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The role of TET-mediated DNA hydroxymethylation in prostate cancer

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Ten-eleven translocation (TET) proteins are recently characterized dioxygenases that regulate demethylation by oxidizing 5-methylcytosine to 5-hydroxymethylcytosine and further derivatives. The recent finding that 5hmC is also a stable and independent epigenetic modification indicates that these proteins play an important role in diverse physiological and pathological processes such as neural and tumor development. Both the genomic distribution of (hydroxy)methylation and the expression and activity of TET proteins are dysregulated in a wide range of cancers including prostate cancer. Up to now it is still unknown how changes in TET and 5(h)mC profiles are related to the pathogenesis of prostate cancer. In this review, we explore recent advances in the current understanding of how TET expression and function are regulated in development and cancer. Furthermore, we look at the impact on 5hmC in prostate cancer and the potential underlying mechanisms. Finally, we tried to summarize the latest techniques for detecting and quantifying global and locus-specific 5hmC levels of genomic DNA.

1 Introduction

Recent large-scale genome sequencing studies have classified primary prostate cancer (PCa) into distinct molecular subtypes, defined by specific genetic alterations (Cancer Genome Atlas Research, 2015). Gene-fusions involving the ETS family of transcription factors occur in over 50% of primary prostate cancers, with the next most common subtype defined by mutations in the SPOP gene (~10% cases) (Cancer Genome Atlas Research, 2015). In addition to these genetic alterations there are recurrent epigenetic changes that occur in over 80% cases and that show less intra-tumor heterogeneity compared to genetic changes (Bastian, Yegnasubramanian, Palapattu et al., 2004,Cooper, Eeles, Wedge et al., 2015,Massie, Spiteri, Ross-Adams et al., 2015), suggesting that these are early and recurrent events. In addition there are also epigenetic

changes that vary between tumor foci and change progressively from early to late stage disease (Brocks, Assenov, Minner et al., 2014,Yegnasubramanian, Haffner, Zhang et al., 2008), highlighting the ongoing epigenetic evolution in prostate tumors.

The epigenetic changes observed in prostate tumors may be shaped by extrinsic factors (e.g. metabolic stress (Sciacovelli, Goncalves, Johnson et al., 2016,Thienpont, Steinbacher, Zhao et al., 2016)) and cell-intrinsic factors (e.g. genetic alterations impinging on epigenetic regulators (Cancer Genome Atlas Research, 2015,Spans, Van den Broeck, Smeets et al., 2016)). For example, a rare molecular subtype of prostate cancer is defined by recurrent point mutations in the IDH1 gene, resulting in increased production of the oncometabolite 2-hydroxyglutarate (2-HG) that inhibits several 2-oxoglutarate (2-OG) dependent deoxygenases including enzymes involved in the metabolism of DNA methylation.

In mammalian cells, DNA methylation occurs on the 5' position of cytosine bases, predominantly at CpG dinucleotides, and is mediated by DNA methyltransferases (DNMT1, DNMT3a/b) using S-adenosylmethionine (SAM) as a methyl-donor (Chiang, Gordon, Tal et al., 1996,Gold, Hurwitz and Anders, 1963). Cytosine methylation (5mC) at transcriptional regulatory elements is associated with reduced gene expression and tissue-specific 5mC profiles are implicated in canalization of cellular differentiation (Razin and Riggs, 1980,Roadmap Epigenomics, Kundaje, Meuleman et al., 2015). 5mC oxidation results in 5-hydroxymethylcytosine (5hmC), a base that was first identified as a constituent of bacteriophage DNA in 1952 (Wyatt and Cohen, 1952) and was subsequently found in mammalian tissues in 1972 (Penn, Suwalski, O'Riley et al., 1972). It was not until 2009 when the ten-eleven translocation methylcytosine dioxygenase (TET) family of proteins were implicated in the oxidation of 5mC to 5hmC that interest in this base began to grow (Tahiliani, Koh, Shen et al., 2009). Initial excitement over 5hmC was due to the implication of this modification in the active demethylation of 5mC during epigenetic reprogramming (He, Li, Li et al., 2011,Ito, Shen, Dai et al., 2011), however much recent attention has focused on 5hmC as a stable epigenetic mark involved in transcriptional regulation and lineage specification (Bachman, Uribe-Lewis, Yang et al., 2014,Koh, Yabuuchi, Rao et al., 2011,Zhang, Su, Jeong et al., 2016).

The functional impact of 5hmC therefore appears to be context-dependent and locus-specific. In this review we explore the mechanisms regulating the expression and function of the TET family and the impact on 5hmC in development and cancer, before focusing on a specific summary of TET and 5hmC alterations in prostate cancer and the mechanisms underlying these changes.

2 The *TET* gene family

The ten-eleven translocation family of proteins (TET1-3) was initially identified because of the involvement of TET1 as a fusion partner of the histone methyltransferase (MLL) gene in acute myeloid leukemia (Lorsbach, Moore, Mathew et al., 2003,Ono, Taki, Taketani et al., 2002). The TET protein family members are mammalian homologs of the trypanosome base J binding proteins JBP1 and JBP2, which were found to oxidize the 5-methyl group of thymine (Iyer, Tahiliani, Rao et al., 2009,Tahiliani et al., 2009). Although 5-hydroxymethylcytosine (5hmC) had previously been detected in mammalian genomes, the functional importance of this modification was not further investigated until Tahiliani and colleagues discovered that TET proteins are dioxygenases that participate in the DNA demethylation pathway by oxidizing 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (Tahiliani et al., 2009). 5hmC is a substrate for deamination into 5-hydroxymethyluridine (5hmU) by activation-induced deaminase (AID) and apolipoprotein B mRNA-editing enzyme complex (APOBEC), followed by base excision repair (BER) (Guo, Su, Zhong et al., 2011). It can also be converted to 5-formylcytosine (5fC) and finally to 5-carboxylcytosine (5caC) with subsequent base-excision repair to yield unmethylated cytosines (He et al., 2011,Ito et al., 2011). Decarboxylation of 5caC by unknown enzymes or removal of the base by thymine DNA glycosylase or other DNA repair enzymes further completes the demethylation pathway (He et al., 2011,Ito et al., 2011,Tahiliani et al., 2009). For these reactions, TET enzymes require 2-oxoglutarate (2-OG or α-ketoglutarate) and Fe(II) as cofactors, rendering their activity acutely dependent on central metabolism and oxygen levels.

The TET family members (TET1-3) all have the same catalytic activity but vary in structural domains (Figure 1). TET proteins harbor a core catalytic oxygenase domain with a double-stranded β-helix (DSβH) containing metal-binding residues which are characteristic for 2-OG and Fe(II)-dependent oxidation (Loenarz and Schofield, 2011). This core domain is typically preceded by a cysteine-rich domain that seems to be required for enzymatic activity (Iyer et al., 2009,Tahiliani et al., 2009). TET proteins catalyze demethylation by binding Fe(II) through conserved His-His-Asp residues. This generates a reactive enzyme-bound Fe(IV)-oxo intermediate by using molecular oxygen from 2-OG, which reacts with 5mC to yield 5hmC.

At their N-terminus, TET1 and TET3 contain a CXXC zinc finger domain, a motif that is also found in DNMT1 and is required for direct DNA-binding of DNMT1 (Pradhan, Esteve, Chin et al., 2008,Xu, Wu, Tan et al., 2011,Zhang, Zhang, Clark et al., 2010). This domain has been reported to recognize unmodified, methylated and hydroxymethylated cytosines and binds preferentially to genomic regions with high CpG content (Pradhan et al., 2008,Risner, Kuntimaddi, Lokken et

al., 2013,Xu et al., 2011,Zhang et al., 2010). TET2 does not contain a CXXC-domain but associates with the IDAX protein which contains a CXXC-domain (Ko, An, Bandukwala et al., 2013). Divergent sequence specificity has been reported for CXXC-domain containing proteins (Frauer, Rottach, Meilinger et al., 2011,Risner et al., 2013), and TET deletion mutants lacking CXXC-domains seem to retain activity to hydroxylate genomic 5mC (Frauer et al., 2011,Liu, Wang, Deng et al., 2013). Together, these data suggest that the recruitment of TET enzymes to specific genomic loci may occur through direct CXXC-domain DNA binding and also indirectly as part of protein-complexes (Nakagawa, Lv, Nakagawa et al., 2015,Yildirim, Li, Hung et al., 2011). The exact combination of direct binding and protein-interaction partners may in part contribute to the differential recruitment of TET proteins in particular cellular contexts.

