Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Review

Loss of 5-hydroxymethylcytosine in cancer: Cause or consequence?

CrossMark

Gabriella Ficz *, John G. Gribben

Centre for Haemato-Oncology, Barts Cancer Institute, EC1M 6BQ London, UK

ARTICLE INFO

ABSTRACT

Discovery of the enzymatic activity that catalyses oxidation of 5-methylcytosine (5mC) to generate 5-hydroxymethylcytosine (5hmC) mediated by the MLL (KMT2A) fusion partner TET1 has sparked intense research to understand the role this new DNA modification has in cancer. An unambiguous picture has emerged where tumours are depleted of 5hmC compared to corresponding normal tissue, but it is not known whether lack of 5hmC is a cause or a consequence of tumourigenesis. Experimental data reveals a dual tumour-suppressive and oncogenic role for TET proteins. *Tet2* mutations are drivers in haematological malignancies but *Tet1* had an oncogenic role in MLL-rearranged leukaemia, where *Tet1* is overexpressed. Over-expression of *Tet2* in melanoma cells re-established the 5hmC landscape and suppressed cancer progression but inhibiting *Tet1* in non-transformed cells did not initiate cellular transformation. In this review we summarise recent findings that have shaped the current understanding on the role 5hmC plays in cancer.

© 2014 Elsevier Inc. All rights reserved.

Contents

1. Introduction

DNA methylation became a cancer research focus in the 1970s when analysis of tumours revealed significant aberrations in 5mC distribution, i.e. hypermethylation of gene promoter CpG islands (or CGIs — genomic regions with dense CG content), and gene silencing as a consequence, against a background of genome-wide hypomethylation [1,2]. In somatic cells DNA methylation occurs in CG dinucleotides and about 70–85% of them are constantly methylated, with the exception of CGIs, which tend to be demethylated. Methylation is catalysed by DNA methyltransferases such as DNMT1, the maintenance methyltransferase, which copies methylation to the newly synthesised strand during cell division and DNMT3A and DNMT3B, de novo methyltransferases — enzymes that can methylate DNA in the absence of a hemimethylated template [3]. This heritable epigenetic modification present in all vertebrates is needed for normal development as demonstrated in knockout mice where functional loss of DNA methyltransferases is either early embryonic lethal (*Dnmt1* and *Dnmt3b*) or perinatal lethal (*Dnmt3a*) [4,5]. Due to the high rate of mutation of methylated CG due to spontaneous deamination to generate TG, the frequency of this CG dinucleotide in the genome is significantly reduced (about 4 fold) compared to other dinucleotides such as GC. Although somatically methylated, the genome undergoes global demethylation in early mammalian embryogenesis (during germ cell differentiation and in the zygote), a process associated with totipotency and pluripotency [6–8]. It has been a long-standing

* Corresponding author.
  E-mail address: g.ficz@qmul.ac.uk (G. Ficz).

question how DNA demethylation occurs and what enzyme catalyses the break of the C–C bond between the cytosine ring and the methyl group, often with claims of activities that proved to be difficult to validate independently [9].

One of the hypotheses for DNA demethylation involved oxidation of 5mC to 5hmC. 5hmC as a modified base had been previously identified in mammalian tissues but ignored and forgotten largely due to controversy [10]. In 2009, the presence of 5hmC was proven unequivocally and the enzymatic process converting 5mC to 5hmC was identified [11,12]. Compared to the relatively constant levels of 5mC in somatic tissues (3–4% of total cytosines), 5hmC levels are significantly lower and vary greatly depending on the cell type (0.1%–0.7% of all cytosines) [13,14]. The Tet family of Fe(II)- and α-ketoglutarate (α-KG) dependent dioxygenases comprises three proteins (TET1, TET2, TET3) each with varying abundance in different cell types. *Tet1* and *Tet2* are relatively highly expressed in embryonic stem cells and early embryogenesis and their levels drop as cells exit pluripotency and undergo differentiation. Later in development *Tet2* is highly expressed in the haematopoietic system while *Tet3* is overexpressed in germ cells/oocyte, brain tissue and more ubiquitously in somatic cells. TET proteins are responsible for generating all of the 5hmC in the genome [15] and lack of all three TET proteins is incompatible with proper development and such cells contribute poorly to a developing embryo in chimera assays [16]. All three proteins catalyse further oxidation steps generating 5fC and 5caC with varying efficiencies [17,18]. The level of these intermediates is ~20 (5fC) and ~3 (5caC) in 10^6 cytosines per genome (or 0.002% 5fC/C and 0.0003% 5caC/C), at least two orders of magnitude fewer than 5hmC indicating that these modifications could be true demethylation intermediates [18]. Both 5fC and 5caC can be excised by TDG [17,19], thus closing the demethylation cycle [20,21]. Acute downregulation of TDG activity generates up to 7 fold increase in 5fC in mouse embryonic stem (ES) cells, indicating lack of excision of the oxidised 5hmC [22]. It is thought that the main role of 5hmC lies in DNA demethylation but 5hmC is relatively stable and abundant in the genome (see Review by Pfeifer and colleagues, this issue). The question thus arises, if TET proteins are capable of iteratively oxidising 5mC to 5caC then why does this reaction stop at 5hmC so frequently? Current data indicates that perhaps 5hmC and other oxidised forms may have epigenetic roles other than functioning as DNA demethylation intermediates (discussed below). There is a possibility that differences among TET proteins might be reflected by their demethylation potential since TET2 seems to be somewhat more efficient in generating 5fC and 5caC [18]. It is notable that the Tet2 gene region has undergone chromosomal inversion and has lost its CXXC domain during evolution, a chromatin binding protein domain which is retained by both *Tet1* and *Tet3* [23]. The separated region encodes a gene called *Cxxc4* or *IDAX* and has been shown to regulate TET2 protein levels [23]. This is interesting since TET1 and TET2 are targeted to different sites in ES cells, and are thought to have different functions in ES cells [24].

