Review Article
DNA methylation in development and disease: an overview for prostate researchers

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Abstract: Epigenetic mechanisms including DNA methylation are critical regulators of organismal development and tissue homeostasis. DNA methylation is the transfer of methyl groups to cytosines, which adds an additional layer of complexity to the genome. DNA methylation marks are recognized by the cellular machinery to regulate transcription. Disruption of DNA methylation with aging or exposure to environmental toxins can change susceptibility to disease or trigger processes that lead to disease. In this review, we provide an overview of the DNA methylation machinery. More specifically, we describe DNA methylation in the context of prostate development, prostate cancer, and benign prostatic hyperplasia (BPH) as well as the impact of dietary and environmental factors on DNA methylation in the prostate.

Keywords: Epigenetics, DNMT1, BPH, aging, prostate cancer, development, demethylase

This review has been designed as an introduction to the field of DNA methylation for prostate researchers. We provide a historical perspective by following advances in DNA methylation research from the inception of the field to the recent discoveries involving DNA methylation and prostate cancer. We summarize the functions of key elements of the DNA methylation machinery and their interactions with the histone modification machinery. The role of DNA methylation as a dynamic regulator of development, homeostasis and disease has been described using research carried out in several organ systems including the prostate. Aging is a major risk factor for prostate cancer and benign prostatic hyperplasia (BPH). This review touches on DNA methylation changes occurring during aging, prostate cancer and BPH. We also summarize research on how environmental toxins affect early prostate development with accompanying changes in DNA methylation and the impact of diets enriched with the methyl donor folic acid on prostate homeostasis and susceptibility to prostate cancer.

Epigenetics: historical perspective

Developmental biologists in the early-twentieth century grappled with the question of how cells containing the same genetic code could behave so differently and develop specialized functions. The term epigenetics, meaning ‘above the genome’, was coined by Waddington in 1942 to explain how non-coding changes to genetic material could direct cell specialization during development [1]. In recent times, definitions of epigenetics have moved beyond developmental biology to encompass other fields including cancer biology and toxicology. One widely accepted definition states that epigenetics is “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail change in DNA sequence” [2, 3]. The modern field of epigenetics has grown to include covalent modifications to histones and DNA as well as non-coding RNAs which act together to finely modulate gene expression.

Introduction to DNA methylation

DNA methylation is the addition of methyl groups to the 5' position of cytosine bases in DNA, preferentially at CpG dinucleotide sites [4]. 60% of CpG dinucleotides in the mammalian genome are methylated [5]. CpG dinucleotides often reside in clusters, or CpG islands, which are generally unmethylated and associa-
DNA methylation in development and disease

DNA methylation is initiated by a class of enzymes called DNA methyltransferases. DNA methyltransferase-1 (DNMT1) preferentially carries out methylation at hemi-methylated DNA sites and preserves methylation patterns during cell replication. DNMT3A and DNMT3B have de novo methylation activity (methyl group addition to unmodified DNA) and are required for mammalian development [6]. Another DNMT3 family member, DNMT3L is catalytically inactive, but cooperates with DNMT3A and DNMT3B during de novo methylation [7, 8].

Transfer of methyl groups to cytosines in DNA is a highly conserved process. Methyltransferases bind to DNA, ‘flip’ out cytosine from the DNA helix and attach the methyl group from S-adenosyl methionine to the 5’ position of cytosine. The N-terminal regulatory domain of the DNMTs contains motifs for protein-DNA interactions, protein-protein interactions and nuclear localization. The C-terminal region contains conserved motifs that define the enzyme active site. The N-terminal region of DNMT3L shares similarities with that of DNMT3A and DNMT3B but lacks the catalytic active site in its C-terminal domain.

**Adding methylation marks**

Broadly, the division of labor between the DNA methyltransferases is clear. DNMT3A and DNMT3B methylate the genome de novo during early development and gametogenesis. DNMT1, with its preference for hemi-methylated DNA, propagates methylation patterns during tissue growth. We will briefly summarize the cellular localization, binding partners and major functions of the DNA methyltransferases below:

**DNMT1**: DNMT1 is a large protein comprised of ~1620 amino acids. During S-phase, DNMT1 localizes to replication forks and assumes a distinct punctate nuclear staining pattern. During other stages of the cell cycle, DNMT1 nuclear staining is diffuse. The N-terminal region of DNMT1 contains a replication foci targeting sequence (RFTS) that targets it to sites of replication [9]. Autoinhibition of the methyltransferase domain of DNMT1 by interaction with the RFTS domain determines substrate specificity. Conformational changes in the N-terminal region inhibit catalytic activity when DNMT1 is bound to unmethylated CpG substrates. This inhibition is released when DNMT1 is bound to hemi-methylated CpG substrates [10]. Preferential localization to replication foci and substrate specificity for hemi-methylated DNA allows DNMT1 to stably propagate tissue-specific methylation patterns. DNMT1 protein abundance changes during the cell cycle (peaking in S-phase) and is present but not particularly abundant in non-replicating cells [11]. During fetal prostate development, Dnmt1 is initially expressed in the urethral mesenchyme but shifts to the developing epithelial prostate buds at later stages [12].