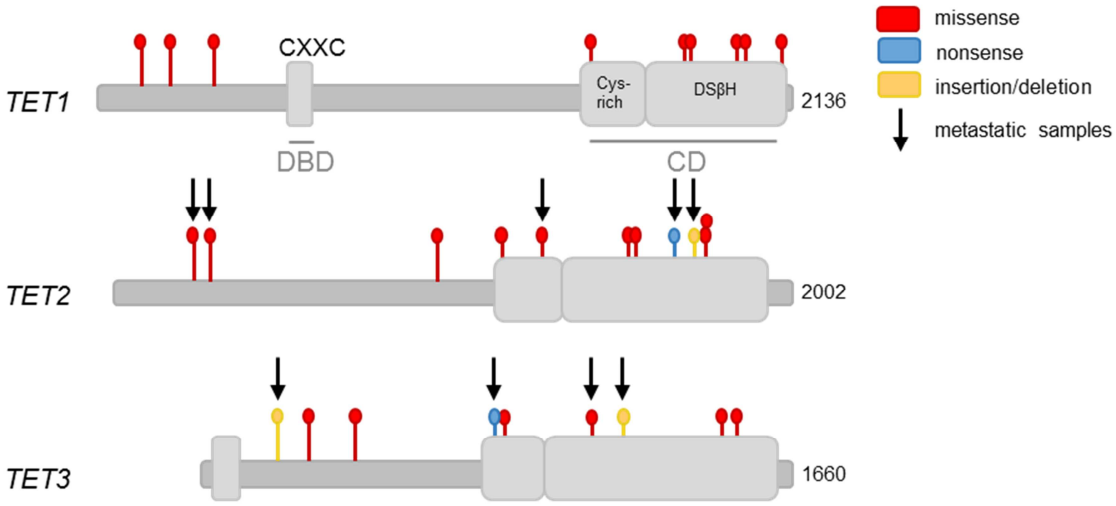

Figure 1: Schematic representation of mutations in TET family in PCa. *TET1-3* missense, nonsense substitutions and insertions/deletions are represented in a simplified protein structure showing the DNA binding domain (DBD) and catalytic domain (CD). The dots represent how frequent the mutations occur in different samples. The scheme contains both primary PCa samples and metastatic samples (indicated with an arrow). This representation is based on data retrieved from cBioPortal (http://www.cbioportal.org/), COSMIC (http://cancer.sanger.ac.uk/cosmic) and (Spans et al., 2016). One sample has been excluded for its hyper mutated genotype (TCGA-XK-AAIW; 6517 nonsynonymous mutations among which 4 mutations are located within *TET* genes).

3 5hmC and the TET enzymes

3.1 Role in transcriptional regulation

One of the best studied covalent DNA modifications is 5mC, which is most prevalent in CpG islands (70-80% of CpGs are methylated). The accumulation of 5mC at gene promoters and enhancers is associated with gene repression (Lee, Morton, Epstein et al., 1994,Nelson, Lee, Nguyen et al., 1997). DNA demethylation occurs in different biological contexts. Passive demethylation refers to the loss of 5mC in a replication-dependent manner during cell division. A process for active demethylation was predicted from studies in early development (Mayer, Niveleau, Walter et al., 2000,Santos, Hendrich, Reik et al., 2002), but this demethylation process remained unknown for decades. The characterization of TET, thymine-DNA glycosylase (TDG) and AID/APOBEC activity defined a pathway for 5mC demethylation (Guo, Li, Liang et al., 2014,He et al., 2011,Morgan, Dean, Coker et al., 2004,Tahiliani et al., 2009), and at least initially, 5hmC was considered as an intermediate of the demethylation pathway (Ito, D'Alessio, Taranova et al., 2010,Williams, Christensen and Helin, 2011). Recent studies have confirmed that in addition to 5hmC being an intermediate step in demethylation, that 5hmC is a stable DNA modification that is present at higher levels in tissues with low replication rates (Bachman et al., 2014), and that conversely 5hmC is decreased in rapidly proliferating tissues and in cancer (Jin, Jiang, Qiu et al., 2011,Lian, Xu, Ceol et al., 2012). Not only are TET proteins involved in the demethylation pathway, but they have also been shown to physically bind to DNA to prevent DNA methylation on specific loci (Wu and Zhang, 2011). Recent publication suggests that TET proteins are not only able to hydroxymethylate DNA, but also RNA (Delatte, Wang, Ngoc et al., 2016). Therefore, it is believed that TET enzymes have a direct influence on gene transcription.

Indeed, TET1 DNA binding and associated 5hmC markers are found in 5' gene regulatory regions and are associated with active gene transcription (Stroud, Feng, Morey Kinney et al., 2011,Williams, Christensen, Pedersen et al., 2011,Wu and Zhang, 2011). Recent studies have shown that 5hmC regulates gene transcription at least in part by directing the dynamic organization and remodeling of chromatin structures. For example, 5hmC can inhibit the binding of the methyl-CpG binding domain of MeCP2, MBDs and RING/zinc-finger domain-containing proteins to DNA (Jin, Kadam and Pfeifer, 2010,Valinluck, Tsai, Rogstad et al., 2004). These chromatin readers repress transcription at loci marked with DNA methylation and therefore, it is suggested that the 5hmC marks can counteract 5mC-mediated transcriptional repression (Tan and Shi, 2012).

An example of the transcriptional activating role of the TET proteins is the enrichment of TET1 and 5hmC at regulatory regions of pluripotency factors such as *Nanog* in mouse embryonic stem cells (mESC) (Ito et al., 2010). Knockdown of *Tet1* or *Tet2* in this system is associated with lowered expression levels of these factors by promoter hypermethylation (Ficz, Branco, Seisenberger et al., 2011,Ito et al., 2010). In addition, a transcriptionally repressive role for TET proteins has been proposed at Polycomb target gene promoters where TET1 contributes to repressive chromatin complexes in ESCs (Williams et al., 2011,Wu, D'Alessio, Ito et al., 2011).

Based on these findings, 5hmC can be considered as a stable epigenetic marker in its own right, in addition to being an intermediate of the demethylation pathway. Clearly, the emerging transcriptional activating and repressing roles of the TET proteins at key genomic loci imply that they play an important function in normal development, pluripotency and tumor suppression.

3.2 Role in development

During the early stages of development there is a role for TET proteins in epigenetic reprogramming. Immediately after fertilization, while the paternal and maternal pronuclei move towards each other, maternal TET3 catalyzes demethylation in the paternal pronucleus (Gu, Guo, Yang et al., 2011,Iqbal, Jin, Pfeifer et al., 2011,Wossidlo, Nakamura, Lepikhov et al., 2011) and a partial demethylation in the maternal pronucleus (Guo et al., 2014). Even in primordial germ cells (PGCs), which migrate into the gonad region at embryonic day 7 and develop into oocytes and sperm cells, genome-wide demethylation as well as chromatin remodeling is observed (Hajkova, Ancelin, Waldmann et al., 2008). This demethylation step is carried out through a replication-dependent mechanism initiated by TET1 and TET2 (Hackett, Sengupta, Zylicz et al., 2013). Expression patterns of TET enzymes suggest functional specificity: *Tet1* and *Tet2* are expressed in PGCs, whereas *Tet3* is highly expressed in oocytes (Gu et al., 2011,Hajkova, 2010,Surani and Hajkova, 2010). However, normal development of PGCs is not ablated by the knockout of individual *Tet* genes (Dawlaty, Ganz, Powell et al., 2011,Gu et al., 2011,Ko, Bandukwala, An et al., 2011), suggesting a level of functional redundancy at least between *Tet1* and *Tet2*.

TET1 also plays an essential role in regulating pluripotency and differentiation in mouse embryonic stem cells (ESCs). Both *TET1* and *TET2* are abundantly expressed in mESCs, accompanied by high 5hmC levels (Ito et al., 2010,Koh et al., 2011,Ruzov, Tsenkina, Serio et al., 2011). It was recently shown that TET1 can functionally replace transcription factors such as OCT4 and SOX2 during somatic cell reprogramming to successfully generate induced pluripotent stem cells (Chen, Gao, Huang et al., 2015,Gao, Chen, Li et al., 2013). Both *Tet1* and

Tet2 are rapidly downregulated upon differentiation (Ito et al., 2010,Koh et al., 2011,Tahiliani et al., 2009), whereas *Tet3* levels rise. Further illustration of selectivity of TET activities came from Ito and colleagues who reported that KO of *Tet1* induced spontaneous differentiation of mESCs into trophoblasts *in vitro,* whereas this was not the case for KO of other *Tet* family members (Dawlaty et al., 2011,Ito et al., 2010). *Tet1* KO in mESCs showed 35% reduction in 5hmC levels and subtle changes in gene expression (Dawlaty et al., 2011). Surprisingly, the homozygous KO of *Tet1* or *Tet2* did not seem to affect normal development in mice (Dawlaty et al., 2011,Li, Cai, Cai et al., 2011,Moran-Crusio, Reavie, Shih et al., 2011,Quivoron, Couronne, Della Valle et al., 2011). No difference in gene expression was found in *Tet3* KO compared to wild type controls (Shen, Inoue, He et al., 2014), suggesting compensation from other TET family members. Most double *Tet1*/*Tet2* deficient mice survive to adulthood, but some die late in embryogenesis or shortly after birth (Dawlaty, Breiling, Le et al., 2013). In contrast, knockout of *Tet3* led to neonatal lethality (Guo et al., 2011), and *Tet1-3* triple deficient mESCs show major defects in differentiation and reprogramming (Dawlaty, Breiling, Le et al., 2014,Hu, Zhang, Mao et al., 2014). This stresses the important involvement of TET3 during embryogenesis and illustrates the partial redundancy between TET proteins.

