The Importance of DNA methylation in Prostate Cancer development

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Abstract

After briefly reviewing the nature of DNA methylation, its general role in cancer and the tools available to interrogate it, we consider the literature surrounding DNA methylation as relating to prostate cancer. Specific consideration is given to recurrent alterations. A list of frequently reported genes is synthesised from seventeen studies that have reported on methylation changes in malignant prostate tissue, and we chart the timing of those changes in the diseases history through amalgamation of several previously published data sets.

We also review associations with genetic alterations and hormone signalling, before the practicalities of investigating prostate cancer methylation using cell lines are assessed. We conclude by outlining the interplay between DNA methylation and prostate cancer metabolism and their regulation by Androgen Receptor, with a specific discussion of the mitochondria and their associations with DNA methylation.

Highlights

- Many DNA methylation changes are observed between benign and cancerous prostate tissue.

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- DNA methylation changes are frequently early and recurrent, suggesting a functional role.
- Androgen-driven metabolic processes in the prostate impinge on DNA methylation.
- Prostate cancer cell lines offer a good model for some methylation changes, but not all.
- Clinical/genomic associations have been reported, but multi-region sampling studies are needed.

Keywords: Prostate cancer, Epigenetic, Methylation, Biomarkers, Metabolism, Mitochondria

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1. Introduction: Why consider the epigenome?

1.1. The origins of prostate cancer are not obviously genomic

Cancer genomics studies have identified recurrently mutated genes and mutation hotspots in a number of cancer types. However, such studies in prostate adenocarcinomas have identified no genes recurrently mutated in more than a seventh of cases [1]. Studies of locally advanced and metastatic prostate cancer have revealed extensive intratumoural clonal heterogeneity [2, 3, 4, 5, 6], in some cases revealing clones with distinct genomic origins [3, 5]. This extensive clonal and spatial heterogeneity creates a significant sampling problem for studies that rely on the use of single tumour specimens. In such studies, intratumoural heterogeneity will amplify intertumoural heterogeneity, contributing to the low recurrence rates of genes affected by point mutations in prostate cancer [1].

Other mutation types have higher levels of recurrence, e.g. 8p deletions (in 40% of cases) and *TMPRSS2-ERG* fusions (in 50% of cases) [7, 8], suggesting that these may be early or convergent events in prostate tumourigenesis. However, many prostate tumours have no definitive genomic driver event [1]. This is consistent with the existence of events that precede the first somatic point mutations and possibly also the acquisition of the first copy number and structural variants. Epigenomic changes are among the candidates for early events. Of these, DNA methylation changes have been

widely studied and found to be the most recurrent events in both locally advanced and metastatic prostate tumours [9, 10].

In prostate cancer, recurrent genome-wide and locus specific DNA methylation alterations have been known for decades [11, 12] and these events impact on gene expression potential [12, 13]. The high recurrence rates of specific somatic alterations in DNA methylation support a strong selective pressure for these events and implicate them in the development of neoplastic phenotypes and as rate limiting steps in disease evolution [12, 13]. Cytosine methylation is the most widely studied epigenetic marker in cancer due to the development of quantitative genomics methods that are compatible with tissue samples obtained from surgical specimens. To date most studies have used prostate cancer cell lines when profiling chromatin structures and histone modifications [14, 15, 16] and other variants of cytosine modification have only been assessed at a global level in primary prostate cancer tissue [17]. Therefore for the purposes of this review we will restrict our focus to the wealth of studies that have profiled cytosine methylation in prostate cancer.

1.2. Considerations for DNA methylation profiling in prostate cancer

DNA methylation is a stable, heritable genome modification that can provide insights into a tumour's origins and evolution. Methylation profiling is aided by the number of well-developed techniques and analysis methods available. It is aided also by the requirement only for standard preparation of genomic DNA as input, making it applicable to routine tumour tissue collections (i.e. in contrast to methods that require cross-linked chromatin).

A range of methylation profiling methods have been developed, from cis-linked, base-pair resolution bisulfite sequencing of the whole genome (WGBS [18]) or GC-base enriched regions (eRRBS [19, 20]), to array based averaging of methylation at specific CpG sites [21, 22], to locus-averaging methods that identify methylation 'peaks' (me-DIP [23, 24]) or that may be a proxy for functional methylation changes (e.g. MBD pull-down [25, 26]). The most widely used platform for studies of clinical tissue samples is the Infinium 450k array [21], that continues to be used due to its reproducibility, well developed analysis methods and consequent potential for integration with a wealth of published data from this platform [27, 28].

Sequencing methods provide the highest resolution profiles and cis-linkage information about the status of adjacent CpGs on the same strand, but have the largest analytical burden. Standard bisulfite sequencing methods do not,

however, discriminate between methyl-cytosine (5-mC) and hydroxy-methyl-cytosine (5-hmC), although these marks are believed to have different functional consequences. To discriminate 5-hmC and 5-mC a two stage analysis is required comparing the results of bisulfite sequencing (for a combined 5-hmC and 5-mC signal) and oxidative bisulfite sequencing (for 5-hmC alone), followed by subtractive analysis. Alternatively, me-DIP approaches using 5-hmC and 5-mC specific antibodies can also discriminate these signals to provide locus-averaged signals. Future improvements in sequencing yields and sensitivities for single molecule sequencing platforms may provide different insights into the epigenetic landscape, for example long read technologies may allow better phasing of epigenetic states along chromosome domains. Recent reports suggest that nanopore-based sequencing technologies may be able directly to read the 5-mc or 5-hmc modifications of cytosine [29].

The DNA methylation landscape varies across the genome, generally showing higher methylation at repeat sequences and retrotransposons compared to lower methylation at active gene promoters and CpG-islands [18, 30]. High levels of DNA methylation at gene promoter regions (and around the transcription start site) correlates with low gene expression [12, 18]. Within a gene locus the methylation profile can vary widely (Figure 1A), meaning that comparisons between samples (i.e. differential analysis) must rely on either comparisons of individual CpG sites or by defining local methylation domains (e.g. differentially methylated regions, DMRs) [31]. CpG-islands have low DNA methylation variance in cancer, while adjacent regions (termed CpG-shelves and CpG-shores; Figure 1B) tend to show higher variation. Most recently locally disordered methylation or epipolymorphisms have been reported [32, 33] and linked to evolutionary plasticity in cancer, as previously suggested for epigenetic variation [34, 35, 33].

The selection of samples for cancer genome sequencing is usually simple because the aim is to identify somatically acquired changes (e.g. comparing tumour tissue with a germline control sample - often blood or buccal swabs). However, tissue specific methylation profiles mean that the most appropriate control sample for cancer methylome studies is normal tissue from the same organ. More stringently, one might aim to match the proportions of cell types (e.g. epithelial, stromal, immune) in the tumour and normal tissue samples. In many cancer types (including prostate cancer) a 'field-effect' change has been observed in the tumour adjacent normal tissue, consistent with a pre-neoplastic state. Therefore, depending on the study aims it may be most appropriate to compare epigenetic state between tumours, tumour adjacent

normal tissue and age-matched tumour-free normal tissue.

1.3. The data used in this review

Through this review we will illustrate key points using previously published data sets. For ease of comparison, and due to their greater number, we will focus solely on data generated using the Illumina Infinium HumanMethylation450 BeadChip. For individual genes, and to relate methylation levels to gene expression we will use the 'TCGA' prostate adenocarcinoma data [28], interrogated and plotted using the TCGA Wanderer interface [36]. For consistency we use Wanderer's associations of probes to genes throughout, although this naturally leads to probes mapping to multiple genes.