Knockout, mutation, and inhibition studies have provided key insights into the role of DNMT1 in maintaining global methylation. Targeted mutation of Dnmt1 in mouse embryonic stem (ES) cells reduces 5-methylcytosine (5mC) 3-fold without affecting cell viability. Mouse embryos carrying the same targeted mutation have a similar 3-fold reduction in 5mC levels but are not viable [13]. In contrast to mouse ES cells, Dnmt1 knockout in human ES cells triggers apoptosis [14]. Conditional deletion of Dnmt1 in mouse fibroblasts leads to severe hypomethylation, p53-dependent apoptosis and aberrant gene expression [15]. Loss of DNMT1 results in elevated mutation rates, genomic instability [16], microsatellite instability [17], DNA damage [18] and cell cycle arrest [19, 20]. DNMT1 activity and expression is upregulated in prostate cancer, where it serves to methylate and suppress key tumor suppressor genes [21].

Mutations in the RFTS domain of DNMT1 have been linked to two neurodegenerative syndromes: autosomal dominant cerebellar ataxia-deafness and narcolepsy (ADCA-DN) and hereditary sensory neuropathy with dementia and hearing loss (HSN1E) [22-25]. These mutations prevent DNMT1 from binding to heterochromatin, leading to aberrant methylation patterns and DNMT1 protein aggregation in the cytosol [25]. There is increasing evidence for the association of single nucleotide polymorphisms of DNA methyltransferases with incidence of different tumor types, including prostate cancer [26-28].

**DNMT3 family**: The DNMT3 family of methyltransferases is comprised of DNMT3A, DNMT3B and DNMT3L. Except for conserved motifs in the C-terminal catalytic region, the DNMT3
DNMT3A and DNMT3B are essential for de novo methylation, transcriptional repression, and early embryonic development. Inactivation of Dnmt3a and Dnmt3b, simultaneously but not individually, blocks de novo methylation, suggesting that these enzymes have overlapping functions. DNMT3B is essential for early embryonic development as Dnmt3b knockout mice are embryonic lethal. In contrast, Dnmt3a knockout mice live up to 4 weeks of age. Dnmt3a and Dnmt3b double knockout embryos die during mid-gestation. DNMT3A and DNMT3B participate in transcriptional repression in association with histone deacetylases [32]. DNMT3L, although lacking in catalytic activity, stimulates de novo methylation by DNMT3A [7]. In the developing prostate, Dnmt3a and Dnmt3b are predominantly expressed in the urethral mesenchyme during fetal stages but localizes to prostate bud tips postnatally [12].

DNMT3A mutations have been observed in acute myeloid leukemia where they are associated with reduced survival [34, 35]. DNMT3B mutations are observed in patients with ICF (immunodeficiency, centromere instability and facial anomalies) syndrome. Loss of DNMT3B function in ICF syndrome is associated with changes in promoter methylation, altered histone marks and aberrant gene expression [36].

Removing methylation marks

DNA methylation is a reversible modification. When DNMT1 is inhibited, cells passively lose methyl marks over several rounds of replication. The ten-eleven translocation (TET) family of methylcytosine dioxygenases catalyze the active removal of methyl marks from DNA independent of replication. During early prostate development, Tet1 and Tet2 are expressed predominantly in the urethral mesenchyme while Tet3 is expressed in the urethral epithelium [12]. TET1, TET2 and TET3 catalyze the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) and subsequently into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [37, 38]. 5caC is recognized and excised by thymine DNA glycosylase (TDG) [39].

There is increasing evidence that 5hmC participates in transcriptional regulation [40]. 5hmC is highly enriched in brain tissue where it is required for neuronal differentiation [41], learning and memory [42]. 5hmC marks are abundant in embryonic stem cells, where they are enriched at enhancers and gene bodies [43]. A recent study has shown that 5hmC localizes to sites of DNA damage and promotes genome stability [44]. 5hmC levels are dramatically reduced in human cancers, correlating with impaired TET activity [45]. 5hmC is significantly reduced in prostate cancer and has been proposed as a biomarker for prostate cancer detection. In contrast, 5fC and 5caC are increased in prostate cancer [46, 47].

Active DNA demethylation can also be catalyzed by 5-methylcytosine deaminases like activation-induced cytidine deaminase (AICDA) and apolipoprotein B mRNA editing enzyme catalytic subunit 1 (APOBEC1) [48]. Deamination of 5mC yields thymine which is immediately replaced with unmethylated cytosine by DNA glycosylases like TDG [49].

Methyl CpG binding domain (MBD) proteins

The MBD proteins are readers of 5mC marks. The mammalian MBD family is comprised of MeCP2, MBD1, MBD2, MBD3 and MBD4 which possess a common Methyl CpG binding domain (MBD). MBD proteins bind to sites of DNA methylation and recruit enzymes for transcriptional repression [50].

DNA methylation and histone modifications

There are two major paradigms of gene silencing by DNA methylation. One, DNA methylation of CpGs in regulatory regions can prevent bind-
ing of transcription factor complexes required for active gene expression. Second, methyl CpG binding domain (MBD)-containing proteins recruit chromatin modifiers to sites of DNA methylation to establish or maintain repressed chromatin states. Conversely, DNA methylation activity can be recruited to repressed chromatin to methylate adjacent DNA and reinforce gene silencing.

Chromatin modifiers add post-translational modifications to histones that relax or compact chromatin. Active transcription occurs from relaxed or euchromatin regions while transcription is repressed in compacted heterochromatin regions. Histone acetyltransferases acetylate lysines on histones, which loosens the interaction between DNA and histones, resulting in an accessible, open chromatin configuration. This is reversed by histone deacetylases (HDACs), resulting in gene silencing. Histone methyltransferases add methyl groups to lysines and arginines of Histone H3 and H4. H3K9 and H3K27 di- and tri-methylation and H4K20 tri-methylation repress transcriptional activity by blocking transcriptional factors from accessing DNA.

