be needed, such as the transrectal ultrasound (TRUS) and prostate biopsy. A stage of the cancer is then determined based on the comprehensive evaluation of all the results from these diagnostic tests, which helps the cancer care team to choose treatment options and to predict a patient's outlook for survival. At early stage, the cancerous epithelial cells are confined to the organ with an intact basement membrane and do not invade the stroma. Active surveillance or watchful waiting is usually recommended for the elderly or those with other serious health problems. If the tumor appears as a large mass, treatment options might include radical prostatectomy (often with removal of the pelvic lymph nodes) and radiation therapy (external beam radiation or brachytherapy or both). As the disease progresses, the tumor extends beyond the prostate capsule and advances to local invasion of surrounding tissues such as seminal vesicle. At this stage, besides the remedies mentioned above, hormone therapy, also called androgen deprivation therapy or androgen suppression therapy, is commonly prescribed, which includes surgical castration (i.e., orchiectomy, a surgery to permanently and irreversibly remove one or both testicles) and chemical castration (i.e., luteinizing hormone-releasing hormone agonists or antagonists, CYP17 inhibitor and anti-androgens, all of which either lower the androgen level or stop the hormone from working). Finally, cancers spread to nearby areas like the bladder, rectum, lymph nodes or even distant organs such as the bones. Unfortunately, no cure is attainable for tumors in this aggressive form, and current treatment merely helps to keep the cancer under control and to improve a man's quality of life. Initially, prostate cancer cells depend on the androgen for their growth and survival, thereby hormone therapy is the most effective way to make prostate tumors shrink or grow slowly when cancer has metastasized beyond the prostate or better effectiveness of radiation therapy is wanted. However, despite the fact that 80–90% of tumors initially respond to the hormone therapy, nearly all patients progress to a more aggressive and lethal form of the disease termed castration resistant prostate cancer (CRPC) with median time to progression of approximately 18–24 months [13]. This means that cancer cells continue to divide perpetually and grow uncontrollable in the presence of castrate levels of testosterone ($\leq$50 ng/dL). Patients with metastatic CRPC retain a guarded prognosis. Without treatment, median survival time ranges from 9.1 to 21.7 months, and most of these patients, if not all, eventually succumb to their disease [14–16]. Over the last two decades, huge advances have been made pertaining to the biology and pathophysiology of CRPC. There have been several models proposed on the causes of CRPC. For instance, AR gene mutations and amplification, which results in altered ligand specificity and increased sensitivity of AR signaling; expression of AR splice variants that lack ligand-binding domain and are constitutively active; aberrant AR reactivation by unbalanced interaction with its co-activators or co-repressors; induction of bypass pathway, which circumvents the AR axis and utilizes other mechanisms to stimulate the proliferation of prostate cancer cell [17]. Better understanding of the disease has

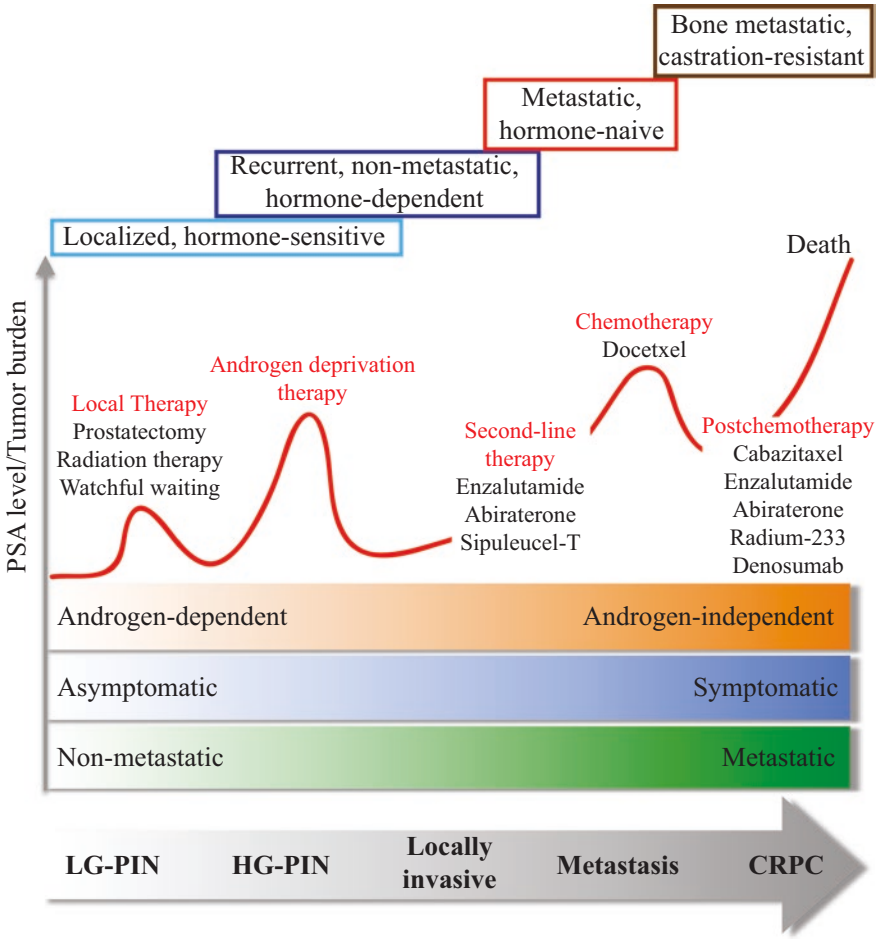

Fig. 1 Prostate cancer progression and treatment options for each stage

enabled the development of new therapeutic modalities including chemotherapy, immunotherapy, novel hormonal and palliative agents, which have gained US FDA approval and significantly improved life expectancy in men with metastatic CRPC (Fig. 1). These innovative treatment options for CRPC include:

(i) Sipuleucel-T. It is a therapeutic vaccine, and generated by first incubating the patient's antigen-presenting cells with a fusion protein consisting of antigen prostatic acid phosphatase and granulocyte-macrophage colony stimulating factor. The activated blood product is then re-infused into the patients and reprograms his immune system to attack the cancer.

(ii) Abiraterone acetate. This is an inhibitor of CYP17A1, the enzyme that catalyzes the synthesis of androgens, and thus decreases circulating levels of the hormone.

(iii) Enzalutamide. The pure anti-androgen is actually a blocker of AR signaling, which inhibits multiple steps along the axis: binding of androgens to AR, AR nuclear translocation, and recruitment of AR to target DNA.
(iv) Cabazitaxel and docetaxel. Both taxane compounds are microtubule inhibitors and thus block the mitotic cellular function, which leads to apoptosis. Cabazitaxel is a dimethyloxy derivative of docetaxel, and is superior to its predecessor because of lower substrate affinity for the drug efflux pump and ability to cross the blood-brain barrier. Therefore, cabazitaxel is the drug of choice in patients with docetaxel-refractory metastatic prostate cancer.
(v) Denosumab. As one of the bone-targeting agents in the management of CRPC, denosumab acts to prevent the maturation of osteoclast cells that break down bone tissues. Receptor activator of nuclear factor-κB (RANK) plays a critical role in osteoclast formation, and it is activated by its specific ligand RANKL. Denosumab is the human monoclonal antibody of RANKL, so it binds to RANKL and blocks the RANK signaling pathway.
(vi) Radium-223. This is another FDA-approved drug that is prescribed to prevent pain and fractures in CRPC patients with bone metastases. It is a "calcium mimetic" radioactive isotope, which means that it accumulates preferentially in areas where bone metastases are forming and emits low levels of α-particle radiation there to cause double-strand DNA breaks and kill cells.

All these new therapies have shown significant clinical improvement in men with metastatic CRPC, however prostate cancer remains the second leading cause of cancer death in American men. Further advances in prostate cancer research require definite mechanistic and molecular analyses, and the most overarching challenges in terms of clinical management include: (1) identification of prognostic markers that distinguish fatal from indolent prostate cancer; (2) exploration of mechanisms that lead to castration resistance; (3) development of strategies to enhance the well-being of men with prostate cancer; (4) recognition of new markers more sensitive and specific than PSA for prostate cancer detection. These studies will facilitate better diagnosis of primary tumors, lead to the development of novel cancer therapies, and improve quality of life for prostate cancer survivors.

2 Genetics and Epigenetics in Prostate Cancer

Like most cancers, prostate cancer is driven by genetic and non-genetic causes. Modern genetic and genome-based technologies have enabled the discoveries of somatic alterations and germline variations, which drive malignant transformation and progression of prostate cancer. Common genetic changes with well-defined roles in the disease include loss of heterozygosity (LOH) of *TP53* (in 10–20% of primary and up to 42% of advanced prostate cancer) [18] and *PTEN* (in approximately 27% of localized and 60% of metastatic tumors) genes [19], fusion of ETS transcription factor genes with androgen-responsive *TMPRSS2* promoter (in about half of prostate

cancer) [20], mutations of *AR* gene (in less than 2% of untreated localized prostate cancer and up to 50% of metastatic hormone-refractory tumors) [21], and mutations of *SPOP* gene encoding the substrate-binding component of a cullin-RING-based ubiquitin ligase complex (in 6–15% of prostate cancer) [22, 23], etc. However, even with all these mutation hotspots in prostate cancer, some cases of prostate tumorigenesis still cannot be explained by definitive driving genomic events. As a consequence of divergent clonal evolution of the disease, the constellation of genetic mutations in prostate cancer can be quite heterogeneous, and many identified mutation types have low levels of recurrence. So genetic change is not the sole contributory factor to the origins of prostate cancer, and it is quite likely that other biological events precede and enforce the malignant transformation of the cells. Epigenetic alteration is one of the candidates for such early events.

Epigenetics refers to any biological process that acts upon the chromatin but does not affect the actual DNA sequences in order to modulate gene expression and subsequently control cell fate [24]. The topics that are covered in the epigenetic study have expanded rapidly, and now include DNA methylation, histone modifications, chromatin remodeling and non-coding RNA processing. A specific epigenetic pattern is highly susceptible to environmental stimuli such as dietary components and life style, hence it undergoes a real-time change upon the stimulation of the external factors and induces biological signaling cascade as an early response. It has been shown that numerous epigenetic alterations appear to be highly recurrent, and sometimes nearly universal, in prostate cancer. These alterations can affect thousands of loci across the cancer genome, reinforcing the establishment of a new transcriptional profile that favors self-renewal, survival, and invasion of prostate cancer cells. It has been demonstrated that accumulation of epigenetic aberrations eventually creates genetic or genomic instability. On the other hand, several genes encoding the enzymes for shaping the epigenetic landscape are found mutated in prostate cancer. Therefore, acquired/inherited genetic mutations and epigenetic aberrations contribute individually and cooperatively to the pathogenesis and progression of prostate cancer. In this chapter, we will mainly focus on two of the most broadly studied epigenetic modifications, DNA methylation and histone methylation. We will not only give a review of the most updated functions of these two epigenetic programs in prostate cancer, but also discuss the prospects for targeting either one of these two marks to better diagnose and treat the disease.