Before the discovery of TET enzymatic activity in 2009, Tet1 was known as the fusion partner of MLL in patients with AML [25,26]. Later it was found that indirect inhibition of TET activity, particularly in AML subclasses with mutations in metabolic enzymes (as discussed below), correlated with promoter hypermethylation. This suggests that lack of TET activity led to increased methylation and subsequent silencing of the majority of genes affected [27,28]. Numerous loss-of-function mutations of *Tet2* have been identified in myeloid cancers where *Tet2* was shown to be a critical tumour suppressor as detailed below. Nevertheless, it has been recently shown that Tet1 plays an oncogenic role in MLL-rearranged leukaemia where the endogenous *Tet1* gene is directly targeted and overexpressed by the MLL fusion proteins [29]. In this study Huang et al. showed in an MLL fusion-induced transformation assay, that downregulation of *Tet1* led to reversal of the tumourigenic phenotype whereas overexpression of *Tet1* exacerbated it. These results suggest contradictory roles for TET proteins in cancer initiation and progression but perhaps such behaviour is more

indicative of a mechanism where TET proteins are participating in reinforcing an existing cellular identity and transcriptional network. Nevertheless, it remains to be seen if aberrant targeting of TET proteins could have a significant role in cellular transformation.

2. Distribution and targeting of TET proteins and 5hmC in development

It took over 50 years from the first observation of 5hmC in bacteriophages to its recognition as a new epigenetic modification [30,31]. Compared to 5mC which is relatively abundant in somatic tissues, absolute levels of 5hmC are highly variable both in genome-wide and in specific genomic single base locations and there seems to be no direct correlation between 5mC levels and 5hmC [32] with the exception of complete lack of 5mC where no 5hmC is generated, as detailed below. This is indicative of a highly controlled targeting mechanism of functional TET proteins to the chromatin to generate the 5hmC patterns observed, mechanisms that might be specific to a particular cellular state [33]. Moreover, this control is exerted differentially among the three TET proteins as evidenced by a series of recent observations. In ES cells, association of TET1 to the chromatin is stronger than that of TET2, which could be explained by the lack of the CXXC domain [34]. Also, TET1 and TET2 are targeted to different genomic regions in ES cells, where TET1 is more associated with promoters while TET2 regulates oxidation of gene bodies as shown in Fig. 1 [24]. Interestingly, Huang et al. found that loss of *Tet2* has a more dramatic effect on global 5hmC loss in both *Tet2* shRNA depleted and Tet2 −/− ES cells, even though it is known that the expression level of *Tet2* is lower in ES cells than that of *Tet1* [15]. This study also showed that different regions are affected in shRNA treated ES cells: while *Tet1* shRNA affected mainly promoter regions, loss of *Tet2* resulted in significant loss of 5hmC in gene bodies and at the boundaries of highly expressed exons [24].

One interesting aspect of the abundance of 5mC and 5hmC in adult tissues is that while the majority of somatic cells have relatively constant 5mC levels, cells in the adult stem cell compartment have significantly lower levels for both modifications, therefore it is thought that the presence of 5hmC in healthy tissues is an indication of the differentiation state, with more differentiated cells having more 5hmC and a tissue specific 5hmC level [35,36].

3. Modulation of TET activity by cofactors and vitamins

TET proteins are dioxygenases that use molecular oxygen, Fe(II) and 2-oxoglutarate to oxidise 5mC and convert it to 5hmC. 2-Oxoglutarate (2OG also known as α-ketoglutarate) is a cofactor produced in the Krebs cycle by isocitrate dehydrogenase (IDH1/2/3) through
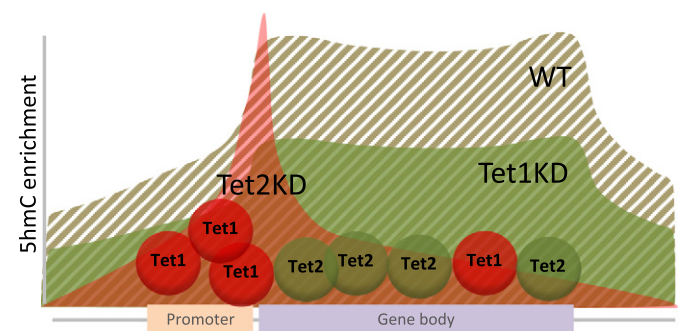

Fig. 1. Distinct distribution patterns for Tet proteins and associated 5hmC loss upon Tet downregulation. The highest density of Tet1 binding sites is located around the gene promoter and loss of Tet1 leads to uniform decrease of 5hmC over the gene body and promoter regions (green relative to the striped area). Loss of Tet2 in mouse embryonic stem cells leads to 5hmC decrease over the gene body indicating specific targeting of Tet2 to gene bodies.

decarboxylation of isocitrate to 2OG. IDH proteins have different roles and locations (IDH1 is cytosolic and IDH2 is mitochondrial) and the importance of these proteins in epigenetic homeostasis is highlighted in gain of function mutations (such as IDH1 R132) which catalyses a neomorphic reaction that converts 2OG to 2-hydroxyglutarate (2HG). 2HG inhibits the catalytic activity of 2OG dependent dioxygenases including TET proteins [28]. Gain-of-function mutations in IDH1 are frequent in cancer as detailed below but since 2OG affects many other epigenetic regulators such as lysine demethylases, subsequent tumourigenic events might be multifactorial and not necessarily TET activity related.

Vitamin C is an essential nutrient for primates (including humans), bats and guinea pigs, which have lost the ability to synthesise it from glucose, unlike most mammals which can synthesise Vitamin C. Recent studies have shown that Vitamin C can enhance DNA demethylation through an increase in TET activity [37,38]. This is possible because Vitamin C acts as an electron donor and recycles the oxidation state of Fe(IV) to Fe(II) therefore adjusting the redox state of iron containing enzymes for subsequent oxidation reactions. In the absence of a substrate (5mC), decarboxylation of 2OG to succinate will lead to inactive TET enzyme with Fe(IV) which can be reduced to Fe(II) by Vitamin C and render the enzyme active again.