MBD proteins (MECP2, MBD1, MBD2, MBD3, MBD4) bridge the gap between DNA methylation and histone modifications. MECP2 and MBD2 binds to methylated DNA and recruit HDAC activity to silence genes [51, 52]. MBD proteins can also recruit histone methyltransferases to sites of DNA methylation. MBD1 recruits the SUV39H1-HP1 heterochromatin complex, which has histone methyltransferase activity, to sites of DNA methylation for transcriptional repression by blocking transcriptional factors from accessing DNA.

DNA methylation in development

Methylation dynamics in early development

For sexual reproduction, DNA methylation marks need to be erased and reset in early germ cells [55]. Following erasure, sperm- or oocyte-specific methylation patterns are re-established. Imprinted genes, which are expressed from either the maternal or paternal allele, are methylated during gametogenesis by the de novo methyltransferases DNMT3A and DNMT3B [8, 33, 56].

Following fertilization, the sex-specific methylation patterns of the sperm and oocyte are erased and reset in the zygote. The zygote undergoes a wave of global demethylation until it reaches the blastocyst stage. De novo methylation is carried out by DNMT3A and DNMT3B in the post-implantation embryo. Cells continue to gain DNA methylation as they differentiate. Inheritable methyl marks are maintained by DNMT1 during cell replication.

Genomic imprinting

In mammals, there are over 100 genes that are expressed solely from the maternal or paternal allele. The expression of genes in a parent-of-origin specific manner is regulated by a process called genomic imprinting [57]. Genomic imprinting controls the dosage of key genes involved in growth and metabolism [58]. Methyl marks are erased during gametogenesis and reset to indicate maternal (oocyte) or paternal (sperm) origin. This results in the generation of differentially methylated regions (DMRs) between the maternal and paternal genomes. The role of DNA methylation in genomic imprinting is best illustrated by the well-studied IGF2/H19 gene locus, a paternal DMR. IGF2 expressed from the paternal allele and H19 expressed from the maternal allele are located close to each other. The transcriptional repressor CTCF binds to the unmethylated maternal chromosome while its binding it blocked on the methylated paternal chromosome. CTCF binding blocks IGF2 expression while driving H19 expression from the maternal allele. The absence of CTCF binding allows IGF2 expression from the paternal allele while blocking H19 expression [59].

Loss of imprinting at the IGF2 locus (expression from both alleles instead of one) occurs during aging and tumorigenesis in the prostate [60]. In mouse models, increased expression of the critical paracrine growth factor IGF2 by loss of imprinting drives prostatic neoplastic growth [61]. Reduction of CTCF expression and binding
activity as a function of age triggers Igf2 loss of imprinting in the prostate [62, 63]. Genomic imprinting is essential for normal development. Prader-Willi syndrome and Angelman syndrome occur from imprinting errors.

**X-inactivation**

To compensate for gene dosage differences, one X chromosome is inactivated in females in a process called X-inactivation. Re-activation of X-linked genes by pharmacological inhibition of DNA methylation provided the first evidence for X inactivation by DNA methylation [64]. The initiation and maintenance of X inactivation is regulated by the long non-coding RNA XIST. XIST is expressed only from the inactivated X-chromosome where it acts in cis to coat the inactive X-chromosome and repress transcription. The methylation status of the XIST promoter corresponds to its expression levels. The XIST gene is demethylated and its expression upregulated in Dnmt1 male mutant embryos [65].

**Silencing of transposable elements**

Transposable elements or transposons can change location and copy number within the genome. Approximately 46% of the human genome and 37.5% of the mouse genome are derived from transposable elements [66]. Methylation suppresses the expression of transposons and these elements can be re-expressed when hypomethylated. If unchecked, transposon expression can lead to mutations and genomic rearrangements. The mouse genome contains ~1000 intracisternal A particle (IAP) retroviruses that have Long Terminal Regions (LTRs) comprised of multiple repeating DNA sequences. Methylation of the LTRs causes transcriptional silencing of IAPs in somatic cells. IAP transcripts are upregulated 50-100 fold in Dnmt1 mutant mouse embryos [67]. Demethylation and activation of IAP genes are observed after treatment with DNA methyltransferase inhibitors [68]. Changes in DNA methylation and expression of retrotransposon elements are observed in prostate cancer [69]. Hypomethylation and re-expression of LINE-1 retrotransposons occur in BPH tissues [70].

**Transcriptional control of differentiation**

As a cell progresses from an undifferentiated to a differentiated state, its DNA methylation landscape changes dramatically. DNMTs play a central role in the transcriptional control of differentiation. DNMTs suppress the expression of differentiation genes to maintain stem cells in an undifferentiated state. Regulation of DNMT1 expression by the pluripotency factors OCT4 and NANOG maintains the undifferentiated state of mesenchymal stem cells [71]. During differentiation, DNMTs methylate pluripotency factors like OCT4 and NANOG to prevent the cell from reverting to an undifferentiated state [72].

Cytidine nucleoside analogs like 5-azacytidine have been used to elucidate the role of DNA methylation in transcriptional control. 5-azacytidine and its 2'-deoxy derivative 5-aza-2'-deoxycytidine can be incorporated into DNA where it inhibits DNA methyltransferase activity and methylation of newly synthesized DNA. Non-myoblast mouse embryo cells change their phenotype drastically to form functional striated muscle cells after 5-azacytidine treatment [73, 74]. Swiss3T3 cells treated with 5-azacytidine or 5-aza-2'-deoxycytidine display new mesenchymal phenotypes of contractile muscle, differentiated adipocytes and chondrocytes [75, 76]. 5-azacytidine treatment causes hypomethylation and reverts cells to a less differentiated state from which new phenotypes can develop.