3 Prostate Cancer and DNA Methylation

3.1 DNA Methylation and Demethylation

DNA methylation is one of the critical epigenetic regulatory mechanisms to control gene expression. The reaction results in the addition of a methyl (−CH3) group to the 5′-carbon position of the cytosine ring (5mC). In mammals, DNA methylation predominantly occurs in the context of CpG dinucleotide (5′-Cytosine-phosphate-Guanine-3′),

and approximately 60–90% of all CpGs are methylated. However, this dinucleotide is found in only 1% of human genome, less than one-quarter of the expected frequency due to the spontaneous deamination of the methylcytosines to thymines. It has been extensively documented that DNA methylation is used as an epigenetic mark for gene silencing, and several models have been proposed to explain the molecular mechanisms [25]. The modification directly retrains the binding of transcription factors to the methylated recognition elements, or it specifically attracts proteins containing a methylated-DNA binding domain (MBD) so that the preoccupied chromatin region is no longer accessible to factors required for gene induction. Besides, methylated DNA establishes a repressive and closed chromatin structure, as suggested by the evidence that methylated chromatin is insensitive to nuclease digestion and histone proteins assembled on it are significantly less acetylated. Finally, a *cis*-acting theory showed evidence that transcriptional repression does not require methylation of promoter sequences but is dependent on the position, length, or density of methylated cytosine residues. All these mechanisms of action indicate how critical and complex DNA methylation can be in terms of gene expression regulation, thus this epigenetic program must be precisely controlled. This covalent chemical modification is catalyzed by DNA methyltransferases (DNMTs), of which 3 active members (DNMT1, DNMT3A and DNMT3B) have been identified [26]. DNMT1 is the first DNA methyltransferase to be discovered and also the most abundant one in all adult human tissues. It is mainly responsible for maintaining DNA methylation patterns after DNA replication, when the parent DNA strand remains methylated while the daughter strand is not. So DNMT1 binds to CpG sites on DNA with only one strand modified, so-called hemi-methylated DNA, and methylates the cytosine on the newly synthesized strand. In contrast, both DNMT3A and B are de novo DNA methyltransferases, which means that they bind with equal affinity to hemi-methylated and non-methylated DNA and that they catalyze DNA methylation from the beginning after embryo implantation. Of course, the maintenance versus de novo function of these enzymes is not absolute, and DNMTs can fulfill the role as one or the other when their levels are modulated. Removal of methyl group from DNA is a more complicated process compared with its methylation, as there are no single enzymes directly catalyzing the reaction [27]. DNA demethylation can be achieved as a passive process simply due to the loss of methylation on daughter strand after several rounds of DNA replication, or it takes place actively by replication-independent processes. Unlike in plants where firm evidence has been identified that direct excision of the methyl group is accomplished by a subfamily of DNA glycosylases specific to 5mC, the active demethylation pathways in animal cells are hotly contested and proposed to involve various mechanisms, none of which have been conclusively proven. So far, accumulating data has suggested an affirmative role for base excision repair (BER) in active demethylation in mammals, which is initiated by either direct excision of 5mC in a locus specific manner or deamination of 5mC to thymine resulting in T-G mismatch. In another hypothetical theory, entire DNA patch containing the methylated CpG sites is removed, and the bulky lesions are then filled with unmodified nucleotides by nucleotide excision repair (NER). Recently, the discovery of Ten-eleven

translocation (TET) family proteins opened up a new mechanistic route for DNA demethylation. Three members, TET1–3, have been currently identified, and all are oxygenases that catalyze the oxidation of 5mC to 5-hydroxymethyl cytosine (5hmC), then 5-formylcytosine (5fC) and finally 5-carboxylcytosine (5caC). TET-mediated removal of DNA methylation could be achieved by several ways: first, DNMT1 does not recognize 5hmC, thus the newly synthesized DNA would not be methylated so that the patterns of methylation will be diluted after several rounds of replication passively; second, BER DNA repair pathway may be activated to process the lesions that are introduced by either a 5hmC-specific or, after deamination of 5hmC to 5hmU, a 5hmU-specific glycosylase; third, the oxidative derivatives of 5hmC (5fC and 5caC) can be ultimately replaced with unmodified cytosine by a decarboxylation reaction similar to the thymidine salvage pathway. Altogether, the whole system for DNA methylation and demethylation cycling is sophisticated, which implies far-reaching effects of these epigenetic programs on the modulation of local and global chromatin structure (Fig. 2). Therefore, any step in these processes going awry may lead to deranged biological conditions, such as genomic imprinting-related diseases, psychiatric disorders and cancers.

3.2 DNA Hypermethylation in Prostate Cancer

Many human diseases, cancers in particular, are found to be associated with aberrant DNA methylation patterns, either globally or locus specifically. One of the common hallmarks in all human malignant neoplasias is the CpG island hypermethylation. By the most updated definition, CpGs are short stretches of DNA that are longer than 500 base pairs in length and have a GC content greater than 55% with an observe-to-expected CpG ratio of at least 65% [28]. In human genome, there are about 29,000 such regions, which occur at or near up to 70% of annotated gene promoters. In normal cells, most promoter CpG islands are unmethylated. However, when cells become transformed or malignant, hypermethylation of certain CpG islands occurs resulting in inappropriate transcriptional repression. This observation has been described in almost every tumor type, including prostate cancer. Although most of the target genes that are inactivated by CpG hypermethylation are supposed to act as tumor suppressors, unique sets of genes with dynamic biological functions are affected when comparing different cancer types. In prostate carcinoma, over 40 genes have been reported to be silenced by hypermethylation, and this number is still increasing probably due to the development of more

Fig. 2 (continued) homocysteine (SAH) and methylated cytosine (5mC). The transferred methyl group is circled. (**B**) DNA demethyation can be achieved by passive demethylation mechanism (*upper panel*) or active demethylation mechanism (*lower panel*). Passive demethylation happens during DNA replication, and the modified cytosines are either missed (5mC) or not recoganized (5hmC) by DNMTs. Active demethylation takes place through nucleotide excision repair (NER) pathway or TETs-involved base excision (BER) pathway

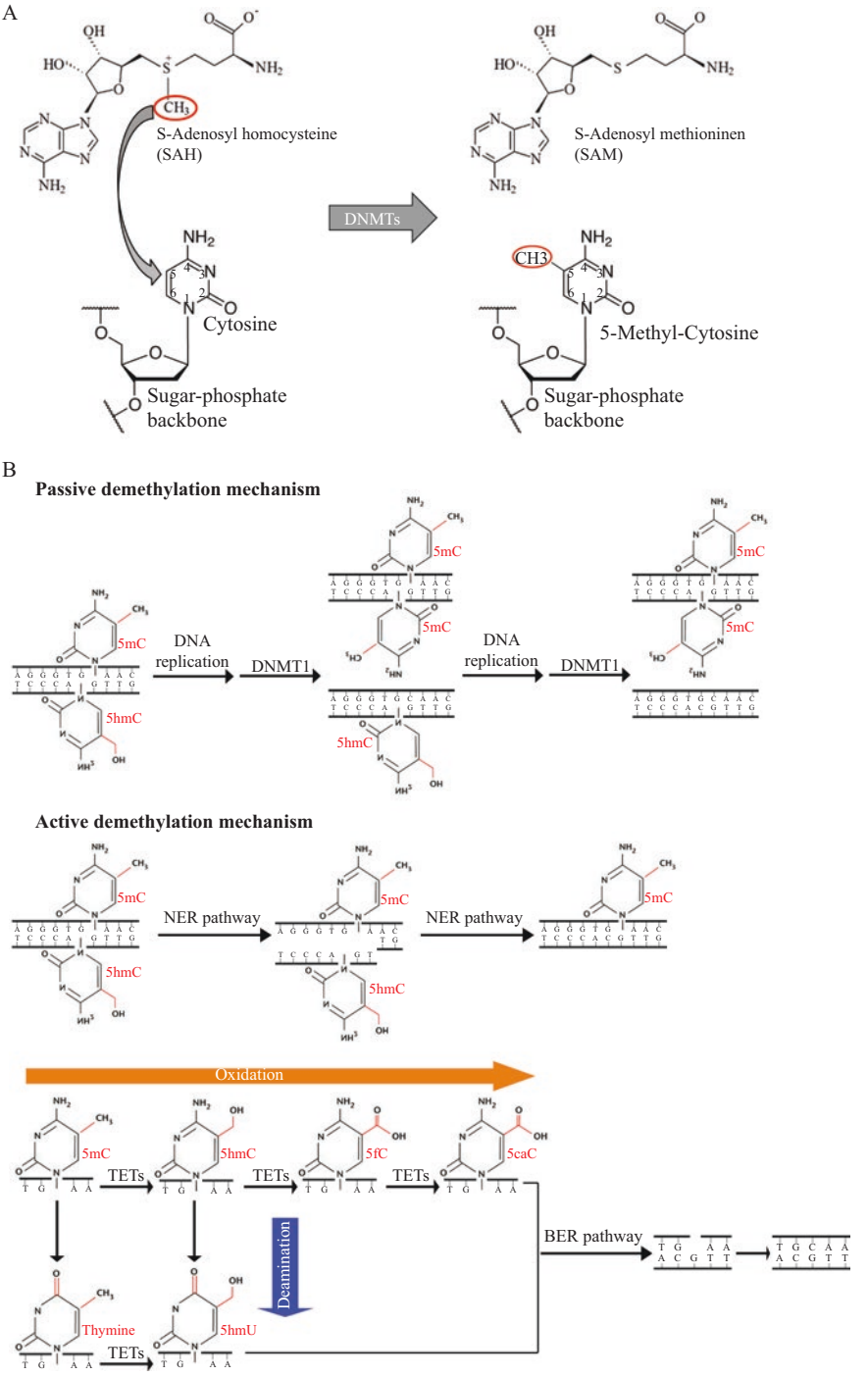

Fig. 2 DNA methylation and demethylation reactions. (**A**) DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which transfers the methyl (−CH3) group from S-Adenosyl-methioninen (SAM) to the 5′-carbon position of the cytosine ring. The final products are S-Adenosyl