4. Mechanism of 5hmC loss in cancer

5mC is the only substrate to generate 5hmC in vivo as evidenced by measuring absolute 5hmC levels in ES cells lacking all three DNA methyltransferases and 5mC as a result [14,39]. In the absence of all DNMTs 5hmC levels are below detection limits, but in cells with normally distributed 5mC throughout the genome, 5hmC levels are highly variable and its level and distribution are cell type specific [33].

Genomic hypomethylation is a key feature in cancer [1,2]. Research for more than a decade has resulted in a more comprehensive picture of DNA demethylation and we now know that loss of methylation can occur through various mechanisms: active loss through iterative oxidation of 5mC to 5hmC, 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) by TET proteins followed by excision of 5fC and 5caC by TDG [20]; active loss through deamination of 5mC to U catalysed by AID (AICDA) and APOBEC1 followed by base excision repair [6]; passive loss whereby methylation is diluted during several cycles of cell divisions in the context of lack of maintenance activity by DNMT1 and its partner UHRF1 [40]. It has been recently confirmed that at least in the case of primordial germ cells (PGCs) passive demethylation is the dominant mechanism to demethylate the genome in combination with active removal of 5mC through iterative oxidation by TET proteins and replicative loss of both 5mC and 5hmC [41–44]. In cells with a functional maintenance methylation system, an accelerated passive demethylation can exist whereby the oxidised 5mC is lost during DNA replication since 5hmC is not recognised by Dnmt1 [45] (see also Review by Hajkova and colleagues, this issue).

Loss of 5hmC in tumours, as discussed below, can occur through two mechanisms reported to date: inactivating mutations of *Tet* and inhibition of TET activity through IDH1/2 mutations [27,28,46,47]. This might be cancer type specific since many solid cancers do not show mutations in either of these genes. Very few cancers exhibit mutations in *Dnmt1* [48] and therefore loss of the substrate 5mC through *Dnmt1* inactivation is not considered to be a significant route to initiating tumourigenesis. Nevertheless, numerous mutations in *Dnmt3a* have been identified in blood cancers as described in more details below.

Loss of TET activity is associated with the cancer phenotype but it is not clear whether TET proteins function as tumour suppressors or oncogenes. While loss of *Tet1* and *Tet2* expression is associated with solid cancers, implying a tumour suppressor role, in the context
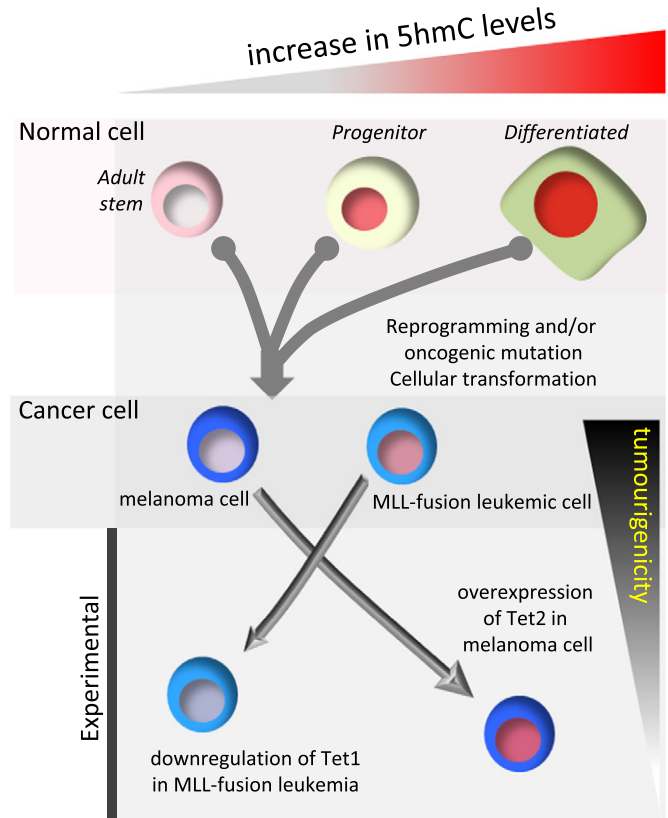

Fig. 2. Dual, cancer-specific functions for TET proteins and 5hmC levels in differentiation stages of adult stem cells. The abundance of 5hmC is low in differentiated adult stem cells and increases over the course of differentiation (intensity of the colour in the nuclei representing 5hmC levels throughout). Cellular transformation can occur through various mechanisms and depending on the type of cancers Tet proteins can have both tumour suppressive or oncogenic roles. Low 5hmC containing melanoma cells can become less tumourigenic by overexpression of Tet2 which also leads to increased 5hmC. MLL-fusion leukemic cells become less tumourigenic upon *Tet1* downregulation which leads to decrease of 5hmC overall.

of genomic rearrangements such as in MLL-fusion rearranged leukaemia, *Tet1* exhibits a clear oncogenic role (Fig. 2) [29].

4.1. Loss of 5hmC in solid cancers

A number of studies have revealed significantly lower 5hmC levels in cancer cell lines [32] or primary tissue, melanoma [49], adenoma and carcinoma [50] compared to the normal surrounding tissue. It is important to highlight that cell culture conditions and the transition from a primary tissue towards establishing a cell line can influence the levels of 5hmC, which is also associated with strong reduction of *Tet* expression [32].