5-aza-2'-deoxycytidine (5AzadC) has been used to study the role of DNA methylation in fetal prostate development. The prostate forms during fetal development as solid epithelial buds arising from the urogenital sinus (UGS) epithelium which grow into the surrounding mesenchyme. Epithelial cells of the developing prostate bud downregulate expression of the epithelial adhesion molecule E-cadherin (Cdh1) to achieve invasive growth into the mesenchyme. Treatment of UGS explants with 5AzadC decreased prostate bud elongation and outgrowth without affecting cell viability. 5AzadC treatment decreased Cdh1 promoter methylation and upregulated Cdh1 mRNA expression in prostate epithelial cells. Increased expression of Cdh1 constrained prostate bud elongation and invasive growth into the mesenchyme. Thus, methylation of the Cdh1 promoter downregulates Cdh1 expression to achieve prostate bud outgrowth [77].

Mesenchymal Androgen receptor (Ar) gene expression is required for prostate bud initia-
Table 1. DNA methyltransferase deletion studies

<table>
<thead>
<tr>
<th>Model</th>
<th>Phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Global DNMT1 null mice</td>
<td>3-fold reduction in genomic 5mC levels</td>
<td>[13]</td>
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<td></td>
<td>Recessive embryonic lethal</td>
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<td></td>
<td>Embryos are developmentally retarded</td>
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<td></td>
<td>Embryos die at mid-gestation</td>
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<tr>
<td>DNMT3A/-</td>
<td>DNMT3A/- mice appear normal at birth but become runted and die at around 4 weeks of age</td>
<td>[6]</td>
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<tr>
<td>DNMT3B/- mice</td>
<td>DNMT3B/- mice exhibit growth impairment, rostral neural tube defects and die at mid-gestation</td>
<td></td>
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<tr>
<td>DNMT1a knockout mice</td>
<td>Heterozygous embryos from homozygous mutant females die during last third of gestation</td>
<td>[178]</td>
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<tr>
<td></td>
<td>Loss of methylation at imprinted loci</td>
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<tr>
<td>DNMT3L knockout mice</td>
<td>Azoospermia in homozygous males</td>
<td>[33]</td>
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<td></td>
<td>Heterozygous embryos from homozygous mutant females die during mid-gestation</td>
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<td></td>
<td>Loss of maternal methylation imprints</td>
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<td>Cre-mediated homozygous DNMT1 deletion in fibroblasts</td>
<td>DNMT1 deletion leads to hypermethylation and aberrant expression of 10% of genes</td>
<td>[15]</td>
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<td></td>
<td>DNMT1 depleted cells undergo p53-mediated apoptosis</td>
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<td>Cre-mediated DNMT1 deletion in neuroblasts (CamK-cre)</td>
<td>95% of cells in the brain hypomethylated</td>
<td>[179]</td>
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<td></td>
<td>Die immediately after birth from respiratory distress; defects in neuronal respiratory control</td>
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<td>Cre-mediated DNMT1 deletion in telencephalic precursors (Emx1-cre)</td>
<td>Mutant mice viable but undergo severe neuronal cell death from E14.5-P21</td>
<td>[180]</td>
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<td></td>
<td>Deregulation of neuronal gene expression</td>
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<td></td>
<td>Defects in neuronal morphology and excitability</td>
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<td>shRNA knockdown of DNMT1 in primary human keratinocytes</td>
<td>DNMT1 depleted cells exit progenitor compartment, undergo premature differentiation</td>
<td>[92]</td>
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<td></td>
<td>DNMT1 loss results in upregulation of cyclin-dependent kinase inhibitors, cell cycle arrest and impaired proliferation</td>
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<td>Cre-mediated DNMT1 deletion in hematopoietic stem cells (HSC) (Mx-cre)</td>
<td>Impaired HSC self-renewal</td>
<td>[181]</td>
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<td>Increased cell cycling and inappropriate expression of differentiation markers in myeloid progenitor cells</td>
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<td>Cre-mediated DNMT1 deletion in pancreatic cells (Pdx1-cre)</td>
<td>DNMT1 loss results in de-repression of p53</td>
<td>[79]</td>
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<td>G2/M cell cycle arrest and apoptosis</td>
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<td>Pancreatic agenesis due to apoptosis of progenitors</td>
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<td>Cre-mediated DNMT1 deletion in retinal cells (Chx10-cre)</td>
<td>Defective photoreceptor differentiation</td>
<td>[182]</td>
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<td>Altered cell cycle kinetics; increased proportion of G1 phase cells</td>
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<td></td>
<td>Increased apoptosis of post-mitotic photoreceptors and other neuronal types</td>
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<td>Cre-mediated DNMT1 deletion in retinal cells (Rx-cre)</td>
<td>Mice have smaller eyes</td>
<td>[183]</td>
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<td>Impaired differentiation of retinal pigment epithelium</td>
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<td></td>
<td>Defects in photoreceptor outer segment morphogenesis</td>
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<tr>
<td>Cre-mediated DNMT1 deletion in intestinal cells (Villin-cre)</td>
<td>Mice die few weeks after birth</td>
<td>[80]</td>
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<td></td>
<td>Induction of differentiation markers in progenitor cells</td>
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<td></td>
<td>Impaired methylation and expression of DNA damage response genes and cell cycle regulators</td>
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<td></td>
<td>Increased DNA damage and apoptosis of progenitor cells</td>
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<tr>
<td>Cre-mediated DNMT1 deletion in Keratin 14 lineage cells (K14-cre)</td>
<td>Uneven epidermal thickness, altered hair follicle size</td>
<td>[184]</td>
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<td>Impaired proliferation at hair follicles, progressive alopecia with age</td>
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<tr>
<td>Cre-mediated DNMT1 deletion in Shh lineage cells (Shh-cre)</td>
<td>Mice die shortly after birth; respiratory complications arising from severe lung hypoplasia</td>
<td>[81, 82]</td>
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<td>Epithelial depletion of urethral and bladder epithelium</td>
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<td>Premature differentiation and loss of bladder progenitors</td>
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<td>Cell cycle arrest and apoptosis of prostate progenitors; reduction in prostate bud number</td>
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tion from the UGS epithelium. The onset of mesenchymal Ar expression corresponds to a reduction in DNMT1 expression and a decrease in Ar promoter methylation. Treatment of UGS explants with 5AzadC inhibits DNMT1, reduces Ar promoter methylation and accelerates the onset of Ar expression, thereby increasing the number of prostate buds specified [78].