Table 1 Hypermethylated and hypomethylated genes in prostate cancer

Categories	Genes
DNA hypermethylation	
DNA repair genes	*GSTP1, MGMT, GPX3, hMLH1*
Hormone signaling genes	*AR, ESRα, ESRβ, RARβ, PR-α, PR-β*
Cell invasion/adhesion genes	*CDH1, CDH13, CD44, LAMA3, LAMB3, LAMC2, TIMP3, S100A2, TIG1, THBS1*
Cell-cycle genes	*CCND2, CDKN1B, RASSF1, CDKN2α, RB1, CDKN1A, CDKN1B*
Apoptotic genes	*GADD45α, PYCARD, RPRM, GLIPR1, DAPK, TNRFSF6, TNRFSR10C, CRBP1, FHIT*
Cell signaling genes	*14–3-3σ, CAV1, APC, PTEN, PTGS1, PTGS2, MDR1, EDNRB, DAB2IP, VEGFR1, HIC1, RUNX3*
DNA hypomethylation	
Gene-locus-specific	*CAGE, HPSE, PLAU, CYP1B1*

sensitive detection technologies. Some representative genes will be discussed in the following section, because their methylation is relatively prevalent in prostate cancer and they involve in a number of pivotal cellular pathways such as hormonal response, tumor cell invasion/metastasis, cell cycle control, apoptosis, and DNA damage repair. A comprehensive list of the methylated genes in prostate cancer is summarized in Table 1. Interestingly, classical tumor suppressor genes, such as *PTEN*, *RB1* and *TP53*, are rarely methylated at their promoter regions in prostate cancer, although genetic alterations like loss of heterozygosity and point mutations are detected in advanced stage cases [29].

As described above, hormones and their corresponding nuclear receptors play significant roles in carcinogenesis and progression of prostate cancer. AR activity is particularly critical for nearly every stage of the cancer growth, from the initiation to the androgen-dependent state till the metastatic, castration resistant status. However, loss of AR protein expression has been seen in as many as 20–30% of androgen-independent tumors, and this is attributed to epigenetic silencing partly by promoter hypermethylation [30–32]. It is reported that the incidence of methylation-mediated AR inactivation ranged from 0%–20% in untreated primary cancer to 13–28% in CRPC tissues. Although the frequency of AR promoter methylation in general appears to be low in prostate cancer and varies from case to case, this type of epigenetic regulation seems to be more prevalent in CRPC than in primary tumor tissues. It is highly clinical relevant to identify this AR-negative subgroup of prostate cancer, and implication of DNA methylation in mediating the downregulation of AR expression will have a profound effect on the treatment regimens for the metastatic, hormone-refractory prostate cancer.

Besides *AR*, other members of the steroid/thyroid hormone receptor superfamily have also been identified as having promoter hypermethylation in some studies of prostate cancer samples. For instance, both estrogen receptors genes, *ESR1* and *ESR2*, which encode two different forms of the receptor ERα and ERβ respectively, are frequently methylated in prostate cancer. Frequencies of *ESR1* methylation are

diverse from 19% to 95% and ESR2 from 65% to 83% in prostate cancer [33–35]. However, the findings on the expression of ERα and ERβ in prostate cancer have been very conflicting [36], especially for ERα levels, although downregulation of both ERs in prostate tumor tissues has been documented in some studies and promoter hypermethylation is the primary mechanism responsible for this transcriptional inactivation [35]. Some evidence showed that higher methylation levels of the ER genes, particularly at some CpG sites, were detected in high-grade and CRPC cancer samples than in low-grade and BPH tissues [34, 35], but it also appears unlikely that alterations in the expression of either ER are associated with the progression of prostate cancer [37, 38]. Therefore, it is still very controversial and remains to be established as for the biological significance of DNA hypermethylation of both ER genes in prostate cancer. One thing we can have certainty about, however, is that DNA methylation is the main reason for gene silencing in any clinical cases when lost or decreased ER expression was noticed. Retinoic acid receptor β (RARβ) is another nuclear receptor that shows abnormal CpG island methylation patterns in prostate cancer, especially in the second promoter region of the gene (*RARβ2*). *RARβ2* methylation varies greatly across studies, for example 0–23% of normal and BPH tissues, 20–94.7% of PIN and 40–97.5% of primary prostate cancer [39–41], and it appears to happen in early stage of prostate cancer, suggesting a role in cancer initiation. There is no clear association between *RARβ2* methylation and pathological stage or Gleason score of prostate cancer [42–44].

DNA damage response (DDR) is an exquisite proofreading mechanism that repairs DNA lesions and prevents the duplication of these errors into daughter cells. Misregulation of DDR pathways leads to the deleterious genomic instability, which is a universal characteristic of cancer cells, and therefore is a major driver for carcinogenesis. So far, two genes that are involved in DNA damage repair have been reported to be hypermethylated in prostate cancer, one is the detoxifier gene glutathione S-transferase Pi (*GSTP1*) and the other is the DNA alkyl-repair gene O^6-methylguanine DNA methyltransferase (*MGMT*). GSTs are a family of metabolic enzymes that catalyze the conjugation of hydrophobic and electrophilic compounds with reduced glutathione for the purpose of detoxification. Thus, inactivation of GST proteins may lead to cell vulnerability to genotoxic foreign compounds and accumulation of DNA base adducts. Indeed, some evidence suggests that mutations or polymorphisms of GST genes can influence BER capacity and subsequent DNA stability, suggesting a potential role for these proteins in DNA damage processing. CpG island hypermethylation of *GSTP1* gene is one of the most common molecular alterations detected in prostate adenocarcinoma. This epigenetic aberrancy is absent or at very low level in nonmalignant prostate tissues, but present in 50–70% of PIN and in nearly all prostate cancers at different stages [45]. Recently, emerging evidence suggests that the extent of *GSTP1* promoter methylation is also positively correlated with the risk of recurrence in prostate cancer patients with early disease [46]. MGMT is one of the few proteins functioning in direct reversal (DR) DNA repair pathway. It transfers the methyl group from O^6-methylguanine to a nucleophilic cysteine residue in its active site. O^6-methylguanine base pairs with thymine instead of cytosine and thus is the major carcinogenic lesion in DNA. The reaction

is irreversible, so the modified cysteine cannot be regenerated and the alkylated MGMT protein is degraded after the direct DNA repair. Results about the association between the status of MGMT methylation and prostate cancer have been inconsistent. Some studies reported low frequency of MGMT promoter methylation in 0–2% of prostate cancer tissues, while others observed moderate to high prevalence of this event in 19–76% of tumor samples [40, 47–49]. This discrepancy may come from technical issues, e.g., various assays used for quantifying methylation levels and different tissue processing methods, so further work or meta-analysis will be needed to resolve the inconsistent results.

Cell proliferation and programmed cell death are two coupling processes that determine the destiny of a cell to either live or die, so deregulation of the balance between cell cycle progression and apoptosis leads to pathological conditions including cancer. *CDKN2A* ($p16^{INK4a}$) is one of the cyclin-dependent kinase inhibitors (CDKIs) and a well-characterized tumor suppressor. Besides genetic changes such as deletion and point mutation, *CDKN2A* is inactivated by DNA hypermethylation in many tumor tissues including prostate [50]. This feature makes the gene unique because other CDKIs, such as *CDKN2B*, *CDKN1A* and *CDKN1B*, are rarely methylated in prostate tumors. However, the frequency of *CDKN2A* promoter methylation varies in prostate tumors across studies, ranging from 0% to 16%, and it appears to be indiscriminate between benign and malignant cases [51]. Interestingly, several reports indicate methylation at exon 2 of *CDKN2A*, which is present in more tumors (73%) relative to normal tissues [52]. Although there was no apparent association between the expression level of *CDKN2A* gene and the extent of its exon 2 methylation, it is plausible that this epigenetic modification may serve as a biomarker for early detection of prostate carcinoma. Another well-known tumor suppressor gene that is frequently silenced by promoter hypermethylation in prostate cancer is RAS association domain family protein 1 isoform A (*RASSF1A*). RASSF1A exerts its tumor suppressive functions by modulating microtubule stability, inducing cell cycle arrest and apoptosis. CpG islands within promoter region of *RASSF1A* gene are highly methylated in a wide range of cancers, and up to 99% of prostate tumors show this epigenetic alteration [53, 54]. In normal epithelial cells and benign prostate tissues, *RASSF1A* promoter methylation is detected in 0–40% of samples, and it also occurs in 64% of PIN [55]. In addition, the relative frequency of methylation is higher in more aggressive tumors with higher Gleason scores compared with less malignant tumors. All these findings suggest that *RASSF1A* promoter methylation may be a common event during prostate carcinogenesis and progression, and hence it can be utilized for the early detection and prognosis prediction of prostate cancer. Many other cell cycle regulators, for example *CCND2* and *SFN*, and apoptosis genes such as *DAPK* and *TNFRSF10C*, have also been found to be aberrantly hypermethylated at their promoter regions in prostate cancer [56].

Most prostate cancer-related deaths are caused by the metastasis of the original tumor cells. The process of tumor invasion and metastasis entails a series of sequential events, including the penetration of original cancer cells into surrounding tissues, spreading to distant organs through the circulatory system, and finally seeding secondary tumors in distinct target locations. During this metastatic cascade, cell

adhesion molecules (CAMs) play important roles in cell-cell and cell-matrix interaction. Therefore, misregulation of CAMs expression is often observed in many human cancers, including prostate. E-cadherin, encoded by *CDH1* gene, is a CAM that distributes at the epithelial cell junctions and mediates cell-cell adhesion. In E-cadherin-negative prostate cancer cell lines, the CpG islands in the promoter of *CDH1* gene are densely methylated, which suggests that epigenetic alteration in DNA methylation contributes to the decreased or loss of E-cadherin expression [57]. Hypermethylation of *CDH1* gene has been detected in 0–77% of prostate tumors, and the overall methylation frequencies are higher in advanced prostate tumors compared with early stage samples [58]. However, several studies reported contradictory results regarding the methylation status of *CDH1* gene in prostate cancer. In two such studies, promoter region of *CDH1* was found no methylation signals in either primary or metastatic prostate tumor samples [47, 59]. In the other, unmethylated *CDH1* gene was detected in metastatic prostate cancer cells in bone, which was significantly associated with the concurrent expression of E-cadherin protein [60]. It is currently unclear why discrepancies were observed in different cases, but epigenetic alteration in promoter methylation appears to be the main explanation for E-cadherin transcriptional inactivation in prostate cancer, rather than *CDH1* gene mutations which lead to loss of E-cadherin function in other cancer types like gastric and breast [61, 62]. Adenomatous polyposis coli (*APC*) gene is also an important molecule that helps control the movement of a cell within or away from a tissue. It associates with the WNT/β-catenin signaling pathway and negatively regulates β-catenin protein stability and interaction with E-cadherin, which is a critical step in cell-cell adhesion. Mutations in *CTNNB1*, the gene encoding β-catenin protein, or truncation in *APC* have been detected in colon cancer and melanoma, which increases the stability of β-catenin. However, these genetic alterations are relatively rare in prostate cancer. In contrast, *APC* gene is commonly methylated at its promoter region, with a prevalence of 27–100% in prostate cancer samples but only 5–6% in noncancerous tissues [63, 64]. Multiple analyses also demonstrated that hypermethylation in *APC* gene is significantly associated with progression of prostate cancer [65, 66], and more frequent in patients who experienced biochemical recurrence, metastasis or death [64, 67]. Many additional genes with critical functions in tumor invasion and metastasis have been reported to undergo methylation-mediated inactivation in prostate cancer, including the cell-surface glycoprotein (*CD44*), H-cadherin (*CDH13*), the scaffolding protein on the caveolae plasma membrane caveolin-1 (*CAV1*), tissue inhibitors of matrix metalloproteinases (*TIMP-2* and *-3*), etc. [68].