Changes in expression of *Tet1* have been reported in solid cancers, where *Tet1* was downregulated while *Tet2* and *Tet3* expression did not show differences compared to normal tissues [32]. Other labs have found strong reduction in *Tet1* expression and significant reduction in *Tet2* and *Tet3* in breast and liver cancers, which could explain the low levels of 5hmC [49–52]. Kudo et al. found that reduction of *Tet1/2/3* expression and subsequently of 5hmC levels was a consequence of BRAF V600E oncogene driven cellular transformation in NIH3T3 cells [53] and excluded the possibility that activation of the MAPK pathway was causing *Tet1* downregulation. Importantly, this study showed that downregulation of *Tet1* using stably integrated shRNA in NIH3T3 cells was not sufficient to induce cellular transformation [53]. This could indicate that loss of 5hmC probably does not have driver properties in cancer initiation but rather reinforces the molecular networks

functioning in cancer cells. Reduction in 5hmC could nevertheless be a consequence of global 5mC loss in cancers. However, colon and prostate adenocarcinoma showed only modest global reduction compared to the loss of 5hmC therefore this cannot account for lower 5hmC seen in these cancers [35].

Very few studies have mapped 5hmC distribution in cancer tissues. Melanoma is associated with strong reductions in absolute 5hmC levels [49] and sequencing of the 5hmC landscape validated wide-spread loss of 5hmC in the genome and revealed increase in 5mC, as a consequence of lack of oxidation, in numerous gene bodies (of genes in Wnt signalling melanogenesis pathways) and promoters (of genes involved in regulation of cell morphogenesis, cytoskeleton organisation etc.). Another report in pancreatic cancer cells found that global loss of 5hmC was accompanied by genomic redistribution of the modification where exons and transcription factor binding sites were enriched for 5hmC compared to normal cells and this enrichment was more variable in promoters, CpG islands and shores in the two cell lines analysed [54].

4.2. Loss of 5hmC in haematological malignancies

Although Tet1 was first identified as one of the MLL fusion partners in leukaemias, it shows the highest expression levels in embryonic stem cells and in early embryogenesis. *Tet2* expression is the highest in the haematopoietic lineage where it is thought to be involved in normal haematopoiesis, exit of haematopoietic stem cells (HSC) from the multipotent niche and lineage determination. A number of *Tet2* knock-out mouse lines have been generated and the overall conclusion is that Tet2 is not essential for normal development as it is not embryonic lethal, but mice lacking *Tet2* develop haematopoietic malignancies with phenotypes similar to human CMML (chronic myelomonocytic leukaemia) patients with mutant *Tet2* [55]. *Tet2* −/− HSCs are skewed towards an undifferentiated state with increased ability to repopulate the haematopoietic compartment during competitive reconstitution assays with normal *Tet2* +/+ cells. Interestingly, this phenotype is similar to conditional *Dnmt1* −/− and *Dnmt3a* −/− HSC, which are also not able to differentiate during serial transplantation assays [56,57]. This strengthens the hypothesis whereby lineage determination in the haematopoietic system is controlled in part by deposition of 5mC and 5hmC, with the principal role being to silence genes used by differentiated myeloid lineages [58]. Hence the number of HSCs in *Tet2* −/− mice is increased and although they can differentiate somewhat, the lineage is skewed towards monocyte/macrophage cell fates [55,59–61].

Numerous *Tet2* mutations have been identified in myeloproliferative neoplasm (MPN), myelodysplastic syndrome (MDS) and other blood cancers [46,62–65], also reviewed in [66]. About 15% of myeloid cancers and 22% of AML have *Tet2* inactivating mutations [46,63] and in AML with normal karyotype (CN-AML) which accounts for 40–50% of cases, mutations in other epigenetic regulators such as EZH2, DNMT3a, TET2, MLL, IDH1 and IDH2 are common. A key question in the case of TET proteins is whether it is the 5hmC generated by the protein, or some different functions of the protein itself that has tumour suppressive roles. To answer this question, one needs to separate the oxidative from the non-oxidative roles of TET proteins. Ko et al. partially addressed this question by analysing myeloid malignancies and the associated 5hmC levels in the context of wild-type or mutant *Tet2* [67]. Within the *Tet2* mutated group where low 5hmC levels were measured, a number of cases fortuitously contained missense mutations in amino acids involved in various aspects of the 5hmC generating TET enzymatic activity with otherwise normal protein sequence [67]. This indicates that in at least some haematological malignancies, the tumour suppressive role of TET2 appears to be correlated to its oxidative activity. A more elegant demonstration of this would be to generate transgenic mice with *Tet2* missense mutations in amino acids involved in oxidative activity, thus providing a causal link between tumour

suppression and TET2 oxidative activity, which has not been reported to date to our knowledge.

5. 5hmC maintenance through cell division

There is no evidence so far that 5hmC is maintained through cell division. Moreover, there is in vitro evidence that DNMT1 does not recognise hemihydroxymethylated DNA to methylate the opposite strand [68]. Therefore, generation of 5hmC can induce 5mC loss during cell division. Combined with subsequent oxidation steps of 5hmC, removal of 5fC and 5caC by TDG is considered to accelerate global demethylation through an active–passive demethylation mechanism [20]. Nevertheless, in certain cell types, particularly with an apparently intact or partially functional methylation system, 5hmC might have a role for deposition of 5mC through an unknown mechanism. This is highlighted by observations that while some genomic regions (such as promoters) accumulate 5mC upon *Tet2* knockdown in ES cells, the vast majority of regions lost both 5hmC and 5mC [24]. In addition, regions such as IAPs (intracisternal A particles) that do not demethylate in ES cells when they are transiting to ground state pluripotency, do accumulate a small amount of 5hmC, but do not demethylate over time [45]. Similar observations have been reported in imprinted control regions and loci escaping reprogramming in PGCs [42,69]. It has been argued that loss of 5hmC through cell division is associated with, and could be caused by, rapid replication cycles in highly proliferating cells [30]. While this is a possibility in primary cell lines expanded in culture [32], mouse ES cells are highly proliferating in culture and maintain a relatively high level of 5hmC over time therefore it does not support this hypothesis.