**Tissue growth and organogenesis**

The mid-gestational lethality of Dnmt1 knock-out embryos prevents the study of organ system development. To overcome this, context-dependent roles of DNMT1 have been studied in several organ systems by employing conditional genetic approaches. This involves the use of tissue-specific Cre drivers to drive Dnmt1 deletion during development. Deletion of Dnmt1 in the developing pancreas results in global hypomethylation, growth arrest and tissue atrophy through p53-dependent apoptosis of pancreatic progenitors [79]. Intestine-specific ablation showed that Dnmt1 is required for proliferation and crypt formation in the perinatal period. Loss of Dnmt1 in the intestinal epithelium led to increased DNA damage and apoptosis [80].

The role of Dnmt1 in the developing lower urinary tract was assessed by conditionally ablating Dnmt1 in the epithelium of the bladder, urethra and prostate. Similar to previous studies on Dnmt1 ablation, widespread DNA damage and p53-mediated apoptosis was observed. This was accompanied by a reduction in prostate bud formation, highlighting the role of Dnmt1 in maintaining cell proliferation and survival of early prostate progenitors [81]. Dnmt1 deletion in the lower urinary tract led to the breakdown of the Wolffian duct-urethra junction which was permissive for the movement of Wolffian duct cells into the urethra and bladder epithelium. These ectopic cells were then reprogrammed to acquire bladder characteristics including Uroplakin expression [82].

DNA methyltransferase deletion studies conducted in several organ systems have been summarized in Table 1.

**DNA methylation in adult homeostasis and disease**

Changes in DNA methylation are associated with the formation of specialized tissue types in the mature organism.

**Genome-wide methylation patterns**

Most methylated CpG nucleotides are scattered throughout the genome and participate in the silencing of retrotransposons and repetitive elements. Clusters of unmethylated CpGs, called CpG islands, are found at the transcription start sites of several housekeeping genes and a few tissue-specific genes. 72% of promoter sequences contain CpG islands [83]. Unmethylated CpG islands are associated with transcriptionally active genes, mostly housekeeping genes and tumor suppressors [84, 85]. The majority of CpG islands are unmethylated irrespective of whether the associated gene is transcriptionally active or inactive. However, methylation of CpGs within CpG islands has been observed and this results in the stable silencing of genes [86].

**Tissue-specific methylation patterns**

Tissue-specific methylation patterns established during development coordinate specialized gene expression programs in multiple tissue types. Demethylation of developmentally methylated regions also contributes to the establishment of tissue-specific methylation patterns. Tissue-specific genes with CpG island promoters are usually unmethylated in all tissue types, even when genes are inactive. In contrast, tissue-specific genes with non-CpG island promoters display tissue-specific methylation patterns. Broadly, DNA methylation at promoter regions is negatively correlated with gene expression [87, 88].

Promoters with “weak” CpG islands, which are intermediate between CpG-rich promoters and non-CpG promoters, display a high frequency of methylation. These weak CpG islands are more prone to de novo methylation during development and contribute to the differential methylation observed between somatic and germ cells [89]. Tissue-specific differentially-methylated regions have also been identified in intergenic and intragenic regions of genes with non-CpG island promoters [90].

**Expression of DNA methyltransferases in adult tissues**

Adult tissues express lower levels of DNA methyltransferases compared to embryonic tissues although DNMT1, DNMT3A and DNMT3B expression is widespread across adult human
DNA methylation in development and disease

DNMT1 + E-cadherin + DNA

Adult mouse prostate lobe
DNMT1 + DNA

Figure 1. DNMT1 expression in the mouse and human prostate. Tissues sections from (A) young human (21 weeks of gestation), (B) young mouse (Postnatal day 9), (C) adult human (23 years) and (D) adult mouse (7 weeks) were labeled with antibodies to DNMT1 (in red) and E-cadherin (in green). Regions enclosed in white boxes within (A), (B), (C) and (D) are magnified in (A'), (B'), (C') and (D'). Prostate tissue from (E) intact, (F) castrated and (G) castrated and testosterone supplemented mice were labeled with antibodies to DNMT1 (in red). DAPI staining is shown in blue. Abbreviations Ur: Urethra, Pr: Prostate.

tissues, with DNMT1 expressed at the highest level [91]. In adult tissues, DNMT1 is highly expressed in rapidly-dividing somatic tissues including epidermis [92] and intestine [93]. DNMT1 regulates the fine balance between self-renewal and differentiation in adult stem cells. Differentiation involves demethylation and activation of tissue-specific genes with concomitant methylation and repression of stem cell genes. DNMT1 expression prevents differentiating cells from reverting back to an undifferentiated state [72]. In self-renewing somatic tissues, DNMT1 methylates and suppresses differentiation of gene promoters and prevents cell cycle exit in tissue progenitors [92]. DNMT1 is highly expressed in developing prostate tissue but its expression is considerably reduced in the adult prostate. DNMT1 expression is upregulated in cycling cells from prostates of castrated mice supplemented with testosterone (Figure 1).