3.3 DNA Hypomethylation in Prostate Cancer

Although DNA hypermethylaion has been focused as an important mechanism for inactivation of tumor suppressor genes in prostate cancer, demethylation of normally methylated genomic regions, also known as DNA hypomethylation, is shown

to associate with prostate cancer development and progression as well. In contrast to DNA hypermethylation that usually occurs at specific regulatory sites of specific individual genes, loss of DNA methylation modification seems to be a genome-wide phenomenon. It predominantly occurs in the intergenic and intronic genomic areas, particularly at repeated sequences including the heterochromatic satellite DNA and interspersed transposable elements. It is postulated that DNA hypomethylation induces genomic instability and mutation events, thus contributing to oncogenesis and cancer progression. For example, aberrations on chromosome 8 were strongly correlated with the presence of hypomethylation in prostate cancer, and such genetic and epigenetic alterations tended to be more frequent in higher-stage tumors [69]. In prostate adenocarcinoma, methylation signals at repetitive DNA elements were dramatically decreased from normal prostate to PIN to cancer [70]. In another study, primary prostate cancer cells from up to 96.7% of patients exhibited dramatic decrease in overall 5mC levels compared with the paired benign and normal sections from the same patient. Interestingly, partial gain of methylation was observed in men with recurrent disease [71]. These results, together with many others, suggest that overall reduction of genomic methylcytosine content appears to occur early in prostate carcinogenesis. Global hypomethylation is thus hypothesized to precede temporally the promoter CpG island hypermethylation that later leads to aberrant silencing of specific tumor suppressor genes critical for cancer progression. However, there is emerging evidence that diffuse genomic hypomethylation in prostate cancer may not adhere to this generalized model. An early report showed that the overall DNA methylation levels were particularly lower in metastatic, androgen-refractory prostate tumors, while the 5mC content in non-metastatic prostate tumors was essentially comparable to that in normal tissues [72]. Similar conclusion was obtained when methylation of repetitive sequences like *LINE-1* retrotransposons was found diminished in 49% of prostate cancer and this hypomethylation was more pronounced in high stage and lymph-node positive tumors [73]. In the same study, hypermethylation at specific genes such as *GSTP1*, *RARB2* and *APC*, however, was neither related to tumor stage nor Gleason score. In an independent report, decreased *LINE-1* methylation was again detected in the primary prostate cancer compared with normal tissues, but the degree of reduction was more dramatic in metastatic prostate cancer. In addition, the overall genomic 5mC content was reduced only in metastatic but not primary cancer or tumors adjacent PIN/normal tissues [74]. All these findings suggest that global DNA hypomethylation may actually occur later than hypermethylation changes and play an important role in prostate cancer progression rather than initiation.

Compared with the focal hypermethylation of CpG islands containing promoters, demethylation of individual genes is much less documented in terms of its role in the initiation and progression of cancers. This type of epigenetic alteration was often ignored because localized DNA hypomethylation seems to be much less frequent in cancer and some theory suggests that specific regional demethylation may occur as a consequence of being swept by the large genomic hypomethylation [75]. Even so, a number of single copy genes have been reported to be derepressed in prostate cancer by the epigenetic mechanism of DNA hypomethylation. For instance, the *PLAU* gene, which promotes extracellular matrix tissue degradation

and cell migration, is highly expressed in most prostate cancer tissues, particularly in the invasive ones [76]. Overexpression of *PLAU* is partly attributed to the unmethylated status of the CpG islands proximal to its transcription start site, which was noticeable in nearly all prostate cancer samples but rare in non-neoplastic tissues or BPH samples. Most interestingly, disruption of the demethylation condition at *PLAU* gene promoter induced higher invasive capacity of prostate cancer cells and larger xenograft prostate tumor volumes in vivo [77, 78]. One unique group of genes with regard to their methylation status in cancers is the cancer/testis antigen (*CTA*), since many of the gene members are hypomethylated in several types of cancers including prostate. As their name indicates, *CTA* genes are typically expressed in germ cells of the testis and most cancers but absent in any other normal tissues. It is well known that this exclusive expression pattern of CTAs is highly correlated with the extent of DNA methylation at their promoters [75]. In prostate carcinoma, a large fraction of *CTA* genes, especially those in the X chromosome-associated subfamily, showed CpG islands hypomethylation. More than that, some report claimed that significant DNA hypomethylation of these genes occurred only in metastatic prostate cancer [74]. Other work showed similar results that some representative *CTA* genes were highly methylated in more than 90% of primary cancer specimens, but severely unmethylated in castration resistant samples [79]. Recently, partial hypomethylation was observed in prostate cancer tissues at the promoter of *XIST* gene, which is transcribed into a non-coding RNA acting as a major effector of the X-chromosome inactivation in females [80]. Although the association between the degree of methylation and transcription of *XIST* gene was not clearly established, it is a perfect example to demonstrate the universal presence of DNA hypomethylation, affecting repeat and unique sequences at specific loci that encode proteins or not. Other hypomethylated genes in prostate cancer include *WNT5A*, *CRIP1*, *S100P*, *CYB1B1* and *HPSE*, etc., overexpression of which have all been implicated in prostate cancer progression [81–83]. Taken together, DNA methylation, both hypo- and hypermethylation, is a critical mechanism that cancer cells adapt to regulate gene expression so as to drive prostate cancer development and progression.

4 Prostate Cancer and Histone Methylation

4.1 Proteins in Regulation of Histone Methylation

Histones are the chief protein components of nucleosome, the basic structural unit of chromatin. They are highly alkaline and positively charged, so they closely associate with DNA, which is negatively charged instead, through a series of electrostatic interactions including hydrogen bonds and salt bridges. Five major families of histone proteins exist: H1/5, H2A, H2B, H3 and H4. Histone H2A, H2B, H3 and H4 are known as the core histones, so two copies of each core protein assemble in an octamerous complex, with which 146–147 base pairs of DNA wrap around in a superhelical manner. This core particle is bound by the linker histones, H1 (or H5 in

avian species), at the entry and exit sites of the DNA, thus locking the DNA into place and organizing nucleosome chains into higher order structures. Interaction between histones and DNA governs the chromatin structure and thus exerts a tremendous amount of influence on gene expression. There are several regulatory mechanisms controlling the dynamic changes in this histone-DNA interaction, one of which is the post-translational modifications (PTMs) on the histone protein tails. Histone proteins feature two structurally and functionally distinct domains: the central globular domain that allows heterodimeric interactions between core histones or mediates the protection of linker DNA, and unstructured terminal tails of various length, on which specific amino acids are subject to various covalent modifications, including acetylation, phosphorylation, ADP-ribosylation, ubiquitination and methylation, etc. These enzyme-assisted modifications primarily occur at N-terminal tails of the histones. They can affect the charge properties of the histone, and thus loosen or tighten the condensed DNA that is wrapped around histones. Such modifications can also recruit other proteins specifically recognizing the modified residues, which act to alter the chromatin structure so that it becomes more closed or more accessible.

Histone methylation is a biochemical reaction by which methyl groups are transferred to specific residues on histone proteins. It can happen on all three basic amino acids: arginine (R), lysine (K) and histidine (H), although lysines on tails of histone H3 and H4 are most commonly targeted, whereas only monomethylation of histidine has been described and it is rarely observed [84, 85]. Because the addition of the methyl group leaves the charge of lysine or arginine intact, methylation of histones can be associated with either transcriptional repression or activation, depending on the specific modified residues in the histones and also the numbers of methyl groups attached. Arginine is able to be either mono- or dimethylated. When it is dimethylated, these two methyls can be added asymmetrically on the same free NH2 group or symmetrically with one on NH2 and one on NH2+ group. Even though the similar reactions end up with molecules in the same chemical formulas, these two types of dimethylation are catalyzed by two different subfamilies of enzymes. Lysine can accept up to three methyl moieties replacing each hydrogen of its NH3+ group. Site-specific methylation is catalyzed by histone modifying enzymes called the histone methyltransferases (HMTs).