For the second data set, the 'Tissue' data set, we amalgamate data from several sources [26, 37, 27, 38, 39] to obtain methylation statuses for prostates from men with no prostate cancer ("Normal"), morphologically normal tissue from men with prostate cancer ("Benign"), benign prostatic hyperplasia ("Hyperplasia"), neoplastic tissue ("Neoplasia"), primary tumours ("Tumour") and metastases ("Metastasis"). We also obtain blood profiles [39] as an additional reference.

For Figure 3A, where space is a constraint, we use only a subset of these drawn from two sources [26, 37]. The third data set, the 'Cell line' data set consists of the combined HumanMethylation450 data detailed later in Table 1. Finally, to annotate genes with androgen receptor (AR) regulation data, we use two previously-published androgen-treated cell line time-course data sets [40, 39].

2. Recurrent epigenetic changes in prostate cancer: markers and drivers of disease evolution

Recurrent alterations in DNA methylation at the *GSTP1* gene promoter and concomitant loss of GSTP1 expression in prostate tumours were reported over 20 years ago [12]. This finding has been replicated in countless independent studies (for example [9, 41, 42]) and well over 1000 samples (reviewed in [43]), providing strong evidence that DNA methylation changes are indeed recurrent across patient cohorts and could be useful markers for the clinical detection of prostate cancer [9, 44].

Several other genes have also been reported to be recurrently hypermethylated in prostate cancer by multiple studies Synthesizing data from 17 studies [26, 45, 27, 28, 10, 46, 2, 47, 48, 20, 24, 49, 38, 22, 50, 51, 52], we

identify 861 genes that are reported in two or more studies, 168 in three or more (detailed in Supplementary Table 1 and Supplementary Figure 1), and 45 that are reported in four or more studies (Figure 2). Some gene families are also recurrently affected, consistent with functional convergence, including multiple changes at the *HOX* gene family loci (Figure 2, Supplementary Figure 1) [10, 49].

2.1. Early epigenetic changes in prostate carcinogenesis

The high recurrence rates of these DNA methylation changes suggest that they may be early events in tumourigenesis. Indeed several studies have detected many such methylation changes in neoplastic samples (PIN) and tumour adjacent, morphologically benign tissue [53, 54, 27]. Indeed the majority of loci that have been suggested as differentiating benign and cancerous prostate appear already to have undergone epigenetic changes in neoplastic tissue (Figure 2) impacting on their potential as prostate cancer markers, but highlighting early or shared events in cancer evolution.

Prospective multi-region sampling studies with good clinical annotations are therefore needed to map tumour specific markers comprehensively, in order to improve diagnostic accuracy from tissue biopsies and non-invasive monitoring. Given the current over-treatment of primary prostate cancer it is also imperative that robust panels of markers are developed to allow patient stratification for active surveillance or clinical intervention.

2.2. An epigenetic 'field-effect' in cancerous prostates

Recent studies suggest that DNA methylation changes in tumour adjacent 'normal' tissue may reflect a 'field effect' in cancerous prostates [50, 24, 27]. DNA methylation profiles have been reported to differ between tumour adjacent benign tissue and benign tissue from cancer-free prostates [50, 24], while benign samples taken at different distances from prostate tumours show similar profiles, supporting a wide clonal expansion of morphologically normal cells [50].

This observation is consistent with the outgrowth of tumour clones originating from clonal benign and PIN tissue, a concept supported by a recent study comparing multiple benign, neoplasia and tumour samples from the same cancerous prostates [27]. In this study a common phylogenetic 'trunk' could be identified using either copy number or DNA methylation profiles, linking tumour samples, PIN and adjacent normal prostate samples. This indication of a shared clonal ancestry contrasts with the more sparse data from

genome sequencing studies, where few point mutations, indels or structural variants link separate tumour foci and pre-cancerous tissues [5], suggesting that the expansion of genetically mutated clones is a later event than the expansion of clones harbouring DNA methylation alterations.

Further studies are required to define more clearly the early neoplastic and tumour initiating events and also comprehensively to distinguish early events from convergent evolution. Longitudinal monitoring through the life-history of a patient with prostate cancer would be required to give a definitive answer to these questions, although this would be very difficult to achieve. An alternative approach would be to combine multi-region tissue sampling cohorts with base-pair resolution methylation sequencing to distinguish early events from convergent evolution. Understanding this would impact on the utility of these changes both as markers of early tumourigenesis and as targets for preventative medicine.

2.3. Underlying clonal stability and ongoing epigenetic evolution in prostate tumours

GSTP1 methylation is present at all stages of prostate cancer development [51], showing that specific epigenetic changes can be maintained throughout disease evolution. In addition, methylome-wide studies have shown stable epigenetic profiles between metastatic deposits within a patient [26] and clear evidence of shared origins for metastatic deposits in primary tumours and premalignant lesions [27]. Interestingly, in most cases neoplastic lesions (PIN) were evolutionarily more similar to localized tumour samples, whereas metastatic deposits were often more closely related to a separate subset of localized tumour samples [27].

Hypomethylation of repeat sequences and cancer testes antigens have been suggested to be relatively late events in prostate cancer development [55, 30]. In addition, metastatic sites have been reported to show greater divergence for DNA hypomethylation within some patients [30].

Studies showing clonal stability of DNA hypermethylation, and evidence of a subset of clones that are more closely related to metastatic disease, support the idea of using DNA methylation markers for prostate cancer detection and prognosis.

2.4. DNA methylation markers for the detection and stratification of prostate cancer

Combined panels of candidate DNA methylation markers have been shown to have high sensitivity and specificity for the discrimination of prostate cancers from benign tissue [51, 38], with more recent studies showing proof of principle in prostate biopsy material [38]. Several studies have shown the potential for non-invasive monitoring of DNA methylation in cell-free DNA to detect prostate cancer [56, 57, 58, 59]. Many of these studies show remarkable sensitivity and support the use of these tests for monitoring disease progression, however larger studies will be required to determine the clinical utility of these promising tests for prostate cancer diagnosis.

A molecular stratification for prostate cancer was proposed recently [28]. This large study found associations between genomic alterations and epigenetic profiles, that may represent a phenotypic difference between these molecular subtypes of prostate cancer. One third of ERG-positive tumours clustered together with a distinct hypermethylation profile [28]. The one percent of prostate cancers that harbour mutations in the gene encoding isocitrate dehydrogenase (IDH1) were found to have a divergent genome-wide hypermethylation profile [28]. This is likely to be a result of IDH1-R132H mutations driving production of the oncometabolite 2-hydroxyglutarate (2HG), with a reciprocal decrease in alpha-ketoglutarate, a key component in the metabolic pathways of DNA methylation (the upstream metabolic processes are described in Section 3). It is currently unclear whether these molecular subtypes have clinical utility, what other factors impact on the observed methylation profiles, or what the phenotypic consequences of these epigenetic differences might be.

More effective diagnosis of prostate cancer would alleviate some of the current burden on health systems and decrease invasive procedures on healthy men. However, it is also critically important to distinguish indolent from aggressive prostate cancers so that aggressive treatments can be appropriately allocated to those patients who require such interventions, sparing other patients unnecessary over-treatment. Associations between DNA methylation changes and prognosis have been reported, including the correlation of *PTGS2* (*COX-2*), *HOXD3* and *ABHD9* hypermethylation with recurrence [51, 22].