6. Reading 5hmC, 5fC and 5caC (5oxiC)

After the discovery of 5hmC, the most studied hypothesis was that 5hmC is an intermediate in the demethylation pathway. This has been validated in various systems, although research has highlighted that it is likely that 5hmC has its own bona fide epigenetic functions (see Review by Pfeifer and colleagues, this issue). First, if 5hmC was an intermediate only in the demethylation cycle, its levels probably would be significantly lower in the genome. Second, known methyl binders [70] do not recognise 5hmC, therefore local oxidation of 5mC could lead to release of these proteins from chromatin. Third, as recent work shows, even though knockout of all three TET proteins leads to some increase in methylation, it does not steadily increase to saturation of methylation in the genome.

A number of labs have attempted to find "readers" for the oxidised forms of 5mC, proteins that would recognise and bind to 5oxiC. The first identified was UHRF1 (known as NP95), which is the protein that recognises 5mC at DNA replication and recruits DNMT1 to methylate the newly synthesised strand. It was shown that UHRF1 recognises 5hmC [71] but the function of this binding is not known, especially since DNMT1 does not recognise hemihydroxymethylated DNA to methylate the opposite strand as it does with hemimethylated DNA in vitro [68]. On the other hand, a similar protein UHRF2 was found to bind to 5hmC [72] and it will be exciting to find out if certain regions in the genome could use an alternative mechanism to maintain 5hmC.

Later, MBD3 was reported to bind to 5hmC [73] but this was not validated by other studies [72,74]. The most comprehensive study so far is by Spruijt et al., where a sensitive SILAC based proteomics approach was used to identify 5oxiC binders. This led to interesting results and identified a number of 5oxiC "readers" [72]. A similar study which used longer promoter sequences as bait [74] followed by protein mass spectrometry highlighted something unexpected. It found that 5fC was recognised uniquely by a higher number of proteins compared to a very few identified as 5hmC binders [74]. Moreover, there was a strong enrichment for transcription factors such as Fox domain transcription factors and the complete NURD complex. Interestingly this pattern was DNA

sequence dependent and common and unique proteins bound significantly to 5fC DNA depending on the bait. It is possible that only a subset of the proteins bound directly, suggested by an independent method used to validate the 5fC binders [74]. It remains to be seen if 5fC can function as a repressive or potentially activating epigenetic modification.

7. Outlook

Understanding the molecular events that drive cellular transformation is essential in order to develop effective cancer therapies. Epigenetic aberrations such as promoter hypermethylation are frequent in tumours and since the discovery of the demethylation mechanism through iterative oxidation of 5mC to generate 5hmC, 5fC and 5caC and recycle C, great attention has been given in understanding the demethylating role of 5hmC in cancer. There have been a series of exciting studies published recently, which answered some of the questions and led to novel diagnostic avenues. There is a strong correlation between loss of 5hmC and cancer development but research to date indicates that it is more likely that TET proteins are reinforcing the molecular circuitries already present in cancer cells, rather than contributing to cellular transformation through loss of TET activity. TET2 in the haematopoietic system is an exception since results in *Tet2* deficient mice demonstrated that loss of *Tet2* is sufficient to induce neoplasm, therefore *Tet2* functions as a bona fide tumour suppressor [55,59,60]. Since Tet1 can have an oncogenic role in MLL-rearranged leukaemia, and lack of oxidation leads to hypermethylation of cancer-relevant sub-classes of genes in melanoma, a key interest for the future would be to know more about how TET proteins are targeted to chromatin and what molecules control this. The observation that overexpression of the Yamanaka pluripotency factors can lead to wide-spread tumour formation in vivo [75], all in the absence of genetic mutations, highlights the fact that epigenetic reprogramming can drive cellular transformation. Indeed, one component of epigenetic reprogramming is global DNA demethylation, a feature frequently observed in cancer together with hypermethylation of CGI promoters. Therefore, understanding targeting of the DNA methylation machinery, in addition to targeting of TET proteins, is a key question to address in the future.

References

[1] M. Esteller, Epigenetic gene silencing in cancer: the DNA hypermethylome, Hum. Mol. Genet. 16 (Spec No 1) (2007) R50–R59, http://dx.doi.org/10.1093/hmg/ddm018.

[2] S.B. Baylin, P.A. Jones, A decade of exploring the cancer epigenome — biological and translational implications, Nat. Rev. Cancer 11 (2011) 726–734, http://dx.doi.org/10.1038/nrc3130.

[3] K.D. Robertson, DNA methylation, methyltransferases, and cancer, Oncogene 20 (2001) 3139–3155, http://dx.doi.org/10.1038/sj.onc.1204341.

[4] E. Li, T.H. Bestor, R. Jaenisch, Targeted mutation of the DNA methyltransferase gene results in embryonic lethality, Cell 69 (1992) 915–926.

[5] M. Okano, D.W. Bell, D.A. Haber, E. Li, DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development, Cell 99 (1999) 247–257.

[6] S. Seisenberger, J.R. Peat, W. Reik, Conceptual links between DNA methylation reprogramming in the early embryo and primordial germ cells, Curr. Opin. Cell Biol. 25 (2013) 281–288, http://dx.doi.org/10.1016/j.ceb.2013.02.013.

[7] W. Reik, W. Dean, J. Walter, Epigenetic reprogramming in mammalian development, Science 293 (2001) 1089–1093, http://dx.doi.org/10.1126/science.1063443.

[8] J.A. Hackett, M.A. Surani, DNA methylation dynamics during the mammalian life cycle, Phil. Trans. R. Soc. B Biol. Sci. 368 (2013) 20110328, http://dx.doi.org/10.1098/rstb.2011.0328.

[9] S.K.T. Ooi, T.H. Bestor, The colorful history of active DNA demethylation, Cell 133 (2008) 1145–1148, http://dx.doi.org/10.1016/j.cell.2008.06.009.