Two major types of HMTs exist, lysine-specific and arginine-specific. Both types of HMTs transfer the methyl groups from S-adenosyl-L-methionine (AdoMet or SAM), which serves as the cofactor and methyl group donor, to either ε-amino group (NH_3^+) on lysine or the guanidine functional group on arginine, forming the methylated products and S-adenosyl-L-homocysteine (AdoHcy). The class of lysine-specific HTMs is subdivided into SET (Su(var)3-9, Enhancer of zeste, Trithorax) domain-containing ones and non-SET domain-containing ones. The SET domain is an evolutionary well-conserved sequence motif of 130–140 amino acid long. It contains a catalytic pocket, where cofactor SAM and the to-be-modified lysine are bound as well as properly oriented. Next, the ε-amine of the lysine substrate is deprotonated, makes a nucleophilic attack on the collinear methyl group on the sulfur of SAM, and finally completes the attachment of the methyl group to the

lysine side chain. The adjacent cysteine-rich regions flanking the SET domain on either side play a crucial role in substrate recognition and maximizing enzymatic activity. Dot1 (Distruptor of telomeric silencing) is the only HMT known to date that does not contain the SET domain. Dot1 and its mammalian homolog, DOT1L (DOT1-Like, also called KMT4), are very special enzymes in terms of its substrate specificity. First, Dot1/DOT1L appears to be solely responsible for methylation of K79 on histone H3; second, unlike SET-domain-containing HMTs that target at the histone tail regions, Dot1/DOT1L is the only enzyme known to methylate a lysine residue in the globular core of the histone; finally, Dot1/DOT1L only methylates histone substrates that are actively engaged in the nucleosome but not the free ones. Despite lacking a SET domain, Dot1/DOT1L share a similar structure with other classical methyltransferases, which surprisingly more resemble histone arginine methyltransferases. However, extensive efforts have failed to demonstrate that Dot1/DOT1L can directly methylate arginine [86]. Amino acids 1–416 at the N terminus of Dot1/DOT1L contain the active histone methyltransferase catalytic sites, where several critical residues (T139, Q168, D161, E186, and D222 of human DOT1L/KMT4) align the methionyl moiety of SAM molecule and the lysine substrate for a methyl transfer reaction. The long, flexible C-terminal tail is important for substrate specificity and nucleosome binding [87]. There are at least nine members of protein arginine methyltransferase (PRMTs) in mammals, which are separated into three main types. Type I PRMTs (e.g., PRMT1, 2, 3, 4, 6, and 8) can all catalyze monomethylation and continue to form asymmetric dimethylarginine. Type II PRMTs (e.g., PRMT5 and 9) produce monomethylarginie and symmetric dimethylarginine. PRMT7 is the single Type III enzyme described to date that generates monomethylation of arginine only [88]. PRMT2 was identified by sequence homology, but demonstrated substantially low enzymatic activity in vitro [89]. Structural comparison suggests that all PRMTs contain a conserved catalytic core where the cofactor SAM binds, and a barrel-like domain where the substrate binds [90]. The sequences at both N- and C-termini are variable among different PRMTs, containing protein-protein interaction modules that may participate in determining substrate specificity or recruiting other proteins critical for enzymatic activity. Like in the methylation reaction mediated by a SET-containing HMT, the nitrogen group on target arginine residue is also first deprotonated and then acts as a nucleophile to attack the methyl group of SAM. It is suggested that a methionine in the active site of Type I PRMTs grants their abilities to catalyze asymmetric methylation, whereas in Type II PRMTs, like PRMT5, the corresponding residue is switched to a serine, so the less bulky side chain of this amino acid now allows for symmetric methylation formation [91].

For many years, histone methylation, unlike acetylation or phosphorylation, was thought to be irreversible, because of the fact that the N-CH3 bond is very stable with a half-life approximately equal to that of histones themselves. The identification of histone demethylases, enzymes that remove methyl groups from histones, completely overturned the dogma (Fig. 3). Two main classes of histone demethylases have thus far been identified, which predominantly target at the lysine residues: the flavin adenine dinucleotide (FAD)-dependent amine oxidase, which includes the Lysine (K) Demethylase 1 (KDM1) family proteins, and the Fe(II) and 2-oxoglutarate

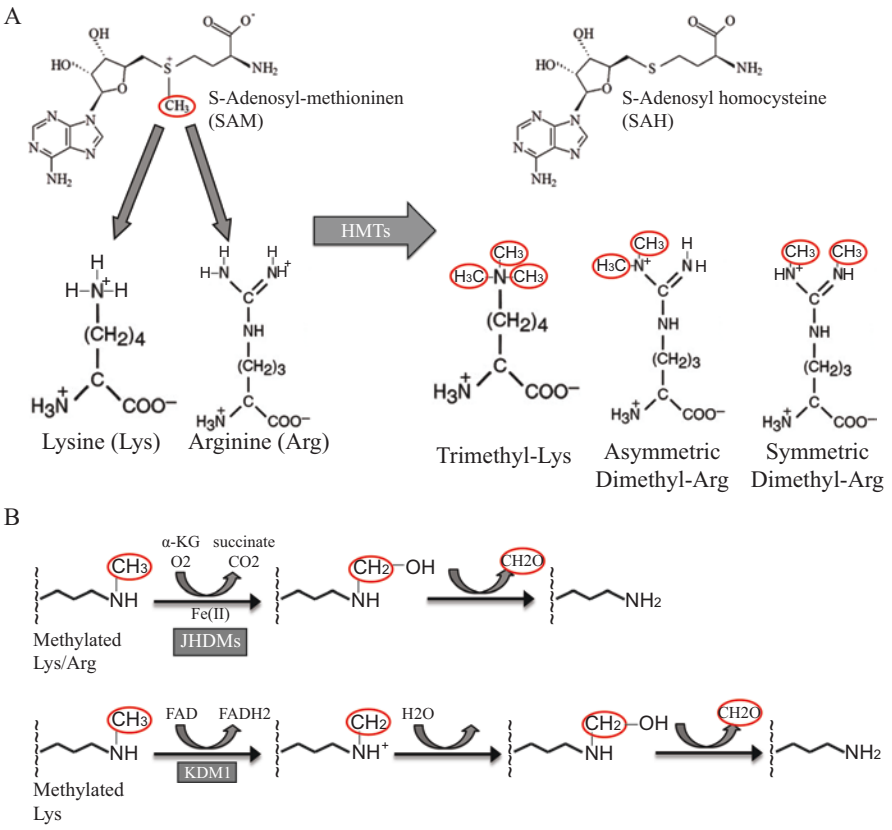

Fig. 3 Histone methylation and demethylation reactions. (**a**) Lysine or arginine can be methylated by histone methyltransferases (HMTs). The transferred methyl (−CH3) group is circled. (**b**) Histone demethylation can be catalyzed by either JmjC-domain-containing histone demethylases (JHDMs) (*upper panel*) or lysine demethylase 1 (KDM1) family proteins (*lower panel*). The removed methyl group is *circled*

(2OG)-dependent dioxygenase, which features a signature motif of JmjC domain. Both families of demethylases operate via an oxidative mechanism that releases formaldehyde as a co-product. KDM1A/LSD1 and KDM1B/LSD2 are the only two members that have been identified so far in the KDM1 family, and KDM1A/LSD1 is actually the first protein demonstrated to possess bona fide histone demethylase activity. Interestingly, both KDM1A and B can demethylate only mono- and dimethylated lysines. The JmjC domain-containing histone demethylases form a larger and more versatile family, which act on multiple histone lysine residues and can accept all three methylation states. Of note, although no arginine-specific demethylases have ever been reported, some of the JmjC KDMs have demonstrated arginine demethylation activity in vitro [92, 93]. There are some other mechanisms of

demethylation, much less common though, such as the nucleophilic demethylation by methylesterases [94]. The dynamic and reversible nature of histone methylation supports the hypothesis that modifications on histone tails, called the histone code, serve as marks for the recruitment of proteins or protein complexes to dictate the information of the genetic code [95, 96]. So, besides the enzymes that add or eliminate the histone modifications, there is another group of proteins that play pivotal roles in deciphering the language of the histone code: the binding partners of specific chemical moieties on histones. A large family of proteins has been identified that can recognize methylated lysine residues, and they are divided into several subfamilies based on the distinct recognition domains they contain, including PHD (plant homeodomain) domain that binds histone H3 in various methylation states, PWWP (named after a conserved Pro-Trp-Trp-Pro motif) domain that is concurrent with other motifs such as PHD, Chromo domain that is known to bind methylated H3K4/9/27, and MBT (Malignant Brain Tumor) domain that mostly binds mono- and dimethylated lysines, etc. In spite of the presence of divergent recognition motifs, their pairs with the corresponding lysine methylation do not simply fit into the "one domain-one mark" model: one single methylated lysine can be recognized by several readers and one reading module can bind multiple separate methylated substrates. Sometimes even different methylation states (mono-, di- or trimethylation) of the same residue can recruit different sets of binders [97], and the more methyl groups attached, the stronger the binding strength will be [98]. Considering all these uncertainties, here comes the question: how are the strength and specificity of one particular lysine methylation reader determined? Firstly, structural evidence suggests that the binding surfaces of distinct domains that recognize the same mark remarkably resemble each other. Secondly, flanking sequences of the methylated lysine are heavily involved in the selective recruitment process and make multiple direct contacts with the reader. Finally, according to the "histone end effects", modified lysine that locates near the end of a histone peptide, like H3K4 methylation, is easy to be read and therefore attracts more diverse binding partners. As for the readers of the methylated arginines, it is still highly ambiguous whether such specific motifs do exist. So far, only two proteins were claimed to recognize methylated arginine, one is the PHD motif within the ADD domain of DNMT3A, which may [99], or may not [100], directly bind symmetrically methylated H4R3; and the other is the Tudor domain of TDRD3 protein, which was spotted using a protein domain microarray approach as a reading module of asymmetrically methylated H3R4 and R17 [101].

All currently known methyltransferases, demethylases and recognition modules of methylated histones are summarized in Table 2, together with the corresponding methylation marks. For years, the diverse array of methylation events on histone proteins is believed to provide exceptional regulatory power of gene regulation in a context-specific manner, and considered to be essential steps in many processes that determine cell fate. Therefore it is not surprising that abnormal expression or activities of the enzymes that write, erase or read methylated histones are implicated in a variety of human disease states including cancers.

Table 2 Proteins in regulation of histone methylation

Histone-modifying enzymes	Epigenetic marks	Proposed functions
Histone Methyltransferases (HMTs)		
Lysine-specific methylation		
EZH2	H1K26me1/2/3	Transcriptional silencing
Unknown	H2BK5me1	Transcriptional activation
MLL	H3K4me1/2/3	Transcriptional activation, permissive euchromatin
G9A/EHMT2, SETDB1	H3K9me1/2/3	Transcriptional silencing, genomic imprinting
EZH1, EZH2, G9A/EHMT2	H3K27me1/2/3	Transcriptional repression, X inactivation
SET2D (tri-Me), ASH1L (mono−/di-Me)	H3K36me1/3	Transcriptional activation/ elongation
DOT1L	H3K79me1/2/3	Transcriptional activation/ elongation, euchromatin
SETDB1, SUV420H, NSD1	H4K20me1/3	Transcriptional silencing (mono-Me)/activation, heterochromatin
Unkonwn	H4K59me1/2/3	Transcriptional silencing
Arginine-specific methylation		
PRMT1/5/6/7	H2AR3me2	Transcriptional activation/ repression
PRMT5/6	H3R2me1/2	Transcriptional repression
PRMT2/5/6	H3R8me2	Transcriptional activation/ repression
CARM1	H3R17me1/2	Transcriptional activation
CARM1	H3R26me1/2	Transcriptional activation
CARM1	H3R42me1/2	Transcriptional activation
PRMT1/5/6/7	H4R3me1/2	Transcriptional activation/ repression
Histone Demethylases		
Lysine-specific demethylation		
KDM2A/JHDM1A	H3K36me2	Transcriptional repression, associated with heterochromatin
KDM2B/JHDM1B	H3K4me3, H3K36me2	Transcriptional repression
KDM3A/JMJD1A	H3K9me1/2	Transcriptional activation
KDM3B/JMJD1B	H3K9me1/2	Transcriptional activation
KDM4A/JMJD2A	H3K9me3, H3K36me3	Transcriptional repression
KDM4B/JMJD2B	H3K9me3	Unknown
KDM4C/JMJD2C	H3K9me2/3	Transcriptional activation, inhibition of heterochromatin
KDM4D/JMJD2D	H3K9me2/3, H1K25me1	Unknown
KDM5B/JARID1B	H3K4me3	Transcriptional activation
KDM5C/JARID1C	H3K4me3	Transcriptional repression