One study aiming to identify prognostic methylation markers for prostate cancer highlighted over one hundred candidate genomic loci [49]. However, the discrimination between relapsed and non-relapsed samples was far weaker

than between tumour and benign samples [49], indicating more subtle differences between these groups. Among the candidate prognostic markers only *PTGS2* (*COX-2*) was validated from the previously mentioned studies. However, other studies have reported more promising findings by combining pilot genome-wide screening with targeted approaches on large test and validation cohorts [60]. A three gene methylation signature (*AOX1*, *C1ORF114*, *HAPLN3*) was able to predict biochemical recurrence with respective hazard ratios of 1.9 and 2.3 in test and independent validation cohorts [60].

By taking a different approach and examining morphologically normal tissue adjacent to tumours, the methylation status of *GSTP1* and *APC* has been reported to have prognostic utility [61]. *GSTP1* and *APC* methylation in tumour adjacent tissue correlated with the methylation of these loci in matched tumour samples [61], consistent with either shared clonal ancestry or convergent evolution. Either hypothesis to explain these DNA methylation changes in tumour adjacent tissue would support a field-effect in a subset of prostate cancers that may impact on outcome. These results are consistent with other reports of an epigenetic field effect (summarized in Section 2.2) and confirm other reports that epigenetic profiling could be a useful tool to avoid false-negatives in diagnostic biopsies [62].

In addition to these important targeted studies in large cohorts there is a need for methylation profiling studies that implement sensitive, genome-wide methods across samples representing the full range of prostate cancer disease stages to provide a clearer picture of the likely diagnostic and prognostic utility of these and other DNA methylation markers for prostate cancer.

2.5. Epigenetic regulation of alternative promoter usage in prostate cancer

In addition to gene silencing, DNA methylation can modulate gene isoform expression by impacting on alternative promoter regions. For example DNA methylation at the *RASSF1*, *APC* and *NDRG2* loci were shown to result in differential isoform expression [46]. This isoform selective expression was actively enforced by the epigenetic status at these loci, as shown by reversal of the isoform expression profile upon treatment with the demethylating agent 5-aza-cytidine [46]. Similar events have been reported in other studies in prostate cancer [63], however the true extent of this feature will only be made clear by large cohort studies combining comprehensive methylation profiling with RNA-sequencing on the same samples. An exemplar study leveraged the large RNA-sequencing gene expression data sets generated by TCGA groups to identify isoform switching in multiple tumour types

(including prostate cancer), and speculate that epigenetic factors could be responsible [64]. In this study, tumour samples could be accurately identified solely by isoform switching signatures, highlighting the potential for isoform switching as a marker for prostate cancer.

2.6. Associations between epigenetic and genetic alterations in prostate cancer

Given the early and recurrent acquisition of *GSTP1* hypermethylation in prostate tumourigenesis many studies have suggested a role for *GSTP1* silencing in driving disease evolution by increasing the mutation rate [65, 66, 67, 68]. Recent studies integrating methylation profiling with genome sequencing have uncovered additional associations between the epigenetic and genetic changes in prostate cancer.

Firstly, it has been shown that the methylation levels in matched benign samples are increased at mutated CpG sites in the tumour in comparison to non-mutated CpGs. [20]. This is consistent with the hypothesis that methylated cytosines are deaminated to uricil (and subsequently copied as thymine), a process believed to drive the observed high C-to-T mutation rates observed in prostate and other cancer types [69, 5].

More surprisingly, it has been reported that sites of tandem duplication events in prostate cancer are frequently hypomethylated, while interchromosomal translocation break points are frequently hypermethylated [20]. These intriguing observations will need to be investigated in larger cohorts with paired methylome sequencing and genome sequencing to better characterize these associations.

Several studies have also suggested a link between *ETS* gene fusion status and DNA methylation profiles [46, 45, 48, 28]. LINE repeats show differential methylation between ERG-positive and ERG-negative prostate tumours [46], differentially methylated regions associated with ERG status have been identified [48] and alternative mechanisms for *EZH2* activation have been proposed in ERG-negative tumours to phenocopy at least some of the consequences of *ETS* gene fusions [45]. The implications of molecular subtype differences in DNA methylation profiles are significant. Differences between *ETS*-fusion positive and negative cases highlight the interplay between epigeneic state, gene rearrangements and hormone signalling, since the AR regulates *ETS*-fusions and AR signalling is altered in ETS-positive tumours [15]. Equally, these effects could lead to misinterpretation of studies that cannot take them into account.

2.7. Epigenetic changes impacting on hormone signalling in prostate cancer

In addition to interactions with genomic events, the epigenetic profile has been linked to AR signalling, both as a modulator of hormone response and a driver event in progression to Castration Resistant Prostate Cancer (CRPC). AR-bound enhancers were observed to show greater intratumoural DNA methylation variation than other enhancer sites [27], suggesting clonal plasticity in the AR regulome.

An integrative analysis of copy number and DNA methylation in CRPC revealed convergence on the androgen synthesis pathway, with copy number and methylation changes converging on *HSD17B2* and other targets that may be involved in hormone therapy resistance [10].

Loss of AR protein expression is a characteristic of hormone-relapsed Prostate Cancer (PCa) that is no longer dependant on AR signalling, a phenotype that is becoming more common following the clinical use of second generation AR targeting therapies [70, 71]. Epigenetic silencing of *AR* gene expression has been reported in prostate tumour samples [72, 73] and treatment of AR negative PCa cells with the global demethylating agent 5-aza-deoxycytadine can induce functional AR signalling in these cells [73, 74, 75, 76]. However, other studies in CRPC have reported no change in *AR* promoter methylation [10], suggesting either differences between analysis methods or possibly patient cohorts treated with different hormone therapies.

Combining androgen signalling blockade with the demethylating agent 5-aza-deoxycytadine increased response in the preclinical models of PCa [77, 75], suggesting that future studies combining demethylating agents with second generation AR blockade may improve patient outcome or delay relapse by targeting the AR, its target genes or upstream pathways.

Oestrogen receptor alpha and beta methylation have also been identified in some studies of prostate cancer samples [78, 51, 46, 26]. Although these methylation changes at *ER* genes are not highlighted in the majority of studies it is noteworthy that studies reporting *ER* gene methylation used MSP [51], MBD-pulldown [26] or restriction enzyme based methods [46] (consistent with either limitations of the more commonly used 450k arrays at these loci or convergent artefacts between the other methods). Future studies should aim to assay DNA methylation at the *ERS1* and *ESR2* loci in prostate tissues to determine whether this could affect the interplay between nuclear hormone receptors and be another mechanism through which epigenetic changes impact on hormone signalling in prostate cancer.

A large number of these putative epigenetic markers were identified by methylation profiling of prostate cancer cell lines. In addition these cell line models are the most widely used tools for functional validation studies of candidate gene silencing or de-repression events identified from genome-wide profiling studies. Therefore we summarize the utility and limitations of these models below.

2.8. Prostate Cancer Cell lines

It has for some time been recognized that immortalized cell lines do not, in respect of their methylation patterns, reflect the cells from which they originate; typically immortalized cells exhibit hypermethylation of CpG islands [79, 80, 81] although it has been noted that this may represent selection pressure where highly methylated cells are more likely to be immortalized successfully [82] and there are suggestions that the methylation changes can predate immortalization [83].

The characteristic methylation changes that occur in immortalized cells are similar to the changes seen in cancers [84, 85] raising the hope that the epigenetics of cancer cell lines may represent malignant tissue well. Recent reports argue that it is specifically the immortalization of cells rather than other oncogenic activity that leads to changes in methylation profile [86]. Intriguingly, sites that are methylated in cancer cell lines are enriched for NANOG binding sites [87]. As well as its role in maintaining stem cell pluripotency, NANOG has been shown to be pro-tumourigenic in prostate cancer cell lines, conferring cancer-stem-cell-like properties [88], and is directly androgen regulated [89].