Postnatally, levels of 5hmC are very tissue- and locus dependent. The highest 5hmC levels have been found in the central nervous system (CNS) (Globisch, Munzel, Muller et al., 2010,Szwagierczak, Bultmann, Schmidt et al., 2010). Hydroxymethylation levels in the cerebellum and hippocampus are higher in adult mice compared to newborns (Szulwach, Li, Li et al., 2011). In general, 5hmC is even higher in terminally differentiated cells than in progenitor cells in the CNS (Haffner, Chaux, Meeker et al., 2011,Haffner, Pellakuru, Ghosh et al., 2013,Orr, Haffner, Nelson et al., 2012). In these cells, the local levels of 5hmC were found to be inversely correlated with MECP2 binding at regions with differential 5-hydroxymethylation (Szulwach et al., 2011).

As previously mentioned, *Tet1* and *Tet2* KO mice are viable and fertile (Dawlaty et al., 2011,Li et al., 2011,Moran-Crusio et al., 2011), and *Tet3* KO leads to neonatal lethality (Gu et al., 2011). However, *Tet1* KO mice do display reduced body mass and smaller litter size (Dawlaty et al., 2011). More detailed analyses of *Tet2* KO mice revealed that TET2 activity is critical for normal hematopoietic lineage differentiation (Madzo, Vasanthakumar and Godley, 2013,Madzo, Liu, Rodriguez et al., 2014). TET2 modulates the balance between self-renewal and differentiation in hematopoietic stem cells. Moreover, mutations in *TET2* have been found in multiple types of leukemia (Ko, Huang, Jankowska et al., 2010,Li et al., 2011,Moran-Crusio et al., 2011,Quivoron et al., 2011), and knockout of *Tet2* leads to hematopoietic phenotypes such as myelomonocytic

leukemia (Albano, Anelli, Zagaria et al., 2011,Chou, Chou, Liu et al., 2011,Ko et al., 2011,Li et al., 2011,Moran-Crusio et al., 2011,Quivoron et al., 2011,Weissmann, Alpermann, Grossmann et al., 2012).

3.3 Role in malignancies

The identification of the *TET1* gene as a recurrent translocation partner in acute myeloid leukemia was a first hint to its role in the oncogenic process. Based on rodent models, TET1 can be considered a tumor suppressor of hematopoietic malignancy and more specifically B cell lymphoma (Cimmino, Dawlaty, Ndiaye-Lobry et al., 2015). *TET2* mutations were found in up to 30% of patients with myeloid malignancies such as chronic myelomonocytic leukemia, acute myeloid leukemia (AML) and myelodysplastic syndromes (Albano et al., 2011,Chou et al., 2011,Ko et al., 2010,Madzo et al., 2013,Weissmann et al., 2012). *TET2* mutations have also been reported in solid tumors including clear-cell renal cell carcinoma, prostate cancer (PCa), and breast cancer (Abdel-Wahab, Mullally, Hedvat et al., 2009,Chung, Schatoff and Abdel-Wahab, 2012,Delhommeau, Dupont, Della Valle et al., 2009,Ko et al., 2010,Nickerson, Im, Misner et al., 2013,Sato, Yoshizato, Shiraishi et al., 2013,Takahashi, 2013,Wu and Ling, 2014). In myeloid malignancies, mutations in *TET2* are mainly loss-of-function whereas in solid tumors missense mutations are clustered in the catalytic domain of the gene, suggesting that disruption of TET2 dioxygenase activity provides a selective advantage in multiple tumor types (Ko et al., 2010). This notion is corroborated by the observation that *TET2* mutations in AML are predominantly associated with genomic hypermethylation (Ko et al., 2010,Madzo et al., 2013).

Recent studies have shown that the overall 5hmC level in genomic DNA is substantially decreased in many solid tumors, which has been linked to reduced expression levels of the TET family members (Jin et al., 2011,Kudo, Tateishi, Yamamoto et al., 2012,Lian et al., 2012,Yang, Liu, Bai et al., 2013). There is an inverse correlation between *TET* expression and robust tumor growth and metastasis in colorectal cancer (Gambichler, Sand and Skrygan, 2013,Hsu, Peng, Kang et al., 2012,Lian et al., 2012). Furthermore, lower TET levels are associated with poor prognosis of patients with early breast cancer and prostate cancer (Spans, Van den Broeck, Smeets et al., 2016,Yang, Yu, Hong et al., 2015). Studies in prostate cancer have reported reduced 5hmC levels in tumor versus normal tissue and suggested this as a potential biomarker for malignant transformation (Haffner et al., 2011,Yang et al., 2013). More recently, 5hmC levels were shown to be significantly reduced in ETS-related gene (ERG) negative PCa compared to normal prostate tissue samples, and this change has been proposed as a potential prognostic marker in ERG negative (but not in ERG positive) prostate cancers (Strand, Hoyer, Lynnerup et

al., 2015). These data are suggestive for a tumor suppressive role of TET proteins in prostate cancer.

4 Regulation of *TET* genes

4.1 Transcriptional control

The TET enzymes are tightly regulated at many levels of gene expression. Here, most relevant pathways in prostate cancer will be discussed. Promoter studies revealed two alternative promoters for mouse *Tet1* as well as *Tet2,* resulting in different isoforms (Neri, Incarnato, Krepelova et al., 2015,Sohni, Bartoccetti, Khoueiry et al., 2015,Zhang, Xia, Wang et al., 2016). The *Tet1a* isoform contains a CXXC DNA-binding domain and is regulated by ESC-cell specific transcription factors including SOX2 and KLF4, while the *Tet1b* isoform lacks the CXXC-domain and is transcribed from an alternative promotor in differentiated cells independently of pluripotency transcription factors (Neri et al., 2015,Sohni et al., 2015). In human cells only one *TET1* isoform has been characterized, however the human *TET1* gene includes an enhancer region that is bound by the pluripotency factors OCT4, SOX2 and NANOG *(Neri et al., 2015)*. Other transcription factors known to regulate *TET* expression are the high-mobility group AT-hook 2 (HMGA2) and hypoxia inducible factor (HIF)-1a (Song, Poliseno, Song et al., 2013,Sun, Song, Huang et al., 2013). Indeed, several groups reported that all three members of the *TET* family can be directly regulated by HIF-1a through binding in the proximal promoter region of *TET* genes (Mariani, Vasanthakumar, Madzo et al., 2014,Thienpont et al., 2016,Wu, Chen, Nieh et al., 2015). Furthermore, regulation can be controlled by epigenetic mechanisms such as hypermethylation of the *TET* promoter (Chim, Wan, Fung et al., 2010,Cimmino et al., 2015).

4.2 Post-translational control

The activity of TET enzymes is also tighly regulated at the protein level. Major influence on TET activity include central metabolism and oxygen availability. TET enzymes are oxygen-dependent dioxygenases and recently tumor hypoxia was shown to reduce TET activity and DNA demethylation (Thienpont et al., 2016). Furthermore, tricarboxylic acid (TCA) cycle intermediate metabolites have been implicated in TET regulation. Isocitrate dehydrogenase (IDH) proteins convert isocitrate to 2-OG using $NADP^+$/NADPH as cofactors (Figure 2). Since 2-OG is an essential cofactor for dioxygenases such as TET proteins, IDH enzymes are important regulators of TET activity. Other rate-limiting steps up- and downstream of 2-OG in central metabolism also have the potential to influence TET activity, including oxygen levels, TCA cycle activity and anabolic processess that utilize TCA metabolites. Mutations in other enzymes

participating in the TCA cycle such as succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been described in cancer too (Adam, Yang, Soga et al., 2014,Xiao, Yang, Xu et al., 2012). Loss-of function mutations in these enzymes result in accumulation of succinate or fumarate respectively, impairing the activity of 2-OG-dependent dioxygenases such as TET, reducing DNA demethylation (Sciacovelli et al., 2016).