(continued)

Table 2 (continued)

Histone-modifying enzymes	Epigenetic marks	Proposed functions
KDM5D/JARID1D	H3K4me2/3	Transcriptional repression, DNA condensation
KDM6A/UTX	H3K27me3	Transcripional activation/repression
KDM6B/JMJD3	H3K27me2/3	Transcriptional silencing
JHDM1D/KDM7A	H3K9me2, H3K27me2	Transcriptional activation
JMJD5/KDM8	H3K36me2	Unknown
KDM1A	H3K4me1/2, H3K9me1/2	Transcriptional activation/ repression
KDM1B	H3K9me2	Transcriptional activation
PHF8/JHDM1F	H3K9me2, H3K4me2, H4K20me, H3K27me2	Transcriptional activation
Arginine-specific demethylation		
JMJD6	H3R2me2, H4R3me1/2	RNA splicing
KDM4E/JMJD2E	H3R2me1/2, H3R8me1/2, H3R26me1/1, H4R3me2	Unknown
KDM5C/JARID1C	H3R2me1/2, H3R8me2, H4R3me2	Unknown
Readers		
Tudor domain (e.g., SHH1)	H3K9me3	
Chromodomain (e.g., HP1, Pc proteins, MRG1/2)	H3K9me3, H3K27me3, H3K4me3, H3K36me3	
PWWP (Pro-Trp-Trp-Pro) (e.g., ZCWPW1)	H3K4me0/1/2/3	
MBT domain (e.g., L3MBTL1/2)	H4K20me1/2	
PHD domain (e.G., BPTF, ING1/2, MMD1)	H3K4me3	
WD40 repeat (e.g., WDR5, CYP71)	H3K4me3, H3K27me3	

4.2 Histone Methylation in Prostate Cancer

Increasing evidence suggests that histone methylation, together with other types of histone modifications, contributes to the onset and progression of prostate cancer. A panel of methylation marks, including mono-, di- and trimethylation of H3K4 (H3K4me, H3K4me2 and H3K4me3) and H3K9 (H3K9me, H3K9me2 and H3K9me3) as well as pan-acetylation of H3 and H4, was stained in a tissue microarray containing 23 nonmalignant prostate tissues and 113 prostate adenocarcinoma samples in various pathological states [102]. H3K9 di- and trimethylation and acetylation of H3 and H4 were all significantly reduced in cancer samples compared to BPH and normal tissues, whereas all three methylation states of H3K4 were upregulated in androgen-independent tumors and correlated with

clinical-pathological parameters. The other histone methylation mark that has been extensively investigated in prostate cancer is H3K27 methylation. Different methylation status (mono-, di- or trimethylation) of H3K27 (H3K27me, H3K27me2 or H3K27me3) showed distinct patterns in normal prostate tissue, clinically localized tumors, hormone-dependent and hormone-refractory prostate cancer [103]. Levels of H3K27 mono- and trimethylation have been reported to positively correlate with aggressive tumor features [103, 104]. Intriguingly, the global concentrations of H3K27me3 in cell-free circulating nucleosome from peripheral blood of prostate cancer patients, detected by an ELISA-based assay, were significantly lower in men with metastatic disease than in those with localized or local advanced tumors [105]. Although it is still deliberative as for how the overall levels of specific histone methylation marks change in prostate cancer, cumulative evidence implies that global patterns of histone methylation may distinguish cancer cells from their normal counterparts or even metastatic disease from organ confined tumors, and it is highly possible that they can be prognostically relevant. Indeed, multiple studies showed that certain methylation marks, either alone or in combination with other types of histone modifications, could serve as independent prognostic markers associated with clinical outcome in prostate cancer patients. In one study, five individual histone modifications, the acetylation of H3K9 (H3K9ac), H3K18 (H3K18ac) and H4K12 (H4K12ac) as well as the dimethylation of H3K4 (H3K4me2) and H4R3 (H4R3me2), were evaluated by immunohistochemical staining in 183 primary prostate cancer tissues [106]. Except H3K9ac, higher level of each one of the rest four histone modifications is correlated with higher grade of cancer samples. Interestingly, combination of the patterns of all these five modifications clearly predicted the clinical outcome of patients with lower grade (Gleason score 2–6) prostate tumors. The prognostic power of specific histone modifications was further confirmed in another prostate cancer cohort [107]. The levels of both H3K18ac and H3K4me2 were quantified immunohistochemically in 279 prostate cancer cases, and stronger intensities of both histone marks were significantly associated with increased risk of tumor relapse. In another study, H3K4 di- and trimethylation (H3K4me2 and H3K4me3), H3K36 trimethylation (H3K36me3), H4K20 trimethylation (H4K20me3) and H3K9 acetylation (H3K9ac) were assessed using immunohistochemistry in 169 primary prostatectomy tissue samples [108]. H3K4me3 alone can serve as an accurate predictor of the biochemical recurrence following radical prostatectomy for low grade (Gleason score ≤ 6) prostate cancer. Taken together, all these studies convincingly demonstrate that changes in overall levels of certain histone methylation events are associated with increased risks of prostate cancer recurrence and poor survival. Therefore, global epigenetic patterns of histone methylation may function as promising biomarkers for prostate cancer prognosis.

Not only the dissimilarity in overall levels, dynamic changes of histone methylation at individual chromatin loci also contribute to prostate cancer initiation and progression by coordinated regulation of cancer-specific gene expression. Because histone methylation has been implicated in both transcriptional activation and repression, a number of oncogenes and tumor suppressor genes were found to be epigenetically switched on or off, respectively, driving the malignant transformation

of prostate epithelial cells. For instance, H3K27 trimethylation (H3K27me3), the methylation mark that is associated with gene silencing, was found to be significantly enriched at the promoter regions of a large number of tumor suppressor genes, such as *ADRB2*, *DAB2IP*, *RARβ2*, etc., in metastatic prostate cancer compared with localized tumors or normal prostates. Presence of this epigenetic mark is correlated with decreased expression of these genes and results in prostate cancer cell growth, survival and metastasis [104, 109–111]. Histone methylation is also intimately involved in controlling the transcriptional activity of AR. Methylation of H3K4 dictates the functionally active chromatin region, and its presence at AR binding sites contributes to the maintenance of the open chromatin architecture and initial recruitment of the pioneer factor FOXA1, which facilitates the transactivation of AR target genes, such as the proto-oncogene *UBE2C*, in CRPC cells [112]. In contrast, methylation of H3K9, another histone mark strongly linked to transcriptional repression, is detected at the regulatory regions of AR target genes, such as *KLK3* that encodes PSA, and constrains the transactivation of these genes. Androgen stimulation leads to transcriptional activation of *KLK3* gene, which is accompanied by a robust decrease in H3K9 methylation levels at its promoter [113]. Silencing of H3K9 demethylases LSD1, JHDM2A or JMJD2C, increased the signals of this repressive mark and subsequently decreased the expression of AR target genes [114, 115]. Recently, AR is found to act as a global transcriptional repressor, and genes being silenced by functional AR are mostly developmental regulators that play important roles in cell differentiation. Surprisingly, AR-repressed genes demonstrated strong enrichment of bivalent H3K4me3 and H3K27me3 modifications at their promoter regions, suggesting that the repressive function of AR is dictated by the status of histone methylation and that this particular epigenetic pattern contributes to prostate cancer progression through cell dedifferentiation and destabilization [116, 117]. All the above evidence offers important insights into the roles of histone methylation in prostate cancer development and progression. No matter if it is at individual genomic locations or at the overall levels, alteration in methylation pattern may directly reflect the aberrant activities or expressions of the enzymes that regulate this epigenetic program. Approximately 50% of the HMTs encoded by the human genome, for example, are now linked to diseases and in particular cancers [118]. In the following parts, only those enzymes that regulate histone methylation marks with clear links to cancer formation and progression will be discussed.

One of the best-characterized histone-modifying proteins in prostate cancer is the enhancer of zeste 2 (EZH2) that specifically methylates histone H3 at lysine 27. It is also reported to have methyltransferase activity towards the linker histone H1.4 at lysine 26. EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which also contains other core components such as EED, SUZ12 and RbAp46/48, for maximum enzymatic efficiency. EZH2 is found to be significantly increased in metastatic, hormone-refractory prostate cancer compared with clinically localized prostate tumors and normal samples. Overexpression of EZH2 is strongly associated with poor clinical outcome and prognosis in prostate cancer patients. Loss of EZH2 expression blocked the aggressive behaviors, like proliferation, metastasis and invasion, of prostate cancer cells, while overexpression of

EZH2 caused the neoplastic transformation of normal prostate epithelial cells [119]. All these observations clearly establish the oncogenic function of EZH2 in prostate cancer. Although there is still much debate about the mechanisms by which EZH2 drives prostate tumorigenesis, it is believed that H3K27me3 at regulatory chromatin regions leading to the downregulation of targeted tumor suppressor genes may in part explain the cancer-driving effects of EZH2. Indeed, a "polycomb repression signature" was identified in metastatic human prostate cancer tissues, which consists of 14 direct targets of EZH2 as they were upregulated upon EZH2 knockdown and contained high H3K27me3 signals in their promoter regions. Interestingly, the signature genes are largely downregulated in prostate cancer and can predict clinical outcome of multiple solid tumors including prostate [109]. In addition, EZH2 is reported to also involve in biological signaling other than epigenetic regulation. For example, EZH2 was recently found to serve as an AR co-activator and facilitate the recruitment of AR to target genes that are critical for the development of androgen-independent prostate cancer [120]. Although this co-activator function of EZH2 is still dependent on its methyltransferase activity, H3K27 methylation is not involved as the specific chromatin loci co-bound by EZH2 and AR was devoid of this epigenetic mark. In addition, cytosolic EZH2 was shown to regulate actin polymerization in prostate cancer cells in a methyltransferase-dependent fashion [121]. EZH2-mediated maintenance of a dynamic actin cytoskeleton controls the shape and motive force of cancer cells, subsequently promoting a metastatic phenotype. All the evidence suggests the possibilities of proteins other than histones being methylated by EZH2, which is also critical for the roles of EZH2 in oncogenic transformation. Several non-histone proteins of EZH2 have been identified albeit not in prostate cancer, such as GATA4 [122], STAT3 [123] and RORα [124]. EZH2-catalyzed methylation modulates either activities or protein stabilities of these transcription factors, which may be broadly relevant to EZH2-dependent normal development and malignancies. All these findings show diverse mechanisms by which EZH2 promotes the aggressive characteristics of cancer cells through methylation of histone or non-histone proteins, and thus pharmacological inhibition of the methyltransferase activity of EZH2 may hold great promise for the treatment of prostate cancer.