Early targeted studies identified good agreement between primary cancers and cell lines [90, 91], but broader differences may mean that only a minority of tumours are well-represented by cell lines. Genome-wide profiling has revealed xenografts to be a better model in head-and-neck squamous cell carcinomas [92], while a recent paper has reported near-complete loss of 5-hydroxymethylcytosine [93] - raising questions about cell lines' value in this regard.

The utility of prostate cell lines is further affected by the fact that the commonly used prostate cancer cell line models (recently reviewed elsewhere [94]) were derived from metastases, and metastasis itself is associated with changes in methylation profiles [95, 96]. Nevertheless, substantial public data exist for a number of prostate cell lines as denoted in Table 1, notably LNCaP, PC3 DU-145 and PrEC, making them an attractive resource.

In Figure 3 A, we cluster the available Illumina Infinium HumanMethylation450 BeadChip cell line data with our example Tissue data set. It is notable that inter-sample heterogeneity increases as one progresses through normal, benign, tumour and metastasis samples. Reflecting their origins, the prostate cancer cell lines are more alike the metastasis samples than they are the primary tumour samples, while the PrEC cells cluster with the normal samples.

It should be noted that all of the cell lines show levels of agreement with primary tumours that are substantially above chance, and that their greater similarity to metastases only requires care to be taken over the interpretation of any results arising. The cell lines still reflect the behaviour of primary tumours at key loci such as *GSTP1* (Figure 3B), although at loci such as the promoter of *TERT* there appears to be progressively greater DNA methylation as one moves from normal tissue, through hyperplastic and neoplastic tissues to primary tumours and metastases, the malignant cell lines showing greater values still (Figure 3C).

Despite the inevitable caveats about the use of cell lines, they offer natural advantages for the inference of function. They enable one to run controlled experiments with identical subjects in each/every arm, and make it possible to measure multiple characteristics (e.g. genome-wide methylation and transcript abundance) on effectively the same samples. Both of these have been exploited to address questions of prostate cancer biology.

To understand better the methylation-driven regulation of the cancer genome, mRNA expression data are the natural orthogonal data to bring into an integrative analysis. In this manner, the methylation-regulated nature of key genes and alternative transcriptional start site usage have been explored in prostate cancer [46, 97].

A substantial body of work has linked DNA methylation with other epigenetic marks better to understand gene regulation in (prostate) cancer. The H3K27me3 mark at promoters is associated with inactivated genes, while methylation of promoters is similarly associated with gene repression, but the two mechanisms have been seen to be neither exclusive nor deterministically linked in LNCaP [98]. Where the two mechanisms do coexist, a dual therapy to reinstate expression of tumour suppressor genes becomes a possibility [99]. Elsewhere it has been shown that a genome-wide restructuring of nucleosome densities is associated with changes in DNA methylation of enhancer regions of PCa cell lines [100].

Other studies have sought to explain epigenetic mechanisms of treatment

and resistance to treatment. Epigenetic silencing of SLFN11 has been associated with resistance to platinum-based chemotherapies in a number of cell lines including DU-145 and PC3 [101], an epigenetic mechanism of the preventative agents sulforaphane and 3,39-diindolylmethane is elucidated [102], and the mechanism of Genistein has been shown not to be dependent on broad methylation changes, but rather histone acetylation [103].

Perhaps most interestingly for our topic, a recent report has shown that dosing cells with S-adenosylmethionine (SAM) inhibits invasion [104]; the same group having previously identified a role for hypomethylation in the metastasis of prostate cancer [105]. SAM is an important methyl donor for histone, DNA and RNA methylation, and all general protein lysine and arginine methylation (as described in Section 3). The demonstration, in vitro and in vivo, that replenishing the reservoir of methyl donors can inhibit metastasis (while only increasing the methylation status of specific loci rather than reversing the genome-wide hypomethylation) provides some evidence of a mechanistic role for the methylation patterns in metastases, highlights the importance of the available metabolic pool for cancer progression, and invites consideration of the broader role of SAM in the metabolic pathways.

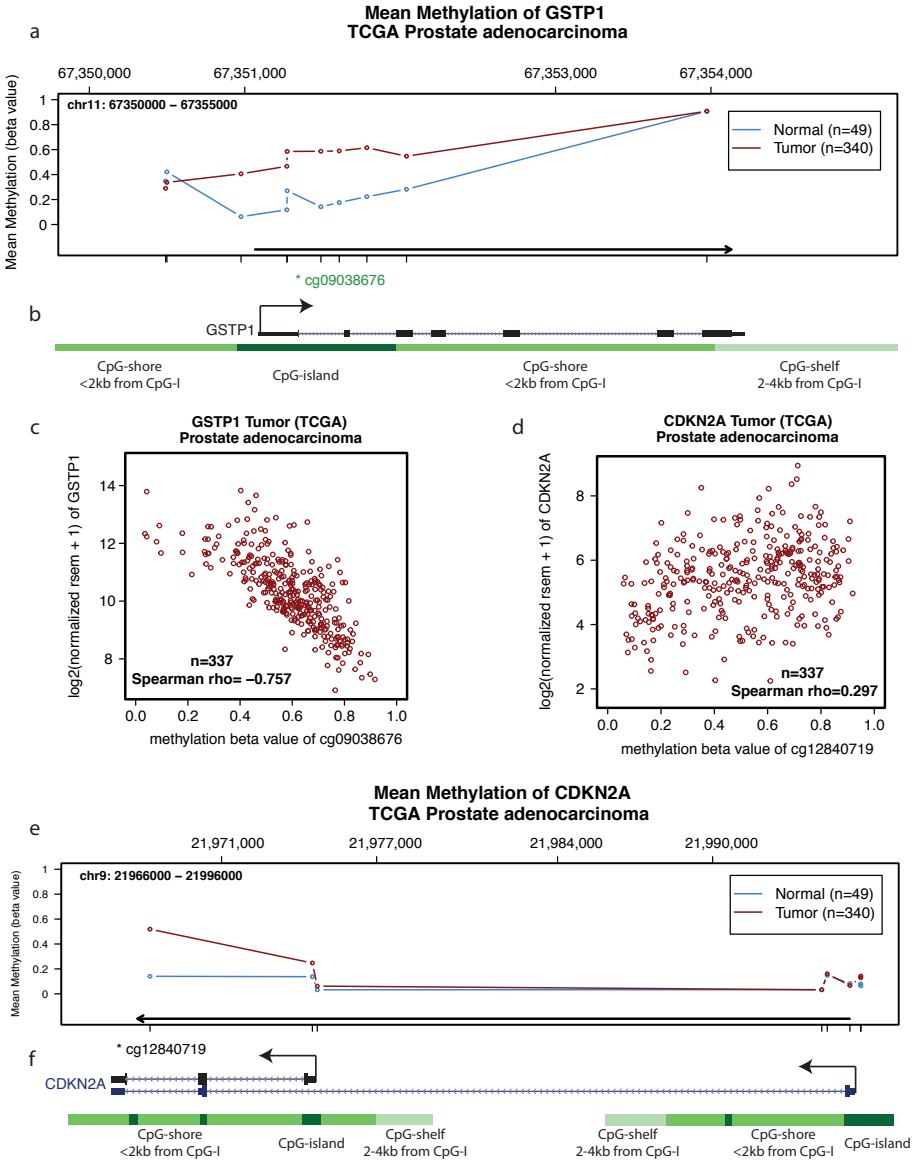

Figure 1: A. Average methylation profiles for prostate tumours (n=340) and normal prostate tissue (n=49) at the *GSTP1* gene locus using the TCGA data set (see section 1.3). B. Schematic showing the *GSTP1* gene locus, indicating the location of the CpG-island, CpG-shore (<2kb from island) and CpG-shelf (2-4kb from island). C. Correlation scatter plot for *GSTP1* expression and methylation (using the 450k array probe highlighted in panel-A). D. Correlation scatter plot for *CDKN2A* expression and methylation (using the 450k array probe highlighted in panel-E). E. Average methylation profiles for prostate at the *CDKN2A* gene locus using the Prostate TCGA data set (see section 1.3). F. Schematic showing the gene and CPGI features at the *CDKN2A* locus.