[10] N.W. Penn, Modification of brain deoxyribonucleic acid base content with maturation in normal and malnourished rats, Biochem. J. 155 (1976) 709–712.

[11] M. Tahiliani, K.P. Koh, Y. Shen, W.A. Pastor, H. Bandukwala, Y. Brudno, et al., Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1, Science 324 (2009) 930–935, http://dx.doi.org/10.1126/science.1170116.

[12] S. Kriaucionis, N. Heintz, The nuclear DNA base 5-hydroxymethylcytosine Is present in Purkinje neurons and the brain, Science 324 (2009) 929–930, http://dx.doi.org/10.1126/science.1169786.

[13] D. Globisch, M. Münzel, M. Müller, S. Michalakis, M. Wagner, S. Koch, et al., Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates, PLoS ONE 5 (2010) e15367, http://dx.doi.org/10.1371/journal.pone.0015367.s005.

[14] A. Szwagierczak, S. Bultmann, C.S. Schmidt, F. Spada, H. Leonhardt, Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA, Nucleic Acids Res. 38 (2010) e181, http://dx.doi.org/10.1093/nar/gkq684.

[15] K.P. Koh, A. Yabuuchi, S. Rao, Y. Huang, K. Cunniff, J. Nardone, et al., Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells, Cell Stem Cell 8 (2011) 200–213, http://dx.doi.org/10.1016/j.stem.2011.01.008.

[16] M.M. Dawlaty, A. Breiling, T. Le, M.I. Barrasa, G. Raddatz, Q. Gao, et al., Loss of Tet enzymes compromises proper differentiation of embryonic stem cells, Dev. Cell 29 (2014) 102–111, http://dx.doi.org/10.1016/j.devcel.2014.03.003.

[17] Y.-F. He, B.-Z. Li, Z. Li, P. Liu, Y. Wang, Q. Tang, et al., Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA, Science 333 (2011) 1303–1307, http://dx.doi.org/10.1126/science.1210944.

[18] S. Ito, L. Shen, Q. Dai, S.C. Wu, L.B. Collins, J.A. Swenberg, et al., Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine, Science 333 (2011) 1300–1303, http://dx.doi.org/10.1126/science.1210597.

[19] A. Maiti, A.C. Drohat, Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites, J. Biol. Chem. 286 (2011) 35334–35338, http://dx.doi.org/10.1074/jbc.C111.284620.

[20] R.M. Kohli, Y. Zhang, TET enzymes, TDG and the dynamics of DNA demethylation, Nature 502 (2013) 472–479, http://dx.doi.org/10.1038/nature12750.

[21] M.R. Branco, G. Ficz, W. Reik, Uncovering the role of 5-hydroxymethylcytosine in the epigenome, Nat. Rev. Genet. 13 (2011) 7–13, http://dx.doi.org/10.1038/nrg3080.

[22] E.-A. Raiber, D. Beraldi, G. Ficz, H.E. Burgess, M.R. Branco, P. Murat, et al., Genome-wide distribution of 5-formylcytosine in embryonic stem cells is associated with transcription and depends on thymine DNA glycosylase, Genome Biol. 13 (2012) R69, http://dx.doi.org/10.1186/gb-2012-13-8-r69.

[23] M. Ko, J. An, H.S. Bandukwala, L. Chavez, T. Äijö, W.A. Pastor, et al., Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX, Nature 497 (2013) 122–126, http://dx.doi.org/10.1038/nature12052.

[24] Y. Huang, L. Chavez, X. Chang, X. Wang, W.A. Pastor, J. Kang, et al., Distinct roles of the methylcytosine oxidases Tet1 and Tet2 in mouse embryonic stem cells, Proc. Natl. Acad. Sci. 111 (2014) 1361–1366, http://dx.doi.org/10.1073/pnas.1322921111.

[25] R. Ono, T. Taki, T. Taketani, M. Taniwaki, H. Kobayashi, Y. Hayashi, LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23), Cancer Res. 62 (2002) 4075–4080.

[26] R.B. Lorsbach, J. Moore, S. Mathew, S.C. Raimondi, S.T. Mukatira, J.R. Downing, TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23), Leukemia 17 (2003) 637–641, http://dx.doi.org/10.1038/sj.leu.2402834.

[27] M.E. Figueroa, O. Abdel-Wahab, C. Lu, P.S. Ward, J. Patel, A. Shih, et al., Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation, Cancer Cell 18 (2010) 553–567, http://dx.doi.org/10.1016/j.ccr.2010.11.015.

[28] W. Xu, H. Yang, Y. Liu, Y. Yang, P. Wang, S.-H. Kim, et al., Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases, Cancer Cell 19 (2011) 17–30, http://dx.doi.org/10.1016/j.ccr.2010.12.014.

[29] H. Huang, X. Jiang, Z. Li, Y. Li, C.-X. Song, C. He, et al., TET1 plays an essential oncogenic role in MLL-rearranged leukemia, Proc. Natl. Acad. Sci. 110 (2013) 11994–11999, http://dx.doi.org/10.1073/pnas.1310656110/-/DCSupplemental/pnas.201310656SI.pdf.

[30] G.P. Pfeifer, S. Kadam, S.-G. Jin, 5-Hydroxymethylcytosine and its potential roles in development and cancer, Epigenetics Chromatin. 6 (2013) 10, http://dx.doi.org/10.1186/1756-8935-6-10.

[31] C.-X. Song, C. He, Potential functional roles of DNA demethylation intermediates, Trends Biochem. Sci. 38 (2013) 480–484, http://dx.doi.org/10.1016/j.tibs.2013.07.003.

[32] C.E. Nestor, R. Ottaviano, J. Reddington, D. Sproul, D. Reinhardt, D. Dunican, et al., Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes, Genome Res. 22 (2012) 467–477, http://dx.doi.org/10.1101/gr.126417.111.