As a functionally important epigenetic mark, methylation of H3K27 is dynamically and precisely controlled. The JmjC domain-containing proteins, UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome) and JMJD3, specifically remove only the di- and tri-methyl groups though, from H3K27, counteracting the action of EZH2. Therefore, it is conceivable that UTX or JMJD3 exerts a tumor-suppressive role in prostate cancer. In support of this supposition, inactivating somatic mutations of UTX have been discovered in many types of human cancers including prostate [125]. Lack of functional UTX may result in increased levels of H3K27 methylation and subsequently have an analogous effect to the phenotypes caused by EZH2 overexpression. Genome-wide study revealed that UTX-occupied promoters were significantly underrepresented for H3K27me3 signals and that majority of the downstream target genes were functionally enriched in RB-centered cell cycle regulation. This suggests a role for UTX-catalyzed demethylation of

H3K27me3 in controlling cancer cell fate through the RB network [126]. This conclusion was further confirmed in another study, which demonstrated that UTX restricted Notch and RB signaling to suppress eye tumor formation in Drosophila, which was dependent on its demethylase activity [127]. More than that, UTX was shown to be localized at the promoters of apoptosis and autophagy genes, upregulated their expressions by removing the repressive methylation marks from H3K27, hence induced cell death of larval salivary glands [128]. In keeping with these findings, it was shown that the other only H3K27 demethylase, JMDJ3, induced the transcriptional activation of several tumor suppressor genes, such as $p16^{INK4A}$ and $p14^{ARF}$ [129, 130]. All the above indications support the idea that demethylation of H3K27 catalyzed by either UTX or JMJD3 impedes tumorigenesis. However, the exact function of UTX or JMJD3 in prostate cancer is insufficiently investigated. Recently, an oncogenic role of UTX was discovered that it cooperates with H3K4 methyltransferase MLL4 in activating transcriptional programs that are required for proliferation and invasiveness of breast cancer cells [131]. This implies that the biological effects of UTX in cancers, either tumor-promoting or tumor-suppressive, are highly tissue-specific. Considering the relatively high rate of loss-of-function mutations of UTX in prostate carcinoma, the demethylase may function as a tumor suppressor in this type of cancer. Adding to the complexity of the situation, JMJD3 was upregulated in prostate cancer with higher expression levels in metastatic samples [132]. Therefore, it is likely that UTX and JMJD3, albeit their same activities against H3K27 demethylation, may produce opposite biological outcomes in prostate cancer. Further investigation is clearly warranted to explore the dynamic and divergent functions of H3K27 demethylation in prostate carcinogenesis and tumor progression.

Unlike H3K27 methylation, which is catalyzed by only two methyltransferases EZH2 or its close homolog EZH1, H3K4 can be methylated by at least ten known or predicted methyltransferases. The major class of such enzymes is the Mixed Lineage Leukemia (MLL) family, which contains six members MLL1–4, SET1A and B. Like EZH2 and most histone-modifying enzymes, MLL-family methyltransferases exist in multiprotein complexes, and the most common components that are shared by all MLL family complexes include WDR5, RBBP5 and ASH2L [133]. Misregulation of *MLL* genes is implicated in prostate cancer development and progression. The recurrent mutations in *MLL2* gene have been identified in 8.6% of prostate cancers [125], and somatic *MLL3* mutations found in African American patients were associated with the aggressiveness of prostate cancer [23, 134]. In addition, translocation of *MLL* gene was found in two metastatic CRPC cases [135]. Besides the genetic alterations, MLL proteins together with the assisting subunits involve intimately in AR signaling through direct epigenetic regulation of AR target genes. It was demonstrated that MLL-containing complex acts as a co-activator of AR signaling, and that pharmacological blockage of MLL-AR axis reduces xenograft tumor growth in CRPC mouse models [136]. WD repeat-containing protein 5 (WDR5), an indispensible subunit of all MLL complexes, is upregulated in human prostate cancer. It directly interacts with the T11-phosphorylated histone H3 at AR-bound chromatin locations, then recruits MLL1 complex that leads to H3K4

methylation at these sites, and subsequent transactivates AR target genes [137]. Using similar mechanism of action, BPTF associated protein of 18 KDa (BAP18), which was also shown to associate with MLL complexes, facilitates the recruitment of MLL1 complex to the androgen-response elements, increases the levels of active epigenetic marks such as H3K4me3 and H4K16ac, and therefore enhances AR-induced transactivation [138]. In addition to AR signaling, MLL complex was shown to activate the transcription of *HOXA9* gene by upregulating H3K4me3 intensity at its promoter region, which induces metastatic phenotype in prostate cancer cells [139]. Another integral subunit of MLL1/2 complexes, menin encoded by the *MEN1* gene, has also been indicated in prostate carcinogenesis. Male mice carrying the loss-of heterozygosity of *MEN1* gene developed prostate cancer, suggesting a possible role of *MEN1* in suppressing tumorigenesis of the prostate gland [140]. However, increased expression of *MEN1* was also detected in metastatic prostate cancer [141–143], and gain at the gene locus was shown to independently predict disease recurrence after radical prostatectomy [144].

Similar to adding the functional methyl onto H3K4, which is catalyzed by multiple methyltransferases, eradiation of this epigenetic mark is tightly controlled by groups of demethylases, such as the JARID1 subfamily proteins (e.g., JARID1A-D) and KDM1 family members (e.g., KDM1A and B). Among them, KDM1A, also known as LSD1, is the most extensively studied demethylase in prostate cancer. Overexpression of LSD1 was detected in prostate cancer compared with benign prostatic hyperplasia, which is positively correlated with high Gleason score, distant metastases and poor prognosis [145, 146]. As the demethylase for the active histone mark H3K4me1/2, LSD1 is expected to mediate transcriptional repression, and indeed, it is found to associate tightly with several corepressors such as NuRD complex, histone deacetylases, and CoREST, etc. [147–149]. How the role of LSD1 as a transcriptional repressor leads to prostate cancer was best explicated by the discovery that LSD1 mediates AR-dependent silencing of target genes such as those involved in androgen synthesis, DNA synthesis and cell proliferation, including *AR* itself [150]. This was concomitant with a decrease of H3K4 methylation intensity at the regulatory chromatin elements of these AR-repressed genes in an androgen-dependent manner. Interestingly, LSD1 has also been repeatedly demonstrated to function as a transcriptional co-activator for AR [113, 115]. Pharmacological inhibition or genetic silencing of LSD1 abrogates androgen-induced gene activation and prostate cancer cell proliferation. In these scenarios, LSD1 stimulates AR-dependent transcription by relieving the repressive histone mark H3K9 methylation. It colocalizes with JMJD2C, the demethylase possessing an enzymatic activity towards H3K9 trimethylation, at AR-binding sites, where JMJD2C initiates the demethylation reaction followed by LSD1-catalyzed removal of remaining mono- and dimethylation marks on H3K9 [115]. Thereby, these two demethylases cooperatively stimulate AR-dependent gene expression. It is postulated that switch in the substrate specificity of LSD1 from H3K4 on AR-repressed genes to H3K9 on AR-activated genes may be determined by phosphorylation status of histone H3. Phosphorylation of H3 on threonine 11 (H3T11ph), which is catalyzed by protein kinase C-related kinase 1 (PRK1), increases the activities of LSD1 (mono- and dimethylation) and

JMJD2C (trimethylation) for H3K9 demethylation [151], while PKCβ1-induced phosphorylation of H3 on threonine 6 (H3T6ph) prevents LSD1 from demethylating H3K4me1/2 [152]. This is an excellent example of the close and dynamic crosstalk among distinct epigenetic patterns in establishing specific chromatin structure for transcriptional regulation. Apart from engaging in AR signaling, LSD1 also controls aggressive features of prostate cancer cells, such as angiogenesis, invasion and metastasis. This attributes to the fact that the demethylase epigenetically activates or represses expression of critical genes in these processes, including lysophosphatidic acid receptor 6 (*LPAR6*) and vascular endothelial growth factor (*VEGF-A*), etc. [153, 154]. Although implied, further mechanisms, such as demethylation of non-histone substrates, need to be explored, which likely contribute to the biological function of LSD1 in prostate cancer [155, 156].

Not only the above mentioned histone-modifying enzymes, many additional proteins that are involved in regulation of histone methylation have been implicated pivotal roles in prostate cancer development and progression. For example, the levels of arginine methyltransferases, such as CARM1, PRMT1 and PRMT2, are elevated in metastatic, hormone-refractory prostate cancer [157–159], and they regulate the transcriptional activity of AR, which is dependent on the arginine methylation states of histone H3 [158–160]. Equally interesting, SET7/9, which can write monomethylation on H3K4, was reported to directly methylate AR at K630 and K632, potentiating transcriptional activity of the nuclear receptor and stimulating prostate cancer cell proliferation [161, 162]. This list of histone-methylation-regulating proteins can keep growing, but their precise functions in prostate cancer development and progression need to be deliberately evaluated. It is intriguing to find that a lot of these enzymes as well as the corresponding histone methylation marks are involved in control of AR activity, further supporting an indispensible role of epigenetics in regulation of central signaling axis that drives prostate carcinogenesis and tumor progression.