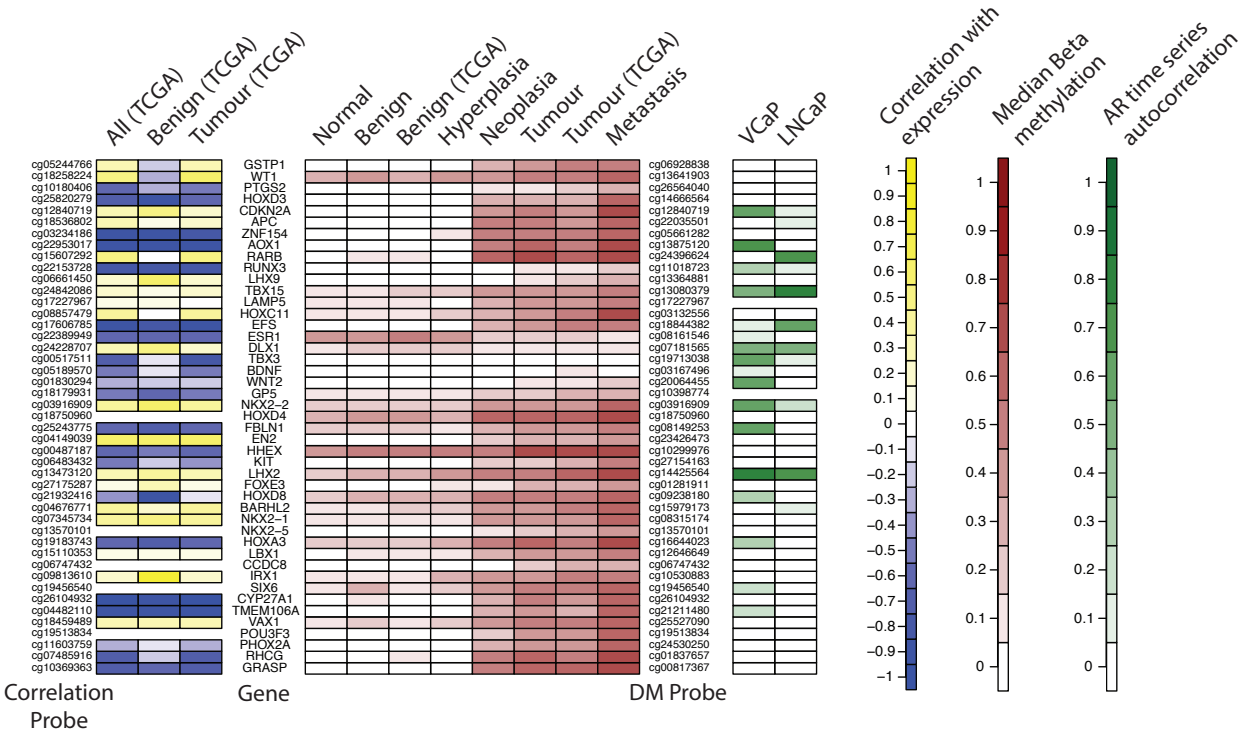

Figure 2: Heatmap summary of genes and marker probes associated with DMRs reported in four or more studies. Left panel, correlations between gene expression (RNA-seq) and DNA methylation (450k arrays), from the PRAD TCGA data set (450k probe IDs indicated on the left). Middle panel, average methylation levels (Beta-values) from multiple studies spanning a range of prostate tissue types (450k probes indicated on the right). Right panel, androgen-stimulated gene expression changes in two prostate cancer cell lines (autocorrelation values denote a change with time following stimulation - a value of zero indicates no systematic change after stimulation). Missing data are indicated with a cross where not obvious.

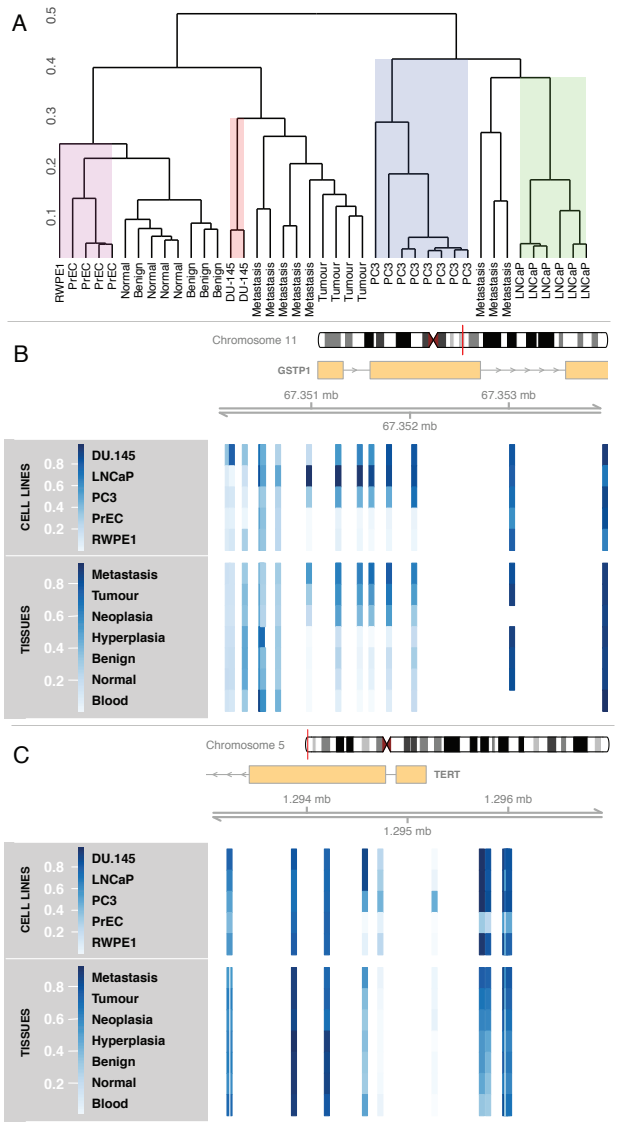

Figure 3: Cell lines as a representation of primary tumours. A) Clustering of our example cell line and tissue data sets (described in Section 1.3). The distance between two samples is defined as Cohen's Kappa measure of agreement (applied after dichotomizing methylation beta values). B) Illustrating, from the cell line and tissue data sets, the median proportion of methylation at loci near the *GSTP1* promoter. As expected, the cancer cell lines are generally hypermethylated in this region, as are the neoplastic and malignant tissues. C) Illustrating, from the cell line and tissue data sets, the median proportion of methylation at loci near the *TERT* gene. An area in the gene promoter shows progressively increased methylation levels, with the cancer cell lines levels most in keeping with metastases.