DNA methylation and aging

5'-methyl cytosine (5mC) levels gradually decline over the lifetime of an organism in a process called epigenetic drift. The rate of loss of 5mC is inversely correlated with the lifespan.
of the organism and is determined by environmental factors like diet [94]. Studies in humans have shown that centenarian DNA has 7% less methylated cytosines than a newborn. Centenarian genomes display loss of methylation at non-CpG island promoters and repetitive DNA sequences like LINE elements and LTR-retrotransposons [95]. Sufficient DNA methylation levels are needed for healthy aging, as demonstrated using DNMT1 haplo-insufficient mice [96].

Alteration of DNA methylation patterns with age occur from cumulative environmental exposures and lifestyle factors [97]. This is best exemplified by the phenotypic changes observed in monozygotic twins with age. Monozygotic twins possess identical genomes and epigenomes early in life but display divergence in DNA methylation and histone modifications with age [98]. Aging-related changes in DNMT expression and availability of methyl donors contributes to epigenetic drift [99, 100]. Aging fibroblasts show a downregulation of DNMT1 and significant overexpression of DNMT3B. These opposing changes in DNMT expression may explain the global hypomethylation and site-specific hypermethylation observed in aging cells [101, 102].

Oxidative stress associated with age has been shown to cause DNA hypomethylation in the prostate [103]. Both global hypomethylation and promoter hypermethylation can result in cellular transformation [97, 99]. Aging-related hypermethylation of autophagy genes has been linked to neurodegenerative diseases and chronic inflammation [104]. In the prostate, aging has been linked to loss of imprinting at the IGF2 locus [63]. Despite widespread changes to the DNA methylome with age, it is not fully understood if these changes contribute to aging-related diseases in the prostate. Changes in DNA methylation have been definitively linked to cancer, an age-related disease. However, it is not fully understood whether these DNA methylation changes are causes or consequences of prostate disease.

DNA methylation aberrations in disease

DNA methylation errors occur in several diseases including cancer, syndromes caused by imprinting errors (Prader-Willi, and Angelman syndromes), Fragile X syndrome and ICF syndrome [105]. Among these, the role of DNA methylation in cancer has been extensively studied. DNMT1 and DNMT3A are modestly overexpressed in several tumor tissues while DNMT3B is significantly overexpressed [91, 106]. Paradoxically, cancer is associated with global hypomethylation and genomic instability, with significant reduction in methylation at repetitive elements. Several cancer types display aberrant hypermethylation at promoter-associated CpG islands. Hypermethylation of genes involved in apoptosis, cell cycle regulation, cell invasion and DNA repair provide cancer cells with survival advantages over normal cells. De novo methylation of imprint control regions can result in aberrant regulation of imprinted genes. Loss of imprinting, allowing biallelic expression of growth promoting genes like Igf2, promotes prostate tumor growth [105].

DNA methylation and prostate disease

In this section we will briefly describe two disease conditions of the prostate: Prostate cancer and Benign Prostatic Hyperplasia (BPH) and the current knowledge of the role of DNA methylation in these diseases.

Prostate cancer and DNA methylation: Prostate cancer is characterized by malignant growth of the prostate and is the third most common cancer type in the United States. Age is a significant risk factor for the development of prostate cancer. Common treatment strategies include anti-androgens (bicalutamide, flutamide, enzalutamide), androgen synthesis inhibitors (ketoconazole, abiraterone acetate), radiation therapy, chemical castration (LHRH agonists) and surgical intervention (www.cancer.gov/types/prostate).

Tissues from metastatic prostate cancer, but not non-metastatic cancer, have drastically reduced 5mC levels compared to normal tissues. This correlation between metastatic capacity and 5mC content suggests that 5mC levels can be used as a biomarker to detect metastatic tumors or that the reduction in 5mC contributes to metastatic progression [107]. Hypermethylation of the regulatory locus of pi-class glutathione S-transferase gene GSTP1 is a recurrent change observed in prostate cancer precursor lesions and prostate adenocarcinoma. GSTP1 encodes an enzyme that cata-
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analyzes cellular detoxification reactions [108, 109]. CpG island hypermethylation of multiple genes, including APC, RASSF1A, and RUNX3, has been detected in prostate cancer [110-112]. Methylation status of several genes, including APC and RPRM, correlate with biochemical recurrence after radical prostatectomy [113]. Hypermethylation of specific CpG islands in prostate cancer correlates with hypomethylation of Alu and LINE-1 repetitive elements [114]. Genome wide methylation studies have identified differentially-methylated regions (DMRs) in prostate cancer compared to benign tissues. These DMRs are enriched for binding by Enhancer of zeste homolog 2 (EZH2), a key driver of prostate cancer [115, 116].

Loss of Androgen receptor (AR) gene expression is a hallmark of castration resistant prostate cancer. CpG methylation of the AR promoter has been observed in prostate cancer cells and is associated with loss of gene expression. Methylation-induced AR inactivation can be reversed by treating cells with DNA methylation inhibitors [117]. The estrogen receptor gene (ESR1) is also frequently methylated and inactivated in prostate cancer [118].