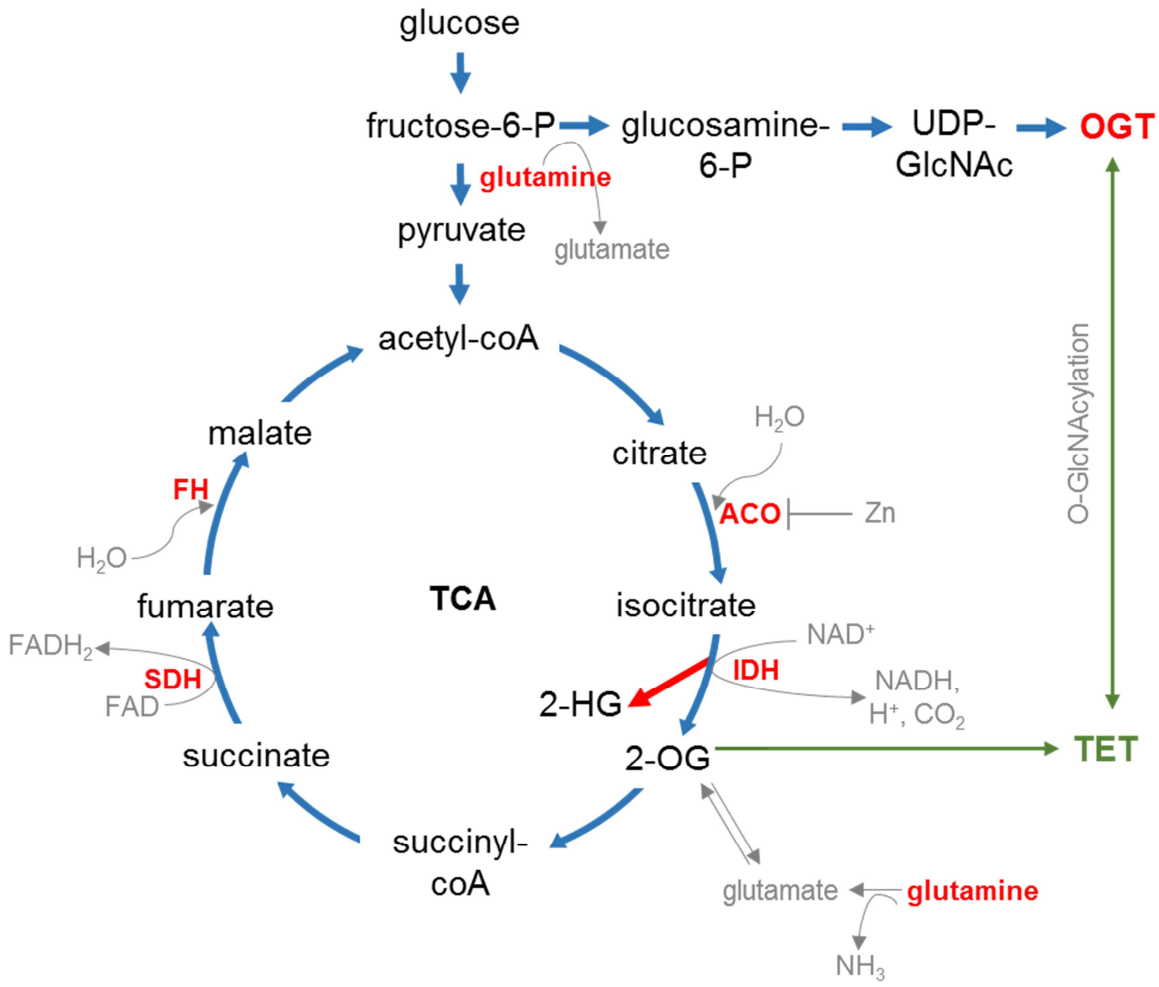

Figure 2: Crosstalk between TET and key enzymes involved in metabolism and glycosylation. Several enzymes involved in the TCA cycle such as isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been reported to influence TET activity in cancer. 2-OG is a product of the TCA cycle and an essential co-factor of TET, thus mutations in *IDH* as well as glutamine deficiency affect its hydroxymethylating capacity. High levels of zinc inhibit aconitase (ACO) resulting in reduced flux through the TCA cycle. Glucose metabolism modulates the activity of OGT

which glycosylates serine and threonine residues of diverse proteins including TET. TET also enables GlcNAcylation by recruitment of OGT to the chromatin.

O-Linked N-Acetylglucosamine (GlcNAc) Transferase (OGT) glycosylates hydroxyl groups of specific serine and threonine residues in many different nuclear and cytoplasmic proteins, thereby controlling their transcription, activity, stability and localization (Ozcan, Andrali and Cantrell, 2010). There is a direct interaction between phosphorylated TET proteins and OGT (Chen, Chen, Bian et al., 2013,Deplus, Delatte, Schwinn et al., 2013,Ito, Katsura, Shimada et al., 2014,Zhang, Liu, Gao et al., 2014), and TET-OGT complexes mainly co-localize on H3K4me3 positive, CpG-rich promoters at sites of active gene transcription (Chen et al., 2013,Deplus et al., 2013,Vella, Scelfo, Jammula et al., 2013). This interaction results in the O-GlcNAc modification of TETs, thereby decreasing available phosphorylation sites and increasing protein stability and promoting DNA demethylation (Bauer, Gobel, Nagaraj et al., 2015,Shi, Kim, Lu et al., 2013,Vella et al., 2013). In addition, TET proteins are essential for efficient recruitment of OGT to chromatin in order to facilitate the modification of histones (Chen et al., 2013,Deplus et al., 2013,Vella et al., 2013). Increased glucose consumption also leads to a higher production of UDP-N-acetylglucosamine (UDP-GlcNAc), which is further used by OGT to glycosylate various proteins. Dysregulation of OGT-TET interaction and co-localization might therefore affect the stability of TET itself, but also of many other target genes such as tumor suppressor genes and oncogenes (p53, c-Myc) (Itkonen, Minner, Guldvik et al., 2013,Yang, Kim, Nam et al., 2006).

Additional post-translational modifications have been reported to influence TET activity including poly (ADP-ribosyl)ation (PARylation) which is catalyzed by enzymes of the poly(ADP-ribose) polymerase (PARP) family. PARPs use NAD^+ as a substrate producing negatively charged polymers of ADP-ribose which are then hydrolyzed by the poly(ADP-ribose) glycohydrolase (PARG) (Schreiber, Dantzer, Ame et al., 2006). PARylation participates in highly diverse cellular processes such as DNA damage response, transcription and apoptosis (Beck, Robert, Reina-San-Martin et al., 2014,Krishnakumar and Kraus, 2010,Schreiber et al., 2006). A direct protein-protein interaction between TET1 and PARP1 has been reported associated with PARylation and enhanced TET1 activity (Ciccarone, Valentini, Zampieri et al., 2015). Recently, *TET1* gene expression has also been found to be positively regulated by PARylation through hypomethylation of CpG islands in the *TET* promoter region (Ciccarone, Valentini, Bacalini et al., 2014).

Lastly, an important nutritional factor has been implicated in TET regulation at the protein level. Vitamin C (or L-ascorbic acid) is an essential antioxidant in mammals which is required for collagen, catecholamine and carnitine biosynthesis (Banhegyi, Benedetti, Margittai et al., 2014,Englard and Seifter, 1986). It is also an essential cofactor of Fe(II)- and 2-OG-dependent dioxygenases (such as TET enzymes), acting as an electron donor, adjusting the redox state of enzymes and reducing Fe(III) to Fe(II). In mouse ES-cells, vitamin C enhances TET activity and therefore promotes demethylation, leading to global increase of 5hmC levels (Blaschke, Ebata, Karimi et al., 2013). This activation is possibly mediated by a direct mechanism whereby vitamin C directly binds to the catalytic domain of TET (Yin, Mao, Zhao et al., 2013).

5 5hmC landscapes in cancer

Hydroxymethylation is inversely associated with cell proliferation as 5hmC levels are decreased in rapidly proliferating tissues and cancer (Jin et al., 2011,Lian et al., 2012). When staining different cancer types such as prostate cancer for 5hmC and proliferation marker Ki67, cells that lost 5hmC present with high levels of Ki67 (Jin et al., 2011). It is believed that 5hmC levels play an important role in gene regulation, but the genomic distribution of 5hmC marks in normal and cancer tissues is still largely unknown.

A global decrease of 5hmC was first observed in hematological malignancies (Coutinho, Monte-Mor, Vianna et al., 2015,Huang and Rao, 2014,Ko, An, Pastor et al., 2015) and later also in solid cancers including breast cancer, colon cancer, melanoma and prostate cancer (Haffner et al., 2011,Lian et al., 2012,Murata, Baba, Ishimoto et al., 2015,Spans et al., 2016,Udali, Guarini, Moruzzi et al., 2015). In melanoma, hypo-5hmC was determined at the gene level for *RAC3*, *IGF1R* and *TIMP2* (Lian et al., 2012). However, while the general trend in tumors is global 5hmC decrease, some cancers such as pancreatic cancer are characterized by increased 5hmC levels (Bhattacharyya, Yu, Suzuki et al., 2013,Huang, Jiang, Li et al., 2013,Navarro, Yin, Ono et al., 2014).