	GoldenGate	27k	450k	Nimblegen	MBD-seq	RRBS	NOMe-seq	MRE-seq	MEDIP
DU-145	GSM1125684, GSM696035	GSM573668	GSM1633638, GSM1323599		SRX118022, SRX118022, GSM1050087, GSM1050090, GSM1050093, GSM1050096, GSM1050099	GSM1050102			
PC3	GSM1125685, GSM696036	GSM573670	GSM1633598, GSM1519011 to GSM1519016*, GSM1323600	GSM1142996 to GSM1143004	GSM1050088, GSM1050091, GSM1050094, GSM1050097, GSM1050100	GSM1050103	GSM1383852		
LNCaP	GSM1125683, GSM696034	GSM573669 (FGC)	GSM1519017, GSM1519018, GSM999368, GSM847569 to GSM847571	GSM1142987 to GSM1142995*	SRX118021, GSM605080, GSM605081	GSM683768, GSM683776, GSM683862, GSM683863, GSM683924, GSM683946		GSM684592, GSM684597 to GSM684600	GSM605948 GSM605950, GSM605954 to GSM605956
ARCaP	GSE35246 (27 samples*)								
PrEC	GSE35246 (4 samples*)		GSM999369, GSM847572 to GSM847574	GSM1142978 to GSM1142986*	GSM605082, GSM605083	GSM683760, GSM683838	GSM1383851	GSM684593 to GSM684596	GSM605945 to GSM605947, GSM605951 to GSM605953
PrED					SRX118020				
RWPE-1		GSM573671	GSM1323601						

Table 1: **Public prostate cell line methylation data (January 2016). Detailing the data available for common prostate cell lines from Gene Express Omnibus (GSM/GSE) and the Short Read Archive (SRX). * - includes control and treated cell lines. Platforms included include Illumina Goldengate Methylation Cancer Panel I ("GoldenGate"), Illumina Infinium HumanMethylation27 BeadChip ("27k"), Illumina Infinium HumanMethylation450 BeadChip ("450k"), Nimblegen Human DNA Methylation 3x720K CpG Island Plus RefSeq promoter array ("Nimblegen"), methyl-CpG binding domain protein-enriched genome sequencing ("MBD-seq"), Reduced representation Bisulfite sequencing ("RRBS"), Nucleosome Occupancy and Methylome sequencing ("NOMe-seq"), Methylation-sensitive Restriction Enzyme Sequencing ("MRE-seq"), and Methylated DNA Immunoprecipitation Sequencing ("MEDIP").**

3. Effects of the metabolic pool on DNA methylation

The prostate gland is a metabolically specialized organ responsible for supporting sperm viability. This specialization is characterized by net secretion of citrate into the seminal fluid [106]. Whilst a significant reduction in the production of these metabolites has been reported in numerous magnetic resonance spectroscopy studies on clinical samples, the molecular drivers for this down-regulation remain controversial [107, 108, 109, 110]. Of the polyamines normally produced by the prostate gland, spermine is particularly abundant [111]. Rat models of castration-induced regression and testosterone-induced regrowth of the prostate gland have shown that polyamine production is tightly regulated by androgens, in part through control over the expression of key biosynthetic enzymes (ornithinedecarboxylase (ODC), S-adenosylmethionine decarboxylase (AMD1) and spermidine synthase (SMS); highlighted in Figure 4 and Figure 5) [112, 113, 114]. The methyl donor in this pathway is S-Adenosylmethioninamine (decarboxy-AdoMet), a metabolite that is directly downstream of the DNA methylation donor S-Adenosylmethionine (SAM, AdoMet). Therefore, alterations in the flux through either polyamine metabolism or DNA methylation would be expected to affect the available pools of methyl-donors, with reciprocal effects on the flux of the other pathway.

The prostate has one of the highest concentrations of polyamines of any tissue in the body and the expression of these enzymes is associated with glandular epithelial cells with significant quantities of polyamines secreted into seminal fluids. Spermine levels in clinical samples have been reported to correlate positively with the differentiation status of the tumour and in preclinical models to promote growth inhibition [115, 116].

Polyamine synthesis requires one-carbon metabolism and in particular methionine metabolism with S-adenosylmethionine (SAM) which upon decarboxylation acts as the primary aminopropyl donor for polyamine synthesis (Figure 4) [117]. Consequently the fate of methionine and its derivatives may be influenced by changes in the specialized secretory functions of the prostate gland as cancer emerges, in particular a decline in polyamine biosynthesis and secretion may enhance the pool of SAM available to support epigenetic modifications (metabolic pathway links shown in Figure 4). Proving causative associations between rates of polyamine biosynthesis and regulation of the epigenetic state via the availability of SAM is extremely challenging, since methylation patterns can be highly locus and cell-type de-

pendent and are supported by a complex regulatory network downstream of metabolite consumption and upstream of DNA substrates. Furthermore, one-carbon metabolism consists of a number of additional interconnected metabolic processes that may impinge on polyamines and the epigenome (folate cycle, methionine cycle and glycine/serine metabolism, Figure 4).

Prostate cancer is characterized by the activity of transcription factors, particularly AR but also others such as c-Myc. A natural question then is how the transcription factors and important enzymes interact with the metabolome and epigenetic status of a tumour. As indicated earlier, the synthesis of polyamines and the expression of the key enzymes required for this and a number of other metabolic processes upstream of SAM production are driven by AR and associated with differentiated prostate cancers (Figure 4 and Figure 5).

Glycine N-methyltransferase (GNMT) is an enzyme which converts glycine to sarcosine and in the process converts SAM to S-adenosylhomocysteine (Figure 4). In cell-lines expression of the GNMT enzyme is androgen dependent and in tissue samples it has been shown to be over-expressed in prostate cancers (Figure 4 and Figure 5) [118, 40]. Sarcosine, the product of the reaction catalysed by GNMT, has also been reported to be elevated and detectable in urine samples and some studies have associated this with the emergence of castrate-resistant disease [119, 120]. Of the enzymes involved in one-carbon metabolism GNMT is therefore currently the most extensively characterized androgen-dependent component of the pathway. By contrast the expression of enzymes required for serine metabolism appears in prostate cancer cell-lines to be repressed by androgens and in other cancer models to be induced by c-Myc [40, 121]. Whilst serine metabolism also impacts on the methionine cycle, the most direct impact of serine consumption appears to be to sustain de novo nucleotide biosynthesis in support of elevated rates of DNA replication and/or transcription and cell proliferation[122]. This in turn is often a feature of cell cycle dysregulation, characteristic of late-stage, metastatic prostate cancer [123]. Hypothetically a phasic transition in one-carbon metabolism may therefore consist initially of reduced rates of polyamine biosynthesis and enhanced DNA and histone methylation in localized disease transitioning into enhanced serine metabolism during metastatic progression. This would at least be consistent with the observations that DNA hypermethylation is an earlier event in prostate tumourigenesis, while hypomethylation may occur in later stage disease (as described in Section 2).

The DNA methylation status of a subset of genes encoding these metabolic

enzymes increases between normal samples compared to benign, tumour and metastasis, consistent with early hypermethylation changes (Figure 5). A separate set of enzyme-encoding genes show hypomethylation in metastatic samples compared to localized and pre-malignant samples (Figure 5), again consistent with genome-wide observations of later stage hypomethylation (Section 2). It is of interest that there appears to be exclusivity of regulation within the metabolism-related genes illustrated here (Figure 5), with AR-regulated genes showing little evidence of differential methylation, and the strongest differentially methylated genes not being AR-regulated. This despite the independence of the data sets from which these characteristics were identified.

In addition to the potential influence of upstream enzyme expression and metabolite pools on DNA methylation, the de-methylation pathway is intricately linked to central metabolism. The TCA cycle metabolite alpha-ketoglutarate is required for TET enzyme activity, the first step in cytosine demethylation (Figure 4). Therefore, mitochondrial function may have a profound effect on both cytosine methylation and the levels of hydroxymethylation in the genome of a cell.