[33] A. Laird, J.P. Thomson, D.J. Harrison, R.R. Meehan, 5-Hydroxymethylcytosine profiling as an indicator of cellular state, Epigenomics. 5 (2013) 655–669, http://dx.doi.org/10.2217/epi.13.69.

[34] P. Vella, A. Scelfo, S. Jammula, F. Chiacchiera, K. Williams, A. Cuomo, et al., Tet proteins connect the O-linked N-acetylglucosamine transferase Ogt to chromatin in embryonic stem cells, Mol. Cell 49 (2013) 645–656, http://dx.doi.org/10.1016/j.molcel.2012.12.019.

[35] M.C. Haffner, A. Chaux, A.K. Meeker, D.M. Esopi, J. Gerber, L.G. Pellakuru, et al., Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers, Oncotarget 2 (2011) 627–637.

[36] Y. Tadokoro, H. Ema, M. Okano, E. Li, H. Nakauchi, De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells, J. Exp. Med. 204 (2007) 715–722, http://dx.doi.org/10.1084/jem.20060750.

[37] K. Blaschke, K.T. Ebata, M.M. Karimi, J.A. Zepeda-Martínez, P. Goyal, S. Mahapatra, et al., Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells, Nature 500 (2013) 222–226, http://dx.doi.org/10.1038/nature12362.

[38] A. Monfort, A. Wutz, Breathing-in epigenetic change with vitamin C, EMBO Rep. 14 (2013) 337–346, http://dx.doi.org/10.1038/embor.2013.29.

[39] G. Ficz, M.R. Branco, S. Seisenberger, F. Santos, F. Krueger, T.A. Hore, et al., Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation, Nature 473 (2011) 398–402, http://dx.doi.org/10.1038/nature10008.

[40] M. Bostick, J.K. Kim, P.O. Esteve, A. Clark, S. Pradhan, S.E. Jacobsen, UHRF1 plays a role in maintaining DNA methylation in mammalian cells, Science 317 (2007) 1760–1764, http://dx.doi.org/10.1126/science.1147939.

[41] J.J. Vincent, Y. Huang, P.-Y. Chen, S. Feng, J.H. Calvopiña, K. Nee, et al., Stage-specific roles for Tet1 and Tet2 in DNA demethylation in primordial germ cells, Cell Stem Cell 12 (2013) 470–478, http://dx.doi.org/10.1016/j.stem.2013.01.016.

[42] J.A. Hackett, R. Sengupta, J.J. Zylicz, K. Murakami, C. Lee, T.A. Down, et al., Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine, Science 339 (2013) 448–452, http://dx.doi.org/10.1126/science.1229277.

[43] N. Okashita, Y. Kumaki, K. Ebi, M. Nishi, Y. Okamoto, M. Nakayama, et al., PRDM14 promotes active DNA demethylation through the ten-eleven translocation (TET)-mediated base excision repair pathway in embryonic stem cells, Development 141 (2014) 269–280, http://dx.doi.org/10.1242/dev.099622.

[44] S. Seisenberger, S. Andrews, F. Krueger, J. Arand, J. Walter, F. Santos, et al., The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells, Mol. Cell 48 (2012) 849–862, http://dx.doi.org/10.1016/j.molcel.2012.11.001.

[45] G. Ficz, T.A. Hore, F. Santos, H.J. Lee, W. Dean, J. Arand, et al., FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency, Cell Stem Cell 13 (2013) 351–359, http://dx.doi.org/10.1016/j.stem.2013.06.004.

[46] F. Delhommeau, S. Dupont, V. Della Valle, C. James, S. Trannoy, A. Massé, et al., Mutation in TET2 in myeloid cancers, N. Engl. J. Med. 360 (2009) 2289–2301, http://dx.doi.org/10.1056/NEJMoa0810069.

[47] C.G. Mullighan, TET2 mutations in myelodysplasia and myeloid malignancies, Nat. Genet. 41 (2009) 766–767, http://dx.doi.org/10.1038/ng0709-766.

[48] Y. Kanai, S. Ushijima, Y. Nakanishi, M. Sakamoto, S. Hirohashi, Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers, Cancer Lett. 192 (2003) 75–82, http://dx.doi.org/10.1016/S0304-3835(02)00689-4.

[49] C.G. Lian, Y. Xu, C. Ceol, F. Wu, A. Larson, K. Dresser, et al., Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma, Cell 150 (2012) 1135–1146, http://dx.doi.org/10.1016/j.cell.2012.07.033.

[50] H. Yang, Y. Liu, F. Bai, J.-Y. Zhang, S.-H. Ma, J. Liu, et al., Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation, Oncogene 32 (2013) 663–669, http://dx.doi.org/10.1038/onc.2012.67.

[51] C.-H. Hsu, K.-L. Peng, M.-L. Kang, Y.-R. Chen, Y.-C. Yang, C.-H. Tsai, et al., TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases, Cell Rep. 2 (2012) 568–579, http://dx.doi.org/10.1016/j.celrep.2012.08.030.

[52] M. Sun, C.-X. Song, H. Huang, C.A. Frankenberger, D. Sankarasharma, S. Gomes, et al., HMGA2/TET1/HOXA9 signaling pathway regulates breast cancer growth and metastasis, Proc. Natl. Acad. Sci. 110 (2013) 9920–9925, http://dx.doi.org/10.1073/pnas.1305172110.

[53] Y. Kudo, K. Tateishi, K. Yamamoto, S. Yamamoto, Y. Asaoka, H. Ijichi, et al., Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation, Cancer Sci. 103 (2012) 670–676, http://dx.doi.org/10.1111/j.1349-7006.2012.02213.x.