Technological improvements and detailed studies have revealed more complex landscapes where 5hmC patterns at individual genomic regions can differ considerably. While tissue and cellular studies have shown that 5hmC is globally decreased in cancer, high-resolution genome-wide studies have shown local enrichment of 5hmC at promoters, enhancers and gene bodies of actively expressed genes (Stroud et al., 2011,Xu et al., 2011). A broad redistribution of 5hmC across the genome was detected in several cancers such as melanoma and pancreatic cancer

(Bhattacharyya et al., 2013,Lian et al., 2012,Thomson, Hunter, Lempiainen et al., 2013). This redistribution generally includes a decrease in 5hmC around transcriptional start sites (TSS) and increased levels in gene bodies, although the overall level is lower in cancer cells compared to normal cells.

To date, few studies have studied 5hmC genome-wide in cancer tissues and further work is needed to map tissue-specific and cancer type-specific 5hmC in order to reveal the diversity of 5hmC changes and to identify molecular signatures.

6 TET and 5hmC in prostate cancer

The clinical behavior of primary as well as metastatic prostate cancer is highly variable with substantial intra- and interpatient heterogeneity and differential outcome. Current risk stratifications of primary tumors combine clinical and pathological parameters including Gleason score, PSA and clinical staging (D'Amico, Whittington, Malkowicz et al., 1998). These risk stratifications may be refined by the inclusion of genetic and epigenetic profiling, such as point mutations (*SPOP*, *TP53*, *PTEN*, and *FOXA1*), copy-number alterations, gene fusions (*TMPRSS2-ERG*) and (hydroxy-)methylation landscapes (Barbieri, Baca, Lawrence et al., 2012,Massie, Mills and Lynch, 2016,Taylor, Schultz, Hieronymus et al., 2010,Tomlins, Rhodes, Perner et al., 2005,Wang, Shankar, Dhanasekaran et al., 2011).

The Cancer Genome Atlas (TCGA) Research Network recently proposed a new molecular taxonomy of primary prostate cancer based on data from 333 primary prostate tumors (Cancer Genome Atlas Research, 2015). They found that 74% of all prostate tumors can be subdivided in one of seven molecular classes, based on fusions involving *ERG* (46%), *ETV1* (8%), *ETV4* (4%) or *FLI1* (1%) and on mutations in *SPOP* (11%), *FOXA1* (3%) or *IDH1* (1%). All of these mutated genes either encode transcription factors or are related to gene expression and epigenetics. Within these different subclasses, epigenetic profiling revealed a diversity of 5mC changes that was not clearly separated by molecular subtypes but did correlate with gene silencing. In addition, approximately one in four prostate tumors could not be classified. Either these unclassified tumors belong to undiscovered subclasses, or there is missing information linking them to any of the existing classes.

6.1 Genetic alterations of *TET* genes in prostate cancer

Genetic alterations are infrequent events in prostate cancer. Mutations that have been found in localized prostate cancer for all three *TET* genes, showing a similar distribution (Figure 1). Strikingly, 70% of these identified mutations are located within the catalytic domain, possibly

having detrimental effects on TET enzymatic activity. Mutations in the *TET* genes were also detected in a large cohort of castration-resistant prostate cancer patients (Robinson, Van Allen, Wu et al., 2015). Again, most of the mutations detected in metastatic PCa are located within the catalytic domain (included in Figure 1).

Spans and colleagues identified a high risk patient with a *TET1* mutation in its catalytic domain showing distinct hydroxymethylation patterns (Spans et al., 2016). In this study, 15% of the high-risk prostate cancer samples presented with a loss of at least one *TET1* copy and 39 out of 40 samples (97.5%) showed decreased TET1 levels, suggesting other mechanisms of TET1 repression in prostate cancer. Further studies are needed to functionally characterize these mutations and the consequences of reduced *TET1* expression in prostate cancer. If validated, we propose that these tumors could be added to the IDH1 molecular group of prostate cancers, since *IDH1* mutants are also known to impair TET1 activity and tumors with *IDH1* mutations present with altered 5mC profiles (Xu, Yang, Liu et al., 2011).

6.2 *TET* transcription in prostate cancer

The mechanisms of transcriptional regulation of the *TET* genes in prostate cancer are still largely unknown. However, many signals are expected to converge on the *TET* genes. The expression levels of the *TET* gene regulating transcription factors OCT4, SOX2 and MYC are frequently altered in prostate cancer (Amini, Fathi, Mobalegi et al., 2014,Gu, Yuan, Wills et al., 2007,Koh, Bieberich, Dang et al., 2010,Sotomayor, Godoy, Smith et al., 2009,Ugolkov, Eisengart, Luan et al., 2011,Yu, Cates, Morrissey et al., 2014), suggesting that there may commonly be alterations on the *TET* transcription levels. The transcriptional repressor HMGA2 is also selectively activated during prostate development and its overexpression in prostate cells promotes formation of prostate intraepithelial neoplasia (PIN) lesions in xenografts (Zong, Huang, Sankarasharma et al., 2012). In breast cancer cell lines, it was shown that knockdown of *HMGA2* induces *TET1* expression which is protective against tumor growth and development of metastases (Sun et al., 2013). Thus, overexpression of *HMGA2* in prostate cancer cells might also downregulate *TET* expression.

TET expression levels are also controlled at the epigenetic level by the DNA-methyltransferases (DNMTs). DNMT1 acts mainly as a maintenance methyltransferase and DNMT3a/b predominantly acts as *de novo* 5mC transferases. DNMT protein levels and activities were both found to be elevated in prostate cancer cells and tissues compared to normal controls (Gravina, Ranieri, Muzi et al., 2013,Patra, Patra, Zhao et al., 2002). *De novo* 5mC by DNMT3a and DNMT3b was reported to be remarkably increased in aggressive PCa cell lines whereas DNMT1

activity appears to be more related to the early stages of malignancy (Gravina et al., 2013). *TET1* promoter hypermethylation has recently been reported in prostate cancer as well as in multiple other cancers (Chim et al., 2010,Cimmino et al., 2015,Kim, Pierscianek, Mittelbronn et al., 2011,Li, Li, Mao et al., 2016). Surprisingly, more detailed investigation of the *TET1* promoter region has shown that the observed hypermethylation in prostate cancer is not correlated with its expression (Figure 3). In contrast, reduced *TET1* expression levels were found to be correlated with hypermethylation throughout the *TET1* promoter region in breast cancer.