5 DNA/Histone Methylation in Prostate Cancer Diagnosis, Prognosis and Treatment

Although in the preliminary stage, epigenetic regulatory mechanisms are gaining strength and proving their potential in terms of risk assessment, diagnosis, and therapy monitoring in prostate cancer. Even though the widespread application of PSA test has a paradigm-shifting impact on the clinical management of prostate cancer, this marker cannot effectively differentiate between cancer and non-cancerous conditions such as prostatitis and BPH. On the other hand, there are frequent occasions when PSA level is detected low but prostate cancer is actually present. Both false-positive and false-negative results of PSA test warrant the discoveries of approaches with high sensitivity and specificity for early detection of prostate cancer. Epigenetic marks hold great promise as useful diagnostic indexes, and DNA hypermethylation seems to especially fulfill this mission as several features of this epigenetic

modification render it promising for risk assessment of prostate carcinogenesis. First, genome-wide and locus specific DNA methylation alterations of certain genes have been recurrently detected in prostate cancer. For instance, *GSTP1* hypermethylation is replicated in tons of independent studies that involve more than thousands of prostate cancer samples [48, 163, 164]. When considered together with methylation status of other genes such as *APC*, the specific epigenetic mark at *GSTP1* gene promoter can distinguish primary prostate cancer from benign tissues with sensitivity approaching 97.3–100% [47]. Second, somatic changes of DNA methylation pattern are usually found to occur early in prostate carcinogenesis. Acquisition of CpG island hypermethylation can already be detected in PIN lesion but is not or rarely present in BPH [49, 165]. Promoter methylation of certain genes helps discriminate between cancerous and non-cancerous prostate cells during the early development of the disease [65, 166]. This highlights the prospective character of DNA hypermethylation as an early event in prostate cancer evolution, and hence suggests that this epigenetic signature may accurately and sensitively diagnose initial stage of the disease. Third, methylated DNA can be detected in cancer tissues and body fluids of prostate malignancy. Moderate or high frequencies of methylation at several gene promoters, such as *RARβ2*, *APC*, *RASSF1A*, and *GSTP1*, were observed in the plasma, serum and urine of patients [167, 168]. One of the biggest advantages of measuring DNA methylation in body fluids is that fluctuation in the levels of this epigenetic modification can be easily and reproducibly quantified using well-developed techniques like methylation-specific PCR and bisulphite sequencing. This enables the feasibility of a non-invasive molecular approach for quick detection of epigenetic changes associated with prostate cancer. Finally, DNA hypermethylation appears to be relatively stable in a defined area of the gene. Somatic alterations of methylation can be repeatedly spotted using specific primers for the particular chromatin regions of particular genes. This is in contrast to genetic mutations, which can take place at a wide range of sites along a gene and therefore be easily missed unless the whole gene is completely sequenced. Besides, DNA is much less susceptible to degradation than protein or RNA, and thus can be maintained at steady levels throughout the sampling process. Due to its relative simplicity, safety and sensitivity, DNA methylation analysis has become a promising tool in molecular diagnostics of prostate cancer, which will substantially reduce mortality and unwanted tension of patients.

In addition to an impact on early detection, epigenetic marks have also been implicated in rapid determination of prognosis and monitor of treatment efficacy in advanced prostate cancer. DNA hypermethylation of several genes is found to correlate with clinicopathological features of poor prognosis like late stage and high Gleason scores, and accurately predict patients who are likely to experience biochemical recurrence [169–171]. While DNA hypermathylation can be an earlier event in prostate tumorigenesis, global hypomethylation and histone methylation seem to happen relatively late in prostate cancer and are more common in metastatic cases. For example, the methylation status of H4R3 positively correlates with increasing tumor grade and can be used to predict the risk of prostate cancer recurrence [106]. Loss of *LINE-1* transposable elements was observed in 67% of prostate

tumors with lymph node metastases but in only 8% of tumors with no metastatic lesions [172]. More interestingly, the prognostic roles of epigenetic marks can be assessed in cell-free circulating tumor DNA (ctDNA) or nucleosome (ctNUC). Methylated ctDNA of *GSTP1* gene was found to be associated with chemotherapy response and overall survival of CRPC patients [173]. H3K27 trimethylation level in intact ctNUC discriminated metastatic prostate cancer from organ confined, locally controlled disease [105]. Taken together, specific epigenetic signatures, such as DNA methylation and histone modifications, represent a new generation of prognostic biomarkers for monitoring of cancer recurrence and therapy response. Although still in its infancy, innovative methodology has been developed so as to detect and validate these epigenetic biomarkers in an efficient and sensitive way using materials originating from body fluids of cancer patients.

Interests in targeting epigenetic modulators for anticancer therapy have never been stopped. So far, six epigenetic drugs, two DNA methyltransferase (DNMT) inhibitors and four histone deacetylase (HDAC) inhibitors, have been approved by FDA for treatment of myelodysplastic syndrome, multiple myeloma and T cell lymphoma [174–178]. In the case of prostate cancer, pharmacological inhibition of DNA methylation and histone modifications show encouraging yet limited antitumor activities. Several nucleoside analogues including those two FDA-approved DNMT inhibitors, 5-Aza-2′-deoxycytidine and 5-Azacytidine, were reported to suppress CRPC cell proliferation, reactivate AR signaling and induce cancer cell differentiation [179, 180]. Compounds that are designed to directly inhibit the enzymatic activity of human DNMTs like RG108 also exhibited some inhibitory efficacy against CRPC in vitro and in vivo [181–183]. Both types of DNMT inhibitors are thought to have the tumor-suppressive effects by specifically demethylating and reactivating tumor suppressor genes. It is indeed the case that exposure of prostate cancer cells to DNMT inhibitors significantly decreased promoter methylation signals of several genes, such as *GSTP1*, *APC*, *RASSF1A* and *RARβ2*, which, in general, is concomitant with the expression restoration of these prostate cancer specific methylated genes [183–185]. Unfortunately, in spite of all these promising results in pre-clinical settings, there are only a few clinical trials testing DNMT inhibitors in prostate cancer patients with either modest activities or severe side effects [186, 187]. A panel of small molecule inhibitors of the enzymes that regulate histone methylation is currently under intensive evaluation to assess their anticancer effectiveness. Compounds that selectively disrupt the catalytic sites of EZH2 are thought to hold great promise for treatment of prostate cancer. The very original prototype of EZH2 inhibitors is 3-dezaneplanocin-A (DZNeP), which later turned out to be a pan-HMT inhibitor [188]. DZNeP downregulates EZH2 protein, decreases the overall levels of H3K27me3, and therefore de-represses several tumor suppressor genes that are epigenetically silenced by PRC2 complex [104, 189, 190]. Exposure of prostate cancer cells to DZNeP resulted in cell cycle arrest, blocked prostatosphere formation, and diminished invasion capacity of the cancer cells [189]. More interestingly, this compound significantly reduced the expression of cancer stem cell markers and therefore abrogated self-renewal ability [189]. More specific inhibitors of EZH2 methyltransferase activity were recently developed, like GSK126,

EPZ-6438, etc. [191–193]. These drugs all demonstrated dose dependent inhibition of H3K27me3 without triggering EZH2 protein degradation. Intriguingly, both H3K27me3-dependent and -independent functions of EZH2 were indicated in mediating the antitumor effects of EZH2 inhibitors, hence the mechanism of drug action in prostate cancer cells needs further investigation [194]. Currently, there are no clinical studies involving EZH2 inhibitors in prostate cancer. Another group of epigenetic drug precursors that have been extensively studied thus far is the inhibitors of histone demethylase LSD1. Because LSD1 catalyzes lysine demethylation via an FAD-dependent monoamine oxidase (MAO) mechanism, majority of currently available compounds targeting LSD1 are actually non-selective MAO inhibitors, which include pargyline, tranylcypromine and phenelzine, etc. Pargyline blocked LSD1-catalyzed demethylation of H3K9 in prostate cancer cells, and subsequently inhibited AR-dependent transcription [113]. Furthermore, this LSD1 inhibitor reduced migration and invasion ability of prostate cancer cells and retarded the epithelial-mesenchymal transition (EMT) process in vitro and in vivo [195]. Inhibition of LSD1 by pargyline and tranylcypromine suppressed proliferation of both androgen-responsive and androgen-independent prostate cancer cells in a dose- and time-dependent manner [154]. However, in another independent study, pargyline treatment induced cell cycle arrest, whereas tranylcypromine had no effect or even promoted proliferation of prostate cancer cells [196]. The conflicting findings prompted comprehensive research on LSD1 function in prostate cancer and urged the generation of more specific inhibitors of the histone demethylase activity. Indeed, several highly selective LSD1 inhibitors have been identified recently, such as NCL-1, HCI-2509 and namoline [197–199]. All of these potent, reversible and selective LSD1 inhibitors suppressed the androgen-independent growth of CRPC cells in vitro and in vivo, with no apparent adverse effects [198–200]. Pan-demethylase inhibitors have also been designed and synthesized, which can simultaneously inhibit both families of KDMs, KDM1 and JmjC-containing demethylases. Several of these compounds caused growth arrest and substantial apoptosis in cancer cells including prostate, but had little effects on nonmalignant cells [201]. Finally, two clinical trials are currently being conducted with the non-specific LSD1 inhibitor phenelzine sulfate, either alone in treating patients with relapsed prostate cancer that has not metastasized (NCT02217709) or in combination with docetaxel to treat patients with progressive prostate cancer after first-line therapy with docetaxel (NCT01253642). Tremendous efforts are ongoing to screen for compounds that target other epigenetic enzymes involving in regulation of histone methylation. For example, selective (e.g., BIX01294, UNC0638 and A-366, etc.) and non-selective (e.g., chaetocin) inhibitors of euchromatic histone methyltransferase 2 (EHMT2, also known as G9a), the HMT that is primarily responsible for H3K9 dimethylation, have been identified [202–205]. Unfortunately, although they efficiently reduce H3K9me2 in prostate cancer cells, their effects on the development and progression of the disease are quite obscure. CARM1 (PRMT4), the protein arginine methyltransferase that methylates H3 on arginines 2, 17, and 26, has been implicated as a transcriptional coactivator of AR signaling [158, 159], and therefore several pharmacological inhibitors of CARM1, such as the 1-benzyl-3,5-bis(3-

bromo-4-hydroxybenzylidene)piperidin-4-one and its analogues, dramatically reduced AR transcriptional activity in a dose-dependent fashion [206]. Additionally, a small molecule inhibitor that dissociates the menin-MLL HMT complex blocked AR signaling and prevented the growth of castration resistant tumors in vivo [136].

Overwhelming evidence supports the idea that solid tumors, such as prostate cancer, may well respond to epigenetic drugs targeting DNA methylation or histone modifications. However, the lack of success in clinical trials testing these drugs in prostate cancer raises the concerns about their potencies, specificities, and side effects. Further work is warranted in order to gain deeper understanding of the global patterns of these epigenetic modifications, such as DNA methylation and histone methylation, during prostate carcinogenesis and tumor progression. Increased insights into these epigenetic regulatory mechanisms will definitely foster successful clinical applications of these epigenetic modifications as biomarkers of cancer diagnosis and risk stratification, in predicting a patient's response to therapy or providing alternative treatment options for prostate cancer.

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