[54] S. Bhattacharyya, Y. Yu, M. Suzuki, N. Campbell, J. Mazdo, A. Vasanthakumar, et al., Genome-wide hydroxymethylation tested using the HELP-GT assay shows redistribution in cancer, Nucleic Acids Res. 41 (2013) e157, http://dx.doi.org/10.1093/nar/gkt601.

[55] K. Moran-Crusio, L. Reavie, A. Shih, O. Abdel-Wahab, D. Ndiaye-Lobry, C. Lobry, et al., Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation, Cancer Cell 20 (2011) 11–24, http://dx.doi.org/10.1016/j.ccr.2011.06.001.

[56] J.J. Trowbridge, J.W. Snow, J. Kim, S.H. Orkin, DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells, Cell Stem Cell 5 (2009) 442–449, http://dx.doi.org/10.1016/j.stem.2009.08.016.

[57] G.A. Challen, D. Sun, M. Jeong, M. Luo, J. Jelinek, J.S. Berg, et al., Dnmt3a is essential for hematopoietic stem cell differentiation, Nat. Genet. 44 (2011) 23–31, http://dx.doi.org/10.1038/ng.1009.

[58] Z.D. Smith, A. Meissner, DNA methylation: roles in mammalian development, Nat. Rev. Genet. 14 (2013) 204–220, http://dx.doi.org/10.1038/nrg3354.

[59] Z. Li, X. Cai, C.L. Cai, J. Wang, W. Zhang, B.E. Petersen, et al., Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies, Blood 118 (2011) 4509–4518, http://dx.doi.org/10.1182/blood-2010-12-325241.

[60] C. Quivoron, L. Couronné, V. Della Valle, C.K. Lopez, I. Plo, O. Wagner-Ballon, et al., TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis, Cancer Cell 20 (2011) 25–38, http://dx.doi.org/10.1016/j.ccr.2011.06.003.

[61] M. Ko, H.S. Bandukwala, J. An, E.D. Lamperti, E.C. Thompson, R. Hastie, et al., Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 14566–14571, http://dx.doi.org/10.1073/pnas.1112317108.

[62] O. Abdel-Wahab, A. Mullally, C. Hedvat, G. Garcia-Manero, J. Patel, M. Wadleigh, et al., Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies, Blood 114 (2009) 144–147, http://dx.doi.org/10.1182/blood-2009-03-210039.

[63] S.M.C. Langemeijer, R.P. Kuiper, M. Berends, R. Knops, M.G. Aslanyan, M. Massop, et al., Acquired mutations in TET2 are common in myelodysplastic syndromes, Nat. Genet. 41 (2009) 838–842, http://dx.doi.org/10.1038/ng.391.

[64] O. Kosmider, V. Gelsi-Boyer, M. Cheok, S. Grabar, V. Della Valle, F. Picard, et al., TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs), Blood 114 (2009) 3285–3291, http://dx.doi.org/10.1182/blood-2009-04-215814.

[65] A.M. Jankowska, H. Szpurka, R.V. Tiu, H. Makishima, M. Afable, J. Huh, et al., Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms, Blood 113 (2009) 6403–6410, http://dx.doi.org/10.1182/blood-2009-02-205690.

[66] L. Cimmino, O. Abdel-Wahab, R.L. Levine, I. Aifantis, TET family proteins and their role in stem cell differentiation and transformation, Cell Stem Cell 9 (2011) 193–204, http://dx.doi.org/10.1016/j.stem.2011.08.007.

[67] M. Ko, Y. Huang, A.M. Jankowska, U.J. Pape, M. Tahiliani, H.S. Bandukwala, et al., Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2, Nature 468 (2010) 839–843, http://dx.doi.org/10.1038/nature09586.

[68] V. Valinluck, LC. Sowers, Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1, Cancer Res. 67 (2007) 946–950, http://dx.doi.org/10.1158/0008-5472.CAN-06-3123.

[69] F.M. Piccolo, H. Bagci, K.E. Brown, D. Landeira, J. Soza-Ried, A. Feytout, et al., Different roles for Tet1 and Tet2 proteins in reprogramming-mediated erasure of imprints induced by EGC fusion, Mol. Cell 49 (2013) 1023–1033, http://dx.doi.org/10.1016/j.molcel.2013.01.032.

[70] B. Hendrich, A. Bird, Identification and characterization of a family of mammalian methyl-CpG binding proteins, Mol. Cell. Biol. 18 (1998) 6538–6547.

[71] C. Frauer, T. Hoffmann, S. Bultmann, V. Casa, M.C. Cardoso, I. Antes, et al., Recognition of 5-hydroxymethylcytosine by the Uhrf1 SRA domain, PLoS ONE 6 (2011) e21306, http://dx.doi.org/10.1371/journal.pone.0021306.s010.

[72] C.G. Spruijt, F. Gnerlich, A.H. Smits, T. Pfaffeneder, P.W.T.C. Jansen, C. Bauer, et al., Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives, Cell 152 (2013) 1146–1159, http://dx.doi.org/10.1016/j.cell.2013.02.004.

[73] O. Yildirim, R. Li, J.-H. Hung, P.B. Chen, X. Dong, L.-S. Ee, et al., Mbd3/NURD complex regulates expression of 5-hydroxymethylcytosine marked genes in embryonic stem cells, Cell 147 (2011) 1498–1510, http://dx.doi.org/10.1016/j.cell.2011.11.054.

[74] M. Iurlaro, G. Ficz, D. Oxley, E.-A. Raiber, M. Bachman, M.J. Booth, et al., A Screen for Hydroxymethylcytosine and Formylcytosine Binding Proteins Suggests Functions in Transcription and Chromatin Regulation, 2013.

[75] K. Ohnishi, K. Semi, T. Yamamoto, M. Shimizu, A. Tanaka, K. Mitsunaga, et al., Premature termination of reprogramming in vivo leads to cancer development through altered epigenetic regulation, Cell 156 (2014) 663–677, http://dx.doi.org/10.1016/j.cell.2014.01.005.