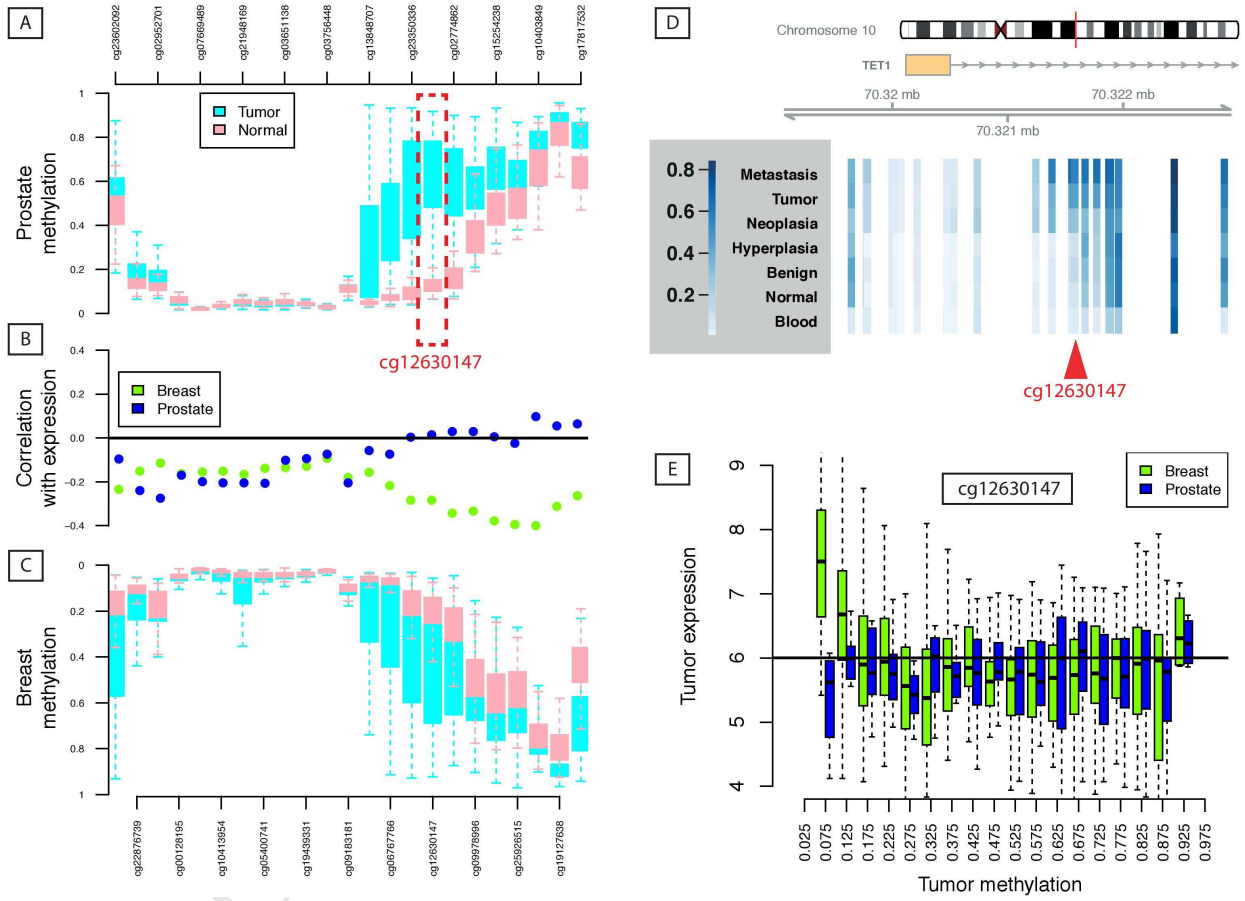

Figure 3: Methylation profile of *TET1* promoter. [A] Depicting methylation profiles for (equally-spaced) probes in the promoter and first intron of TET1 for TCGA prostate samples (cg12630147 is highlighted)(Cancer Genome Atlas Research, 2015). The pattern is the same as [C] seen in Breast samples from TCGA. [D] Aggregated public data show that the difference in methylation seen in TCGA between Tumor and Normal tissue of the first-intron regulatory region of *TET1* is part of a larger trend. Depicted are average levels (Beta values) for Blood (from PCa patients), 'Normal' prostate tissue (from non-PCa patients), 'Benign' apparently normal tissue (from PCa patients), Hyperplastic tissue, PIN, PCa

and Metastasis. For a number of probes in the first intron, including cg12630147 (highlighted), there is a general trend of greater methylation with malignancy. This figure is based on data retrieved from: (Aryee, Liu, Engelmann et al., 2013,Brocks et al., 2014,Cancer Genome Atlas Research, 2015,Massie et al., 2015,Naeem, Wong, Chatterton et al., 2014,Paziewska, Dabrowska, Goryca et al., 2014) [B] The correlation profiles of each methylation probe and TET1 gene expression from TCGA are presented, showing that methylation of the first intron does not regulate TET1 expression in prostate cancer as it does in breast. [E] The patterns of association between methylation and expression are highlighted for probe cg12630147. TCGA data were obtained from the Wanderer tool.

Recent studies have proposed an interaction between the androgen receptor (AR) and *TET* regulation. Dhiman and colleagues reported a role for the AR in the regulation of DNA methylation patterns in gene regulatory regions (Dhiman, Attwood, Campbell et al., 2015). MicroRNAs such as the miR-29 family are induced upon androgen stimulation and repress *TET2* in a high risk prostate cancer model (Takayama, Misawa, Suzuki et al., 2015) (Figure 4). Moreover, *TET2* transcription can be directly repressed by binding of activated AR to the distal enhancer region of *TET2* (Takayama et al., 2015). Furthermore, the *AR* itself can also be silenced by DNA methylation in a subset of androgen-deprivation treated prostate cancers, promoting the development of the disease towards an androgen-independent phenotype (Jarrard, Kinoshita, Shi et al., 1998,Kinoshita, Shi, Sandefur et al., 2000,Yamanaka, Watanabe, Yamada et al., 2003). Therefore, it is possible to predict a model whereby a balanced network of links between AR signaling and TET activity are disrupted at different stages of tumorigenesis to drive first epigenetic changes and subsequently evolution of resistance to therapy.

Hypoxia has been recognized as an important factor in promoting tumor malignancy (Harris, 2002,Ljungkvist, Bussink, Kaanders et al., 2006,Semenza, 1999). Preclinical data have shown that hypoxia can induce the selection of aggressive cancer which is characterized by reduced sensitivity to apoptosis and DNA repair and an increased angiogenesis, proliferation and metastatic potential (Subarsky and Hill, 2003). Hypoxic primary tumors also form a greater risk of developing progressive disease (Milosevic, Fyles, Hedley et al., 2004), and prognostic signatures for poor outcome in prostate cancer include tumor hypoxia (Lalonde, Ishkanian, Sykes et al., 2014). Increased angiogenesis preceded by hypoxia signaling has been reported in PCa as well (Milosevic, Chung, Parker et al., 2007). Several groups have shown that *TET* is directly regulated by HIF-1a through binding near its promoter region (Mariani et al.,

2014,Thienpont et al., 2016,Wu et al., 2015) in addition to the direct effects of oxygen availability on TET enzyme activity. In PCa, there might also be an additional role for androgens in hypoxia control through the transcriptional regulation of angiogenesis factors, providing another potential link between PCa biology and *TET* regulation (Boddy, Fox, Han et al., 2005,Fernandez, Reece, Ley et al., 2015,Horii, Suzuki, Kondo et al., 2007,Woodward, Wachsberger, Burd et al., 2005).

6.3 TET protein activity in prostate cancer

In addition to the transcriptional regulation of *TET* genes, the activity and stability of these enzymes is regulated at the protein level (as outlined in section 4.2). In PCa, androgen signaling regulates both the expression of *TET* genes and the recruitment of TET proteins to methylated DNA. In prostate epithelial cell lines, stimulation of the AR has been reported to induce dynamic patterns of 5mC in the vicinity of the androgen responsive elements (AREs) at AR target genes resulting in selective access of regulatory factors to these regions (Dhiman et al., 2015). Strikingly, TET1 and TDG seem to be co-recruited to these regions upon AR stimulation, indicating an important role for local demethylation in the transcriptional regulation of AR target genes. However, these findings requires further validation and investigation of whether the AR itself is responsible for the recruitment of TET and TDG to the target genes. A recent publication even shows that TET2 and the AR can form a complex to regulate the expression of androgen-responsive genes (Nickerson, Das, Im et al., 2016).

Furthermore, there is a clear link between metabolic alterations in PCa and TET protein activity. As oxygen and 2-OG dependent enzymes, TET proteins are acutely regulated by the microenvironment and cellular metabolism. For example prostate tumors frequently present with regions of hypoxia, and a recent study showed that TET activity is reduced in hypoxic conditions which was associated with hypermethylation of many gene promoters (Thienpont et al., 2016). This provides one explanation for the early and recurrent 5mC alterations observed in PCa tumors.

Mutations in TCA enzymes *IDH1* (cytosolic) and *IDH2* (mitochondrial) are frequently identified in acute myeloid leukemia, glioma, chondrosarcoma, enchondroma and thyroid cancer patients, and have also been found in 1% of patients with localized PCa (Chan, Milosevic and Bristow, 2007,Kang, Kim, Oh et al., 2009). A recent TCGA study defined PCa cases with *IDH1* mutations as a separate molecular subclass (Cancer Genome Atlas Research, 2015). Importantly, mutant IDH enzymes are often not catalytically inactive, but have an altered catalytic activity resulting in

the conversion of isocitrate to the oncometabolite 2-hydroxyglutarate (2-HG) instead of 2-OG (Amary, Bacsi, Maggiani et al., 2011,Dang, White, Gross et al., 2009,Hemerly, Bastos and Cerutti, 2010,Mardis, Ding, Dooling et al., 2009,Murugan, Bojdani and Xing, 2010,Pansuriya, van Eijk, d'Adamo et al., 2011,Yang, Ye, Guan et al., 2012). Accumulation of this alternative metabolite prevents catalytic activity of TET enzymes by competing with 2-OG for binding in the catalytic site of 2-OG-dependent dioxygenases (Xu et al., 2011). Furthermore, a link was also proposed between the IDH and hypoxia. 2-OG is required for proper function of prolylhydroxylases (PHD) which are responsible for hydroxylation and promotion of HIF1α degradation. When conversion of isocitrate is deflected towards the formation of 2-HG, PHD won't be able to degrade HIF1α and favoring angiogenesis (Zhao, Lin, Xu et al., 2009). Given the spatial heterogeneity of hypoxia in PCa (Boutros, Fraser, Harding et al., 2015) and its potential to apply selective pressure, foci with mutations in *IDH1* might be favored in this environment. This raises the question whether the 1% of PCa samples defined in the IDH1 subclass of the TCGA classification are representative or if there might be more cases wherein *IDH1* mutations are not sampled.

High levels of zinc in the prostate inhibit the activity of the TCA enzyme aconitase (ACO) (Costello, Liu, Franklin et al., 1997) and some reports have shown that intracellular zinc concentrations fall during PCa development (Desouki, Geradts, Milon et al., 2007,Singh, Desouki, Franklin et al., 2006). The resulting alterations in the TCA cycle upstream of 2-OG production may impact TET activity as well as the 5hmC dynamics during PCa development. Future studies will be needed to assess the functional consequences of this prostate-specific phenotype at different stages of prostate malignancy.