4. Mitochondrial methylation and prostate cancer

4.1. Importance of mitochondria in PCa

While of general interest in cancer (reviewed, for example by Wallace [124]), mitochondria are of specific interest in prostate cancer beyond the characteristic zinc-inhibition of TCA cycle and AR regulation of certain metabolites described in the previous section. Recent 'pan-cancer' analyses have identified many mutations in mitochondrial DNA, without showing them to be drivers [125, 126], but animal models have demonstrated the functional impact of mitochondrial genetic mutations on prostate cancer [127] and a cohort study in humans has identified associations with proliferation [3].

The case for DNA methylation changes in the prostate cancer mitochondria themselves is not as clear cut as that for genetic changes. Methylation of mitochondrial DNA has been controversial since it was first reported [128]. Subsequent papers variously confirmed this result [129] or reported no such methylation [130]. The discussion has continued since with arguments for and against based on sequence analysis [131, 132], and use of methylation sensitive and insensitive restriction enzymes [133, 134]. One aspect of the argument against the existence of methylation in the mitochondria has

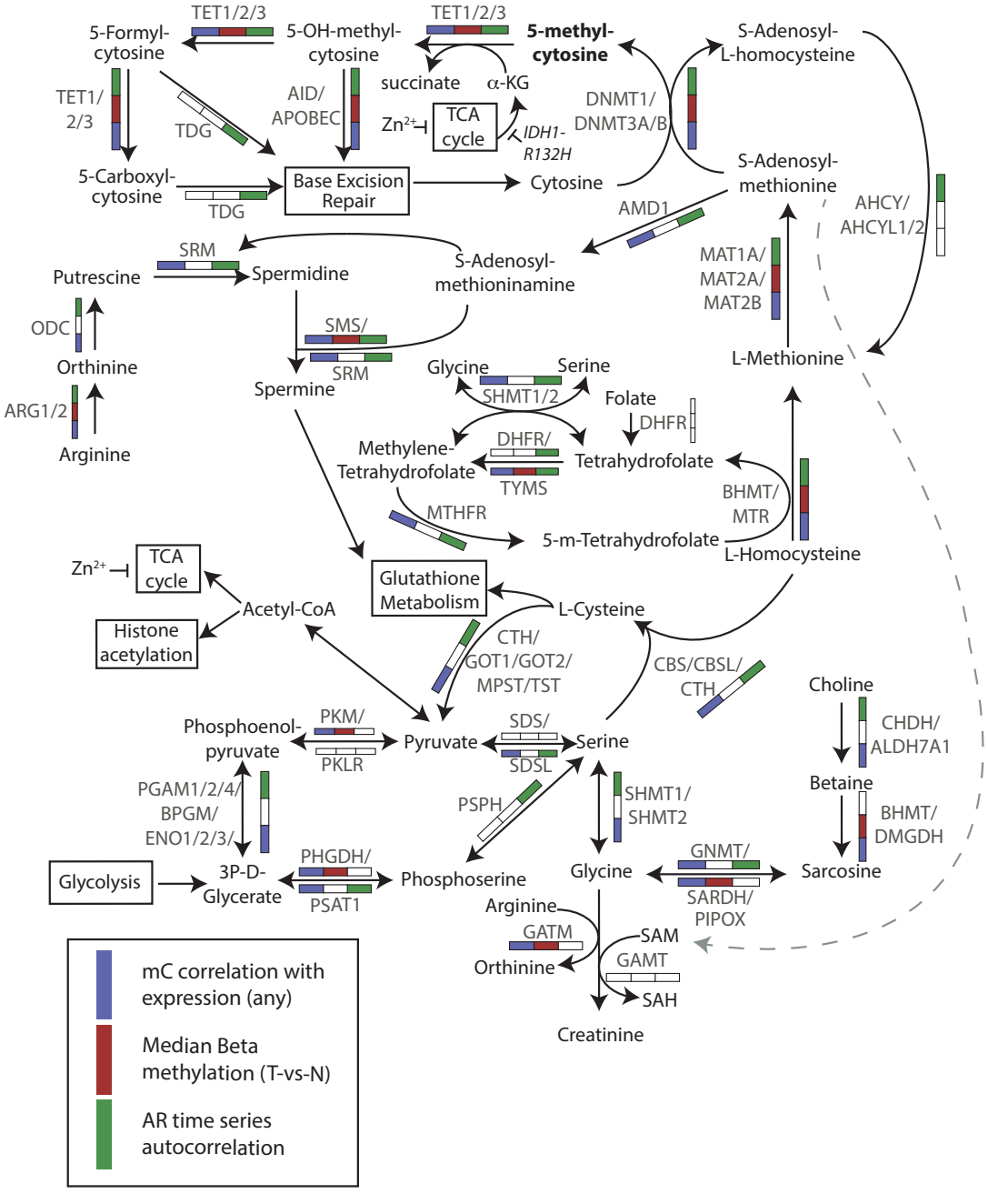

Figure 4: Schematic representation of metabolic pathways that impact on DNA methylation. The core DNA methylation pathway is represented at the top of the schematic, with connected pathways of relevance to prostate cancer shown below. Colour key relates to metadata annotations shown in Figure 5.