DNMT1 excess has been described in prostate cancer cell lines and tissues [21, 119]. DNMT1 expression is significantly upregulated in localized and metastatic prostate cancer tissues compared to normal tissues [120]. Protein and mRNA expression of Dnmt1, Dnmt3a and Dnmt3b are increased in the transgenic adeno-carcinoma of mouse prostate (TRAMP) model of prostate cancer [121]. Overexpression of the isoform DNMT3A2 and DNMT3B has also been observed in prostate tumors [122]. DNMT1 inhibition with 5-azacytidine kills prostate tumor cell lines at high doses [123] while promoting metastatic invasion at lower doses [124]. Due to its effectiveness in reversing gene silencing, Azacytidine treatment is being studied in combination with other therapeutic options for the treatment of advanced prostate cancer [125-127]. DNMT inhibition was shown to block tumorigenesis in the TRAMP mouse model of prostate cancer [128]. In a cohort of prostate tissue samples, DNMT1 expression was highest in poorly-differentiated prostate cancer and lowest in well-differentiated prostate cancer. DNMT1 expression was negatively correlated with expression of GSTP1 and APC, which are hyper-methylated in prostate cancer. This suggests a role for DNMT1 in the methylation and repression of key genes during prostate cancer progression [129].

Benign prostatic hyperplasia and DNA methylation: Benign Prostatic Hyperplasia (BPH) is a non-malignant enlargement of the prostate. BPH growth initiates by the age of 30 and continues throughout the individual’s lifetime. The major risk factor for the development of pathological BPH is age. By the age of 50, 50% of men develop pathological BPH [130]. Enlargement of the prostate in BPH results in urethral constriction and manifestation of lower urinary tract symptoms (LUTS). LUTS include storage (increased frequency, urgency, nocturia) and obstructive symptoms (weak stream, incomplete voiding, urinary obstruction, overflow incontinence) [131]. BPH in association with LUTS are a significant financial burden [132, 133] and negatively impacts quality of life but does not lead to significant morbidity if treated. BPH is treated with three major classes of drugs, alpha adrenergic receptor blockers, 5-alpha reductase inhibitors and phosphodiesterase-5 inhibitors [134-136].

Prostates with BPH have significantly lower levels of global 5mC compared to normal tissue [107]. Dnmt1 is expressed at low levels in BPH tissue compared to prostate cancer. GSTP1 and APC are hypermethylated in prostate cancer but hypomethylated in BPH [129]. Although BPH is characterized by global hypomethylation, recurrent hypermethylation of the tumor suppressor gene 14-3-3Sigma occurs in BPH tissues [137]. DNMT1 regulates the methylation and repression of SRD5A2 gene expression in BPH samples. DNMT1 expression in SRD5A2-silenced BPH tissues is regulated by the inflammatory mediators IL-6, TNFa and NF-kB [138, 139]. Methylation and repression of SRD5A2 results in an androgenic to estrogenic switch. Decreased reliance of androgenic pathways in BPH could be behind the reduced efficacy of 5-alpha reductase inhibitors in some patients [140].

Impact of dietary and environmental factors on DNA methylation

DNA methylation is influenced by environmental factors including diet [141, 142], particulate pollution [143-145], carcinogens and benzene exposure [146]. The Developmental Origins of
Heath and Disease hypothesis (DOHaD) states that early life environmental influences can impact adult disease. DNA methylation is one mechanism by which signatures of early life exposures can be embedded in the genome. The heritable and stable nature of DNA methylation would allow these early changes to persist into adulthood, where additional insults can trigger disease onset. The DOHaD hypothesis originated from observations made in low birthweight infants. Infants with low birthweight were more prone to cardiovascular disease later in life, suggesting that poor fetal nutrition can increase adult disease susceptibility [147, 148].

Maternal diet can influence DNA methylation patterns in offspring. This is best illustrated by the seminal Agouti mouse studies. Transcription from the agouti gene locus is associated with increased obesity and yellow coat color. Maternal diet enriched in methyl donors causes methylation and repression of agouti gene transcription and produces lean mice with brown coat color [149, 150]. The Dutch famine studies have demonstrated differential methylation of several metabolism genes, including the IGF2 locus, with prenatal exposure to poor nutrition. Exposed individuals have poor metabolic regulation, increased body mass index and higher risk for developing schizophrenia in later life [151, 152].

Commonly occurring environmental toxins like Bisphenol A (BPA), Dioxin (TCDD), and phthalates are capable of interfering with endocrine signaling and are called endocrine disruptors. Developmental exposures to hormones and endocrine-disrupting chemicals have been shown to alter prostate development. Early prostate development is dependent on androgens (testosterone and dihydrotestosterone). Disrupting androgen action or exposure to estrogenic compounds alters early prostate development with long-term consequences to the adult prostate. Exposing pregnant mice to low doses of estrogen or estrogenic compounds (BPA or diethylstilbestrol) increases the number of prostate glands formed during fetal life and increases adult prostate size [153-155]. Developmental exposure to estrogens or BPA increases susceptibility to prostate cancer in an adult rat model of prostate cancer [156]. Prenatal exposure to the endocrine disruptor ethinylestradiol, found in oral contraceptives, pre-disposes male and female gerbils to prostate lesions with aging [157]. Developmental exposure to estradiol and BPA alters DNA methylation patterns of several genes in the prostate including phosphodiesterase type 4 variant 4 (PDE4D), Pitx3, Wnt10b, Paqr4, Sox2, Chst14, Tpd52 and Creb3l4. These DNA methylation changes persist into adulthood [156, 158, 159]. Additional research is required to ascertain whether differential regulation of these genes contributes to prostate cancer. Maternal exposure to the persistent environmental contaminant TCDD alters prostate bud specification by disrupting Aryl hydrocarbon receptor signaling [160, 161]. Exposure to TCDD during in utero and lactational stages decreases adult rat prostate size by impairing early prostate proliferation and differentiation [162]. In utero and lactational exposure to TCDD predisposes mice to urinary dysfunction in later life, but the exact mechanism is not known [163]. Early TCDD exposure can alter DNA methylation at imprinted loci in preimplantation embryos [164]. Further research is required to link TCDD induced DNA methylation changes to defects in prostate growth and urinary tract function.