Together, there are many mechanisms acting at different levels to regulate TET activity. In this section we discussed the majority of these mechanisms applicable for prostate cancer (Figure 4). Future and ongoing research will help to identify new mechanisms and elucidate the role of pathways already reported in PCa or other cellular contexts.

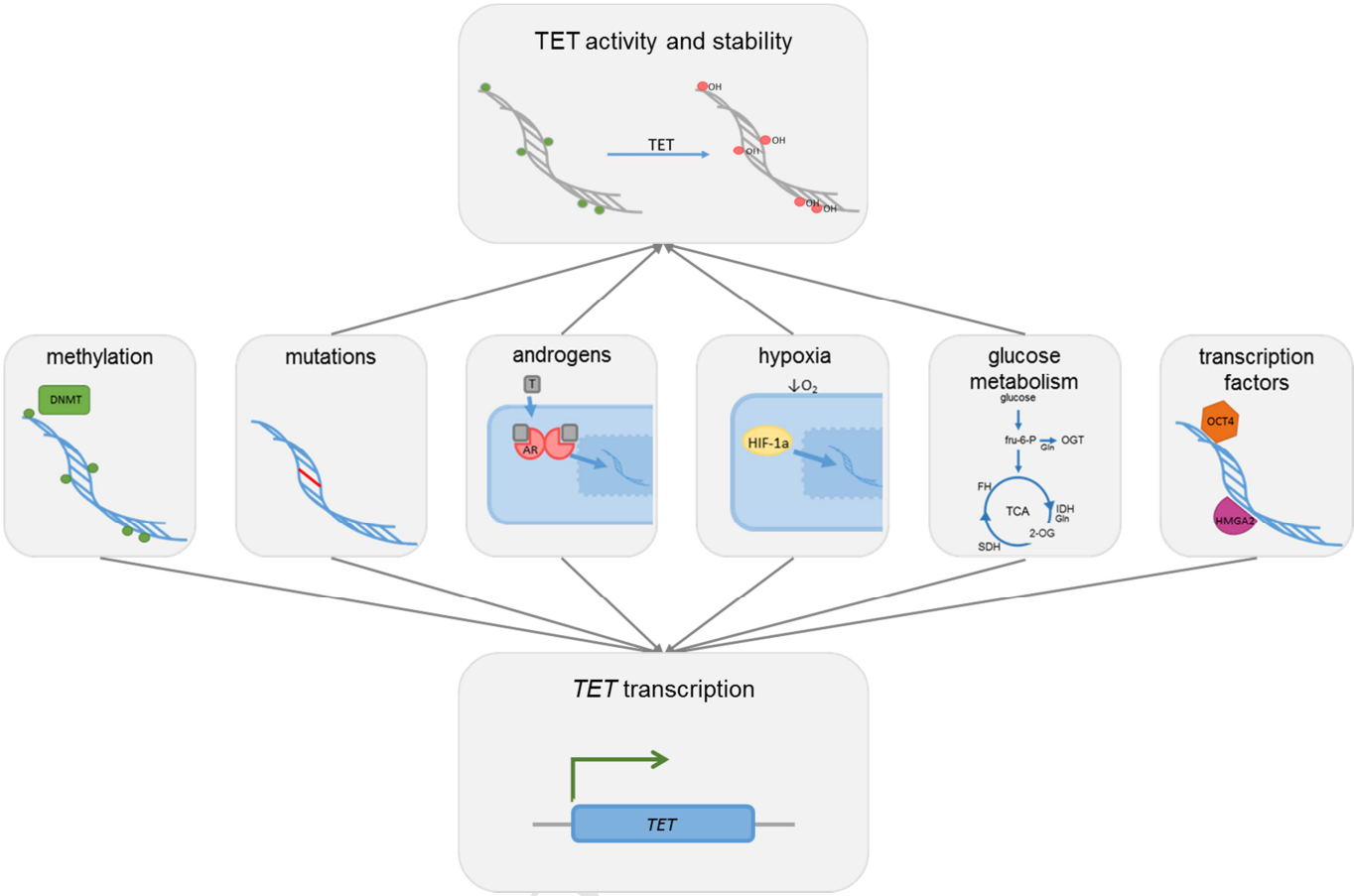

Figure 4: Overview of the important mechanisms influencing TET transcriptional activity and activity and stability in prostate cancer. Many different pathways are altered during PCa development and/or progression. TET activity and stability is dependent on mutations in the *TET* encoding genes, transcription, androgen regulation and metabolic state such as hypoxia and glucose metabolism. On the other hand, TET transcriptional activity can be regulated by methylation of CpG islands in its regulatory regions, *TET* mutations, androgen signaling partly via miRNAs, hypoxia, products of the TCA cycle and altered transcription factor levels and activities such as OCT4 and HMGA2.

7 Methods for epigenetic profiling of prostate samples

In order to better understand the spatial and temporal regulation of TET proteins numerous methods have been developed to detect and quantify the 5hmC content of genomic DNA. These methods leverage specific chemical conversions, enzymatic labeling, affinity enrichment or direct detection (Table 1). Each 5hmC analysis method has specific advantages and limitations; the appropriate choice of method for a given application depends on factors including: the required genomic coverage; the amount of input material available; the number of

samples to be analyzed; and the optimal sequence resolution (Skvortsova, Zotenko, Luu et al., 2017). Careful consideration should be given to the balance between genomic resolution and analytical complexity. Methods that provide a quantitative signal across a genomic locus can allow a high-throughput analysis of differences between tumor and normal, or groups of tumors with different clinical features. In contrast, methods that allow single base resolution are more costly and have a much higher analytical burden, but can identify differences (and patterns of differences) that would be missed using locus-averaging methods.

7.1 Chemical conversion of 5hmC

Methods using sodium bisulfite conversion (BS) may be considered the current 'gold standard' for the analysis of cytosine modifications (Frommer, McDonald, Millar et al., 1992). BS treatment results in the deamination of unmethylated cytosines to uracil (that is subsequently converted to thymine), whereas both 5mC and 5hmC are protected from BS-conversion (and subsequently read out as cytosines; Table 1). Oxidative bisulfite conversion (oxBS) is specific for 5mC alone and therefore the subtraction of oxBS from BS signals allows the specific identification of 5hmC-modified sites (Booth, Branco, Ficz et al., 2012). This subtractive analysis can provide digital genome-wide base-pair resolution analysis of both 5mC and 5hmC, albeit at significant financial and computational cost. The original methods for BS-seq and oxBS-seq require large amounts of input material (micrograms of genomic DNA) (Booth et al., 2012,Frommer et al., 1992,Lister, O'Malley, Tonti-Filippini et al., 2008). However, optimized protocols have been developed that allow lower amounts of starting material (~100ng) (Miura, Enomoto, Dairiki et al., 2012), opening the way for BS/oxBS analysis of precious clinical samples. It is currently not practical to use genome-wide sequencing implementations of these methods in studies with large clinical sample collections due to prohibitive costs and the absence of standardized analysis pipelines for quantitative differential analysis. Array hybridization platforms for BS analysis have been widely used in several large-scale studies (e.g. TCGA and ICGC) including several prostate cancer cohort studies (Brocks et al., 2014,Cancer Genome Atlas Research, 2015). However, the most popular platform (Illumina 450k array) is no longer in production and the cost of dual analysis (BS/oxBS) to differentiate 5mC and 5hmC marked loci on newer array platforms is similar to sequencing based approaches. Where prior knowledge of informative loci exists, as is the case for prostate cancer (Cancer Genome Atlas Research, 2015,Spans et al., 2016), a targeted BS/oxBS-sequencing approach is both practical and affordable for use in large cohort studies (BlueprintConsortium, 2016).

7.2 Affinity enrichment of 5hmC

In contrast, affinity enrichment methods offer a practical high-throughput alternative to map genomic loci marked with 5mC or 5hmC (Table 1). These approaches enrich (or 'pull-down') DNA fragments containing the modification of interest using specific antibodies (MeDIP/hMeDIP) (Ficz et al., 2011,Pomraning, Smith and Freitag, 2009,Weber, Davies, Wittig et al., 2005) or base-modification binding proteins (e.g. MDB-seq/JBP1-seq) (Robertson, Dahl, Vagbo et al., 2011,Serre, Lee and Ting, 2010). The relative enrichment at specific genomic loci provides a quantitative read-out of cytosine modifications at that locus and allows comparative analysis between conditions or cell types. For example, hMeDIP has been applied to clinical prostate cancer samples and has identified extensive heterogeneity in 5hmC profiles between tumor and normal tissue (Spans et al., 2016). Lower costs and less complex downstream analysis make affinity enrichment methods a practical option for large-scale studies that aim to identify or monitor differences between cell types or treatment conditions (Uribe-Lewis, Stark, Carroll et al., 2015,Yegnasubramanian, Wu, Haffner et al., 2011). The resolution of affinity enrichment methods is limited by the input DNA fragment length (e.g. 100-400bp) and in most methods the output provides an average signal across a locus, meaning that focal and low-level differences may be missed in such analyses. Most affinity-based methods also require relatively large amounts of input material (micrograms of DNA) to analyze both 5mC and 5hmC genome-wide.