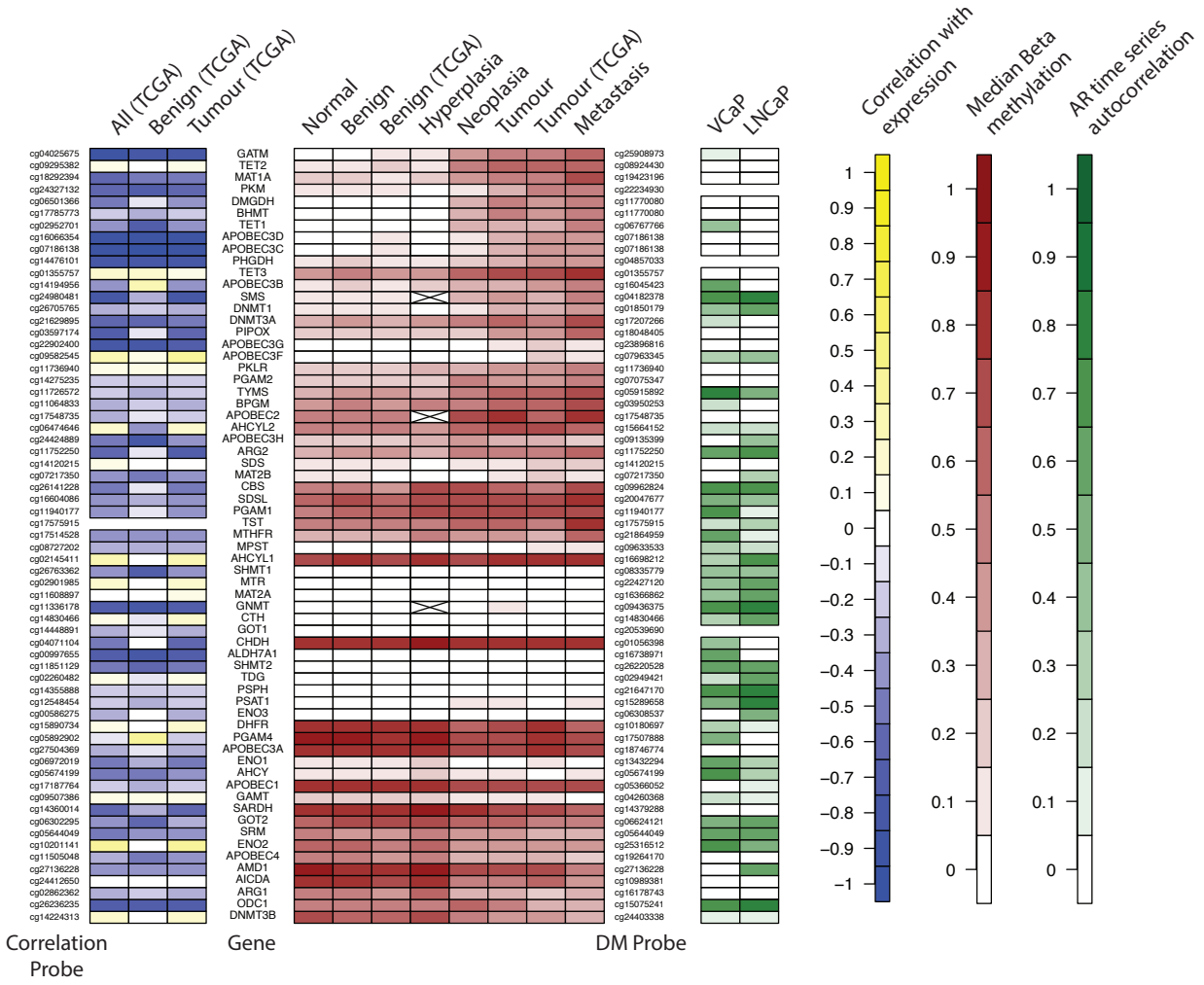

Figure 5: Heatmap summary of genes in metabolic pathways that impact on DNA methylation (relating to Figure 4). Left panel, correlations between gene expression (RNA-seq) and DNA methylation (450k arrays), from the PRAD TCGA data set (450k probe IDs indicated on the left). Middle panel, average methylation levels (Beta-values) from multiple studies spanning a range of prostate tissue types (450k probes indicated on the right). Right panel, androgen-stimulated gene expression changes in two prostate cancer cell lines (autocorrelation values denote a change with time following stimulation).

been the absence of the actors that facilitate methylation of nuclear DNA. However, recent years have seen the identification of methyl donors [135], methyltransferases [136, 137], and even TET1 present in the mitochondria [138]. This latter observation supporting reports of 5-hydroxymethylcytosine in the mitochondrial DNA [136, 139]. The history of this topic is reviewed more thoroughly elsewhere [140].

Recently there have been studies that provide stronger evidence that epigenetic modifications of mitochondrial DNA do indeed take place. For example, one study identified methylated bases in nucleus-free platelets [141], while another used orthogonal and complementary technologies to profile the mitochondrial methylation in a wide range of tissue and cell types [142]. Given the unique behaviour of mitochondria in prostate cancer, if mitochondrial DNA can be methylated, this is an aspect that may reward investigation.

4.2. Reciprocal regulation of nuclear methylation and mitochondria

Apart from direct epigenetic changes to the mtDNA, the mitochondria have a complex relationship with epigenetic alterations to the nuclear DNA. Of the order of a thousand coding genes have products that are active in the mitochondria, and only 13 originate from the mitochondrial DNA. It follows that any epigenetic regulation of the remaining mitochondrial actors in the nucleus will likely influence mitochondrial behaviour. One study identified tissue-specific differentially methylated regions in mitochondrial-acting nuclear-encoded genes [143] while another has concluded that epigenetic regulation of mitochondrial-acting nuclear-encoded genes was higher than other nuclear-encoded genes [144]. This latter result can be replicated in prostate cancer using a single tumour sample (Figure 6A). While this is representative of the other samples in our example set, with $> 5,000$ probes from regions around the transcription start sites of mitochondrial actors showing consistent hypomethylation and approximately 400 showing consistent hypermethylation, there is also evidence of changes in the regulation of these regions with the progression of disease. Figure 6B shows a heatmap of the 114 most variable probes in these regions, and it is apparent that again there are clusters of probes that variously gain methylation in neoplastic tissue and continue to do so in tumour and metastases, others with the opposite pattern, and smaller numbers where the changes are primarily defined by the metastases samples.

DNA polymerase gamma is responsible for the replication of mitochondrial DNA and is regulated by the methylation of the *POLG* gene [145, 146]

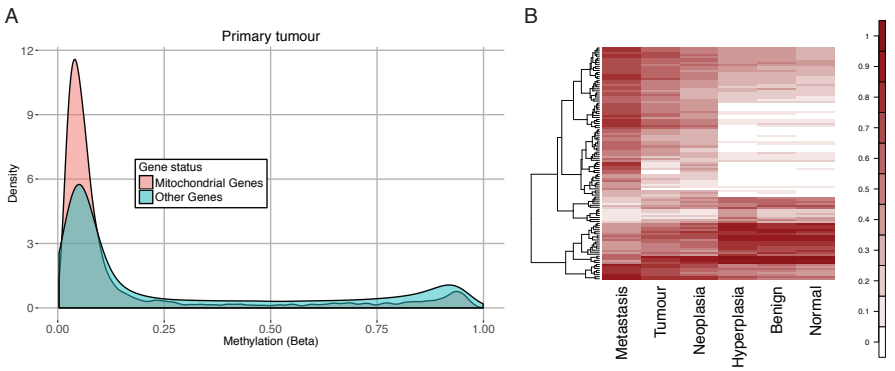

Figure 6: A. Reproducing Figure 4 of Chinnery et al. for a high-cellularity prostate cancer sample. Probes on the Infinium HumanMethylation450 BeadChip that lay within 1000 bases of transcription start sites (TSS) were divided between genes listed in MitoCarta and those that are not. B. A heatmap of median beta methylation values for different tissues across probes that show most variation in our data.

leading to associations between mtDNA copy number and *POLG* methylation levels. Methylation of the *PPARGC1A* gene has also been seen to correlate negatively with mtDNA copy number.

While the methylation of some genes can regulate mtDNA copy number, it has also been shown that mtDNA copy number can regulate some nuclear genes [147, 144]. Furthermore, studies using cybrids have shown that the mitochondria can affect nuclear methylation patterns [139, 148]. Given the importance of epigenetics to prostate cancer regulation, and the characteristic behaviour of mitochondria (and broader metabolic pathways) in the disease, these associations demand attention in attempts to unravel prostate cancer biology.

5. Current perspectives and future directions for the role of epigenomic changes in prostate cancer

It is clear that clonal expansions of cells with stable epigenomic changes occur in prostate cancer. DNA methylation changes are the most recurrent events so far identified in prostate cancer, and specific changes may associate with outcome. The epigenome continues to evolve throughout the life history of prostate cancer, with distinct features presenting at different stages and interacting with specific genomic changes. It will be crucial to overlay other epigenetic changes within the same cohorts of samples to build up a picture

of the epigenetic landscape in prostate cancer. Future studies are also needed to integrate both genomic and epigenomic data in large cohorts of samples, to elucidate the interaction between genomic and epigenetic changes, to provide a more comprehensive view of the pathways affected in each prostate tumour sample, and to assess clinical associations with specific sets of changes.

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Supplementary Material

SupplementaryTable1-Genelists.xls

A spreadsheet summarizing all genes reported in the 17 published studies relating to the prostate cancer tissue summary in main text.

SupplementaryTable2-ReccurentlyCalledGenesAnnotated.xlsx

For all genes reported in at least three of the 17 studies, we present correlation with gene expression and median beta methylation values for several tissues for the probe showing greatest correlation with expression and the probe showing greatest differential methylation. Additionally. autocorrelations from time-course experiments are used to annotated genes as being AR regulated as detailed in the main text.

SupplementaryFigure3-ReccurentlyCalledGenesHeatmap.pdf

A figure equivalent to Figure 2 in the main text, but presenting all genes that were reported in at least three of the 17 studies (in contrast to Figure 2 which presented genes reported in at least four of the studies).

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