DNA methyltransferase co-factor S-adenosyl methionine (SAM) is the source of methyl groups for DNA methylation. Following removal of the methyl group, SAM is converted to S-adenosyl homocysteine (SAH) and homocystine. Homocysteine re-methylation to replenish pools of SAM requires several methyl group donors like methyl-folate, methionine and betaine. Availability of methyl donors in the diet can influence DNA methylation. This is elegantly illustrated by studies in the Agouti mouse model. Early life exposure to BPA hypomethylates the IAP retrotransposon element upstream of the agouti gene and shifts coat color to yellow. Maternal diet supplementation with methyl donors reverses BPA-induced hypomethylation and shifts coat color back to brown [165].

Dietary folate is a well-studied methyl donor. Folates are water soluble vitamins that participate in one-carbon transfer, DNA synthesis, cell growth, hematopoiesis and metabolism. While folates occur naturally in the diet, folic acid is the synthetic form that is supplemented in fortified food stuffs or consumed as dietary supplements. Folates and folic acid are converted to 5-methyl tetrahydrofolate which provides methyl groups for the conversion of homocysteine to...
Table 2. Studies examining the effects of folic acid on prostate homeostasis and disease

<table>
<thead>
<tr>
<th>Study</th>
<th>Findings</th>
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<td>Animal models of folic acid supplementation</td>
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<tr>
<td>4 mg/kg or 24 mg/kg folic acid throughout gestation and lifetime</td>
<td>Folic acid supplemented males mice show improvement of urinary symptoms</td>
<td>[176]</td>
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<td>12-fold higher than recommended in mice</td>
<td>in a hormone induced model of urinary obstruction</td>
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<tr>
<td>4 mg/kg or 24 mg/kg folic acid throughout gestation and lifetime</td>
<td>Folic acid supplemented mice show reduced response to castration</td>
<td>[177]</td>
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<td>12-fold higher than recommended in mice</td>
<td>induced prostate involution. Prostates of castrated folic acid</td>
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<td>supplemented mice are larger than prostates from castrated control</td>
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<td>10-fold higher than recommended fed to TRAMP mice after weaning</td>
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<td>Clinical studies on the interaction of folic acid with prostate cancer</td>
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<td>Plasma levels of folates were tested for association with prostate</td>
<td>Increasing levels of plasma folate positively correlated with prostate</td>
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<td>cancer risk (Northern Sweden Health and disease cohort)</td>
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<td>(Aspirin/ Folate polyp prevention study)</td>
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<td>Plasma levels of folates were tested for association with prostate</td>
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<td>cancer risk (ProtecT study)</td>
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<tr>
<td>Serum folate levels were tested for association with prostate cancer</td>
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<td>cell proliferation in biopsies (University of Pittsburgh)</td>
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<td>Oral folic acid supplementation was tested for association with</td>
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<td>risk (JANUS cohort, Norway)</td>
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<tr>
<td>Meta-analysis of published randomized trial data to study the</td>
<td>Folic acid supplementation does not have a significant association with prostate cancer incidence</td>
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<td>association of folic acid supplementation on cancer risk</td>
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methionine. Methionine is converted to SAM, the methyl donor for DNA methylation reactions. Adequate consumption of folates is required to maintain SAM pools.

In 1998, the United States mandated folic acid fortification in cereal grains. Folic acid fortification has raised serum folate levels in populations of several age groups, most significantly in children and older populations [166, 167]. In addition to participating in methylation, folates are important players in nucleotide biosynthesis and polyamine synthesis. The complex role of folates in the body could yield protective or deleterious effects depending on the context. High gestational folic acid has been shown to have adverse effects on offspring in rodent studies [168, 169]. Multiple clinical trials have associated folic acid supplement consumption with increased risk of prostate cancer [170-172]. Moreover, studies in the TRAMP prostate cancer mouse model have shown that dietary folic acid deficiency can suppress tumor growth [173]. However, a meta-analysis using combined data across multiple randomized trials have failed to identify an association between folic acid supplementation and prostate cancer risk [174, 175].

Despite contradictory data, the prevalence of folic acid supplement consumption warrants further research into the interaction between folic acid supplementation and prostate disease. Maternal supplementation, food grain fortification and consumption of multi-vitamin supplements have resulted in populations that have been exposed to high levels of folic acid throughout their lifetime. A study modeling this lifetime folic acid exposure in mice, showed that folic acid supplementation from gestation relieves lower urinary tract symptoms in mice with hormone-induced urinary dysfunction [176]. Further, folic acid supplementation slows down prostate shrinkage in mice after castration [177]. It is important to fully understand the consequences of folate supplementation at various stages of development and during disease processes in the prostate. Rodent models and clinical studies examining the impact of folic acid on the prostate are summarized in Table 2.

Conclusions

This review provides an overview of the DNA methylation machinery and the dynamic changes in DNA methylation that occur during development, tissue growth and ultimately aging and disease. DNA methylation is at the confluence of the genome and environment. Early life changes in DNA methylation patterns are overlaid on our genomes and can affect disease susceptibility later in life. We hope that this review can be used as an introduction to the field by prostate researchers interested in studying DNA methylation.

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Disclosure of conflict of interest

None.

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