7.3 Enzymatic labeling of 5hmC

Several 5hmC analysis methods have employed enzymatic covalent tagging of 5hmC bases using T4 bacteriophage β-glucosyltransferase (β-GT) (Bhattacharyya et al., 2013,Pastor, Pape, Huang et al., 2011,Petterson, Chung, Tan et al., 2014,Song, Szulwach, Fu et al., 2011,Yu, Hon, Szulwach et al., 2012). The β-GT enzyme labels 5hmC residues with glucose molecules, either protecting these residues from chemical conversion (TAB-seq) (Yu et al., 2012) or restriction enzyme digestion (HELP-GT, RRHP) (Bhattacharyya et al., 2013,Petterson et al., 2014). Alternatively, β-GT can incorporate azide-glucose moieties at 5hmC positions, allowing subsequent tagging with N-hydroxysuccimide-esters to incorporate either biotin for enrichment methods (hm-Seal) (Song et al., 2011) or fluorophores for direct single molecule quantification (Song, Diao, Brunger et al., 2016). Modifications of these methods allow analysis from nanograms of input DNA (nano-hmC-Seal) (Han, Lu, Shih et al., 2016), allowing 5hmC analysis in small samples (~1000 cells). Most implementations of β-GT tagging of 5hmC result in quantitative signals at a locus-wide level and therefore may miss focal and low-level differences between samples similar to affinity enrichment methods.

7.4 Direct detection of 5hmC

Direct analysis of 5hmC would circumvent many of the limitations inherent to the methods outlined above: inaccuracies from chemical conversion or enzymatic labeling efficiencies; amplification bias and lack of complete genomic coverage; the analytical burden of indirect and subtractive, relative analysis. Single molecule sequencing platforms (e.g. Nanopore and PacBio) offer the capability for direct detection of modified bases in native DNA molecules (Laszlo, Derrington, Brinkerhoff et al., 2013,Schreiber, Wescoe, Abu-Shumays et al., 2013,Wanunu, Cohen-Karni, Johnson et al., 2011). The long read lengths of these platforms also allow analysis of genomic regions that are inaccessible to short read and array-based platforms. For example, a recent study using single molecule real-time sequencing mapped base modifications within specific LINE repeats in the human genome (Suzuki, Korlach, Turner et al., 2016). Alternative single molecule approaches are also being developed, including β-GT fluorophore labeling of single native DNA molecules and direct detection and quantification (Song et al., 2016). Current implementations of these single molecule technologies have limited throughput (e.g. number of reads per sequencing run), making genome-wide analysis difficult and also require large amounts of starting material. In addition, the analysis methods for these newer technologies are not standardized and continuing platform improvements make standardization unlikely in the short-term. However, these methods are constantly improving and with increased throughput are likely to offer the deepest insights into DNA base modifications (including 5hmC) in the future. A particular advantage with respect to prostate cancer analysis is the fast sample-to-data turnaround times, opening the possibility of real-time analysis of patient samples in the clinic to improve diagnostic workflows, prognostication or treatment selection.

	Method Name	Description	Advantages [a]	Disadvantages [b]	References
7.1 Chemical conversion	BS and OxBS-sequencing	Bisulfite conversion (5mC + 5hmC) minus Ox-bisulfite conversion (5mC)	Digital signal, base-pair resolution	DNA fragmentation, input >1µg, BS + OxBS analysis for subtract. analysis	(Booth et al., 2012,Frommer et al., 1992)
	Tn5mC-seq	Tagmentation library prep, followed by BS or OxBS	Efficient library prep, low input (10ng)	Requires high quality input DNA, BS/oxBS after tagmentation reduces complexity	(Adey and Shendure, 2012,Wang, Gu, Adey et al., 2013)
	PBAT	Bisulfite treatment, followed by library prep	100ng DNA for WGBS, base pair resolution	Would require BS and OxBS analysis for subtractive analysis	(Miura et al., 2012)
Affinity enrichm	CMS-pulldown	BS conversion of 5hmC to CMS and anti-CMS antibody pull-down	Quantitative, high-throughput, lower analytical burden	BS conversion and antibody specificity/ efficiency, locus avg.	(Pastor et al., 2011)

	hMeDIP-seq	5hmC-specific antibody pull-down	Direct 5hmC test, quantitative, high-throughput, lower analytical burden	Locus wide signal, resolution limited by fragment length, Ab-specificity (1µg input)	(Ficz et al., 2011,Williams et al., 2011)
	JBP1-pulldown	T4 β–GT tagging and JBP1 pull-down	Reagents generated in-house, HTP method	Fragment resolution, high input (1-100µg DNA)	(Robertson et al., 2011,Robertson, Dahl, Ougland et al., 2012)
7.3 Enzyme conversion	GLIB, hmC-Seal, nano-hmC-Seal	T4 β-GT biotin-tagging of 5hmC	Quantitative, low input (ng DNA), HTP	Resolution limited by fragment length, locus average signal	(Han et al., 2016,Pastor et al., 2011,Song et al., 2011)
	TAB-seq	T4 β-GT 5hmC protection, Tet 5mC oxidation, BS convert.	Base pair resolution, 5hmC readout (not BS / oxBS subtractive)	Dependent on enzymatic and BS conversion efficiency	(Yu et al., 2012)
	MspI/HpaII digest +/- β-GT variants: HELP-GT, RRHP, HMST	HpaII / MspI digest +/- β-GT protection of 5hmC	Up to base-pair resolution, sensitive, 100ng-1µg input	Single CpG/fragment, GC-rich loci, relative analysis (5hmC/5mC)	(Bhattacharyya et al., 2013,Khare, Pai, Koncevicius et al., 2012,Petterson et al., 2014)
	Aba-seq or Pvu-seal-seq	5hmC-specific restriction digest with β-GT tagging and biotin pulldown	Up to base pair resolution, high sensitivity	Single CpG analyzed per fragment, digest and tagging efficiency	(Sun, Dai, Borgaro et al., 2015)
	Thiol, selenol or aldehyde enzyme tagging	M.HhaI/M.SssI tag with R-SH/R-SeH donor, then NHS-ester labeling	Direct 5hmC labeling, flexible tagging workflow	Not extensively validated, ~20% recovery and ~1% FP	(Liutkeviciute, Lukinavicius, Masevicius et al., 2009,Liutkeviciute, Kriukiene, Grigaityte et al., 2011)
7.4 Single molecule analysis	Direct single molecule sequencing	Direct 5hmC detection in single molecule SMRT or nanopore sequencing	Direct detection, digital readout, long reads, low consensus error, rapid output	High input DNA, no standardized analysis, low reads per run for WGS analysis	(Flusberg, Webster, Lee et al., 2010,Laszlo et al., 2013,Schreiber et al., 2013,Wanunu et al., 2011)
	Tagged single molecule sequencing	T4 β-GT biotin tag and SMRT-seq or nanopore seq	Improved signal with bulky Glc. modification	Dependent on tagging efficiency	(Song, Clark, Lu et al., 2011,Zahid, Zhao, He et al., 2016)
	BS and Ox-BS single molecule sequencing	Bisulfite conversion and SMRT-seq	Longer read analysis: captures regions not possible by short-reads	Ox-BS conversion efficiency, DNA fragmentation	(Yang, Sebra, Pullman et al., 2015)
	Thiol labeling (BS mediated)	Thiolation of 5hmC in ssDNA with nanopore seq	Improved detection of 5hmC	Efficiency, specificity and throughput not well characterized	(Lu and He, 2013)

Table 1: Summary of selected published methods for the analysis of 5hmC. Subset: BS-conversion; affinity enrichment; enzymatic tagging; direct analysis. [a] Base-pair resolution methods allow the assessment of individual cytosine residues at each locus analyzed. [b] Locus average methods provide a global view of 5mC and 5hmC at a given locus (in most cases the resolution is defined by DNA fragment lengths in the input material).

8 Conclusions

The importance of TET enzymes and 5hmC in development and disease is clear, as are the multiple levels of regulation of these important epigenetic regulators. By contrast, the role of these proteins in PCa is only beginning to be understood, but the combination of insights from studies in other systems and high-throughput analysis methods offer great potential for future studies. Over the coming years we will no doubt learn more about the role of TET and 5hmC in PCa biology, and the potential for TET proteins and their targets to act as disease markers and therapeutic targets to improve patient outcomes.

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Highlights

- Description of the link between prostate cancer and TET-mediated hydroxymethylation
- Mapping of the hydroxymethylation landscape in prostate cancer
- Overview of mechanisms influencing TET expression and activity in the prostate
- Methods for detecting and quantifying global and locus-specific 5(h)mC levels

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